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M. B. H. Youdim, M. Gerlach (eds.)

Parkinson's Disease and Related Disorders

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Preface

It is our pleasure to present the Proceedings of the 16th International Congress on Parkinson's Disease (PD) and Related Disorders (16th ICPD) which took place in Berlin from June 5–9, 2005. This congress was the most successful congress ever with more than 3500 participants in the roaring German capital, consisting of an innovative program and with emphasis on bringing basic and clinical scientists together. Special attention has was paid in inviting young scientists. Therefore, the major aspect of scientific sessions was to identify young and up coming individuals in the field, with novel approaches to PD and novel models as a whole. The congress gave us the opportunity to present Germany and its capital after the burden of recent history in the new light of a reunified and peaceful country. We have succeeded in presenting the country as an important part of Europe and as a country of arts, architecture and renewal. The Congress attracted new friends from more than 75 countries worldwide. For this reason, we are most thankful to the World Federation of Neurology (WFN), Research Group on Parkinsonism and Related Disorders (RGPD), chaired by Professor Donald Calne for bringing this congress to Germany!

The Congress had many highlights with lectures covering all the major fields in PD and Related Disorders. The opening ceremony was highlighted by the inspiring presentation of Nobel Laureate Paul Greengard who lectured on dopamine-related signalling pathways in the brain, followed by the welcome addresses by Professor Riederer, President of the 16th ICPD, Professor Calne, President of the WFN-RGPD, Dr. Slewett, President emeritus of the National Parkinson Foundation, Miami, USA (NPF), Professor Kimura, President WFN, Professor Reichmann, President German Parkinson Society and Professor Einhäupl, Chairman of the Germany Science Council. The speeches were followed by a musical interlude of the ''Sunday Night Orchestra'' and the award ceremony of the WFN Research Committee on Parkinsonism and Related Disorders. The welcome reception presented typical German dishes and drinks.

In total the congress included 4 plenary lectures, 20 symposia, 6 hot topics, 4 video sessions, 1 workshop with demonstration, 29 educational seminars, more than 600 posters which were presented throughout the congress, 44 guided poster tours, 4 poster symposia, and 14 satellite symposia.

There were many scientific highlights and this proceeding intends to give a representative overview of congress programme. In this preface we are only able to give a glimpse of the outstanding lectures and scientific events during the 5 days.

The congress started with a satellite symposium on the significance of neuromelanin in the human brain. This symposium was dedicated to Prof. Youdim on the occasion of his 65th birthday. These contributions are published separately in a Special Issue of the Journal of Neural Transmission. Professor Carlsson, 2000 Nobel Laureate, spent significant time at the congress site and was often seen discussing topics of mutual interest with congress participant's. There was an interesting new study presented by Professor Deuschl, Kiel, in which he demonstrates that deep brain stimulation results in even better outcome of motor function than regular medication. For this reason, he advocated earlier use of deep brain stimulation in PD. New medications were discussed in detail both in the plenary lectures and satellites and new drugs such as rasagiline, the new MAO-B-inhibitor and rotigotine, the new dermal patch, were discussed in detail. There were satellite meetings on apomorphine, COMT-inhibitors, levodopa, spheramine (a new promising cell therapy for the treatment of PD in the advanced stage), dopamine transporter scanning, dopamine agonists such as pramipexole, ropinirole and cabergoline, adenosine antagonists, restless legs, deep brain stimulation, botolinum toxine A, and the new lisuride dermal patch. All satellites were of highest quality and delivered valuable insights in present and new therapy of PD. Special lectures addressed the advent of gene therapy and stem cell therapy, although it is apparent that there is still a long way to go until this therapy cab be safe and affordable for many PD patients longing for disease modifying treatment.

Professors Schapira and Olanow gave an overview on the ever contradictory aspects of neuroprotection. While neuroprotection is generally accepted in animal models and cell culture, there is still discussion on whether SPECT- and PET-analyses and the delayed start design, as employed in the rasagiline study indicated neuroprotection in man. For neuroprotection to be successful earlier diagnosis of PD is mandatory. For this reason, groups from Amsterdam, Dresden, Tübingen and Würzburg are working on early diagnosis procedures such as olfactory tests, parenchymal sonography, REM sleep analyses, and biochemical markers.

There were lectures on treatment of PD and many on genetic abnormalities causing PD, mitochondrial abnormalities and other disturbances of cell function which lead to dopaminergic cell death.

The other major aspect of the scientific session was the field of basic neuroscience to illuminate our current understanding of how neurons die in sporadic and familial PD. This included symposia on development of midbrain dopaminergic neurons, the role of iron in neurodegeneration, and the progress on genetics and proteomics and the concept of developing novel multifunctional neuroprotective drugs for such a complex disease.

Twenty nine educational seminars covered the most important topics and problems in clinical science bringing theory to practice and treatment strategies.

The guided poster tours allowed exchange of scientific ideas and shed light on new findings in etiology, diagnosis and treatment of PD and related disorders.

A special highlight of the Congress was the Art Exhibition, demonstrating the creativity of our patients with movement disorders. This exhibition was organized by the German Parkinson's lay organisation as well as by the Austrian lay organisation. Professor Maurer, Frankfurt, presented Art from an Alzheimer's patient, the Carolus Horn Exhibition, which impressively demonstrated change in the way to paint during a dementive process.

Another highlight was the Medical Historical Exhibition which was organised by Dr. Ch. Riederer, Würzburg, which focused on the history of the treatment of PD and emphasized the Berlin contributions by H. Lewy, W. v. Humboldt, R. Hassler and others.

A special tribute was paid to Melvin Yahr who sadly passed away in early 2005 shortly before this Congress. He was greatly missed.

Due to generous educational grants from the industry the organizers were able to honour outstanding scientists and clinicians, Toshiharu Nagatsu, Yoshikuni Mizuno, Japan (Award of the WFN Research Group on Parkinsonism and Related Disorders), Saskia Biskup, Germany and Andrew B. Singleton, USA (16^{th} ICPD Junior Research Award), Jonathan Brodie, Canada and Alan Crossman, UK (Merck KGaA Dyskinesia Research Award). GE Healthcare sponsored the 16^{th} ICPD Senior Researcher Award given to Silvia Mandel, Israel and Vincenzo Bonifati, The Netherlands. Both companies gave educational grants for the 12 Poster Prizes while the Melvin Yahr Foundation sponsored 26 Fellowships. In addition the congress made it possible to bring numerous young scientists to the congress by giving them financial support for travelling and accommodation.

The Senator Dr. Franz Burda Award presented by Helmut Lechner, Austria, and Franz Gerstenbrand, was given to Laszlo Vecsei, Hungary and Tino Battistin, Italy.

We thank all the participants who gave us their creative input to organize a World Congress on PD (as indicated in the First Announcement) which fulfilled the criteria of excellence and made the congress so successful. This was YOUR congress and which many of you influenced by letting us know your wishes and expectations. New concepts, formats and innovations, the active and constructive cooperation by the participating industry and the lay organisations made all this possible. This can measured by the numerous complimentary letters and emails we have received since then and we hope it sets the standards for future meetings! By doing all this we tried to come close to our milestone ''Present and Future Perspectives of Parkinson's Syndrome''.

Our special thanks go to CPO Hanser Congress Organisation, the programme committee and the WFN Research Group which all worked so hard to make this Congress so successful.

Finally the congress proceedings are published and we thank all those who contributed to this volume. Special thanks go to Springer Verlag, Vienna, New York for their efficient and splendid ability in being able to publish the proceeding so rapidly.

Peter Franz Riederer, Heinz Reichmann, Moussa Youdim, Manfred Gerlach

Würzburg, Dresden, Haifa, spring 2006

Contents

2. Iron and neuromelanin

3. Genetics

4. Imaging

5. Models

6. Clinical approaches

7. Neuroinflammation

8. Neurosurgery

Listed in Current Contents/Life Sciences

J Neural Transm (2006) [Suppl] 70: 1–4

Melvin Yahr (1917–2004). An appreciation

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Melvin Yahr (1917–2004)

I am honoured yet somewhat wary in being invited to write an appreciation of Melvin David Yahr. Can an outsider, a non-neurologist and a non-American, really grasp his contribution to movement disorder clinical practice, to the specialty of neurology, and to the larger world of Medicine? In so far as Melvin Yahr's importance extended beyond the borders of Neurology into all corners of the world, the answer is, perhaps, yes. Given such a long period of consistent and extensive activity (first paper in Journal of Pediatrics in 1944, 357th in 2003), much of the customary academic and professional rivalry and anguish, well described by Hornykiewicz (2004), will be unknown to an outsider and perhaps is better left that way until some future disinterested biographer intervenes. Here follow brief comments on some of his papers.

Duvoisin et al. (1963) give an insight into some 1960s thinking. Yahr and his colleagues studied a clinical sample from Columbia-Presbyterian Medical Center (225 subjects attending in 1962 of whom 195 had classical paralysis agitans) and refuted authors who asserted that, with the passing of the post-encephalitic cohort, Parkinsonism would largely disappear [by 1980] . . . thereafter constituting a numerically insignificant disease entity.

Melvin Yahr (Yahr et al., 1969) was important in those early years showing the efficacy of L-dopa (from Birkmayer and Hornykiewicz, 1961 onwards) and affirming that enough L-dopa would produce and sustain clinical response. (Hornykiewicz, 2004 engagingly and courageously records the chronology and conflict of those papers and their authors.) In a placebo controlled, double blinded study, with careful evaluation (more later about the Scale used for evaluation), 60 subjects, 56 with Parkinson's Disease, aged 44–78 years of at least 3 years duration and followed for 4 to 13 months, were given 750 mg to 1 gram of L-dopa 3 to 5 times daily. All these patients had been hospitalized for the study – those were the days! After initial symptomatic improvement, objectively there was 'renewed ability to perform simple movements which had been lost for several years, such as turning over in bed or rising from a chair'. They noted that some subjects did not reach ultimate functional improvement until treated for 3 to 4 months. Abrupt cessation of the drug led to loss of effect in the ensuing week and restoration took at least a further week.

Younger clinicians will have no memory of the excitement produced when L-dopa was introduced (in today's parlance, ''Awakenings'' is a 'must-read'). It is in the same league as witnessing the original clinical

response to penicillin (Fletcher, 1984) or the present writer's joy as an intern at the effectiveness of oral diuretics replacing parenteral mercurials.

In his 1970 presidential address to the American Neurological Association's 95th Annual General Meeting, Melvin Yahr began: ''I'm not a philosopher or historian, much less a prophet'' and then described ''Neurology's position in the present crisis in American medicine''. His analysis bears repetition and response even today. He bluntly asserted: ''the public is disaffected with the health care we are giving them'' ... the affluent complain about their waits to see physicians, the indigent complain they have no access to physicians. He warned that the false dichotomy of ''medical research'' *or* ''medical care'' ignored research as the catalyst for both clinical care and teaching. While recognizing the complementary nature of basic and applied research, he pleaded that their funding should not be in direct competition. Where he differs from many presidential addresses, which focus on the clinician and his (certainly *his* in 1970) preoccupations, is his dissection of the (American) health care delivery system ''which is about as unhealthy, uncaring and unsystematic a delivery system as one can imagine''. He emphasized the context: ''the senseless war in Vietnam, poverty, hunger, environmental pollution, divisions between the races, alienation of our young people. And somewhere in that group ... inadequate medical care.'' He challenged then and now: ''the large sums of money expended by our government on misguided military adventures should, instead, be serving the cause of human betterment and as physicians we have an obligation to say so''.

''100 years ago we were unable to exist with half slave half free, so we cannot now continue to exist with half our people barred from decent health care''. He envisaged developing ''a comprehensive health plan for all to which ability to pay will cease to be a barrier to participation''. He then applied these principles to neurological practice and training. He perceptively commented on: urban/rural practice (''the irresistible ambience of West Coast living'' – very pertinent for a former Winnipeg physician when read during a January sabbatical in Vancouver); the needed continuum of care required ''through the various phases of the many long-term diseases with which we are involved''; and he made an impassioned plea for ''one class of care – first class''. Other topics included telehealth (not his term) consultations, relevant CME in neurological matters for primary care physicians, and the relationship between the academic health centre and its medical hinterland. To this writer, this address was unexpectedly refreshing, revealing, and still relevant.

The Hoehn and Yahr Scale (1967)

It is a truism that Parkinson's Disease was and is a clinical diagnosis: there are no laboratory tests, no imaging techniques, no genetic markers to confirm the diagnosis. It is the clinician's decision. This judgment nicely combines the art and science of medicine but the first attempt to supply a scientific basis for this judgment appeared in Hoehn and Yahr (1967). This is Melvin Yahr's most famous paper (at least 2886 citations by mid-January, 2004) because it laid the foundation for *measuring* Parkinsonism.

The Hoehn and Yahr Scale appeared before the obligatory application of psychometric and clinimetric measures to clinical scaling, before sensitivity and specificity, before predictive values, before receiver operating curves and the rest of the scientific apparatus ensuring those twin pots of gold: validity and reliability (albeit tempered with simplicity, acceptability, accuracy, cost – Cochrane and Holland, 1971 – sensibility – Feinstein, 1987 – and responsiveness – Rockwood et al., 2003). The main objective of the paper was to determine the clinical variability, progression

and mortality of Parkinson's Disease given the then paucity of information about the natural history of the condition. This would subsequently give the background upon which to judge the effectiveness of the newly introduced L-dopa therapy.

Hoehn and Yahr reported on 802 subjects derived from a retrospective clinical sample of 856 patients diagnosed with paralysis agitans, Parkinson's Disease and Parkinsonism seen at the Columbia-Presbyterian Medical Center from 1949 to 1964. Nearly 85% had classic Parkinson's Disease and 13% had post-encephalitic associated Parkinsonism. This was the largest clinical sample hitherto studied. Two hundred and sixty three subjects attending in 1963–4 were examined more closely and it was from this subsample that the famous clinical stratification was derived. They found only 10% free of tremor at onset and incidentally note the continuing occasional clinical conundrum of Parkinsonism and essential tremor; 14% exhibited ''mild-to-moderate organic mental syndrome . . . usually characterized by recent memory defects and some impairment of judgment and insight''; 4% were ''moderately to severely depressed'' (no further details given).

They wisely point out that the presence of the classical signs of tremor, rigidity and akinesia varies with respect to disability – its presence and progress; hence the need to quantify this interaction of physical signs and functional consequences into clinical stages. Hoehn and Yahr recognized that these stages may not correlate with pathology but they claimed a clinimetric basis for ''reproducible assessments by independent examiners of the general functional level of the patient''.

Five clinical stages, ''based on the level of clinical disability'' were reported on 183 patients with ''primary parkinsonism'' (viz. Parkinson's Disease, paralysis agitans or idiopathic Parkinsonism) – a subset of the 263 'more closely examined'. They dichotomized these stages into: mildly affected (Stages 1–III) and severely affected (Stages IV–V).

Five clinical stages: degrees of disability

Stage I: unilateral involvement only, usually with minimal or no functional impairment.

Stage II: bilateral or midline involvement, without impairment of balance.

Stage III: first sign of impaired righting reflexes. This is evident by unsteadiness as the patient turns or is demonstrated when he is pushed from standing equilibrium with the feet together and the eyes closed. Functionally the patient is somewhat restricted in his activities but may have some work potential depending on the type of employment. Patients are usually capable of leading independent lives and their disability is mild to moderate.

Stage IV: fully developed, severely disabling disease; the patient is still able to walk and stand unassisted but is markedly incapacitated.

Stage V: confinement to bed or wheelchair unless aided.

It is unfair to criticize a 1967 paper in terms of current epidemiological standards (McDowell, 1996). Clinically derived scales (e.g. Rankin, 1957 for stroke rehabilitation) are still used inspite of academic strictures. Ramaker et al. (2002) in their recent comprehensive review of measuring Parkinson's Disease, regret that the Hoehn and Yahr Scale is frequently chosen ''as the gold standard for testing other scales'' because of its lack of psychometric and clinimetric properties – but at the time it emerged it was groundbreaking.

Melvin Yahr contributed to many major textbooks of Medicine, Neurology and Movement Disorders. In his 357 publications, themes included: amino acid biology, the

continuing relationship of central nervous system infection and Parkinsonism, autonomic nervous system failure with special attention to orthostatic hypotension, and every aspect of the drug management of Parkinson's Disease. His experience and expertise (in others not necessarily the same thing) were highly valued. No part of movement disorder neurology was untouched by his presence: as an explorer, quantifier, analyser, teacher and practitioner. An obituary by former students (Di Rocco and Werner, 2004) expresses the richness of his contribution to neurology and neurologists. He was an exemplar of successful ageing. Rejecting the curse of mandatory retirement, he continued his clinical and academic work into the last weeks of his life: he was the *compleat* physician.

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J Neural Transm (2006) [Suppl] 70: 5–7

Melvin D. Yahr, 1917–2004. A personal recollection

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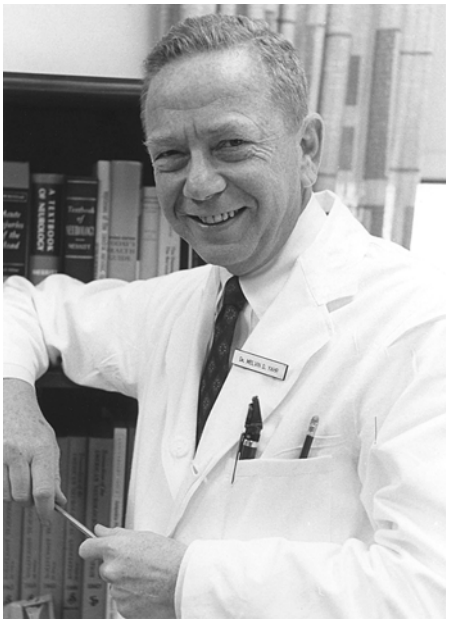

Melvin D. Yahr, one of the giants of 20th century neurology died on January 1st 2004, aged 87, of lung cancer, at his home in Scarsdale, New York. His was an intense and long life of uninterrupted scientific productivity. His first paper, on myasthenia gravis, was published in 1944 and his last one, of course on Parkinson's disease, appeared in press in 2005, sixty one years later. Born in 1917 in New York City, Yahr was the youngest of six children of immigrant parents. His family lived in Brooklyn where his father owned a bakery. He went to New York University School of Medicine and completed an internship and residency at Lenox Hill Hospital and Montefiore Hospital in New York City. As a student he played the clarinet in a jazz combo to earn extra money, but insisted that he was not a talented musician. Later, when questioned about the origin of the phenomenal musical talent of his daughters, he attributed all to his wife Felice, whom he married when she was a 23-year-old writer working at Fortune Magazine. Yahr served in the US army from 1944 to 1947 and was discharged with the rank of Major. Back in NY, he joined the faculty at Columbia University College of Physicians and Surgeons where he began his work as an academic neurologist. He had wide clinical interests

but after a few years he began focusing on Parkinson's disease. Building on the work of Carlsson, Hornykiewicz and Cotzias, in the 1960's Yahr conducted the first double blind randomized large clinical trials of Ldopa in the treatment of Parkinson's disease. The success and impact of this treatment was tremendous; patients were ''unfrozen'' from statue-like rigidity and brought back to life. In 1967, together with Peggy Hoehn, he devised a 5-stage scale, simplicity itself, to determine the severity of Parkinson disease. The Hoehn and Yahr rating scale is still the gold standard and levodopa remains the most widely used medication for the treatment of Parkinson's disease.

Melvin Yahr became H Houston Merritt professor of neurology at Columbia University before moving downtown, as he used to say, to Mount Sinai School of Medicine, where he become professor of neurology and chairman of the department. Yahr brought to Mount Sinai the country's first multidisciplinary center for research in Parkinson's disease and related disorders, a pioneering example of translational research. Under his leadership, basic scientists and clinical investigators working in close proximity, made significant contributions to the understanding and treatment of these disorders.

He chaired study sections for the National Institute of Neurological Communicative Disorders and Stroke, he was an adviser for the National Research Council, the National Academy of Sciences, and the New York City Board of Education. He was president of the American Board of Neurology and Psychiatry, the American Neurological Association, and the Association for Research in Nervous and Mental Diseases. He received many prizes and awards and was an honorary member of the British, French, Belgium and Argentine Neurological societies.

Melvin Yahr was an imposing presence. I first met him in 1982 during my neurology residency at Mount Sinai. He was 64, famous and at the top of his game. He had a low baritone voice and a very characteristic way of speaking that we all used to imitate. He was impeccably dressed and always wore a crisp shirt and tie under his white lab coat. And he smoked a pipe, an indispensable tool for the neurologist-detective of his generation.

Yahr was first and foremost a clinician; but believed strongly in basic research. He loved neurology and he got great satisfaction from his work. He was a superb teacher. I remember vividly Morning Reports as a senior neurology resident; every day of the week at 9 in the morning, after rounding the neurology ward, the senior residents went into his office in the 14th floor of the Annenberg Building, junior residents were not allowed. The 5 or 6 seniors sat in couches and chairs facing him who was sitting behind his desk, reclined backwards, almost always smoking a pipe. The curtains were usually lowered, so the room was dark. Many times we couldn't see his face because it was covered by the desk lamp and by a journal he was reading and holding in front of him. One could only see the smoke from his pipe coming up from behind the journal. We felt we were in front of the oracle. We presented each new patient trying to be brief and to the point. At the end of each patient's presentation we heard his voice saying: next! or some short comment. But sometimes it was different. He would put the journal down and ask a few more questions and then go through the differential diagnosis or focus on one particular aspect of the history and what it meant. For us it was magic, it all made sense when he explained it. He left us mesmerized and we walked out of his office full of ideas and imagining that we actually knew what we were all doing. Clinical neurology was an exciting job with Melvin Yahr.

Twice a week he also did ''Chief of Service Rounds.'' With all the residents and medical students sitting around him, he interrogated and examined a patient from the Neurology ward. With Melvin Yahr this was high theatre. He was a master performer.

Melvin Yahr was outspoken and blunt and was used to be in charge. He was not easily convinced (– of anything), and his most typical questions were – ''What do you want?'' to his students and ''What is it that you cannot do?'' to his patients. He was frequently gruff and stern but had a fine sense of humor and compassion.

Almost everything that is necessary for a neurological diagnosis is in the history, he used to say and he mostly stuck to that. Of course he used radiology and electrophysiology extensively, but he had a deep distrust for all forms of testing. He asked patients very clear questions and had the ability to make them talk and reveal information that nobody else seemed to have been able to obtain. He listened intently, rarely interrupting with his gaze locked on the patient. His neurological examinations were very focused brief and revealing: as residents, we entertained the possibility that Yahr could actually alter plantar responses in patients at will, and we believed that he always knew what he was going to find, as he never appeared surprised. He kept the tradition of clinical neurology training one on one, almost like an apprentice. Neurology was his passion. He was a methodical thinker, disciplined, focused and persistent.

Melvin Yahr did not believe in retirement. When he stepped down as chairman at Mount Sinai in 1992, his office was demolished, literally. I guess the powers to be thought he would have stayed there otherwise. Undeterred, he got a new office, and a new endowed chair remaining as active as ever.

He appreciated beauty, loved red wine and cognac. A favorite line of his was ''It's a racket!'' applied to a variety of senseless medical or everyday life schemes. He was a Democrat, which in the US, means liberal.

He is survived by Nancy his companion after the death of his wife Felice in 1992, and by 4 brilliant daughters, Carol an opera singer, Barbara, an orchestra conductor, Laura a pathologist and Nina a social worker, and 5 grandchildren.

Melvin Yahr died 200 years after James Parkinson. He would have pointed that out. Until the end Yahr remained intellectually vibrant. He was writing and seeing patients just a few weeks before his death. He will be missed.

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The discovery of dopamine deficiency in the parkinsonian brain

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Summary. This article gives a short historical account of the events and circumstances that led to the discovery of the occurrence of dopamine (DA) in the brain and its deficiency in Parkinson's disease (PD). Some important consequences, for both the basic science and the patient, of the work on DA in the PD brain are also highlighted.

Early opportunities

In 1951, Wilhelm Raab found a catecholamine (CA)-like substance in animal and human brain (Raab and Gigee, 1951). He knew that this CA was neither noradrenaline (NA) nor adrenaline; today, we know that it was, at least in part, dopamine (DA). Raab examined its regional distribution in the brain of humans, monkeys and some "larger animals", and found highest levels in the caudate nucleus. He found no changes of this CA in the caudate in 11 "psychotic" patients. He did not try to look for this compound in the caudate nucleus of patients with Parkinson's disease (PD).

In 1952, G. Weber analyzed brains of patients with PD, obtained postmortem, for cholinesterase activity (Weber, 1952). He found a reduction of the enzyme activity in the putamen, and hypothesized about the significance for PD. Had Weber known of Raab's study published the year before, he might have measured Raab's CA-like compound in his PD postmortem material. In his report, Weber does not refer to Raab's study.

In 1952–1954, Marthe Vogt performed her landmark study of the regional distribution of NA and adrenaline in the brain of the dog (Vogt, 1952, 1954). She isolated the amines from brain tissue extracts by paper chromatography and eluted the corresponding "spots" for (biological) assays. Marthe Vogt was well aware of Raab's work. However, for practical reasons, she did not stain the CA (with ferricyanide) on the chromatograms of regions that contained little NA, such as the caudate; thus she let pass the opportunity of detecting DA's presence in the brain and its striatal localisation.

Setting the stage for the DA/PD studies

In August 1957, Kathleen Montagu reported on the presence of DA, identified by paper chromatography, in the brain of several species, including a whole human brain (Montagu, 1957). In November 1957, Hans Weil-Malherbe confirmed this discovery and examined DA's intracellular distribution in the rabbit brain stem (Weil-Malherbe and Bone, 1957). Neither he, nor Montagu, offered any speculations on the physiological role of brain DA. At the same time as Weil-Malherbe, in November 1957, Arvid Carlsson observed that in naïve and reserpine treated animals "*3,4-dihydroxyphenylalanine caused*

central stimulation which was . . . markedly potentiated by iproniazid" (Carlsson et al., 1957). He concluded that the study "*supports the assumption that the effect of 3,4-dihydroxyphenylalanine was due to an amine formed from it"* – leaving the question of whether this amine was NA or DA, unconsidered. In the Fall of 1957, a few weeks before Carlsson's report, Peter Holtz published observations on, *inter alia*, L-dopa's central stimulant and "awakening" (from hexobarbital anesthesia) effects, and clearly suggested, apparently for the first time, that this could be due to the accumulation of "*the dopamine formed in the brain from L-dopa"* (Holtz et al., 1957). (Raab, in 1951, was the first to observe increased brain levels of his CA-like substance after i.p. L-dopa; but he does not mention any behavioral L-dopa effects [Raab and Gigge, 1951].)

Holtz's conclusion was soon confirmed in two biochemical studies. In February 1958, Carlsson reported that reserpine depleted, in addition to NA and serotonin, brain DA, and L-dopa replenished it while causing central excitation (Carlsson et al., 1958). In May 1958, Weil-Malherbe obtained, independently, the same biochemical results in a well documented study (Weil-Malherbe and Bone, 1958). Neither Carlsson nor Weil-Malherbe ventured any explicit statements about brain DA's possible physiological role or its involvement in the reserpine syndrome.

More than a year before these first brain DA studies, in the Fall of 1956, Blaschko had already proposed that DA – until then seen as being merely an intermediate in the biosynthesis of CA – had "*some regulating functions of its own which are not yet known"* (Blaschko, 1957). In early 1957, Hornykiewicz, in Blaschko's Oxford laboratory, tested this idea experimentally. He analyzed DA's vasodepressor action (in the guinea pig) and proved that DA had actions distinct from NA and adrenaline and thus qualified as a biologically active substance in its own right; L-dopa behaved exactly like DA (Hornykiewicz, 1958). In 1958, Hornykiewicz (now back in Vienna) examined (in the rat) the central actions of several substances, including the parkinsonism-inducing chlorpromazine and bulbocapnine, as well as cocaine and MAO inhibitors, and showed that only the latter affected (increased) the levels of brain DA (Holzer and Hornykiewicz, 1959).

Marthe Vogt, in her 1954 NA study in the dog brain, inferred NA's possible role in brain function from the amine's specific distribution pattern. In January 1959, Åke Bertler and Evald Rosengren, patterning themselves on Marthe Vogt's NA study, published a study, also in the dog, on the regional distribution of brain DA (Bertler and Rosengren, 1959a); a few weeks later, Isamu Sano reported on DA's regional distribution in the human brain (Sano et al., 1959) (followed by Bertler and Rosengren, 1959b). Both research groups found that DA was mostly concentrated in the nuclei of the basal ganglia, especially caudate and putamen. Bertler and Rosengren (1959a) concluded that their "*results favour*[ed] *the assumption that dopamine is connected with the function of the corpus striatum and thus with the control of movement"*; and Sano "*considered DA to function in the extrapyramidal system which regulates the central motoric function"* (Sano et al., 1959). Although Bertler and Rosengren pointed out DA's possible involvement in reserpine parkinsonism, neither they nor Sano suggested the possibility of striatal DA being directly involved in diseases of the basal ganglia.

DA is severely reduced in PD striatum

Several eyewitness accounts have recently been written about the historical events and consequences of the discovery of the DA deficiency in PD (Sourkes, 2000; Hornykiewicz, 2001a, b, 2002a, b).

Early in 1959, Hornykiewicz, aware of DA's localisation in the basal ganglia, started

a study on DA in postmortem brain of patients with PD and other basal ganglia disorders. He and his collaborator Herbert Ehringer analyzed the brains of 17 adult non-neurological controls, 6 brains of patients with basal ganglia disease of unknown etiology, 2 brains of Huntington's disease, and 6 Parkinson brains. Of the 14 cases with basal ganglia disease, only the 6 PD cases had a severe loss of DA in the caudate and putamen (Ehringer and Hornykiewicz, 1960). Ehringer and Hornykiewicz concluded that their observations "*could be regarded as comparable in significance* [for PD] *to the histological changes in substantia nigra"* . . . so that "*a particularly great importance would have to be attributed to dopamine's role in the pathophysiology and symptomatology of idiopathic Parkinson's disease"*. This discovery was published in December 1960. Ever since, it has provided a solid, rational basis for all the following research into the mechanisms, the causes, and new treatments of PD.

It is interesting to note that in none of the brain DA and/or L-dopa studies preceding the Ehringer and Hornykiewicz 1960 paper, is there any hint to be found that such a study should be done. The first such suggestion was made in an article from Montreal, submitted for publication end of November 1960, reporting on reduced urinary DA in PD patients. The authors concluded that future investigations should "*include analysis of the catecholamine content in the brains of patients who have died with basal ganglia disorders"*, so as to "*help determine whether the concentration of cerebral dopamine itself undergoes major changes"*. The article was published in May 1961; a "note added in proof" informed the readers that the suggested study has, in the meantime, been done (Barbeau et al., 1961).

The fact that the Montreal group quoted the paper from Vienna so soon after it was published on December 15, 1960, deserves a comment. This article was written in German and published in a German language journal. Theodore Sourkes, the leading biochemist of the Montreal group, must have read it almost immediately after it came out. He contacted Hornykiewicz about this article by letter dated February 10, 1961. For the Vienna discovery, there were, obviously, neither language nor information transfer barriers. This was opposite to what happened to a (lecture) overview article of Sano, published in Japanese in 1960. Independently from Hornykiewicz, Sano had analyzed the brain of a single PD patient, but was "*reluctant to speculate, from that single experience* [low putamen DA] *about the pathogenesis of Parkinson's disease"* (Sano, 1962). The publication remained unnoticed until it was recently reprinted in English translation (Sano, 2000).

The question arises: Why did none of the pioneers of the early brain DA research think of studying the PD brain? It appears that the main reason was their too exclusive preoccupation with the central effects of reserpine. This is surprizing because even then it was obvious that reserpine, like most pharmacological animal models, was not a perfect centrally acting drug; it depleted, to the same degree as DA, also the brain NA and serotonin, making a clear decision about the relative importance of these changes impossible. The exclusive "fixation" on reserpine made leading monoamine researchers of that period overlook the most obvious, that is, PD as the ultimate "brain DA experiment of Nature".

Two practical consequences

Inaugurating the nigrostriatal DA pathway

When the DA deficiency in PD was discovered, nothing was known about DA's cellular localisation in the brain. In Huntington's disease, Ehringer and Hornykiewicz (1960) had found normal striatal DA. Since in Huntington's disease there is a severe loss of striatal neurons accompanied by marked gliosis, the normal striatal DA suggested that the amine was probably contained in terminals of fibre tracts originating outside the striatum. Rolf Hassler

had proved, back in 1938, that in PD, loss of the substantia nigra compacta neurons was the most consistent pathological change (Hassler, 1938). Thus, in 1962, Hornykiewicz started a study of the substantia nigra in 10 PD brains. The outcome of such a study was by no means certain. Hassler himself rejected the possibility of a nigro-striatal connection (see page 869 in: Jung and Hassler, 1960); and Derek Denny-Brown declared, in 1962, that "*we have presented reasons against the common assumption that lesions of the substantia nigra are responsible fo parkinsonism"* (Denny-Brown, 1962). In his study, Hornykiewicz found markedly reduced nigral DA, similar to the DA loss in the striatum. In the report published in 1963, Hornykiewicz concluded from his observation that "*on the other hand, cell loss in the* [PD] *substantia nigra could well be the cause of the dopamine deficit in the striatum"* (Hornykiewicz, 1963).

At the time of Hornykiewicz's DA/substantia nigra study, two research groups were already trying to tackle the question of brain DA's cellular localization. In Montreal, Poirier and Sourkes were using electrolytic brain lesions, in the primate; in Sweden, Fuxe, Dahlström (and others) were applying, in the rat, the just developed CA histofluorescence method. A year after Hornykiewicz published his study, each of the two research groups was able to report on the existence of a DA-containing nigrostriatal connection. Both groups referred, in their first publications, to Hornykiewicz's 1963 nigral DA study (Andén et al., 1964; Dahlström and Fuxe, 1964; Poirier and Sourkes, 1965). This contribution to the discovery of the nigrostriatal DA pathway had for Hornykiewicz yet another consequence. Several years later, Hassler wrote him a letter in which he expressed his candid opinion on the nigrostriatal DA pathway. He wrote: "*I believe that your interpretation of your observations does not agree with many known facts, this being so because you accept the American* [?!] *opinion about the direction of the nigrostriatal connections. I believe that all your observations can be equally well, or even better, explained by the striatonigral direction* [of that pathway]*"* (Hassler, 1967).

L-dopa for the PD patient

The discovery of the severe striatal DA deficiency in PD had also a far-reaching clinical consequence. Hornykiewicz immediately took the step "from brain homogenate to treatment" and asked the neurologist Walther Birkmayer to do clinical trials with i.v. L-dopa. After a delay of eight months, in July 1961, Birkmayer injected 50–150 mg L-dopa i.v. in 20 PD patients, most of them pretreated with an MAO inhibitor. The first report, published in November 1961, conveys, even today, the excitement about what since has been called "the dopamine miracle"; it reads as follows:

The effect of a single i.v. administration of L-dopa was, in short, a complete abolition or substantial relief of akinesia. Bed-ridden patients who were unable to sit up; patients who could not stand up when seated; and patients who when standing could not start walking, performed after L-dopa all these activities with ease. They walked around with normal associated movements and they even could run and jump. The voiceless, aphonic speech, blurred by pallilalia and unclear articulation, became forceful and clear as in a normal person. For short periods of time the patients were able to perform motor activities which could not be prompted to any comparable degree by any other known drug. (Birkmayer and Hornykiewicz, 1961).

Simultaneously with, and independently from, the trials in Vienna, Sourkes and Murphy, in Montreal, proposed to Barbeau a trial of oral L-dopa. They observed, with 200 mg L-dopa, an amelioration of rigidity that "*was of the order of 50 percent"* (Barbeau et al., 1962). Interestingly, Sano in his overview in 1960 also mentioned that he

had injected 200 mg L-dopa i.v. in two patients; however, he did not evaluate the effect clinically, being "*more interested in subjective complaints"* (Sano, 1962). Sano concluded that "*treatment with dopa has no practical value"* (Sano, 2000).

Today, especially thanks to Cotzias's introduction of the high dose oral treatment regimen (Cotzias et al., 1967), L-dopa is recognized as the most powerful drug available for PD. As Sourkes very aptly expressed it, the discovery of L-dopa "*proved to be the culmination of a century-and-a-half search for a treatment of Parkinson's disease"* (Sourkes, 2000).

Despite the unprecedented success, doubts were expressed about L-dopa's "miraculous" antiparkinson effect. Many neurologists suspected a placebo effect of the i.v. injected L-dopa, ignoring the fact that Birkmayer and Hornykiewicz (1962) had described, already in 1962, the ineffectiveness of i.v. injected compounds related to L-dopa, such as: D-dopa, 3-O-methyldopa, DA, D, L-dops, and also 5-HTP. This should have convinced the doubters that the L-dopa effect could not have been a placebo effect.

Especially counterproductive were various statements by some rather prominent brain scientists. Thus, some claimed that "*the actions of DOPA and DOPS* [the direct precursor of NA] *were similar"*, cautioning that "*dopamine can activate not only its own receptors* [in the brain], *but also those of noradrenaline, and vice versa"* (Carlsson, 1964, 1965); others felt that "*the effect of L-dopa was too complex to permit a conclusion about disturbances of the dopamine system in Parkinson's disease"* (Bertler and Rosengren, 1966), still others compressed all their doubts in the terse phrase that L-dopa "*was the right therapy for the wrong reason"* (Ward, 1970; Jasper, 1970); and, finally, there was the statement that "*since L-dopa floods the brain with dopamine, to relate its* [antiparkinson] *effects to the natural function of dopamine neurons may be erroneous"* (Vogt, 1973). These and similar critical statements diminished the status of L-dopa as a specific DA replacing agent and put in doubt the very concept of DA replacement in PD.

Viewed against the background of the initial skepticism, today's opinion has substantially changed, as reflected, for instance, in a recent "Editorial":

The identification of the dopaminergic deficit in Parkinson's disease and the development of dopamine replacement therapy by Hornykiewicz and his contemporaries profoundly influenced research into Parkinson's disease, and perhaps even all neurological disorders. This is especially true for Alzheimer's disease, in which current cholinergic therapy is the intellectual heir of dopamine replacement therapy for Parkinson's disease. (Hardy and Langston, 2004).

Thus has theoretically based research led, in an amazingly straight line, to very practical results. As Immanuel Kant, that eminent philosopher of the Age of Enlightenment, put it some 200 years ago: "There is nothing more practical than a sound theory".

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Synchronizing activity of basal ganglia and pathophysiology of Parkinson's disease

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Summary. Early physiological studies emphasized changes in the discharge rate of basal ganglia in the pathophysiology of Parkinson's disease (PD), whereas recent studies stressed the role of the abnormal oscillatory activity and neuronal synchronization of pallidal cells. However, human observations cast doubt on the synchronization hypothesis since increased synchronization may be an epi-phenomenon of the tremor or of independent oscillators with similar frequency. Here, we show that modern actor/critic models of the basal ganglia predict the emergence of synchronized activity in PD and that significant non-oscillatory and oscillatory correlations are found in MPTP primates. We conclude that the normal fluctuation of basal ganglia dopamine levels combined with local cortico-striatal learning rules lead to non-correlated activity in the pallidum. Dopamine depletion, as in PD, results in correlated pallidal activity, and reduced information capacity. We therefore suggest that future deep brain stimulation (DBS) algorithms may be improved by desynchronizing pallidal activity.

Introduction: The computational roles of the basal ganglia and dopamine

Modeling of the basal ganglia has played a major role in our understanding of the physiology and pathophysiology of this elusive group of nuclei. These models have undergone evolutionary and revolutionary changes over the last twenty years, as ongoing research in the fields of anatomy, physiology and biochemistry of these nuclei has yielded new information. Early models dealt with a single pathway through the basal ganglia nuclei (cortex-striatum-internal segment of the globus pallidus; GPi) and focused on the nature of the processing performed within it, convergence of information vs. parallel processing of information. Later, the dual (direct and indirect) pathway model (Albin et al., 1989) characterized the inter-nuclei interaction as multiple pathways while maintaining a simplistic scalar representation of the nuclei themselves. The dual pathway of the basal ganglia networks emphasized changes in the discharge rates of basal ganglia neurons. The model predicts that in the dopamine depleted Parkinsonian state firing rates in the external segment of the globus pallidus (GPe) are reduced, whereas cells in the internal segment (GPi) and the subthalamic nucleus (STN) display increased firing rates (Miller and DeLong, 1987; Bergman et al., 1994). This model resulted in a clinical breakthrough by providing key insights into the behavior of these nuclei in hypo- and hyper-kinetic movement disorders, and lead

to subsequent findings showing that inactivation of STN and GPi can improve the motor symptoms in Parkinsonian animals (Bergman et al., 1990) and human patients. Finally, in line with the model predictions many studies have demonstrated reversed trends of pallidal discharge rates in response to dopamine replacement therapy (DRT) in both human patients and primates (e.g. Heimer et al., 2002). The next generation of models elaborated the intra-nuclei interactions and focused on the role of the basal ganglia in action selection and sequence generation which form the most current consensus regarding basal ganglia function in both normal and pathological conditions (Mink, 1996).

The dual pathway rate and the action-selection models represent the most common delineation of the basal ganglia functional anatomy and physiology. Nevertheless, new findings challenge these models. Thus, several primate studies have failed to find the expected significant changes of firing rates in MPTP monkeys. Similarly, biochemical and metabolic studies indicate that GPe activity does not change in Parkinsonism. Whereas the rate model strongly predicts that the enhanced GPi inhibitory output in Parkinsonism should reduce thalamic and motor cortex firing rates, several studies in dopamine-depleted primates have shown no change in spontaneous thalamic and motor cortical firing rates (e.g. Goldberg et al., 2002). Finally, both the dual-pathway rate and the action-selection model predict strong (positive or negative) correlations between pallidal neurons. However, all correlations studies (e.g. Raz et al., 2000; Bar-Gad et al., 2003a) of pallidal neurons in the normal monkey revealed lack of correlation between the spiking activity of these neurons.

The complex anatomy of the basal ganglia and the physiological correlation studies point to a different neural network approach to information processing in the basal ganglia. One example is the *Reinforcement Driven Dimensionality Reduction (RDDR)* model (Bar-Gad et al., 2003b). The RDDR model postulates that the basal ganglia can be modeled as an actor/critic reinforcement learning neuronal network whereas the goal of the system is to maximize the (discount) expectation of all future reinforcements by dynamic modification of behavior. The reinforcement signal is provided by the midbrain dopaminergic (the critic) projections to the striatum, i.e., to the actor networks of the basal ganglia. The dopamine-reinforcement signal represents the mismatch between expectations and reality or the temporal difference (TD) error. In the normal primate the background dopamine activity (5–10 spikes/s) represents a match between the animal's prediction and reality, whereas elevation or suppression of dopaminergic activity represents a situation where reality is better or worse than predictions, respectively (Morris et al., 2004). The actor part of the basal ganglia network (cortex; striatum and STN; GPe and GPi) compress cortical information using reinforcement controlled cellular (Hebbian and anti-Hebbian) learning rules. The ultimate goal of basal ganglia actor is to achieve optimal behavior or policy, e.g., optimal state-action (stimulus-response) associations, by modification of the efficacies of the gigantic matrix of $>10^{13}$ cortico-striatal synapses. This setting of the cortico-striatal functional efficacy leads to optimal connectivity between the sensory and the frontal cortices and optimal behavior which maximize expected future reward. Optimal representation of the state-action matrix in the actor part of the basal ganglia networks is achieved by decorrelation of the spiking activity of the pallidal neurons (output stage of the basal ganglia). The model suggests that the chronic dopamine depletion in the striatum of PD patients is perceived as encoding a continuous state whereas reality is worse than predictions. This lead to modifications in the delicate high-dimensional matrix of efficacies of the cortico-striatal synapses and abnormal synchronization of

the basal ganglia networks (in additions to changes in firing rate and pattern). Furthermore, inappropriate dopamine levels – as, for example during pulsatile dopamine replacement therapy – will cause abnormal random organization of the cortico-striatal network, and eventually would lead to dyskinesia (inappropriate state-action pairing).

Results: Synchronized activity in the basal ganglia

Multiple electrode studies analyzing for correlations of pallidal neurons in the normal monkey revealed lack of correlation between the spiking activity of these neurons (e.g. Raz et al., 2000; Bar-Gad et al., 2003a). These studies have shown an increase in both oscillatory activity and in neuronal correlation of pallidal cells in MPTP primates (Raz et al., 2000; Heimer et al., 2002). This increase in pallidal synchronization has been shown to decrease in response to dopamine replacement treatment (Heimer et al., 2002).

However recent human studies have found oscillatory neuronal correlation only in tremulous patients and raised the hypothesis that the increased neuronal synchronization in Parkinsonism is an epi-phenomenon of the tremor or of independent oscillators with similar frequency (Levy et al., 2000). Human studies are limited by constraints related to recording duration, selected anatomical targets and clinical state of the patients (e.g., most surgical patients have undergone many years of dopamine replacement therapy (DRT) and have already developed dyskinesia). We therefore investigated the role of oscillatory activity and of neuronal correlation throughout the different clinical states of PD in the MPTP primate models of this disease. The tremulous vervet monkey and the rigid-akinetic rhesus monkey were selected to imitate tremulous and non-tremulous subtypes of human patients. We combined multi-electrode recordings with a newly improved tool for spectral analysis of both single cells discharge and interneuron relations (Rivlin et al., 2006) and distinguished between neuronal correlations of oscillatory nature and non-oscillatory correlations. We found that a major fraction of the primate pallidal cells develop both oscillatory and non-oscillatory pair-wise synchronization, following the induction of dopamine depletion and PD clinical signs. Non-oscillatory burst oscillations were mainly found in the GPe, whereas 10 Hz synchronous oscillations were dominant in the GPi. In contrast with the study of human patients, we found oscillatory activity in both the tremulous and the non-tremulous monkey. Clearly, non-oscillatory synchronized burst cannot be the result of a common tremor drive or of independent oscillators with similar frequencies. Moreover, our theoretical analysis of coherence functions revealed that small changes – such as of 0.1% of the basic oscillatory frequency – between the oscillation frequencies of two simulated neurons would result in non-significant coherence value if the recording duration is equal or longer than 10 minutes. Therefore, we can rule out the possibility of false detection of significant coherence in the typical recording duration applied in our primate recordings.

Discussion

The basal ganglia networks can be modeled as an actor/critic reinforcement learning network. The actor networks connect all cortical areas through the basal ganglia with the executive areas of the frontal cortex. The midbrain dopaminergic neurons (the critic) provide a temporal-difference error message to the striatum controlling the efficacies of the cortico-striatal synapses. The distribution of the cortico-striatal efficacies represent the state-action matrix (policy) implanted by basal ganglia. Under normal dopamine influence, the basal ganglia provide an optimal representation of state-action matrix, resulting in non-correlated activity of neurons in

the output structures of the basal ganglia. However, in dopamine depleted subjects the striatum faces an unremitting message of ''reality worse than predictions'' leading to modification of the efficacies of the cortico-striatal synapses and abnormal synchronization of basal ganglia activity. Current DBS methods overcome this probably by imposing a null spatio-temporal firing in the basal ganglia enabling the thalamo-cortical circuits to ignore and compensate for the missing basal ganglia. We therefore suggest that future DBS methods should be directed towards manipulation of the abnormal synchronization of the basal ganglia in PD. This may be achieved by multiple micro-contacts within the DBS targets, rather than the single macro-contact used today.

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Basal ganglia discharge abnormalities in Parkinson's disease

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Summary. In the traditional model of the pathophysiology of parkinsonism, parkinsonian motor signs are viewed as the result of changes in discharge rates in the basal ganglia. However, not all experimental findings can be explained by rate changes alone, and changes in discharge patterns in these nuclei are increasingly emphasized as pathophysiologically important, including changes in burst discharges, in synchrony, and in oscillatory activity. This brief review highlights the pathophysiologic relevance of these rate and pattern changes in the pathophysiology of parkinsonism.

Introduction

In early Parkinson's disease selective degeneration of a small number of dopaminergic cells in the lateral portion of the substantia nigra pars compacta (SNc) leads to the signs of parkinsonism. Both the behavioral specificity and the seemingly disproportionate magnitude of the effect of such a small lesion in the midbrain are artifacts of the anatomy of the basal ganglia. The functional specificity arises from the fact that the basal ganglia are topographically organized, and that the early degenerative process in Parkinson's disease affects predominately those SNc neurons that project to the motor portion of the striatum (the putamen) with relative sparing of other striatal areas. The magnitude of the behavioral effect of SNc lesions is explained by the fact that the basal ganglia are major components of circuits involving specific regions of the cerebral cortex and thalamus (Alexander et al., 1986) so that loss of dopamine in the motor portion of the striatum (the putamen) exerts strong effects on all other elements of the 'motor' circuit, including motor areas of the extrastriatal basal ganglia, and movement-related areas in thalamus and precentral motor fields. Thus, although pathologically a localized problem (at least initially), Parkinson's disease is pathophysiologically a disorder engaging the entire motor circuit. We here review the changes in neuronal activity that occur in this circuit in Parkinson's disease.

Changes in discharge rate

Inputs from cortical sensory-motor areas reach the basal ganglia through the putamen and the subthalamic nucleus (STN), while motor portions of the internal pallidal segment (GPi) and the substantia nigra pars reticulata (SNr) serve as output stations of the basal ganglia, projecting to the ventral anterior and ventrolateral nuclei of the thalamus. Information is conveyed between putamen and GPi/SNr through a monosynaptic GABAergic projection ('direct' pathway), and a polysynaptic ('indirect') pathway which involves the external pallidal segment (GPe) and STN.

Tonic GABAergic output from neurons in GPi/SNr is thought to inhibit their projection targets in thalamus, thereby reducing cortical activation. Dopamine, released from terminals of the nigrostriatal projection, is thought to facilitate transmission along the direct pathway, and to reduce transmission along the indirect pathway. These dual dopamine actions lead to a net reduction of inhibitory basal ganglia output, which may facilitate cortical activity, and eventually movement (Wichmann and DeLong, 2003).

Traditional models of the pathophysiology of parkinsonism are strongly influenced by this anatomic arrangement (reviewed in Wichmann and DeLong, 2003; Albin et al., 1989), which suggests that the overall amount of movement is in some way inversely related to the magnitude of basal ganglia output. According to the scheme mentioned above, loss of striatal dopamine within the motor circuit would result in increased STN activity, and increased basal ganglia output, reduced thalamocortical activity and the development of parkinsonian motor signs, such as akinesia or bradykinesia. Conversely, dopamine-induced dyskinesias have been postulated to result from decreased basal ganglia output.

The prediction that the discharge rates of neurons in the basal ganglia output nuclei and in the STN are increased in Parkinson's disease has been generally confirmed in animal models of Parkinson's disease, and is also supported by recording studies in humans undergoing functional neurosurgery as treatment for Parkinson's disease. Moreover, decreases in discharge in GPi have been found in animals with dyskinesias induced by administration of dopaminergic drugs.

It is noteworthy, however, that the predicted rate changes have not been found in all studies of parkinsonian subjects. More importantly, in a recent monkey study designed to test the rate-based model, we showed that increased STN and GPi rates (in this case produced by ibotenic acid lesions of GPe) are not *per se* responsible for parkinsonian signs (Soares et al., 2004). Despite rate changes similar to those in parkinsonian animals, the GPe-lesioned animals showed only minor motor impairments. Lesion studies have also demonstrated problems with rate-based models of parkinsonian pathophysiology. For example, based on the model, pallidal lesions would be expected to result in involuntary movements, but, in fact, have little effect on normal motor behavior. Similarly, thalamic inactivation, although predicted to induce parkinsonism, does not.

Pattern changes

Given that changes in discharge rate of basal ganglia neurons cannot fully explain the generation of parkinsonism or the production of drug-induced dyskinesias, changes in the activity patterns of basal ganglia neurons have been intensely studied. Although we will discuss here different types of pattern changes separately, all of them tend to occur in parallel, and may result from the same underlying mechanism(s).

Burst discharges

One of the firing properties that has been studied in detail is the incidence of burst discharges. Such burst discharges are a normal feature of basal ganglia discharge, and the timing and 'strength' of bursting may represent aspects of external events or behavior, probably representing the increased synchronization of cortical inputs to the subthalamic nucleus or striatum (Magill et al., 2000). In parkinsonism, the incidence of bursts is increased (e.g., Soares et al., 2004), most often in the context of synchronized oscillatory activity (see below). In part, this may occur because of an enhanced interaction between cortical areas and basal ganglia areas, particularly the STN, but other pathologic phenomena may also play a role. For instance, in dopamine-free co-cultures of STN and GPe cells, synchronized bursting

develops because of network properties (Plenz and Kitai, 1999). Prominent bursts in STN discharge have been shown to occur as rebound phenomena in response to prolonged or synchronized inhibitory GPe inputs to the STN (e.g., Bevan et al., 2002). Although less well studied, rebound bursts are also known to occur in other basal ganglia areas. Changes in inhibitory inputs to these areas may increase rebound bursting.

Synchrony

A second important phenomenon affecting the firing properties of basal ganglia cells in parkinsonism are changes in the synchrony of firing between neighboring neurons. Under physiologic conditions, the firing of neighboring basal ganglia neurons is largely uncorrelated (Bergman et al., 1994; Wilson et al., 2004). In the dopamine-depleted state, however, increased synchrony is observed in the STN (Bergman et al., 1994; Levy et al., 2002), in the pallidum (e.g., Heimer et al., 2002), in the striatum (between tonically active neurons, most likely corresponding to cholinergic interneurons) (Raz et al., 2001), and in frontal cortical areas (Goldberg et al., 2002). The link between synchrony and dopamine depletion is most clearly revealed by the fact that treatment with dopaminergic agents rapidly reduces the interneuronal synchronization observed in parkinsonism in the monkey pallidum (Heimer et al., 2002) and in the human STN (Levy et al., 2002). However, the mechanisms by which dopamine exerts these effects remain unclear. It has been proposed that dopamine loss in the striatum may trigger enhanced electrotonic coupling between neighboring striatal cells (see, e.g., Onn and Grace, 2000), or activity changes in interneurons or axon collaterals (Guzman et al., 2003). In the STN, synchrony is more likely to be the result of synchronous (or divergent) inputs from external sources rather than locally generated (Wilson et al., 2004). The potential synchronizing effects of local dopamine loss in GPe, GPi, or SNr, has not been explored in detail.

Oscillations

A third major change in the discharge patterns of basal ganglia neurons in parkinsonism is that dopamine loss enhances the tendency of neurons in the basal ganglia-thalamocortical circuitry to discharge in an oscillatory pattern (Soares et al., 2004; Bergman et al., 1994). These oscillatory changes are rapidly reversed by systemic treatment with dopaminergic agents (Levy et al., 2002), and are therefore likely to represent a direct effect of dopamine deficiency. Such oscillations are mostly confined to the extrastriatal basal ganglia, and characteristically occur in the alpha- and beta frequency bands. For instance, in parkinsonian monkeys and patients, oscillatory bursting typically emerges in both the 3–8 Hz band, and a power spectral band around 10 Hz (Bergman et al., 1994; Levy et al., 2000). Local field potential (LFP) recordings through implanted deep brain stimulation (DBS) electrodes in GPi and STN of untreated parkinsonian patients have shown similar oscillatory activity in the 10–30 Hz range, likely reflecting synchronized oscillatory neuronal spiking (Levy et al., 2002; Brown, 2003). Given the relation between the basal ganglia, thalamus and cortex, it is not surprising that pathologic oscillatory activity in the 'antikinetic' 10–30 Hz band has also been observed in areas of cortex that are related to the basal ganglia in parkinsonian patients and animals (Goldberg et al., 2002). MEG studies have also identified an oscillatory network with pathologic coupling at 10 Hz in patients with parkinsonian tremor, which included multiple cortical motor areas, as well as diencephalic and cerebellar areas (Timmermann et al., 2003).

Global engagement of the basal ganglia-thalamocortical circuits in synchronized oscillatory bursts may severely disrupt processing

at all levels of the circuitry. This may affect cortical activities such as event-related modulation of beta-band oscillations, or functions such as motor planning or sequence learning. In support of this concept, 10-Hz stimulation of the STN area has been shown to exacerbate akinesia (Timmermann et al., 2004). It has been speculated that DBS may act to desynchronize neurons in the basal ganglia (Brown et al., 2004). A recent study in MPTP-treated primates demonstrated that high-frequency cortical stimulation may also have antiparkinsonian effects, perhaps by the same mechanism (Drouot et al., 2004).

Although it is tempting to speculate that parkinsonian tremor may directly result from synchronized oscillatory bursting in the basal ganglia, studies of the correlation or coherence between tremor and basal ganglia oscillations have not been conclusive, perhaps resulting from the fact that different limbs of parkinsonian patients may engage in tremor of different frequencies (Bergman et al., 1998), making it difficult to identify a 'causative' basal ganglia oscillation for a given tremor movement. Although often associated with tremor (Levy et al., 2002), synchronized oscillations do not always result in tremor (Soares et al., 2004; Heimer et al., 2002). Additional anatomic or physiologic factors (poorly defined at this moment) may be necessary for tremor to occur.

It remains unclear at this point whether clinical symptoms such as tremor, akinesia or bradykinesia result from the impairment of specific cortical regions or motor loop sub-circuits (e.g., those centered on the supplementary motor area, motor cortex or other pre-central motor fields) or from incomplete functional compensation through less affected areas of frontal cortex.

Conclusion

Earlier models of the pathophysiology of Parkinson's disease that emphasized the importance of changes in discharge rates in the basal ganglia of parkinsonian subjects, have been supplanted by a more complex scheme in which pattern changes take center stage. Rate and pattern changes are not, however, mutually exclusive. In fact, some of the rate changes are probably explained by the emerging pattern abnormalities, such as bursts in discharge. It appears that these rate and pattern changes in the basal ganglia-thalamocortical pathways actively disrupt cortical processing. The strongest argument in favor of this view is the fact that neurosurgical interventions which eliminate the disordered activity in the basal ganglia either through chronic electrical stimulation or lesions result in immediate improvements of parkinsonian motor signs, and in a relative 'normalization' of activity in cortical motor areas (as judged by functional imaging).

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Bad oscillations in Parkinson's disease

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Summary. Recordings in humans as a result of functional neurosurgery have revealed a tendency for basal ganglia neurons to oscillate and synchronise their activity, giving rise to a rhythmic population activity, manifest as oscillatory local field potentials. The most important activity is synchronised oscillation in the beta band (13–30 Hz), which has been picked up at various sites within the basal ganglia-cortical loop in PD. Dopaminergic medication and movement suppress this activity, with the timing and degree of suppression closely correlating with behavioural performance. Accordingly synchronisation in the beta band has been hypothesised to be essentially antikinetic in nature and pathophysiologically relevant to bradykinesia.

The major model explaining the function of the basal ganglia (BG) in health and disease was that proposed by Albin and Delong at the end of the 1980s, which has been highly influential ever since. This model effectively synthesised neurochemical and anatomical data to explain how the BG might sway cerebral cortical activity. The influence of BG output over cortical motor areas was viewed as an increase or decrease in tonic excitation of the cortex by the thalamus, brought about by serial inhibition and excitation at earlier stages in the BG-cortical loop. Subsequently, however, it has become clear that neuronal discharge rate may not change in disease as predicted by the model, neither can it account for the major therapeutic benefits of functional neurosurgery in Parkinson's disease (PD). The implication is that it is not the degree of collective excitation or inhibition brought to bear at different stages of the BG-cortical loop, but rather the patterning of activities that lead to disease. Recent work has confirmed that synchronised oscillatory activity appears to be a fundamental feature of the BG, particularly in the diseased state.

Two possibilities exist for recordings of BG activity in humans as a result of functional neurosurgery: either single neuron recordings can be made intra-operatively through microelectrodes, or local field potentials (LFPs) can be recorded from the deep brain electrodes used for stimulation in the few days that follow implantation, while the electrode leads are externalized prior to connection to the subcutaneous stimulator. Both approaches have revealed a tendency for BG neurons to oscillate and synchronise their activity, giving rise to a rhythmic population activity, manifest as oscillatory LFPs (Kühn et al., 2005). The most consistent finding is synchronised oscillation in the beta band ($\sim$20 Hz), which has been picked up at various sites within the BG-cortical loop in PD.

Behaviourally related modulations of oscillatory activity

Synchronisation in the beta band has been hypothesised to be essentially antikinetic in

nature and pathophysiologically relevant to bradykinesia. This is supported by a number of observations relating changes in beta activity with behaviour and treatment. One of the earliest behavioural observations was that the beta LFP activity picked up in the subthalamic nucleus (STN) and globus pallidus interna (GPi) was reduced in PD patients prior to and during self and externally paced voluntary movements (Cassidy et al., 2002). Indeed, the mean timing of the drop in activity following a cue to move positively correlates with the mean reaction time across patients, which it precedes (Kühn et al., 2004). This relationship is so strong it may even be observed in individual subjects across single trials (Williams et al., 2005). If the reduction in beta activity is linked specifically to the facilitation of subsequent movement, an augmentation of power might also be predicted in this frequency band when a pre-prepared movement requires cancellation. This has been confirmed in subthalamic recordings during the 'Go-NoGo' paradigm (Kühn et al., 2004). In addition, a strong relationship between motor processing and beta suppression has been suggested by experiments that compare the suppression of beta activity following warning cues in reaction time tasks. These cues can be either fully informative or uninformative about the direction indicated by subsequent imperative cues eliciting movement with either the left or right hand. In the case of uninformative cues there is no prospective information about which hand will be called upon to move, so motor selection can only occur after the go cue, as confirmed by longer reaction times. Under these circumstances the suppression of the beta activity following the warning cue is far less than following an informative warning cue, indicating that the beta suppression related to the amount of motor preparation that was possible rather than to any non-specific alerting effect of the warning cue (Williams et al., 2003).

Treatment related modulations of oscillatory activity

The earliest observation relating to beta band oscillations in BG LFPs in patients with PD was that they were increased after the withdrawal of levodopa and suppressed following its return (Fig. 1). Thus background levels of beta activity were increased as motor performance deteriorated in the off medication state. More recently, it has also become apparent that the relative degree of beta suppression prior to and during movement is diminished after PD patients have been withdrawn from levodopa (Doyle et al., 2005). The increase in background levels of beta and the decrease in the reactivity of beta oscillations prior to and during movement might contribute to the paucity and slowness of voluntary movements, respectively.

Of course another effective treatment for akinesia is high frequency deep brain stimulation (DBS). Here it has proven technically difficult to record beta activity during stimulation of the same site, but it has been possible to record beta activity from the GPi during stimulation of the subthalamic area in patients with PD. This has confirmed that

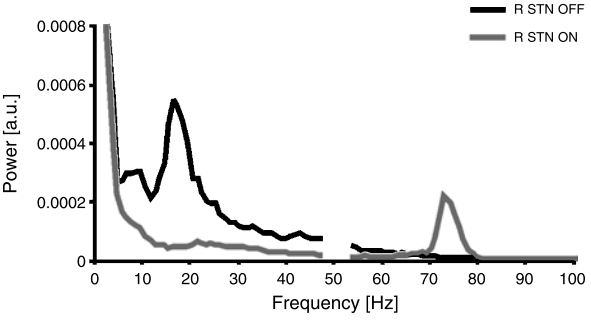

Fig. 1. Power spectra of LFP activity recorded from the contacts of a DBS electrode in the subthalamic nucleus of a patient with PD on and off their antiparkinsonian medication. Off medication, the LFP is dominated by oscillations with a frequency of around 20 Hz, in the so-called beta band. After treatment with levodopa there is suppression of the beta band activity and a new oscillation arises in the gamma band, – peaking at 75 Hz. Mains artefact at 50 Hz has been omitted

high frequency DBS also suppresses background levels of beta activity, in tandem with clinical improvement (Brown et al., 2004).

Earlier, it was stressed that increased synchronisation in the beta frequency band was a characteristic of activity throughout the BG-cortical loop. Accordingly, the same relationship between beta synchrony and motor impairment would be anticipated at the level of the cerebral cortex in patients with PD. A recent study in patients with chronically implanted DBS electrodes found that the degree of synchronisation in the beta band between cortical sites over central motor areas correlated with motor impairment, when patients were withdrawn from medication and therapeutic stimulation. In addition, both the reduction in beta synchronisation effected by high frequency stimulation of the STN, and that achieved with levodopa, correlated with treatment induced improvements in motor performance (Silberstein et al., 2005a).

Finally, if beta activity is essentially antikinetic in nature, could its excessive suppression following antiparkinsonian therapy or lesioning of the STN help explain hyperkinesias? Recent recordings in the GP of PD patients during levodopa-induced dyskinesias demonstrate that dyskinetic muscle activity may inversely correlate with pallidal beta activity, in keeping with the latter's posited antikinetic character (Silberstein et al., 2005b).

Why is beta activity inversely correlated with motor processing?

The above observations suggest that there is an inverse relationship between beta band synchronisation and motor processing. However, the relationship appears a generic one, inconsistent with an explicit role of synchronous population activity at these frequencies in motor processing. Thus BG LFP activity in the beta band is suppressed following behaviourally relevant stimuli, such as warning and go cues, and prior to and during self-paced movements. This inverse relationship between beta band synchronization and motor processing raises the possibility that the novel processing necessary for renewed movement may be actively antagonised by synchronisation in the beta band. Recordings in primates confirm an inverse relationship between oscillatory LFP activity in the beta band and local task-related rate coding, so that oscillations are preferentially suppressed in the local area of the striatum showing task-related increases in discharge rate (Courtemanche et al., 2003).

So one possibility is that synchronisation in the beta band impairs rate coding in the BG-cortical system. The finding of synchronisation at frequencies above 60 Hz in the STN LFP raises an additional possibility (Fig. 1). These gamma band oscillations are focal, increased by movement and appear after treatment with levodopa (Cassidy et al., 2002), suggesting that they might relate to specific coding of movement related parameters. Synchronisation at 60–90 Hz, in particular, is phase locked to similar activity in the motor areas of the cerebral cortex (Cassidy et al., 2002) and, at subcortical and cortical levels, may share a similar role to that posited for gamma band synchronization in the visual cortex. Given the apparent reciprocal relationship between beta activity and oscillations above 60 Hz in STN LFPs with respect to dopaminergic stimulation and movement, it is possible that extensive synchronisation under 30 Hz precludes the involvement of neuronal assemblies in a different pattern of synchronisation that is directly involved in information transfer. Consistent with this beta and gamma activities in the STN are inversely correlated at rest over time (Fogelson et al., 2005a).

There is, however, an alternative explanation for the beta activity and this is that it is a passive characteristic of basal ganglia-cortical networks when they are not engaged in active processing. In this formulation the oscillatory activity is viewed as a characteristic of the resting or idling state, rather than

a phenomenon that may actively impede novel processing. Several observations would argue against this possibility. First, there is the rebound synchronisation of beta activity following movement and the premature synchronization of this activity when movement is to be voluntarily suppressed (Cassidy et al., 2002; Kühn et al., 2004). Although there may be degrees of active suppression of dynamic movement related processing, it seems unlikely that the BG-cortical system would enter into a deeper idling state than at rest when movement has to be inhibited or terminated. Second, direct stimulation of the BG in the beta band may be antikinetic, although effects so far have been small (Fogelson et al., 2005b).

The above reviews the evidence that excessive synchronisation in the beta band in the BG-cortical system might antagonise motor processing, contributing to akinesia in PD. Much of this evidence, however, is correlative in nature, so that there remains a need for the direct demonstration of causality between synchronisation in the beta band and the suppression of novel movement related processing.

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Cortical muscle coupling in Parkinson's disease (PD) bradykinesia

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Summary. *Objectives:* To determine if novel methods establishing patterns in EEG–EMG coupling can infer subcortical influences on the motor cortex, and the relationship between these subcortical rhythms and bradykinesia. *Background:* Previous work has suggested that bradykinesia may be a result of inappropriate oscillatory drive to the muscles. Typically, the signal processing method of coherence is used to infer coupling between a single channel of EEG and a single channel of rectified EMG, which demonstrates 2 peaks during sustained contraction: one, ∼10 Hz, which is pathologically increased in PD, and a ∼30 Hz peak which is decreased in PD, and influenced by pharmacological manipulation of $GABA_A$ receptors in normal subjects. *Materials and methods:* We employed a novel multiperiodic squeezing paradigm which also required simultaneous movements. Seven PD subjects (on and off L-Dopa) and five normal subjects were recruited. Extent of bradykinesia was inferred by reduced relative performance of the higher frequencies of the squeezing paradigm and UPDRS scores. We employed Independent Component Analysis (ICA) and Empirical Mode Decomposition (EMD) to determine EEG/EMG coupling. *Results:* Corticomuscular coupling was detected during the continually changing force levels. Different components included those over the primary motor cortex (ipsilaterally and contralaterally) and over the midline. Subjects with greater bradykinesia had a tendency towards increased ∼10 Hz coupling and reduced ∼30 Hz coupling that was erratically reversed with L-dopa. *Conclusions:* These results suggest that lower ∼10 Hz peak may represent pathological oscillations within the basal ganglia which may be a contributing factor to bradykinesia in PD.

Introduction

Bradykinesia/hypokinesia

An interesting aspect of Basal Ganglia (BG) function is how activity in the widespread cortical projections to the striatum (numbering ∼100 times the number of striatal neurons (Oorschot, 1996)) can be efficiently summarized (compressed). Computational neural net models that perform this type of statistical operation, called dimension reduction, attempt to remove correlations from

output neuronal units (Foldiak, 1990). Uncorrelated outputs result in the most efficient means for information transfer (Nadal and Parga, 1994), as highly correlated outputs implies redundancy, and therefore inefficient compression (Bell and Sejnowski, 1995). Some computational models of the striatum and basal ganglia incorporate more than simple dimension reduction; they include a phasic dopaminergic component which adds behaviour saliency to the process (Bar-Gad et al., 2000; Bar-Gad and Bergman, 2001).

Consistent with theoretical results, experimental evidence confirms that primate BG neurons normally tend to fire independently from one another during motor tasks (Bar-Gad et al., 2003). Correlations between neighbouring neurons in the primate globus pallidus are usually very weak, however, after MPTP application, neighbouring neurons in GPi dramatically increase their correlation, and to a lesser extent neighbouring neurons in GPe. The ineffective dimension reduction in the Parkinsonian state may result in abnormal feedback within the basal ganglia loops and contribute to widespread pathological oscillations throughout the basal ganglia (Bar-Gad and Bergman, 2001; Raz et al., 2001; Bar-Gad et al., 2003).

Abnormal synchronization within the basal ganglia may, in fact, be a characteristic of the Parkinsonian state. Raz et al. (2001) examined the relation between tonically-active neurons in the striatum and pallidum in the vervet monkey, both before and after injection with MPTP. After MPTP injection, there was a marked increase in the number of neuron pairs which displayed significant peaks in cross-correlograms corresponding to $\sim$10 Hz. They concluded that ''coherent oscillations of the whole basal ganglia circuitry underlie the clinical features of Parkinson's disease'' (Raz et al., 2001).

A cardinal symptom of PD that may be a consequence of pathological BG oscillations is bradykinesia (Raz et al., 2001). However, the clinical sign of bradykinesia is merely an indicator for much broader areas of motor disability in PD such as gait disturbances and micrographia. Since pathological BG oscillations have been suggested to be the key underlying feature of many of the clinical manifestations of the disease (Raz et al., 2001), demonstration of a quantitative measure indicative of these oscillations (e.g. abnormal increase in $\sim$10 Hz EEG/EMG coupling) that can be obtained non-invasively and inexpensively would be beneficial. Demonstration of an electrophysiological marker for bradykinesia will provide a target to monitor various pharmacological and surgical therapies.

Oral pharmacotherapy is typically used in treatment in PD, and while this may partly reverse tonic dopamine levels (Heimer et al., 2002), it will not be able to provide the precise phasic changes normally a feature of dopaminergic neuronal firing. Yet in other dopaminergic systems, such as the ventral tegmental/prefrontal region, dopaminergic neurons demonstrate precise timing of their firing patterns, especially with respect to expectation of reward (Schultz et al., 1997). In contrast, Deep Brain Stimulation (DBS) methods would theoretically have the capability to modulate their stimulation on a second-by-second basis if the appropriate cues for doing so could be determined.

A non-invasive assay of abnormal BG oscillations influencing motor cortex in PD patients would hence prove valuable. It would enable an assessment of how much of the motor deficits commonly observed in PD are due to pathological BG oscillations that are observed in animal models of PD, and the influences of tonic drug therapy. Moreover, because measurements of oscillations could be done on a second-by-second basis, behavioural paradigms could be developed to determine the properties of different sensory stimuli that may result in abnormal BG oscillations (or a pathological increase in normally-present physiological oscillations).

Corticomuscular coupling

A number of recent studies have investigated oscillatory activity around 15–30 Hz in the primary motor cortex (M1), both in humans using MEG (Salenius et al., 1997), and in monkeys using local field potential recordings (LFP) (Murthy and Fetz, 1992; Brown et al., 1998; Feige et al., 2000; Kilner et al., 1999, 2000, 2002; Baker et al., 2001, 2003; Jackson et al., 2002). These oscillations appear to be strongest during rest or steady contractions, but may be diminished or modulated during dynamic movements (Kilner et al., 2000; Pfurtscheller and Neuper, 1994; Salmelin and Hari, 1994; Pfurtscheller et al., 1996). Both theoretical and experimental work support that these widespread oscillations are critically related to inhibitory systems and therefore amenable to pharmacological manipulations (Whittington et al., 1995; Wang and Buzsaki, 1996; Pauluis et al., 1999).

That synchronization between two separate neural systems is important for normal functioning is widely accepted in various studies on the sensorimotor system (Bressler et al., 1993; Classen et al., 1998; Grosse et al., 2002, 2003). More recently there has been interest in determining the coupling between ongoing cortical rhythms (using Local Field Potentials, EEG or MEG) and oscillations in the electrical activity of the muscles (measured by surface EMG). Although the function of normal oscillations in the cortex, basal ganglia and cerebellum is far from clear (Farmer, 1998), these different oscillation-frequencies may be important for linking the primary motor cortex and the basal ganglia and cerebellum (Grosse et al., 2002).

The majority of studies investigating neural synchrony or corticomuscular coupling have used ''coherence'' as a measure of coupling between ongoing oscillations. Coherence, crudely speaking, is a normalized quantity measuring the degree of time-locked correlation between two signals as a function of frequency. Thus if two noisy waveforms receive a common input of say, 30 Hz, one would expect a peak in their coherence at 30 Hz.

Nevertheless there are a number of technical limitations associated with the current method of measuring the coherence between a single EEG lead and a single rectified EMG lead:

1. It is often assumed that there is temporal stationarity of the EEG and EMG spectra. Previous studies have suggested that EEG/EMG coherence is maximal during sustained contractions and disappears during changing of movements. However, as motor movements are naturally dynamic and non-stationary, it would be desirable to have assays that can track dynamic changes in muscle activity. Newer approaches, such as the wavelet coherence (Lachaux et al., 2002; Saab et al., 2005) may address this issue.
2. The common formulation of coherence relies on pairwise comparisons. Nevertheless the biology suggests that the mapping between the brain and musculature is many-to-many, as opposed to one-to-one (Murthy and Fetz, 1992). Recent analyses on real and simulated data have emphasized that the multivariate approach is much more accurate than pairwise analyses, which can be misleading (Kus et al., 2004).
3. Rectification (taking the absolute value) of the EMG to estimate the envelope. While rectification of the EMG when it clearly consists of individual motor units separated in time may result in the frequency spectrum approaching that of the envelope frequency (Myers et al., 2003), such a situation rarely occurs in practice with surface EMG during anything more than a minimal contraction – the typical scenario. Others have argued that rectification is not warranted on theoretical grounds (for a review, see (Farina et al., 2004)).

Despite these limitations, corticomuscular coherence has proved valuable to investigate different features of the motor system. The effects of sensory input on corticomuscular coupling have been investigated by temporary de-afferentation with ischemia or digital nerve block (Fisher et al., 2002; Pohja and Salenius, 2003). No significant change in the dominant frequencies of the corticomuscular coherence or EMG–EMG coherence (implying a common cortical signal in both muscles) was detected, suggesting that the sensory feedback loop is not necessary for the generation of corticomuscular coherence.

The 15–30 Hz coherence may be modulated by a number of factors. Kristave-Feige et al. found that corticomuscular coherence was decreased when attention was divided between motor and arithmetic tasks (Kristeva-Feige et al., 2002), but the full extent upon that higher cognitive functions may modulate corticomuscular coherence is still unclear. Pharmacological manipulations that affect the GABA system affect 20 Hz cortical oscillations, but the computed corticomuscular coherence is relatively invariant to pharmacologic interventions, suggesting that that corticomuscular coherence itself may exhibit homeostasis, and have a functional role in motor control (Baker and Baker, 2003; Salenius et al., 2002).

We prefer the more general term EEG/EMG ''coupling'' as opposed to ''coherence'', as coherence is a specific mathematical operation which is fundamentally limited to comparing two waveforms. More general techniques that we have employed are able to provide multiple EEG to multiple EMG comparisons (McKeown, 2000; McKeown and Radtke, 2001).

Recently, we have suggested that Empirical Mode Decomposition (EMD) is a way to estimate the envelope of motor unit potentials without having to use rectification (Liao et al., 2005) (Fig. 1). Like ICA, EMD attempts to decompose a time series into

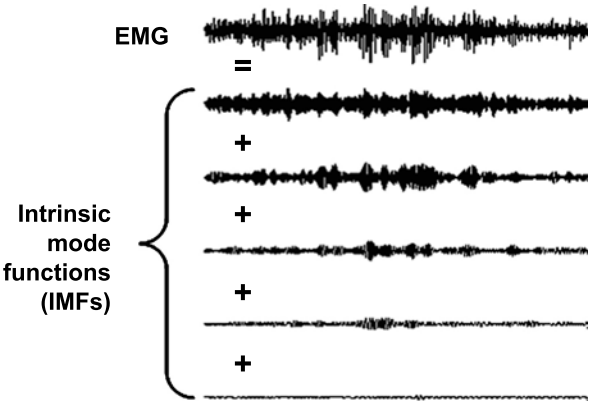

Fig. 1. With EMD, the EMG signal is decomposed into components, called intrinsic mode functions (IMFs)

individual components, so that the linear sum of the components approximates the original signal (Huang et al., 1998). However, unlike ICA which examines linear combinations of simultaneous-recorded univariate time series, EMD works only on a univariate time series. In EMD, the extracted components are referred to as Intrinsic Mode Functions (IMFs).

IMFs form a complete and 'nearly' orthogonal basis for the original signal. In some

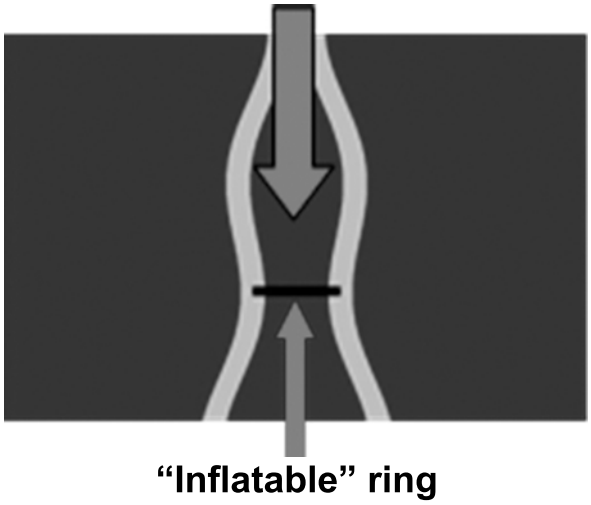

Fig. 2. The motion of the undulating sides of the ''tunnel'' is downward. The ''inflatable'' ring is under the subject's control

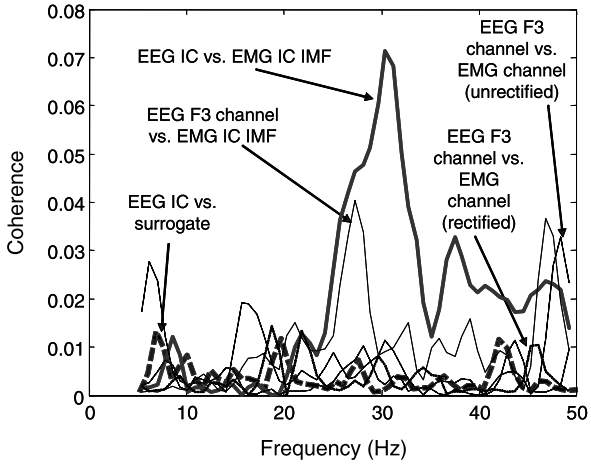

Fig. 3. Coherence between different EEG measures and different EMG measures. Note the prominent peak at around 30 Hz for the coherence between the one EEG IC and one IMF. The F3 channel from the EEG had less coherence with the single EMG channel (either with or without rectification). Also note the much reduced coherence between the EEG IC and the surrogate, which had the identical frequency spectrum of the IMF. The $p < 0.001$ level (determined by Monte Carlo simulation) is approx 0.02

situations, different components may have sections with similar frequencies at different time durations, but locally, any two components will tend to be orthogonal. Because the IMFs are formed by explicitly fitting the envelope of a time series, we posited that IMFs derived from EMG recordings would more closely reflect underlying muscle firing activity, and therefore would better correspond with ongoing brain rhythms (see Fig. 3) (Liao et al., 2005).

Corticomuscular coupling in PD

It is perhaps not surprising that corticomuscular coherence analysis has been proposed to monitor cortical dysfunction in Parkinson's disease. The normal coherence between MEG signals and simultaneously recorded EMG activity at 15–30 Hz is disrupted in PD patients withdrawn from L-Dopa (Salenius et al., 2002), suggesting a disruption of cortical drive. Coherence between muscles, i.e. EMG–EMG inter-muscular coherence, may indirectly measure cortical influence. Improvements of bradykinesia correspond with increases in EMG–EMG coherence (Brown et al., 2001).

Salenius et al. (2002) found that 3 out of 8 PD patients off L-Dopa medication had abnormally strong MEG–EMG coherence at the much lower frequencies of 5–12 Hz compared with medicated or eight healthy age-matched control subjects. The demonstration that PD patients have a reduction in the corticomuscular coherence at 15–30 Hz, and that L-Dopa improves the coherence in the 15–30 Hz bands (Salenius et al., 2002) support the contention that the basal ganglia contribute to the modulation of 15–30 Hz coherence.

One explanation for the presence of these generally non-overlapping frequency bands is that while the higher frequency band ($\sim$15–30 Hz) represents direct corticomuscular coupling, the coherent lower frequency band ($\sim$10 Hz) may represent some temporal aspect of ''online'' motor updating. This has been suggested by Grosse et al., who demonstrated that slow finger movements are actually composed of intermittent ($\sim$9 Hz) velocity bursts, and on the basis of MEG recordings, suggested that these bursts may be modulated by the cerebello-thalamo-cortical loop (Gross et al., 2002). These intermittent changes in velocity may therefore reflect cerebellar and/or basal ganglia influences on the final motor output (Welsh et al., 1995). We note that the lower frequency band is differentially modulated from the 15–30 Hz band by pharmacological manipulation (Baker and Baker, 2003), suggesting a different mechanism.

Here we suggest a method, based on ICA and Empirical Mode Decomposition (EMD) to infer corticomuscular coupling. We demonstrate that this provides a means to infer $\sim$10 Hz oscillations in PD, and that these correlate with UPDRS bradykinesia scores.

Methods

Subjects

Seven right-handed L-Dopa-treated patients with clinically definite mild-moderate PD (Hoehn & Yahr stage 1–2 (Hoehn and Yahr, 1967)) were recruited, and were asked to use their right hand. All subjects had clinical signs affecting their right hand. Exclusion criteria included atypical parkinsonism, concurrent dementia precluding informed consent, and other significant concurrent medical or psychiatric illness. PD subjects were examined off L-Dopa medication after overnight withdrawal (at least 12 hours). The experiment was performed in the off L-Dopa state, and then repeated $\sim$1 hr after the morning dose of immediate-release formulation of L-Dopa.

Participants sat facing a 17″ computer screen $\sim$80 cm away with stimuli subtending a visual angle of 2 degrees. The subjects held a rubber squeeze bulb in their right hand with their arm stabilized in one position. Each subject had their maximum voluntary contraction (MVC) tested, and all subsequent forces were scaled to this amount. The subject was instructed that they must control an "inflatable" ring (by way of the squeeze bulb) that must move through an undulating tunnel without touching the sides (see Fig. 2). Their goal was to prevent scraping the craft on the ring on the tunnel. Calibration was performed so that squeezing the force bulb between 5–15% of MVC was required to prevent scraping of the tunnel. Each subject performed three 5-minute trials. During one trial, the subject used the squeeze bulb only with the right hand. In another trial, the subject was instructed to press a computer mouse button with the left hand when cued by a colour change of the inflatable ring. Finally the subject was asked not to squeeze the squeeze bulb, but still press the mouse button when the colour change in the ring was noted. The three tunnels were identical for all three trials: they consisted of the sum of two frequencies: $1/8$ and $1/11$ Hz. The order of the three trials was counterbalanced across subjects.

Data collection and analysis

Subjects wore an electrode cap and surface EMG electrodes on their abductor pollicus brevis and 1st dorsal interosseus. Data were sampled at 512 Hz. For this study, only the portions of the data where the subject was squeezing the bulb (with and without button press) were used for analysis. EEG data were bandpass filtered from 1–60 Hz using a 4th order Butterworth filter.

The EEG is often contaminated with artefacts, such as eyeblinks, eye movements, temporalis muscle activation and EKG. In addition, Independent Component Analysis (ICA) has been shown to be a powerful way to unmix different brain rhythms that may be temporally independent, but associated with highly spatially overlapping topographical distributions by the time they are recorded out on the scalp electrodes (Jung et al., 2000).

Corticomuscular coupling was determined by first applying infomax ICA (Bell and Sejnowski, 1995) to the EMG independent components (ICs). EMD was applied to each EMG IC separately using software developed by Smith et al. (Rilling et al., 2003) as previously described (Liao et al., 2005).

An augmented data consisting of time-lagged IMFs derived from the EMG ICs, and the EEG was then created. ICA was then applied to the augmented matrix and components that loaded significantly on both sections of the data (i.e. the loading on one section of the data did not exceed five times that on another section) were examined. See reference (McKeown and Radtke, 2001) for a more detailed discussion of the methods.

Results

Behavioural data

All subjects were able to perform the task without difficulty. When the frequency ratio (FR) of the higher frequency to lower frequency of the pressure generated by the subject was computed, and this tended to correlate well with UPDRS bradykinesia scores but did not reach significance ($r = 0.597$ $p < 0.15$).

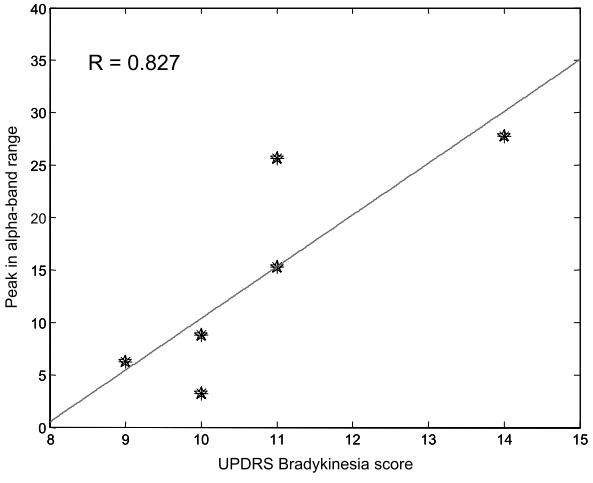

Fig. 4. Comparison between amplitude of peak in alpha range of Left motor cortex component and UPDRS bradykinesia scores in the "off" state

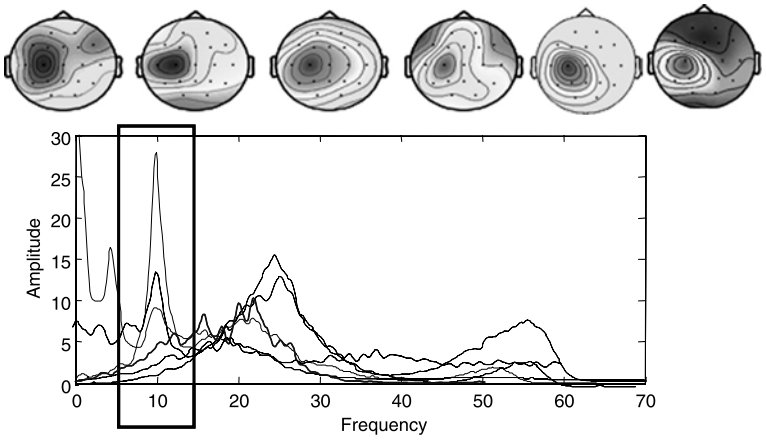

Fig. 5. In 6/7 PD subjects, a component was derived from leads over the left motor cortex. Some of these demonstrated peaks in the alpha range, which correlated with the extent of bradykinesia

EEG/IMF coupling

Some components loaded heavily over the left primary motor cortex, and had prominent peaks in their frequency spectra at about 10 Hz (Fig. 5). The peak in their frequency spectra in the alpha range correlated with their UPDRS scores ($R = 0.827$, $p < 0.02$, Fig. 4). In one subject, with prominent tremor, there was a peak at 5 Hz, exactly half of their other peak in the alpha band. Other components were in the midline and appeared to be modulated by the expectation of pressing the button, but not the button press itself (Fig. 6). Still others (not shown) were modulated with the periods of 8 and 11 Hz, the periods that the subjects pressed.

After L-Dopa, the effect on their behavioural performance (as measured by their FR described above) was erratic. The effects on the 10 Hz peak (not shown) were less consistent.

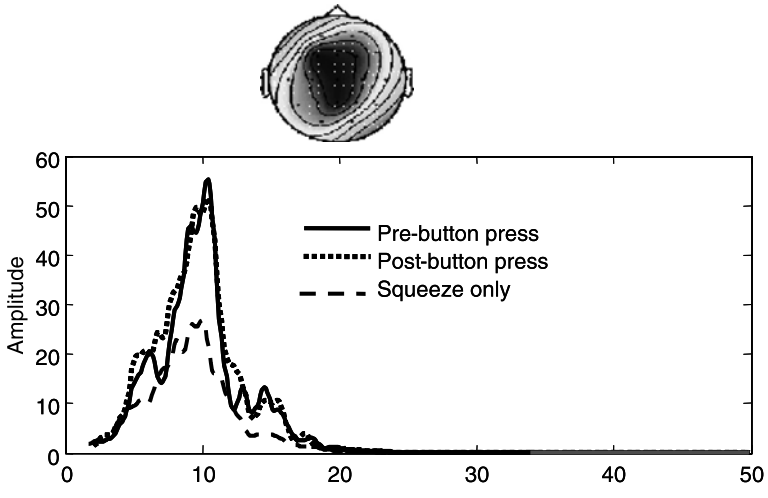

Fig. 6. An example of an ICA component derived from the augmented EEG/EMG data set from a single normal subject. Note that this component was continually active during the "dual" task (as suggested by the higher amplitudes at ~10 Hz), but was not modulated by the button press itself, possibly reflecting an attentional phenomenon from the cingulate gyrus

Discussion

We have demonstrated that a combined EMD/ICA approach is able to isolate combined brain/muscle electrical activity that is modulated by motor performance, Parkinson's bradykinesia (Fig. 4) and attention (Fig. 6). We note that prior studies have suggested that EEG/EMG coupling is only evident during sustained contraction and disappears during changes in force (Kilner et al., 1999), such as ramping. However, in our case the subjects' force was continually changing. There was little evidence of the EEG/EMG coupling being discontinuous or abruptly ceasing during the continuous performance. It is therefore tempting to speculate that the lack of coherence detected by other groups may be a result of the analysis method.

The method we employed here is similar in spirit to PLS (partial least squares). In PLS the cross-covariance between two data sets (in this case EEG and EMG) is first computed and then SVD (singular value decomposition) is used to find the prominent directions in the intersection between data sets. However, the described method is fundamentally different: by using infomax ICA, we effectually used mutual information as opposed to 2^{nd} order statistics (i.e. covariance) to determine coupling. The success of ICA over PCA in decomposing EEG suggests that this might be more fruitful.

We used EMD to estimate the envelope of the sEMG recordings. This has the benefit of tracking non-stationary signals (such as the EMG) and does not first require high-pass filtering, with the possibility of losing useful information as with the case of rectification. A further benefit of using EMD is that it allows the instantaneous estimate of frequency via the Hilbert transform (the Huang-Hilbert transformation (Huang et al., 1998)). Further work is required to explore the benefits of using the Huang-Hilbert transformation in the non-stationary problem of EEG/EMG coupling.

The effect of L-Dopa was varied, as has been shown in previous studies. L-Dopa would presumably affect tonic dopamine firing, but may have varied effects on phasic firing which might be required for the continuously changing motor task performed here.

The midline component in Fig. 6 may result from the cingulate cortex related to the increase attentional load required during the combined squeeze/button press task.

Conclusion

We have demonstrated a significant peak at $\sim$10 Hz in PD subjects prior to medication that correlated significantly with UPDRS bradykinesia scores. As bradykinesia may be related to abnormal synchronization of cortical signals driving the musculature, it suggested that this link is not spurious.

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GDNF as a candidate striatal target-derived neurotrophic factor for the development of substantia nigra dopamine neurons

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Summary. Glial cell line-derived neurotrophic factor (GDNF) has been known for many years to protect and restore dopamine neurons of the substantia nigra (SN) in lesion models of parkinsonism, but much less has been known of its normal physiologic role. We have found that GDNF injected into the striatum postnatally suppresses naturally-occurring cell death in SN dopamine neurons, and neutralizing antibodies augments it. Neutralizing antibodies augment cell death during the first phase, which occurs during the first postnatal week, but not during the second phase in the second week. To further explore the possible neurotrophic role of GDNF, we created double transgenic mice which overexpress GDNF exclusively in the target regions of mesencephalic neurons, particularly the striatum. As anticipated for a limiting, target-derived factor, this resulted in an increased surviving number of SN dopamine neurons after the first phase of cell death. However, this increase did not persist into adulthood. We conclude that GDNF is the leading candidate for a target-derived neurotrophic factor for SN dopamine neurons during the first phase of cell death, but that other factors must play an essential role in later development.

Since its original discovery, GDNF has been considered to be a candidate target-derived neurotrophic factor for the development of substantia nigra (SN) dopamine neurons (Lin et al., 1993). In support of this possibility, its mRNA is expressed in striatum, most abundantly during the early postnatal period (Schaar et al., 1993; Stromberg et al., 1993; Blum and Weickert, 1995; Choi-Lundberg and Bohn, 1995; Golden et al., 1999; Cho et al., 2003). GDNF protein is also expressed within postnatal striatum (Lopez-Martin et al., 1999). In addition, the abundant expression of mRNA for the GDNF receptor, GFRα1, in SNpc, and for the signaling tyrosine kinase Ret as well, offers further support for GDNF as a possible neurotrophic factor for developing SN dopamine neurons (Widenfalk et al., 1997; Yu et al., 1998). The principal evidence marshaled against a possible neurotrophic role is that GDNF and GFRα1 homozygous null mice show no decrease in the number of SN dopamine neurons on the day of birth (Treanor et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). However, these mice die shortly after birth due to developmental abnormalities of the kidney and enteric nervous system. We have previously shown that the naturally occurring cell death event for SN dopamine neurons takes place largely within the first two postnatal weeks (Janec and Burke, 1993; Oo and

Burke, 1997). Therefore, the early death of these mice makes it difficult if not impossible to define a phenotype related to trophic factor regulation of the natural cell death event. Furthermore, none of these null mutations were temporally regulated, so compensatory changes may have taken place. These considerations offer ample grounds for not accepting the negative observations in the homozygous null mice as definitive, insofar as a phenotype affecting the development of the SN dopaminergic system is concerned.

To further evaluate GDNF as a possible neurotrophic factor for SN dopamine neurons we assessed its ability to support their viability in a unique postnatal primary culture model (Rayport et al., 1992), established when these neurons would normally be undergoing their natural cell death event, and when they would be elaborating their striatal target contact (Burke, 2003). We found that among factors which had previously been reported to support mesencephalic dopamine neurons in embryonic culture, including BDNF, TGFβ1,2,3, NT3, bFGF, TGFα and EGF, GDNF alone augmented survival, and it did so by suppressing apoptosis (Burke et al., 1998). To determine whether these observations could be confirmed in the *in vivo* context, we assessed the effect of GDNF injected into the striatum on PND2 on the level of natural cell death in SN dopamine neurons. We observed a 60% suppression of natural cell death by intrastriatal GDNF injection (Oo et al., 2003). To determine whether endogenous GDNF may play a role in regulating natural cell death, we injected GDNF neutralizing antibodies into the striatum. Two different neutralizing antibodies both induced natural cell death in dopamine neurons by 2–3 fold (Oo et al., 2003). We assessed the developmental dependence of these anti-GDNF antibodies to induce death, and found that this effect was limited to the first postnatal week. Therefore, although our earlier studies had suggested that SN dopamine neurons are dependent on striatal target until PND14, i.e., throughout the first and second phases of natural cell death (Kelly and Burke, 1996), dependence on GDNF was observed only through the first phase (Oo et al., 2003).

Thus, in its ability to acutely regulate the natural cell death event of SN dopamine neurons both *in vitro* and *in vivo*, GDNF fulfills important criteria for a neurotrophic factor for these neurons. However, classic neurotrophic theory would also predict that a sustained increase in the supply of a limiting target-derived factor should augment the number of neurons which survive the natural cell death period. It is important to emphasize that an adequate test of this prediction requires a *sustained* increase in expression. Single intrastriatal injections of GDNF on PND2 have been shown to not have a lasting effect on the number of surviving dopamine neurons (Beck et al., 1996), but single injections are unlikely to have a lasting effect, given that the natural cell death event takes place over a two week period. In order to achieve a sustained overexpression of GDNF in the target regions of the mesencephalic dopaminergic projections, we utilized a double transgenic approach as described by Mayford et al. (1996). We crossed mice transgenic for a CaMKII-tTA construct with mice transgenic for a BiTetO-LacZ-rGDNF construct. The double transgenic mice (CBLG-DT) demonstrated expression of LacZ specifically in striatum (where it was most abundant), hippocampus and cortex, as previously described for CaMKII-tTA mice (Mayford et al., 1996; Yamamoto et al., 2000). The CBLG-DT mice overexpress GDNF in forebrain structures throughout the period of natural cell death, and within the striatum, at a cellular level, strictly within medium striatal neurons (Kholodilov et al., 2004), as it is for endogenous GDNF (Oo et al., 2005).

Increased expression of GDNF within striatal medium-sized neurons throughout development leads to a 46% increase in the number of SN dopaminergic neurons surviving the first phase of natural cell death, thus

confirming the hypothesis that striatal target expression of GDNF regulates this phase. This increase does not, however, persist into adulthood. Therefore, although striatal GDNF is both necessary and sufficient for regulation of SN dopamine neuron survival during the first phase of natural cell death, it alone is not sufficient to lead to a lasting increase in their adult number. At some time between PND7 and adulthood, the number of these neurons must revert to their normal, wildtype number. This does not, however, occur as a ''rebound'' phenomenon, with an augmented level of natural cell death, during the second phase of death on PND14; we have, in fact, shown that on the contrary, levels of apoptosis are reduced in the double transgenic mice on that day. Therefore, the time course and mechanism of ''normalization'' of the adult number of SN dopamine neurons in the CBLG-DT mice is unknown.

Just as there is no lasting increase in the adult number of SN dopamine neurons in the CBLG-DT mice, there is no increase in nigral dopaminergic innervation of the striatum. We assessed morphologic features of TH-positive and dopamine transporter (DAT)-positive fibers; TH and vesicular monoamine transporter (VMAT2) protein expression, biochemical measures of dopamine and its metabolites, and physiologic measures of dopamine release and re-uptake, and found no changes.

The response of the ventral tegmental area (VTA) dopaminergic system to sustained overexpression of GDNF in targets was quite different from that of SN dopamine neurons. In the CBLG-DT mice, there was a 55% increase in the number of VTA dopamine neurons as compared to wildtype controls in adult animals (Kholodilov et al., 2004). In addition, adult CBLG-DT mice demonstrated increased dopaminergic innervation of cortical regions, assessed by both TH and DAT-positive fiber staining. This morphologic phenotype was accompanied by a behavioral phenotype: CBLG-DT mice demonstrated an augmented motor activity response to amphetamine. Thus, there is a fundamental difference between the SN and VTA dopaminergic systems in their developmental response to GDNF expression in target.

Thus, for the first phase of natural cell death in SN dopamine neurons, GDNF fulfills many of the criteria specified by classic neurotrophic theory. The ability of exogenous GDNF, when injected into the striatum, to reduce apoptosis, and for overexpression to augment the surviving number of dopamine neurons clearly suggests that there is competition for GDNF during the first phase of cell death. The cellular basis for this competitive regulation strategy is unknown.

The question arises, what factors after the first phase of natural cell death are important regulators of SN dopamine neuron survival? It appears that in the absence of an increase in such factors, increased target GDNF alone is unable to change the surviving number of dopamine neurons. One obvious possibility is that abundance of the receptor, GFRα1, may become limiting. While such regulation may occur at the level of the SN, autonomous to dopamine neurons, there is an alternate possibility, that it may occur in a non-cell autonomous fashion, at the level of striatal target. The possibility that GFRα1 may act *in trans* to influence in a non-cell autonomous fashion incoming projection systems which express the signaling kinase Ret was proposed by Yu et al. (1998) when they observed a discrepancy in some developing regions between GFRα1 and Ret mRNA expression. They noted that often regions which expressed abundant GFRα1 mRNA, but little Ret, were the targets of systems abundant in Ret expression (Yu et al., 1998). In support of the concept that GFRα1 may act in a non-cell autonomous fashion to regulate neural development, Paratcha et al. demonstrated that GFRα1 can be released by neurons and modulate neurite outgrowth, guidance, and neuron survival (Paratcha et al., 2001). These investigators have also shown that, in the

presence of saturating concentrations of GDNF, immobilized, exogenous GFRα1 acting *in trans* can influence the strength and direction of neurite outgrowth (Ledda et al., 2002). We have found that GFRα1 is expressed not only in SNpc, as previously shown, but also in striatal medium-sized neurons (Cho et al., 2004). In the striatum, it is maximally expressed between PND10 and 14. In this location it theoretically could act *in trans* to regulate the development of the nigro-striatal projection, and the viability of SN dopamine neurons. This will be an important future area of investigation.

In conclusion, we have presented evidence that GDNF is currently the leading candidate for a striatal target-derived neurotrophic factor for SN dopamine neurons. However, it must also be recognized that our principal evidence that endogenous GDNF plays such a role derives from acute studies with neutralizing antibodies, which show an induction of apoptosis. Such an effect, based on intracerebral injections, may have alternate interpretations. It is therefore now imperative to develop conditional null mice for GDNF and its receptor GFRα1, to determine the effect of regionally selective null mutations, which are compatible with postnatal survival, on the final number of SN dopamine neurons (Enomoto, 2005).

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The engrailed transcription factors and the mesencephalic dopaminergic neurons

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Summary. The *engrailed* genes belong to a large family of homeobox transcription factors. They are found throughout the animal kingdom, are highly conserved in the DNA binding domain and have been investigated for more than half a century. In the murine genome, two *engrailed* genes exist, called *Engrailed-1* and *Engrailed-2*. Here, we summarize the properties of the *engrailed* genes and their functions, such as conserved structures, cellular localisation, secretion and internalisation, transcription factor activity, potential target genes and review their role in the development of mesencephalic dopaminergic neurons. During early development, they take part in the regionalization event, which specifies the neuroepithelium that provides the precursor cells of the mesencephalic dopaminergic neurons with the necessary signals for their induction. Later in the postmitotic neurons, the two transcription factors participate in their specification and are cell-autonomously required for their survival.

Introduction

The *engrailed* genes have been investigated in species throughout the animal kingdom, including annelids (Wedeen and Weisblat, 1991), molluscs (Wanninger and Haszprunar, 2001), insects and crustaceans (Patel et al., 1989; Scholtz et al., 1994; Duman-Scheel and Patel, 1999), echinoderms (Lowe and Wray, 1997) and chordates, such as amphioxus (Holland et al., 1997) and multiple vertebrates (Joyner et al., 1985; Ekker et al., 1992; Logan et al., 1992; Koster et al., 1996; Lopez-Corrales et al., 1998) including man (Zec et al., 1997; Barak et al., 2003). They belong to a large class of transcription factors with a conserved DNA binding domain, the homeobox. This group of genes is characterized by homeotic tissue transformation when their expression is altered during early embryogenesis. Some of them also take part in the specification of neuronal properties, such as directed axonal outgrowth, target recognition and synaptogenesis.

Engrailed genes across evolution and their molecular properties

The first mentioning of the *engrailed* genes was a spontaneously occurring mutation in *Drosophila melanogaster* characterized by the malformation of thoracic segments, scutellar modifications and distorted wings (Eker, 1929). The cloning of the underlying genes was achieved more than 50 years later (Fjose et al., 1985; Kuner et al., 1985), its DNA binding activity revealed (Desplan et al., 1985) and its function, as a transcriptional regulator, established (Han et al., 1989; Ohkuma et al., 1990). In vertebrates, two *engrailed*

homologues exist (Ekker et al., 1992; Force et al., 1999) with one exception, zebrafish, where genome duplication led to four paralogues, each with distinct functional properties (Ekker et al., 1992; Force et al., 1999). On the protein level, sequence differences between homologues (orthologues and paralogues) are significant, but the homeobox domain is always highly conserved. Despite the large sequence differences outside the homeobox, the biochemical conservation of the *engrailed* genes is impressive. They show functional homology over more than 100 million years of evolution and the paralogues are in most aspects functionally redundant. When mouse *Engrailed-1* (*En1*) is replaced by homologous recombination with its paralogue *Engrailed-2* (*En2*) or with its drosophila homologue, the otherwise lethal phenotype, with significant defects in mid- and hindbrain, is rescued. The resulting animals are viable and fertile with normal brain morphology (Hanks et al., 1995, 1998), suggesting that a common conserved genetic pathway exists in protostomia and deuterostomia.

The protein sequence analysis revealed five distinct subdomains in the engrailed proteins, designated as engrailed homology (EH) regions (EH1-5) (Logan et al., 1992). The EH4 region represents the homeodomain and includes approximately 60 amino acids, that form three alpha helices, common with all classes of homeodomain proteins (Manak and Scott, 1994). The third helix is responsible for the binding to specific sequences in the large groove of double stranded DNA (Desplan et al., 1988; Kissinger et al., 1990; Ades and Sauer, 1994). Besides the DNA binding activity, the engraileds can also establish proteic interactions through the EH1, 2, 3 and 5 domains. The proteins act mostly as transcriptional repressors via the EH1 and EH5 domains (Han et al., 1989; Ohkuma et al., 1990; Smith and Jaynes, 1996), however this inhibitory function is not a fixed property. Protein interactions mediated by EH2 and EH3 can determine the regulatory target by affinity adjustment, but can also switch the transcriptional activity from repressor to activator (Serrano and Maschat, 1998; Gemel et al., 1999; Kobayashi et al., 2003). Examples for engrailed associated molecules are the *hepatocyte nuclear factor 3 beta* (*HNF3*β/*Foxa2*) (Foucher et al., 2003) and the *pre B-cell leukemia transcription factor 1* (*Pbx1*) (Gemel et al., 1999).

Cell localization and secretion/internalisation of engrailed proteins

As transcriptional regulators, the engrailed proteins are localized in the nucleus, however, a small proportion (∼5%) of the intracellular protein is found in the cytoplasm associated to membrane vesicles, that can be transported anterogradely in the axon (Joliot et al., 1997). Surprisingly, this cytoplasmic proportion of the proteins can be secreted and internalised by other cells. Export from the nucleus, secretion and internalisation depends on the third helix of the homeobox and the first few aminoacids N-terminal to it (Joliot et al., 1998; Maizel et al., 1999). This seemed to happen via an energy- and receptor-protein-independent mechanism without involving classical exo/endocytosis (Han et al., 2000; Cosgaya et al., 1998; Joliot et al., 1998), but it is nevertheless a highly regulated process, since phosphorylation of the serine-rich domain inhibits secretion and nuclear export, and simultaneously increases DNA binding by several fold (Maizel et al., 1999, 2002). The nuclear export opens the possibility, that engrailed can function outside the nucleus and act as intercellular polypeptidic messengers (Joliot et al., 1998; Maizel et al., 1999) and might for example locate mRNAs into axonal terminals (Joliot et al., 1997). Most of these studies have been performed in cell culture and they still need confirmation in vivo, however they suggest that homeoprotein transcription factors may have non-cell-autonomous signaling properties and act in a paracrine

manner (Prochiantz, 1999; Prochiantz and Joliot, 2003).

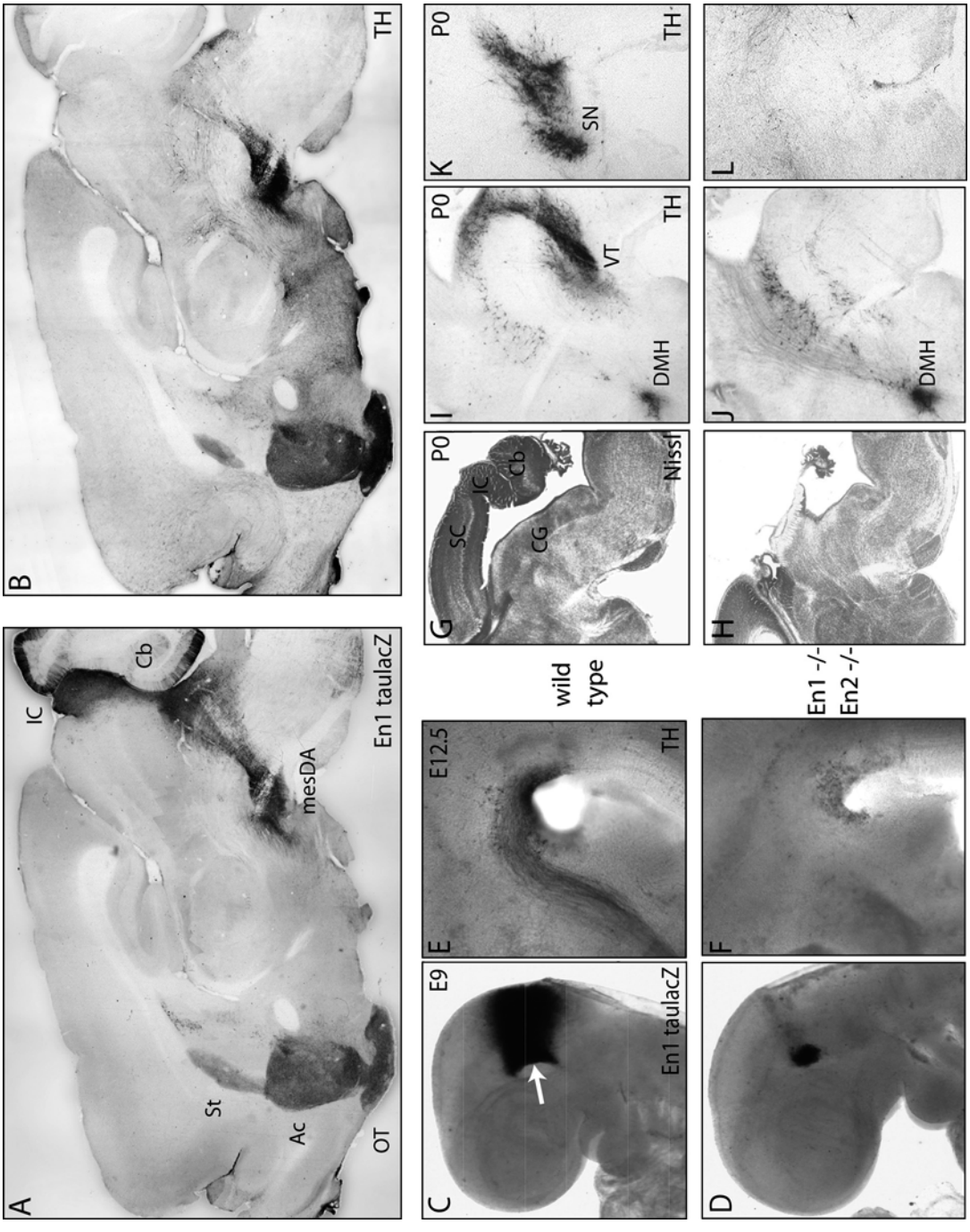
Expression of engrailed in the murine brain, pattern formation of mid/hindbrain and neurogenesis of mesDA neurons

The most common function of the *engrailed* genes in all investigated phyla is an involvement in pattern formation during early embryogenesis (Hidalgo, 1998; Wurst and Bally-Cuif, 2001). Later in development, they take part in neurogenesis (Condron et al., 1994; Harzsch et al., 1998) and neuronal differentiation (Saueressig et al., 1999; Marie et al., 2000). In vertebrates, the early developmental role of *En1* and *En2* arises from their expression in a broad band rostral and caudal to the isthmus, the border between mid- and hindbrain (Fig. 1C, D). Targeted inactivation of the genes results in a gene-dose dependent deletion of mid- and hindbrain structure mostly affecting the colliculi, the cerebellum (Fig. 1G, H) and ventrally located neuronal populations like the serotonergic neurons of dorsal raphe nucleus, the noradrenergic cells of the locus coeruleus and the motor nuclei of the III and IVth nerve (Wurst et al., 1994; Simon et al., 2005). Their regional expression is probably determined by other, earlier expressed transcription factors, like *Otx2*, *Pax2*, *Pax5* and *Gbx2* (Wurst and Bally-Cuif, 2001), since their mutant phenotype in respect to the midbrain is rather similar (McMahon and Bradley, 1990; Acampora et al., 1995; Ang et al., 1996; Schwarz et al., 1997) and their ectopic expressions by domain shifts alter the *engrailed* expression domains and mid/hindbrain structures in manner concordant with this notion (Hidalgo-Sanchez et al., 1999; Brodski et al., 2003; Puelles et al., 2003, 2004). Another molecule that plays a key role in the regulation of *engrailed* expression during early embryogenesis is Wnt1, a diffusible signaling protein. Its expression has no influence on the initial engrailed expression in the midbrain, however it is essential for the maintenance of this expression (Danielian and McMahon, 1996).

Differentiation of mesDA neurons and survival

Briefly after the rostral to caudal neuroaxis has been laid down, mesDA neurons are induced in the ventral midbrain by the combined interaction of two diffusible molecules, sonic hedgehog (SHH) and the fibroblast growth factor 8 (FGF8), released by the floor plate and isthmus, respectively (Ye et al., 1998). Thereafter, cells become postmitotic (Altman and Bayer, 1981) and begin to express the first markers specific for their neurotransmitter phenotype (Perrone-Capano and Di Porzio, 2000). Three regulatory pathways involved in the differentiation of the mesDA neurons have been identified so far. The key transcription factors for these pathways are Nurr1, Lmx1b and the *engrailed* genes (reviewed in Simon et al., 2003). In contrast to Nurr1, the *engrailed* expression in the postmitotic neurons begins relatively late and is initially only detectable in the surrounding midbrain tissue. The onset of expression in the postmitotic neurons occurs gradually over 72 hours, with the first TH positive cells in the ventral midbrain expressing the two genes at E11, the latest start to express them at E14 (Alberi et al., 2004). This expression persists from then on throughout the life of the animal (Fig. 1A, B).

It seems that the neuroepithelial precursor cells never express the *engrailed* genes. The role of the two transcription factors is indirect, arising from the fact that they regulate the *FGF8* expression, which is essential for the induction of these precursor cells. In embryos homozygous null for *En1* and *En2*, the initial *FGF8* expression at E8 is wild type-like, however no *FGF8* is detectable at the mid/hindbrain border at E9 (Liu and Joyner, 2001). The lower number of postmitotic mesDA neurons in E12 *engrailed* double mutant embryos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(Fig. 1E, F) (Simon et al., 2001; Alberi et al., 2004) could be explained by this altered *FGF8* expression. A conserved region in the large intron of the *FGF8* gene with an active En1 binding site exists in the genome of higher vertebrates (Gemel et al., 1999 and our own unpublished database search; Simon et al., 2004) suggesting a direct regulation of *FGF8* by the *engrailed* genes. Ectopic *FGF8* expression in chicken embryonic midbrain infected with retroviruses expressing *En1* supports this idea (Shamim et al., 1999).

The most noticeable function of the *engrailed* genes in respect to mesDA neurons is their close connection to the survival of these cells. Despite the early morphological deletion of mid/hindbrain structures, mesDA neurons are generated in the *engrailed* double mutant mice and start to express markers typical for their neurotransmitter phenotype. However, shortly thereafter the mesDA neurons die with signs of apoptosis and at E14 all are lost (for P0 Fig. 1I–L), suggesting that in parallel to the onset of genes expression in the wild type, a requirement for the *engrailed* genes sets in which leads to death of the cells if not met in the mutant (Simon et al., 2001; Alberi et al., 2004). By performing cell mixing in vitro and in vivo (chimera), we demonstrated that the *engrailed* requirement for the survival of mesDA neurons is cell-autonomous and not attributable to the deletion of midbrain tissue in the mutant. Furthermore, RNA interference experiments on primary midbrain cell cultures showed that cell death is induced in less than 24 hours after transfection, indicating that the *engraileds* may regulate one or more genes in the apoptosis pathway.

Engrailed regulatory targets

The close connection of mesDA neurons to one of the most prominent human neurodegenerative disorders makes it of high interest to identify genes that are involved in the survival of these cells. Since the role at the *engrailed* genes in the survival of mesDA neurons cannot be a direct consequence of their DNA binding property or protein–protein interactions with other transcriptional regulators, the effector genes must be downstream to them. The most interesting candidate target gene to our opinion is α-*synuclein*, a presynaptic protein, not expressed in mesDA neurons of the engrailed double mutants (Simon et al., 2001). α-*Synuclein* was the first gene associated to familial form of Parkinson's disease (Polymeropoulos et al., 1997; Kruger et al., 1998), however its inactivation by homologous recombination in mice leads only to minor changes in the nigrostriatal system (Abeliovich et al., 2000).

An approach to isolate genes downstream of the engrailed was recently attempted in our laboratory (Thuret et al., 2004a, b). We

Fig. 1. Engrailed expression in mouse brain and loss of mesDA neurons in absence of both engrailed genes. Sagittal sections of adult (**A**, **B**) and P0 brains (**G**–**L**) and two whole mount preparation (**C**–**F**), immunostained against the β-gal, to detect the En1/tauLacZ reporter (**A**, **C**, **D**) and against tyrosine hydroxylase (TH) (**B**, **E**, **F**, **I**–**L**), and Nissl stained (**G**, **H**). **A**, **B** The expression pattern of the tauLacZ reporter genes demonstrates that in adult animals En-1 is expressed in the inferior colliculus, the cerebellum and the mesDA neurons. The axonal projection to the basal ganglia revealed by the reporter is identical to TH. **C**, **D** At E9, En-1 is expressed in a broad band rostral and caudal to the isthmus (arrow) in the wild type, while in the engrailed double mutant embryos (En1-/-;En2-/-) part of mid/hindbrain is absent and the expression of the En1/tauLacZ reporter is reduced to a small domain in the ventral midbrain (**E**, **F**). At E12, TH positive cells are detectable in the ventral midbrain of wild type and double mutants. The mutant domain is smaller and no axonal outgrowth is detectable (**G**, **H**). The Nissl staining of the P0 brain section reveals loss of inferior (IC) and superior colliculus (SC), cerebellum (Cb) and large parts of the periaqueductal central grey (CG) in the mutant (**I**–**L**). The sagittal sections reveal that all mesDA neurons are lost in the *engrailed* double mutants at PO. *Ac* nucleus accumbens, *OT* olfactory tubercle, *SN* substantia nigra, *St* striatum, *VT* ventral tegmentum

compared the expression pattern of ventral midbrain between wild type and *engrailed* double mutant embryos with a PCR based differential display technique at the age when the mesDA neurons are disappearing, and isolated *synaptotagmin 1* (Syt1), *v-erb-a erythroblastic leukemia viral oncogene homolog 4* (ErbB4) and *microtubule-associated protein 1 B* (Mtap1b). Syt1 is an integral membrane protein of synaptic vesicles and acts as Ca^{2+} sensor in vesicle exocytosis (Fernandez-Chacon et al., 2001). ErbB4 is involved in neuronal development as one of the high affinity neuregulin receptors and it seems to play a role in regulating the dopamine release in the striatum (Yurek et al., 2004) Mtap1b is expressed at high levels in the developing brain and is involved in microtubule assembly and dendrite morphogenesis (Teng et al., 2001). There are two evolutionary conserved overlapping binding sites for the Engrailed genes and HNF3β (Foxa2) in the promoter of Mtap1b. The two genes antagonize each other when regulating the Mtap1b promoter in primary cultures of embryonic mes/metencephalic neurons (Foucher et al., 2003).

Direct evidence about genes involved in axonal outgrowth in the mesDA neurons and regulated by engrailed is still missing. Nevertheless, ectopic expression of En1 in mice induces EphrinA2 and EphrinA5 (Lee et al., 1997) leading in chicken to ectopic projections of retinal nasal axons (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996). EphA5-ephrinA5 system has been recently shown to be involved in the development of the mesDA axonal projections to the striatum, suggesting a role of the Engrailed gene in this process (Sieber et al., 2004).

Conclusion

The *engrailed* genes have a dual function in the development of mesDA neurons. They take part in the regionalization event, which gives rise to the neuroepithelium that provides the precursors cells in the ventral midbrain with the FGF8 signal necessary for their induction. In the postmitotic neurons, the two transcription factors are essential for their survival by preventing the induction of apoptosis. Furthermore, the continuous expression of the *engrailed* genes from midgestation to adulthood suggests that they may participate in the long-term maintenance of this neuronal population.

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The role of Pitx3 in survival of midbrain dopaminergic neurons

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Summary. Dopamine belongs to the most intensively studied neurotransmitters of the brain, because of its implications in psychiatric and neurological disorders. Although, clinical relevance of midbrain dopaminergic (mDA) neurons is well recognized and dopaminergic dysfunction may have a genetic component, the genetic cascades underlying developmental processes are still largely unknown. With the advances in molecular biology, mDA neurons and their involvement in psychiatric and neurological disorders are now subject of studies that aim to delineate the fundamental neurobiology of these neurons. These studies are concerned with developmental processes, cell-specific gene expression and regulation, molecular pharmacology, and genetic association of dopamine-related genes and mDA-associated disorders. Several transcription factors implicated in the post-mitotic mDA development, including Nurr1, Lmx1b, Pitx3, and En1/En2 have contributed to the understanding of how mDA neurons are generated in vivo. Furthermore, these studies provide insights into new strategies for future therapies of Parkinson's Disease (PD) using stem cells for engineering DA neurons in vitro. Here, we will discuss the role of Pitx3 in molecular mechanisms involved in the regional specification, neuronal specification and differentiation of mDA neurons.

The heterogeneity of midbrain dopaminergic (mDA) neurons

The mDA system (A8–A10 cell groups) is involved in many brain functions including motor control, reward, emotional and motivated behavior, and is of clinical importance because of its implication in neurological and psychiatric disorders. The A9 cell group located in the substantia nigra pars compacta (SNc) has preferred projections to the dorsal striatum forming the nigrostriatal pathway, which is involved in the control of movement. The mDA system further includes the ventral tegmental area (VTA), located in the A10 group and the retrorubral field located in the A8 group. Dopamine neurons of the VTA with their efferents to the nucleus accumbens, other limbic brain areas and the cortex form the mesolimbic/cortical pathways, and are involved in the control of emotional behaviors and reward. In addition, specific mDA subpopulations have been described based on pharmacology, gene expression and electrophysiological properties. A neuropathological enigma is posed by the selective degeneration of the SNc dopamine neurons in PD and many animal models of PD, whereas mDA neurons in the VTA are largely spared. Gene expression profile studies of discrete adult mDA subpopulations revealed distinct molecular features that might underlie the differential susceptibility (Grimm

et al., 2004; Greene et al., 2005). Therefore, one may expect that the difference between dopamine neurons of the SNc and those of the VTA roots in the molecular make-up of these neurons, which might originate from subpopulation-specific developmental pathways. Interestingly, the recent discovery of a brain phenotype in Pitx3-deficient mice indicates that Pitx3 drives molecular pathways that are essential for the development and/or survival of specific mDA subsets (Smidt et al., 2004a, b). Thus, these data suggest that differentiation of specific mDA subpopulations is controlled by different developmental pathways/factors, or that different subpopulations differentially respond to the same factor.

Pitx3 and its role in mDA development

The development of mDA neurons follows a number of stages marked by distinct events. After preparation of the region by signals that provide induction and patterning, cascades of transcription factors involved in specification and differentiation enroll towards fully matured mDA neurons (Hynes and Rosenthal, 1999). Molecular studies into the developmental pathways of these neurons and analysis of mutant animals defective in mDA development have identified several key transcription factors, including Nurr1, Lmx1b and En1/En2, with a function in specification of transmitter identity, neuronal identity and survival of mDA neurons (Smidt et al., 2004a; Perlmann and Wallen-Mackenzie, 2004; Simon et al., 2004).

The paired-like homeodomain transcription factor Pitx3 is uniquely expressed in the brain in post-mitotic mDA neurons during the late differentiation phase from E11.5 onwards, and its expression is conserved among species including human. Genetic analysis of the Aphakia (*ak*) mouse mutant revealed deletions in the Pitx3 gene, causing the ablation of Pitx3 expression (Smidt et al., 2004a, b). These Pitx3-deficient (*ak*) mice display neuroanatomical alterations in the mDA system from E12.5 onwards, characterized by the absence of mDA neurons in the SNc, whereas mDA neurons in the VTA and the most lateral tip of the SNc are largely spared (Smidt et al., 2004a, b). As a consequence of the neuronal loss in the SNc, connections to the dorsal striatum are virtually absent resulting in a dramatic decrease of dopamine. Initial behavioural analysis of *ak* mice revealed inconsistent reports on their motor impairments. Although it was stated that *ak* mice display the akinetic subtype of PD and motor deficits that are reversed by L-DOPA (van den Munckhof et al., 2003; Hwang et al., 2005), we and others observed no characteristic neurological PD symptoms in *ak* mice (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004a, b).

The mechanism by which Pitx3 influences specifically the survival of SNc mDA neurons is unknown and intriguing. Post-mitotic mDA neurons start to express Pitx3 at the most ventral position of the developing midbrain after they have migrated ventrally from the neuroepithelium. Therefore, Pitx3 is not directly involved in the proliferation and/or migration of young mDA neurons, but rather in the terminal differentiation and maintenance. A possible explanation for the selective vulnerability, observed in *ak* mice may be that Pitx3 is not expressed in all mDA neurons. However, we and others found complete overlap between Pitx3 and tyrosine hydroxylase (TH), the key enzyme in dopamine synthesis (Smidt et al., 2004a, b; Zhao et al., 2004). Thus, although all mDA neurons depend on identical signals for their early specification, the specification of neuronal fate of mDA subsets is probably maintained, in part, by independent regulatory cascades.

Origin and specification of mDA neurons

Specification of neuronal fates begins with the acquisition of anterior-posterior (A/P)

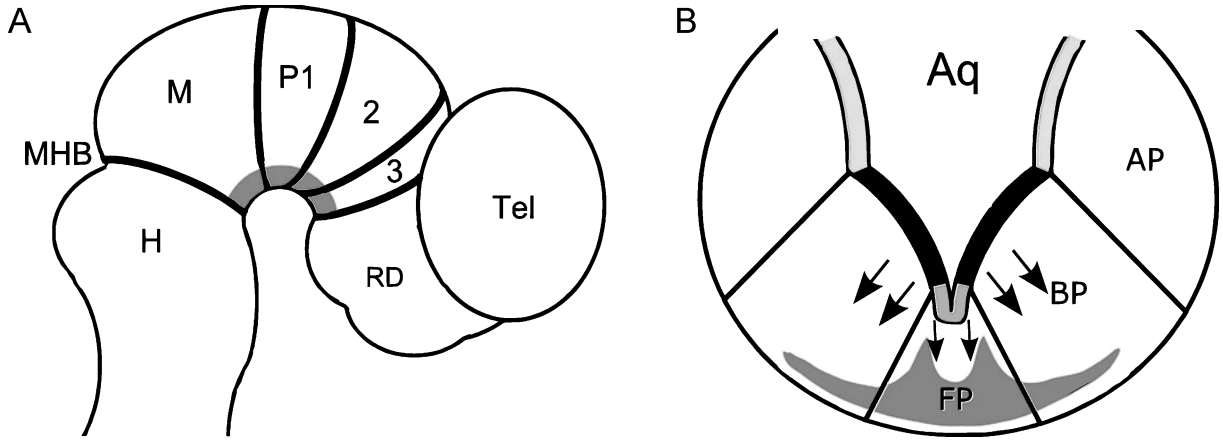

Fig. 1. Schematic representation of the anterior/posterior (**A**) and dorsal/ventral (**B**) patterning of the brain and the emergence of mDA neurons (red) with specific identity to regional molecular coding. **A** Drawing of an E12.5 mouse brain in a sagittal plane showing the location of fully differentiated mDA neurons (red) in specific brain segments (M-P3). **B** Drawing of an E12.5 mouse midbrain in a coronal plane, showing the ventral localization of fully differentiated mDA neurons. Neurons are born in the ventricular zone across specific longitudinal domains (floor plate (FP, green), basal plate (BP, blue) or alar plate (AP, yellow)) and migrate ventrally (arrows) where they adopt the full dopaminergic phenotype and start to express Pitx3. *Aq* aqueduct; *H* hindbrain; *M* midbrain; *MHB* mid-hindbrain border (Isthmus); *P1-3* prosomere *1–3*; *RD* rostral diencephalon; *Tel* telencepalon

and dorsal-ventral (D/V) patterning in restricted domains of the neuronal plate (Fig. 1). D/V patterning causes longitudinal subdivisions in the brain (floor plate, basal plate and alar plate), whereas A/P patterning leads to neuromeric domains (forebrain, midbrain, isthmus and hindbrain; Puelles, 2001). The commitment of neuronal identity by a molecular code within progenitor cells in the ventricular zone and region-specific developmental cascades ultimately results in induction of distinct neuronal cell types, including mDA neurons (Fig. 1). In human embryos, mDA neurons in the SNc and VTA originate independently across several neuromeric domains and longitudinal subdivisions, and thus are not primarily unitary (Verney et al., 2001). Thus, the developmental origin of mDA neurons with respect to the longitudinal subdivisions and neuromeric domains in the brain, and the molecular codes within mDA subsets might determine the distinct features of these cells.

Concluding remarks

It becomes more and more clear that the mDA system harbors a multitude of specific functional neuronal units exemplified by region-specific molecular codes during development and in the adult. The role of Pitx3 in the development of SNc mDA neurons might link molecular codes to survival of mDA subsets, which can be exploited in the treatment of PD. Recently, it was shown that Pitx3 facilitates differentiation of mouse embryonic stem cells into the A9 cell group of mDA neurons, without affecting the total number of dopamine neurons (Chung et al., 2005), illustrating the importance of identifying the appropriate signals and factors that influence normal mDA development. Until now no molecular target genes of Pitx3 are identified and molecular processes initiated by Pitx3 remain unidentified. Therefore, further investigation is warranted to elucidate the role of Pitx3 in mDA neuronal development and maintenance.

Note added in proof

Recent development in specification of the dopamine neurons of the SNc and VTA have led to the new nomenclature of these neurons as the meso-diencephalic dopamine (mdDA) neurons. This is highlighted in the following recent review: Smits et al., 2006 in "Progress in Neurobiology".

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Genetic analysis of dopaminergic system development in zebrafish

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Summary. Zebrafish have become an important model organism to study the genetic control of vertebrate nervous system development. Here, we present an overview on the formation of dopaminergic neuronal groups in zebrafish and compare the positions of DA neurons in fish and mammals using the neuromere model of the vertebrate brain. Based on mutant analysis, we evaluate the role of several signaling pathways in catecholaminergic neuron specification. We further discuss the prospect of identifying novel genes involved in dopaminergic development through forward genetics mutagenesis screens.

Introduction

An important avenue for biomedical research for Parkinson's Disease involves analysis of differentiation and circuit formation of the dopaminergic system in animal models. Several features predispose the zebrafish (*Danio rerio*) as an excellent model organism to study neural development of vertebrates. Zebrafish embryos develop rapidly, and a functional larval nervous system is established within four days of development. Due to their external development, these embryos are easily accessible to experimental manipulation at all stages. The relatively short generation time of three months makes efficient genetic analysis and mutagenesis screens possible. Moreover, during the past decade, the international community of zebrafish researchers has successfully established a centralized collection of resources (www.zfin.org), and the sequencing of the zebrafish genome is nearly complete (http://vega.sanger.ac.uk/Danio_rerio/). The small embryos and larvae are particularly popular among developmental neurobiologists since genetic analysis, cell biological manipulations, and pharmacological interference can easily be combined in a single embryo. Further, the transparent embryos allow direct visualization of the results *in vivo* using transgenic marking of cells with fluorescent proteins. Taken together, these features make zebrafish an attractive model system to study dopaminergic system development. However, as the evolutionary distance between zebrafish and human is about 350 million years, differences in neuroanatomy and circuit formation needs to be carefully considered.

Overview of dopaminergic development in zebrafish

The formation of dopaminergic groups has been studied by analysis of expression of *tyrosine hydroxylase* (*th*), *dopamine transporter* (*dat*), and *dopamine beta hydroxylase* (*dbh*) (Holzschuh et al., 2001, 2003a) as well as by immunohistochemistry for Tyrosine hydroxylase (TH) (Kaslin and Panula, 2001; Rink and Wullimann, 2002). We will focus here on the embryonic and larval DA systems – additional small DA groups may be present

in adult zebrafish. While we try to use the prosomere model (Puelles and Verney, 1998) to compare positions of DA neurons in fish and mammals, we will not use the A1–A17 numbering established for mammalian systems (Smeets and Gonzalez, 2000), as there is so far little information on potential functional similarities. The first dopaminergic neurons differentiate at about 18 hours post fertilization (hpf) in the prospective posterior tuberculum (basal plate area of prosomere 3, for comparison to human see distribution of human catecholaminergic groups in the prosomere model of Puelles and Vernier (1998)). Successively, additional groups of DA neurons are specified to build the full complement of DA groups, which is complete by four days post fertilization (dpf) and include the following groups. (i) Within the ventral diencephalon, several DA groups can be distinguished: the ventral portion of the posterior tuberculum contains two groups of DA cells with a large soma and high levels of DA expression, one close to the alar-basal plate boundary and one further basal. Between these groups, a cluster of small DA cells situated close to the ventricle can be found. An additional group of small DA cells develops at the alar-basal border and extends from the posterior tuberculum into the ventral thalamus. This group expands significantly in cell number during larval development. Another group develops at the ventral base of the posterior tuberculum, and extends into the hypothalamus. Within the hypothalamus, two DA groups can be detected by 5 dpf. (ii) A cluster of DA cells that expands significantly during late larval development can be detected in the pretectum. (iii) Groups of DA cells form in the preoptic and paraventricular region. (iv) The olfactory bulb group. (v) A group in the subpallium. Dopaminergic neurons are further present in the retina (dopaminergic amacrine cells). Based on the absence of *dat* expression from areas that express *th* in the hindbrain, it can be surmised that DA neurons do not develop in the hindbrain. In contrast, noradrenergic neurons develop in the locus coeruleus and in the medulla oblongata area postrema in the hindbrain. DA neurons do not develop in the zebrafish mesencephalon. This is a major difference between fish and mammals, which has been attributed to a caudal-ward shift of dopaminergic activity during evolution from fish to mammals (Smeets et al., 2000). Retrograde labeling experiments revealed ascending projections from the posterior tuberculum into the pallium and subpallium in zebrafish (Rink and Wullimann, 2000), which led the authors to suggest that these DA groups may contribute to ascending regulatory circuits similar to those to which mammalian DA neurons of the substantia nigra contribute to.

Signaling requirements

The availability of many mutations affecting components of signaling pathways involved in brain patterning and cell differentiation allowed us to address signaling requirements for DA neuronal specification in zebrafish. In mammalian embryos, many mutations affecting major signaling pathways are lethal prior to DA system development. In contrast, most mutant zebrafish embryos survive for at least two days since a functional cardiovascular system is not required for early development and store of maternal proteins enables cells to survive. As experiments in mammalian systems indicated a requirement of Shh and FGF8 signaling for dopaminergic neuron development (Ye et al., 1998), we tested contribution of these signaling pathways (summarized in Table 1; (Holzschuh et al., 2003b). For the Shh pathway, both *syu* (shh) and *smu* (Shh co-receptor Smoothened) mutant embryos have been analyzed. In Shh signaling deficient embryos, the early ventral diencephalic and the olfactory bulb DA groups form, but pretectal and retinal amacrine DA cells are reduced or absent. Thus the floorplate-derived Shh signal may not contribute to specification of early ventral diencephalic DA

Table 1. Impact of signaling pathways on specification and differentiation of zebrafish catecholaminergic neurons

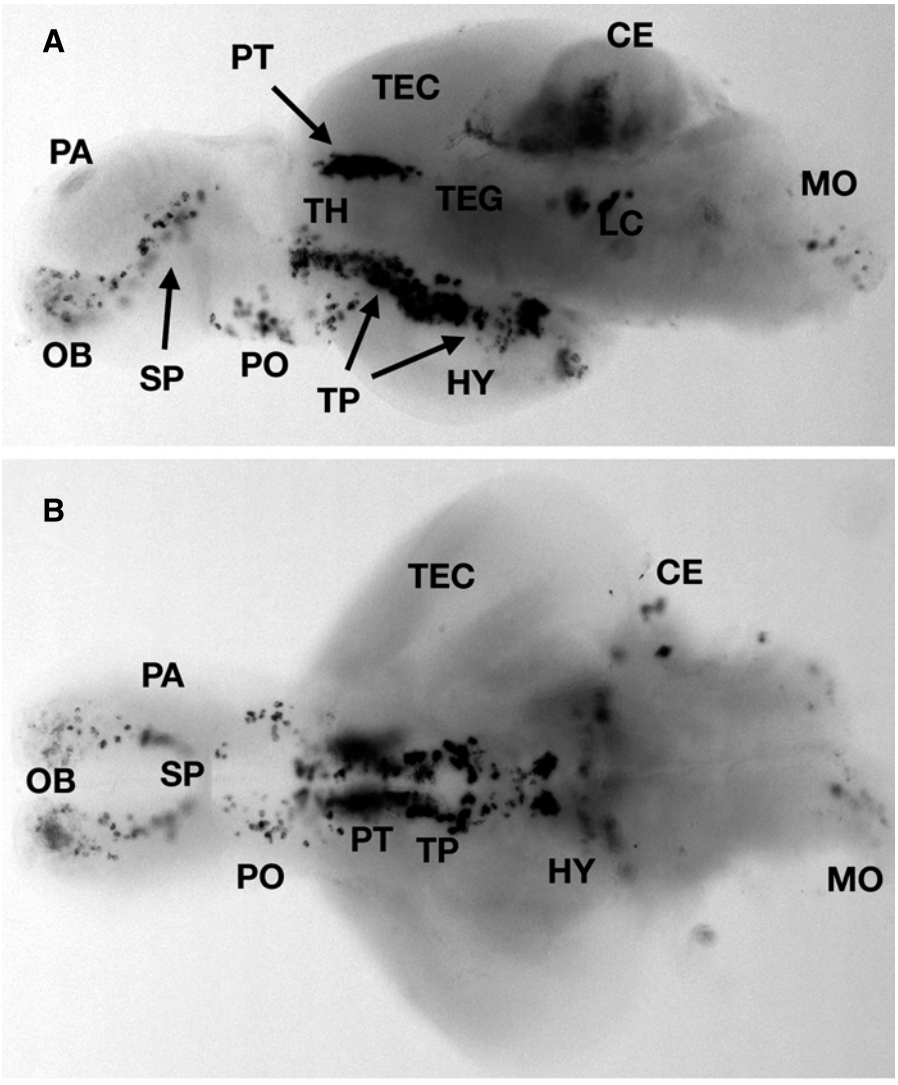
Signal	Catecholaminergic groups		
	Affected	Non affected	Not determined*
Shh (*smu*, *syu*)	PT(−0), vDC(−), AC(−)	OB, LC, MO	PO, SP, HY
FGF8 (*ace*)	LC(0), vDCa(−)	OB, PT, vDCp, AC, MO	PO, HY
Nodal/TGF (*MZsur*)	vDC(−0)	LC, MO, AC	PT, OB, PO, SP, HY
Nodal/TGF (*oep* or *cyc*)	PT(0), vDC(0), HY(0), AC (*cyc*: 0), OB (0)	LC, MO	PO, SP
Retinoic acid (>24 hpf)	MO (0)	PT, OB, vDC, PO, SP, LC	HY, AC

Abbreviations for DA groups: *PT* pretectal DA neurons, *OB* olfactory bulb DA neurons, *vDCa* anterior DA group ventral diencephalon (group 1 according to Rink and Wullimann), *vDCp* posterior DA groups in the ventral diencephalon (groups 2–6 according to Rink and Wullimann), *PO* preoptic group, *SP* DA group of subpallium, *HY* hypothalamic DA groups, *AC* amacrine, *DA* cells of retina, *LC* locus coeruleus NA neurons, *MO* medulla oblongata area postrema NA neurons, (−) – reduced number of cells, (0) – absent, (−0) – reduced or absent, *not determined because embryos show global defects

neurons, but Shh derived from the zona limitans intrathalamica at the border of prosomers 2 and 3 may be involved in specification of precursors of DA neurons in the pretectum.

Analysis of *ace* mutant zebrafish, which are devoid of FGF8, revealed that FGF8 contributes both to specification of DA and NA neurons. Locus coeruleus NA neurons are completely absent in *ace* mutant embryos. Within the ventral diencephalon, the caudal DA groups form, but the anteriormost cluster of DA cells, corresponding to group 1 neurons in Rink and Wullimann (2002), appears to be absent in mutant embryos. An expression domain for FGF8 has been reported to exist in the posterior tuberculum, and may be the source of FGF signaling required for the development of these neurons. The pretectal DA group appears to develop independently of FGF8, even though an FGF8 expression domain has been reported in close proximity of this DA group in the dorsal thalamus. The fact that the majority of early differentiating ventral diencephalic DA neurons develop even in *ace* and *smu* double mutant embryos, which should be devoid of both FGF8 and Shh signaling, led us to consider additional pathways that may contribute to DA development. Nodal signals of the TGFbeta family play an important role in development of the ventral diencephalon. However, a complete depletion of Nodal signals in *cyc* mutants (affecting Nodal related protein 2), or in *oep* mutant embryos (affecting the Oep Nodal co-receptor) also deletes a significant portion of the ventral diencephalon. This makes it difficult to distinguish whether the absence of ventral diencephalic DA neurons in these mutants is caused by early patterning defects or defects in specification of DA neurons. The analysis of the zebrafish *sur* mutation, which affects Fast1/FoxH1, a transcription factor and transducer of Nodal signals, was more informative. In *MZsur* mutant embryos, which lack both maternal and zygotic expression of Fast1/FoxH1, DA neurons are often completely absent from the ventral diencephalon. Analysis of the expression pattern of *dbx1a*, a genes expressed in the basal plate of prosomere 3, indicates that the posterior tuberculum still forms in *MZsur* embryos. This argues for the role of Nodal/TGFbeta family signals in specification of the ventral DA groups. Whether this involves direct induction of ventral diencephalon (DC) DA cells, or regulation of the prepattern of this region remains to be addressed.

Retinoic acid is directly involved in neuronal differentiation as well as in hindbrain patterning. When zebrafish embryos older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medulla oblongata
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prosomeres
rhombomeres
hypothalamus
posterior tuberculum

than 24 hpf are exposed to retinoic acid, segment identity, as judged by establishment of the Hox gene code is already complete and a shift in segment identities can not be induced. However, exogenous retinoic acid induces an expansion of the medullary NA group often well into rhombomere 3 (Holzschuh et al., 2003a). These findings argue that within a dorsoventral domain competent to form NA neurons, the rostro-caudal retinoic acid gradient determines the anterior limit of NA differentiation. Other CNS DA or NA groups are not affected by RA under these conditions.

Delta/Notch signaling is also involved in DA specification, but its role has not been analyzed in detail. Mutations in *mib*, a ubiquitin ligase required for proper Delta/Notch signaling, generate supernummary DA neurons in all clusters, indicating that this neurogenic switch is broadly involved in restricting precursors from differentiating into DA neurons (Holzschuh and Driever, unpublished).

Several other signaling systems play important roles in neural differentiation. However, for some, including the Wnt signaling pathway, manipulation of the signaling pathway in the whole embryo causes severe early anterioposterior patterning defects in the neural plate which makes it impossible to analyze its specific contributions to DA or NA specification. For some other signaling pathways, mutations are currently not available.

Genetic approaches

Zebrafish present an ideal system for so-called ''forward'' genetic mutagenesis screens to identify additional genetic components contributing to specification and differentiation of DA neurons. The short generation time and high fecundity allow large scale genetic screens where thousands of mutagenized genomes can be analyzed for new mutations affecting DA system development. Such screens have been performed using either Tyrosine hydroxylase immunohistochemistry (Guo et al., 1999) or *th* mRNA *in situ* hybridization (Holzschuh et al., 2003a). Following mutagenesis of male zebrafish (G0) with an alkylating agent, ethylnitrosourea (ENU), two or three generation breeding schemes are feasible in zebrafish: In the two generation scheme, eggs from F1 progeny females are fertilized *in vitro* with UV inactivated sperm, and F2 embryos develop as haploids with no genetic contribution from the father. If an F1 female is heterozygous for a new mutation, 50% of the haploid F2 should express a mutant phenotype. Haploid screens are quick and require less animal facility space. However, towards the second and third day, the development of haploid embryos deviates from that of normal embryos, and it is difficult to screen for defects in late developing neuronal groups. In the three generation scheme, F1 males and females are crossed to each other to generate F2 families. If a new mutation was bred into an F2 family, 50% of the fish are heterozygous, and every fourth sibling cross should reveal the mutant phenotype in a quarter of the F3 embryos. Three generation screens are time consuming and costly, but are well suited to discover subtle defects affecting late aspects of DA system development. Zebrafish are currently the only vertebrate in which genetic screens

Fig. 1. Distribution of zebrafish catecholaminergic neurons with respect to the neuromeric organisation of the brain: relating teleost to mammalian organisation of the DA system. **A**, **B** Brain prepared from 28 day old wild type zebrafish larvae was processed by *in situ* hybridization to detect expression of *tyrosine hydroxylase* mRNA. Blue signal indicates presence of catecholaminergic neurons. **C** Relative location of catecholaminergic groups projected onto the neuromere model of the vertebrate brain (see also Puelles and Verney, 1998; Smeets and Gonzales, 2000; Rink and Wulliman, 2002). Lateral (**A**, **C**) and dorsal (**B**) views with anterior at left. *CE* cerebellum, *HY* hypothalamus, *LC* locus coeruleus, *MO* medulla oblongata, *OB* olfactory bulb, *PA* pallium, *PO* preoptic region, *PT* pretectum, *SP* subpallium, *TEC* tectum, *TEG* tegmentum, *TH* thalamus, *TP* posterior tuberculum

for new genes involved in DA neuronal development are performed. Such screens should identify factors which are expressed at very low levels and have thus escaped biochemical or molecular biology approaches.

Conclusions

Genetic analysis in zebrafish has already revealed an unexpected complexity in signaling requirements for the different dopaminergic groups that form in the zebrafish di- and telencephalon. It appears that local patterning of the dorsoventral and anterioposterior axis of the CNS may generate a ''prepattern'', which in combination with different local signals serves to specify neural cells to take on a dopaminergic fate. As such, the regulatory inputs which control DA differentiation, may be convergent rather than following one or two instructive signals only. The rapid genetics and other experimental possibilities available in zebrafish will help to further our understanding of dopaminergic differentiation. A careful comparison with mammalian systems will reveal which aspects of DA differentiation are conserved among vertebrates. Since circuits from basal ganglia into striatum or subpallium are essential for movement control, and circuits with similar function (albeit in different neuroanatomical locations) exist from fish to mammals, one would expect that a significant portion of their molecular determinants may also be conserved.

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Striatal plasticity in parkinsonism: dystrophic changes in medium spiny neurons and progression in Parkinson's disease

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Summary. Striatal dopamine loss in Parkinson's Disease (PD) sets into play a variety of compensatory responses to help counter dopamine depletion. Most of these changes involve surviving dopamine neurons, but there are also changes in striatal medium spiny neurons (MSNs), which are the major target of dopamine axons. Among these changes are decreases in MSN dendritic length and spine density, which may dampen excessive corticostriatal glutamatergic drive onto MSNs that occurs secondary to dopamine loss. An increasing knowledge of dendritic changes in PD suggests strategies for tracking progressive worsening of symptoms and is opening new ideas on novel therapeutic strategies for PD.

Overt symptoms of Parkinson's Disease (PD) are not seen until there has been a sharp decrease in the number of dopamine neurons in the substantia nigra and an extensive depletion of striatal dopamine. The ability of those surviving dopamine neurons to compensate for large decreases in striatal dopamine by increasing their firing rate, synthesis of dopamine, and fractional release of dopamine have contributed considerably to our understanding of the normal and pathophysiological function of dopamine neurons. However, the dramatic changes in dopamine neurons have also led investigators to an almost exclusive focus of PD research on dopamine neurons as a means of uncovering new therapeutic strategies for PD and related disorders.

Dopamine axons that innervate the striatum synapse with the medium spiny neuron, named for its size and dendrites that are densely-studded with dendritic spines. Dopamine axons form a characteristic triadic arrangement with medium spiny neurons (MSNs) and corticostriatal glutamatergic axons, such that DA synapses are usually directed to the neck of the dendrite, with the spine head being the target of a single glutamatergic axon from the cortex (Bolam, 1984; Arbuthnott et al., 2000). Striatal DA receptors, including both D_1 and D_2 receptors, are localized to spines, with D_2 heteroceptors also being present on cortical axons innervating the striatum. The ultrastructural arrangement involving dopamine and glutamate axons and MSN dendritic spines suggests that DA may modulate or gate excitatory cortical information that funnels through the striatum. Consistent with this notion is the observation that DA-denervated MSNs are physiologically hyperexcitable.

If the function of DA is to modulate the ability of MSNs to gate glutamatergic drive, what compensatory mechanisms might the MSN exhibit in response to DA denervation? One change might be to alter glutamatergic transmission by decreasing the expression of glutamate receptors on MSNs. Alternatively, a change in the actual density of glutamater-

gic synapses, which are formed between cortical axons and the spines of MSNs, would delimit excessive corticostriatal glutamatergic drive onto MSNs. Both mechanisms appear to be at play (Arbuthnott, 2000; Dunah et al., 2000; Brown et al., 2005), and are probably interrelated.

Because cortical glutamatergic projections to the striatum synapse onto the heads of dendritic spines, a decreased density of glutamatergic synapses could occur because of a loss of the cortical projection neurons to the striatum, which has not been observed in PD, or may occur because of a decrease in the density of dendritic spines on MSN dendrites. In both animal models of parkinsonism (intranigral 6-hydroxydopamine lesions of the rat and MPTP treatment of mice) (see Arbuthnott, 2000) and postmortem studies of PD patients (Stephens et al., 2005; Zaja-Milatovic et al., 2005) a decrease in dendritic spine density and total dendritic length has been observed.

The loss of dendritic spines does not appear immediately after striatal DA denervation, but takes several days to develop; once present, the decrease in dendritic spine density does not appear to return to normal. Moreover, the decrease in dendritic spine density is seen in the putamen of PD patients treated with levodopa (Stephens et al., 2005; Zaja-Milatovic et al., 2005) and in the dopamine-depleted striatum of rats that were treated with levodopa (unpublished observations). These findings suggest that striatal dopamine loss may be the proximate cause of the dendritic remodeling, which once present can only be remedied by non-dopaminergic therapies.

The changes in dendrites of MSNs that occur in response to striatal DA denervation are not found on all MSNs, but instead are restricted to striatopallidal neurons that express the D_2 receptor (Day et al., 2005), i.e., are found on striatal neurons that contribute to the indirect pathway. This finding is consistent with previous studies that have found that chronic treatment with D_2 dopamine receptor antagonists such as haloperidol also causes dsytrophic changes in MSNs (Kelley et al., 1997). The observation that haloperidol causes the same dendritic changes in MSNs as striatal dopamine denervation underscores the argument that disruption of dopamine signaling is the proximate cause of the morphological changes in MSNs.

What are the mechanisms subserving the dendritic changes in MSNs that occur in response to dopamine depletion? As noted earlier, there are changes in certain glutamatergic receptors that probably represent a compensatory response to cope with increased corticostriatal drive onto MSNs (Dunah et al., 2000; Brown et al., 2005). A different but major change in MSNs involves the key intracellular signaling integrator calcium calmodulin-dependent protein kinase II (CaMKII). Striatal dopamine denervation leads to large and sustained increases in CaMKIIα activation, as reflected by phosphorylation of the enzyme at Thr^{286} (Brown et al., 2005). Because CaMKIIα phosphorylates a number of downstream targets, including the glutamatergic AMPA receptor subunit GluR1, we also followed changes in some of these proteins, but were surprised to observe no significant changes in either levels of a large number of postsynaptic density associated-proteins, including total or phosphorylated levels of GluR1 (Brown et al., 2005).

However, when we examined animals that sustained lesions of the striatal dopamine innervation one or two years earlier, we found that levels of GluR1 phosphorylated at the Ser^{831} residue were increased starting one year after striatal dopamine denervation, but that $pSer^{845}$-GluR1 levels did not change. This suggests that there is an interaction between aging and the consequences of dopamine denervation on striatal neurons. Because PD is an age-related disorder, it is clear that more work is needed on how aging permits physiological changes to emerge that are not seen in young animals with the same pathology. Alternatively, our data may suggest that duration of illness rather then age is the critical variable. It is clear that these findings

suggest that the biochemical changes reported in postmortem studies of PD must be carefully correlated with both age and duration of illness.

Because levodopa treatment does not appear to reverse the dsytrophic changes in MSN dendrites in either animal models of parkinsonism or in postmortem studies of PD, are there any other means of intervening to stop dendritic changes? Our understanding of the dendritic remodeling in MSNs suggests that glutamate (AMPA or NMDA) receptor antagonists or group II metabotropic glutamate receptor agonists might be effective, although these modifiers of glutamatergic transmission have not yet been studied in this context. We have taken a different approach, based on the question of how CaMKIIα is activated after striatal dopamine depletion.

CaMKII activation depends on an increase in intracellular Ca^{2+}; interesting, changes in intracellular Ca^{2+} are a key determinant of dendritic spine formation and morphology (Segal, 1995). There are several potential sources of the increase in calcium that drive phosophorylation of Thr^{286} on CaMKIIα. Among these are NMDA receptors, but levels of these receptors are decreased by striatal DA depletion. AMPA receptors do not normally conduct Ca^{2+}. Although AMPA receptors lacking the GluR2 subunit do conduct Ca^{2+}, our preliminary data suggests decreased levels of GluR2 and its anchoring protein GRIP in the dopamine-denervated striatum. Among other sources that contribute to increased intracellular levels of Ca^{2+} are intracellular stores linked to ryanodine, and L-type voltage gated calcium channels (LVGCCs).

LVGCCs are of particular interest because D_2 dopamine receptors, which appear to be critical for spine maintenance, inhibit the activity of these calcium channels (Hernandez et al., 2000). We therefore examined the effects of chronic administration of the LVGCC antagonist nimodipine on dopamine depletion-elicted decreases in dendritic spine density, and found that nimodipine completely blocked the spine loss (Day et al., 2005). Thus, although dopamine replacement by levodopa could not restore spine loss, targeting a downstream activator (LVGCCs) that was set into play by dopamine depletion could reverse dystrophic dendritic changes in MSNs.

The morphological changes in striatal MSNs that occur in response to dopamine denervation can be conceptualized as a plastic response that may function to dampen excessive corticostriatal glutamatergic drive that could cause cell death. The mechanisms involved are becoming more clear, and there are tantalizing clues from the animal data of potential treatment strategies. For example, we have seen that dendritic spine loss is maintained after dopamine depletion, and is still present one year after the initial insult when compared to age-matched control animals. This suggests that dendritic changes in MSNs may be very useful markers for studies of novel strategies to slow the progressive worsening of symptoms–but not dopamine loss–in PD. Clinical trials of neuroprotective drugs have largely been initiated based on clinical observations, and have been hampered by the lack of animal models that permit accurate assessment of progression. An animal model with which one could predict progression would allow more focused clinical trials of new therapies, particularly important in a time of dwindling financial resources with which to conduct clinical trials. Moreover, because there has been considerable controversy concerning the appropriate marker for progression, studies following spine loss in postmortem as well as preclinical studies may be illuminating.

The loss of dendritic spines may be protective and allow survival of MSNs, but only at a cost. If there is a progressive loss of dendritic spines there should be a point at which dopamine receptors, which are normally localized to spines, decrease in density. This would have the effect of rendering a PD patient less response to levodopa or agonist treatment. Although the idea that there is a decreased responsiveness to dopamine

replacement therapy late in the course of PD has been discussed for some time, the suggestion has been controversial because treatment-associated dyskinesias may set a ceiling above which escalating doses of levodopa is counterproductive. The dystrophic changes in MSN dendrites suggest that there is a pathophysiological basis for a decrease in responsiveness that grows more important over time. If the dsytrophic changes in MSN dendrites can be prevented or slowed, the loss of responsiveness to dopamine replacement may be correspondingly prevented or slowed. We found that a L-type voltage gated calcium channel antagonist prevented dendritic spine loss in animals with striatal dopamine denervation. LVGCC antagonists are in wide use for the treatment of hypertension. Although PD patients often have autonomic dysfunction, as well as orthostatic hypotension associated with dopamine replacement therapy, appropriate specific LVGCCs may still be useful because they reduce blood pressure in hypertensive patients but do not appear to do so in normotensive subjects.

We have become increasingly aware that PD does not just involve the degeneration of dopamine neurons, but is marked by a far more extensive pathological involvement of the brain. The involvement of the striatum in PD has been viewed primarily in the context of the loss of dopamine and the attendant changes in cortical inputs to the striatum, with little recognition of changes in the neurons of the striatum. As knowledge concerning the plasticity of striatal medium spiny neurons in both normophysiological and pathophysiological grows, so will new approaches to the treatment of Parkinson's and related diseases.

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The nigrostriatal DA pathway and Parkinson's disease

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Summary. The discovery of the nigrostriatal DA system in the rat was made possible by the highly specific and sensitive histochemical fluorescence method of Falck and Hillarp in combinations with electrolytic lesions in the substantia nigra and removal of major parts of the neostriatum. Recent work on DA neuron evolution shows that in the Bottlenose Dolphin the normal DA cell groups of the substantia nigra are very cell sparse, while there is a substantial expansion of the A9 medial and A10 lateral subdivisions forming an impressive ''ventral wing'' in the posterior substantia nigra. The nigrostriatal DA pathway mainly operates via Volume Transmission. Thus, DA diffuses along concentration gradients in the ECF to reach target cells with high affinity DA receptors. A novel feature of the DA receptor subtypes is their physical interaction in the plasma membrane of striatal neurons forming receptor mosaics (RM) with the existence of two types of RM. The ''functional decoding unit'' for DA is not the single receptor, but rather the RM that may affect not only the integration of signals in the DA neurons but also their trophic conditions. In 1991 A2A receptor antagonists were indicated to represent novel antiparkinsonian drugs based on the existence of A2A/D2 receptor–receptor interactions and here P2X receptor antagonists are postulated to be neuroprotective drugs in treatment of Parkinson's Disease.

Historical introduction

The development of the Falck-Hillarp method for the localization of catecholamines (CA) and serotonin (5-hydroxytryptamine, 5-HT) at the cellular level (see Carlsson et al., 1962) led to the demonstration of CA cell bodies in the substantia nigra (Dahlström and Fuxe, 1964a). These CA cell bodies most likely represented dopaminergic (DA) neurons, this statement being based on the biochemical correlates of this region of the brain (see Anden et al., 1964). Processes from these then putative DA cells were found to enter the zona reticulata and were mainly interpreted as DA axon bundles; however, they were later shown to mainly represent DA dendrites (Björklund and Lindvall, 1975). In the neostriatum, nucleus accumbens and olfactory tubercle, a densely packed punctate to diffuse CA fluorescence was found, this probably representing densely packed DA nerve terminals based on a pharmacological analysis and biochemical correlates (Fuxe, 1965a, b). With the development of a method for demonstration of monoamine containing nerve fibres based on axotomy with the accumulation of monoamines in the fibres on the

cell body side of the lesion (Dahlström and Fuxe, 1964b), it became possible, using strategically placed lesions, to trace the DA axons from the DA cells of the substantia nigra to the DA terminal regions of the striatum. The DA axons reached the crus cerebri, formed tracts in the capsula interna and entered the fibrae capsulae internae (Anden et al., 1964, 1965). These studies represented the first mapping of the nigrostriatal DA pathway in the rat, and subsequently it was shown that the monkey DA pathway was quite similar (Battista et al., 1972). The nigrostriatal DA pathway in the rat is schematically shown in Fuxe et al. (1985). The islandic striatal DA nerve terminal system was described in detail in 1972 (Olson et al., 1972; Tennyson et al., 1972), but was first reported by Fuxe in 1970 (see Fuxe et al., 1971). It mainly originates from the ventral tier of the substantia nigra, and contains calbindin negative nigral DA cells (see Gerfen, 2004), appears to be mainly involved in motivational learning (see Agnati et al., 2003a), and effects its action mainly via D1 and D4 receptors (Rivera et al., 2002; Agnati et al., 1988).

In the course of this early work plastic responses were found in the nigral DA cells after striatal ablation, which demonstrated early increases (2 days) in size and DA fluorescence intensity, followed in time (28 days) by shrinkage and disappearance of DA fluorescence in the DA cells of the zona compacta (Anden et al., 1965). These qualitative descriptions of plasticity in DA nerve cells were later on quantitatively described by Agnati et al. (1984) using morphometry and microdensitometery techniques (see also Janson et al., 1991). In the latter paper hypertrophy of DA cells could be observed in the vervet monkey after long term ventromedial tegmental lesions, representing compensatory responses to partial lesions of the nigrostriatal DA pathway. One of the most beautiful examples of plasticity in CA and 5-HT terminal systems are found in the cerebellum following endothelin 1 induced ischemic lesions causing hyperinnervation of the remaining granular cell islands in the lesioned cerebellar area (Fuxe et al., 1993). The microdensitometry and microfluorimetry of DA fluorescence disappearance after tyrosine hydroxylase inhibition in DA cell groups and DA terminal systems has had a strong impact on the discovery of discrete DA turnover changes in the brain in pharmacolcogical, physiological and pathophysiological experiments (Agnati et al., 1980a), providing a novel understanding of their function, and as targets for drug action.

The nigrostriatal DA pathway and brain evolution

Recently, Manger et al. (2004) have studied the distribution and characteristics of DA cells in the midbrain of the bottlenose dolphin revealed with tyrosine hydroxylase immunohistochemistry. It was observed that many components of the A9 DA nuclear complex were only weakly developed, with a minimal number of cells in the pars compacta, pars lateralis and pars ventralis. In contrast, the medial A9 and lateral A10 cell groups had merged and expanded to form a massive ventral wing of DA cells in the posterior substantia nigra, which may reflect the need for a DA modulation of whole body movements versus the specialized limb movements of other mammalian orders. As discussed already by Manger et al. (2004, see also Manger, 2005) it seems possible that the subdivision of CA cell groups and of other immunohistochemically identifiable transmitter cell groups may be the same within the same phylogenetic order of mammals. The above architecture of DA cell groups may therefore be typical for all cetaceans and represent an evolutionary trend that would be independent of brain size, phenotype and lifestyle. In support of this view Manger's group has recently found that the highveld molerat exhibits the same nuclear parcellation

of DA, 5-HT, and cholinergic cell groups as the laboratory rat, in spite of a significantly regressed visual system, an unusual circadian rhythm, and a subterranean behavioural phenotype (Da Silva et al., 2006). This line of investigation is beginning to allow us to understand how the DA system may be changing in the course of brain evolution and how this specifically relates to the emergence of novel phylogenetic orders of mammals. The next step will be to see how these changes affect the DA axonal trajectories and the DA terminal network, including the size and DA contents of their varicosities in various target regions and thus regional DA transmission. Understanding the evolutionary processes and occurrence of changes in the DA system is of importance when attempting to project results obtained in animal models to pathological conditions found in humans.

The nigrostriatal DA pathway and communication

Since the first indications of their existence (Fuxe, 1965a, b), the DA varicosities have been regarded as the sites for storage, synthesis and release of DA, all being originally regarded as representing synaptic terminals. However, after action of DA releasing compounds like amphetamine, a diffuse specific DA fluorescence, probably representing an extracellular fluorescence, appeared around DA cell bodies and dendrites in the midbrain (Fuxe and Ungerstedt, 1970), indicating that DA released by amphetamine action could reach the extracellular space. In 1975 Descarries et al. showed that the majority of the cortical 5-HT varicosities were asynaptic. In view of the indications that CA can be released from all varicosities (Malmfors, 1965; Fuxe, 1965a, b) it seemed likely that DA may not only operate via synapses but also via asynaptic varicosities, which seemed likely to be the major mode of communication in the partially DA denervated striatum (Fuxe, 1979). Based on a lack of correlation of the regional distribution of beta endorphin and enkephalin terminals and their opiate receptors, Agnati et al. (1986) introduced the concept of two principal modes of communication in the CNS: (1) volume; and (2) wiring, transmission (VT and WT). VT mainly takes place via the extracellular fluid and WT mainly via synapses. The electronmicroscopic observations that extrasynaptic striatal DA receptors and asynaptic striatal DA varicosities were in the majority made it clear that VT was the major mode of communication in the nigrostriatal DA pathway (Jansson et al., 2002), involving leaking DA synapses and asynaptic DA varicosities. These two transmission modes enables the DA filtering action on glutamate inputs to the striatal neurons, acting as a high pass filter (Agnati et al., 2005a).

In the nucleus accumbens shell, D1 receptor and TH immunoreactive (IR) terminal mismatches have been observed, where high densities of TH IR terminals, representing DA nerve terminals, surround D1 receptor rich rostrocaudal columns containing only few DA terminals, seen as patches in the transverse sections (Jansson et al., 1999). These results open up the possibility that DA may diffuse via concentration gradients into these patches to activate high affinity DA receptors involving distances of 100–200 μm. This migration process may be accelerated by the existence of pressure waves and temperature gradients causing movements of the ECF (Agnati et al., 2005b). Uncoupling protein 2, present in mitochondria, predict thermal synapses, since it produces a disappearance of the H^+ gradient with generation of heat (Horvath et al., 1999). It was therefore very significant that UCP2 rich terminal islands were in good register with the TH IR nerve terminals surrounding the D1 rich patches, with a high degree of overlap, but co-storage in the same DA terminal could not be determined (Rivera et al., 2006). It therefore seems possible that UCP2 may act to enhance the migration of DA into the D1 rich mismatch region. Strong UCP2 IR is

only located in discrete DA terminal systems in the ventral striatum and cerebral cortex which seem specialized for VT with large intensely TH IR varicosities (Rivera et al., 2006). These observations raise several novel aspects regarding the dynamics of DA VT in the brain.

By analyzing the migration of molecules in the brain with dual probe microdialysis, both probes in the striatum with an inter-probe distance of 1 mm, Hoistad et al. (2000) could not obtain evidence for long distance migration of intact 3H-DA, at least at physiological concentrations as studied in the out probe after chromatographic separation. Only indications for long distance migration of 3H-DOPAC and 3H-HVA were obtained. It is of interest that after DA denervation observations were obtained indicating an increased and specific clearance of 3DA derived compounds from the extracellular fluid into the brain circulation with changes in the blood-brain barrier, which is in line with the view that DA participates in the regulation of brain microcirculation (Iadecola, 1998).

The nigrostriatal DA pathway, receptor–receptor interactions and development of A2A antagonists for treatment of PD

The first indications for the existence of intramembrane receptor–receptor interactions were obtained in 1980 with substance P modulating the binding characteristics of high affinity 5-HT agonist binding sites in membrane preparations of the CNS, reflecting a possible increase in the number of high affinity 5-HT agonist binding sites (Agnati et al., 1980b). In 1981 it was possible to demonstrate that CCK-8 modulated the affinity and number of striatal D2 antagonist binding sites (Fuxe et al., 1981). In 1982 the receptor mosaic hypothesis of the engram was introduced (Agnati et al., 1982). Formation of supramolecular aggregates of receptors in the plasma membrane was postulated. This could affect the synaptic weight and be a mechanism for engram formation and thus represent the molecular basis for learning and memory. This work indicated that conformational changes in membrane receptors, induced via other receptors, could be produced not only via changes in membrane potential or via changes in their state of phosphorylation, but also via direct physical receptor–receptor interactions. The molecular basis was postulated to be heteromerization of the seven-transmembrane spanning G protein coupled receptors (Zoli et al., 1993). The first evidence came with the discovery of the GABA B heterodimer (see Marshall, 2001).

Two types of DA receptor mosaics (RM) can now be distinguished, namely DA RM1, formed by the same type or subtype of DA receptors and DA RM2, formed by DA receptors directly interacting with other types of transmitter receptors (GPCR or ion channel receptors) (see Agnati et al., 2003b, 2005a). DA receptor mosaics of a mixed type may also exist, these being formed by different receptors but with an RM1 present. RM1 can show cooperativity, since all receptors bind the same transmitter, in this case DA (Agnati et al., 2005a). These RM are located in the lipid rafts of the DA cells, where flottilin-1 seems to play an important role as an adapter protein (Jacobowitz and Kallarakal, 2004).

The A2A/D2 heteromeric receptor complex is of particular interest in relation to Parkinson's disease (PD), since it is located in the striato-pallidal GABA pathway, being the first neuron in the indirect pathway mediating motor inhibition when activated, and offers new treatment strategies for PD, as A2A inhibits D2 receptor recognition and signalling in the heteromeric complex (Ferre et al., 1991; Fuxe et al., 1998). Based on this work, A2A antagonists were postulated to be potential antiparkinsonian drugs acting by enhancement of D2 signaling. Unspecific adenosine receptor antagonists, like caffeine and theophyllamine, were in fact already shown in 1974 to enhance the motor activat-

ing effects of l-dopa and DA receptor agonists in rat models of PD (Fuxe and Ungerstedt, 1974), but the underlying mechanism was unknown. In view of the existence of the A1/D1 heteromeric complexes (Gines et al., 2000) in the direct GABA pathway inhibiting D1 recognition and signaling, the antiparkinsonian effects of these methylxanthines may be caused by a combined blockade of A1 and A2A receptors, enhancing D1 and D2 signaling, respectively. The evidence for the existence of A2A/D2 heteromers is based on coimmunoprecipitation experiments (Hillion et al., 2002), and by qualitative and quantitative assessment of fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) (Canals et al., 2003; Kamiya et al., 2003). Zones of interaction for A2A/D2 heterodimers have been analyzed, and an involvement of epitope–epitope electrostatic interactions have been demonstrated. By means of mass spectrometry and pull-down experiments a positively charged Arg-rich epitope from the N terminal part of the third intracellular loop of the D2 receptor has been shown to electrostatically interact with two adjacent aspartate residues or a phosphorylated serine, both negatively charged in the C terminal part of the A2A receptor (Ciruela et al., 2004). It is of significance that Woods and coworkers (Woods et al., 2005) have found that the interface in the D1/NMDA heteromeric complex (Lee et al., 2002) may involve similar electrostatic interactions. Thus, the C terminus of the NR1-1 subunit contains an Arg-rich epitope that can interact with adjacent glutamates or a phophorylated serine in the C terminal part of the D1 receptor. This electrostatic interaction could therefore represent a fundamental mechanism for heteromerization and receptor–receptor interactions.

Apart from the intramembrane A2A/D2 interaction in the heteromeric receptor complex, with A2A reducing the D2 decoding mechanism inter alia over adenylate cyclase (AC) and L type voltage dependent Ca channels, there exists a crosstalk at the AC. At this level D2 inhibits via Gi/o the A2A activation of the AC and thus A2A signalling (Fuxe et al., 2001). In advanced PD, when D2 signaling is very low, the A2A antagonist may therefore still show certain antiparkinsonian actions by counteracting the exaggerated A2A signaling set free by the low D2 tone. However, A2A antagonists may not modulate the other D2 effectors, like the activation of inwardly rectifying potassium channels and inhibition of calcium channels. When discussing the A2A/D2 interaction at the membrane level it should be considered that the stoichiometry of the A2A/D2 heteromeric complex, as for the A2A/D3 heteromeric complex (Torvinen et al., 2005), is unknown in the striatum. However, even a DA receptor tetramer can become modulated by an A2A monomer or homodimer, since they may regulate the cooperativity in the tetramer (Agnati et al., 2005a; Torvinen et al., 2005). Finally, it may be that the integration of the adenosine and dopamine induced conformational changes in these RM may be different when geometry (spatial organization) is different, due to differential modulation of the cooperativity in the DA receptor tetramer (Fuxe et al., 2006).

The major antiparkinsonian action of A2A antagonists appears to be the increase in the therapeutic ratio of l-dopa and D2 agonists allowing, for example, a lowering of the l-dopa dose with reduced development of dyskinesias (see Fuxe et al., 2001, 2003a; Chase, 2004), which may be explained by the existence of the striatal A2A/D2 heteromeric complex, where A2A inhibits D2 signaling. However, it is difficult to understand why A2A antagonists alone can exert antidyskinetic actions in models of PD (Kanda et al., 1998), since D2 signalling will become increased. However, a hypothesis has been advanced (Antonelli et al., 2006) that l-dopa induced dyskinesias may substantially be produced by a change in the balance of A2A/D2 heteromers versus A2A homomers in the

plasma membrane due to l-dopa induced internalization of A2A/D2 heteromers. Thus A2A homomers become dominant and abnormal increases in A2A signalling will develop with increases in inhibition of protein phosphatase 1. The excessive phosphorylation of the abnormal RM formed by the l-dopa induced panorama of transcription factors will assist in their stabilization involving phosphorylated ionotropic glutamate receptors (Chase, 2004), and abnormal motor programs will be formed with the appearance of dyskinesias. In this way we can begin to understand the multiple actions of A2A antagonists responsible for its antiparkinsonian and antidyskinetic actions primarily involving the striato-pallidal GABA neurons in the indirect pathway.

The D1 enriched direct pathway overexpresses D3 receptors upon development of l-dopa induced dyskinesias (Bordet et al., 2000). It therefore seems possible that antidyskinetic drugs can be developed based on a D3/D1 receptor interaction in the direct GABA pathway (Fuxe et al., 2003b).

Increases in the understanding of DA RM and their interactions with DA receptor interacting proteins forming a local horizontal molecular network and acting as an integrated recognition-transducing system in the lipid rafts of the membrane (Agnati et al., 2005a), will open up new targets for treatment of PD. Thus, there can be drugs developed acting inter alia on the synthesis and release of the DA receptor oligomeric building blocks in the endoplasmic reticulum, on adapter and scaffolding proteins for DA receptors, on DA receptor cotrafficking, on the insertion of the DA RM building blocks into the membrane, and on DA induced receptor assemblies.

The nigrostriatal DA pathway and PD

In sporadic PD there is increasing evidence that mitochondrial dysfunction, with reduced formation of ATP and increased formation of reactive oxygen species (ROS) leading to oxidative damage, plays an important role in the pathogenesis (Dauer and Przedborski, 2003; Beal, 2000). Both genetic susceptibility factors and environmental factors such as toxins, virus infections and hypercaloric diet may be involved in the etiology (Barja, 2004; Fuente-Fernandez and Calne, 2002).

Braak et al. (2004) have made the discovery that PD may be a multisystem disorder, where projection neurons with unmyelinated or weakly myelinated axons are especially vulnerable. The nigrostriatal DA neurons represent such a type of neuron. In presymptomatic stages mainly the medulla oblongata with the dorsal motor nucleus of the vagus nerve and the olfactory bulb are affected shown inter alia with alfa synuclein immunoreactivity, indicating a possible neuro-invasion by an unknown pathogen (Braak et al., 2003). It also seems possible however that these vulnerable neurons may be especially susceptible to mitochondrial dysfunction since there is high demand for ATP to make it possible for the Na/K ATPase to restore the resting membrane potential after each action potential depolarizing the entire axon as it travels long distances down to the terminals. The deficiencies in complex I activity in PD may also enhance the misfolding of proteins and their aggregation, resulting in abnormal protein–protein interactions (Agnati et al., 2005a), contributing to the neurodegeneration (protein conformational disorders). The ubiquitin-proteosomal pathway cannot cope with this increased demand for protein degradation, especially since this process is ATP dependent. The misfolding of proteins also takes place in the DA axons and terminals that may lead to interference with axoplasma flow that also is highly ATP dependent, explaining, for example, the swollen alfa-synuclein IR neurites found early in PD (Braak et al., 2004). These protein aggregates give rise to the Lewy bodies in PD.

There may be several reasons why, for example, certain DA nerve cells in the zona compacta, like those in the ventral and lateral

parts, are more vulnerable to neurodegeneration in PD than other DA cells in the zona compacta. It may be that they belong to different types of trophic units (Agnati et al., 1995) and therefore can't receive the same trophic support from extracellular FGF-2 (Fuxe et al., 1996) and GDNF (Grondin et al., 2002). It may also be that the DA cells with highest vulnerability have lower amounts of, or different types of, ATP sensitive potassium channels (KATP) that are not as sensitive to metabolic stress. Therefore, they cannot spare sufficient amounts of neuronal energy as they cannot effectively shut down the firing of the DA cells in response to the reduced ATP/ADP ratio via opening of the K^+ channels leading to hyperpolarization (see Liss and Roepner, 2001).

It is of importance that hydrogen peroxide (H_2O_2) can activate the sulfonylurea receptor 1 that contains KATP channels, causing glutamate dependent inhibition of striatal dopamine release (Avshalumov and Rice, 2003). This may be an additional reason why DA cells with these types of KATP channels, activated also by ROS, may show increased protection against PD (Liss and Roepner, 2001).

Based on the mitochondrial hypothesis of PD, it must be emphasized that the reduction of ATP signalling may be a significant factor in causing the degeneration of the nerve cells in PD, but this has not been previously discussed. Thus, ATP is known to be an extracellular activity dependent signalling molecule in neuron/glia communication

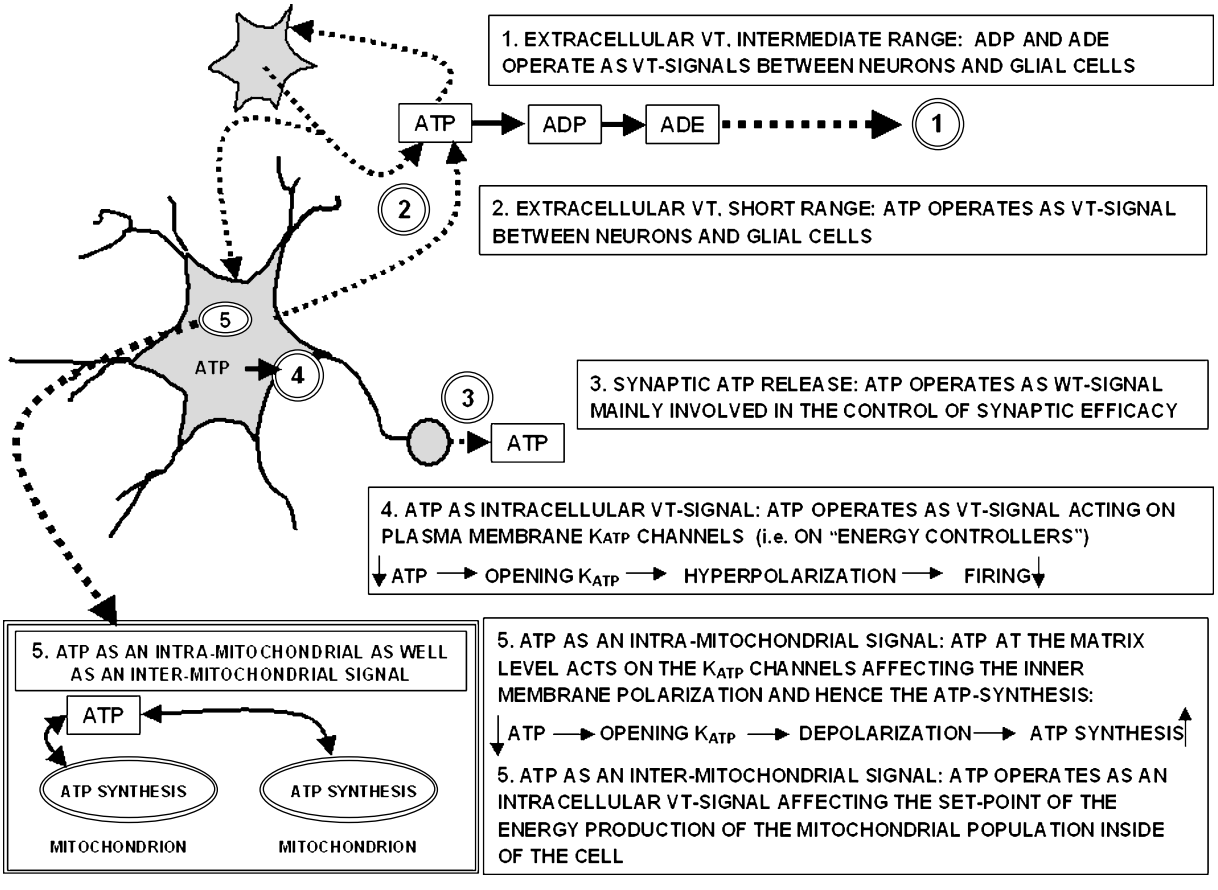

Fig. 1. Schematic illustration of ATP as a volume transmission (VT) and wiring transmission (WT) signal, and as an intracellular and intra-inter mitochondrial signal

acting via many subtypes of P2x (ion channel coupled) and P2y (G protein coupled) receptors and may exert trophic actions (Fields and Stevens, 2000; Burnstock and Knight, 2004). As seen in Fig. 1, ATP can operate as a short-range volume transmission (VT) signal between neurons and glia while its metabolites ADP and adenosine (ADE) can operate as intermediate VT signals. ATP may also operate as a synaptic signal. Thus, ATP may be released from DA cells and dendrites as a transmitter, like DA.

Reduction of ATP as an intracellular signal can, as discussed, lead to the opening of KATP channels on the plasma membrane with hyperpolarization and reduction of the firing rate. As also illustrated, ATP can act as an intramitochondrial and intermitochondrial signal. Its reduction here activates mitochondrial ATP sensitive potassium channels, leading to depolarization and an increase of ATP synthesis, in this way affecting the set point of energy production (Busija et al., 2004). Taken together the combined actions on KATP channels by ATP reductions will allow a balance to develop between firing and ATP synthesis (Fig. 2). If this energy balance is disrupted the nigrostriatal DA function wears off.

Such a disruption may be brought about if subtypes of ATP P2x receptors (see North, 2002) exist on the nigral DA nerve cells. These ATP receptors are permeable to small monovalent cations and certain subtypes to Ca ions. It is of significance that certain subtypes of P2x receptors may be activated by ROS, especially H_2O_2 and hydroxyl radicals, as demonstrated in vagal lung afferent fibres (Ruan et al., 2005) and such P2x receptors may be postulated to exist on DA nerve cells (Fig. 3). The resulting sodium influx will produce a depolarization of the DA cell since the Na/K-ATPase cannot maintain the ion gradient, and the KATP channels close upon depolarization (Bryan et al., 2004) and the inhibitory D2 autoreceptor activated inwardly rectifying K^+ current mainly operate at resting membrane potential (Fig. 3). The most interesting P2x receptor relating to this hypothesis would be the P2x7 receptor, as it fails to desensitize and larger currents are found upon repeated application. After several

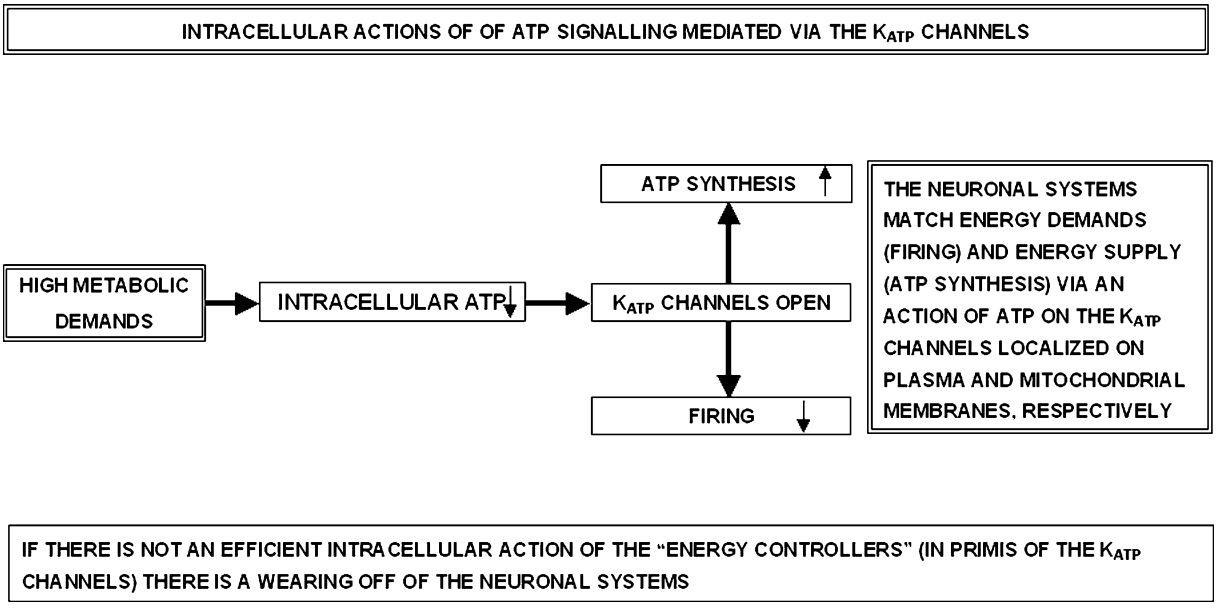

Fig. 2. Scheme of plasma membrane and mitochondrial KATP channel activation by ATP depletion leading to energy balance in conditions with high metabolic demands

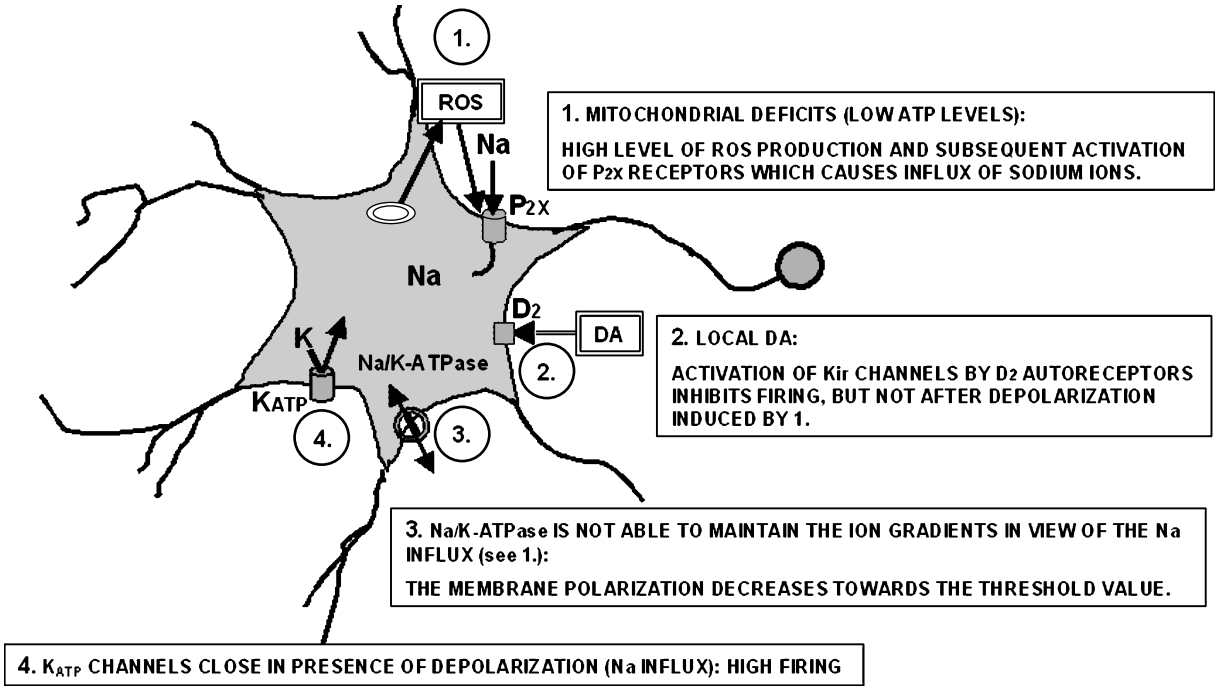

Fig. 3. Hypothesis on the possible role of P2x receptors in nigral DA nerve cells as to their degeneration in PD. Due to mitochondrial dysfunction in PD, ROS may be formed thereby activating the P2x receptors together with ATP released from degenerating nerve cells and glial cells with influx of especially Na ions, leading to depolarization, since the Na/K ATPase cannot maintain the ion gradients. The KATP channels close as the membrane depolarizes and cannot act as a brake nor can the D2 autoreceptors that activate inwardly rectifying K^+ channels operating close to the equilibrium membrane potential. The resulting high firing rate will cause a progressive ATP depletion of the DA neuron leading to cell death. Similar events may take place in other vulnerable neurons in PD and P2x receptors have been demonstrated in the dorsal motor nucleus of the vagus (Burnstock and Knight, 2004) where the pathology begins (Braak et al., 2004)

seconds of P2x7 activation, permeability to larger organic cations increases. This may either be related to a dilation of the pore of the P2x7 channel or to the activation of a distinct channel protein (North, 2002). After prolonged agonist application, the P2x7 activation leads to membrane blebbing, seen as large hemispherical protrusions, and finally to cell death (North, 2002). The pore formation with influx of extracellular chloride ions seems to play a major role for the apoptotic cell death induced (Tsukimoto et al., 2005). Antagonists of P2x7 receptors are presently being developed against inflammatory processes (Baraldi et al., 2004).

Based on the present hypothesis it will be of substantial interest to study the distribution pattern of P2x and P2y receptors in the nigrostriatal DA pathway and its subsystems, as well as in the adjacent astroglia and microglia, and how the receptor distribution pattern may change in models of PD, and in PD with focus on P2x7 receptors. This approach may reveal new aspects of the mechanisms of neurodegeneration in the nigrostriatal DA neurons in PD, and initiate novel strategies for neuroprotective treatments of PD based on, for example, the development of P2x receptor antagonists.

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Relationship between axonal collateralization and neuronal degeneration in basal ganglia

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Summary. In this paper we evaluate the hypothesis of a possible link between the degree of axonal collateralization of neurons located within the different components of basal ganglia and the vulnerability of these neurons to neurodegenerative or neurotoxic events. Our results stemmed from single-cell labeling experiments in rodents and primates, immunohistochemical study of the dopaminergic nigrostriatal pathway in parkinsonian monkeys, and immunocytological analysis of the human striatum in normal individuals and in patients with Huntington's disease. Our results indicate that projection neurons within virtually all basal ganglia components are endowed with a widespread and highly collateralized axon that yields a fixed number of terminals. Such a high degree of axonal collateralization allows exquisitely precise interactions between the various basal ganglia nuclei. However, the maintenance of this unique morphological trait implies high-energy consumption and renders basal ganglia neurons highly vulnerable to neurodegenerative, metabolic or neurotoxic insults.

Introduction

The pathological hallmark of Parkinson's disease (PD) is the presence of Lewy bodies in the dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) and the progressive loss of such neurons that project to the striatum via the nigrostriatal pathway. Although the degeneration of the nigrostriatal DA pathway is directly involved in motor symptoms of PD, a significant number of basal ganglia neurons other than those of the SNc show cytoskeletal damages in the sporadic form of PD. However, the causes of progressive cell losses and the mechanisms whereby neurons die in PD are still unknown. The aim of this study was to explore the possibility that the degree of axonal collateralization of neurons located within the different components of primate basal ganglia might render these elements more vulnerable to neurodegenerative, metabolic or neurotoxic insults.

Material and methods

This study is based on: (1) analysis of material from single-cell labeling experiments in rodents and monkeys; (2) immunohistochemical studies of the DA nigrostriatal pathway in parkinsonian monkeys; and (3) investigation of human striatum in normal individuals and in patients who suffered from Huntington's disease (HD). Animal and human experiments were undertaken in accordance with the Canadian guidelines for the use and handling of animal and human brain material and Laval University Ethic Committee approved our protocols. Single-cell labeling experiments were undertaken in Sprague-Dawley rats and cynomolgus monkeys (*Macaca fascicularis*) of both sexes. The animals were anesthetized and received bilateral microiontophoretic injections of anterograde tracer biotin dextran amine in different components of the basal ganglia. After a survival period of 8–10 days, the animals were

sacrificed and their brain were sectioned along diverse planes with a freezing microtome. The neuronal tracer was revealed by using diaminobenzidine as the chromogen (see Parent et al., 2001; Prensa and Parent, 2001). The nigrostriatal DA pathway was examined in 2 normal cynomolgus monkeys and 4 cynomolgus monkeys that were rendered parkinsonian by intravenous injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP; total dose: 5 mg). The MPTP-injected monkeys became severely bradykinetic and were sacrificed 4 months after initial MPTP exposure. Their brains were processed according to the standard avidin-biotin complex immunohistochemical method for the demonstration of the DA marker tyrosine hydroxylase, which was visualized by using diaminobenzidine as the chromogen. Observations were also made on human post-mortem striatum from 4 patients with HD (grades 2 to 3) and 4 age-matched controls. A standard immunohistochemical procedure was used to verify the status of the various types of chemospecific neurons in the human striatum in HD cases compared to controls, as described in detail elsewhere (Cicchetti et al., 2000).

Results

Single-cell labeling studies

These studies showed that virtually all components of primate basal ganglia and associated thalamic nuclei, including the striatum, external (GPe) and internal (GPi) segments of globus pallidus, subthalamic nucleus (STN), centre median/parafascicular thalamic complex (CM-Pf), and substantia nigra (SN), harbor several types of projection neurons endowed with a long, widespread and highly branched axon. The most extreme example is GPi neurons, the axon of which arborizes profusely within the thalamus and brainstem (Fig. 1). Despite their relatively small cell body (about 30 μm in diameter), these neurons support an axon of about 30 cm long. Hence, the cell body/axon length ratio is such a case is of about 1/10,000. Single-cell labeling studies have also provided the first clear picture of the complexity of the axonal arborization of single DA neurons of the SNc in both rodents and primates. The data revealed that, irrespective of their location in the ventral or dorsal tiers of the SNc, DA nigral neurons project to either the striosomes (patches) or the extrastrisomal compartments of the striatum by means of a long and highly arborized axon. The pattern of axonal arborization varies markedly from one neuron to the other. Some axons arborizes profusely in a large sector of the striatum, but provide very few axon collateral to extrastriatal structures, such as the pallidal complex and STN, whereas other axons branched profusely in non-striatal structures but provide only a few thin collaterals to the striatum. These two types of axons appear to represent the two extremes of a widespread morphological continuum that contains many intermediate or mixed forms of axonal arborizations. Or data suggest that the pattern of axonal arborization of nigrofugal DA neurons obeys a simple rule, that is, the more the axon arborizes in the striatum the less it does so in non-striatal structures, and vice versa.

Parkinsonian monkeys

Observations in PD monkeys have revealed that nigrostriatal DA neurons aborizing profusely in the striatum are much more vulnerable to degeneration than nigral neurons with an axon that branches abundantly in non-striatal structures and relatively little in the striatum. In accordance with neuroanatomical findings described above, the data obtained in PD monkeys indicate that the nigrostriatal DA system is composed of several subsystems, each having its specific origin in the SNc, its particular terminal region in the striatum and its own degree of vulnerability to neurodegenerative processes that are at play in PD. The most highly vulnerable neurons are those whose cell body is located in the ventral tiers portion of the SNc and whose axon arborizes principally in the sensorimotor territory of the striatum. These are the DA neurons that contains neuromelanin pigments and which are known to degenerate massively in sporadic PD. Much less sensitive to degeneration are the DA neurons with a cell body in the ventral tegmental areas whose axon arborizes principally in the ven-

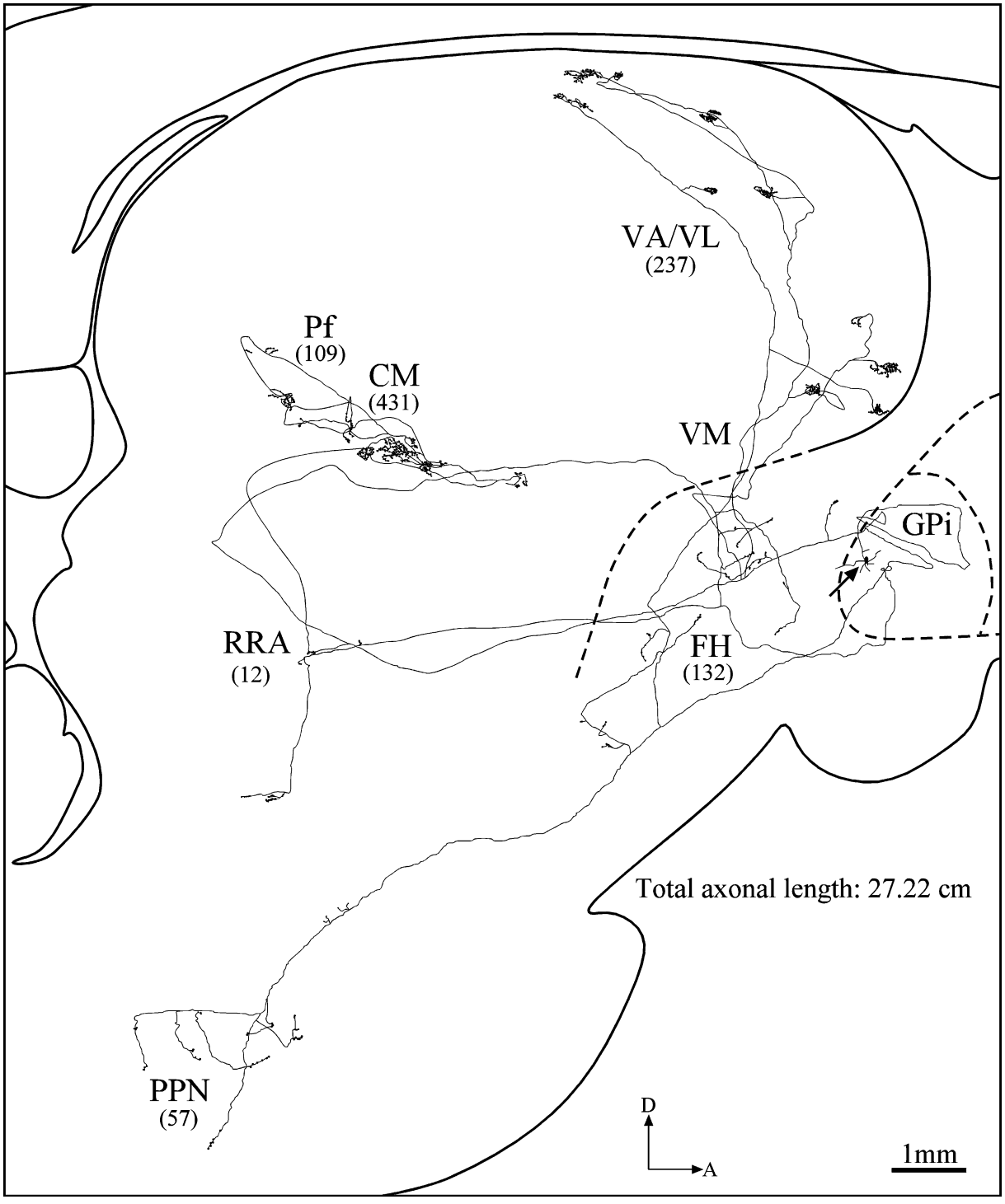

Fig. 1. Axonal arborization of a GPi neuron that projects to the ventral tier thalamic nuclei, centre médian/parafascicular thalamic complex, pedunculopontine tegmental nucleus, Forel's field H, zona incerta and retrorubral area, as viewed on sagittal plane. The number of axonal varicosities observed in each target site is indicated in parentheses, whereas the arrow points the location of the cell body. The axon was entirely reconstructed from serial sections using camera lucida drawings and a computerized image-analysis system. *CM* centre médian thalamic nucleus; *FH* Forel field H; *GPi* internal segment of the globus pallidus; *Pf* parafascicular thalamic nucleus; *PPN* pedunculopontine tegmental nucleus; *RRA* retrorubral area; *VA/VL* ventral anterior/ventral lateral thalamic nuclei; *VM* ventromedial thalamic nucleus

tral (limbic) territory of the striatum. Also less sensitive to degeneration are the DA neurons that project directly to GPi and much less so to striatum. The latter neurons, as well as those at the origin of the mesolimbic dopaminergic pathway, express calbindin, a calcium-binding protein that maintains intracellular calcium homeostasis and which may thus confer some resistance against neurodegenerative processes.

Huntington's disease

The pathological hallmark of HD is a gross atrophy of the striatum, which is the result of a massive neuronal loss of the striatal medium spiny projection neurons. Our observations indicate that, in contrast to medium-sized spiny projection neurons, virtually all the large and medium-sized aspiny local circuit neurons of the striatum are spared in HD.

Discussion

The long, widespread and highly collateralized axon of basal ganglia projection neurons allows exquisitely precise interactions between the various basal ganglia components and related thalamic nuclei. However, the maintenance of this unique morphological trait imposes upon neurons a metabolic burden that may render them highly vulnerable to neurodegenerative, neurotoxic or ischemic insults. This is particularly the case for pallidal neurons, which degenerate massively in cases of toxic encephalopathy. Laplane et al. (1984) have shown that toxic lesions involving mainly the pallidal complex can produce severe signs of psychic akinesia, without significant motor deficits. These findings indicate that basal ganglia, and principally the pallidal complex, have important non-motor functions that may be severely altered by toxic or hypoxic insults.

Congruent with the results of our single-cell labeling experiments in primates, the data obtained in PD monkeys suggest that, in contrast to what is commonly believed, the nigrostriatal DA pathway is not a monolithic entity. Instead, this important projection system appears to be composed of different neuronal subunits, each having its own site of origin, its specific terminal fields within the striatum, and its particular degree of vulnerability to neurodegenerative processes. Here again, the most sensitive subsystems are those composed of neurons whose axon is highly collateralized and widely distributed within the striatum.

Likewise, striatal neurons destined to die in HD are exclusively projection neurons, the majority of which possesses a long axon that arborizes in the three major striatal target structures. In contrast, striatal neurons with a short, intrinsic axon are specifically spared in the striatum of HD patients. These findings suggest that the main pathological lesion in HD might lie not in the striatum, as commonly believed, but in striatal targets.

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Pathology associated with sporadic Parkinson's disease – where does it end?

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Summary. Parkinson's disease (PD) is a multisystem disorder in which predisposed neuronal types in specific regions of the human peripheral, enteric, and central nervous systems become progressively involved. A staging procedure for the PD-related inclusion body pathology (i.e., Lewy neurites and Lewy bodies) in the brain proposes that the pathological process begins at two sites and progresses in a topographically predictable sequence in 6 stages. During stages 1–2, the inclusion body pathology remains confined to the medulla oblongata, pontine tegmentum, and anterior olfactory structures. In stages 3–4, the basal mid- and forebrain become the focus of the pathology and the illness reaches its symptomatic phase. In the final stages 5–6, the pathological process is seen in the association areas and primary fields of the neocortex. To date, we have staged a total of 301 autopsy cases, including 106 cases with incidental pathology and 176 clinically diagnosed PD cases. In addition, 163 age-matched controls were examined. 19 of the 301 cases with PD-related pathology displayed a pathological distribution pattern of Lewy neurites and Lewy bodies that diverged from the staging scheme described above. In these cases, olfactory structures and the amygdala were predominantly involved in the virtual absence of brain stem pathology. Most of the divergent cases (17/19) had advanced concomitant Alzheimer's disease-related neurofibrillary changes (stages IV–VI).

Introduction

Sporadic Parkinson's disease (PD) entails the selective degeneration of circumscribed portions of the human nervous system (Wakabayashi et al., 1990; Takahashi and Wakabayashi, 2001, 2005; Jellinger and Mizuno, 2003; Braak et al., 2004).

At neuropathological examination, all of the patients suffering from this disorder reveal the presence of α-synuclein-immunoreactive inclusion bodies in vulnerable neuronal types. These inclusions occur as spindle-shaped and, in part, branching Lewy neurites (LNs) within the dendrites and axons of involved nerve cells. Somatic inclusions may appear as punctate aggregations close to the deposits of neuromelanin or lipofuscin granules. Larger but more weakly immunoreactive globular inclusions can also be observed in the form of pale bodies, which probably go on to evolve into strongly labeled Lewy bodies (LBs) (Lowe, 1994;

Goedert, 2001; Takahashi and Wakabayashi, 2001, 2005; Kuusisto et al., 2003; Saito et al., 2003; Braak et al., 2004; Mikolaenko et al., 2005).

Individuals with clinical symptoms of ''parkinsonism'' in the absence of LNs or LBs form a heterogeneous group of motor dysfunctions, including multiple system atrophy, progressive supranuclear palsy, neurodegeneration with brain iron accumulation I, and some forms of familial PD (Galvin et al., 2001; Lantos and Quinn, 2003; Jellinger, 2003a; Pankratz and Foroud, 2004), so that neuropathological confirmation of the clinical diagnosis is essential in each instance.

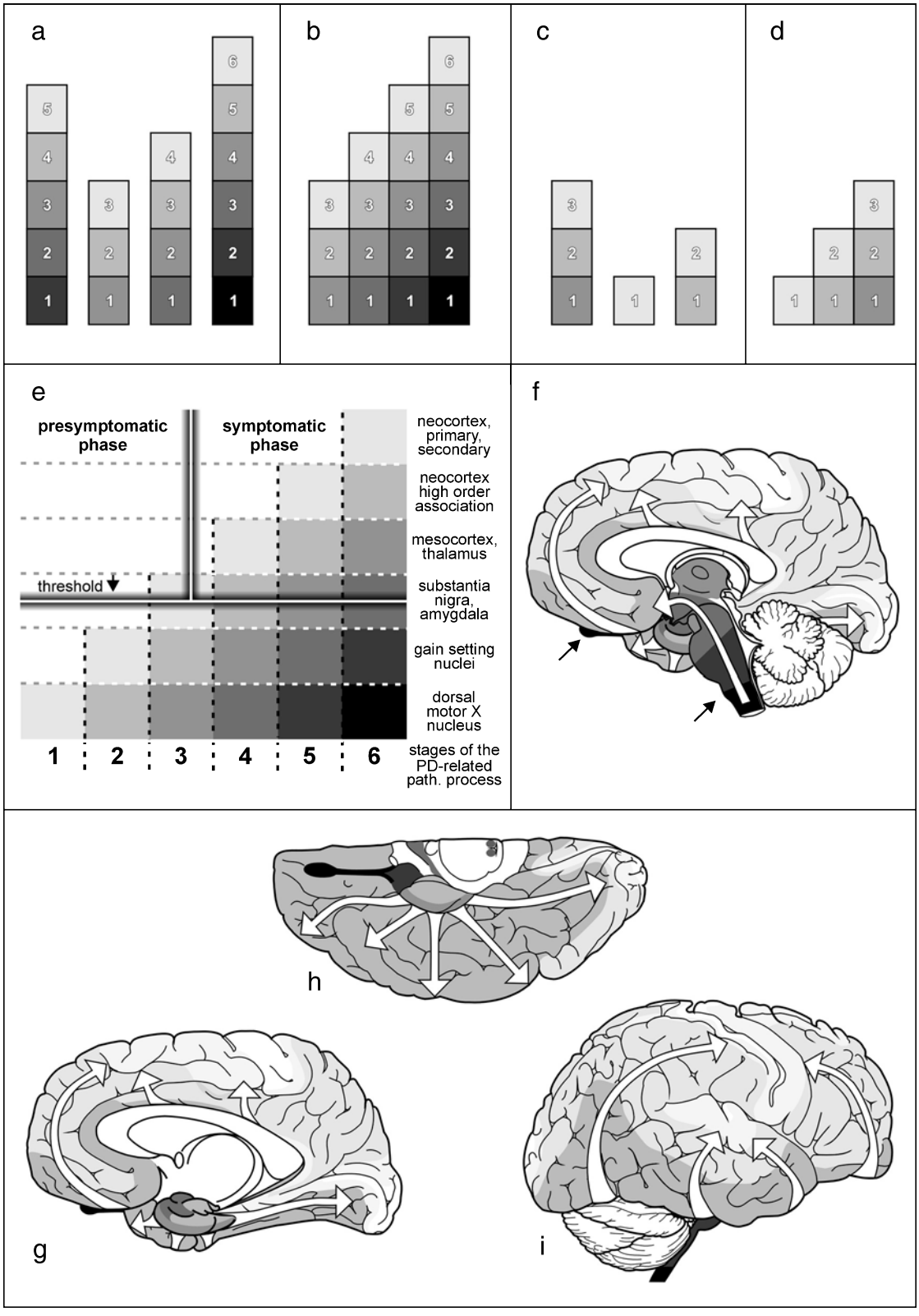
Only a few of the many neuronal types within the human nervous system are prone to develop the PD-related lesions. This selective involvement is reflected in the regional distribution pattern of the pathology. The disease process is not haphazard (Braak et al., 2003a, 2004; Del Tredici and Braak, 2004). These features enable the observer to recognize the presence of the pathological process even in individuals without PD-related motor symptoms.

Although LNs and LBs are more prevalent with advancing age, they do not occur in all elderly non-symptomatic individuals or age- and gender-matched controls. Thus, we refrain from viewing incidental LB pathology as a normal concomitant of aging (Braak et al., 1995; Dickson, 1998; Del Tredici et al., 2002; Mikolaenko et al., 2005).

Sporadic PD requires years to develop. As in nearly every illness, some individuals arrive at, and cross, the threshold from a subclinical phase to the symptomatic manifestation of disease (Thal et al., 2004). The symptoms begin almost imperceptibly and worsen over time. In the autopsy material available to us, symptomatic cases can be assigned to one of four subgroups that can be differentiated based on changes in the topographic distribution pattern of the lesions in the brain. Each subgroup displays newly affected regions in addition to the lesions in previously involved ones (Fig. 1a). The majority of sporadic PD autopsy cases can be grouped in this way. Given the consistency of this finding and assuming that the disease process increases in extent and severity in a predetermined manner, the four clinical subgroups can be arranged in an ordered sequence (Fig. 1b). Support for this hypothetical reconstruction comes from a number of non-motor symptoms that become manifest during the clinical disease course and possibly correspond to the gradual progress of the pathological process (Abbott et al., 2001; Doty, 2001; Tissingh et al., 2001; Hawkes, 2003; Ponsen et al., 2004; Ahlskog, 2005; Stiasny-Kolster et al., 2005; Braak et al., 2005).

By comparison, relatively little is known about the presymptomatic phase in PD (Koller et al., 1991; Sawle, 1993; Wolters et al., 2000; Ahlskog, 2005). That such a phase in PD exists, however, has been shown

Fig. 1. a Virtually all clinically diagnosed PD cases can be assigned to one of four subgroups based on the topographic distribution pattern of the brain pathology. Each subgroup displays newly affected regions in addition to the inclusion body pathology in previously involved regions. **b** Given the assumption that the disease process increases in extent and severity, the four clinical subgroups can be arranged in an ordered sequence. **c** The presymptomatic cases fall into one of three different subgroups. **d** Once again, the subgroups can be arranged such that the pathological process can be seen to progress in extent and severity. **e** In both the presymptomatic and the symptomatic phases of sporadic PD, inclusion bodies occur in the same vulnerable neuronal types. Taken together, both phases presumably reflect the entire spectrum of the disease process in six neuropathological stages. Increasing density of the gray shading in areas underneath the diagonal indicates the growing severity of the pathology in vulnerable brain regions indicated at right hand margin. **f–i** Diagrams showing the gradual ascent of the pathological process underlying sporadic PD (white arrows) that begins in the lower brain stem and olfactory bulb (black arrows in f) and ends in the cerebral cortex


a
b
c
d
e
presymptomatic phase
symptomatic phase
neocortex, primary, secondary
neocortex high order association
mesocortex, thalamus
threshold
substantia nigra, amygdala
gain setting nuclei
dorsal motor X nucleus
1 2 3 4 5 6
stages of the PD-related path. process
f
h
g
i

by PET studies (Morrish et al., 1998; Brooks, 2000). In our material, non-symptomatic cases that show PD-associated brain pathology fall into one of three subgroups (Fig. 1c). As in the symptomatic cases, these subgroups differ with respect to their lesional distribution patterns (Del Tredici et al., 2002; Braak et al., 2003a). Once again, it is the changes in the regional distribution pattern of the inclusion bodies that provide the logic for arranging the subgroups such that the disease process is seen to increase in extent and severity in a methodical sequence (Fig. 1d). According to this view, incidental LNs and LBs, even when minimally present, are innately pathological entities (Forno, 1969; Del Tredici et al., 2002; Thal et al., 2004; Takahashi and Wakabayashi, 2005).

By the time subtle symptoms become recognizable, susceptible brain stem nuclei, including the dorsal motor nucleus, the lower raphe system, and locus coeruleus, are already heavily involved. Assuming that all of these sites are not affected with identical severity from the very outset of the disease process, a more likely explanation would be that a mild to moderate burden of pathology develops over time in each of these nuclei until the cumulative threshold from presymptomatic to symptomatic disease manifestation is crossed. The pathological process underlying both phases is marked by the presence of the same types of inclusion bodies in the same susceptible neuronal types in specific brain regions. Insofar as the lesional pattern of the last presymptomatic subgroup closely resembles that of the first symptomatic subgroup, both sets of subgroups combined reflect the entire spectrum of the pathology associated with sporadic PD (Fig. 1e).

Stages of sporadic PD-associated pathology in the brain

Each of these considerations prompted the design of a staging procedure that included assessment not only of clinically diagnosed sporadic PD cases but also cases with incidental LNs/LBs because such lesions probably represent the first step along a disease continuum. And, in fact, spontaneous remission seems not to occur after formation of the initial lesions or after the threshold to clinical disease has been crossed (Braak et al., 2003a). A methodological limitation to this approach is that the progression of the pathological process was reconstructed postmortally using cross-sectional data. Thus, the conclusions drawn from these data are not definitive, although it can be argued that they rest upon admissible assumptions. Clearly, the discovery of suitable biological markers that can be measured longitudinally can help to refine or refute the proposed staging procedure (Rachakonda et al., 2004).

For practical reasons, staging initially was confined to the brain. In addition to 301 brains from incidental and clinically diagnosed PD cases (Table 1A–C), 164 age-matched controls were examined and assessed semiquantitatively (Table 1I). The lesional distribution pattern in all known selectively vulnerable brain regions was studied in thick sections (100 μm) immunostained for α-synuclein (Braak et al., 2003a). With exception of the study by Parkkinen et al. (2005), other autopsy-based studies to date report that it has been possible, for the most part, to replicate the staging procedure described here (Jellinger, 2003b, 2004; Saito et al., 2004; Neumann et al., 2005).

Stage 1

Within the brain, the earliest signs of α-synuclein immunoreactivity occur simultaneously in the dorsal motor nucleus of the vagus nerve and anterior olfactory structures (Fig. 1f, arrows). They do not occur in any of the other brain regions known to become involved at later disease stages. This means that the PD-related pathology does not begin anywhere whatsoever in the brain or at multiple brain sites but, rather, in all probability only in the

Table 1. Demographic data of the 301 cases examined

	N	Age	SD
A. All cases with sporadic Parkinson's disease (PD)-related pathology			
total	**301**	77.3	±8.1 years
female	139	78.1	±7.9 years
male	162	76.6	±8.2 years
B. Cases with predominantly incidental PD-related pathology (PD stages 1–3)			
total	**106**	77.3	±7.8 years
female	46	78.2	±7.6 years
male	60	76.6	±8.0 years
C. Predominantly clinically diagnosed cases of sporadic PD (PD stages 4–6)			
total	**176**	76.8	±8.0 years
female	83	78.0	±7.7 years
male	93	75.7	±8.2 years
D. Cases with distribution patterns of PD-related pathology diverging from PD stages 1–6			
total	**19**	81.7	±9.5 years
female	10	78.9	±11.7 years
male	9	84.8	±5.2 years
E. Cases with Alzheimer's disease (AD)-related neurofibrillary pathology (NF) stages I–III and concomitant PD-related pathology (PD stages 1–6)			
total	**230**	76.4	±7.7 years
female	102	77.2	±7.2 years
male	128	75.8	±8.0 years
F. Cases with AD-related NF stages I–III and concomitant PD-related pathology but with a distribution pattern diverging from the PD stages 1–6			
total	**2**	87.0	±7.1 years
G. Cases with AD-related NF stages IV–VI and concomitant PD-related pathology (PD stages 1–6)			
total	**49**	79.5	±9.0 years
female	25	82.0	±8.3 years
male	24	76.8	±9.0 years
H. Cases with AD-related NF stages IV–VI and concomitant PD-related pathology but with a distribution pattern diverging from the PD stages 1–6			
total	**17**	81.1	±9.7 years
I. Cases without PD-related pathology in vulnerable regions known to be affected in sporadic PD (controls)			
total	**163**	79.0	±10.0 years
female	96	79.6	±9.8 years
male	67	78.2	±10.5 years

lower medulla oblongata and in anterior olfactory structures.

The olfactory pathology is seen chiefly in the anterior olfactory nucleus within the olfactory tract (Pearce et al., 1995; Mesholam et al., 1998; Hawkes et al., 1999; Doty, 2001; Hawkes, 2003). A few LNs appear there at first and then develop into a dense network interspersed with LBs. The olfactory pathology is not prone to make incursions into non-olfactory sites. Accordingly, the disease process appears to begin chiefly in the dorsal motor nucleus of the vagus nerve. From there, it progresses steadily upwards towards the cerebral cortex (Figs. 1f–i) (Braak et al., 2003a, b). Stage 1 cases exhibit mainly a few isolated LNs in the dorsal motor nucleus and some faintly immunoreactive α-synuclein aggregations in preganglionic vagal axons connecting the central nervous system with the postganglionic neurons of the enteric nervous system (Huang et al., 1993; Hopkins et al., 1996).

Stage 2

In stage 2, the lesions in the dorsal motor nucleus worsen and inclusion bodies also occur in the lower raphe nuclei, notably the great raphe nucleus, and magnocellular portions of the adjoining reticular formation. LNs also may occur for the first time in the locus coeruleus.

During the first two stages, the pathology in non-olfactory sites is confined to the medulla oblongata and pontine tegmentum. The nosological process that ultimately leads to the clinical picture of PD does not begin in the substantia nigra (Del Tredici et al., 2002; Braak et al., 2003a; Lang and Obeso, 2004; Ahlskog, 2005) and nigral involvement presupposes obvious affection of lower brain stem nuclei. Were it possible to diagnose PD already in the early, presymptomatic stages 1 or 2, and were a causal therapy to become available, the subsequent destruction of the substantia nigra could be prevented.

Stage 3

In stage 3, the disease process crosses the upper limit of the pontine tegmentum and the lesions are seen in basal portions of the mid- and forebrain.

LNs occur for the first time in the substantia nigra, pars compacta, followed by the appearance of LBs within the melanized dopaminergic projection neurons located there. Macroscopically, the nucleus appears to be intact and, even microscopically, shows no clear signs of neuronal loss (Braak et al., 2004).

The pathological process also is evident in the amygdala – above all in the central subnucleus (Braak et al., 1994) – as well as the cholinergic tegmental pedunculopontine nucleus and cholinergic magnocellular nuclei of the basal forebrain.

Stage 4

In stage 4 cases, the picture is increasingly dominated by the heavy involvement of the amygdala. Moreover, a specific portion of the cerebral cortex, namely the anteromedial temporal mesocortex (Braak, 1980), shows the first visible signs of the pathology. A thick network of LNs emerges in the superficial layers of this transitional cortical region and many deep layer projection cells contain LBs. The temporal mesocortex is highly susceptible to both sporadic PD and AD (Braak and Braak, 1992; Braak and Del Tredici, 2004; Braak et al., 2004).

To the extent that the clinical protocols of some stage 3 and most stage 4 cases mention beginning PD-related motor symptoms, it can be postulated that, at about this time, the presymptomatic phase yields to the clinically recognizable disease phase (Braak et al., 2003a).

Stages 5 and 6

In the final stages 5 and 6, the neurodegenerative process attains its greatest topographic extent (Figs. 1f–i). Vulnerable portions of the substantia nigra appear macroscopically nearly denuded of melanoneurons. With the temporal mesocortex as its starting point, the inclusion body pathology gradually extends into the neocortex. Pyramidal cells that contain LBs appear first in high-order association areas of the neocortex (stage 5), then in first order association areas, and, finally, in some instances, even in the primary sensory and motor fields.

During stages 5 and 6, patients usually manifest the full range of PD-related clinical symptoms. Damage to the autonomic, limbic, and somatomotor systems can become compounded by impairment of superordinate limbic system centers and functional deficits attributable to the cerebral cortex (Apaydin et al., 2002; Braak et al., 2005).

Concomitant PD-related α-synuclein and AD-related neurofibrillary pathologies

Previous studies have described an amygdala-predominant pattern of α-synuclein pathology without affection of the lower brain stem – mainly in cases with concomitant neurofibrillary pathology (Arai et al., 2001; Kotzbauer et al., 2001; Jellinger, 2003b). The question arises whether the proposed staging procedure can be applied to all cases with PD-related inclusion bodies. Clearly, there appear to be some exceptions (Table 1D).

We recently examined α-synuclein immunoreactivity in two autopsy groups with mild (NF stages I–III) and advanced (NF stages IV–VI) neurofibrillary changes (Table 1E–H). In the group with mild changes (N = 232), the regional distribution pattern of the PD-related lesions diverged from the proposed staging procedure twice (Table 1F). The brain stems of these two individuals were intact or nearly intact. All remaining cases could be assigned to a PD stage (Table 1E, N = 230).

The proportion of divergent cases increased to 17, however, when severe intraneuronal

changes associated with advanced Alzheimer's disease (N = 66) also were present (Table 1G, H). The α-synuclein pathology in these 17 cases occurred predominately in olfactory structures and the amygdala. Although it is too soon to tell, our working hypothesis is that the concomitant pathology, equivalent to stages IV to VI of Alzheimer's disease, may influence the development of a supervening Parkinson process (Hamilton, 2000; Geddes, 2005).

In closing, the staging procedure describes the hypothetical origins and progression of the disease process in the brain. According to this protocol, the neocortex appears to be the final destination of the Parkinson-related pathology. It still needs to be determined, however, not only how the pathological process begins and progresses in extent and severity with disease duration but also, and perhaps more importantly, whether the disease process commences within the central nervous system at all (Braak et al., 2003b; Del Tredici and Braak, 2004).

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Critical appraisal of brain pathology staging related to presymptomatic and symptomatic cases of sporadic Parkinson's disease

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Summary. Clinical Parkinson's disease (PD) is a well-characterised syndrome that benefits significantly from dopamine replacement therapies. A staging procedure for sporadic PD pathology was developed by Braak et al. assuming that the abnormal deposition of α-synuclein indicates the intracellular process responsible for clinical PD. This paradigm has merit in corralling patients with similar cellular mechanisms together and determining the potential sequence of events that may herald the clinical syndrome. Progressive pathological stages were identified – 1) preclinical (stages 1–2), 2) early (stages 3–4, 35% with clinical PD) and 3) late (stages 5–6, 86% with clinical PD). However, preclinical versus early versus late-stage cases should on average be progressively older at the time of sampling, a feature not observed in the cohort analysed. In this cohort preclinical cases would have developed extremely late-onset PD compared with the other types of cases analysed. While the staging scheme is a valuable concept, further development is required.

Requirement for staging disease progression

Parkinson's disease (PD) is currently characterised by several cardinal motor symptoms, including rigidity, bradykinesia and/or resting tremor, that benefit significantly from dopamine replacement therapies. However, it is clear that considerable neuronal destruction occurs prior to the onset of these motor symptoms with commensurate compensatory mechanisms accounting for the delay in clinical onset (Jankovic, 2005). It is therefore necessary to recognise this presymptomatic disease stage to advance knowledge on the cellular mechanisms underlying the disease process.

Clinical demographics of sporadic Parkinson's disease

Sporadic PD is one of the most reliable and stable neurological diagnoses with 92% of the clinical features remaining unchanged for 5–10 years (Jankovic et al., 2000). While the incidence of sporadic PD significantly increases with age, the average age of onset for levodopa-responsive motor symptoms is around 60 years old. Fewer studies have assessed the average age at death for sporadic PD patients, but recent large-scale risk analysis using death and mortality as outcomes suggest that patients survive to 76 years of age on average (Wirdefeldt et al., 2005). These data show that, in the relatively homogeneous group of patients with dopa-

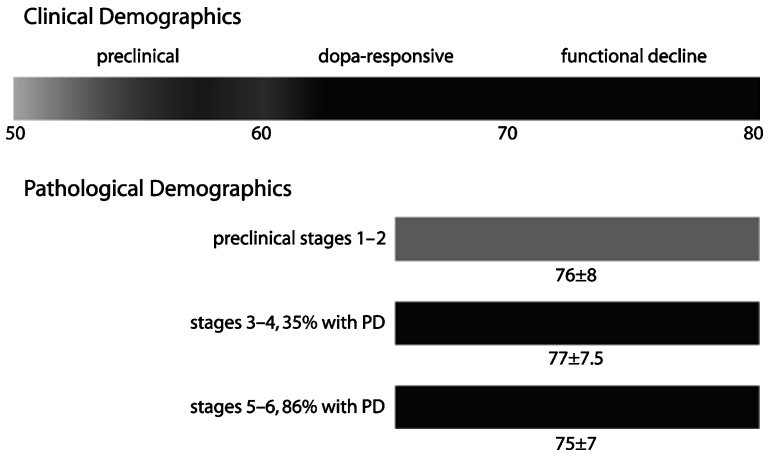

Fig. 1. Stages identified for sporadic PD and average demographics compared with the stages identified in the Braak staging scheme for sporadic PD and the demographics of the cases fulfilling criteria for these pathological stages. Note the considerable overlap in demographics for the pathological stages suggesting that, potentially, only the cases in stages 5–6 are comparable with the majority of clinical cases analysed

responsive deficits, the disease is very slowly progressive with several stages clearly defined (Fig. 1). The presymptomatic period is estimated at 5–10 years most commonly occurring prior to the age of 60 (Jankovic, 2005). There is a 5–10 year period of relatively stable motor deficits occurring for most patients in their 60s (Jankovic et al., 2000), which is followed by a 5–10 year period of functional decline to death.

Staging procedure for sporadic Parkinson's disease and pathological demographics

Braak and colleagues devised a neuropathological staging scheme under the assumption that the abnormal intraneuronal accumulation of α-synuclein is the common pathology for sporadic PD. By analysing the distribution and severity of α-synuclein cytoplasmic inclusions in brain specimens from the population at large (168 cases selected for detailed analysis, including 41 PD cases, 69 with α-synuclein inclusions, and 58 controls; the last two groups derived from screening 413 cases), cases can be categorised according to different hierarchical stages of disease infiltration (Braak et al., 2003). The most mildly affected cases (7% of the population) displayed lesions in the dorsal motor nucleus of the vagus nerve, the intermediate reticular zone of the medulla oblongata, the magnocellular portions of the reticular formation, the locus coeruleus and, frequently, the anterior olfactory nucleus (stages 1–2). Cases with moderate involvement (11% of the population) exhibit additional lesions in select mesencephalic and prosencephalic nuclei (stages 3–4), and all cases with severe pathology (6% of the population) show additional deterioration of the neocortex (stages 5–6).

Analysis of the demographics of the pathological stages observed shows considerable overlap rather than progression (Fig. 1). While this may be expected in pathological series due to average life expectancies, it suggests that most cases dying with this type of stage 1–2 pathology may never have gone on to develop clinical symptoms due to the very late timing of their preclinical period, a finding recently validated (Parkkinen et al., 2005). Thus, it is now important to confirm the existence of the same preclinical phenomena in autopsy material from populations within the predicted preclinical age range, or

determine the alternate preclinical pathologies at these predicted time points.

Comparison with other studies

As may be expected, a number of subsequent studies analysing α-synuclein pathology in populations of autopsy specimens have now been published using variable methods on variable populations with variable results (Table 1). Identification of α-synuclein-positive lesions for neuropathological staging was performed on 100 μm free floating sections with most of the brain available for analysis (Braak et al., 2003), a method that produces superb resolution of even minor lesions. Unfortunately, all other published studies to date have used the CERAD protocol for tissue preparation and sampling which is likely to compromise results, particularly for early, low frequency lesions.

In addition to the methods used, the populations analysed have differed significantly. Apart from staging study by Braak et al. (2003), only two other studies have included a population of sporadic PD cases in their analysis (Jellinger, 2004; Saito et al., 2004). To evaluate the progressive stages of PD pathology, studying cases with PD would seem to be a prerequisite. These studies have all evaluated large hospital based autopsy samples where bias towards disease is ensured. The large study by Saito and colleagues (Saito et al., 2004) shows very similar results with 20% of the sample overall having α-synuclein inclusions and a proportion of hospital cases without clinical PD or dementia also having α-synuclein lesions (13–15% in both studies).

Whereas the other samples analysed cannot inform on the progression of sporadic PD due to the absence of such cases, they do confirm that a proportion of people without clinical disease have α-synuclein pathology (Table 1). In the community samples analysed, the proportion of these preclinical cases is somewhat less (8–10%) than in the hospital based samples (Table 1), suggesting alternate clinical features may occur in the hospital patients sampled. These studies also confirm that these preclinical cases are considerably older than would have been predicted based on the clinical pattern of sporadic PD, particularly in the Japanese populations (Table 1). An additional feature addressed by these alternate studies that was not addressed in the original staging study (but see Braak et al., 2005) is the interaction between α-synuclein pathology and Alzheimer pathology. In all studies it has been shown that there is a considerable synergistic effect between these pathologies with a much

Table 1. Comparison between publications analysing the prevalence of α-synuclein inclusions in the aged

	Sectioning	Cases	Outcomes
Braak et al. (2003)	100 μm serial sampling	454, hospital 41 PD, 4 AD	24%, 6 stages, 3 categories most PD cases stages 5–6 preclinical 76 ± 8 years old
Parkkinen et al. (2003)	6 μm CERAD	904, community 0 PD, 82 AD	13% but 10% in aged synergistic AD effect preclinical 76 ± 1 years old
Wakisaka et al. (2003)	6 μm CERAD	102, community 0 PD, 29 demented	22.5% but 15% in aged synergistic AD effect
Jellinger (2004)	6 μm	260, hospital	50% of AD, 30% of aged
Saito et al. (2004)	6 μm CERAD	1241, hospital 66 PD, 479 demented	20%, increase with age similar/varied PD stages preclinical 83 ± 9 years old
Mikolaenko et al. (2005)	10 μm CERAD	117, community 7 PD, 34 AD	25% but only 8% in aged preclinical 83 years old

higher prevalence of α-synuclein pathology in cases with Alzheimer's disease (Table 1). This consistent finding then calls into question the validity of using α-synuclein pathology as an accurate marker for sporadic PD alone and reflects the need for better concepts concerning multiple disease pathologies. The older preclinical cases identified may herald a later mixed dementia syndrome rather than typical sporadic PD.

Further analyses of clinical features in cases with Lewy bodies

The concept that α-synuclein pathology occurs preclinically presupposes that considerable quantities are required prior to clinical deficits, as recently suggested (Parkkinen et al., 2005). The known compensatory mechanisms that also occur further support the concept that any neuronal dysfunction could be masked in this disease phase (Jankovic, 2005). This has been highlighted recently in a study of the later cognitive deficits in patients with clinical PD (Braak et al., 2005). While on average increasing PD disease stage correlated with cognitive status, a number of cases do not fit this concept with some having no cognitive deficits and many cortical α-synuclein inclusions while others are clearly demented without cortical α-synuclein pathology (Braak et al., 2005). In the medulla oblongata, the link with clinical deficits appears to be related to the degree of neuronal loss in addition to the deposition of α-synuclein (Benarroch et al., 2005). Thus, the supposition that all α-synuclein pathology indicates significant and constant clinical deficits needs to be tempered. Perhaps α-synuclein pathology associates with fluctuating clinical features, whereas sustained clinical deficits may occur only following an additional critical degree of neuronal loss.

It should be highlighted that the progressive association of clinical deficits with a progressive pattern of PD pathology has not been found. The recent analysis of late cognitive deficits in sporadic PD showed no correlations between disease duration and pathological stage or between disease duration and increasing cognitive decline (Braak et al., 2005). The lack of any relationship between the timing of dementia and the cortical infiltration of α-synuclein pathology has been a point of discussion for some time now, with many studies recognising this dilemma and some studies suggesting the correlation is to the related visual hallucinations (Harding et al., 2002). Further work is definitely required to determine the clinical correlates of α-synuclein pathology and their predictable timing in relation to lesion severity.

Areas requiring further clarification and recommendations

The staging paradigm proposed by Braak et al. (2003) has considerable merit in corralling patients with similar cellular mechanisms together and, in the case of sporadic PD, determining the potential sequence of events that may herald the clinical syndrome. However, the concept of progressive stages relies on similar clinical cases being sampled along a disease continuum and, therefore, preclinical versus early versus late-stage cases should on average be progressively older at the time of sampling. In this way, the preclinical symptoms and phases of the disease will be determined. Autopsy screening of cases in the predicted preclinical age range is now required and the evaluation of cell loss associated with α-synuclein pathology may assist in differentiating PD from later-onset dementia syndromes.

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A short overview on the role of α-synuclein and proteasome in experimental models of Parkinson's disease

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Summary. The Ubiquitin Proteasome System is a multi-enzymatic pathway which degrades polyubiquinated soluble cytoplasmic proteins. This biochemical machinery is impaired both in sporadic and inherited forms of Parkinsonism. In the present paper we focus on the role of the pre-synaptic protein α-synuclein in altering the proteasom based on the results emerging from experimental models showing a mechanistic chain of events between altered α-synuclein, proteasome impairment and formation of neuronal inclusions and catecholamine cell death.

Introduction: the role of the Ubiquitin Proteasome System in PD

Parkinson's disease (PD) is pathologically characterized by a selective degeneration of the nigrostriatal dopaminergic (DA) system, and by the presence of a pathological hallmark, the Lewy Bodies (LB), in the spared DA neurons of the substantia nigra pars compacta (SNpc). LB are inclusion bodies with a polymorphic shape, and constituted by a lipidic core surrounded by protein aggregates of polyubiquinated neurofilaments. In post-mortem tissue obtained from PD patients, LB are routinely identified by immunostaining with antibodies against the protein α-synuclein (Spillantini et al., 1997). Moreover, LB contain also other proteins, such as parkin and ubiquitin C-terminal hydrolase L1 (Lowe et al., 1990; Schlossmaker et al., 2002).

These observations, joined with recent discoveries on the genetic basis of rare forms of PD, disclosed a potential role for several, previously non explored proteins in the degeneration of nigrostriatal neurons. Some genetic forms of PD share the involvement of the Ubiquitin Proteasome System (UPS). More recently, UPS impairment has been demonstrated also in idiopathic PD (McNaught et al., 2003). UPS is a cellular, multi-protein structure involved in the degradation of polyubiquinated soluble cytoplasmic proteins. Ubiquitin tagging to proteins to be degraded by the UPS involves the so-called E1, E2 and E3 enzymes. Polyubiquinated proteins are substrate of the 26S proteasome, a proteolytic complex with a catalytic core formed by two outer and two inner heptameric rings. In the latter, there is a pore into which the polyubiquinated proteins are carried, the hydrolytic function of the UPS (a chymotripsin- a trypsin-like, and a peptidylglutamyl-peptide hydrolytic site) resides in the same pore.

Parkin is an E3 ubiquitin-protein ligase catalyzing the covalent binding of ubiquitin

to proteins to be degradated by the UPS. Inherited PD due to a mutation in the parkin gene represents an early-onset severe form of the disease (for a review, see Huang et al., 2004). The role of the UPS in the pathogenesis of PD has also been suggested by the inherited mutation of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), a protein widely diffused in the brain, which has the role of transforming polyubiquinate chains into monomeric forms of ubiquitin (for a review, see Huang et al., 2004).

Finally, α-synuclein, although not forming a constituent of the UPS, is associated with UPS activity and it is relevant for the pathogenesis of PD. This is substantiated by the parallel finding that α-synuclein is a constituent of LB, and that in at least two separate families containing cases of autosomal dominant juvenile Parkinsonism there are mutations (A53T and A30T) in the gene coding for this protein (for a review, see Huang et al., 2004). In line with these findings, it was hypothesized that mutated α-synuclein interferes with the functioning of DA nigrostriatal cells with a ''gain of function'' (in both families PD was autosomal dominant). Moreover, the discovery of an inherited form of PD associated with over-expression of α-synuclein (Singleton et al., 2003), added new evidence to the role of α-synuclein in PD. In particular these data demonstrate that α-synuclein per se is detrimental even when it is over expressed with a normal conformation. This is confirmed by replicating nigrostriatal degeneration in mice over expressing the wild-type form of synuclein (Masliah et al., 2000), and by data obtained in wild-type mice treated with neurotoxic doses of amphetamine (Fornai et al., 2005, see below).

Moreover, inhibition of UPS activity in mesencephalic and PC12 colture, induces the accumulation of α-synuclein in the form of cytoplasmic aggregates (for a review, see Betrabet et al., 2005).

New data from experimental models of PD

A critical issue in developing hypothesis on the pathogenesis of human diseases, is represented by the availability of reliable *in vivo*/*in vitro* experimental models. In the case of PD, appropriate *in vivo* models should share with the human disorder at least two main features: selective impairment of DA nigrostriatal and noradrenergic (NA) locus coeruleus (LC) neurons, and the formation of LB.

Amphetamines toxicity

Substituted amphetamines, namely methamphetamine (MA) and methylen-dioxymethamphetamine, are widely used to induce selective nigrostriatal DA degeneration in mice. Both amphetamines induce the formation of multilamellar inclusion bodies (''whorls'') in nigral DA neurons (Figs. 1, 2), which can be stained by antibodies against α-synuclein and ubiquitin (Fornai et al., 2004). These effects occur after few repeated, two h apart, neurotoxin administration, as opposed to what observed with bolus administration of another widely used neurotoxin, MPTP (see below). The neurotoxic effect of amphetamines has been related to an increased cytoplasmic release of DA from DA vesicles; excessive amounts of cytoplas-

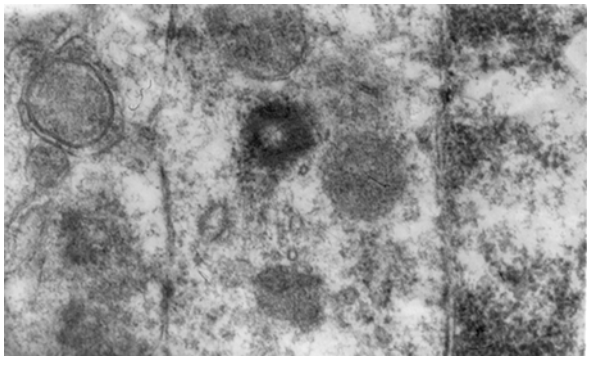

Fig. 1. Neuronal aggregate visualized at transmission electron microscopy in a dopamine neuron of the substantia nigra of a male C57 Black mouse following methamphetamine administration

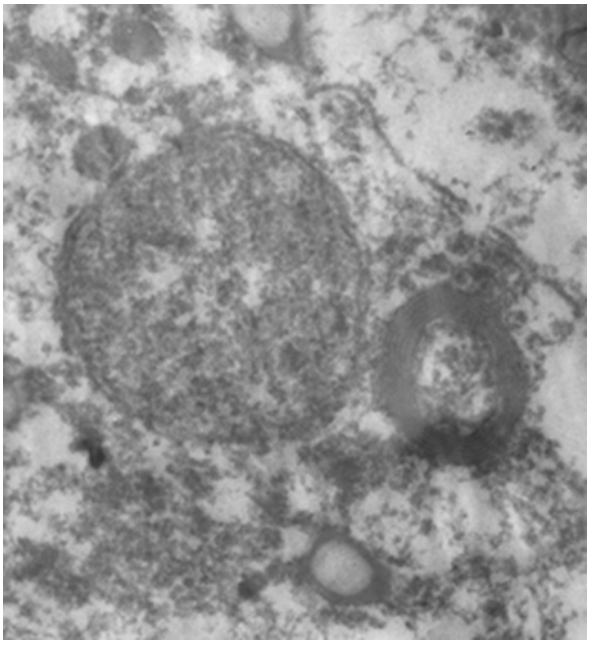

Fig. 2. Neuronal whorl visualized at transmission electron microscopy in a dopamine neuron of the substantia nigra of a male C57 Black mouse following methamphetamine administration

mic DA induce the formation of toxic by-products, free radicals, in a cascade of events culminating in significant oxidative stress for DA neurons. We speculated that in these experimental conditions the formation of multilamellar inclusions could represent a defense reaction of the DA neurons since: a) whorls can be observed only in DA nigrostriatal neurons and in GABAergic cells of the dorsal striatum, where DA terminals impinge, and where a massive, non-physiologic DA release is induced by amphetamines; b) they occur before the formation of nigrostriatal degeneration; c) their onset can be occluded by antioxidant substances. Moreover, time-dependency of whorls formation and its DA-dependency is confirmed in a more controlled setting, such as cultured PC12 cells which possess the machinery for DA synthesis, and release DA. The close relationship between whorls and LB-like, multifibrillar inclusions could be observed when examining PC12 cells exposed to MA at different time points. And again, the close relationship between inclusion formation and DA availability was demonstrated by showing that inhibition of tyrosine-hydroxylase, before exposure to MA attenuates inclusion bodies formation, and that a similar effect is obtained by pre-treating these cells with a powerful antioxidant, such as *S*-apomorphine.

A relevant role of α-synuclein in the neurotoxic effects of substituted amphetamines has been suggested. We recently demonstrated that both amphetamines derivatives, at doses inducing selective nigrostriatal degeneration in mice, induce also an over-expression of α-synuclein in the spared DA nigrostriatal neurons (Fornai et al., 2004). This phenomenon is selective for this subclass of SNpc neurons, since it does not occur in pars reticulata GABAergic cells. This observation lends substance to the link between α-synuclein over-expression and DA degeneration (Fornai et al., 2005a), and it confirms the ''gain of function'' for the mechanism of PD due to α-synuclein. As already mentioned, this is in line with the observation of a form of PD associated with over-expression of α-synuclein (Singleton et al., 2003), and DA nigrostriatal degeneration in mice over-expressing the wild-type form of α-synuclein (Masliah et al., 2000).

The MPTP model

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin which is widely used as an experimental tool to induce nigrostriatal degeneration in mice. After administration of neurotoxic regimens of MPTP a marked loss of striatal DA can be observed. However, with the schedules of MPTP administration usually employed, i.e. 2–3 bolus injections a few h apart, nigrostriatal degeneration is not associated with the onset of inclusion bodies in DA neurons. The mechanism of action of MPTP is related to the inhibition of the complex I of mitochondrial respiratory chain by its catabolite

MPP+. The discovery of this mechanism suggested a pivotal role of mitochondrial impairment in the pathogenesis of Parkinson's disease. Accordingly, a new experimental tool in the late 80's was used to induce experimental parkinsonism, consisting in the administration of rotenone, a selective inhibitor of the complex I. It was been recently observed that continuous, i.v. administration of low dose of rotenone can induce, concomitantly with nigrostriatal loss, the onset of inclusion bodies in the SNpc reminiscent of LB (Betarbet et al., 2000). The caveat of the discrepancy in the effects of the two tools, sharing a similar mechanism of action, has been solved recently (Fornai et al., 2005b). Mice were chronically implanted with osmotic minipumps, releasing MPTP continuously throughout at least three weeks. After 30 days of this regimen of administration, even at daily dosages as low as 5 mg/kg of MPTP, we observed the appearance of electron-dense, fibrillary inclusion bodies in the SNpc, which could be stained by anti-ubiquitin and anti- α-synuclein antibodies. These structures were preceded, at earlier time points after starting of MPTP infusion, by the appearance of ''whorls'', which were immunopositive for ubiquitin and α-synuclein (similarly to what observed after amphetamine administration, see above). It is likely that the regimen of MPTP administration used in this study mimics the progressive insult occurring in SNpc DA neurons of parkinsonian patients better than the acute, bolus, administration of MPTP usually used. The chronic administration of MPTP induces a prolonged inhibition of the UPS, as opposed to the acute, reversible effect observed after single i.p. MPTP administration.

Chronic inhibition of the complex I of mitochondrial respiratory chain is therefore associated with a chronic inhibition of UPS. Such a sustained decreased activity of the proteasome seems fundamental in the formation of inclusion bodies. By incidence, the usefulness of such a model is confirmed by the observation of neuronal degeneration, and occurrence of inclusion bodies, at the level of the noradrenergic nucleus locus coeruleus, a typical feature of PD (Forno et al., 1996).

Concerning the hypothesis of a pivotal role of α-synuclein in DA degeneration and LB formation, recently it was demonstrated that continuous, administration of MPTP in α-synuclein knock-out (KO) mice, even with high daily dosages of MPTP (30 mg/kg/die), was unable to produce nigrostriatal toxicity and formation of LB-like inclusion. Moreover, in α-synuclein KO mice the inhibitory effect of continuous MPTP administration on UPS activity was significantly attenuated as compared with wild-type mice (Fornai et al., 2005b). This additional piece of information: a) strengthens the hypothesis of a significant relation between the prolonged inhibition of UPS and the development of nigrostriatal degeneration and LB disease; b) suggests that α-synuclein has a topic role in neurodegeneration.

Conclusions

Nigrostriatal DA degeneration in PD takes place in a time-span of years, and it is likely to be due to repeated chronic sub-toxic oxidative insults towards DA neurons. This process is associated with chronic impairment of UPS activity: its capability to remove ubiquinated proteins is overwhelmed by the high amounts of misfolded proteins produced in the stressed DA neurons, and oxidative stress itself inhibits UPS activity amplifying this phenomenon. Such an impairment might lead to the accumulation of catabolic products into the neurons, whose appearance and composition maturate from ''whorls'', or autophagic bodies, up to overt LB-like inclusions. Whether these inclusions represent simply an epiphenomenon of such a catabolic impairment, or they also concur to the ongoing neuronal degeneration, it is not clear at present. α-Synuclein, a specific component of

LB, is likely to play a pivotal role both in such a selective degeneration and in LB formation. The elucidation of the relationship of α-synuclein and UPS in physiological and pathological conditions will help to better understand the pathogenesis of PD and to disclose new therapeutic approaches for halting the progressive DA degeneration.

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The role of protein aggregates in neuronal pathology: guilty, innocent, or just trying to help?

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Summary. Protein aggregates such as Lewy bodies have done much for the scientists in the field of neurodegenerative diseases: They have highlighted the affected cell populations and they have trapped the mutant disease protein. Instead of a good reputation, however, protein aggregates have received incriminations, because they are consistently seen at the site of crime. Reviewing the arguments, crucial evidence has become known that (a) the specific neuronal pathology precedes the appearance of protein aggregates in mouse models of disease, (b) the neurodegenerative disease in patients occurs with comparable severity when visible protein aggregates remain absent, (c) the neurotoxicity in vitro is best reproduced by oligomers, not polymers of the mutant disease protein. Is it feasible that protein aggregates are inert byproducts of the disease protein malconformation, or that they even represent beneficial cellular efforts to sequestrate the soluble toxic disease protein, together with normal or aberrant interactor proteins? Whatever the answer will be, one positive role of protein aggregates seems clear: In contrast to earlier speculations that random cytoplasmic proteins are trapped within the aggregates, scientists now believe that the composition of the Lewy body reflects the network of interactions between crucial players in disease pathogenesis, such as the PARK1, PARK2 and PARK5 protein.

Specificity of protein aggregates for degenerating cell populations

Since the first description of neurodegenerative disorders such as Alzheimer's, Parkinson's, Creutzfeldt-Jakob's disease (AD, PD, CJD) about 100 years ago, histological stains of protein aggregates with eosin, silver impregnation, congo-red, thioflavine, anti-ubiquitin antibodies were crucial to define the disease hallmarks. A location of the aggregates in the temporal cortex was found typical for Alzheimer's, a location in the substantia nigra typical for Parkinson's disease. The neuron populations affected by aggregates displayed severe cell death at the same time. This topographic specificity has made protein aggregates the most helpful marker of neurodegenerative processes so far. Indicating the increased vulnerability of dopaminergic substantia nigra neurons in PD, they have paved the way for the therapeutic successes of dopaminergic substitution in PD. Highlighting the increased vulnerability of cholinergic basal forebrain neurons in AD, the protein aggregates have also been the starting point for the cholinergic therapy of AD (Francotte et al., 2004). However, over the past decades

several neurodegenerative disorders caused by an unstable polyglutamine domain have been characterized, and surprisingly certain degenerating neuron populations in several Spinocerebellar Ataxias were spared from visible aggregate formation (Koyano et al., 2002), while surviving neuron populations in Huntington's disease patient brains could show particularly high aggregate formation (Kuemmerle et al., 1999). In the hereditary Spinocerebellar Ataxia type 2 (SCA2) and the sporadic multi-system atrophy (MSA), almost identical patterns of severe neuronal degeneration develop, although the protein aggregates are extremely sparse in SCA2 (Koyano et al., 1999; Huynh et al., 2000), while aggregate formation is prominent in MSA – but in oligodendrocytes (Dickson et al., 1999).

Protein aggregates usually contain the mutant disease-causing protein

The principal disease-causing proteins, beta-amyloid and tau for Alzheimer's (Fassbender et al., 2001; Mandelkow and Mandelkow, 1998), alpha-synuclein and parkin for Parkinson's (Spillantini et al., 1997; Farrer et al., 2001), the prion protein in Creutzfeldt-Jakob's disease (Prusiner, 1998) and different polyglutamine proteins in autosomal dominant Spinocerebellar Ataxias (Zoghbi and Orr, 2000) are all contained in the protein aggregates which are typical for each disorder: Amyloid plaques and neurofibrillary tangles in AD, Lewy bodies and neurites in PD, infectious prion particles in CJD, and neuronal nuclear inclusions in polyglutamine diseases. Therefore, the aggregates are helpful to molecular scientists through the identification of proteins, whose mutation initiates pathogenesis. Purification and peptide-sequencing of pathological aggregates would seem useful to define candidate proteins, which might be disease-causing. Apart from the technical difficulties inherent to such an approach, however, we have to remember that amyloid plaques are formed in AD variants that are caused by mutations of presenilin proteins, without the presenilins becoming part of the aggregates (Tandon and Fraser, 2002). It is widely assumed, that only toxic proteins with gain-of-function mutations are contained in aggregates, while other proteins with loss-of-function mutations promote aggregation and disease without becoming part of the inclusion bodies.

Aggregates indicate that hereditary and sporadic forms of disease share pathogenesis

A third important contribution to our molecular understanding was achieved by protein aggregates: Whether rare monogenic neurodegeneration disorders with early-onset can represent the same pathogenic mechanisms as frequent sporadic diseases of old-age like Alzheimer's and Parkinson's, has been questioned many times. The finding of amyloid plaques and neurofibrillary tangles with identical characteristics both in presenile and senile cases of AD, both in cases with hereditary beta-amyloid mutations and in cases without family history (Murrell et al., 1991) has given much credibility to the investigators of disease genes and to the genetic mouse models of disease. The observation of infectious prion particles from brain tissue of sporadic cases and cases with hereditary prion mutations (Tateishi and Kitamoto, 1995) came as a surprise and clearly argues in favour of common pathogenetic mechanisms. More recently, mutations or overexpression of alpha-synuclein were found to cause hereditary PD accompanied by aggregation of alpha-synuclein (Polymeropoulos et al., 1997), and identical alpha-synuclein aggregates were found to be induced by unknown causes in sporadic PD (Duda et al., 2000).

Pharmaceutical efforts to prevent aggregates seem beneficial

Experimental therapies of neurodegeneration have lately been guided by hypotheses, that

(1) the protein aggregates are stable and sequester proteins irreversibly, (2) the aggregates are the cause of pathogenesis and that (3) the reduction of visible aggregates would be beneficial (Wang et al., 2005). With similar assumptions, efforts have been undertaken to stimulate aggregate formation through inhibition of the proteasomal protein degradation, and thus induce neurodegeneration in rodents (McNaught et al., 2004). It is not clear, however, through which mechanisms a universal intoxication of proteasomes should result in a selective vulnerability of individual neuron populations. Recent experiments indicate that the intracellular protein aggregates of polyglutamine diseases can be quite dynamic under physiological conditions (Kim et al., 2002), and that soluble oligomers rather than the less soluble visible aggregates of alpha-synuclein are the toxic principle correlated to neurodegeneration in PD (Conway et al., 2000; Rochet et al., 2004). The therapeutic small molecule Congo Red, which inhibits aggregation at the early step of oligomerization (Sanchez et al., 2003), has been able to reduce the toxicity of beta-amyloid (Lorenzo and Yankner, 1994; Crowther et al., 2005) and huntingtin (Heiser et al., 2000).

Neuronal pathology occurs without visible aggregates in knock-in mouse models

Some scientists have argued that a faithful mouse model of a human neurodegenerative disease must show visible protein aggregates, and indeed there are well known mouse mutants where the aggregates are more prominent than in the human disorder (Davies et al., 1997; Neumann et al., 2002). However, in these models the mutant disease protein has usually been introduced as a transgene and has been strongly overexpressed so as to maximize the phenotype. When mouse mutants with a normal dosage or only slightly elevated dosage of the disease protein are considered, then the neuronal pathology occurs before or without appearance of visible aggregates (Menalled et al., 2003; Bowman et al., 2005). When we introduced low levels of transgenic A53T-mutant alpha-synuclein into mice, we found PD-reminiscent behaviour alterations, Lewy-neurite-like degenerative changes, and a selective alteration of striatal dopamine levels at old age, before the advent of demonstrable alpha-synuclein aggregates or nerve cell loss (Gispert et al., 2003). Only once the wildtype mouse alpha-synuclein was removed from these transgenic lines through crosses with an alpha-synuclein knock-out line (Cabin et al., 2002), axonal loss and visible alpha-synuclein aggregates became apparent and the mice showed paralyses and reduced survival (Cabin et al., 2005).

Disease in patients is comparably severe when visible aggregates remain absent

The conspicuous absence of Lewy bodies from autosomal-recessive variants of PD has initially been taken to indicate different pathogenetic pathways from sporadic and autosomal-dominant PD variants: In autosomal-recessive PARK2 the particularly early onset age in absence of Lewy pathology has been used as an argument for the protective role of visible protein aggregates (Shimura et al., 2000). And in autosomal-recessive PARK6 we found early-onset and prominent mitochondrial pathogenesis (Valente et al., 2004), but no clinical signs such as anosmia/dysautonomia/early sleep alteration as would be typical in early stages of Lewy pathology (Braak et al., 2002). However, this is not exclusive of autosomal-recessive PD. In autosomal-dominant PARK8 one family has been documented now, where the clinical course of disease is comparably severe in affected family members, whether they show alpha-synuclein-aggregates, tau-aggregates, or no detectable aggregates at all (Zimprich et al., 2005).

Lewy bodies contain the PARK1, PARK2 and PARK5 proteins with their protein interactors, and may thus define a pathway between crucial players of PD pathogenesis

Although Lewy pathology is absent from brains of PARK2 patients, the wildtype PARK2 disease protein, parkin, is aggregated in Lewy bodies of sporadic PD cases (Shimura et al., 1999). Direct protein interactors of parkin, such as Pael-R, are also contained in Lewy bodies (Murakami et al., 2004) and influence the progression of neuron loss. Similarly, the wildtype PARK1 protein, alpha-synuclein, is also present in Lewy bodies of sporadic PD cases. Again, its direct interactor protein, synphilin-1, forms part of the Lewy inclusions (Engelender et al., 1999), influences cell survival (Lee et al., 2002) and is ubiquitinated by parkin (Chung et al., 2001). Therefore, several constituents of Lewy bodies have now been searched for disease-causing mutations in PD families and collectives (Bandopadhyay et al., 2001; Krüger et al., 2003) – a task like searching for a needle in a haystack, when the enormous genetic heterogeneity of PD is considered. The best validated example is the cortical Lewy body constituent UCH-L1 (Dickson et al., 1994), which we found mutated in one German PD family (Leroy et al., 1998), and which was discovered in subsequent analysis to interact directly with alpha-synuclein in vitro (Liu et al., 2002), to protect against PD as a polymorphic variant in man (Maraganore et al., 2004), and to protect against neurodegeneration in mouse (Saigoh et al., 1999). UCH-L1 has therefore been named PARK5. Preliminary evidence indicates, that the PARK1, PARK2 and PARK5 disease protein not only coexist in Lewy bodies, but also have a common functional role in the assembly of ubiquitin lysine 63-linked multiubiquitin chains (Doss-Pepe et al., 2005). In polyglutamine disease, the disease proteins together with their interaction partners such as CREB – binding protein and ubiquitin-binding-domains are sequestered into the inclusion bodies (MacCampbell et al., 2000; Donaldson et al., 2003).

Conclusion

Thus, the appearance of detectable protein aggregates does not seem to be a prerequisite for a faithful model of neurodegeneration. Animal models with ubiquitously enhanced aggregation have widespread pathology and reflect the tissue specificity of diverse neurodegenerative disorders quite poorly. However, protein aggregates can identify affected neurons and may shed light on the pathogenetic mechanism: It appears that the proteins contained in aggregates are not random cytoplasmic or nuclear molecules, but are mutant or wildtype variants of the disease proteins themselves with their direct protein interactor network. Aggregates therefore represent the crucial pathway of neurodegenerative pathogenesis, and define the optimal protein targets for experimental therapies. The purification of amyloid plaques and its main protein constituents has given major insights into Alzheimer's disease in the past. Efforts to purify protein aggregates in PD, other neurodegenerative disorders and psychiatric diseases have been slow to get started (Iwatsubo et al., 1996; Mitsui et al., 2002), but they hold great promise.

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New face of neuromelanin

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Summary. The massive, early and relatively circumscribed death of the dopaminergic neurons of the substantia nigra in Parkinson's disease has not yet been adequately explained. The characteristic feature of this brain region is the presence of neuromelanin pigment within the vulnerable neurons. We suggest that neuromelanin in the Parkinson's disease brain differs to that in the normal brain. The interaction of neuromelanin with iron has been shown to differ in the parkinsonian brain in a manner consistent with an increase in oxidative stress. Further, we suggest an interaction between the lipoprotein α-synuclein and lipidated neuromelanin contributes to the aggregation of this protein and cell death in Parkinson's disease. The available data suggest that the melaninisation of the dopaminergic neurons of the substantia nigra is a critical factor to explain the vulnerability of this brain region to early and massive degeneration in Parkinson's disease.

Parkinson's disease (PD) is a progressive neurodegenerative disease which afflicts approximately 1% of the population aged 65 years and older and 4–5% of those over 85 years of age. It is characterised clinically by severe motor dysfunction, including the typical resting tremor, cogwheel rigidity and bradykinesia or poverty of movement. These characteristic symptoms are known to result from the progressive death of dopaminergic neurons in the substantia nigra pars compacta, an important relay region in the brain motor circuit. These clinical symptoms only develop following the death of at least 60% of the dopaminergic neurons in the substantia nigra, leading to a severe reduction of tissue dopamine levels in the caudate and putamen. While a diagnosis of probable PD based upon the typical motor symptoms can be made during life, a definitive diagnosis is possible only post mortem and is based upon two criteria: the marked loss of substantia nigra dopaminergic neurons, and the presence of abnormal inclusions (Lewy bodies and Lewy neurites) in the cell bodies of some surviving neurons. Lewy bodies consist of abnormally aggregated cellular proteins, primarily α-synuclein, a soluble presynaptic protein of uncertain function in the healthy brain. Interestingly Lewy bodies are found in only a small proportion (approximately 5%) of the surviving neurons in PD and are not exclusive to the substantia nigra pars compacta but are found in a number of brain regions in PD patients in a relatively consistent pattern (Braak et al., 2003). Studies of various forms of genetically inherited PD have shown that many forms of inherited PD are not characterised by aggregated α-synuclein (Huang et al., 2004), indicating that the deposition and aggregation of α-synuclein is not a prerequisite for the development of the clinical syndrome of PD. Furthermore, in all forms of PD (genetic and sporadic) neuronal cell loss early in the disease process is restricted to the

dopaminergic neurons of the substantia nigra but does not occur in the nearby dopaminergic nuclei (McRitchie et al., 1997) nor in non-dopaminergic neurons, despite the more widespread Lewy body pathology. From these data two important but often overlooked conclusions must be drawn: firstly, that the dopaminergic neurons of the substantia nigra are selectively vulnerable to degeneration early in PD and secondly, that the abnormal aggregation of proteins such as α-synuclein, while undoubtedly important in the disease process, cannot be the primary mechanism leading to the especial vulnerability of these neurons to cell loss early in the disease. These conclusions thus raise the question of what is it about the dopaminergic neurons of the substantia nigra which results in this early and circumscribed cell loss?

Recent studies on the many inherited forms of PD have identified a number of gene products as abnormal in these genetically determined forms of PD (Huang et al., 2004). These gene products affect several cellular pathways, resulting in α-synuclein deposition and proteosomal dysfunction, oxidative stress and mitochondrial dysfunction (Huang et al., 2004). As a result of these findings, hypotheses regarding each of these mechanisms as the primary mechanism for cell death in PD have developed. While these four mechanisms cannot be assumed to be independent, as each mechanism appears to be capable of impinging upon the other, they are generally discussed as the most likely candidates for degenerative changes within the substantia nigra in PD. While a body of experimental data supports the idea of changes in cellular pathways in the substantia nigra in PD the reason for the especial vulnerability of this region has not been resolved. A search for a feature of the dopaminergic neurons of the human SN which distinguishes them from nearby dopaminergic neurons would find it difficult to avoid the most characteristic feature of these neurons, that is the presence of large amounts of the pigment neuromelanin (NM) which gives the region its characteristic dark appearance and for which the substantia nigra (Latin: dark body) is named.

NM has been a focus of PD research for some time. In 1988 it was reported that the NM-containing cells of the SN are more vulnerable in PD (Hirsch et al., 1988). In fact a direct correlation was reported between cell loss in the individual cell groups which make up the SN and the percentage of NM-positive cells found in them (Hirsch et al., 1988). This suggests that in the normal brain NM may confer an advantage upon the cells in which it is found. The idea that NM may play a protective role in the dopaminergic cells of the SN in the normal brain is attractive and would reflect a corresponding role of melanins in other bodily tissues, such as the skin and the eye. Indeed there are good reasons why such a pigment has evolved within this brain region as the neurochemical environment of the dopaminergic SN is highly oxidative as a result of the catabolism of dopamine, via both enzymatic and non-enzymatic pathways, and a naturally high concentration of tissue iron. While we have recently demonstrated that some published data based upon a synthetic dopamine melanin may be erroneous while applied to the human pigment because of the differing behaviour of these synthetic and native pigments (Li et al., 2005), melanins can exhibit radical scavenging properties and we have shown that human neuromelanin can inhibit iron-induced lipid peroxidation of rat brain in vitro (Double et al., 1999). Further, we have demonstrated that human neuromelanin can attenuate the death of primary mesencephalic cultured cells by an oxidative stimulus (Li et al., 2005).

In view of these findings supporting the hypothesis that NM can play a protective function it is interesting to revisit the early data of Kastner and colleagues (Kastner et al., 1992) who reported that pigmented cells in the PD brain contain less NM than those in control brains. We suggest that NM

in the PD brain may differ to that in the normal brain. The massive loss of pigmented neurons, and therefore NM, in the parkinsonian brain at post mortem means that investigations of NM in the PD brain are technically difficult. Nevertheless a review of the literature reveals some data to support this thesis. It is of interest that one of the primary pathological hallmarks of PD, the Lewy body, is known to form within the boundaries of pigment in the cells in which these are found. As noted above, one of the primary constituents of the Lewy body is α-synuclein, a protein which has been shown to aggregate under conditions of oxidative stress and high iron concentrations (Kaur et al., 2004). In this context it is pertinent that we have shown that NM pigment is an effective binder of iron (Double et al., 2003) in a manner analogous to the iron binding core of the major iron binding protein ferritin (Gerlach et al., 1995). Studies using a variety of sophisticated biophysical techniques have established that the iron signal associated with NM granules in the PD SN is higher than that in the normal brain, suggestive of increased NM-bound iron in this brain region (Götz et al., 2004). Recently Faucheux and colleagues (Faucheux et al., 2003) have demonstrated that the redox activity of NM aggregates, attributed by these authors to Fe^{2+}, is significantly increased in parkinsonian patients, a finding not observed in the surrounding non-melanised tissue. Further redox activity of the NM aggregates was positively correlated with the severity of neuronal loss (Faucheux et al., 2003). These findings suggest that changes in NM precede cell loss in PD.

Structural analyses using nuclear magnetic resonance spectroscopy and electron paramagnetic resonance spectroscopy indicate that NM isolated from the parkinsonian brain may have a decreased ability to bind iron (Aime et al., 2000; Lopiano et al., 2000). Our work has shown that NM appears to maintain a reserve for binding additional iron in the healthy brain (Double et al., 2003), but in the PD brain any increase in iron might result in saturation of NM's iron binding capacity, leading to increased free iron concentrations in the surrounding tissue. These data support the idea that iron concentrations are increased in the parkinsonian SN and that this increase stimulates a localised increase in the oxidative environment of the melaninised neurons. Certainly there is a body of data which indicates that the parkinsonian substantia nigra exists in an environment of oxidative stress (Fasano et al., 2003). A localised increase in iron and/or iron-induced oxidative stress resulting from changes in the amount of iron in the substantia nigra or a reduced ability of NM in the PD to chelate iron could stimulate aggregation of α-synuclein within the boundaries of NM pigment. An early study using nuclear magnetic resonance spectroscopy reported that NM isolated from the parkinsonian brain differed from that in the healthy brain in that it was mainly composed of a highly cross-linked, protease-resistant, lipo-proteic material (Aime et al., 2000). This suggests that the composition of NM may differ in the PD brain, an idea supported by the more recent finding that α-synuclein is covalently bound to NM isolated from the parkinsonian brain but not in NM from the normal brain (Fasano et al., 2003).

In this context it is relevant that α-synuclein can take the structural form of a lipoprotein and we have recently demonstrated that NM granules consist of a significant proportion of lipids, a feature of NM which distinguish it from melanins in peripheral tissues in which no lipids are present (Fedorow et al., 2005). We have recently identified the primary lipid species in NM to be the polyisoprenoid dolichol (Fedorow et al., 2005). Accumulation of dolichol is associated with neurodegeneration in disorders such as the neuronal ceroid lipofuscinoses, but this is the first time that concentrated dolichol has been identified in the normal

brain. Further dolichol is known to increase in grey matter regions of the brain with normal aging, particularly after the sixth decade of life (Fedorow et al., 2005). Given that the greatest risk factor for PD is increasing age it is not unreasonable to suggest that an interaction between α-synuclein and lipidated NM pigment might stimulate the aggregation of this protein and the eventual formation of Lewy bodies within melaninised nigral neurons. This hypothesis would explain the especial vulnerability of the melanised neurons in PD and the formation of the final product of α-synuclein aggregation, the Lewy body, in close association with this cellular pigment. Of course Lewy bodies are not an inevitable consequence of aging, as well as being found in other cellular types, for example in cortical neurons in Dementia with Lewy bodies. It is therefore likely that an additional, as yet unidentified change, early in the parkinsonian SN is required to trigger aggregation of α-synuclein. The limited data available to date however inextricably link changes in NM in PD with two of the most accepted hypotheses regarding the mechanism of cell death in Parkinson's disease; α-synuclein deposition and oxidative stress.

The available data suggest that the melanisation of the dopaminergic neurons of the substantia nigra is a critical factor to explain the vulnerability of this brain region to early and massive degeneration in PD. We hypothesise that changes in NM precede cell death in this disorder, a suggestion that concords with the modest quantity of data gathered to date on changes in NM in PD. Further analyses of the structure and function of this pigment in the parkinsonian brain are required to identify such changes and to characterise their influence on cell survival.

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The effect of neuromelanin on the proteasome activity in human dopaminergic SH-SY5Y cells

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Summary. In Parkinson's disease (PD), the selective depletion of dopamine neurons in the substantia nigra, particular those containing neuromelanin (NM), is the characteristic pathological feature. The role of NM in the cell death of dopamine neurons has been considered either to be neurotoxic or neuroprotective, but the precise mechanism has never been elucidated. In human brain, NM is synthesized by polymerization of dopamine and relating quinones, to which bind heavy metals including iron. The effects of NM prepared from human brain were examined using human dopaminergic SH-SY5Y cells. It was found that NM inhibits 26S proteasome activity through generation of reactive oxygen and nitrogen species from mitochondria. The mitochondrial dysfunction was also induced by oxidative stress mediated by iron released from NM. NM accumulated in dopamine neurons in ageing may determine the selective vulnerability of dopamine neurons in PD.

Abbreviations

DMSO dimethyl sulfoxide, *DTT* dithiothreitol, *ECF* enhanced chemofluorescence, *MEM* minimum essential medium, *NM* neuromelanin, *PBS* phosphate-buffered saline, *SOD* superoxide dismutase, *PD* Parkinson's disease, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *UPS* ubiquitin-proteasome system, *ZsGFP* a green fluorescence protein homologue from Zoanthus sp.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorders in the aged and recently several genes responsible for the familial PD have been reported. However, the etiology of sporadic PD is still an enigma, and oxidative stress, impairment of mitochondrial function and the ubiquitin-proteasome system (UPS) are suggested to initiate a ''malignant cycle'' resulting in the cell death (Dexter et al., 1994). Especially, recent evidences suggest that failure of the UPS leads to aggregation and accumulation of abnormal proteins to form the inclusion bodies and induce neuronal cell death in the familial case of PD (Polymeropoulos et al., 1997; Shimura et al., 2000; Leroy et al., 1998). Dysfunction of the UPS is found to occur also in the sporadic form of PD also

(McNaught and Olanow, 2003). However, the factors, which determine the selective vulnerability of dopamine neurons are still unknown. One possibility is the involvement of dopamine and the metabolites themselves. Dopamine is a highly reactive catecholamine and is oxidized enzymatically and non-enzymatically. Autoxidation of dopamine produces speroxide and quinones, and polymerized quinones are interacted with proteinacious components and lipids (Gerlach et al., 1995; Dzierzega-Lecznar et al., 2004; Fedorow et al., 2005), to produce highly aggregated conjugates, neuromelanin (NM).

NM is a dark pigmented granule, which accumulate in catecholaminergic neurons of the substantia nigra and locus ceruleus. NM has been supposed to be a mere ''waste box'' in the neurons, but recently it was found to function as a reservoir of trace metals, such as iron (Zecca et al., 2001a). The involvement of iron in dopaminergic degeneration during ageing has been suggested repeatedly and recent studies show the content of iron in the substantia nigra was higher than that in the locus ceruleus (Zecca et al., 2004). Iron possibly induces cell death in nigral dopamine neurons through oxidative stress, as confirmed by studies on postmortem parkinsonian brains and on the cellular and animal PD models (Jenner, 2003). In the substantia nigra of PD brains, increased oxidative stress is suggested by increase in oxidative modification of lipids, proteins and DNA (Yoritaka et al., 1996). These results suggest that the effect of NM and iron should be examined on mitochondrial dysfunction, oxidative stress and the UPS.

In this paper we report the effects of NM prepared from control human brain on the production of reactive oxygen and nitrogen species (ROS, RNS) in mitochondria, the UPS activity using neuroblastoma SH-SY5Y cells. The in situ ubiquitin-26S proteasome activity was examined using the cells transfected with a proteasome sensor vector. The results are discussed in relation to the role of NM in selective vulnerability of dopaminergic neurons in PD and ageing.

Materials and methods

Materials

NM was isolated and purified from the substantia nigra of control human brains, as described previously (Gerlach et al., 1995) and was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration (Shamoto-Nagai et al., 2004). L-Cysteine was purchased from Sigma (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA), deferoxamine mesylate (DFX) and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma (St. Louis, MO, USA), and anti-polyubiquitin monoclonal antibody from NBT (Tokyo, Japan). A proteasome sensor vector, pZsProSensor-1, was purchased from BD Biosciences (Palo Alto, CA, USA). 2′,7′-Dichlorofluorescein (DCF), N-acetyl cysteine (NAC), L-cysteine and catalase from bovine liver, minimum essential medium (MEM) and other reagents were from Wako (Kyoto, Japan).

Measurement of in situ 26S proteasome activity in SH-SY5Y cells expressing a proteasome sensor vector

SH-SY5Y cells were cultured as reported (Shamoto-Nagai et al., 2004). Transfectant with a proteasome sensor vector was established using a pZsProSensor-1 eukaryotic expression vector, designed to express ZsGFP fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome, by lipofection technique as reported previously (Shamoto-Nagai et al., 2004). The culture medium was changed with the medium containing L-Cyst-DMSO solution without (control) or with NM and the cells were cultured for 3 days. In addition, the cells transfected with proteasome sensor vector was incubated with various concentrations of iron in the presence or absence of 25 μM DFX or antioxidants for 20 h. The fluorescence of ZsGFP in the living cells was measured as described before (Shamoto-Nagai et al., 2004), and the fluorescence intensity of the cells was expressed as arbitrary fluorescence unit/mg protein. The protein amount was measured according to Bradford (1976).

Detection of polyubiquitinated proteins in the cells treated with NM

After treatment with NM for 1 or 3 days, the cells were gathered, washed with PBS, and lyzed in the RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Fifty μg of protein was subjected to SDS-polyacrylamide electrophoresis

using 12.5% polyacrylamide gel (Bio Craft, Tokyo, Japan), and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Plolyubiquitinated proteins were visualized using anti-polyubiquitin antibody and enhanced chemofluorescence (ECF) Western blotting kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously (Shamoto-Nagai et al., 2003).

Isolation of mitochondria from SH-SY5Y cells

Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999). The cells were gathered, washed with PBS and suspended in the isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.5) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannhein, Germany). The mitochondrial fraction was prepared by homogenization and two steps of centrifugation.

Measurement of ROS-RNS with H_2DCFDA

The mitochondria were suspended in PBS and the production of ROS-RNS was quantified fluorometrically by measuring DCF produced from H_2DCFDA (Crow, 1997). H_2DCFDA was added to be 50 μM to the mitochondria suspension in the presence or absence of NM suspension (1–5 μg/ml) in dark at 37°C. The increase in DCF fluorescence at 504 nm with excitation at 520 nm was measured at every 30 min for 3 h in a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan). The generation of ROS-RNS was expressed as mol DCF per min per mg protein. The effects of DFX and anti-oxidants were also examined in the same way, after 15 min pre-incubation with DFX or the antioxidants.

Statistics

Experiments were repeated at least 3 times. The data was expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.

Results

The effect of NM on in situ 26S proteasome activity

After treatment with NM or iron the accumulation of ZsGFP was observed in cytoplasm by fluorescence microscopy (Fig. 1). After 3 days' incubation, the fluorescence intensity in the cells treated with 0.1 μg/ml

Fig. 1. Effects of NM on the in situ activity of 26S proteasome in SH-SY5Y cells transfected with the proteasome sensor vector. The cells were cultured in the presence of 0.1 μg/ml of NM for 3 days. Left: morphological observation of the cells treated with NM. Right: Accumulation of ZsGFP (white arrows) was observed by fluorescence microscopy

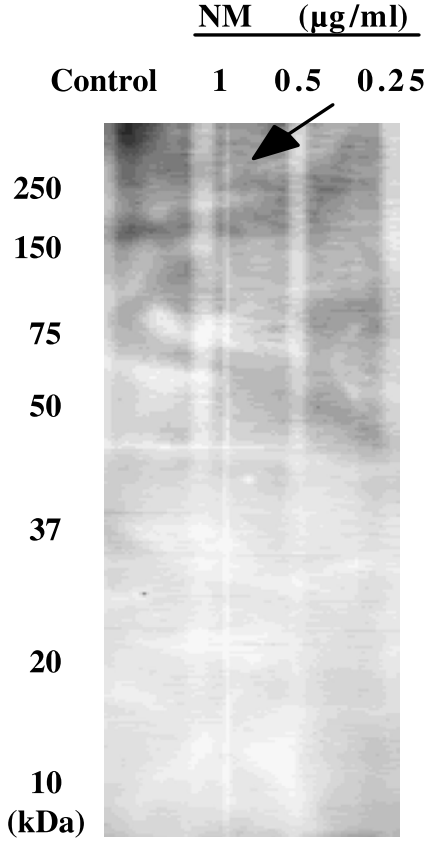

Fig. 2. Accumulation of poly-ubiquitinated proteins in SH-SY5Y cells treated with NM for 3 days (arrow). The cells were cultured with or without NM and lysed, then proteins were separated by SDS-PAGE and immunoblotted using anti-polyubiquitin antibody as described in the Materials and methods

NM increased to be that of 161% of control cells. The accumulation and the increase of fluorescence intensity was confirmed by measuring its fluorescence intensity by Fluorospectrometry also (Fig. 4). The increase of ZsGFP, that reflects the reduced activity of 26S proteasome by iron, was restored by DFX.

Accumulation of polyubiquitinated proteins in SH-SY5Y cells treated with NM

The cells treated with various concentration of NM was gathered and applied to Western blotting using anti-polyubiquitin antibodies. As shown in Fig. 2, in the cells treated with 1 μg/ml of NM, the accumulation of polyubiquitinated proteins, which is the specific substrate for 26S proteasome, was detected (arrow).

Increase of ROS-RNS in isolated mitochondria by NM

The production of $^{\bullet}OH$, NO and $ONOO^-$ in mitochondria was quantified fluorometrically using DCF cleaved from H_2DCFDA as an indicator (Fig. 2). In the presence of mitochondria, NM increased DCF fluorescence. In addition, SOD, but not catalase, reduced DCF production in mitochondria themselves. DFX significantly reduced the DCF production from mitochondira enhanced by NM. These results indicate that $O_2^{\bullet -}$ plays the key role in ROS-RNS production and the involvement of iron in the ROS-RNS production by NM.

Discussion

In this paper, it was clearly shown that NM increased ROS and RNS generation especially superoxide, in mitochondira, which

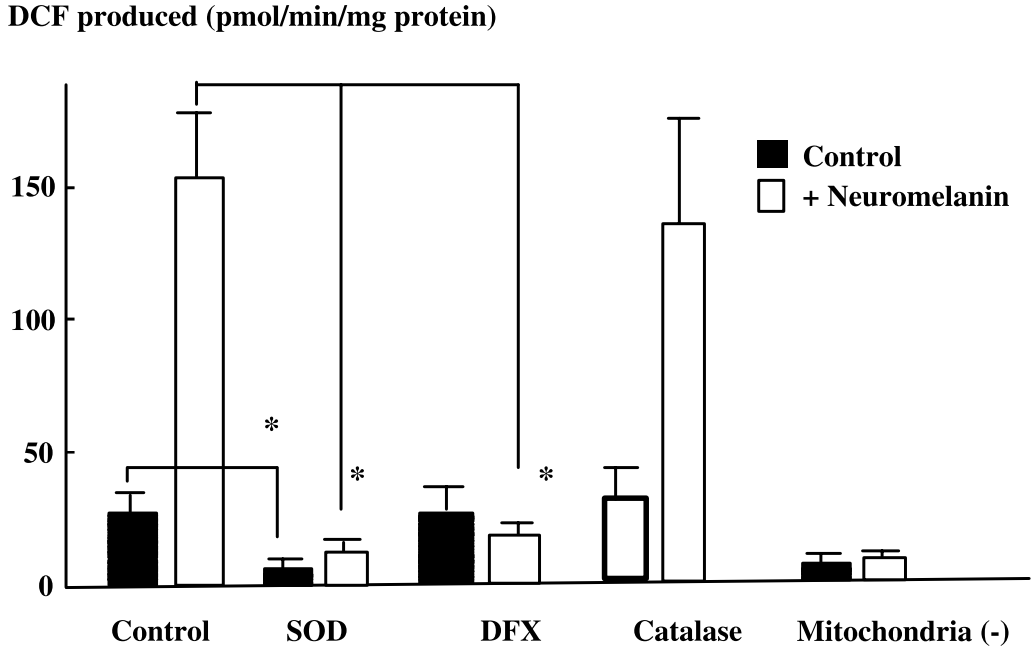

Fig. 3. Effects of SOD, deferoxamine mesylate (DFX), and catalase on DCF production in mitochondria prepared from SH-SY5Y cells. Mitochondria (30 μg protein) were incubated with or without 2.5 μg/ml NM in PBS, and the effects of SOD (1000 units), DFX (1 μM), and catalase (500 units) were examined. Generated ROS-RNS was quantitatively measured as DCF produced from H_2DCFDA and expressed as pmol/hr. The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. $^*P < 0.01$

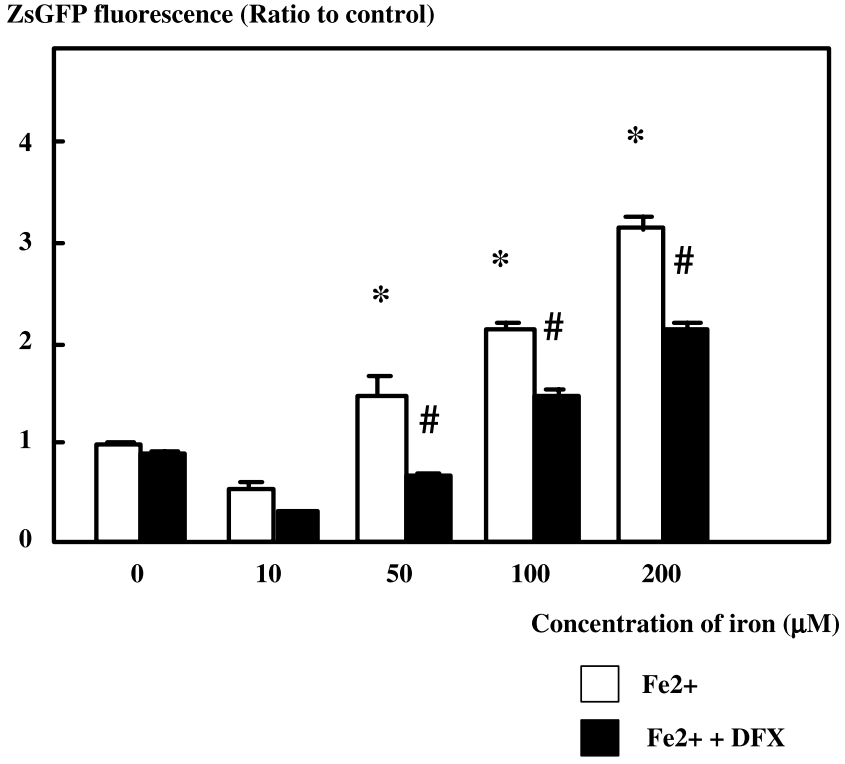

Fig. 4. Iron inhibited in situ activity of 26S proteasome in SH-SY5Y cells transfected with a proteasome sensor vector. After the treatment with iron with or without deferoxamine mesylate (DFX) for 20 h, the fluorescence intensity of ZsGFP, which is coded by a proteasome sensor vector, at 505 nm with excitation at 493 nm was quantified and expressed as arbitrary fluorescence unit/mg protein. The column and bar represent mean and SD of 3 experiments. After the treatment with iron, the fluorescence intensity increased in a dose-dependent manner. $^{*}p<0.05$ compared to the control. This increase was suppressed by 25 μM DFX significantly. $^{\#}p<0.05$ compared to the cells treated with iron without DFX

was mediated by released iron, as shown by the complete suppression of DFX. The onset of oxidative stress may deteriorate the function of mitochondria, in addition to the direct inhibition of mitochondrial respiratory chain enzymes. NM reduced the in situ activities of 26S proteasome, as shown using a green fluorescent protein homologue targeted to 26S proteasome. The mitochondrial toxins, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), were reported to induce neuronal death selective to dopamine neurons with formation of Lewy body-like inclusion body in in vivo models of PD (Betarbet et al., 2000; Kowall et al., 2000). The mechanism of cell death has not been elucidated, but the involvement of reduced UPS activity was suggested. Recently we found that mitochondrial dysfunction caused by rotenone, a complex I inhibitor, increased abnormal oxidative modification of proteins with acrolein, and reduced the activity of proteasome, through binding of aggregated oxidized protein to the catalytic site of 20S proteasome and direct adduction of acrolein to 20S protosome itself (Shamoto-Nagai et al., 2003). It may be reasonable to assume that mitochondrial dysfunction plays a central role in the pathogenesis of sporadic PD, and impairment of the UPS may be a final executor in the neural degeneration.

Iron is known to induce oxidative stress by enhancing electron transfer in a Fenton

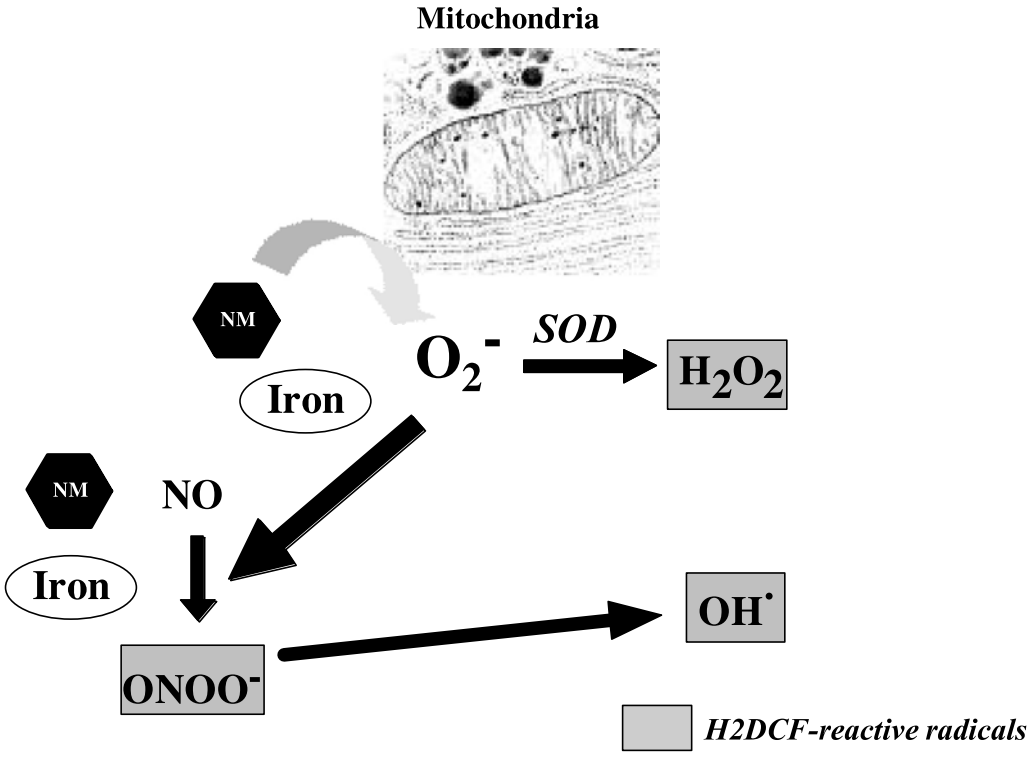

Fig. 5. The scheme how iron increases oxidative stress in mitochondria. Iron released from NM generates superoxide (O_2^-), which is catalized by SOD. O_2^- also reacts with nitric oxide (NO) by near-diffusion limit to produce peroxinitrite ($ONOO^-$). $ONOO^-$ is a strong radical itself and is decomposed into hydroxyl radical ($OH^{\bullet}$) also

reaction (Fig. 5). In the dopaminergic neurons, almost all the iron exists as bound with ferritin or NM, suggesting that NM may be neuroprotective, by chelating iron and other trace metals (Zecca et al., 2001b). Iron content in the substantia nigra increases by ageing but remained stable after the fourth decade of age, but NM content increases further according to ageing (Zecca et al., 2001a). In the postmortem PD brain, increased iron content was detected (Riederer et al., 1989) and recent results using transcranial ultrasonography revealed that in the substantia nigra of parkinsonian patients, not only the iron content increased, but that of NM decreased (Zecca et al., 2005). These results indicate that in the dopaminergic neurons in PD, the binding capacity of NM with iron was decreased and as a result, cytosolic free iron increased to inhibit ubiquitin-26S proteasome system, as shown in this paper (Gerlach et al., 2003).

Recent proteomics studies indicate the involvement of endosome-lysosome system as a source of proteinacious components in human NM (Tribl et al., 2005). This means NM production is not only ''passive'' accumulation of oxidative products, but some enzymatic system is required for the formation of high molecular aggregates. It requires further studies to elucidate whether the characteristics of NM itself or its synthetic pathway are changed in PD. The future studies on the intracellular mechanisms underlying the selective cell death by iron released from NM may bring out new strategies to prevent or rescue the decline in nigral dopamine neurons in PD.

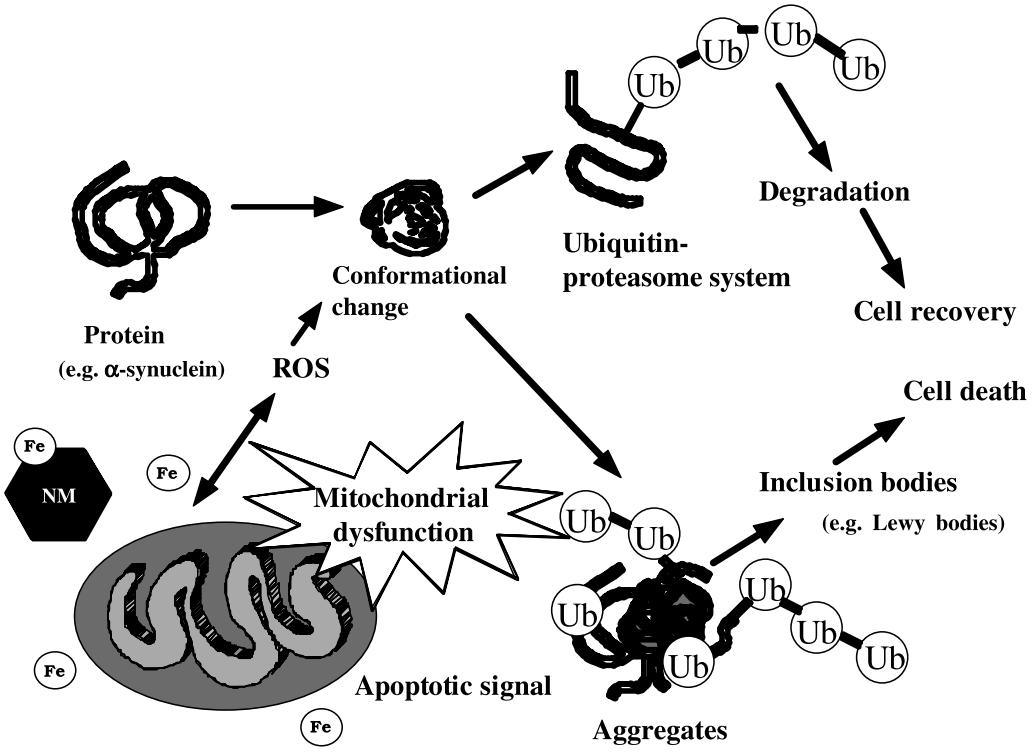

Fig. 6. The possible mechanism of dopaminergic neuronal death in Parkinson's disease. Iron released from NM increases oxidative stress in the mitochondria, and inhibit 26S proteasome system in the cells. Impairment of 26S proteasome increases accumulation of abnormal proteins to induce cell death

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Potential sources of increased iron in the substantia nigra of parkinsonian patients

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Summary. Histopathological, biochemical and *in vivo* brain imaging techniques, such as magnetic resonance imaging and transcranial sonography, revealed a consistent increase of substantia nigra (SN) iron in Parkinson's disease (PD). Increased iron deposits in the SN may have genetic and non-genetic causes. There are several rare movement disorders associated with neurodegeneration, and genetic abnormalities in iron regulation resulting in iron deposition in the brain. Non-genetic causes of increased SN iron may be the result of a disturbed or open blood–brain-barrier, local changes in the normal iron-regulatory systems, intraneuronal transportation of iron from iron-rich area into the SN and release of iron from intracellular iron storage molecules. Major iron stores are ferritin and haemosiderin in glial cells as well as neuromelanin in neurons. Age- and disease dependent overload of iron storage proteins may result in iron release upon reduction. Consequently, the low molecular weight chelatable iron complexes may trigger redox reactions leading to damage of biomolecules. Additionally, upon neurodegeneration there is strong microglial activation which can be another source of high iron concentrations in the brain.

Introduction

Iron is one of the most abundant metals in the human body. It is essential for various brain functions and it is involved in neuronal communication. There is increasing evidence that iron is involved in the mechanisms that underlie many neurodegenerative diseases (Gerlach et al., 1994; Zecca et al., 2004). Biochemical, histopathological, and *in vivo* brain imaging techniques, such as magnetic resonance imaging and transcranial sonography, revealed a consistent increase of substantia nigra (SN) iron in Parkinson's disease (PD) (Riederer et al., 1985, 1988, 1989; Sofic et al., 1988, 1991; Gerlach et al., 1994, 2005; Berg et al., 2001; Götz et al., 2004). While the cause of the increased iron concentration in the degenerating parkinsonian SN is unclear, the interaction of this metal with cellular constituents, such as α-synuclein and neuromelanin, appear to be important for the development of the characteristic neuropathology

characterising the disease and possibly, oxidative-mediated neurodegeneration. Recent work investigating the time course of dopaminergic cell death and iron accumulation in animals models of PD (He et al., 2003) suggest that these pathways may not represent the initial trigger of the disease process but may reflect as yet unidentified alterations in iron homeostasis and represent secondary but important mechanisms involved in the progressive nature of the disease. This paper will review potential sources of increased SN iron in PD.

Regulation of iron brain homeostasis

The ability of the brain to store a readily bioavailable source of iron is essential for normal neurological function because both iron deficiency and iron excess in the brain have serious neurological consequences. Iron homeostasis in the brain is regulated by

- mechanisms for transport of iron into the brain,
- mechanisms for transport of iron into neurons and
- molecules involved in brain iron storage.

Transferrin, a bilobal glycoprotein of approximately 88 kDa with its two homologous domains both containing one high-affinity Fe(III)-binding site (Ponka, 1999) is the most important iron transport protein in the human brain. Most studies on transferrin within the brain focus upon its role in iron uptake via the transferrin receptor, although it is also involved in intracellular iron processing and possibly in iron efflux (Aisen et al., 1999; Ponka, 1999). In addition, it has been suggested that lactotransferrin (synonym lactoferrin) have a role in the transport of iron into dopaminergic neurons in the SN. Lactotransferrin is an 80 kDa glycoprotein belonging to the transferring family with a transferrin-similar structure (Anderson et al., 1989) that has been localized in the human brain to neurons, glial cells and microvasculature (Aisen and Leibman, 1972). Lactotransferrin crosses the blood–brain-barrier (BBB) in an iron saturated and native form (Fillebeen et al., 1999a) and is also synthesized within the brain (Fillebeen et al., 1999b). Iron binds more avidly to lactotransferrin than to transferrrin (Birgens, 1991), and in contrast to transferrin, the binding of lactotransferrin to its receptor is independent of its degree of iron saturation (Davidson and Lonnerdal, 1989). In addition, the lactotransferrin expression is not regulated by intracellular iron (Yamada et al., 1987). In this absence of intracellular feedback the expression of the lactotransferrin receptor could become uncontrolled (Faucheux et al., 1995).

The most important molecules involved in brain iron storage are ferritin and neuromelanin (NM). Ferritin is a 450 kDa protein with 24 subunits forming a cavity which can store up to 4500 atoms of iron (III). Ferritin-bound iron is compartmentised within the brain so that it cannot participate in redox reactions and acts as a protective mechanism against iron-induced oxidative damage (Halliwell and Gutteridge, 1986). Ferritin is highly expressed within the glial compartment, predominantly in oligodendrocytes but also in microglia and astrocytes (Connor et al., 1994), while ferritin staining of neurons is absent in both the young and aged brain (Connor et al., 1990).

NM is a dark pigment produced in dopaminergic and noradrenergic neurons of the human SN and locus coeruleus, respectively. The loss of NM-containing dopaminergic neurons and the resulting pallor of the SN is one of the most striking features of PD (Jellinger, 1989; Sian et al., 1999). A relationship between the loss of the dopaminergic SN cells and their NM content has been reported (Hirsch et al., 1988), suggesting a role of NM for neurodegeneration in PD. The function of NM has yet to be established, but it is considered as an endogenous iron-binding molecule in pigmented neurons (Double et al., 2000; Federow et al., 2005).

It may therefore play a physiological role in intraneuronal iron homeostasis.

At the post-transcriptional level cellular iron uptake and storage are regulated by cytoplasmic factors, the iron-regulatory protein 1 and 2 (IRP-1 and IRP-2). When intracellular iron levels fall, IRPs bind to iron-responsive elements (IREs) in the 5′-untranslated region of ferritin mRNA and the 3′-untranslated region of the transferrin receptor mRNA, inhibiting the translation of ferritin RNA to decrease iron storage capacity and stimulates the translation of the transferrin receptor mRNA by stabilisation of the mRNA to up-regulate iron uptake. When sufficient intracellular iron is present the opposite situation develops to down-regulate intracellular iron levels (Thomson et al., 1999).

Potential sources of increased SN iron in PD

Iron misregulation in the brain may have genetic and non-genetic causes. There are several rare movement disorders associated with neurodegeneration, and genetic abnormalities in iron regulation resulting in iron deposition in the brain (Thomas and Jankovic, 2004). For example, patients with neuroferritinopathy suffer from parkinsonism and the iron deposition in the basal ganglia is caused by a mutation in the gene encoding the ferritin light chain on chromosome 19q13.3. Non-genetic causes of increased SN iron may be the result of

- a disturbed or open BBB,
- local changes in the normal iron-regulatory systems,
- intraneuronal transportation of iron from iron-rich area into the SN and
- release of iron from intracellular iron storage molecules.

A disturbed or open BBB as a cause of increased SN iron in PD

A potential source of increased iron is from peripheral influx through a disturbed or open BBB in the SN (Riederer, 2004). This has been suggested from studies in rats, in which the dopaminergic neurons were destroyed by infusion of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle and higher iron concentrations were measured in SN using both histochemical and neurochemical methods (Oestreicher et al., 1994). Indeed, recently a BBB dysfunction in the midbrain of PD patients was demonstrated by using radiolabelled verapamil hydrochloride and positron emission tomography (Kortekaas et al., 2005). Verapamil is a specific substrate for the P-glycoprotein multidrug resistance system in the cell membrane. P-glycoprotein functions as an efflux pump, and verapamil does not cross the BBB. Kortekaas et al. (2005) found a high level of uptake of verapamil in the midbrain of patients with PD but no uptake in age-matched healthy controls. These data suggest a dysfunction of the P-glycoprotein system in vulnerable brain regions in PD, suggesting the BBB might render the midbrain accessible to serum iron.

Local changes in the normal iron-regulatory system as a potential cause for increased SN iron in PD

A change in the normal iron regulatory systems, such as a local increase in transferrin receptor number could also result in an increase in SN iron. Results from studies investigating the density and distribution of the transferrin binding site in the midbrain in post-mortem PD suggested that transferrin receptor number, while increased in the caudate and putamen, are actually decreased on the perikarya of melanised neurons in the SN (Faucheux et al., 1995, 1997; Morris et al., 1994). In the serum, transferrin (the iron transport protein) levels were either unchanged (Riederer et al., 1988) or even decreased (Logroscino et al., 1997). However, in postmortem ventricular CSF transferrin levels were lower (Riederer et al., 1988). In addition,

serum ferritin concentrations are increased in PD (Riederer et al., 1988). Further, serum iron is reported to be either unchanged (Torsdottir et al., 1999) or even appears to be decreased in the parkinsonian patients compared with controls (Riederer et al., 1988), even when iron intake is equivalent (Torsdottir et al., 1999). Such results point to a general change in iron regulation in PD, which is not restricted to the brain. In contrast, another iron-binding glycoprotein, lactotransferrin, is reported to be increased in surviving neurons in the SN and ventral tegmental area in the PD brain (Leveugle et al., 1996). The increase in lactotransferrin is associated with increased numbers of lactotransferrin receptors on neurons and microvessels in the parkinsonian SN (Faucheux et al., 1995). The observed increases in this iron mobilization system could represent one mechanism by which iron might concentrate within the PD SN.

Intraneuronal transportation of iron from iron-rich area into the SN as a potential cause of increased SN iron in PD

Another possibility for increased iron in PD SN is that iron might be transported intraneuronally from iron-rich areas into the SN. Many areas of the basal ganglia normally contain high concentrations of iron (Hallgren and Sourander, 1958): The globus pallidus (21.30 mg iron/100 g fresh weight) contains the highest concentration of iron in the brain and is directly connected to the SN pars compacta via afferent γ-aminobutyric acid (GABA) neurons. To date, however, there is no known mechanism which would explain the translocation of iron from one area of the brain to another, although such a phenomena has been demonstrated in the immature rat where the BBB, however, is not fully developed (Dwork et al., 1990). In addition, there is no evidence for decreased iron concentrations in such assumed iron-rich areas in PD.

Release of iron from intracellular iron storage molecules as a potential cause of SN iron in PD

A fourth possibility is that the increased iron levels might result from the redistribution of intracellular iron. Early work attributed the increased iron primarily to nigral glial cells (Jellinger et al., 1990); glial cells are known to store iron and the gliosis occurring in the parkinsonian SN is associated with the degenerating dopaminergic neurons (Jellinger et al., 1990, 1992). The migration of iron-containing activated microglia and macrophages into the degenerating SN represents a normal immune response to the degenerative process but could also pose another source of increased reactive oxygen species production in the SN. Significantly, the glial cells contain ferritin, the major iron binding protein within the brain. Ferritin has been found by using a dissociation-enhanced lanthanide solid phase two-site fluoroimmunoassay based on the direct sandwich technique with polyclonal antibodies (DELFIA) to be significantly increased in the SN but not in the putamen of PD (Riederer et al., 1988). Indeed, an increase in the number of ferritin-immunoreactive microglia in the PD SN has been demonstrated (Jellinger et al., 1990); the presence of an abundance of these scavenger cells in the degenerating brain region might be expected. The logical consequence of the migration of ferritin-expressing glial cells into the degenerating SN would be an increase in total ferritin in this region. The concentration of ferritin in the parkinsonian basal ganglia has been reported to be slightly increased (Riederer et al., 1989) or reduced (by between 25 to 53%; Dexter et al., 1990) or unchanged when compared with controls (Mann et al., 1994) depending on the method of measurement and quantification of L- and H-ferritin, respectively, used. These latter findings are, however, surprising, given that intracellular iron levels regulate ferritin levels; an increase in intracellular iron would normally result in an

up-regulation of ferritin expression, rather than a decrease, suggesting that normal iron regulatory systems are dysfunctional in PD. The work of Connor et al. (1992, 1995) supports this hypothesis; this research group has studied changes in iron regulatory systems in both normal ageing and in disease in detail and have reported changes in both PD and Alzheimer's disease (AD) which are at variance with the changes occurring in normal aging. This suggests that iron homeostasis may be disrupted in both the AD and PD brain, but the focus of these changes appears to differ between the two diseases. AD is characterised by a decrease in the iron mobilisation protein transferrin (Connor et al., 1992), in contrast, PD is characterised by a decrease in iron storage capacity. Normal ageing is reported to be accompanied by an up-regulation of ferritin expression for reasons that are unknown; in PD this normal up-regulation response appears to fail (Connor et al., 1995).

As discussed above at the post-transcriptional level cellular iron uptake and storage are regulated by IRP-1 and IRP-2. Of particular interest is the fact that the activation of IRPs increases the cell's potential to take up iron (Meneghini, 1997). IRPs, predominantly IRP-1, have been described in the human brain (Hu and Connor, 1996), and changes in IRP-2 are reported to be associated with the pathological hallmarks of AD, suggesting that changes in this iron regulatory system might be linked to the disease process (Smith et al., 1998). Possible changes in this system in PD are yet to be investigated.

Iron can be released from ferritin by various exogenous and endogenous substances via reductive mechanisms (Boyer et al., 1988; Monteiro and Winterbourn, 1989; Lapenna et al., 1995; Double et al., 1988). Of particular interest are mechanisms that might be physiologically relevant. Glial cells produce significant amounts of superoxide ($^{\bullet}O_2^-$) and also nitric oxide ($NO^{\bullet}$) from L-arginine, and both of these free radicals are reported to release iron from ferritin stores (Biemond et al., 1984; Rief and Simmons, 1990; Youdim et al., 1993; Yoshida et al., 1995). We have further demonstrated that a variety of catechol-based substances, including the dopaminergic neurotoxin 6-OHDA, can release iron from ferritin *in vitro* (Double et al., 1998). The release of ferritin-bound iron by 6-OHDA is associated with lipid peroxidation, a response abolished by the addition of an iron chelator; thus we have suggested that this release is important for 6-OHDA toxicity. Interestingly, we showed that the native neurotransmitter dopamine is also capable of releasing iron from ferritin, although whether this release is elicited by dopamine itself or after its oxidation to 6-OHDA is unclear. Comparative studies demonstrated that the release of ferritin-bound iron is dependent upon the substance containing an ortho-dihydroxyphenyl structure and upon the redox potential of the substance (Double et al., 1998). Such in vitro work is of interest as significant amounts of 6-OHDA can be formed in vitro from the oxidation of dopamine by H_2O_2 (Napolitano et al., 1995), and it has been suggested that 6-OHDA can also be formed in vivo under conditions of oxidative stress and may contribute to degeneration in PD (Jellinger et al., 1995; Linert et al., 1996). Certainly "6-OHDA-like substances" have been identified in the urine of PD patients (Andrew et al., 1993).

As discussed above, however, ferritin is located mainly in the glial compartment in the brain; neurons mostly do not stain positive for ferritin (Connor et al., 1990). As free radicals are highly reactive, it is unlikely that glial-derived free radicals diffuse across the intracellular space in sufficient quantities to damage neuronal constituents. If intracellular iron release contributes to neuronal damage it seems more probable that an intraneuronal iron source is responsible for oxidant-mediated damage. Such an iron source is the intraneuronal pigment NM.

The function of NM has yet to be established, but it is considered as an endogenous

iron-binding molecule in pigmented neurons (Double et al., 2000; Federow et al., 2005). It may therefore play a physiological role in intraneuronal iron homeostasis. Support for this theory comes from changes in NM in the PD brain where significantly less iron is bound to NM than that seen in the normal brain (Lopiano et al., 1999). This suggests that changes in iron-binding to NM result in increased levels of intraneuronal free iron and the subsequent cell damage observed in PD.

Isolated human NM consists of 2.8% iron as estimated by Mössbauer spectroscopy (Gerlach et al., 1995), while the concentration of iron(III) in the SN has been estimated using electron paramagnetic resonance at 6780 ng iron/mg intact SN tissue or 11300 ng iron/mg isolated NM (Shima et al., 1997). This measurement is in agreement with the estimate of 9700 ng iron/mg isolated NM using total reflection X-ray fluorescence (Zecca and Swartz, 1993). Iron binding studies using NM isolated from the human SN demonstrated that NM contains high ($K_d = 7.18 \pm 1.08$ nM) and low-affinity binding sites ($K_d = 94.31 \pm 6.55$ nM) for iron(III) (Double et al., 2003). Our recent data demonstrates that a purely iron(III) signal can be measured from intact frozen SN tissue using Mössbauer spectroscopy (Double et al., 2003). These data support reports that iron is directly bound to NM granules in the SN (Good et al., 1992; Jellinger et al., 1992; Zecca et al., 1996) and that this signal is increased in PD (Kienzl et al., 1995).

The interaction of iron with NM is of interest because the behaviour of NM changes in the presence of iron; instead of inactivating free radicals, it begins to act as an effective pro-oxidant. It is unclear whether iron bound to NM can contribute to free radical-producing mechanisms or whether the presence of NM within the dopaminergic cells represents a pool of iron which, under certain circumstances, can be released to interact in free radical-producing pathways. Nevertheless such mechanisms are of interest as they represent an intraneuronal source of free radicals that could increase the oxidative load within the vulnerable dopaminergic neurons. While the physiological relevance of these proposed mechanisms is unclear we have demonstrated the functional consequences of NM's influence on the cell and its modulation by iron in vitro (Double et al., 1999). In the absence of iron, isolated human NM significantly decreased membranal damage in rat cortical homogenates in vitro as measured by lipid peroxidation. Further when NM was added together with iron the amount of lipid peroxidation measured was significantly less than that induced by iron alone. These results support the hypothesis that NM has antioxidant properties and can protect the cell from radical-induced damage. It is possible that NM may serve a similar function in binding iron in neurons, as does ferritin in the glia, thus representing an as yet unrecognised mechanism by which the cell can protect itself against oxidative damage. In contrast, when iron-saturated NM was added to the membrane homogenate, cell damage was significantly increased to 264% of that induced by NM alone; this damage was significantly attenuated by the addition of the iron chelator desferoxamine (Double et al., 1999). These results support the hypothesis that NM can have a protective influence on the cell, but can be detrimental when iron levels rise above a certain level.

Increased iron deposits in the SN may also result from heme oxygenase-1, a 32 kDa stress protein that degrades heme to biliverdin, free iron, and carbon monoxide (Schipper, 2004). In the normal mammalian brain heme oxygenase-1 mRNA and protein are confined to small populations of scattered neurons and glia. An increased heme oxygenase-1 staining was found in astrocytes and Lewy bodies in SN of patients with PD (Schipper, 2000). The cause for heme oxygenase-1 overexpression is unknown. It has been demonstrated that pro-oxidant effects of dopamine, hydro-

gen peroxide and proinflammatory cytokines stimulate the expression of heme oxygenase-1 (Schipper, 2004). Therefore, it was suggested that glial heme oxygenase-1 induction is a final common pathway leading to pathological iron sequestration and mitochondrial disturbances in neurodegenerative disorders.

Conclusion

Increased iron deposits in the SN in PD may have several reasons that may reside in disturbances of iron uptake, storage and transport as neurodegeneration progresses. Major iron stores are ferritin and haemosiderin in glial cells as well as NM in dopaminergic neurons. Age- and disease dependent overload of iron storage proteins may result in iron release upon reduction. Consequently, the low molecular weight chelatable iron complexes may trigger redox reactions leading to damage of biomolecules. Additionally, upon neurodegeneration there is strong microglial activation which can be another source of high iron concentrations in the brain. Although the current evidence suggests that increased brain iron may be a secondary result of neuronal degeneration in PD, the question of whether iron-associated degenerative pathways are a significant factor driving progressive neuronal death is yet to be definitively answered (Youdim et al., 1989; Riederer, 2004).

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Iron and Friedreich ataxia

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Summary. Friedreich ataxia is due to insufficient levels of frataxin, a mitochondrial iron chaperone that shields this metal from reactive oxygen species (ROS) and renders it bioavailable as Fe II. Frataxin participates in the synthesis of iron–sulfur clusters (ISCs), cofactors of several enzymes, including mitochondrial and cytosolic aconitase, complexes I, II and III of the respiratory chain, and ferrochelatase. It also plays a role in the maintenance of ISCs, in particular for mitochondrial aconitase. A role of frataxin in heme synthesis has been postulated, but is controversial. Insufficient frataxin leads to deficit of ISC enzymes and energy deficit. Iron levels increase in mitochondria. Oxidative stress may result from respiratory chain dysfunction and from direct reaction between iron and ROS. Stress pathways are activated that may lead to apoptosis or other forms of cell death. The basis for the selective vulnerability of specific neurons, like sensory neurons, is still unknown.

Introduction

Friedreich's ataxia (FA) is an autosomal recessive disease characterized by progressive neurological disability, cardiomyopathy, and increased risk of diabetes mellitus (Pandolfo, 2003). The disease, which currently has no treatment, affects roughly 1 in 50,000 people. The first symptoms usually appear in childhood, but age of onset may vary from infancy to adulthood. Atrophy of sensory and cerebellar pathways causes ataxia, dysarthria, fixation instability, deep sensory loss and loss of tendon reflexes. Corticospinal degeneration leads to muscular weakness and extensor plantar responses. A hypertrophic cardiomyopathy may contribute to disability and cause premature death. Kyphoscoliosis and pes cavus are common.

The FA gene (FRDA) encodes a small mitochondrial matrix protein, frataxin, that is highly conserved in evolution. FA is caused by a so far unique mutation mechanism: the expansion of an intronic GAA triplet repeat sequence. Most patients are homozygous for expanded GAA repeats, rare patients are heterozygous for an expanded repeat and a point mutation affecting the frataxin coding sequence. In all cases, FA patients have a profound but not complete frataxin deficiency, with a small residual amount of normal protein (Campuzano, 1996).

Most of our initial knowledge about the functional role of frataxin came from the investigation of yeast cells in which the frataxin homolog gene (YFH1) was deleted (Babcock, 1997). A mouse model of FA was difficult to generate because complete loss of frataxin causes early embryonic lethality. Viable mouse models have been so far obtained only through a conditional gene targeting approach (Puccio, 2001).

These investigation have unequivocally revealed that frataxin deficiency leads to loss

of function of Fe–S center containing enzymes (in particular respiratory complexes I, II and III, and aconitase), excessive free radical production in mitochondria and progressive iron accumulation in these organelles. Ongoing biochemical and structural studies are aimed to understand the specific function of the protein.

Frataxin function and pathogenesis

Knock-out of the yeast frataxin homolog gene (YFH1) has provided the first information on frataxin function (Babcock, 1997). Most YFH1 knock-out yeast strains, called *ΔYFH1*, lose the ability to carry out oxidative phosphorylation, cannot grow on non-fermentable substrates, and lose mitochondrial DNA. In *ΔYFH1*, iron accumulates in mitochondria, more then 10-fold in excess of wild type yeast, at the expense of cytosolic iron. Loss of respiratory competence requires the presence of iron in the culture medium, and occurs more rapidly as iron concentration in the medium is increased, suggesting that permanent mitochondrial damage is the consequence of iron toxicity. Iron in mitochondria can react with reactive oxygen species (ROS) that form in these organelles. Even in normal mitochondria, a few electrons prematurely leak from the respiratory chain, mostly from reduced ubiquinone (or probably its semiquinone form), directly reducing molecular oxygen to superoxide (O_2^-). Mitochondrial Mn-dependent superoxide dismutase (SOD_2) generates hydrogen peroxide (H_2O_2) from O_2^-, then glutathione peroxidase oxidizes glutathione to transforms H_2O_2 into H_2O. Iron may intervene in this process and generate the hydroxyl radical ($OH^\bullet$) through the Fenton reaction ($Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^\bullet + OH^-$). $OH^\bullet$ is highly toxic and causes lipid peroxidation, protein and nucleic acid damage. Occurrence of the Fenton reaction in *ΔYFH1* yeast cells is suggested by their highly enhanced sensitivity to H_2O_2 (Babcock, 1997).

Disruption of frataxin causes a general dysregulation of iron metabolism in yeast cells. Because iron is trapped in the mitochondrial fraction, a relative deficit in cytosolic iron results, causing a marked induction (10- to 50-fold) of the high-affinity iron transport system of the cell membrane, normally not expressed in yeast cells that are iron replete. As a consequence, iron crosses the plasma membrane in large amounts and further accumulates in mitochondria, engaging the cell in a vicious cycle.

The reason why *ΔYFH1* cells accumulate iron in the mitochondrial fraction may in principle involve increased iron uptake, altered utilization or decreased export from these organelles. Experiments involving induction of frataxin expression from a plasmid transformed into *ΔYFH1* yeast cells indicate that the protein stimulates a flux of non-heme iron out of mitochondria.

Several mitochondrial enzymes are known to be impaired in *ΔYFH1* yeast cells, in particular respiratory chain complexes I, II, and III and aconitase (Rötig, 1997). These enzymes all contain iron–sulfur clusters (ISCs) in their active sites. ISCs are synthesized in mitochondria and are highly sensitive to free radicals. Remarkably, all defects in ISC synthetic enzymes in yeast lead to mitochondrial iron accumulation, apparently due to defective iron export out of mitochondria, as it occurs in *ΔYFH1*. Recent data point to a direct involvement of frataxin in ISC synthesis. Frataxin appears not to be essential, but to strongly stimulate an early step of ISC synthesis. It appears to interact with the scaffold protein Isu1, where the first ISC assembly takes place (Stehling, 2004). Therefore, the following pathogenic cascade may be proposed: 1) frataxin deficiency primarily impairs ISC synthesis; 2) reduced ISC synthesis leads to inhibited iron export and mitochondrial iron accumulation; 3) iron generates free radicals that cause further damage to ISCs and decrease the enzymatic activities of ISC-containing proteins (ISPs);

4) decreased activities of several repiratory complexes (I, II, III) and of Krebs cycle enzymes lead to a further increase free radical production and the establishment of a vicious cycle.

It has been proposed that frataxin stimulates ISC synthesis because it is an iron chaperone. In the mitochondrial environment, frataxin would deliver iron to biosynthetic enzyme complexes and protects it from free radical attack. When mitochondrial iron is high, frataxin would also be able to detoxify and store the metal in a redox inactive form (O'Neill, 2005).

Several observations support the hypothesis that the above findings in experimental models have their counterpart in the human disease and are therefore relevant for its pathogenesis. Altered iron metabolism, free radical damage, and mitochondrial dysfunction all occur in FA (reviewed in Babcock et al., 1997).

Involvement of iron in FA was suggested twenty years ago by the finding of deposits of this metal in myocardial cells from FA patients. Iron accumulation has also been reported in the dentate nucleus and in the mitochondrial fraction from cultured fibroblasts has been reported. A general abnormality of iron metabolism may also be occurring in FA patients, as suggested by the high level of circulating transferrin receptor, the principal carrier of iron into human cells, which may reflect a relative cytosolic iron deficit as observed in the yeast model.

Oxidative stress has been demonstrated in FA cells and patients (Pandolfo, 2003; Calabrese, 2005). In particular, patients with FA have increased plasma levels of malondialdheyde, a lipid peroxidation product as well as increased urinary 8-hydroxy-2′-deoxyguanosine (8OH2′dG), a marker of oxidative DNA damage (Pandolfo, 2003). Patients' fibroblasts are sensitive to low doses of H_2O_2, that induce cell shrinkage, nuclear condensation and apoptotic cell death at lower doses than in control fibroblasts. Increased free radical production was directly shown in mouse P19 embryonic carcinoma cells with reduced levels of frataxin, adversely affecting their neuronal differentiation and increasing apoptosis, an observation that may have implication for the human disease (Santos, 2001).

Mitochondrial dysfunction has been proven to occur *in vivo* in Friedreich ataxia patients. Phosphorus-MRS analysis of skeletal muscle and heart shows a reduced rate of ATP synthesis (Lodi, 1999). Biochemically, the same multiple ISP enzyme dysfunctions found in *ΔYFH1* yeast are found in affected tissues from FA patient (Puccio, 2001).

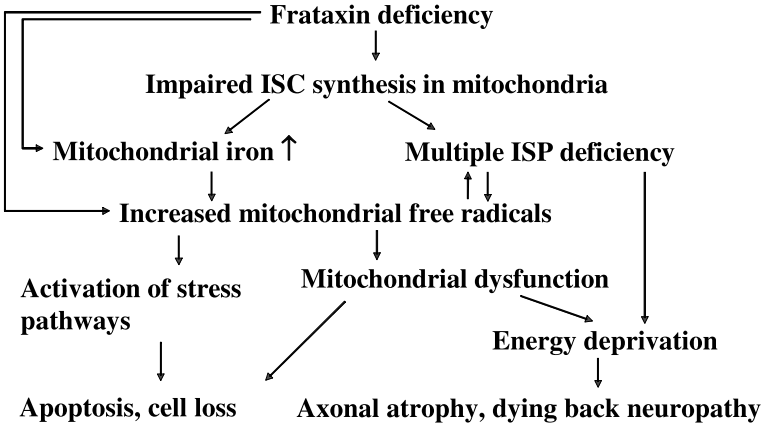

Fig. 1. Hypothesis for the molecular pathogenesis of Friedreich ataxia

Conclusions

FA is an example of a systemic disease with a major neurodegenerative component that is due to mitochondrial dysfunction related to abnormal iron handling in these organelles. A general hypothesis on its pathogenesis, that tries to keep into account all our current knowledge on frataxin function, is shown in Fig. 1.

Our current knowledge on FA pathogenesis can provide important clues to develop treatments for this so far incurable disease. Molecular therapies aimed to restore frataxin levels by gene replacement, by protein therapy, or by acting on the triplet repeat expansion are fascinating possibilities, but they require much basic research to determine feasibility and to develop appropriate approaches. The same can be said of cellular therapies to repair damage resulting from neurodegeneration and cardiomyopathy. However, the findings on mitochondrial dysfunction, oxidative stress and iron abnormalities suggest therapeutic options that may be developed in a shorter time frame. In this regard, encouraging results with Coenzyme Q derivatives suggest that antioxidant treatment targeted to mitochondria and stimulating the respiratory chain may be beneficial. Searching for small molecules that mimic some or all of frataxin function is now possible and may lead to simple and effective treatments.

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Nongenetic causes of Parkinson's disease

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Summary. Study of the nongenetic causes of Parkinson's disease (PD) was encouraged by discovery of a cluster of parkinsonism produced by neurotoxic pyridine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the 1980s. Since that time, epidemiologic investigations have suggested risk factors, though their results do not establish causality. Pesticide exposure has been associated with increased risk in many studies. Other proposed risks include rural residence and certain occupations. Cigarette smoking, use of coffee/caffeine, and non-steroidal anti-inflammatory drugs (NSAIDs) all appear to lower risk of PD, while dietary lipid and milk consumption, high caloric intake, and head trauma may increase risk. The cause of PD is likely multifactorial. Underlying genetic susceptibility and combinations of risk and protective factors likely all contribute. The combined research effort by epidemiologists, geneticists, and basic scientists will be needed to clarify the cause(s) of PD.

Introduction

Parkinson's disease (PD) is a common, disabling and increasingly prevalent neurodegenerative disorder of middle and late life with unknown causes. Although some genes causing familial parkinsonism have been identified, these account for only a fraction of cases. Most cases of PD are not found in familial clusters, and twin studies find heritability to be low (Tanner, 1999, Wirdefeldt, 2004).

Etiologic clues from the investigation of disease clusters

An observed disease cluster may indicate cause if the excess of cases is due to identifiable factors. However, there are many uncertainties regarding the appropriate definition of a cluster, and excesses of disease are expected to occur by chance. Nevertheless, much of the work investigating the nongenetic causes of parkinsonisms was prompted by investigation of a cluster of parkinsonism produced by the neurotoxic pyridine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston, 1983). MPTP-induced parkinsonism has many clinical and pathologic similarities to PD, suggesting that substances with similar mechanisms may cause PD. For example, the pesticides rotenone and paraquat have mechanistic and/or structural similarities to MPTP, and both, like MPTP, can produce experimental parkinsonism in animals (DiMonte, 2003).

Etiologic clues from descriptive epidemiology

In epidemiologic investigations, the characteristics of a disease are investigated in entire populations, rather than in individuals, to provide etiologic clues. Comparing disease

frequencies among populations with differing characteristics and identifying unique patterns of disease within populations may indicate possible causes. In all populations studied, PD is rare before age 50, then increases rapidly with increasing age (Van den Eeden, 2003). This pattern could reflect either the cumulative effects of an environmental exposure or an age-associated genetic factor. PD is also more common in men than in women (Korell and Tanner, 2004), and male-associated environmental exposures or X chromosome-linked genetic susceptibility factors could explain this. Studies in multiethnic populations have suggested that PD may be less common in Blacks and Asians and more common in Hispanics (Van den Eeden, 2003), but the populations studied were too small to be conclusive. If real, these differences could reflect different environmental or genetic risk factors, although differences in health care utilization may also contribute. Geographic variations have been suggested, including an excess of PD in rural North America and a north-south gradient of mortality, but these findings require replication (Korell and Tanner, 2004). PD may differ internationally, with North America and Europe showing higher rates of disease, but there are too few comparable studies to allow a definitive conclusion (Korell and Tanner, 2004). PD prevalence has increased recently in certain areas (Zhang, 2005; Kuopio, 1999), but not others (Rocca, 2001), perhaps reflecting region-specific increases in PD-causing toxicant exposure, or improved recognition of PD.

Clues from analytic studies

In analytic epidemiologic studies, proposed risk factors for disease are compared between persons with PD (cases) and those without PD (controls). A critical limitation of this approach is the inability to directly determine whether an association between a risk factor and PD is causative. In humans, ethical considerations limit experiments to prevention and treatment; experiments investigating causes are performed in animals, and results may not easily be extrapolated to humans.

Pesticides

In general, pesticide exposure is associated with an increased risk of PD. A meta-analysis of 19 published studies found a combined odds ratio (OR) of 1.94 (95% C.I. 1.49–2.53) for pesticide exposure (Priyadarshi, 2000). However, the category pesticide is very broad, and includes chemicals with many different mechanisms. Only a few studies have identified specific compounds or compound classes, including herbicides, insecticides, alkylated phosphates, organochlorines, wood preservatives, dieldrin and paraquat (Korell and Tanner, 2004; Firestone, 2005). Gene-environment interaction may also be important, and those with impaired pesticide metabolism may be most vulnerable (Elbaz, 2004).

Farming

Numerous studies world-wide have identified rural living, farming, gardening and drinking well water as risk factors for PD (Korell and Tanner, 2004). These associations may represent pesticide exposure, or other rural-related risks. Although the specific associations are varied, the consistency of the general finding is remarkable.

Other occupations

A higher frequency of PD has been reported among teachers and health care workers (Schulte, 1996; Tsui, 1999), carpenters and cleaners (Fall, 1999) and in workers chronically exposed to metals (Gorell, 2004). Welding has been proposed as a risk factor (Racette, 2005), but this finding is controversial (Fryzek, 2005).

Smoking

Cigarette smoking, on average, appears to lower the risk of developing PD by about half, with greater protection associated with more smoking (Hernan, 2002). This inverse association has been reported in nearly every population studied over more than 30 years (Quik, 2004). Animal studies suggest that nicotine may protect against experimental parkinsonisms (Quik, 2004), although others have suggested that not smoking is an early behavioral manifestation of PD (Menza, 2000).

Diet

Oxidative stress may be increased by lipid consumption and higher caloric intake, and eating foods high in animal fat has been associated with increased risk of PD in several studies (Korell and Tanner, 2004). Milk consumption is associated with an increased risk of PD in two prospective studies (Chen, 2002; Abbott, 2005). Milk can contain toxicants such as organochlorine pesticides, as well as high levels of the MPTP analogue, tetrahydroisoquinoline.

Coffee and caffeine

An inverse association of both coffee and caffeine consumption and PD has been reported in many case control and cohort studies (Ross, 2000; Ascherio, 2001). In men, a dose-dependent association between higher consumption and lower risk is observed, but a less clear pattern is seen in women. Caffeine may be neuroprotective via antagonist action on the adenosine A2A receptor.

Head trauma

Prior head trauma has been associated with PD in numerous case control studies (Bower, 2003). The head injury typically occurs decades before the diagnosis of Parkinson's disease, minimizing the chance that disease-related disability caused the injury. Medical record validation suggests that this is a real association, not explained by recall bias.

Nonsteriodal Anti-inflammatory Drugs (NSAIDs)

An inverse association of NSAID use with risk of PD has been observed in two prospective studies for non-aspirin NSAIDs, as well as for aspirin (Chen, 2003; Abbott, 2003). Inflammatory mechanisms appear to contribute to neurodegeneration in PD, and animal studies suggest that NSAIDs have neuroprotective properties (McGeer, 2004).

Other associations

Various other factors have been associated with PD, many of these are shown in the Table 1. In general, these findings require further investigation.

Table 1. Factors associated with the risk of Parkinson's disease

Factors directly associated
Increasing age
Diet: Animal fat, Milk
Drinking well water
Physical and emotional stress
Male gender
White race
Family history of PD
Rural residence
Pesticides
Farming
Metals
Factors inversely associated
Smoking
Caffeine intake
NSAID use
Alcohol
Greater physical activity

PD Parkinson's disease, *NSAID* non-steroidal anti-inflammatory drug

Conclusions

Recent advances in epidemiological, genetic, and basic science research are generating plausible hypotheses regarding the cause of Parkinson's disease and suggest PD is a complex disorder with multiple causes, likely due to interactions of genes and environment. Successful future therapies and preventive measures will depend on a sound understanding of the interaction of these factors.

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Is atypical parkinsonism in the Caribbean caused by the consumption of Annonacae?

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Summary. An abnormally frequent atypical levodopa-unresponsive, akinetic-rigid syndrome with some similarity to PSP was identified in the Caribbean island Guadeloupe, and was associated with the consumption of plants of the Annonacea family, especially *Annona muricata* (corossol, soursop) suggesting a possible toxic etiology. Annonaceae contain two groups of potential toxins, alkaloids and acetogenins. Both alkaloids and annonacin, the most abundant acetogenin, were toxic *in vitro* to dopaminergic and other neurons. However we have focused our work on annonacin for two reasons: (1) annonacin was toxic in nanomolar concentrations, whereas micromolar concentrations of the alkaloids were needed, (2) acetogenins are potent mitochondrial poisons, like other parkinsonism-inducing compounds. We have also shown that high concentrations of annonacin are present in the fruit or aqueous extracts of the leaves of *A. muricata*, can cross the blood brain barrier since it was detected in brain parenchyma of rats treated chronically with the molecule, and induced neurodegeneration of basal ganglia in these animals, similar to that observed in atypical parkinsonism. These studies reinforce the concept that consumption of Annonaceae may contribute to the pathogenesis of atypical parkinsonism in Guadeloupe.

Introduction

A cluster of atypical parkinsonsim was reported in Guadeloupe (Caparros-Lefebvre et al., 1999, 2002). Whereas 30% of parkinsonian patients examined in the Neurology Department of the University Hospital (Pointe-a-Pitre) had idiopathic Parkinson's Disease (PD), 70% had atypical forms with levodopa unresponsive parkinsonism, early postural instability, cognitive decline and, in half of the cases, supranuclear gaze palsy and/or hallucinations. The number of patients, the absence of family histories of parkinsonism in most cases and their cross-ethnic origins suggested that an environmental factor might be responsible.

This was supported by a case-control study showing that, compared to idiopathic PD or controls, patients with atypical parkinsonism consumed significantly more fruit and infusions or decoctions of leaves from plants of the Annonacea family, particularly *Annona muricata* (soursop, corossol), *A. muricata* and other plants of the Annonaceae contain potential neurotoxins:

isoquinolinic alkaloids and acetogenins, a large, unique and structurally homogenous class of polyketides (fatty acid derivatives) specific to the Annonaceae, some of which are among the most potent inhibitors of complex I of the mitochondrial respiratory chain known. Because complex I inhibitors such as MPP^+ or the pesticide rotenone kill dopaminergic neurons in the substantia nigra (Langston et al., 1999; Betarbet et al., 2000; Hoglinger et al., 2003), we tested *in vitro* and *in vivo* the neurotoxicity of annonacin, the most abundant acetogenin of *A. muricata*, and compared it to reticuline and coreximine, the two major isoquinolic alkaloids in the fruit. To determine whether alimentary and medicinal consumption of annonaceous products results in exposure to toxic concentrations of these substances, we quantified annonacin in fruit and traditional leaf extracts of *A. muricata*.

Material and methods

Mesencephalic cell cultures

Mesencephalic cell cultures were prepared from the ventral mesencephalon of day 15.5 rat embryos (Lannuzel et al., 2002, 2003). Survival of dopaminergic neurons was quantified by counting the number of cells immunoreactive for tyrosine hydroxylase (TH) and all neurons, regardless of their neurotransmitter immunoreactive for microtubule-associated protein-2 (MAP-2).

Extraction of alkaloids and annonacin

The roots of *A. muricata* were dried, powdered and extracted with methanol (Lannuzel et al., 2002, 2003). The purity of the compounds was confirmed by analytical high-pressure liquid chromatography (Lannuzel et al., 2003).

Animal model

Male Lewis rats (Janvier Breeding Center, Le Genest St Isle, France) were implanted with Alzet osmotic mini pumps (2 ML4; IFFA CREDO, Arbresle, France) to deliver annonacin for 28 days through a catheter in the left femoral vein (Hoglinger et al., 2003; Champy et al., 2004). For histological analysis, the rats were anaesthetized (30 mg/kg sodium pentobarbital i.p.), killed by transcardial perfusion and the brains treated as described (Champy et al., 2004). For ATP assay and annonacin detection, rats were perfused with phosphate-buffered saline, before removing the brains that were frozen immediately in isopentane at $-30°C$ and stored at $-80°C$.

Detection of annonacin

Annonacin in plant preparations and brain extracts from treated animals was purified by reverse-phase HPLC and analyzed by matrix associated laser desorption ionization-time of flight (MALDITOF) mass spectrometry (Champy et al., 2005).

Measurement of complex I activity and intracellular ATP levels

Complex I activity was evaluated by spectrophotometry (Lannuzel et al., 2003; Champy et al., 2004) and intracellular ATP levels by chemluminescence (Lannuzel et al., 2003).

Results

Toxicity of Anonaceae for dopaminergic neurons in culture

We extracted the most abundant alkaloids (reticuline and coreximine) and the main acetogenin (annonacin) from *A. muricata* and tested their neurotoxicity in primary neuronal cultures. After a 24 hours treatment, 50% of the dopaminergic neurons degenerated with 13 μM coreximine and 304 μM reticuline. Annonacin was much more potent than the alkaloids, with an effective concentration [EC50] of 18 nM. Annonacin was approximately 50-fold more potent than the classic complex I inhibitor MPP^+ and 2-fold

Table 1. Comparison of the toxic effects of annonacin, rotenone and MPP^+ for TH^+ and MAP-2^+ neuronal cells in mesencephalic cultures

Toxins	TH^+ cells EC_{50} (μM)	MAP-2^+ cells EC_{50} (μM)
Annonacin	0.018 ± 0.004	0.027 ± 0.005
Rotenone	0.034 ± 0.009	0.041 ± 0.011
MPP^+	1.9 ± 0.3	72 ± 11

The cultures were exposed to each toxin for 24 h and then processed for TH or MAP-2 immunocytochemistry

more potent than rotenone in inducing neuronal death (Table 1). Unlike MPP^+ but similar to rotenone, cell death induced by alkaloids or annonacin did not require toxin uptake by the dopamine transporter and, consequently, was not restricted to dopaminergic cells. Raising glucose concentrations largely restored intracellular ATP levels and prevented neuronal demise. Thus, alkaloids, but more so annonacin, extracted from *A. muricata* promote dopaminergic neuronal death by impairing energy production.

Annonacin, induces nigral and striatal neurodegeneration in rats

Because of its toxicity *in vitro*, we asked whether chronic systemic exposure of a living animal to annonacin would result in neurodegeneration similar to that observed in atypical parkinsonian patients. We therefore, infused annonacin intravenously (3.8 and 7.6 mg per kg per day for 28 days) in rats and demonstrated that annonacin can cross the blood brain barrier intact, since unmetabolized annonacin was detected in the brain

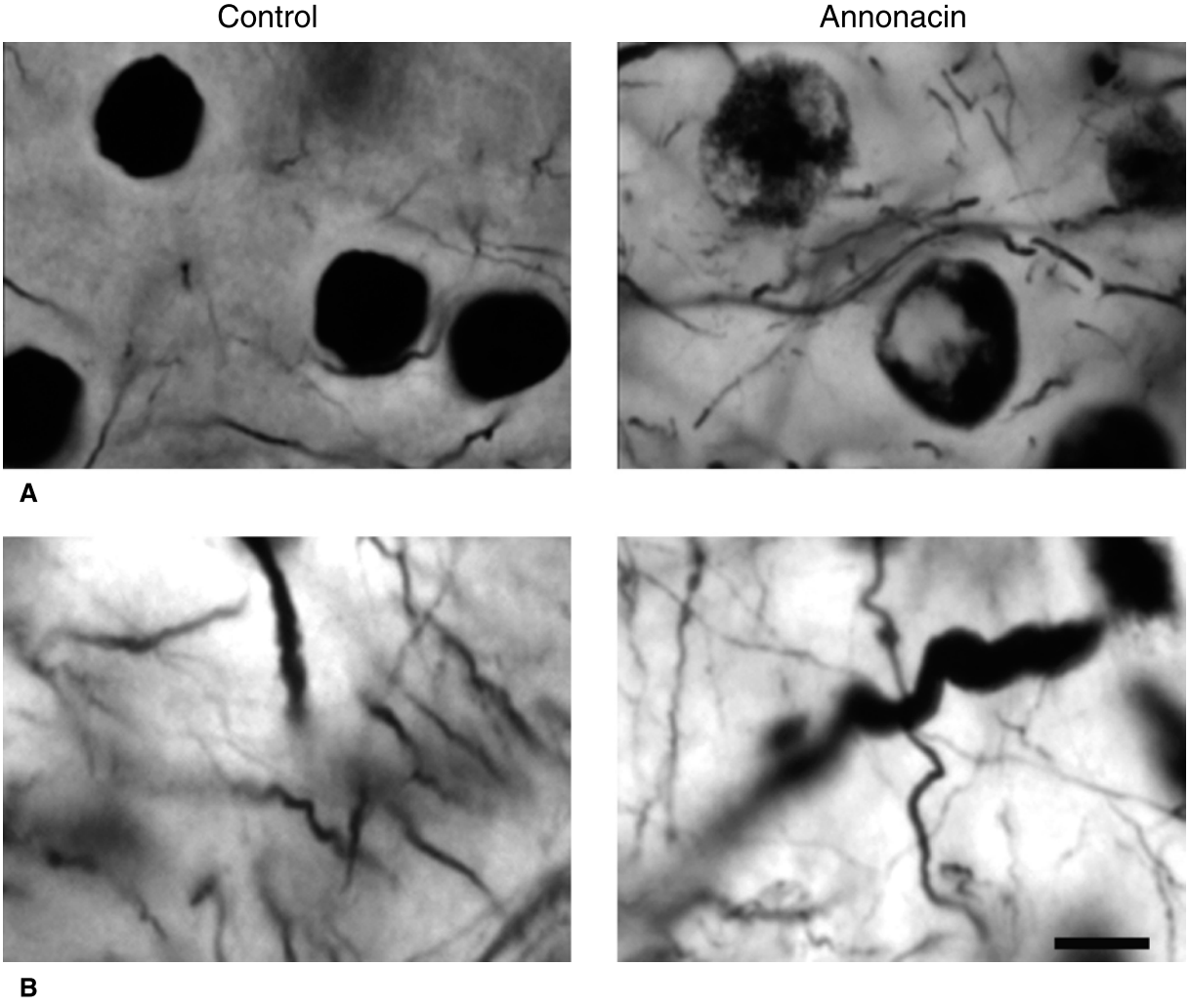

Fig. 1. Histological alterations visualized by Bodian silver impregnation in the striatum of annonacin (7.6 mg/kg/day)-treated rats. Enlarged and dystrophic nuclei (**A**) and dystrophic fibres (**B**) can be observed in annonacin-treated animal but not in control animal. Scale bar: 10 µm

parenchyma, and decreased brain ATP levels by 44%. Annonacin induced morphological alterations and a dose-dependent loss of nigral dopaminergic neurons and striatal dopaminergic fibers (Fig. 1). Neurodegeneration was not restricted to nigrostriatal neurons but was pronounced and widespread in basal ganglia and brainstem nuclei, whereas hippocampus, cerebellum, and cerebral cortex were only moderately affected. The distribution of the lesions, including nigral and striatal neuronal cell loss, was similar to that of patients with atypical parkinsonism, reinforcing the hypothesis that systemic exposure to complex I inhibitors in fruit and extracts of Annonaceae can induce atypical parkinsonism.

Quantification of acetogenins in A. muricata

To quantify the exposure to acetogenins through consumption of *A. muricata*, we determined the concentrations of annonacin in extracts and preparations of fruit and leaves by matrix-assisted laser desorption-ionization mass spectrometry. An average fruit was estimated to contain about 15 mg of annonacin, a can of commercial nectar 36 mg and a cup of infusion or decoction 140 µg. As an indication of its potential toxicity, an adult who consumes one fruit or can of nectar a day is estimated to ingest, over one year, the amount of annonacin that induced brain lesions in rats receiving purified annonacin by intravenous infusion.

Discussion

We showed that *A. muricata* contains alkaloids and mitochondrial complex I inhibitors, acetogenins, are toxic to dopaminergic and other neurons. Annonacin, the most abundant acetogenin can cross the blood brain barrier and induce neurodegeneration in the basal ganglia as in atypical parkinsonism. Our results, therefore, support the hypothesis that consumption of annonaceous products may contribute to the neuronal degeneration responsible for the atypical forms of parkinsonism observed in Guadeloupe and reinforce the concept that mitochondrial dysfunction might play a role in the aetiology of Parkinson's disease or PSP-like syndromes, a concept born twenty years ago with the discovery that the complex I inhibitor MPP^+ is selectively accumulated in dopaminergic neurons causing degeneration of nigrostriatal dopaminergic neurons and parkinsonism in humans (Langston et al., 1999). Our findings indicate that natural, lipophilic complex I inhibitors such as annonacin that distribute homogenously throughout the brain are also capable of inducing a PSP-like form of atypical parkinsonism. The consumption of Annonaceae in Guadeloupe and other tropical areas (Angibaud et al., 2004), which is a source of these neurotoxins potentially constitutes a serious public health problem. Further investigations are therefore urgently needed.

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CYP450, genetics and Parkinson's disease: gene × environment interactions hold the key

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Summary. The ecogenetic theory contends that most cases of Parkinson's disease (PD) result from the actions of environmental factors in genetically susceptible individuals on a background of normal ageing. This notion is supported by epidemiologic data; family history of PD and exposures to environmental toxins such as pesticides increase risk, while cigarette smoking reduces risk. As a result, polymorphic genes that code for metabolic enzymes have been considered as candidates for conferring differential risk for PD. Given their prominence in xenobiotic metabolism, the cytochrome P450 (CYP) genes have come under great scrutiny. The activity of CYP2D6 is largely determined by genetic variability and common sequence variants exist in human populations that lead to poor metaboliser (PM) phenotypes. These have been extensively studied as genetic risk factors for PD with inconsistent results. However, these studies have disregarded interactive effects (e.g. gene × environment interactions) despite the assertions of the ecogenetic theory. Data from our group and others suggest that the CYP2D6 PM genotype interacts with certain environmental factors such as pesticide exposure and cigarette smoking to confer differential risk for PD. Previous failure to consider such interactions might, in part, explain the inconsistencies observed in the CYP2D6 genetic risk-factor literature. Our data illustrate, using CYP2D6 as an exemplar, that it is crucial to consider both genetic and environmental factors, and their interactions, in any examination of risk factors for PD.

Introduction

Parkinson's disease is a late onset neurodegenerative disease affecting 1.6% of the population over 60 years of age and with an average age at onset in the late 7^{th} or early 8^{th} decade. The triggers for the disease remain unknown although a complex aetiology is universally accepted. This symposium discusses the cytochrome P450 (CYP) family of metabolic enzymes, their role in the CNS and the implications for PD. This paper will provide background to this discussion. Why might modulators of xenobiotic metabolism be important in PD? What role(s) do the CYPs play? How do we interpret the existing literature? What important considerations must be addressed in the study of risk factors in human populations? These issues will be addressed in this discourse using real data from the study of the CYP2D6 gene in PD as an exemplar.

Ecogenetic theory

The ecogenetic theory of PD (see Fig. 1), adapted from the cancer literature and first

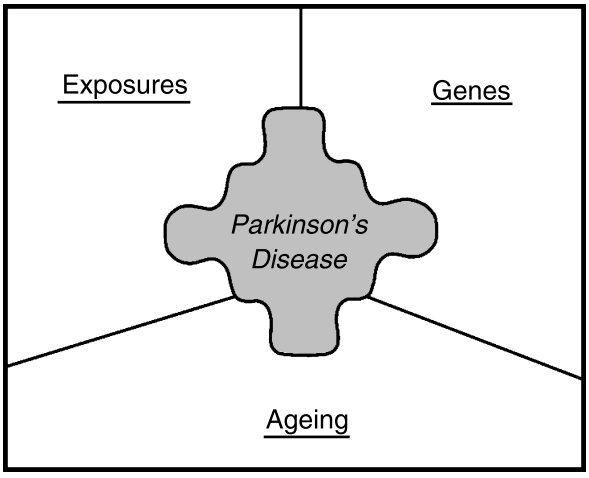

Fig. 1. The ecogenetic theory of PD

outlined by Barbeau and colleagues in their 1985 Lancet paper suggests that:

"... Parkinson's disease is the result of environmental factors acting on genetically susceptible individuals against a background of normal ageing" (Barbeau, 1985).

This statement, written 20 years ago remains totally consistent with all available current research evidence. Let's now briefly look at each aspect of this theory.

Environmental factors and PD

Intense interest in environmental exposures as risk factors for PD developed in the 1980s after reports confirmed that intoxication with the synthetic meperidine derivative, MPTP, could reproduce the features of PD in humans and animals. This stimulated a search for exogenous or endogenous molecules with similar effect. This also prompted a multitude of epidemiological surveys to assess potential environmental risk factors for Parkinson's disease. It is now clear that a number of classes of molecules have an ability to induce parkinsonism in animals and humans. Examples include the mitochondrial toxin rotenone, proteasomal pathway inhibitors and certain heavy metals. In addition, epidemiological surveys, mostly using a case-control experimental design, have identified environmental exposures that may modify risk for the disease. Exposures to pesticides, neurotoxic metals, solvents, well water and rural residency reportedly increase risk; cigarette smoking and coffee consumption appear to be associated with a reduced risk. Despite considerable noise, three consistent findings emerge from such studies: (1) Exposure to pesticides is associated with increased risk; (2) Exposure to cigarette smoke is associated with a decreased risk; (3) A family history of PD is associated with increased risk.

These findings are consistent with the ecogenetic theory. Readers interested in further information about these risk factors are directed to meta-analyses that review the literature for these risk factors in more detail. Pesticide exposure yields a summary odds ratio (OR) of 1.94 (95%CI = 1.49–2.53) (Le Couteur et al., 1999). Smoking's effect is likewise modest (OR = 0.59, 95%CI = 0.54–0.63) (Hernan et al., 2002). These effect-sizes, which constitute a less than two-fold change in risk, require large sample sizes to detect.

With the caveat that family members tend to share environments and occupations, the family history story points to inherited factors (this will be discussed below).

Genetic factors and PD

That genes are important in the aetiology of PD is unequivocal. Rare monogenic forms of parkinsonism, resulting from genetic abnormalities in the so-called *PARK* genes are now well known. These are helping to define the important biological pathways leading to the neurodegeneration seen in PD. Twin studies, family studies and more complex genetic segregation analyses also point to a genetic component for typical idiopathic PD. It is, however, pertinent to remind the reader that the genetic component of PD is relatively modest. Moreover, the vast majority of individuals with PD have no apparent family history. Thus it is logical that any examination

of genetic risk factors for PD should also consider their environmental context.

Ageing and PD

The final protagonist in the ecogenetic theory of PD is normal ageing. Ageing results in a multitude of physiological changes that could contribute to increased risk for neurodegenerative disease. Well characterised changes in the central nervous system (CNS), cardiovascular system (CVS), metabolic pathways and gene-expression profiles, many of which are highly inter-related, all contribute to this increased risk. In terms of PD, the effect of ageing appears to shift the aetiological balance more towards environmental risk factors. In other words, the older an individual gets, the greater the environmental component to PD risk. This idea is illustrated in the results of twin studies and family studies.

This extremely rudimentary summary of the major concepts of the ecogenetic theory of PD seeks only to provide a flavour for the complex and multifactorial nature of the aetiology of PD. A more comprehensive summary of age-environment and gene-environment interactions in the pathogenesis of PD can be found in a recent review article on the topic (Le Couteur et al., 2002).

Metabolic pathways link all three aspects of the ecogenetic theory

One link between all three main components of the ecogenetic theory for PD involves the body's ability to deal with endogenous or exogenous molecules that may be directly or indirectly neurotoxic; this ability is manifest in metabolic or detoxification pathways. Such pathways are responsible for the removal of environmental exposures including certain pesticides and components of cigarette smoke. They are made up of enzymes such as the CYP enzymes, the activity of which are subject to the influences of commonly occurring genetic polymorphism. Furthermore, the influence of normal ageing can result in significant physiological and gene-expression alterations, leading to important perturbations in these metabolic pathways. These have important potential implications for the risk of diseases such as PD.

Metabolic pathways

Metabolic pathways can be simply classified into three different phases. Phase I metabolism involves oxidations, phase II enzymes conjugate these oxidised substrates into more polar ones, while phase III processes actively transport these conjugated molecules across membranes and out of cells. Examples of phase I enzymes include the flavin monooxygenases (FMO), paraoxonases and the cytochrome P450 family which is the focus of this symposium. The glutathione transferases (GSTs), N-acetyl-transferases (NATs) and glucuronidases are all phase II enzymes, while the multi-drug resistance associated proteins (MRPs) are in the phase III category.

For reasons outlined above, it is possible that enzymes in any or all of these categories could be regarded as potential candidates to influence the risk for PD. Indeed, genetic polymorphism in many of them has been assessed for association with disease. In historical terms, the CYP2D6 gene has arguably received the most attention; the remainder of this paper will concentrate on these studies. However, the various issues raised in relation to the study of CYP2D6 are equally applicable to the study of other metabolic enzymes in association to differential risks for PD.

The Cytochrome P450s

Recent genomic analyses highlight the natural diversity of this family of enzymes. Approximately 4000 different cytochrome P450 genetic isoforms exist in nature, being represented in all forms of life from simple bacteria through to complex eukaryotes. There are at least 50 different human CYPs isoforms, divided into 10 different subfamilies. These enzymes are generally defined as

mix-function mono-oxygenases that act together with the cofactor NADPH-P450. In the course of this symposium you will hear of several of these human isoforms including, CYP1A1, CYP2C6, CYP2C13, CYP2D6, CYP2D7 and CYP2E1. The genes coding for these isoforms exhibit commonly occurring genetic variability in human populations that contribute to individual variability in enzyme activity. A review by A. K. Daly summarises the currently known and functionally relevant ones (Daly, 2003).

Influencers of metabolic activity

We have already mentioned that normal human ageing and common genetic sequence variation are two important influencers of metabolic enzyme activity. However, it is also pertinent to highlight that there is a complex web of interacting determinants which can modulate the gene-expression and protein function of metabolic enzymes. In particular, an individual's exposure to various external factors can have massive effects. Many of the CYP genes exhibit inducibility, suppression and competitive influences. For example, CYP1A1 expression in the lung is induced by components or cigarette smoke and CYP2E1 in liver is alcohol inducible. Conversely, the ingestion of certain dietary components (such as grapefruit juice) or drugs can result in considerable reductions in the CYP3A4 metabolism of other substrates. These effects may also be influenced by genetic background. Even for CYP2D6, a so-called non-inducible isoform, certain medications including anti-parkinsonian agents have been shown to be associated with reduced activity. These points highlight the importance of non-genetic factors resulting in phenotypic variability.

CYP2D6 phenotypic variability

CYP2D6 has attracted great attention to PD researchers because it participates in the metabolism of the parkinsonism-inducing toxin MPTP, herbicides (like atrazine and paraquat) and organophosphate pesticides. Moreover, it has been long known that there is considerable phenotypic variability in the enzyme activity of CYP2D6. CYP2D6 activity (or phenotype) was traditionally measured pharmacokinetically in terms of a metabolic ratio (MR), defined as the ratio of parent substrate (usually spartine or debrisoquin) to hydroxyl-metabolite in the urine of patients. Population frequencies of CYP2D6 phenotype can be grouped into three categories, namely extensive metaboliser (EMs, ''normals''), intermediate metabolisers (IMs) and poor metabolisers (PMs). There are also a very small number of individuals who have an extremely rapid enzyme activity (ultra-rapid metabolisers). The phenotypic activity of CYP2D6 is primarily (but not exclusively) controlled by the different versions (or alleles) of the CYP2D6 gene that exist in human populations. Thus genetic analyses using DNA from patients can be used to define the specific nucleotide sequences at the CYP2D6 locus (genotypes) and thus infer the phenotypic category of an individual (phenotype). There are several different CYP2D6 alleles that result in a PM phenotype, however three of these make up the vast majority, namely: (1) CYP2D6*3 (2549A>del frameshift); (2) CYP2D6*4 (the most common PM variant resulting from a 1846 G>A spice site variation); (3) CYP2D6*5 (a full deletion of the gene). Individuals carrying one PM allele usually act as IMs while two copies of a variant allele lead to the PM phenotype. Traditional PCR-RFLP methods have been used to genotype these alleles, but these methods are fast being replaced by newer methods such as the affymetrix CYP-chip which can simultaneously screen for 18 CYP PM variants (including 10 in CYP2D6). In this age of the $1000 genome there is an explosion of available genetic sequence information from CYP genes. It will be a tremendous challenge to determine the most appropriate ways to use this information to learn more about disease risk.

CYP2D6 PMs and PD

There have been dozens of studies that have tested the question of whether CYP2D6 PMs are over-represented in PD cases. The first such analysis, published in the Lancet in 1985, was conducted by Barbeau and colleagues who showed that IM and PM phenotypes were over-represented in their PD group (Barbeau, 1985). A series of replicate studies based on phenotyping methods yielded inconsistent results. Smith and colleagues were the first to use a purely genetic characterisation of metaboliser status (Smith et al., 1992). They showed that PMs were over-represented in their PD group with an OR = 2.54 (95%CI 1.51–4.28).

Subsequent studies again yielded equivocal findings. Five meta-analyses have now summarised the published data (McCann et al., 1997; Christensen et al., 1998; Rostami-Hodjegan et al., 1998; Joost et al., 1999; Persad et al., 2003). Interestingly, all Caucasian analyses show a slightly increased frequency of CYP2D6 PMs in PD cases compared to controls. However, the summary data appears highly influenced by the original genetic study of Smith and colleagues (Smith et al., 1992) and, taken together, the general consensus of PD researchers is reflected in the statement of Riedl and colleagues:

"As yet there is no conclusive evidence to suggest that CYP2D6 polymorphisms confer susceptibility to PD" (Riedl et al., 1998).

While it is clear from the published literature that PM status does not confer a particularly strong "main effect" on PD risk, three important facts should ensure that CYP2D6 remains on the research radar of PD researchers: (1) Current thinking suggests that there are many determinants of PD risk, most with modest effect sizes similar to the apparent PM effect (consider pesticides, cigarette smoking and family history as "main effects" of similar magnitude); (2) None of the previous literature reports seriously considered adjustment of analyses for important potential confounders such as age, gender, ethnic background or environmental factors (the most critical being smoking which may be related to CYP genotype); (3) These studies may not be asking the right question with respect to CYP2D6. Clearly, if a polymorphism at the CYP2D6 locus influences susceptibility to PD by altering the metabolism of an environmental neurotoxin, then studies will only show an association if they examine those subjects that have had such neurotoxin exposure. Thus studies need to examine interactive, as opposed to main effects if we are to better understand the impact of CYP genotype on PD risk.

Do interactions between CYP2D6 PM genotype and environmental exposures influence the risk for PD?

To explore this question we examined 400 neurologist diagnosed PD cases and 400 unrelated, unaffected aged individuals for whom we had well characterised epidemiological information (including cigarette smoking and pesticide exposure data) and DNA for CYP2D6 genotype analysis. The results are summarised in Table 1.

PD cases were more likely to have claimed regular pesticide exposures as defined as weekly exposure for a period of six months or more (adjusted OR = 3.57, 95%CI = 1.15–11.07). However, a particularly interesting result is observed when this data was stratified for CYP2D6 PM genotype. We considered unexposed, EMs as the reference. For individuals who have never been exposed to pesticides, PM genotype appears protective, suggesting that perhaps CYP2D6 might actually be involved in the metabolism of as yet unidentified substrates with a protective effect. EMs, who are exposed to pesticides, don't appear to be at substantially increased risk. However as exposure dose is increased, and the ability to metabolise CYP2D6 substrates (such as pesticides) decreases the risk for PD increases;

Table 1. Studying CYP2D6 PM gene × environment interactions in 400 Australian PD cases and 400 unaffected aged controls

	Exposure to Pesticides (regular = weekly > 6 months)	
	Never	Regular
Cases	151 (38%)	61 (16%)
Controls	208 (54%)	21 (5%)
	OR* (95%CI) = 3.57 (1.15–11.07)	

	Exposure to Pesticides		
Group	Never OR	Occasional OR	Regular OR
	(95%CI)	(95%CI)	(95%CI)
EMs	1.00	1.24 (0.82–1.86)	1.3 (0.63–2.83)
IMs	0.95 (0.59–1.54)	0.89 (0.54–1.47)	3.27 (1.21–8.80) P > 0.02
PMs	0.29 (0.11–0.80) P > 0.02	2.09 (0.80–5.43)	8.47 (1.01–69.76), P > 0.05

All OR calculated using logistic regression adjusted for: age, sex, family history of PD & smoking. The respective freq. of the *3 & *4 alleles were 1.15% & 22.3% in cases and 1.29% & 2.3% in controls. 3 cases and 1 control were homozygous *5. All genotypes were in HWE

PMs who claim regular exposure exhibit an OR of 8.47 (95%CI = 1.01–69.76). In isolation this result was considered interesting but probably simply a chance finding. Therefore, it was with much interest to discover that almost an identical finding was reported by the group of Alexis Elbez and colleagues, who were studying a cohort of French farmers, also with known pesticide exposures (Elbaz et al., 2004). Their data also clearly show a reduced OR for unexposed PMs and an increase in risk with increasing pesticide exposure and reduced metabolic capacity. Clearly these interesting findings warrant further examination.

Given that CYP2D6 participates in the metabolism of nicotine and possibly other components of cigarette smoke, we thought that we should also consider the possibility for interactions between PM genotype and cigarette smoking influencing risk for PD. While these data are in an extremely preliminary stage, we have made two interesting observations: (1) PM genotype may be related to smoking status in healthy normal subjects (we examined 540 unaffected aged Australians and showed that PMs are less likely to smoke and smoke less if they happen to be smokers); this highlights the possibility that cigarette smoking could be a crucial confounding variable in any examination of PM genotype and PD and (2) An analysis of 434 PD cases who claimed no pesticide exposure, revealed that PMs who smoke exhibit a later age-at-onset of disease (69 ± 5ys) compared to non-smokers and EMs who smoke (61 ± 11ys) $P = 0.02$; this relationship is dependent on dose. One explanation for this tantalizing result is that PMs, who have a reduced ability to eliminate nicotine or other potential neuroprotective components of cigarette are somewhat protected from the development of PD. It is important to note, however, that we did not observe any direct effect of PM status on the OR for smoking in this analysis. Both of these results are speculative and require further serious examination in replicate cohorts. None-the-less this provides further evidence for the existence of relevant gene-environment interactions influencing an individual's risk for PD.

Conclusions

This paper summarises the ecogenetic theory for the aetiology of PD. It provides arguments for why genetic variables in enzymes involved in the metabolism of endogenous and exogenous substrates (particularly the CYP genes) are valid candidates for conferring differential risk to the development of PD. Using CYP2D6 PM genotypes as an exemplar, data are presented to highlight the importance of considering the environmental context when examining genetic variability in metabolic pathways. Failure to consider crucial gene × environment interactions in such analyses may result in the inappropriate dismissal of important risk-altering factors. The aetiology of PD is, by its nature, complex and single main effects are unlikely to account for major proportions of the attributable risk. Single gene × environment interactions are also likely to be poor oversimplifications of the reality. However, with the concerted efforts of collaborative researchers, examining well-defined study cohorts of adequate sample size, with standardised and validated instruments for assessing environmental exposures, and an appreciation that neither genetic nor environmental risk factors for PD can be examined with mutual exclusivity, there is much to be gained from the continued development of the ecogenetic theory for PD.

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Unique cytochromes P450 in human brain: implication in disease pathogenesis

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Summary. Cytochromes P450 is a family of heme proteins that metabolize xenobiotics including drugs. Unique human brain cytochrome P450 enzymes metabolize xenobiotics including drugs to active/inactive metabolites through biotransformation pathways that are different from the well-characterized ones in liver. We have identified an alternate spliced functional transcript of CYP2D7 containing partial inclusion of intron 6 in human brain but not in liver or kidney from the same individual. Genotyping revealed the presence of the frame-shift mutation 138delT only in those subjects who expressed the brain variant CYP2D7, which metabolizes codeine exclusively to morphine unlike hepatic CYP2D6 that metabolizes codeine to nor codeine and morphine. CYP1A1 bioactivates polycyclic aromatic hydrocarbons to reactive DNA binding metabolites and initiates carcinogenesis. We have identified a unique splice variant of CYP1A1 having deletion of 87 bp of exon 6 which is present in human brain but not in liver of the same individual. We present evidence for the existence of biotransformation pathways in human brain that are dissimilar from known pathways in liver. Identification and characterization of novel CNS-specific P450 enzymes generated by alternate splicing of known genes or as yet unidentified genes may help predict consequences of exposure to xenobiotics including pesticides in the brain.

Metabolism of foreign compounds is an important prerequisite for detoxification of xenobiotics. A major enzyme involved in the metabolism of foreign compounds is cytochrome P-450 (P450). Generally, P450 mediated metabolism of xenobiotics leads to the formation of hydrophilic, non-toxic metabolites that are easily excreted from the body. However, there are instances wherein an inert, non-toxic compound is bioactivated to a reactive, toxic metabolite that can interact with cellular macromolecules leading to cell damage. Multiple forms of P450, which are selectively induced or inhibited by a variety of drugs are known to exist in liver, the major organ involved in P450 mediated metabolism (de Montellano, 1996). P450 enzymes, such as CYP2D6 exhibit genetic polymorphism and 7–10% of caucasians are poor metabolizers of debrisoquine (prototype substrates for P4502D6) while the remaining 90% are extensive metabolizers. The significance of P450 metabolism in extrahepatic organs (such as lung, kidney, skin, nasal epithelium) and pharmacological and toxicological consequences of *in situ* metabolism in target organs has been increasingly recognized (Gram et al., 1986; MeLemore et al., 1990). These studies have revealed the preferential localization of drug metabolizing enzymes within specific cell types in these organs rendering them vulnerable to damage

by bioactivation, in situ, within these cells (Boyd, 1980).

Human brain is perhaps one of the most complex organs both functionally and anatomically. Brain exhibits a multitude of diversity both with respect to its distinct anatomical regions and cellular elements and is highly vulnerable to damage by toxic compounds due to the limited regenerative capability of the neurons, the major cell type involved in specialized functions of the brain. The distinctive features of the capillary endothelial cells surrounding the cerebral blood vessels render protection to the brain by preventing entry of circulating molecules. The blood–brain barrier, as this hypothetical barrier is commonly known, results from the presence of tight junctions and the paucity of pinocytic vesicles. However, xenobiotics that are lipophilic in character can diffuse through endothelial cells of brain capillaries and enter neuronal cells. Thus, bioactivation, in situ, in neuronal cell can have far-reaching consequences causing irreversible disruption of neuronal function. Further, metabolism of drugs in brain can lead to local pharmacological modulation at the site of action and result in variable drug response. Minor metabolic pathways, which are not of significance in liver could potentially produce significant pharmacological responses, if they were to occur at the site of action, within specific nuclei in the brain. This raises the question – can the brain metabolize xenobiotics? What is the P450 content in brain and where is the enzyme localized?

P450 content in rat brain is approximately 3–10% of the corresponding level in liver. The enzyme is not uniformly distributed amongst different regions of the brain and highest P450 levels have been detected in olfactory lobes, cerebellum and brain stem. Cytochrome P450 content and associated monooxygenase activities have been measured in microsomes from human brain tissue obtained at autopsy. Regional differences have been noted in the distribution of P450 in human brain and the hemeprotein levels are highest in the brain stem and cerebellum and lowest in striatum and hippocampus. Immunocytochemical localization of brain P450 has been performed using antisera to hepatic P450 enzymes and the preferential localization of P450 in neuronal cells has been documented (Ravindranath and Boyd, 1995).

The presence of P450 in neuronal cells, which have very limited regenerative capability brings forth the potential consequences of bioactivation of xenobiotics in situ in the CNS. In neurodegenerative diseases such as, Parkinson's, Alzheimer's and motor neuron disease specific cell populations within specialized regions of the brain are affected leading to selective loss of function. A role for environmental toxicants has been proposed in the pathogenesis of these disorders. The above hypothesis has gained ground following the incidence of Parkinson's disease (PD) in young adults who were exposed to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). The observations made with MPTP have demonstrated how the unique features of the CNS can potentially aid bioactivation of inert compounds to ultimate toxins, sequester them in target cells leading to irreversible damage to specific regions of the brain and thus bring about selective loss in function. The localization of P450 in specific cell types, such as the neurons of substantia nigra, which are affected in Parkinson's disease points to the possible role of P450 mediated bioactivation or impaired detoxification of xenobiotics within these cells.

Following these discoveries several studies were carried out to ascertain the association between polymorphism in P450 enzymes, such as CYP2D6, 1A1, 1A2 and 2E1 and incidence of PD. These were based on the hypothesis that defective hepatic metabolism of environmental toxicants could potentially result in increased burden in the CNS leading to neurodegeneration. Several studies have been carried out in diverse population across the world with confounding

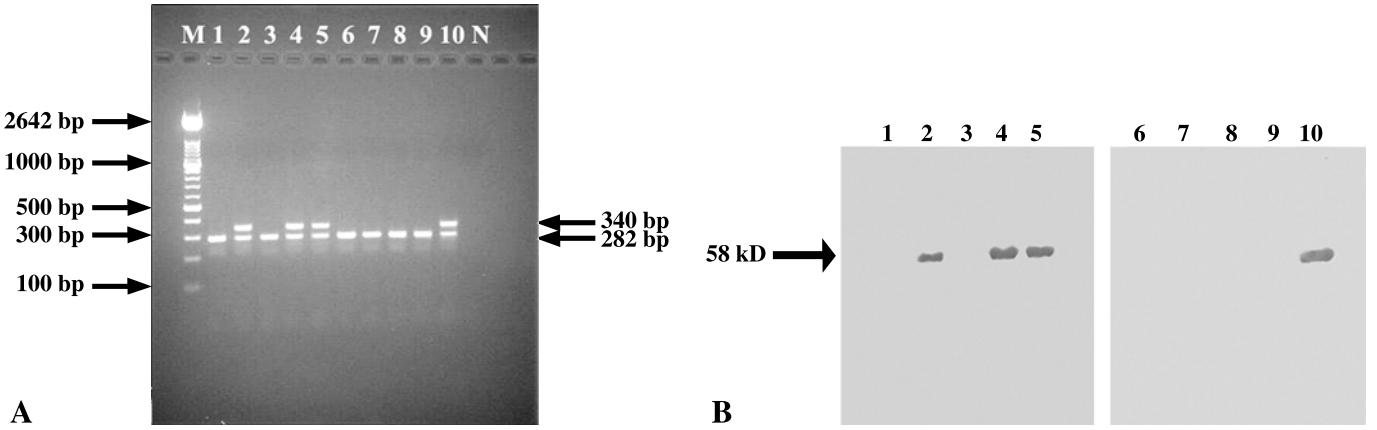

Fig. 1. Expression of brain variant CYP2D7 and CYP2D6 in human samples. **A** RT-PCR analysis identifying brain variant CYP2D7 in 10 human brain autopsy samples (lanes 1–10). Human brain was obtained from Human Brain Tissue Repository, NIMHANS, India. The average age of the individuals was 30.7 ± 4.1 years and postmortem delay between death and autopsy was 8.2 ± 1.7 hrs. Brain variant CYP2D7 could be detected in 4 samples wherein a band of 340 bp was detected. The PCR amplified product of 282 bp representing CYP2D6 was detected in all samples. **B** Membrane preparations from the 10 human autopsy brain samples (lanes 1–10) were subjected to immunoblotting and stained with antiserum to brain variant CYP2D7. Brain variant CYP2D7 protein could be detected only in 4 brain samples, which also showed the expression of the transcript by RT-PCR. 'M' represents molecular weight markers

results and no clear association has emerged. This raises an important question: Are there brain-specific biotransformation pathways that differ from the well-characterized one in liver? While many P450 enzymes are expressed in brain, is there anything unique about their presence there?

One of the first evidence to emerge demonstrating the difference between brain and liver in the metabolism xenobiotics was the observation of differential metabolism of alprazolam. Alprazolam, an anti-anxiety agent, is metabolized in liver by P4503A to 4-hydroxy alprazolam (4-OHALP, pharmacologically less active) and α-hydroxy alprazolam (α-OHALP, pharmacologically more active). We observed that the relative amount of pharmacologically active metabolite, α-OHALP formed in brain was higher than liver. Since hydroxy metabolites of alprazolam are hydrophilic and not easily cleared through blood-CSF barrier, α-OHALP would potentially have a longer half-life in brain. While P450 levels in the brain are one-tenth to one-fifteenth of the corresponding hepatic levels and it is generally acknowledged that the capability of the brain to carry out P450 mediated metabolism of xenobiotics is substantially lower, the above considerations not withstanding bioactive metabolites can be formed in significantly high amounts in the brain (Pai et al., 2002).

Conclusive evidence for the presence of unique, brain-specific P450 enzymes was provided following the identification of a splice variant of CYP2D7 in human brain. A frame-shift mutation 138delT generates an open reading frame in the pseudogene, CYP2D7 and an alternate spliced functional transcript of CYP2D7containing partial inclusion of intron 6 was identified in human brain but not in liver or kidney from the same individual. mRNA and protein of the brain variant CYP2D7 was detected in 4 out of 10 human autopsy brains (Fig. 1). Genotyping revealed the presence of the frame-shift mutation 138delT only in those human subjects that expressed the brain variant CYP2D7. In liver, the major organ involved in drug metabolism, a minor metabolic pathway mediated by CYP2D6 metabolizes codeine (pro-drug) to morphine (active drug) while nor-codeine, is the major metabolite. In contrast, when expressed in Neuro2a cells, brain variant CYP2D7 metabolized codeine to morphine with greater efficiency compared to cells expressing CYP2D6. Morphine binds to μ-opioid receptors in certain regions of the central nervous system, such as periaqueductal gray and produces pain relief. The brain variant CYP2D7 and μ-opioid receptor colocalize in neurons of periaqueductal gray area in human brain indicating that metabolism of codeine to morphine could occur at the site of opioid action (Pai et al., 2004).

CYP1A1, the cytochrome P450 enzyme, bioactivates polycyclic aromatic hydrocarbons to reactive metabolite(s) that bind to DNA and initiate carcinogenesis. We have identified in human brain a unique splice variant of cytochrome CYP1A1 cDNA having deletion of 87 bp of exon-6 (Chinta et al., 2005). This splice variant was present in human brain, but not in the liver from the same individual and was absent in rat brain and liver. Structural modelling of the putative protein indicated broadening of the substrate access channel indicating that it could potentially have broader substrate specificity. The presence of distinct cytochrome P450 enzymes in human brain generated by alternate splicing that are different from well-characterized hepatic forms indicates that metabolism of xenobiotics could occur in brain by pathways different from those known to occur in liver.

Current research on the pathogenesis of PD and its relationship to xenobiotic metabolizing enzymes, such as, P450 have been restricted to identifying association between genetic polymorphisms in P450 enzymes and incidence of sporadic PD (Riedl et al., 1998). Assessment of gene-environment interaction in PD, eg. exposure to pesticides and polymorphisms in P450 has provided evidence

for increased incidence of PD in CYP2D6, PM (poor metabolizer) phenotype who have been exposed to pesticides (Elbaz et al., 2004). Although genetic polymorphism of CYP2D6 is one of the important determinants of inter-individual variation in drug response, functional polymorphism of P450 does not always correlate with outcome. We now present evidence for the existence of a pathway that can potentially mediate metabolism of xenobiotics at the site of action by mechanisms that are dissimilar from known pathways in liver.

Recent evidence indicating the presence of unique P450 enzymes in brain generated by alternate splicing indicates that potentially these unique P450 enzymes could play a role in determining the consequence of pesticide exposure in brain leading to neurodegeneration and potentially sporadic PD. Since the alternate spliced P450 enzymes are specifically generated in brain but not in other organs in the same individuals they are not related to differences in the genomic sequence. Nervous system has a propensity for generating alternate spliced genes and their expression is governed by the spliceosomal complex and RNA binding proteins, which are poorly understood. This discovery adds a new dimension to the role of environmental toxins, such as, pesticides in the pathogenesis of PD, wherein the metabolism of the toxin, in situ, in the brain by brain-specific P450 enzymes would govern the outcome. Identification and characterization of novel histio-specific isoforms of P450 generated by alternate splicing of known genes or as yet unidentified genes may help predict outcome of exposure to xenobiotics including drugs that act on the CNS.

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Cytochrome P450 and Parkinson's disease: protective role of neuronal CYP 2E1 from MPTP toxicity

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Summary. Elucidation of the biochemical steps leading to the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)-induced degeneration of the nigro-striatal dopamine (DA) pathway has provided new clues to the pathophysiology of Parkinson's Disease (PD). In line with the enhancement of MPTP toxicity by diethyldithiocarbamate (DDC), here we demonstrate how other CYP450 (2E1) inhibitors, such as diallyl sulfide (DAS) or phenylethylisothiocyanate (PIC), also potentiate the selective DA neuron degeneration in C57/bl mice. In order to provide direct evidence for this isozyme involvement, CYP 2E1 knockout mice were challenged with MPTP or the combined treatment. Here we show that these transgenic mice have a low sensitivity to MPTP alone, similarly to the wild type SVI, suggesting that it is likely that transgenic mice compensate for the missing enzyme. However, in these CYP 2E1 knockout mice, DDC pretreatment completely fails to enhance MPTP toxicity; this enhancement is instead regularly present in the SVI control animals. This study indicates that the occurrence of CYP 2E1 in C57/bl mouse brain is relevant for MPTP toxicity, and suggests that this isozyme may have a detoxificant role related to the efflux transporter of the toxin.

Abbreviations

DA dopamine; *PD* Parkinson's Disease; *DDC* diethyldithiocarbamate; *PIC* phenylethylisothiocyanate; *DAS* diallyl sulfide; *MPTP* 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; *MPP*$^+$ 1-methyl-4-phenylpyridinium; *SN* substantia nigra; *TH* tyrosine hydroxylase; *SVI* Cyp 2e1+/+ (129S1/SvImJ); *GONZ* Cyp 2e1−/− (129/SV-Cyp 2e1^{tm1Gonz}).

Introduction

In 1985, a member of our group serendipitously achieved unexpected data on MPTP toxicity by demostrating, for the first time, that a compound, diethyldithiocarbamate (DDC), potentiates MPTP toxicity in the mouse model (Corsini, 1985). The DDC-induced enhancement of MPTP toxicity in mice has been extensively confirmed by numerous reports in which Authors give the results of their studies on MPTP metabolism in general and on MPP$^+$ kinetics (the toxic metabolite) in particular, pursuing the definition of the enzymes responsible for the neurotoxic versus neuroprotective pathways in the liver and brain as well.

More recently, the discovery of the occurrence of cytochrome P450 in the brain and in DA neurons as well, the function of which is still unknown (Warner et al., 1994), led to a

new hypothesis in this respect (Corsini et al., 2002). In particular, cytochrome P450 2E1 (CYP 2E1) was identified in the rat brain and in DA neurons of the substantia nigra as well (Watts et al., 1998) and, at the same time, DDC was found to be a fairly specific inhibitor of this isozyme (Stott et al., 1997).

In order to understand the role played by CYP 2E1 in the DDC-induced enhancement of MPTP toxicity in mice, we studied the effects of diallyl sulfide (DAS) and phenylethylisothiocyanate (PIC), two specific inhibitors of CYP 2E1 enzymatic activity (Brady et al., 1991; Nissbrandt et al., 2001), on MPTP toxicity. CYP 2E1 knockout mice were challenged with MPTP or the DDC-combined treatment.

Materials and methods

Animals

Male C57/bl mice (Harlan, Italy), 8 weeks old and weighing 20 to 24 gr, were kept under environmentally controlled conditions (12 hrs light/dark cycle with light on between 07.00 and 19.00 hrs; room temperature +21°C) with food and water *ad libitum*. The animals were treated in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health. The experiments described in this article were formally approved by the Committee for Scientific Ethics of the University of Pisa.

Knockout mice

Male *Cyp 2e1* knockout mice (*129/SV-Cyp 2e1^{tm1Gonz}*) (*Cyp 2e1−/− Stock number: 002910*) and their wild type counterparts (*129S1/SvImJ*) (*Cyp 2e1+/+ Stock number: 002448*) were obtained from The Jackson Laboratory (Bar Harbor ME, USA). *Cyp2e1* (−/−) mice in 129/Sv-*Ter* background were generated in the Gonzalez laboratory (Lee et al., 1996) back-crossed four times into the wild type 129/Sv-*Ter* strain. These animals were screened for viral infection by Charles River Laboratories and all tests were negative. Confirmation of the *Cyp 2e1−/−* status was confirmed by the absence of CYP 2E1 as determined by liver DNA PCR phenotyping by Charles River Laboratories.

Experimental protocol

Twelve mice per group were treated i.p. with either MPTP hydrochloride (36 mg/kg) or distilled water. The animals were pretreated i.p. with DDC (400 mg/kg) or DAS (25 mg/kg) or PIC (25 mg/kg) or the vehicle, one hr before MPTP administration. DDC was easily dissolved in distilled water, whereas the liquid DAS was given already dissolved in a mixture of DAS plus tween 80 plus propylene glycol plus distilled water in 1, 1.5 and 10 volume proportions, respectively. The liquid PIC plus distilled water and one drop of tween 80 was made up to the appropriate volume and then homogenized with an ultrasonic disrupter prior to the injection. DA has been measured according to Vaglini et al. (2004). For statistical evaluation ANOVA with Sheffe-F analysis was used.

Results

Striatal modification of DA in C57-bl

The effect of the acute administration of CYP 2E1 inhibitors on the dopamine (DA) content in the mouse striatum one hour before a single exposure to MPTP is shown in Fig. 1.

In agreement with previous data (Corsini et al., 1985), 7 days after the combined treatment (DDC + MPTP), striatal DA content fell to 8.9% of controls (10.3 ± 2.8 and 115.2 ± 4.5 ng/mg protein, respectively). The animals treated with DDC alone did not show any change compared with control values. Figure 1 also shows the effects of pretreatment with DAS. The combined treatment

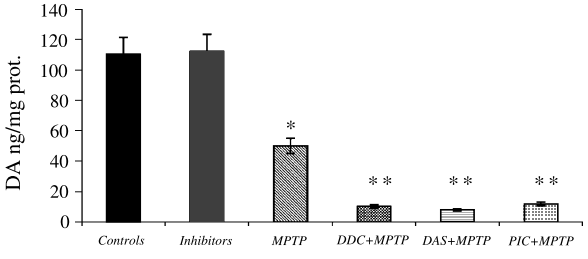

Fig. 1. Effect of DDC (400 mg/Kg), DAS (25 mg/kg) and PIC (25 mg/kg) on striatal tissue levels of dopamine (DA) in MPTP-treated mice. The results are the mean ± s.e. of 3 experiments (n = 6–8 mice for each experiment). C57-bl were treated with MPTP (30 mg/kg i.p.) or saline 60 min. after DDC, DAS or PIC. The animals were sacrified 7 days later. Respective controls received saline instead of inhibitors or MPTP. $^{*}p < 0.05$ in comparison with control mice; $^{**}p < 0.05$ in comparison with the animals treated with MPTP

Table 1. Effect of DDC on striatal tissue levels of dopamine (DA) in wild type (SVI) and knockout (GONZ) (Cyp 2e1−/−) male mice treated with MPTP. The results are the mean ± s.e. of N(10–20) animals for each group. Wild type and knockout were treated with MPTP (30 mg/kg i.p.) or saline solution 60 min after DDC at the dose of 400 mg/Kg i.p. The animals were sacrified 7 days later

Treatments	SVI (CYP 2E1+/+) DA (ng/mg prot.)	%	GONZ (CYP 2E1−/−) DA (ng/mg prot.)	%
Controls	114.1 ± 8.3		109.4 ± 9.8	
DDC	105.8 ± 9.0		108.8 ± 4.1	
MPTP	80.2 ± 4.1*	29	76.5 ± 6.3*	30
DDC + MPTP	48.7 ± 5.1**	57	73.7 ± 5.7***	32

*$p < 0.05$ in comparison with control mice; **$p < 0.05$ in comparison with MPTP-treated animals; ***n.s. in comparison with MPTP-treated animals

(DAS + MPTP) reduced the striatal DA content to 7.2% whereas MPTP alone caused a reduction of only 45.0% compared with controls. (Control values were 110.2 ± 8.2 ng/mg protein; 7.9 ± 1.0, 49.5 ± 4.4 ng/mg protein DAS + MPTP and MPTP respectively). As shown in Fig. 1, PIC also potentiated MPTP toxicity. The combined treatment significantly reduced the striatal DA content in comparison with MPTP alone ($p < 0.05$).

MPTP toxicity in CYP 2E1 knockout mice (GONZ)

The effect of MPTP treatment on the striatal DA content was studied in *CYP 2E1−/−* (GONZ) and wild type (SVI) male mice. As shown in Table 1, the single usual dose of 30 mg/kg i.p. produced a significant reduction in GONZ (30%) and SVI (29%) as well animals. The DDC pretreatment, at the usual time and dose schedule, completely failed to potentiate the DA fall in knockout mice (32%) whereas in wild type animals the combined treatment significantly enhanced the DA fall up to 57%.

Discussion

In this study we have demonstrated that, similarly to DDC, the CYP 2E1 inhibitors, such as DAS and PIC, markedly enhance MPTP toxicity, as measured by the dramatic fall in striatal DA content. This finding cannot be considered a fortuitous event, since for many years a great number of compounds have been tested as 'enhancers' but none of them have proved to increase MPTP toxicity, except for ethanol and acetaldehyde.

In order to provide direct evidence of toxicity, we have also performed the usual procedure of tyrosine-hydroxylase immunoreactivity in midbrain coronal slices of our treated mice (not reported here). Our results clearly indicate that while MPTP, at the dose we used, produced a minimal loss of DA perikaria in the SNpc (about 10%), the combined treatments induced at least 50% damage of the DA neurons, as previously observed with DDC (Vaglini et al., 2004). All these data confirm that DAS and PIC also strongly potentiate MPTP toxicity in this animal species, suggesting a specific role of CYP 2E1 in this toxic event.

In order to provide direct evidence for CYP 2E1 involvement, CYP 2E1 knockout mice (GONZ) and their respective wild type animals (SVI) were challanged with MPTP or the combined DDC + MPTP treatment. GONZ mice revealed a sensitivity to MPTP neurotoxicity similar to that one of SVI animals, but significantly lower than C57/bl strain. This suggests that it is likely that transgenic mice compensate the lack of CYP 2E1 with other isozyme. A similar compensation among

different P450 enzymes in this strain was observed for acetominophene toxicity (Lee et al., 1996). However in these knockout mice, DDC completely failed to enhance MPTP toxicity; this effect was instead regularly observed in the wild type animals. This further confirms the direct role of CYP 2E1 in DDC-induced enhancement of MPTP toxicity.

This study adds new insight into the role of CYP isozymes in MPTP-induced lesions of the nigro-striatal DA pathway, drawing attention more towards the metabolism in the brain than in the liver.

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Nicotine induces brain CYP enzymes: relevance to Parkinson's disease

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Summary. Brain expression of cytochromes P450 2B6, 2D6 and 2E1 is higher in smokers, and is induced by nicotine in animals. These enzymes can metabolize many of the neurotoxins associated with Parkinson's disease. Since smoking is known to be protective against Parkinson's disease, we hypothesise that nicotine-induced elevation of brain CYPs in smokers may contribute to neuroprotection against Parkinson's disease. This supports the therapeutic use of nicotine to delay the progress of this disease.

Cytochrome P450 enzymes (CYPs) metabolize a variety of compounds including drugs, food components, environmental carcinogens, and endogenous compounds such as neurotransmitters. Xenobiotic metabolism occurs mainly in the liver, but CYPs are expressed in most tissues examined, including brain (Miksys and Tyndale, 2002). The overall expression of brain CYPs is much lower than in liver, but these enzymes are expressed at different levels among brain regions, and are highly concentrated in specific cells, reaching levels that are as high if not higher than in individual hepatocytes (Miksys et al., 2000). While CYPs in brain are unlikely to contribute to overall metabolism, they are regulated differently from hepatic CYPs (e.g. Miksys et al., 2000), and respond to inducers with complex region- and cell-specific patterns which may impact the local metabolism of compounds that cross the blood–brain barrier.

Many of the CYP2 family enzymes are highly polymorphic, and some studies have associated genetic variation in CYP2D6 with neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Miksys and Tyndale, 2002). Many of these studies were inconclusive due to potential problems including insufficient population size, inappropriate controls, and undetected or unidentified alleles. However a meta analysis indicated that genetically deficient CYP2D6 poor metabolizers, lacking functional CYP2D6 enzyme, are at higher risk for Parkinson's disease (Odds Ratio 1.47, 95% CI: 1.18–1.96, $P = 0.01$) (McCann et al., 1997), especially if exposed to environmental toxins (Elbaz et al., 2004). However extensive metabolizers, with genetically normal activity, can also develop Parkinson's disease. CYP enzymes are highly regulated, and their expression can be inhibited or induced by xenobiotics. An individual with a CYP2D6 extensive metabolizer genotype may therefore have a poor metabolizer phenotype (inhibited CYP2D6 activity), and be at higher risk for developing Parkinson's disease, or may have higher than normal (induced) CYP2D6 levels, and be at lower risk.

It is thought that Parkinson's disease is caused, at least in part, by exposure of genetically susceptible individuals to endogenous and/or environmental toxins (Elbaz et al., 2004). Genes associated with Parkinson's

disease have been identified, as have some toxins that can cause a Parkinson's disease-like syndrome, such as catecholamines, amphetamine, pesticides, tetrahydroisoquinoline (TIQ) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Since 1985, efforts have been made to elucidate the role of drug-metabolizing CYPs in the development of Parkinson's disease, as these enzymes, in particular CYP2D6, are able to inactivate or activate many of these neurotoxins. CYP-mediated reactions can also create neurotoxic free oxygen radicals, and the balance of the neuroprotective and neurotoxic roles of specific brain CYPs requires elucidation for Parkinson's disease and other CNS disorders. Parkinson's-inducing toxins, or their neurotoxic metabolites, can be taken up into dopaminergic neurons by the dopamine transporter, where they cause oxidative stress, mitochondrial dysfunction, and eventually cell death. CYPs are expressed in neurons in human brain (Miksys and Tyndale, 2002, 2004), including dopaminergic neurons of substantia nigra and their terminals in the striatum, where these enzymes are ideally situated to detoxify Parkinson's disease causing agents, and so contribute to protection against the disease.

The neuroprotective effects of smoking for some neurodegenerative diseases are well documented; smokers are 50% less likely to develop Parkinson's disease, and this effect is dose-dependent, where a higher number of cigarette-pack-years is correlated with lower risk for developing the disease (Quik, 2004). Pilot therapeutic studies have been initiated using nicotine alone to slow the progress of Parkinson's disease, as this would be preferable to smoking. Nicotine protects against dopaminergic neuronal degeneration in vitro and nicotine and tobacco smoke are neuroprotective in animal models of Parkinson's disease (Quik, 2004), but the mechanism(s) are not clear. Nicotine's neuroprotective effects may be mediated by activation of nicotinic acetylcholine receptors (Quik, 2004) resulting in increased dopamine release. Alternately, tobacco smoke may inhibit monoamine oxidases in the brain that activate Parkinson's disease-causing toxins such as MPTP. We have shown that smoking in humans (Miksys et al., 2002, 2003; Miksys and Tyndale, 2004) and nicotine treatment in animals (Howard et al., 2003; Miksys et al., 2000) increase CYP expression in brain. We postulate that nicotine's neuroprotection against Parkinson's disease may be mediated, at least in part, by induced brain CYPs. Higher levels of CYP enzymes in the brain, due to genotype and/or due to CYP induction by xenobiotics such as nicotine in tobacco smoke, may protect against development or progression of Parkinson's disease by increasing the inactivation of neurotoxins. The following sections summarize our findings on the effects of nicotine in animal models, and smoking in humans, on brain expression of three members of the CYP2 family, CYP2D6, CYP2E1 and CYP2B6.

CYP2D6

This CYP has been most often associated with Parkinson's disease; CYP2D6 inactivates the neurotoxins MPTP and TIQ as well as a variety of pesticides and endogenous neurochemicals. Genetic variation affects CYP2D6 expression levels in brain in the same way as in liver, for example individuals who are heterozygous for the *4 deletion allele have lower brain CYP2D6 levels than wild type individuals, and individuals homozygous for the *4 allele do not express CYP2D6 in brain, as found in liver (Miksys et al., 2002). We have shown that rat brain CYP2D is enzymatically active, and this activity correlates with CYP2D protein expression among brain regions (Miksys and Tyndale, 2004). In human brain, CYP2D6 protein and mRNA expression vary among regions and are restricted to specific cells such as pyramidal neurons in the CA1 region of hippocampus and frontal cortex layer III,

and pigmented cells of the substantia nigra (Miksys et al., 2002). Smokers have significantly more CYP2D6 than non-smokers in some brain regions such as globus pallidus, substantia nigra and cerebellum; this increase can be specific to cell types such as the Purkinje cells in the cerebellum and the pigmented cells in the substantia nigra. Tobacco smoke has many components, and in order to determine whether nicotine is responsible for the elevated CYP2D6 levels observed in smokers, we examined brain CYP2D6 levels in African green monkeys treated chronically with nicotine. Our preliminary data show an increase in frontal cortex and in Purkinje cells of the cerebellum after more than two weeks of nicotine treatment at 0.6 mg/kg per day, a dose that produces plasma nicotine levels similar to those seen in smokers. These findings support the hypothesis that nicotine increases brain CYP2D6 levels. Nicotine may be neuroprotective against Parkinson's disease through increased inactivation of neurotoxins by induced CYP2D6 in brain.

CYP2E1

Studies in mice suggest that CYP2E1 plays a protective role in Parkinson's disease, probably through a mechanism involving detoxification in the brain (Vaglini et al., 2004). CYP2E1 metabolizes many xenobiotics such as ethanol, acetaminophen, halothane, aromatic hydrocarbons and endogenous compounds such as arachidonic acid. This enzyme is expressed in human brain, and the levels vary among brain regions and cell types (Howard et al., 2003; Miksys and Tyndale, 2002, 2004). We found that CYP2E1 expression was higher in some brain regions of alcoholic smokers when compared to alcoholic non-smokers, for example in pyramidal neurons of the frontal cortical layers III–VI, and in glial cells (Howard et al., 2003).

We also determined that nicotine alone could induce CYP2E1 by several fold in a human neuronal cell line that expressed both nicotinic acetylcholine and $GABA_A$ receptors (Howard et al., 2003). In addition, in rats treated chronically with nicotine, brain CYP2E1 was elevated 2–3 fold in 5 of 7 regions examined. This increased expression was restricted to specific cells such as pyramidal neurons and glial cells of the frontal cortex, and neurons in the caudate putamen (Howard et al., 2003).

Rodent and primate brain neuroanatomies are different as are their constitutive CYP2E1 expression patterns. Therefore, to test whether nicotine could contribute to the higher CYP2E1 in brains of human smokers, we are investigating the effect of chronic nicotine treatment on brain CYP2E1 in African green monkeys. Our preliminary data suggest that nicotine can induce CYP2E1 in some brain regions of primates, such as frontal cortex and cerebellum, and that this induction is cell specific, for example in the Purkinje cells of the cerebellum. Increased brain levels of CYP2E1 through smoking could enhance the protective role of CYP2E1 against Parkinson's disease, supporting our hypothesis that nicotine exerts its protective effects in part through induction of brain CYPs.

CYP2B6

This enzyme has not been directly associated with Parkinson's disease, however it can inactivate organophosphate insecticides and paraquat, a herbicide related in structure to MPTP. CYP2B6 is expressed throughout human brain in both neurons and glial cells (Miksys et al., 2003). Expression is highest in the caudate nucleus, putamen and cerebellum. When we examined smokers compared to non-smokers, we found that CYP2B6 expression was higher in pyramidal cells of the hippocampus, neurons of the caudate nucleus and putamen, and in the Purkinje cells of the cerebellum (Miksys et al., 2003; Miksys and Tyndale, 2004). To investigate the effects of nicotine alone, we examined the effect of chronic treatment on brain CYP2B levels in rats. We found that CYP2B

was higher in nicotine treated rats compared to saline treated controls in five of seven brain regions examined (Miksys et al., 2000; Miksys and Tyndale, 2004). Since the rat brain regions that showed CYP2B induction by nicotine were different from the regions that showed elevated CYP2B6 levels in smokers, we are currently investigating the effect of chronic nicotine on brain CYP2B6 levels in African green monkeys. Our initial data indicate that nicotine does induce CYP2B6 in monkey brain, suggesting that elevated CYP2B6 in at least some brain cell types of smokers may be due to nicotine in cigarette smoke. Increased CYP2B6 expression in brains of smokers, or individuals treated with nicotine, may enhance the central inactivation of neurotoxins associated with Parkinson's disease symptoms, such as organophosphate pesticides and paraquat.

The brain expression of at least three CYPs is higher in smokers and induced by nicotine in animals. Neither nicotine nor smoking increases the hepatic levels of CYP2D6 or CYP2B6, therefore overall metabolism remains largely unaffected. Smoking and nicotine modestly increase hepatic levels of CYP2E1. These CYPs are elevated in smokers in specific brain cell types, where they may be neuroprotective through inactivation of neurotoxins. However, high brain CYP enzyme activity for some CYPs has also been associated with neural oxidative damage. If, on further investigation, the overall balance between these processes in smokers proves to be neuroprotective, particularly in cell-types affected in Parkinson's disease, then these findings support the current therapeutic proposal to use nicotine to delay the onset or progression of Parkinson's disease. Further investigation into the role of specific CYPs in neuroprotection by nicotine is warranted and may lead to development of novel therapies that could replicate the protective role of nicotine through manipulation of CYP levels in the brain but not the liver.

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Genetic causes of Parkinson's disease: extending the pathway

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Summary. The functional characterization of identified disease genes in monogenic forms of Parkinson's disease (PD) allows first insights into molecular pathways leading to neurodegeneration and dysfunction of the nigrostriatal system. There is increasing evidence that disturbance of the ubiquitin proteasome pathway is one important feature of this process underscoring the relevance of protein misfolding and accumulation in the neurodegenerative process of PD. Other genes are involved in mitochondrial homeostasis and still others link newly identified signalling pathways to the established paradigm of oxidative stress in PD. Additional factors are posttranslational modifications of key proteins such as phosphorylation. Also, molecular data support the role of altered iron metabolism in PD. Here we describe known genes and novel genetic susceptibility factors and define their role in neurodegeneration.

Mapping and identification of PARK genes: an overview

Today, 11 gene loci responsible for or associated with PD have been mapped and genetic alterations in genes have been identified for 7 of these loci (Table 1). As numerous familial cases of PD can not be traced back to the currently known gene loci, one has to assume that more gene loci will be discovered in the future. It is likely that the function of the genes not yet identified will even further extend our understanding of the underlying pathogenic mechanisms causing neuronal cell loss. Our groups identified mutations in PD patients in several other genes, such as *synphilin-1* (Marx et al., 2003), its interacting protein periphilin (Soehn et al., submitted), *Omi/HtrA2* (Strauss et al., 2005), *14-3-3zeta-like* (Mosbacher et al., unpublished), and *ceruloplasmin* (Hochstrasser et al., 2005) which will direct our search for pathomechanisms in neurodegeneration also towards phosphorylation, proteolytic digestion, and iron metabolism.

Protein aggregation

The identification of α-*synuclein* as the first gene responsible for inherited PD was the starting point for studies defining a general role of this protein in neurodegeneration. First it turned out that ubiquitin was not the most specific marker for Lewy bodies. Indeed, α-synuclein was shown to be the most specific component in these pathognomonic protein lesions not only in brains of carriers of mutations in the α-synuclein gene, but also in the common sporadic form of the disease. This suggested a potential role of α-synuclein in molecular mechanisms leading to neurodegeneration also in non-familial PD (Spillantini et al., 1997).

Table 1. Genetically defined gene loci causing PD

Gene locus	Inheritance	Onset	Pathology	Map position	Gene	Reference
PARK1	Dominant	40s	Nigral degeneration with Lewy-bodies	4q21	*α-Synuclein*	Polymeropoulos et al., 1997
PARK2	Recessive	20s	Nigral degeneration without Lewy-bodies	6q25	*Parkin*	Kitada et al., 1998
PARK3	Dominant	60s	Nigral degeneration with Lewy-bodies, plaques and tangles	2p13	unknown	Gasser et al., 1998
PARK4	Dominant	30s	Nigral degeneration with Lewy-bodies, vacuoles in neurons of the hippocampus	4q21	*α-Synuclein* triplication and duplication	Singleton et al., 2003
PARK5	Dominant	∼50	Pathological data not reported yet	4p14	UCH-L1	Leroy et al., 1998
PARK6	Recessive	∼40	Pathological data not reported yet	1p35-37	*PINK1*	Valente et al., 2001
PARK7	Recessive	∼30	Pathological data not reported yet	1p38	*DJ-1*	Bonifati et al., 2002
PARK8	Dominant	∼50	Variable α-synuclein and tau pathology	12cen	*LRRK2*	Zimprich et al., 2004; Paisán-Ruiz et al., 2004
PARK9	Recessive	∼10	Striatal atrophy	1p36	unknown	Hampshire et al., 2001
PARK10	Dominant	50–60	Pathological data not reported yet	1p32	unknown	Hicks et al., 2002
PARK11	Dominant	Late	Pathological data not reported yet	2q36	unknown	Pankratz et al., 2003

The potency to form fibrils is strongest for the Ala53Thr α-synuclein followed by the Ala30Pro substitution. In addition wild-type α-synuclein is, to a lesser degree, able to convert oligomers and aggregates into fibrils (Giasson et al., 1999). The fact that even wild type α-synuclein forms aggregates has major implications for the understanding of sporadic PD, where no mutations in the α-synuclein gene were observed (Farrer et al., 1999). This represents an analogy to Alzheimer's disease (AD), where mutations in the *APP* gene result in an increased Aß fibrillization. To date there are different concepts for factors leading to aggregation of wild type α-synuclein in Lewy bodies: First, an increased expression of wild type α-synuclein might lead to intracellular accumulation and 'choking' of protein degradation mechanisms. This concept is supported by the identification of a promoter polymorphism in the *α-synuclein* gene as a risk factor in sporadic PD (Krüger et al., 1999; Tan et al., 2000), which might regulate gene expression. Recent studies demonstrated such a regulatory function of the promoter polymorphism on gene expression resulting in an increased expression of α-synuclein (Touchman et al., 2001). Even more intriguing evidence for a dosage effect of α-synuclein expression came from the observation that *α-synuclein* gene triplication does cause familial PD (Singleton et al., 2003). Therefore, increased levels of wild type α-synuclein contribute to neurodegeneration via excess amounts of protein overwhelming the degradation system and leading to increased accumulation and finally aggregation of α-synuclein.

Subsequent identification of mutations in the *Parkin* and *UCH-L1* (*ubiquitin carboxy-terminal hydrolase L1*) gene revealed that the proteins encoded by these genes are also components of the characteristic Lewy bodies (UCH-L1) and/or play a functional role in the ubiquitin-proteasome degradation pathway (Parkin, UCH-L1), underlining the relevance of this pathway in the pathogenesis of PD. Functional studies on UCH-L1 revealed two opposite functions of the physiological UCH-L1: Besides its hydrolytic activity in the degradation of polyubiquitin chains necessary for ubiquitin recycling, it may act as an ubiquitin-ligase mediating K63-linked ubiquitination (Liu et al., 2002). Whereas linkage of proteins with ubiquitin in position K48 leads to targeting to the proteasome, K63-linked conjugates mediate intracellular signalling and interfere with proteasomal degradation (Hofmann et al., 1999). Interestingly, the disease-associated Ile93Met mutation shifted the role of UCH-L1 towards ubiquitin-ligase activity with predominant linkage of ubiquitin residues in position K63. The respective ubiquitinated substrates interfere with protein degradation via the 26S proteasome and re-utilization of ubiquitin is hampered (Liu et al., 2002). Whether the ubiquitin E3-ligase Parkin predominantly mediates ubiquitination in position K48 or K63 of the ubiquitin protein is currently a subject of debate.

Parkin, but also the E3-ligases SIAH1 and SIAH2 (seven in absentia homologue 1 and 2) ubiquitinate an interactor of α-synuclein, called synphilin-1 (Engelender et al., 1999; Chung et al., 2001; Liani et al., 2004), a protein of unknown function which aggregates within Lewy bodies (Murray et al., 2003). We have recently identified a R621C missense mutation in two independent sporadic PD patients and demonstrated that C621 synphilin-transfected cells were more susceptible to staurosporine-induced cell death (Marx et al., 2003). We also identified periphilin as a novel binding partner of synphilin-1 and identified a missense mutation in two cousins suffering from PD (Soehn et al., submitted). Although the role of periphilin needs still to be investigated, it is interesting to note that it reveals a high degree of overlap with tyrosine hydroxylase expression in the substantia nigra in rodent brain (Soehn et al., submitted).

Role of proteolytic processing and mitochondrial dysfunction in PD

Mutations in the *parkin* gene are thought to cause a loss of ligase function preventing the ubiquitination of its substrates. This supported the hypothesis of impaired ubiquitin-mediated protein degradation causing neurodegeneration in PD via accumulation of Parkin substrates. However, existing loss-of-function mouse models do not display any neuronal loss and do not exhibit an accumulation of any of the known Parkin substrates (Itier et al., 2003; Palacino et al., 2004; Goldberg et al., 2003). An interesting feature in parkin knockout mice was an effect on mitochondrial respiratory capacity and indirect markers for oxidative stress (Palacino et al., 2004). Mitochondrial dysfunction is a common feature of PD (Beal, 2000). A specific and selective loss of mitochondrial complex I activity in the substantia nigra of PD patients reflects an important mechanism of mitochondrial pathology in PD (Shapira, 1999). Neuronal mitochondria function as integrators of diverse cellular stresses and mediators of apoptosis and therefore represent a major interface between endogenous or exogenous toxins and neurodegeneration. Mitochondrial dysfunction was also observed in a *parkin* knockout *Drosophila* model displaying morphologically altered mitochondria and increased susceptibility to oxidative stress (Pesah et al., 2004; Greene et al., 2003). These observations concerning mitochondrial dysfunction might further link Parkin to the other genes identified in autosomal recessive PD.

One of these, *PINK1*, encodes a serine/threonine kinase that is induced by PTEN in response to oxidative stress. The protein is

encoded in the nucleus and translocated from the cytoplasm to the mitochondria to exert its physiological function. Overexpression of G30D mutant *PINK1* significantly reduced cell viability in the paradigm of proteasome inhibitor MG132-induced cellular stress *in vitro*, suggesting an important protective function of the wild type protein in mitochondria (Valente et al., 2004). Another functional study on two point mutations (G309D and L347P) revealed a loss of kinase activity as a possible pathogenic mechanism (Beilina et al., 2005). A recent study indicated that a substantial portion of the PINK1 protein may be exported to the cytosol after mitochondrial processing. Moreover, overexpression of PINK1 in cell culture led to intracytoplasmic inclusions reminiscent of insoluble aggregating proteins, which might indicate a contribution of disturbed protein degradation to the pathogenic mechanism (Beilina et al., 2005). Future studies will unravel the substrates of PINK1 in mitochondria and in the cytosol and how PINK1 may interfere with mitochondrial homeostasis and/or the ubiquitin proteasome system.

The most interesting feature of DJ-1 that is mutated in another form of autosomal recessive PD is its function as a sensor of oxidative stress. Under oxidative conditions, an acidic form of the DJ-1 protein accumulates and mediates its translocation to the mitochondria *in vitro* (Canet-Aviles et al., 2004). Indeed, a protective function of DJ-1 in terms of oxidative stress and inhibition of mitochondrial complex I activity has been shown (Taira et al., 2004). In this context, the oxidative modification of the sulfhydryl group of cystein in position 106 of the peptide sequence seems to be critical, since a mutation of this amino acid leads to a dominant negative effect in the paradigm of mitochondrial damage (Canet-Aviles et al., 2004). Therefore, loss of function mutations in the *DJ-1* gene, as suggested from the autosomal recessive mode of inheritance, might lead to increased susceptibility to oxidative stress conditions.

Recently, another genetic susceptibility factor was described that links mitochondrial pathology with PD. Omi/HtrA2 is a nuclear encoded mitochondrial protein involved in cellular stress response and mitochondrial homeostasis. In German PD patients, two novel variants of the Omi/HtrA2 protein were identified that result in defective activation of the protease activity of Omi/HtrA2. Immunohistochemistry and functional analysis in stably transfected cells revealed that S399 mutant Omi/HtrA2 and to a lesser extent the risk allele of the A141S polymorphism induced mitochondrial dysfunction associated with altered mitochondrial morphology. Cells overexpressing S399 mutant Omi/HtrA2 were more susceptible to stress-induced cell death than wild type expressing cells (Strauss et al., 2005).

In summary, the functional characterization of identified genes in PD reveals mitochondrial pathology and defective protein degradation processes. Interestingly, biochemical data and pathological studies in sporadic PD patients show that mechanisms identified in inherited forms of the disease are also involved in the pathogenesis of the common sporadic form.

Role of phosphorylation in the pathogenesis of PD

Posttranslational modifications play a major role in neurodegenerative diseases. Various animal models of PD based for instance on the application of toxins such as 6-hydroxydopamine (Kulich and Chu, 2001), MPP+ (Gearan et al., 2001) or the overexpression of α-synuclein (Yamada et al., 2004) revealed kinase phosphorylation induction of various pathways and proteins. Current PD research does focus on posttranslational phosphorylation of proteins even more intensely, as two of the genes mutated in PD, PINK1 and LRRK2, encode kinases. The resulting altered function and their implication in the pathogenesis of PD, however, might differ in both

genetically defined subgroups. For instance, both alleles of *PINK1* (phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1) have to be deleted to cause autosomal recessive PD (Valente et al., 2004) whereas only one aberrant allele of *LRRK2* (leucine-rich repeat kinase 2) is sufficient to cause the most frequent form of autosomal dominant PD (Zimprich et al., 2004; Paisán-Ruiz et al., 2004). Furthermore, PINK1 is one of the few kinases which are targeted to mitochondria and is predicted to be a Ser/Thr kinase (Beilina et al., 2005). In contrast, LRRK2 has a predicted nonreceptor tyrosine kinase-like domain (Sehn, 2004). The subcellular distribution of the LRRK2 gene product awaits first characterizations; however, there is currently no indication of a mitochondrial localization or of predicted mitochondrial localization signals (Zimprich et al., 2004). A direct link between these two kinases and other proteins known to be involved in the PD pathway has not been explored in detail yet. However, PINK1 has been shown to bind synphilin-1 (Bandopadhyay et al., 2005), which interacts with α-synuclein. Both proteins have been shown to be phosphorylated by casein kinase II (Lee et al., 2004). Notably, the interaction between both proteins and the tendency of aggregate formation in cell culture is greatly dependent on phosphorylation. In case of Parkin, phosphorylation by casein kinase 1 modulates its E3 ubiquitin ligase activity which is reduced by ER stress (Yamamoto et al., 2005).

Current evidence also implies an involvement of 14-3-3 proteins in PD, a protein family which usually binds to phosphorylated proteins and has been implicated in the pathogenesis of neurodegenerative diseases (Berg et al., 2003a). Indeed, 14-3-3 proteins have been shown to inhibit dephosphorylation leading to a prolonged stimulation of tyrosine hydroxylase (Toska et al., 2002), the key enzyme in dopamine synthesis. 14-3-3η suppresses Parkin activity, an effect that is abrogated by α-synuclein (Sato et al., 2005). Most importantly, two mutations in the α-synuclein protein, A30P and A53T, did not bind 14-3-3η leading to a failure to activate Parkin (Sato et al., 2005). We and others showed that 14-3-3 proteins are components of Lewy bodies (Berg et al., 2003b; Kawamoto et al., 2002) further strengthening the pathogenic importance of 14-3-3 proteins in PD.

Role of iron metabolism in the pathogenesis of PD

Iron is increasingly implicated in the pathogenesis of PD. However, it is not clear yet, whether the increase of iron in PD is a secondary phenomenon or a causal factor of neuronal death. Results of transcranial ultrasound studies indicate that this method is valuable to detect an alteration of the substantia nigra (SN) in PD that is associated with increased SN iron content (''SN hyperechogenicity'') (Berg et al., 2002). A familiar predisposition to SN hyperechogenicity indicates a possible genetic background for the ultrasound marker in adults associated with susceptibility for nigrostriatal injury (Ruprecht-Dörfler et al., 2002). Variations in some genes involved in brain iron metabolism (*CP* and *ferritin-L* gene) have been found to be associated with extrapyramidal symptoms and even PD (Klomp and Gitlin, 1996; Kohno et al., 1999; Bosio et al., 2002; Curtis et al., 2001). Moreover, a recently developed mouse model underscores the important role of H-ferritin in brain iron metabolism (Thompson et al., 2003). While null mutants were not viable, mice deficient in H-ferritin had a protein profile mimicking the one seen for iron management in PD and AD with signs of oxidative stress. Similarly, disruption of the mouse homologue of *IRP2* leads to iron accumulation and neurodegeneration (La Vaute et al., 2001). Screening for mutations in PD patients revealed a K54R substitution in the *ferritin-H* gene, $-77C>T$ in the *IRP2* gene, and K91N and I217T in the *HFE* gene

(Felletschin et al., 2003; Deplazes et al., 2004, Akbas et al., in preparation), but not in an equally large cohort of controls. Except for variations in the *CP* gene (Hochstrasser et al., 2004), none of the other variations was found to be associated with PD or SN hyperechogenicity as marker for the increased iron content.

For three variations detected in the *CP* gene, an association with PD and/or SN hyperechogenicity could be demonstrated. While the I63T mutation was only found in a single PD patient with SN hyperechogenicity, the D544E variation was significantly associated with PD and hyperechogenicity, and for the R793H mutation, segregation with SN hyperechogenicity could be shown. Functional relevance could be demonstrated for the I63T and D544E mutation (Hochstrasser et al., 2005): Western blot analyses of transfected HEK cells showed impaired N-glycosylation of the I63T CP protein. Serum analyses of the patient heterozygous for this mutation revealed half of the normal control values of CP and markedly reduced ferroxidase activity indicative of ER-associated degradation (Helenius and Aebi, 2004). In addition, reduced serum iron and transferrin saturation were associated with this mutation demonstrating reduced iron efflux due to impared ferroxidase activity.

PD patients with the D544E mutation had also significantly decreased serum CP concentrations and ferroxidase activity compared to controls unaffected by this missense mutation. In patients and controls carrying the D544E mutation, the normal correlation between ferroxidase activity and iron as well as between ferroxidase activity and transferrin saturation could not be shown, which might be due to an impairment of CP ferroxidase activity that involves iron oxidation for binding to transferrin. In addition, D544E ceruloplasmin was mainly synthesized as apo-ceruloplasmin in HEK293 cells, indicating failure to incorporate copper.

A reduction of CP concentration and ferroxidase, although to a lesser extent, was also found in patients with the R793H mutation. A link between CP and PD may also be seen upon comparing the clinical presentation of aceruloplasminemia and PD: Patients with aceruloplasminemia present in their middle ages with symptoms of progressive neurodegeneration of the basal ganglia and retinal neurons, and to a lesser extend of the cerebellum and cortex (Klomp and Gitlin, 1996). Moreover, we could show that CP is a prominent component of Lewy bodies (Hochstrasser et al., 2004).

Outlook

Eight years after the identification of the involvement of α-synuclein in the pathogenesis of PD, the genetic cause of a large number of familial PD cases has been identified. The number of PD genes has been grown to six but will increase even further. In addition, several susceptibility genes such as *Omi/HtrA2*, *synphilin-1*, *ceruloplasmin*, and *periphilin* have been identified and extend our understanding of the neurodegenerative process. With the generation of numerous animal models specifically mimicking the genetic alterations in humans, we now have the chance to investigate pathogenesis and treatment options of PD.

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Progress in familial Parkinson's disease

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Summary. To date 11 forms of familial Parkinson's disease (PD) have been mapped to different chromosome loci, of which 6 genes have been identified as the causative genes, i.e., *alpha-synuclein* (*SNCA*), *parkin*, *UCH-L1*, *PINK1*, *DJ-1*, and *LRRK2*. For *UCH-L1*, additional families with this mutation are necessary before concluding that *UCH-L1* is the definite causative gene for PARK5, as only one family so far has been reported. *SNCA*, *UCH-L1*, and *LRRK2* mutations cause autosomal dominant PD and the remaining gene mutations autosomal recessive PD. Age of onset tends to be younger in familial PD compared with sporadic PD, particularly so in autosomal recessive PD. Generally familial cases respond to levodopa quite nicely and progression of the disease tends to be slower. It is an interesting question how familial PD-causing proteins are mutually related each other. In this article, we review recent progress in genetics and molecular biology of familial PD.

Introduction

To date 11 forms of familial Parkinson's disease (PD) have been mapped to different chromosome loci (Table 1). In this article we review recent progress in these familial forms of PD.

PARK1

PARK1 is an autosomal dominant familial PD caused by mutations of *alpha-synuclein* (*SNCA*). Clinical features of PARK1 were first described by Golbe et al. (1990) on large autosomal dominant kindreds immigrated to USA from Contursi, a village in the hills of Salerno Province in southern Italy. Ancestors of this family are believed to have moved to Italy from Greece. Clinical features consist of L-dopa-responsive parkinsonism and variable degrees of cognitive impairment. The average age of onset of the original families reported by Golbe et al. (1990) was 46.5 ± 10.8 years (range, 28–68, $N = 33$).

Alpha-synuclein has been mapped to the long arm of chromosome 4 at 4q21-q23. To date, 3 missense mutations, i.e., A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), and A53T (Polymeropoulos et al., 1997) and triplication (Singleton et al., 2003) and duplication (Chartier-Harlin et al., 2004; Ibenez et al., 2004) of the entire *alpha-synuclein* are known (Fig. 1). Alpha-synuclein is a neuron-specific protein localized mainly in the presynaptic terminal membranes and synaptic vesicles. Although the function of alpha-synuclein is not well known, aggregated alpha-synuclein is accumulated in the nigral neurons in PD indicating that alpha-synuclein plays an important role in the pathogenesis of PD. Recently reported families with triplication and duplication of *alpha-synuclein* suggest that overexpression of normal alpha-synuclein per se is neurotoxic to nigral neurons.

Table 1. Inherited forms of Parkinson's disease

Name	Inheritance	Locus	Gene
PARK1	AD	4q21-23	*α-synuclein (SNCA)*
PARK2	AR	6q25.2-27	*parkin*
PARK3	AD	2p13	*unknown*
PARK4	AD	4q21-23	*α-synuclein*
PARK5	AD	4p14	*UCH-L1*
PARK6	AR	1p35-36	*PINK1*
PARK7	AR	1p36	*DJ-1*
PARK8	AD	12p11.2-q13.1	*LRRK2/ dardarin*
PARK9	AR	1p36	*unknown*
PARK10	AD/AR/SP	1p32	*unknown*
PARK11	AD	2q36-37	*unknown*

AD autosomal dominant, *AR* autosomal recessive, *SP* sporadic

Regarding the relationship between the types of mutation and clinical features, triplication and E46K mutations are associated with dementia in addition to parkinsonism and wide-spread neuropathologic changes with cortical Lewy bodies in addition to nigral neurodegeneration (Farrer et al., 1999; Zarranz et al., 2004). Actually neuropathological characteristics are consistent with those of diffuse Lewy body disease. On the other hand, duplication was associated with pure L-dopa-responsive parkinsonism without dementia. Ala53Thr mutation is associated with variable degrees of cognitive impairment. Ala30Pro is less likely to show cognitive impairment.

Functions of alpha-synuclein are not well known. Alpha-synuclein is a natively unfolded brain specific protein consisting of 140 amino acids without significant amount of secondary structure (Weinreb et al., 1996). From its localization in presynaptic terminals, it has been speculated that it may be related to neurotransmitter regulation. Alpha-synuclein has a tendency for self-aggregation and oligomer formation. Soluble oligomers ultimately form insoluble aggregates, which are the major component of Lewy bodies (Spillantini et al., 1998). Particularly, oligomers of alpha-synuclein are toxic to neurons inducing release of dopamine into the cytoplasm from synaptic vesicles (Volles and Lansbury, 2002), impairment of 26S proteasome (Snyder et al., 2003), and mitochondrial dysfunctions (Tanaka et al., 2001). Mitochondrial impairment results in reduced ATP synthesis. As 26S proteasome is an ATP-dependent protein degrading enzyme, mitochondrial impairment reduces its catalytic activity. Thus vicious cycles are formed within nigral neurons leading them to slowly progressing neuronal death. Mutated alpha-synuclein proteins show increased tendency for self-aggregation (El Agnuf et al., 1998).

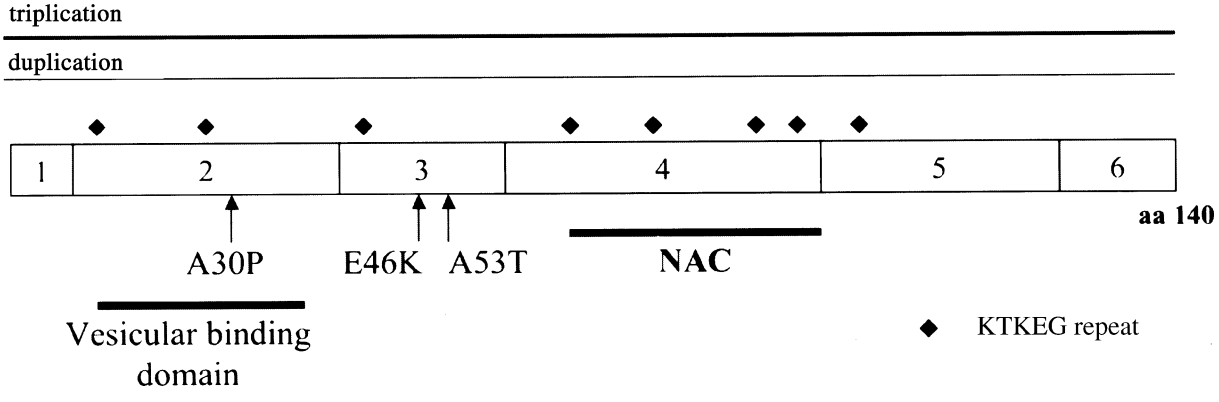

Fig. 1. Schematic presentation of the exons of *alpha-synuclein* and its mutations. Three missense mutations, duplication (thin line) and triplication (thick line) are known. Closed diamonds indicate approximate positions of the KTKEGV repeats. NAC represents non-amyloid component of the senile plaque

This is likely to be a reason for earlier onset of ages in familial PD due to alpha-synuclein mutations compared with sporadic PD. Furthermore, aggregated insoluble alpha-synuclein proteins are likely to impair transport of vital substances within nigral neurons. The insoluble aggregate of alpha-synuclein is highly phosphorylated at Ser-129 (Fujiwara et al., 2002).

PARK2

PARK2 is an autosomal recessive familial PD caused by mutations of *parkin*. Clinical features of PARK2 were first described by Yamamura et al. (1973). They reported 16 patients (13 familial patients in 5 unrelated families and 3 sporadic cases); clinical features of 11 patients from the initial 4 families were essentially identical. Ages of onset were between 17 and 28 years in 10 out of 11 patients and 42 in the remaining one. All the patients showed tremor, rigidity, bradykinesia, and postural instability. Atypical features included sleep benefit, temporary improvement of parkinsonism after a nap or sleep, and dystonic postures in the feet during walking (talipes equinovarus). Dementia was absent.

These patients show good response to L-dopa but they frequently develop motor fluctuations (wearing off and dyskinesia) sooner than late onset PD patients; usually two to three years after the initiation of L-dopa. Since the gene analysis became possible, many atypical features have been reported. For instance, age of onset can be as late as 72 (Lincolon et al., 2003). Other atypical features reported include dementia (Benbuman et al., 2004), psychosis and behavioral problem (Kahn et al., 2003), cerebellar ataxia (Kuroda et al., 2001), peripheral neuropathy (Tassin et al., 1998; Okuma et al., 2003; Kahn et al., 2003), hyperhidrosis (Yamamura et al., 1998), orthostatic hypotension (Kahn et al., 2003), urinary urgency and impotence (Kahn et al., 2003), and hemiparkinsonism-

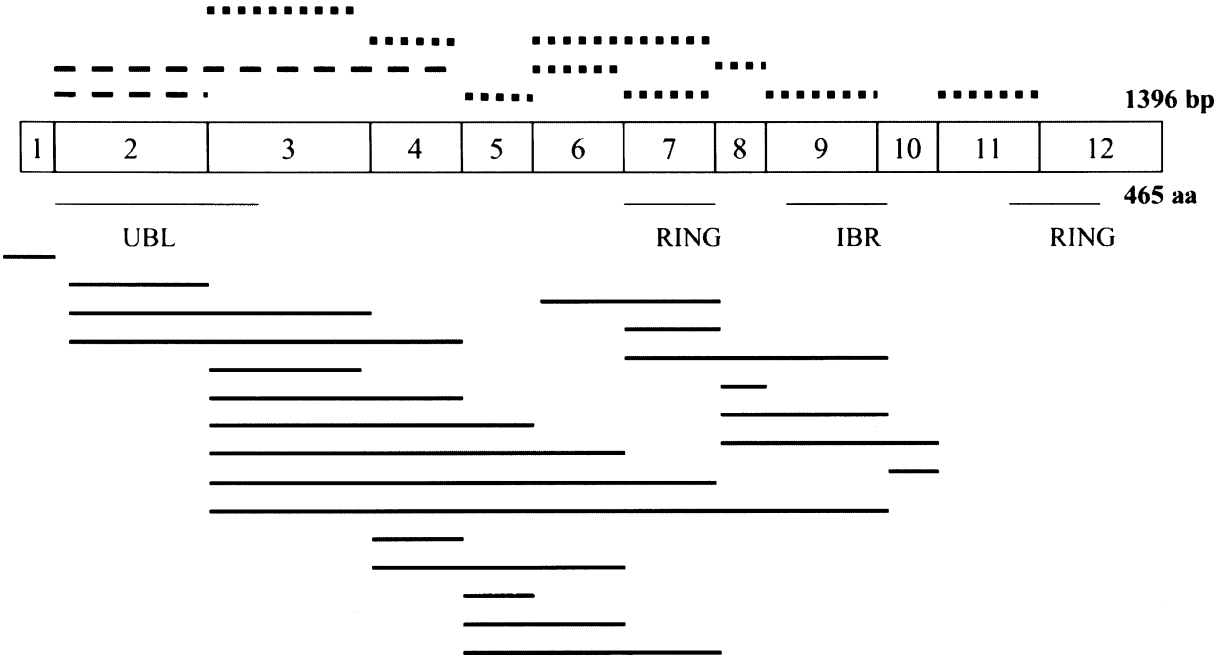

Fig. 2. Schematic presentation of exons of parkin and its exon rearrangements. summarized from the following literature, i.e., Hattori et al. (1998), Abbas et al. (1999), Lücking et al. (2000), Oliviera et al. (2003), and Hedrick et al. (2004). Broken lines indicate triplication, dotted lines duplication, and solid lines deletions of exons. *UBL* ubiquitin-like domain, *RING* RING domain, *IBR* in-between RINGs

hemiatrophy (Pramistaller et al., 2002) have been reported.

PARK2 is caused by mutations of *parkin* (Kitada et al., 1998), which has been mapped to the long arm of chromosome 6 at 6q25.2-q27. To date more than 30 different exon rearrangements (deletion, duplication, and triplication) (Fig. 2), 30 missense mutations, and 8 nonsense mutations, and close to 20 small deletions or insertions (Fig. 3) have been reported (Hattori et al., 1998; Abbas et al., 1999; Lücking et al., 2000; Oliviera et al., 2003; Hedrick et al., 2004). These numbers are still increasing. Usually PARK2 patients harbor either homozygous mutations or compound heterozygous mutations of *parkin*. But at times single heterozygous mutations are seen. According to our hand, approximately 20% of patients with *parkin* mutations were single heterozygotes, in that only one allele of *parkin* showed a mutation and we could not find the second mutation. Question is how they could have got the disease. As PARK2 is an autosomal recessive disorder, it is expected that both of parkin alleles are mutated. Although exact mechanism is not known, numbers of possibilities can be considered. For instance, single normal parkin may not be suffice to its complete function (haploinsufficiency); mutated parkin protein in some way may interfere with the function of normal parkin protein (dominant-negative effect); single mutated parkin may predispose to late onset Lewy body-positive PD.

Parkin protein was found to be an ubiquitin-protein ligase (E3) of the ubiquitin system (Shimura et al., 2000). The ubiquitin-proteasome system (UPS) is an important intracellular proteolytic system responsible for wide variety of biologically important cellular processes, such as cell-cycle progression, signaling cascades, developmental programs, the protein quality control system, DNA repair, apoptosis, signal transduction, transcription,

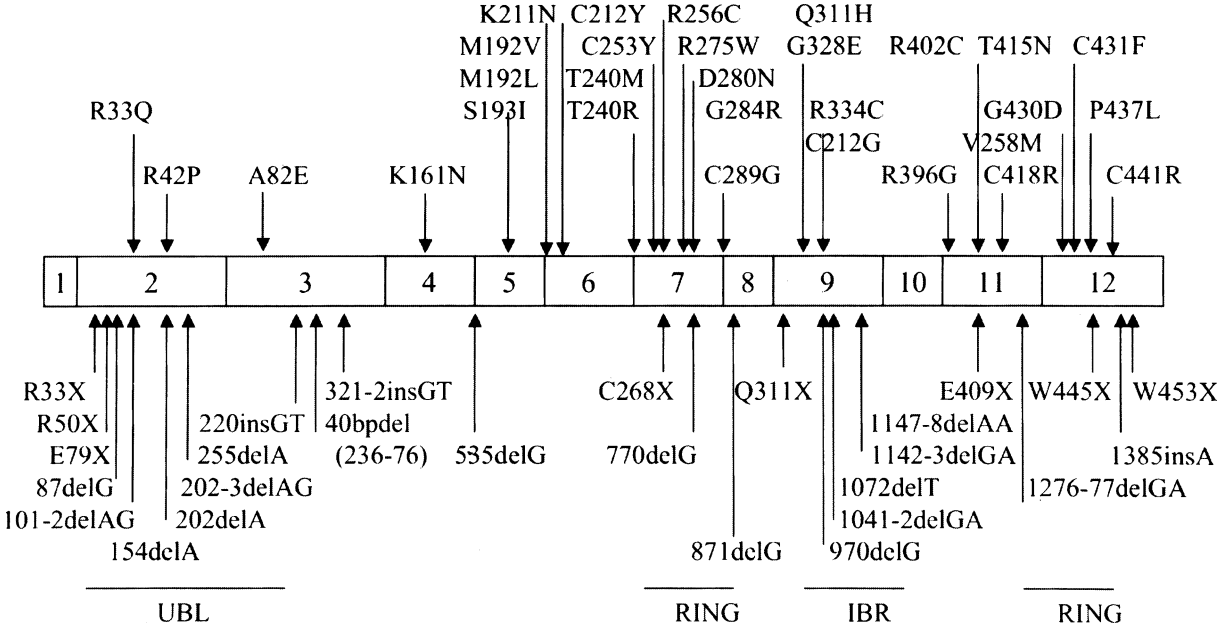

Fig. 3. Schematic presentation of exons of parkin and missense mutations, nonsense mutations, and small deletions summarized from the following literature, i.e., Hattori et al. (1998), Abbas et al. (1999), Lücking et al. (2000), Oliviera et al. (2003), and Hedrick et al. (2004). Missense mutations are shown above the exons and nonsense mutations and small deletions below the exons. *UBL* ubiquitin-like domain, *RING* RING domain, *IBR* in-between RINGs

metabolism, immunity, and neurodegeneration (Tanaka et al., 2004). The ubiquitin system consists of three enzymes, i.e., the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2), and the ubiquitin-protein ligase (E3). The E3 transfers ubiquitin molecules to target proteins forming a poly-ubiquitin chain which is recognized by 26S proteasome as the proteolytic signal. Therefore, in the presence of mutated parkin proteins, accumulation of parkin-substrate proteins is expected to be the major cause of nigral neuronal death. However, to date there is no clear immunohistochemical evidence to indicate accumulation of parkin-substrates in PARK2 patients, despite many parkin-interacting proteins have been reported such as CDCrel-1 (Zhang et al., 2000), glycosylated alpha-synuclein (Shimura et al., 2001), PAEL receptor (Imai et al., 2001), and synphilin-1 (Chung et al., 2001). We recently reported that parkin-knock down SH-SY5Y cells showed increased formation of dopamine/dopa-derived quinones and apoptotic cell death (Machida et al., 2005); these quinones appeared to be the mediator of cell death. Thus parkin appears to have a potent anti-oxidative property. As in the case of sporadic PD, oxidative damage may be an important mechanism of nigral neuronal death in PARK2.

Other mechanism that has been postulated is polyubiquitylation at the lysine-63 residue of the ubiquitin molecules. Polyubiquitin chains formed via the lysine-48 residue of the ubiquitin molecule mainly become a marker for proteolytic attack by the 26S proteasome. On the other hand, lysine 63-linked polyubiquitylation has many biological roles other than proteolysis, such as endocytosis, DNA repair, translation, IkB activation, DNA silencing, virus budding, protein sorting, and protein trafficking (Tanaka et al., 2004). Parkin promotes not only polyubiquitylation at lysine-48 but also at lysine-63. Recently, Lim et al. (2005) reported that parkin enhanced lysine-63 mediated polyubiquitylation of synphilin-1. Thus this is a novel aspect of the functions of parkin protein, however, exact molecular mechanism of nigral neurodegeneration in PARK2 is still open to question.

PARK3

PARK3 is an autosomal dominant familial PD linked to the short arm of chromosome 2 at 2p13 (Gasser et al., 1998). The disease gene has not been identified yet. Clinical features are essentially similar to those of sporadic late onset PD; the age of onset was 36 to 89. Interestingly, penetrance was 40% suggesting that some apparently sporadic PD patients may represent PARK3. Dementia developed in two out of six original families (Gasser et al., 1998). Autopsy findings from those families showed nigral neurodegeneration and neurofibirillary tangles in cortical neurons.

Recently, Strauss et al. (2005) reported a missense mutation (G399S) in *HtrA2/Omi*, which has been mapped to the same locus (2p13), in 4 sporadic PD patients; cells overexpressing S399 mutation was reported to be more susceptible to stress-induced cell death than wild type. But this mutation was negative in the original families of PARK3.

HtrA2 is a serine protease that has extensive homology to bacterial heat shock endoprotease (Faccio et al., 2000). Interestingly this is a mitochondrial protein localized in the intermembrane space and is released from mitochondria upon apoptotic stimuli initiating apoptosis cascade by activating caspase 3 (Suzuki et al., 2001). This is a proapoptotic protein; nonetheless, its mutation in its PDZ domain (carboxy-terminal side) was associated with familial PD. Further interestingly, a mutation in the protease domain caused motor neuron degeneration type 2 in mice (Jones et al., 2003). Knockout mice were reported to have shown striatal neuronal loss (Martins et al., 2004). This gene appears to be an interesting addition to the research on familial PD.

PARK4

PARK4 is an autosomal dominant familial PD caused by triplication of *alpha-synuclein* (Singleton et al., 2003). This mutation was found in the large kindred, which has been designated as Spellman–Muenter–Waters–Miller family or Iowanian family. Initial family was reported by Spellman (1962) who reported an autosomal dominant family with PD in the United States. Then Muenter et al. (1998) made extensive clinical studies on this family. Another autosomal dominant family later reported by Waters and Miller (1994) was found to be another branch of the kindred reported by Spellman and Muenter. Clinical features consist of L-dopa responsive parkinsonism and dementia, which are consistent with clinical diagnosis of diffuse Lewy body disease. In autopsied patients, many cortical Lewy bodies were found in addition to nigral neurodegeneration with Lewy body formation.

This family was reported to be linked to the short arm of chromosome 4 (Farrer et al., 1999) but in fact the causative gene of this family was found to be triplication of *alpha-synuclein* (Singleton et al., 2003); the 1.5 Mb region including introns on both sides of *alpha-synuclein* was triplicated in a tandem fashion. Therefore, PARK4 should be reclassified as a form of PARK1.

PARK5

PARK5 is an autosomal dominant familial PD linked to the short arm of chromosome 4 at 4p14-p15.1. To date only one family is reported (Leroy et al., 1998). Clinical features are essentially similar to those of late onset sporadic PD; the age of onset was 49 to 50.

Leroy et al. (1998) found I93M missense mutation in the ubiquitin carboxyterminal hydrolase-L1 gene (*UCH-L1*) (Fig. 4). UCH-L1 is a neuron specific enzyme that cleaves carboxyterminal peptide bond of polyubiquitin chains; UCH-L1 is an ubiquitin recycling enzyme. I93M-mutated UCH-L1 has half of the catalytic activity of the wild enzyme (Leroy et al., 1998). The supply of ubiquitin for proteins that have to be destroyed by 26S proteasome may be reduced with this mutation. Interestingly homozygous deletion of exon 7 and 8 in mouse UCH-L1 causes gracile axonal dystrophy (*gad*) mouse; this is an autosomal recessive condition characterized by axonal degeneration and formation of spheroid bodies in motor and sensory nerve terminals (Saigho et al., 1999).

PARK6

PARK6 is an autosomal recessive young onset familial PD caused by mutations of *PINK1* (*PTEN-induced kinase 1*) (Valente et al. (2001). Clinical features of PARK6 are essentially similar to those of PARK2; the age of onset of the original family studied by Valente et al. (2001) ranged from 32 to 48, somewhat older than those of PARK2. Reflecting this later age of onset, dystonia and sleep benefit which are common to young onset PARK2 are usually not seen in PARK6 unless the age of onset is young.

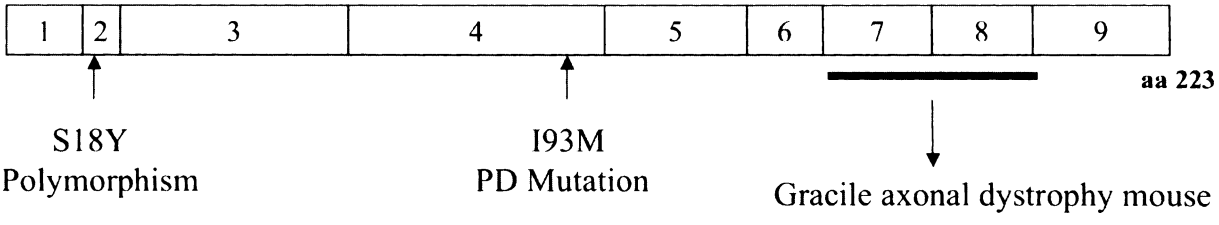

Fig. 4. Schematic presentation of exons of *UCH-L1* and its mutations. Only one mutation is known. I93M is associated with autosomal dominant PD. Interestingly homozygous exonic deletion involving exon 7 and 8 induces gracile axonal dystrophy (gad) mouse. S18Y polymorphism is said to confer neuroprotection for sporadic PD, but controversies exist

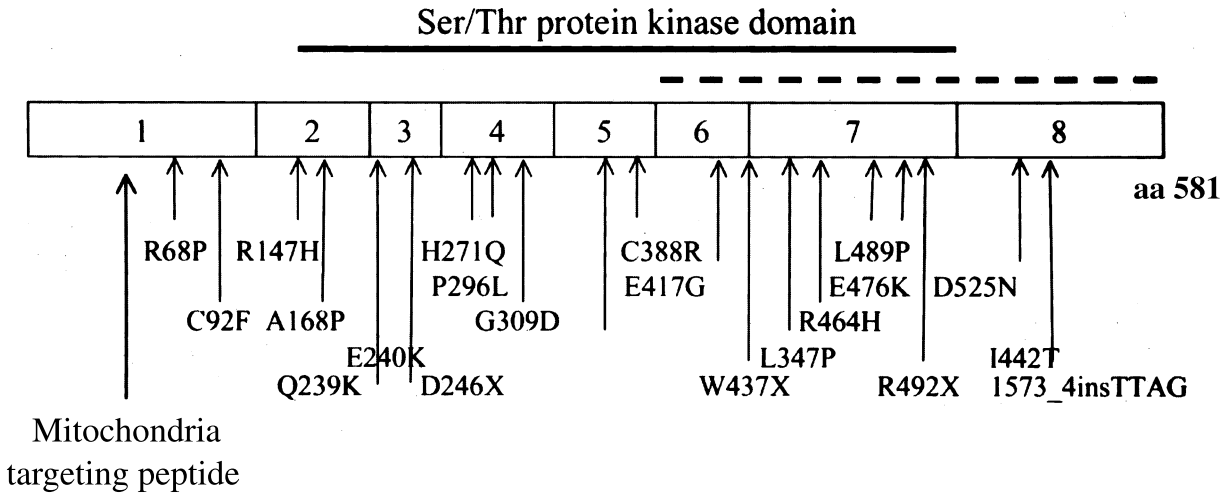

Fig. 5. Schematic presentation of exons of *PINK1* and its mutations summarized from the following literature, i.e., Valente et al. (2004), Hatano et al. (2004), Heary et al. (2004), Rohe et al. (2004), and Li et al. (2005). As PINK1 is a mitochondrial protein, it has a mitochondria-targeting sequence (exon 1). Two mutations in this targeting sequence are also known. Many missense and nonsense mutations are reported. Recently, we found an exonic deletion involving exon 6 to 8 indicated by the broken line. The solid line indicates the catalytic domain

PINK1 has been mapped to the short arm of chromosome 1 at 1p35-p36 (Valente et al., 2004). To date, 17 missense mutations, 3 nonsense mutations, one insertion, and one exon deletion are known (Valente et al., 2004; Hatano et al., 2004; Heary et al., 2004; Rohe et al., 2004; Li et al., 2005) (Fig. 5). We recently found a novel missense mutation (C388R) and an exonic deletion from exon 6 to 8; the latter was the first documented case with exonic deletion mutation in PARK6 (Li et al., 2005). PARK6 appears to be the second most common autosomal recessive PD after PARK2.

PINK1 is a mitochondrial matrix protein and has a protein kinase activity, however, its exact functions are not known. PINK1 stands for PTEN-induced kinase 1. PTEN stands for protein tyrosine phosphatase with homology to tensin: *PTEN* is a tumor suppressor gene on chromosome 10 mutated in many human tumors (Steck et al., 1997).

PARK7

PARK7 is an autosomal recessive familial PD caused by mutations of *DJ-1* (Bonifati et al., 2003). Clinical features are essentially similar to those of PARK2 including the age of onset, which is younger than that of PARK6. Affected patients show L-dopa-responsive parkinsonism of varying severity and drug-induced motor fluctuation and dyskinesia. Interestingly, three out of four patients in the original family showed psychiatric disturbances (anxiety attacks) (Dekker et al., 2003). Atypical clinical features include short statue and brachydactyly, which were found in Dutch kindred (Dekker et al., 2004).

DJ-1 has been mapped to the short arm of chromosome 1 at 1p36 and was identified as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated Ras (Nagakubo et al., 1997). To date, 6 missense mutations, 1 intronic mutation, 1 small deletion, and 2 exonic deletions (exon 1 to 5 and exon 5 to 7) are known (Bonifati et al., 2003; Abou-Sleiman et al., 2003; Hague et al., 2003; Hering et al., 2004) (Fig. 6). *DJ-1* mutations are rare compared with *parkin* and *PINK1* mutations. We could not find *DJ-1* mutations among Japanese PD families studied.

DJ-1 is a potent anti-oxidative protein and this character depends on its 106-cysteine residue (Taira et al., 2004). DJ-1 is a cytoplas-

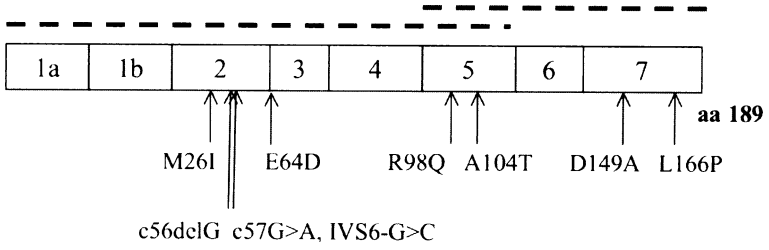

Fig. 6. Schematic presentation of exons of *DJ-1* and its mutations summarized from the literature, i.e., Bonifati et al. (2003), Abou-Sleiman et al. (2003), Hague et al. (2003), and Hering et al. (2004). Exon 1 and 2 are spliced out in the mature protein. Broken lines indicate exonic deletions

mic protein (Bonifati et al., 2003); however, oxidized DJ-1 is relocated to mitochondria (Canet-Aviles et al., 2004). DJ-1 undergoes dimmer formation to become active (Honbou et al., 2003; Tao and Tong, 2003). One of the PD-inducing missense mutations, L166P, interferes with dimmer formation (Wilson et al., 2003) and is degraded more rapidly than wild DJ-1 by ubiquitin-proteasome-system (Macedo et al., 2003; Miller et al., 2003) or by autoproteolysis (Gorner et al., 2004). This mutant DJ-1 is also mislocalized to mitochondria. Further interestingly, parkin interacts with mutated DJ-1 (L166P) but not with wild one (Moore et al., 2005), suggesting that parkin might be acting as a quality control protein for DJ-1. Thus molecular mechanism of nigral neuronal death in PARK7 appears to be at least in part related to dysfunction of anti-oxidative property of DJ-1.

PARK8

PARK8 is an autosomal dominant PD caused by mutations of *LRRK2/dardarin*. Clinical features were first described in large Japanese kindred (Nukada et al., 1978). They reported 36 patients in 5 generations. The age of onset ranged from 38 to 68 (mean = 53). Later the mean age of onset was reported as 51 ± 6 as the number of affected members increased (Funayama et al., 2002). Initial symptom was either gait disturbance or rest tremor. All of them showed L-dopa-responsive parkinsonism. Motor fluctuations and psychiatric side effects from L-dopa treatment can be seen. Clinical features are essentially similar to those of late onset sporadic PD except for slightly younger age of onset. Post-mortem examination in four patients from the original family showed pure nigral degeneration without Lewy body formation (Funayama et al., 2002). But later on another patient who came to autopsy from the same family showed nigral degeneration with Lewy bodies (Personal communication with Dr. K. Hasegawa).

The Western Nebraska family (Family D) reported by Wszolek et al. (1995), which inluded 18 patients in 5 generations, turned out to be PARK8. The age of onset was 48 to 78 (mean 63). Neuropathological features of this family are very interesting in that among the four patients who came to autopsy, one patient showed brain stem type Lewy body pathology; the second patient showed diffuse Lewy body disease pathology; the third patient showed nigral neuronal loss and gliosis with neurofibrillary tangles in the remaining nigral neurons without Lewy body formation; the fourth patient showed marked neuronal loss and gliosis in the nigra and locus coeruleus without any inclusions or tau-positive accumulations (Wszolek et al., 2004). Four different pathological findings in the same family would indicate the difficulty of defining a disease entity by neuronal inclusions. Family A reported by Denson and Wszolek (1995) was also turned out to be PARK8.

PARK8 has been mapped to the centromeric region of chromosome 12 (Funayama

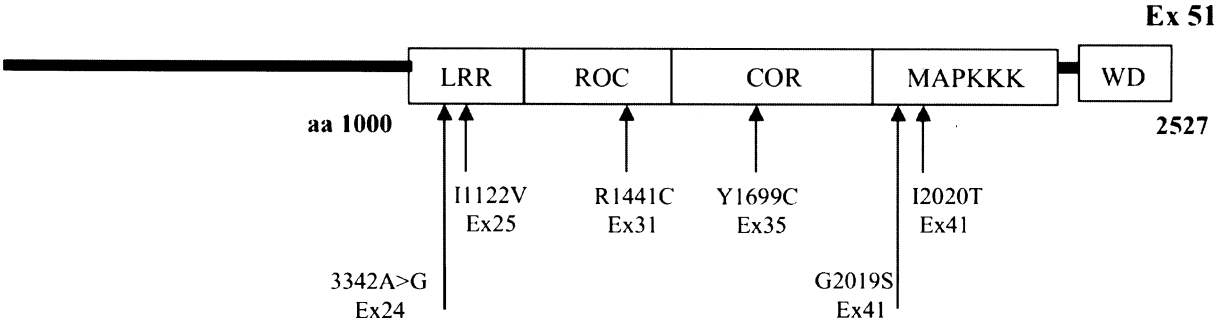

Fig. 7. Schematic presentation of *LRRK2* and its mutations summarized from the literature, i.e., Zimprich et al. (2004), Paisan-Ruiz et al. (2004), and Kachergus et al. (2005). LRRK2 protein belongs to ROCO protein family, which is characterized by the presence of ROC domain and COR domain. Many of the ROCO proteins also have LRR, MAPKKK, and WD domains. See the text for the explanations of these domains. To date 6 missense mutations have been reported in the homology region. Exon 41 appears to be a mutational hot spot

et al., 2002). The causative gene was identified as *LRRK2/dardarin* (Zimprich et al., 2004; Paisan-Ruiz et al., 2004). LRRK2 stands for leucine-rich repeat kinase 2 and dardar means tremor in the Bask language where families of PARK8 are found. *LRRK2* is a huge gene encompassing 144 kb and the open reading frame consists of 1449 base pairs in 51 exons. LRRK2 protein consists of 2527 amino acids and it is ubiquitously expressed in the cytoplasm of many organs. To date 6 missense mutations have been reported (Zimpricht et al., 2004; Paisan-Ruiz et al., 2004; Nichols et al., 2005) (Fig. 7).

LRRK2 protein belongs to the ROCO protein family. ROCO proteins are a group of proteins which has ROC and COR domain (Bosgraaf and Haastert, 2003). ROC stands for Ras in complex proteins belonging to the Ras/GTPase superfamily, and COR stands for carboxy terminal of ROC. In addition, many ROCO proteins have a LRR (leucine-rich repeat) domain, which has 3 to 16 leucine-rich repeats, a MAPKKK (mitogen-induced protein kinase kinase kinase) domain, and a WD domain, which is rich in tryptophan and aspartate repeats. The function of LRRK2 is still unknown but as it has protein kinase domain, it is likely that its role is phosphorylation of proteins that are important for the survival of nigral neurons. It is interesting to note that alpha-synuclein aggregates in PD are highly phosphorylated in Ser-129 (Fujiwara et al., 2002); therefore, it is an interesting question whether or not LRRK2 is in some way related to phosphorylation of alpha-synuclein.

PARK9

PARK9 is an autosomal recessive familial PD linked to the short arm of chromosome 1 at 1p36 (Hampshire et al., 2001). The causative gene has not been identified. Clinical features consist of L-dopa-responsive parkinsonism, supranuclear gaze palsy, pyramidal sign, and dementia, called Kufor-Rakeb syndrome. The age of onset is 10 to 20. Neuropathologically not only the substantia nigra but also the pyramidal tract, putamen, and the pallidum show neurodegeneration. PARK9 appears to be a form of multiple system atrophy.

PARK10

The PARK10 is linked to the short arm of chromosome 1 at 1p32. This locus was found by genome wide scanning on familial as well as sporadic cases of PD in Iceland (Hicks et al., 2002); they studied 117 PD patients and 168 of their unaffected relatives within 51 families using 781 microsatellite markers. The mean age of onset was 65.8. They showed linkage to chromosome 1p32 with a lod score of 4.9. The disease gene has not been identi-

fied yet. As expected from the source of the clinical subjects, clinical features are essentially similar to those of sporadic PD.

PARK11

PARK11 is an autosomal dominant familial PD linked to the long arm of chromosome 2 at 2q36 to q37 (Pankratz et al., 2003). The causative gene has not been identified yet. Clinical features are essentially similar to those of sporadic PD with the mean age of onset at 58. Neuropathological findings are not known.

Other forms of familial PD

There are many families in which linkage analysis failed to show linkage to any one of the known loci that are associated with familial forms of PD. Such reports are increasing every year. According to our hands, we have analyzed 347 families for known PD-causing genes including non-Japanese families with either autosomal dominant or recessive inheritance. We found 116 families with *parkin* mutations, 8 families with *PINK1* mutations, no *DJ-1* mutation, 10 families with *LRRK2* mutations, and 2 families with *alpha-synuclein* duplication. Overall mutation rate was 136 positive families out of 347 (39.2%). In another word, approximately 60% of familial patients with PD did not have known mutations. Mutual relationship among the familial PD causing proteins is an interesting and important subject to study. Identifying new genes for familial PD would give us important information on this topic. Such information would also give us important clues to investigate pathogenesis of sporadic PD.

Acknowledgements

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Molecular mechanisms of nigral neurodegeneration in Park2 and regulation of parkin protein by other proteins

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Summary. Most of the patients with Parkinson's disease (PD) are sporadic. However, Since identification of monogenic forms of PD, the contribution of genetic factors to the pathogenesis of sporadic PD is proposed as one of major risk factors. Indeed, this is supported by the demonstration of the high concordance in twins, increased risk among relatives of PD patients in case control and family studies. Thus, the functional analysis for the gene products for familial PD provides us a good hint to elucidate the pathogenesis of nigral degeneration. For example, although α-synuclein is involved in a rare dominant form of familial PD with dopa responsive parkinsonian features, this molecule is a major component of and Lewy bodies (LBs). In contrast, Park2 (parkin-related disease) is the most frequent form among patients with young-onset PD. However, Park2 brains generally lack the formation of LBs. In the other word, parkin responsible for Park2 is essential for the formation of LBs. Thus, both α-synuclein and parkin are speculated to share a common pathway. Here, we reviewed the parkin function and molecular mechanisms of Park2.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder primarily caused by selective dopaminergic cell loss in the midbrain substantia nigra pars compact. However, the exact cause of PD is still unknown. Since identification of monogenic form of PD, the functions of gene products for familial PD (FPD) have provided us good information for studying the mechanisms underlying neurodegeneration in PD. To date, eleven loci have been mapped and among them, six causative genes have been identified as causative genes in familial PD, which have significantly enhanced our understanding of the genetic mechanisms of not only FPD but also sporadic PD. Among the forms of FPD, the causative gene, *parkin* of AR-JP, representing the most prevalent form of familial PD (Kitada et al., 1998), is of a special interest, because it is liked to ubiquitin-proteasome system (UPS) as an E3 ubiquitin-protein ligase (Shimura et al., 2000), which covalently attaches ubiquitin to target proteins, designating them for degradation by the 26S proteasome (Pickart et al., 2001). These findings suggest that accumulation of the parkin substrate(s) due to loss-of-function of parkin induces loss of dopaminergic neurons. Thus, Park2 is caused by failure of proteolysis mediated by UPS (Dawson and Dawson, 2003). Since then, our knowledge of the substrate(s) for parkin has expanded, and indeed at present various putative substrates, such as

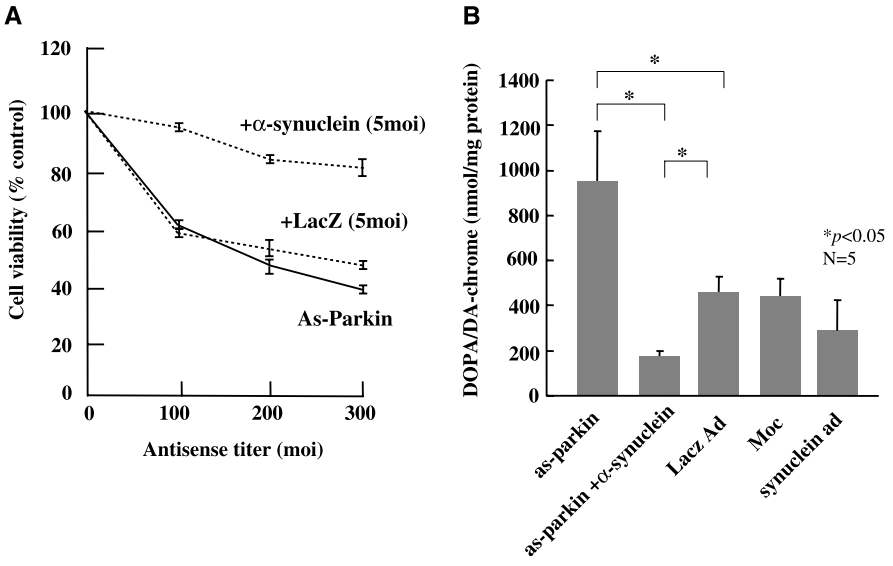

Fig. 1. α-Synuclein inhibits parkin knockdown-induced apoptosis and accumulation of DOPA- and DA-quinones. **A** Effects of overexpression of wild α-SN on as-parkin induced deterioration of cell viability. Differentiated SH-SY5Y cells were treated for 48 hours with as-parkin adenovirus. Cells were coinfected with LacZ and α-SN adenovirus (5 moi) and at the 150 moi titers of as-parkin adenovirus. The cell viability was measured and represented. **B** Cellular level of DOPA/DA-chromes. After the differentiated SH-SY5Y cells were treated for 36 hours with as-parkin, wild α-SN, LacZ and adenoviruses, cellular DOPA/DA-chromes were measured. Note the profound decrease of DOPA/DA-chromes in α-SN-expressing SH-SY5Y cells. Data are the mean ± SEM of 10 determinations. $^{*}P < 0.05$ versus control group (Tukey's multiple t test)

CDCrel-1, synphilin-1, α-SN22 (*O*-glycosylated form of α-SN), Pael-R etc, have been identified, but the pathophysiological role of parkin is still poorly understood (see review Hattori and Mizuno, 2004). Furthermore, null mice have no phenotypes for PD although several changes including dopamine metabolism have been reported so far. However, a direct link between these factors and dopaminergic cell death has not yet been reported. The important question of why dopaminergic neurons in the SN are particularly vulnerable to the loss-of-function effect of parkin remains to be determined. Although parkin is expressed ubiquitously in the brain, the pathologic findings of Park2 brains show severe neuronal loss with gliosis in the SN and mild neuronal loss in the locus coeruleus (LC), suggesting that the pathological change of Park2 brain is mainly in the SN. To investigate such selective neuronal loss, we established a good *in vitro* model by parkin knock down using full length antisense parkin.

Molecular mechanisms of Park2

Recently, two groups independently reported the generation of model mice lacking the *parkin* gene, which display certain abnormalities of dopamine metabolism (Itier et al., 2003; Goldberg et al., 2003). However, these parkin knockout mice had only subtle phenotypes exhibiting grossly normal brain morphology. In contrast, full-length human parkin antisense knocked-down endogenous parkin protein in differentiated human neuroblastoma cells (SH-SY5Y), 12 to 36 hours after infection and reduced cell viability (Machida et al., 2005). In addition, control β-galactosidase expressing adenoviruses could not knockdown

parkin and failed to affect cell viability. Thus, this system itself using adeno virus is not cytotoxic to the culture cells. The specificity of the antisense effect was confirmed by the result of co-infection of sense *parkin* expressing adenovirus, which abrogated reduction of cell viability. On the other hand, *parkin* antisense had no effect on cell viability of HeLa cells, suggesting that *parkin* antisense exert a specific effect on the cell viability of differentiated SH-SY5Y cells. Thus, this *in vitro* model is a powerful tool for elucidating the several issues as mentioned before.

Although *in vitro* system could induce the cell loss, why do parkin knockout mice lack abnormalities like AR-JP patients? One plausible explanation is the presence of a putative molecule(s) that suppresses the defect induced by loss-of-function of parkin, and such molecule(s) present abundantly in the brain should be linked to the pathogenesis of PD. Here we propose that α-SN is the molecule that compensates for the loss of parkin, since α-SN prevented apoptotic cell death induced by as-parkin. In this regard, Western blotting analysis showed that the dopaminergic SH-SY5Y cells did not express α-SN at significant levels, which is in marked contrast to the high abundance of dopaminergic neurons *in vivo*. Regardless of the compensating role of α-SN for the loss-of-function of parkin in Park2, α-SN is probably unable to cope with the accumulation of toxic molecules in the absence of parkin and thus apoptotic neuronal death perhaps occurs gradually, leading to degeneration of dopaminergic neurons. This is the first evidence for the anti-apoptotic role of α-SN and its involvement in the common pathway of parkin.

To date, several studies have demonstrated that α-SN could exert protective effect against various cellular stresses such as oxidative damage and related apoptosis of neurons. Considering the reason why mutation of α-SN causes familial PD, it is clear that this type of disease is due to the gain-of-toxic function of the mutant α-SN, differing from neuroprotective roles of the wild-type α-SN. In this context, it is noteworthy that α-SN is a major component of Lewy bodies, the pathological hallmark of PD, and at high concentrations, it oligomerizes to β-pleated sheets known as protofibrils (i.e., fibrillar polymers with amyloid-like characteristics). In addition, α-SN proteins with disease-causing mutations tend to generate protofibrils, suggesting that protein misfolding including α-SN plays a key role in the pathogenesis of PD. In addition to our finding, α-SN could play dual function such as neuroprotection and or neurotoxicity. Considering the presence of the patients with α-SN multiplication, overproduction of this molecule could cause for developing PD, and lower level expression would be also cytotoxic to the dopaminergic neurons. Indeed, α-SN knock out mice displayed the impairment of the dopamine release although neuronal loss has not been reported so far.

It remains unclear why dopaminergic neurons of the substantia nigra and locus coeruleus are selectively vulnerable to the loss of parkin in AR-JP patients. In the present study, we provided a clue for this enigmatic puzzle. Considering the specificity of the lesions in PD, it is possible that the high oxidative state associated with DA metabolism may cause deterioration of dopaminergic neurons. The mechanism underlying increased oxidative stress may involve DA itself, because oxidation of cytosolic DOPA/DA may be deleterious to neurons. Indeed, DA causes apoptotic cell death with morphological nuclear changes and DNA fragmentation. In this regard, we showed here that as-parkin directed loss of parkin leads to abnormality of DOPA/DA metabolism, which generated DOPA/DA-quinones in SHSY5Y cells. Thus, DA and its metabolites seem to exert cytotoxicity mainly by generating highly reactive quinones through auto-

oxidation. On the other hand, the toxicity of DOPA and DA is due to the generation of reactive oxygen species that could disrupt cellular integrity, causing cell death. However, the reason for the production of oxidative DOPA/DA-metabolites following loss of parkin is not clear at present.

The loss-of-function of parkin by full length antisense strategy could lead to the cell death of differentiated dopaminergic cells *in vitro*. In addition, the increasing of DOPA/DA-quinones was associated with the cell death, suggesting that quinines derived from dopamine metabolism are killer molecules in Park2 brains. This cell-based experiment enhances our understanding of the pathophysiology of PD and is potentially useful for drug screening in the future. Our results also showed that α-SN and parkin are involved in DA metabolism and its aberrant regulation is accompanied by accumulation of oxidative DOPA/DA metabolites.

Recently, parkin has been negatively regulated by S-nitrosylation modification and BAG5 (Kalia et al., 2004; Chung et al., 2004). Thus, loss-of-function through parkin mutation as in Park2, nitrosylation or binding with BAG5 results in nigral degeneration in not only Park2 brains but also sporadic PD. In the other word, such negative regulation system for parkin ubiquitin ligase suggests a possible mechanistic link between the familial and sporadic forms of PD. As s-nytrosylation for parkin has been reported in sporadic PD, DOPA/DA metabolites could be also involved in the pathogenesis for sporadic form of PD as well as Park2 brains. Finally, several gene products have been reported so far, the relationship among them is unclear at present. However, considering clinical similarities including neuropathologic findings between sporadic and FPD, most of the gene products may share a common pathway on the pathogenesis for PD.

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Parkin and defective ubiquitination in Parkinson's disease

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Summary. Parkinson's Disease (PD) is a common neurodegenerative disorder that is characterized by the progressive loss of dopamine (DA) neurons. Accompanying the loss the of DA neurons is the accumulation of Lewy bodies and neurites, intracytoplasmic proteinaceous inclusions that contain alpha-synuclein, synphilin-1, components of the ubiquitin proteasomal pathway and parkin. Recent advances indicate that PD is due in some individuals to genetic mutations in alpha-synuclein, DJ-1, PINK-1, LRRK2, and parkin. Understanding the molecular mechanisms by which mutations in familial-linked genes cause PD holds great promise for unraveling the mechanisms by which DA neurons degenerate in PD. Parkin is E3-ubiquitin-protein ligase that ubiquitinates itself and promotes its own degradation. Familial associated mutations of parkin have impaired ubiquitin ligase function suggesting that this may be the cause of familial autosomal recessive PD. Parkin might be required for formation of Lewy bodies as Lewy bodies are absent in patients with parkin mutations. Parkin interacts with and ubiquitinates the alpha-synuclein interacting protein, synphilin-1. Formation of Lewy-body-like ubiquitin-positive cytosolic inclusions occurs upon co-expression of alpha-synuclein, synphilin-1 and parkin. Nitric oxide inhibits Parkin's E-3 ligase activity and its protective function by nitric oxide through *S*-nitrosylation both *in vitro* and *in vivo*. Nitrosative and oxidative stress link parkin function with the more common sporadic form of Parkinson's disease and the related α-synucleinopathy, DLBD. Development of new therapies for PD and other disorders associated with nitrosative and oxidative stress may follow the elucidation of the pathways by which NO *S*-nitrosylates and inhibits parkin. Moreover, parkin and alpha-synuclein are linked in common pathogenic mechanism through their interaction with synphilin-1 and parkin may be important for the formation of Lewy bodies.

Parkin is an ubiquitin E3 ligase

Parkin is common cause of autosomal recessive (AR) Parkinson's disease (PD). A large number of mutations in parkin are linked to PD, including deletions of single or multiple exons, duplications or triplications of exons, frame shift mutations, and point mutations. Haploinsufficiency of parkin, due to loss of one copy of the gene, also constitutes a risk factor for the onset of PD (von Coelln et al., 2004a).

Parkin contains 465 amino acid protein and is a RING-type E3 ubiquitin ligase that contains an N-terminal ubiquitin-like domain, SH2-like domain that links the N-terminus to a C-terminal RING-finger box composed of two RING finger domains separated by an In-Between-Region (von Coelln et al., 2004a).

E3 ubiquitin ligases act as scaffolding proteins and confer substrate specificity to the ubiquitin proteasome system (UPS) where they interact with a ubiquitin E2 conjugating enzyme and the E1 activating enzyme to ubiquitinate specific substrates. Parkin appears to use UbcH7, UbcH8, Ubc6 and Ubc7 as its E2 ubiquitin conjugating enzymes. The majority of protein turnover within the cell is handled by the UPS (Pickart, 2001). Proteins destined for degradation are covalently tagged with ubiquitin by the E1, E2 and E3 complex of enzymes. Polyubiquitinated proteins are ultimately targeted to the 26S proteasome to be enzymatically degraded. Typically individual ubiquitin monomers are covalently bonded to one another via lysine-48 (K-48) chains. However, ubiquitin contains a total of seven lysine residues at positions 6, 11, 27, 29, 33, 48 and 63 and it is now clear that polyubiquitination could also occur through alternative lysine residues on ubiquitin (Pickart, 2001).

Familial associated mutations are thought to lead to the loss of parkin's E3 ligase activity potentially resulting in the accumulation of substrates that are not properly targeted for degradation by the UPS. Several substrates have been identified including the synaptic vesicle-associated septins CDCrel-1 and CDCrel-2; the α-synuclein interacting protein, synphilin-1; the putative G protein-coupled parkin-associated endothelin-like receptor (Pael-R); the microtubule proteins, α- and β-tubulin; the cell cycle protein, cyclin E; the p38 subunit of the aminoacyl-tRNA synthetase complex; the vesicle-docking protein synaptotagmin XI; and the dopamine transporter, DAT and an O-glycosylated form of α-synuclein (von Coelln et al., 2004a). The large number of substrates is surprising as most E3 ligases only interact with and ubiquitinate a small number of substrates and as such it is not clear, which if any of the above substrates is involved in the pathogenesis of PD due to parkin mutations. Moreover, several animal models deficient in parkin have been developed and none of the parkin substrates appear to accumulate in parkin-null mice (Goldberg et al., 2003; Itier et al., 2003; Perez and Palmiter, 2005; Von Coelln et al., 2004b). Although a detailed and careful analysis of the potential accumulation of parkin substrates has yet to be performed. Thus, it is unclear whether any of them are involved in the pathogenesis of PD due to parkin mutations (Giasson and Lee, 2003).

Parkin is a dual function ubiquitin ligase

The general absence of the signature LB inclusions in post-mortem brain samples of patients is an interesting feature of parkin-related PD and suggests that functional parkin is required for LB formation (von Coelln et al., 2004a). Our identification of the alpha-synuclein interacting protein, synphilin-1, as a parkin substrate, together with our demonstration that parkin mediates ubiquitination of proteins within LB-like inclusions formed by the co-expression of the alpha-synuclein interacting protein, synphilin-1, with alpha-synuclein fit with this notion (Chung et al., 2001). Moreover synphilin-1 is a component of LBs in brains of sporadic PD patients (Wakabayashi et al., 2000) and a point mutation in synphilin-1 was recently found in a number of German individuals with sporadic PD (Marx et al., 2003). Taken together, these results suggest a functional molecular link between parkin, synphilin-1 and α-synuclein in LB biogenesis as well in PD pathogenesis. Since parkin functions as a ubiquitin ligase, substrates ubiquitinated by parkin are thought to be destined for proteasomal degradation. Surprisingly, we found that parkin ubiquitinates synphilin-1 in a non-classical, proteasomal-independent, manner that involves K63-linked polyubiquitin chain formation that leads to enhanced inclusion formation (Lim et al., 2005a). However, at an unusually high parkin to synphilin-1 expression ratios or when primed for K48-linked ubiquitination

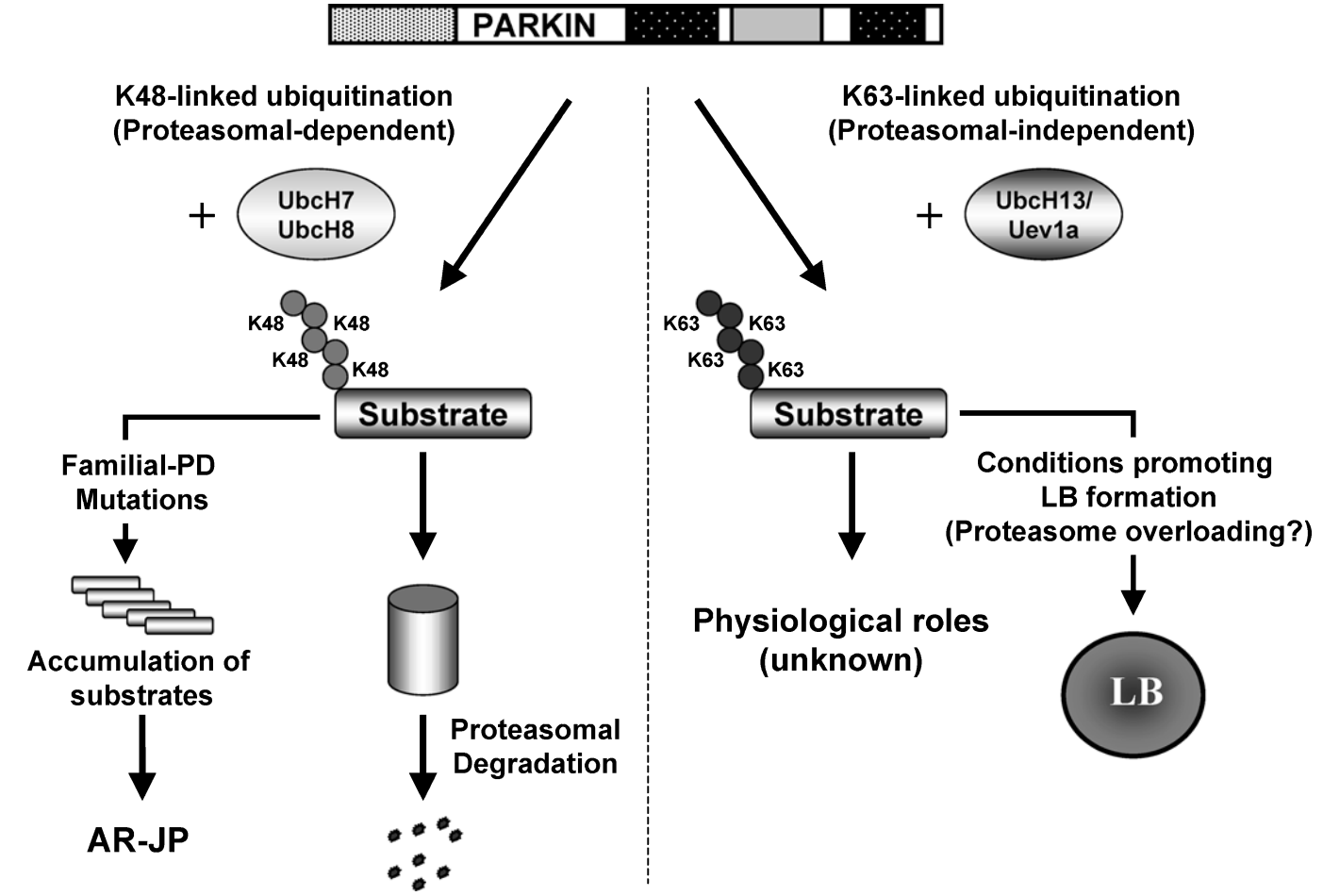

Fig. 1. Parkin is a dual function E3 ligase. Different outcomes result from Parkin's dual function polyubiquitin chain formation. K48-linked polyubiquitination is responsible for substrate degradation via the proteasome. K63-linked polyubiquitination of substrates may be involved in Lewy Body formation. Reproduced with permission (Lim et al., 2006)

parkin can mediate the degradation of synphilin-1 (Lim et al., 2005a). Consistent with our findings, Madura and co-workers also demonstrated recently the ability of parkin to assemble both K48- and K63-linked chains under different conditions (Doss-Pepe et al., 2005). Thus parkin appears to function as a dual function ubiquitin ligase (Fig. 1). The dual specific activity of parkin is apparently governed by members of the E2s it recruits. Parkin apparently interacts with the E2 complex composing of UbcH13 and Uev1a in the assembly of K63-linked chain (Doss-Pepe et al., 2005). The UbcH13/Uev1a heterodimer is the only E2 enzyme that is known to mediate K63-linked polymerization of ubiquitin. On the other hand parkin utilizes UbcH7 and UbcH8 for its degradative function (von Coelln et al., 2004a). It is not clear what governs the extrinsic or intrinsic factors that determine parkin's choice of E2. Thus, parkin may play important roles in the degradation and sequestration of toxic proteins in PD.

Nitrosylation and Parkin

The observation of widespread nitration of pathological inclusions both in neurodegenerative synucleinopathies and tauopathies (Ischiropoulos and Beckman, 2003) suggests that nitric oxide (NO) is an important player in neurodegeneration. The formation of peroxynitrite is thought to mediates most of NO's action in neurodegeneration. Peroxynitrite damages proteins by reacting with tyrosine residues to form 3-nitrotyrosine (Ischiropoulos and Beckman, 2003). Recent studies also suggest that NO can also contribute to the pathogenesis of PD through *S*-nitrosylation. Parkin is *S*-nitrosylated by NO both in vitro and in vivo (Chung et al., 2004; Yao et al., 2004). The cysteine rich RING box represents a potential target for *S*-nitrosylation to control the enzymatic activity of parkin. We and others found that parkin is biphasically modified by NO through *S*-nitrosylation and *S*-nitrosylation of parkin results in initial enhancement of it's E3 ligase activity followed by inactivation (Chung et al., 2004; Yao et al., 2004). The role of the biphasic modulation of parkin's E3 ligase activity is not clear, but the chronic inhibition of parkin's activity by the high level of nitrosative stress in PD likely leads to decreased survival of dopaminergic neurons. Parkin is *S*-nitrosylated in postmortem brain tissues from PD patients (Chung et al., 2004; Yao et al., 2004). Moreover, in the MPTP model of PD, parkin *S*-nitrosylation is increased in a biphasic manner due to the activity of iNOS and nNOS (Chung et al., 2004). Biochemical and ESI-MS studies revealed that the sites of modification are located within the first RING finger and IBR domains, which are known to be important for the function of parkin (Chung et al., 2004; Yao et al., 2004). Chronic *S*-nitrosylation of parkin leads to inhibition of its protective function and it is conceivable that this may account for neuronal loss in PD. The finding that parkin is *S*-nitrosylated and this modification is substantially increased in PD patients provides a common pathogenic pathway for both sporadic and familial forms of PD. Inhibition of parkin E3 ligase activity, which is critical for the survival of dopaminergic neurons may play an important role in sporadic PD as environmental contaminants could result in increased levels of nitrosative stress leading to chronic inhibition of parkin E3 ligase activity by constant elevated levels of nitrosative stress.

Conclusions

Parkin is an E3 ubiquitin ligase that is the major cause of familial autosomal recessive PD. It is a dual function E3 ligase involved in the degradation and sequestration of toxic proteins via ubiquitination with K48 or K63 ubiquitin chains, respectively. Moreover, parkin is regulated by nitrosative stress through *S*-nitrosylation that ultimately impairs

its ubiquitination and protective functions. Elucidating the relative contributions of K48 versus K63 chain formation and the identification of pathogenic substrates will lead to a better understand of the role of parkin dysfunction in the pathogenesis of PD due to parkin mutations.

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PINK-1 and DJ-1 – new genes for autosomal recessive Parkinson's disease

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Summary. Our genetic knowledge of Parkinson's disease (PD) is moving forward at an impressive speed. In less then 10 years family-based linkage analysis and positional cloning have led to the identification of several genes for familial forms of PD, which has been of critical importance to the scientific advance of PD research as the causal genes have offered new tools to model and understand pathways leading to neurodegeneration in PD.

New genes for autosomal recessive PD

Combining the evidence observed through pathology and laboratory studies, suggests that PD is a disorder of the cellular protein quality control system, which is associated with neuronal accumulation of misfolded proteins and presence of protein aggregates. Although there is indeed strong evidence of the involvement of the protein quality control system in PD there is also biochemical evidence that suggests that oxidative stress and mitochondrial dysfunction can lead to the disease, as oxidation-modified proteins have been shown to accumulate in the context of normal aging and PD, and may participate in the generation of protein aggregates in neurodegenerative disorders.

The recent identification of new genes for autosomal recessive Mendelian forms of PD, such as DJ-1 and PINK-1, indeed provides evidence for other pathways then the disturbance of protein quality control system, which can lead to the disease.

PINK-1 encodes the PTEN-induced kinase 1 and is mitochondrially located providing a direct molecular link between mitochondria and the pathogenesis of PD (Valente et al., 2004). The protein may exert a protective effect on the cell that is abrogated by the mutations, resulting in increased susceptibility to cellular stress. Unfortunately detailed functional studies are not yet available.

In contrast, for the DJ-1 protein a large number of studies have already become available. A homozygous deletion found in a Dutch family represents a natural knockout of DJ-1, indicating that the loss of function of DJ-1 is pathogenic (Bonifati et al., 2003). The L166P mutation probably also induces a loss of DJ-1 function because this mutant protein is unstable and rapidly degraded by the 20S/26S proteasome system, resulting in much lower steady state levels in both transfected cells and patient lymphoblasts (Macedo et al., 2003; Miller et al., 2003; Moore et al., 2003). DJ-1 crystal structures and gel filtration experiments show that the L166P mutation disrupts the C-terminal domain and the dimerization capability of DJ-1 suggesting that the dimerization is functionally important

(Baulac et al., 2004; Honbou et al., 2003; Huai et al., 2003; Lee et al., 2003; Olzmann et al., 2004; Tao et al., 2003; Wilson et al., 2003).

Important clues for the role of DJ-1 in neurodegeneration have come from the evidence that human DJ-1 is converted into a variant having more acidic isoelectric point in response to sublethal doses of paraquat (Mitsumoto et al., 2001a, b), a toxin which generates reactive oxygen species (ROS) within cells and has been associated with dopamine neuron toxicity. The possible function of DJ-1 as molecular chaperone is intriguing in the light of the role of the molecular chaperones in the pathogenesis of PD and other neurodegenerative diseases since ROS and proteasomal inhibition have been correlated with PD pathology. These proteins function in assisting the proper folding of nascent polypeptides and the refolding of damaged proteins; they are also involved in targeting and/or delivering of protein to the proteasomal system for degradation.

DJ-1-deficient embryonic stem (ES) cell derived dopamine neurons (DNs) and primary DNs with DJ-1 levels reduced by RNAi ''knockdown'', display increased sensitivity to oxidative stress in the form of H_2O_2 (Martinat et al., 2004). The initial accumulation of H_2O_2 induced ROS in these appears normal in DJ-1-deficient dopamine neurons (DNs), but over time the cellular defenses to ROS seem impaired, leading to increased apoptosis. Additionally, DJ-1 deficiency sensitizes cells to the proteasomal inhibitor lactacystin but not other toxic stimuli such as tunicamycin. Proteasomal inhibition induces the accumulation of short-lived and misfolded cytoplasmic proteins, leading to oxidative stress and apoptosis and one might hypothesize that DJ-1 mutations lead to PD because of an increased sensitivity of DJ-1-deficient DNs to such stressors.

There is evidence that DJ-1 can function as an ATP-independent cytoplasmic redox-sensitive molecular chaperone for multiple targets (Canet-Aviles et al., 2004; Shendelman et al., 2004). L166P mutant DJ-1 fails to function as a molecular chaperone *in vivo* or *in vitro* and consistent with this, this mutant fails to complement DJ-1 knockout cells *in vivo*, even when overexpressed at artificially high levels (Marimat et al., 2004). The L166P loss of function seems not due simply to reduced levels of DJ-1 protein, but might also be a consequence of altered structure and resultant loss of function.

Candidate substrates for DJ-1 chaperone activity in the context of PD include α-synuclein and neurofilament proteins (NFL), based on their presence in PD protein inclusions. Indeed DJ-1 inhibits the aggregation of α-synuclein in differentiated cells *in vivo*, and loss of DJ-1 leads to increased accumulation of insoluble α-synuclein. The DJ-1 chaperone activity is inhibited by reducing conditions, and can be stimulated by oxidation. Thus, in the normal reducing environment of the cell, DJ-1 may be inactive. Production of ROS and alteration of the redox state of the cytoplasm may activate DJ-1 chaperone activity as a mechanism of coping with protein aggregation and misfolding.

Pathological analysis of brains from patients with DJ-1 related forms is of great importance, but brain material is not currently available. However, investigating the presence of the DJ-1 protein in brain from patients with Lewy body disease and other neurodegenerative diseases provides clues on the involvement of DJ-1 in neurodegeneration. While no convincing evidence of DJ-1 immunoreactivity in Lewy bodies has been reported (Bandopadhyay et al., 2004; Rizzu et al., 2003), in transfection studies DJ-1 appears to associate with α-synuclein. However, DJ-1 does not colocalize with the punctate protein aggregates visible by immunostaining in the case of either α-synuclein or NFL, supporting the notion that DJ-1 functions at an early step in the aggregation process, when the substrate protein may be misfolded, but has not yet formed a mature aggregate. It can be

hypothesized that DJ-1 may promote the degradation of such misfolded proteins, either through the proteasome or through other cellular pathways such as chaperone-mediated autophagy.

In human post-mortem material DJ-1 immunoreactivity colocalizes within a subset of pathological tau inclusions in various neurodegenerative disorders. At first this might seem contradictory however, these findings could represent and endstage of protein aggregates in the situation when DJ-1 is not able to cope with the amount of misfolded proteins.

These data suggest that DJ-1 could function to suppress protein aggregates in the cytoplasm possibly in an early step in the formation of protein aggregates. It has been suggested that such protofibrils, rather than the large fibrillar aggregates, may underlie toxicity *in vivo* in several neurodegenerative diseases. A strong signal for DJ-1 was also observed in activated astrocytes both in neurodegenerative disorders and in material from other brain diseases such as Schizophrenia, in agreement with a more general role for DJ-1 as a chaperone.

On the basis of all the available evidence one can propose that DJ-1 is involved in the cellular response to stress at multiple levels. First, it might directly react to stress signals (e.g., redox changes, misfolded proteins) being a molecular chaperone. Second, DJ-1 might modulate the gene expression of the stress response at the post-transcriptional level by the known interaction with RNA-binding protein complexes. Third, DJ-1 might translocate to the nucleus in response to stress signals. In the nucleus it might interact with cofactors and modulate the gene expression at the transcriptional level. Although the exact involvement of human DJ-1 in the oxidative stress response, or in the response to misfolded protein stress remains to be shown, the proposed model is intriguing in the light of the evidence of oxidative stress and protein misfolding documented in the brains of patients with PD. Especially since recent studies have shown that mutations in α-synuclein and parkin, two other PD-related genes, might also be linked to oxidative stress supporting the view that different forms of PD may have similar pathogenetic mechanisms, which likely includes a role for DJ-1.

Recently a mouse model was reported bearing a germline disruption of DJ-1 (Goldberg et al., 2004). These DJ-1 null mice are healthy and reproduce normally. No reduction of dopaminergic neurons in the substantia nigra was observed in the brains of DJ-1 mutants up to 12 months of age. The mice are also did not show an increased sensitivity to paraquat. However, they did show a dopaminergic deficit. Real-time electrochemical measurements of acute striatal slices revealed reduced evoked dopamine overflow in the knockout mice. This reduction was primarily due to an increased reuptake of dopamine by the dopamine transporter in the absence of an upregulation of this gene at the mRNA or protein level.

No effects were detected on induction of corticostriatal long-term potentiation (LTP) but the induction of long-term depression (LTD) was impaired. Since induction of LTP requires activation of Dopamine 1 receptors (D1R) and LTD the activation of both D1 and D2 receptors this suggests that DJ-1 is required for normal dopamine D2 receptor (D2R) regulation of dopamine reuptake in the presynaptic terminal of nigral neurons. In agreement with this, behavioral and electrophysiological studies showed a blunted response of the D2R to pharmacological challenges and the mice showed impaired performance in behavioral and locomotor tests. This study demonstrates a link between DJ-1 and dopaminergic physiology in the nigrostriatal system in which DJ-1 plays a role in mediating the downstream effects of the Dopamine D2 receptor activation in nigral neurons.

The discovery that mutations in DJ-1 cause autosomal recessive early-onset forms of PD

establishes that the loss of the DJ-1 function leads to human neurodegeneration. Clarifying the mechanisms of DJ-1 related disease might also potentially shed light on novel mechanisms of brain neuronal maintenance and promote the understanding of pathogenesis of common forms of PD. The chaperone activity of DJ-1 and/or its possible role as oxidative sensor are intriguing in the light of the current pathogenetic scenarios for PD. However, the importance of identifying a novel gene causing PD is even greater if this leads to innovative ideas about pathogenesis. In the case of DJ-1 potentially novel insights are the focus on the nuclear and cytoplasmic control of gene expression in PD pathogenesis.

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Clinical and pathologic features of families with *LRRK2*-associated Parkinson's disease

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Summary. The etiology for Parkinson's disease (PD) remains unknown. Genetic causes have been identified with several distinct mutations. Recently, 9 mutations involving a novel gene, leucine-rich repeat kinase 2 (*LRRK2*), have been identified as the cause of autosomal dominant PD in kindreds, with some of them previously linked to the *PARK8* locus on chromosome 12. *LRRK2* mutations are relatively common genetic causes of familial and sporadic PD. In addition, these mutations have been identified in diverse populations. The clinical and pathologic features of *LRRK2*-associated PD are indistinguishable from idiopathic PD; however, considerable clinical and pathologic variability exists even among kindreds. This short review highlights the clinical and pathologic features in *LRRK2*-associated parkinsonism.

Abbreviations

^{18}F-dopa ^{18}F-6-fluoro-L-dopa, *LB* Lewy bodies, *LRRK2* leucine-rich repeat kinase 2, *PD* Parkinson's disease, *PET* positron emission tomography, *SN* substantia nigra.

Introduction

Parkinson's disease (PD) is typified clinically by bradykinesia, rigidity, postural instability, and rest tremor and pathologically by both the presence of Lewy bodies (LB), eosinophilic inclusions containing protein aggregates, and the loss of dopaminergic neurons in the *pars compacta* of the *substantia nigra* (SN). PD affects 1% of individuals aged 65 years or older, a prevalence second only to Alzheimer's disease among neurodegenerative disorders. The etiology of idiopathic PD is unknown. However, genetic causes have now been identified with several distinct mutations (Eriksen et al., 2005).

Recently, 9 mutations involving a novel gene, leucine-rich repeat kinase 2 (*LRRK2*), have been identified as the cause of autosomal dominant PD in kindreds, with some of them previously linked to the *PARK8* locus on chromosome 12 (Paisan-Ruiz et al., 2004; Zimprich et al., 2004a, b; Di Fonzo et al., 2005; Gilks et al., 2005; Kachergus et al., 2005; Nichols et al., 2005). The *LRRK2* gene encodes LRRK2, a large protein comprising 2,527 amino acids and belonging to the ROCO group within the Ras/GTPase superfamily (Zimprich et al., 2004a). The function of LRRK2 protein is currently unknown.

In this review, we describe the clinical and pathologic features in families with *LRRK2*-associated PD.

Sagamihara Family

In 2002, Funayama et al. (2002) identified a novel locus, *PARK8*, on chromosome 12q12

Table 1. Clinical and pathologic features of families with *LRRK2*-associated Parkinson's disease*

Feature	Family			3 Unrelated English patients (Gilks et al., 2005)	Family 111
	Sagamihara family (Funayama et al., 2002)	Family D (Wszolek et al., 1995, 2004)	Family A (Wszolek et al., 1997)		
Ancestral origin	Japan	USA	Canada, Germany	UK	USA
Mutation, substitution†	6059T > C, I2020T	4321C > T, R1441C	5096A > G, Y1699C	6055G > A, G2019S	6055G > A, G2019S
Gene domain	MAPKKK	Roc	COR	MAPKKK	MAPKKK
No. affected	47	22	16	NA	2
Average age at onset, y	54	65	53	NA	68
Additional features	None	SnGP	D, A, Dys	NA	D, RLS, OH
No. of autopsies; pathologic features	4; "pure" ND	4; ND in all, LB in brainstem in 1, widespread in 1, tau in 1	3; ND in all, LB in limbic cortex in 1, ubiquitin inclusions in 2, anterior horn axonal spheroids in 1	3; ND and LB in all, LB in limbic cortices in 2, SP and NFT in 1	1; ND, LB in SN, brainstem, amygdala, and basal nucleus of Meynert

A amyotrophy; *D* dementia; *Dyst* dystonia; *LB* Lewy bodies; *NA* not available; *ND* nigral degeneration; *NFT* neurofibrillary tangles; *OH* orthostatic hypotension; *RLS* restless legs syndrome; *SN* substantia nigra; *SnGP* supranuclear gaze palsy; *SP* senile plaques. *Only *LRRK2* families with previously published postmortem neuropathologic examinations are included. †Official *LRRK2* gene/protein nomenclature derived from GenBank Accession: AY792511

that cosegregates with autosomal dominant parkinsonism in a family from Sagamihara, Japan, consisting of 31 individuals from 4 generations. Recently, the presence of a *LRRK2* mutation, 6059T>C (I2020T), was confirmed in 19 affected members of this family (Funayama et al., 2005).

Clinical features

The mean age of affected family members at onset of symptoms was 51 years. The disease affected men and women equally. The parkinsonian features were unilateral at onset and responded favorably to dopaminergic agents. All affected members had the constellation of rest tremor, bradykinesia, rigidity, and postural instability. No dementia or other neurologic features were reported.

Pathologic features

Postmortem examination performed on 4 affected members revealed pure nigral degeneration with the absence of LB (Table 1).

Family D (Western Nebraska) and Family A (German–Canadian)

Zimprich et al. (2004b) searched for the *PARK8* locus in 167 individuals (84 affected and 83 unaffected) derived from 21 Caucasian families with autosomal dominant PD. Significant linkage to the *PARK8* locus was observed in 2 large families previously described clinically and pathologically by Wszolek et al. (1995, 1997, 2004) and referred to as Family D (Western Nebraska) and Family A (German–Canadian). Zimprich et al. (2004a) first identified the *LRRK2* mutation for Family D (4321C>T [R1441C]) followed shortly by that for Family A (5096A>G [Y1699C]) in April 2004. Both families are Caucasian with Family D probably of English extraction and Family A of German origin. At present, the Family D pedigree includes 190 family members with 22 affected individuals. The Family A pedigree now includes 208 family members with 16 affected individuals.

Recently, 5 members from Family A and 10 from Family D were examined using positron emission tomography (PET) (Adams et al., 2005). The PET findings in these patients with autosomal dominant parkinsonism were indistinguishable from those typical of idiopathic PD. The findings on PET included reductions in ^{18}F-6-fluoro-L-dopa (^{18}F-dopa) uptake, binding of ^{11}C-(+/)α-dihydrotetrabenazine to the vesicular monoamine transporter, and binding of ^{11}C-*d-threo*-methylphenidate to the dopamine transporter; intact postsynaptic dopamine D2 receptors; and dopaminergic neuron dysfunction involving the putamen with sparing of the caudate nucleus.

Clinical features of Family D

The clinical features included all 4 cardinal signs of PD – rigidity, bradykinesia, asymmetric rest tremor, and postural instability. Nine individuals were treated with levodopa and responded favorably. The average age of symptom onset was 65 years (range, 48–78 years). The first sign of PD was bradykinesia in 9 patients and resting tremor in 6 patients. One patient had both bradykinesia and resting tremor. This information was not available for 6 patients from older generations. No additional clinical features such as pyramidal, cerebellar, sensory, ocular, or autonomic dysfunction were identified. However, 1 affected family member with PD subsequently developed supranuclear gaze palsy at the age of 83 years, 5 years after onset of her motor symptoms. She died 6 years later. She remained responsive to levodopa therapy until her death.

Pathologic features of Family D

Four autopsies of affected Family D members were performed (Wszolek et al., 2004). All 4 had neuronal loss and gliosis in the SN. LB were seen in 2 individuals, which was limited to the SN in 1 case and with more

widespread, general distribution in the other. No distinctive pathology was identified in the third case. The fourth subject who presented with PD and supranuclear gaze palsy had tau-positive neurofibrillary tangle pathology without LB (Table 1).

Clinical features of Family A

The average age of symptomatic onset was 53 years (range, 35–65 years). Unilateral resting tremor was the initial sign. Bradykinesia, rigidity, and resting tremor were observed in all family members affected with PD. Amyotrophy (muscle weakness, atrophy, and fasciculations) was seen in 1 affected individual who also had features of PD. Dementia alone was observed in 2 family members from the German branch. Those treated with dopaminergic agents benefited clinically but later experienced dose wearing off, peak-dose dyskinesia, and unpredictable on–off phenomenon.

Pathologic features of Family A

Detailed postmortem examinations were performed on 2 Canadian members of Family A. A single microscopic slide of brain tissue (basal portion of the pons) was available for examination for 1 additional affected member and showed no abnormalities. Results of the 2 more extensive neuropathologic examinations revealed severe loss of pigmented neurons with extracellular melanin in the SN. Neuronal loss and gliosis in SN with ubiquitin-immunoreactive neuronal lesions were also identified. LB were not seen in SN. This subject also had Alzheimer's disease pathology. The other case with clinical features of amyotrophy had mild motor neuron disease (Table 1).

Basque Families and Family PL

Paisan-Ruiz et al. (2004, 2005) described an autosomal dominant form of parkinsonism with a *LRRK2*/dardarin mutation in 4 apparently unrelated kindreds from the Basque region of Spain. In addition, they described Family PL, a British pedigree comprising 22 members, 12 of whom were affected and found to have an autosomal dominant parkinsonism caused by a different *LRRK2* mutation.

Clinical features of the Basque families

All 4 Basque families demonstrated the cardinal clinical signs of PD with good responses to dopaminergic agents and typical levodopa-related complications. In general, the disease started with a unilateral rest tremor.

Family UGM03 was a pedigree of 62 individuals spanning 4 generations with 9 affected members. The mean age of onset was 66 years (range, 50–79 years). In addition to rest tremor, a minority of individuals had initial symptoms of gait dysfunction. Dementia was not observed in this family. Family UGM04 comprised 11 individuals spanning 2 generations with 3 affected members. The mean age of symptomatic onset was 62 years (range, 59–64 years). No dementia was observed in affected members. Other features reported were hyperreflexia and Babinski signs. Family UGM05 had 30 members from 3 generations with 8 affected individuals. The mean age of onset was 58 years (range, 57–60 years). In addition to rest tremor, the disease began as unilateral clumsiness in most, with foot dystonia in half the affected individuals. Postural instability was not reported, although 1 individual had severe gait abnormalities. This member also had paranoid delusions that preceded dopaminergic therapy. Family UGM06 was the largest of the 4 pedigrees, with 106 members spanning 4 generations with 15 affected members. The mean age of symptomatic onset was 61 years (range, 59–64 years). No dementia was reported in the affected members.

Clinical features of Family PL

The clinical features of Family PL were not specified but were reportedly similar to the

phenotype observed among the Basque kindreds (Paisan-Ruiz et al., 2004).

Pathologic features

Autopsies were not performed for any of the Basque families or for Family PL.

Family 292 and Family 415

Recently, Hernandez et al. (2005) described the clinical and PET characteristics of 2 North American families with autosomal dominant parkinsonism, Family 292 of English descent and a Russian Ashkenazi Jewish family referred to as Family 415. Probands from both families have a *LRRK2* 6055G>A (G2019S) mutation. The authors reported only abridged pedigrees for both families.

Clinical features

Five members from Family 292 were examined. The average age at onset of symptoms was 58 years. In general, the presenting symptom in these patients was unilateral resting tremor. Rigidity and bradykinesia were also typical among the affected members. Although the occurrence of postural instability was not reported, gait problems developed 10 years after disease onset in 3 of the 5 family members examined. Four of the 5 individuals responded well to dopaminergic agents.

PET with ^{18}F-dopa imaging was performed on 2 affected members, 1 genetically unaffected sibling, and 3 next-generation family members. Imaging demonstrated uptake reductions in the caudate nucleus and putamen of the 2 affected family members. These responses were typical but less than expected compared with individuals of similar disease duration with idiopathic PD. No abnormalities were seen on PET in the family members studied.

One member of Family 415 was examined. At onset of symptoms, she was 54 years old. The ages of symptomatic onset for 5 affected members who were not examined ranged in age from their mid 50s to late 70s. The initial symptom in all affected members was a unilateral rest tremor. The member studied responded well to levodopa for 15 years after which she developed a drug-induced on–off phenomenon, which interfered with gait, and dyskinesias, which lead her to opt for a pallidotomy. The surgery alleviated some drug-related fluctuations and dyskinesia; however, 10 years later her gait dysfunction worsened, necessitating use of a wheelchair.

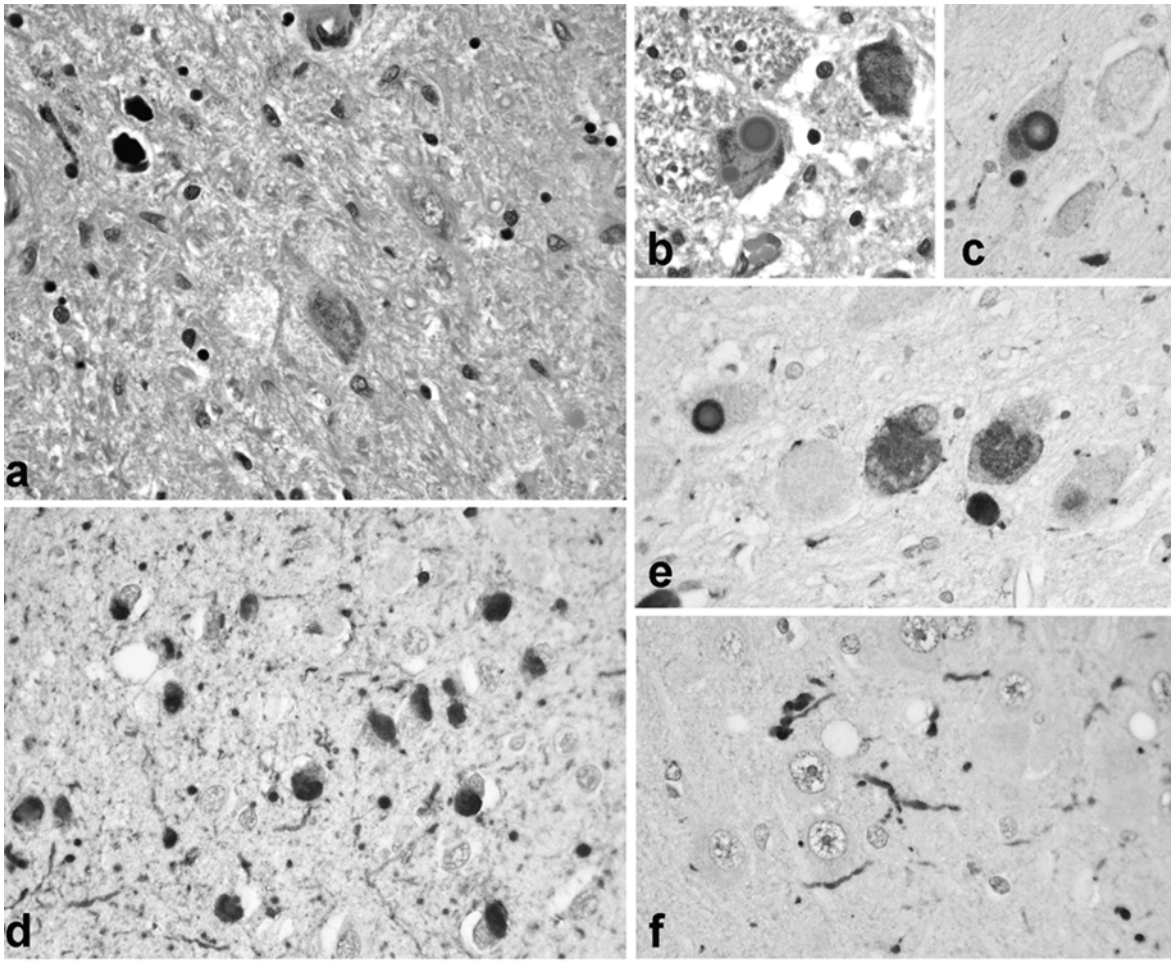
Pathologic features

Autopsies were not performed on members of either family.

LRRK2 in diverse populations

Pathogenic *LRRK2* mutations are relatively common and present in diverse geographic populations. The following studies describe individuals with parkinsonism that is clinically indistinguishable from PD. In all studies, the individuals with *LRRK2*-associated parkinsonism had variable age of disease onset, even among kindreds. A slower disease progression compared with idiopathic PD has been reported (Nichols et al., 2005). Incomplete age-dependent penetrance was also documented (Di Fonzo et al., 2005; Kachergus et al., 2005; Nichols et al., 2005).

Zimprich et al. (2004a) identified 4 (9%) of 44 PD families each with a different *LRRK2* mutation. These families originated from Austria, Germany, and the United States. Subsequently, Kachergus et al. (2005) identified the commonest known *LRRK2* mutation, 6055G>A (G2019S), by sequencing multiplex families with autosomal dominant parkinsonism linked to *PARK8*. They reported that 7 (2.8%) of 248 affected individuals with familial PD had a G2019S *LRRK2* substitution. These individuals were derived from families with ancestral roots in the United States, Norway, Ireland, and Poland. Di Fonzo et al. (2005) reported that 4 (6.6%)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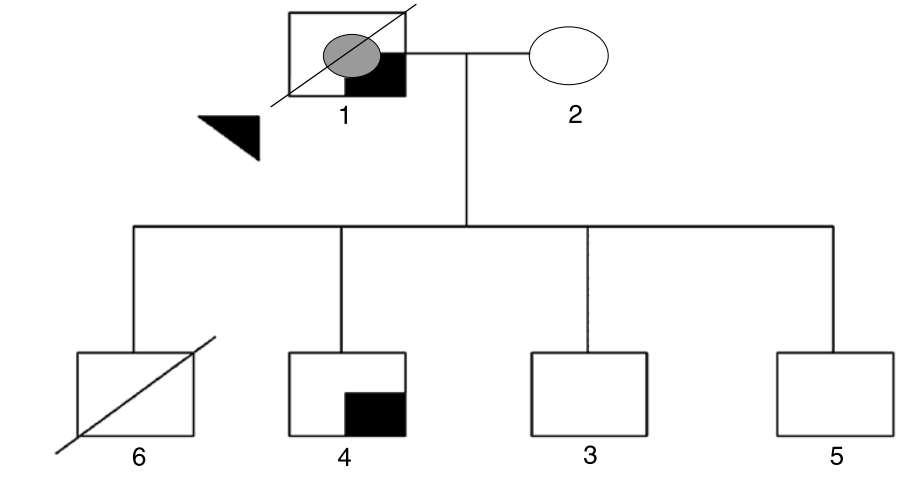
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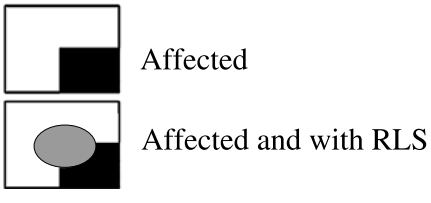
Affected
Affected and with RLS

of 61 individuals from geographically diverse PD families, including some asymptomatic carriers. The frequency of a *LRRK2* G2019S mutation in familial parkinsonism was examined by Nichols et al. (2005). Thirty-five (5%) of 767 affected individuals from 358 multiplex families tested positive for *LRRK2* 6055G>A (G2019S) mutation.

The *LRRK2* 6055G>A (G2019S) substitution has been reported in individuals with apparent idiopathic PD. Six individuals were observed to have the G2019S substitution among a cohort of 806 European descendants with idiopathic PD (Kachergus et al., 2005). Three of the 6 did not have a family history of PD. Gilks et al. (2005) estimated that the mutation may be responsible for idiopathic PD in a clinic-based study where 8 (1.6%) of 482 individuals tested positive for the 6055G>A (G2019S) mutation. Three of these individuals had autopsies performed (Table 1).

Short clinical and pathologic descriptions of an affected individual from one of our families (Family 111) with the *LRRK2* 6055G>A (G2019S) mutation are presented in Fig. 1 and Table 1. The index case from this family developed restless legs syndrome during the course of his illness, suggesting that restless legs syndrome may be part of the phenotype in G2019S substitution carriers.

Discussion

This short review highlights some of the clinical and pathologic features in *LRRK2*-associated parkinsonism, a relatively common genetic cause of familial and sporadic PD. In general, mutations in the *LRRK2* gene are associated with disease that is, for the most part, indistinguishable from idiopathic PD. Clinically, *LRRK2*-associated PD is characterized by the presence of cardinal features of PD, responds to levodopa therapy, and has a similar age of onset. However, additional signs such as dementia, pyramidal signs, and amyotrophy have been reported. Some patients also exhibited postural tremor, dystonia, and restless legs syndrome. The age of symptomatic onset for *LRRK2*-associated PD is variable even in kindreds, causing both early- and late-onset disease. Penetrance appears to be incomplete and age dependent, which could explain the large range of age of disease onset and the presence of *LRRK2* substitutions in asymptomatic family members (this is particularly evident for G2019S carriers). Disease progression with *LRRK2*-associated PD may be slower than that of idiopathic PD (Nichols et al., 2005). Pathologic variability has been demonstrated in family members that have come to autopsy and in recent Brain Bank series (Wszolek et al., 2004; Zimprich et al., 2004a; Gilks et al., 2005; Ross et al., 2005). While nigral degeneration is universal, and LB are often reported, alternate pathologies are possible even with the same family. Most notably, tau pathology (neurofibrillary tangles) and ubiquitin pathology have been described (Zimprich et al., 2004a). This variability in pathology

Fig. 1. Family 1244 (*LRRK2* G2019S mutation). The proband presented with gait dysfunction at the age of 68 years. Therapy with levodopa/carbidopa was initiated at the age of 78 years with good response. Subsequently, restless legs syndrome (RLS), dyskinesias, and cognitive impairment developed. Examination at 81 years revealed asymmetric rigidity, bradykinesia, and severe postural instability. No rest tremor was noted. Mini-Mental Status Examination score was 26/30 at age 81 years and 18/30 at age 82 years. Orthostatic hypotension was confirmed by autonomic studies. However, he was on combined therapy with levodopa/carbidopa, 1,500 mg/d, and pramipexole, 0.75 mg/d. He died of pneumonia at the age of 84 years. Postmortem neuropathologic examination showed transitional Lewy body disease affecting the limbic structures, with severe neuronal loss, gliosis, and axonal spheroids in the substantia nigra (*a*). Lewy bodies were detected in brainstem nuclei (*b*, *c*), the amygdala (*d*), and the basal nucleus of Meynert (*e*). Neuritic pathology was marked in amygdala and also present in hippocampal CA2 region (*f*)

would suggest that mutations leading to LRRK2 dysfunction may interfere with cellular pathways ultimately resulting in tau, ubiquitin, or α-synuclein depositions, all thought to be associated with neurodegeneration.

Genetic testing can now aid in the diagnosis of PD. Future studies are needed to further the understanding of *LRRK2*-associated PD, which may lead to symptomatic therapies for idiopathic PD. The development of transgenic animal models is paramount in the investigation of *LRRK2*-associated PD. These may be used to study the efficacy and adverse effects of novel pharmaceutical approaches before initial clinical trials in genetically defined patients and asymptomatic carriers.

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Molecular genetic findings in LRRK2 American, Canadian and German families

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Summary. A locus for a dominant form of PD has been mapped to the pericentromeric region of chromosome 12 in a Japanese family. We have confirmed linkage in two families of European ancestry and identified mutations in the gene for LRRK2 in these two and four additional families with dominantly inherited PD. All mutations are located in highly conserved domains of the gene. The LRRK2 protein belongs to the ROCO protein family, and includes a ras domaine (ras of complex proteins) and a protein kinase domain of the MAPKKK class and several other major functional domains. Within affected carriers of Families A and D, six *post-mortem* diagnoses reveal brainstem dopaminergic degeneration accompanied by strikingly diverse pathologies. These include abnormalities consistent with Lewy body Parkinson's disease, diffuse Lewy body disease, nigral degeneration without distinctive histopathology and progressive supranuclear palsy-like pathology. Hence, *LRRK2* may be central to the pathogenesis of several major neurodegenerative disorders associated with parkinsonism.

Introduction

Parkinsonism is a clinically defined syndrome, characterized by variable combinations of akinesia, rigidity, tremor and postural instability. The most common cause of parkinsonism is Parkinson's disease (PD). In PD, parkinsonism is caused by a degeneration of dopaminergic neurons of the substantia nigra, leading to a deficiency of dopamine in their striatal projection areas. Characteristic eosinophilic inclusions, the Lewy bodies, are found in surviving dopaminergic neurons but also, though less abundantly, in other parts of the brain, and have been considered to be essential for the pathologic diagnosis of PD.

Genetic research of the past years, in particular the mapping and cloning of a number of genes which cause, when mutated, monogenically inherited forms of the disorder has shown that PD is actually not a disease entity, but rather a heterogeneous group of diseases associated with a spectrum of clinical and pathological changes.

Pathologically, all forms have in common a predominant degeneration of dopaminergic neurons of the substantia nigra, although in some forms the pathologic process appears to be more selective (as in parkin-associated parkinsonism, PARK2), whereas in others the degenerative process is more widespread. In some forms there is typical Lewy body pathology (PARK1, PARK3), whereas in others, the pathology differs from that considered to be typical for PD in some cases but not in others (PARK8). Remarkably,

pathology can even vary within single families. These observations indicate that different, and probably interrelated pathogenic pathways are likely to lead to the process of nigral cell death.

Although the different mutations and loci identified so far appear to be directly responsible in only a relatively small number of families each, there is accumulating evidence that the molecular pathways identified may be common to more than one genetic form of parkinsonism and may, in one way or another, also play a role in the common sporadic disease. This will – eventually – allow to the development of novel protective and therapeutic strategies.

Parkinson's disease caused by mutations in the gene for Leucine-rich repeat kinase 2 (LRRK2, PARK8)

Recently, a locus for a dominant form of PD has been mapped in a large Japanese family to the pericentromeric region of chromosome 12. Affected in this family showed typical l-dopa responsive parkinsonism with onset in their fifties. Pathologically, nigral degeneration was found, but no Lewy bodies or other distinctive inclusions (Funayama et al., 2002).

We have confirmed linkage to this region in two families of European ancestry and refined the disease gene containing interval to about 3 cM (Zimprich et al., 2004b). Following a positional cloning approach, we and others have identified the disease gene in this region as the gene for leucine-rich repeat kinase 2 (LRRK2) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004a). The encoded protein has also been called ''dardarin'' (Paisan-Ruiz et al., 2004). The gene spans a genomic region of 144 Kb, with 51 exons encoding 2527 amino acids (Fig. 1). The gene is expressed in all brain regions and also in all peripheral tissues examined so far, although at low levels.

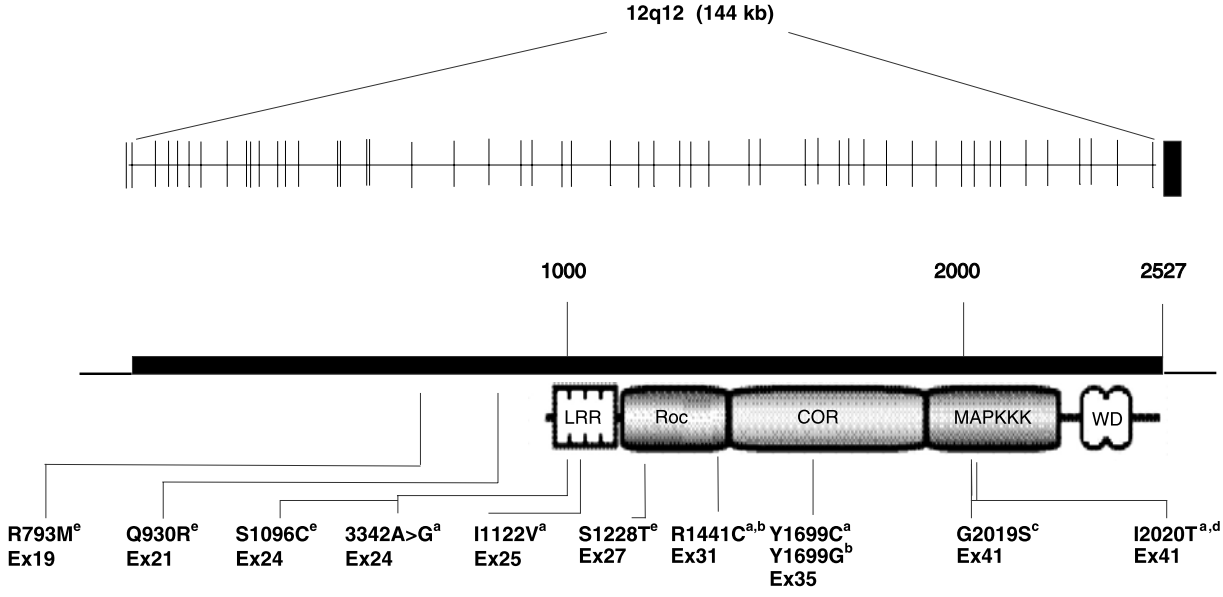

Fig. 1. Genomic structure and functional domains of LRRK2. The gene spans a genomic distance of 144 kb and contains 51 exons. *LRR* leucine rich repeat; *Roc* Ras of complex proteins: *COR* C-terminal of Roc; *MAPKKK* mitogen activated kinase kinase kinase; *WD* Beta-Propeller. Mutations as published in: a: Zimprich et al. (2004a); b: Paisan-Ruiz et al. (2004); c: Kachergus et al. (2005), Di Fonzo et al. (2005), Gilks et al. (2005), Nichols et al. (2005); d: Funayama et al. (2005); e: Berg et al. (2005)

LRRK2-associated PD is remarkable for several reasons. First, mutations in the LRRK2 gene appear to be the most common cause of autosomal-dominantly inherited parkinsonism discovered so far. Four different mutations were detected in five of 34 dominant families studied by Zimprich et al. (in two of the families, the same mutation, R1441C, arose independently, based on the analysis of polymorphisms closely surrounding the gene). The same codon was affected in the group of Basque families studied by Paisan-Ruiz et al. (Paisan-Ruiz et al., 2004), but this mutation resulted in a different amino-acid exchange. These cases and other series show that LRRK2 mutations account for approximately 10 to 20% of dominantly inherited PD.

One particularly common mutation, Gly2019Ser, was detected on a founder haplotype across several European populations (Kachergus et al., 2005) and in up to 5 to 6% of several large cohorts of families with dominant parkinsonism (Di Fonzo et al., 2005; Nichols et al., 2005), and even in 1 to 2% of patients with sporadic late-onset disease (Gilks et al., 2005). Penetrance of this mutation is age-dependent, being about 20% with the age of 50 and rising to 80% at the age of 80. This reduced penetrance probably accounts for the finding of a negative family history in a significant proportion of cases.

Second, clinical signs and symptoms resemble typical sporadic PD in most families. This is true also for age of onset, which is on average in the late fifties and sixties in the families described so far. Therefore, of the PD-genes identified, LRRK2 mutations are by far the most common genetic cause of inherited PD and are likely to play a role also in the setting of typical sporadic late-onset disease.

Third, although the clinical picture appears to resemble typical PD, the associated pathology is remarkably variable. Pathologic changes include abnormalities consistent with Lewy body Parkinson's disease (this is found in the vast majority of cases), diffuse Lewy body disease, nigral degeneration without distinctive histopathology and progressive supranuclear palsy-like tau aggregation. LRRK2 mutations may therefore be an upstream event in the cascade leading to neurodegeneration with different pathologies.

By sequence homology, LRRK2 can be assigned to the group of recently identified ROCO-proteins (Bosgraaf and Van Haastert, 2003) and contains a protein kinase domaine of the MAPKKK class, suggesting a role in intracellular signaling pathways, but its precise function remains to be determined. Mutations appear to be clustered in functionally important regions, which are highly conserved through the species.

Conclusions

The genetic findings in rare inherited forms of PD have contributed to our understanding of the clinical, neuropathologic, and genetic heterogeneity of PD. The variability of clinical features, such as age at onset, occurrence of dementia, or other associated features found within single families, suggests that a single genetic cause (the pathogenic mutation in a given family) can lead to a spectrum of clinical manifestations.

The elucidation of the normal function of the protein encoded by LRRK2 and its dysfunction in disease will provide new insight into the molecular pathogenesis of PD and possibly also open up new therapeutic possibilities.

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Genetic and DAT imaging studies of familial parkinsonism in a Taiwanese cohort

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Summary. We here summarize the results of genetic investigations on a series of 82 parkinsonian patients from 60 families in Taiwan. We found 13 *parkin* patients in 7 families (12%), 2 *PINK1* sibs from 1 family, and 1 *LRRK2* patient from 1 family with I2012T mutation. We also identified SCA2 in 8 patients from 5 families (8%) and SCA3 in 3 patients from 1 family, all presenting with parkinsonian phenotype.

In the available patients with *parkin*, *PINK1*, SCA2 and SCA3, the dopamine transporter (DAT) scan revealed that the reduction of uptake was primarily observed in the bilateral putamen, basically sharing a similar pattern with that in idiopathic Parkinson's disease.

We concluded that the genetic causes contributed to about 25% of our series of familial parkinsonism. The *parkin* mutations and SCA2 were the most frequent genetic causes in our series with Chinese ethnicity. The results of DAT scan indicated that bilateral putamen was essentially involved in various genetically-caused familial parkinsonism.

Introduction

In recent investigations on the familial parkinsonism have led to the identification of a series of Parkinson genes. There are 6 genes including α-*synuclein*, *parkin*, *UCHL1*, *DJ1*, *PINK1* and *LRRK2* which have been linked to familial parkinsonism (Polymeropoulos, 1997; Kidata, 1998; Leroy, 1998; Bonifeti, 2003; Valente, 2004; Paisan-Ruiz, 2004; Zimprich, 2004). Although the genetic causes are responsible for about 10% of Parkinson's disease population, those have contributed significantly to the understanding of the pathogenesis of IPD. *Parkin* mutations linked to autosomal recessive early onset parkinsonism have been documented most commonly in familial parkinsonism and also often in sporadic early onset Parkinson's disease worldwide (Kidata, 1998; Hatorri, 1998; Abbas, 1999; Lucking, 2000). More reports on mutations in the newly-discovered *LRRK2* gene will be expected in patients with an autosomal dominant inherited family or late onset IPD in the coming years (Paisan-Ruiz, 2004; Zimprich, 2004).

We have initially reported homozygous deletion of exon 3 in the *parkin* gene in 2 sibs from a Taiwanese family (Lu, 2001). Subsequently, compound heterozygous mutation in *PINK1* gene was described in 2 sisters in the other family (Hatano, 2004). Other early-onset familial parkinsonism with *parkin* mutations has also been reported from Taiwan (Wu, 2002, 2005).

Interestingly, in our series we have also identified abnormally increased CAG repeats in SCA2 and SCA3 families with dominant

parkinsonian phenotype responded well to levodopa (Lu, 2002; Tuite, 1995). Particularly, SCA2 presenting with parkinsonian phenotype was found in Taiwanese population with Chinese ethnicity (Gwinn-Hardy, 2000; Shan, 2001; Lu, 2004).

To access the pattern of impairment of dopamine neurons, we have previously used ^{99m}Tc-TRODAT-1 SPECT imaging to measure the DAT activities in IPD (Yen, 2002). Here, we will apply the same imaging technique to evaluate the damage of the dopamine terminal in the striatum in those genetic parkinsonism.

Materials and methods

Patients

The research protocol was approved by Human Ethic Committee of Chang Gung Memorial Hospital and Institute of Review Board. A series of consecutive 82 patients coming from 60 families which have at least 2 or more affected members, were included in this study. The other affected member was either the first or second degree relative of proband. None revealed parental consanguinity. All patients fulfilled the criteria of possible or probable Parkinson's disease proposed by Gelb et al. (1999) with a good and sustained response to levodopa. Patients with a possibility of secondary parkinsonism caused by toxins, drugs, or other degenerative causes were excluded. The detailed clinical history, family history, clinical manifestations, levodopa induced dyskinesias and motor fluctuations were obtained in all patients. Clinical evaluations included the Hoehn and Yahr stage and Unified Parkinson's Disease Rating Scale.

Genetic analysis

Venous blood sample was collected from each patient after the informed consent was signed. The genetic analysis was conducted according to those published methods included single strand complement polymorphism, quantitative polymerize chain reaction, and direct sequence of the suspicious and hot exon in 5 genes including *α-synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*. The number of CAG repeats of *ataxin 2* and *ataxin 3* was also analyzed by using the modified techniques as previously reported (Lu, 2002; Tuite, 1995).

^{99m}Tc-TRODAT-1 SPECT imaging

The SPECT imaging was performed as that described previously (Yen, 2002). In brief, 2 ml of ligand, ^{99m}Tc-TRODAT-1 containing a dose of 925 MBq (25 mCi) was injected intravenously. SPECT images were acquired 4 hours later by using a Siemens MULTISPECT 3 gamma camera. For analysis of uptake of ^{99m}Tc-TRODAT-1 in the striatum, the specific uptake ratio (UR) was calculated by the summation of three adjacent transversal slices representing the

Table 1. Clinical demography and genetic analysis of 60 familial parkinsonism

	Parkin	*PINK1*	*LRRK2*	SCA2	SCA3	Unknown
P. No./F. No.	13/7	2/1	1/1	8/5	3/1	55/45
Gender (F/M)	4/9	2/0	0/1	5/3	3/0	19/36
Examined age (range), yrs	40.5 ± 10.3 (25–65)	41.5 ± 2.1 (40–43)	61	57.8 ± 11.1 (41–75)	54.7 ± 5.5 (49–60)	60.4 ± 11.5 (33–81)
Onset age (range), yrs	27.4 ± 10.5 (15–55)	17.5 ± 0.7 (17–18)	47	44.9 ± 10.9 (34–69)	38.7 ± 5.1 (33–43)	47.5 ± 13.0 (18–75)
Disease duration (range), yrs	13.6 ± 5.6 (6–22)	24.0 ± 2.9 (22–26)	14	11.1 ± 8.0 (3–35)	16.0 ± 10.5 (6–27)	12.9 ± 6.6 (3–29)
L-dopa/day (range), mg	355 ± 211.4 (100–700)	300 ± 100 (200–400)	900	514.3 ± 377.2 (150–1300)	666.7 ± 351.2 (300–1000)	
L-dopa induced dyskinesia	+++ (n = 6)	++ (n = 1)	−	−	+ (n = 1)	
Genetic mutation or CAG number (range)	com-heter (n = 6), heter (n = 5), homo (n = 2)	com-heteo (n = 2)	I2012T	36.0 ± 1.1 (35–38)	70	

P. No. patient number, *F. No.* family number, *com-heter* compound heterozygous, *heter* heterozygous, *homo* homozygous, +++ = marked, ++ = moderate, + = mild, − = none

most intense striatal DAT binding. A standard region of interest (ROI) template was constructed according to a stereotactic shape from a MRI atlas and included regions for the putamen, caudate nucleus and occipital cortex. In short, the specific UR represented (ROI counts – occipital cortex counts)/occipital cortex counts. We also calculated the P/C ratio, from counts in the relevant regions, using the formula [(putamen-occipital cortex)/(caudate-occipital cortex)], to investigate the regional variability of DAT in the caudate nucleus and putamen. The asymmetrical index was also analyzed for evaluation of the difference between both side uptakes in the striatum. The paired-t test was used for the asymmetry evaluation of various uptake ratios in different parkinsonism caused by genetic mutations.

Results

The findings of our clinical and genetic studies were summarized in Table 1. We found 13 *parkin* patients from 7 families (mean age at onset 27.4 ± 10.5 years), 2 *PINK1* sibs from 1 family (17.5 ± 0.7 years), 1 *LRRK2* patient from 1 family (47 years). We also found 8 SCA2 patients from 5 families with parkinsonian phenotype (44.9 ± 10.9 years), and 3 SCA3 patients from 1 family with parkinsonian phenotype (38.3 ± 5.1 years). At present, *parkin* mutations were the most common genetic cause of familial parkinsonism in our series, and were followed by SCA2, *PINK1* and SCA3. No mutation was detected in the remaining 55 patients from 45 families. Compound heterozygous and heterozygous mutations rather than homozygous mutation were most frequently found in the *parkin* gene. Compound heterozygous mutation was also found in the *PINK1* patients. One patient with I2012T mutation in *LRRK2* gene was identified. His mother died 15 years ago was a victim of Parkinson's disease either. Presumably, this was an autosomal dominant inheritance. The mean CAG repeat number was 36.0 ± 1.1 (range, 35–38) in SCA2 families. The mean CAG repeat number was 70 in SCA3 family.

PINK1 and *parkin* patients were of younger age at onset, compared to those of SCA2 and SCA3. The clinical features included typical parkinsonism in *parkin*, *PINK1* and *LRRK2*

Table 2. Mean uptake ratios in different mutated parkinsonism

Group	Disease duration	C S/O	C C/O	C P/O	I S/O	I C/O	I P/O	C P/C	I P/C
Parkin	14.33 ± 3.72	0.35 ± 0.10	0.68 ± 0.16	0.22 ± 0.08	0.39 ± 0.10	0.63 ± 0.13	0.31 ± 0.12	0.33 ± 0.08	0.50 ± 0.21
N = 6	(12–21)	(0.19–0.47)	(0.48–0.87)	(0.10–0.35)	(0.25–0.55)	(0.45–0.85)	(0.14–0.44)	(0.21–0.44)	(0.23–0.78)
PINK1	22.00 ± 4.24	0.37 ± 0.18	0.75 ± 0.33	0.21 ± 0.08	0.45 ± 0.28	0.89 ± 0.28	0.27 ± 0.24	0.28 ± 0.01	0.27 ± 0.18
N = 2	(19–25)	(0.24–0.50)	(0.51–0.98)	(0.15–0.27)	(0.25–0.64)	(0.69–1.09)	(0.10–0.44	(0.28–0.29)	(0.14–0.40)
SCA2-P	14.75 ± 11.87	0.27 ± 0.03	0.48 ± 0.12	0.17 ± 0.05	0.29 ± 0.12	0.47 ± 0.14	0.21 ± 0.11	0.39 ± 0.19	0.44 ± 0.17
N = 4	(5–32)	(0.23–0.29)	(0.38–0.62)	(0.11–0.23)	(0.17–0.45)	(0.38–0.67)	(0.08–0.34)	(0.17–0.62)	(0.20–0.55)
SCA3-P	10.50 ± 6.36	0.38 ± 0.18	0.61 ± 0.11	0.30 ± 0.20	0.45 ± 0.13	0.65 ± 0.16	0.38 ± 0.11	0.47 ± 0.24	0.58 ± 0.02
N = 2	(6–15)	(0.25–0.51)	(0.53–0.68)	(0.16–0.44)	(0.35–0.54)	(0.53–0.76)	(0.30–0.45)	(0.30–0.65)	(0.57–0.59)

C contralateral, *I* ipsilateral

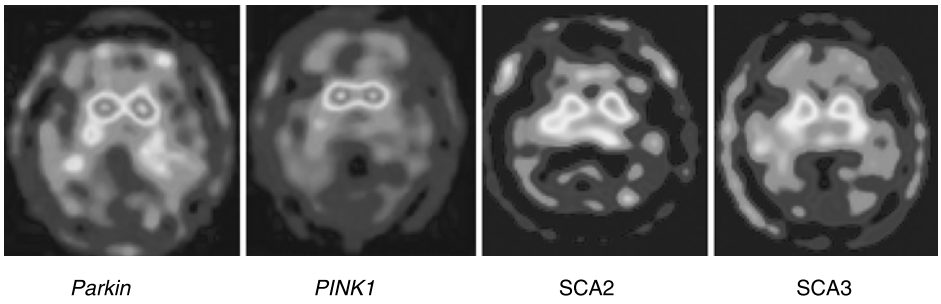

Fig. 1. ^{99m}Tc-TRODAT-1 images of representative patients from each different genetic parkinsonism. Symmetrically reduced uptakes were observed in *parkin*, *PINK1*, SCA2, and SCA3 patients with similar age and disease duration

patients. Sleep benefit and severe levodopa induced dyskinesias were observed in *parkin* and *PINK1* patients. Focal dystonia was an initial and dominant symptom in *parkin* patients. Mild cerebellar signs such as dysarthria, mild ataxia, and gait disturbance were observed in SCA2 and SCA3 families 10 years after the onset of parkinsonism.

DAT scan using ^{99m}Tc-TRODAT-1 SPECT imaging was performed in limited patients who were available (Table 2). The uptakes of ligand were markedly reduced in the putamen and caudate nucleus, slightly worse in the putamen contralateral to the dominant side of parkinsonism. The preferential involvement of contralateral putamen in 4 groups was similar to that in IPD patients (Fig. 1). Therefore, the pattern of reduction in uptakes is similar in 4 groups, but most severely affected was noted in SCA2 patients. Bilateral P/C ratios were similar among 4 groups, which indicated a relatively symmetrical impairment of DAT activities. This further implied a relatively symmetrical loss of dopamine neurons in these genetically-caused familial parkinsonism.

Discussion

In our small series of familial parkinsonism, mutations could only be identified in about 25% of families. The genetic cause in the majority of our families (75%) remains unknown. This low acquirement is probably due to ethnic reasons. However, a further evaluation with other new approaches is mandatory. *Parkin* mutations contributed about 12% of the total 60 families, and are the most common genetic cause. All the *parkin* patients were with an autosomal recessive inheritance without parental consanguinity. It is different from those reported from Japanese often in association with consanguineous parents (Kidata, 1998; Hatorri, 1998). No consanguineous marriage was also found in the other *parkin* families reported from Taiwan (Wu, 2002, 2005).

The clinical presentations of our *parkin* patients were similar to those reported previously in Japanese and Caucasian (Kidata, 1998; Hatorri, 1998; Abbas, 1999; Lucking, 2000), as well as to other *parkin* patients reported from Taiwan (Wu, 2002, 2005). They were inherited as an autosomal recessive mode. The characteristic early-onset parkinsonism, focal dystonia, sleep benefits, good response to levodopa and easily-induced dyskinesias were also observed.

Most interestingly, we found SCA2 and SCA3 families with dominant parkinsonian phenotype in our series. Particularly in SCA2 families, the parkinsonian symptoms were so dominant that let us unable to distinguish the patients from IPD in the early stage (Lu, 2002, 2004; Gwinn-Hardy, 2000; Shan, 2001; Yen, 2002). The cerebellar signs developed in the late stage, except mild dysarthria might be detected earlier. Their Parkinsonism responded

well to the levodopa therapy. Although, similar patients have been documented in other races (Furtado, 2002), most reported patients were from Chinese population. These facts have further implied that ethnicity is an important contributing factor. Likewise, parkinsonian phenotype of SCA3 was predominantly reported from African population (Gwinn-Hardy, 2001; Subramony, 2002).

We have identified I2012T mutation of *LRRK2* gene in one patient, presumably with an autosomal dominant inheritance (Di Fonzo, 2005; Nicolas, 2005). In an unpublished data, we have not identified this mutation in a series of 652 IPD patients and 624 controls. Neither G2019S nor I2020T mutations was found.

Using ^{99m}Tc-TRODAT-1 scan, we found that the DAT concentrations in genetic-mutated parkinsonism was markedly reduced in the putamen and caudate nucleus, especially in the putamen contralateral to the dominant side. These findings are similar to those of IPD, but with a more symmetrical involvement in the bilateral striatum. Interestingly, these finding are similar in all 4 groups; *parkin*, *PINK1*, SCA2 and SCA3. Those findings might be nonspecific, just a final common pathway of dopamine neuron death, or it might have further implications.

In conclusion, genetic mutations were responsible for about a quarter of our cohort of familial parkinsonism in Taiwan. *Parkin* mutation and SCA2 are the most common genetic causes. The parkinsonian phenotype of SCA2 is most interesting and might be contributed to Chinese ethnicity. However, the genetic causes of the rest three quarters of familial parkinsonism remain unknown.

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Neuroimaging in Parkinson's disease

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Summary. Structural imaging studies often reveal relatively limited findings in Parkinsonian disorders, as the most profound changes are neurochemical and hence better revealed by functional studies such as PET or SPECT. However, newer magnetic resonance techniques such as spectroscopy, diffusion weighted imaging, diffusion tensor imaging and magnetization transfer have shown promise in differentiating between idiopathic Parkinson's and the atypical parkinsonian disorders such as multiple system atrophy and progressive supranuclear palsy. We review here recent advances in functional imaging as well as in structural studies of basal ganglia disorders. Functional studies may give insights into mechanisms underlying disease pathogenesis, as well as neurochemical alterations.

Magnetic resonance imaging and movement disorders

Magnetic resonance imaging (MRI) allows the *in vivo* visualization of brain structures, providing vital information such as the brain volume and tissue content. It facilitates not only the diagnosis, but also the understanding of disease process in movement disorders.

Volumetric analysis

Conventional MRI may aid in the differentiation of atypical parkinsonism. The presence of the ''hot-cross bun'' sign (arising from a combination of atrophy and increased signal) in the pons, putaminal atrophy, and a hyperintense rim at the edges of the putamen suggest a diagnosis of multiple system atrophy (MSA), whereas the presence of midbrain atrophy with dilatation of the third ventricle is more likely associated with progressive supranuclear palsy (PSP) (Schrag et al., 2000). However, most of these signs lack sensitivity, and by themselves, are not specific. A mild degree of putaminal hypodensity and a hyperintense rim at the edges of the putamen have been reported in some cases of Parkinson's disease (PD) (Bhattacharya et al., 2002). Volumetric analysis may better discriminate PD from atypical parkinsonism. MSA patients have smaller striatal and brainstem volumes compared to PD patients (Schulz et al., 1999). Using voxel-based morphometry, Brenneis et al. (2003) showed cortical atrophy in MSA patients compared to PD and control subjects. There was also significant reduction in volume of the left caudate head in PD patients compared to control subjects. Others have demonstrated significant atrophy in the anterior cingulate gyrus, hippocampus, and superior temporal gyrus in PD patients (Summerfield et al., 2005). Demented PD patients had several cortical and subcortical areas affected, but the most significant change was in the anterior cingulate gyrus, hippocampus, and thalamus (Summerfield et al., 2005). The superior cerebellar peduncle volume, corrected for total intracranial volume, was significantly reduced in PSP patients

compared to control, MSA, and PD subjects (Paviour et al., 2005).

Magnetic resonance spectroscopy

Proton magnetic resonance spectroscopy (MRS) allows the *in vivo* measurement of brain metabolism. Four major hydrogen-containing metabolites may be identified: a) N-acetylaspartate (NAA), a marker of neuronal integrity; b) creatine (Cr), which relates to general metabolism; c) choline (Cho), which is altered by membrane turnover; and d) myo-inositol (MI), a marker of glial cells. There was a widespread reduction in NAA/Cr ratio in the pons, putamen, and cortical white matter in MSA patients compared to control subjects, representing widespread neuronal and axonal involvement (Watanabe et al., 2004). Compared to PD patients, MSA patients had significantly reduced NAA/Cr ratio in the pons and putamen. Of interest, the pontine NAA/Cr ratio was reduced in MSA patients even in the absence of structural MRI findings such as the ''hot-cross bun'' sign and putaminal rim hyperintensity (Watanabe et al., 2004). Both increased and decreased NAA/Cr ratios have been reported in the substantia nigra (SN) of PD patients. Quantitative analysis confirmed no significant difference in MRS metabolites between PD and control subjects, except for a reduction in CSF-corrected creatine in the SN of PD patients (O'Neill et al., 2002).

MR techniques to define nigral pathology

With special pulse sequence and imaging techniques, subtle changes in the midbrain and SN may be defined in PD patients. Segmented inversion recovery ratio imaging (SIRRIM) is a new technique that uses two inversion recovery sequences, one to suppress white matter and the other to suppress gray matter, to display the SN as an isolated structure for a more detailed analysis (Hutchinson et al., 2003). With SIRRIM, a gradient of radiological change in SN may be observed that is consistent with postmortem findings in PD (Fearnley and Lees, 1991; Hutchinson et al., 2003; Hutchinson and Raff, 1999). It should be noted, however, that using a similar technique, Hu and colleagues found that while structural changes detected using inversion recovery MRI correlated with the degree of striatal dopamine deficiency assessed using ^{18}F-dopa PET, PET was more sensitive in discriminating moderately severe PD from controls (Hu et al., 2001).

Sohmiya et al. (2004) used T2-weighted morphometric study to demonstrate an increase in the maximum distance of SN and a decrease in distance between the red nucleus and SN in PD patients. The authors interpreted these findings as a reflection of neuronal loss and an increase in iron content. However, in subjects older than 70 years, there was no significant difference in measurements between PD and control subjects.

Diffusion-weighted imaging

Diffusion-weighted imaging (DWI) is a relatively new technique that measures the diffusion of water molecules in tissue. Water molecules tend to diffuse along the directions of the fibre tracts in the brain. Any disruption of tissue architecture will increase the freedom of diffusion of water molecules, leading to greater diffusion isotropy. Quantification of diffusion is possible by calculating the apparent diffusion coefficient (ADC). DWI requires a very short acquisition time and has relatively high spatial resolution. In recent years, investigators have applied DWI technology to define areas of neuronal loss in neurodegenerative diseases. MSA patients have significantly higher putaminal ADC values reflecting the greater neuronal loss in this region compared to PD and control subjects (Schocke et al., 2002). However, there was no significant difference in ADC values between PD and control subjects. PSP patients have higher ADC values in the putamen, caudate and globus pallidus compared

to PD patients, but a significant difference in ADC values between PSP and MSA patients was not present (Seppi et al., 2003). Nevertheless, using a step-wise logistic regression analysis, the authors found that putaminal ADC was able to discriminate between PSP and PD with a sensitivity of 90% and a positive predictive value of 100%. In another DWI study, there was a significant increase in ADC values in PSP patients compared to controls in the prefrontal and precentral white matter (Ohshita et al., 2000).

Diffusion tensor imaging

DWI measures the diffusion of water molecules in one plane, and may underestimate the diffusion-related pathologic process in the brain. A modified technique, known as diffusion tensor imaging (DTI), allows the ADC in three orthogonal directions to be calculated and averaged as the diffusion tensor Trace(D). Schocke et al. (2004) reported a significant increase in Trace(D) in the putamen and pallidum of MSA patients compared to PD patients. There was a significant increase in putaminal ADC in the y- and z-directions in MSA patients. The putaminal Trace(D) completely discriminated MSA patients from PD and control subjects. Using another measure of diffusion known as fractional anisotropy (FA), Yoshikawa et al. (2004) demonstrated early changes in the nigrostriatal projections in parkinsonian brains. The authors found a reduction in FA values in PD patients compared to control subjects, in regions of interest placed along a line from the SN to the lower part of the striatum. In advanced cases of PD, the FA values were also reduced in the subcortical white matter. PSP patients had reduced FA values in most subthalamic structures and in subcortical white matter.

DTI has also provided new insights into the pathologic process in idiopathic dystonia. While conventional MRI is usually unhelpful in idiopathic dystonia, FA values were reduced in the subgyral white matter of the primary sensorimotor cortex in DYT-1 gene mutation carriers compared to control subjects (Carbon et al., 2004). Furthermore, the FA values were significantly lower in manifesting DYT-1 mutation carriers than non-manifesting DYT-1 mutation carriers. Although the significance of this finding is uncertain, it may represent altered anatomical connectivity of the supplementary motor area, resulting in an increased susceptibility of DYT-1 mutation carriers to develop clinical dystonia.

Magnetization transfer imaging

Magnetization transfer imaging (MTI) is another novel imaging technique that measures the transfer of energy between highly bound protons (such as in the myelin and cell membranes) and the highly mobile protons of free water. Pathological changes in these structures will reduce the exchange of magnetization between bound and free protons, resulting in a reduced magnetization transfer ratio (MTR). The MTRs were reduced in the putamen, globus pallidus, thalamus, and subcortical white matter of PSP subjects compared to controls (Hanyu et al., 2001). In MSA, the MTRs were reduced in the pons, middle cerebellar peduncle, putamen, and white matter of the precentral gyrus (Naka et al., 2002). Eckert et al. (2004) were able to completely discriminate control and PD subjects from MSA and PSP patients using a step-wise linear discriminant analysis that included globus pallidus, putamen, and caudate nucleus in the final model. However, the discrimination between PD and control subjects was less than perfect (with misclassification rates between 20% to 25%), and up to one-third of MSA patients were misclassified as PSP.

Functional MRI

Functional MRI (fMRI) monitors brain functions with relatively good spatial and temporal resolutions. It measures blood oxygenation that indirectly reflects neuronal activity. In an fMRI study on task-specific

dystonia, compared to healthy subjects, dystonic patients had significantly larger activation of the contralateral primary sensorimotor cortex, and underactivation of bilateral premotor areas (Pujol et al., 2000). These findings support the hypothesis of an abnormal recruitment of cortical areas in dystonia.

In an event-related fMRI study comparing PD patients to control subjects, PD patients in the ''off-levodopa'' state showed decreased activation of the supplementary motor area, but increased activation in the primary motor cortex and the lateral premotor cortex bilaterally (Haslinger et al., 2001). Following oral levodopa replacement, there was partial normalization of the abnormal patterns of cortical activation. In another fMRI study, the effects of dopaminergic therapy on cognitive and motor functions in PD patients were evaluated (Mattay et al., 2002). Motor tasks activated the supplementary motor area, lateral premotor cortex, sensorimotor cortex, parietal cortex, and cerebellum bilaterally. The cortical activations were greater in the ''on-levodopa'' state, and the increase in activation correlated with better motor performance. Working memory tasks activated the prefrontal cortex, pericingulate cortex, anterior cingulate cortex, and parietal cortex bilaterally. The cortical activations, however, were greater in the dopamine-depleted state, and correlated with errors in task performance. Taken together, these findings suggest different dopaminergic systems are involved in the control of motor functions and working memory. The nigrostriatal dopaminergic system modulates motor functions indirectly via thalamic projections to motor cortex, whereas the mesocortical dopaminergic system facilitates cognitive tasks via direct inputs to the prefrontal cortex.

Recent PET findings in neurodegenerative disorders

Attention has recently been drawn again to the role of inflammation in the pathogenesis of PD. Activated microglia express the peripheral benzodiazepine binding site, which can be assessed using radiolabelled analogs of PK11195. Ouchi and coworkers studied 10 drug-naïve early stage PD patients using [^{11}C]PK11195 and PET, as well as [^{11}C]CFT, a marker of dopamine transporter binding. After injection, [^{11}C]PK11195 binding was higher in the midbrain than in other analyzed brain regions, such as the thalamus and putamen, suggesting accumulation within microglia in this region. An age-related increase in BP in the thalamus and midbrain of healthy controls was also observed, in keeping with former studies. Interestingly, microglial activation correlated with standard measures of disease severity: increased [^{11}C]PK11195 binding correlated negatively with ipsilateral striatal [^{11}C]CFT and positively with UPDRS scores (Ouchi et al., 2005). These exciting findings support an association between neuroinflammatory mechanisms and PD. However, it is still not possible to determine whether this represents a causative relationship or a secondary effect.

It has been suggested that substances or toxins present in the environment may be involved in the pathogenesis of PD. Experimental investigations disclose pro-apoptotic and pro-oxidative effects of certain types of pesticides. Similarly, levels of organochlorine compounds are increased in PD midbrains. In normal brains, an intact blood–brain barrier (BBB) can actively prevent the concentration of pesticides. In this context, Kortekaas and colleagues assessed the *in vivo* properties of the BBB in PD using [^{11}C]verapamil PET. This ligand is a substrate for the P-glycoprotein transporter, one of the BBB structures responsible for transporting substances from the brain to the blood. In this study, 5 PD patients with mild disease were compared to 5 controls. The authors found an increase in [^{11}C]verapamil distribution volume (DV) of up to 18% in the brainstem of PD subjects. It could be argued that antiparkinsonian medications could possibly have an effect on the BBB. However, one of the patients was drug-naïve and it is indeed note-

worthy that no DV overlap was observed between patients and controls (Kortekaas et al., 2005). This provocative finding is yet to be confirmed by other centres and in larger numbers of subjects. It is furthermore necessary to clarify whether the increase in [^{11}C]verapamil binding is exclusively indicative of impaired BBB function, as opposed to other phenomena, such as increased Ca^{++} channel binding.

Alzheimer's disease

Relatively localized abnormalities of regional cerebral glucose metabolism have been recognized for some time in Alzheimer's disease, and the distribution of such changes appears to be different from that associated with Lewy Body Dementia. A method to detect extracellular β-amyloid deposits (which constitute senile plaques) *in vivo* recently became available. By modifying thioflavin-T, an amyloid-binding dye, a collaborative research group obtained a suitable PET ligand, *N*-methyl-[^{11}C]2-(4′-methylaminophenyl)-6-hydroxybenzothiazole, or "Pittsburgh Compound-B" (PIB). In a preliminary study, 25 AD patients (mild to moderate disease) underwent dual PIB and FDG PET and were compared to controls. PET identified a significant accumulation of PIB in the frontal and temporoparietal cortices, compatible with postmortem studies of amyloid deposition. There was no significant correlation between PIB uptake and cognitive impairment, but this may reflect the preponderance of individuals with early and intermediate disease. Interestingly, 3 patients with a clinical diagnosis of AD and relatively preserved Minimental State Examination showed PIB uptake within normal limits, as well as a largely unaffected glucose metabolic rate (FDG) (Klunk et al., 2004). This work opens new possibilities for diagnosis in AD. However, it should be noted that the method is not sensitive to neurofibrillary tangles, the other pathological hallmark of AD. Factors such as changes in blood flow to the frontal cortex may also play a role, and should be further investigated. Should therapies aimed at slowing deposition of β-amyloid be developed, this is a potential tool for observations on disease progression.

PET in familial PD

Most cases of PD are sporadic and the etiology unknown. Recently identified mutations resulting in parkinsonism have provided valuable clues to the pathogenesis of this common neurodegenerative disease (Vila and Przedborski, 2004). However, the clinical expression in some inherited forms of parkinsonism differs from that of sporadic PD in features such as age of onset, rate of progression, associated neurological features, and the incidence of complications. Furthermore, neuropathological and neurochemical characterization of most of these forms of parkinsonism has been limited, and thus their relationship to sporadic PD has for the most part remained unresolved.

By providing quantitative information on pre- and postsynaptic striatal dopaminergic function, positron emission tomography (PET) can be invaluable for the *in vivo* investigation of PD. In some forms of inherited PD, PET findings have been similar to those of sporadic PD. Clinically, subjects with autosomal-recessively inherited PD due to *parkin* (PARK2) mutations present with levodopa-responsive parkinsonism, typical therapy-related complications, diurnal fluctuations, hyperreflexia and focal dystonia of young onset; although late onset and sporadic cases have been reported. ^{18}F-dopa studies of young and late-onset PD patients with *parkin* mutations have revealed a rostrocaudal gradient and asymmetry typical of sporadic PD, although the caudate was affected more severely in parkin-associated parkinsonism than in sporadic PD (Scherfler et al., 2004). In a recent study, reduced dorsal and ventral midbrain ^{18}F-dopa uptake suggested impairment of catecholaminergic and serotonergic pathways in *parkin*-associated PD. However, some studies have suggested findings different from

sporadic PD. Reduced striatal D2 receptor binding has been demonstrated in PD patients with *parkin* mutations in comparison to asymptomatic family members, sporadic idiopathic PD patients and controls, suggesting post-synaptic dysfunction in this group (Hilker et al., 2001; Scherfler et al., 2004). Similarly, although the clinical features are reminiscent of sporadic PD, patients with young-onset, autosomal recessively-inherited parkinsonism with linkage to PARK6 display more severe involvement of the anterior striatum than do disease duration and severity-matched sporadic PD controls (Khan et al., 2002b). As with some subjects with *parkin* (PARK2) mutations, PARK6 affected individuals demonstrate a less severe phenotype than their scans would suggest and heterozygous mutation carriers have demonstrated striatal pre-synaptic dopaminergic dysfunction without clinical signs of disease (Hilker et al., 2001; Khan et al., 2002).

In studies of dominantly inherited parkinsonism related to mutations in the α-synuclein gene, the PET findings have been similar to sporadic PD (Samii et al., 1999), although the clinical course may be somewhat more aggressive than typical PD.

The most recent addition to defined dominantly-inherited causes of PD is PARK8, in which multiple mutations in the LRRK2 (leucine-rich repeat kinase 2) gene encoding a newly described large, multifunctional protein (known as LRRK2 or dardarin, from the Basque for tremor), have been described (Di Fonzo et al., 2005; Kachergus et al., 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Gly2019Ser mutations in this gene may account for between 1–2% and 5–7% of sporadic and familial PD, respectively (Aasly et al., 2005; Gilks et al., 2005; Nichols et al., 2005). Patients with mutations in this gene have clinical features similar to sporadic PD, but while loss of dopamine-producing neurons of the substantia nigra is seen in all, the ultrastructural pathology may include diffuse or brainstem-restricted Lewy bodies, abnormal tau or amyloid deposition, or no specific ultrastructural pathology.

To date, PET studies of affected families, including a large series from our own centre (Adams et al., 2005), have demonstrated findings similar to sporadic PD (Hernandez et al., 2005; Paisan-Ruiz et al., 2005). More specifically, reductions in striatal ^{18}F-dopa uptake, DAT and VMAT2 binding are asymmetric with greater severity contralateral to the more clinically affected side and a rostrocaudal gradient with the putamen more severely affected than the caudate. Striatal D2 receptor binding was preserved. Such findings suggest that the most important pathological correlate of PD is nigral dopaminergic cell loss rather than specific ultrastructural findings. Furthermore, study of asymptomatic mutation carriers at our centre has revealed early reduction in DAT binding followed by VMAT2 binding levels and relative sparing of ^{18}F-dopa uptake. Our findings suggest upregulation of dopa-decarboxylase and relative downregulation of the DAT may provide compensatory mechanisms delaying the onset of clinical disease.

The similarities and differences detected on PET (and SPECT) studies of familial and sporadic PD patients may reflect variations in technique to some degree, but within a specific modality more likely represent the phenotypic similarities and differences seen clinically and pathologically. The further study of these patient groups using PET will provide a better understanding of the underlying mechanisms that contribute to disease pathogenesis as well as the compensatory mechanisms, while permitting exploration of potential neuroprotective therapies.

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Transcranial sonography in the early and differential diagnosis of Parkinson's disease

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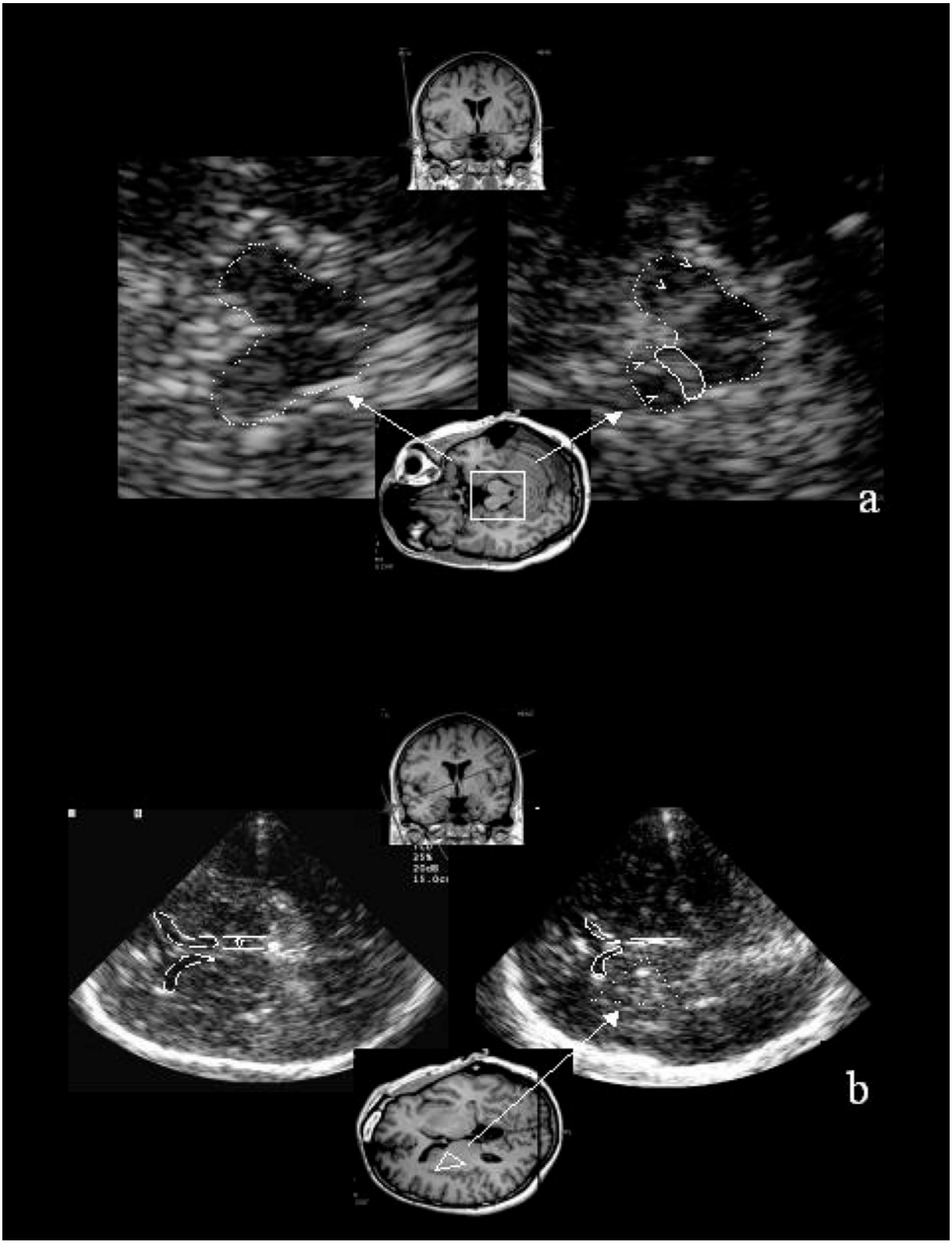
Summary. In recent years transcranial sonography (TCS) has become a widely used method for the visualization of the brain parenchyma through the intact scull. Using TCS, our group discovered changes of the echotexture – namely increased echogenicity – at the substantia nigra (SN) in about 90% of patients with Parkinson's disease (PD). These results assessed with an interrater reproducibility of $r = 0.8$ in several studies have been confirmed by several other groups. In contrast increased SN echogenicity is rarely found in patients with atypical or symptomatic Parkinsonian syndromes, providing a valuable tool for differential diagnosis.

Interestingly, increased SN echogenicity can also be found in about 8 to 10% of healthy subjects. In PET analyses more than 60% of these clinically healthy individuals show a subclinical reduction of the striatal ^{18}F-Dopa uptake indicating an alteration of the dopaminergic nigrostriatal system and nigral cell loss. Furthermore, it was possible to demonstrate that this ultrasound finding has a functional impact as subjects with an increased echogenicity of the SN (i) showed more frequently clinical symptoms of asymmetric hypokinesia with increasing age and (ii) developed more often and more severe Parkinsonian side effects when treated with neuroleptic therapy for neuropsychiatric disorders. Longitudinal studies indicate that the ultrasound signal does not change in the course of the disease. Moreover, presymptomatic carriers of mutations causative for monogenetic PD display the same echofeature as their relatives already affected by the disease. These findings indicate that increased SN echogenicity constitutes a biomarker for vulnerability of the nigrostriatal system in healthy subjects and eventually PD in a subgroup of persons with additional risk factors.

Introduction

Although more than half of the dopaminerigc neurons at the subtantia nigra (SN) have already degenerated when the first motor syptoms of Parkinson's disease (PD) occur, it is – especially in the beginning of the disease – sometimes difficult to differentiate between idiopathic PD and atypical Parkinsonian syndromes (PS) or idiopathic PD (iPD) and secondary PS.

In recent years, transcranial sonography (TCS) has become a valuable tool in the differential diganosis of Parkinsonian syndromes, especially in the early course of the disease. Moreover, recent data indicates that TCS may even be helpful for the preclinical identification of people at risk for PD.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Application of TCS in the diagnosis of PD

To visualize the brain in standardized scanning plans, a 2.5 MHz phased ultrasound probe is pressed onto the temporal bone window of the subject to be investigated.

For the diagnosis of PD the axial scanning plane through the mesencepahlic brainstem is chosen. In this plane the butterfly shaped brain stem is normally depicted as a hypoechogenic structure accept of a slightly hyperechogenic midline. This is different in PD, where marked areas of hyperechogenicity are visible at the anatomical sites of the SN. SN hyperechogenicity is not quantifieable. Therefore, the area of hyperechogenic signal is encircled and measured (Fig. 1) (Berg et al., 1999).

The finding of first studies, implicating a prevalence of SN hyperechgoenicity in more than 90% of PD patients (Becker et al., 1995; Berg et al., 2001b), has been replicated by many others. Interestingly, although changes of SN echogenicity in the course of the disease could be expected – e.g. a reduction of echogeniciy with a decreasing number of neurons or an increase of echogenicity with increasing amounts of toxic substances, a five year follow up study of PD patients revealed that there were no changes in the extension of SN echogenicity although disease progression was quite severe in some patients (Berg et al., 2005). Moreover, investigations of patients at different disease stages imply that there is no association of area of SN echogenicity and disease severity. Therefore SN hyperechogenicity may rather be a vulnerability marker than a marker for disease progression in PD.

TCS in the differential diagnosis of PS

SN hyperechogenicity is therefore typical for iPD no matter what stage of the disease. What about atypical PS? A study comparing patients with striato nigral degeneration (SND) and progressive supranuclear palsy (PSP) with iPD showed that patients with these aPS have significantly smaller areas of SN echogenicity than patients with iPD (Walter et al., 2003). The same holds true for secondary PSs like vascular Parkinsonism.

Another echofeature, that may be detected in the third ventricle scanning plane might be additionally helpful in the differential diagnosis of atypical PS like SND and PSP (Fig. 1): Normally, the basal ganglia contralateral to the ultrasound probe appear hypoechogenic. In patients with SND or PSP, however, frequently hyperechogenicity at the medial lentiform nucleus (NL) can be detected, a finding typical for dystonia, in which the increased signal intensity was shown to be associated with increased copper content. Taking together the ultrasound features SN hyperechogenicity and normal echogenicity of the NL or normal SN echogenicity and hyperechogenicity of the NL a high positive predictive value of more than 90% can be achieved for iPD or atypical PS like SND or PSP respectively (Behnke et al., 2005). However, similar to iPD, marked SN hyperechogenicity is detected in about 90% of patients with corticobasal degeneration (CBD) and is the main finding differentiating CBD from PSP or SND (Walter et al., 2004a).

Therefore TCS is indeed valuable to differentiate between iPD and CBD on the one hand and PSP and SND on the other hand. The same holds true for the differential diag-

Fig. 1. Corresponding transcranial sonography (TCS) and MRI images of the mesencepahlic (a) and third ventricle (b) scanning plane. The respective upper graphs depict angulation of the transducer for visualization of landmarks in the standardized scanning plane. **a** The normally hypoechogenic mesencepahlic brainstem (left, encircled with dotted line) is characterized by marked areas of hyperechogenicity at the anatomical site of the SN (arrow heads and encircled on one side) in Parkinson's disease. **b** The basal ganglia region next to the ventricular system (encircled) is normally hypoechogenic (left). Atypical Parkinsonian Syndromes are often associated with areas of hyperechogenicity in projection to the lentiform nucleus (right, encircled with dotted lines)

nosis of tremor. In a study published by Niehaus et al., only 10% of patients with essential tremor showed SN hyperechogenicity in comparison to 93% of the tremor dominant PD patients. There was no significant difference between SN echogenicity in patients with tremor and controls, enabling a good prediction for the diagnosis of PD (Niehaus et al., 2004).

The possibility to get very quickly and side effect free a ''glance'' into the brain provides an easy way also for the differential diagnosis of secondary PS. Enlargement of the ventricular system typical for hydrocephalus can easily be depicted by TCS. Also, basal ganglia or frontal tumors that may become clinically apparent by symptoms like bradykinesia resembling PD may be dected as hyperechogenic lesions.

Therefore, it may be concluded that SN hyperechogenicity is indeed valuable for the diagnosis of PD as it occurs in more than 90% of PD patients irrespective of disease stage and is quite specific in the differentiation of iPD versus PSP, SND or sPSs.

SN hyperechogenicity as preclinical marker for nigrostriatal impairment

In all studies on the prevalence of SN hyperechogenicity in PD this ultrasound feature was also detected in a small percentage of healthy subjects. This finding gained crucial interest when in a group of 30 healthy controls one out of two subjects with SN hyperechogenicity developed PD, two years after the initial examination. Did this patient develop PD only by chance or may increased SN echogenicity indeed be regarded as a vulnerability marker for nigrostriatal impairment?

To answer this question the prevalence of SN hyperechogenicity in the healthy population was evaluated. In two consecutive studies comprising more than 400 adult subjects of all age groups SN hyperechogenicity was found in 8–10% irrespective of age (Berg et al., 1999, 2002).

^{18}F-Dopa PET examinations in 20 young healthy persons with SN hyperechogenicity and in 20 young healthy persons without this ultrasound feature were chosen for evaluation of a possible subclinical alteration of dopamine metabolism.

Indeed, in spite of some overlap between the groups a marked reduction in ^{18}F-Dopa uptake was seen in more than 60% of healthy individuals with SN hpyerechogenicity both in the caudate nucleus and putamen of both sides (Berg et al., 1999, 2002).

However, the percentage of healthy individuals displaying increased SN echogenicity definitely exceeds the prevalence of iPD. Interestingly, however, it is quite similar to the prevalence of incidental Lewy body disease – an alteration of the SN supposed to represent a preclinical form of PD.

Therefore, even if not all subjects with SN hyperechogenicity develop iPD a functional relevance might still be possible. To test this hypothesis more than 90 people older than 65 years of age were investigated with TCS and a standardized motor examination according to the Columbia University Rating Scale (CURS), excluding subjects with the diagnosis of iPD prior to the investigation. In accordance with the hypothesis, we found that subjects with areas of SN echogenicity similar to the extension in PD had more often signs of motor retardation according to the CURS, although only two of them presented with the clinical picture of iPD, which had not been diagnosed beforehand (Berg et al., 2001c).

Another possibility to detect a subclinical impairment is further stressing of the nigrostriatal system. In clinical practice this may be achieved by blocking dopminergic transduction by application of neuroleptics that bind to dopamine receptors.

It is known, that some patients develop an extrapyramidal syndrome (EPS) under neuroleptic therapy. However, time point and severity of EPS differ by large among patients receiving neuroleptics. Therefore, it may be

hypothesized that patients with SN hyperechogenicity might be more vulnerable to the development of extrapyramidal features than subjects without this echofeature.

A retrospective analysis of psychiatric patients confirmed this hypothesis. Patients who experienced an EPS under neuroleptic therapy had larger areas of SN echogenicity than subjects without EPS (Berg et al., 2001a). This finding was substantiated by a prospective investigation. Here patients who were about to receive high dosage neuroleptic therapy for the first time were investigated by TCS. Clinical follow up examination under neuroleptic therapy revealed that subjects with larger areas of SN echogenicity developed more often and more severe EPS than subjects with a smaller area of SN echogenicity (Berg et al., 2001a).

Causes of SN hyperechogenicity and predisposition

Postmortem ultrasound examinations compared with biochemical and histological investigations of the SN disclosed that increasing areas of SN echogenicity correspond to an increasing amount of iron measured by spectroscopy and verified histologically (Berg et al., 2002). This association could also be confirmed in vivo by MRI investigations determining T2-relaxation times, which is used for the detection of elevated tissue iron content. Here, patients with PD and increased area of SN echogenicity show decreased T2-relaxation time, corresponding to increased iron levels. Also, healthy subjects with SN hyperechogenicity had a significant decrease of T2-relaxation times when compared to subjects without this echofeature, although values were less reduced compared to PD patients. Taken together a good correlation for TCS, MRI and PET data could be shown, as healthy subjects with increased SN echogenicity had a reduced T2-relaxation time as well as a reduced ^{18}F-Dopa uptake compared to healthy controls without this echofeature, but these changes were less obvious than in patients with idiopathic PD and SN hyperechogenicity (Behnke et al., 2005).

If TCS is suitable to detect preclinical changes of the SN the question whether there are any preclinical alterations in mutation carriers for monogentic PD is at hand. A first study by U. Walter could show that symptomatic Parkin mutations carriers all had either moderate or marked SN hyperechogenicity. This was also true for asymptomatic carriers with abnormal PET findings and even half of the aymptomatic carriers with normal PET findings displayed SN hyperechogenicity, indicating, that TCS might be even more sensitive to identify presymptomatic mutation carriers than PET (Walter et al., 2004).

Examination of mutation carriers of four further genes responsible for mononogenetic PD showed SN hyperechogenicity in all of them, although to a different extent, possibly related to differently weighted pathophyiology.

A predisposition for SN hyperechogenicity could also be shown for family members of patients with iPD in a study comprising 58 first degree relatives of iPD patients with almost half of them showing the same echofeature as their affected relatives. Several of these SN positive subjects showed signs of motor retardation, however, without the clinical picture of full blown PD (Ruprecht-Dörfler et al., 2003).

Relation of SN echogenic size and course of PD

Although an increased area of SN echogenicity is typical for PD, the size of SN echogenicity is not the same comparing affected patients. Knowing that there is a predisposition for this ultrasound feature, that it is associated with a subclinical impairment of the nigrostriatal system and that the signal alteration does not change with progression of the disease, the relation of SN echgogenic size and course of PD needs to be established.

A retrospective analysis of PD patients who underwent sequential PET-examinations disclosed, that patients with larger areas of SN echogenicity have a slower progression of the disorder but an earlier age of disease onset.

According to this finding different subgroups of PD may be proposed: i) those having a stronger predisposition documented by marked SN hyperechogenicity who might represent the more genetically determined PD-courses with earlier onset and slower progression and ii) those with smaller SN echogenic sizes showing later disease onset and faster disease progression that might be mainly caused by other factors like exo- and endotoxins (Schweitzer et al., in press).

Conclusion

According to these studies, TCS is useful

- in the early diagnosis of PD
- in the differential diagnosis versus SND, PSP, tremor, and sPSs
- in the detection of a subclinical impairment
- to evaluate pathophysiologic processes in PD
- to identify subgroups of the disorder.

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How to judge animal models of Parkinson's disease in terms of neuroprotection

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Summary. Ideally, animal models of Parkinson's should reproduce the clinical manifestation of the disease, a loss of some but not all dopaminergic neurons, a loss of some non dopaminergic neurons and alpha-synuclein positive inclusions resembling Lewy bodies. There are at least three ways to develop animal models of PD. The first two are based on the etiology of the disease and consist in 1) reproducing in animals the mutations seen in inherited forms of PD; 2) intoxicating animals with putative environmental toxins causing PD. The last method currently used, which is not exclusive of the first two, is to try to reproduce the molecular or biochemical changes seen post-mortem in the brain of patients with PD. In this review we discuss the advantages and the drawbacks in term of neuroprotection of the currently used models.

What are the requirements for the development of animal models of Parkinson's disease?

Before discussing the advantages and the drawbacks of the various animal models of Parkinson's disease (PD), one has first to set the scene and try to identify the major characteristics of PD that should be reproduced in these models. If one asked patients suffering from PD what they would like to see in an animal model for testing neuroprotective strategies, they would almost certainly say that it should reproduce the clinical manifestations of the disease, thereby enabling putative neuroprotective treatments to be tested to see whether they alleviate the clinical manifestations of the disease and restrict its development. Thus, animal models of the disease should reproduce akinesia, rigidity and tremor. Whereas many models faithfully reproduce akinesia and rigidity, most do not exhibit a true parkinsonian rest tremor with a frequency of 4 to 5 Hz. True parkinsonian rest tremor is only seen in some species of monkey intoxicated by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), baboons and green monkeys being the ones most commonly affected. These symptoms are very likely due to the loss of dopaminergic neurons projecting to the striatum. It is thus essential for animal models to reproduce the lesion of nigrostriatal dopaminergic neurons. Yet, other dopaminergic neurons are also affected in PD albeit to a lesser extent (Hirsch et al., 1988). For instance, about 50% of the dopaminergic neurons degenerate in the ventral tegmental area and catecholaminergic cell group A8. Animal models of the disease should therefore also reproduce this differential vulnerability of dopaminergic neurons. Furthermore, despite the fact that degeneration of non-dopaminergic neurons has been reported in PD

for almost 30 years, considerable attention has recently been focused on non-dopaminergic lesions because they still represent a major limitation of current therapies. Unfortunately, most of the currently available animal models of PD do not reproduce these non-dopaminergic lesions. The third characteristic of PD which should be reproduced in animal models is the progression of the neuronal degeneration, with a presymptomatic phase before a certain threshold of neuronal degeneration has been reached and a symptomatic phase thereafter. Last but not least, animal models should reproduce intra-neuronal inclusions in the form of Lewy body-like structures consisting of a protein accumulation of mainly α-synuclein and ubiquitin. Various groups of investigators have tried to develop animal models with these four characteristics in various species. Some have used very simple species such as flies (*Drosophila*), worms (*C. elegans*) or fish (goldfish, zebra fish). These very simple models have the great advantage of a relatively short life span allowing interactions between different genes to be tested by crossing genetically manipulated animals. Yet, from a behavioral standpoint, they differ dramatically from human beings. That is why most animal models of PD have been developed in mammals and especially in rodents. The mouse is an extremely useful species in which genetic manipulation has been developed. Nevertheless, the behavioral repertoire of MPTP-intoxicated monkeys is much closer to the one seen in patients with PD.

After having discussed the major characteristics of animal models of PD and the species in which they can be developed, the different methodologies to develop these models will be discussed in the next two sections. There are at least three ways to develop animal models of PD. The first two are based on the etiology of the disease and consist in 1) reproducing in animals the mutations seen in inherited forms of PD; 2) intoxicating animals with putative environmental toxins causing PD. The last method currently used, which is not exclusive of the first two, is to try to reproduce the molecular or biochemical changes seen post-mortem in the brain of patients with PD. These changes include increased oxidative stress, mitochondrial dysfunction, defects in protein handling, protein accumulation, gliosis and inflammatory changes. In the next section we will briefly discuss the animal models developed on a genetic basis.

Animal models of Parkinson's disease based on a genetic etiology

Several genes and loci involved in inherited forms of PD have been identified during the past ten years. It is anticipated that more mutations will be identified in the coming years for already identified loci or newly characterized inherited forms of the disease. Animal models based on a mutation in the PARK-1 gene (α-synuclein) have attracted great interest because the protein that is modified in this form of the disease accumulates in Lewy bodies in sporadic cases of PD. Thus, even though PARK-1 mutation is observed in very few families around the world, the mechanism of α-synuclein accumulation in the brain may be of much more general relevance. A model based on α-synuclein over-expression reproducing most of the characteristics of PD has been developed in the fly by Feany and co-workers (Feany et al., 2000). They reported reduced climbing activity in flyes over-expressing the wild-type or mutated form of α-synuclein. This motor impairment was associated with the loss of dopaminergic neurons and the presence of intracellular inclusions immunoreactive for α-synuclein and ubiquitin. This model may thus satisfy all the required characteristics. However, a note of caution should be added because mutations in genes unrelated to PD, such as those involved in an inherited form of Alzheimer's disease, provoke similar behavioral changes. Other groups of investigators

have therefore tried to develop transgenic mice over-expressing the wild-type α-synuclein or mutated forms of α-synuclein. Most of these models show α-synuclein-positive inclusions but no loss of dopaminergic neurons, whatever the age at which the animals are analyzed. Thus, this model may be extremely useful to analyze the early phase of α-synuclein fibrillation and accumulation but will not allow the testing of strategies to prevent the death of dopaminergic neurons. In order to avoid such limitations, Kirik and co-workers used a gene transfer methodology to over-express α-synuclein in rats (Kirik et al., 2002). They showed that, using a strong promoter (prion promoter), α-synuclein over-expression induced both a death of dopaminergic neurons in the substantia nigra and an accumulation of intraneuronal α-synuclein. As far as PARK-2 is concerned, parkin knock-out animals have also been developed by several groups of investigators. Yet, again, no loss of dopaminergic neurons has been identified in these animals. However, Itier and co-workers reported an increased dopamine turn-over in the striatum of the parkin knock-out animals (Itier et al., 2003). Because such changes have also been reported in the early phase of PD in humans, they may well represent an early alteration of dopaminergic neurons. Models based on other identified mutations in genes encoding PINK-1 or DJ-1 have also been developed but preliminary data reported in abstracts presented at several meetings (Society for Neuroscience, Movement Disorders Meeting, International Symposium on Parkinson's Disease and Related Disorders) indicate that no loss of dopaminergic neurons is seen in these animals. For this reason most of the models of PD used so far are based on the use of neurotoxins.

Animal models of Parkinson's disease based on the use of neurotoxins

Most of the models of PD based on the use of neurotoxins try to mimic the effect of environmental toxins or reproduce the biochemical changes seen in the brain of patients post mortem. Yet, these two approaches are not mutually exclusive. In line with this, the vast majority of models are based on the finding that the activity of complex-1 in the mitochondria is decreased in PD. The toxin most commonly used to induce a complex-1 deficiency is MPTP, which induces a parkinsonian syndrome in both humans and animals. Recently, we re-evaluated this model in C57Bl6 mice from both a behavioral and a biochemical standpoint (Rousselet et al., 2003). Using a chronic injection protocol and various cumulative doses of MPTP (60 mg, 420 mg, 540 mg), we showed that MPTP induced a dose-dependent loss of dopaminergic neurons in the substantia nigra. Furthermore, at the highest dosage, MPTP also partially destroyed dopaminergic neurons in the ventral tegmental area, thus reproducing the differential vulnerability of dopaminergic neurons in PD. Dopamine measurements made in the striatum and in the frontal cortex of these animals evidenced a major loss of dopamine in the striatum and a more moderate but significant loss in the prefrontal cortex of about 50%. In terms of behavior, the animals surprisingly displayed a hyperactivity that was dependent on the dose of MPTP injected (the higher the MPTP dose, the higher the locomotor activity). These results are at first sight puzzling since they suggest that the increased locomotor activity was associated with the dopaminergic denervation, in contrast to what is seen in patients with PD. Yet, reports in rat have indicated that dopamine deficiency in the frontal cortex is associated with increased locomotor activity. Furthermore, despite the chronic intoxication, no α-synuclein- or ubiquitin-positive inclusions were seen in these animals. However, Fornai and co-workers recently reported that a continuous infusion of mice with MPTP using an osmotic pump not only induced a degeneration of dopaminergic neurons but also the formation of α-synuclein- and ubiquitin-positive

inclusions (Fornai et al., 2005). Given the behavioral limitations of MPTP-intoxicated mouse model, species more closely related to humans have been used to mimic the symptoms of PD. We recently developed a model of PD in green monkeys with progressive MPTP intoxication at very low doses. After a few MPTP injections, the monkeys were assessed as normal on a monkey parkinsonian rating scale but already displayed subtle behavioral changes in a reach and grasp test (Pessiglione et al., 2003). Indeed, whereas control animals had a fully appropriate trajectory when asked to take some food with or without an obstacle in front of their hand, MPTP-intoxicated monkeys had aberrant initial trajectories which were corrected by visual guidance. This suggests that at the early phase of the disease, some compensatory mechanisms may occur. Then, with further MPTP injections, the animals became symptomatic recovered and after more injections became parkinsonian with a stable disease. Neurochemical analysis performed post-mortem evidenced a minimal degeneration of dopaminergic terminals in the dorsal striatum of the presymptomatic animals and an almost total wipe out of dopaminergic innervation in the severely parkinsonian animals. Furthermore, in another group of monkeys chronically intoxicated with MPTP we found that two years after the last MPTP intoxication a major glial reaction was still detectable in the substantia nigra (Barcia et al., 2004). This glial reaction consisted in an astrogliosis, considered as a scar after the degeneration of dopaminergic neurons. Furthermore, a microgliosis identified by HLA-DR immunoreactivity was also seen. Because this microgliosis is generally considered as an index of an ongoing pathological process these data suggest that neuronal alteration may progress in this model. Taken together, these data indicate that MPTP-intoxicated monkeys constitute an interesting model of PD reproducing neuronal loss, behavioral changes and perhaps the progression of the lesion. However, no Lewy body-like inclusions have yet been observed in this model. It would therefore be tempting to use in monkeys the intoxication protocol developed by Fornai and co-workers in rodents that induced the accumulation of proteins in Lewy body-like structures.

Other complex-1 inhibitors have been used to develop animal models of PD. In particular, Betarbet and co-workers used rotenone in rats to develop such a model (Betarbet et al., 2000). They showed a loss of dopaminergic neurons in the substantia nigra and the presence of α-synuclein-positive inclusions in the remaining neurons of the treated animals. More recently, using an identical protocol, we showed that non-dopaminergic neurons, mostly in the basal ganglia, were also affected by rotenone (Höglinger et al., 2003). The fact that rotenone is a well-known insecticide for plants led to the notion that environmental toxins may be involved in the development of the parkinsonian syndrome. In line with this, an atypical form of PD has been described in the French Caribbean. This syndrome may be due to the consumption of a beverage of an infusion from certain tropical plants or the use of such plants for medicinal purposes. These plants contain acetogenins among which annonacin is a very potent complex-1 inhibitor. On this basis, using a similar protocol to that used for rotenone, we tested the possible deleterious effect of annonacin in rats (Champy et al., 2004). We observed a loss of dopaminergic and non-dopaminergic neurons and behavioral changes reminiscent of the symptoms seen in this atypical form of PD, resembling progressive supranuclear palsy. These data indicate that environmental toxins might be responsible for some atypical forms of PD and that these toxins might therefore be used to develop animal models of the disease.

Other compounds have been used to mimic environmental toxins or biochemical changes seen in the brain of patients with

PD. In particular, altered protein processing and protein accumulation have attracted considerable interest in recent years. For example, a proteasome dysfunction having been reported specifically in the substantia nigra of patients with Parkinson's disease, McNaught and co-workers induced an inhibition of the proteasome in rats and demonstrated a hypokinetic behavior in these animals associated with a loss of dopaminergic neurons (McNaught et al., 2004). This model may be of interest but has not yet been used to test neuroprotective strategies.

Conclusions

Several animal models have been used to test neuroprotective strategies. For instance, we and others have tested the use of anti-inflammatory drugs such as pioglitazone (a peroxisome proliferator-activated receptor gamma agonist) in a mouse model and found an almost complete preservation of dopaminergic neurons in the substantia nigra (Breidert et al., 2002). Several other neuroprotective strategies have been tested in animal models (antioxidant, monoamine oxidase B inhibitors, anti-inflammatory drugs, energy restoration, etc.), yet none of the drugs tested in animals has so far produced as strong a neuroprotective effect in patients with PD as in the treated animals. There are several likely reasons for this lack of predictability of the animal models in terms of neuroprotection. The most important one is probably that the four main characteristics of the disease are hardly ever reproduced simultaneously in existing animal models of the disease. The second reason is that, due to species differences, specific changes may occur in humans but not in animals. On the other hand, PD may have several different causes and should perhaps not be considered as a single disease. Neuroprotective strategies in humans should consequently be tested on subgroups of patients characterized by a specific etiology or a specific type of symptoms. Further work is probably needed to develop better animal models of the disease and to better characterize subgroups of patients.

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Limitations of cellular models in Parkinson's disease research

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Summary. Cell cultures for Parkinson's disease research have the advantage of virtually unlimited access, they allow rapid screening for disease pathogenesis and drug candidates, and they restrict the necessary number of animal experiments. Limitations of cell cultures, include that the survival of neurons is dependent upon the culture conditions; that the cells do not develop their natural neuronal networks. In most cases, neurons are deprived from the physiological afferent and efferent connections. In Parkinson's disease research, mesencephalic slice cultures, primary immature dopaminergic neurons and immortalized cell lines – either in a proliferating state or in a differentiated state – are used. Neuronal cultures may be plated in the presence or absence of glial cells and serum. These different culture conditions as well as the selection of outcome parameters (morphological evaluation, viability assays, biochemical assays, metabolic assays) have a strong influence on the results of the experiments and the conclusions drawn from them. A primary example is the question of whether L-Dopa is toxic to dopaminergic neurons or whether it provides neurotrophic effects: In pure, neuronal-like cultures, L-Dopa provides toxicity, whereas in the presence of glial cells, it provides trophic effects when applied. The multitude of factors that influence the data generated from cell culture experiments indicates that in order to obtain clear-cut and unambiguous results, investigators need to choose their model carefully and are encouraged to verify their main results with different models.

Introduction

Most new experimental therapeutic strategies for Parkinson's disease (PD) are first evaluated in cellular models. The most prominent advantages are (i) easy access of cells in culture to genetic and pharmacological interventions, (ii) easy and repeated access to cells for imaging and various biochemical assays to determine viability, metabolic state, protein aggregates and other parameters, (iii) the possibility to implement high-throughput screening for drug candidates and (iv) they restrict the number of animal experiments that need to be performed. Of course, these advantages come at the cost of several limitations. In this review, we will briefly discuss some cellular models of Parkinson's disease and methods used with them.

Cultures

Immortalized cell lines

Immortalized cell lines display the advantages of cell culture models to the greatest extent. They represent homogenous populations of continuously proliferating cells. This

allows large-scale experiments with reproducible results in a variety of tests. Genetic material can be brought into the cell effectively by chemical and physical means and cells can be frozen at −150°C. This allows to generate banks of cell lines, stably expressing various proteins of interest. Overexpression of a newly identified disease-causing gene or if a new mutation within a known gene is commonly the first step in its characterization since various biochemical and molecular studies can be readily performed. Freezing aliquots of low passage cell stocks is crucial to reproduce results since even cell lines are known change their appearance and behaviour with increasing passages as they accumulate genetic and epigenetic alterations. This effect, known as ''replicative senescence'', can

Table 1. Culture systems of dopaminergic neurons

Name	Origin	Neuronal differentiation	Dopaminergic phenotype[1]	α-Synuclein pathology
SH-SY5Y	human neuroblastoma	retinoic acid ±BDNF	DAT, D2R, DA, TH, vesicles, DA release[2]	eosinophilic cytoplasmic inclusions upon co-overexpression of α-synuclein and synphilin-1[3]
PC12	rat neuroblastoma	NGF	DAT, D2R, DA, TH, vesicles, DA release[4]	synuclein is upregulated following NGF[5]; cytoplasmic inclusions upon proteasome inhibition[6] and methamphetamine[7]
MN9D	mouse hybrid mesencephalic cells	GDNF, retinoic acid, overexpression of Nurr1[8]	DA, TH, D2R. no DAT[9] but sensitivity to low doses of MPP^{+}[10]	no record found
CSM14.1[11]	rat embryonal mesencephalon	39° (temperature-sensitive immortalization)	TH	no record found
MESC2.10[12]	8 week old human mesencephalon	tetracycline, GDNF, dibutyryl cyclic AMP	TH, DAT, DA release, electrically active	express endogenous human WT α-synuclein, inclusions following overexpression of synuclein and administration of amphetamine
primary midbrain cultures	E13 rodent embryo midbrain	without special treatment	5–10% dopaminergic neurons	endogenous synuclein-1[13]; inclusions following proteasome inhibition[14]
organotypic slice cultures	postnatal rat substantia nigra pars compacta	already differentiated	postnatal dopaminergic neurons in their natural environment	no record found

[1]Abbreviations: *DAT* Dopamine transporter, *D2R* D2 dopamine receptor, *DA* dopamine, *TH* tyrosine hydroxylase; [2]Presgraves et al. (2004a, b); [3]Marx et al. (2003); Smith et al. (2005); [4]Pothos et al. (2000); Beaujean et al. (2003); [5]Stefanis et al. (2001); [6]Rideout et al. (2001); [7]Fornai et al. (2004); [8]Transcription factor that is active in dopaminergic neurons; [9]Hermanson et al. (2003); [10]Kim et al. (2001); [11]Haas and Wree (2002); [12]Lotharius et al. (2002); [13]Rat homologue of human alpha-synuclein; [14]McNaught et al. (2002)

make it hard to reproduce results generated with one special batch of cells. Another drawback of proliferating cells is the difficulty to differentiate between growth arrest and real cell death as both ultimately result in a difference in cell number between ''treated'' and ''untreated'' cells. In our hands, SH-SY5Y exposed to 250 μM 1-methyl-4-phenylpyridium (MPP^+) displayed only growth arrest after 48 and actual cell death not before 72 h (Soldner et al., 1999). We therefore encourage to establish the occurrence of cell death in each paradigm using a morphological marker (see below) before drawing conclusions from biochemical data or overall cell numbers.

As researchers in Parkinson's disease pathogenesis we are interested in the survival of mature dopaminergic neurons of the human substantia nigra pars compacta (SNc). Major characteristics of some widely used or upcoming ''dopaminergic'' cell lines are summarized in Table 1, assuming that neuronal differentiation, dopaminergic phenotype and the ability to form aggregates of alpha-synuclein – the histological hallmark of Parkinson's disease – are relevant traits to model PD. It is important to keep in mind, however, that even midbrain dopaminergic neurons are not a homogeneous population. Neurons in the SNc are much more strongly affected in PD patients and more vulnerable to MPP^+ toxicity than neurons in the ventral tegmental area (VTA). Gene expression profiles of these two populations have been performed recently (Chung et al., 2005) and may indicate some further susceptibility traits specific for PD.

All of the immortalized cell lines listed in Table 1 can be differentiated to a more neuron-like phenotype including cellular processes. Neuroblastoma cells can be differentiated by retinoic acid and/or growth factors. CSM14.1 cells have been immortalized by a temperature-sensitive SV40 large T antigen. These cells differentiate at the imperimissive temperature of 39°C. Similarly, MESC2.10 cells have been immortalized with a LINX v-myc retroviral vector under control of a tet-system, allowing differentiation by tetracycline. Neuronal differentiation and in particular the growth of neuronal processes is an important feature to make a better model for neurodegenerative diseases. We and others have shown that neurotoxins such as MPP^+ damage neurites long before they kill neuronal somata and therapeutic strategies may vary greatly in their effect on neuritic integrity and functional parameters on one hand and survival of the cell body on the other hand (Herkenham et al., 1991; von Coelln et al., 2001; Berliocchi et al., 2005). After differentiation, however, cells are generally more delicate and gene transfer is made more difficult, often leaving viral vectors as the only possibility. They are also no longer amenable to FACS sorting (see below) and some other techniques requiring cell suspensions.

Primary immature dopaminergic neurons

Primary mesencephalic cultures are typically prepared from E13 rouse or rat embryos. They contain midbrain dopamine neurons cultured in the context of their physiological neighbours. As other primary neuronal cultures, neurons readily differentiate and form neurites and synapses. Even though these cultures are often referred to as ''primary dopaminergic neurons'', TH positive neurons actually make up only 5 to 10 percent of the total population. This constitutes an often invincible obstacle for viability assays and even more for many biochemical assays because significant changes that may occur in dopaminergic neurons are diluted or cancelled by changes in non-dopaminergic neurons. Moreover, there is more variability between cultures prepared on different days and by different investigators than in cell lines due to variations in preparation and processing of the tissue.

Gene transfer in primary neuronal cultures is more difficult than in cell lines. Calcium

phosphate transfection works in some cases; in most cases viral gene transfer has to be used. In our hands, lentiviruses have been successfully used whereas adenoviruses and adeno-associated viruses infect GABAergic but not dopaminergic neurons in primary midbrain cultures.

Help for the use of primary mesencephalic cultures may come from the growing field of functional and automated fluorescence microscopy (for a recent review see Bunt and Wouters, 2004). These techniques will eventually allow to specifically follow individual cells in culture, determine on a single cell level activation of second messenger cascades or protein aggregation – features that thus far relied on western blots and other ''bulk'' techniques. Once this has been accomplished, the low percentage of dopaminergic neurons in primary midbrain cultures and the often low transfection efficacy in these cultures will not be a problem for productive research on these neurons anymore.

In all cellular models, neurons are evidently deprived of their natural environment, of afferent and efferent connections. As these connections are an integral part of a neuron's life in the brain, this is no trivial change. Even though cultured cell do engage in cellular contacts, these are two dimensional only. One side of the cell is usually occupied by the glass or plastic support and another side by the liquid cell culture medium. Much of the ''natural'' environment is substituted by this culture medium. Consequently, the exact culture conditions largely determine the survival of specific types of cells. For example, primary cultures are almost purely neuronal when plated without serum. With serum, there is glial growth as well, helping the survival of neurons but complicating biochemical and viability assays even further – unless cocultures of neurons and glia are intended to study their reciprocal interactions. Another critical factor in primary cultures is cell density with cells plated at a higher density surviving better (Falkenburger and Schulz, unpublished observation) and being more resistant to serum withdrawal (Collier et al., 2003).

Organotypic cultures

Some of the drawbacks just mentioned may be avoided by culturing not dissociated neurons but brain slices (Sherer et al., 2003b; Jakobsen et al., 2005). Typically, brain slices from postnatal rat pups are used, cultured either on coverslips in ''roller tubes'' as originally described by Gähwiler (1988) or on membranes at the interface between medium and the air of the incubator (Stoppini et al., 1991). Organotypic cultures combine features of cell culture and intact animals: Neurons are more easily accessible for pharmacology, gene transfer and – most importantly – repeated imaging than inside the intact animal. Gene transfer has to rely on viral vectors, however, which are either injected to locally in the slice or added to the culture medium (for a recent review see Teschemacher et al., 2005). By choosing appropriate slicing planes, important neuronal projection (such as the nigrostratal tract) can be preserved in the culture, in addition to the local neuron-glia microenvironment. Moreover, postnatal neurons are more fully differentiated than embryonic tissue used for primary cell cultures. Similar as in primary dopaminergic cultures, ''bulk'' techniques to determine viability and biochemical alterations are generally difficult, measuring dopamine production can be useful in some paradigms. It may be expected that with the advancement of imaging techniques such as two-photon microscopy this culture will gain relevance for some applications.

Methods

Inducing oxidative stress

The use of 6-hydroxydopamine (6-OHDA) and MPP^+ to study cell death of dopaminergic neurons in culture has been reviewed recently by others (Collier et al., 2003; Dauer and Przedborski, 2003). Therefore, we want to focus on two critical aspects of these widely

used paradigms. Both neurotoxins are taken up into dopaminergic neurons through the dopamine transporter (DAT). Inside the cell, they lead to the production of reactive oxygen species, inhibition of oxidative phosphorylation, and cell death. The mode of cell death depends on the concentrations that are used and is controversially discussed. As they very selectively kill dopaminergic neurons, 6-OHDA and MPTP (which is metabolized into MPP^+) are commonly used to generate animal models of PD. Interestingly, the regional distribution of neuronal cell death following systemic MPTP treatment parallels that observed in PD patients which has been correlated to the regional distribution of the DAT (Uhl et al., 1994). Therefore, a large part of the selectivity of MPP^+ and 6-OHDA results from their uptake through the dopamine transporter. As a consequence, inhibitors and modulators of the DAT will give false positive results in a neuroprotection study using MPP^+ or 6-OHDA. In addition, it cannot be inferred from MPP^+ and 6-OHDA data alone whether dopaminergic neurons are just sensitive to these toxins because they are accumulated by the DAT or whether these neurons are also particularly vulnerable to this mechanism of toxicity – that is mitochondrial impairment and oxidative stress. Since oxidative damage and mitochondrial impairment have been observed in the substantia nigra of PD patients (Dauer and Przedborski, 2003), this is nonetheless most likely the case. In addition, it is convenient to selectively challenge dopaminergic neurons in a mixed primary or organotypic culture and appealing to move from the MPP^+ cell culture to the MPTP mouse model.

To overcome this dependence on the dopamine transporter, the non selective mitochondrial complex I inhibitor rotenone has been used by several groups. Rotenone induces oxidative stress in cell lines, slice cultures and animals (Sherer et al., 2003b). Dopaminergic neurons appear to be particularly vulnerable to rotenone toxicity again indicating that oxidative stress may represent an important pathogenic factor in PD.

Inducing protein aggregates

Histologically, PD is characterized by depletion of dopaminergic neurons in the substantia nigra and by cytoplasmic inclusions of aggregated protein, so called Lewy bodies. As mentioned above, selective degeneration of dopaminergic neurons can be mimicked by MPP^+. Additionally, chronic but not acute exposure to MPP^+ and rotenone have been shown to induce Lewy-body like inclusions in animals (Sherer et al., 2003a; Fornai et al., 2005). In cell culture, protein aggregation is commonly induced by proteasome inhibition or by overexpression of mutant genes hat have been linked to PD in patients (see Table 1). The intriguing result that aggregates may also be induced by cytoplasmic dopamine following methamphetamine treatment (Fornai et al., 2004) along with the role of dopamine adducts for protein aggregation indicate that dopaminergic neurons are particularly susceptible to aggregate formation.

Protein aggregates can be assayed and defined in various ways: (i) high-molecular smear on gel electrophoresis from cell lysates, (ii) SDS insoluble pellet or proteinase K resistant fraction of cell lysates, (iii) ''bright fluorescent spots'' following overexpression of GFP fusion proteins, (iv) areas of FRET between two fluorescently labelled proteins, (v) thioflavine-S or thiazin red positive inclusions, (vi) aggregates of fibrils in electron microscopy. Which of these methods is used depends on the experimental design and the technical equipment available at the institution. In general, fluorescent imaging has the advantage that live cells can be repeatedly assayed whereas techniques such as the assay of oligomeres on gel electrophoresis or of fibrils in electron microscopy provide a better picture of what we are actually looking at. To what extent the different methods target overlapping species has unfortunately not been investigated in detail. Interestingly, protein aggregation seems to be a specific process occurring more readily in differentiated than in undifferentiated neurons (Hasegawa et al., 2004), suggesting that undifferentiated and non-neuronal cell lines most likely are less suited models to study protein aggregation.

Assays of cell viability

In most studies concerning the pathogenesis or experimental therapies of PD, cell viability or survival is the main target outcome. Therefore, the method used to assay cell viability must be carefully chosen. In undifferentiated cell lines, surviving cells can be counted using a Neubauer chamber or automated devices. This does, however, not differentiate between effects on cell proliferation and effects on cell viability (see above). Viable cells are characterized by their capacity to exclude and prevent staining by trypan blue (blue staining in light microscopy) and propidium iodine (red fluorescent nuclear staining) and their capacity to contain overexpressed proteins such as GFP and its fusion proteins. Other viability stainings use cellular enzymes to generate fluorescent, membrane-impermeable molecules from membrane-permeable precursors such as fluorescein diacetate or different calcein probes. Biochemical assays measure LDH which is contained in viable and released from dying cells or probe mitochondrial function (MTT and alamar blue assay for example). Generally, biochemical assays are better suited for large-scale and high-throughout analysis be-

cause they can be performed in a mircotiter plate format. Morphological methods have the advantage that they look more directly at what we are interested in: the survival of neurons. Also, they allow differentiation between effects on proliferation and viability as mentioned above (Soldner et al., 1999).

Fluorescence activated cell sorting (FACS) is increasingly used to conveniently measure a variety of parameters. It allows assays of (i) cell number: total cell number, number of fluorescently labelled cells, fraction of double-labeled cells, etc. (ii) cell cycle analysis by quantifying the amount of propidium-iodine-DNA staining per (permeabilized) cell (iii) detection of dying cells by propidium iodine staining of unpermeabilized cells, (iv) detection of apoptosis through the recognition of surface expression of annexin. It is therefore a very powerful technique to assay cell proliferation and cell death, which has been used in primary neuronal cultures (Garcia et al., 2005). However, we and others have not been able to use FACS in differentiated neuronal cultures since neurites tend to rupture and neurons die when they are manually detached from their support.

In differentiated cell lines, primary and organotpyic cultures, most of the morphological and biochemical analysis mentioned above habe been used, in addition to more more specific functional test such as dopamine uptake. In primary mesencephalic cultures, staining or co-staining for tyrosine hydroxylase is commonly performed to assay surviving dopaminergic neurons since assays of total viability are dominated by the 95% non-dopaminergic neurons in these cultures. However, numbers get very low (and variability is high) when one has to compare the viability of TH-positive and successfully transfected cells between two different conditions. Advances in automated microscopy and statistics coming from clinical trials might provide an escape to this. Recently developed systems allow us to automatically and repeatedly find a large number of specific areas within a multiwell plate, take a picture and analyze it offline. It is therefore conceivable that we will in the future not compare the number of surviving neurons in treated and untreated cultures but rather follow a large number of defined cells in culture and correlate their survival with the treatment they have obtained. However, the statistical analysis of these data will also be considerably more challenging (for a recent example, see Arrasate et al., 2004).

Conclusions

In this review, we briefly mentioned some cellular models used in PD research. We discussed some of the methodological limitations associated with them and in some cases indicated possible solutions. The selection of models and limitations was necessarily subjective and incomplete. However, similar arguments may apply to other models as well. We strongly believe that cellular models have an important role for investigating general principles of cell stress and cell death, but also for exploring new treatment strategies. Undifferentiated cell lines can be used for initial and screening experiments. Positive results should be validated in differentiated or primary cultures before moving to animal models. It should be kept in mind, however that some aspects of neuronal degeneration are specific to postmitotic neurons and therefore have to be investigated in primary or organotypic cultures.

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The Rotenone model of Parkinsonism – the five years inspection

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Summary. Treatment of rats with rotenone has been proposed in the year 2000 to provide an animal model of idiopathic Parkinson's disease. We review here the experience that has been gained meanwhile with this model. The published data suggest that the model does not ideally reproduce the pathophysiology of Parkinson's disease, that Rotenone treatment does not cause a purely neurodegenerative concondition, that the Rotenone model does not ideally recapitulate the motor symptoms of Parkinson's disease, that degeneration of the dopaminergic neurons is highly variable, that striatal neurons appear to degenerate more consistently than neurons in the substantia nigra, and that cytoplasmic accumulation of the tau protein is more abundant than alpha-synuclein aggregation in severely lesioned animals. In summary, these data suggest that Rotenone-treated rats model atypical Parkinsonism rather than idiopathic Parkinson's disease.

Introduction

Idiopathic Parkinson's disease (IPD) is a chronic neurodegenerative disorder affecting primarily, though not exclusively, the dopaminergic neurons of the substantia nigra. Reduced activity of complex I of the mitochondrial respiratory chain has been measured in the postmortem substantia nigra of IPD patients. Additionally, exposure to pesticides, many of which are mitochondrial toxins, has been identified as a risk factor for IPD. Based on these observations, Greenamyre and coworkers have developed an experimental model of IPD by exposing rats systemically for a 28 day period to the pesticide Rotenone, a lipophilic complex I inhibitor (Betarbet et al., 2000).

Materials and methods

We review here the experience gained with this model, based on publications listed in the pubmed database, published from 2000 to July 2005.

Results

The model does not ideally reproduce the pathophysiology of IPD

Several authors reported reduced activity of complex I in peripheral tissues of IPD patients, such as platelets, suggesting a generalized metabolic fault affecting the entire body. An equal number of reports, however, were unable to find evidence for such peripheral alterations. Given the controversy regarding this question, it is not justified up to now, scientifically speaking, to claim the existence of a generalized complex I defect in IPD. In 1990, Schapira et al. performed a careful analysis of the complex I activity in the brain of IPD patients. He clearly demonstrated that complex I dysfunction within the brain of IPD patients affects specifically the substantia

nigra, but not other brain areas such as the caudate nucleus, cerebral cortex, cerebellum, or globus pallidus. Kingsbury and coworkers (2001) recently strengthened the concept, that complex I dysfunction within the brains of IPD patients affects specifically the substantia nigra. The observations of Schapira (1990) and Kingsbury (2001) have not been contradicted. In contrast to the pattern described in IPD brains, Rotenone treatment leads to a homogenous reduction of complex I activity throughout the brain (Betarbet et al., 2000). Thus, Rotenone treatment does not reproduce the pattern of complex I inhibition described in IPD. In addition, reduced activity of complex I is not specific to IPD, but has also been described to be implicated in other neurodegenerative conditions (Friedreich ataxia, Steele Richardson Olszewski Syndrome, Atypical Parkinsonism of Guadeloupe) in which neuronal cell loss follows a completely different pattern than in IPD.

Rotenone treatment does not induce a purely neurodegenerative condition

Rotenone treatment does not induce a purely neurodegenerative condition, as it is the case in IPD. Instead, there is significant peripheral toxicity. Mortality rises up to 50% when rats are exposed to Rotenone doses necessary to induce neuronal cell loss (Sherer et al., 2002; Höglinger et al., 2003; Fleming et al., 2004). Liver necrosis and degeneration of the gastrointestinal tract have been documented in Rotenone-treated rats (Lapointe et al., 2004). Peripheral toxicity can be explained by the fact that liver mitochondria are much more sensitive to Rotenone than brain mitochondria (Betarbet et al., 2000). Typically, Rotenone treatment is associated with severe weight loss and muscle wasting, making it difficult to differentiate if motor symptoms arise from neurodegeneration or from muscle wasting (Sherer et al., 2002; Höglinger et al., 2003; Fleming et al., 2004; Lapointe et al., 2004).

The Rotenone model does not recapitulate the motor symptoms of IPD

Reduced locomotion has been uniformly reported in Rotenone-treated rats (Betarbet et al., 2000; Sherer et al., 2002; Höglinger et al., 2003; Fleming et al., 2004; Lapointe et al., 2004). The extent of dopaminergic cell loss in the substantia nigra and of dopaminergic fiber loss in the striatum in Rotenone-treated rats is only moderate. A similar degree of dopamine depletion induced by other toxins such as 6-OHDA is not sufficient to reduce locomotor activity in rats, as documented in numerous publications on this subject. Thus, it is unlikely that hypo-locomotion observed in Rotenone-treated rats results from dopamine depletion, as it is the case in IPD. Consistently, Fleming et al. (2004) found no correlation between the loss of TH-immunoreactivity in the striatum and scores of hypo-locomotion in Rotenone-treated rats. Lapointe et al. (2004) found the motor impairment to be related to peripheral toxicity rather than to neurodegeneration. The IPD-typical symptoms rigidity and tremor are not observed in Rotenone-treated rats (Höglinger et al., 2003; Lapointe et al., 2004). Instead, severe postural instability and dystonia, both untypical for IPD, but frequently observed in atypical forms of Parkinsonism, are frequently seen in Rotenone-treated rats (Lapointe et al., 2004; Höglinger et al., 2005). Postural instability and dystonia appear to be correlated with the severity of intrinsic striatal lesions in Rotenone-treated rats (Lapointe et al., 2004; Höglinger et al., 2005).

Degeneration of the dopaminergic neurons is highly variable

In the Rotenone-model, the degree of degeneration of the nigrostriatal dopaminergic projection neurons is, unfortunately, highly variable and at present unpredictable. In one study, only 20% of rats that survived the Rotenone treatment developed a pronounced loss of dopaminergic fibers in the striatum

(Lapointe et al., 2004). In some studies, the degree of dopaminergic denervation in the striatum did not reach statistical significance due to the high variability (Lapointe et al., 2004; Zhu et al., 2004). Most studies did not provide any quantification of dopaminergic neurodegeneration due to the high variability (Betarbet et al., 2000; Sherer et al., 2003; Fleming et al., 2004). Presently, only four studies have reported cell counts of the dopaminergic neurons in the substantia nigra of Rotenone-treated rats (Höglinger et al., 2003; Lapointe et al., 2004; Zhu et al., 2004; Garcia-Garcia et al., 2005), two of which did not find significant cell loss (Lapointe et al., 2004; Zhu et al., 2004). However, experimental animal models need a high degree of reproducibility, otherwise it is difficult to evaluate the effect of experimental interventions.

Striatal neurons appear to degenerate more consistently than neurons in the substantia nigra

Four independent groups reported a degeneration of intrinsic striatal neurons, such as dopaminoceptive projection neurons and cholinergic interneurons, in Rotenone-treated rats (Ferrante et al., 1997; Höglinger et al., 2003; Lapointe et al., 2004; Zhu et al., 2004). These neurons are lost in some atypical Parkinson syndromes, but not in IPD.

Tau-pathology occurs in the Rotenone-model

Chronic Rotenone-treatment has been reported to result in cytoplasmic aggregation of the presynaptic protein alpha-synuclein, as it occurs in IPD (Betarbet et al., 2000; Höglinger et al., 2003). Recent evidence, however, demonstrates that in rats with severe striatal pathology, cytoplasmic accumulation of the microtubule-associated protein Tau largely exceeds the amount of alpha-synuclein aggregation, as it is the case in some atypical Parkinson syndromes, but not in IPD (Höglinger et al., 2005).

Discussion

Chronic Rotenone infusion is an interesting model to study the consequences of systemic complex I inhibition. The scientific interest in the model is justified given the broad spectrum of natural and synthetic compounds in our environment that affect complex I. Regarding the behavioral deficits, the pattern of neurodegeneration and the protein-pathology induced by Rotenone, the similarities with IPD are limited. We believe that Rotenone-treated rats model atypical Parkinsonism rather than IPD, but further work will be necessary to convincingly demonstrate this.

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Controversies on new animal models of Parkinson's disease Pro and Con: the rotenone model of Parkinson's disease (PD)

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Summary. A general complex I deficit has been hypothesized to contribute to neurodegeneration in Parkinson's disease (PD) and all toxins used to destroy dopaminergic neurons are complex I inhibitors. With MPTP or 6-OHdopamine, this hypothesis can not be tested since these toxins selectively accumulate in the dopaminergic neurons. However with rotenone, which penetrates all cells, the hypothesis can be tested. Thus, the proof of the hypothesis is whether or not rotenone-induced neurodegeneration mimics the degenerative processes underlying PD.

Low doses of rotenone (1.5 or 2.5 mg/kg in oil i.p.) were administered to Sprague Dawley rats on a daily basis. After about 20 days of treatment, signs of parkinsonism occurred and the concentrations of NO and peroxidase products rose in the brain, especially in the striatum. After 60 days of treatment, rotenone had destroyed dopaminergic neurons. Behaviourally, catalepsy was evident, a hunchback posture and reduced locomotion. Other transmitter systems were not, or much less affected. L-DOPA-methylester (10 mg/kg plus decarboxylase inhibition) potently reversed the parkinsonism in rats. Also when infused directly into the dopaminergic neurons, rotenone produced parkinsonism which was antagonized by L-DOPA. Some peripheral symptoms of PD are mimiced by rotenone too, for example a low testosterone concentration in the serum and a loss of dopaminergic amacrine cells in the retina.

These results support the hypothesis of an involvement of complex I in PD and render the rotenone model as a suitable experimental model. The slow onset of degeneration make it suitable also to study neuroprotective strategies. Evidence that rotenone-induced neurodegeneration spreads beyond the dopaminergic system is not contradictory given that, according to the new staging studies, also degeneration in PD is not confined to dopamine neurons.

Introduction

The primary cause for neurodegeneration in idiopathic Parkinson's disease (PD) is still unknown. Furthermore, so far it was not possible to demonstrate unequivocally a neuroprotective effect in clinical studies. Thus, as long as the cause for neurodegeneration is not understood, we do not know whether or not an animal model, mimicking the processes underlying the disease, exists and we do not know how to create one. It could well be that several and different causes contribute to the disease (multiple hit hypothesis) accounting for the differences in symptomatology and progression.

Table 1. Neurotoxins

	6-OH-Dopamine	MPTP	Rotenone
Solubility	in water	in water	in oil
Uptake into neuron	by transporter	by transporter	penetration
Crossing of blood–brain-barrier	no	yes	yes
Alpha-synuclein formation	no	no	yes
Peripheral administration	no	yes in monkeys and some mice strains	yes
Selectivity for dopamine	high	high	less

Modeling degeneration of dopaminergic neurons

6-hydroxy-dopamine (6-OHDA)

Historically, 6-OHDA was the first toxin that quite selectively destroys catecholaminergic neurons due to uptake by the respective transporter. However, neurodegeneration induced by 6-OHDA is acute (within 24 hours) and thus does not mimic the slow neurodegeneration seen in PD (Table 1).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP crosses the blood-brain-barrier and is converted by monoamine-oxidase B to MPP^+ within astrocytes. MPP^+ is taken up by the dopamine (DA) transporter, thereby it is enriched in DA neurons where it inhibits mitochondrial complex I. Today, the MPTP treated monkey is a frequently used animal model for PD (for review see Gerlach and Riederer, 1996). Like with 6-OHDA, also MPTP does not model slow neurodegeneration.

Rotenone

Among the neurotoxins rotenone is of specific importance because this substance might help to discover one of the mechanisms responsible for neurodegeneration in PD. A well based hypothesis posits that a general mitochondrial complex I deficiency underlies the disease and that DA neurons (and the other neurons degenerating in PD) are especially vulnerable for complex I inhibition. Probably auto-oxidation of DA, which results in the formation of reactive oxygen species, contributes to the specific vulnerability of DA neurons. Indeed a general complex I deficiency (about 25%) in all tissues including blood platelets has been reported for PD and all toxins that destroy DA neurons inhibit complex I. But 6-OHDA and MPTP could not test this hypothesis because they are taken up by the DA transporter and accumulate selectively in the DA neurons. However rotenone which is lipophilic, enters every body cell and does not accumulate selectively in DA neurons.

It has been well known for a long time that rotenone is toxic for DA neurons however the in vivo specificity has not been investigated in depth. Betarbet et al. (2000) infused 2–3 mg/kg i.v. rotenone during 28–36 days in rats using minipumps. They reported a quite selective substantia nigra degeneration. Additionally they found α-synuclein immuno-reactivity in the affected neurons.

In order to develop a technically less demanding rat model Alam and Schmidt (2002) investigated the effects of rotenone (1.5 and 2.5 mg/kg), given systemically i.p. in oil on a daily basis. After about 20 days of treatment, signs of parkinsonism occur (catalepsy in the rat) and the concentration of nitric oxide (NO) as well as of peroxidation products rose in the brain, especially in the striatum (Bashkatova et al., 2004). After

daily administration over 60 days rotenone destroyed DA neurons, DA concentrations in the striatum and prefrontal cortex were reduced, as well as tyrosine hydroxylase concentration in the striatum. Behaviourally, a full blown catalepsy was evident, together with hunchback posture and reduced locomotion (Alam and Schmidt, 2002). Concentrations of other transmitters assessed so far, such as serotonin and its metabolites are not, or much less, changed by rotenone (Alam and Schmidt, 2004a).

A strong criterion that a rotenone model of PD must fulfill to be accepted as predictive, is that the induced effects are antagonized by the clinically effective antiparkinsonian drug L-DOPA. This has been tested and it was found that L-DOPA methylester (10 mg/kg i.p. plus peripheral decarboxylase inhibitor benserazide) potently reversed the parkinsonian signs in rotenone pretreated rats (Alam et al., 2004). When infused stereotaxically into the medial forebrain bundle of rats, rotenone destroys dopaminergic neurons and produces PD symptoms. Rotenone is about 2–3 times as potent as 6-OHDA in this procedure. The so induced parkinsonism is consistently counteracted by L-DOPA (Alam and Schmidt, 2004a).

These data clearly support the above mentioned hypothesis stating that a weak mitochondrial complex I deficiency may underlie PD which does not kill most cells of the body except the DA neurons and those which degenerate in PD. However there are still arguments not supporting this view: Sherer et al. (2003) conclude from their findings, that not the complex I inhibition and subsequent ATP loss is responsible for the neuronal damage, but that an additional unknown mechanism, causing oxidative stress, accounts for the toxicity. As this is also the case for MPTP, this additional mechanism should be unraveled. In conclusion, the rotenone data support the hypothesis that a general complex I deficiency my represent one of the factors underlying PD. This however does not substantiate the hypothesis unequivocally. Thus, does rotenone represent a valid model of PD? There is much evidence in favor of this view but some arguments are also against it. Höglinger et al. (2003) replicated the experiments of Betarbet et al. (2000) and found neurodegeneration beyond the dopaminergic system which they consider to be not typical for idiopathic PD. Their conclusion is that the destruction caused by rotenone does not fully mimic early idiopathic PD which is confined to the DA system, but rather atypical PD with additionally non-dopaminergic lesions (Hirsch et al., 2003). Thus at least for PD with associated non-dopaminergic lesions, rotenone-induced parkinsonism represents a very promising animal model.

PD patients also suffer from disturbances in the peripheral autonomic nervous system. In line with this are the findings of Braak and Del Tredici (2005) showing that first signs of PD occur in the dorsal nucleus of vagus nerve. Most patients with PD, and all people suffering from PD-associated orthostatic hypotension, have a loss of cardiac sympathetic innervation, probably due to overproduction of α-synuclein. It remains to be established, but there are some indications that rotenone, due to its general complex I inhibition, can mimic these peripheral symptoms of PD too. The low testosterone concentrations which are typical for PD, are mimicked by rotenone in the rat (Alam and Schmidt, 2004b) as well as the DA-cell loss in the retina.

Assets and drawbacks of the model

Rotenone can be administered peripherally and locally in the rat. It thus represents an easy model to induce parkinsonism. The degeneration is slow (about 60 days) which makes the model suited to study neuroprotective agents. Rotenone-induced parkinsonism is the only model in which α-synuclein is formed (Table 1). The model mimics general

complex I deficiency which is considered to play a role in PD. The drawback of the model could be the extent of the non dopaminergic damage, this is a matter of current discussion: According to the viewpoint of Hirsch et al. (2003) rotenone does not model early idiopathic PD that is believed to be confied to the DA system. However Braak and Del Tredici (2005) showed neurodegeneration in PD well beyond the DA system.

There are rat-strain differences in the sensitivity to rotenone: In the experiments by Alam and Schmidt (2002, 2004a) Sprague Dawley rats have been used, studies of others have been done with Lewis rats. In our hands Long Evans rats were much more resistant to peripherally administered rotenone. The mechanisms underlying these differences are not clear so far. In comparison to 6-OHDA or MPTP the lesions induced by rotenone are more variable, the extent of the lesion varies considerably. Further, rotenone does not induce parkinsonism in all animals, about 2–3% of Sprague Dawley rats are not susceptible to rotenone after 60 days of treatment. Also in the rats that respond to rotenone, the time span during which the individual rats develop the symptoms, varies considerably. This variability raises some problems for the laboratory researcher but basically, it shares similarities with the situation in human PD.

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Proposed animal model of severe Parkinson's disease: neonatal 6-hydroxydopamine lesion of dopaminergic innervation of striatum

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Summary. Rats lesioned shortly after birth with 6-hydroxydopamine are posed as a near-ideal model of severe Parkinson's disease, because of the non-lethality of the procedure, near-total destruction of nigrostriatal dopaminergic fibers, near-total dopamine (DA)-denervation of striatum, reproducibility of effect, and relative absence of overt behavioral effects – there is no aphasia, no adipsia, and no change in motor activity. In vivo microdialysis findings reinforce the utility of the animal model, clearly demonstrating L-DOPA beneficial actions without an increase in hydroxyl radical production.

Introduction

An adulthood lesion of either *pars compacta* substantia nigra (pcSN) or nigrostriatal dopaminergic tracts produces a reasonable animal model of Parkinson's disease (PD), but the animal is beset with major behavioral deficits that weaken and threaten survival: aphagia, adipsia, immobility, and lack of grooming. Also, there is often large variation in effect resulting from small differences in positioning of a needle tip or cannula tip (or electrode) in pcSN or along the nigrostriatal tracts (Zigmond and Keefe, 1998).

Breese and colleagues, in an extensive series of studies, found that a 6-hydroxydopamine (6-OHDA) lesion of neonatal rats produced near-total destruction of the nigrostriatal dopaminergic tract (Breese and Breese, 1998). Moreover, gross behavior of the rats was little changed from control – mobility and grooming were unaltered; and there was an absence of aphasia and adipsia. Through a series of large studies in my laboratory – involving more that a thousand rats – we recognize that this preparation is a near-ideal animal model of severe PD. In particular, there is 99% destruction of dopamine (DA) innervation of striatum, the extent of DA neuronal damage is reliable and consistent (∼1% variation of effect) and 99% or the rats survive the procedure. Ongoing *in vivo* microdialysis studies reinforce the view of this preparation as an excellent model of severe PD.

Methods

At 3 days after birth rat pups are pretreated with desipramine HCl (20 mg/kg IP), 1 h before bilateral ICV administration of 6-hydroxydopamine (6-OHDA; 67 µg, base, on each side) or saline-ascorbic acid (0.1%) vehicle. To administer the latter substances, rat pups are individually immersed in ice (∼60 sec) to produce cold-anesthesia. Pups are then placed on a flat surface under a bright light. In this manner, the sagittal and

transverse sinuses overlying the cranium, as well as bregma and lambda, can be seen through the transparent intact dermis. A 26-gauge needle, attached to a microliter syringe, is positioned 1.5 mm anterior to lambda and 2 mm lateral to the sagittal plane. The needle, equipped with a polyethylene sleeve up to 2 mm from the tip, is then lowered to the stop position (i.e., sleeve), with the needle in the lateral ventricle. After injection of 5 µl of 6-OHDA or vehicle, the needle is left in place for at least 30 sec. Immediately afterward, an injection is made in the same manner into the other lateral ventricle. Pups are warmed to promote recovery, and each pup is then returned to the litter. In adulthood, litters treated with 6-OHDA are generally ~5% smaller than controls – although this can be minimized if desired by exchanging dams (Brus et al., 1994). When these rats are used as a model of PD, there are no additional treatments during development or before the time of the PD study.

Results

Neurochemical and neuroanatomical effects on striatal DA innervation

In rats treated at 3 days after birth with 6-OHDA, there is an marked reduction in adulthood levels of striatal DA (~99% ± 1%), DOPAC (~99%) and HVA (~99%). Anatomically, there is ~99% reduction in the number of tyrosine hydroxylase (TH) immunopositive fibers innervating striatum. This serves as a near-ideal neurochemical and neuroanatomical template of severe PD (Kostrzewa et al., 1998).

General behavior of 6-OHDA-lesioned rats

Rats lesioned with 6-OHDA at 3 days after birth are behaviorally indistinguishable from control. Eating, drinking, grooming, and motor activity levels are equivalent to that of control rats – except that these rats are ~5% smaller. The latter alteration can be obviated by exchanging dams before the time of weaning (Brus et al., 1994).

DA-Agonist-induced behaviors of 6-OHDA-lesioned rats

When challenged with the first or second dose (1 wk interval) of the DA D_1 agonist SKF 38393, locomotor and stereotyped behaviors were identical in control and lesioned rats. However, the third and subsequent dose of SKF 38393 produced a many-fold increase in locomotor and stereotyped activities in 6-OHDA-lesioned rats (Kostrzewa, 1995). The gradual induction of D_1 receptor supersensitivity is characterized as a *priming* phenomenon (Breese and Breese, 1998).

When challenged with the first or repeated doses (1 wk interval) of the DA D_2 agonist quinpirole, locomotor and stereotyped behaviors were identical in control and lesioned rats. However, the initial two doses of quinpirole produced heterotypic *priming* of D_1 receptors. Accordingly, if rats received two challenge treatments with quinpirole, the first dose of the D_1 agonist SKF 38393 produced a many-fold increase in locomotor and stereotyped activities (Breese and Breese, 1998).

Striatal in vivo microdialysate alterations in 6-OHDA-lesioned rats

When challenged with the first dose of 3,4-L-dihydroxyphenylalanine (L-DOPA), there was a many-fold increase in locomotor and stereotyped activities in 6-OHDA-lesioned rats (Breese and Breese, 1998). When these rats were studied by *in vivo* microdialysis, L-DOPA acutely produced a marked increase in striatal extraneuronal (*i.e.*, microdialysate) levels of both DA and DOPAC in the 6-OHDA-lesioned rats. In fact, the overall L-DOPA-induced increase in striatal extraneuronal DA and DOPAC was much greater in lesioned vs control rats (Kostrzewa et al., 2005).

In an ongoing series of studies we have further found that acute L-DOPA treatment did NOT increase the levels of hydroxyl radical ($HO^{\bullet}$) in either striatal tissue or in striatal *in vivo* microdialysates (Kostrzewa et al., 2000). The implication of these findings is that L-DOPA is not likely to accelerate DA neuronal damage and therefore, L-DOPA

is not likely to accelerate the progression of PD.

Neurochemical and neuroanatomical effects on striatal 5-HT innervation

In rats treated at 3 days after birth with 6-OHDA, there is 5-HT (serotonin) fiber hyperinnervation of striatum, observed histochemically as an increase in the number of tryptophan hydroxylase-immunopositive fibers. Accompanying this neuroanatomical change is a 50–100% increase in the striatal content of both 5-HT and its major metabolite 5-HIAA (5-hydroxyindoleacetic acid). These rats are behaviorally supersensitive to 5-HT_2 agonists (Brus et al., 1994; Kostrzewa et al., 1998). It is possible to prevent 5-HT fiber proliferation by adding a small dose of the 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), to the 6-OHDA solution at 3 days post-birth.

Discussion

Rats lesioned with high-dose 6-OHDA at 3 days after birth represent a good model of severe PD, because the neuroanatomical and neurochemical indices in this model are representative of dopaminergic alterations in a human with severe PD. The rat model can be easily utilized, because 6-OHDA does not produce lethality. Also, the approx. 99% DA-denervation of striatum is reliable ($\pm 1\%$) and consistent. Survivability of the rats is not compromised, because eating, drinking, grooming, and locomotor activities are virtually unaltered – rats in adulthood are indistinguishable from controls. Moreover, the *in vivo* microdialysate studies with L-DOPA confirm that the L-DOPA-induced extraneuronal levels of DA and DOPAC are virtually identical to effects observed in rats that are DA-lesioned in adulthood.

Conclusion

Rats lesioned neontally with 6-OHDA represent a near-ideal model of severe PD because of the reproducibility of the extent of DA fiber denervation, and absence of variability in effect and survivability of animals.

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α-Synuclein overexpression model

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Summary. *Objectives.* To elucidate the role of α-synuclein in the pathogenesis of Parkinson's disease (PD), both human α-synuclein transgenic mice and targeted overexpression of human α-synuclein in rat substantia nigra (SN) by viral vector-based methods have been studied, however little is known about the pathogenetic changes of dopaminergic neuron loss. Therefore, it is necessary to address whether the pathogenetic changes in the brains of patients with PD are recapitulated in these models.

Methods and results. We used the recombinant adeno-associated viral (rAAV) vector system for human α-synuclein gene transfer to rat SN and observed approximately 50% loss of dopaminergic neurons in SN at 13 weeks after infection. In the slower progression of neurodegeneration, we identified several important features in common with the pathogenesis of PD, such as phosphorylation of α-synuclein at Ser129 and activation of caspase-9. Both findings were also evident in cortical tissues overexpressing α-synuclein via rAAV.

Conclusions. Our results indicate that overexpression of α-synuclein via rAAV apparently recapitulates several important features of brains with PD and dementia with Lewy bodies (DLB), and thus α-synucleinopathy described here is likely to be an ideal model for the study of the pathogenesis of PD and DLB. This model is also useful for the gene therapy research.

Introduction

Parkinson's disease (PD) is characterized by degeneration of dopaminergic neurons in substantia nigra (SN), which causes movement disorders such as rigidity, tremor, bradykinesia and postural instability. However, the exact mechanism(s) of dopaminergic neuron degeneration is not clear at present. Considerably many factors, such as genetic factors, aging, inflammation and chemicals have been implicated in the pathogenesis of PD and many studies of genes linked to familial forms of the disease have provided insights into the pathogenesis.

However, the physiological functions of α-synuclein protein have yet to be fully defined. The protein α-synuclein is a major component of the Lewy body (LB), the pathological hallmark of PD, and is therefore likely to play an important role in the pathogenesis of PD. Indeed, overexpression of α-synuclein in cultured neuronal cells or dopaminergic neurons in rat SN induces neuronal cell death. Moreover, missense mutations in α-synuclein gene, which cause PD in an autosomal dominant manner, accelerate α-synuclein toxicity.

To confirm the function of α-synuclein in vivo, we create the hemiparkinsonian model by the direct injection of rAAV-α-synuclein in the dopaminergic neurons of the substantia nigra in rat (Kirik, 2002; Yamada, 2003).

Methods

Construction of plasmids for rAAV and preparation of rAAV

The human α-synuclein gene was cloned into plasmid pAAV-MCS (Stratagene, La Jolla, CA, [pAAV α-synuclein]). Using the protocol provided by the manufacturer, rAAV-α-synuclein and rAAV-enhanced green fluorescent protein (EGFP) were prepared using pAAV-α-synuclein and pAAV-IRES-EGFP (Stratagene) respectively, and the final titres were 4.2×10^{11} genomes/ml (rAAV-α-synuclein) and 5.6×10^{11} genomes/ml (rAAV-EGFP). The number of rAAV genome copies was semi-quantified by PCR within the cytomegalovirus (CMV) promoter region using primers 5′-GACGT CAATAATGACGTATG-3′ and 5′-GGTAATAGCGAT GACTAATACG-3′.

Injection of rAAV vector

Rats (10-week-old) were anesthetized with pentobarbital (40 mg/kg body weight, intraperitoneally) and each viral vector suspension was injected into the anterior end of the left SN (3 μl, 5.3 mm posterior and 2.4 mm lateral from bregma, 7.4 mm below the dural surface, tooth bar = 4.0 mm) or left lateral cortex (Cx, 2 μl, 1.0 mm anterior and 5.0 mm lateral from bregma, 3.0 mm below the dural surface, tooth bar = 4.0 mm). The injection rate was 1 μl/min.

Immunofluorescence staining

Double immunofluorescence staining was performed by simultaneous incubation of sections with two primary antibodies (4°C for 14 hours), followed by two secondary antibodies (room temperature for 1 hour). The primary antibodies used here were anti-tyrosine hydroxylase (TH, rabbit IgG, 1:1000, Calbiochem), anti-green fluorescent protein (GFP, mouse IgG, 1:100, Sigma), anti-human α-synuclein (clone LB509, 1:500), anti-cleaved caspase-9 (1:500), anti-α-synuclein phosphorylated at Ser129 (clone Pser129, rabbit IgG, 1:500, Fujiwara et al., 2002) and anti-histone H1 (mouse IgG, 1:500, Santa Cruz Biotechnology).

Results

In the present study, expression of the gene of interest via rAAV was driven by the CMV promoter, whereas other studies used the chicken beta-actin (CBA) promoter, which is more than 20-fold more effective. Therefore, the expression level of EGFP and α-synuclein via rAAV here was expected to be considerably lower than that reported in earlier studies. To evaluate the duration required to reach the peak level of EGFP expression, the number of EGFP-expressing ($EGFP^+$) cells was counted at 2, 8 and 13 weeks after rAAV-EGFP injection. The number of $EGFP^+$ cells increased gradually during 13 weeks.

We next counted the number of dopaminergic neurons in SN at 13 weeks after rAAV-EGFP or rAAV-α-synuclein infection. Using the level of SN most effectively infected with rAAV, we observed approximately 50% decrease in the number of dopaminergic neurons in SN injected with rAAV-α-synuclein (α-synuclein group), while only a small decrease was observed in SN injected with rAAV-EGFP. The efficacy of infection and interest gene expression in both groups was likely to be the same because the expression level of EGFP and α-synuclein were similar. Furthermore, $EGFP^+$ or α-synuclein-expressing cells were mostly identified as dopaminergic neurons.

In brains with PD, α-synuclein phosphorylated on the serine residue at position 129 (phospho-α-synuclein) accumulates as a component of insoluble aggregations such as LB in dopaminergic neurons of SN. To confirm the presence of phospho-α-synuclein in SN affected with α-synucleinopathy in the present study, we performed immunofluorescence staining of phospho-α-synuclein using a Pser129 antibody (kindly gift from Dr. Iwatsubo). The specificity of the antibody for α-synuclein phosphorylated on serine 129 was already confirmed. At 13 weeks after rAAV-α-synuclein infection, phospho-α-synuclein was detected throughout the neurons, including in the cytoplasm, nuclei and axons. In several neurons, phospho-α-synuclein accumulated in the nucleus. However, in contralateral SN, images immunoreactive for phospho-α-synuclein were never detected. A more careful observation revealed inclusions or aggregations of phospho-α-synuclein. Phospho-α-synuclein did not always form

aggregates. In contrast to images acquired by LB509, Pser129 did not stain primarily the cell membrane. In this regard, the epitopes for LB509 and Pser129 do not overlap but are adjacent to each other, therefore both antibodies can be used simultaneously for detecting the same protein although they can partially disturb each other.

To further elucidate the mechanisms underlying α-synucleinopathy, activation of caspase-9 was investigated immunohistochemically. First, we examined the specificity of the antibody used here for rat activated (cleaved) caspase-9. Caspase-9 is activated in neuronal cells treated with staurosporin, which was specifically identified by the antibody used in the present study. At 13 weeks after rAAV-α-synuclein infection, when approximately half of the dopaminergic neurons were lost, caspase-9 was highly activated in the remaining α-synuclein$^+$ neurons, and activated caspase-9 was more evident in a larger proportion of α-synuclein-accumulated neurons. In contrast, activated caspase-9 was not detected in EGFP$^+$ neurons. This finding indicated that rAAV infection *per se* did not cause caspase-9 activation.

Discussion

Advantages of AAV vector

We previously confirmed significantly *in vivo* higher expression of GFP in the striatum by using an AAV vector without any inflammatory reaction (Mochizuki et al., 2001) and also detected its expression in the neurons of the SN. Such positive staining in the SN was clearly distinct from that at the injection site and was consistent with the known striatal target structures, suggesting that AAV injection resulted in retrograde transport of EGFP. We have previously shown that employment of a strong promoter allows effective and long-term expression of transferred genes using this system. Other advantage of AAV vectors includes a broad host cell range, integration of the gene into the host genome, and lack of pathogenicity. For *in vivo* studies, we believe that AAV virus vectors are the most useful tools for gene therapy.

Table 1. Advantages of virus mediated overexpression model

1. Decrease a time to create model
2. A high level expression of interest gene
3. Multiple genes introduction
4. Hemiparkinsonian model
5. The specific brain regions as you like
6. Primate model
7. Low cost

Advantages of α-synuclein overexpression model

To create such α-synuclein overexpression model, AAV vector or lentivirus vector (Lo Bianco, 2002; 2004) were used. There are several advantages of α-synuclein overexpression model as the genetic modified Parkisnon's diseased model (Table 1). Kirik et al. already created the genetic modified primate model for Parkinson's disease by AAV vector methods. This primate model is also useful tool as the preclinical study (Kirik, 2003). Furthermore, the slow progressive α-synucleinopathy induced in the present study reproduced the SN changes seen in PD with regard to several important events that have not been fully confirmed in other models. A model in which the pathogenetic conditions of α-synucleinopathy most closely resemble those of PD is required to enhance our understanding of the mechanisms of PD and also help in the design of gene therapy research for PD (Lo Bianco, 2004; Yamada, 2005).

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Kynurenines, Parkinson's disease and other neurodegenerative disorders: preclinical and clinical studies

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Summary. The kynurenine pathway is the main pathway of tryptophan metabolism. L-kynurenine is a central compound of this pathway since it can change to the neuroprotective agent kynurenic acid or to the neurotoxic agent quinolinic acid. The break-up of these endogenous compounds' balance can be observable in many disorders. It can be occur in neurodegenerative disorders, such as Parkinson's disease, Huntington's and Alzheimer's disease, in stroke, in epilepsy, in multiple sclerosis, in amyotrophic lateral sclerosis, and in mental failures, such as schizophrenia and depression. The increase of QUIN concentration or decrease of KYNA concentration could enhance the symptoms of several diseases. According to numerous studies, lowered KYNA level was found in patients with Parkinson's disease. It can be also noticeable that KYNA-treatment prevents against the QUIN-induced lesion of rat striatum in animal experiments. Administrating of KYNA can be appear a promising therapeutic approach, but its use is limited because of its poorly transport across the blood–brain barrier. The solution may be the development of KYNA analogues (e.g. glucoseamine-kynurenic acid) which can pass across this barrier and disengaging in the brain, then KYNA can exert its neuroprotective effects binding at the excitatory glutamate receptors, in particular the NMDA receptors. Furthermore, it seems hopeful to use kynurenine derivatives (e.g. 4-chloro-kynurenine) or enzyme inhibitors (e.g. Ro-61-8048) to ensure an increased kynurenic acid concentration in the central nervous system.

Introduction

Kynurenine (KYN) is an intermediate in the pathway of the tryptophan (TRP) metabolism. This pathway is known to be responsible for nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Fig. 1). KYN is formed in the mammalian brain (40%) and is taken up from the periphery (60%). The rate of cerebral KYN synthesis was $0.29\,\mathrm{nmol/g/h}$ (Gal and Sherman, 1978). In the brain, KYN can be converted to other components of the pathway: the neurotoxic 3-hydroxy-kynurenine (3-HK) or quinolinic acid (QUIN) and the neuroprotective kynurenic acid (KYNA).

Some 25 years ago it was found that intermediates of the kynurenine pathway (KP) have neuroactive properties: convulsions were appeared after mice received QUIN (Lapin, 1978). QUIN is a selective ligand of N-methyl-D-aspartate (NMDA) receptor (Stone and

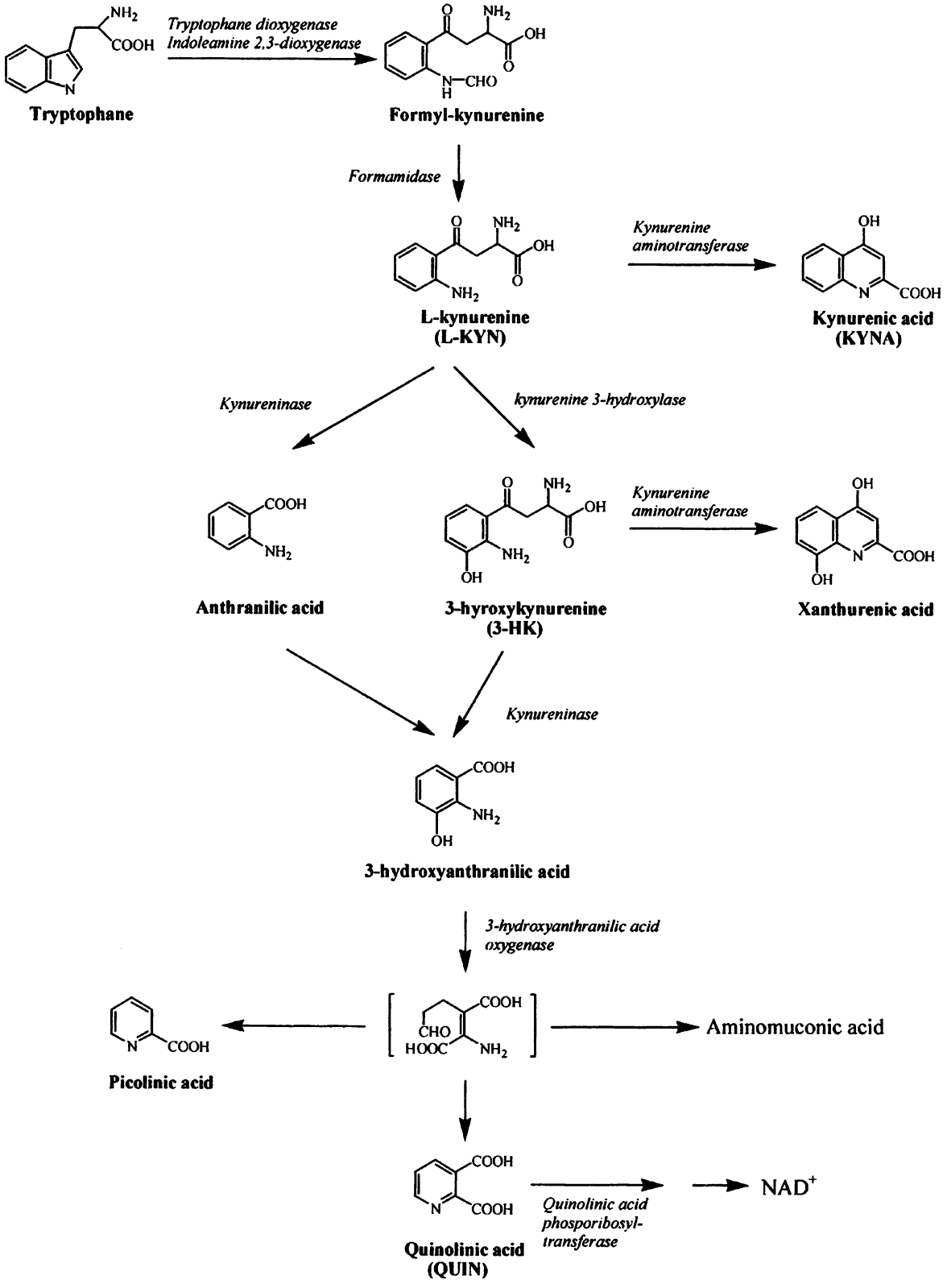


Fig. 1. The kynurenine pathway

Perkins, 1981), which can therefore cause neuronal damage (Schwarcz and Kohler, 1983; Schwarcz et al., 1984). It has similar neurotoxic effects to those of glutamate in the neocortex, striatum and hippocampus (Perkins and Stone, 1983).

It was hypothesized already in 1983 that QUIN plays an important role in Huntington's disease (Schwarcz et al., 1983). Three years later, it was proved that the injection of QUIN into the rat striatum duplicated the neurochemical features of this disorder (Beal et al., 1986).

Intrastriatal injection of QUIN induces a substantial neuronal loss, which is potentiated by the administration of 3-HK (Guidetti and Schwarcz, 1999). It produces early changes in the activity of the striatal neurons and movements of several cations, which may contribute to subsequent abnormalities in energy metabolism and to cell death (Bordelon et al., 1998).

The other important metabolite of KP is kynurenic acid (KYNA) which is one of the few known endogenous broad-spectrum antagonist of excitatory amino acid (EAA) receptors (Swartz et al., 1990), especially the NMDA receptors. KYNA behaves as a neuroprotective agent: it can inhibit the overexcitation of these receptors by binding the glycine allosteric site. Pharmacological studies have proved that KYNA is derived primarily from kynurenine aminotransferase II (KAT II) activity in most brain regions (Guidetti et al., 1997), which is located primarily in the glia (Du et al., 1992). Moreover, KYNA non-competitively blocks the activity of presynaptic α7-nicotinic acethylcholine

Table 1. Observations with kynurenine and related compounds in animal experiments

1947	The kynurenine pathway was recognized as a major route of tryptophan metabolism	(Beadle et al., 1947)
1978	Convulsions were appeared after quinolinic acid injection into the brain of mice	(Lapin, 1978)
	40% of KYN is formed in the brain and 60% is taken up from the periphery	(Gal and Sherman, 1978)
1981	Quinolinic acid is a selective ligand of N-methyl-D-aspartate (NMDA) receptor	(Stone and Perkins, 1981)
1982	Kynurenic acid may be a neuroprotective agent	(Perkins and Stone, 1982)
1984	Seizures activity and lesions were found after intrahippocampal quinolinic acid injection	(Schwarcz et al., 1984)
1987	Blockade of the excitatory amino acid receptors protects anoxic hippocampal slices	(Clark and Rothman, 1987)
1988	7-Chloro-kynurenic acid is a selective ligand of the glycine site of NMDA receptors	(Kemp et al., 1988)
	The concentration of the kynurenic acid is 10–150 nM in the mammalian brain	(Moroni et al., 1988)
1990	Kynurenine produces slight, but kynurenic acid marked behavioural changes in rats	(Vecsei and Beal, 1990b)
1991	Kynurenine can cross easily via the blood–brain barrier by neutral amino acid carriers	(Fukui et al., 1991)
	Quinolinic acid produces toxic free radicals	(Rios and Santamaria, 1991)
1992	Kynurenine-aminotransferase (KAT) II is located primarily in the glia	(Du et al., 1992)
1997	Kynurenic acid derives from KAT II activity in most brain regions	(Guidetti et al., 1997)
2001	Kynurenic acid non-competitively block the activity of α-nACh receptors	(Hilmas et al., 2001)

(nACh) receptors and can increase the expression of non-α7-nACh receptors (Hilmas et al., 2001). Cross-talk between KYNA and the nicotinic cholinergic system has been presumed to play a role in the pathogenesis of numerous brain disorders, including Alzheimer's disease and schizophrenia, in which brain KYNA levels are elevated and nicotinic functions are impaired (Alkondon et al., 2004).

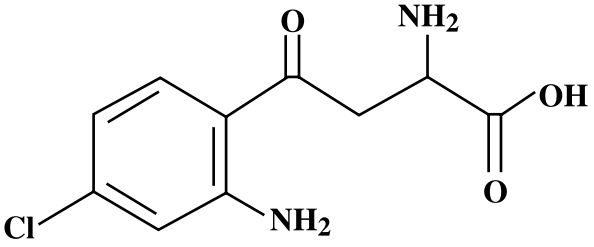
Earlier observations are summarized in Table 1 concerning the kynurenine and its derivatives.

Changes in the absolute or relative concentration of KYNA or QUIN in the brain have been implicated in numerous neurological and psychiatric disorders, e.g. Parkinson's disease, Huntington's and Alzheimer's diseases, stroke, epilepsy, multiple sclerosis, depression and schizophrenia. Elevated QUIN level or decreased KYNA concentration causes impairment in the cellular energy metabolism by overexciting the glutamate receptors, in particular the NMDA receptors. Glutamate-mediated excitotoxic damage decreases the voltage-dependent Mg^{2+} blockade, causes abnormalities in cellular Ca^{2+} homeostasis and elevated production of reactive oxygen species (Greene and Greenamyre, 1996).

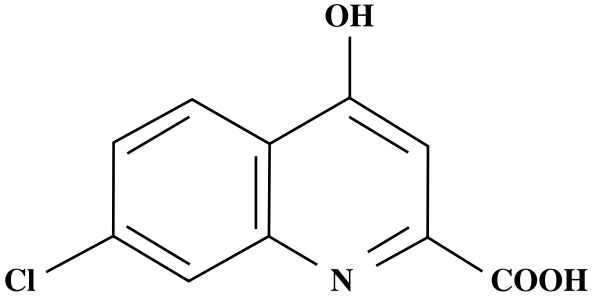
It is extremely important that the suitable prevention or correction of the KP's abnormality should be used which could attenuate the pathological processes.

There are at least two ways in which therapeutic agents are being developed with the aim of modulation of the KP.

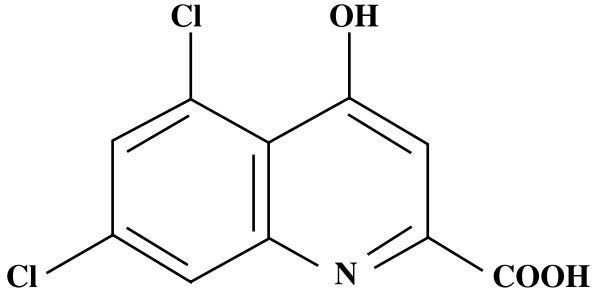
One approach is to use analogues of KYNA as antagonists at glutamate receptors, because KYNA is able to pass the BBB only poorly that makes its use difficult. The aim here is to develop different analogues of KYNA, which can cross the BBB easily and display similar effectiveness on the affected receptors to that of KYNA. Some synthetic KYNA derivatives can behave as antagonists of NMDA receptors and provide an attractive strategy for the development of novel neuroprotective and anticonvulsive agents. 7-chloro-KYNA (7-Cl-KYNA) is a potent selective antagonist at the glycine site (Kemp et al., 1988), but its penetration through the BBB is poorly. Its prodrug, 4-chloro-KYN (Wu et al., 1997) readily enters the brain from the circulation and prevents QUIN-induced neurotoxicity in the rat hippocampus (Wu et al., 2000a) and striatum (Lee and Schwarcz, 2001) after systemic administration (Fig. 2). Furthermore, D-glucose or D-galactose esters of 7-chloro-



4-chloro-kynurenine



7-chlorokynurenic acid



5,7-dichlorokynurenic acid

Fig. 2. Chemical structures of 4-chloro-kynurenine, 7-chlorokynurenic acid and 5,7-dichlorokynurenic acid

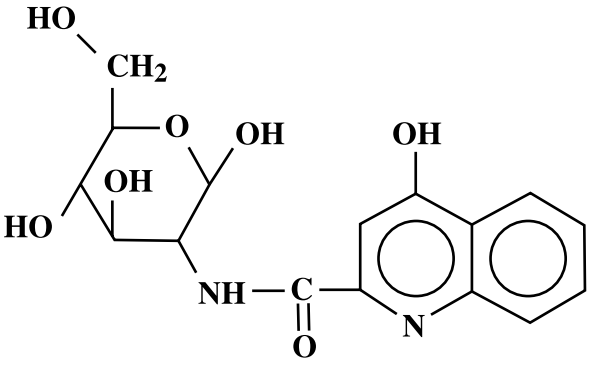


Fig. 3. The chemical structure of glucoseamine-kynurenic acid

KYNA were synthetised to facilitate the transport of 7-Cl-KYNA across the BBB (Battaglia et al., 2000). A new KYNA analogue, the glucoseamine conjugate of kynurenic acid, (Fig. 3), synthetized in the Institute of Medical Chemistry, University of Szeged, was used in electrophysiological experiments: this new compound administered intraperitoneally can decrease the population spike amplitude in the CA1 area of the hippocampus (Robotka et al., 2005) indicating the ability to cross the blood–brain barrier.

The second approach to fend off the effects of a KP disturbance is to use enzyme inhibitors, decreasing the activities of the enzymes which facilitate the QUIN formation. These enzyme inhibitors can therefore shift the KP towards the neuroprotective KYNA and inhibit the accumulation of QUIN and other neurotoxic metabolites. Kynurenine 3-hydroxilase inhibitors (nicotinylalanine, PNU 156561 and Ro-61-8048) and kynureninase inhibitors (S-aryl-L-cysteine-S,S-dioxide and ortho-methoxybenzoylalanine) can block the synthesis of the neurotoxic 3-HK and QUIN, thus can cause an indirect enhancement of KYNA concentration in the brain.

These are some of the most promising possibilities as novel therapeutic strategies for the treatment of neurodegenerative diseases in which the hyperactivation of amino acid receptors could be involved.

Kynurenines and Parkinson's disease

KYNA and Parkinson's disease

The kynurenine pathway accounts for 80% of non-protein tryptophan metabolism. As it was discussed earlier, it includes an agonist (QUIN) and an antagonist (KYNA) at the NMDA receptors, which can behave as an excitotoxin and as a neuroprotectant agent in the central nervous system. It is well known that endogenous excitotoxins have been implicated in the degeneration of dopaminergic neurons in the substantia nigra pars compacta of patients with Parkinson's disease. Miranda et al. (1997) investigated whether an increased level of the endogenous KYNA can protect nigrostriatal dopamine neurons against QUIN-induced excitotoxin damage. They found that 1.5-fold increasing of the KYNA in the substantia nigra prevented the QUIN-induced reduction in striatal tyrosine hydroxylase. However, 9 hours following the administration of nicotinylalanine (kynureninase and kynurenine hydroxylase inhibitor) with KYN (KYNA precursor) and probenecid (inhibitor of organic acid transport), a time when whole brain KYNA levels had decreased 12-fold, QUIN injection produced a significant depletion in striatal tyrosine hydroxylase. Thus, it was demonstrated that increases in endogenous KYNA level can prevent the loss of nigrostriatal dopaminergic neurons resulting from a focal infusion of the excitotoxin QUIN (Miranda et al., 1997). This elevated KYNA level could also prevent the contralateral turning behaviour seen following QUIN administration (Miranda et al., 1999).

Altered KYNA metabolism was found in red blood cells and in plasma in patients with Parkinson's disease: KAT I and II activity were lower in the plasma while KAT II activity were increase in red blood cells. This enhancement of KAT II activity may mediate a protective response against excitatory neurotoxic effects (Hartai et al., 2005).

Altered glutamatergic neurotransmission appears to be central to the pathophysiology of Parkinson's disease. Both NMDA-sensitive and non-NMDA-sensitive glutamate receptors contribute to excitatory postsynaptic currents of dopamine-containing neurons and may play a critical role in the physiology as well as pathology related to these neurons (Mereu et al., 1991). Glutamate may act via ionotropic receptors within striatum to regulate dopamine synthesis, whereas it may influence dopamine release via an action on receptors in substantia nigra (Zigmond et al., 1998). Both spontaneous and evoked dopamine release in the striatum are under the local tonic excitatory influence of glutamate (Kulagina et al., 2001).

Wu et al. (2000) supported the hypothesis that ionotropic receptors can inhibit impulse-dependent dopamine release by the mechanism that acts locally within the striatum. This finding contrasts with previous reports that glutamate can excite impulse-independent dopamine release. This extends earlier findings that glutamate may both excite and inhibit subcortical dopamine systems by suggesting that the excitatory and inhibitory actions of striatal ionotropic glutamate receptors are specifically associated with impulse-independent and impulse-dependent dopamine release (Wu et al., 2000c).

Different doses of glutamate receptor antagonists were tested against MPP+ neurotoxicity on dopaminergic terminals of rat striatum. KYNA, which is a non-selective antagonist of glutamate receptors, partially protected dopaminergic terminal degeneration while dizocilpine, a non-competitive channel blocker of the NMDA receptors, failed to protect dopaminergic terminal from MPP+ toxicity. These findings suggest that protective effect of KYNA against the toxic MPP+ on dopaminergic terminals are mediated mainly through the AMPA-kainate subtype (Merino et al., 1999).

Homeostatic interactions between dopamine and glutamate are central to the normal physiology of the basal ganglia. This relationship is altered in Parkinsonism and in levodopa-induced dyskinesias, resulting in an upregulation of corticostriatal glutamatergic function. Using Ro 61-8048, a kynurenine 3-hydroxylase inhibitor which resulted in an increased level of KYNA, with levodopa produced a moderate but significant reduction in the severity of dyskinesias while maintaining the motor benefit. This result suggests a promising novel approach for managing levodopa-induced dyskinesias in Parkinson's disease (Samadi et al., 2005). Nerve cells in the substantia nigra pars compacta are known to express tyrosine hydroxylase. It was shown that the dopaminergic neurons of this region express also kynurenine aminotransferase (KAT), the enzyme taking part in the formation of KYNA. It was demonstrated that after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment, KAT-I expression was decreased in the substantia nigra (Knyihar-Csillik et al., 2004).

Dopaminergic stimulation caused a functionally significant decrease in the concentration of KYNA: L-DOPA effected a dose-dependent, transient reduction in striatal kynurenate, reaching the nadir of -37.5% 1.5 after the drug administration. This effect was abolished in animals with a 6-hydroxydopamine-induced lesion of the nigrostriatal pathway, but was not influenced by a prior striatal quinolinate lesion. These data confirm the dopaminergic control of striatal KYNA formation and suggest that the interactions are mediated by astrocytic dopamine receptors. This might provide a link among the dopaminergic, glutamatergic and cholinergic neurotransmission in the normal and diseased striatum (Wu et al., 2002).

It is well known that Parkinson's disease results from a progressive loss of dopaminergic neurons of the substantia nigra. Clinical symptoms only appear when neuronal cell death exceeds 50–60%. This late appearance is due to compensatory mechanisms. It is possible that glutamatergic inputs to the

substantia nigra may be implicated in this masking of this disease. Reversible pharmacological blockage of this input effect the appearance of motor disturbances. This blockade could lead to presymptomatic diagnosis of Parkinson's disease (Bezard et al., 1997a, b).

Ogawa et al. (1992) investigated the concentration of the tyrosine and tryptophan metabolites in the frontal cortex, putamen and substantia nigra pars compacta in Parkinson's diseased and control brain tissue. Dopamine concentration was significantly decreased in the putamen and substantia nigra of diseased tissue, regardless of L-DOPA therapy. KYN and KYNA concentrations were lowered in each region of the diseased groups (with or without L-DOPA-treatment) than in the control group, but the molar ratios of TRP to KYN and KYN to KYNA were unchanged among three groups. In contrast, 3-hydroxykynurenine concentration was increased in the putamen in the Parkinson's disease without L-DOPA-group and in the three regions of the brain tissue with Parkinson's disease with L-DOPA therapy (Ogawa et al., 1992). However, the concentration of total serotonin, 3-hydroxytryptophan, KYN and 3-hydroxykynurenine decreased significantly in diseased patient according to the study of Tohgi. 3-Hydroxykynurenine concentration had significant positive correlations with L-DOPA doses (Tohgi et al., 1993a, b). Neopterin concentration and KYN/TRP ratios were increased both in serum and cerebrospinal fluid of patients as compared to controls. Furthermore, significant correlations existed between the neopterin level and KYN/TRP ratio. This suggests the activated cell-mediated immune response in subgroup of patients with advanced Parkinson's disease (Widner et al., 2002b).

Catalepsy-akinesia with rigor- and reduced locomotion show similarities with symptoms of Parkinson's disease. 7-chloro-KYNA dose-dependently counteracts dopamine D2 receptor-mediated catalepsy, induced by haloperidol, but it has not influence on locomotion and on dopamine D1 receptor-mediated catalepsy. These findings are surprising since NMDA receptor antagonists counteract both dopamine D1 and D2 receptor-mediated catalepsy. D1 and D2 receptors are located on different populations of neurons, so it may be supposed that these different neuronal populations have different sensitivity for ligands binding at the glycine site of the NMDA receptors (Kretschmer et al., 1994; Ossowska et al., 1998).

The subthalamic nucleus has been implicated in movement disorders in Parkinson's disease because of its pathological mixed burst firing mode and hyperactivity. In physiological conditions the bursty pattern of this nucleus has been shown to be dependent on slow wave cortical activity, thus glutamate afferents might be involved in this bursting activity. But according to a recent study glutamatergic-receptors blockade does not regularize the slow wave sleep bursty pattern of subthalamic nucleus (Urbain et al., 2004).

QUIN and Parkinson's disease

Excitotoxins constitute a group of agents are capable of activating excitatory amino acid receptors and producing axon-sparing neuronal lesions. QUIN is a pyridine-dicarboxylic acid which is localized to glia and immune cells, and its content increases with ages. Focal injections of QUIN into the nucleus basalis magnocellularis produced sustained loss of cholinergic neuron markers in the neocortex and amygdala. This resulted in an impairment of performance of memory-related tests. In the striatum focal QUIN injections have been found to largely replicate the neurotransmitter deficits prevailing in Huntington's disease. QUIN is also highly damaging to the striatopallidal encephalinergic neurons (Jhamandas et al., 1994). Autoradiographic techniques were used to study distribution of histamine H2-receptors in the brains of patients affected by human neurodegenerative pathologies compared with control cases. The highest level of

histamine binding sites in control cases were found in the caudate, putamen and accumbens nuclei. In Huntington's chorea the levels of histamine H2-receptor binding sites were found to be markedly decreased in virtually all region examined, while in Parkinson's disease the levels of histamine H2-receptor binding sites were not different from those of control cases. These results were comparable with those obtained from unilaterally neurotoxin-lesioned guinea pigs. Similar losses of binding sites were observed in QUIN-lesioned striatal intrinsic neurons, whereas lesioning dopaminergic cell bodies in the substantia nigra with 6-OH-dopamine did not produce any significant change. These results strongly suggest that histamine H2-receptors are expressed by striatal neurons, which degenerate in Huntington's chorea, but not by nigral dopaminergic neurons and may play a role in the regulation of the intact striatonigral pathway (Martinez-Mir et al., 1993).

Macaya et al. (1994) observed that an axon-sparing injury to the developing striatum induced by excitotoxin QUIN resulted in a decrease in dopaminergic neurons in the substantia nigra pars compacta of the adult. As the striatum is a major target for the substantia nigra pars compacta dopaminergic system, they have hypothesized that a decrease in the size of the striatal target during development may result in an induced regressive event in this region. It was concluded that developmental striatal excitotoxic injury is associated with induced apoptotic cell death in substantia nigra (Macaya et al., 1994). Furthermore, it was found that induction of apoptotic cell death was largely restricted to the first 2 postnatal weeks. After that time induction of the death was abated (Kelly and Burke, 1996). Increased expression of cyclin-dependent kinase 5 was observed in this induced apoptotic neuron death (Henchcliffe and Burke, 1997). It was demonstrated that destruction of the intrinsic striatal neurons by a local injection of QUIN dramatically enhanced the magnitude of substantia nigra pars compacta apoptosis and resulted in a low number of adult dopaminergic neurons, strengthening the apoptotic nature of the observed developmental cell death in this region. Later, it was supported that the overexpression of the anti-apoptotic protein Bcl-2 attenuated both natural and QUIN-induced apoptosis. Thus, developmental neuronal death plays a critical role in regulating the adult number of dopaminergic neurons in the substantia nigra pars compacta (Jackson-Lewis et al., 2000).

Multiple system atrophy of the striatonigral degeneration (MSA-SND) type is increasingly recognized as major cause of neurodegenerative Parkinsonism. Previous attempts to mimic MSA-SND pathology in rodents have included sequential injections of 6-OH-dopamine and QUIN into medial forebrain bundle and ipsilateral striatum (''double toxin-double lesion'' approach). Preliminary evidence in rodents subjected to such lesions indicates that embryonic transplantation may partially reverse behavioural abnormalities. Intrastriatal injections of MPP+ (a mitochondrial toxin) results in (secondary) excitotoxic striatal lesion and subtotal neuronal degeneration of ipsilateral substantia nigra pars compacta producing MSA-SND-like pathology by a simplified ''single toxin-double lesion'' approach. Comparative studies of human SND pathology and rodent striatonigral lesions are required in order to determine the rodent models most closely mimicking the human disease process (Wenning et al., 1999, 2000).

Injection of excitotoxin QUIN into the striatum has been extensively used as an experimental model of Huntington's disease, while injection of 6-OH-dopamine into the dopaminergic nigrostriatal pathway provides a well established model of Parkinson's disease. Intrastriatal application of QUIN produced major changes in cytochrome oxidase (decrease) and active glycogen phosphorylase (increase) histochemistry at the level of striatum and of most of the other basal ganglia

nuclei. Although attenuated over time, these changes persisted up to one year after the lesion. On the contrary, after intrastriatal injection of 6-OH-dopamine no remarkable changes were observed in these 2 metabolic markers staining. This study illustrates the discrepancies between the morphological changes and metabolic changes that are induced when using these experimental models of neurodegenerative disorders (Levivier and Donaldson, 2000).

Alteration in the isoprenoid metabolite digoxin has been reported in neuronal degeneration (Parkinson's disease), functional neuropsychiatric disorders (schizophrenia and epilepsy) and immune-mediated disorders (e.g. multiple sclerosis). Digoxin, an endogenous Na^+–K^+ ATPase inhibitor, secreted by the hypothalamus, was found to be elevated and red blood cell membrane Na^+–K^+ ATPase activity was found to be reduced in all these disorders. Digoxin can also preferentially upregulate tryptophan transport over tyrosine, resulting an increased levels of depolarizing tryptophan catabolites, serotonin, QUIN, nicotin, and decreased levels of hyperpolarizing tyrosine catabolites, dopamine, noradrenalin, morphine, contributing to membrane Na^+–K^+ ATPase inhibition. It can result in increased level of intracellular Ca^{2+} and reduced level of Mg^{2+} leading to glutamate excitotoxicity (Kurup and Kurup, 2002, 2003a). Hence, the dysfunctional isoprenoid pathway and related cascade are very important in the pathogenesis of Parkinson's disease. This hypothalamic digoxin-mediated model for this disease is also postulated (Kurup and Kurup, 2003b).

Behavioural and electrophysiological methods were used to investigate the effects of combining a unilateral QUIN lesion of the entopeduncular nucleus with a striatal transplant of fetal ventral mesencephalic tissue in 6-OH-dopamine hemi-lesioned rat model for Parkinson's disease. The results of the experiments show enhanced motor and neuronal sensitivity to amphetamine after the intervention suggesting that such a multiple approach might prove more beneficial than one-site intervention targeting either the entopeduncular nucleus or the striatum (Olds et al., 2003). Parkinson's disease (hypokinetic disorder) and Huntington's disease (hyperkinetic disorder) share the fact that in the motor pathways the dysfunction starts in the striatum. A study aimed to determine whether the introduction of a mild Huntington's disease condition in the Parkinson's disease striatum can counter the hypokinetic condition. The findings suggest that the shift from ipsilateral rotation to oral stereotypy after QUIN administration in medial, central and lateral striatum was due to reduced striatal output caused by a loss of projection neurons, insufficient to induce Huntington's disease symptoms, but sufficient to counter the Parkinson's disease condition (Olds et al., 2005). This result was found when QUIN was injected in globus pallidus internus (Lonser et al., 1999).

Kynurenines and other neurodegenerative disorders

The kynurenines are known to exert noteworthy neuroactive properties on the excitatory glutamate receptors, in particular on the NMDA receptors. The neurotoxic effects of excitatory amino acids play an important role in the pathogenesis of various neurodegenerative disorders, causing impairment in the cellular energy metabolism. Glutamate-mediated excitotoxic damage decreases the voltage-dependent Mg^{2+} blockade, causes abnormalities in cellular Ca^{2+} homeostasis and elevates the production of reactive oxygen species (Greene and Greenamyre, 1996). The glial uptake of amino acids, and hence reduction of the extracellular concentrations of glutamate and aspartate, and receptor desensitization could allow neuronal survival.

Clinical observations are summarized in Table 2 concerning kynurenine and its derivatives.

Table 2. Pre-clinical and clinical observations related to some neurological and psychiatric disorders

1973	Kynurenine derivatives are probably involved in depression	(Lapin, 1973)
1979	Elevated excretion of xanthurenic acid in psychiatric patients	(Hoes, 1979)
1983	Quinolinic acid was hypothesized to play an important role in Huntington's disease	(Schwarcz et al., 1983)
1986	Quinolinic acid injection into the rat striatum duplicated the neurochemical features of Huntington's disease	(Beal et al., 1986)
1990	Intracerebral injections of kynurenic acid decreased the symptoms of akinesia, tremor and rigidity in parkinsonian monkey	(Graham et al., 1990)
1992	Abnormalities of kynurenine pathway in Parkinson's disease	(Ogawa et al., 1992)
	Kynurenic acid concentrations are reduced in cerebral cortex of Huntington's diseased patients	(Beal et al., 1992)
1993	Kynurenine derivatives may play an important role in chronic epileptogenesis	(Du et al., 1993)
1994	Inhibitors of kynurenine hydroxylase and kynureninase have sedative activities	(Carpenedo et al., 1994)
	Excitatory amino acids induce excitation in dopamine-containing neurons of the substantia nigra	(Wu et al., 1994)
1997	Pre-treatment of kynurenine suppresses epileptiform activity in combined enthorinal/hippocampal slices	(Scharfman and Ofer, 1997)
2003	Quinolinic acid may be one of the factors involved in the pathogenesis of neuronal damage in Alzheimer's disease.	(Guillemin et al., 2003b)
2004	Systemically administered kynurenine together with probenecid markedly inhibits pentylenetetrazol-induced seizures	(Nemeth et al., 2004)

Changes in the absolute or relative concentrations of these compounds (in particular KYNA and QUIN) in the brain have been implicated in numerous neurodegenerative disorders, e.g., Huntington's diseases, Alzheimer's disease, stroke, epilepsy multiple sclerosis, depression and schizophrenia. Both agents can act on the NMDA receptor complex and influence the glutamatergic transmission; as discussed earlier KYNA is an NMDA receptor antagonist, and neuroprotective agent, whilst QUIN is an agonist and neurotoxic agent.

Huntington's disease

When QUIN was infused chronically into the rat striatum deficits were induced of spatial learning in radial arm water maze task, leading the authors to propose that chronically raised QUIN could induce the related deficits seen in Huntington's disease (Shear et al., 1998). When acute intrastriatal injection was used and the animals studied in a range of behavioural paradigms together with a crude histological assessment, it was concluded that QUIN provided a good model of the earlier symptoms of this disease (Bordelon and Chesselet, 1999).

Huntington's disease is characterized by gradually evolving selective neuronal death. Several lines of evidence suggest the important role of an excitotoxic mechanism. Beal et al. found increased KYN/KYNA ratio in diseased post-mortem putamen and decreased KYNA concentration in CSF and in the brain (Beal et al., 1992). The intrastriatal injection of QUIN leads to a similar pattern of neuronal death to that seen in this human disease by producing a progressive mitochondrial dysfunction, the impairment of cellular energy homeostasis (Bordelon et al., 1997) and major decreases in SOD activity. The total SOD and Cu/ZnSOD activities increased in young transgenic mice (Huntington mutation), but decreased in older mice. This suggested a compensatory mechanism to protect cells from free radical-induced damage, but the

system becomes insufficient in older animals (Santamaria et al., 2001).

Besides the loss of neurons with NMDA receptors in post-mortem brains of Huntington's disease patients the proliferation of astrocytes with glutamate transporter transcripts may serve as a compensatory mechanism protecting the neostriatal neurons from glutamate excitotoxicity (Arzberger et al., 1997).

The levels of KYNA in the caudate nucleus are reduced in this disease, more, both isoform of KAT are also reduced in the striatum (Jauch et al., 1995). There is, therefore, an apparently selective impairment of KYNA synthesis in the striatum of Huntington's disease, possibly due to the absence of an activator or a co-factor for KAT.

It is well known that dopamine, a major neurotransmitter used in the striatum, is involved in movement disorders such as Huntington's and Parkinson's diseases. With the loss of neurons in the striatum of patients with Huntington's disease, there is an associated downregulation of dopamine receptors, which may alter dopamine-mediated responses. In a recent study, dopamine-mediated electrophysiological depression was studied in QUIN-induced experimental disease of rats which results in QUIN-induced reduced responses to dopamine in the striatal neurons (Chen et al., 2002).

Alzheimer's disease

Disturbances of the KP have been described in relation to Alzheimer's disease. Amyloid-β-1-42, a cleavage product of the amyloid precursor protein, induces the expression of indoleamine-2,3-dioxygenase (IDO) and results in a significant increase in QUIN production (Guillemin et al., 2003a). A major aspect of QUIN toxicity is lipid peroxidation, and markers of lipid peroxidation are found in Alzheimer's disease. These data imply that QUIN may be one of the critical factors in the pathogenesis of neuronal damage in Alzheimer's disease (Guillemin and Brew, 2002). Amyloid precursor protein can suppress the elevation of intracellular Ca^{2+} induced by glutamate and this suppression of NMDA receptor current is apparently mediated by cyclic guanosine monophosphate (Furukawa and Mattson, 1998). However, amyloid beta-peptide impairs the function of membrane ion-motive ATPases and glucose and glutamate transporters, and can enhance Ca^{2+} influx through voltage-dependent and ligand-gated calcium channels. Abnormalities in calcium regulation in astrocytes, oligodendrocytes and microglia have been documented in some experimental models of Alzheimer's disease, suggesting that the cellular calcium homeostasis plays a prominent role in the pathogenesis of this disease (Furukawa and Mattson, 1998).

Stroke

It is well known that massively release of excitatory amino acids play a major role in ischemic neuronal degeneration, and KYNA has a neuroprotective effect against ischemic brain damage. Its altered metabolism could be implicated in the pathogenesis of neurodegeneration during ischemia/anoxia. However, the level of KYNA in area CA1 is not altered 24 and 72 h following transient global ischemia in the gerbil hippocampus, which indicates that KYNA production is preserved in this region during the early stages after ischemic insults (Luchowska et al., 2003). KYN-3-mono-oxygenase inhibitors reduce the neuronal death in region CA1 of organotypic hippocampal slices exposed to 30 min of oxygen and glucose deprivation by decreasing the local synthesis of 3-HK and QUIN (Carpenedo et al., 2002).

Rat aortic slices produce and liberate KYNA upon exposure to KYN. It has been found that the aortic KYNA production is diminished by modification of the ionic milieu, hypoxia and hypoglycemia (Stazka et al., 2002). Administration of KYN produces

a significant increase in the normal and ischemic corticocerebral blood flow (cCBF), which can be prevented by pretreatment with either atropine or N-omega-nitro-L-arginine-methyl-ester. It is suggested that the cCBF-increasing effect of KYN might be mediated by the activation of cholinergic and NO pathways (Sas et al., 2003).

Epilepsy

Even low concentrations of endogenous KYNA can reduce the number of hippocampal slices with spontaneous epileptiform discharge after exposure to a buffer lacking magnesium. It is suggested that endogenous KYNA plays an important role in the suppression of seizure-like activity (Scharfman et al., 2000). In WAG/Rij rats, which are a genetic model of absence epilepsy, significantly lower concentrations of KYNA have been found in the frontal cortex than in non-epileptic controls. This indicates that selective deficits of endogenous KYNA may account for increased excitability in the frontal cortex (Kaminski et al., 2003).

Natsume et al. (2003) investigated whether the metabolism of the serotoninergic system in the brain, including the KP, is involved in temporal lobe epilepsy (TLE). Their studies of patients with intractable TLE by positron emission tomography (PET) using alpha-(11C)-methyl-L-TRP (α-MTRP) revealed a significantly increased α-MTRP uptake in the hippocampus ipsilateral to the seizure focus in patients with normal hippocampal volumes as compared with patients with hippocampal atrophy and healthy controls. This demonstrates a dysfunction of the serotoninergic system, which could include metabolism through the KP in TLE patients with normal hippocampal volumes (Natsume et al., 2003). Moreover, there is a significant correlation between the α-MTRP uptake and the frequency of interictal spikes in patients with tuberous sclerosis complex (Fedi et al., 2003).

Multiple sclerosis

KYNA levels were measured in the CSF of patients with relapsing-onset multiple sclerosis (MS) during remission or not progressing for at least 2 months, and were found to be significantly lower as compared with subjects with non-inflammatory neurological diseases, and those with inflammatory disease. These results provide further evidence of the alterations in the KP during remitting-onset MS (Rejdak et al., 2002). In a recent study, KAT I and KAT II activity were significantly higher in the red blood cells and KYNA level was elevated in the plasma of the patients with multiple sclerosis. This indicates a compensatory mechanism against excitatory neurotoxic effects in these patients (Hartai et al., 2005).

Amyotrophic lateral sclerosis

The oxidative degradation of TRP to KYN and to N-formyl-KYN results in the covalent cross-linking and aggregation of human superoxide dismutase (hSOD1). The cytotoxicity of the motor neurons is increased in amyotrophic lateral sclerosis. The implication of oxidant-mediated aggregation of hSOD1 in this serious disorder is discussed in a recent review (Zhang et al., 2004). The glutamate transporter-1 isoform, member of the glutamate transporter gene family, is the most important transporter involved in keeping extracellular glutamate concentration below neurotoxic levels. Its loss and the increase an extracellular glutamate level has been documented in cases of sporadic and familial amyotrophic lateral sclerosis (Vanoni et al., 2004).

Schizophrenia

Another aspect of neuropsychiatry is the elevated level of the KP metabolism in schizophrenic patients. Studies on postmortem frontal cortices obtained from individuals with schizophrenia and controls have demonstrated

that the concentration of mRNA for tryptophan dioxygenase (TDO) is 1.6-fold higher in the schizophrenia group, whereas the concentration of mRNA for IDO is not significantly different between the two groups (Miller et al., 2004). In schizophrenia, many patients show in filtering the sensorium. While total blockade of the NMDA receptors disrupts the auditory gating in rat, the partial blockade achieved by antagonism of its glycine co-agonist binding site does not. This observation indicates that the disruption in auditory processing is not attributable to greatly elevated KYNA levels (Shepard et al., 2003). An increased phasic activity of the dopaminergic neurons of the ventral tegmental area (VTA) has been noted after pharmacologically elevated levels of endogenous KYNA, a pathophysiological condition analogous to that seen in schizophrenic patients (Erhardt and Engberg, 2002). Nicotine leads to both the inhibition and the excitation of VTA dopamine neurons. The inhibitory action of this drug may play a prominent role in causing a profound dysregulation of the mesocorticolimbic dopamine system and may help to explain the high prevalence of tobacco-smoking in schizophrenics (Erhardt et al., 2002). By a recent study, increased KYNA concentration induced by non-steroid anti-inflammatory drugs displaying an inhibitory action on cyclooxygenase-1 contributes to their analgetic efficacy as well as to their psychiatric side effects (Schwieler et al., 2005).

Depression

A disturbed metabolism of TRP affects the biosynthesis of the neurotransmitter 5-HT and this appears to be associated with an increased susceptibility to depression. Thus, the activation of IDO could be an important link between the immunological network and the pathogenesis of depression (Widner et al., 2002a). A decreased serotoninergic transmission and increased levels of KYN derivatives, mainly the neurotoxic 3-HK and QUIN, result from an enhanced IDO activity. In consequence the overproduction of ROS and hippocampal atrophy can be found, which be associated with depression (Wichers and Maes, 2004). Reduced TRP availability plays a role in interferon alpha (INFα)-induced depressive symptoms, and paroxetine attenuates the behavioral consequences of an INFα-mediated TRP depletion (Capuron et al., 2003).

Discussion

It is widely accepted that activation of the excitatory amino acid receptors plays a role in neuronal death in Parkinson's, Huntington's and Alzheimer's disease, stroke, epilepsy and psychiatric disorders. Excitotoxicity is caused by overexcitation of these receptors by exogenous or endogenous receptor agonists. The kynurenine pathway involves both an agonist (QUIN) and an antagonist (KYNA) acting mainly at the NMDA receptors. Impairments of this pathway are strongly involved in neuronal death of the above-mentioned disorders. The intrastriatal injection of QUIN (an animal model of Huntington's disease) causes progressive mitochondrial dysfunction, impairment in cellular energy homeostasis and major decreases in SOD activity. The total SOD and Cu/Zn SOD activities were increased in young transgenic mice (Huntington mutation) but decreased in older mice. This suggests a compensatory mechanism to protect cells from free radical-induced damage but the system becomes insufficient in older animals (Santamaria et al., 2001). QUIN is able to induce a wide variety of toxic effects, such as GABA depletion, ATP exhaustion, neuronal oxidative stress enhancement and cell death. Oxidative stress is recognized as accountable for redox regulation involving reactive oxygene and nitrogen species. Its role is pivotal for the modulation of critical cellular functions such as apoptosis program activation and the ion transport involved in excitotoxicity. QUIN can increase the

generation of reactive oxygene and nitrogen species by activating NMDA receptors which increase intracellular Ca^{2+} levels and result in the activation of xanthine oxidase and nitric oxide synthase. At low arginine concentrations, neuronal NO synthase generates NO and superoxide, favouring the production of the toxin peroxynitrite. The NMDA-induced excitotoxicity in neuronal cells is therefore dependent on the arginine availability (Do et al., 2002). However, kainic acid-induced striatal lesions produced more pronounced behavioural effects than QUIN lesions (Vecsei and Beal, 1991a). Kynurenine could slightly attenuate the kainic acid-induced behavioural changes (Vecsei and Beal, 1990a).

The relationships with oxidative stress, free radicals and neurodegenerative diseases have been well reviewed (Lewen et al., 2000; Emerit et al., 2004).

KYNA is a broad-spectrum ionotropic glutamate receptor antagonist, which can act in particular at the strychnine-insensitive glycine binding site of the NMDA receptors. KYNA also non-competitively blocks the activity of presynaptic α7-nACh receptors and can increase the expression of non-α7 nACh receptors (Hilmas et al., 2001). Cross-talk between KYNA and the nicotinic cholinergic system has been presumed to play a role in the pathogenesis of numerous brain disorders, including Alzheimer's disease and schizophrenia in which brain KYNA levels are elevated and nicotinic functions are impaired (Alkondon et al., 2004). KYNA can inhibit the overexcitation of glutamatergic transmission, and may therefore influence physiological and pathological processes and have therapeutic effects in neurological disorders (Stone, 2000, 2001; Stone et al., 2003). However, KYNA in high doses induces ataxia, stereotype behaviour (Vecsei and Beal, 1990b) and marked changes in behavioural tasks (Vecsei and Beal, 1991b).

There are striking similarities between the effects of QUIN and Huntington's disease which can lead the investigators to propose a causative role for QUIN in this disorder. QUIN-induced lesions in the striatum of monkeys produced dyskinesias and dystonia resembling those of the human disease (Storey et al., 1994; Burns et al., 1995) and distribution of changes in the levels of glutamate, GABA and other amino acids produced by QUIN is similar to that seen in disease (Storey et al., 1992). The levels of 3-HK, which can lead the formation of QUIN, are elevated in the putamen and substantia nigra of brains obtained from patients with Parkinson's disease. The ratio of KYN/3-HK is reduced in the substantia nigra, in the frontal cortex and in the putamen (Ogawa et al., 1992). This would imply not only an increased synthesis of the neurotoxic 3-HK and QUIN, but also a decreased availability of KYN for the synthesis of KYNA. The plasma concentration of KYN is increased in patients with clinically diagnosed anxiety, but a marked anxiolytic activity of KYNA was also shown using a variety of anxiogenic agents (Lapin, 1998). Therefore, the role of kynurenines in anxiety has already been unclear. Lowered level of plasma KYN was exhibited in depressed patients which increased to control values on treatment (Orlikov et al., 1994). Since repeated administration of amphetamine is known to induce a psychosis closely resembling that of schizophrenia, it is possible that this disorder itself involves a decline of KYNA levels with a secondary hyperactivation of NMDA receptors and a loss of neurons in limbic regions.

It has been seen that the sufficient concentration of the kynurenine pathway's metabolites is extremely important to maintain the normal brain function. Prevention or correction of the impairment of this pathway could attenuate the pathological processes of several neurodegenerative disorders.

KYNA use as a neuroprotective agent is rather limited because it has a very limited ability to cross the blood–brain barrier. However, its prodrug, L-kynurenine, can cross easily this barrier (Fukui et al., 1991) and can

convert to KYNA in the brain. This effect could be affirmed by co-administrating with probenecid which is an inhibitor of the organic acid transport (Vecsei et al., 1992; Nemeth et al., 2004). Another way to be able to use KYNA as a protectant agent is to conjugate with a compound which can transport the barrier. 4-chloro-KYN, prodrug of 7-chloro-KYNA, readily enters the brain from the circulation and provides antiexcitotoxic neuroprotection after systemic administration. It resulted in a prolonged and functionally relevant blockade of the hippocampal glycine site of NMDA receptors (Wu et al., 2000b). Other derivatives of KYNA can also promote passage through the BBB to allow the systemic administration of this neuroprotective agent. In an earlier study, D-glucose or D-galactose esters of 7-Cl-KYNA were synthesized to facilitate the transport of 7-Cl-KYNA across the BBB. Intraperitoneal administration of these substances revealed that these prodrugs are of potential interest in the experimental therapy of epilepsy and acute or chronic neurodegenerative disorders (Battaglia et al., 2000).

A newly synthesized KYNA analogue, glucoseamine-kynurenic acid (KYNA-NH-GLUC), can cross the BBB more easily and can probably modify the glycine allosteric site of NMDA receptors effectively. Intravenous administration of this drug results in appreciable reductions in the amplitudes of the somatosensory-evoked responses, although the intracerebroventricular application of KYNA-NH-GLUC and KYNA caused similar effects on behaviour (Fuvesi et al., 2004). KYNA-NH-GLUC significantly decreases the evoked activity in the CA1 region of the rat hippocampus, the effect being more powerful on coadministration with PROB (Robotka et al., 2005).

The results discussed above indicate the importance of the normal function of the KP and the possibilities of neuroprotection with KYN derivatives. Elevated levels of KYNA in the brain using enzyme inhibitors or KYNA analogues may reduce the overactivation of EAA receptors and may modify or arrest the progression of various neurodegenerative disorders. This can offer a novel therapeutic opportunity where the development of these powerful new compounds promises a key for brain neuroprotection.

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What's new? Clinical progression and staging of Parkinson's disease

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Summary. Several new advances facilitate current understanding of the progression of Parkinson's disease. The application of statistical modeling techniques has helped to estimate rates of clinical decline in the context of symptomatic interventions. These approaches may allow a new means for testing neuroprotection effects even when patients are on dopaminergic treatment. Further, the development of new rating scales, specifically the Movement Disorder Society-initiated revision of the Unified Parkinson's Disease Rating Scale has capitalized on a greater clinical appreciation of non-motor elements of Parkinson's disease. Finally, adaptations of new technologies that are computer-based and enable data transmission from at-home environments allow researchers to capture disease impairment and disability with potentially greater precision and much more frequently than permissible in a hospital clinic or practice setting.

Introduction

Observations from as early as the nineteenth century and modern longitudinal studies have established that Parkinson's disease (PD) is a chronic and progressive neurological illness (Jankovic and Tolosa, 2002). Clinical decline is usually slow but relentless, though several clinical patterns exist. For example, a slower progression occurs with tremor-predominant disease compared to the akinetic-rigid form, and a faster progression occurs in subjects with very elderly disease onset compared to those who develop PD in early middle-age. In considering ''What's New'' in studies of clinical progression, this essay focuses on advances in rating Parkinson's disease: the application of statistical modeling to estimate rates of clinical decline in spite of symptomatic interventions; the development of new rating scales; and adaptations of new technologies to capture disease impairment and disability with greater precision. In harmony with neurophysiological and neuroimaging advances, these clinical contributions to rating and staging Parkinson's disease will allow a more comprehensive assessment of the disease and its response to therapeutic interventions aimed at ameliorating symptoms and slowing natural decline.

Statistical modeling to document disease progression

The Unified Parkinson's Disease Rating Scale is the most widely utilized tool for rating PD impairment and disability (Fahn et al., 1987). It has gained world-wide acceptance and is the primary end-point measure for most PD-related clinical trials. Its clinimetric profile has been established over two decades since its development, and reliability/validity testing has corroborated its value (Goetz et al., 2003). The scale consists of four sub-scales with scores that can be summed for a Total

UPDRS score , or each part can be analyzed separately (Part I: Cognition, Mood and Behavior; Part II: Activities of Daily Living; Part III: Motor Examination; and Part IV: Complications of Disease and Therapies). Its major application has focused on motor aspects of PD, highlighted primarily by Parts II and III with less emphasis placed on the non-motor elements captured in Part I and, to some extent, Part IV. Analyses of clinical trials examining early PD patients without symptomatic treatment demonstrate an annual increase of approximately 10 points on the Total UPDRS score (Kieburtz, 2003). The scale is highly responsive to levodopa and dopamine agonists with reductions in UPDRS scores estimated usually between 40–50% six months after introduction of therapy. The fact that symptomatic treatment dramatically improves UPDRS scores and patient function, however, has confounded the analysis of underlying disease progression once treatment is initiated. To address this issue and develop reasonable sample size calculations for measuring disease progression in the face of symptomatic treatments, longitudinal clinical trials have been re-analyzed for calculating UPDRS progression rates in this context. As part of the large NET-PD Statistical Core effort, Guimaraes et al. examined UPDRS scores from three patient series over 2–3 years under treatment with either levodopa or agonists, often supplemented with levodopa (Guimaraes et al., 2005). Following an initial improvement in UPDRS scores, continued dopaminergic therapy was associated with clinical decline and an annual linear 3-point progression of total UPDRS scores. This model predicts a return to pre-treatment UPDRS scores by five years. Such calculations define a base rate of progression in the context of best medical care. Subjects already treated with symptomatic therapies have traditionally been excluded from neuroprotection clinical trials because of concerns that symptomatic care confounds the interpretation of data. The 3-point annual progression in UPDRS scores across different studies and across different dopaminergic therapies is strong evidence that neuroprotective programs aimed at slowing disability/impairment progression rates can include subjects already on symptomatic care.

UPDRS revision: (MDS-UPDRS)

As part of a program to evaluate scales in use for assessing PD, a Movement Disorder Society Task (MDS) Force conducted a critique of the UDPRS in 2003. Because several advances in the current understanding of PD had occurred since the scale was originally developed, the Task Force recommended a revision (Goetz et al., 2003). Based on this critique, the MDS initiated this process in 2004, has developed a new scale, termed the MDS-UPDRS, and clinimetric testing will conclude in 2007 (Goetz et al., 2005). The MDS-UPDRS retains the UPDRS structure of four parts with a total summed score, but the parts have been modified to provide a section that integrates non-motor elements of PD: I: Non-motor Experiences of Daily Living; II: Motor Experiences of Daily Living; III: Motor Examination; IV: Motor Complications. To link the responses to clinically pertinent designations and to provide a consistency across questions and parts, all items have five response options with uniform anchors of 0 = normal, 1 = slight, 2 = mild, 3 = moderate, 4 = severe. Parts I and II are envisioned to be self-administration instruments, so that the total rater time should remain below 30 minutes. Detailed instructions for testing and data acquisition accompany the MDS-UPDRS in order to increase uniform usage. Multiple language editions are planned and teaching tapes. The three-part clinimetric program will provide testing of reliability, validity and responsiveness to interventions.

Although the new version primary assesses PD as a motor disorder, the MDS-UPDRS places a new emphasis on non-motor elements of PD. It coalesces all non-motor aspects of

PD into one section, rather than spreading them across Part I and IV as in the original scale. Part I covers topics in the original scale, but also assesses several new areas, including screening questions on anxious mood, nighttime sleep problems, urinary and bowel difficulties and daytime sleepiness. Because the non-motor questions are screening tools, an official UPDRS Appendix accompanies the revision to guide clinicians and researchers on recommended in-depth scales that can be used for detailed assessments of non-motor problems.

To reflect the field's increasing emphasis on early disease and neuroprotection, the MDS-UPDRS has shifted its emphasis with the goal of detecting very mild impairments and disabilities rather than emphasizing the documentation of severe dysfunction. Retaining five-item options for all questions (0–4), the new scale introduces ''slight'' and separates this rating from ''mild'' using the contruct that the former refers to symptoms/signs with sufficiently low frequency or intensity to cause no impact on function whereas the latter causes modest impact. Because clinicians consider very severe impairments poorly responsive to therapeutic interventions, the new scale absorbs the two former highest ratings ''moderate'' and ''severe'' into a single category.

Although the MDS-UDPRS will not be published until it has successfully passed clinimetric testing, dissemination of the revision process, explanation of the key changes and description of the clinimetric programs allow clinicians and researchers to understand and to actively participate in the revision process (contact person: Christopher G. Goetz cgoetz@rush.edu).

Development of new rating tools

Whereas the UPDRS in its current and its revised versions are likely to remain the clinical anchors of PD evaluation, other tools are being developed. Some are independent of the UDPRS and some have adapted the gold standard to accommodate new technological advances. To assess non-motor elements in PD exclusive of motor dimensions, a patient-based inventory, the Non-Motor Symptom Screening Questionnaire, has been developed and clinimetrically tested (Chaudhuri, 2004). Video-based techniques to assess individual components of the UPDRS at home in the patient's ambient environment have been tested in small series of patients (Nyholm et al., 2005). Likewise, for capturing motor fluctuations, rather than relying on paper-based diaries, new electronic-based diaries monitor time of data entry and remind the patient to enter data with signals throughout the day (Nyholm et al., 2004). These advances utilize standard assessment measures but capitalize on electronic advances to maximize accurate data collection, often without requiring the patient to come to the doctor's office.

Over the past two decades, multiple appliances or computer based technologies have been developed to measure objectively the hallmarks of PD, tremor, bradykinesia, rigidity and gait/balance impairments. These tools have never been widely applied in clinical trials nor adopted as useful measures for detecting natural disease progression or response to interventions. In concert with the growing emphasis on early disease and neuroprotection, however, a new interest has emerge to evaluate computer-based tools that could be used by patients to monitor their parkinsonian impairments on a weekly basis at home. Working with the Kinetics Foundation, Palo Alto, California USA, a combined team of US and European researchers is currently developing a single portable computer module that patients will keep at home and twice weekly enter data documenting tremor, multiple dimensions of bradykinesia, with both simple and complex movements, reaction time/movement time, and voice volume (Goetz et al., 2005). The pilot program will primarily assess feasibility, examining patient

compliance and data transfer dimensions, including efficiency and accuracy. Longitudinal studies of changes in these measures in conjunction with the UPDRS will allow investigators to establish if these indices decline in parallel to the UDPRS and whether the computer-based data capture more subtle and earlier change than the standard clinical ratings.

Future perspectives

Although a major research effort has been launched to identify biomarkers of PD progression, the clinical distinction between decline, stabilization, and improvement is the ultimate issue to patients and their physicians. The prioritization of accurate disease monitoring crosses the interface of government agencies, private donors and industries investing in PD, because new rating scales, new tools, and new methods of analysis are linked to PD itself and not to any single therapy. Developing methods that will accurately detect small but clinically meaningful changes in patient function remains an ongoing priority in PD research. A clear and simple battery of tests that monitor disease progression across large populations will extend research and clinical care outside the restrictions of academic movement disorders units to wider medical spheres.

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Parkinson's disease: premotor clinico-pathological correlations

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Summary. Parkinsonism is a clinical syndrome characterized by bradykinesia, hypo-/akinesia, muscular rigidity, and resting tremor, mainly caused by Parkinson's disease (PD). Progressive loss of nigral neurons with Lewy bodies is considered an essential neuropathological feature. Recent studies, however, indicate that nigral degeneration is only a part of this synucleinopathy, and clinical symptoms go far beyond motor parkinsonism. Olfactory disturbances, autonomic dysfunction, pain, sleep fragmentation, depression, and dementia with or without psychosis are frequently seen. The variability in the expression of these signs and symptoms suggests multiple causes and/or pathogeneses within the present diagnostic disease entity. In this article, a recently proposed staging of PD-related brain pathology will be correlated with the various clinical expressions. It will be argued that the specific topographical sequence of the pathology, depending on the extent and progression of the degenerative process at defined sites, may explain the individually variable expression of this disease.

Introduction

Parkinsonism is defined as a syndrome, manifesting with bradykinesia, hypo-/akinesia, rigidity, tremor, and postural instability, chiefly caused by a significant depletion in brain dopamine. Historically, it was thought to be a sporadic condition, idiopathic Parkinson's disease (PD), but in time the clinical spectrum was also recognized to be the result of a variety of gene defects as well as a possible consequence of trauma, infections, drugs, intoxications, and several other neurodegenerative diseases, such as multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) (Calne, 2005).

During the last decades, more attention has been given to PD-related non-motor symptoms, such as hyposmia, autonomic dysfunctions, mood disorders, sleep disorders, sensory disorders, and cognitive deficits, which sometimes are noticeable even before motor parkinsonism becomes overt. PD, therefore, is now considered the clinical manifestation of a multi-system degenerative process, comprising not only the dopaminergic but also the noradrenergic, serotoninergic, cholinergic, and other systems.

Recently, doubts have been raised about PD as constituting a single disease entity. These doubts are fed by the clinical heterogeneity (Lewis, 2005; Uitti, 2005), suggesting at least two different clinical forms: tremulous PD and akinetic-rigid PD, the latter often accompanied by mood disorders, cognitive dysfunction, and psychosis. Although hyposmia, sensory disorders, and mood disorders antedating motor parkinsonism are generally accepted as symptoms within the framework of PD, in cases where dementia and psychosis

precede motor parkinsonism another disease entity was suggested: dementia with Lewy bodies (DLB). Not only the variability in the scope of initial symptoms, but also the rate of progression and additional characteristics might suggest genotypic heterogeneity.

In the past years, Braak and colleagues studied the course of the pathology in a considerable number of incidental cases and symptomatic PD patients, and proposed a staging procedure based upon the readily recognizable topographical extent of the lesions (Braak, 2000, 2003a, 2004). The intracerebral formation of abnormal proteinaceous Lewy bodies and Lewy neuritis in PD was found to start at clearly defined sites and to progressively advance in a topographically predictable sequence. During the preclinical stages 1–2, alpha-synuclein inclusion body pathology is confined to the medulla oblongata, pontine tegmentum, olfactory bulb, and anterior olfactory nucleus. The nigral substance and other nuclear grays of the basal midbrain and forebrain become progressively involved in the following stages 3–4, and the process, in stages 5–6, finally enters the neocortex.

The clinical course of the disease can also be subdivided into two different phases: the 'preclinical,' or preferably 'premotor,' phase and the clinical, motor phase. During the premotor phase, individuals might be asymptomatic or display non-motor signs and symptoms with, as a rule, a normal neurological examination. In that phase though, functional imaging may indicate a loss of the integrity of the presynaptic dopaminergic system associated with eventual PD (Berendse, 2001; Ponsen, 2004). Better recognition of these signs as PD-related manifestations will allow earlier diagnosis and better treatment, especially when a causal/protective therapy becomes available.

The premotor phase

Extrapolating from the extent of the degeneration of the nigrostriatal dopaminergic system in PD, as visualized by positron emission tomography or single photon emission computed tomography using radioligands for the dopamine transporter (Booij, 1999), and also as shown by post-mortem cell counts of melanoneurons in the substantia nigra, the onset of nigral dopaminergic neuronal loss seems to antedate the clinical diagnosis (based on levodopa-responsive motor parkinsonism) of PD by about 4–6 years (Fearnley, 1991; Morrish, 1998; Marek, 2001). During this period between the presumed onset of loss of dopaminergic neurons in the substantia nigra and the clinical diagnosis of PD, i.e., during part of the premotor phase of PD, more than half of the nigral neurons projecting to the putamen are lost (Tissingh, 1998a, b; Braak, 2004). The initial PD-related pathological changes in the substantia nigra, however, are always accompanied by extra-nigral pathology (Braak, 2000). Actually, the neuropathological process may begin in extra-nigral structures: the dorsal motor vagus nucleus and olfactory bulb with related portions of the anterior olfactory nucleus (Braak, 2003a, 2004; Del Tredici, 2002). Assuming that PD-related pathology indeed first occurs outside the nigra, the premotor phase may be even substantially longer than imaging data of the nigrostriatal system suggest. In this premotor phase, PD patients were found to consult their general physicians more frequently than age-adjusted controls (Gonera, 1997).

Clinical premotor symptoms

The premotor phase may be passed without any substantial medical problems because the impact on daily life due to eventual premotor symptoms varies greatly, and many patients might not notice problems or take them for granted as an inevitable consequence of aging. As a rule, however, PD patients will, to some extent, suffer non-motor signs and symptoms before the first signs of motor parkinsonism become overt. These symptoms comprise hyposmia (Tissingh, 2001), autonomic nervous system disorders (Chaudhuri, 2005), sensory

disorders (Giuffrida, 2005), depression (Montgomery, 1999) sleep disorders (Fantini, 2005) and cognitive (executive) impairment (Dubois, 1997). Evidence suggests that such symptoms may be markers of a preclinical stage of PD (or DLB) (Oppenheimer, 1980; Larner, 2000; Stoffers, 2001; Micieli, 2003; Ponsen, 2004). All these premotor signs and symptoms, however, are *per se* neither specific, nor sensitive for PD, although in combination they might be highly suggestive for the disease (Koller, 1998; Przuntek, 2004).

1. Olfactory dysfunction is a common finding in patients with PD. As a rule, odor detection, discrimination, and identification tests in PD patients are significantly lower as compared to controls, notably in *de novo* patients (Tissingh, 2001). Therefore, it was hypothesized that olfactory tests could be useful in the early diagnosis of PD. In an effort to determine whether otherwise unexplained (idiopathic) hyposmia is associated with an increased risk of developing PD, a recent prospective study with olfactory tests and sequential single-photon emission computed tomography using [I^{123}]β-CIT as a dopamine transporter ligand (Booij, 1999) showed that, two years from baseline, 10% of elderly asymptomatic PD-relatives with idiopathic hyposmia (a cohort of 10% of all participating relatives) had developed clinical PD as opposed to none out of a cohort of the other, non-hyposmic relatives. Inasmuch as in the remaining asymptomatic hyposmic relatives the average rate of decline in dopamine transporter binding was also found to be significantly higher than in the normosmic relatives, it was concluded that in elderly individuals idiopathic hyposmia induces a risk of at least 10%, and probably as high as 22%, of developing PD (Ponsen, 2004).
2. Autonomic dysfunctions are common in neurodegenerative diseases with intraneuronal alpha-synucleinopathic aggregations, such as PD, DLB, MSA, and the related Lewy body disorder characterized by apparently isolated severe autonomic failure (pure autonomic failure). In PD, both the central and peripheral autonomic nervous systems have been found to be affected. Resulting dysautonomia variably includes cardiovascular symptoms, gastrointestinal, urogenital, sudomotor, and thermoregulatory dysfunctions, pupillary abnormalities, as well as sleep and respiratory disorders, each of which compromises patients' activities of daily life in the motor and premotor phases of the disease (Chaudhuri, 2005; Magerkurth, 2005).

   Neurovegetative problems in patients with PD have been recognized since the original description by James Parkinson in 1817. In sporadic but also familial PD patients, the prevalence of clinically relevant dysautonomia was found to be over 50%. Signs of autonomic dysfunction may arise during the premotor phase but, as a rule, come to the patients' attention only later. Most of these patients suffer orthostatic hypotension with baroreflex failure (with deficits of at least 20 mm Hg), usually combined with impaired cardiovascular regulation due to cardiac sympathetic denervation (Bonucelli, 2003; Singleton, 2004; Amino, 2005). In time, PD-related (but also dopaminomimetic drug-related) gastrointestinal problems, such as dysphagia and delayed gastric emptying, may be noticed by patients and induce nutritional depletion. Videofluoroscopic studies in these PD patients show a variable dysphagia with or without aspiration (Waxman, 1990).

   Further autonomic disorders comprise sphincter-related bladder dysfunction in 30–90% of patients, with anticholinergic but not levodopa-responsive hyperactivity of the detrusor muscle, leading to nocturia, urinary frequency, and urgency (Soler, 2004). In addition, profound impairment within the realms of sexual behavior, arousal, orgasm, and drive, but not fantasy, are commonly reported by PD patients (Yu,

2004). Female PD patients mainly report difficulties with arousal, whereas males complain about erectile dysfunction and premature ejaculation, all of which result in difficulties reaching orgasm and/or attaining sexual satisfaction in half of the patients (Bronner, 2004).

Pupillomotor abnormalities also have been reported as a result of the PD-related degeneration of cholinergic brainstem nuclei, e.g., the Westphal-Edinger nucleus, which controls pupillomotor function (Jellinger, 1999). Owing to sympathetic dysregulation during the course of the disease, probably at the hypothalamic level, thermoregulation can also be disturbed in PD patients. In this instance, patients suffer severe, intermittent, drenching sweats, associated with 'off-phase' sub-therapeutic levodopa plasma levels (Sage, 1995).

3. Sensory disorders, mainly diffuse pain in the shoulder, or tingling sensations in the limbs heralding motor parkinsonism in the involved side of the body, may be presenting symptoms of PD or can occur during pharmacotherapy-related wearing-off and/or 'on-off' fluctuations. In a clinical study, Giufrida and colleagues found that 67% of about 400 consecutive PD patients complained of sensory or painful syndromes, mostly muscular pains. More than 25% of these patients experienced these complaints already in the premotor phase (Giuffrida, 2005).
4. Depression and anxiety are additional frequently complicating non-motor problems, arising not only in the motor but also in the premotor phase of the disease: a strong association exists between depression and the subsequent incidence of PD (Schuurman, 2002). The reasons for the high frequency of depression and anxiety in PD are poorly understood. Although most PD patients suffer psychomotor retardation, it has been suggested that in many cases psychosocial factors also may play a role. Degeneration of neurotransmitter systems other than the dopamine system might play a role in the development of these affective disorders (Remy, 2002).
5. In the premotor phase, sleep disorders, such as insomnia and sleep fragmentation as well as excessive daytime sleepiness and rapid eye movement behavior disorder (RBD) have been reported (Arnulf, 2002). RBD is characterized by the lack of motor inhibition during REM sleep, which leads to a vigorous and potentially harmful dream-enacting behavior. This disorder often is seen during the premotor phase and is marked by a delayed emergence in up to 40% of individuals with PD or DLB (Schenck, 1996; Fantini, 2005). Especially in this last category of cases, RBD is associated with cognitive dysfunctions comparable to those seen in early PD and DLB: progressive premotor cognitive, mainly executive, dysfunction with impaired verbal fluency and visuospatial functions (Mahieux, 1998; Simard, 2000). Furthermore, RBD patients also score significantly worse on olfactory threshold, odor discrimination, and odor identification tests. In a recent study confirmed by FP-CIT SPECT, 15% of hyposmic RBD patients were actually found to be suffering from PD. RBD, especially in combination with hyposmia and/or cognitive dysfunction, is considered a common and early premotor manifestation of PD and DLB (Schenck, 1996; Fantini, 2005; Stasny-Kolster, 2005).
6. Neuropsychological investigations of patients with PD have shown specific executive impairments even in the initial phase of the disease, including a deficit of behavioral regulation in sorting or planning tasks accompanied by subtle mnemonic disturbances due to a defective use of memory storage as well as impaired manipulation of internal representation of visuospatial stimuli owing to problems in movement (Morris, 1988; Dubois, 1997; Brown, 1998; Hocherman, 1998). In approximately 20–40% of patients, these

problems eventually may proceed during the course of the disease to dementia, which constitutes an important risk factor for caregiver distress, decreased quality of life, and nursing home placement. Dementia in PD (PDD) is typically characterized by a progressive dysexecutive syndrome with attentional deficits and fluctuating cognition, often accompanied by psychotic symptoms (Bosboom, 2004). In a number of patients, however, dementia with additional delusional and hallucinatory behavior occurs already in the premotor phase, a condition known as DLB. Clinical symptoms in PDD and DLB, except for the debut of motor symptoms, however, are the same: there are prominent executive dysfunctions with visuospatial dysfunction and mild mnemonic deficits combined with psychotic phenomena and fluctuating consciousness, as well as an invariable hypersensitivity to antipsychotics. PDD is, for the most part, a condition that develops during the motor phase, whereas DLB is a typical feature during the premotor phase (Bosboom, 2004).

Staging-based neuropathological findings

Stage 1

The progressive neurodegenerative process associated with idiopathic PD is marked by the formation of proteinaceous inclusion bodies, Lewy bodies and Lewy neuritis, that involve susceptible neuronal types of the human nervous system (Braak, 2000, 2003a, 2004). Most probably, the substantia nigra is not the first site in the brain to become involved in PD (Del Tredici, 2002). Instead, the formation of Lewy pathology in PD appears to begin in the olfactory system as well as in the central autonomic nervous system, more precisely in the dorsal glossopharyngeus-vagus complex (Vanderhaeghen, 1970; Oppenheimer, 1980; Hague, 1997; Iwanaga, 1999; Del Tredici, 2002; Braak, 2003b).

The olfactory bulb, olfactory tract, and/or anterior olfactory nucleus can be involved very early in the disease, e.g., in incidental cases (Del Tredici, 2002; Braak, 2003b, 2004). In a quantitive analysis using tyrosine hydroxylase immunohistochemistry, Huisman and colleagues found that the number of dopaminergic cells in the olfactory bulb of PD patients was twice as high compared to age- and gender-matched controls. They suggested that increased levels of dopamine in the olfactory glomeruli, known to inhibit olfactory transmission, may be responsible for PD-related hyposmia, thus explaining the fact that hyposmia in these patients is not levodopa-responsive (Huisman, 2004).

In PD and also in DLB, Lewy bodies have been seen in autonomic regulatory regions, such as the hypothalamus and the sympathetic (intermediolateral nucleus of the thoracic spinal cord, sympathetic ganglia) and parasympathetic (dorsal motor vagus, sacral spinal cord) nuclei, as well as in the enteric nervous system, cardiac plexus, pelvic plexus, and adrenal medulla, thereby making it clear that both central and peripheral autonomic nervous systems are involved (Vanderhaeghen, 1970; Oppenheimer, 1980; Hague, 1997; Iwanaga, 1999; Braak, 2003b). To the extent that the large projection neurons of the dorsal motor vagus cell complex in the medulla oblongata connect the central nervous system with postganglionic nerve cells of the enteric nervous system, it has been hypothesized that PD may even start in these autonomic postganglionic neurons outside the CNS (Braak, 2003b; Kaufmann, 2004). Nonetheless, involvement of the peripheral nervous system in stage 1 still awaits confirmation.

Stage 2

Stage 1-related pathology worsens in this stage, and additional lesions occur in the medulla oblongata and pontine tegmentum, including the lower raphe nuclei, the magnocellular portions of the reticular formation – in particular the gigantocellular reticular nucleus – and

Table 1. PD-related non-motor signs and symptoms in relation to neuropathology (Vanderhaeghen, 1970; Oppenheimer, 1980; Hague, 1997; Iwanaga, 1999; Del Tredici, 2002; Braak, 2000, 2003a, b, 2004; Huisman, 2004; Braak and Braak, 2005)

Non-motor signs and symptoms	Braak & colleagues	Extra-nigral pathology
Hyposmia	Stage 1	Olfactory bulb, anterior olfactory nucleus
Dysphagia		Dorsal motor glossopharyngeal nucleus
Constipation		Dorsal motor vagal nucleus
Bladder dysfunction		Postganglionic parasympathetic neurons*
Sexual dysfunction		Preganglionic and postganglionic
Orthostatic hypotension		sympathetic neurons*
Pain, inattention	Stage 2	Coeruleus-subcoeruleus complex
Depression, sleep disorders		Lower raphe nuclei
Executive dysfunction		Gigantocellular reticular nucleus
Autonomic dysfunctions	Stage 3	Central subnucleus of the amygdala
Executive dysfunction		Ventral tegmental area
REM sleep behavior disorder		Pedunculopontine nucleus
Inattention, drowsiness and		Magnocellular cholinergic nuclei
cognitive dysfunction		of the basal forebrain
fluctuating consciousness with		Oral raphe nuclei
Executive dysfunction, apathy,	Stage 4	Anteromedial temporal mesocortex
mnemonic, and emotional problems		Basolateral subnuclei of the amygdala
Agnosia and apraxia	Stage 5	Prefrontal and high order sensory association fields of the neocortex
Sensorimotor dysfunctions	Stage 6	Premotor and first order sensory association fields of the neocortex, as well as primary areas of the neocortex

*Awaits confirmation

the coeruleus-subcoeruleus complex. These cellular complexes function together as the 'gain-setting' or 'level-setting' system of the reticular formation. Noradrenergic coeruleus cells modulate the cortical signal-to-noise ratio and are important for attentional mechanisms. As part of the medial pain system, these neurons also regulate the pain-control system that inhibits the relay nuclei for somatosensory and viscerosensory input (Scherder, 2005). Via the limbic system, serotoninergic cells of the raphe nuclei play a role in the regulation of mood and affection. Coeruleus and raphe neurons also inhibit the tegmental pedunculopontine nucleus, which stimulates REM sleep. Reticular cholinergic cells project to the thalamus, hypothalamus, and the basal nucleus of Meynert that cholinergically innervates the cerebral cortex. Together with the serotoninergic raphe cells, the reticular cholinergic neurons also regulate the activity of the forebrain, thereby having a major influence on arousal mechanisms and states of consciousness.

Stage 3

As it moves upwards through the lower brainstem, the process crosses the upper limit of the pontine tegmentum and enters the basal portions of midbrain and forebrain. The nigral substance and ventral tegmental area become involved together with the limbic central subnucleus of the amygdala, which regulates the gain setting system as well as the dorsal motor vagal nucleus. In this stage, the cholinergic tegmental pedunculopontine

nucleus becomes affected as well as the serotoninergic oral raphe nuclei, the cholinergic magnocellular nuclei of the basal forebrain, and the hypothalamic tubermamillary nucleus.

Stage 4

The process continues to advance upwards to the anteromedial temporal mesocortex including the transentorhinal region, the most highly vulnerable portion of the transition zone between the allocortex and neocortex. The temporal mesocortex mediates the transfer of neocortical high-order sensory association areas (via the amygdala, entorhinal cortex, and hippocampal formation) to the prefrontal neocortex. Thus, impairment of this neocortico-limbic-neocortical system that is essential for adequate selective data processing may be accompanied by executive and mnemonic dysfunction, loss of self-initiative, and apathetic behavior. In the majority of cases with stage 4 pathology, Lewy neuritis can be seen for the first time in the second sector of the Ammon's horn.

Stages 5 and 6

During the last two disease stages, the inclusion body pathology progresses from the temporal mesocortex to the neocortex. Mesocortical and neocortical areas that undergo late myelination degenerate first, whereas those that myelinate earlier are spared or become involved only later (Braak, 2003b, 2004). As such, Lewy neuritis and Lewy bodies first occur in the prefrontal and high-order sensory association areas of the neocortex, followed by their appearance in the premotor and first-order sensory association areas and, finally, in the primary sensory fields, such as the auditory field in Heschl's gyrus (Braak, 2003a, 2004). In the final stages, the progressive degeneration seen at earlier stages is more severe and capable of contributing to additional functional deficits related to late-stage neocortical degeneration.

Clinico-pathological correlations

Not only the impact of premotor symptoms on the activities of daily living but also our awareness of them changes. For instance, patients are only seldom aware of their hyposmia and their mild autonomic dysfunctions, such as orthostatic and postprandial hypotension, bowel and bladder disturbances, and sexual dysfunction. They react to these inconveniences as daily discomforts within tolerable limits or attribute another explanation to seemingly minor ailments, such as diffuse pains. Depression, for instance, might be considered to be a consequence of mid-life crisis, and sleep fragmentation as well as mild cognitive impairment as inevitable in aging. This underscores the necessity to adequately search for these symptoms. Again, such signs and symptoms are *per se* neither specific nor sensitive for PD but in combination they might be highly suggestive for the disease (Koller, 1998; Przuntek, 2004). In summary, the severity of signs and symptoms caused by degeneration of olfactory and autonomic systems depends on the exact location and extent of the local degeneration as well as on the local rate of progression. Thus, the first clinical expression associated with the initially predisposed lesion sites may become overt years before motor symptoms emerge, but, by the same token and depending the nature of the local process, in some cases later.

Hyposmia, autonomic dysfunctions, pain, and sleep fragmentation are common findings in patients with PD. It is plausible that stage 1 and 2-related pathology in most patients comes to clinical expression well before stages 3–4 when lesions in the nigral substance have sufficient impact (a functional loss of 50%) to induce the first signs of motor parkinsonism (Ponsen, 2004). Based on detailed neuropathologic studies in patients with pure autonomic failure, Oppenheimer, already in 1980, was the first to suggest that progressive degeneration with Lewy bodies in the central and peripheral autonomic system was

associated with PD (Oppenheimer, 1980). In fact, his observations gave birth to the suggestion that the phenotype of this disease correlates with the regional localization of the Lewy bodies: a brainstem distribution in parkinsonism, a cortical distribution in dementia, and Lewy bodies in the autonomic pathways in autonomic failure (Oppenheimer, 1980). Based on the same observations, combined with the idea of a stereotypical topographic pathological progression, Braak and colleagues (Braak, 2003b) hypothesized that PD might originate outside of the central nervous system. In that case, they reasoned, a yet unidentified pathogen could be capable of passing the mucosal barrier of the gastrointestinal tract to enter the central nervous system by retrograde axonal and transneuronal transport along post- and preganglionic neurons (Braak, 2003b).

Depression is significantly associated with the subsequent incidence of PD (Schuurman, 2002). In patients suffering from depression and anxiety, these conditions were found to be related to both a specific loss of dopamine and serotonin/noradrenaline innervation. In the premotor phase, depressive symptoms most probably are related to the Lewy body pathology in serotoninergic raphe cells known to play a role in the regulation of mood and affection.

Up to 30% of a consecutive series of PD patients was found to suffer REM-sleep disorders with daytime sleepiness and fluctuating attention, mostly in combination with executive deficits and hyposmia. As a matter of fact, the presence of RBD was also associated with an increased risk of manifesting hallucinations and delusions. In one-third of the patients, these problems became overt in the premotor phase (Pacchetti, 2005). REM sleep disorders, therefore, and especially RBD, with underlying stage 2 and 3-related pathology in the medulla/pons, might be regarded as specific premotor manifestations of both, PD and DLB (Schenck, 1996; Mahieux, 1998; Simard, 2000; Stiasny-Kolster, 2004).

Premotor signs of mild executive cognitive dysfunction are seen in nearly all PD patients and might be explained by the stage 2 noradrenergic and serotoninergic neuronal degeneration with sleep fragmentation as well as daytime sleepiness with inattention. Executive functions represent a range of cognitive functions under the control of the frontal lobes and generally require some sort of working memory and optimal attention. Degeneration of the ascending cholinergic and catecholaminergic neuronal systems may contribute, at least in part, to the occurrence of frontal-lobe-like symptomatology associated with PD (Fantini, 2005).

Stage 3-related cortical cholinergic denervation may or may not precede cortical dopaminergic denervation, but sooner or later both conditions may accumulate, and choline deficiency-related fluctuating inattention and drowsiness can attenuate dopamine-deficiency-induced executive processes or *vice versa*, and mild cognitive impairment usually proceeds to dementia, often in combination with delusions and hallucinations. Accordingly, further cognitive deterioration may develop with the occurrence of the first stage 3–4-related signs of involvement of the ventral tegmental area (with dopaminergic projections to the frontal lobes) as well as the tegmental pedunculopontine nucleus and the basal nucleus of Meynert, amygdala, and anterior temporal mesocortex. Degeneration of the pedunculopontine nucleus, for example, may induce RBD with significant executive dysfunction as well as dementia with delusions and hallucinations, a condition known as DLB (Schenck, 1996; Mahieux, 1998; Simard, 2000). Cognitive deterioration can be accelerated by aging, with the further loss of the integrity of the ascending cholinergic innervation of the cerebral cortex. All of this may occur well before – but also after – the first motor signs and symptoms become manifest when nigral degeneration finally reaches the clinical threshold. Stage 3-related degeneration of the nigral system requires

some time to exceed the threshold for induction of the first motor signs (Tissingh, 1998a, b), and additional stage 3 and even stage 4–6 pathology-related clinical signs, depending on local disease progression, may become overt in the interim. Eventually, concomitant Alzheimer-related pathology also may contribute to this process.

Owing to the stage 4 involvement of the basolateral amygdala, emotional and/or motivational disorders might also become manifest together with loss of initiative, apathy, executive dysfunction, and mnemonic problems. Furthermore, given the modest involvement of the mesocortex and Ammon's horn, further mnestic disturbances might arise. When the pathological process reaches the neocortex in stages 5 and 6, however, more cortical cognitive dysfunction with dementia develops.

Thus, dementia with delusions and hallucinations may occur both in the premotor phase as well as in the motor phase. Cummings considers both conditions (the one beginning with RBD and dementia complicated by visual hallucinations and late parkinsonism, the other beginning with motor parkinsonism and progressing to PDD) to be manifestations of DLB, inasmuch as the principle PDD pathology is posited to consist of cortical Lewy bodies, which suggests that PDD is a form of DLB (Cummings, 2004). Because in some individuals cognitive decline can develop in the presence of only mild PD-related cortical pathology and, conversely, widespread cortical PD lesions do not necessarily lead to cognitive decline, Cummings' conclusion seems premature (Waxman et al., 1990). On the contrary, it makes more sense to consider both conditions (DLB and PDD) as manifestations of PD. It appears necessary to posit the existence of a translational period – with mild to moderate non-motor impairments – that includes cognitive dysfunction with or without psychotic symptoms related to stage 1–4 pathology, beginning around (and sometimes prior to) motor signs and symptoms, which require several years to become clinically overt (Braak, 2005).

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Detection of preclinical Parkinson's disease along the olfactory trac(t)

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Summary. The association of Parkinson's disease (PD) with an impaired sense of smell was first reported about thirty years ago. Since then, it has become quite firmly established that olfactory dysfunction is one of the first and most prevalent clinical manifestations of this disorder. Recent data from an ongoing prospective study indicate that otherwise unexplained hyposmia in first degree relatives of patients with sporadic PD is associated with an increased risk of developing clinical PD of at least 13%. In particular, a combination of impaired olfactory function and reduced striatal [^{123}I]β-CIT binding on a baseline SPECT scan appears to be a strong predictor of a subsequent diagnosis of PD. Pathological studies support these observations by demonstrating that the anterior olfactory structures may be one of the induction sites of PD pathology. Considering that there is a doubling rather than a loss of dopaminergic neurons in the olfactory bulb in PD patients, the pathophysiology of olfactory dysfunction in PD is far from being elucidated. Studying prodromal manifestations of PD, such as olfactory dysfunction, and their underlying pathophysiology may greatly contribute to the development of treatment strategies that focus on preclinical detection and slowing down disease progression.

Introduction

In Parkinson's disease (PD), the second most prevalent neurodegenerative disorder, the appearance of the classical motor triad marks the end of a lengthy preclinical phase in which the degenerative process already afflicts much damage to the brain. Any type of treatment aimed at slowing down disease progression should therefore preferably be applied prior to the onset of parkinsonism. This would both increase the number of neurons remaining to be protected from degeneration as well as lengthen the time frame available for neuroprotection. Preclinical detection of PD has become one of the most challenging goals to achieve in clinical neurology. The olfactory approach to this issue appears to be particularly promising. Although the association of PD with an impaired sense of smell has been known for about thirty years, the true significance of these olfactory impairments is only beginning to be fully appreciated. Recent data emphasize the potential early diagnostic and etiological implications of the presence of olfactory deficits in PD patients.

Olfactory deficits in Parkinson's disease

Psychometric testing of olfactory function in PD patients has revealed that the prevalence of olfactory impairments is as high as 80–90% (Doty et al., 1988; Hawkes et al., 1997), thus at least equaling the prevalence of the characteristic resting tremor. The olfactory deficits are not restricted to any single functional modality but include impairments of odor detection, discrimination and

identification. Moreover, deficits in olfactory function can be detected from the earliest clinical stage of disease onwards (Tissingh et al., 2001). Evidence is accumulating that olfactory testing can also be used to distinguish PD from several other disorders, such as progressive supranuclear palsy, vascular parkinsonism and essential tremor.

Olfactory deficits as a risk factor for Parkinson's disease

Patients with an established clinical diagnosis of PD often recall a loss or reduction of the sense of smell that started years before the onset of motor signs. Reports of olfactory dysfunction in asymptomatic first degree relatives of patients with a familial form of parkinsonism or sporadic PD further strengthened the idea that olfactory impairments might be a preclinical or prodromal sign of PD (Markopoulou et al., 1997). Some six years ago, we designed a prospective study, involving a cohort of first-degree relatives of PD patients, to determine whether olfactory impairments are indeed a sign of incipient PD. After exclusion of individuals with (suspected) parkinsonism, memory dysfunction or potential alternative causes of olfactory disturbances, the total number of relatives in the cohort was 361. At baseline, a combination of olfactory detection, identification and discrimination tasks was used to select groups of hyposmic ($n = 40$) and normosmic ($n = 38$) individuals for single-photon emission computed tomography (SPECT), using [^{123}I]β-CIT as a dopamine transporter ligand, to assess nigrostriatal dopaminergic function. Baseline SPECT scans demonstrated a subclinical loss of striatal [^{123}I]β-CIT binding in some of the hyposmic but in none of the normosmic relatives (Berendse et al., 2001). A subclinical degeneration of the nigrostriatal dopaminergic system was later also observed in a small group of hyposmic individuals that were selected for [^{123}I]FP-CIT SPECT scanning using a combination of olfactory testing and transcranial sonography of the substantia nigra (Sommer et al., 2004).

Two years from baseline in our prospective study, a clinical neurological examination and a second [^{123}I]β-CIT SPECT scan were performed in the groups of hyposmic and normosmic relatives. In addition to measuring striatal [^{123}I]β-CIT binding at two years, we calculated the rate of decline of striatal [^{123}I]β-CIT binding from baseline. A Dutch translation of a validated questionnaire, sensitive to the presence of parkinsonism, was used in the follow-up of the relatives that were not selected for baseline SPECT scanning and in a few relatives ($n = 4$) that chose to withdraw from the groups of scanned individuals. A total of ten out of 361 individuals were lost to follow-up, six of whom died. At the two year follow-up visit, 10% of the individuals with unexplained hyposmia, who also had strongly reduced baseline striatal [^{123}I]β-CIT binding, had developed clinical PD as defined by the United Kingdom PD Brain Bank criteria (Ponsen et al., 2004). By contrast, none of the other relatives in the cohort (i.e. those in the normosmic or questionnaire groups) fulfilled these criteria. Furthermore, analysis of the SPECT scans revealed that among the hyposmic relatives who had not developed parkinsonism, the average rate of decline in [^{123}I]β-CIT binding was significantly higher than in the group of normosmic relatives (Ponsen et al., 2004). The latter finding would seem to indicate that at least some hyposmic individuals had developed a subclinical degeneration of the dopaminergic system that might be expected to ultimately result in the development of clinical motor signs.

At present, we are in the midst of the four year follow-up of the cohort that again includes a clinical neurological examination and [^{123}I]β-CIT SPECT scanning. Having re-evaluated little more than half of the cohort, our preliminary clinical data indicate that

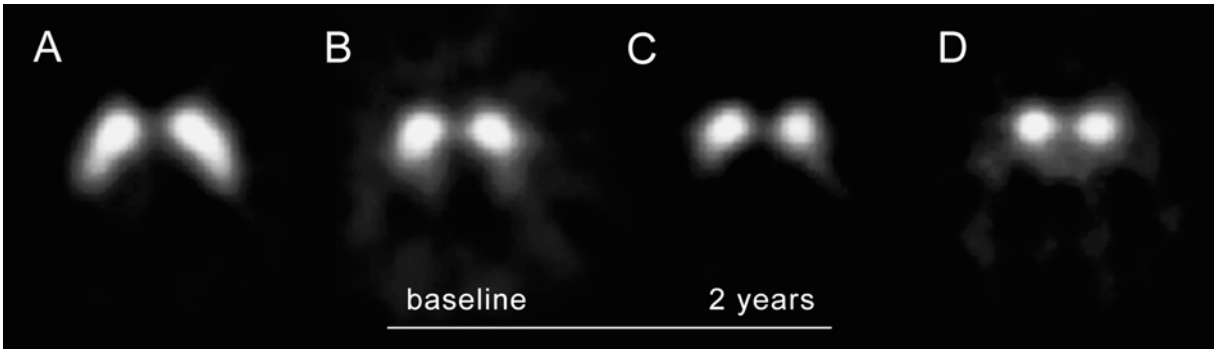

Fig. 1. [^{123}I]β-CIT SPECT images. **A** Two year follow-up scan of normosmic relative. **B**, **C** Baseline and two year follow-up scans of hyposmic relative, asymptomatic two years from baseline, clinical diagnosis of PD four years from baseline. **D** Two year follow-up scan of hyposmic relative, clinical diagnosis of PD two years from baseline

so far one more hyposmic relative has developed the classical motor signs of PD. Interestingly, baseline striatal [^{123}I]β-CIT binding in this individual was within the range of control values. Two years from baseline, [^{123}I]β-CIT binding in this case had dropped below control values (Fig. 1). In this single individual, hyposmia apparently developed even before the appearance of a significant loss of nigrostriatal dopaminergic terminals. The data obtained thus far in this ongoing prospective study are compatible with the notion of a slowly developing degenerative process that affects the olfactory system prior to the onset of clinical motor signs and quite possibly prior to the onset of nigral dopaminergic cell loss.

Parkinson's disease pathology in anterior olfactory structures

Recent neuropathological studies in PD have provided compelling evidence in favor of a topographically predictable spreading of degenerative changes over the brain (Braak et al., 2003). Based on the distribution of alpha-synuclein containing Lewy bodies and Lewy neurites, six different pathological stages of PD have been proposed. The most striking observation is that Lewy body pathology in a number of extranigral brain areas, including the olfactory bulb and tract, appears to precede degeneration of nigrostriatal dopaminergic neurons. Although the presence of neuropathological changes typical of PD in the olfactory bulb and tract has been described previously (Pearce et al., 1995), these novel data suggest that the anterior olfactory system may actually be one of the induction sites of the neuropathological process in PD.

Pathophysiology of olfactory deficits in Parkinson's disease

Oxidative stress-induced degeneration of dopaminergic neurons in the substantia nigra is an important pathological feature of PD. The presence of numerous dopaminergic neurons in the olfactory bulb might lead one to assume that olfactory dysfunction in PD is related to a loss of these neurons. In an effort to substantiate this assumption, anatomists in our research institute performed a quantitative analysis of the number of dopamine neurons in the post-mortem human olfactory bulb. Quite contrary to the original expectations, a doubling of the number of TH-positive periglomerular neurons was found in the olfactory bulb of patients with a pathologically confirmed diagnosis of PD relative to age-matched controls (Huisman et al.,

2004). Considering that dopamine is known to inhibit neurotransmission between the axons of olfactory receptor cells and the dendrites of mitral cells within the glomeruli, it is tempting to speculate that the increase in dopaminergic neurons in the olfactory bulb is responsible for the hyposmia in PD. Along these lines, it would seem logical that olfactory dysfunction in PD does not improve with the administration of dopaminergic agents (Doty et al., 1988). Apparently, one of the earliest clinical signs of PD, i.e. impaired olfactory function, is unrelated to dopaminergic hypofunction.

Many questions raised by these observations remain to be answered. Firstly, one may wonder why dopaminergic neurons in the olfactory bulb are resistant to the degenerative process in PD. Perhaps their resistance is related to the absence of melanin, a possibility supported by the fact that several other populations of non-melanised dopaminergic neurons remain intact in PD. Secondly, it is unclear why there should be an increase in the number of dopaminergic neurons in the olfactory bulb. The tentative hypothesis that this may be a compensatory mechanism for the loss of nigral dopamine neurons is at odds with recent pathological observations that the olfactory bulb is affected by the disease process before the substantia nigra. Ultimately, we need to know in what way the known pathological changes, i.e. the increase in the number of dopaminergic neurons, the presence of Lewy bodies and neurites in the olfactory bulb and tract, and the neuronal loss in the anterior olfactory nucleus contribute to the olfactory deficits in PD.

Conclusions

In summary, several lines of evidence seem to converge to show that in PD degenerative changes and functional disturbances in the olfactory system already become apparent during a prodromal or preclinical period that ends with the appearance of the classical motor triad. Olfactory testing is easy to perform and will therefore become a central element of strategies aimed at the preclinical detection of PD patients. Since olfactory loss may occur in many other conditions including Alzheimer's disease and head trauma, it is unlikely that olfactory testing will be able to stand alone as a screening tool for PD. Additional elements of a successful future screening strategy might include genetic or other susceptibility markers on the one hand, and highly specific biological markers of the disease process such as SPECT or PET scanning of the nigrostriatal dopaminergic system on the other hand. Widespread screening for preclinical cases of PD can of course only be justified once we have an effective neuroprotective treatment to offer affected individuals. Future studies into the pathophysiology of olfactory dysfunction in PD may draw our attention to novel pathogenetic mechanisms and thus contribute to the development of neuroprotective treatments.

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The clinical approach to gait disturbances in Parkinson's disease; maintaining independent mobility

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Summary. Gait is affected in all stages of Parkinson's disease (PD) and is one of the hallmarks for disease progression. The fear of getting into the wheel chair is one of the first thoughts many patients ask about when the diagnosis of PD is given. At the early stages of the disease gait disturbances are present and can be measured but in most patients it does not cause significant functional disturbances. In contrast, as the disease progress, gait disturbances and postural control abnormalities are becoming major causes for lost of mobility and falls. These unfortunate consequences should be forecasted at the early stages of the disease and a preventive approach should be taken. Treatment of gait disturbances at the early stages of the disease is mainly to encourage patients to exercise and walk daily and by drugs in those with disabling symptoms. At the advanced stages, treatment should be aggressive in order to keep the patient walking safely. Drugs, physiotherapy and functional neurosurgery should be used wisely for best outcomes and least side effects. When time comes and the risk of falls is very significant, walking aids should be suggested and if no other option is left, wheel chair is a very reasonable option to maintain mobility out of home, preserving quality of life and avoiding falls with all it severe consequences.

Introduction

Gait disturbances are among the most important motor problems associated with Parkinson's disease (PD). They are the presenting symptom in 12–18% of the cases and will affect all patients as the disease progress (Martin et al., 1973; Pahwa and Koller, 1997). Gait disturbances can lead to falls, insecurity, fear and loss of mobilization, independence, and institutionalization (Martignoni et al., 2004; Balash et al., 2005). Hoehn and Yahr in their original motor staging of PD already stated that most if not all patients will develop postural instability (stage 3) and gait disturbances (stage 4) as the PD progress (Hoehn and Yahr, 2003). As such, prevention, delaying or treating parkinsonian gait disturbances should start right from the time of diagnosis and continue throughout the course of the disease. The therapeutic strategy should be based on the current understanding that postural instability and locomotion disorders will play a major role in the advanced stages when the fight to maintain mobility and independency will be at the base of the entire treatment. Another indirect factor in the battle for secured and independent walking is the mental state. Cognitive decline, anxiety or depression can affect the patient's drive as well as the interaction with the environment. Misjudgment of possible danger or true

obstacles in the environment or the patient's actual functional state can frequently lead to falls. Similarly, excessive fear of falling or panic attacks is frequently leading to avoidance behavior and self chosen ''home arrest''. Assessing and treating affective and cognitive aspects are pivotal factors in the fight to keep patients walking secured and independent.

Treating PD patients in general and gait disturbances in particular, should take into consideration the stage of the disease and the degree of disability.

Clinical approach when the risk to fall is low and mobility is well preserved

The clinical approach to the parkinsonian patient at the early stages of the disease when there are objective gait disturbances but their impact on daily function is still minor or moderate should be conservative. All patients at these stages are fully independent but are understandably worried about the future. The most common problems at the early stages are complaints of slow walk, shuffling gait and decreased arm swing, mainly on the more affected side of the body. These symptoms develop slowly and, as a result, most patients are not aware of the functional deterioration. It is frequently the spouse who first notices such changes and organizes the first appointment with the doctor. The fact that there is no significant disability and that the patient can adjust his/her daily activities according to the difficulties, can give the treating physician and the family the options if to treat the gait disturbances medically or physically. If medically, all antiparkinsonian drugs can ease some of the burden at this stage. If physically, a daily walk and exercise can give significant benefit and some times postpone the need for medications. Observing a disciplined regimen of daily exercise has many positive outcomes, several of which were mentioned above. It is a common belief that exercise during the early stages of PD will delay or slow down physical deterioration and loss of mobility, even though such data has never been published base on class IA evidenced based research. The recommendation to exercise daily has additional benefits like paying attention to the general fitness and weight, building the muscles, and strengthening the bones. Furthermore, daily exercise is an active self dependent task which leaves some control in the patient's hands in addition to its positive effect on mood and cognition.

Based on the general knowledge that as PD will progress and that gait and balance problems will inevitably develop, a ''delaying'' approach should be taken from the time of diagnosis. The therapeutic plan should be geared to deal with the patient's general physical condition, general affective and cognitive aspects, strategies for the prevention of falls and associated injuries, as well as adopting a positive attitude of being active and taking responsibility in the fight for independency and mobility.

Many non-neurological problems can affect mobility and balance among these adult patients. They should be urged to aggressively treat any existing hyperlipidemia, diabetes mellitus, cardiac problems and hypertension (Skoog and Gustafson, 2003). They should be encouraged from the very early stages of the disease to keep their body weight down to BMI = 25 or less, considering the deleterious contribution of overweight to instability and immobilization (Mc Graw et al., 2000) as well as to brain dysfunction and the development of dementia (Gustafson et al., 2003). Special attention should be given to the feet, joints and spinal column because of the significant role of the musculoskeletal system in mobility and gait. In general, patients in the early stages of the disease do not realize the extent to which their general health status will effect their future mobility, and it is the responsibility of the neurologist to make the patient aware of these preventive aspects. This approach should be maintained throughout the

course of the disease, and every visit should start with a discussion on the assessment and control of non-neurological issues.

Gait disturbances and falls are closely related to the individual's affective state and cognition (Hoehn and Yahr, 1967; Adkin et al., 2003; Jantti et al., 1995; Whooley et al., 1999; Lenze et al., 2004). Depressed people fall and break bones as a result of their falls more frequently than non-depressed people (Jantti et al., 1995; Whooley et al., 1999; Lenze et al., 2004). Aggressive treatment of depression can have a significant impact on the willingness of the PD patient to exercise and take steps to enhance his/her physical fitness. It is vitally important to treat depression either medically or by psychosocial support or both. Among its many benefits, physical activity can also improve mood with its recognized positive consequences.

Dementia is a widespread complication of advanced PD and a significant contributing factor to the occurrence of falls. Dementia is the end result of many slowly progressive pathological processes, such as atherosclerosis, obesity, depression, lack of cognitive stimulation or head trauma. Treating all secondary risk factors can delay or slow down the rate of cognitive decline, with significant impact on the mental performance of PD patients at the more advanced stages of the disease.

Another aspect of delaying potential consequences of PD is the early detection and aggressive treatment of osteoporosis. Osteoporotic bone is significantly more vulnerable to injury, and even minor trauma can sometimes cause fractures that require surgery and lead to loss of mobility. All PD patients in all stages of the disease should be educated to assess their bone density regularly throughout the course of the disease and follow professional advice how to protect or treat osteoporosis.

Symptomatic medical treatment aimed specifically for gait disturbances should be given at the early stages only if it causes significant disability or may lead to fall. A mild to moderate gait slowness or a decreased arm swing do not justify the use of drugs. In contrast, a history of frequent falls or shuffling gait with low ground clearance are danger and should be treat aggressively.

Clinical approach when the risk to fall is high and mobility is compromised

Disturbed gait and postural control represent major and very disabling aspects of advanced parkinsonism affecting most if not all patients (Hoehn and Yahr, 1967). Gait disturbances initially appear at the ''Off'' state, when dopaminergic treatment is less effective. As the disease progresses, even the ''On'' state is associated with gait and postural disturbances which classically manifest as short stride, low speed, shuffling gait as well as stooped posture and freezing of gait (FOG) or propulsion and festinations (Morris et al., 1994, 1996; Baatile et al., 2000; Giladi et al., 1992, 2001). In addition, significant gait dysrhythmicity with increased stride-to-stride variations (Schaafsma et al., 2003) and left/right steps asymmetry (Plotnik et al., 2005) has been recorded by sensitive gait assessment tools. The importance of those sub clinical measures is their predictability for FOG and falls (Bloem et al., 2004). Most gait disturbances can initially be improved up to the level of a normal gait during the ''On'' state when medications are effective. Other common problems of advanced parkinsonian stages are involuntary leg movements in the form of ''Off'' dystonia and ''On'' dyskinesia. At the advanced stages of parkinsonism, cognitive disturbances play a major role in the fight for mobility and independence without falls. Dementia can significantly influence the therapeutic options as well as the risk to fall.

Fine-tuning of the anti-parkinsonian medications can decrease the total daily ''Off'' time and ''On'' dyskinesia with a direct and immediate effect on secured mobility and stability.

Aside from optimal control and fine-tuning of ''Off'' and ''On'' periods, specific treatments can improve specific problems and non-motor disturbances that might have a significant impact on gait. Orthostatic hypotension, depression, and dementia should all be treated aggressively medically and behaviorally. All can be improved by medications and appropriate exercise and support with significant impact on patients mobilization safely.

At the most advanced stages of PD when ''Offs'' are very frequent and very disabling with dyskinesias which can cause major disability, functional neurosurgery at the level of the basal ganglia should be considered (Giladi and Melamed, 2000). Pallidotomy and deep brain stimulation of the sub-thalamic nucleus (STN) or the internal globus pallidum (GPi) have been very effective in avoiding motor response fluctuations with the elimination of ''Off'' periods and dyskinesias (Allert et al., 2001; Ferrarin et al., 2004).

Posture, balance, gait and transfers could be targeted by physiotherapists (Rubinstein et al., 2002; Plant et al., 1997). Physical therapy may induce small but significant improvements in gait speed and stride length (Plant et al., 1997). A sensory, cue-enhanced physical therapy program showed improvements lasting up to 3 months after the therapy had ended (Rubinstein et al., 2002; Nieuwboer et al., 1997).

General fitness can be maintained by daily exercise which should be recommended to every patient but even more persuasively to those at the more advanced stages of PD. A daily walk for 30–45 minutes during the ''On'' period is highly recommended for general health as well as for specific physical and mental needs. Daily walking has been shown to improve stride length and walking speed with a carryover effect of several months, even when the exercise was stopped (Sunvisson, 1997; Lokk, 2000; Scandalis et al., 2001).

Mobilization should be maintained for as long as possible but not at the price of risking the individual to dangerous falls. Walking aids should be considered if drugs and behavioral treatment cannot maintain safe walking. Only rarely will the patient be the first to suggest the use of walking aids, so the obligation of raising this issue falls upon the doctor or the physical therapist. When walking becomes extremely difficult and dangerous and demands much effort and energy but does not substantially improve the patient's quality of life, it is time to switch the patient's mindset to now regard walking as an exercise without any mobilization goal. This is the time to introduce the use of wheelchair for actual mobilization and represents the end of the fight for ambulatory independence. When instability becomes a major risk for falls, walking aids can decrease the risk and preserve mobility. Use of a wheelchair is a practical and effective option when all others possible interventions have failed. Beside its stigma and lost of independency the wheelchair can let the patient get out of home and get every where in a safe and easy way.

Conclusions

Walking is affected by parkinsonism throughout the course of the disease. The most significant risk of gait disturbances in PD is falls with its deleterious consequences like fractures, fear of falling and ''self chosen home arrest''. At the early stages of the disease when falls risk is low, patients and their care givers should be encourage to adapt healthy lifestyle, and instruct to treating all risk factors for atherosclerosis, dementia and deterioration of physical fitness. Daily exercise can be adopted at this stage to prepare for the future when physical deterioration is inevitable. As the disease progressed, and falls risk become a real danger medical, surgical, mental and physical interventions should be all aimed towards the preservation of independent mobilization and avoid falls. It is a long-term task, which needs a multidisciplinary team of neurologists, internists, ophthalmologists,

physical therapists, and many others. Beside the role of the multidisciplinary team it is the role of the patient and the caregivers tounderstand right from the early stages that voiding falls is a long term task which required constant attention and effort. Only when all forces are combined and talk with each others, falls can be delayed and even prevented.

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Getting around and communicating with the environment: visual cognition and language in Parkinson's disease

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Summary. Vision in PD. In PD an impairment of dopaminergic neurons of the preganglionic retina and a defect of the retinal nerve fibers (axons of the retinal ganglion cells) has been demonstrated and a correlation of loss of spatial contrast sensitivity, with the progression of motor impairment in PD has been described. These low level visual deficits contribute but do not directly explain behavioural visual deficits in PD involving spatial cognition, internal representation, space navigation and visual categorization.

Language deficits in non-demented PD patients can include impairments in comprehension, verbal fluency, and naming. Comprehension deficits become evident when patients are required to process sentences with non-canonical, irregular grammatical structures. Semantic memory deficits may result in the impairments in category fluency and confrontational naming. Selective language deficits may be due to impaired dynamics of the "phonological loop" connecting the pre-frontal cortex and the basal ganglia. A more encompassing linguistic and functional model of PD specific language impairments would be useful for evaluating language deficits in the context of motor dysfunction.

Visual-cognition and language processing are significant non-motor constituents of impaired activities of daily living (ADL) in Parkinson's disease (PD). They present a challenge to functional neuroanatomical models and management of PD patients.

Vision

When asked about vision (Lee and Harris, 1999) PD patients report problems 1) judging distance and motion in the street, 2) reaching for objects, and 3) moving through narrow spaces within their homes. "It's not as simple as it looks . . . I am still going, but I have run out of space to move in . . . my space, our space, is nothing like your space: our space gets bigger and smaller . . . " (from a parkinsonian patient, in Sacks, 1990).

Primary visual processes

Primary visual processes in PD have been summarized in recent reviews (Bodis-Wollner, 2003). Dopaminergic neurons control the preganglionic organization of the receptive field of ganglion cells (Fig. 1). The PD specific preganglionic retinopathy, shown in MPTP monkeys, is quantified with the ERG which can be also performed in patients. The consequence of dopaminergic deficiency of the human retina is apparently retinal nerve fiber (axons of the retinal ganglion cells) thinning. This can be clinically seen and quantified in PD patients. Retinal dopaminergic deficiency leads to specific loss of spatial contrast sen-

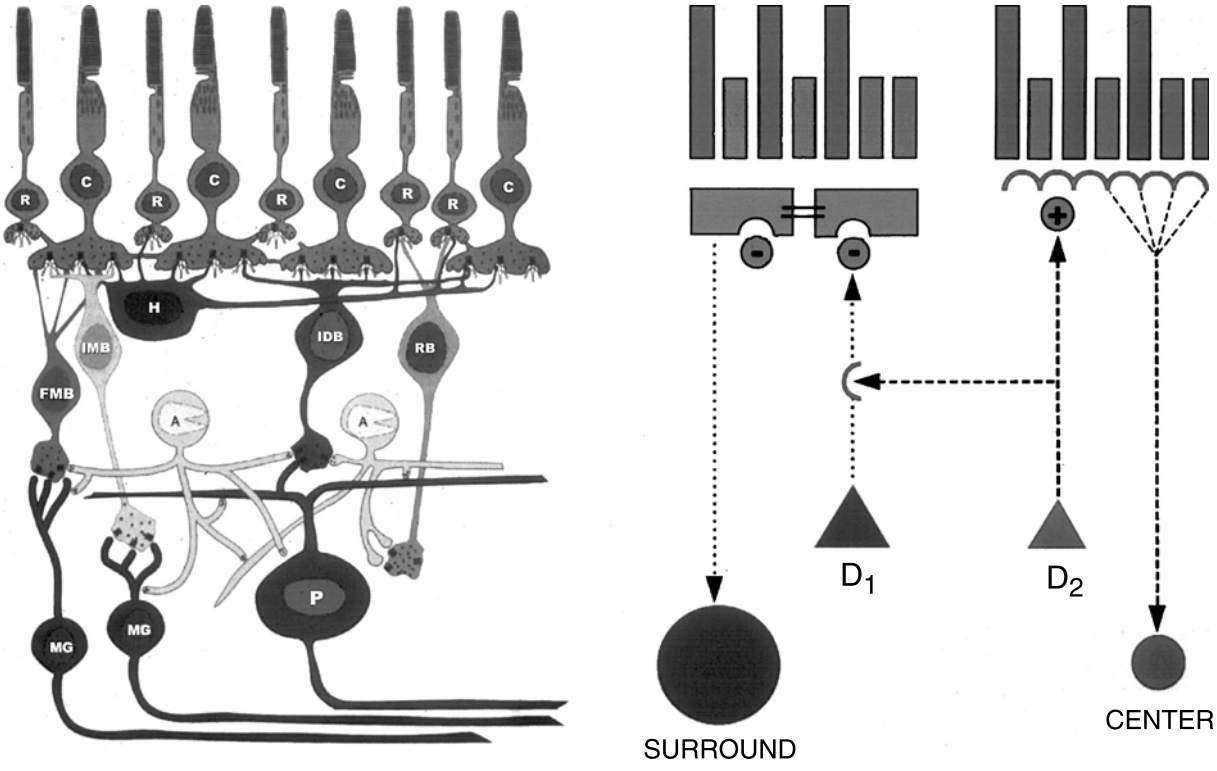

Fig. 1. Left: The network of the mammalian retina consists of five different classes of neurons arranged in different layers: photoreceptors (rods R and cones C), horizontal cells (H), bipolar cells of different classes (invaginating midget bipolars IMB, flat midget bipolars FMB, invaginating diffuse bipolars DB, and rod bipolars RB), amacrine cells (A), and ganglion cells (falling into two main classes, midget ganglion cells MG, and parasol ganglion cells P). An orderly subset of amacrine cells are dopaminergic. The relative proportion of DA amacrine cells is higher in the fovea. There are two sizes of DA amacrines: large and small (not shown). **Right**: Simplified schema of the D1–D2 interaction in the retina. The D1 dopamine pathway locally enhances the ''surround'' signal for individual ganglion cells, while the D2 pathway enhances the ''center'' signal by coupling photoreceptors. Experimental results suggest that these two dopamine pathways are not independent from each other. Consistent with the results we postulate that D2 pre-synaptic receptors are located in the D1 pathway. Thus D2 ligands postsynaptically amplify the ''center'' response while presynaptically attenuate the ''surround''. The effect is functionally synergistic. Conversely postsynaptic D2 blockade attenuates the center response and presynaptic blockade enhances the surround. Thus ganglion cells with large surrounds are particularly affected by D2 blockade while neurons with small centers are easily amplified by D2 agonists. Conversely D1 agonists have a larger effect on large ganglion cells' and negligible effect on small ganglion cells' receptive fields (after Bodis-Wollner, 1990; Bodis-Wollner and Tzelepi, 2002)

sitivity (CS). CS, a measure of contrast vision apart from visual acuity and color vision worsens with progressive motor impairment.

Many tests of visual-spatial deficits show sensitivity to visual and other cognitive processes. Given the hierarchical nature of the visual system, degraded visual information may cause cognitive visual dysfunction. Difficulty seeing and discriminating visual stimuli could be due to impaired CS and could occur as the result of dopaminergic deficiency of the retina. For goal directed movements PD patients have an increased dependence on visual information compared to someone without the disease, suggesting the behavioural consequence and importance of retinal visual impairment. Yet, contrast sensitivity losses in other diseases which affect only the retina do not cause the kind of visuo-cognitive deficits observed in PD.

We summarize below those select cognitive visual defects in PD which are unlikely to be caused **only** by primary visual impairment.

Visuospatial deficits

Visuospatial deficits should lead to difficulty navigating oneself around one's environment. Space exploration is comprised of tasks such as the ability to scan the environment and to move the body to interact with this environment. Scanning the world is impaired in PD but how scanning problems interact with visuospatial deficits has not been established.

Spatial cognition

Spatial cognition is the mental manipulation of spatial information, and this requires visual memory **and** egocentric representation. Oliver Sacks (1990) describes parkinsonian patients who walk on a tilt but perceive their own body and the environment to be perfectly upright. It was shown some thirty years ago by Bowen and colleagues that PD patients show deficits in judging the visual vertical and horizontal, and indeed, advanced PD patients do demonstrate very exaggerated bent postures. However, it has not been established whether the problem is due to visual impairment, to a distorted body centered coordinate system, to a visual working memory deficit, or all of the above. In a simple experimental paradigm of visuo-spatial ability, patients are asked to judge the orientation of lines (Benton). This test is very sensitive to PD; however it may depend on primary visual dysfunction of orientation selective visual neurons.

Visual categorization

Dopaminergic mechanisms in the neostriatum play an important role in categorization. Visual categorization can be quantified with event-related potentials (ERPs) obtained when subjects have to decide whether a briefly presented image contains, for instance, animals or non-animals. In normals, the mean amplitudes of N1 (150–250 ms) and N2 components (400–600 ms) are more negative for non-animal scenes as compared with stimuli containing animals, whereas P2 (250–350 ms) is more positive for animal scenes. In PD, N1 and N2 components were similar for both animal and non-animal stimuli, and P2 is reduced. Apparently, both perceptual (N1) and **semantic** (N2) processes related to the categorization of natural scenes are specifically impaired in PD. In the temporal domain however the slowed semantic processing is preceded by a relatively normally paced perceptual analysis. These electrophysiological results are consistent those hypothesis which emphasize the importance of striatal dopaminergic mechanisms in classification functions.

Internal visual representation

PD patients have difficulty with the mental rotation of objects. This deficit, in the absence of concurrent visual input, is unlikely to depend on primary visual dysfunction. Brown and Marsden (1998) hypothesized that the link between voluntary effort, sensory input and sequencing of motor movements or thought suffers in PD due to a deficient role of the basal ganglia in neuronal binding. Gamma-frequency rhythms of discharge activity from thalamic and cortical neurons are facilitated by cholinergic arousal and resonate in thalamocortical networks. Experimental evidence shows the visual perceptual role of synchronized gamma range brain activity when illusory contours are perceived and when blindfolded subjects execute voluntary saccades. It is probable that gamma recorded over the visual cortices is modulated by prefrontal attentional mechanisms. The role of gamma oscillatory deficits in many aspects of PD has not been widely researched.

Language

Language has traditionally been thought to be unaffected in Parkinson's disease (PD) given

the primarily frontal-striatal pathology of the disease that typically spares the language centers. However, select language impairments, independent of IQ or general verbal abilities, are affected in PD. Although it is uncommon to find non-demented PD patients with frank aphasia syndromes, impairments of language comprehension, verbal fluency, and naming have been shown to occur in the PD patients.

Comprehension

Parkinson's patients usually evidence deficits when processing sentences with non-canonical, irregular grammatical structures (ex., ''sleep–slept'', ''eat–ate'', ''the hat was found by the girl'', ''the dog was hugged by the boy''). Investigations into the specific role of the basal ganglia have helped gain insight into some of the mechanisms involved in processing complex, irregular grammatical structure.

The basal ganglia is not seen to play a primary role in sentence comprehension but rather a secondary/supportive one. In Parkinson's patients, frontal-striatal dysfunction appears to lead to hyperactivation and difficulties with inhibition during late stage language processing (Longworth et al., 2005). Using event-related brain potentials (ERP) components ELAN (early left anterior negativity, present in early stage integration) and P600 (late centroparietal positivity, present in late stage integration), Friederici and colleagues (2003) found the basal ganglia to be more involved in late stage syntactic integration. They also found P600 abnormality in Parkinson's patients and others patient groups with basal ganglia dysfunction.

Fluency

Verbal fluency requires the ability to search for and generate multiple responses within specific time and criteria constraints. Deficits in both letter and category fluency may be a good indictor of incipient dementia in non-demented PD patients and may have utility in predictions of dementia onset in PD patients (Piatt et al., 1999). The most commonly reported fluency deficit in non-demented Parkinson's patients is impaired category fluency, usually with relatively intact letter fluency. Category fluency and letter fluency are seen to have different lexical retrieval demands and although the exact nature of the cause of category fluency deficits in PD patients is not clearly understood, the deficit has been attributed to deficits in working memory, semantic memory, and retrieval from semantic store. It should be noted though that Piatt and colleagues (1999) reported verb generation (vs. noun generation) rather than letter or category fluency to be affected in their PD patients. Neuroimaging and lesion studies have shown verb retrieval to be primarily an anteriorly mediated task, heavily reliant on the fronto-striatal system, while noun retrieval has been shown to access more posterior regions (Bertella et al., 2002). Hence, deficits were hypothesized to be due to either executive dysfunction or grammatical impairment.

Naming

Naming tests, unlike fluency tests, do not have high demands on speed and effortful retrieval in accessing items from semantic store. Hence, it can provide a picture of semantic access mostly independent of executive processes. Naming deficits comparable to verbal fluency deficits are often found in PD, suggesting that semantic memory deficits are present even when executive demands of a task are minimized. Portin and colleagues (2000) acknowledged that although frontal dysfunction can disrupt semantic processing, that alone does not account for the impairment of semantic knowledge in PD. They gave their PD patients external guidance as a compensation for impaired ''self-directed search strategies'' and failed to find improvement in performance.

Discussion

The functional anatomy of cognitive visual skills in PD

Some of the visuo-cognitive deficits may result from caudal involvement, but on the whole the evidence doesn't support that impaired visuo-cognitive deficits would result from simply transmitting distorted retinal signals to the cortex. The evidence is for non-linear transformation between retina and cortex. Furthermore, one has to consider top-down effects from non-occipital cortices on visual processing and the reciprocal modulating effect of the basal ganglia and cortical processing.

One theory of cognitive dysfunction in PD suggests that they are in some way related to disruption of frontal-basal ganglia neural circuits, crucial for executive functions. However, the instruments developed for measuring executive function do distinguish patients with frontal lobe damage from PD patients. Visual categorical deficits suggest that impaired visual working memory in PD has a posterior location (Antal et al., 2002). Brown and Marsden (1998) hypothesized that the link between voluntary effort, sensory input and sequencing of motor movements or thought suffer in PD due to a deficient role of the basal ganglia in neuronal binding. Microelectrode studies established that ''gamma'' frequency, roughly around 40 Hz, reflects synchronization of distinct neuronal groups and their functional binding. Our studies in normal subjects provided evidence that power in the gamma frequency band of the human EEG is modulated by voluntary saccades. Gamma is not a simple correlate of non-specific alertness or diffuse attention. For instance, intrasaccadic gamma may reflect a mechanism for internal monitoring of the movement and short-term plasticity in neuronal connectivity prior to new fixation. The evidence we very briefly summarized raises the question of whether impaired internal predictive control and motor output in PD suffer due to the lack of timely interaction between distributed neuronal groups which include both the basal ganglia and various cortices.

Language in PD and dementia

Language impairment in Parkinson's disease may overlap with dementia, but some PD based language deficits are different in severity and type from those typically found in Alzheimer's dementia (AD). Selective language deficits seen in PD may be due to impaired dynamics of the ''phonological loop'' connecting the pre-frontal cortex and the basal ganglia. There is a clear need for a more complex and extensive cortical/subcortical neuronal model for language processing, such as one which helps to account for the ability to encode an item and its position in a sequence. Language deficits are important constituents of impoverished communication and likely to contribute significantly to impaired activities of daily living. It will require further work to create a more encompassing linguistic and functional model of PD specific language impairments.

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Cardiovascular aspects of Parkinson disease

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Summary. This chapter provides an update about cardiovascular aspects of Parkinson disease (PD), with the following topics: (1) Orthostatic hypotension (OH) as an early finding in PD; (2) neurocirculatory abnormalities in PD + OH independent of levodopa treatment; (3) cardiac and extracardiac noradrenergic denervation in PD + OH; (4) progressive loss of cardiac sympathetic innervation in PD without OH.

Orthostatic hypotension as an early finding in PD

In PD, OH can pose a major management problem. Studies have varied widely in reported frequencies of OH in PD. In 5 large studies involving more than 80 patients each, the frequency of OH ranged from 30 to 58% (Briebach et al., 1990; Magalhaes et al., 1995; Senard et al., 1997; Allcock et al., 2004; Korchounov et al., 2004). A substantial minority of PD patients therefore have OH.

More than a half century ago Nylin and Levander reported the case of a patient with OH who developed orthostatic intolerance at the age of 67. Eight years later he was diagnosed with OH from ''asympathicotonic orthostatism'' and over the course of the next year a resting unilateral tremor, masked face, and ''cogwheel'' rigidity, findings recognized by the authors as typical of PD (Nylin and Levander, 1948). The notion that OH can be an early finding in PD and even precede the movement disorder is therefore by no means new. Of 3 post-mortem case reports about PD + OH patients, where the timing of onset of OH with respect to the movement disorder was reported, in all 3 OH had developed first (Vanderhaeghen et al., 1970; Schober et al., 1975; Kaufmann et al., 2004).

Carrying out an analysis of the frequency of OH as an early finding in PD + OH would require evidence that the patients did not have multiple system atrophy (MSA), which is almost always associated with OH. Diagnosing MSA differentially from PD + OH can be very difficult clinically. Autopsy studies have revealed a disappointingly high frequency of erroneous diagnosis, even by well experienced academicians. To exclude patients with MSA, we took a novel tack based on results of cardiac sympathetic neuroimaging. As discussed below, remarkably consistent and by now abundant literature shows that cardiac sympathetic neuroimaging distinguishes PD from MSA, with cardiac sympathetic denervation in the former but not the latter. We reviewed medical history data from patients with PD + OH evaluated at the NIH, to determine the frequency with which OH was an initial or early finding. Neuroimaging evidence of cardiac sympathetic denervation was used to exclude MSA.

We found that 21 (60%) of 35 PD + OH patients had an early onset of OH. In 4

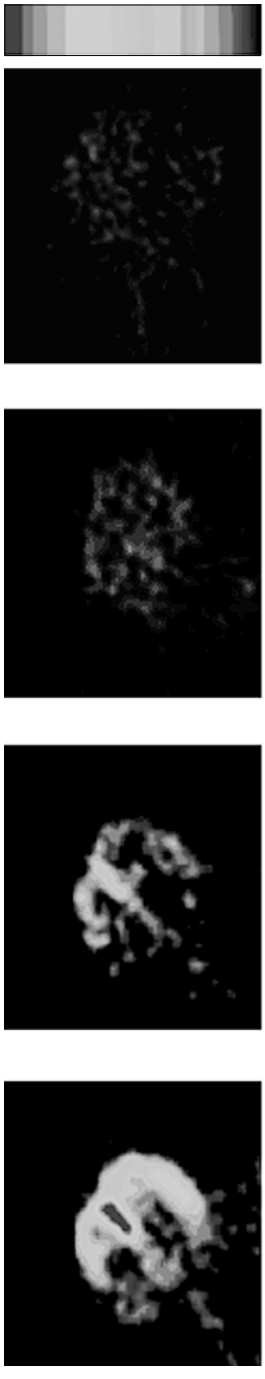

Fig. 1. Progressive loss of cardiac sympathetic innervation, as indicated by 6-[^{18}F]fluorodopamine-derived radioactivity, in a patient with Parkinson disease without orthostatic hypotension

(13%), OH had developed before symptoms of a movement disorder. In 4 others the patients had no symptoms of a movement disorder at the time of evaluation but nevertheless had sufficient clinical signs to diagnose PD (Goldstein, 2005).

Neurocirculatory abnormalities in PD + OH independent of Levodopa treatment

Levodopa is a precursor of dopamine and therefore of norepinephrine (NE), the sympathetic neurotransmitter. In the treatment of PD, levodopa is usually combined with an inhibitor of L-aromatic-acid-decarboxylase that does not penetrate the blood–brain barrier. The combined treatment augments delivery of levodopa to the brain and mitigates nausea and vomiting thought to result from occupation of dopamine receptors outside the blood–brain barrier. Such treatment attenuates but does not prevent catecholamine synthesis from levodopa outside the brain. Neurocirculatory abnormalities underlying OH might therefore might reflect levodopa treatment.

To test this hypothesis, we carried out tests of reflexive cardiovagal gain (decrease in interbeat interval per unit decrease in systolic pressure during the Valsalva maneuver; orthostatic increase in heart rate per unit decrease in pressure); and reflexive sympathoneural function (decrease in pressure during the Valsalva maneuver; orthostatic increment in plasma NE) in PD patients without or with OH who were off or on levodopa at the time of testing. Severity of orthostatic hypotension did not differ between the levodopa-untreated and levodopa-treated groups with PD + OH. The two groups had similarly low reflexive cardiovagal gain during the Valsalva maneuver and during orthostasis and had similarly attenuated reflexive plasma NE responses during both manipulations. Low values for reflexive cardiovagal gain and sympathoneural responses were strongly related to

orthostatic hypotension. PD + OH therefore features reflexive cardiovagal and sympathoneural failure, independently of levodopa treatment (Goldstein et al., 2005).

Cardiac and extracardiac noradrenergic denervation in PD + OH

More than 25 studies over the past several years have agreed remarkably on the finding that virtually all patients with PD have a loss of sympathetic innervation of the heart, as demonstrated by low myocardial concentrations of radioactivity after injection of the sympathoneural imaging agents, ^{123}I-metaiodobenzylguanidine and 6-[^{18}F] fluorodopamine, neurochemical assessments during right heart catheterization (Goldstein et al., 2000), and post-mortem histopathology (Orimo et al., 2001, 2002; Amino et al., 2005).

Whether PD + OH entails a loss of extracardiac noradrenergic nerves has been less clear. In a recent study we asked whether 6-[^{18}F]fluorodopamine can visualize sympathetic innervation in extracardiac organs and if so whether patients with PD + OH have neuroimaging evidence of extracardiac noradrenergic denervation (Tipre and Goldstein, 2005). To validate the method, healthy volunteers underwent 6-[^{18}F]fluorodopamine scanning of the head, thorax, and abdomen, with or without treatment with desipramine to block sympathoneural uptake of catecholamines. Desipramine treatment was associated with decreased 6-[^{18}F]fluorodopamine-derived radioactivity in the heart, renal cortex, and thyroid but not in the liver, spleen, renal pelvis, or salivary glands. The PD + OH group had decreased 6-[^{18}F]fluorodopamine-derived radioactivity in the heart ($p < 0.0001$), renal cortex ($p = 0.02$), and thyroid ($p = 0.01$), compared to healthy controls. PD + OH therefore seems to involve decreased noradrenergic innervation that is most prominent in the heart but is also detectable in extracardiac organs.

Consistent with this notion, we also found that plasma NE during supine rest PD + OH patients off levodopa had lower mean plasma NE than did healthy controls but higher levels than did pure autonomic failure patients. PD + OH patients also had low plasma levels of dihydroxyphenylglycol, the main neuronal metabolite of NE, but higher levels than in patients with pure autonomic failure (PAF). Therefore, the extracardiac noradrenergic denervation in PD + OH is not so severe as in PAF (Goldstein et al., 2005).

Progressive loss of cardiac sympathetic innervation in PD without OH

About half of the patients with PD without OH have been found to have a loss of 6-[^{18}F]fluorodopamine-derived radioactivity diffusely in the left ventricular myocardium, and a bit less than half had loss localized to the lateral or inferior walls, with relative preservation in the septum or anterior wall (Goldstein et al., 2002). Only a very small minority have had entirely normal cardiac 6-[18F] fluorodopamine-derived radioactivity. Thus, virtually all patients with PD have evidence for at least some loss of cardiac sympathetic innervation.

We have followed PD patients with partial loss of cardiac sympathetic innervation, to examine what happens to such innervation over time. After a mean of 2 years, 6-[^{18}F]fluorodopamine-derived radioactivity in the interventricular septal myocardium had declined by 16% from that at the time of initial evaluation (Li et al., 2002). A longer follow-up study over up to 5 years has confirmed about an 8% yearly decline (unpublished observations).

In PD without OH, cardiac noradrenergic denervation therefore seems to progress over years. If so, then cardiac sympathetic neuroimaging might provide a biomarker for detection of early PD and for testing the efficacy of potential neuroprotective treatments.

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Multiple system atrophy and autonomic failure

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Summary. Multiple system atrophy (MSA) is a sporadic neurodegenerative disorder that affects adults. It is characterised by autonomic failure affecting many systems; cardiovascular, urinary, sexual, gastrointestinal and sudomotor, amongst others. In addition there are motor deficits, resulting in both parkinsonian and/or cerebellar features. This review will outline the clinical features, investigations and management of MSA, with a particular emphasis on autonomic failure.

Introduction

Multiple system atrophy (MSA) is a sporadic disorder affecting adults usually over the age of 50. Autonomic failure is an integral component of this disorder. There are three major forms, based on the motor deficit; parkinson (MSA-P), cerebellar (MSA-C) and mixed (MSA-M). A characteristic of MSA is the unpredictability about which system is affected and the rate of progression of disease. This chapter will focus on various aspects regarding autonomic failure in MSA, with an outline of investigative approaches and treatment.

Clinical features & investigation

Every organ in the body has an autonomic innervation. Thus, a variety of clinical features occur in MSA as a result of autonomic failure; these will be described under different systems. Investigation includes screening tests of cardiovascular autonomic function, together with evaluation of other systems (Table 1). Investigation is designed to determine if autonomic function is normal or abnormal, and if the latter, ideally to assess the functional deficit, site of lesion and pathophysiological mechanisms.

An important reason for testing is making a precise diagnosis, especially in the early stages of parkinsonism, as autonomic function is impaired in various parkinsonian syndromes, including idiopathic Parkinson's disease (IPD) (Mathias, 2005). A recent hypothesis suggests that in the initial phase of IPD, unlike previously considered, autonomic centres such as glossopharyngeal and vagal nuclei in the brain stem are affected (Braak et al., 2003). Tests that help currently to distinguish MSA from IPD include the clonidine-growth hormone stimulation test, and cardiac meta-idobenzylguanadine scintigraphy (Kimber et al., 1997; Courbon et al., 2003).

These may enable diagnosis even at an early stage of the disease. An accurate diagnosis aids the many aspects of management – from discussion, prognosis and choice of therapy to anticipation and avoidance of complications.

Table 1. Outline of autonomic and allied investigations in MSA

Cardiovascular	
Physiological	Head-up tilt (60 degrees)*; standing*; Valsalva's maneouvre*
	Pressor stimuli* (isometric exercise, cold pressor, mental arithmetic)
	Heart rate responses – Deep breathing*, hyperventilation*, Standing*, head-up tilt*
	Liquid meal challenge
	Modified exercise testing
	Carotid sinus massage
Biochemical	Plasma noradrenaline: supine and head-up tilt or standing
Imaging	Cardiac sympathetic innervation – MIBG scintigraphy and 18F-Dopa PET scanning
Endocrine	Clonidine – alpha-2 adrenoceptor agonist: growth hormone stimulation
Gastrointestinal	Video-cine-fluoroscopy, barium studies, endoscopy, gastric emptying studies, lower gut studies and anal sphincter electromyography
Renal function & urinary tract	Day and night urine volumes and sodium/potassium excretion
	Urodynamic studies, intravenous urography, ultrasound examination, urethral sphincter electromyography
Sudomotor	Thermoregulatory sweat test
	Sweat gland response to intradermal acetylcholine, sympathetic skin response
Eye & Ocular Function	Pupil function, pharmacological and physiological. Schirmer's test
Respiratory	Laryngoscopy
	Sleep studies to assess apnoea and oxygen desaturation

*Indicates screening autonomic tests used in our London Units
Adapted from Mathias and Bannister (2002)

Cardiovascular system & blood pressure control

In MSA, the baroreceptor reflex pathways which control blood pressure on a beat-by-beat basis are impaired. This often results in orthostatic (postural) hypotension, a cardinal feature, leading to recognition of autonomic failure and consideration of MSA. Orthostatic hypotension is defined as a fall in systolic blood pressure of 20 mmHg or more, or in diastolic blood pressure of 10 mmHg or more, on either standing or head-up tilt to 60° for 3 minutes. Reduced perfusion of organs results in a variety of symptoms (Mathias et al., 1999). Hypoperfusion of the brain can cause dizziness, visual disturbances, loss of consciousness and transient impairment of cognition. Pain in the neck muscles ('coathanger' ache), and lower back result from muscle hypoperfusion. A low level of blood pressure reduces renal blood flow, causing oliguria during the day, and nocturia at night when the blood pressure is restored while supine. Non-specific symptoms such as weakness, lethargy, fatigue and falls (the last, especially in the elderly), may occur. Symptoms occur when upright, as blood pressure usually is promptly restored on returning to the horizontal (Fig. 1), with relief of symptoms.

In addition to postural head-up change, a variety of factors may lower blood pressure further while supine; these often unmask or worsen orthostatic hypotension. These include the speed of positional change, time of day, a warm environment, raising intrathoracic pressure during straining while micturating and defaecating, food ingestion, alcohol consumption and even mild physical exertion. In autonomic failure, drugs with usually mild vasodilatory effects may cause hypotension; these include certain anti-parkinsonian agents, and drugs used to treat autonomic deficits, such as sildenafil (Hussain et al., 2001).

The treatment of orthostatic hypotension is based on a combined approach, utilising

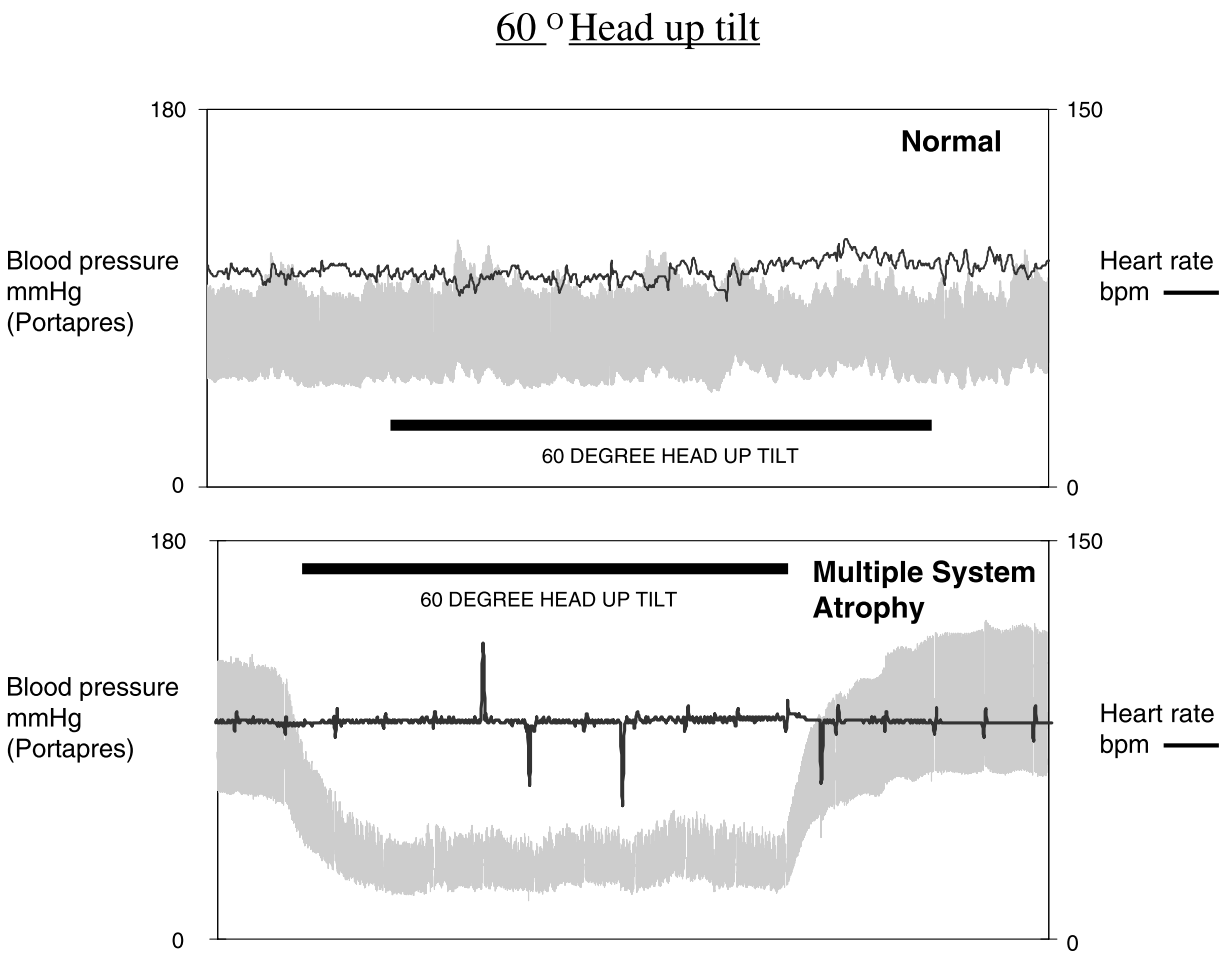

Fig. 1. Blood pressure and heart rate before, during and after head-up tilt in a normal subject (upper panel), and in a patient with autonomic failure due to multiple system atrophy (MSA; lower panel). In the normal subject there is no fall in blood pressure during head-up tilt, unlike the patient with MSA in whom blood pressure falls promptly and remains low with a blood pressure overshoot on return to the horizontal. In the patient with MSA there is only a minimal change in heart rate despite the marked blood pressure fall, because of cardiac parasympathetic failure. In each subject continuous blood pressure and heart rate was recorded with the Portapress II

non-pharmacological and pharmacological measures, based on understanding of provoking factors and the mechanisms involved (Mathias, 2003).

Autonomic failure

Urinary system

Symptoms from frequency to incontinence, often occurs early in the disease and may cause much distress. However, urinary symptoms are not uncommon in the over 50's, in males with prostatic hypertrophy and females with pelvic floor dysfunction. A variety of treatments, both pharmacological and non-pharmacological are available. Intermittent self-catheterisation is of benefit, especially when there is a high residual urine volume. Anticholinergics reduce frequency and urgency.

Sexual function

Erectile failure is common in males, often at an early stage of the illness (Kirchof

et al., 2003). It is important to exclude non-neurogenic causes. A variety of measures, pharmacological and interventional, provide benefit. Orally effective drugs include the phosphodiestrase inhibitors, although these have the potential to lower blood pressure substantially.

Alimentary tract & lower cut

Salivation usually is not affected in MSA, unlike in PD where excessive salivation is common. Oropharyngeal dysphagia often is troublesome as the disease progresses. The patient may not be aware of difficulty in swallowing. Cinevideofluroscopic examination, together with suitable advice based on the findings, often is of value.

Constipation is a common feature and a change in bowel habit suggests autonomic involvement of the lower gut.

Sudomotor function & thermoregulation

Hypohidrosis is a feature of autonomic impairment of sweat gland function and may cause heat intolerance. Overheating can lower blood pressure substantially.

Ocular system

Partial ptosis, together with miosis as part of Horner's syndrome may occur. Lachrymation usually is not affected.

Respiratory system

Clinical features include inspiratory stridor, apnoeic periods and involuntary inspiratory sighs.

Management strategies in MSA

In MSA, autonomic failure results in a multitude of problems which may be compounded by the motor deficit and side effects of drugs. Specific treatment is needed for orthostatic hypotension and bladder, bowel and sexual dysfunction, using a combination of pharmacological and non-pharmacological measures. Certain deficits warrant specific treatment, such as tracheostomy for respiratory abnormalities, and a percutaneous endogastrostomy tube for oropharyngeal dysphagia, especially when there is a risk of tracheal aspiration. In addition to treatments specifically addressing failure of autonomic and allied systems, problems such as depression need treatment. The drugs used for such problems may themselves worsen orthostatic hypotension.

A important component is education of patients and their partners, relatives and carers. This helps ensure that therapeutic approaches are more likely to be effective and beneficial. Education should also include the wide spectrum of health care workers, from medical practitioners to support therapists (dieticians, physiotherapists, occupational and speech therapists). Linking specialist hospital care with the patient, carers and community can be co-ordinated by Autonomic Nurse Specialists and Autonomic Liaison Nurses, ideally to provide a seamless service.

An important means of disseminating information and increasing awareness of these disorders is patient support groups; in the UK the Autonomic Disorders Association Sarah Matheson Trust, in Denmark the MSA Trust and in the USA the Shy-Drager Association, provide such support.

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Sleep disturbances and excessive daytime sleepiness in Parkinson disease: an overview

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Summary. Sleep disturbances are frequent in Parkinson disease. These disorders can be broadly categorized into those that involve nocturnal sleep and excessive daytime sleepiness. The disorders that are often observed during the night in PD include sleep fragmentation that may be due to recurrent PD symptoms, sleep apnea, Restless Leg Syndrome/periodic limb movements and REM sleep behavior disorder. Excessive daytime sleepiness is also a common occurrence in PD. EDS can arise from several etiologies, and patients may have more than one etiology responsible. The causes of EDS include nocturnal sleep disorder with sleep deprivation and resulting daytime somnolence, the effect of drugs used to treat PD, and possibly neurodegeneration of central sleep/wake areas. Appropriate diagnosis of the sleep disturbance affecting a PD patient can lead to specific treatments that can consolidate nocturnal sleep and enhance daytime alertness.

Sleep fragmentation

Nocturnal sleep disturbances and excessive daytime sleepiness are frequent in Parkinson's disease. Nocturnal sleep disturbance affects from 60 to 98% of patients with Parkinson's Disease (PD) (Kales et al., 1971; Lees et al., 1988; Larsen, 2003). Of these, approximately 25% report moderate to severe nighttime sleep problems (Tandberg et al., 1999). The most common nighttime sleep complaint of PD patients is frequent nocturnal awakenings or sleep fragmentation (Factor et al., 1990). In comparison to normal elderly controls and elderly subjects with a chronic disorder (diabetes), PD patients complain of greater sleep fragmentation despite the increased use of sleeping medications (Tandberg et al., 1998). Nocturnal awakenings may be prolonged resulting in a reduction of total sleep time with consequent daytime fatigue and sleepiness. Sleep fragmentation in PD have many etiologies including nocturnal recurrence of PD symptoms, medications, coexistent sleep apnea, and periodic limb movements of sleep (Comella, 2003).

Sleep fragmentation due to recurrence of PD symptoms can occur during any sleep stage, but is more common in light sleep (Stages 1 and 2) (Stern et al., 1968; Mouret, 1975; Askenasy and Yahr, 1985; Factor et al., 1990). PD patients may complain of night awakenings and inability to fall back to sleep (Lees et al., 1988; Tandberg et al., 1998; Happe et al., 2001). Treatment with levodopa preparations can improve sleep fragmentation due to recurrent symptoms (Askenasy and Yahr 1985; Juncos et al., 1987; Lees, 1987) and may also improve early morning function (Jansen and Meerwaldtt, 1990; Pahwa et al., 1993). The nocturnal administration of

apomorphine is has been found to increase nocturnal sleep time and reduce nighttime movements in PD (Priano et al., 2003). Deep brain stimulation surgery has also been demonstrated to improve sleep quality and sleep fragementation (Iranzo et al., 2002). Paradoxically, despite observations that PD medications alleviate night time sleep fragmentation, some studies have shown that these drugs, particularly the direct dopamine agonists, cause increased nocturnal activity (van Hilten et al., 1994) as measured by polysomnography (Hogl et al., 2003) or actigraphy (Comella et al., 2005).

Sleep apnea

Obstructive sleep apnea is typically associated with an increased body mass index and was not considered a major issue in PD. However, the occurrence of sleep apnea in PD is more frequent than previously suspected. In one study, 31% of PD patients assessed with polysomnography were shown to have a significant apnea (Ferini-Strambi et al., 1992), and in another, 20% had moderate to severe sleep apnea (Arnulf et al., 2002) despite a normal body mass index. Snoring, one of the symptoms of sleep apnea, has been shown to predict the occurrence of daytime sleepiness (Hogl et al., 2003; Braga-Neto et al., 2004). Polysomnographic studies in PD demonstrate a greater frequency of sleep apnea than normal controls, with obstructive events being most common (Maria et al., 2003).

Restless legs syndrome and periodic limb movements

Restless legs syndrome (RLS) and Periodic limb movement in sleep (PLMS) can cause sleep disruption in PD. RLS is marked by an urge to move accompanied by dysethesias in the limbs that occurs at rest and is alleviated by movement. Symptoms occur in a circadian pattern with onset usually in the evening hours when lying in bed to sleep. RLS has been reported to be more frequent in PD (Ondo et al., 2002) although some studies indicate the frequency to be no greater than that found in normal age-matched controls (Tan et al., 2002). PLMS may occur in association with RLS or independently. Whether PLMS is more common in PD has not been clarified (Happe et al., 2003; Happe and Trenkwalder, 2003). One study found a correlation between dopaminergic cell loss and the number of PLMs in a group of PD patients using single photon emission computed tomography (SPECT) and evaluating striatal [(123)I]beta-CIT binding (Happe et al., 2003). Although the association of RLS and PLM in PD has been suggested, it may be that the advanced age of the patient population or a secondary condition, such as iron deficiency could account for the apparent increase (Askenasy et al., 1987; Wetter et al., 2000; Ondo et al., 2002; Garcia-Borreguero et al., 2003; Happe and Trenkwalder, 2003). The long acting direct dopamine agonists have been shown to provide effective relief of RLS symptoms and PLMS (Silber et al., 1997; Ferini-Strambi, 2002). Carbergoline, a dopamine agonist, reduces PLMS in PD (Hogl et al., 2003). Other agents that may be useful in PD patients with RLS/PLMS include gabapentin and opioid drugs, although nocturnal confusion may arise as a side effect of the latter (Schapira, 2004). Treatment studies have not yet addressed the efficacy of these agents for RLS in PD.

REM sleep behavior disorder

REM behavior disorder (RBD) (Schenck et al., 1986, 1987) is a sleep disorder characterized by the occurrence of muscle activity during REM sleep with the occurrence of dream enactment (Schenck et al., 2002). REM sleep without atonia (RWA) shows abnormal muscle activation during REM sleep without manifest behaviors. Polysomnographic findings of RBD include excessive

chin muscle tone and limb jerking during REM. Complex, vigorous and sometimes violent behaviors may occur (Schenck and Mahowald, 1991; Comella et al., 1998; Chiu et al., 2000). Patients and bedpartners have sustained ecchymosis, lacerations, fractures and dislocations as a result (Dyken et al., 1995; Comella et al., 1998; Olson et al., 2000). Although the clinical features of RBD appear to be unique, sleep apnea can produce similar phenomenon (Iranzo and Santamaria, 2005). Clinical criteria alone is only about 33% sensitive in making the diagnosis, and polysomnography is needed to determine the presence of RBD (Eisensehr et al., 2001).

RBD or RWA is present in 25–50% of PD patients (Comella et al., 1998; Boeve et al., 2001; Gagnon et al., 2002). PET scans have shown reduced striatal dopamine innervation and loss of brain vesicular monoamine transporter (Albin et al., 2000; Gilman et al., 2003). Animal experiments implicate specific brainstem nuclei as the anatomic basis for RBD (Lai and Siegel, 2003). Cross sectional and longitudinal evidence suggests that idiopathic RBD may in fact be a harbinger of PD or other synucleinopathy (Schenck et al., 1996; Boeve et al., 2003; Eisensehr et al., 2003) and that the association of PD and RBD indicates more extensive brainstem pathology in certain groups of PD patients. Pathologic studies in moderate to severe PD have shown a 40% reduction of cholinergic neurons and Lewy body formation in the pedunculopontine tegmental nucleus, a nucleus directly involved in modulating REM sleep (Hirsch et al., 1987).

In many patients, pharmacologic intervention for RBD may not be necessary if symptoms are mild and intermittent. In cases where behavior is more violent, putting either the patient or the caregiver at risk for injury, protective measures and treatment are indicated. Although lacking controlled trials, small doses of clonazepam have been found to reduce or eliminate the symptoms (Schenck et al., 2002). A dose as small as 0.25 mg of clonazepam may allow both patient and care giver peace during the night and prevent additional sleep related injuries (Olson et al., 2000). Donezepil has been reported to improve RBD in three patients (Ringman and Simmons, 2000). Melatonin was effective at doses ranging from 3–12 mg in 14 patients with parkinsonism (Boeve et al., 2003) and pramipexole was beneficial in 5 patients (Fantini et al., 2003). To date, there are no published controlled trials of any therapy for RBD.

Sleep and hallucinations

Approximately 40% of PD patients have dopaminergic drug induced hallucinations (Fenelon et al., 2000). Up to 82% of PD patients with drug-induced hallucinations (Pappert et al., 1999) have a sleep disturbance. A recent clinic based study showed that three factors are independently predictive of visual hallucinations: severe cognitive impairment, duration of PD and daytime sleepiness (Fenelon et al., 2000). Abnormalities in REM sleep have also been associated with the occurrence of hallucinations in PD. A current hypothesis suggests that hallucinations in PD reflects degeneration of brainstem areas involved in the control of REM sleep, and represent intrusions of dream imagery into waking hours (Comella et al., 1993; Arnulf et al., 2000; Manni et al., 2002). By using polysomnography and the multiple sleep latency test (MSLT) in PD patients with hallucinations, investigators have shown an increased occurrence of nighttime RBD and abnormal REM sleep during daytime napping (Arnulf et al., 2000; Rye et al., 2000). Daytime hallucinations often occurred coincident with daytime REM intrusions (Arnulf et al., 2000). The simultaneous occurrence of RBD and REM-related hallucinations suggests that in some PD patients, dysregulation of REM sleep may be the primary factor in the pathogenesis of dopaminergic drug induced hallucinations. The occurrence of REM sleep

during the MSLT is a characteristic polysomnographic finding in narcolepsy, which has hypnogogic and hypnopompic hallucinations as one of its clinical characteristics. A narcolepsy-like phenotype was found in up to 39% of sleepy PD patients (Arnulf et al., 2002) and ventricular cerebrospinal fluid (CSF) hypocretin levels in PD patients are reduced (Drouot et al., 2003), although lumbar CSF samples did not show a similar reduction (Overeem et al., 2002). In contrast to narcolepsy, however, cataplexy has not been described in PD and a recent study suggested that there is little overlap between these disorders (Baumann et al., 2005). Discontinuation of drugs including anticholinergic agents, anxiolytics, centrally active pain medications, antidepressants and other drugs may be effective. The atypical antipsychotics such as clozapine or quetiapine reduce hallucinations without worsening PD (Fernandez et al., 1999; Friedman and Fernandez, 2002; Factor et al., 2003).

Hypersomnolence: excessive daytime sleepiness (EDS) in PD

Excessive daytime sleepiness (EDS) is a frequent complaint of PD patients. The description of ''sleep attacks'' and motor vehicle accidents in PD patients taking either pramipexole or ropinirole published in 1999 stimulated interest in this topic (Frucht et al., 1999). In one community based study (Tandberg et al., 1999), EDS was seen in 15.5% of PD patients compared to 4% of patients with diabetes mellitus and 1% of controls. EDS was associated with more severe PD, greater PD-related disability, cognitive decline, more frequent hallucinations and a longer duration of levodopa therapy. Longitudinal assessments of EDS in PD over a 4 year span show that 8% had EDS at baseline. In these patients EDS persisted and an additional 21% developed new symptoms of EDS. The factors associated with development of EDS in this study included dementia, and more rapid progression of parkinsonism (Gjerstad et al., 2002). Additional studies have confirmed the frequent occurrence of EDS in PD and examined the role of dopaminergic therapy in its genesis (Arnulf et al., 2002; Hobson et al., 2002; Roth et al., 2003; Braga-Neto et al., 2004; Pal et al., 2004; Razmy et al., 2004). Levodopa has been known to cause sleepiness since its introduction in the 1960's. In one early series of 131 PD patients (Lesser et al., 1979), levodopa monotherapy caused somnolence that limited levodopa dosage in 14% of patients. Comparisons between dopamine agonists and levodopa have shown that both classes of drug cause somnolence (Hauser et al., 2000). Others have found a variety of factors associated with EDS in PD, including severity of PD and total dose of all dopaminergic agents (Hobson et al., 2002; Razmy et al., 2004; Stevens et al., 2004) and confirm that any antiparkinson medication can cause daytime sleepiness in PD patients.

Several studies using polysomnography and MSLT suggest that daytime sleepiness may be a primary feature of PD, unrelated to PD treatments, or nocturnal sleep disturbance (Rye et al., 2000; Arnulf et al., 2002; Roth et al., 2003). A current hypothesis is that sleepiness or a susceptibility to EDS may be an integral part of PD, reflecting the extent of the neurodegenerative process.

The diagnosis of excessive daytime sleepiness is begins with patient and caregiver interview. The interview should include sleep habits, presence of nocturnal sleep disruption (snoring, respiratory pauses, movements in sleep), and a complete drug history. The ESS provides a useful tool that is practical in an office setting for evaluating the presence and severity of EDS. When combined with the Inappropriate Sleep Composite index, it serves to identify those PD patients at risk for falling asleep at the wheel. Although anecdotal reports of PD patients involved in driving mishaps have appeared (Frucht et al., 1999, 2000; Hauser et al.,

2000), in the absence of a systematic investigation, whether PD patients who drive are at greater risk for motor vehicle accidents whether or not on dopamine agonists, remains to answered (Homann et al., 2003).

Treatment of daytime sleepiness in PD includes assessment of the patient for possible nocturnal sleep disturbance. Sleep apnea, periodic limb movements and other disorders that disrupt nocturnal sleep should be considered. This often involves referral to a sleep specialist. Drugs that can cause sleepiness should also be assessed. If a patient reports onset of EDS following initiation or increased dosage of dopaminergic therapy, either a reduction in dose or a switch to another drug may be necessary. Likewise, discontinuation or change in drugs prescribed for another medical condition or diagnosis and treatment of conditions such as depression may be beneficial for treatment of EDS. The administration of daytime stimulant medications is reserved for those patients who are unresponsive to other medication adjustments. The amphetamine metabolites of selegiline may increase alertness. Modafinil, a stimulant drug approved for treatment of EDS in narcolepsy, may also be of modest benefit as has been shown in two controlled studies in a small number of PD patients with excessive daytime sleepiness (Hogl et al., 2002; Adler et al., 2003).

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Sleep and wakefulness disturbances in Parkinson's disease

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Summary. Patients with Parkinson's disease experience prominent difficulties in maintaining sleep, painful night-time abnormal movements, and daytime sleepiness, sometimes culminating in sleep attacks. Recent insights into the pathophysiology of sleep disorders in PD points to a complex interaction between movement disorders, side-effects of dopamine agents and lesions in sleep-wake regulating systems. Treatment with dopamine agonists provides a twice higher risk of daytime sudden sleep episodes than levodopa, with no difference between ergotic and non ergotic compounds. Insomnia can be improved by a better control of night-time disability, restless legs syndrome and dystonia using subthalamic nucleus stimulation or night-time levodopa. A specific REM sleep disorder contributes to REM sleep behavior disorder and also to hallucinations (suggesting they could be awake dreams) and excessive daytime sleepiness. The management of sleep and alertness problems requires to analyze their potential causes, to monitor night-time and daytime sleep, and to subtly adjust psychotropic and dopaminergic treatment.

Introduction

Non-motor manifestations of Parkinson's disease (PD), such as sleep and wakefulness disorders, may considerably alter the quality of life of the patients and their spouse. Patients indeed complain of prominent difficulties maintaining sleep, cumbersome and painful night-time abnormal movements, and daytime sleepiness, sometimes culminating in sleep attacks. The interest for the pathophysiology of these troubles has only occurred recently. Major advances have, however, been done in this field in the past decade, pointing to a complex drug-disease interaction and showing evidences of dopaminergic and non-dopaminergic lesions in systems regulating sleep and alertness.

Insomnia

As already stressed by James Parkinson in 1817, when patients have an advanced disease, "*The sleep becomes much disturbed*". Two-third to 81% of PD patients indeed complain of difficulty maintaining sleep, with long periods of wakefulness lasting 30 to 40% of the night (Tandberg et al., 1998). As a comparison, this frequency of insomnia is double of that found in age- and sex-matched diabetic patients, although both groups report similarly frequent nocturnal pain. Motor problems, mood and anxiety disorders, lesions in sleep regulating systems, and side-effects of dopaminergic treatment may all contribute to insomnia. Depressed patients with advanced disease more often report insomnia than others. They also suffer

from major nocturnal motor problems, including long lasting episodes of ''Worst Off'' condition, difficulty urinating and turning in bed, painful dystonia, restless legs syndrome and involuntary legs movements. Motor disability can be dramatically improved by subthalamic nucleus stimulation. Such a motor treatment provides an important benefit on sleep duration and quality, strongly suggesting that night-time motor disability is the primary cause of insomnia in advanced patients (Arnulf et al., 2000a). This functional surgery had, however, no effect on REM sleep behavior disorder, periodic leg movements during sleep and on restless legs syndrome (Kedia et al., 2004), suggesting that these last two symptoms could be caused by dopaminergic or non dopaminergic dysfunction out of the basal ganglia loop. Recently, it has been shown that 10 to 20% of patients with PD may still suffer from restless legs syndrome, despite being treated with high doses of dopaminergic drugs. These patients have lower serum ferritin levels ($<50\,mg/L$), suggesting that a possible central iron deficiency, as described in idiopathic restless legs syndrome, may modulate the sensory symptoms (Ondo et al., 2002).

Most of nocturnal motor symptoms should be alleviated by dopaminergic treatment, but levodopa prescribed in the evening (even slow release forms) is usually insufficient to totally cover the 8–10 hours of night-time need. In addition, the use of important doses of dopamine agonist before sleep may, on the contrary, worsen sleep quality, with more frequent and longer arousals. Patients can, however, in our experience, benefit from additional small doses of immediate release form of levodopa during their nocturnal awakenings. Treatments usually aimed at reducing sleep maintenance insomnia, such as mianserin 5 mg, or amytryptilline 5–40 mg, may also benefit to PD patients. An benefit of olanzepin, clozapine and quietapine in insomniac patients have also been occasionally reported.

REM sleep behavior disorder (RBD)

Patients suffering from RBD enact their dreams and nightmares during periods of REM sleep devoid of the physiological muscle atonia. They show purposeful hand and arm movements which are sometimes violent and may lead to injuries of the patient or their bed companion. They talk, shout, fall out of bed, but only rarely do they walk like sleepwalkers do when emerging from slow wave sleep. Such RBD can be observed in all types of parkinsonism, whether they result from synucleinopathies (affecting one third of PD patients and almost all patients with Lewy body dementia or multisystemic atrophy), tauopathy, as in supranuclear palsy (Arnulf et al., 2005), or *Parkin* gene mutation (Kumru et al., 2004). In one quarter of the patients, this behavior disorder has heralded other symptoms of PD by 5 to 10 years. The lesion responsible for RBD is unknown, but lesions of the locus subcoeruleus in the pontine tegmentum led to typical RBD (initially named ''oneiric behavior'') in animal models of RBD and was observed in single human RBD cases (Arnulf et al., 2000b). In addition, PD patients with RBD have slower EEG rhythms when awake, suggesting they have concomitant cortical, thalamic or brainstem lesions (Gagnon et al., 2004). Finally, an intriguing aspect of patients during RBD is their clinically normal motor behavior and speech, together with the almost total absence of tremor and dystonia. This suggests a transient functional normalization of the basal ganglia motor loop during REM sleep. Understanding how this ''sleep benefit'' occurs during REM sleep could help to find new treatments in Parkinson's disease.

All classes of antidepressants drugs worsen RBD, while clonazepam and possibly high doses of melatonin usually alleviate the symptoms.

Hallucinations

The association between sleep fragmentation, vivid dreams and hallucinations in PD was

reported early in cross-sectional studies, but not confirmed in a recent prospective longitudinal study by Goetz et al. (2005). On the other hand, Pachetti et al. (2005) recently reported in a large group of PD patients that hallucinations were two to three times more frequent in patients with RBD than without. This was the first time this association was examined, making RBD an independent risk factor for psychosis in PD (Pachetti et al., 2005). Interestingly, direct sleep monitoring during hallucinations and sometimes delusions in PD patients indicated that these events were in the majority of patients timely associated with REM sleep at night or during the day, and all of them had RBD (Arnulf et al., 2000b). This suggests that hallucinations could be awakened dreams similar to the severe hypnagogic hallucinations reported by patients with primary narcolepsy.

Excessive daytime sleepiness

As many as one third of PD patients may be inappropriately sleepy during daytime (for a review, see Arnulf et al., 2005). Excessive daytime sleepiness could be caused by non restorative nocturnal sleep (with significant sleep apnea in 20% of the patients and severe periodic leg movement syndrome in 15% of them, although the impact of periodic leg movements during the night on subsequent daytime alertness is still a matter of debate), lesions in sleep-wakefulness systems and side-effects of drugs. A narcolepsy-like pattern without cataplexy, characterized by inappropriate sleep onsets in REM sleep during the daytime has been evidenced in up to 40% of sleepy PD patients (Arnulf et al., 2002). It has occasionally been reported in untreated, young patients, in patients with multisystem atrophy, in Lewy body dementia and in *Parkin* gene mutation. This narcolepsy-like pattern (but not RBD) can be reproduced in animal (non-human primates and cats) models of PD, suggesting that the dopamine depletion partly causes it. A decrease of hypocretin (a hypothalamic neurotransmitter that is deficient in primary narcolepsy) secretion has been evidenced in the lateral ventricle of patients with advanced PD but not in the cerebrospinal fluid obtained through lumbar puncture in otherwise sleepy PD patients.

Sleep attacks

The most concerning aspect of patients being abnormally sleepy during the daytime is the risk of road accident while driving, especially if the sleep episodes are sudden and devoid of prodroma (sleep attacks, also named ''sudden onset of sleep''). Such sleep attacks behind the wheel are rare (4–8% of patients) but severe events. Accidents (or near-accidents) by driving off the road, or single vehicle accidents are generally believed to be caused by drowsiness. In contrast, accidents occurring at crossroads, in parking area, and involving other road users more often result from difficult driving tasks such as turning, changing lane, parking and backing up, and would be caused by movement impairment such as freezing. Since the ability to drive when one is affected by PD is a crucial question in patients with reduced mobility, there is a necessity to identify only patients at risk of road accident. In all studies, sleep attacks in active conditions (such as driving, walking or speaking), occurred on a background of abnormal sleepiness already present in daily passive conditions, such as watching TV or being a passenger in a car. Abnormal scores (greater than 10) on the standardized Epworth sleepiness scale detect at risk patients with a sensitivity of 52–72%, with the exception of a subgroup of patients who is unaware of falling asleep and underscore on this scale. In these last cases, the care-giver assessment of the patient's sleepiness is useful. Sleep attacks have been reported in patients treated with dopamine agonists, sometimes more frequently when using non-ergoline agonists, but also to a lesser degree in patients newly treated with levodopa. Dopaminergic drugs

have stimulant or on the contrary sedative effects, but the reason for this last unexpected effect in some patients remains unknown, although it is more frequently observed in patients with more advanced disease. Sleep attacks can be partly prevented by a close follow-up and a warning to patients with each drug change. Removal or replacement of a recently-introduced dopamine agonist may relieve daytime sleepiness. If not, the adjunction of modafinil, which shows neuroprotective effects in animals models of PD, may benefit some patients. There is however a need for more active stimulants in PD.

Conclusion

Recent insights into the pathophysiology of sleep disorders in PD points to a complex interaction between movement disorders, dopamine agents' side-effects and lesions in sleep-wake regulating systems. A specific REM sleep disorder contributes to RBD and also to hallucinations and excessive daytime sleepiness. The management of sleep and alertness problems requires thorough analysis of their potential causes, through clinical interviews, standardized scales such as the Epworth Sleepiness Scale and the Parkinson Sleep Disorder Scale, and monitoring nighttime and daytime sleep. Each treatment can be weighed regarding its sleep/wakefulness effects: e.g. small doses of sedative antidepressant can improve the sleep continuity but worsen an underlying RBD; clonazepam alleviates RBD but may worsen sleep apnea; dopamine agonists in the evening can improve restless legs symptoms, levodopa at night may dramatically alleviate akinesia and dystonia, but higher dosages may, on the contrary, increase wakefulness; a nocturnal ventilation using continuous positive airway pressure can reduce daytime sleepiness and nycturia in patients with obstructive sleep apnea, providing the patient can tolerate the mask without additional insomnia. As for other motor and non-motor symptoms in PD, subtle adjustments may lead to major changes in the quality of night and daytime alertness of patients.

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Parkinson's disease dementia: what's in a Lewy body?

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Summary. This brief review deals with pathological aspects of dementia associated with Parkinson's disease (PDD). PDD has been variably linked with cortical Lewy body topography and density. α-Synuclein and Alzheimer-type pathology frequently co-exist, suggesting that a combination of pathology related to protein dysmetabolism, possibly with synergistic protein–protein interaction, underpins the cognitive impairment in PDD. Dementia may therefore ensue when a ''toxic threshold'' is reached, irrespective of the combination of pathologies involved in reaching that threshold. The nature of this putative protein–protein interaction needs to be further elucidated, and also whether there are specific clinical correlates of the pathological substrate. Serum and cerebrospinal fluid proteins or imaging techniques may be useful in future as biomarkers to identify the relative contribution of Lewy-related and Alzheimer-type pathology in a given case of PDD and to inform the rational use of drugs that can reduce α-synuclein aggregation and β-amyloid production.

Introduction

Dementia associated with Parkinson's disease (PD) is common and difficult to manage. Community-based studies have indicated that up to 80% of elderly people with PD may develop cognitive impairment sufficiently severe to fulfil DSM criteria for dementia (PDD). There is no clear and consistent pathological correlate with PDD; specifically, the relative role for the Lewy body and associated Alzheimer-type pathology and the distribution of these pathologies has not been resolved. It seems likely that the pathological basis (like the clinical phenotype) is heterogeneous (Table 1). Clearly, elucidating the pathophysiological mechanisms underpinning PDD will assume even greater significance when disease-modifying treatments become available.

The relationship of PDD to dementia with Lewy bodies (DLB) is a matter of ongoing debate, but many now consider these disorders to be a spectrum of disease (sometimes referred to collectively as ''Lewy body dementias''), differing in temporal and spatial evolution of the disease process. This brief synopsis will concentrate upon PDD, although many of the concepts and conclusions apply equally to DLB.

What's in a Lewy body?

Chronic axonal transport blockage is implicated in the development of cortical Lewy bodies (CLBs) (Katsuse et al., 2003). Intraneuronal Lewy pathology begins in the axonal terminal, before spreading to the cell body and finally to the dendrite. α-Synuclein accumulates initially in neuronal cytoplasm,

Table 1. Possible pathophysiological mechanisms for dementia in PD

Anatomical substrate	Subcortical • loss of ascending projections from pigmented brainstem nuclei • prefrontal-caudate nucleus ''disconnection'' Intrinsic cortical pathology
Neurochemical substrate	Cholinergic deficiency Dopaminergic hyper-/hypo-function Other monoaminergic neurotransmitter deficiencies
Neuropathological substrate	Lewy bodies & dystrophic neurites Alzheimer-like changes (plaques & neurofibrillary tangles) Vascular pathology

without filamentous components. Lewy bodies and Lewy-related neurites then form, composed of granulo-filamentous components, before the inclusions are degraded to extracellular Lewy bodies. Amyloid precursor protein, chromogranin-A, synphilin-1 and synaptophysin also accumulate in CLBs. Torsin A, a protein with homology to yeast heat-shock protein 104 co-localizes with α-synuclein in Lewy bodies (McLean et al., 2002). Tau co-aggregates with α-synuclein in neurones most vulnerable to neurofibrillary tangle (NFT) formation, for example locus coeruleus and basal nucleus of Meynert. Lewy bodies sequester the ubiquitin-activating enzyme, E1, and the E3 ubiquitin ligase, parkin, which are also recruited to aggresomes for enhanced proteolysis. Inhibition of proteasomal function or generation of mis-folded proteins seems to induce formation of aggresome/Lewy body-like inclusions and cytotoxicity in dopaminergic neurones in culture (McNaught et al., 2002).

Evidence against cortical Lewy bodies as the sole cause of dementia

All PD brains, from demented cases or not, may have CLBs. In one study, 17 PD cases were reported where no history of cognitive impairment was ever recorded in life yet pathologically these cases, typical of PD, also fulfilled diagnostic criteria for either limbic or neocortical pathological sub-types of DLB (Colosimo et al., 2003). Recently, as few as 20% of cases with cortical α-synuclein pathology were retrospectively diagnosed in medical records as having PDD or DLB and this pathology was the ''sole cause'' for dementia in only 45% of those cases (Parkkinen et al., 2005).

Alzheimer-type pathology has also been linked to the aetiopathogenesis of PDD. In one series, moderate to severe dementia was reported in 33% of 200 consecutive autopsied PD cases, with degree of cognitive impairment significantly correlated with AD pathology (Jellinger et al., 2002). AD lesions corresponding to CERAD B or C were seen in 84% of the demented PD cases. Regional NFT counts have also been correlated with dementia in PD. In one small series of PD cases, although there were no significant group differences in allocortical or neocortical LB counts between demented and non-demented PD groups, mean entorhinal NFT severity ratings were more than twice those for non-demented cases (SantaCruz et al., 1999). Senile plaque (SP) densities were also greater in every cortical region measured for demented versus non-demented PD cases.

Vascular amyloid-β deposition is more common in elderly (demented and non-demented) PD brains than in age-matched controls. Cognitive impairment in PD is, however, largely independent of coexistent vascular pathology, except in cases with severe cerebrovascular disease (Jellinger, 2003).

Evidence for cortical Lewy bodies as a primary cause of PDD

In contrast with the above, other series have found that CLB density in several cortical areas correlates with degree of cognitive impairment, independent of or in addition to Alzheimer-type pathology. In one series, when CERAD class C cases were excluded from the analysis, the number of cortical LBs in the frontal gyrus was the most significant predictor of cognitive impairment in 45 PD patients (Mattila et al., 2000). Parahippocampal Lewy body density may differentiate PDD and DLB from non-demented PD cases with over 90% sensitivity and positive predictive value (Harding and Halliday, 2001). In another study, CLBs, demonstrated using α-synuclein immunohistochemistry, were reported to be highly sensitive (91%) and specific (90%) neuropathological markers for PDD, and better indicators of dementia than NFTs, SPs or dystrophic neurites (Hurtig et al., 2000). Notably, however, 10% of patients in this series with neuropathological changes typical of PD and judged during life to be demented had no cortical pathology of note (*i.e.* no CLBs, NFTs or SPs).

Apaydin and colleagues reported that mean and median Lewy body counts were increased nearly 10-fold in neocortex, limbic cortex and amgdala in demented versus non-demented PD cases (Apaydin et al., 2002). Patients with high CLB counts in one region were likely to have high counts in other areas. Although Alzheimer-type pathology was described as ''modest'' in this series, there were significant correlations between CLB counts and SP and (to a lesser extent) NFT counts. In 22 prospectively assessed PD patients, dementia rating and regional Lewy body scores were highly correlated in entorhinal and anterior cingulate cortical areas (Kövari et al., 2003). Most recently Braak and colleagues demonstrated that MMSE score correlated with Braak PD stage in a series of 88 PD patients, although two-thirds of patients with stage 4 pathology were moderately or severely demented, implying that abundant CLBs are not necessary for dementia (Braak et al., 2005). Furthermore, higher burdens of NFT and SP pathology were also noted in the cognitively impaired cases.

Reconciling the differences: synergism not polarization?

Methodological reasons may account in part for disparity in the above studies. Thus several series reported clinicopathological correlations before the advent of more sensitive α-synuclein immunohistochemical techniques, and may therefore have under-estimated CLB and Lewy neurite densities. Pathological diagnostic criteria for Alzheimer's disease now place greater emphasis upon the presence of NFTs, rather older criteria in which SP density was regarded as more important. Additionally, retrospective review of medical records may be insensitive to mild or even moderate levels of cognitive impairment, while clinical definitions used in the diagnosis of PDD are currently variable and sub-optimal.

Notwithstanding these methodological issues, a majority of studies have commented upon co-existing pathology in the cerebral cortex of PDD cases, with CLBs and a variable admixture of Alzheimer-like pathology. Furthermore, significant correlations between CLB counts and SP density, in particular, have been reported by several groups.

Recent observations suggest that processes involved in the mis-folding and formation of CLBs and abnormalities in the accumulation of β-amyloid protein (Aβ) and metabolism of tau-protein may not be independent. Regarding Aβ doubly transgenic mice expressing the human form of this protein, as well as α-synuclein, develop severe memory and learning deficits in addition to motor problems (Masliah et al., 2001). Doubly transgenic mice also develop more α-synuclein-immunoreactive inclusions than α-synuclein singly transgenic mice. Furthermore, Aβ

peptide promotes aggregation of α-synuclein in cell-free systems and intraneuronal accumulation of α-synuclein in cell culture (Masliah et al., 2001). PD and Alzheimer disease patients with the apolipoprotein E ε4 allele have a significantly greater numbers of CLBs than those without the allele (Mattila et al., 2000; Tsuang et al., 2005).

Tau, associated with NFTs, is a microtubule-associated protein involved in intra-axonal microtubular assembly and stabilisation. Tau immunostaining may be present at the periphery of the Lewy body (Ishizawa et al., 2003). Co-incubation of tau and α-synuclein synergistically promotes fibrillization of both proteins (Giasson et al., 2003). A recent meta-analysis indicated that homozygosity for the tau H1 haplotype is associated with an increased risk of Parkinson's disease (Healy et al., 2004), although studies have not differentiated between demented and non-demented PD cases. It may be, therefore, that the association with tau haplotype is stronger for PDD rather than PD.

Possible drug effects on disease progression and dementia

Drugs currently used symptomatically in the management of PD and its complications may have adverse effects upon rate of cognitive decline. Thus, PD patients exposed to long term (defined as more than two years) anticholinergic medication have 2.5-fold higher cortical densities of SPs, compared to PD patients with short-term or no exposure to these drugs (Perry et al., 2003). In this study, NFT densities were also increased in the chronically treated PD group. These data raise the possibility that anticholinergic drugs, currently commonly used to treat bladder dysfunction, or tricyclic antidepressants with anticholinergic properties, may induce a pathological substrate for dementia in PD.

Observational studies indicate that rate of cognitive decline is doubled in demented patients (not PD) taking typical antipsychotics. Most recently, the atypical antipsychotic, quetiapine, has been associated with a significantly more rapid rate of cognitive decline in Alzheimer disease patients compared to those taking placebo (Ballard et al., 2005). Suppression of BDNF would be one plausible pathophysiological mechanism underpinning this apparent effect. These results may also, of course, be relevant to PDD patients, although further studies are required to confirm this.

Conclusion

Dementia associated with PD has been variably linked with CLB topography and density. The common co-occurrence of α-synuclein and Alzheimer-type pathology suggests that a combination of pathology related to protein dysmetabolism, possibly with a synergistic protein–protein interaction, is the most probable explanation underpinning the cognitive impairment in these disorders. Dementia may therefore ensue when a ''toxic threshold'' is reached, irrespective of the combination of pathologies involved in reaching that threshold. Future studies should elucidate further the nature of the putative protein–protein interaction, whether there are specific clinical correlates of these pathological processes, identify robust biomarkers to reflect the relative contribution of Lewy-related and Alzheimer-type pathology to the dementia and explore the rational use of drugs that can reduce α-synuclein aggregation and β-amyloid production.

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Role of microglia in inflammation-mediated degeneration of dopaminergic neurons: neuroprotective effect of Interleukin 10

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Summary. Inflammation in the brain has been recognized to play an increasingly important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease. Inflammation-mediated neurodegeneration involves activation of the brain's resident immune cells, the microglia, which produce proinflammatory and neurotoxic factors including cytokines, reactive oxygen species (ROS), nitric oxide, and eicosanoids that directly or indirectly cause neurodegeneration. In this study, we report that IL-10, an immunosuppressive cytokine, reduced the inflammation-mediated degeneration of dopaminergic (DA) neurons through the inhibition of microglial activation. Pretreatment of rat mesencephalic neuron–glia cultures with IL-10 significantly attenuated the lipopolysaccharide (LPS) induced DA neuronal degeneration. The neuroprotective effect of IL-10 was attributed to inhibition of LPS-stimulated microglial activation. IL-10 significantly inhibited the microglial production of tumor necrosis factor α (TNF-α), nitric oxide, ROS and superoxide free radicals after LPS stimulation.

Introduction

The pathogenesis of several neurological disorders, including Parkinson's disease, Alzheimer's disease (Dickson et al., 1993; Liu and Hong, 2003), is now thought to be mediated by an inflammatory response by resident cells in the brain. Microglia, the resident immune cells of the brain, contribute to this inflammation by serving the role of immune surveillance and host defense (Kreutzberg, 1996). Activated microglia produce a variety of pro-inflammatory factors and reactive oxygen species (ROS), all of which serve immune surveillance functions by removing foreign microorganisms (Aloisi, 1999). Our previous results have shown that over-activation of microglia and overproduction of pro-inflammatory factors may lead to neuronal degeneration in the CNS (Liu et al., 2002).

Interleukin (IL)-10 is a cytokine produced by a variety of cell types including type 2 helper T cells, B cells, and macrophages. IL-10 has been shown to suppress inflammation in many experimental models of inflammatory disease. Mizuno et al. (1994) have found that cells in the CNS also produce IL-10. Microglia, which are cells in the CNS very similar both phenotypically and functionally to macrophages, express IL-10 receptor mRNA, and consequently they may be strongly regulated by IL-10. IL-10 produced in the CNS, therefore, may play an important role in the pathophysiology

of CNS disorders by inhibiting the function of microglia.

The goal of the present study is to evaluate the effects of IL-10 on LPS-induced neurotoxicity in rat primary midbrain cultures. Here, we show that IL-10 attenuate microglial pro-inflammatory cytokine and ROS production and protect DA neurons from LPS-induced neurotoxicity.

Materials and methods

Reagents

The recombinant rat IL-10 (rrIL-10; R & D system, Minneapolis, MN). LPS were purchased from Sigma-Aldrich (St. Louis, MO). All the cell culture ingredients were obtained from Invitrogen (Carlsbad, CA). The [^{3}H] DA (30 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA), The fluorescence probe DCFH-DA was obtained from Calbiochem (La Jolla, CA).

Rat mesencephalic neuron–glia cultures

Primary mesencephalic neuron–glia cultures were prepared from the brains of embryonic day 14/15 Fischer 344 rats, following our previously described protocol (Liu et al., 2002). Briefly, the ventral mesencephalic tissues were removed and dissociated by a mild mechanical trituration. Cells were seeded at 5×10^5/well to 24-well culture plates pre-coated with poly-D-lysine (20 ng/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in 0.5 ml/well maintenance medium. The medium consisted of minimum essential medium containing 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum, 1 g/l glucose, 2 mM [SCAP]L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 ng/ml streptomycin. Three days after the initial seeding, 0.5 ml of fresh maintenance medium was added to each well. Seven-day-old cultures were used for treatment. The composition of the cultures at the time of treatment was approximately 48% astrocytes, 11% microglia, 40% neurons, and 1 to 1.5% TH-immunoreactive (ir) neurons.

Primary microglia-enriched cultures

Rat microglia-enriched cultures, with a purity of >98%, were prepared from whole brains of 1-day-old Fischer 344 rat pups, following our described protocol (Liu et al., 2002). For superoxide assays, 10^5 cells/well/0.2 ml medium were grown overnight in 96-well culture plates before use.

Uptake assay

[^{3}H]DA uptake assays were performed as described previously (Liu et al., 2002). Cultures were incubated for 20 min at 37°C with 1 μM [^{3}H]DA in Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold Krebs-Ringer buffer, cells were collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

Nitrite and TNFα assays

The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent, and release of TNFα was measured with a rat TNFα enzyme-linked immunosorbent assay kit from R & D Systems (Minneapolis, MN).

Superoxide assay

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1. Microglia-enriched cultures in 96-well culture plates were washed twice with Hanks' balanced salt solution without phenol red (HBSS). Cultures were then incubated at 37°C for 30 min with vehicle control (water) or IL-10 in HBSS (50 μl/well). Afterward, to each well was added 50 μl of HBSS with and without SOD (50 U/ml, final concentration), 50 μl of WST-1 (1 mM) in HBSS, and 50 μl of vehicle or LPS (10 ng/ml). Thirty minutes later, absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The difference in absorbance observed in the absence and presence of SOD was considered to be the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Statistical analysis

The data were expressed as the mean ± S.E.M. Statistical significance was assessed with an analysis of variance followed by Bonferroni's t test using the Stat View program (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Effect of IL-10 on LPS-induced degeneration of DA neurons

Mesencephalic neuron–glia cultures were pretreated with IL-10 for 1 h and then stimulated with LPS for 7days. The degeneration of DA neurons was then determined by [^{3}H] DA uptake assay. The [^{3}H] DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 40% of the vehicle control (Fig. 1A). At 30 ng/ml IL-10, the LPS-induced decrease in DA uptake was completely restored, and IL-10 alone at this concentration range did not affect DA uptake levels in the cultures.

IL-10 treatment inhibits LPS-induced production of NO, TNFα and intracellular and extracellular reactive oxygen species

The LPS-stimulated activation of microglia was suppressed by pretreatment with IL-10 in neuron–glia cultures. Accumulation of nitrite, an indicator of LPS stimulated production of NO, was determined 24 hrs and 48 hrs after LPS stimulation. As shown in Fig. 1B, pretreatment with 30 ng/ml IL-10, completely blocked LPS-stimulated NO production. As shown in Fig. 1C, pretreatment with 30 ng/ml IL-10 significantly reduced LPS-induced production of TNFα determined at 3 h after LPS stimulation.

To test the effect of IL-10 on the microglial generation of ROS, enriched-microglial

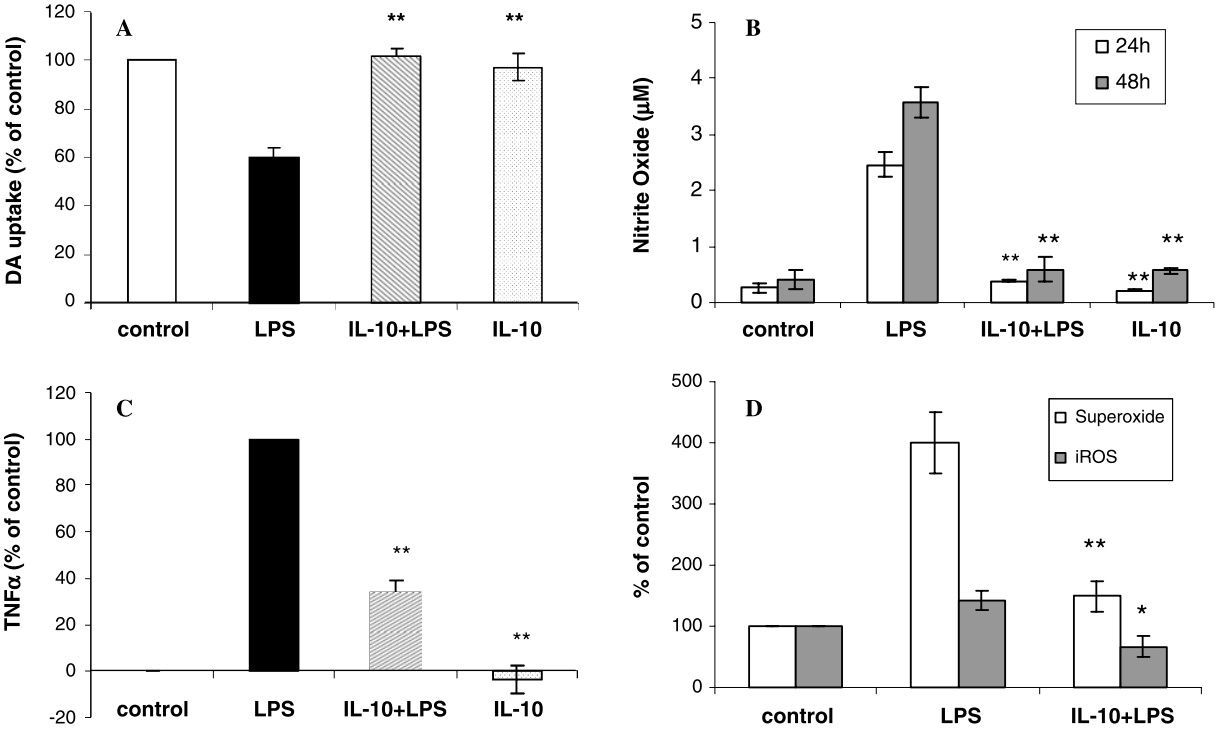

Fig. 1. IL-10 is neuroprotective against LPS-induced neurotoxicity and inhibits LPS-induced microglia activation. Rat primary mesencephalic neuron–glia cultures seeded in a 24-well culture plate at 5×10^5 cells were pretreated with IL-10 (30 ng/ml) for 1 h before the addition of 10 ng/ml LPS. Eight days later, the LPS-induced dopaminergic neurotoxicity was quantified by the [^{3}H] DA uptake assay (**A**); Effects of IL-10 on LPS-induced production of nitrite oxide (**B**); TNF-α (**C**); superoxide and iROS (**D**) as % of control. The results are the mean ± SE of 4 individual experiments in triplicate in each experiment. $^*P < 0.05$, $^{**}P < 0.01$, compared with LPS culture

cultures were pretreated with IL-10, then exposed to LPS. IL-10 significantly inhibited intracellular ROS production and microglial superoxide response to nearly control levels. (Fig. 1D). Based on our previous data that indicates a central role for ROS in microglial-mediated destruction of DA neurons, it appears that the neuroprotective effect of IL-10 is at least partially due to a reduction in LPS-induced oxidative stress.

Discussion

Degeneration of the nigrostriatal DA pathway is a hallmark of Parkinson's disease. LPS-induced degeneration of DA neurons in mesencephalic neuron–glia cultures is a useful *in vitro* model for the identification of potential therapeutic agents. Our data confirms that microglia play an important role in Parkinson's disease by secreting pro-inflammatory mediators such as TNFα, nitric oxide, and ROS. And this inflammatory response can be inhibited by the anti-inflammatory cytokine, IL-10. Concomitantly, IL-10 also can protect the DA neurons from LPS-induced DA neuronal degeneration. This further supports the notion that pro-inflammatory products from micorglia are responsible for all or most of the neurodegenerative phenotype seen in Parkinson's patients.

IL-10 is predominantly an immunosuppressive and anti-inflammatory cytokine, and plays a critical role in limiting tissue injury during infections by limiting the duration and intensity of immune and inflammatory reactions (Moore et al., 2001; Berg et al., 1995), including the response to LPS. LPS has been shown to activate macrophages and microglial cells through the TLR-4-mediated signaling pathway (Olson and Miller, 2004), leading to the production of pro-inflammatory mediators such as cytokines, reactive oxygen species, reactive nitrative species, NO, and eicosanoids. IL-10 has been shown to strongly regulate that pathway in macrophages, also to predominantly inhibit the NF-κB pathway through interference in transcriptional regulation (Schottelius et al., 1999), and it is likely that activation of this pathway is a major player in the inflammatory response seen in Parkinson's patients. Therefore, it is likely that this pathway could serve as an excellent target for therapy directed at decreasing neuronal destruction in Parkinson's disease.

We show here that IL-10 regulates a large number of pro-inflammatory mechanisms in microglia that may have a role in the destruction of DA neurons, including TNFα, NO, and ROS. However, the exact mechanism by which the neuroprotective effect of IL-10 on activated microglia is unclear, since each of these neurotoxic factors may have similar effects on DA neurons. Our previous results have shown that microglial NADPH oxidase plays a crucial role in causing DA neuronal death by over-activated microglia through the direct or indirect induction of ROS and TNFα production (Qin et al., 2004). Therefore, it appears that IL-10 may mediate its neuroprotective effect either directly or indirectly through the inhibition of NADPH oxidase activity, perhaps by regulating the NF-κB-dependent activation or function of PHOX. Experiments to test these hypotheses are currently underway.

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Role of cytokines in inflammatory process in Parkinson's disease

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Summary. We investigated whether the cytokines produced in activated microglia in the substantia nigra (SN) and putamen in sporadic Parkinson's disease (PD) are neuroprotective or neurotoxic. In autopsy brains of PD, the number of MHC class II (CR3/43)-positive activated microglia, which were also ICAM-1 (CD 54)-, LFA-1 (CD 11a)-, TNF-alpha-, and IL-6-positive, increased in the SN and putamen during progress of PD. At the early stage activated microglia were mainly associated with tyrosine hydroxylase (TH)-positive neurites in the putamen, and at the advanced stage with damaged TH-positive neurons in the SN. The activated microglia in PD were observed not only in the nigro-striatal region, but also in various brain regions such as the hippocampus and cerebral cortex. We examined the distribution of activated microglia and the expression of cytokines and neurotrophins in the hippocampus of PD and Lewy body disease (LBD). The levels of IL-6 and TNF-alpha mRNAs increased both in PD and LBD, but those of BDNF mRNA and protein drastically decreased specifically in LBD, in which neuronal loss was observed not only in the nigrostriatum but also in the hippocampus. The results suggest activated microglia in the hippocampus to be probably neuroprotective in PD, but those to be neurotoxic in LBD. As an evidence supporting this hypothesis, two subsets of microglia were isolated from mouse brain by cell sorting: one subset with high production of reactive oxygen species (ROS) and the other with no production of ROS. When co-cultured with neuronal cells, one microglia clone with high ROS production was neurotoxic, but another clone with no ROS production neuroprotective. On the other hand, Sawada with coworkers found that a neuroprotective microglial clone in a culture experiment converted to a toxic microglial clone by transduction of the HIV-1 Nef protein with increasing NADPH oxidase activity. Taken together, all these results suggest that activated microglia may change in vivo from neuroprotective to neurotoxic subtsets as degeneration of dopamine neurons in the SN progresses in PD. We conclude that the cytokines from activated microglia in the SN and putamen may be initially neuroprotective, but may later become neurotoxic during the progress of PD.

Toxic change of activated microglia may also occur in Alzheimer's disease and other neurodegenerative diseases in which inflammatory process is found.

Introduction

Parkinson's disease (PD) is characterized by specific degeneration of the dopamine

neurons in the substantia nigra (SN) pars compacta and the resulting loss of the nerve terminals in the striatum (the putamen and caudate nucleus), which is accompanied by a deficiency in the neurotransmitter dopamine in the striatum. This dopamine deficiency is responsible for most of the movement disorders called parkinsonism, i.e., muscle rigidity, akinesia, and resting tremor. The causative genes and their chromosomal locations of Familial PD (PARK) have been identified; PARK 1 (alpha-synuclein), PPRK 2 (parkin), PARK 5 (UCH-L1), PARK 6 (PINK 1), PARK 7 (DJ- 1), and PARK 8 (LRRK2) (Mizuno, 2005). However, most PD is sporadic without hereditary history. The pathogenesis of sporadic PD is still enigmatic (Foley and Riederer, 1999), but reactive free radicals produced by oxidative stress are speculated to play an important role (Youdim and Riederer, 1997).

We (Mogi and Nagatsu, 1999; Nagatsu et al., 1999, 2000; Nagatsu, 2002) previously reported by enzyme-linked immunosorbent assay (ELISA) increased levels of pro-inflammatory cytokines, decreased levels of neurotrophins, and changes in the levels of apoptosis-related factors in the nigro-striatal region of postmortem brain and/or ventricular or spinal cerebrospinal fluid in Parkinson's disease (PD) or in animal models of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or by 6-hydroxydopamine. Other workers (Hartmann et al., 2000; Hirsch et al., 1998, 1999, 2002) also reported changes in pro-inflammatory cytokines and their receptors, and apoptosis-related factors in the nigro-strialtal regions in PD, suggesting the presence of inflammatory process called neuroinflammation in parkinsonian brain. These changes in pro-inflammatory cytokines, neurotrophins and apoptosis-related factors in PD suggest apoptotic death of the nigro-striatal dopamine neurons in PD (Jellinger, 2000; Nagatsu et al., 1999, 2000; Hartmann et al., 2000; Hirsch et al., 1998, 1999, 2002).

Cytokines, neurotrophins, reactive oxygen species (ROS), and reactive nitrogen species (RNS) may be most probably produced by activated microglia in the brain with neuroinflammation. Although the causative relation with neuroinflammation is not clear, the presence of alpha-synuclein-positive intracellular inclusions, called Lewy bodies, in dopamine neurons in the substantia nigra is another feature of sporadic PD. In Lewy body disease (LBD) (Kosaka, 2000), also called dementia with Lewy bodies (DLB), both parkinsonian movement disorder and dimentia are observed, and Lewy bodies are widely distributed not only in the nigrostriatum but also in the cerebral cortex and hippocampus.

In the brain from patients with PD an increased number of major histocompatibility complex (MHC) class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive activated microglia were first reported by McGeer et al. (McGeer et al., 1998; McGeer and McGeer, 1995), which suggests inflammatory process to occur in the brain in PD patients and the origin of cytokines most probably to be activated microglia.

We (Imamura et al., 2003) first examined whether activated microglia in the brain in PD produce pro-inflammatory cytokines. Pro-inflammatory cytokines such as TNF-alpha and IL-6 are pleiotropic and can be either neuroprotective and neurotoxic. Therefore, we further aimed at asking the question whether increasing levels of cytokines and the presence of activated microglia in the brain in PD is neuroprotective rescuing dopamine neurons or neurotoxic causing dopamine cell loss.

Increasing levels of cytokines are produced from activated microglia in the putamen in PD

We (Imamura et al., 2003) identified by Western blot analysis TNF-alpha protein as a 21-kDa band, IL-6 protein as a 17-kDa

band, and MHC-II (CR3/43) protein as 34- and 28-kDa bands in homogenates of the putamen and peripheral blood mononuclear cells from PD patients, in agreement with our previous results by ELISA (Mogi and Nagatsu, 2000; Nagatsu et al., 1999). We then showed by immunohistochemistry that almost all activated microglia in the putamen from PD brains are positive for both ICAM-1 and LFA-1. We further proved by double immunofluorostaining the coexistence of TNF-alpha and IL-6 with MHC class II (CR3/43) in ICAM-1- and LFA-1-positive activated microglia in the putamen from PD patients. These results confirm that TNF-alpha and IL-6 are produced by activated microglia in the putamen in PD (Imamura et al., 2003).

Activated microglia are observed not only in the nigro-striatal region but also in various regions of the brain in PD

The presence of activated microglia and the absence of reactive astrocytosis in the substantia nigra of patients with PD suggest microglial involvement in the pathological process of dopamine neurons (McGeer et al., 1988; McGeer and McGeer, 1995; Mirza et al., 2000). We (Imamura et al., 2003) showed MHC class II (CR3/43)-positive activated microglia to be widely distributed not only in the substantia nigra and putamen, but also in various brain regions of PD patients, frequently in association with alpha-synuclein-positive Lewy neurites and monoaminergic neurons. In normal brains, many Ki-M1p-positive resting microglia, but only few MHC class II (CR3/43)-positive activated microglia were seen in the substantia nigra and putamen. In PD brains, however, MHC class II (CR3/43)-positive ramified microglia were seen in those regions. PD patients were shown to have a significantly higher number of MHC class II (CR3/43)-positive microglia compared with normal controls. The cell count of MHC class II (CR3/43)-positive microglia in PD increased as the neurodegeneration of pigmented cells in the substantia nigra advanced. Moreover, a significantly higher number of MHC class II (CR3/43)-positive microglia were also observed in the hippocampus (HC), transentorhinal cortex (TC), cingulate cortex (CC) and temporal cortex (TC) in PD compared with normal controls. In the early stages in PD, MHC class II (CR3/43)-positive microglia in the putamen were associated with intensively tyrosine hydroxylase (TH)-positive dopamine neurites without degeneration. In the advanced stage in PD, MHC class II (CR3/43)-positive microglia in the substantia nigra were associated with damaged TH-positive dopamine neurons and neurites. MHC class II (CR3/43)-positive microglia were also associated with non-degenerated serotonin (WH-3)-positive nerve terminals without degeneration in the substantia nigra. In the cingulate cortex in PD, activated microglia were frequently associated with alpha-synuclein-positive Lewy neurites. These immunohistochemical observations on PD brains suggest that activated microglia may act for either neuroprotection or neurotoxicity depending upon the brain regions and the stage of disease. We speculate that there may be neuroprotective and neurotoxic subtypes of microglia producing different kinds and different amounts of cytokines, neurotrophins, reactive oxygen species (ROS), and reactive nitrogen species (RNS), and that activated microglia in the nigro-striatal region in PD may be non-toxic subtype acting for neuroprotection at least in the early stage but may change to neurotoxic subtype causing neurodegeneration during the progression of the disease.

Our immunohistochemical results suggesting the neuroprotective or neurotoxic dual roles of activated microglia associated with healthy or damaged neurons and neurites in various brain regions in PD are schematically summarized in Fig. 1.

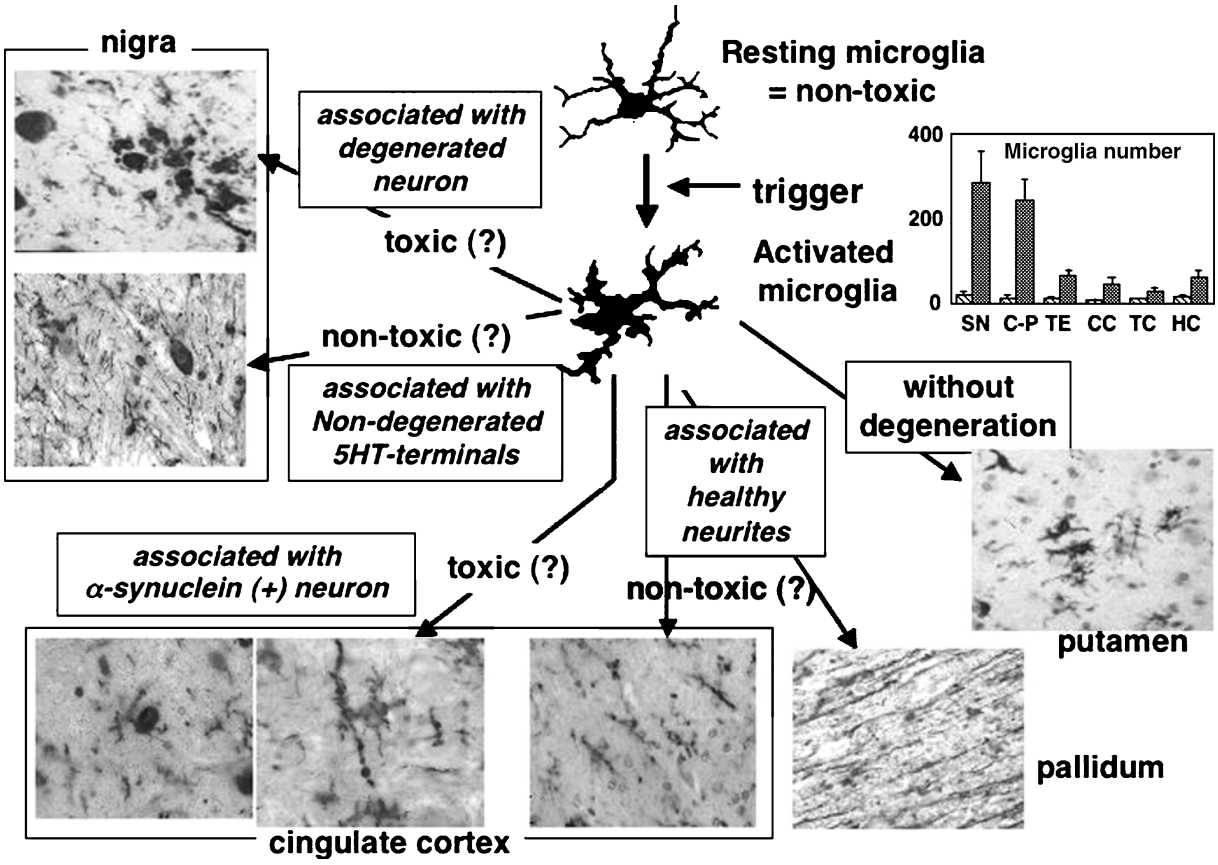

Fig. 1. Schematic diagram showing the dual potential roles of microglia in PD. In parkinsonian brains, activated microglia are observed not only in the substantia nigra (SN) and caudate-putamen (C–P) but also in other brain regions such as pallidum and cingulate cortex. Activated microglia associated with neurons or neurites without degeneration may be non-toxic and act for neuroprotection, whereas activated microglia associated with degenerated neurons and neurites may be neurotoxic and promote neurodegeneration. *TE* transenthorhinal cortex; *CC* cingulate cortex; *TC* temporal cortex; *HC* hippocampus

Expression of cytokines in the hippocampus and putamen in PD is different from that in LBD

Activated microglia have multiple roles. First, MHC II-positive activated microglia act for antigen presentation. Second, activated microglia phagocytose damaged cells. Third, activated microglia produce neurotoxic substances such as pro-inflammatory cytokines that are pleiotropic and either neurotoxic or neuroprotective like TNF-alpha, IL-1beta and IL-6, superoxide anions (ROS), nitric oxide (RNS), and glutamate. Fourth, activated microglia also produce neurotrophic substances such neurotrophins as BDNF, IL-6 and TNF-alpha that can also act for neuroprotection. As described above, we (Imamura et al., 2003) observed activated microglia in PD brain not only in the nigrostriatum but also in the hippocampus. We (Imamura et al., 2003, 2005) also observed in LBD activated microglia in the nigro-striatum and hippocampus. Neuronal degeneration in the putamen was observed in both PD and LBD, whereas neuronal loss in the hippocampus was observed in LBD, but not in PD without dementia. In order to examine whether activated microglia in the putamen and hippocampus in PD and LBD are neuro-

toxic or neuroprotective, we (Imamura et al., 2005) compared the expression of cytokines and neurotrophins in the hippocampus and putamen in postmortem brains in PD, LBD/DLB, and normal controls.

In normal controls, neuronal loss and activated microglia were not observed in the hippocampus CA 2/3 region, and neurons were strongly BDNF-positive. Immunohistochemical examination of the hippocampus CA 2/3 region in PD showed that the number of MHC II (R3/43)-positive microglia increased, which were also ICAM-1 (CD54)-, LFA-1 (CD11a)-, TNF-alpha-, and IL-6-positive. Alpha-synuclein-positive cells were also observed. BDNF-positive neurons were only slightly decreased in PD as compared with normal controls. In the hippocampus in LBD, the number of MHC-II (CR3/43)-positive microglia and alpha-synuclein-positive cells increased more than those in PD. Furthermore, in LBD all neurons were very weakly stained by anti-BDNF. These immunohistochemical data on the hippocampus CA 2/3 indicate that activated microglia increase both in PD and LBD, but that the content of neurotrophic BDNF drastically decreases specifically in LBD.

Expression of mRNAs of cytokines and neurotrophins was examined by RT-PCR in the hippocampus and putamen in normal controls, PD, and LBD. In the hippocampus, mRNA levels of IL-6 and TNF-alpha increased in both PD and LBD, but mRNA levels of BDNF greatly decreased in LBD, as compared with those of normal controls and PD. In the putamen, mRNA levels of IL-6 increased in both PD and LBD. In contrast, mRNA levels of BDNF increased in PD, but decreased in LBD. We (Mogi and Nagatsu, 2002) previously reported by ELISA that the content of BDNF protein decreased in the striatum in PD. Therefore, increased level of BDNF mRNA in PD is speculated to be a compensatory change probably by activated microglia. These different changes in mRNA levels of IL-6 and BDNF in PD and LBD suggest that activated microglia in the hippocampus and putamen in PD and LBD may be different in properties and may secrete different kinds and different amounts of cytokines and neurotrophins such as IL-6 and BDNF. We speculate that activated microglia in the hippocampus may be neuroprotective in PD and neuroltoxic in LBD (Imamura et al., 2005).

Two subsets of microglia and two clones of microglia with neurotoxic and neuroprotective properties are isolated in terms of intracellular ROS production induced by phorbol myristate acetate (PMA) stimulation

We separated two subsets of microglia from mouse brain by cell sorting based on profiles of intracellular ROS production induced by PMA. One subset of microglia produced greater amounts of ROS than another subset of microglia. The results suggest that there are at least two subsets of microglia in mouse brain; one active subset and another inactive subset in production of ROS upon stimulation by PMA. In supporting this hypothesis, two cell lines of microglia, Ra2 cells and 6-3 cells, were generated by spontaneous immortalization of primary mouse microglia. Both clones were dependent on granulocyte macrophage colony-stimulating factor (GM-CSF). The GM-CSF-dependent Ra2 microglia did not produce ROS by PMA stimulation. In contrast, the GM-CSF-dependent 6-3 microglia showed increasing ROS production upon stimulation by PMA. N18 neuronal cells were sensitive to oxidative stress by hydrogen peroxide, and showed dose-dependent apoptotic cell death by the addition of hydrogen peroxide. N18 cells cultured in the presence of 50 mM hydrogen peroxide died almost completely by apoptosis. When the N18 neuronal cells were co-cultured with macrophage RAW264.7 cells or 6-3 microglia cells and stimulated with PMA, cell viability of the neuronal cells decreased as determined by

cell viability assay (WST assay, PI dye exclusion assay, and TUNEL staining). On the contrary cell viability of the neuronal N18 cells increased by co-culture with Ra2 microglia. These results show 6-3 microglia to be neurotoxic and Ra2 microglia to be neuroprotective when these cells are co-cultured with neuronal cells, supporting our concept that there may exist neurotoxic and neuroprotective subsets of microglia in the brain.

In agreement with our hypothesis, Hirsch et al. (1998) also proposed separate populations of microglia; one subpopulation of glial cells may play a neuroprotecrive role by metabolizing dopamine and scavenging oxygen free radicals and another that may be deleterious to dopamine neurons by producing NO and toxic proinflammatory cytokins.

Lentiviral transduction of neuroprotective microglia with HIV-1 Nef protein induces toxic change

AIDS patients frequently develop an human immunodeficiency virus type 1 (HIV-1)-associated abnormalities in cognition and parkinsonian motor dysfunction. HIV-infected macrophages were observed in the striatum, and dopamine concentrations were significantly reduced in the striatum (Sarder et al., 1996).

Nef is the first viral protein detectable after human HIV-1 infection, enhances virus production and infectivity, and exerts pathologic effects independently of viral replication. Microglia are phagocytes of myeloid origin and the principal target of HIV infection in the brain. Microglia produce superoxide, and express all components of the superoxide generating phagocyte NADPH oxidase (Vilhardt et al., 2002). We transduced Nef protein using lenti virus vector into nontoxic Ra2 microglia. Both Ra2 and nefRa2 microglia were similar in GM-CSF dependency. Ra2 microglia did not produce ROS by stimulation with PMA. In contrast, nefRa2 robustly produced ROS owing to activation of NADPH oxidase. When N18 neuronal cells were co-cultured with Ra2 or nefRa2 microglia, Ra2 microglia were shown to be neuroprotective, but nefRa2 neurotoxic, indicating toxic change of Ra2 microglia by transduction of Nef protein. Addition of superoxide diamutase (SOD) partially recovered the neurotoxicity of nefRa2 to change the glial cell line to be neuroprotective. These results suggest that toxic change in nefRa2 microglia may be partially due to increased ROS production by increased NADPH oxidase. Another possibility of toxic change is increased production of myeloperoxidase (MPO).

The toxic change of reactive microglia suggests two step activation of microglia in PD

Based on these in vitro results suggesting the presence of neuroprotective or neurotoxic subsets of activated microglia, we propose a hypothesis of two-step activation of microglia in the brain in PD in vivo, as schematically shown in Fig. 2. Ramified resting microglia in the normal brain support neurons for control of neuronal activity, development, and homeostasis in the brain. The observation on activated microglia associated with intensely tyrosine hydroxylase (TH)-positive neurites in the striatum in the early stage of PD and with other non-degenerated neurons and neurites in various brain regions suggest that microglia activated by the first stimuli may act for neuroprotection by producing neurotrophins, neurotrophic cytokines, and antioxidant at the first step. The activated microglia at this first step may be neuroprotective. As described above, Sawada with coworkers found that microglia in a cell culture experiment are converted from the neuroprotective to neurotoxic forms upon expression of the HIV-1 Nef protein (Vilhardt et al., 2002). Similar toxic change of activated microglia may occur in PD brain as the second step by other factors such as in-

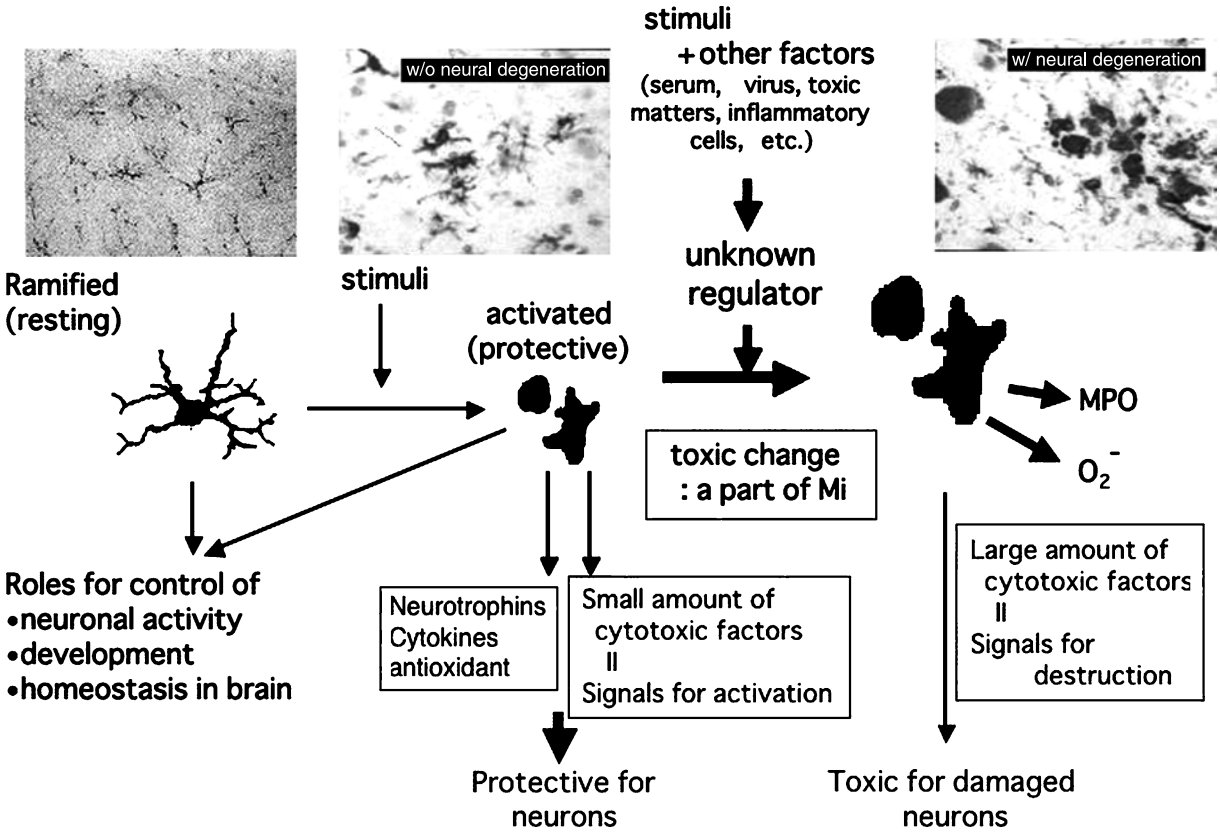

Fig. 2. Schematic diagram showing a hypothesis of two-step activation of microglia. We isolated neuroprotective and neurotoxic subsets of microglia, and also neuroprotective and neurotoxic clones from mouse brain. In addition, Sawada and coworkers (Vilhardt et al., 2002) found in a cell culture experiment that a neuroprotective microglial clone converted from the protective to toxic cells upon transduction of the HIV-1 Nef protein with activation of NADPH oxidase. Based on these results, we propose a hypothesis of two step activation of microglia. Activated microglia by the first stimuli may initially act for neuroprotection by producing protective neurotrophins, cytokines, and antioxidants, but by the second stimuli and unknown regulators may change to be neurotoxic by producing ROS and MPO. This toxic change of activated microglia may promote the progress of PD

vasion of serum, viruses, toxic matters, or inflammatory cells in a part of neuroprotective microglia in a specific brain regions such as the nigro-striatum in PD. As the results of toxic change of activated microglia, large amounts of cytotoxic factors such as ROS and RNS produced by NADPH oxidase or MPO may promote neuronal loss.

Conclusion and future prospects

Oxidative stress is thought to play a key role in sporadic PD (Youdim and Riederer, 1997). Presence of neuroinflammation and oxidative stress may have a causative link in PD. Oxidative stress may trigger microglia activation and neuroinflammation (Hald and Lutharius, 2005).

In the brain from patients with PD, activated microglia are observed not only in the nigro-striatum where cell loss of dopamine neurons occurs, but also in various brain regions such as the hippocampus. The activation of microglia may occur in tow steps. At the first step, the activated microglia produced by unknown stimuli may act for neuro-

protection at least in the early stages of PD. At the second stage by other unknown factors, neuroprotective microglia may be subjected to toxic change that convert microglia from neuroprotective to neurotoxic type to promote the progression of neurodegeneration.

There remain several points to confirm this hypothesis on the role of activated microglia and cytokines in PD. First, the presence of neuroprotective and neurotoxic microglia in the human brain should be confirmed. Second, in vivo evidences of toxic change of microglia are required in some experimental models of PD. Third, the stimuli to activate microglia at the first stage must be identified. Since the causative factors of sporadic PD are speculated to be multiple, the stimuli may also be multiple. Fourth, the factors and unknown regulators for the toxic change of activated microglia must be identified.

The present hypothesis is expected to be useful for developing drugs against PD. Anti-inflammatory drugs have been considered for the treatment of PD. However, such anti-inflammatory drugs should inhibit the toxic change of microglia or act only to toxic subtype of microglia.

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Surgical therapy for Parkinson's disease

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Summary. High frequency stimulation (HFS) has become the main alternative to medical treatment, due to its reversibility, adaptability, and low morbidity. Initiated in the thalamus (Vim) for the control of tremor, HFS has been applied to the Pallidum (GPi), and then to the subthalamic nucleus (STN), suggested by experiments in MPTP monkeys. STN-HFS is highly efficient on tremor, rigidity and bradykinesia and is now widely applied. Criteria for success are correct patient selection and precise electrode placement. The best outcome predictor is the response to Levodopa. The mechanisms of action might associate inhibition of cell firing, jamming of neuronal message and exhaustion of synaptic neurotransmitter release. The inhibition of glutamate STN release could be neuroprotective on nigral cells. Animal experiments support this hypothesis, not contradicted by the long term follow up of patients. Neuroprotection might have considerable impact on the management of PD patient and warrants clinical trials.

Introduction

Surgery was the first treatment for Parkinson's disease (PD), before the introduction of the levo-dopatherapy. In the years 1950, as a product of serendipity and random walk, thalamotomy and pallidotomy were used to suppress essential as well as parkinsonian tremors. The effects were usually very satisfactory but, depending on the size of the lesion, they could decrease along time or they could be associated to side effects and complications, particularly when surgery was performed bilaterally. In this context, during the sixties, the advent of levodopa, because also of its efficiency, sent surgery to archives. One has to wait until the early years 1980, to see the levodopa side effects raising the need for alternate solutions and the brain graft's saga starting (Bjorklund et al., 2003; Winkler et al., 2005). In 1987, the discovery of high frequency stimulation (HFS) reintroduced surgery in the field of the therapy of PD and movement disorders (Benabid et al., 1987; 2005). In 1992, Laitinen reintroduced pallidotomy (Laitinen et al., 1992) which was redesigned from the previous approach from Leksell (Svennilson et al., 1960). In 1993; HFS of the subthalamic nucleus (STN) becomes a treatment of advanced stages of PD (Pollak et al., 1993; Limousin et al., 1998). Since the year 2000, there is a real blossoming of surgical therapies for PD and movement disorders, based on HFS. Overall, HFS of STN is currently the prevalent surgical therapy for PD (Krack et al., 2003).

HFS mimics lesion in all available targets and might act through functional inhibition

This is based on the fact that the effects of HFS mimic those of ablative surgery. This was observed, or at least related to HFS, for

the first time in 1987 (Benabid et al., 1987) during a thalamotomy for essential tremor where it became clear that, in a frequency dependant manner, there was a paradoxical lesion like effect of stimulation at frequencies around or above 100 hertz. This led to the surgical concept that ''HFS is equivalent to lesion''. As a consequence, HFS has replaced ablative surgery in all available targets, including the thalamus, the pallidum, and the STN nucleus, where it is considered, although we do not know exactly how this happens, that HFS induces a functional inhibition.

The effects are immediate and reversible

This is easily observed in the thalamus where the tremor can stop within seconds after the onset of stimulation and recur as quickly when the stimulation is stopped. This is also the case during pallidal stimulation which suppresses levodopa induced dyskinesias immediately as well as reversibly (Siegfried et al., 1994). Finally, a similar observation can de done in STN, following the demonstration in MPTP monkeys in 1990 (Bergmann et al., 1990) and in 1991 (Aziz et al., 1991), that it could be a new as well as an efficient surgical target (Pollak et al., 1993; Limousin et al., 1998), after it has been shown also that HFS in monkeys would produce the same effect than lesions without inducing the hemiballistic expected side effects (Benazzouz et al., 1993). Since, we learnt that STN HFS is efficient on akinesia, rigidity and tremor as well as, indirectly, on levodopa induced dyskinesias in a very acute and reversible manner.

Clinical benefits of HFS of STN in advanced stages of PD

The long term effect of STN HFS are stable and a three year follow up in 1998 (Limousin et al., 1998), as well as a five year follow up in (Krack et al., 2003), were published, showing that most of the symptoms, such as tremor, rigidity and akinesia, and to a lesser but still very significant degree, instability, gait and activities of daily living, as well the indexes of Schwab and England and total UPDRS III are stably improved, at least at 50% for most of them and around 65 to 70% for tremor and rigidity. However, speech and writing are not so spectacularly improved. Besides of these cohort studies after 3 and 5 years, one might take into account that in 2005, we gathered 12 years of experience in more than 250 bilateral STN cases, confirming the stability and the quality of those long term results. In addition, drug doses, and therefore the iatrogenic dyskinesias, can be decreased because of the quality of the stimulation effects, which allow reducing the levodopa equivalent doses by an average of 60%, which in turn as a consequence decreases disability due to dyskinesias by more than 70% and duration of dyskinesias by an average of 65% along these five years. There is also a progressive decrease of choreoballic dyskinesias induced by a levodopa challenge as well as by STN HFS which, as compared to preoperative control values, are decreased at 6 months and even more at 12 months, and barely inducible around 2 years after surgery. The speech is less improved than motricity: according to the rating scales, the average improvement of speech is around 35%, as compared to the 60% (UPDRS III) observed in the same series of patients for the motor symptoms (Gentil et al., 2001). This insufficient benefit on speech and language might be due to a possible existence of a ''symptomatotopy'': the target is explored mainly by the clinical evaluation of the improvement of the rigidity of the wrist during the intraoperative tests, which determines the final placement of the chronic stimulating electrode. One might imagine that, if symptoms were depending on various subparts of the STN, one might systematically miss the language area, if any, explaining why this function is often, but not always, less improved than the rest of the motor functions. Dysarthria, which appears usually at higher

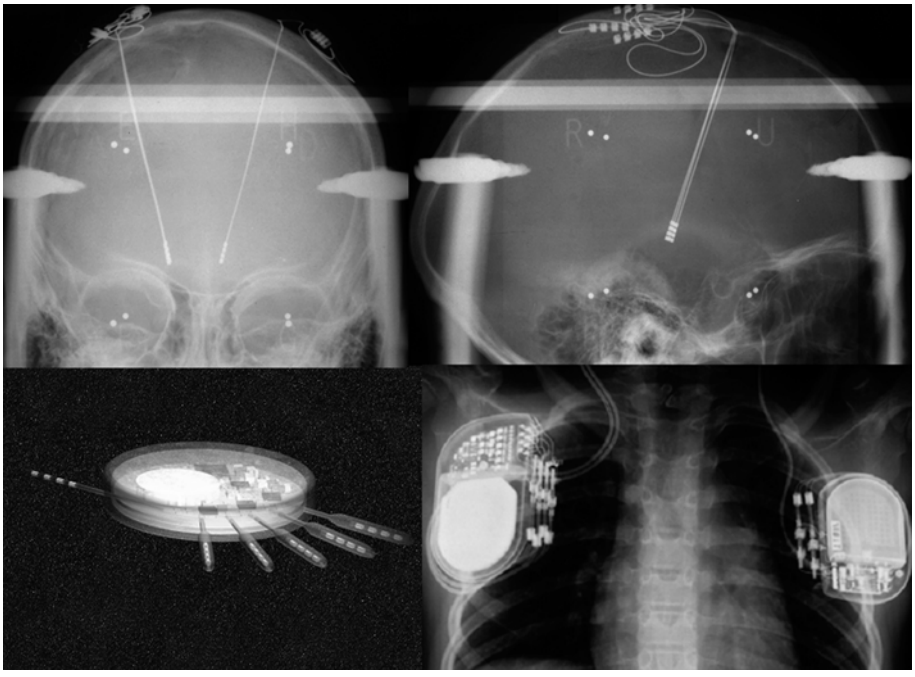

Fig. 1. *Upper Lane*: Anteroposterior and Lateral X-Ray views of a bilateral implantation in STN with 2 electrodes on the right side and one electrode in the left side. *Lower Lane*: Lower right corner: Connection of the 2 electrodes on the right side to a Kinetra and of the left electrode to a Soletra. Lower left corner: Sketch of the prototype of a programmable multiplexer

voltages, is probably due to the spreading of current to the corticobulbar fibres of the internal capsule and the solution could be provided by multiple electrode implantations, allowing a finer coverage of the volume of the STN. An implantable and programmable multiplexer is being designed and tested to achieve this goal (Fig. 1). Actually, multiple electrodes can be implanted, as it has been done in several instances, either with multiple electrodes in one target (in some cases the choice for the best of 5 electrodes on one STN was difficult and in 25 patients 2 electrodes were implanted, sometimes with connexion of the 2 electrodes on one side to a Kinetra® and the other one to Soletra®) (Fig. 1) or in some cases in two different targets for the same patient (as it has been done in 2 cases of dystonia with 2 electrodes implanted bilaterally in STN and implanted also bilaterally in the internal pallidum). The hypothesis that there might be a ''symptomatotopy'' into the STN nucleus, may explain why some symptoms such as speech are not so well improved. The main symptom on which the electrodes are targeted is the rigidity of the wrist. Rigidity might be taken care of by other parts of the STN nucleus than the one controlling the speech. The idea has come to implant several electrodes into the STN and particularly to put the five electrodes into the five channels explored by micro-electrodes. A prototype of a programmable implantable multiplexer has been developed and will be submitted to clinical trial. There is no cognitive decline as shown with follow ups of 3 years and 5 years (Ardouin et al., 1999; Jahanshahi et al., 2000) using the Mattis, Beck and frontal score scales at 3 years and then confirmed at 5 years follow up. Depression is observed post-operatively in 20% of the cases and one suicide has been observed in the entire series of 250 cases. Their causes might be multifactorial. Part of the mechanism

could be due to the decrease (in average about 50 to 65%) in levodopa doses which might induce strong withdrawal effects of this potent psychotonic drug. The role of the pre-existing neuropsychological background should not be neglected.

Also, the strong improvement provided by DBS stimulation in STN is responsible for profound changes in patients' live (the patients quite often say ''Surgery was my second birthday''). However a possible role of the limbic part of the STN, which could be involved by the spreading of the current, cannot be ruled out and will be further explored.

STN DBS does not cure PD but might be neuro-protective

This has been shown in experimental animals using the Sauer and Oertel model (Sauer and Oertel, 1994) where intra-striatal injection of 6 OHDA in rats induces by retrograde transportation an ipsilateral degenerescence of the nigral cells in the substantia nigra compacta (Piallat et al., 1996). This is prevented if prior to 6 OHDA intra-striatal injection, a lesion is made into the STN using intra STN kainic acid lesion. Similar data have been observed by Paul et al. (2004). In primate experiments done in our laboratory, and so far not published, studies have been done on 30 monkeys either receiving a kainic acid lesion or DBS into the STN using the 3389 Medtronic electrode and a stimulator, before or after MPTP administration to make those animals parkinsonian. The primary outcome of those studies is the ratio of the right to the left side (the right side being the one where STN has been altered either by kainic acid lesion or STN stimulation) of the counts of the total number of cells stained by the Nissl stain or of the preoperative UPDRS III score. The remaining 38% of the patients improve, from UPDRS III 58 in pre-op to 38 at one year follow-up. Half of those patients, 19% of the total population of 89 patients so far tested, show a continuing and progressive decrease in the UPDRS III score showing a significant improvement along 5 years. However, this does not allow us to consider that neuroprotection is demonstrated as the period of off medication off stimulation has always been too short, for 2 to 4 hours in addition to the off during the night. This can be criticized as it might be possible that much longer off period (which is neither ethically possible nor accepted by most of the patients) might lead those patients to a level of their UPDRS III score which might be equivalent or even worse than their pre-operative basic score. Therefore, other studies are needed, including in particular non clinical outcome measures, such as metabolic outcomes provided by PET scan studies.

The complications are mild, most of the time reversible, but not absent

DBS surgery, although less risky than the ablative surgery, cannot be considered as risk less.

Related to ventriculography, there is 1.5% of asymptomatic bleeding and 2.1% of superficial infection at the level of the skin.

Related to the implantation of the electrode, 1.2% of bleeding has been symptomatic, 14.2% were asymptomatic (revealed by systematic post-operative MRI control). Confusion and bradyphrenia are observed in 21.8% of the cases and all resolved within 3 days to 3 weeks.

Infection related to the hardware was observed in 1.4%, most of it being observed after a long time due to erosion of the skin in front of the bulky connectors and never propagated to the brain or cerebrospinal fluid spaces.

Related to stimulation, complications are dyskinesias which always happen at voltages higher than the threshold for clinical improvement. Eyelid opening apraxia is observed at 15.6%, often appearing in patients who already had it before surgery and which

rarely needed additional treatment with injection of botilinum toxin.

However, surgical complications are most of the time related to the surgical practice and therefore can and must be solved. Confusion is temporary, probably related to the caudate nucleus, which is bilaterally traumatized by the guide tubes for micro-recording. This happens less often if the tracks are parallel to the midline, going through the ventricle or far lateral, avoiding not only the ventricle itself but also its border, where is the caudate nucleus.

Criteria for success are essentially the result of team cooperation

The patient's selection is extremely important, concerning essentially idiopathic Parkinson's disease and the quality of the levodopa response is one of the major predictors of success (Charles et al., 2002). The surgical procedure depends on the quality of the targeting which is based on neuroradiology but also on electrophysiology using micro recording as stimulation and the quality of the surgical steps. The microrecording is quite often debated and, depending on the teams, might be considered as either dangerous or useless. Largest studies are needed to evaluate the benefit and the risk of this approach which in our opinion is valuable and participates to the optimal positioning of the final electrode. The use of intraoperative stimulation requires clinical examination under local anaesthesia and the presence of a neurologist in the neurosurgical operating room is always beneficial for the quality of the results. The follow up and tuning of the patient is creating a heavy burden on the shoulders of neurologist. There is a delicate stimulation-drug interplay which is sometimes critical to manage during the first three weeks. Then, further adjustments of parameters are usually needed along the evolution of the patients. This makes actually their follow up easier but because of the lack of a widespread education of general neurologists, this relies essentially on the shoulder of academic teams. This becomes sometimes a limiting factor. There is a clear need for education of neurologists, particularly those involved in movement disorders, to take care of this follow up, which however is much lighter than dealing unsuccessfully with the difficulties encountered by advanced Parkinson's patients. The patient's selection is critical: the best patients have idiopathic Parkinson's disease with levodopa responsiveness (the STN induced improvement and the Levodopa induced improvement shows a strong linear relationship). Patients with motor fluctuation and dyskinesias are the most improved patients because of the disappearance of their fluctuations and levodopa induced dyskinesias. The patients should not have already reached any level of neurocognitive alteration and there should not be any general contraindication. It seems reasonable to consider that STN stimulation could be proposed to patients when medication fails to maintain their quality of life, and particularly before their professional activity is jeopardized. One might define a quality index which would be the ratio of the medical improvement to the surgical improvement. This would allow the surgical teams to monitor the quality of their surgery, trying to keep this quality index as close as possible to 1.

The quality of the surgery is a key factor

The surgical procedure is a matter of debate and one should leave the surgical teams choosing what they feel the most comfortable according to their situation, expertise and equipment. There are different ways to skin a cat: however the stereotactic method ensures precision, the MRI, with or without ventriculography, provides the landmarks, the microrecording identifies the target, the stimulation simulates intraoperatively the final outcome, particularly as far as side effects are considered, the fixation to the skull

of the electrode is a very crucial step, which prevents migration of the electrodes, and hardware implantation should be performed extremely carefully in order to prevent infection. But the cat must be properly skinned out, and variations, deviations, innovations are welcome but they should be validated if they provide equal or even better outcome, using again the quality index which should be used as a criterion to validate those differences from the main classical techniques. The precision of the surgical procedure is the key and there is a direct relationship between the distance of a given contact to the coordinate of the theoretical best target (which is the point defined by the average coordinates of the clinically defined best contacts in a large series of patients). In our series, the average distance of 394 clinical contacts to the target is equal to 2.03 ± 0.9 millimetres. Measuring and calculating those distances for each patient allow us to predict correctly in 54% of the cases the contact which will be chosen and in 91% including the up or lower contact, next to it. This might help the neurologists in their screening for the best contact.

All efforts must be spent to reach the optimum and surgeons' hours which are ''saved'' might cost years of patient's discomfort.

The tuning and follow-up are most of the time done by the neurologists

There are not 64 parameter combinations as often stated. Most of the time, stimulation can be monopolar (the case positive and only one contact activated) at 130 Hz frequency, 60 microsecond pulse width, and around 2.5 volts amplitude. Any need for change because of insufficient effect and/or side effects means more or less electrode misplacement. The tuning needs training. There is a non simple optimal compromise between symptoms, side effects, drug dosages and parameters. STN high frequency stimulation plus levodopa induces dyskinesias, increasing the voltage induces a current spread and then the recruitment of side effects. The battery end of life must be prevented by regular checking to avoid dramatic situations when the battery finally fails abruptly. This must be performed or carefully controlled by the neurologist. The concept of ''spatial overdose'' can be described in a case of a patient with simulation at 130 Hz, 60 microseconds and 3 volts on contact no. 2, which highly improved the patient but then induced apathy. Then, in order to improve this apathy, which was considered as a remaining part of the Parkinson's symptoms, the contact no. 3 has been added, keeping the other parameters equal. This led to hypomania and laughter. Then adding a third contact (contact no. 1) again without changing parameters induced aggressivity and loss of temper. This should teach us that complications, and particular neuropsychological complications, might be in part due to inadequate tuning of the parameters and to inadequate choice of contacts.

One must absolutely locate the sites of side effect

Where do they come from? Is STN responsible for all those changes? The case by the group of Paris (Bejjani et al., 1999) of a patient who was acutely and strongly depressed by deep brain stimulation in the STN area was initially related to the position of the electrode in STN and then to the Substantia nigra reticulata. This explanation are not necessarily easy to state: this is probably not occurring in STN as patients exhibiting these side effects are exceptional, as well as we have now the knowledge, through STN stimulation for epilepsy, that this type of behaviour is not induced in those cases. Similarly the laughter which has been reported in several situations by simulation at the STN site, but at higher voltages, makes difficult to relate exactly this abnormal behaviour considered as a side effect and a real location where this neurophysiological effect is induced. On the contrary, the dyskinesias or

hemiballistic type which are induced very easily during surgery or during the tuning of the patient by STN stimulation are clearly equivalents of hemiballism or of dyskinesias: there are due to too strong parameters of stimulation of this structure. However it is not possible to rule out the role of the limbic part of the STN nucleus.

What did we learn from this experience along 12 years of STN stimulation in Parkinson's disease and of 17 years of stimulation of the thalamus?

We know for sure that high frequency stimulation induces functional inhibition which is frequency dependent, within a plateau area from 100 to about 2,500 Hz, that ganglia and not bundles are involved by this functional frequency dependent inhibition. This does not create a de novo process but it seems that HFS disrupts a wrong information processing. There are conflicting data which need clarification and a generalized theory. The mechanism is unknown but the causes are probably multiple. This mechanism could be due, as we initially suggested it in the thalamus, to a jamming which is supported by experiments in the monkey, (Hashimoto et al., 2003) where STN stimulation induced at the level of GPI dual effects mixing silencing and bursting. Silencing of STN neurons has been observed in human during stimulation (Welter et al., 2004) at frequencies starting about 80 Hz, as well as after the stimulation period (Filali et al., 2004). These data are difficult to obtain and to analyze, because of the stimulation artefact. Similar effects have been observed in GPI (Dostrovsky et al., 2000) and they have been interpreted as the results of activation of afferent GABAergic terminals.

It could be neurotransmitter depletion by exhaustion of the biochemical process ensuring the replenishment of the synaptic vesicles. Non published data from our group have shown that stimulation of cells in culture was able to diminish the prolactine release of GH3 cells as well as the release of dopamine, epinephrine and norepinephrine in PC12 cells, suggesting that exhaustion of the neurotransmitter production could be part of the mechanism involved. This leads to a putative model of the mechanism of HFS encompassing the globality of these mechanisms, which might all of them play altogether a specific role leading to this very clinically efficient effect, acute and reversible as well as titrable, of deep brain stimulation.

What did we learn also in terms of global functioning?

It seems clear that there are therapeutical networks and nodes, but not simple targets: for instance the tremor can be altered by stimulation in Vim, CM-Pf, STN and GPI, while bradykinesia and rigidity are improved by stimulation of STN and GPI, and that the recent observation of OCD improvement might encompass the STN, the GPI, the accumbens as well as CM-Pf. These circuits overlap in part with those used by dopamine and subcomponents may account for subtle differences in effects.

What are the alternatives?

There is no better future for a method than being replaced by an even better one. This could be gene transfer as it has been already tried (Luo et al., 2002) using AAV mediated gene transfer of GAD in STN: this nucleus has been transformed from a glutamatergic to gabaergic nucleus. Similarly the infusion of growth factors such as GDNF directly in the caudate nucleus and putamen has been reported (Gill et al., 2003). Although this has not been confirmed by a multicenter clinical trial (Nutt et al., 2003), there is probably something to use in this approach and new methods should be designed and refined. Motor cortex stimulation has been reported in MPTP monkeys to improve rigidity at high

frequency by mechanisms which are not fully understood but might involve a decrease in activity in STN and in the pallidum (Drouot et al., 2004). Finally the neural grafts are still the ultimate elegance. An extremely large and skillful amount of work has been done during the last decades, unfortunately not leading to a currently routine therapeutic approach, but there is no doubt that in the future the improvement of the method and may be the use of new paradigms, particularly using solutions such as stem cells or genetically modified host cells, could be providing solutions which would be truly therapeutic and curative instead of substitutive methods (Bjorklund et al., 2003).

There is a need for a consensus: we must compare our targets and our results, although we know that neurosurgical methodological consensus is an utopy. We need a consensus on data communication: for instance coordinates on MRI or on ventriculography should be provided with x, y, z coordinates versus common standardized landmarks such as anterior commissure, posterior commissure, AC-PC line, the height of the thalamus and the midline and means and standard deviations as well as the number of cases should be provided to allow fair comparison between results of different teams Similarly, the outcomes should be provided as percentages of improvement on global scales used and agreed on by all teams such as UPDRS, and quality of life scales. The quality index (ratio of the improvement of stimulation versus the improvement of medication) and the outcome should provide the percentage of improvement on commonly agreed scales, the quality index should be also used to rationalize the results of the patients according to their initial status. At this level again means of the deviation and number of cases should be provided. There should be comparison between series and coordinates and we should not see anymore sentences such as ''our results are similar to what is reported in the literature''.

Conclusion

We do have a tool as HFS of STN is a current surgical option for treatment of Parkinson's disease. It is safe, reversible, and adaptable and leaves open the future. There is no cognitive decline, and psychological side effect are multi factorial and should benefit from psychological support or prevention. Its mechanism is unknown and it provides access to the pathophysiology of movement disorders. It should and will be replaced by new methods if these new methods provide better outcome.

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Deep brain stimulation for the treatment of Parkinson's disease

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Summary. Approximately 30,000 patients have been treated throughout the world with deep brain stimulation for Parkinson's disease and other conditions. With accumulating experience, there has been an appreciation of the important benefits of this procedure, including the alleviation of disability and improvement in the quality of life. We have also become aware of some limitations of DBS surgery. Among the important issues that remain to be resolved are the timing of surgery, whether early or late in the course of the disease, and the best target for the individual patient, including a reassessment of the relative merits of globus pallidus versus subthalamic nucleus surgery. A better understanding of the symptoms that are resistant to both levodopa therapy and DBS surgery is also required.

Introduction

The introduction of deep brain stimulation (DBS) as a therapeutic alternative for the treatment of advanced Parkinson's disease (PD) has revolutionized the clinical management of this condition. In the last decade, the treatment has matured from one of ''last resort'' to a valuable therapeutic modality that is now offered routinely to patients. It has become an important contributor to helping these patients live an active and functional life. In this review we will discuss the anatomical targets, technical aspects, clinical results and adverse effects of DBS for the treatment of PD.

Anatomical target

Targets for DBS treatment of PD are the thalamus, globus pallidus internus (GPi), and the subthalamic nucleus (STN). There is still debate regarding target selection.

Thalamic DBS significantly improves contralateral arm tremor, but it is not effective for the treatment of other parkinsonian motor symptoms (Benabid et al., 1996; Pollak et al., 2002). As a result, thalamic surgery for PD has virtually been replaced by GPi and particularly STN DBS surgery.

The GPi is a large structure, which can lead to significant variation in the site of DBS implantation. This may be one of the factors responsible for the variability in outcome reported in different surgical series (30–55% improvement with bilateral stimulation) (Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Brown et al., 1999; Burchiel et al., 1999; Durif et al., 2002; Ghika et al., 1998; Rodriguez-Oroz et al., 2005). The reduction in tremor with GPi surgery is in the order of 70–80%, while rigidity and akinesia improve by approximately 40–60% (Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Brown et al., 1999; Burchiel et al., 1999; Durif et al., 2002; Ghika et al., 1998; Rodriguez-Oroz et al., 2005). Gait and posture

have a smaller degree of improvement that significantly declines over time (approximately 40% at 1 year and 25% at 3–4 years). There is a striking decrease in the involuntary movements induced by levodopa, in the order of 70–90% (Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Brown et al., 1999; Burchiel et al., 1999; Durif et al., 2002; Ghika et al., 1998; Krack et al., 1998; Rodriguez-Oroz et al., 2005).

The compact size of the subthalamic nucleus and the reproducibility of surgical results (Hamani et al., 2004, 2005) has made the STN the most popular surgical target to treat patients with PD. STN DBS not only improves the cardinal manifestations of the disease and levodopa-induced dyskinesias, but also postural instability and gait to a certain extent (Hamani et al., 2005; Kleiner-Fisman et al., 2003; Krack et al., 2003; Rodriguez-Oroz et al., 2005). Although not entirely clear, it has been suggested that DBS in the STN might pose a higher risk of cognitive and psychiatric side effects compared to GPi stimulation. A final comparison of GPi and STN as the proper target for the treatment of advanced Parkinson's disease will only be properly addressed in future prospective randomized trials. Details on the surgical technique, clinical outcome and adverse effects of STN DBS are presented below.

Subthalamic nucleus stimulation

Selection criteria

Perhaps the most critical factor in determining surgical outcome in DBS surgery is the selection of appropriate surgical candidates. To be considered an optimal candidate, patients have to be diagnosed with PD, present disabling motor fluctuations with a prolonged ''off'' state, significant dyskinesias, and demonstrate a good clinical response to L-DOPA (Chen et al., 2003; Daniele et al., 2003; Herzog et al., 2003; Nasser et al., 2002; Pahwa et al., 2003; Patel et al., 2003; Simuni et al., 2002; Thobois et al., 2002; Welter et al., 2002). The last item is worth stressing, as the response to L-DOPA during the L-DOPA challenge test seems to predict surgical outcome (Charles et al., 2002). During this test, a patient's United Parkinson's Disease Rating Scale (UPDRS) motor scores are evaluated before and after receiving a large standardized dose of L-DOPA. The improvement measured in this challenge is what a patient might expect from DBS therapy if a good surgical outcome is attained.

The main exclusion criteria for STN DBS are the presence of a significant cognitive dysfunction, concomitant neurological disorders, and medical problems that might pose a risk for the patient during the procedure (i.e. coagulopathies) (Daniele et al., 2003; Herzog et al., 2003; Nasser et al., 2002; Pahwa et al., 2003; Patel et al., 2003; Simuni et al., 2002; Thobois et al., 2002; Vingerhoets et al., 2002; Welter et al., 2002). There is no consistent age limit for the operation but several centers do not offer surgery to patients older than 70 years.

Surgical technique and parameters of stimulation

As the STN and surrounding structures can be directly visualized with magnetic resonance imaging (MRI), this has been the most commonly employed imaging technique for targeting the nucleus (Barichella et al., 2003; Daniele et al., 2003; Dujardin et al., 2001; Figueiras-Mendez et al., 2002; Gomez et al., 2003; Herzog et al., 2003; Kleiner-Fisman et al., 2003; Krack et al., 2003; Lopiano et al., 2001; Nasser et al., 2002; Ostergaard et al., 2002; Pahwa et al., 2003; Patel et al., 2003; Pinter et al., 1999; Simuni et al., 2002; Starr et al., 2002; Thobois et al., 2002; Vesper et al., 2002; Vingerhoets et al., 2002; Volkmann et al., 2001; Welter et al., 2002). Ventriculography and/or computed tomography scans (CT) are also used to precisely target the anterior and posterior commissures (the former technique) and correct possible imaging distortions related to the MRI

(Daniele et al., 2003; Dujardin et al., 2001; Figueiras-Mendez et al., 2002; Krack et al., 2003; Pinter et al., 1999; Thobois et al., 2002). To date however, the use of neuro-navigation planning stations for surgical targeting has also been able to correct MRI distortions and several centers are using only this imaging modality to target the STN.

Although there remains some controversy over the benefits of microelectrode recording (MER) to map the correct DBS target, most centers that have reported their experience in the literature do use MER to target the STN (Dujardin et al., 2001; Figueiras-Mendez et al., 2002; Gomez et al., 2003; Herzog et al., 2003; Kleiner-Fisman et al., 2003; Krack et al., 2003; Lopiano et al., 2001; Pahwa et al., 2003; Simuni et al., 2002; Starr et al., 2002; Thobois et al., 2002; Vesper et al., 2002; Vingerhoets et al., 2002; Welter et al., 2002). The objectives of this technique are to identify the sensorimotor territory of the subthalamic nucleus (characterized by the presence of movement-related cells) and the transition between the STN and the substantia nigra (Abosch et al., 2002; Hutchison et al., 1998). Once the surgical target is mapped, the DBS electrode is implanted at the established coordinates and each contact is tested for efficacy and stimulation-induced adverse effects. Such stimulation-induced effects are often caused by the electrical stimulation of structures that are adjacent to the STN. Typical findings include stimulation of oculomotor fibers (medial to the STN), the corticospinal tract (anterior and lateral), and fibers of the medial lemniscus (posterior). If adverse effects are noticed when low voltages are applied, the electrodes may have to be repositioned.

Implantation of the pulse generator and battery constitutes the second step of the procedure. The pulse generator is most commonly implanted in the subcutaneous tissue of the infraclavicular region. This can be done on the day the electrodes are implanted or after a brief period of time (days to weeks). Programming of the device often starts a few weeks after surgery. Most centers are currently using monopolar stimulation (one of the electrode contacts is used as the cathode and the pulse generator as the anode). Stimulation parameters typically vary between 2.5 to 3.5 V, 60 or 90 microseconds of pulse width, and 130–185 Hz of frequency (Hamani et al., 2005).

Clinical outcome

A summary of the clinical outcome with STN DBS may be found in Table 1.

With STN DBS, UPDRS part II scores (activities of daily living) improve by 50–66%

Table 1. Clinical outcome following subthalamic nucleus stimulation in Parkinson's disease

	1 year follow-up		3–5 years follow-up	
	"on" stim; "off" meds	"on" stim; "on" meds	"on" stim; "off" meds	"on" stim; "on" meds
UPDRS II	50–66%	60–76%	40–45%	45–65%
UPDRS III	50–66%	65–80%	45–60%	55–75%
Tremor	75–95%	85–95%	75–90%	95–98%
Rigidity	60–75%	70–85%	55–74%	70–85%
Bradykinesia	45–65%	65–80%	40–55%	50–65%
Gait	50–70%	65–81%	40–61%	55–74%
Postural Inst.	55–70%	65–80%	30–50%	40–60%

"on" stim; "off" meds: Patients evaluated with stimulation and no drugs. *"on" stim; "on" meds*: Patients evaluated with stimulation and drugs. *UPDRS* Unified Parkinson's disease rating scale. *Postural Inst.* Postural instability

after 1 year (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005), and by 40–45% after 3–5 years (Krack et al., 2003; Rodriguez-Oroz et al., 2005). When both stimulation and medications are used, UPDRS II scores improve by 60–76% after 12 months (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005), and 45–65% after 3–5 years (Krack et al., 2003; Rodriguez-Oroz et al., 2005).

Improvement in UPDRS motor scores with stimulation alone is 50–66% after 1 year (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005), and 45–60% after 3–5 years (Krack et al., 2003; Rodriguez-Oroz et al., 2005). When both stimulation and medications are used, UPDRS motor scores improve by 65–80% after 1 year (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005), and 55–75% after 3–5 years (Krack et al., 2003; Rodriguez-Oroz et al., 2005). Improvements in tremor and rigidity using only DBS at 1 year (without medications) are 75–95% and 60–75%, respectively (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005). Benefits of stimulation to treat these symptoms are sustained at long-term (Krack et al., 2003; Rodriguez-Oroz et al., 2005).

In contrast, the efficacy of STN DBS to control gait, postural instability, and to a certain extent bradykinesia declines over time (45–70% improvement at 1 year with DBS alone vs. 30–61% at 3–5 years) (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005).

The deterioration that has been reported at long term in the quality of life and overall motor scores in PD patients treated with STN DBS (Krack et al., 2003; Rodriguez-Oroz et al., 2005) is partly the result of worsening of gait, posture, and akinesia that occurs at long-term. In addition however, non-dopaminergic symptoms begin to play a significant role in the morbidity of the condition. Symptoms that are resistant to levodopa, such as speech problems, cognitive and psychological difficulties, bladder, bowel and sexual dysfunction, among others, are also resistant to surgery and lead to a major disability in patients with advanced PD. At this time there is no effective treatment to address these symptoms.

Dyskinesias

The ability of STN DBS to improve dyskinesias is likely related to two mechanisms: The reduction in patients' levodopa intake after surgery or a direct antidyskinetic effect. The mean levodopa-equivalent dose used pre-operatively is reduced by approximately 50–60% with STN DBS at 1 year (Hamani et al., 2005; Krack et al., 2003), results are sustained at long-term (Krack et al., 2003; Rodriguez-Oroz et al., 2005). At 1 year, the mean reduction in dyskinesias is around 70–80% and seems to be sustained at long term (Hamani et al., 2005; Krack et al., 2003; Rodriguez-Oroz et al., 2005). This feature of reducing levodopa requirement is not usually seen with GPi DBS surgery.

Adverse effects

There are several types of complications and adverse effects that can occur with DBS surgery. These include 1) surgical and hardware-related complications, 2) adverse effects related to stimulation, and 3) neurological complications.

Surgical and hardware-related complications

The most fearsome complications of STN DBS are intracranial hemorrhages produced by the electrode penetration. The incidence of hemorrhages is approximately 2–3%. These are most commonly located in the brain parenchyma but can also be subdural or intraventricular (Hamani et al., 2005). Most hemorrhages are asymptomatic, and are identified only by post-operative scanning (Terao et al., 2003). However, in some patients the effects of a bleeding can be serious, leading

to transient or permanent sequelae. There is some suggestion in the literature that multiple passes of microelectrodes for mapping may increase the risk of hemorrhage. In addition, some feel that the increased operative time for microelectrode mapping may increase the infection risk. In contrast, the use of MER is presumed to decrease the adverse effects associated with targeting inaccuracy. A definitive study weighing benefits and risks of MER has not yet been conducted.

Infections occur in 3–4% of the patients treated with STN DBS (Hamani et al., 2005). This infection rate is roughly equal to that of other neurosurgical procedures. Though controversial, it has been estimated that approximately half of these wound infections can be treated with antibiotics alone, while the other half require the removal of parts or the entire DBS system.

Lead replacement or repositioning is required in approximately 5% of the patients treated with STN DBS (Hamani et al., 2005). Half of these are leads that need to be repositioned due to an initially poor clinical effect. One-quarter are replaced after lead migration from an initially effective position. The remaining 1/4 are replaced after breakage of the wires. Postoperative swelling in the region of the internal pulse generator or extension cables occurs in less than 1% of patients.

Current battery technology has developed compact and long-lived batteries. Still the constant stimulation required to treat PD eventually exhausts the batteries and several replacements may be needed. The estimated time for the batteries to fail in patients with PD is 4–5 years (Bin-Mahfoodh et al., 2003). Emerging technologies should produce batteries that can periodically be recharged, obviating the need for replacement.

Other miscellaneous complications include CSF leaks, meningitis, venous phlebitis, pneumonia, urinary infections, pulmonary embolism, and perioperative seizures. These complications totaled an additional 3.1% of patients (Hamani et al., 2005).

Stimulation-induced adverse effects and neurological complications

Dyskinesias, paresthesias, diplopia, dystonia, and motor contractions are relatively common stimulation-induced side effects (particularly when higher voltages are used during the programming of the patients). In addition, hypophonia, eyelid apraxia, increased libido, sialorrhea, and decreased memory have also been reported, mostly unrelated to stimulation.

Weight gain is a common feature following STN surgery and may be attributed to several changes, including the control of dyskinesias (Barichella et al., 2003), the increased motility experienced by the patients, and the greater social engagement that often occurs after surgery. Perioperative confusion occurs is approximately 15% of the patients (Hamani et al., 2005). It is usually transient and may be related to reductions in levodopa perioperatively (Berney et al., 2002; Lang et al., 2003). Depression has been reported in 5–25% of the patients treated with STN DBS (Berney et al., 2002; Hamani et al., 2005; Houeto et al., 2002). However, these numbers are likely underestimated if one acknowledges that the general incidence of depression in patients with PD is in the order of 40–50% (McDonald et al., 2003; Murray, 1996). As suicide attempts have been described in PD patients that underwent STN DBS (Burkhard et al., 2004; Doshi et al., 2002), careful attention must be paid to patients that develop psychiatric complications in the post-operative period. Future studies are still needed to identify predictive factors of these complications before the procedures.

Conclusion

Bilateral subthalamic nucleus stimulation improves motor outcomes, activities of daily living and dyskinesias in patients with PD. Despite of the impact of this potent therapy, symptoms that are resistant to levo-dopa do not respond very well to surgery. The better understanding of the mechanisms of the

disease and the identification of new surgical targets are current objects of study and may help to treat patients with PD in the future.

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Deep brain stimulation in Parkinson's disease patients: biochemical evidence

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Summary. Deep brain stimulation (DBS) of the subthalamic nucleus (STN) in Parkinson's disease (PD) patients augments STN-driven excitation of the internal globus pallidus (GPi). However, other DBS-induced changes are largely unknown. Here we report the biochemical effects of STN–DBS in two basal ganglia stations (putamen – PUT – and GPi) and in a thalamic relay nucleus, the anteroventral thalamus (VA).

In six advanced PD patients undergoing surgery, microdialysis samples were collected from GPi, PUT and VA before, during and after one hour of STN–DBS. cGMP was measured in the GPi and PUT as an index of glutamatergic transmission, whereas GABA was measured in the VA.

During clinically effective STN–DBS, we found a significant decrease in GABA extracellular concentrations in the VA (−25%). Simultaneously, cGMP extracellular concentrations were enhanced in the PUT (+200%) and GPi (+481%).

DBS differentially affects fibers crossing the STN area: it activates the STN–GPi pathway while inhibiting the GPi–VA one. These findings support a thalamic dis-inhibition, as the main responsible for the clinical effect of STN–DBS. This, in turn, re-establishes a more physiological level of PUT activity.

Introduction

It has been recently shown that subthalamic nucleus (STN) deep brain stimulation (DBS) increases cGMP in the internal pallidus (GPi) while it induces a motor improvement similar to l-dopa treatment, in Parkinson's disease (PD) patients (Stefani et al., 2005). In the central nervous system, extracellular cGMP levels are considered as a marker of excitatory activity, being enhanced either by increased glutamatergic or by reduced GABA transmission (Fedele and Raiteri, 1999; Pepicelli et al., 2004). Paradoxically therefore, the recent report (Stefani et al., 2005) suggests that STN–DBS increases excitatory activity in the GPi; this finding is at odds with the expected effects, based on previous understanding of basal ganglia circuitry. If the pathways reported in the monkey models

of Parkinson's disease (Albin et al., 1989), also apply to human PD brain, then a motor improvement is expected when GPi activity is reduced and, in turn, motor thalamus activity increased. Thus, the cGMP increase, detected in GPi during STN–DBS seems at variance with basal ganglia changes expected to be related to motor improvement in PD.

DBS-induced motor improvement should be coupled to an GABA-decrease-driven increase in VA/VL activity. Hence, the assessment of GABA changes in the VA/VL during STN–DBS should be useful to clarify STN–DBS mechanism of action. Following VA/VL dis-inhibition, a cortical activation is expected which, in turn, could increase the activity of the cortico-putaminal pathway. Therefore, in the present study, we have measured the extracellular levels of GABA, the main inhibitory transmitter released by GPi fibers, in the VA and, simultaneously, cGMP in the PUT and GPi, before, during and after STN–DBS.

Methods

Six, out of ten, advanced PD patients were included in this study according to selection criteria previously reported (Peppe et al., 2004) to implant bilaterally permanent stimulating electrodes both in the STN and in the GPi. In each patient, microdialysis was performed only in one of the two hemispheres. Patient clinical characteristics are reported in Table 1. Written, informed consent was obtained from each patient who participated in the study. The Local Ethics Committee approved the protocol and consent form describing the risks and potential benefits of the study. Briefly STN and GPi target areas were identified preoperatively by means of ventriculography and intra-operatively by means of single unit recordings on two different trajectories, each performed with a multi-electrode holder, one aimed at the STN and the second aimed at the GPi (Peppe et al., 2004). The STN trajectory passed through the VA, the GPi trajectory passed through the PUT. After electrophysiological identification of the targets, the recording electrodes in the GPi, VA and PUT were replaced by microdialysis probes.

Probes were infused (5 μl/min) for stabilization (90 min) (Fedele et al., 2001). During stabilization, the permanent stimulating electrode (Medtronic mod 3389) was implanted in ipsilateral STN. Then, basal microdialysis and clinical data were collected for 50 minutes. Afterwards, STN–DBS was switched on for 60 minutes. Then, 60 minutes of recovery were performed. Clinical changes, contralateral upper limb rigidity and akinesia were continuously assessed by an expert neurologist utilizing selected items of UPDRS (Fahn et al., 1987) (rigidity 0–4, finger tapping 0–4, hand movement 0–4; total = 0 corresponds to "normal", 12 maximum score), while remaining blind to the stimulus intensity between 0 and 3 V. GABA and cGMP concentration were determined by high performance liquid chromatography and by radio-immuno assay (RIA), respectively (Stefani et al., 2005; Fedele et al., 2001).

Significance of GABA and cGMP changes was assessed by performing the mean of single determinations obtained under basal conditions, during STN–DBS and during recovery conditions. Mean values were compared by non-parametric Friedman ANOVA followed by Wilcoxon test. To assess significance of variations in the single fractions after switching DBS on or off, single fraction means were compared to the mean of the previous section (i.e. DBS vs. basal or recovery

Table 1

Patient no.	Age (years)	Disease duration (years)	LD therapy (years)	LTTS duration (years)	LD therapy before DBS (mg)
1	54	7	7	3	800
2	45	6	5	3	1200
3	54	11	6	5	800
4	65	13	9	6	1100
5	62	10	8	3	750
6	68	8	5	3	800
Mean	**58.00**	**9.17**	**6.67**	**3.83**	**908.33**
SD	**8.56**	**2.64**	**1.63**	**1.33**	**190.83**

vs. DBS). All these comparisons were performed by Wilcoxon test. Clinical scores were compared using similar non parametric methods.

Results

Extracellular cGMP concentration was examined in ten subjects. In four patients, cGMP basal values (in the first 5 fractions), either in the GPi or in the PUT, were below the RIA detection limit (<1 fmol) and, therefore, data were eliminated. In the other six patients, cGMP basal concentrations were clearly detectable and were relatively similar among patients (see Table 2).

Clinical data

In all the six selected patients with detectable basal cGMP levels, STN–DBS produced a significant (Friedman ANOVA, $Chi^2 = 14.25$, $p < 0.001$) and clear decrease of the UPDRS items score in the contralateral part of the body. The mean basal score (8.27 ± 1.05) significantly decreased to 5.67 ± 0.70 (Wilcoxon test, $Z = 2.52$, $p < 0.05$) after only ten minutes of STN–DBS. Once STN stimulation was switched off, the UPDRS score increased significantly to 8.06 ± 0.88 just 10 minutes after DBS (Wilcoxon test, $Z = 2.52$, $p < 0.05$).

Microdialysis data

STN–DBS produced a significant change of GABA extracellular concentrations in the VA ($n = 6$, mean -24.5%, Friedman ANOVA $Chi^2 = 9.75$, $p < 0.05$). The decrease in GABA levels was significant (Wilcoxon test $Z = 2.52$, $p < 0.05$) from the first fraction following STN–DBS in comparison to the mean basal value (Table 2). During STN–DBS, the mean value of GABA concentration, was significantly lower than mean basal value (Wilcoxon test $Z = 2.52$, $p < 0.05$). These changes paralleled the amelioration of clinical performance. After cessation of DBS, GABA levels remained decreased for at least

Table 2

	Basal					Mean	STN–DBS						Mean	Recovery					Mean
Fractions	1	2	3	4	5		1	2	3	4	5	6		1	2	3	4	5	6	
Patient no.																				
GABA-VA																				
1	4.30	4.00	4.50	4.90	4.30	**4.40**	3.90	3.70	4.00	4.00	3.80	3.90	**3.88**	3.70	3.70	3.90	3.50	3.40	3.70	**3.65**
2	4.90	4.30	4.10	4.50	4.70	**4.50**	4.10	4.20	3.90	3.80	4.10	4.10	**4.03**	1.50	2.30	3.00	3.50	4.00	3.40	**2.95**
3	4.00	3.90	3.70	4.30	4.10	**4.00**	3.00	4.00	3.50	3.90	3.20	3.00	**3.43**	3.00	3.30	4.10	5.40	6.30	5.00	**4.52**
4	5.10	4.70	4.50	4.80	5.30	**4.88**	3.20	3.10	3.30	3.50	3.40	3.10	**3.27**	3.70	3.80	4.00	4.00	4.20	4.50	**4.03**
5	4.20	4.10	4.00	4.70	4.50	**4.30**	3.90	4.10	3.90	4.10	3.80	3.80	**3.93**	3.40	4.00	5.80	6.20	5.30	5.70	**5.07**
6	5.10	5.60	5.40	5.30	5.50	**5.38**	3.50	2.09	2.00	2.04	2.13	1.70	**2.24**	5.00	5.00	5.50	5.30	6.30	6.00	**5.52**
Mean	**4.60**	**4.43**	**4.37**	**4.75**	**4.73**	**4.58**	**3.60**	**3.53**	**3.43**	**3.56**	**3.41**	**3.27**	**3.46**	**3.38**	**3.68**	**4.38**	**4.65**	**4.92**	**4.72**	**4.29**
SD	**0.49**	**0.64**	**0.59**	**0.34**	**0.56**	**0.49**	**0.44**	**0.81**	**0.75**	**0.77**	**0.70**	**0.89**	**0.67**	**1.14**	**0.88**	**1.06**	**1.14**	**1.24**	**1.05**	**0.94**

(continued)

Table 2 (*continued*)

	Basal					Mean	STN–DBS						Mean	Recovery						Mean
Fractions	1	2	3	4	5		1	2	3	4	5	6		1	2	3	4	5	6	
cGMP PUT																				
1	1.10	1.50	1.90	1.60	1.20	**1.46**	2.00	3.50	5.40	4.40	5.50	4.70	**4.25**	4.30	1.40	1.00	1.90	1.40	1.50	**1.92**
2	1.90	1.70	1.20	1.30	1.30	**1.48**	1.50	1.60	1.40	1.70	2.10	2.10	**1.73**	2.20	1.50	1.70	1.10	1.20	1.50	**1.53**
3	1.40	1.10	1.40	1.30	1.20	**1.28**	1.70	2.20	2.70	2.50	2.80	2.30	**2.37**	2.90	2.10	1.60	1.10	1.40	1.70	**1.80**
4	1.70	1.50	1.70	1.40	1.30	**1.52**	1.40	2.30	2.50	2.30	3.20	3.50	**2.53**	3.20	1.40	1.50	1.70	1.70	1.60	**1.85**
5	1.20	1.50	1.70	1.30	1.70	**1.48**	1.30	2.90	3.20	2.90	3.20	3.10	**2.77**	2.50	1.50	1.20	1.70	1.60	1.40	**1.65**
6	1.40	1.50	1.60	1.30	1.50	**1.46**	1.40	5.60	6.40	5.90	6.90	6.30	**5.42**	6.10	2.10	1.40	1.50	1.00	1.50	**2.27**
Mean	**1.45**	**1.47**	**1.58**	**1.37**	**1.37**	**1.45**	**1.55**	**3.02**	**3.60**	**3.28**	**3.95**	**3.67**	**3.18**	**3.53**	**1.67**	**1.40**	**1.50**	**1.38**	**1.53**	**1.84**
SD	**0.30**	**0.20**	**0.25**	**0.12**	**0.20**	**0.08**	**0.26**	**1.42**	**1.90**	**1.57**	**1.84**	**1.59**	**1.38**	**1.45**	**0.34**	**0.26**	**0.33**	**0.26**	**0.10**	**0.26**
cGMP GPi																				
1	2.40	2.20	1.90	2.40	2.10	**2.20**	4.40	9.70	24.50	22.10	29.70	30.10	**20.08**	28.50	2.40	1.30	1.10	2.10	2.30	**6.28**
2	1.70	1.30	1.70	1.50	1.40	**1.52**	2.70	2.30	2.50	3.20	3.10	3.40	**2.87**	3.90	2.10	1.50	1.40	1.20	1.30	**1.90**
3	2.50	1.90	2.40	1.60	2.00	**2.08**	3.00	2.70	2.30	2.30	2.50	2.90	**2.62**	3.20	2.00	1.50	1.10	2.10	2.30	**2.03**
4	1.00	1.50	1.10	1.00	1.00	**1.12**	2.50	9.40	10.70	12.60	10.90	11.20	**9.55**	12.10	1.90	1.10	1.40	1.10	1.40	**3.17**
5	1.30	1.10	1.70	1.30	1.20	**1.32**	4.40	8.20	16.40	15.50	17.20	16.10	**12.97**	15.20	2.20	1.20	1.10	1.30	1.40	**3.73**
6	1.90	2.10	2.80	2.30	2.40	**2.30**	3.10	2.80	3.50	5.90	6.40	5.10	**4.47**	13.40	2.70	1.80	1.50	2.20	1.50	**3.85**
Mean	**1.80**	**1.68**	**1.93**	**1.68**	**1.68**	**1.76**	**3.35**	**5.85**	**9.98**	**10.27**	**11.63**	**11.47**	**8.76**	**12.72**	**2.22**	**1.40**	**1.27**	**1.67**	**1.70**	**3.49**
SD	**0.59**	**0.45**	**0.60**	**0.56**	**0.56**	**0.50**	**0.84**	**3.60**	**9.05**	**7.81**	**10.41**	**10.47**	**6.89**	**9.21**	**0.29**	**0.25**	**0.19**	**0.52**	**0.47**	**1.60**
UPDRS																				
1	7.0	8.0	7.0	7.0	8.0	**7.40**	6.0	5.0	5.0	5.0	5.0	5.0	**5.17**	5.0	8.0	8.0	8.0	8.0	9.0	**7.67**
2	8.0	7.0	7.0	8.0	8.0	**7.60**	7.0	6.0	5.0	5.0	5.0	5.0	**5.50**	5.0	8.0	7.0	8.0	8.0	8.0	**7.33**
3	9.0	10.0	10.0	9.0	11.0	**9.80**	9.0	7.0	7.0	6.0	6.0	6.0	**6.83**	6.0	9.0	9.0	10.0	11.0	11.0	**9.33**
4	8.0	7.0	8.0	8.0	8.0	**7.80**	6.0	6.0	5.0	5.0	5.0	5.0	**5.33**	6.0	8.0	8.0	7.0	8.0	9.0	**7.67**
5	9.0	9.0	10.0	10.0	9.0	**9.40**	8.0	7.0	6.0	6.0	5.0	5.0	**6.17**	5.0	9.0	9.0	10.0	11.0	10.0	**9.00**
6	7.0	8.0	7.0	8.0	8.0	**7.60**	5.0	5.0	5.0	5.0	5.0	5.0	**5.00**	5.0	8.0	7.0	8.0	8.0	8.0	**7.33**
Mean	**8.00**	**8.17**	**8.17**	**8.33**	**8.67**	**8.27**	**6.83**	**6.00**	**5.50**	**5.33**	**5.17**	**5.17**	**5.67**	**5.33**	**8.33**	**8.00**	**8.50**	**9.00**	**9.17**	**8.06**
SD	**0.89**	**1.17**	**1.47**	**1.03**	**1.21**	**1.05**	**1.47**	**0.89**	**0.84**	**0.52**	**0.41**	**0.41**	**0.70**	**0.52**	**0.52**	**0.89**	**1.22**	**1.55**	**1.17**	**0.88**

20–30 minutes in all the subjects and then returned to pre-stimulation values in the subsequent fractions.

STN–DBS produced also a significant change in cGMP concentration in the PUT ($n = 6$, mean +219%, Freidman ANOVA $Chi^2 = 14.25$, $p < 0.001$). In each patient, the rise in cGMP was significant from the second fraction following STN–DBS (Table 2). During STN–DBS, the mean value of cGMP was significantly (Wilcoxon test, $Z = 2.52$, $p < 0.05$) higher than mean basal and recovery values. After cessation of DBS, cGMP concentration remained elevated for only 10 minutes in all the six subjects and, like clinical amelioration, returned to pre-stimulation values in the following fractions.

In the same subjects, STN–DBS produced a significant increase of cGMP concentration in the GPi ($n = 6$, mean +497%. Friedman ANOVA $Chi^2 = 14.25$, $p < 0.001$). In each patient, the rise in cGMP was significant (Wilcoxon test, $Z = 2.52$, $p < 0.05$) from the first fraction following STN–DBS (Table 2). During STN–DBS, the mean value of cGMP was significantly (Wilcoxon test, $Z = 2.52$, $p < 0.05$) higher than basal and recovery values.

Discussion

It has been postulated that stimulation leads to inhibition of STN – hence, of basal ganglia output – because STN–DBS alleviates parkinsonian symptoms as does STN lesion (Gill and Heywood, 1997) or STN inactivation caused by local injection of lidocaine or muscimol (Levy et al., 2001).

In the present study, we show that clinically effective STN–DBS induced (i) a significant decrease of GABA extracellular levels in the VA and (ii) a significant increase of cGMP extracellular concentrations in the PUT; (iii) moreover, we confirm our previous report (Stefani et al., 2005) of a significant increase in cGMP extracellular levels in the GPi.

In line with the view that increased glucose metabolism in afferent inhibitory fibers from GPi occurs in VA–VL of MPTP-lesioned monkeys (Mitchell et al., 1989), the present study shows that motor improvement by STN–DBS is paralleled by a net decrease of GABA in motor thalamus. Indeed, it has been shown that GPi firing activity, far from decreasing, is increased during STN–DBS (Hashimoto et al., 2003). In addition, a recent PET study in humans has reported a STN–DBS-induced elevation of blood flow in the GPi, suggesting an increased metabolic activity in this area (Hershey et al., 2003). Accordingly, our previous and present findings, showing an increase of excitatory neurotransmission in the GPi, may represent the biochemical counterpart of these electrophysiological and PET data.

A first hypothesis to explain how STN–DBS may promote a reduced GABA release in the VA/VL complex together with an increased cGMP within the GPi would involve the possible activation of presynaptic glutamate receptors increasing GABA release in the GPi during STN–DBS (Bruet et al., 2003) and thus inhibiting GPi–VA/VL transmission. This explanation is conflicting with the clear increase of firing activity in the GPi during clinically efficacious STN–DBS reported by Hashimoto et al. (2003).

Recent electrophysiological findings in humans during surgery may provide alternative hypotheses. The Toronto group reported STN cell inhibition by STN–DBS (Filali et al., 2004) and Welter et al. (2004) described analogous firing changes but associated with the appearance of a ''burst-like'' firing. Actually, a DBS-induced STN firing decrease should result in a reduced excitatory transmission to the GPi possibly accounting for the reduced GABA release in the VA/VL. However, reduced STN activity should produce a decrease of cGMP in the GPi, which is the opposite of what we observed. As acknowledged by the authors themselves

(Welter et al., 2004), it is possible that, STN–DBS stimulation may simultaneously excite the thin fibers (0.8–1.6 μm) (Kita et al., 1983) originating from the STN and innervating GPi, while inhibiting spontaneous firing from STN cell bodies recorded during surgery. This ''decoupling'' between somatic and axonal firing is based on the different sensitivity to a cathodal stimulation of large elements like cell body in comparison to thin fibers, according to their different diameter (Rattay, 1999; Plonsey and Barr, 2000; Basser and Roth, 2000). Alternatively, the increased cGMP extracellular concentrations in GPi could be induced by the shift of STN firing pattern to a stimulus-driven ''burst firing'' mode (Welter et al., 2004) coupled to inhibition of the spontaneous inter-stimulus firing. Nevertheless, we find that increased GPi excitation (i.e. increased cGMP concentration in the GPi) does not produce an increased GABA release in the target VA–VL complex, as one would expect on the basis of basal ganglia circuitry, but instead a fall in thalamic GABA levels.

A possible explanation for this paradox is that the electric field around the STN–DBS electrode may preferentially affect the large (1.5–5.0 micron) GPi fibers (Sidibé et al., 1997) directed to the thalamus and passing along the dorsal border of the STN, where the active contact is often located, inhibiting action potentials propagation along this pathway, similarly to the effect on large cell somata in the STN. Alternatively the ''burst firing'' induced by DBS in the STN may driven GPi firing into a pattern antagonist to the pathological ''high frequency'' dominating basal ganglia in off condition (Brown et al., 2004).

Thalamic nuclei relay form a crucial link between the basal ganglia and cortex by transmitting basal ganglia output to specific cortical areas. Once VA–VL is dis-inhibited by STN–DBS, the activation of the thalamo-cortical pathway is likely to produce an increased excitatory drive to the cortex at rest and/or during movement. PET studies, performed before and following effective STN stimulation, reported a re-activation of fronto-temporo-parietal cortex at rest (Hilker et al., 2004) or of dorsolateral prefrontal cortex and supplementary motor area during movement (Limousin et al., 1997). Several electrophysiological studies have pointed out that cortical functions, impaired in PD patients, are improved during STN–DBS (Pierantozzi et al., 1999, 2002). A direct STN–DBS-induced activation of the thalamo-cortical pathway, producing cortical potentials, has been recently demonstrated for low frequency stimulation (MacKinnon et al., 2005). This finding reinforces the theory that STN–DBS activates the thalamo-cortical pathway.

There are few reports concerning cortico-putaminal pathway changes following pharmacological treatments in PD state. During clinical improvement we found an increase of cGMP in the PUT, an effect that is reminiscent of the l-dopa-induced striatal glutamate increase reported in animals rendered parkinsonian (Jonkers et al., 2002). The cGMP increase we observed is likely due to an increased glutamatergic excitatory input. Large glutamate inputs to PUT come from the cortex, as well as from the centromedian (CM)-parafascicular (Pf) thalamic complex and from the VA/VL complex itself. Both these possibilities are in line with the classical view of the basal ganglia circuitry.

We conclude that: (i) DBS-induced motor improvement in PD is linked to a disinhibition of the thalamo-cortical pathway; (ii) that the second event is a re-activation of putaminal activity, marked by cGMP level increase in putamen, probably linked to increased glutamatergic cortico-putaminal or thalamo-putaminal activity.

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Neurosurgery in Parkinson's disease: the doctor is happy, the patient less so?

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Summary. Despite the overall excellent outcome of neurosurgery in patients with Parkinson's disease, there is often a contrast between the improvement in motor disability and the difficulties of patients to reintegrate a normal life. In this study, the personal, familial and professional difficulties experienced by patients two years after bilateral high frequency stimulation of the subthalamic nucleus were carefully analyzed. To avoid such socio-familial maladjustment, we strongly suggest taking into consideration the patients' psychological and social context before the operation and during the post-operative follow-up.

Despite levodopa treatment, parkinsonian motor disability worsens during the course of the disease, due to the appearance of levodopa-induced adverse reactions and symptoms unresponsive to levodopa treatment. In severe levodopa-responsive forms of the disease, bilateral high-frequency stimulation of the subthalamic nucleus (STN) can be envisaged, which and results in a marked reduction of motor disability and levodopa-related complications in most patients (Limousin et al., 1998). No significant changes in cognitive function are observed provided strict inclusion criteria are used (Welter et al., 2002). Although psychic disturbances can occur shortly after the operation (confusion, mania), or later as a relapse of a pre-existing disorder (major depression, personality disorders), depression and anxiety are improved (Funkiewicz et al., 2004). The overall outcome of neurosurgery is therefore an improvement in activities of daily living and quality of life (Lagrange et al., 2002). Nevertheless, those of us who take care of the operated patients are struck by the contrast between the dramatic improvement in parkinsonian motor disability and the inability of certain patients to reintegrate into a normal familial and social life. Some are unexpectedly dissatisfied, while others become apathetic and are therefore confronted with familial conflicts and maladjustment at work. To try to understand the personal, familial and social difficulties experienced by patients who underwent neurosurgery, the reasons for this maladjustment were assessed in Parkinson's disease (PD) patients before and 24 months after bilateral STN stimulation, using a semi-structured psychological and psychiatric interview.

The patients ($n = 29$) were selected for treatment by continuous bilateral high frequency stimulation of the subthalamic nucleus (STN) (Bejjani et al., 2000). All had an advanced, levodopa-responsive form of the dis-

ease (mean age: 52.4 years; disease duration: 10.8 years; parkinsonian motor disability [UPDRS III] ''off'' drug: 36.0, ''on'' drug: 5.0; levodopa-equivalent dosage [1215 mg/day]). Patients were considered mentally normal: there was no intellectual impairment (mean Mattis Dementia Rating Scale score: 140), nor were patients particularly depressed (mean MADRS score: 7.1) or anxious (mean BAS: 7.5). Quality of life was assessed using the PDQ-39 scale (Peto et al., 1995). The Social Adjustment Scale (SAS) (Paykel et al., 1971), a semi-structured interview exploring six dimensions (work, social life, family life, marital relations, interaction with children and a general score) was used to evaluate the patients' psycho-social condition. Repeated in-depth open interviews, aimed at obtaining qualitative psychological observations were backed up by a semi-structured psychiatric interview (MINI 500: mini international neuropsychiatric inventory) (Sheehan et al., 1998). The patients underwent neurosurgery for bilateral placement of stimulating leads within the STN in a single operative session, as previously described (Bejjani et al., 2000).

A comparison of the patients' state 24 months after neurosurgery with their preoperative state showed a dramatic improvement in parkinsonian motor disability, as expected in such carefully selected patients (mean reduction of 67%, in UPDRS-III score, 67% in UPDRS-IV score and 64% in levodopa-equivalent dosage). The patients' neuropsychological status was unchanged (Mattis score: 140); there was a significant improvement in mood and anxiety (+35%, +6%) as well as in the quality of life score (PDQ 39: +24%). Despite this general improvement in motor disability and mental functions, there was no significant improvement in the global and sub-dimensional SAS scores. This result was unexpected, but should be seen in the light of the following two points: a) before surgery, only 21% of the patients had a normal global social adjustment score; b) changes in social adjustment score varied from patient to patient (8/29 improved, 11/29 worsened, 10/29 were unchanged). The only two sub-dimensions of the SAS that tended to be worse were 'work' and 'marital relations'. The latter observation very likely explains why 64% of the patients who were working before surgery wanted to stop their professional activity, and that 65% of those who were married (or lived with a partner) experienced a conjugal crisis after the operation.

These results thus confirm our impression – also shared by other international teams used to treating these particular forms of PD by bilateral stimulation of the STN – that there is a striking contrast between the often spectacular improvement in patients' motor disability and quality of life (see above) and the absence of mean improvement in social adjustment, whether at a personal, familial or professional level. Any conclusion that neurosurgical treatment for PD does not have a beneficial effect on social adjustment must, however, be qualified for the following reasons. 1) The small number of patients in this preliminary study means that the overall conclusions do not necessarily apply to each individual patient. Thus, while some patients experienced a complete lack of social integration, others enjoyed a total reintegration into their social environment. 2) At the time of surgery, our population of patients had problems of socio-familial adjustment that were intentionally not taken into account, and the outcome of our study might well have been different had the patients and their families benefited from psychological management. Be that as it may, how can one explain the relative lack of success of the operation on a personal, familial and socio-professional level given the patients' recovery, or near recovery, of normal motor function? One cannot implicate the patients' intellectual state, since it remained normal after the operation, nor was there any development of anxio-depressive symptoms, since the patients' state in this respect was rather improved. As in other

surgical operations leading to a radical change in the patient's health, for example after a heart transplant (Boudrez et al., 2001), or surgery for epilepsy (Derry et al., 2000), the sudden improvement in the pathology, which abruptly transforms patients from a state of severe disability to one where they have in theory recovered the capacity to return to a normal life, would clearly seem to play a crucial role. If the reasons for the lack of sufficient improvement in social integration were not motor, intellectual or psychiatric, what, then, could they be? This is where the many psychological interviews that we conducted before and after the neurosurgery have proved so helpful since they provide the necessary material to be able to discuss the repercussions of the neurosurgery from three different angles: the effect on the patient (the ''self''), on the patient's family, and more particularly the patient's spouse (the ''other''), and on the patient's socio-professional life (''the others'').

1) The repercussions of the neurosurgery on the patient: the ''self''

Six different contributory factors were evidenced.

a) *The self-image and the feeling of strangeness*. After the temporary euphoria following the operation, some patients had difficulty in adopting a new self-image. For some, the quasi-normality of their physical state aroused a feeling of strangeness (''I don't seem to recognise myself without the problems I had before'').
b) *The loss of an aim in life*. Before surgery, the patient's life revolved around his or her disease in such a way that fighting the disease was an end in itself. After surgery, it was as if the patient's life had lost all meaning. For example, one patient said: ''before stimulation, every day was a struggle. Now, I miss the time when I used to fight. Nowadays, I'm like a soldier when the war is over, there's no longer anything to fight against. My life seems empty. I get up in the morning without any aim or prospects''.
c) Several patients felt that the disease had caused a ''*retrospective disaster*'' in their lives. Despite the motor improvement (i.e. a ''repaired body''), these patients kept an image of themselves as having been damaged by the consequences of the disease (''distressed mind''). One of our patients, for example, said: ''Now, I'm capable of leading a normal life, go out, visit friends, go swimming, have a sexual life. Yet, PD has demolished everything. Today, my life is like a forest without trees: I no longer have any friends I can see, or places to visit. I don't even have the opportunity to go back to work. So what's the point! Now that I feel ready to get going, there's nothing, everything's dull''.
d) *The loss of vital force ('élan vital')*. Even though the patients were not depressed or apathetic (except in 5 cases), some had difficulty in initiating complex actions or thoughts and planning, a problem that occurred in a context of attention deficit. ''Aren't there any more precise places where you could stimulate me? Can't you move the electrodes a little, a millimetre or just a half, to make me a bit more euphoric? I want to get back some of my enjoyment in life, that's what I miss'' said one patient, for example.
e) The persistence of ''*negative anticipation*''. After neurosurgery, the parkinsonian patient may still feel the need to anticipate the potential problems that the disease used to cause before the operation, notably the unpredictability of the symptoms. One of our patients expressed this quite clearly: ''Even though I know my motor state is perfectly normal, I just can't get rid of the thoughts I used to have, when I was ill. I still have the same reflexes to initiate walking and the rituals before taking the medicine. I always refuse invitations to go out because I imagine I'll have

freezing attacks and walking problems, even though it's no longer the case. *My body is cured but my mind is still sick*''. Curiously, this difficulty in looking ahead, and the restrictions that it causes, can persist long after the operation.

f) *The altered body image*. After the operation, a small number of patients had the impression that their body had been ''dehumanised'' because of the metallic device inserted in their brain: ''I'm an electronic doll'' said one; ''I feel like a 'Robocop''' said another; ''I'm under remote control'', said yet another. This impression of a body transformed into a machine that no longer belongs to the person and is dependent on a device worthy of a science fiction story is very different from the impression of an altered body attacked from within that some patients experience before neurosurgery, when they suffer periods of dyskinesia or freezing. ''I feel I'm forced to live like a prisoner in an alien body that's out of control'', said one patient.

2) The repercussions of the neurosurgery on the couple: the ''other''

Even though in the majority of cases marital problems existed before neurosurgery, generally speaking they became worse after the operation, with two main scenarios.

a) *The patients reject their spouse* because they have recovered their autonomy and feel ''cured''. The spouse is surprised by the suddenness of the change and continues to behave as a care-giver, which in some cases leads to ''overprotectiveness'' with the patient being maintained in a state of dependence on his or her spouse. In such cases the spouse finds it difficult to give up the role of care-giver. One spouse, for example, felt ''lost'': ''When he was ill, we were the perfect couple. Since the operation, he wants to live like a young man: going out, meeting new people... It's unbearable! I prefer him as he was before, always nice and quiet.'' Whereas her spouse, whose PD had been remarkably improved, said: ''During all these years, I let myself be treated like a child because I didn't have the strength to fight back. That period is over now. I'm going to claim my old place back again. Now I want to have control over my life again, recover the life I had before PD''.

b) The opposite situation is that of *the patient rejected by his/her spouse*, who expects the patient to return to a normal life after stimulation. ''During all the years he was ill, I had to grin and bear it, trying not to do or say anything that could hurt him, but I can't stand it any more. He won't try, he just sits there and expects me to do everything'', said the wife of one patient, and it was difficult for the patient to make his wife understand that, even though the operation was a success, he was no longer able to run his own life.

3) The repercussions of the neurosurgery on other people: the ''others''

After the operation, some patients still wanted to be recognised as sick and many of those who had a professional activity no longer wanted to work. We distinguished two types of behaviours, that were not mutually exclusive, however:

a) *Patients for whom work became of secondary importance in their lives after the operation*: ''Before, I thought work was the most important thing in my life. Now I intend to do other things. I realise that my presence at the office is not indispensable and that the work gets done even when I'm not there'', said a company director, happily. This suggests that there may have been an overinvestment in work before the operation, as a way of fighting the disease and showing that he was no

different from anyone else. After, such patients may consider that society owes them a social debt.

b) In the second category are those *patients in need of recognition*. One patient, while awaiting the operation, expressed her despair in the following terms: ''If I don't have the operation in the next few months, I'll just curl up and die. As long as I have my work, I can go on. If the day comes when I can't go to work any more, it will be as if a curtain has come down on my life''. Six months after the operation, even though there was no longer any trace of motor handicap, she made several attempts to go back to work, without success: ''I don't have the same powers of concentration as before, I have a lot of work and I'd rather spend my time doing other things''. The patient repeatedly asked to be put on sick leave without any valid reason, preferring to go for walks and spend time shopping and chatting on the Internet. She announced her disease to her entourage, became involved in the activities of associations and wished ''to be recognised as ill''. Before the disease, these patients felt recognised as a result of the effort they made to maintain a respectable position in the eyes of society. After the operation, they felt freed from these constraints, no longer needing to fight for the right to live a normal life despite their disability; it was as if they were at last able to expressing their true feelings.

In conclusion, this psychological analysis has helped to evidence problems experienced by a certain number of successfully operated patients, in whom various types of social and professional maladjustment without any particular psychiatric or intellectual cause are in stark contrast to the clinical improvement. One should no doubt take into account in this study the strict inclusion criteria for neurosurgery (Welter et al., 2002). Nevertheless, the reasons, for this personal, familial and socio-professional maladjustment remain unclear. The possibility of a direct role of high-frequency electrical stimulation in the STN cannot be entirely ruled out, particularly since this type of surgery can lead to mood changes (hypomania, which is usually transitory) or severe psychiatric complications if the patients have had serious psychiatric manifestations during their life prior to the operation (Houeto et al., 2002). This was not, however, the case in the patients included in this study, even if we had the impression that some of them had transient subtle difficulties of intellectual concentration and planning, or certain states of mild psychic excitation (impatience, logorrhoea). It seems more likely that the difficulty in social integration experienced by our operated patients resulted, not directly from a modification of the patients' personality, but rather indirectly from a difficulty of reintegrating into the socio-familial and professional environment. The sudden change in the patients' motor state, enabling most of them to return to a virtually normal life in a world much changed after all the years of their disease, also probably played a role. The latter hypothesis is important to consider since a large number of parkinsonian patients will undoubtedly be operated in the years to come given the excellent clinical results achieved so far. If these psychological difficulties can be anticipated and even alleviated once patients have been selected for surgery and during the postoperative follow-up, this should greatly facilitate the integration of operated patients into the new context of their existence. That is why it is so important in future to include a psychological interview for patients selected for neurosurgery, during the preoperative procedure and again during the postoperative follow up.

The unquestionable success of this type of neurosurgery (bilateral high-frequency stimulation of the STN) in the treatment of certain specific forms of Parkinson's disease is now widely acknowledged, due to the very

often spectacular motor improvement without any intellectual or psychiatric side effects, on condition that candidates for neurosurgery are very carefully selected. In view of patients' postoperative problems of adjustment evidenced in this study – and already well-known to the medical surgical teams that look after such patients – a new dimension in the management of operated patients should in future be carefully considered, namely the psycho-social status of the patient and the patient's entourage, and the socio-professional context.

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Placebo effect and dopamine release

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Summary. The placebo effect can be encountered in a great variety of medical conditions, but is particularly prominent in pain, depression and Parkinson's disease. It has been shown that placebo responses play a part in the effect of any type of treatment for Parkinson's disease, including drug therapy, deep brain stimulation and dopamine tissue transplantation. Recent studies have demonstrated that the placebo effect in Parkinson's disease is related to the release of substantial amounts of endogenous dopamine in both the dorsal and ventral striatum. As the ventral striatum is involved in reward processing, these observations suggest that the placebo effect may be linked to reward mechanisms. In keeping with this placebo-reward model, most recent experiments have shown activation of the reward circuitry in association with placebo responses in other disorders. In addition, as dopamine is the major neurotransmitter in the reward circuitry, the model predicts that the release of dopamine in the ventral striatum could be involved in mediating placebo responses not only in Parkinson's but also in other medical conditions.

Introduction

The ability of the mind to influence healing of the body has long puzzled the scientific community. It is clear that most treatments used in ancient times would now be considered placebos. Yet, they seemed to be effective at the time, at least to some extent (de la Fuente-Fernández et al., 2002b). Unfortunately, however, the placebo effect has often been regarded as a mere nuisance in the medical literature.

The power of placebos was spurred in the 1950s, when Beecher reported consistent placebo responses (Beecher, 1955). About one-third of patients were found to feel better after placebo administration. The mechanisms implicated in such placebo responses were unknown at the time. In the late 1970s, Levine and colleagues showed that placebo analgesia could be reversed by the administration of naloxone (Levine et al., 1978). This key observation clearly suggested that the effect of placebos on pain disorders was mediated by the release of endogenous opioids. The first firm evidence for a biochemical basis of the placebo effect was thus born. Nonetheless, perhaps due to the idea that opioids could hardly explain placebo responses encountered in other medical conditions, the placebo effect was again disregarded and mostly forgotten for decades.

However, there has been a resurgence of interest in the mechanisms implicated in mediating placebo responses. Based on the results from placebo investigations in Parkinson's disease, we have recently proposed an integrated model of the placebo effect, where

the pivotal neurochemical is dopamine (de la Fuente-Fernández et al., 2004). Essentially, the model establishes a link between placebo responses and reward mechanisms. This article will review the evidence for such a placebo-reward model.

Placebo effect in Parkinson's disease

Although placebo responses can be encountered in a great number of medical conditions, there are three disorders in which the placebo effect is particularly prominent: pain, depression and Parkinson's disease (de la Fuente-Fernández et al., 2002b, 2004). In fact, objective placebo responses have been reported in any form of therapy for Parkinson's disease, including deep-brain stimulation (de la Fuente-Fernández, 2004) and sham surgery (McRae et al., 2004). The study by McRae et al. (2004) is particularly remarkable. In a double-blind sham surgery-controlled trial of human fetal mesencephalic transplantation for Parkinson's, the authors found that the patients' perception of which group they were assigned to had a greater impact on both quality of life and motor function than the actual treatment (McRae et al., 2004).

PET studies with raclopride have shown that the placebo effect in Parkinson's disease is mediated by the release of endogenous dopamine in the dorsal striatum (de la Fuente-Fernández et al., 2001). In other words, the clinical effect observed in patients with Parkinson's disease in response to placebo administration is related to the release of endogenous dopamine in the motor part of the striatum, which receives nigrostriatal dopamine projections. Hence, the placebo-induced clinical benefit is virtually identical to the benefit obtained after exogenous administration of levodopa. In both cases there is an increase in synaptic dopamine levels in dorsal striatum. It should be emphasized that, in contrast to placebo investigations in pain disorders or depression, which rely on subjective ratings, the clinical effect of placebos on Parkinson patients can be evaluated objectively by a blinded examiner.

Naturally, these PET observations on placebo-induced dopamine release also suggested that placebos should induce the same downstream changes in the basal ganglia circuitry as those obtained after exogenously derived dopaminergic stimulation. This prediction was later proved true. Thus, in keeping with current models of basal ganglia function, placebos were found to induce a decrease in the activity of subthalamic nucleus neurons in placebo-responsive Parkinson patients (Benedetti et al., 2004). But, what triggers the release of dopamine in response to placebo administration?

Placebo effect and reward

It was hypothesized that placebo-induced dopamine release in Parkinson patients was related to the expectation of clinical benefit (de la Fuente-Fernández et al., 2001). Indeed, there is growing recognition of the critical role of expectation in the mechanism of the placebo effect (de la Fuente-Fernández, 2004). If one admits that the clinical benefit experienced by a patient must have rewarding properties, expectation of clinical benefit can be considered equivalent to expectation of reward. On the other hand, it is well known that the release of dopamine in the ventral striatum plays a crucial role in reward mechanisms (de la Fuente-Fernández et al., 2004). Hence, if the placebo effect is related to the activation of the reward circuitry, there should be dopamine release in the ventral striatum in response to placebo administration. PET studies with raclopride demonstrated that this was indeed the case (de la Fuente-Fernández et al., 2002a). The placebo effect in Parkinson's disease is therefore associated with the release of dopamine in both the dorsal (motor) and ventral (reward-related) striatum. Intriguingly, while the placebo-induced release of dopamine may

follow an ''all-or-none'' pattern in the ventral striatum, in the dorsal striatum the amounts of dopamine released after placebo administration is quantitatively related to the clinical benefit (i.e., larger amounts of dopamine are associated with greater placebo responses).

Dopamine and placebo effect

The connection between the placebo effect and reward mechanisms predicts that the reward circuitry should be activated whenever a placebo response occurs, not only in Parkinson's disease, but also in other medical disorders. There is mounting evidence supporting this notion. Thus, for example, placebos also activate reward-related brain structures in conditions such as pain and depression (de la Fuente-Fernández et al., 2002b, 2004). It should be noted that, in addition to the ventral striatum, placebos also activate cortical areas known to respond to

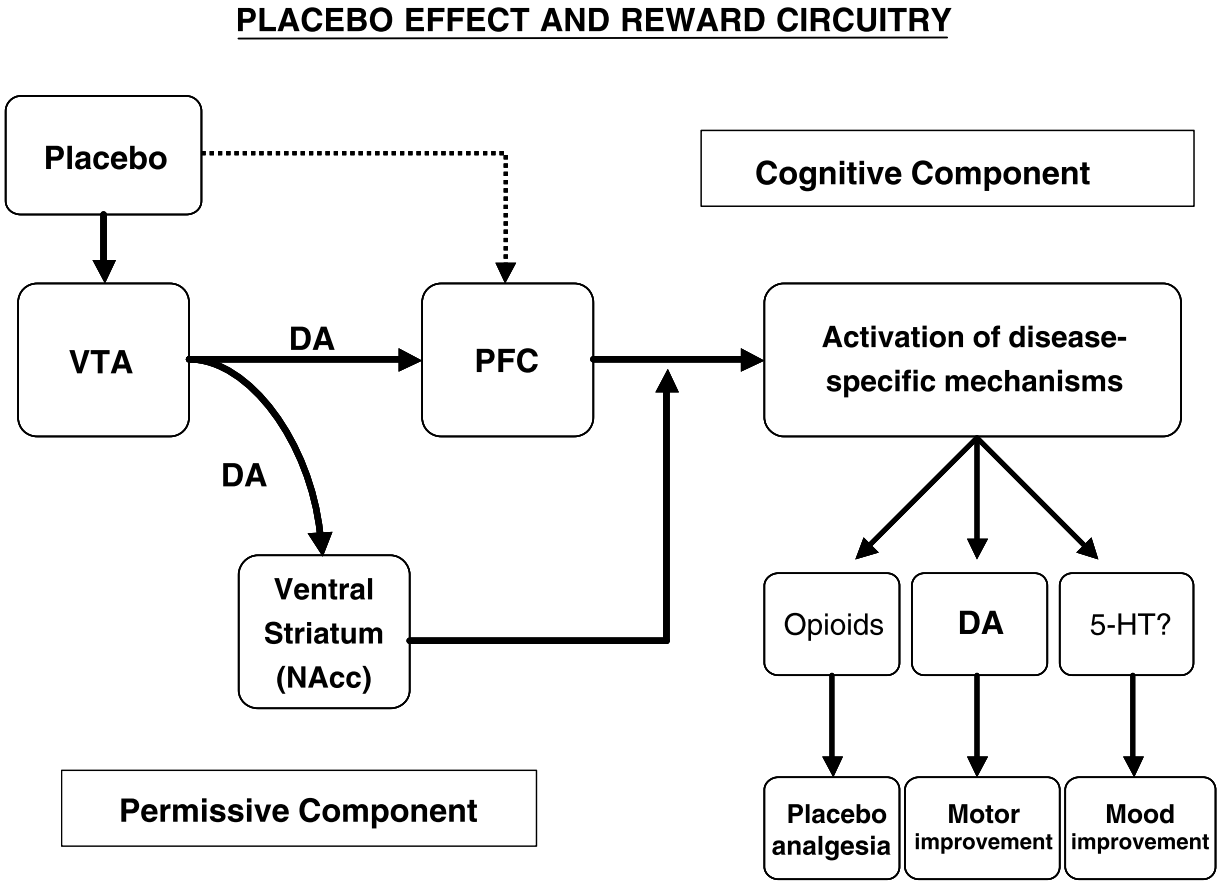

Fig. 1. Theoretical model for the placebo effect: The expectation of reward (i.e., expectation of clinical benefit) after placebo administration activates dopamine (DA) neurons of the ventral tegmental area (VTA), which leads to the release of dopamine (DA) not only in the ventral striatum (nucleus accumbens, NAcc), but also in the prefrontal cortex (PFC). This PFC activation, in turn, sets in motion disease-specific mechanisms, such as DA release in the dorsal striatum to improve motor function (which explains the clinical placebo effect in Parkinson's disease), the release of opioids to alleviate pain (placebo analgesia) and, perhaps, activation of serotonin (5-HT) pathways to reduce depression. While the release of dopamine in the ventral striatum may function as a gate (permissive component) for placebo responses, the activation of PFC-dependent pathways represents a higher cognitive component of the placebo effect (modified from Lidstone et al., 2005)

reward expectation such as the orbitofrontal cortex, the dorsolateral prefrontal cortex, and the anterior cingulated gyrus, which suggests the involvement of high cognitive processing in placebo responses (de la Fuente-Fernández et al., 2002b, 2004). On the other hand, as dopamine is a major neurotransmitter in reward processing (Schultz, 1998), the release of dopamine may be a common phenomenon in any placebo response. There are studies underway to prove (or disprove) this prediction.

There is evidence to suggest that, in addition to dopamine, other neurotransmitters and neuropeptides are also involved in both reward and placebo mechanisms (de la Fuente-Fernández et al., 2004). Indeed, it is tempting to speculate with the possibility that different endogenous neuroactive substances contribute to placebo responses in a disease-specific fashion (de la Fuente-Fernández et al., 2002b) (Fig. 1). For example, while opioids could be particularly implicated in placebo analgesia (Levine et al., 1978), serotonin might play a major role in mediating the placebo effect in depression.

Conclusions

The placebo effect in Parkinson's disease reflects the release of dopamine in the striatum. It has been proposed that the placebo effect is related to reward mechanisms. As dopamine is a major neurotransmitter in reward processing, it follows that the release of dopamine in the ventral striatum may be a common phenomenon in placebo responses encountered in other medical disorders. Further research is necessary to determine how cortical processing interacts with the reward circuitry to elicit a disease-specific placebo response.

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A new look at levodopa based on the ELLDOPA study

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Summary. Levodopa has been the gold standard for Parkinson's disease (PD) therapy since it was successfully introduced in 1967. But in the years since then, after recognizing that levodopa often leads to the motor complications of wearing-off and dyskinesias, there have been debates among clinicians as to when levodopa therapy should be started. Delaying therapy was advocated for the purpose of delaying the development of these motor complications. This became more popular as the dopamine agonists became available. Although less potent than levodopa in ameliorating the symptoms of PD, they were much less likely to produce the unwanted motor complications, even though they had their own adverse effects. When it was recognized that dopamine, itself, might be a factor leading to the death of dopaminergic neurons through its contributing to the formation of oxyradicals, a new concern arose, namely that levodopa, through its conversion to brain dopamine, might add to the existing oxidative stress and possibly enhance neurodegeneration of dopaminergic neurons. Though widely debated and without definite evidence, this possibility was sufficient to make some clinicians have further reason to delay the start of levodopa therapy. The ELLDOPA study was created to test this hypothesis. The clinical component of the study failed to find an enhancement of PD symptoms after levodopa was withdrawn following 40 weeks of levodopa therapy. Rather, the clinical results indicated that the symptoms had progressed much less than placebo, and in a dose-response manner. This suggests that levodopa may actually have neuroprotective properties. The uncertainty that a 2-week withdrawal of levodopa may not have entirely eliminated its symptomatic benefit and the discordant results of the neuroimaging component of the ELLDOPA study have created even more uncertainty that levodopa is neuroprotective. A survey of neurologists who treat PD patients showed that the vast majority of these clinicians do not believe levodopa is neuroprotective, and they remain concerned about the drug's likelihood of inducing motor complications. Thus, the ELLDOPA study failed to change the treating pattern of PD, and the clinicians require more convincing evidence of either neuroprotection or neurotoxicity of levodopa before they would alter their treatment approach.

The Fluctuating History of Levodopa in the treatment of Parkinson's disease

Following the discovery of striatal dopamine deficiency in Parkinson's disease (PD) by Hornykiewicz and colleagues (Ehringer and Hornykiewicz, 1960; Bernheimer et al., 1973), Birkmayer and Hornykiewicz (1961) injected small doses of levodopa (up to 150 mg) intravenously and reported a transient reversal of akinesia. Barbeau et al. (1962) also reported benefit with small oral doses of

levodopa (200 mg). Subsequently, many other investigators using small oral or intravenous doses reported similar results in very brief communications (Friedhoff et al., 1964; Umbach and Bauman, 1964; Hirschmann and Mayer, 1964; Pazzagli and Amaducci, 1966; Bruno and Bruno, 1966). However, not every investigator reported benefit from such small doses of levodopa. Greer and Williams (1963) failed to find benefit in two patients after 1 gm of D,L-dopa orally. Aebert (1967) saw no benefit after 70 to 100 mg L-dopa intravenously, nor did Rinaldi et al. (1965) even with inhibition of monoamine oxidase. Double-blind trials with low dosage levodopa also failed to provide benefit (Fehling, 1966; Rinne and Sonninen, 1968) using up to 1.5 mg/kg of intravenous levodopa. McGeer and Zeldowicz in 1964 were the first to use high doses of D,L-dopa that were later found to be successful by Cotzias et al. in 1967. They used up to 5 gm per day in ten patients for several days, and in one patient, 3 gm daily for 2 years, but only two patients showed any objective improvement.

The breakthrough in establishing levodopa as a therapeutically useful drug was the report of Cotzias et al. (1967). They treated 16 patients with doses of D,L-dopa of 3–16 gm per day, building the dosage up slowly to avoid anorexia, nausea and vomiting, which had been the dose-limiting complications with previous investigators. They reported marked improvement in eight patients and less improvement in two others. Of the eight who received 12 g/or more per day, seven showed marked benefit. Granulocytopenia was seen in four patients, and bone marrow examination revealed vacuoles in the myeloid cells in four of the 12 patients with bone marrow examinations.

Because of the hematologic problems and because D-dopa in not metabolized to form dopamine, Cotzias and his colleagues subsequently used L-dopa (1969), and these problems were no longer encountered. Yahr et al. (1969) carried out the first double-blind study with high dosage levodopa. This and many subsequent reports showed significant improvement in approximately 75% of patients with parkinsonism. Although a complete reversal of symptoms is rarely obtained, akinesia and rigidity were generally most benefited, and many who had been unable to turn in bed or arise from a chair became able to do so. Tremor has a more variable response; sometimes it is eliminated by levodopa, and in other patients, the tremor is resistant. A number of other symptoms, including postural instability and speech disturbance, are typically unaffected by levodopa therapy, suggesting these symptoms are not solely due to dopamine deficiency. The introduction of levodopa therapy by Cotzias was a revolutionary treatment for PD, not just an evolutionary one.

The development of inhibitors of L-aromatic amino acid decarboxylase that do not cross the blood–brain barrier was the next major step. Carbidopa and benserazide are such peripheral decarboxylase inhibitors. When given with levodopa, they allow for a 4-fold increase in the effectiveness of a given dose because peripheral metabolism of L-dopa to dopamine is blocked. More importantly, these agents block the gastrointestinal side effects, which are due to peripheral dopamine acting upon the vomiting center of the area postrema, which is not protected by the blood–brain barrier. The combination of levodopa with carbidopa was commercially marketed under the trade name of Sinemet, to indicate without (''sine'') emesis. The combination of benserazide and levodopa is marketed under the brand name of Madopar.

Levodopa remains today the most powerful drug available to treat PD, and this drug is considered the ''gold standard.'' The absence of a robust response to high-dose levodopa essentially excludes the diagnosis of PD and suggests there must be another explanation for the parkinsonian symptoms. In contrast,

a marked and sustained response strongly supports the diagnosis of PD (Marsden and Fahn, 1982). Although numerous other treatment options are available in early PD when the disease is mild, virtually all patients will eventually require levodopa therapy as the disease worsens.

Early in the course of the disease, levodopa provides a long-duration response that can last several days even if levodopa is discontinued. This continuous response occurs in the presence of a short plasma half-life of a little more than 30 minutes (Muenter and Tyce, 1971; Tolosa et al., 1975).

As PD worsens (or with long-term usage of levodopa), more serious and persistent complications, such as ''wearing off'' fluctuations and dyskinesias (abnormal involuntary movements) emerge; these motor complications affect 75% of patients after 6 years of levodopa therapy (Fahn, 1992). These problems markedly impair the quality of life and functional status of affected patients, and prove challenging not only for the patient, but also for the treating physician. Today, these motor complications, especially clinical fluctuations and abnormal involuntary movements (dyskinesias), have limited the usefulness of levodopa. In fact, this has led many clinicians to consider a dopa-sparing strategy, using instead dopamine agonists, monoamine oxidase inhibitors, amantadine, and anticholinergics in the milder stages of disease, especially in younger patients who are more prone to develop these motor complications (Quinn, 1994; Fahn, 1998, 1999; Montastruc et al., 1999). The dopa-sparing strategy became more popular as the dopamine agonists became available. Although less potent than levodopa in ameliorating the symptoms of PD, they are much less likely to produce the unwanted motor complications, even though they have their own adverse effects profile (Rascol et al., 2000; Parkinson Study Group, 2000).

Early in the evolution of developing a treatment strategy for PD – in the early 1970s – I had advocated using levodopa early in the course of the disease as an attempt to spare dopaminergic neurons from overworking to produce more endogenous dopamine. However, half a decade later, I reversed my opinion and advocated the strategy of delaying the introduction of levodopa to avoid an early onset of wearing-off, on–off and dyskinesias (Fahn and Calne, 1978) as these motor complications were becoming increasingly common with high dosage levodopa therapy. Since 1980 there have been continual debates at neurology meetings and in the literature about whether to start levodopa early or late (Fahn et al., 1980; Hoehn, 1983; Muenter, 1984; Fahn and Bressman, 1984; Melamed, 1986; Markham and Diamond, 1981, 1986; Caraceni, 1994; Quinn, 1994; Montastruc et al., 1999; Weiner, 1999; Factor, 2000; Montastruc, 2000). These debates mostly centered around the higher degree of clinical benefit seen with levodopa compared with other anti-PD medications versus the risk of developing motor complications that were particularly due to levodopa and not other medications. In double-blind clinical trials directly comparing starting treatment with levodopa or one of the dopamine agonists, pramipexole and ropinirole, levodopa was statistically significantly more likely than the agonists to induce both motor fluctuations and dyskinesias (Parkinson Study Group, 2000, 2004a; Rascol et al., 2000).

In 1983 a new element was added to the debate. In that year Cohen (1983, 1986) reported that oxyradicals produced by dopamine would cause oxidative stress in dopaminergic neurons and could contribute to their degeneration, leading to the developing of PD. Cytosolic dopamine can be autoxidized to produce dopamine quinone and enzymatically oxidatively deaminated by monoamine hydroxylase to produce hydrogen peroxide, which in turn can lead to oxyradicals. Oxidative stress contributing to the pathogenesis of PD has much support, including postmortem biochemical evidence (Fahn, 1989; Fornstedt

et al., 1990; Olanow, 1990, 1992; Jenner, 1991, 2003; Jenner et al., 1992; Fahn and Cohen, 1992; Zigmond et al., 1992; Spencer et al., 1995; Alam et al., 1997; Jenner and Olanow, 1998). Debates now include the potential of levodopa to enhance the progression of PD when considering whether levodopa should be started later rather than earlier.

The results of many *in vitro* studies have suggested that levodopa may be injurious to dopaminergic neurons (see reviews by Fahn, 1996, 1997). These findings have raised concerns that chronic levodopa exposure might hasten disease progression in PD patients. Accordingly, some physicians and patients have opted to defer the use of levodopa for as long as possible (Fahn, 1999). Others physicians have continued to use levodopa as first-line therapy, arguing that it is inappropriate to withhold the most potent symptomatic treatment for PD in the absence of clinical evidence of toxicity (Agid, 1998; Weiner, 1999; Factor, 2000).

Has the ELLDOPA study changed the way we perceive of levodopa therapy?

Because of the ongoing controversy about whether levodopa is toxic, a large, multicenter, randomized controlled clinical trial comparing three different doses of levodopa with placebo treatment in patients with early PD (the ELLDOPA study) was designed to answer this question (Parkinson Study Group, 2004b). This was a double-blind, pla-

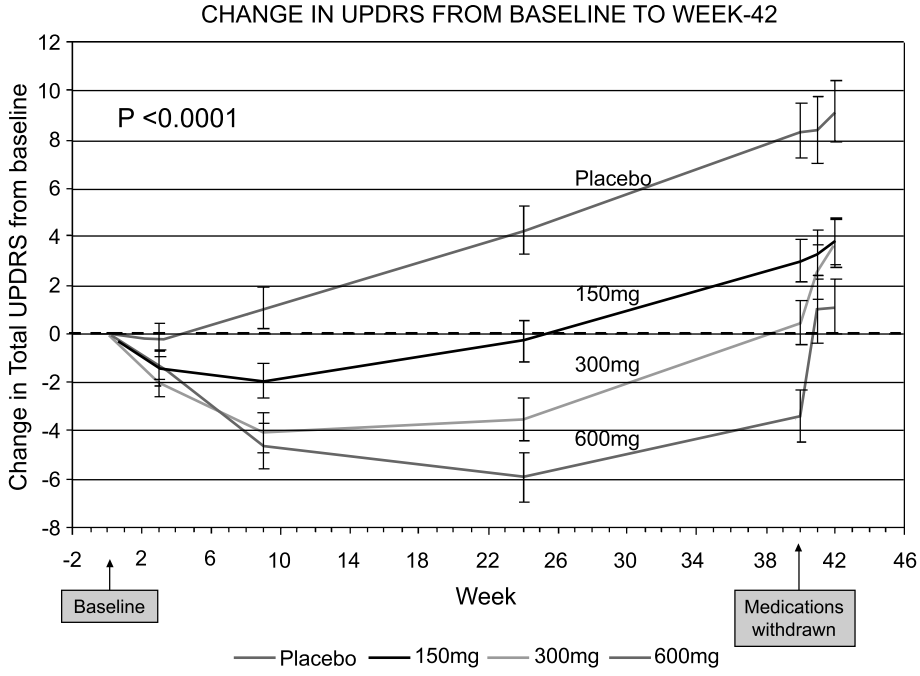

Fig. 1. Changes in Unified Parkinson's Disease Rating Scale (UPDRS) from baseline to Week-42 in the ELLDOPA study. The changes in subjects treated with levodopa at different dosages or with placebo were determined on the basis of the total score of UPDRS. The scores were obtained by the blinded treating investigator who performed the evaluations before the morning dose of the daily dose of the study drug. The points on the curves represent mean changes from baseline in the total scores at each visit. Improvement in parkinsonism is represented by lower scores, and worsening by higher scores. Negative scores on the curves indicate improvement from baseline. The bars indicate standard error. Reproduced from Parkinson Study Group (2004b) with permission from the Massachusetts Medical Society (© 2004)

cebo-controlled, parallel group, multicenter trial of patients with early PD who had not been previously treated with symptomatic therapy. A total of 361 patients were enrolled, and were randomized equally to receive treatment with low- (150 mg/day), middle- (300 mg/day), or high- (600 mg/day) dosage levodopa, or placebo. After forty weeks of treatment, the subjects underwent a three-day taper of their medications, followed by a two-week washout period during which they received no treatment for their PD. The primary outcome measure was the change in the total Unified Parkinson's Disease Rating Scale (UPDRS) score between baseline and after the washout period at Week-42. The goal of the study was to determine whether levodopa treatment affects the rate of progression of PD.

At the end of the two-week washout period, the UPDRS scores of patients treated with all three doses of levodopa were lower (better) than those of the placebo-treated group, in a dose-response pattern (Fig. 1). These findings suggest that levodopa is not neurotoxic, and may even be neuroprotective, though the possibility that patients were experiencing a longer duration of symptomatic response to levodopa that had extended beyond the two-week washout period could not be excluded. The highest dosage of levodopa was, however, associated with a statistically significantly higher incidence of motor complications, including dyskinesias (16% in the highest dose group) and a trend to develop the ''wearing off'' phenomenon.

In addition to the clinical data, a subset of patients in the ELLDOPA trial was also evaluated with β-CIT SPECT imaging, which (β-CIT binds to the dopamine transporter, DAT) was used as a marker for intact nigrostriatal dopaminergic neurons. These neuroimaging studies showed that there was a larger decrease in striatal DAT binding in patients treated with levodopa, in a dose-response pattern (Fig. 2). Thus, these results are discordant with the clinical results. In contrast with the clinical data, the imaging findings suggest that levodopa may hasten the progression of PD. However, it is possible

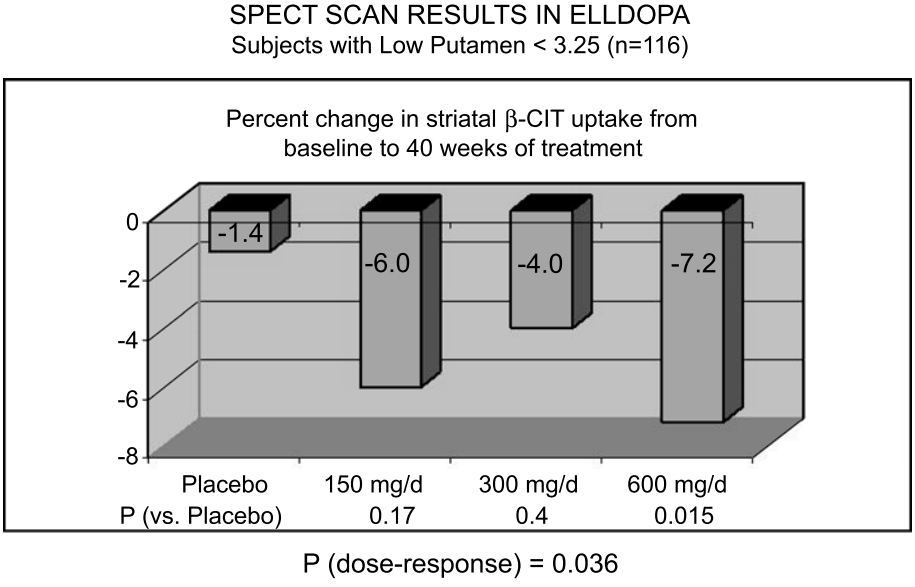

Fig. 2. Percent changes in striatal binding of β-CIT binding using SPECT from baseline to Week-40 in 116 subjects with low putamen binding (<3.25) at baseline in the ELLDOPA study. The bar graphs reveal the percent reduction in β-CIT binding from baseline to Week-40 when subjects were taking the highest assigned dosage of study drug. Data from Parkinson Study Group (2004b)

that the observed changes in the levels of uptake of this marker reflected a pharmacological effect of levodopa on DAT activity, rather than evidence of injury to dopaminergic neurons.

Thus, intriguing as the results of the ELLDOPA study are, it remains unclear whether levodopa may (either positively or negatively) affect the natural history of PD. Based on the clinical results, which indicate that a dosage of at least 600 mg/d of levodopa would be most likely to slow the progression of PD, it would be logical for physicians to now consider starting therapy with this dose. However, the uncertainty whether a 2-week washout was sufficient to eliminate the symptomatic effect of levodopa, the discordant result from the imaging component of ELLDOPA, and that the dosage of levodopa is important in the development of motor complications, it is reasonable to customize the dose of levodopa to fit the specific needs of each patient.

The question as to whether knowing the results of the ELLDOPA study would influence physicians' treatment strategy was addressed in a survey (Fahn and Mazzoni, 2006). The results showed that no physician would start levodopa at 600 mg/d and most would require a definite proof that levodopa slowed the rate of progression by at least 50% in order to start with this dosage. The concern of levodopa's known likelihood to induce motor complications is the primary reason for not altering treatment patterns for most clinicians. It is interesting that the results of ELLDOPA did not convince physicians that levodopa is neuroprotective. Eighty-eight percent of both those who were aware or not aware of the results of ELLDOPA believed that levodopa is not neuroprotective. This is most likely the main factor that prevents physicians from starting treatment of PD with levodopa. It is clear from this survey that we need to establish with a high degree of certainty that 600 mg/d of levodopa slows the rate of progression before subjecting patients to the motor complication risks of such a dosage. This would require a different type of clinical trial design, the so-called delayed-start design (Leber, 1997).

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Thirty five years of experience in the treatment of Parkinson's disease with levodopa and associations

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Introduction

For those devoted to Parkinson's disease and related disorders this periodical meeting is the most famous and attractive since many years ago. The organisation is perfect, the display of topics almost explosive especially in etiopatogenic and therapeutic aspects. We have the privilege to participate in this historical event for the advance of neurology. We have difficulties to resume our rich experience of 36 years; we present some flashes of the clinics, methodology and therapeutics. We like to present our homage to one leader in this field: Prof. Rolf Hassler who was pathologist and devoted to stereotactic surgery and especially an outstanding researcher. In 1938 he published in the same city of the Congress of Berlin a paper with 89 pages on Parkinson's disease and Parkinson post-enciphalitic. Professor Hassler was a friend of our Institute of Neurology and it is an honour for us to remind his figure and his scientific production.

Methodology for evaluating parkinsonism

1) Hoehn and Yahr stage

2) Webster scale

It was the first scale used in most centers. It is fast, precise and coherent and very useful for rapid and repeated evaluations, also in the private office and continuous clinical observation.

3) UPDRS

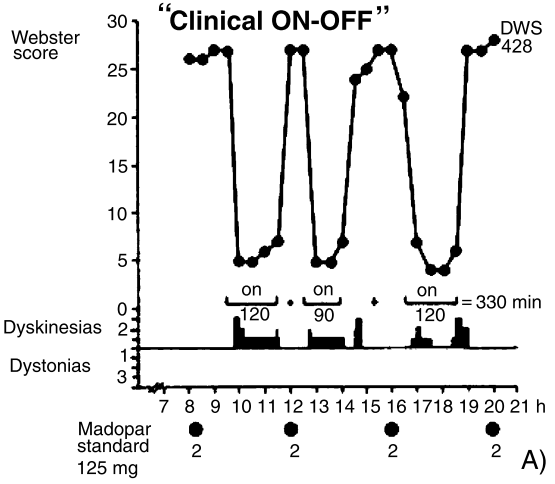
United Parkinson's Disease Rating Scale is the most complete and the best for research

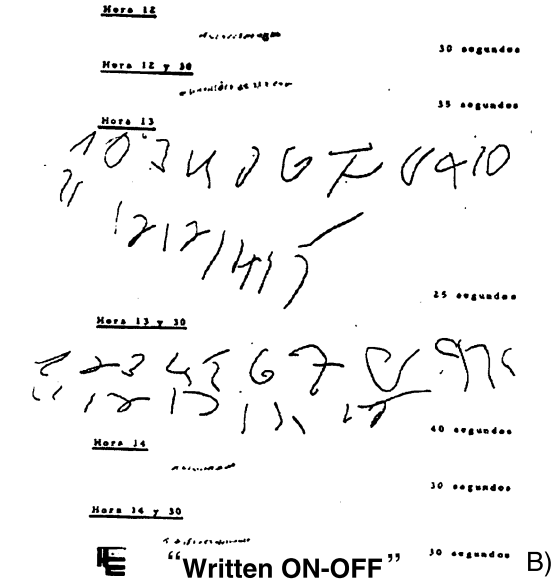
4) Continuous clinical observation and registration in graphics of all clinical information

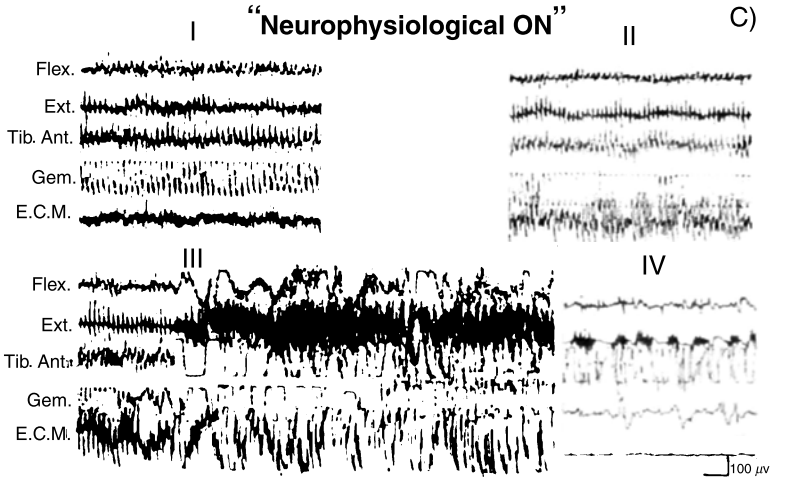
It is essential for learning about the phenomens specially motor complications. Our group works with 10 neurologists and 20 or 22 patients simultaneously during 12 hours. The Welster Scale is applied every 30 minutes. We collect, then, 25 values of parkinsonism: the Day Webster Score (DWS) that means the amount of parkinsonism of the Day (twelve hours). If the patient shows the ON–OFF effect as motor complication we can add the time and duration of the ON periods (ON DURATION) as well as the sum of the OFF periods (OFF DURATION). Dyskinesia and dystonia are registered according to appearance intensity, duration and quality (Fig. 1A).

New clinical expressions of Parkinson's disease under chronic dopatherapy

The basic triad akinesia, rigidity and tremor can show a transient disociation presenting as


"Clinical ON-OFF"
Webster score
DWS 428
on 120
on 90
on 120
= 330 min
Dyskinesias
Dystonias
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 h
Madopar standard 125 mg
A)


"Written ON-OFF"
B)


"Neurophysiological ON"
C)
I
II
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Flex.
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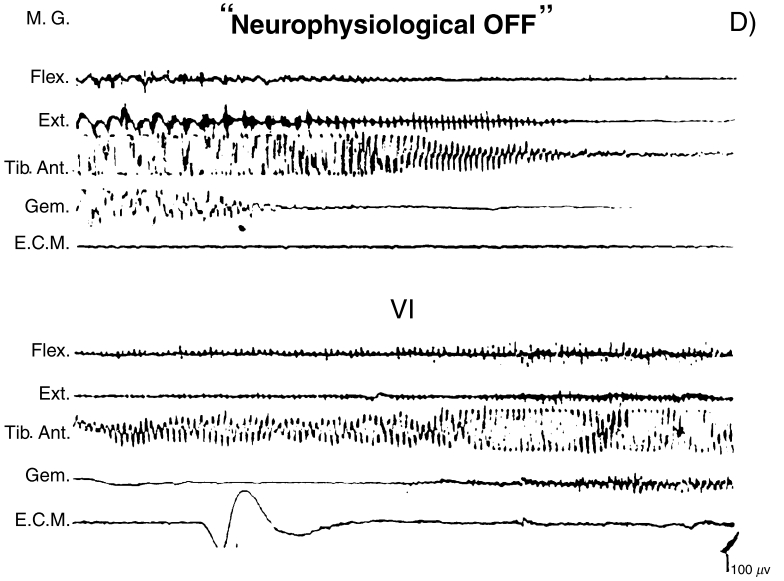
M. G.
"Neurophysiological OFF"
D)
Flex.
Ext.
Tib. Ant.
Gem.
E.C.M.
VI
100 µv

– Paradoxal akinesia without rigidity
– Severe tremor episodes

New signs:

– Abnormal movements
– Dystonic manifestations
– Motor fluctuations
  – Benign: "End of dose impairment" severe: on–off effect
  – ON–OFF effect: Random oscillations, Yo–Yo ing.

Simultaneous combination of dyskinesias ("hiperdopaminergic") and parkinsonism ("hypodopaminergic").

Dynamic, repetitive and alternating states.

The most dramatic are the sudden changes of parkinsonism to chorea and vice versa.

ACTA NEUROL. LATINOAMER. 1970, *16*: 170-183

Tratamiento del parkinsonismo con L-Dopa

C. CHOUZA, C. MARÍN, S. ROMERO y J. B. GOMENSORO

(Montevideo)

ACTA NEUROL. LATINOAMER. 1975, *21*: 108-125

"Long-Term Syndrome" en el tratamiento del Parkinsonismo con L-Dopa

C. CHOUZA, SUSANA ROMERO y J. B. GOMENSORO

(Montevideo)

Fig. 2. First paper

Fig. 1. B The "WRITTEN ON–OFF". We can see the alternance of severe micrography (OFF period) and the irregular and macrographic numbers (ON periods) similar to the choreic writing. **C** These records were performed before the intake of Levodopa. We can see the activity of tremor (4–5 c/s). Suddenly begins the "ON" phenomenon as a bioelectrical storm including interferencial activity (dystonias, dyskinesias, artefacts) that will be fragmented appearing periodical rhythmic activity (myoclonus) and the ON period is consolidated. The frequency of these dischardes is indreasing rapidly (1–2 c/s) as well as its amplitude, arriving to a Parkinsonian tremor (5 c/s). This is the beginning of the "OFF" period (**D**)

Significance of Levodopa

Levodopa was discovered by the multidisciplinary conjunction. We can conclude by this reason a rational therapeutic of Parkinson's disease.

The Levodopa divided the history of this disease in the Prelevodopa and Postlevodopa periods.

It was the first time of administration by oral route of a precursor of neurotransmitter (Dopamine).

Levodopa was the key to open the way for treating other neurodegenerative diseses.

The treatment with Levodopa is:

- The most physiological, because we are administrating the neurotransmitter in deficit.
- A substitution therapy or compensatory therapy.
- Potent-therapy.
- Global effect on most symptoms and signs.
- Then, it is a symptomatic therapy and not etiopategenic.
- Good balance on the D_1 and D_2 dopaminergic receptors.
- Increase life expectance.
- Better quality of life.
- In a doubtful case the response to Levodopa is an argument in favour of the diagnosis of Parkinson's disease.
- Good or excellent response of dystonia in DRD cases.

Continuous dopaminergic stimulation

The Long-Term Syndrome is the group of adverse effects induced under chronic dopatherapy and they are originated in the central nervous system in general.

Abnormal movements and dystonias, motor oscillations ''wearing off'' and ''ON–OFF'' effect; psiquiatric disturbances, hallucinations, difficulties, in gait and speech orthostatic hypotension, loss of efficacy of levodopa. This symtomathology depends in most cases of pharmaco-kinetics and pharmacodynamic factors (Chouza et al., 1970, 1975).

The administration per os of the intake of levodopa each 4–6 hours induce plasmatic oscillations with marked peaks of the dopaminergic agent.

These peaks are not physiologic because they induce motor fluctuations and dyskinesias.

The aim of the research nowadays is to avoid substances or ways of administration that induce these plasmatic oscillations.

The most important drugs

a) Controlled release levodopa
   - levodopa/benserazide HBS (Hydrodinamically Balanced System).
   - levodopa/carbidopa CR (Controlled Release).

b) Long acting dopaminergic agonists
   - Pramipexole (half life of elimination: 8 hours).
   - Pergolide (half life of elimination: 25 hours).
   - Cabergoline (half life of elimination: 65 hours) (Bracco et al., 2004).

c) Bromocriptine retard 50 mg i/m (experimental)

d) Prolongation of the ON period
   - Selegiline (IMAO-B), Tolcapone (ICOMT), Entacapone (INCOMT).

e) Other associations:
   - Levodopa/benserazide HBS + selegiline (Chouza et al., 1989).

Levodopa/benserazide HBS + lisuride in subcutaneous infusion (Chouza et al., 1988).

Conclusion

Levodopa has been a milestone in the history of PD treatment and it is the axis of therapeutic strategies. It continues to be the ''gold standard'', being the most physiological and potent antiparkinsonian agent, with good balance of action on D_1–D_2 receptors. The asso-

ciation of dopaminergic agonists, the ''silver standard'' is the best combination in the therapeutic of this disease.

The new drugs, with different receptorial profiles will enable both monotherapy and multiple pharmacological associations, as well as combinations with advanced technics.

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Concerning neuroprotective therapy for Parkinson's disease

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Summary. Studying potential neuroprotective therapy for Parkinson's disease is conceptually problematic because of the heterogenous nature of the Parkinson's syndrome and complexities in operational definitions for neuroprotection. The current literature concerning neuroprotection provides no convincing evidence of any treatment as definitively neuroprotective in Parkinson's disease. Recent clinical trials and novel trial designs are reviewed that may identify meaningful therapy, resulting in maintenance of neurological function and quality of life for persons with Parkinson's disease.

The concept of pursuing neuroprotective therapy for Parkinson's disease (PD) is nearly universally embraced. Unfortunately, it is increasingly unclear what is being embraced. Both ''neuroprotection'' and ''Parkinson's disease'' require further clarification before rational arguments and conclusions can be made.

There is no consensus regarding what neuroprotection is or how it can be operationally defined. Without measurement, the efficacy of any treatment aimed at influencing the course of a progressive syndrome cannot be adequately determined. Furthermore, given that PD is a syndrome with potentially numerous causes and pathogeneses, critical thought must be given to experimental designs and analyses purportedly studying neuroprotection in PD.

Parkinson's disease

The current definition of PD entails only motor manifestations of symptomatic complaints. The clinical definition requires a constellation of two of three cardinal features: resting tremor, bradykinesia, rigidity, with or without postural instability, occurring in the absence of obvious causes (neuroleptic exposure, etc.) or additional findings (supranuclear gaze palsy, dysautonomia, etc.). The true clinical spectrum of PD is probably far greater and includes cognitive, behavioral, olfactory, sleep, gastrointestinal, dermatological, etc. disturbances.

PD is likely a syndrome with potentially multiple causes or scenarios leading to a clinical syndrome. Genetic and environmental events or exposures (or lack thereof) may contribute to the development of PD. Some of these causes may be preventable, avoidable, or potentially influenced, while others may be unavoidable.

While clinically defined for the purposes of epidemiological and therapeutic study, the syndrome also has functional imaging characteristics. The functional imaging of PD through PET and SPECT studies, employing targeted radioligands, can demonstrate patterns suggestive of PD versus atypical parkinsonian syndromes. However, there is substantial overlap and no imaging study can be considered diagnostic at this time. Furthermore, although

some ''control'' subjects (no symptoms or clinical findings) with ''abnormal'' functional imaging studies exist, likely reflecting an early disease state, the reverse may also be found. Namely, there are patients with symptomatic states in whom current functional imaging is thought to be normal. Consequently, the ability of imaging to provide diagnostic certainty regarding diagnosis is imperfect.

Finally, although neuropathological findings have been considered the diagnostic ''gold standard,'' as indicated by the term ''idiopathic Lewy body Parkinson's disease,'' recent findings suggest that neuropathological diagnostic criteria are imperfect, just as every other measure. Variable neuropathological diagnoses in affected individuals from a PD kindred found to have a single gene abnormality (PARK 8 – LRRK2 gene), including differing inclusion body hallmarks, leads one to the conclusion that PD is imperfectly defined even with neuropathological evaluation (Uitti et al., 2004, Zimprich et al., 2004).

In summary, PD is a syndrome. Specific disease entities, implying singular cause and pathogenesis, encompassed under the PD syndrome heading, will require different diagnostic criteria than are currently available. When considering a ''PD cohort,'' one must assume that the group has heterogeneous clinical, functional imaging, and pathological findings. These variabilities may or may not contribute to confusion in interpreting the natural course of progression in a cohort.

Neuroprotection

Most would agree that neuroprotection entails some combination of preventing, forestalling, delaying, stopping, arresting, minimizing or slowing the otherwise usual progression of a neurodegenerative disorder. Conceptually, neuroprotection is generally considered as an action whose impact on neuronal health translates into a clinically recognizable consequence. Neuroprotection is typically mechanistically envisioned as taking place at the molecular/cellular level. Slowing or minimizing decline implies the ability to protect or slow neuronal demise or dysfunction, which manifests with positive clinical, functional imaging, or neuropathological consequence.

In practice, the ability to count neurons (the location of which would also be disputed), measure physiological biomarkers, neuropathologically sample cellular tissue are all outside of current available technology or practice. Indirect measures of neuroprotection that are available relate to functional imaging and clinical outcomes. Functional imaging and clinical outcome measures are the only methods that have been used in PD-neuroprotective studies.

Neuroprotection also implies altering a course that is otherwise inevitable. To prove that a therapy is clinically relevant in a neuroprotective manner, the usual course must be defined or predictable. For a given disease, the outcome measures over time may decline or progress linearly, exponentially or in any mathematical model. Depending upon the model of progression and timing of intervention, neuroprotective therapy may or may not be helpful. Additionally, if a neuroprotective intervention is introduced to a heterogenous cohort, with variable courses treated at varying stages within those courses, the effects of intervention may be difficult to ascertain. Studying large numbers of subjects over extended periods of time may be the only method to extricate the true effects of therapy. Unfortunately, such specifications for studies make them extremely costly and logistically difficult to complete.

In the case of PD, monitoring outcome measures of progression are also complicated by symptomatic therapy that may, to a variable degree, mask or influence clinical signs, symptoms of parkinsonism and other surrogate markers of disease severity (such as functional imaging and electrophysiological measures). Therefore, analyses of neuropro-

tection in PD is a complicated enterprise that presents many operational difficulties.

Neuroprotective studies in PD

No study of neuroprotective therapy has proven clinically effective for PD. Numerous compounds have been suggested as potential neuroprotective agents (Table 1). Only two agents have been associated with improved survival in PD (levodopa and amantadine) (Uitti et al., 1993, 1996), but meaningful neuroprotective studies in the future will require clinical as well as quality of life functional outcome measures (including motor, behavioral, cognitive, socioeconomic, etc. measures).

The largest neuroprotective trial completed (DATATOP) assessed the effects of selegiline, a selective MAO-B inhibitor, and α-tocopherol in a double-blind placebo-controlled trials employing a 2×2 factorial design. Over 800 PD patients were randomized and followed to a clinical end-point of requirement for levodopa therapy because of increasing parkinsonism (Parkinson Study Group, 1989). The study identified a delay in end-point in subjects treated with selegiline (10 mg/day) compared to placebo but further long-term studies demonstrated that there was a substantial symptomatic effect and that any benefit in terms of motor progression was not sustained. Analysis of long-term data took advantage of variable durations of treatment with selegiline and disclosed that those treated with selegiline longer tended to reach a disability endpoint sooner (Parkinson Study Group, 1996). Studies with selegiline identified the complicating factor of a small, but measurable, symptomatic effect of a treatment when employing a clinical outcome measure (motor scoring) as a primary outcome.

Table 1. Potentially neuroprotective agents for PD

Bioenergetic agents
Coenzyme Q10, creatine
Antioxidants
Vitamin E, C, iron chelators
MAO-B inhibitors
rasagiline, selegiline, lazabemide
Anti-apoptotic agents
mixed lineage kinase inhibitors -CEP-1347, TCH346, rapamycin selegiline, rasagiline, caspase inhibitors, mRNA interference
NMDA-receptor antagonists
amantadine, memantine
Hormones
estrogen
Dopaminergic agents
nicotine, dopamine agonists, levodopa
A_{2A} (adenosine receptor) antagonists
istradefylline, caffeine
Anti-inflammatory agents
minocycline, aspirin, COX-2 inhibitors, tetracycline
Growth/Neurotrophic factors
neuroimmunophilins -GPI-1485, GDNF, GM-1 ganglioside, SR57667, proprarygylamines

Bioenergetic agents have also received attention as potentially neuroprotective. Coenzyme Q10 was shown to be safe in a trial lasting up to 16 months. 60 de novo PD patients received 300, 600, or 1200 mg/d and compared to 20 subjects receiving placebo. There was a trend suggesting less increase in total UPDRS score in treated subjects (Shults et al., 2002). However, this study failed to show any substantial differences in decline of the motor subscore of the UPDRS. This has led to other studies in progress evaluating doses of up to 2400 mg/d of coenzyme Q10.

Studies of dopamine agonists, namely pramipexole and ropinirole, in comparison to levodopa have been well publicized. These studies have included reviews of clinical outcomes and functional imaging. The Comparison of the Agonist Pramipexole versus Levodopa on Motor Complications in PD (CALM-PD) study compared the two agents effects on UPDRS and single photon emission CT (SPECT) employing β-CIT (a radioligand thought to reflect density of dopamine transporters, an indirect measure of nigrostriatal integrity) (Parkinson Study Group, 2002). The Ropinirole as an Adjunct to Levodopa (REAL) study employed striatal fluorodopa-PET as a

surrogate biomarker of nigrostriatal function (Whone at al., 2003). While both of these studies concluded that the functional imaging data suggested a slowing of decline in nigrostriatal function with dopamine agonists compared to levodopa, these findings were not borne out by clinical observations (UPDRS measures) which favored levodopa despite long-term follow-up. Additionally, alternative explanations, including variable pharmacological effects on the imaging radioligands themselves, appear to be an equally plausible explanation for the imaging data which appears to be counterintuitive to the clinical data (Ahlskog, 2003). Similarly, the Early versus Late L-Dopa (ELLDOPA) study compared clinical and functional imaging findings in patients randomized to receive placebo or one of three doses of levodopa. The clinical data after 40 weeks of treatment and 2 weeks of washout suggested benefit in the levodopa arms (by UPDRS) while at the same time showing greater decline by β-CIT-SPECT (Parkinson Study Group, 2004).

Study design

Rasagiline, a selective MAO-B inhibitor, has also been studied in a novel manner (Parkinson Study Group, 2004). A delayed-start design was employed that randomized subjects to receive active treatment or placebo followed by a period after which placebo patients were switched to active treatment while maintaining blindness. After further follow-up the groups were compared and found to maintain differences, with those receiving rasagiline without delay maintaining greater benefit. This treatment study design may minimize any differences between groups that are predominantly related to relatively acute symptomatic benefit.

Another research design, the futility study design, has been employed recently in U.S. government-funded studies underway. The fundamental difference in the futility study design is that the null hypothesis is that treatment is better than placebo (as opposed to the typical efficacy study where the null hypothesis is that active treatment and placebo are equivalent). Such a design allows smaller sample sizes and duration of follow-up, with the expectation that only large differences or benefits between treatment and placebo will be identified (and pursued further). This may be an effective screening design to identify therapy that has potential for substantive clinical benefit.

Finally, identification of genetically-derived PD, such as LRRK2 patients (Zimprich et al., 2004), although likely explanatory for a minority of PD patients, may offer the opportunity to identify patients in a pre-symptomatic state. Treatment of such individuals with effective neuroprotective therapy may result in a scenario where a given individual never becomes symptomatic. The pre-symptomatic genetically-derived PD state, in transgenic animals and humans, promises to offer opportunities to delineate utility of many potentially neuroprotective treatment strategies.

Conclusion

Neuroprotection for PD is conceptually problematic because of the heterogenous nature of the PD syndrome and complexities in operational definitions for neuroprotection. No currently available treatment is known to be definitively neuroprotective. Presently, the pragmatist may argue for treatment with multiple agents (addressing multiple causes/pathogeneses) while others may suggest none or a single agent based upon hypothesizing one particular disease process. At this time, neither approach (recommending single or multiple agents) can avoid justifiable criticism. Sound clinical trial designs may provide meaningful information relating to identifying therapeutic treatments that lead to improved quality of life over time, regardless of whether such improvements can ever be ascribed to ''neuroprotection.'' Another ongoing challenge is to design studies that may identify

subsets within PD cohorts who may respond to neuroprotective therapy, resulting in maintenance of neurological function and quality of life.

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Triggering endogenous neuroprotective mechanisms in Parkinson's disease: studies with a cellular model

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Summary. Glial cell line-derived neurotrophic factor (GDNF) has been implicated in the protection of dopamine (DA) neurons from oxidative stress in animal models of Parkinson's disease (PD). We have now shown that GDNF can also protect against the effects of 6-hydroxydopamine (6-OHDA) in a dopaminergic cell line and in cultures of primary DA neurons prepared from rat substantia nigra (SN). This appears to involve a rapid and transient increase in the phosphorylation of several isoforms of extracellular signal-regulated kinase (ERK). Our evidence indicates that ERK activation also can be modulated by reactive oxygen species (ROS), including those generated by endogenous DA. Identification of the ways by which these pathways can be triggered should provide insights into the pathophysiology of PD, and may offer useful avenues for retarding the progression of the disorder.

Introduction

Although PD is likely to involve the loss of many types of neurons, it is the loss of DA neurons of the SN that appears responsible for the motor symptoms typically used to define the disorder. Thus, reducing the loss of these cells should provide substantial clinical benefit. Evidence suggests that one reason for SN degeneration is an excess of oxidative stress. We have used an experimental model of PD that involves treating adult rats with an intracerebral, unilateral injection of 6-OHDA to produce a high level of such stress. Normally this would result in a marked reduction in the use of the contralateral limbs and the loss of DA neurons in the ipsilateral nigrostriatal projection. However, working with Tim Schallert and his colleagues at the University of Texas, Austin, we found that these consequences could be attenuated by placing the ipsilateral forelimb in a cast for one week immediately before or after the 6-OHDA treatment, thereby forcing the use of the normally affected limb (Tillerson et al., 2001; Cohen et al., 2003). We now wish to understand the processes that underlie these phenomena in the hope that this will lead to a rational basis for reducing disease progression.

GDNF can attenuate the death of dopaminergic cells normally caused by exposure to oxidative stress

Exercise had already been shown by Carl Cotman and his colleagues at the University of California, Irvine, to increase trophic factors in the brain (Cotman and Bechtold, 2002). Thus, we hypothesized that forced motor activity reduced the vulnerability of DA neurons through an increase in the avail-

ability of such factors. One likely candidate was GDNF. In 1993, GDNF was identified as a survival factor for DA neurons in culture, and this observation was soon followed by the demonstration that the neurotrophic factor also could protect DA neurons in adult animals against the effects of DA toxins such as 6-OHDA (Bohn, 1999). Moreover, Annie Cohen and Amanda Smith in our group observed that forced exercise caused an increase in GDNF in the contralateral striatum that peaks after 3 days of treatment (Cohen et al., 2003). Thus, we have set about to determine how GDNF protects DA neurons from oxidative stress.

We have begun our studies with two in vitro models. Yun Min Ding, Juliann Jaumotte, and Armando Signore observed that we could produce a specific degeneration of dissociated DA neurons from the SN region of rat pups (P0) if the cultures were briefly exposed to 6-OHDA (40–500 μM; 15 min). This effect was attenuated by the addition of GDNF (100 ng/ml) to the medium (Ding et al., 2004). Using the dopaminergic cell line MN9D, Susana Ugarte, Eva Lin, and Ruth Perez found that 6-OHDA (250 μM, 15 min) also produced a concentration- and time-dependent death of these cells, and that this, too, could be attenuated by the addition of GDNF (10 ng/ml) (Ugarte et al., 2003).

GDNF failed to block completely the effects of 6-OHDA in either experimental model. However, we reason that an understanding of the mechanism of the protective effects that are seen will provide us with important insights into determinants of DA neuron survival. Thus, using the MN9D model we have begun to dissect out the intracellular events responsible for the GDNF-induced protection that occurs. Eva Lin and Jane Cavanaugh observed that exposure of MN9D cells to GDNF (10–100 ng/ml for 15 min) resulted in the phosphorylation of three isoforms of ERK – ERK1/2 and ERK5, as well as an increase in pCREB (Cavanaugh et al., 2004; Lin et al., 2004).

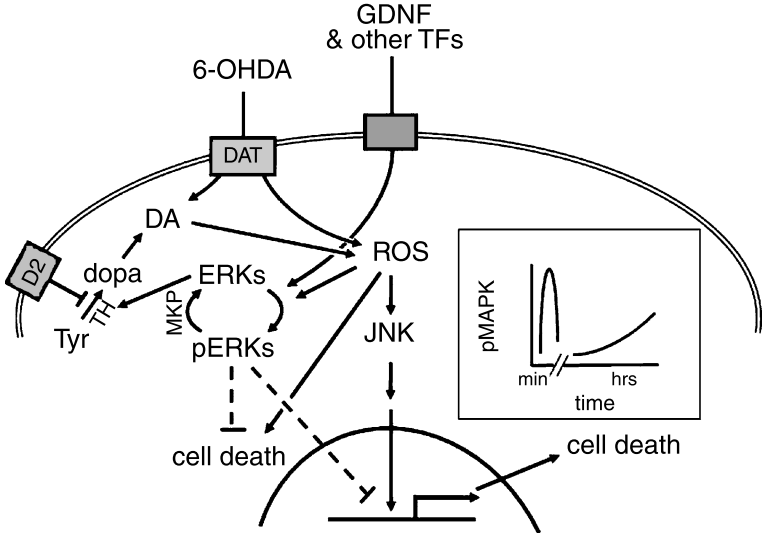

Fig. 1. A simplified version of our hypothesis for the actions of 6-OHDA, DA, and GDNF. Pro-survival pathways in green; pro-death pathways in red. Solid, excitation; dotted, inhibition. Components not specified include are the role of BAD, CREB, and nF-κB, and the importance of protein anchors. The ***inset*** is provided to indicate that the kinetics of changes in MAP kinases is critical to their impact on survival, with short term increases in kinase activity often being neuroprotective (shown for ERK) and more prolonged increases often being neurotoxic (shown for JNK)

We believe that the apparent activation of ERK participates in the neuroprotective effects of GDNF since the MEK inhibitor U0126 (5–10 μM) eliminated the protective effects of GDNF against 6-OHDA toxicity in the MN9D cell line (Ugarte et al., 2003).

Members of our research group have found parallels to these findings in animal models. Amanda Smith has examined the effect of forced limb use on activation of ERK1/2 within the nigrostriatal pathway. As in the case of exercise-induced changes in the striatum, Dr. Smith observed that forced limb use increased pERK1/2 in the nigrostriatal pathway associated with the overused forelimb (Smith et al., 2004). In the striatum, forced use increased ERK1/2 (4-fold), which was sustained during the 7-day casting period. In the SN, the increase in pERK1/2 was more gradual, with maximum increases in pERK1/2 of 5-fold at day 7 post-cast (Smith et al., 2004). Niklas Lindgren and Rehana Leak have further shown that the direct injection of GDNF into the striatum produces a marked activation of pERK1/2 in SN neurons within 24 hr. Thus, both in vivo and in vitro data are consistent with the hypothesis that increases in the availability of GDNF protect DA neurons from oxidative stress acting in part via an activation of ERK isoforms (see Fig. 1). Of course, there is still much to be done. For example, we have not yet shown that in vivo blockade of GDNF or ERK signaling will block the neuroprotection we observed in our animal model.

ERK activation may also be triggered by oxidative stress

In our studies of the role of ERK in the neuroprotective effects of GDNF, we also observed that ERK1/2 and 5 was activated by 6-OHDA itself (Lin et al., 2003; Cavanaugh et al., 2004). To examine this phenomenon more carefully, Eva Lin treated MN9D cells with 6-OHDA (500 μM). We observed that pERK1/2 increased by 25-fold at 15 min but returned to baseline by 30 min. After removing the 6-OHDA, a second, smaller, but sustained peak arose by 3–6 hr and persisted for several more hours. This late pERK1/2 peak was correlated with activation of caspase-3 and cell death. Phosphorylation of ERK's downstream substrate CREB followed the activation profile of ERK1/2.

To begin to determine the relation between ERK phosphorylation and the toxic effects of 6-OHDA, we re-examined the response to 6-OHDA in the presence of U0126 (5 μM). When this inhibitor was added to the medium before and during toxin exposure so as to block the first pERK peak, vulnerability of cells to 6-OHDA was increased 2-fold. No such effect was seen if U0126 was provided after this initial peak (Lin et al., 2003). This suggests that the initial transient activation of ERK after oxidative stress is a compensatory response, functioning to reduce cellular vulnerability.

Jane Cavanaugh has since shown that 6-OHDA increased pERK5 as well as pERK1/2 and that U0126 inhibits the activation of all three ERK isoforms. Thus, experiments are now in progress to determine which of the isoforms is involved in retarding the toxic effects of oxidative stress in this model.

The role of endogenous DA in neuroprotection

Having observed that 6-OHDA activated ERK isoforms in MN9D cells and that this was associated with a reduction in 6-OHDA-induced toxicity, we reasoned that DA might have a similar impact. It is well known that DA, like 6-OHDA, is highly electroactive and readily oxidizes to form several ROS. Thus, Eva Lin examined the effects of incubating MN9D cells for 24 hr with α-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in DA biosynthesis. We observed that decreasing DA levels in

this way was associated with a decrease in pERK1/2. This was associated with an increase in basal cell death and in the toxic effects of 6-OHDA (Lin et al., 2003). Our observations suggest that the vulnerability of DA neurons is regulated in part by changes in DA turnover and that GDNF acts in part through this mechanism.

Can PD be affected via alterations in GDNF and/or activation of ERK signaling?

Clinical trials with GDNF have produced conflicting results. A report appeared in 2003 by Stephen Gill and his colleagues suggesting that GDNF had great promise. This led to a much larger, multi-center blinded study. Unfortunately, that study has been halted because of reports of a lack of clinical improvement and evidence of potentially toxic effects. Yet, given the clear evidence for the efficacy of GDNF in laboratory models as well as some patients, it seems clear that we must pursue an understanding of how this and related molecules exert their neuroprotective effects. Moreover, studies of trophic factors, oxidative stress, and related intracellular signaling cascades may provide insights into how endogenous neuroprotective processes can be stimulated to retard the progression of PD.

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Ladostigil, a novel multifunctional drug for the treatment of dementia co-morbid with depression

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Summary. Ladostigil is a novel drug that inhibits acetyl and butyrylcholinesterase, and monoamine oxidase (MAO) A and B selectively in the brain. It reverses memory deficits induced by chronic inhibition of cortical cytochrome oxidase in rats and has anxiolytic and antidepressant-like activity in prenatally-stressed rats. Ladostigil also prevents oxidative–nitrative stress induced in astrocytes in the hippocampal CA1 region following icv injection of STZ in rats which also impairs their episodic memory. The unique combination of ChE and MAO enzyme inhibition combined with neuroprotection makes ladostigil a potentially useful drug for the treatment of dementia in subjects that also have extrapyramidal dysfunction and depression.

Introduction

Progressive neuronal loss resulting in deficits in cortical cholinergic transmission occurs in the three major forms of dementia, Alzheimer (AD) vascular (VD) and dementia with Lewy bodies (DLB). These deficits are strongly correlated with impairment of attention and cognitive function (Ellis, 2005). A significant proportion of patients with each of these forms of dementia have extrapyramidal dysfunction and depression. Although the aetiology of these dementias is not known, the oxidation of cell constituents, membrane lipids and DNA are seen in the ageing brain resulting from overproduction of oxidative free radicals (ROS) because of its diminished capacity to remove them. ROS could either be the cause or the result of the mitochondrial dysfunction in AD indicated by the reduction in cytochrome oxidase (COx) activity in several cortical and hippocampal brain regions (Hirai et al., 2001). Impaired mitochondrial activity together with reduced levels of ATP can lead to apoptosis and cell death. ROS may also be the cause of activation of glial cells with increased regional expression of cytokines and nitric oxide (NO) in the brains of patients with dementia that both results from and contributes to the production of β-amyloid and neurodegeneration (Katsuse et al., 2003).

Pharmacological rationale

To date, acetylcholinesterase (AChE) inhibitors are the only drugs that have been shown to produce significant symptomatic improvement in cognitive performance and behavioural abnormalities in subjects with AD, VD and DLB (Bullock, 2004). While AChE inhibitors slow the decline in cognitive impairment and executive function, there is no evidence that they can reduce oxidative stress *in vivo* or ameliorate depression. Ladostigil, (TV3326) was designed to increase choliner-

gic transmission, prevent the formation of ROS or their actions and ameliorate depression. It is an aminoindan containing the propargylamine group found in the selective, monoamine oxidase (MAO)-B inhibitor rasagiline and the carbamate moiety of rivastigmine. Ladostigil inhibits both AChE and butyrylcholinesterase (BuChE) and has a longer duration of action than rivastigmine. In contrast to other irreversible MAO-A inhibitors ladostigil has very little or no effect on the enzyme in the gastro-intestinal tract at doses that block MAO-A and B in the brain by more than 80%. It therefore causes minimal potentiation of the cardiovascular effect of orally-administered tyramine. This selective inhibition probably results from the formation in the brain of the decarbamylated metabolite which is a much more potent inhibitor of MAO than ladostigil itself (Weinstock et al., 2003).

Antagonism of memory induced in rats by inhibition of brain cytochrome oxidase

In order to mimic the reduction in COx activity seen in the cortex and hippocampus of AD patients we administered sodium azide (NaAz) (1 mg/kg/hour) for 4 weeks to male rats via Alzet minipumps. This resulted in a selective decrease of 20–25% in enzyme activity in the cingulate and parietal cortices and in the dentate gyrus and CA1 region of the hippocampus. The rats showed a loss of episodic memory in the object recognition test that was restored by a single oral dose of ladostigil (50 μmol/kg) administered two hours before the test (Table 1). This dose inhibits AChE in the frontal cortex by $39 \pm 3\%$ which agrees well with the values of inhibition obtained with both rivastigmine and donepezil in patients with AD (Kaasinen et al., 2002). When administered chronically to rats for 4 weeks from one day after instillation of the NaAz pump, ladostigil (50 μmol/kg) prevented the deficit in working memory in the Morris water maze test even when the rats were tested 20–22 hours after its administration when brain AChE was no longer inhibited. These findings suggest that ladostigil can reverse existing memory impairment and may also be able to prevent its development if given early enough.

Anxiolytic and antidepressant-like activity of ladostigil

Antidepressants are at least as effective as benzodiazepines in the treatment of generalized anxiety disorder that may precede or accompany depressive symptoms. Anxiolytic behaviour with features of depression can be

Table 1. Reversal by ladostigil of deficit in episodic memory induced in rats by chronic inhibition of cerebral cytochrome oxidase

Treatment	Trial 1: Two identical objects	Trial 2: One familiar and one new object		
	Total exploration time (sec) ± sem	Total exploration time (sec) ± sem	Diff new-familiar (sec) ± sem	Discrimination[#] index ± sem
Sham pump (10)	17.4 ± 1.7	21.3 ± 3.2	$7.7 \pm 1.9^*$	$0.32 \pm 0.06^*$
NaAz (10)	29.8 ± 6.2	24.3 ± 3.9	-3.1 ± 3.2	-0.05 ± 0.12
NaAz + ladostigil 50 μmol/kg (10)	20.4 ± 2.5	25.8 ± 2.9	$8.7 \pm 1.3^*$	$0.36 \pm 0.07^*$

(Number of rats) [#]Discrimination index: Time exploring new object-time exploring familiar object/total exploration time. [*]Significantly different from 0 (i.e. show discrimination or spend more time exploring new object)

induced in rats by prenatal stress (Weinstock, 2001). These can be detected in the elevated plus maze (EPM) test by an avoidance of the open arms, and in the forced swim test (FST) by a lomger duration of immobility than in control rats. Ladostigil (50 μmol/kg) administered daily for 6 weeks from puberty to prenatally-stressed rats abolished their hyperanxiety and depressive-like behaviour in the EPM and FST tests in adulthood. This dose inhibited brain MAO-A and B by 60–65% but had no effect on the behaviour of control rats. In human subjects, amelioration of symptoms by antidepressant treatment is usually accompanied by restoration to normal of the feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Prenatal stress in rats also disrupts the regulation of the response of the HPA axis to stress, as seen in the delay in return to normal of plasma corticosterone. Chronic treatment with ladostigil normalized the response of the HPA axis in these rats (Poltyrev et al., 2005).

Reduction of oxidative–nitrative stress *in vivo* by ladostigil

Ladostigil, (0.1–1 μM) prevents cytotoxicity in cell culture induced by the NO donor, Sin 1 or 6 hydroxydopamine in SHSY5Y and PC12 cells. The cytoprotective activity of ladostigil is due to the presence of the propargylamine moiety and occurs at concentrations that are too low to inhibit either AChE or MAO (Youdim and Weinstock, 2002). We have found that intracerebroventricular injection of streptozotocin (icv-STZ) in rats causes deficits in spatial memory 4–6 weeks later that result from pathological processes in the septo-hippocampal system that include selective damage to myelin. The memory deficits are significantly reduced by chronic treatment with ladostigil (Weinstock and Shoham, 2004). We now report that icv STZ causes activation of astrocytes, as indicated by GFAP staining, one week after its injection before memory deficits are seen. These active astrocytes display oxidative–nitrative stress (indicated by a specific antibody to nitrotyrosine) in the alveus, stratum oriens and stratum radiatum in the hippocampus. Ladostigil (2.9 μmol/kg) given once daily for one week before, until one week after icv STZ virtually abolished the astrocyte activation and nitrotyrosine staining (Fig. 1). This dose does not inhibit AChE or MAO in the brain but is compatible with that of other propargylamino-indan compounds that reduce oxidative stress in cell culture (Youdim and Weinstock, 2002).

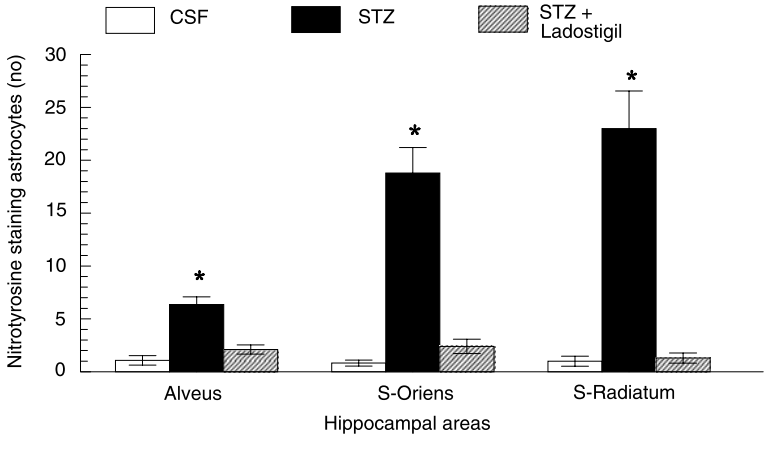

Fig. 1. Prevention by ladostigil of oxidative–nitrative stress in astrocytes in hippocampal CA1 regions induced in rats by icv STZ. *Significantly different from other groups, $P<0$

Conclusions

Ladostigil is a multifunctional drug designed to answer the therapeutic requirements of progressive neurodegenerative diseases with features of dementia, depression and extrapyramidal symptoms. It inhibits AChE and BuChE and has a slower onset and longer duration of action than rivastigmine. Ladostigil antagonizes memory deficits induced by scopolamine, icv STZ or chronic COx inhibition in rats, closed head injury in mice or advanced age in monkeys (Buccafusco et al., 2003). It may therefore be expected to provide similar beneficial effects on cognitive function and attention in patients with AD, VD and DLB to those seen by AChE inhibitors currently in use.

In addition, the unique ability of ladostigil to inhibit both MAO-A and B selectively in the brain enables the drug to exert anxiolytic and antidepressant-like activity without causing clinically significant potentiation of the pressor response to oral tyramine. MAO inhibition by ladostigil prevented the decline in striatal dopamine induced by the neurotoxin MPTP in mice and should also help to maintain dopamine levels in subjects with Parkinson's disease and dementia. Since oxidative stress and gliosis occur in neurodegenerative disorders like AD, the ability of ladostigil to reduce these features in a rat model *in vivo* suggests that it may also be able to reduce neurodegeneration and slow disease development. These multiple pharmacological actions of ladostigil make it a potentially useful drug for the treatment of dementia with extrapyramidal dysfunction and depression.

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M30, a novel multifunctional neuroprotective drug with potent iron chelating and brain selective monoamine oxidase-ab inhibitory activity for Parkinson's disease

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Summary. Iron and monoamine oxidase activity are increased in brain of Parkinson's disease (PD). They are associated with autoxidation and oxidative deamination of dopamine by MAO resulting in the generation of reactive oxygen species and the onset of oxidative stress to induce neurodegeneration. Iron chelators (desferal, Vk-28 and clioquinol) but not copper chelators have been shown to be neuroprotective in the 6-hydroxydoapmine and MPTP models of Parkinson's disease (PD), as are monoamine oxidase B inhibitors such as selegiline and rasagiline. These findings prompted the development of multifunctional anti PD drugs possessing iron chelating phamacophore of VK-28 and the propargylamine MAO inhibitory activity of rasagiline. M30 is a potent iron chelator, radical scavenger and brain selective irreversible MAO-A and B inhibitor, with little inhibition of peripheral MAO. It has neuroprotective activity in *in vitro* and *in vivo* models of PD and unlike selective MAO-B inhibitors it increases brain dopamine, serotonin and noradrenaline. These findings indicate beside its anti PD action, it may also possess antidepressant activity, similar to selective MAO-A and nonselective MAO inhibitors. These properties make it an ideal anti PD drug for which it is being developed.

Introduction

The role of iron in neurodegeneration and its accumulation in various brain regions associated with neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease. sHuntington disease, amytrophic lateral sclerosis and iron gene mutated neurological disorders such as aceruloplasminemia, PARK2 and Friedreich ataxia has put this metal on center stage (Zecca et al., 2004; Youdim et al., 2004a, b). Parkinson's Disease (PD) brain studies point to an on going oxidative stress and inflammatory processes, that may be involved in the increase in iron deposition and increased MAO activity in the substantia nigra pars compacta (SNpc) in the pathology of nigrostriatal dopamine neuron degeneration (Berg et al., 1999; Dexter et al., 1989a, b; Gerlach et al., 1994; Jellinger, 1999; Jenner and Olanow, 1996; Riederer et al., 1989; Youdim et al., 1993; Youdim and Riederer, 2002; Grunblatt et al., 2004). The iron also accumulates within melanized dopamine neurons of SNPC (Jellinger et al., 1992). Animal studies with 6-hydroxydopamine (6-OHDA) and MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) support the oxidative stress hypothesis of nigrostriatal dopamine neuron degeneration in Parkinson's disease (Cohen,

2000; Langston, 1996). Both neurotoxins increase the iron content of substantia nigra pars compacta in rats and monkeys (Hall et al., 1992; Oestreicher et al., 1994; Temlett et al., 1994; Goto et al., 1996; Mochizuki et al., 1994) and in mice (Lan and Jiang, 1997a, b). It is well established that chelatable (ionic) iron can induce oxidative stress because of its interaction with hydrogen peroxide and to generate reactive hydroxyl radical, which is known to initiate a process of membrane lipid peroxidation that results in cell membrane fluidity and finally cell death by a further cascade of events (Halliwell, 2001; Ben Shachar et al., 1991) and more recently Youdim et al. (2004b), reported that intraventricular pretreatment with the prototype iron chelator, desferal, was able to be protective against 6-OHDA in rats, with confirmation coming also from Lan and Jiang (1997a, b) in the MPTP model. More recently these results have been confirmed pharmacologically with the copper and iron chelator, clioquinol and by over-expression of ferritin in mice that results in neuroprotection against MPTP neurotoxicity (Kaur et al., 2003). The fact that desferal has an extremely low blood brain barrier penetration led us to develop a number of blood brain barrier permeable iron chelators such as VK-28 (Warshawsky et al., 2000) for ironing iron out of the brain. Studies employing systemic pretreatment with the brain permeable iron chelator, VK-28, have shown neuroprotection against 6-OHDA lesion of striatal dopamine neurons in rats (Ben Shachar et al., 2004).

These drugs would have several important advantages; first they would prevent iron-

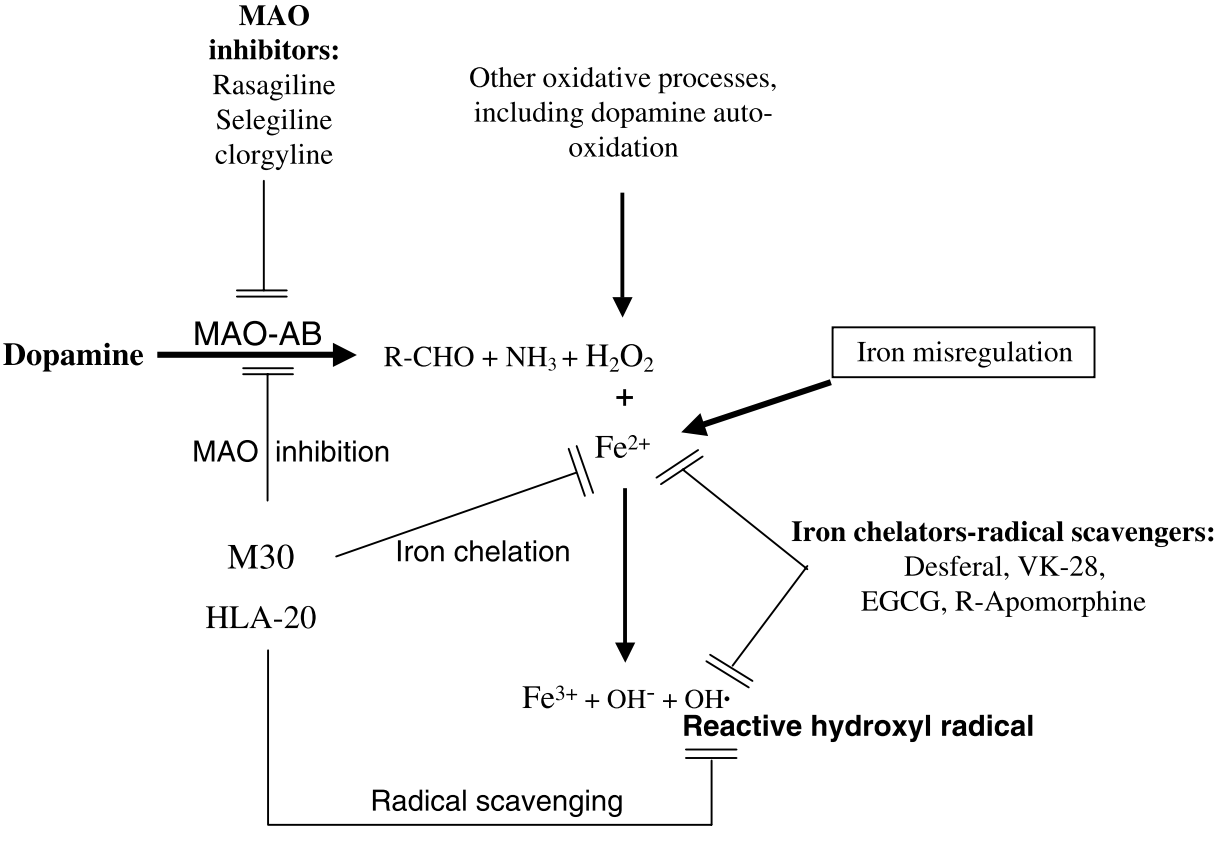

Fig. 1. The pathway of dopamine metabolism by monoamine oxidase and autoxidation and the its interaction with iron to induce oxidative stress induced neuronal death

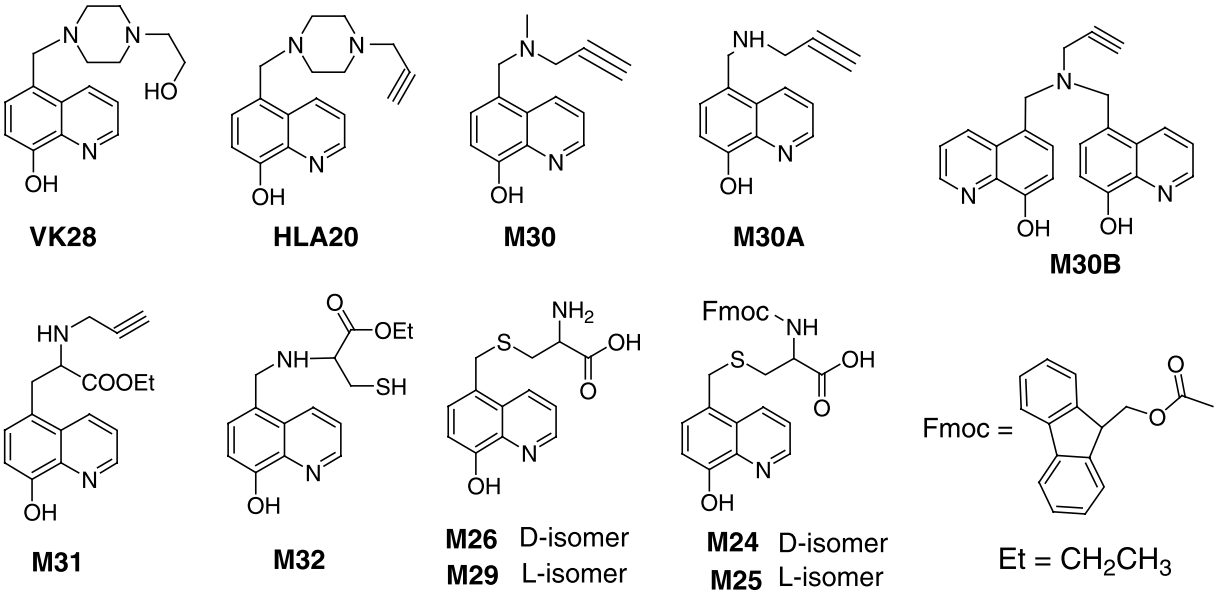

Fig. 2. Structures of brain permeable novel iron chelators derived from iron chelator VK-28 (Zheng et al., 2005)

hydrogen peroxide initiated generation of reactive $OH^{\bullet}$ (Fenton chemistry), secondly they might mobilize out chelatable iron from the brain and thirdly they prevent the autoxidation of dopamine to cytotoxic semiquinone, with the resultant liberation of oxygen radicals. On several occasions dopamine metabolism via autoxidation and by the reaction of MAO has been implicated to induce degeneration of nigrostriatal dopamine neurons (Riederer et al., 1989; Paris et al., 2005) (Fig. 1). Such a hypothesis is not far fetched, since D-penicillamine, a copper chelator, has been successfully employed in the treatment of Wilson disease for removal of neuronal copper. Aceruloplasminemia is a neurodegenerative disease associated with increased deposition of iron in the globus pallidus and substantia nigra. A recent study has shown that chronic desferal given in relatively large doses resulted in a decrease in iron in both regions, as shown by MRI, which correlated with clinical improvements (Miyajima et al., 1997). A recent report has shown that chelatable iron is a potent releaser of excitotoxic glutamate, as a consequence of acotinase activation, which has consistently been implicated in the neurodegenerative diseases including PD and Alzheimer's disease (Mcgahan et al., 2005).

These previous studies, together with the fact that systemic injection of VK-28, unlike that of desferal, is brain permeable and neuroprotective in the 6-OHDA and MPTP models. (Ben Shachar et al., 2004, Youdim et al., 2004b) led us to modify VK-28 by introducing the MAO-inhibitory-neuroprotective propargylamine moiety at different sites in the molecule. The result has been development of multifunctional neuroprotective M30 series of brain permeable iron chelators (Fig. 2). These compounds have similar iron chelating potency to desferal, possess potent irreversible brain selective MAO-AB inhibitory activity and neuroprotective and neurorescue properties similar to rasagiline (Youdim, 2005) as anti PD (Zheng et al., 2005a).

Iron chelating and antioxidant properties of multifunctional M30 derivative iron chelators

Several novel antioxidant-iron chelators bearing 8-hydroxyquinoline moiety were synthe-

sized, and various properties related to their iron chelation, and neuroprotective action were investigated (Zheng et al., 2005a, b) (Fig. 1). All the chelators including M30, M30A, M30B and HLA-20 exhibited strong iron(III) chelating and high antioxidant properties with ability to inhibit mitochondrial membrane lipid peroxidation. Chelator M30 and HLA20 had good permeability into K562 cells and displayed the highest protective effects against differentiated P19 cell death induced by 6-hydroxydopamine. EPR studies suggested that these chelators also act as radical scavenger to directly scavenge hydroxyl radical (Zheng et al., 2005a, b).

In vitro MAO-A and B inhibitory activity of M30

These novel multifunctional chelators were further examined for their activity as antioxidants, MAO-B inhibitors, and neuroprotective agents *in vitro*. Determination of Vk-28, desferal and a large number of iron and copper chelators indicated that these compounds do not inhibit MAO-A or B significantly *in vitro* (Youdim et al., 2004a; Zheng et al., 2005b). Three of the selected chelators possessing propargylamine moiety of rasagiline (M30, M30A and M30B and HLA20 and M32) were the most effective in inhibiting iron-dependent lipid peroxidation in rat brain mitochondrial homogenates with IC_{50} (12–16 μM) value comparable to that of desferal, a prototype iron chelator which does not cross the blood brain barrier. Their antioxidative activities were further confirmed using electron paramagnetic resonance spectroscopy, demonstrating similarity to desferal. In PC12 cell culture, the three above novel chelators at 0.1 μM were able to attenuate cell death induced by serum-deprivation and neurotoxicity induced by 6-hydroxydopamine and hydrogen peroxide. M30 possessing the N-propargyl MAO inhibitory moiety of the antiparkinson drug rasagiline displayed more neuroprotective potency than that of rasagiline as did N-propargyl piperazine chelator, HLA-20. In addition, the *in vitro* brain mitochondrial MAO assays showed that M30 was a highly potent MAO-A and B inhibitor (IC_{50}, MAO-A, 0.037 ± 0.02; MAO-B, 0.057 ± 0.01), while M30A and M30B showed MAO-A and B inhibitory potency with one-two orders of magnitude less and HLA20 was a moderately selective MAO-B with IC_{50} of 110 μM. These data suggested that M30 and HLA20 might serve as leads in developing drugs with multifunctional activities for treatment of various neurodegenerative disorders including PD and AD.

In vivo MAO-A and B inhibitory activity and prevention of MPTP neurotoxicity

The examination of acute (1–5 mg/kg) and chronic (5–10 mg/kg) IP or PO once daily for 14 days) *in vivo* studies in mice and rats, have shown M30 to be a potent brain selective (striatum, hippocampus and cerebellum) MAO-A and -B inhibitor (Gal et al., 2005). It has little effects on the enzyme activities of the liver and small intestine. This is similar to what we have observed with our anti Alzheimer-antiparkinson drug ladostigil (TV3326) (Weinstock et al., 2000; Youdim and Buccfesco, 2005). Its N-desmethylated derivative, M30A and M30B are significantly less active *in vivo*, confirming the *in vitro* results. Acute and chronic treatment with M30 results in increased levels of dopamine (DA), serotonin (5-HT) and noradrenaline (NA) and decreases in DOPAC (dihydroxyphenylacetic acid), HVA (homovanillic acid) and 5-HIAA (5-hydroxyindole acetic acid) as determined in striatum, hippocampus and hypothalamus. In the MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease it attenuates the dopamine depleting action of the neurotoxin, the loss in tyrosine hydroxylase activity and increases striatal levels of dopamine, serotonin and noradrenaline, while decreasing their metabolites (Fig. 3a, b). Since dopamine is equally well metabolized

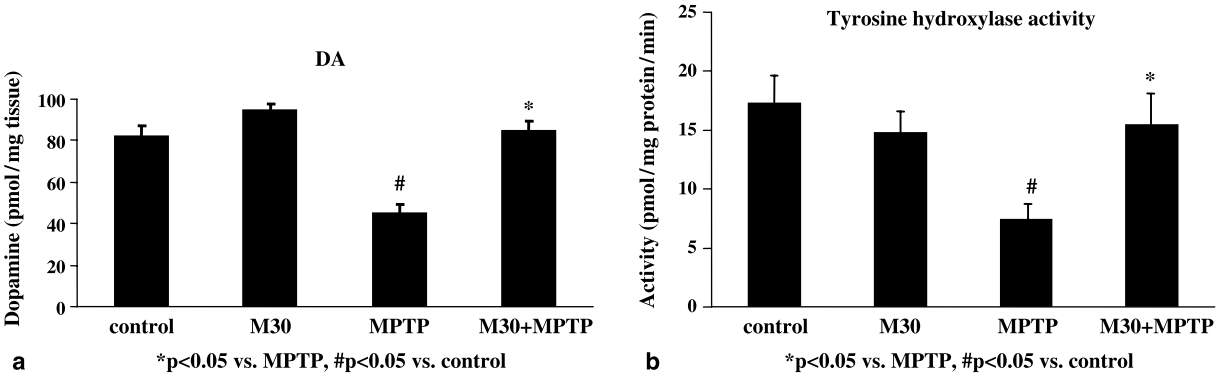

Fig. 3. Neuroprotective effect of M30, an iron chelator-brain selective monoamine oxidase-AB inhibitor in MPTP mouse model of Parkinsonism (Zheng et al., 2005; Gal et al., 2005)

by MAO-A and -B, it is expected that M30 would have a greater dopamine neurotransmission potentiation in Parkinson's disease. This is important since selective MAO-A (clorgyline, moclobemide) or MAO-B (selegiline, rasagiline lazabemide) inhibitors do not alter brain dopamine. Only when both forms are inhibited does brain dopamine increase.

The importance of iron chelation and MAO-A and B inhibition by M30 for dopamine neurotransmission in Parkinson's disease

Pretreatment of rats, with the iron chelator VK-28, which is a relatively potent inhibitor of membrane lipid peroxidation and penetrates the brain, protected against 6-OHDA induced lesion of striatal dopamine neurons (Ben Shachar et al., 2004) similar to results obtained using desferal (Ben Shachar et al., 1991; Youdim et al., 2004c). This was confirmed by prevention of the reduction in striatal dopamine, DOPAC and HVA and the reduction in increased dopamine turnover normally seen with 6-OHDA. VK-28-induced protection is observed whether the chelator is given IVC or IP. Its neuroprotective activity is relatively more potent as compared to some other radical scavenging agents, such as vitamin E (Acuna-Castroviejo et al., 1997; Cadet et al., 1989; Ferger et al., 1998; Moussaoui et al., 2000; Perry et al., 1985; Roghani and Behzadi, 2001) used in 6-OHDA or the MPTP animal models. However, desferal's major limitation as a neuroprotective drug is its inability to cross the BBB. It is well established that intranigral or intraventricular 6-OHDA initiates an increase of total iron in the substantia nigra and striatum, at the sites of neurodegeneration, both in monkeys, rats and mice (Hall et al., 1992; Lin et al., 1997; Lin and Lin, 1997; Oestreicher et al., 1994). The process by which 6-OHDA or MPTP initiates iron accumulation in substantia nigra pars compacta is not fully known. It may depend on their abilities to release iron from ferritin, as well as to down regulate transferrin receptors and up regulate divalent metal transporter at the cell surface membrane, which can be prevented by iron chelators such as desferal (Monteiro and Winterbourn, 1989; Double et al., 1998; Linert et al., 1996; Lode et al., 1990; Mash et al., 1991; Pezzella et al., 1997). These neurotoxin also inhibit mitochondrial complex I activity (Glinka and Youdim, 1965; Glinka et al., 1996) which can lead to oxidative stress dependent release of iron. Similar features have also been reported in the MPTP induced neurotoxicity in mice. The exact mechanism by which VK-28 produced neuroprotection is unclear, but we assume that similarly to desferal it chelates the iron and prevents complex I inhibition (Glinka et al., 1996).

Neither desferal or VK-28 have appreciable MAO inhibitory activities either *in vitro*

or *in vivo*. M30 is an iron chelator, with equivalent potency to that of Vk-28 desferal (Zheng et al., 2005a, b). Similar to VK-28 it has radical scavenging activity and iron-induced membrane lipid peroxidation inhibitory potencies close to those of desferal (Zheng et al., 2005a, b). This is an advantage, since radical scavengers (Perry et al., 1985) such as vitamin E (Cadet et al., 1985), melatonin (Acuna-castroviejo et al., 1977), and the green tea polyphenols EGCG wich is an anti oxidant as well as a potent iron chelator (Mandel et al., 2005) have neuroprotective activity against MPTP and 6-OHDA neurotoxicity *in vivo*.

The M30 series of drugs were thus developed for two purposes. On the one hand, it was designed to prevent the ability of iron to induce oxidative stress, as a consequence of reactive hydroxyl radical generation via its interaction with hydrogen peroxide (Fenton Reaction). Secondly, M30 was designed to inhibit the formation of reactive hydroxyl radical from hydrogen peroxide generated by MAO and potentiate the pharmacological action of accumulated dopamine formed from L-dopa (L-dihydroxyphenylalanine).

MAO is considered one of the major enzymes that generate hydrogen peroxide which is also generated by several other oxidative reactions such as xanthine oxidase or by conversion of superoxide generated. If not adequately removed by brain glutathione peroxidase, it can accumulate and interact with labile (ionic) iron. In PD substantia nigra (SN) GSH (reduced glutathione), the rate limiting co-factor of glutathione peroxidase diminishes significantly with the progression of the disease (Riederer et al., 1989; Sian et al., 1994). This together with accumulation of iron in SN would make dopamine neurons vulnerable to oxidative stress, unless iron is removed and MAO inhibited, thus preventing Fenton Reaction.

Besides its potent iron chelation and prevention of membrane lipid peroxidation, M30 is a potent inhibitor of MAO-A and B in all brain regions examined, with a slight preference for MAO-B (Gal et al., 2005). However, it is a poor inhibitor of the liver and small intestine MAO-A and B. This is a highly important pharmacological advantage of the drug, since irreversible inhibition of MAO-A which is prominent in these tissues is associated with the ''cheese reaction'' (potentiation of tyramine-induced cardiovascular activity), as has been observed with tranylcypromine, iproniazid and clorgyline. However, it is expected that M30 would not show such property. This is borne by the observation that our multifunctional cholinesterase-brain selective MAO-AB inhibitor, ladostigil (Weinstock et al., 2000) exhibits similar tissue MAO inhibitory properties and shows highly significant limited potentiation of tyramine-induced cardiovascular effect (Weinstock et al., 2002). The mechanism underlying the preference of M30 for the brain enzyme inhibition it not known. It is possible that in the brain, the inhibitor is metabolizm to active metabolite(s) that accumulate and are retained by the brain. The other possibility is that this drug inhibits its metabolized by a brain specific cytochrome P-450, which is absent in the liver and small intestine.

MAO-A and B enzyme inhibitory activity of M30 results in increased brain levels of dopamine, serotonin and noradrenaline. Thus, it is likely to have not only antiParkinson activity but also antidepressant property, similar to other non-selective MAO-AB (tranylcypromine) and selective MAO-A inhibitors (moclobemide) (see Youdim and Weinstock, 2004 for review). Similar to non selective MAO inhibitors and selective MAO-B inhibitors such as selegiline, rasagiline, lazabemide and milacemide (Foley et al., 2000), M30 prevents MPTP induced nigrostriatal dopaminergic neurotoxicity and dopamine depletion in mice, by protecting against the loss of dopamine neurons, by the presence of normal tyrosine hydroxylase activity. In contrast to the selective MAO-B or MAO-A inhibitors which do not increase brain levels

of striatal dopamine (see Green et al., 1977; Lamensdorf et al., 1996; Finberg and Youdim, 2002), M30 caused significant increase in striatal DA, as well as 5-HT and NA levels, when given acutely either IP or PO. Thus, only when both enzyme forms are inhibited does brain dopamine increase and its metabolites DOPAC and HVA decrease (Green et al., 1977), as has been observed with M30. This is not unexpected since O'Carroll et al. (1983) showed that dopamine is equally well metabolized by both forms of the striatal human brain enzymes. Thus, when one enzyme form is inhibited, the other can continue to metabolize it (Green et al., 1977). Thus we have advocated on several occasions that an MAO inhibitor which inhibits both forms of brain MAO, and does not potentiate the cardiovascular action of tyramine, may have a superior dopamine neurotransmission potentiation and antiParkinson activity as compared to the selective MAO-A or -B inhibitors (Youdim and Weinstock, 2004). Chronic M30 treatment does not cause an increase in striatal dopamine. However, it significantly reduces DOPAC and HVA. Resulting in increased dopamine turnover as reflected in DOPAC plus HVA/DA, and indicates increased DA release, as has been reported previously with other MAO inhibitors (Lamensdorf et al., 1996).

Conclusion

Free (chelatable) iron, more than any other transitional metal, plays a pivotal role in the processes of oxidative stress, inflammatory processes and cell death in many non neuronal and neuronal diseases. This is due to its cell abundance in brain regions (substantia nigra, globus pallidus, dentate gyrus, thalamus) associated with neuronal degeneration in neurodegenerative diseases, profound redox state and decompartmentation from ferritin, which can result in oxidative stress, induce cell death as a consequence of reactive hydroxyl radical generation (Youdim and Riederer, 2004; Zecca et al., 2004) and its ability to release the excitotoxin glutamate (McGahan et al., 2005). The present study shows that the iron chelator M30 has potent iron chelating, radical scavenging and brain selective MAO-AB inhibitory activity (Fig. 2). In neuronal cell culture it has neuroprotective and neurorescue activity by regulating the anti apoptotic Bcl-family proteins and PKC activation similar to what has been established for rasagiline (Youdim, 2003; Avramowich et al., 2005). *In vivo* it induces neuroprotection against MPTP neurotoxicity and prevents the fall in striatal dopamine without affecting serotonin or noradrenaline as a consequence of its MAO inhibitory and iron chelating properties. The fact that it does this with IP treatment shows it crosses the blood brain barrier. M30 treatment increases the basal levels of the neurotransmitters dopamine, serotonin and noradrenaline in the absence and presence of MPTP indicating that it does not interfere with the iron dependent enzymes tyrosine hydroxylase and tryptophan hydroxylase activity. Thus, the multifunctional M30 and its other derivatives could be novel drug for the treatment of Parkinson's disease with and without depressive illness for which they are being developed.

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Involvement of multiple survival signal transduction pathways in the neuroprotective, neurorescue and APP processing activity of rasagiline and its propargyl moiety

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Summary. Our recent studies aimed to elucidate the molecular and biochemical mechanism of actions of the novel anti-Parkinson's drug, rasagiline, an irreversible and selective monoamine oxidase (MAO)-B inhibitor and its propargyl moiety, propargylamine. In cell death models induced by serum withdrawal in rat PC12 cells and human SH-SY5Y neuroblastoma cells, both rasagiline and propargylamine exerted neuroprotective and neurorescue activities via multiple survival pathways, including: stimulation of protein kinase C (PKC) phosphorylation; up-regulation of protein and gene levels of PKCα, PKCε and the anti-apoptotic Bcl-2, Bcl-xL, and Bcl-w; and up-regulation of the neurotrophic factors, BDNF and GDNF mRNAs. Rasagiline and propargylamine inhibited the cleavage and subsequent activation of pro-caspase-3 and poly ADP-ribose polymerase. Additionally, these compounds significantly down-regulated PKCγ mRNA and decreased the level of the pro-apoptotic proteins, Bax, Bad, Bim and H2A.X. Rasagiline and propargylamine both regulated amyloid precursor protein (APP) processing towards the non-amyloidogenic pathway. These structure-activity studies have provided evidence that propargylamine promoted neuronal survival via neuroprotective/neurorescue pathways similar to that of rasagiline. In addition, recent study demonstrated that chronic low doses of rasagiline administered to mice subsequently to 1 methyl-4 phenyl 1,2,3,6 tetrahydropyridine (MPTP), rescued dopaminergic neurons in the substantia nigra pars compacta via activation of the Ras-PI3K-Akt survival pathway, suggesting that rasagiline may possess a disease modifying activity.

Introduction

Rasagiline (N-propargyl-1-(R)-aminoindan), a potent selective irreversible inhibitor of monoamine oxidase (MAO)-B, is a novel anti-Parkinsonian drug, which may have disease modifying properties. In light of recently reported benefits in patients with early illness, rasagiline is a promising new treatment for Parkinson's disease (PD) (Parkinson Study Group, 2005). Recent multi-center double-blind mono-therapy with rasagiline by the Parkinson Study Group (Parkinson Study Group, 2004) and as adjunct therapy to L-DOPA (Rabey et al., 2000), has shown that rasagiline confers significant symptomatic improvement and suggested possible alterations in disease progression at doses of 1 and 2 mg (Chen and Swope, 2005). Rasagiline has been shown to have a broad neuroprotective activity against a variety of neurotoxins in neuronal cell cultures and in animal models.

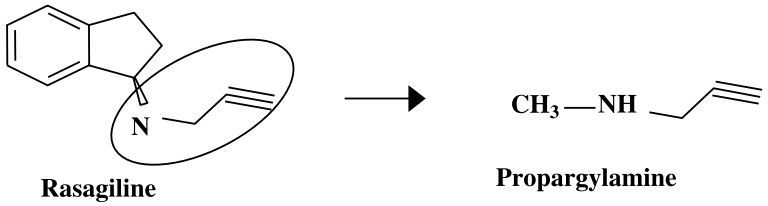

Fig. 1. The chemical structures of the anti-Parkinson's drug/MAO-B inhibitor, rasagiline and its propargyl moiety, propargylamine

This includes attenuation of cell death in partially differentiated rat pheochromocytoma (PC12) cells deprived of serum and nerve growth factor (NGF) (Maruyama et al., 2000b) and neuroprotection against the endogenous neurotoxin N-methyl-(R)-salsolinol (N-M-(R)-Sal) (Akao et al., 2002; Maruyama et al., 2001a, b), 6-hydroxydopamine (6-OHDA) (Maruyama et al., 2000a), SIN-1 (a peroxynitrite donor) (Maruyama et al., 2002a, b) and glutamate toxicity (Finberg et al., 1999) in neuronal cells. In vivo studies have described the protective effect of rasagiline in N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in mice and monkeys, preventing its conversion to 1-methyl-4-phenylpyridinium (MPP^+) (Heikkila et al., 1985), in focal ischemia model in rats (Speiser et al., 1999) and in neurotrauma model of head injury in mice (Huang et al., 1999). In addition, rasagiline suppresses the cell death cascade initiated by pro-apoptotic mitochondrial proteins, prevents decline in mitochondrial membrane potential ($\Delta\Psi m$), inhibits the apoptotic processes including activation of caspase3, nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DNA fragmentation (Maruyama et al., 2002b; Youdim et al., 2003).

Our recent studies investigated new aspects of the pro-survival effects of rasagiline and its propargyl moiety, propargylamine (Fig. 1), in serum deprivation-neuroprotective/neurorescue-models, using rat PC12 cells and human SH-SY5Y cells. The molecular mechanisms of the neuroprotective/neurorescue effects of rasagiline and propargylamine on pro- and anti-apoptotic proteins, neurotrophic factors and amyloid precursor protein (APP) regulation are discussed in this review.

The mechanisms underlying neuroprotective activity of rasagiline and propargylamine

A major pathway implicated in neuronal cell survival is the intrinsic or mitochondrial signaling, triggered and mediated by the Bcl-2 family members that may either support cell survival (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1) or promote cell death (Bax, Bak, Bcl-Xs, Bad, Bid, Bik, Hrk, Bok) (Cory and Adams, 2002). The pro-apoptotic proteins of the Bcl-2 family members may trigger the opening of the mitochondrial mega-channel, or a specific channel in the outer mitochondrial membrane, both of which promote the fall in mitochondrial membrane potential, leading to cytochrome *c* release (Bernardi et al., 2001; Kroemer and Reed, 2000). The competitive action of the pro-and anti-survival Bcl-2 family proteins regulates the activation of the caspases which dismantle the cell (Adams and Cory, 1998; Evan and Littlewood, 1998; Zamzami et al., 1998). Bcl-2 overexpression was shown to prevent cell death, probably by inhibiting Bax translocation and insertion into mitochondrial membrane, or via a direct interaction with the channels (Cheng et al., 2001). Rasagiline was recently shown to prevent the fall in mitochondrial membrane potential ($\Delta\Psi m$) and the opening of mitochondrial voltage depen-

dent anion channel via the increase in Bcl-2 and Bcl-xL proteins (Maruyama et al., 2000a, 2002a). This is consistent with recent study (Weinreb et al., 2004) provided evidence that the neuroprotection effect of rasagiline is mediated by gene regulation of the Bcl-2-related protein family. Neuroprotection experiments were done in serum-starved partially differentiated rat PC12 cells. Cell viability was markedly reduced by 24 h serum withdrawal. Both rasagiline and its propargyl moiety, propargylamine, significantly reduced cell death induced by serum deprivation. The finding that propargylamine (Weinreb et al., 2004) had similar effects with the same potency as rasagiline, enlightens the importance of the propargyl moiety for the neuroprotection activity of rasagiline.

These drugs also decreased the mRNA of the pro-apoptotic members, Bax and Bad and increased the mRNA of the cell survival members, Bcl-2, Bcl-w and Bcl-xL. Additionally, the involvement of protein kinase C (PKC) pathway in rasagiline-induced inactivation of the BH3-only pro-apoptotic Bcl-2 family member, Bad, was demonstrated (Weinreb et al., 2004). This was consistent with PKC-dependent promotion of cell survival via phosphorylation and inactivation of Bad-mediated cell death (Tan et al., 1999). Thus, PKCα is known to phosphorylate Bcl-2 in a site that increases its anti-apoptotic function (Ruvolo et al., 1998) and overexpression of PKCε results in increased expression of Bcl-2 (Gubina et al., 1998). Moreover, suppression of PKCα triggers apoptosis through down-regulation of Bcl-xL (Hsieh et al., 2003). The role for PKC activation in the neuroprotective mechanism of rasagiline and propargylamine, is supported by the results that both compounds can activate p-PKC levels and up-regulate essential PKC isoforms involved in cell survival pathways, PKCα and PKCε, in mice hippocampus (Bar-Am et al., 2004) and in starved PC12 cells (Weinreb et al., 2004). Rasagiline also down-regulated detrimental PKCγ in serum-deprived PC12 cells (Weinreb et al., 2004). The inhibition of PKC activity has blocked the neuroprotective action of rasagiline and its propargyl moiety compound in serum-deprived PC12 cells. The specific broad spectrum PKC inhibitor, GF109203X, which exhibits high affinity for the conventional PKCs (α, β, γ), as well as the novel isoenzyme PKCε (Gekeler et al., 1996; Ku et al., 1997), markedly reversed rasagiline-suppressive effects on the protein expression of the pro-apoptotic regulator/cell death machinery, Bad, and on the cleavage and activation of pro-caspase-3 and poly (ADP-ribose) polymerase (PARP), in serum withdrawal-induced programmed cell death (Weinreb et al., 2004). Similarly, GF109203X, and the ERK1/ERK2 inhibitor, PD98059, prevented rasagiline-activation/phosphorylation of p42 and p44 mitogen-activated protein kinase (MAPK), thus indicating that rasagiline directly activates PKC-MAPK pathway (Yogev-Falach et al., 2003). The importance of PKC pathway in rasagiline-neuroprotective activity is supported also by previous data demonstrating that rasagiline (Yogev-Falach et al., 2003) induced the release of the neuroprotective-neurotrophic non-amyloidogenic soluble APP (sAPPα) by MAPK-and PKC-dependent mechanisms in vitro (Yogev-Falach et al., 2002).

Rasagiline has been shown to cause up-regulation of antioxidative proteins, such as superoxide dismutase (SOD) and glutathione (Youdim, 2003; Youdim and Weinstock, 2002). Nonetheless it is unlikely that the neuroprotective effect of rasagiline is related to MAO-B inhibition, because PC12 cells contain only MAO type A (Youdim, 1991; Youdim et al., 2001). Moreover, the S-isomer of rasagiline, TVP1022, which is not an inhibitor of MAO-A or -B also protected serum-deprived PC12 cells from cell death, suggesting that the mode of action is independent of MAO inhibition. These results are consisting with previous reports providing clear evidence that the neuroprotection by rasagiline and its

derivatives does not depend on inhibition of MAO-B (Youdim et al., 2003), but rather is associated with some intrinsic pharmacological action of the propargyl moiety in these compounds acting on the mitochondria cell survival proteins.

Additional neuroprotective mechanism of rasagiline involves the up-regulation of the expression levels of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), as demonstrated in neuronal cells (Maruyama et al., 2004; Weinreb et al., 2004). Indeed, both neurotrophic factors have been shown to induce neuroprotective and neurorescue activity in dopaminergic and cholinergic neurons, and promote survival of major neuronal types affected in Alzheimer Disease (AD) and PD (Murer et al., 2001; Wang et al., 2002). Recently, rasagiline was proved to increase the protein and mRNA levels of GDNF in neuroblastoma cells through activation of nuclear factor-kappaB (NF-kappaB) transcription factor (Maruyama et al., 2004). These results indicate that the induction of neurotrophic factors by rasagiline might suppress apoptosis in neurodegenerative diseases and may support a possible disease-modifying activity of rasagiline (Blandini, 2005).

Neurorescue activity of rasagiline and propargylamine

The widespread hypothesis suggests that apoptosis is the predominant mode of neuronal death in neurodegenerative disorders, such as AD and PD. Since diagnosis of neurodegenerative diseases is based on the appearance of clinical characteristics, patients have already suffered massive neuronal degeneration. Only 10% to 30% of normal neuronal population is surviving in the clinical stage of the disease. Therefore, there is a significant effort to develop drugs with neurorescue activity for therapy in the clinically diagnostic patients.

Consistent with the finding of the neuroprotective mechanism of rasagiline and propargylamine, recent study (Bar-Am et al., 2005) demonstrated that the neurorescue activity of these two drugs are also mediated by the Bcl-2 family proteins and the neurotrophic factors GDNF and BDNF. In this study, using human SH-SY5Y neuroblastoma cells, a prolonged serum deprivation-neurorescue model was established, in which the cells were exposed to serum free media 3 days prior to the administration of rasagiline and propargylamine. In these extreme serum deprived-conditions, the severe loss of viability was due to apoptotic cell death, detected by a significant up-regulation of apoptotic-associated parameters, including cleaved caspase-3 and PARP and the early apoptosis-associated phosphorylated protein, H2A.X. The neurorescue effects of rasagiline and propargylamine (Bar-Am et al., 2005), was characterized by the reduction in the cleavage of caspase-3 and PARP, as well as in phosphorylated histone, H2A.X levels. The pro-survival effects of both drugs were mediated by the Bcl-2 family proteins, thus increasing both Bcl-2/Bax ratio of mRNA and protein levels and reducing the ''BH3-only'' proteins, Bad and Bim.

As another mechanism of neurorescue, propargylamine was found to up-regulate gene expression levels of GDNF and BDNF following long-term serum deprivation of SH-SY5Y cells (Bar-Am et al., 2005). These results indicate that the induction of neurotrophic factors by propargyl-related derivatives and propargylamine and might suppress apoptosis in neurodegenerative diseases. Indeed, controlled clinical studies with rasagiline suggested the possible disease modifying action of rasagiline in PD patients (Blandini, 2005). In recent study, chronic low doses of rasagiline administered to mice subsequently to MPTP, rescued dopaminergic neurons in the substantia nigra pars compacta (Sagi et al., 2005). Employing proteomic and genomic screening tools combined with a biology-based clustering method, we demonstrated that rasagiline induced a number of cell signaling

mediators associated with the tyrosine kinase receptor (Trk) pathway, including ShcC, SOS, AF6, Rin1, and Ras, in parallel with a specific increase in the Trk-downstream effecter phosphatidylinositol 3 kinase (PI3K) proteins. Confirmatory immunohistochemical analysis indicated that this effect was associated with activation of the substrate of PI3K, Akt and phosphorylation/inactivation of glycogen synthase kinase-3β and Raf1. These results demonstrated the essentiality of the activation of Ras-PI3K-Akt survival pathway in rasagiline-mediated neurorescue effect.

Regulation of holo-APP levels and sAPPα release by rasagiline and propargylamine

In AD, increased expression and/or altered processing of APP causing an increase in generation of β-amyloid peptides, may play a central role in the amyloidogenesis process (Bush, 2003), leading to the formation of the senile plaque (Mills and Reiner, 1999; Turner et al., 2003). A common mechanism leading to up-regulation of holo-APP expression and Aβ formation is the shortage of energy

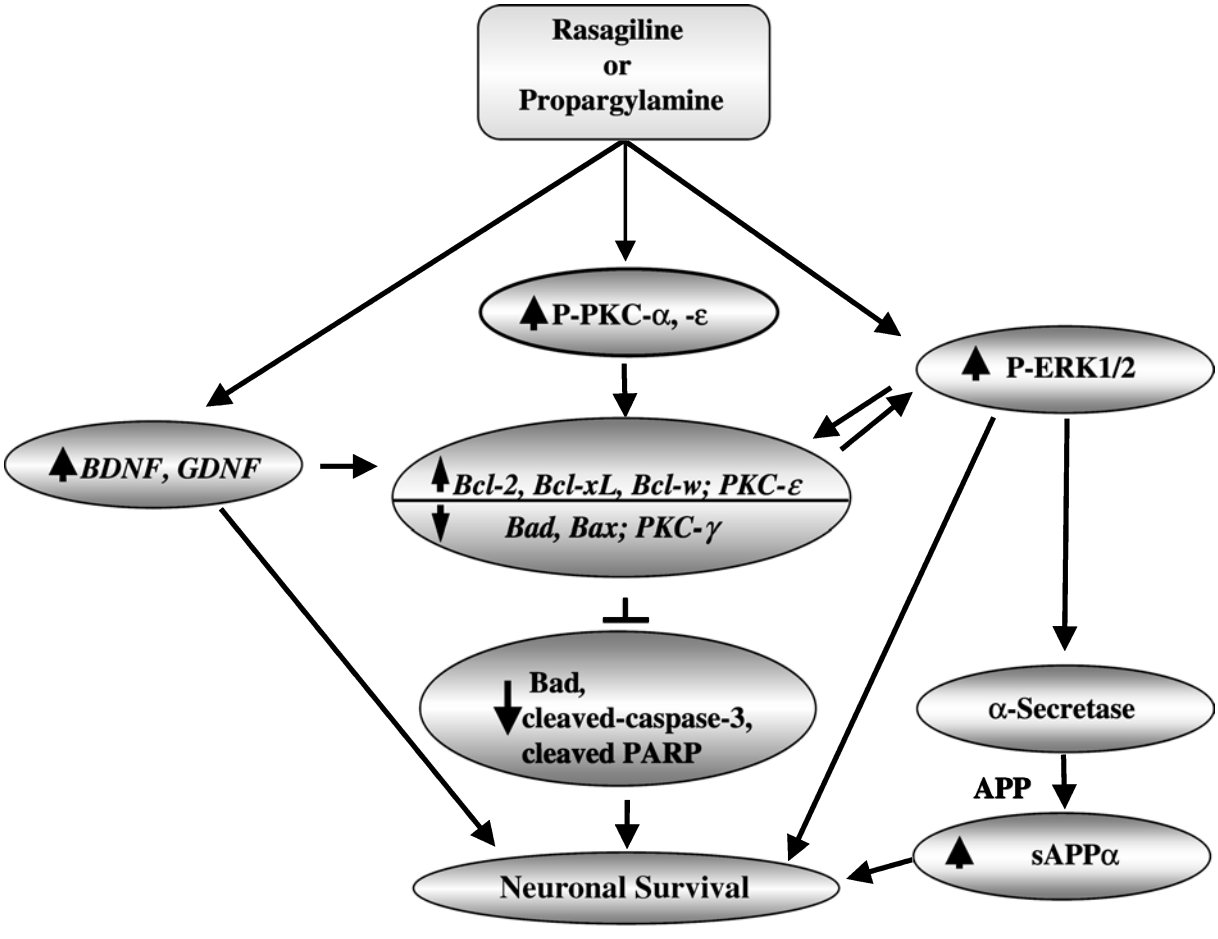

Fig. 2. Schematic overview demonstrating protein and gene targets involved in the neuroprotective activity of the anti-Parkinson drug rasagiline and propargylamine, with respect to their proposed modulating effect on signal transduction pathway, neurotrophic factors, and APP regulation in vitro and in vivo

supply, related oxidative stresses and apoptosis. Indeed, rasagiline significantly protected rat PC12 cells against amyloid-beta peptide (Aβ1-42)-toxicity (Yogev-Falach et al., 2002). In addition, rasagiline and propargylamine both significantly down-regulated holo-APP protein levels in the neurorescue model, in human neuroblastoma SH-SY5Y cells (Bar-Am et al., 2005). Thus, this observation could be of a value in reducing Aβ formation. In agreement, recent in vivo studies demonstrated that rasagiline and its anti-Alzheimer cholinesterase-brain selective MAO inhibitor derivative, ladostigil (Youdim et al., 2005) reduced the levels of cell-associated holo-APP in mice hippocampus (Bar-Am et al., 2004). Structure-activity has clearly shown that α-secretase dependent processing of APP is associated with the propargyl moiety of rasagiline, since propargylamine itself has similar action, but not aminoindan, the metabolite of rasagiline (Bar-Am et al., 2004). The regulatory mechanism of these drugs on holo-APP expression is presumably post-transcriptional, since they suppress holo-APP protein levels without altering mRNA expression. Additionally, both rasagiline and propargylamine increased sAPPα levels in the medium of serum-deprived SH-SY5Y cells, via activation of PKC and MAPK pathway (Yogev-Falach et al., 2002, 2003).

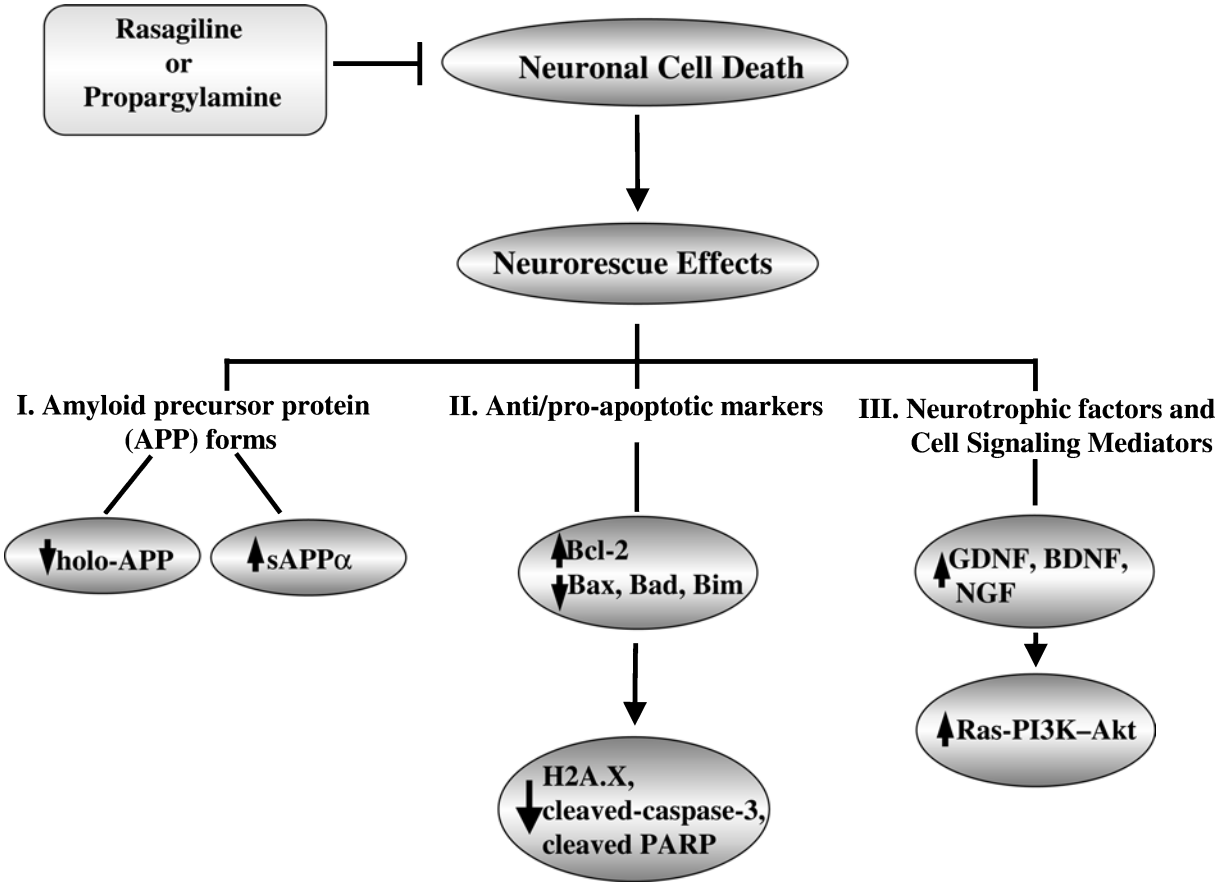

Fig. 3. Proposed schematic model for the neurorescue effects of rasagiline and propargylamine involving regulation of the expression levels of anti/pro-apoptotic proteins and genes, neurotrophic factors and APP processing

Conclusions

Multiple signaling pathways have been identified (Figs. 2 and 3) that may be involved in the neuroprotective/neurorescue mechanism of action of rasagiline and propargylamine. The significant novel findings in these described studies is that although the propargylamine, the three carbon active moiety of rasagiline, which is an extremely poor inhibitor of MAO (IC50 $>50\,\mu M$) (Zheng et al., 2005a), it has a similar potency of neuroprotective/neurorescue and APP processing activity as the parent drug rasagiline. We have recently demonstrated (Zheng et al., 2005b) that introduction of the propargyl moiety into other drugs, such as iron chelators (e.g.VK-28) and radical scavengers, which we have developed for the treatment of neurodegenerative diseases (Youdim and Buccafusco, 2005; Zecca et al., 2004), render them multifunctional drugs targeting various central nervous system disorders. The molecular mechanism which is described in the present paper (Figs. 2 and 3) enlighten the crucial pharmacological activity of the propargyl moiety in rasagiline. Future research on structure-activity relationship of aminoindan, the second metabolite of rasagiline, will indicate its role in the mechanism of action of rasagiline and clarify the possible disease-modifying activity of the drugs, as suggested in clinical trials of early PD patients (Parkinson Study Group, 2005).

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Anti-apoptotic gene therapy in Parkinson's disease

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Summary. Apoptosis, whether caspase-dependent or caspase-independent, has been implicated as one of the important mechanisms leading to the death of dopaminergic neurons in the substantia nigra of Parkinson's disease patients. Major advances of our understanding of apoptosis have been achieved in studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in mice and monkeys and 6-hydroxydopamine (6-OHDA) toxicity in rats and monkeys. The use of viral vectors to either express anti-apoptotic proteins or to downregulate pro-apoptotic proteins has the major advantage of addressing selective molecular targets, bypassing the blood–brain–barrier to specifically target the nigrostriatal pathway by their stereotaxic application and by the choice of the appropriate virus and promotor. Used thus far have been virus-mediated overexpression of inhibitor of apoptosis proteins, inhibitors of the c-jun-N-terminal kinase (JNK) pathway, inhibitors of calpains and dominant negative inhibitors of the protease activating factor (APAF)-1 and cdk5. Most studies implicate the endogenous, mitochondrial pathway in the apoptosis of dopaminergic neurons. The results suggest that only an inhibition of this pathway upstream of caspase activation will also result in the protection of nigrostriatal dopaminergic terminals and behavioral benefit, whereas an inhibition of caspases alone may not be sufficient to prevent the degeneration of terminals, although it may promote the survival of neuronal cell bodies for some time.

Cell death mechanisms

In recent years, several cell death mechanisms have been implicated in the death of dopaminergic neurons. They include mitochondrial dysfunction, generation of free radicals, inflammation, apoptosis, excitotoxicity, necrosis and autophagy. The death of dopaminergic neurons leads to a deficit of dopamine in the striatum, producing symptoms in the patient of rigidity, akinesia and tremor. It has been hypothesized recently that axonal pathology may actually pre-date the degeneration of neurons (Berliocchi et al., 2005) especially in Alzheimer's disease (Cash et al., 2003; Stokin et al., 2005), Huntington's disease (Trushina et al., 2004; Charrin et al., 2005) and amyotrophic lateral sclerosis (Jablonka et al., 2004; Kieran et al., 2005). In addition, Parkinson's disease causing mutations in α-synuclein alter axonal transport (Jensen et al., 1998; Jensen and Gai, 2001; Saha et al., 2004) of α-synuclein. The transgenic overexpression of α-synuclein leads to accumulation in neurites (Kahle et al., 2000; Rathke-Hartlieb et al., 2001) and death of dopaminergic neurons. If loss of axons, terminals and striatal dopamine concentrations pre-date death of dopaminergic

neurons then a therapy that prevents the execution of cell death and promotes axonal recovery would not only stop the progression of the disease but also lead to an improvement of the symptoms. Indications for such a possibility of improvement come from transgenic mouse models of Huntington's disease in which in the conditional model of a tetracycline-induced interruption of the extended polyglutamine track of huntingtin leads to a reversal of pathology and symptoms (Yamamoto et al., 2000).

Protein aggregation

Genetic data suggests that failures of the ubiquitin proteasome system could lead to intracytosolic deposition of insoluble protein aggregates consisting of α-synuclein and other proteins (Krüger et al., 2002; Bossy-Wetzel et al., 2004). It is currently debated, whether the protein aggregates themselves or their precursors, protofibrils, induce neuronal dysfunction and death (Caughey and Lansbury, 2003). Gene therapeutic approaches inducing the expression of heat shock protein (HSP)70 (Dong et al., 2005), or β-synuclein (Hashimoto et al., 2004), which interact with α-synuclein and prevent its aggregation, have protected from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity and α-synuclein aggregation in transgenic animals. Interestingly, conditional expression of parkin in drosophila (Haywood and Staveley, 2004) or virus mediated overexpression of parkin in rats (Yamada et al., 2005) protected against transgene-induced α-synucleinopathies.

Because transgenic overexpression of wild-type or mutant α-synuclein leads to a synucleinopathy but – at least in mice – does not reliably induce dopaminergic death, toxin-induced animal models are widely used to study cell death mechanisms in dopaminergic neurons (Schober, 2004). Furthermore, the exact pathogenic mechanisms are still unknown for the newly identified mutations in DJ1 (Bonifati et al., 2003), Pink1 (Valente et al., 2004), and LRRK2 (Zimprich et al., 2004), and in the sporadic cases, which still account for the majority of Parkinson's disease patients. In contrast, intoxication of humans, monkeys and mice with MPTP leads to most of the pathological, biochemical and behavioral characteristics of Parkinson's disease including death of dopaminergic substantia nigra neurons, reduction of the concentration of dopamine in the striatum, an inhibition of mitochondrial function, the generation of reactive oxygen species, induction of inflammation, and a reduction of spontaneous locomotion. If indeed the downstream mechanisms and the execution of death in dopaminergic neurons are similar in the genetic and sporadic cases of Parkinson's disease, toxin-induced models of Parkinson's disease will enable the identification of important pathogenetic pathways as well as therapeutic strategies.

Apoptosis

Apoptosis was initially defined by distinct morphological and biochemical alterations. In the classical case, condensation and fragmentation of chromatin, compaction of cytoplasmic organelles, a decrease in cell volume and alterations of the plasma membrane are observed, resulting in the recognition and phagocytosis of apoptotic cells. Caspases have been identified as the major proteases to execute apoptosis. However, several non-caspase-mediated forms of apoptosis have also been reported. The following cell death forms have been identified and summarized (Leist and Jaattela, 2001):

(i) classical apoptosis;
(ii) apoptosis-like programmed cell death, in which the chromatin condensation characteristically is less compact and complete than in apoptosis (the typical feature found in non-caspase-mediated forms of programmed cell death including autophagy – characterized by the

formation of large, lysosome-derived cytosolic vacuoles – and ''dark cell death'');
(iii) necrosis-like programmed cell death, in which chromatin condensation is lacking (the initiation of this cell death may be dependent on caspase activation, however the execution is independent of caspases); and
(iv) accidental necrosis and cell lysis.

Based on morphological grounds, the results to detect apoptosis in dopaminergic substantia nigra neurons *post mortem* in brains from patients that had suffered from Parkinson's disease were controversial. Reported figures of apoptotic dopaminergic neurons range from 5% to 8% on the one hand (Mochizuki et al., 1996; Anglade et al., 1997; Tompkins et al., 1997), whereas others failed to detect apoptotic changes at all (Kosel et al., 1997; Banati et al., 1998; Wüllner et al., 1999). Given the slow and chronic nature of the disease course, the chances to detect morphological alterations of apoptosis or to find evidence for apoptotic DNA fragments will be low, if apoptosis *in vivo* is executed as quickly as apoptosis *in vitro*. However, the detection of molecular apoptotic markers in human brain tissue (Hartmann et al., 2000, 2001a) and animals (Tatton and Kish, 1997; Eberhardt et al., 2000) strongly supports the hypothesis that apoptotic mechanisms play an important role in the death of dopaminergic neurons. Interestingly, the rate of apoptotic death compared with non-apoptotic death is higher in paradigms that utilize a chronic rather than an acute paradigm of MPTP intoxication (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). A biochemical hallmark of Parkinson's disease is a reduced activity of complex I of the electron transport chain in the substantia nigra (Schulz and Beal, 1994). Among the first targets of activated caspases are mitochondria themselves, leading to disruption of electron transport, loss of mitochondrial transmembrane potential, reduction of ATP concentrations, generation of reactive oxygen species and morphological alterations of mitochondria (Ricci et al., 2003). Recently, the 75 kDa subunit of complex I was identified as a caspase substrate accessible to the intermembrane space of the mitochondria (Ricci et al., 2004). Expression of a noncleavable mutant of p75 protected against mitochondrial dysfunction in response to apoptotic stimuli and delayed cell death but left cytochrome c release from mitochondria and DNA fragmentation unaffected.

Caspases as a target for gene therapeutic intervention

In mice that are chronically intoxicated with MPTP, caspase-3 activation is observed in tyrosine hydroxylase (TH)-positive neurons of the *substantia nigra pars compacta* (SNpc; Eberhardt et al., 2000; Hartmann et al., 2000; Mochizuki et al., 2001). Until recently, no peptide inhibitors of caspases that pass the blood brain barrier were available. Therefore, intracerebroventricular application offered the only option to deliver the peptide inhibitors to the brain. This approach was successfully used in a transgenic mouse model of amyotrophic lateral sclerosis (Li et al., 2000). Recently, Beal and colleagues reported for the first time that the systemic application of a novel peptidyl broad-spectrum caspase inhibitor, Q-VD-OPH, offered protection against MPTP toxicity (Yang et al., 2004). However, systemic treatment with broad spectrum caspase inhibitors has the disadvantage that physiological functions of caspases involved in tumor and immunological defense mechanisms may be compromised as well.

We therefore developed gene based therapies to inhibit caspases. The family of inhibitor of apoptosis proteins, of which one is the X-chromosome linked inhibitor of apoptosis protein (XIAP) are endogenous inhibitors of caspase-3 and caspase-7 (Deveraux

and Reed, 1999), and likely caspase-9 as well (Robertson et al., 2000). The anti-caspase activity is mediated by baculoviral-inhibitor of apoptosis repeat domains (BIRs; Clem and Miller, 1994). Stereotaxic injection of an adenovirus into the striatum that mediated the expression of XIAP in the striatum and – after retrograde transport – in dopaminergic neurons of the SNpc also protected from MPTP-induced loss of TH-positive neurons in the SNpc (Eberhardt et al., 2000). However, in this paradigm of 5×30 mg/kg MPTP injected i.p. over a period of 5 consecutive days, XIAP expression did not prevent the degeneration of nigrostriatal terminals and the loss of dopamine and its metabolites in the striatum (Eberhardt et al., 2000). These results were confirmed in mice overexpressing XIAP (Crocker et al., 2003a). In contrast, in the rat 6-hydroxydopamine model, adenovirally mediated overexpression of the neuronal inhibitor of apoptosis protein (NAIP) (Crocker et al., 2001) protected not only from the loss of dopaminergic SNpc neurons but also from the degeneration of terminals in the striatum (Crocker et al., 2001). In the MPTP model we only achieved an improvement of synaptic function when we combined the adenovirus-mediated expression of XIAP with the expression of GDNF (Eberhardt et al., 2000). This combined gene therapeutic approach provided synergistic effects and prevented the degeneration of TH-positive neurons in the SNpc and their terminals in the striatum. Our data also implicate that the terminals degenerate independently of caspase activity. This hypothesis is supported by preliminary data showing that, on the C57BL/6 background, caspase-3-deficient mice (Houde et al., 2004) are protected from MPTP-induced loss of TH-positive neurons in the SNpc but not against the reduction of dopamine concentrations in the striatum (Rathke-Hartlieb and Schulz, unpublished). More generally, Nicotera and colleagues (Berliocchi et al., 2005) demonstrated a temporal and mechanistic dissociation of cell death mechanisms underlying the degeneration of neurites and neuronal bodies. Neurites degenerate at an early stage by an active caspase-independent fragmentation characterized by segregation of energy competent mitochondria. Later, the cell body mitochondria release cytochrome c, which is followed by caspase activation, morphological changes and cell demise (Berliocchi et al., 2005).

Therapeutic interference with the initiation of apoptosis

The activation of caspases may occur through the external, death receptor-mediated pathway or the internal, mitochondria-mediated pathway (Fig. 1). In the external pathway, the activation of Fas/CD95 or other members of the tumor necrosis factor (TNF) superfamily leads to a direct activation of caspase-8 via interaction with death domains. Activated caspase-8 may activate executioner caspases directly, e.g. caspase-3, or may cleave Bid, a pro-apoptotic member of the Bcl-2 family, which then promotes cytochrome c release (Hengartner, 2000), providing a cross link between the mitochondrial and the death receptor pathway of caspase activation. MPTP application does not only lead to the activation of the effector caspase-3 but also the initiator caspases (caspase-8 and caspase-9) and Bid cleavage, and to mitochondrial cytochrome c release (Hartmann et al., 2001b; Viswanath et al., 2001). These changes were attenuated in transgenic mice neuronally expressing the general caspase inhibitor protein baculoviral p35 (Viswanath et al., 2001). Caspase-9 inhibition prevented the activation of both caspase-3 and caspase-8 and also inhibited Bid cleavage, but not cytochrome c release. These data would be compatible with a model of cytochrome c-induced caspase-9 activation leading to caspase-3 activation that mediates the effector phase of apoptosis, and with an amplification loop involving caspase-8.

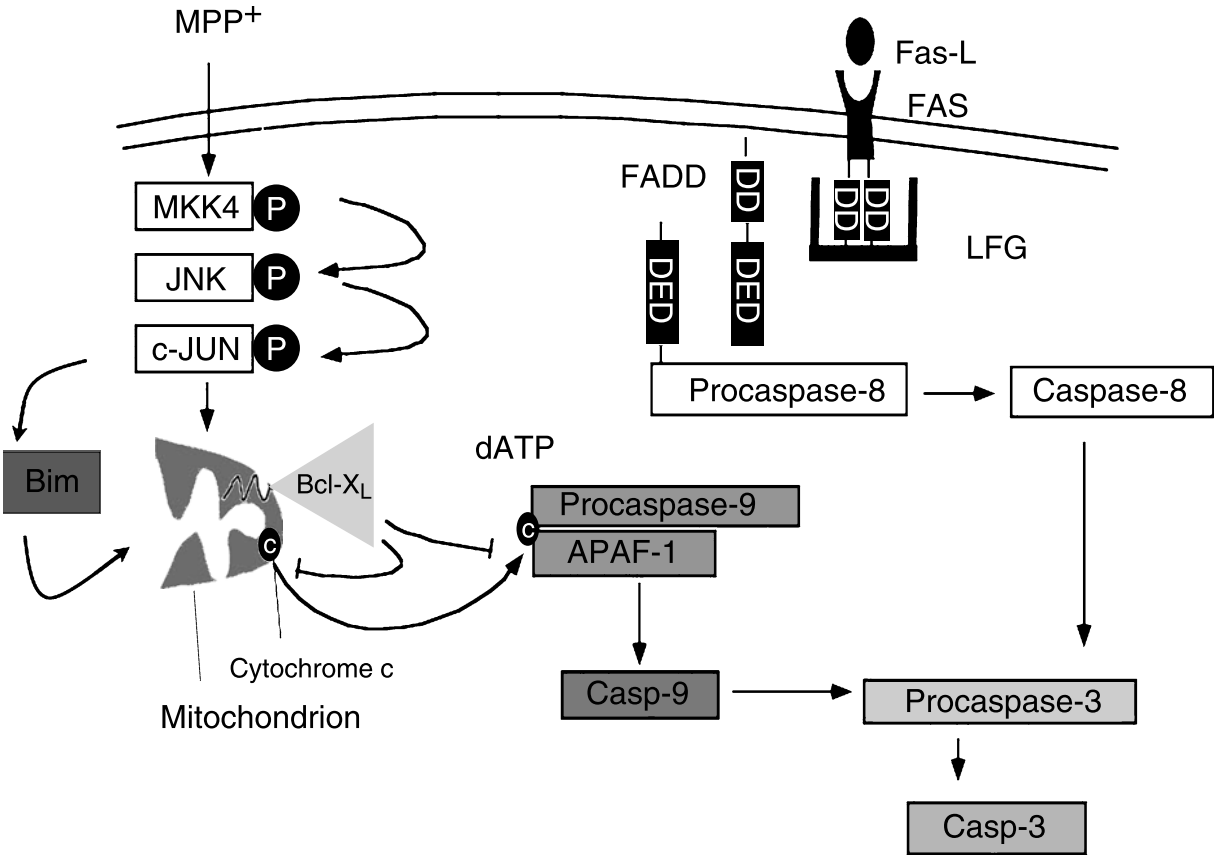

Fig. 1. Pathways of MPTP/MPP+-mediated caspase-dependent apoptosis. Apoptosis may be induced by the endogenous, mitochondria-mediated pathway (left side) or by the exogenous, cell death receptor mediated pathway (right side). In healthy neurons, the sensitivity to FasL/CD95L is blocked by the expression of LFG. The JNK pathway appears to play a crucial role for the activation of the mitochondrial pathway. The release of cytochrome c and other factors from mitochondria is probably induced by the transcriptional regulation of pro-apoptotic factors, e.g. the BH3 only, proapoptotic members of the Bcl-2 family which include Bim

Death receptor, exogenous pathway of apoptosis

In accordance with the results and hypotheses mentioned and in contrast to results obtained in lymphocytes and glial tumor cell lines, exogenous application of FasL/CD95L did not induce death in dopaminergic SH-SY5Y cells (Gomez et al., 2001) or primary mesencephalic cultures (von Coelln and Schulz, unpublished). It was therefore unexpected that Hayley and colleagues found protection from MPTP-induced loss of dopaminergic neurons in Fas/CD95 deficient mice (Hayley et al., 2004). However they did not find protection against the loss of terminals. In addition they observed increased expression of Fas in the *substantia nigra* after MPTP application. Because c-jun is a transcription factor for Fas/CD95, the authors investigated the effects of an adenovirus mediating the expression of dominant-negative c-jun. Expression of dominant-negative c-jun in the nigrostriatal system protected from the induction of Fas/CD95 and loss of dopaminergic SNpc neurons. Interestingly, Fas-deficient mice displayed a pre-existing reduction in striatal dopamine levels and locomotor behavior when compared with wild-type mice. This finding is in line with a recent demonstration

of mice with absent Fas/CD95 (*lpr* mice) or FasL/CD95L (*gld* mice) function that exhibited a reduced number of dendritic branches *in vivo* at the time when synapse formation took place (Zuliani et al., 2006) without affecting the number of neurons. The branching increase occurred in a caspase-independent and death domain-dependent manner. Furthermore, lentivirus-mediated expression of CD95L/FasL *in vitro* and *in vivo* did not induce apoptosis. We recently confirmed that neurons express Fas but are resistant to FasL/CD95L *in vitro* (Beier et al., 2005). This resistance was mediated by the lifeguard (LFG)/neuronal membrane protein (NMP)35 that co-localizes and physically interacts with Fas/CD95, thereby preventing the induction of the exogenous, Fas/CD95 mediated apoptosis pathway (Somia et al., 1999; Beier et al., 2005). Downregulation of LFG expression by *antisense* oligonucleotides or small interfering RNA led to increased sensitivity of neurons to FasL/CD95L-induced apoptosis and re-installed the caspase-8 dependent cell death pathway. Because the expression of LFG is transcriptionally regulated by the phosphatidylinositol 3-kinase (PI 3-kinase)-Akt/protein kinase B (PKB) pathway (Beier et al., 2005), neuronal sensitivity to FasL/CD95L may occur under pathological conditions in which the PI3-kinase and/or Akt activity is inhibited. This may explain the resistance of *gld* and *lpr* mice against cerebral ischemia and spinal trauma and why neutralizing antibodies against FasL/CD95L CD95L provide protection in these paradigms (Martin-Villalba et al., 1999, 2001; Demjen et al., 2004).

An inhibition of the PI3-kinase/Akt pathway under pathological conditions may also be the reason for the observed resistance of Fas/CD95-deficient mice to MPTP toxicity. To date, however, this possibility has not been tested. Alternative explanations arise from the specific requirements of the MPTP model. To become toxic, MPTP requires the conversion to its active, toxic metabolite, MPP^+ through non-neuronal monoamine oxidase B activity, and subsequently the uptake of MPP^+ into dopaminergic neurons by the dopamine transporter. Although Hayley and colleagues (Hayley et al., 2004) ruled out that in Fas/CD95 deficient mice the striatal concentration of MPP^+ was lower than in wild-type controls, they did not study the density of dopamine uptake sites. Because they themselves reported a reduced density of nigrostriatal fibers, a reduction in the density of the dopamine transporter appears to be a likely explanation for the resistance against MPTP in Fas/CD95-deficient mice.

Mitochondrial, endogenous pathway of apoptosis

Together with procaspase-9 the apoptotic protease activating factor (APAF)-1 forms the apoptosome that is activated and leads to the release of active caspase-9 after binding of cytochrome c, which is translocated from mitochondria to the cytosol after an apoptotic stimulus. Using an adeno-associated virus, Mochizuki and colleagues (Mochizuki et al., 2001) delivered an APAF-1 dominant negative inhibitor to the striatum of C57Bl/6 mice and demonstrated protection against the loss of dopaminergic SNpc neurons induced by subsequent MPTP toxicity. In contrast, virus mediated expression of a dominant negative inhibitor of caspase-1 was not protective. These results emphasize the importance of the endogenous, mitochondrial death pathway in MPTP toxicity. In addition, transgenic mice overexpressing Bcl-2 are protected from MPTP toxicity (Offen et al., 1998; Yang et al., 1998) as well as mice with deficient for Bax (Vila et al., 2001).

Persistent activation of the c-jun-N-terminal kinase (JNK) pathway has been demonstrated in various apoptotic cell death paradigms (Davis, 2000) and is considered to be one of the major pathways to initiate

the release of pro-apoptotic factors from the mitochondria. The exact mechanisms how JNK initiates this pathway are still hypothetical and may be diverse depending on the paradigm. This may involve the transcriptional induction of pro-apoptotic Bcl-2 family members, e.g. Bid or Bim. JNK-dependent expression of Bim and its importance for the induction of neuronal apoptosis has been shown in primary cerebellar granule neurons (Harris et al., 2002).

The phosphorylation and thereby activation of JNK is a multi-step process that involves the activation of several upstream kinases of the mixed lineage kinase (MLK) family and the formation of protein complexes to ensure signaling specificity. Scaffold proteins have been identified that facilitate the formation of these complexes required for the activation of JNK. One important member is the JNK interacting protein (JIP)-1. We reasoned that the forced expression of the JNK binding domain of JIP-1 would prevent the phosphorylation of JNK. Adenovirus-mediated forced expression of JIP-1 in the nigrostriatal pathway blocked the MPP^+/MPTP-induced activation of JNK and c-jun, the activation of caspase-9 and caspase-3 in SH-SY5Y cells *in vitro* as well as in the SNpc *in vivo* (Xia et al., 2001). Dopaminergic neurons and their striatal terminals were protected from MPTP-induced death and the mice showed behavioral improvement in amphetamine-induced rotational behavior. These data suggest that interference with the apoptotic cascade upstream of mitochondria may result in better functional protection than just inhibiting the executioner part of apoptosis, e.g. by caspase inhibition. Because MPTP leads to the induction of Bim expression, this JNK-mediated pathway is likely to be functional in MPTP toxicity but may not be the only one. In addition, JNK has been shown to upregulate the expression of COX2 (Hunot et al., 2004), whose neuronal expression is instrumental for MPTP toxicity (Teismann et al., 2003). The functional significance of the JNK pathway for MPTP toxicity is further supported by the effects of the mixed lineage kinase (MLK)-3 inhibitor, CEP-1347 (Saporito et al., 1999, 2000).

Other anti-apoptotic gene therapeutic strategies

In an attempt to inhibit non-caspase mediated apoptosis, Park and colleagues induced forced expression of calpastatin (an inhibitor of calpains) and dominant-negative cdk5 by adenoviral infection of the nigrostriatal pathway. Both strategies provided protection against MPTP toxicity and point to an additional, alternative cell death pathway (Crocker et al., 2003a, b). Further, infection with adeno-associated viruses to express HSP70 resulted in protection from MPTP-induced apoptosis, suggesting that this therapy with chaperons may have direct anti-apoptotic properties as well as affecting the pathological aggregation of proteins (Dong et al., 2005).

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The discovery of the pressor effect of DOPS and its blunting by decarboxylase inhibitors

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Summary. In the 1950s it was found that an artificial aminoacid, 3,4-threo-dihydroxyphenylserine (DOPS), was converted to norepinephrine (NE) in a single step by the enzyme L-aromatic amino acid decarboxylase (AADC), bypassing the need for the rate limiting enzyme dopamine beta hydroxylase. Trying to replicate the success of dihydroxyphenylalanine (DOPA) in the treatment of Parkinson disease, treatment with DOPS was attempted in patients with autonomic failure who have impaired NE release. DOPS improved orthostatic hypotension in patients with familial amyloid polyneuropathy, congenital deficiency of dopamine beta hydroxylase, pure autonomic failure and multiple system atrophy. DOPS pressor effect is due to its conversion to NE outside the central nervous system because concomitant administration of carbidopa, an inhibitor of AADC that does not cross the blood–brain barrier, blunted both the increase in plasma NE and the pressor response. DOPS pressor response is not dependent on intact sympathetic terminals because its conversion to NE also occurs in non-neuronal tissues.

Introduction

Melvin Yahr played a pioneering role in the use of levodopa to restore dopamine neurotransmission in patients with Parkinson disease (PD). Trying to replicate that remarkable therapeutic success, he was also involved in a less well-known, still unfolding story, using a similar pharmacological paradigm to restore noradrenergic neurotransmission in patients with autonomic failure.

Orthostatic hypotension, the most disabling feature of autonomic failure, is due to deficient release of norepinephrine (NE) by postganglionic sympathetic neurons. As shown in Fig. 1, this defect in NE release can be due to degeneration of peripheral postganglionic sympathetic neurons, as it occurs in the Lewy body disorders (pure autonomic failure, PD and dementia with Lewy bodies), and in some peripheral neuropathies (e.g. amyloid and diabetic polyneuropathy); or may be caused by lesions in central autonomic neurons, as it occurs in patients with multiple system atrophy (MSA) (Kaufmann and Biaggioni, 2003). Rarely, the defect in NE release is caused by congenital deficiency of the enzyme dopamine beta hydroxylase (Kim et al., 2002). Finally, in autoimmune autonomic neuropathy, autoantibodies against ganglionic nicotinic cholinergic receptors prevent neurotransmission at the autonomic ganglia and block NE release (Vernino et al., 1998).

Normally, NE is synthesized from levodopa (Fig. 2), which is first decarboxylated to dopamine by L-aromatic amino acid decarboxylase (AADC) and then hydroxylated to norepinephrine by dopamine beta hydroxylase (DBH). The rate-limiting enzyme for

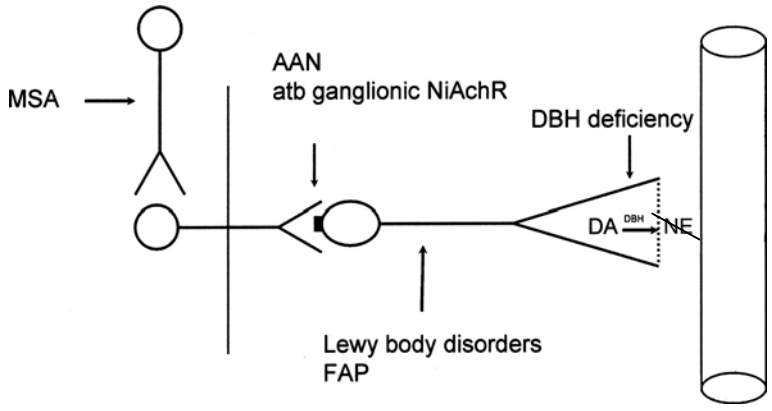

Fig. 1. Site of involvement in the sympathetic efferent pathway in different types of autonomic failure. *AAN* autoimmune autonomic neuropathy, *MSA* multiple system atrophy, *FAP* familial amyloid polyneuropathy, *DA* dopamine, *DBH* dopamine beta hydroxylase, *NE* norephinephrine, *atb* antibody, *NiAChR* nicotinic cholinergic receptor

this reaction is DBH. Thus, oral administration of levodopa leads to an increase in dopamine but no significant increase in norepinephrine. However, a synthetic amino acid identical to levodopa but with an added beta hydroxyl group (Fig. 2), dyhidroxyphenylserine, DOPS, was found, in the 1950s, by Blaschko et al. (1950) and Schmiterlow (1951) to be converted, in vivo, to norepinephrine after a single decarboxylation step by AADC, bypassing the need for DBH.

DOPS has four steroisomers (Bartholini et al., 1975). Early studies used a racemic mixture that contained both the D and L isoforms of DOPS but only the L-isoform is converted to biologically active L-norepinephrine. Furthermore, the D-stereoisomer of DOPS might competitively inhibit the decarboxylation of the L stereoisomer to L-norepinephrine. (Inagaki et al., 1976) Thus, the pure L isoform of DOPS is the preferred formulation for treatment.

Administration of DOPS could increase the synthesis of NE in sympathetic neurons and in other tissues as well (Fig. 3). Using the neutral aminoacid transporter, DOPS could be taken up by sympathetic neurons, converted to norepinephrine, stored and released during sympathetic activation, thus acting as a neurotransmitter. DOPS may also be con-

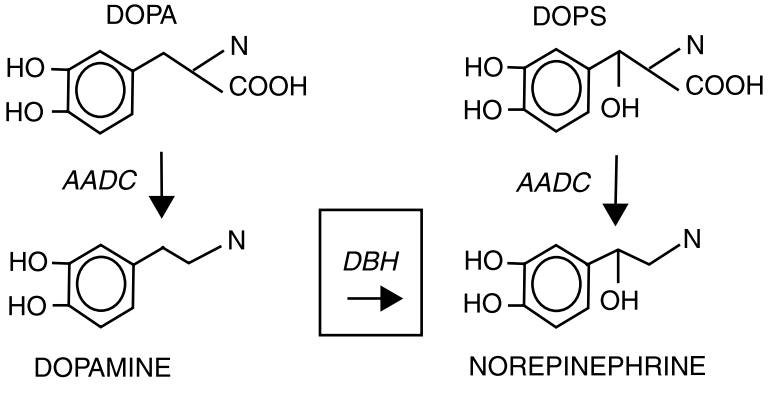

Fig. 2. The pathway for the physiological synthesis of norepinephrine from DOPA and non-physiological synthesis of norepinephrine from DOPS. *DOPS* 3,4,-L-threo-dihydroxyphenyserine, *DOPA* Dihydroxyphenylalanine, *AADC* L-aromatic aminoacid decarboxylase, *DBH* dopamine beta hydroxylase

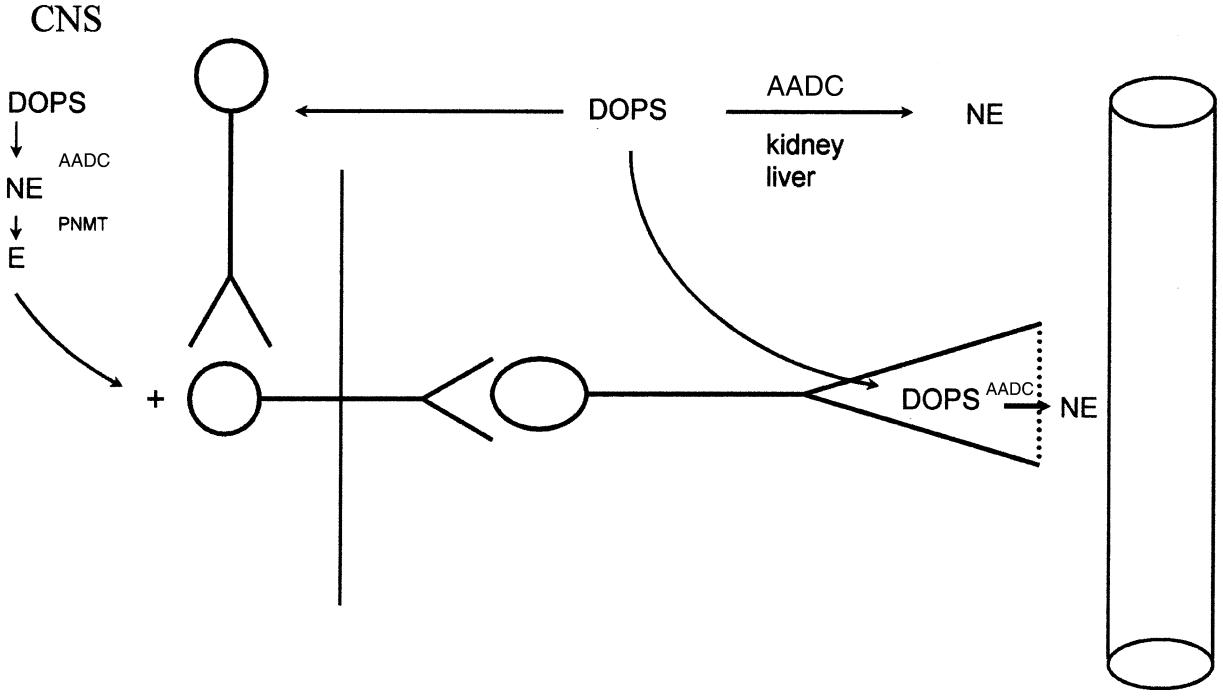

Fig. 3. Sites of decarboxylation of DOPS to NE. Central, pre and postganglionic sympathetic neurons and a blood vessel are depicted. *CNS* central nervous system, *NE* norepinephrine, *DOPS* 3,4-threo-dihydroxy-phenylserine, *E* ephinephrine, *AADC* L-aromatic aminoacid decarboxylase, *PNMT* phenylethanolamine N-methyltransferase

verted to NE outside sympathetic neurons, because AADC is widely expressed in the cytoplasm of different tissues including kidney, gut and liver, and these cells also express the neutral aminoacid transporter in their surface. NE synthesized from DOPS in non-neural tissues is rapidly released into the bloodstream where it acts as a circulating vasoconstrictor hormone. Finally, DOPS crosses the blood–brain barrier (Kato et al., 1987) and it has been suggested that its pressor effect could be due to central activation of sympathetic outflow (Kachi et al., 1988). This, however, proved not to be the case (see below).

Clinical uses of DOPS

DBH deficiency

DOPS proved extremely useful in patients with DBH deficiency. These patients have severe orthostatic hypotension, their serum dopamine levels are high and they have undetectable plasma NE. In 1987, Biaggioni and Roberstson (1987) and Man in't Veld (Man in't Veld et al., 1987) showed that administration of DOPS had a dramatic normalizing effect on the blood pressure of these patients. DOPS was directly converted to norepinephrine, bypassing the need for dopamine beta hydroxylase. They also showed that DOPS was taken up by postganglionic neurons where it was converted to NE stored and released when appropriate. After treatment with DOPS, NE increased upon standing, and infusion of tyramine produced release of NE, whereas before treatment, tyramine had induced release of dopamine.

Other types of autonomic failure

In 1980, Suzuki et al. reported that administration of DOPS had a pronounced pressor effect and induced a marked increase in urinary excretion of norepinephrine in patients with familial amyloid polyneuropathy, a dis-

order with orthostatic hypotension due to destruction of sympathetic fibers (Suzuki et al., 1980). In 1981, Araki and collaborators (Araki et al., 1981) reported that in rats oral administration of DOPS increased blood pressure but the pressor effect was much more pronounced in rats made hypotensive by chemical sympathectomy with the neurotoxin 6-hydroxydopamine. Interestingly, L-threo-DOPS produced the same increase in plasma NE concentrations in sympathectomized rats as in the controls, indicating that norepinephrine was synthesized from DOPS in places other than sympathetic nerves. The pressor effect was markedly reduced by inhibition of peripheral decarboxylase and by blockade of alpha-adrenoceptors.

In 1983, Birkmayer et al. (1983) reported successful use of DOPS in a small study of parkinsonian patients with orthostatic hypotension. In the last decade, with few exceptions (Hoeldtke et al., 1984), several small studies have reported success using DOPS in the treatment of orthostatic hypotension in patients with different types of autonomic failure (Kaufmann et al., 1991; Freeman et al., 1999) including a recent report of its use in a patient with autoimmune autonomic neuropathy (Gibbons et al., 2005). Recent work has clarified the mechanism of the pressor effect of DOPS in patients with autonomic failure (Kaufmann et al., 2003) and confirmed the earlier findings in rats (Araki et al., 1981). Better understanding of its mechanism of action and pharmacokinetics should allow proper clinical use of this compound in patients with autonomic failure.

Mechanism of action of DOPS

Central vs. peripheral

Although DOPS crosses the blood–brain barrier (Karai et al., 1987; Kachi et al., 1988) its pressor effect is only due to its conversion to norepinephrine outside the brain. Concomitant administration of DOPS with carbidopa, an inhibitor of AADC that does not cross the blood–brain barrier and thus inhibits NE synthesis only outside the CNS, blocked both the pressor effect and the increase in plasma norepinephrine (Fig. 4). This is important because many patients with orthostatic hypotension have parkinsonism and are treated with a combination of DOPA and a decarboxylase inhibitor, which will prevent norepinephrine formation from DOPS outside the brain, blunting its pressor effect.

Neurotransmitter vs. circulating hormone

Rather than being taken up by postganglionic sympathetic neurons, converted to NE intraneuronally, as it occurs in patients with DBH deficiency, and released when sympathetic fibers discharge during orthostasis, in degenerative autonomic disorders, DOPS appears to exert its pressor effect mostly by conversion to NE outside sympathetic neurons (in the kidney, gut and liver), acting as a circulating vasoconstrictor hormone. This is suggested by the finding that administration of DOPS increased blood pressure and venous plasma NE levels both in the supine and upright position and that the magnitude of the increase did not change during orthostasis (Fig. 4).

This peripheral extraneuronal mechanism of action of DOPS renders it very effective in patients with PAF, a disorder with widespread loss of sympathetic terminals (Goldstein et al., 1997; Hague et al., 1997) and markedly exaggerated pressor responses to circulating norepinephrine (Polinsky et al., 1981). Indeed, Kaufmann et al. (2003) found that the pressor response to DOPS in patients with PAF was more pronounced than in patients with MSA, despite a lower dose of DOPS being taken by PAF patients. Thus, non-neural tissue most likely stomach, liver or kidney, where AADC is extensively expressed, appears to be the major site of NE generation from DOPS. There was a clear relationship between the pressor response and venous plasma norepinephrine levels.

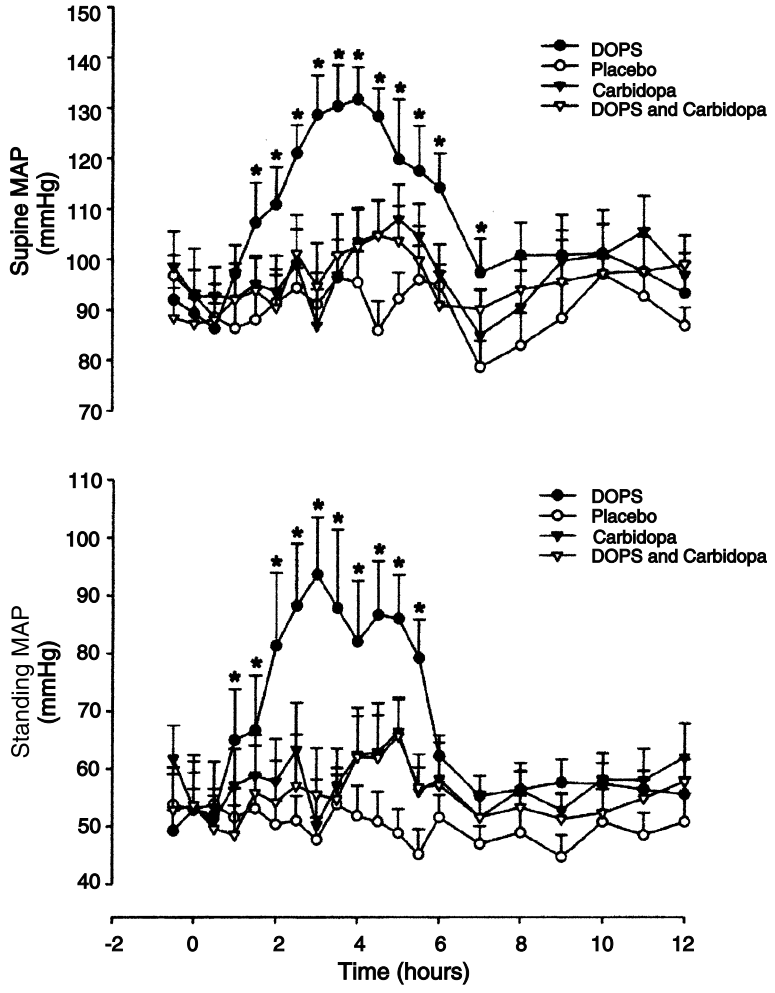

Fig. 4. The effect of 3,4-threo-dihydroxyphenylserine (DOPS), carbidopa, DOPS + carbidopa and placebo on mean blood pressure (MBP) when supine and after 3 minutes standing. Data = mean ± SE. *denotes $p < 0.05$ (vs. placebo). From Kaufmann et al. (2003)

In patients with autonomic failure, the peak blood pressure effect occurred around 4 hours after DOPS administration. Blood pressure remained higher than placebo for another 3 hours, for a total of 7 hours. Plasma NE levels increased progressively. As shown in Fig. 5, the threshold NE venous plasma level required to exert a pressor effect was 700 pg/mL.

Pharmacokinetics

The peak DOPS plasma levels occurred around 3 hours after oral administration of DOPS (Fig. 6), a similar time frame and levels to those reported for DOPA (Nutt and Woodward, 1986). NE peaked at 6 hours after DOPS ingestion.

Patients with PAF had significantly lower plasma NE and DHPG levels than did the patients with MSA, both at baseline and after DOPS administration. The volume of distribution was smaller in PAF than in MSA, possibly reflecting neuronal uptake in MSA, and lack of it in PAF.

The peak levels of NE were only about one-thousandth the peak level of DOPS. This might be interpreted as indicating low

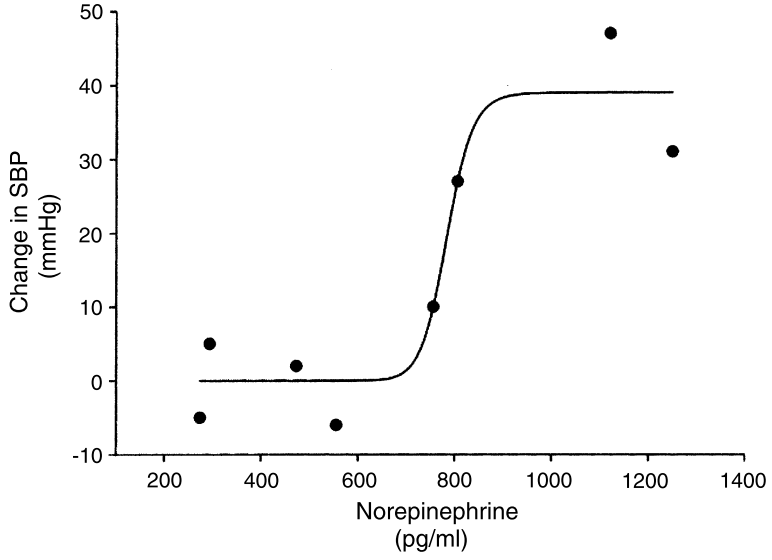

Fig. 5. Plasma concentration of norepinephrine (NE) vs. changes in systolic blood pressure (SBP). Data = mean ± SE. Solid curve represents the best fit ($r^2 = 0.903$). From Kaufmann et al. (2003)

efficiency of conversion of DOPS to NE; however, the difference is mostly explained by much longer half-time of DOPS in the plasma – about 140 minutes – which exceeded by about a hundred-fold the known half-time of NE in the plasma, which is 1.5 minutes or less (Goldstein et al., 1983). Therefore, even if DOPS were converted completely and instantaneously to NE, from the large difference in half times of clearance from the plasma, the peak DOPS level in venous plasma would exceed the peak NE level several fold (Goldstein et al., 2004).

Intracellular storage, neuronal vs. extraneuronal

The slow decline in plasma NE from the peak level probably reflects ongoing production of NE from DOPS and ongoing entry of the produced NE into the bloodstream from a cellular storage site. Cells that express the

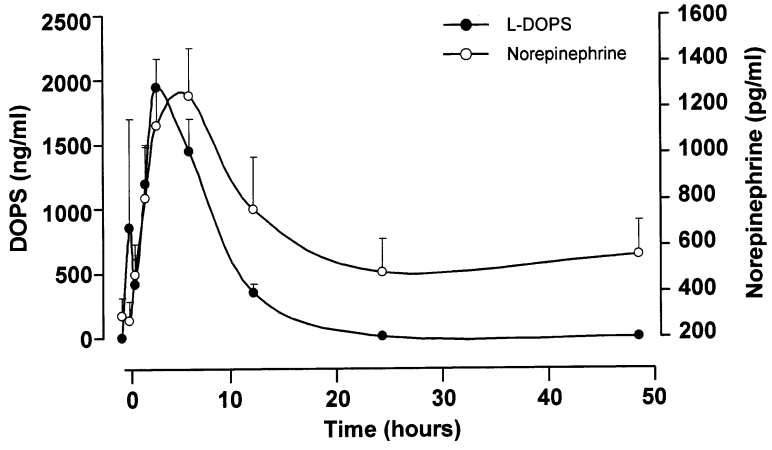

Fig. 6. Levels of DOPS and norepinephrine after DOPS administration. Data = mean ± SE. DOPS = 3,4,-L-threo-dihydroxyphenyserine

neutral amino acid transporter and also contain AAAD, including sympathetic nerves and parenchymal cells in the liver and kidneys, would be expected to take up DOPS and convert it to NE. In patients with PAF, storage of DOPS and NE is likely to occur in non-neuronal tissue as sympathetic terminals are severely affected but in MSA patients both sympathetic nerves and extraneuronal production of NE from DOPS is likely.

In conclusion

DOPS increases blood pressure and alleviates the symptoms of orthostatic hypotension in patients with multiple system atrophy and pure autonomic failure. After oral administration, DOPS is converted to NE even in patients with severe destruction of peripheral sympathetic nerves, through decarboxylation to norepinephrine in non-neural tissues, in the kidney, gut and the liver, where AADC is widely expressed. The pressor effect of DOPS is due to its conversion to NE outside the central nervous system because concomitant administration of carbidopa, an inhibitor of AADC that does not cross the blood–brain barrier, blunted the pressor response. This finding has implications for the use of DOPS in parkinsonian patient already taking DOPA and a decarboxylase inhibitor.

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Pathophysiology of dystonia

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Summary. Understanding of the pathophysiology of dystonia derives primarily from studies of focal dystonias. Physiological investigations have revealed a number of abnormalities that may reflect the genetic substrate that predisposes certain individuals to develop dystonia. There is a loss of inhibition in the central nervous system, and a loss of surround inhibition specifically. Plasticity is increased, and there are sensory abnormalities. Which of these disorders is primary is uncertain.

Introduction

Dystonia is the term for a set of disorders characterized by abnormal postures and unwanted muscle spasms that interfere with motor performance. There are many types of dystonias. Primary focal dystonias are the most common. These disorders tend to come on in adult life and include spasmodic torticollis or cervical dystonia, blepharospasm and focal hand dystonia, most commonly writer's cramp. Most of the physiological studies have used patients with focal dystonias, and while there may be similarities among dystonias of different types, there should be caution in overgeneralization.

Writer's cramp is generally seen in persons who have spent much time writing. Repetitive activities in other tasks are precedents for other task specific disorders. A long period of stereotyped, repetitive behavior seems to be important. Clearly all persons who do considerable writing do not develop writer's cramp. Likely, analogously, many patients who have blepharospasm have a history of eye symptoms, such as dry eye, before the onset of the dystonia. Patients with focal laryngeal dystonia, spasmodic dysphonia, often have a history of a sore throat. Hence, the most likely scenario is that, like most diseases, focal dystonias are products of a genetic background and an environmental insult. That is, for example, writer's cramp develops with excessive writing only in those persons who are genetically predisposed. There is evidence that there is a genetic influence in the focal dystonias.

There are three general lines of work at the present time that may indicate the physiological substrate for dystonia.

Loss of inhibition

A principal finding in focal dystonia is that of loss of inhibition (Hallett, 2004). Loss of inhibition is likely responsible for the excessive movement seen in dystonia patients. Excessive movement includes abnormally long bursts of EMG activity, co-contraction of antagonist muscles, and overflow of activity into muscles not intended for the task. Loss of inhibition can be demonstrated in spinal and brainstem reflexes. Examples are the loss of reciprocal inhibition in the arm in patient with focal hand dystonia and

abnormalities of blink reflex recovery in blepharospasm. Loss of reciprocal inhibition can be partly responsible for presence of co-contraction of antagonist muscles that characterizes voluntary movement in dystonia.

Loss of inhibition can also be demonstrated for motor cortical function including short intracortical inhibition, long intracortical inhibition, and the silent period.

There is an animal model for blepharospasm that supports the idea of a combination of genetics and environment, and, specifically, that the background for the development of dystonia could be a loss of inhibition (Schicatano et al., 1997). In this model, rats were lesioned to cause a depletion of dopamine; this reduces inhibition. Then the orbicularis oculi muscle was weakened. This causes an increase in the blink reflex drive in order to produce an adequate blink. Together, but not separately, these two interventions produced spasms of eyelid closure, similar to blepharospasm. Correlating with this animal model, patients with blepharospasm after a Bell's palsy were reported (Chuke et al., 1996). This could be a human analog of the animal experiments. The idea is that those patients who developed blepharospasm were in some way predisposed.

A principle for function of the motor system may be ''surround inhibition''. When making a movement, the brain must activate the motor system, and it is likely that when one specific movement is generated, other possible movements are suppressed. The suppression of unwanted movements would be surround inhibition, and this should produce a more precise movement. For dystonia, a failure of ''surround inhibition'' may be particularly important since overflow movement is often seen and is a principal abnormality.

There is now good evidence for surround inhibition in human movement. Sohn et al. have shown that with movement of one finger there is widespread inhibition of muscles in the contralateral limb. Sohn et al. have also shown that there is some inhibition of muscles in the ipsilateral limb when those muscles are not involved in any way in the movement (Sohn and Hallett, 2004b). TMS was delivered to the left motor cortex from 3 ms to 1000 ms after EMG onset in the flexor digitorum superficialis muscle. MEPs from abductor digiti minimi were slightly suppressed during the movement of the index finger in the face of increased F-wave amplitude and persistence, indicating that cortical excitability is reduced.

Surround inhibition was studied similarly in patients with focal hand dystonia (Sohn and Hallett, 2004a). The MEPs were enhanced similarly in the flexor digitorum superficialis and abductor digiti minimi indicating a failure of surround inhibition.

Abnormal plasticity

There is an abnormal plasticity of the motor cortex in patients with focal hand dystonia (Quartarone et al., 2003). This has been demonstrated using the technique of paired associative stimulation (PAS). In PAS a median nerve shock is paired with a TMS pulse to the sensorimotor cortex timed to be immediately after the arrival of the sensory volley. This intervention increases the amplitude of the MEP produced by TMS to the motor cortex. It has been demonstrated that the process of PAS produces motor learning similar to long-term potentiation (LTP). In patients with dystonia, PAS produces a larger increase in the MEP than what is seen in normal subjects.

The possibility of increased plasticity in dystonia had been suspected for some time given that repetitive activity over long periods seems to be a trigger for its development. An animal model supported this idea (Byl et al., 1996). Monkeys were trained to hold a vibrating manipulandum for long periods. After some time, they became unable to do so, and this motor control abnormality was interpreted as a possible dystonia. The sensory cortex of these animals was studied, and sensory receptive fields were found to

be large. The interpretation of these results was that the synchronous sensory input caused the receptive field enlargement, and that the abnormal sensory function led to abnormal motor function. The results suggested that the same thing might be happening in human focal dystonia; repetitive activity caused sensory receptive field changes and led to the motor disorder.

Abnormal sensory function

Stimulated by the findings of sensory dysfunction in the primate model, investigators began examining sensory function in patients with focal hand dystonia and found it to be abnormal. Although there is no apparent sensory loss on a clinical level, detailed testing of spatial and temporal discrimination revealed subtle impairments (Molloy et al., 2003). The abnormality is present on both hands of patients with unilateral hand dystonia and also on hands of patients with cervical dystonia and blepharospasm. The identification of abnormality of sensation beyond the symptomatic body parts indicated that the sensory abnormality could not be a consequence of abnormal learning, but is more likely a pre-existing physiological state.

Sensory dysfunction can also be demonstrated with somatosensory evoked potential (SEP) testing. The dipoles of the N20 from stimulation of individual fingers show disordered representation in the primary sensory cortex and these abnormalities are present on both hands of patients with focal hand dystonia. The bilateral SEP abnormality was the first indication in the literature that the sensory abnormality was more likely endophenotypic than a consequence of repetitive activity (Meunier et al., 2001). PET studies show that the sensory cortex is more activated than normal with writing and is more activated when patients are experiencing more dystonia. Voxel-based morphometry studies in patients with focal hand dystonia show an increase in gray matter in the primary sensory cortex (Garraux et al., 2004). Such observations indicate that dystonia is a sensory disorder as well as a motor disorder.

There have been other implications that sensorimotor integration is abnormal. One piece of evidence comes from evoked potential studies during a reaction time task. In this experiment, the imperative stimulus was a median nerve stimulus used to trigger the movement. In normal subjects the N30 peak is gated in the reaction time, but this does not happen in patients with focal hand dystonia. Other evidence comes from studies of the influence of a sensory stimulus on the MEP induced by TMS. At intervals of about 200 ms between a median nerve stimulus and the TMS, there is a normal inhibition of the MEP (called long afferent inhibition, LAI). In patients with focal hand dystonia, the inhibition is converted to facilitation. It is conceivable that a loss of inhibition in sensory systems could give rise to this abnormality so that, once again, loss of inhibition could possibly be the most fundamental disorder.

There are also data from sensory function that are compatible with loss of surround inhibition.

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Genetics of dystonia

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Summary. Primary torsion dystonia (PTD) has a broad clinical spectrum, with earlier onset of symptoms associated with more generalized muscle involvement. The causes for most dystonia are unknown although several monogenic subtypes have been identified. One important genetic cause of PTD is DYT1; a three base pair deletion in this gene is a major cause for early-onset dystonia. Its identification has allowed the development of cellular and animal models; it has also permitted studies that identify both ''manifesting'' and ''non-manifesting'' DYT1 mutation carriers. These studies have expanded our understanding of clinical expression to include psychiatric symptoms and also have enabled imaging studies of endophenotypes. These advances provide a widened platform for future research.

Introduction

Genetic etiologies have long been suspected for many subtypes of dystonia. Recent molecular advances have lead to the identification of an increasing number of genes for primary and secondary dystonia subtypes (see Table 1). This information has opened the way for studies aimed at characterizing basic pathogenic mechanisms, including cellular and animal models. It has also allowed for a broader analysis of phenotype and endophenotype to further characterize the spectrum of gene expression. Defining genetic etiologies has altered the way neurologists diagnose and counsel patients, including the important need to provide genetic counseling to patients and their families. Ultimately, understanding the genetic causes of dystonia, and the effects of these alterations, holds the promise of rational, targeted therapies.

Primary dystonia

There are many classification schemes to organize the causes of dystonia. Most create at least two broad categories: primary torsion dystonia (previously named idiopathic torsion dystonia), and secondary (or non-primary) dystonia (see Table 1 and Fig. 1). Primary torsion dystonia (PTD) is defined as a syndrome in which dystonia is the only clinical sign (except for tremor) and there is no evidence of neuronal degeneration or an acquired cause. Secondary (non-Primary) dystonias include all other dystonia subtypes and can further divided into inherited, complex, and acquired etiologies.

Primary torsion dystonia (PTD)

Early-onset PTD and DYT1

Early-onset PTD is 3–5 times more common in Ashkenazi Jews compared to other populations and is transmitted in an autosomal dominant fashion with reduced penetrance

Table 1. Causes of dystonia

Primary

- Autosomal dominant
  - Early limb (DYT1, other genes to be determined)
  - Mixed (DYT6, DYT13, other genes to be determined)
  - Late Focal (DYT7, other genes to be determined)
- Other genetic causes
  - ?autosomal recessive,
  - complex

Secondary

- Inherited
  - Dystonia plus (non-degenerative)
    - DRD (DYT5-GCH1, DYT14, other biopterin deficiencies, tyrosine hydroxylase deficiency)
    - Myoclonus – Dystonia (DYT11-epsilon sarcoglycan, 18p locus)
    - Rapid-Onset Dystonia Parkinsonism (DYT12, ATP1A3)
  - Degenerative
    - Autosomal Dominant (e.g., Huntington's disease, SCA's especially SCA3)
    - Autosomal Recessive (e.g., Wilson's, NBIA1, GM1, GM2, Parkin)
    - X-linked (e.g., X-linked Dystonia-Parkinsonism/Lubag, DDP)
    - Mitochondrial
- Complex/unknown
  - Parkinsonism (e.g., Parkinson's disease, multisystem atrophy, progressive supranuclear palsy, corticobasal degeneration)
- Acquired
  - e.g., drug-induced, perinatal injury, head trauma, cervical trauma, peripheral trauma, infectious and post infectious, tumor, AVM, stroke, central pontine myelinolysis, multiple sclerosis

DRD dopa-responsive dystonia; *GCH1* GTP cyclohydrolase1; *SCA* spinocerebellar ataxia; *NBIA1* neurodegeneration with brain iron accumulation; *DDP* deafness dystonia peptide

of 30–40% in both Ashkenazi Jews and non-Ashkenazim (Bressman, 1989; Pauls, 1990). The difference in disease frequency is thought to be the result of a founder mutation in DYT1 (see below) that was introduced into the Ashkenazi population at the time of a ''bottleneck'' in the 1600s, followed by a period of tremendous population growth (Risch, 1995).

The gene at locus DYT1 was identified in 1997 (Ozelius, 1997) and is responsible for a large proportion of early limb onset PTD (also known as dystonia musculorum deformans or Oppenheim's disease) across many different populations (Valente, 1998; Leube, 1999; Bressman, 2000). Only one recurring mutation in DYT1, an in – frame GAG deletion, has been associated unequivocally with PTD. The DYT1 gene encodes a novel protein, torsinA, that is 332 amino acids long ($\sim$38 kD), with potential sites for glycosylation and phosphorylation, as well as an amino terminal hydrophobic leader sequence consistent with membrane translocation/targeting (Ozelius, 1997). The GAG deletion results in the loss of one of a pair of glutamic acid residues near the carboxy terminus of the protein. TorsinA shares functional regions with the AAA+ superfamily. These proteins are characterized by Mg++-dependent ATPase activity and typically form six-membered, homomeric ring structures. Many serve as chaperones that mediate conformational changes in target proteins. They are associated with a number of functions including protein folding and degradation, cytoskeletal dynamics,

Dystonia classification: genetic loci (includes all primary and dystonia plus)				
DYT1	9q34	AD	Early limb-onset PTD - Oppenheim's	TorsinA (1997)
DYT2	Not mapped	AR	Early-onset	Not identified
DYT3	Xq13.1	XR	X-linked Dystonia - Parkinsonism (lubag)	Multiple transcript system (2003)
DYT4	Not mapped	AD	Whispering dysphonia	
DYT5	14q22.1	AD	Dopa Responsive Dystonia (DRD)	GCH1 (1994)
DYT6	8p	AD	"mixed"	Not identified
DYT7	18p	AD	Adult cervical	Not identified
DYT8	2q	AD	Paroxysmal dystonic choreoathetosis	Myofibrillogenesis regulator 1 (2004)
DYT9	1p21	AD	Episodic choreoathetosis / ataxia with spasticity	Not identified
DYT10	16	AD	Paroysmal kinesigenic dystonia(EKD1 & 2)	Not identified
DYT11	7q21	AD	Myoclonus Dystonia	ε Sarcoglycan (2001)
DYT12	19q13	AD	Rapid-Onset Dystonia Parkinsonism	Na+/K+ -ATPase alpha3 (2004)
DYT13	1p36	AD	Cervical/cranial/brachial	Not identified
DYT14	14q13	AD	DRD	Not identified
DYT15	18p11	AD	Myoclonus Dystonia	Not identified

Fig. 1. Dystonia (DYT) genetic loci

membrane trafficking and vesicle fusion and response to stress.

TorsinA is widely distributed in normal adult brain and brain tissue from patients with a variety of movement disorders including Parkinson's disease and DYT1 dystonia (Augood, 1998, 2002; Shashidharan, 2000; Walker, 2002). There is intense expression in substantia nigra compacta dopamine neurons, cerebellar dentate nucleus, Purkinje cells, basis pontis, locus ceruleus, numerous thalamic nuclei, the pedunculopontine nucleus, the oculomotor nuclei, hippocampal formation and frontal cortex. TorsinA immunolocalization in the human CNS appears to be restricted to the cytoplasm of neurons (Konakova, 2001), and when transfected into mammalian cells behaves as lumenally oriented glycoproteins (Kustedjo, 2000; Hewett, 2003). Overexpression of wild-type torsinA in cultured cells prevents protein aggregation and protects against cytotoxicity after proteasomal inhibition, oxidative stress, or trophic factor withdrawal (Shashidharan, 2004; Mclean, 2002; Kuner, 2003); in contrast, mutant torsinA fails to protect cells from these toxic insults and leads to the formation of perinuclear inclusion bodies (Shashidharan, 2004; Hewett, 2000; Gonzalez-Alegre, 2004).

Despite these findings, suggesting torsinA protects against various stressors, examination of DYT1 brains have been relatively unrevealing (Walker, 2002; Furukawa, 2000; Rostasy, 2003), except for one recent study of four clinically affected DYT1 brains (McNaught, 2004). This study utilized highly sensitive antibodies to ubiquinated proteins and found perinuclear inclusion bodies in the midbrain reticular formation and periaqueductal grey, specifically involving cholinergic neurons of the pedunculopontine nucleus. In addition tau/ubiquitin immunoreactive aggregates were observed in pigmented neurons of the substantia nigra compacta and locus coeruleus. These pathological findings are consistent with the notion that mutated torsinA

may impair protein handling; they also point to involvement of the pedunculopontine nucleus and other brainstem structures, which have extensive connections to the basal ganglia. Recent transgenic mouse model results support these findings as pathological for DYT1. Shashidharan and colleagues reported that 40% of four overexpressing transgenic lines developed hyperkinesias; those with hyperkinesias were found to have decreased striatal dopamine compared to increased dopamine levels in transgenic mice without hyperkinesias. Immunohistochemistry revealed perinuclear brainstem inclusions similar to those identified in DYT1 human brains (Shashidharan, 2005).

Although all DYT1 PTD has a single molecular basis, clinical expression is extraordinarily broad, even within families; 70% of gene carriers have no definite signs of dystonia and among the remaining 30% dystonia ranges from focal to severe generalized (Bressman, 2000). There are however common DYT1 clinical characteristics: the great majority of people with dystonia due to DYT1 have early onset (before 26 years) that first affects an arm or leg. About 65% progress to a generalized or multifocal distribution, the rest having segmental (10%) or only focal (25%) involvement. When viewed in terms of body regions ultimately involved, one or more limbs are almost always affected (over 95% have an affected arm). The trunk and neck may also be affected (about 25–35%) and they may be the regions producing the greatest disability (31); the cranial muscles are less likely to be involved (<15–20%). Rarely, affected family members have late-onset (up to age 64 years) (Opal, 2003). Also, although the arm is the body region most commonly affected in those with focal disease, the neck or cranial muscles have been reported as isolated affected sites (Tuffery-Giraud, 2001). Because of the founder effect, the DYT1 GAG deletion is more important in the Ashkenazi population, where it accounts for about 80% of early (less than 26 years) onset cases; this compares with 16–53% in early-onset non-Jewish populations.

With identification of the DYT1 gene it has become possible to more fully investigate the clinical features and expression of the gene using a variety of approaches, including imaging, psychological, and neurophysiological testing. By comparing DYT1 gene carriers, including non-manifesting carriers, to non – carriers, gene associated features can be distinguished. Using this paradigm, ^{18}F-fluorodeoxyglucose positron emission tomography (PET) and network analysis, Eidelberg and colleagues demonstrated an abnormal pattern of glucose utilization characterized by covarying metabolic increases in the basal ganglia, cerebellum, and supplementary motor cortex (SMA) that was present in both ''manifesting'' and ''non-manifesting'' gene carriers (Eidelberg, 1998). Glucose PET studies have also been utilized for psychomotor testing in ''non-manifesting'' gene carriers and show subtle abnormalities in sequence learning both in the motor performance and recruitment of brain networks (Ghilardi, 2002). Other imaging studies of DYT1 gene carriers have found decreased striatal D2 receptor binding (Asanuma, 2005), and microstructural changes involving the subgyral white matter of the sensorimotor cortex (Carbon, 2004). Non-imaging studies using this paradigm include an assessment of possible psychiatric manifestations; an equal and increased risk for major recurrent depression in DYT1 gene carriers manifesting and not manifesting dystonia was found (Heiman, 2004). Electrophysiological analyses have also identified abnormalities, namely reduced intracortical inhibition and a shortened cortical silent period (Edwards, 2003). These studies strongly support the presence of wider clinical gene expression, abnormal brain processing and associated structural brain changes in gene carriers regardless of overt motor signs of dystonia, expanding the notion of penetrance and phenotype.

Early-onset but not DYT1

A large group of early-onset PTD, especially among non-Jewish populations, is not due to the DYT1 GAG deletion. Two loci, DYT6 (Almasy, 1997) and DYT13 (Valente, 2001), have been mapped in families having an average age-onset in adolescence. However, neither locus has been confirmed in other families and they are suspected to account for only a minority of non-DYT1 early-onset cases. Further, overall clinical features in these two families differ from DYT1 (although features in any single family member may overlap with DYT1). The family phenotypes for DYT6 and 13 are marked by prominent involvement of cranial and cervical muscles with variable spread; also, compared to DYT1 a greater proportion of family members have later adolescent and adult onset. To distinguish this phenotype from the typical early-onset phenotype associated with DYT1 and typical late-onset focal phenotypes the term "mixed" has been applied.

Late-onset PTD

Like early-onset PTD, late-onset PTD also appears to have autosomal dominant inheritance (Defazio, 1993). However, unlike early-onset dystonia, most studies show that penetrance is even more reduced (about 12%–15% compared to 30% for early-onset); alternatively, penetrance may be higher in a subset with the remainder sporadic. Consistent with the notion of increased penetrance in a subset of late-onset PTD, are descriptions of large families with more highly penetrant autosomal dominant disease. One such family with adult-onset torticollis was studied resulting in the mapping of DYT7 (Leube, 1996). Other clinically similar families have been excluded from DYT7 (47) suggesting yet other loci for adult onset focal PTD. Most recently a polymorphism in the D5 dopamine receptor (DRD5) gene and a DYT1 haplotype (but not the GAG deletion) have been associated with adult-onset focal dystonias but the role of DRD5 and DYT1 as a susceptibility genes remain to be elucidated (Misbahuddin, 2002; Clarimon, 2005).

Secondary dystonia and dystonia plus syndromes

Etiological subgroups for secondary dystonias include: 1) inherited causes; 2) a group of primarily parkinsonian disorders including Parkinson's disease that are thought to have complex etiologies and; 3) environmental or acquired causes. In addition most classifications also include other movement disorders that may display dystonic phenomenology such as tics and the paroxysmal dyskinesias and the pseudo-dystonias. The latter are not considered true dystonia but muscle contractions mimicking dystonia such as seen in Sandifer's syndrome, orthopedic conditions and psychogenic dystonia.

Among the inherited forms of secondary dystonia is a relatively newly defined category of dystonia-plus syndromes consisting of three clinically defined entities: dopa – responsive dystonia (DRD), myoclonus – dystonia (M-D), and rapid – onset dystonia-parkinsonism (RDP). The dystonia-plus category was distinguished from both primary dystonia and other inherited secondary dystonias because it shares some but not all features of both groups. That is, like primary dystonia these three syndromes do not appear to be degenerative. Although pathology is limited, evidence to date supports genetic defects that result in functional brain changes not associated with progressive neuronal death. Further, unlike primary dystonia, but similar to the other degenerative secondary dystonias, the dystonia plus group has as characteristic clinical features signs other than dystonia including parkinsonism for DRD and RDP and myoclonus for M-D.

As our understanding of these syndromes is expanding the complexity of their genetic and clinical heterogeneity is being detailed. For example, for DRD there are currently

several known genetic biochemical etiologies each with protean clinical manifestations, and for myoclonus – dystonia there appear to be at least two genetic etiologies (see Table 1).

Conclusions

There has been tremendous recent growth in our knowledge of the clinical and genetic complexity of dystonia, including PTD and the dystonia-plus syndromes. The delineation of the varied clinical subtypes of dystonia has aided gene identification, e.g., DYT1, GCH1, epsilon sarcoglycan. With the identification of genes it has been possible to develop cellular and animal models; it has also allowed the identification of populations harboring mutations and expanded our understanding of endophenotypes. These advances have provides a widened platform for future research.

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Index