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E. Koutsilieri (eds.)

Neuropsychiatric Disorders

An Integrative Approach

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Journal of Neural Transmission
Supplement 72

SpringerWienNewYork

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SpringerWienNewYork is part of Springer Science + Business Media
springer.at

Typesetting: Thomson Press India Ltd., Chennai
Printing: Druckerei Theiss GmbH, 9431 St. Stefan im Lavanttal, Austria

Printed on acid-free and chlorine-free bleached paper

SPIN: 12089468
Library of Congress Control Number: 2007931042

With 109 (partly coloured) Figures

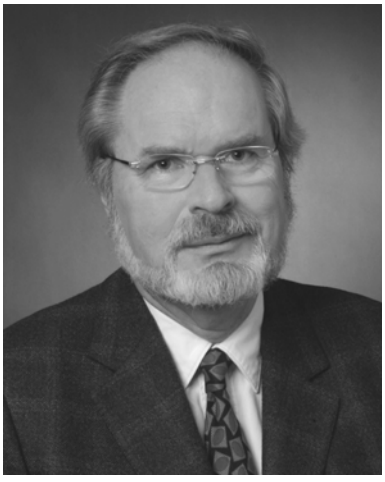
ISSN 0303-6995
ISBN 978-3-211-73573-2 Springer-Verlag Wien New York

J Neural Transm (2007) [Suppl 72]: V–VI

Printed in Austria

Journal of Neural Transmission

Preface



This supplementum of the *Journal of Neural Transmission* was conceived as a celebration of the professional life and work of Professor Peter Riederer, Professor of Clinical Neurochemistry in the Clinic for Psychiatry and Psychotherapy at the University of Würzburg, Germany, on the occasion of his retirement in April 2007. Many of Peter's colleagues and friends have contributed manuscripts to this issue. The international character of this issue, and the broad range of topics it includes, is recognition in itself of the high regard enjoyed by Peter in the field of neurochemistry.

Peter Riederer was born in Königsberg (Germany) in 1942 but completed most of his early schooling and university education in Vienna, where he completed his doctorate in chemistry at the Vienna University of Technology in 1970. He first made his mark on the field of neurochemistry as a young Postdoctoral Fellow, with his instrumental and initiating role in the discovery in 1975 of the beneficial effects of L-deprenyl (selegiline), the first selective monoamine oxidase B inhibitor, in the therapy of Parkinson's disease (PD). This discovery, made jointly with Professor Walther Birkmayer – who had introduced the combined L-DOPA/decarboxylase inhibitor (benserazide) strategy into antiparkinsonian therapy in 1967 – and his young colleague Moussa Youdim – who also proceeded to a stellar career in PD research and became a close friend of Peter's – cemented his lifelong interest in the investigation of PD.

Rising to the position of Head of Clinical Neurochemistry at Ludwig Boltzmann Institute for Clinical Neurobiology at Lainz Hospital (Vienna) in 1971, Peter achieved a series of significant insights into the nature of PD and other neurological disorders, resulting in a number of attractive offers to head neurochemistry departments at various Universities. In 1986, Peter assumed his current position as Professor of Clinical Neurochemistry at the University of Würzburg. During his two decades in Franconia he has assiduously built the international reputation of his department, the success of which was recognized in 1999 by its being awarded the status of a National Parkinson Foundation Center of Excellence (USA). Among his significant contributions from this period, is his body of work on the neurochemistry of schizophrenia and mechanisms of neurodegeneration in Alzheimer's disease (AD) and PD. Most recently the role of iron in PD and the possibility of neuroprotective and neurorestorative strategies in neurodegenerative disease (together with Moussa Youdim and two of us, M.G. and K.D.) attracted his attention.

Peter has authored over 900 scientific papers and chapters, a body of work which has contributed much to our understanding of the aetiology and possible new treatment strategies for PD and other brain disorders, including AD, depression and schizophrenia. As an example of his standing it is worth noting that in 2004 he was the most cited chemist in medical research. He has been recognized personally with numerous international and national awards and honours, including the Burda Prize for research in PD, the Eli Lilly Prize in Neuropsychopharmacology (which he was awarded twice). He shared the Claudius Galenus Gold Prize (for his deprenyl research) and was awarded with the honorary membership of the Hungarian Pharmacology Society. He has served on numerous journal editorial boards, most prominently as Editor-in-Chief of the

Journal of Neural Transmission. He has also organized many successful congresses in his areas of expertise, most notably serving as the Congress President of the Sixteenth International Congress of Parkinson's Disease and Related Disorders in Berlin in 2005 and serving as Chairman of the local organising committee of the World Congress on Biological Psychiatry in Berlin 2001.

Popular with his students, Peter inspired many students to enter neurochemistry research, and he has mentored many young scientists embarking upon successful research careers: K. Double (Australia), N. Durany (Spain), M. E. Götz (Germany), E. Grünblatt (Germany), C. Konradi (USA), E. Koutsilieri (Germany), G. Münch (Australia), W.-D. Rausch (Austria), G Reynolds (UK) and E. Sofic (Bosnia-Herzegovina) to name only a few. His international reputation is underlined by the number and breadth of his collaborators from countries around the globe. The fact that many of these collaborations, including those with the editors of this issue, as well as those with other long-term colleagues such as S. Hoyer (Germany), K. Jellinger (Austria), W. Maruyama (Japan), T. Nagatsu (Japan), M. Naoi (Japan), T. Saito (Japan) and M.B.H. Youdim (Israel) have been fruitfully pursued for years, even decades, says much about the esteem and admiration with which he is regarded. As a colleague and person, Peter is valued for his wide-ranging knowledge, his sense of fairness and last but not least his Viennese charm. Many of his colleagues and collaborators have also been lucky enough to benefit from Peter's generous hospitality and that of his equally charming wife, Inge.

On the occasion of his retirement we congratulate Peter for a lifetime of achievement, for his many valuable contributions to neurochemistry, and for enriching the lives of all the students and colleagues who have worked with him. We also thank him for the friendship and warmth which he has unstintingly extended to the editors of this special issue, and to so many others, throughout the years. We hope to continue both our professional and personal relationships with Peter for many years to come.

Manfred Gerlach, Würzburg, May 2007
Jürgen Deckert, Würzburg, May 2007
Kay Double, Sydney, May 2007
Eleni Koutsilieri, Würzburg, May 2007

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Printed in Austria

Journal of Neural Transmission

The meeting of minds and times with Peter Riederer: an appreciation

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"The harder you work, the harder it is to surrender."
Vince Lombardi

It is fair to say that if I had not received a phone call at Oxford University from Prof. Merton Sandler in summer of 1973, that a young chap by the name of Peter Riederer, from Prof. Walter Birkmayer Department, was in London and wanted to discuss some aspects of monoamine oxidase (MAO) inhibitors for Parkinson's disease (PD), I probably would not be where I am today. If there were two contrasting people, that were us. Here was this large but soft spoken, rather gentle and austere Viennese, meeting a small rather assertive individual from Iran. It was meeting of the minds and an instant connection that has lasted some 34 years, with a result of some near one hundred joint publications, some 25 books and hopefully advancing the prospect for treatment of PD. Peter wanted to know was there an MAO inhibitor that did not cause a ''Cheese Reaction'', a side effect of first generation of non-selective MAO inhibitors, that could be employed in the treatment of PD. In 1961 Birkmayer and colleagues had used MAO inhibitors to treat PD, gastrointestinal and blood pressure problems in such patients was a limiting factor. My instant reaction to his request was that the Hungarian pharmacologist, Joseph Knoll, whom I had met at the MAO meeting in Sardinia in 1971, in honour of Hugh Blaschko, had described a failed MAO-B inhibitor anti-depressant called L-deprenyl, that did not give a cheese reaction in isolated pharmacological preparations and in vivo (Knoll and Magyar, 1972). The other logic of using L-deprenyl was that in 1970 with Merton Sandler we had studied MAO activity in different human brain regions (Collins et al., 1970; Youdim et al., 1972). The basal ganglia, had a higher activity towards the MAO-B substrate benzylamine and dopamine than the other regions, suggesting that predominance of MAO-B in this brain region. It was decided I should stop over in Vienna to give a lecture on MAO for Birkmayer sake, since I was going to meet Joseph Knoll in Budapest, and present a paper at the Hungarian Pharmacology Society.

I had once before been in Vienna as a stop over, but this time Peter was a great host. After the lecture at the Neurological Institute we landed in a Heurige restaurant, drinking a significant amount of the young wine and thinking that the Hungarians might have a gold mine in L-deprenyl that they were not aware of. The ensuing headache that evening was worth what was to come eventually with L-deprenyl. Peter explained to me why he was looking for an MAO-inhibiting substance without major side effects and we decided to convince Birkmayer to try L-deprenyl in parkinsonian patients with on–off-phases. I let have some 5 mg of L-deprenyl, which I had received from Joseph Knoll in Budapest and transferred to Peter Riederer and Birkmayer at a lunch in the Sacher restaurant in Vienna, with the emphasis that if it should cause hypertension in the PD subject, we should abandon the project. Later Joseph Knoll did not appreciate our hypothesis about the usefulness of L-deprenyl as dopaminergic drug for PD, when I told our intention. He insisted that that L-deprenyl acted as psychoenergizer like amphetamine with phenylethylamine being its major action. Some months later at Oxford our secretary informed me that I have a call from an excited person by the name of Riederer from Vienna. My first reaction was that the use of L-deprenyl by Birkmayer has had a major side effect in PD subjects. But Peter assured me that L-deprenyl was given to 44 PD subjects and the drug works (Birkmayer et al., 1975, 1977; Lees et al., 1977).

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The lack of video in those days resulted in making a film of some of the patients. The clinical results were presented at the 5th International Congress of PD in Vienna and Melvin Yahr, who was the second investigator to study L-deprenyl, asserted in the summary of the congress that this is a novel new direction for the treatment of PD (Yahr, 1975). The rest is history except that it took nearly 15 years before L-deprenyl, renamed in USA as selegiline, reach its shores.

The next stage in this collaboration was the long term effect of L-deprenyl, which we (Birkmayer et al., 1983, 1985) had studied in more than 800 subjects since in 1975. It was apparent that L-deprenyl as adjuvant to L-DOPA (L-3,4-dihydroxyphenylalanine, levodopa) may alter the progression of the disease and we wrote in the summary of the paper presented at the MAO meeting in Heidelberg *"The prolongation of the evolution of Parkinson's disease with long-term (−)-deprenyl treatment shows for the first time that the degeneration of the dopaminergic nigrostriatal fibers can be depressed to some extent"* (Birkmayer et al., 1983). This was prior to the identification of MPTP (N-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine) as a dopaminergic neurotoxin and its prevention by L-deprenyl in 1984 (Heikkila et al., 1984). The rest is history resulting in the publication of thousands of publications on various aspects of L-deprenyl, pharmacology and neuroprotection. It also led to the development of a number of other MAO-A and B inhibitors as anti-Parkinson drugs by many pharmaceutical companies, which all failed to reach the market. The exception being rasagiline (Azilect®) a restricted analogue of L-deprenyl, which John Finberg and I co-developed with Teva Pharmaceutical Company (Youdim et al., 2005).

The other aspect of our collaboration has been the work we initiated on brain iron metabolism in PD, a subject I started at Oxford in 1974, at the time when no one had paid much attention to the role of iron in brain function and dysfunction (Youdim, 1985). It was Sheila Callender, the Reader in Department of Haematology at Oxford University who had read one of my earlier papers on iron metabolism and MAO and asked why I had not continued the work on brain iron metabolism. As a consequence my group started the work on brain iron metabolism, which was presented at the Ciba Foundation symposium (Youdim and Green, 1976) and summarized in the Handbook of Neurochemistry (Youdim, 1985) with some emphasis on role of iron in oxidative stress and PD. 1985 was another turning point for Peter and I when we met at the International Society for Neurochemistry in Copenhagen, where Paul Mandel had invited me to talk about iron and brain function. The subject of human brain iron metabolism came up, I imparted to Peter that several publications from 1924 and 1968 had shown that iron is increased in substantia nigra of PD and this could be relevant to the pathology of the disease. His response was that he had similar data and which were presented at meeting in Austria in May 1985, well before the published letter of Dexter et al. in the Lancet (1987). So for the second time we had the meeting of the minds to explore the role of iron in PD and that led to extensive collaborations and publications on brain iron in PD and its animal models, which has now become a major topic of interest in neurodegenerative processes and other neurodegenerative diseases, including Alzheimer's disease (Riederer et al., 1989; Gerlach et al., 1994; Berg et al., 2002, 2004; Götz et al., 2004; Zecca et al., 2004). This topic led to my collaboration with Avraham Warshawsky in 1989 for the development of brain permeable iron chelators as therapeutic agents for PD and other neurodegenerative disorders including Alzheimer's disease, which we have done very successfully (Youdim et al., 2004; Gal et al., 2005; Zheng et al., 2005a, b), demonstrating that iron chelators are neuroprotective in 6-hydroxydopamine kainate and MPTP models of PD.

The collaboration with Peter did not weaned there and took another turn when we decided to study the mechanism of dopamine neurodegeneration in the MPTP model of PD and in sporadic PD brains employing for the first time transcriptomics and proteomic profiling of substantia nigra pars compacta. With out Peter's ability to obtain PD brains from the brain banks in Austria and Germany this project would have never have got of the ground and it was the first time that this approach was made for the study of sporadic PD. I consider this as probably the most important and significant work we done, since it brings a new dimension to the study of neurodegenerative processes in PD and opens up novel avenues on the mechanism of neurodegeneration, novel drug development and even development of phenotypic model of sporadic PD (Grünblatt et al., 2004; Mandel et al., 2005).

The meeting of the minds between Peter and I was not directed entirely in our research interests. It included exchange of students, post-doctoral fellows, organization of numerous symposia, conferences and congresses and editing of many books and traveling to all corners of the world and presenting the results of our collaboration.

Above all these events, there is the human side of Peter Riederer as a friend, colleague and teacher. His humanity can be measured by the constant support he has given to his colleagues, the young students, Israeli scientists, Israel and myself. He is the only person who has never failed to visit Israel, to participate in conferences and support it no matter if there was a conflict in Israel with its Arab neighbors.

No one has been as fortunate as I, to have known, worked and observe him from the distance of Israel and so closely. His capacity to shoulder extensive amount of work diligently has always astonished me. Never in the extensive years of collaborations did we ever exchange a harsh word and disagreed about any aspects of the works, publications or what we set out to do. He has always been ready to listen and give sound judgements. I have learnt much from him and will continue to do so and I have no doubt that we shall continue the meeting of our minds well after his so called ''official retirement''. All members of my center to wish him further successes in the future and no doubt will continue to be so.

"Don't think of retiring from the world until the world will be sorry that you retire. I hate a fellow whom pride or cowardice or laziness drive into a corner, and who does nothing when he is there but sit and growl. Let him come out as I do, and bark."

Samuel Johnson

"Life is no brief candle to me. It is a sort of splendid torch which I have got a hold of for the moment, and I want to make it burn as brightly as possible before handing it on to future generations."

George Bernard Shaw

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J Neural Transm (2007) [Suppl 72]: 5–15

Printed in Austria

Journal of Neural Transmission

Succi nervorum: a brief history of neurochemistry

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Summary The nature of intracellular communication and integration in the central nervous system remained a source of controversy long after it had been accepted that the brain is intrinsically involved in the reception of external and internal sensory impressions, in the control of both voluntary and involuntary physiological functions, and in the processes associated with consciousness and psychic function in humans. The role of the specific chemistry of the brain in these functions was specifically addressed only in the 20th century, although chemical examination of brain tissue can be traced at least as far back as 1719 to Hensing's *Cerebri examen chemicum*. Throughout the 1940s and 1950s evidence accumulated from a variety of laboratories that certain chemical substances, such as acetylcholine, noradrenaline and histamine, might be involved in central nervous system neurotransmission, but conclusive evidence for such communication was difficult to obtain. Commencing with Carlsson's 1957 paper on the antireserpine effects of DOPA and culminating in the successful amelioration of parkinsonian akinesia by Birkmayer and Hornykiewicz via administration of L-DOPA in 1961, followed by the identification of specific nervous tracts which utilized dopamine as a transmitter, chemical neurotransmission in the brain was ultimately demonstrated through a combination of pharmacological, physiological and clinical research. Neurochemistry had thereby graduated from a branch of general physiology to being centrally involved in models of central nervous system function.

Keywords: History, neuroscience, levodopa therapy, neurotransmission

The nature of intracellular communication and integration in the central nervous system remained a source of controversy long after it had been accepted that the brain is intrinsically involved in the reception of external and internal sensory impressions, in the control of both voluntary and involuntary physiological functions, and in the processes associated with consciousness and psychic function in humans. By the early twentieth century it was recognized that, in contrast to the heart or liver, discrete regions of the brain were associated with specific functions, rather than its acting as a homogenous organ, and the Ramón y Cajal neuron hypothesis had essentially displaced the Golgi reticular model of central nervous system structure. Neurophysiologists had demonstrated the electrical properties of nervous communication; advances in techniques for the preparation and staining of nervous tissue and the efforts of diligent neuroanatomists had illuminated the structure of the brain at both the macroscopic and microscopic levels, and neuropathologists identified structural changes associated with various neurological disorders, such as the degeneration of the substantia nigra in parkinsonism. Important as these discoveries were for understanding brain architecture, however, they were not able to explain precisely *how* the individual cells of the brain communicated and coordinated their activities with one another. It is salient that for two-thirds of the twentieth century three strands of neuroscientific investigation were pursued in parallel, only occasionally interacting to produce a significant practical outcome: clinical neuroscience, neuropathology and neurochemistry. Only at the end of the 1960s were these three strands weaved together to yield a more complete and fruitful view of central nervous system function. The present paper attempts a brief and by no means comprehensive overview of some significant aspects of the development of one of these strands, neurochemistry.

The pioneers: pre-20th century

The earliest specific work in this area was probably the 1719 dissertation of Johannes Thomas Hensing (1683–1726), Professor of Medicine and later also of *Philosophia naturalis chymica* in Giessen, entitled *Cerebri examen chemicvm, ex eodemqve phosphorum singularem omnia inflammabilia accendentem*, wherein the presence of a specific substance (phosphorus) in brain was described for the first time (Tower, 1983). This report had largely been for-

This paper is dedicated with gratitude and affection to my friend and colleague Peter Riederer, a long-term and stalwart supporter of my research.

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gotten by the time further preliminary investigations of the chemical constitution of the brain were undertaken towards the end of the eighteenth century by pioneers including Michel-Augustin Thouret (1748–1810), Antoine-Francois de Fourcroy (1755–1809) and Nicolas-Louis Vauquelin (1763–1829), culminating in the publication by Vauquelin in 1811 of *Analyse de la matière cérébral de l'homme et de quelques animaux*. Their work had been fostered by the confluence in France of the Enlightenment and the rapid advances in chemistry spearheaded by Antoine Lavoisier (1743–1794), as well as the opportunity afforded by the exhumation of bodies from the *Cimetière des SS. Innocents* from 1785 (see Sourkes, 1992; Tower, 1994).

In neighbouring Germany, also at the forefront of developments in chemistry, interest in brain chemistry accelerated during the mid-19th century. The term *Nervenchemie* was introduced in 1856 by the Tübingen pioneer of physiological chemistry Julius Eugen Schlossberger (1819–1860), who devoted a major section of his *Erster Versuch einer allgemeinen und vergleichenden Thier-Chemie* to the chemical nature of nervous tissue. He cited Vauquelin's 1811 report as his principal source. Schlossberger assayed the organic and inorganic constituents of both central and peripheral nervous tissue (including spine), partly with the aim of attempting to explain its function; he concluded, for example, on the basis of differential vascularisation, that metabolism in grey was greater than in white matter. Many subsequent texts on physiological chemistry similarly included sections on the chemistry of nerve tissue, but the preferred term for this subfield of comparative animal chemistry was *Gehirnchemie* (as employed by Kühne in his 1868 *Lehrbuch der physiologischen Chemie*) or 'brain chemistry'. This underscored the fact that, notwithstanding the significance of chemical assessment of the brain for understanding neural function, brain chemistry was not regarded as a field of enquiry distinct from general physiological chemistry. The same chemical mechanisms which underlay peripheral function were ultimately expected to suffice for explanations of the nervous system activities (reviewed: McIlwain, 1988, 1990).

The chemist and oenologist Johann Ludwig Wilhelm Thudichum (1829–1901) is often designated the 'father of neurochemistry' (he himself used the term 'brain chemistry'), not because he was the first active investigator in this field, but because he was the first to apply himself to its investigation over an extended period. Thudichum was born near Frankfurt am Main in Germany, had studied medicine and chemistry at Giessen and Heidelberg, but moved to London in 1853 to escape employment difficulties probably related to his expression of pro-revolutionary sympathies in 1848. Thudichum's interest in clinical chemistry was particularly fostered in England by his association with John Simon (1816–1899), Medical Officer of the Privy Council and Local Government Board, and his first publication on brain chemistry, *Researches on the chemical constitution of the brain*, appeared in 1874 (seven years after he had initiated his investigations) as a long appendix to Simon's report for that year. His work was motivated from the beginning by the philosophy he enunciated ten years later in *A treatise on the chemical constitution of the brain*:

> When the normal composition of the brain shall be known to the uttermost item, then pathology can begin its search for abnormal compounds or derangement of quantities . . . it is probable that by the aid of chemistry many derangements of the brain and mind, which are present obscure, will become accurately definable and amenable to precise treatment, and what is now an object of anxious empiricism will become one for the proud exercise of exact science (Thudichum, 1884, p. 259f.).

Thudichum particularly emphasized the significance of phosphatides in brain tissue, describing them as the "centre, life, and chemical soul of all bioplasm whatever" (Thudichum, 1884, p. xii). He also recognized that nervous tissue might present unique problems to the chemical physiologist:

> . . . the brain is . . . the most diversified chemical laboratory of the animal body; . . . all other organs . . . are relatively much more simple and very much less specific in their chemical constitution than the organs producing and conducting nerve-power (Thudichum, 1884, p. 27).

Thudichum hypothesized that disorders ranging from headache to insanity might be the result of endogenous noxa produced either by the brain or transported there by the blood, or by exogenous toxins such as alcohol and morphine. Perhaps surprisingly, however, he rejected attempts at an immediate integration of brain chemistry and physiology; he was particularly vehement in his polemics against the work of investigators such as Wilhelm Kühne (who coined the term 'enzyme') and Felix Hoppe-Seyler, leading European chemist and editor of the *Zeitschrift für physiologische Chemie*. Until the catalog of normal constituents in human brain had been completed, such extrapolation was, in his mind, dangerous speculation (McIlwain, 1975).

Although accepted by many, Thudichum's results were not universally applauded by his contemporaries. The most long-lasting and particularly bitter controversy in which he was involved concerned the proposal in 1864 by Oscar

Liebrich (1839–1908), then working with Hoppe-Seyler in Tübingen, that the brain essentially consisted of a single substance, the lipid 'protagon'. Thudichum contended as early as 1874 that the proposed substance was nothing but a mixture of smaller lipid molecules, but Thudichum died before the debate was ultimately decided in his favour (Sourkes, 1995). Nevertheless, the precise methods and meticulous details recorded in his 1884 volume and its revised, German language edition (1901) ensured recognition of the magnitude of his contributions to the exploration of brain chemistry (Drabkin, 1958; Sourkes, 2003).

First thoughts on chemical communication in the brain: 1900–1950

During the first third of the twentieth century, it was established that certain substances – acetylcholine and adrenaline (epinephrine) – were employed in peripheral tissues as messenger substances between nerve cells, as well as for communication between nerve and muscle cells. The significance of these findings was recognized by the award of the 1936 Nobel Prize for Physiology or Medicine to the Austrian pharmacologist Otto Loewi (1873–1961) and the British physiologist Henry Dale (1875–1968). For an extended period after Thudichum's death, 'brain chemistry' remained, on the other hand, remained fairly well within the boundaries set by Thudichum, although discoveries with future significance – such as the identification of gangliosides by Ernst Klenk (1896–1971) in the 1930s – were made (Sourkes, 2006). There were certainly voices early in the 20th century which suggested that 'humoral transmission' – chemically mediated information transfer between nerve cells – might occur in brain as it did in the periphery, but this phenomenon was much more difficult to demonstrate in the central nervous system than in neuromuscular junctions. Further, precise localization of putative humoral agents was impractical with available techniques, so that the concept of a pathway associated with a particular neurosubstance could not be developed. 'Neurochemistry' as such remained in its embryonic stages, and was still understood in the sense of Thudichum: cataloging and measurement of constituents, without producing a synthesis which might explain specific neural functions or disorders.

The first major new textbook on the subject, *Chemistry of the Brain*, was published by the American chemist Irvine Page (1901–1991) in 1937, but was concerned principally with general metabolic pathways, most of which the author conceded had not been extensively studied in nervous tissue. More surprising for the modern reader would be the thoughts included in his final chapter ('The brain and thought'):

> Mind and matter may be two aspects of universal stuff . . . thought is a spiritual manifestation . . . energy itself may be of spiritual origin (Page, 1937, p. 430).

But Page also noted that ''the various parts of the brain differ markedly in [chemical] composition, which leads one to suspect differences in function'' (p. 425). Similarly, the neuroanatomists Cécile (1875–1962) and Oskar Vogt (1870–1959), on the basis of their detailed investigations of basal ganglia architecture in the second decade of the 20th century, had concluded that the striatal system was especially prone to particular types of damage, and that this vulnerability was not due to differential perfusion, as commonly supposed, but was instead attributable to specific chemical characteristics of the striatal system which differentiated it from other central nervous system regions:

> By recognizing this non-homogenous chemical constitution [*Chemismus*], we have, however, established the necessary prerequisite for its detection, and further – as we have emphasized many times – will have thus also laid the essential basis for chemotherapy, which we believe appears to promise more success in the control of striatal disorders than any other therapeutic approach (Vogt and Vogt, 1920).

A similar thought was expressed by the eminent British neuropathologist, Joseph Godwin Greenfield (1884–1958) some 35 years later:

> Anatomical and histological studies seem unlikely to reveal much more of the pathogenesis of [parkinsonism]. The cause of the neuronal degeneration remains a problem whose solution may be found in enzyme chemistry, or some other new field of investigation (Greenfield, 1955).

The limits of neuroanatomy and neuropathology were clear: although neuropathologists could clearly demonstrate the neuropathological features of basal ganglia disease, this knowledge contributed little to understanding the etiology of such disorders, let alone to developing means for ameliorating their effects. Surgical interventions were necessarily experimental in nature, while pharmacological approaches were entirely empirical. Understanding the *Chemismus* of the brain would be required before rational therapeutic approaches could be developed.

This situation would not change significantly for some time, but by the 1940s there was a growing awareness of

the possibility that altered brain metabolism might be involved in neurological disease:

> Hedged about as it is with delicate restrictions, surely it is more possible to understand how pathologically the brain in its metabolism may not only be subjected to the action of toxins (the usual view), but occasionally fail owing to self poisoning with its own misguided machinery. So should we envisage a possible occasional development of mental abnormality (Peters, 1940).

The author of these lines, the Oxford biochemist Rudolph Albert Peters (1889–1982), commenced his essay (in an industry journal) with: ''Brain tissue is the most important biological invention in Nature.'' His review of the 'biochemistry of brain tissue', however, was concerned principally with factors modulating oxidative respiration, so that even acetylcholine was mentioned only in passing (Peters, 1940). A few years later, Derek Richter (1907–1995; Central Pathological Laboratory and Mill Hill Emergency Hospital, London) included in his review of the ''[b]iochemistry of the nervous system'' a section on acetylcholine, noradrenaline and related enzymes in the brain; the physiological significance of such molecules, however, was still controversial and their function in the brain a complete mystery: ''other investigators are unwilling to consider acetylcholine as anything more than an incidental by-product of nerve metabolism''. There was some discussion of the effects of pharmacological agents in the central nervous system, but even this was limited to the impact of convulsants and narcotics on central respiration (Richter, 1944).

But the move towards a more intimate analysis of central nervous system biochemistry had already begun. At the invitation of Richard Willstätter (1872–1942), Irvine Page had established in 1928 a department for brain chemistry at the Kaiser-Wilhelm-Institut für Psychiatrie in Munich, where he worked for three years with the aim of establishing a laboratory of neurochemistry and undertaking research which that gave ''fats and sterols a better name'' (Dustan, 1996). This was the first department specifically devoted purely to neurochemistry, and Page later remembered his disappointment that he had been unable to inaugurate such a facility in his home country. After his return to the United States, Page commenced work with Donald Van Slyke (1883–1971) in cardiovascular research, ultimately leading to the isolation in 1947 of the vasoconstrictor they named 'serotonin' (Page, 1957).

Dale had asked himself in 1934 whether acetylcholine or an acetylcholine-like substance might be concerned with normal transmission at central synapses, but had noted: ''With no direct experience of central nervous physiology, I cannot properly allow myself merely to speculate.'' Evidence supporting the hypothesis was obtained from studies of the effects of various acetylcholine-related substances on the electroencephalogram, but even then chemical transmission was not necessarily invoked. In his comprehensive 1945 review of ''present views on the mode of action of acetylcholine in the central nervous system'', the Cambridge physiologist Wilhelm Feldberg (1900–1993) presented evidence that atropine and acetylcholine produced opposite effects when applied to the central nervous system. In considering the effects of atropine when given alone, Feldberg commented:

> There is another well known action of atropine, its sedative effect on the rigidity and tremor of parkinsonism. It is tempting to regard this effect as a central atropine-acetylcholine antagonism, similar to that observed when both drugs are applied artificially to the central nervous system (Feldberg, 1945).

As atropine had only a minor effect on spontaneous and reflex activity in the central nervous system, Feldberg assumed that acetylcholine was released inside or at least very close to its target tissue, so that it was difficult to block its activity by the application of an antagonist, a solution which had been suggested by Dale and Gaddum to explain the same problem in the peripheral nervous system. For Feldberg, a role for acetylcholine in the central nervous system was thus highly probable; he was not, however, opposed to the idea that it might not be the universal central transmitter, and that electrical transmission might be important at some synapses.

Attempts to demonstrate the presence of acetylcholine in the brain had commenced at the end of the 19th century, but the first reliable report of its presence in brain tissue was published in 1931 (Chang and Gaddum, 1931). Although the broad regional distribution of acetylcholine in cat and dog brain had been reported by MacIntosh in 1941, it would not be until the 1960s that methods would be developed which allowed precise quantification of acetylcholine levels in nervous tissue. The Indian pharmacologist Dikshit used bioassays to demonstrate an acetylcholine-like substance in the cat basal ganglia in 1933, and suggested that acetylcholine release in the central nervous system by sensory elements of the vagus might be involved in central transmission. In place of direct measurement of acetylcholine, assay of enzyme activity involved in acetylcholine metabolism was employed to quasi map the transmitter. The catabolic enzyme choline esterase

was first mapped in the central nervous system by David Nachmansohn (1899–1983) in 1939 (then in Paris; see also Nachmansohn, 1972), and by Hans Birkhäuser (Medizinische Universitätsklinik, Basel) in 1940. Birkhäuser concluded:

> It is probable that high [choline] esterase levels in a particular tissue are indicative of significant nervous activity. Certain motor disturbances, as observed, for example, in schizophrenia, might be attributable to problems in enzymatic activity. Reduced AChE levels would lead to the accumulation of ACh, resulting in constant stimulation of the affected region of the central nervous system . . . Before one can investigate pathologic brains for enzyme levels, the occurrence of these substances in the normal organ must be established (Birkhäuser, 1940).

The next major step would thus be the mapping of potentially neuroactive substances in the brain, as it was accepted (although not unequivocally: see Burn et al., 1950) that localization was probably an indicator of function. Birkhäuser suggested that the distribution of monoamine oxidase (MAO) in the human brain might yield clues regarding central nervous system disease, and, employing a modification of the Warburg manometric method, measured high levels in thalamus and caudatus, somewhat lower activity in putamen, and lower values in pallidum and cortex. A quarter century would pass before the next report on regional MAO activity in human brain (Birkhäuser, 1940).

The distribution of brain esterase suggested to G. Weber (Neurochirurgische Klinik des Kantonspitals Zürich) in 1951 that the enzyme might be involved in ''the function of these regions [putamen, caudatus, pallidum] as transfer stations for incoming impulses''. He subsequently found that choline esterase activity was not detectable in putamen, pallidum and nucleus rubber of two post-encephalitic parkinsonian brains, perhaps explaining the effectiveness of anticholinergic drugs in such patients (Weber, 1952). In the meantime, Feldberg and Marthe Vogt (1903–2003) examined the enzyme choline acetyltransferase in forty distinct regions of the dog brain, and also identified high levels were found in the caudatus:

> the caudate nucleus belongs to a group of basal ganglia which inhibit voluntary impulses to the skeletal muscles. The fact that administration of atropine in Parkinsonism can partly compensate for the loss of these centres is interesting in this respect, although it is not possible at the moment to offer any explanation, since the mechanism of the inhibitory action of these centres is anything but understood (Feldberg and Vogt, 1948).

Chemical maps of the brain: catecholamines and serotonin

Technical developments between 1920 and the 1950s made possible the reintegration of concepts of brain chemistry and neural function, achieving what McIlwain (1991) later described as the recapture by neurochemistry of its specific biological components. Neurochemistry was ceasing to be a laboratory curiosity: it was developing into system which would allow direction manipulation of brain function by pharmacological intervention, thus opening the road to neuropharmacology.

Further, it was emerging as a defined field of enquiry in its own right. The term 'neurochemistry' appears to have been coined by Kenneth Allan Caldwell Elliott (1903–1986). Elliott established a brain chemistry research laboratory in the Montreal Neurological Institute in 1944; as the other departments already bore titles prefixed with 'neuro-', he decided that 'neurochemistry' would be an appropriate description for his section. Further, he defined this field of research as being ''the chemistry of brain and nerve'', aiming for ''the solution of problems concerned with injury and disease of the brain and nervous system and the mind'' (Elliott, 1949). Also pointing in the direction of the emergence of neurochemistry as an identifiable field was the subtitling of the proceedings of the 1954 ''Symposium on the Developing Nervous System'' with ''Proceedings of the First International Neurochemical Symposium'' (Waelsch, 1955). The term 'neurochemistry' was then employed by editors Elliott, Page and Judah Hirsch Quastel (1899–1987) as the uncomplicated title of their 1955 textbook. Although initially conceived as essentially being the new edition of Page's 1937 text, it developed into much more over the four years of its compilation:

> The rate of advance in the past 16 years has far outpaced that of the period between the previous two texts (Thudichum, 1884; Page, 1937). This recent rapid accumulation of information is widely scattered in many journals, some of them not obviously connected with Neurology. The time for integration of available data into broader truths and the recognition of Neurochemistry as a specific fields of research has arrived (Elliott et al., 1955, p. ix).

In the same year, McIlwain published *Biochemistry and the central nervous system*, in the preface to which he noted that around 3000 papers ''which concern chemical substances or processes and the central nervous stytem'' were appearing each year in various types of journals, as there was a clear overlap between biochemistry and several

other areas, particularly pharmacology and endocrinology (McIlwain, 1955, p. v) in physiological and chemical journals. A reviewer noted that the time was right for such books, as ''Biochemistry is now providing one of the main approaches to an understanding of many of the problems of neurology and psychiatry'' (Thompson, 1956).

The founding in 1956 of the *Journal of Neurochemistry* (later the official journal of the International Society for Neurochemistry, established 1967) was a further significant development, both marking the emergence of neurochemistry as a field distinct from general physiology and biochemistry as well as the acceptance of the term 'neurochemistry' itself. The journal, like neurochemistry itself, was not clinically oriented, but the productive coming together of clinic and laboratory was made possible by this forum dedicated to the specific chemistry of the brain. The focus of the journal changed with time as that of neurochemistry shifted: in 1956/7–1960, around 40% of papers concerned basic brain constituents, under 25% transmitters; in 1975–1980, these figures were about 15 and 40%, respectively, and in 1985–90 15 and 60% (Curzon, 1993).

Attention with regard to the brain shifted in the mid-1950s from acetylcholine to serotonin and the catecholamines. It was also during this period time that the connection between neurochemical findings and brain disease first received wider attention, a shift was facilitated by the introduction of the first neuroleptic agents, chlorpromazine (1949) and reserpine (1952). A range of pharmacological research tools available at this point favoured investigation of serotonin or 5-hydroxytryptamine (5-HT), previously regarded primarily as a vasoactive hormone, notwithstanding the fact that they would later prove to be less than specific for serotonergic systems: the psychotropic effects of lysergic acid diethylamide (LSD) and other indole-based compounds, the depletion of central 5-HT by reserpine, the elevation of its levels by the precursor 5-hydroxytryptophan (5-HTP) and the MAO inhibitor iproniazid. The result was that investigators asked whether altered human brain 5-HT levels might be involved in neurologic or psychiatric disease and whether pharmacological manipulation of its levels might provide solutions to these problems (Woolley, 1957). But interest in the catecholamines was also marked by the gradual elucidation of the noradrenaline synthetic pathway, a problem which had occupied biochemists and physiologists since the beginning of the century.

In 1954, Marthe Vogt published a landmark paper which would serve as example for those who followed her: ''The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs''. 'Sympathin' was the term employed for the mixture of adrenaline and noradrenaline in brain, the presence of which had previously been demonstrated by Ulf von Euler (1905–1983) and Peter Holtz (1902–1970). The aim of Vogt was simple but radical:

> The present work is concerned with the question whether these sympathomimetic amines, besides their role as transmitters at vasomotor endings, play a part in the function of the central nervous tissue itself (Vogt, 1954).

Vogt had reported briefly in 1952 (in German) that sympathin exhibited a distinct distribution pattern, and now undertook the detailed analysis of its localization in dog brain. Biological assays were used: measuring the effect of noradrenaline on rat blood pressure and adrenaline on the rat uterus, detection limits of about 10 ng (noradrenaline) and 5 ng (adrenaline) per gram wet tissue could be achieved. The highest sympathin concentrations were found in regions containing the diencephalic, mesencephalic and bulbar representations of sympathetic activities, as well as in the area postrema. She found that drugs which depleted peripheral catecholamines via central stimulation could sometimes also deplete central catecholamines if applied for prolonged periods. Vogt concluded that nothing could be surmised about the function of brain sympathin on the basis of her report; it was ''tempting'' to assign it a transmitter role corresponding to its function in the periphery, but she felt that the evidence for this interpretation was insufficient at this point (Vogt, 1954).

Meanwhile, Bernard Brodie and his group (Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda) were the driving force in 5-HT research, and were attempting to integrate the available neurochemical information into a bipolar schema which extended the divisions of the peripheral autonomic system into the central nervous system. In this model, noradrenaline was the neurohormone of the Hessian ergotropic system and serotonin that of the trophotropic system (Brodie et al., 1959; see also Costa et al., 1989; Kanigel, 1993). Brodie also remarked upon another important feature of neurochemistry as it was then emerging:

> The pharmacologist has long sought biochemical reasons for the action of drugs on various organs. Similarly the physiologist has striven to explain the function of organs in terms of biochemical processes. However, the gulf separating physiology and pharmacology on one side from biochemistry on the other is still precariously bridged. Perhaps the reason for this has been the rather common conviction that the specific organ function can be explained in terms of the ''universal'' reactions of intermediary metabolism (Brodie et al., 1959).

The limitations of models based on general biochemical principles for explaining the operation of the central nervous system were thus becoming evident.

In 1957 the Swedish pharmacologist Arvid Carlsson (*1923) published the first of a series of papers which would not only revolutionize brain chemistry, but also lead to its first directed clinical application. As mentioned above, the alkaloid reserpine had been employed since the mid-1950s as a sedative, and this action was attributed by Brodie to its depletion of brain 5-HT. Carlsson proposed that it was actually due to catecholamine depletion, and sought to resolve the issue by replenishing the central stores of individual monoamines in reserpinized animals. As serotonin and catecholamines themselves do not readily penetrate the blood–brain barrier, Carlsson administered their amino acid precursors, 5-hydroxytryptophan (5-HTP) or DOPA, respectively. 5-HTP alone did not relieve reserpine-induced sedation, whereas DOPA completely reversed tranquilization within half an hour. Carlsson also described the dramatic effect of intravenous DOPA in reserpinized rabbits: within 10–15 minutes, its sedative effects had been relieved, an effect recorded in a film with which he astounded conference participants in the following years. The DOPA dose required could be reduced by pre-treating the animals with iproniazid, a MAO inhibitor. Carlsson, however, was initially surprised by the discovery that noradrenaline levels were not markedly restored by DOPA administration in these experiments, and drew the bold conclusion that dopamine, hitherto regarded only as an intermediate in noradrenaline synthesis, might itself possess neuroactive properties.

Carlsson's report occupied about two-thirds of a page in the letters to the editor section of the 30 November 1957 issue of *Nature* (Carlsson, 1957a), and would later be nominated by many researchers as the paper which excited their interest in the possibility of an effective, rational biochemical therapy for Parkinson's disease. This and related publications from Carlsson's group (including Carlsson, 1957b, 1959; Carlsson et al., 1958; Bertler and Rosengren, 1959) and by Isamu Sano in Japan (1959) describing the localization of dopamine in the brain and its probable role in basal ganglia function paved the way to neurochemical analysis of central nervous function.

Despite these results, many senior researchers were of the opinion that chemical transmission played only a subordinate role in the brain, and when possible transmitters were discussed, the catecholamines were usually excluded. John Crossland (Department of Physiology, The University, St. Andrews) reviewed in 1957 the possibilities for chemical transmission in the CNS, including ACh, 5-HT, histamine and substance P, but specifically remarked that:

> It is remarkable that, although noradrenaline is known to be one of the non-cholinergic effector agents in the autonomic nervous system, it appears to have no such function at central synapses (Crossland, 1957).

At the First International Symposium on Catecholamines, held at the National Institutes for Health in Bethesda (October 1958), Seymour Kety (1915–2000), scientific director of the United States National Institute of Mental Health, nevertheless commented:

> It is quite apparent . . . that definitive knowledge [on the central action of catecholamines] has not kept pace with our comprehension of the metabolism and action of these important substances elsewhere in the body (Kety, 1959).

Perhaps most surprising was that Marthe Vogt was particularly cautious with regard to the interpretation of Carlsson's (and Brodie's) results:

> It will be clear from the foregoing discussion that our ignorance as regards the function of brain sympathin could not be more complete (Vogt, 1957).

Vogt was still sceptical when she again addressed the issue at the 1960 Ciba Symposium on Adrenergic Mechanisms:

> I am only trying to bring forward the evidence which is incompatible with the view that the level of catechol amines has some consistent correlation with behaviour, and the evidence that the level of 5-HT in the brain may determine certain aspects of behaviour. My personal view is that neither of these theories will have a long life (Vane et al., 1960, p. 578).

But many of the established pharmacologists and physiologists were of this view. Expressing his own assessment of the catecholamines discussion, the doyen of British pharmacology, John Gaddum (1900–1965) commented in his summation of the symposium:

> The meeting was in a critical mood, and no-one ventured to speculate on the relation between catechol amines and the function of the brain (Gaddum, 1960).

This view stood in abject contradiction to the sometime passionate discussion of just such 'speculations' which were recorded in the 40 pages of the record of the discussion (Vane et al., 1960, pp. 548–587). But the mood was changing, and the philosophy of this change was summarized by Hermann ('Hugh') Blaschko (1900–1993), who

had played a major role in the elucidation of the catecholamine synthetic pathway, at the same meeting:

> Biochemistry has ceased to be a refined kind of cookery; we no longer destroy all the structural elements in attempts to separate the chemical constituents of the tissues as pure compounds. This must still be done, but we also try to break up the tissues in a more controlled fashion, so as to keep the structures of subcellular size intact; and there is the parallel study by cytological methods . . . to find out where the structures isolated are situated in the intact cell (Vane et al., 1960, p. 578).

Blaschko noted, however, that little had emerged to date from the convergence of physiology and pharmacology, as the active substances occur at concentrations too small to localize precisely in the brain; nonetheless, methods were emerging which would overcome this problem.

Neurology, neurochemistry and pharmacology converge: the L-DOPA experiment

The final stage in this phase of the journey was initiated in 1960–1961 in Vienna. As Kety noted in 1961 at the Bel-Air Symposium on *Monoamines et système nerveux central*:

> One of the speakers yesterday mentioned that the court of last appeal is the practical effect on patients. We must not forget, however, that basic research can also be done in man, and clinical studies can contribute a great deal to a fundamental understanding of how these agents act and what are the rôles of the monoamines in the brain (Kety, 1962).

After studying with Blaschko in Oxford, Oleh Hornykiewicz (*1926) had established himself as the dopamine specialist in Vienna, and in 1960 he undertook the first neurochemically based neuropathological investigation of parkinsonism. He assessed dopamine and noradrenaline levels in the brainstem and basal ganglia of parkinsonian and normal brains. Confirming Carlsson's and Sano's results in the normal brain, Hornykiewicz and his assistant Ehringer also discovered the dramatic loss of striatal dopamine in parkinsonism:

> Instead of the pink color given by the relatively high concentrations of dopamine in the control samples, the reaction vials containing the extracts of the Parkinson's disease striatum showed hardly a tinge of pink discoloration. The brain dopamine deficiency in Parkinson's disease, today standard textbook knowledge . . . at that moment I literally could see it with my own naked eye! (Hornykiewicz, 1992)

Ehringer and Hornykiewicz (1960) had thereby provided the first evidence which linked a particular disorder with a specific central neurochemical defect. There had been earlier reports linking brain disorders to possible chemical anomalies, based on the effects of therapeutic drugs; but these remained hypothetical in the absence of direct proof.

Ehringer and Hornykiewicz had provided provocative evidence for the 'dopamine deficiency hypothesis' of parkinsonism, but one more step was required to confirm its clinical significance. Hornykiewicz suggested to Walter Birkmayer (1910–1996) that administration of the dopamine precursor, L-DOPA, to parkinsonian patients might improve their condition, and provided 2 *g* of the expensive amino acid for this purpose. In July 1961 Birkmayer administered 50 mg L-DOPA intravenously to a female postencephalitic parkinsonian patient, achieving dramatic results which he recorded on film:

> The effect of a single intravenous injection of L-DOPA in Parkinson's disease was, in short, the total abolition or the substantial reduction of akinesia. Patients who, when lying in their beds, could not sit themselves up; who could, when sitting, could not stand; or who, when standing, could not start walking, were able to accomplish these tasks with ease after L-DOPA. They walked with the normal associated swinging movements, they could even run and spring. The voiceless, aphonic speech, with its unclear, palilalic articulation, became as strong and clear as that of normal persons. The patients could, for a short period, carry out motor activities to a degree which had been thus far achieved under the influence of no other medicament. This DOPA effect reached its peak within 2.3 hours and lasted (to a lesser degree) for 24 hours (Birkmayer and Hornykiewicz, 1961).

The drama captured in the film of Birkmayer's first patient marked the beginning of the road to success of L-DOPA as the gold standard in the therapy of Parkinson's disease. But also significant was the fact that the administration of L-DOPA to parkinsonian patients was, in effect, a human experiment which confirmed the dopamine deficiency hypothesis of parkinsonism. The neurochemical change detected by Hornykiewicz was not linked in any way to the standard (anticholinergic) therapy of parkinsonism, his results could not have been predicted on the basis of previous clinical experience. The successful treatment of parkinsonian patients with L-DOPA by Birkmayer was thus the crucial proof of the clinical significance of their discovery. Equally importantly, however, was that it confirmed and underscored the validity and utility of the neurochemical approach to the investigation and therapy of neurolo-

gical disorders; not only mood and arousal, but also motor deficits attributed to irreversible and probably progressive damage of the central nervous system could, in principle, be managed – and perhaps cured – by the administration of specific neurochemical compounds. The revolutionary nature of this development can hardly be overstated. In the space of less than a decade – between Vogt's mapping of brain sympathin in 1954 and the successful therapy of parkinsonian patients with L-DOPA in 1961 – both psychiatric and neurological disease of the central nervous system had become valid targets for directed neurochemical investigation and, ultimately, therapy.

But the change in neuroscientific paradigm was not immediately embraced without reservation. Further investigation of catecholamine biochemistry and physiology – it should be borne in mind that 'receptors' were still essentially hypothetical constructs at this point, for instance – as well as the demonstration of the existence of catecholamine-utilizing pathways in the brain – both by means of functional and biochemical changes following disruption of these pathways (see for example, Poirier and Sourkes, 1965, as well as Portig and Vogt, 1969: wherein Vogt provides evidence for dopamine release in the caudatus!) and through visualization of these pathways with the fluorescent techniques then being developed in Sweden (Falck, 1962) – were required before the new approach to neurological function could be completely accepted by the biomedical community. But as early as 1965, Uvnäs could comment at the Symposium on ''Mechanisms of release of biogenic amines'' (Stockholm, February 1965):

> [Biogenic amines] have already been the principle topics at a number of Symposia and other scientific gatherings. That may be so, but there is one aspect which has been relatively little discussed. Not only do these amines play an important role as chemical mediators in the peripheral and central nervous system; more and more drugs are found to exert a similar action via the release of amines or by interfering with the release. Release of amines is implicated in disturbances characteristic of various diseases (Uvnäs, 1966).

The introduction of L-DOPA therapy for parkinsonism represented the provisional culmination of this phase in the evolution of neurochemistry: it was at this stage that clinic, neurochemistry and neuroanatomy converged, although it was not immediately recognized. Were Ehringer and Hornykiewicz to report the dopamine deficiency for the first time today, there would be a rush of papers from other workers in a very short time confirming, refuting and qualifying their findings. The early successes of Birkmayer and others with L-DOPA, though not entirely satisfactory, would stimulate much greater interest in further examination of the therapy today than they did in 1961.

It is also interesting that, at about the same time, the lead in pharmacological research moved definitively from the clinic to the laboratory. Whereas the history of the therapy of brain disorders had previously been punctuated by the introduction by individual clinicians of novel or modified therapies, established more on the basis of empiricism, personal experience serendipity than theoretical considerations, the move to 'rational therapeutics' placed the emphasis on laboratory testing and theoretical underpinning of new approaches. This was not only the result of advancing knowledge and technology, but also of the new style of management of drug development, as well as the gradual legal recognition of patients' rights and the need to set limits to the experimental élan of medical innovators. The requirement for long term large scale trials before a drug could be marketed with specific claims placed such investigations well outside the scope of individual scientists or clinicians; cooperation between various types of investigators and between different institutions became an absolute requirement. This also meant that the role of the drug company was altered; the cost and scale of such projects could only be managed by a large organization. Further, it is rather unusual today for a drug promoted by an individual or extracted by a plant collector to attract much attention in the general scientific or medical community; the pharmaceutical firms thus control to a significant degree the direction in which new development occurs.

These changes were not universally welcomed by clinical researchers. Frustration with the increased degree of regulation of medical research was clearly expressed, for example, by Lewis Doshay (1896–1965), largely responsible for the introduction of the anti-parkinsonian drug 'Artane' in the early 1950s. In 1965 he commented that a ''competent and careful investigator'' could achieve evaluations which were at least as informative as objective measuring devices by careful long-term, large scale clinical investigations, and that the outcome of the new regulations was that ''freedom of investigation no longer exists and the patients wait in vain for new and better remedies'' (Doshay, 1965).

It is also appropriate here to recapitulate the question of what constitutes a 'rational therapy'. Empirically determined therapies of neurological disorders were by no means 'irrational' in the sense of 'without basis' or 'arbitrary'. Earlier therapies functioned to a degree, and the continued employment of these approaches, is attributable to their

being the subject of rational and scientific trial and selection. Both this success and the problems associated with these therapies, in turn, had an impact on the development of the neurosciences; this impact, however, was limited until the concept of the central nervous system as a chemical system had been developed and, equally importantly, accepted.

The manner in which the L-DOPA therapy for parkinsonism emerged turned this relationship on its head. A string of findings in the laboratory – from Brodie's discovery of the amine-releasing properties of reserpine to Carlsson's discovery that DOPA countered the behavioural effects of reserpine, and finally the identification of dopamine accumulation in the basal ganglia of animals and then of humans (which depended on the availability of suitable techniques), combined with clinical observations that firstly, reserpine induced a parkinsonism-like state in man and secondly, that the nature of the motor and vegetative symptoms of natural parkinsonism suggested that the neurochemical substances being examined in the laboratory might be involved – hinted that a basal ganglia dopamine deficiency might underlie parkinsonism. This deficiency was then sought and found, and finally, as the practical outcome of this chain of events, L-DOPA was finally administered to patients. In other words, pathological states yielded clues regarding the function of the normal or healthy brain, and parkinsonian patients were, in a certain sense, the experimental subjects in an extended experiment. That was the real scientific triumph of L-DOPA therapy: the successful extrapolation of basic research into the clinic, the demonstration that medical research by chemists, biologists and others would play a far more active role in the development of novel therapeutic strategies than had previously been possible with the invaluable but largely descriptive research of the neuropathologist.

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J Neural Transm (2007) [Suppl 72]: 17–28

Printed in Austria

Journal of Neural Transmission

Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y

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Summary Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder in industrialized countries. Present cell culture models for PD rely on either primary cells or immortal cell lines, neither of which allow for long-term experiments on a constant population, a crucial requisite for a realistic model of slowly progressing neurodegenerative diseases.

We differentiated SH-SY5Y human dopaminergic neuroblastoma cells to a neuronal-like state in a perfusion culture system using a combination of retinoic acid and mitotic inhibitors. The cells could be cultivated for two months without the need for passage. We show, by various means, that the differentiated cells exhibit, at the molecular level, many neuronal properties not characteristic to the starting line.

This approach opens the possibility to develop chronic models, in which the effect of perturbations and putative counteracting strategies can be monitored over long periods of time in a quasi-stable cell population.

Keywords: Dopaminergic neurons, mitotic inhibitors, neuronal differentiation, neuronal markers, perfusion culture, retinoic acid

Abbreviations

araC	cytosine β-D-arabinofuranoside
BDNF	brain derived neurotrophic factor
BrdU	bromodeoxyuridine
DA	dopamine
DAT	dopamine transporter
DRD2	dopamine receptors type 2
FBS	fetal bovine serum
FdUr	5-fluoro-2′-deoxyuridine
HMBS	hydroxymethylbilane synthase
HRP	horseradish peroxidase
MAP-2	microtubule-associated protein 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NeuN	neuronal nuclei
NeuroD1	neurogenic differentiation 1
PD	Parkinson's disease
PDL	poly-D-lysine
RA	retinoic acid
SN	*substantia nigra*
TH	tyrosine hydroxylase
Ur	uridine

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Introduction

PD is a slowly progressive degenerative neurological disorder resulting from a degeneration of dopamine-producing neurons in the *substantia nigra* (SN) (Dauer and Przedborski, 2003). Various *in vivo* and *in vitro* models exist for PD. The most prevalent *in vivo* models rely on rodents and primates. However, such models are inherently expensive, there is an interspecies variability and also animal-to-animal variation in sensitivity to specific neurotoxins and drugs used (Bove et al., 2005).

The present *in vitro* (cell culture) models use primary cells or immortal cell lines. Neither cell type, however, represents a suitable model for a chronic, progressive disease such as PD. Primary cells cannot be cultured for a sufficiently long period due to the onset of replicative senescence (Blander et al., 2003), while immortal cells replicate too quickly for long-term effects on a cell to be determined. In the latter case, the cells are typically differentiated for 2–3 days, until then they sprout neurite-like processes. Regardless of the source, cells are treated with neurotoxins for a short period of time, on the order of 3–5 days. This is far from optimal if one wants to establish a chronic model.

Usage of rodent cells (be it primary or immortalized lines, such as PC12) faces the added problem of slight but relevant metabolic differences between rodents and humans (Herman, 2002). Human dopaminergic neuroblastoma cell lines are better suited for developing PD models

because they have biochemical properties of human neurons *in vivo* (Sherer et al., 2001). Moreover, since they are tumor derived cell lines, they continuously divide and can provide the required quantity of cells for different experiments, without exhibiting a large variability (Biedler et al., 1973). However, these cell lines do not have all the characteristics of adult neurons in the brain, and, due to immortality, still have the disadvantage of a short doubling time (Biedler et al., 1973). One way to circumvent these shortcomings is differentiation of these cells to dopaminergic, neuron-like, cells.

Neuronal differentiation can be induced *in vitro* by exposure to different agents such as: tetradecanoylphorbol acetate, brain derived neurotrophic factor (BDNF), norepinephrine, retinoic acid (RA) etc. (Encinas et al., 2000; Laifenfeld et al., 2002; Presgraves et al., 2004). In the case of RA-induced differentiation, one can observe the formation of neurites whose length increases with time of exposure. Moreover, there is an increased synthesis of neurospecific enzymes (such as acetylcolinesterase), neurotransmitters (catecholamines like dopamine, DA), changes in the cytoskeleton markers (neurofilaments) and electrophysiologic modifications as seen in normal neurons (Melino et al., 1997). All these effects are due to RA induction of numerous gene products, including transcription factors, structural proteins, neurotransmitters, neuropeptide hormones, growth factors, enzymes and cell surface receptors (Maden and Hind, 2003). After treatment with RA, cells arrest in the G1-phase of the cell cycle, DNA synthesis is inhibited and growth inhibition can be detected already at 48 h after treatment (Melino et al., 1997).

Most differentiation protocols for the SH-SY5Y cell line involve usage of RA as sole differentiation factor, with differentiation performed over a few days. After this differentiation period, cells were considered to be differentiated based primarily on their morphology, without much additional characterization. In several studies, SH-SY5Y cells were treated 48 h with 10 μM RA and the differentiation was assessed by measuring the neurite length, i.e. the neurites had to be longer than 50 μm (Nicolini et al., 1998). Due to the short differentiation protocol (which is insufficient for a terminal differentiation), the follow-up experiments with neurotoxins had to be performed over 24 h, which necessitated high doses of neurotoxins, not physiologically relevant. Similarly, Maruyama et al. (1997) differentiated SH-SY5Y cells for 3 days with 10 μM RA, but differentiation was appreciated purely on the basis of morphological changes and arrest of proliferation. It is unclear whether cells differentiated this way accurately exhibit neuronal characteristics without a detailed molecular analysis.

During *in vivo* neurodifferentiation various proteins experience changes in their expression levels as a consequence of cellular specialization. In order to compare undifferentiated with differentiated cells, the following neuronal markers were interesting for us. Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in the synthesis of DA and other catecholamines, namely the conversion of tyrosine to dihydroxyphenylalanine. This makes TH the marker of choice for dopaminergic neurons (Gates et al., 2006). At the subcellular level, TH is found in small, punctate structures (Hashemi et al., 2003). Synaptophysin, which is an integral membrane glycoprotein, is a marker for synaptic vesicles that store and release classical neurotransmitters. Thus, its presence indicates secretory activity typical for neurons and neuroendocrine cells (Gaardsvoll et al., 1988). Dopamine receptors type 2 (DRD2) are expressed in neurons of the midbrain, caudate and limbic system (Nestler and Aghajanian, 1997). Dopamine transporter (DAT) is a sodium-dependent DA reuptake carrier expressed only in dopaminergic neurons and has higher levels of expression in SN *pars compacta* (Storch et al., 2004). Microtubule-associated protein 2 (MAP-2) is an abundant neuronal cytoskeletal phosphoprotein that binds to tubulin and stabilizes microtubules, essential for the development and maintenance of neuronal morphology, cytoskeleton dynamics and organelle trafficking (Binder et al., 1985). Tau is a heterogeneous group of microtubule stabilizing proteins associated with several diseases. In the normal brain, Tau is localized in the axons of neurons (Wood et al., 1986). βIII-tubulin is a neuron-specific class of tubulin. During development, the relative abundance of this protein increases with the rate of neuronal differentiation (Lee et al., 1990). Nestin is a member of the family of intermediate filaments and is expressed mainly in neuroepithelial stem cells/precursors. Nestin is not expressed in mature cells and terminal neuronal cell differentiation is associated with down-regulation of this protein (Duggal and Hammond, 2002). Laminin is a major glycoprotein component of basement membrane involved in neuronal survival, differentiation, growth cone guidance and neurite growth (Timpl and Brown, 1994). Neuronal nuclei (NeuN) is a vertebrate neuron-specific nuclear antigen with unknown function. Developmentally, NeuN immunoreactivity is observed after the neurons become postmitotic and no reactivity has been observed in the proliferative zones (Mullen et al., 1992). Neurogenin is a transcription factor that induces neurogenesis and inhibits the differentiation of neural stem cells into astrocytes (Ma et al., 1996). Neurogenic differentiation 1 (NeuroD1) is a member of the basic helix-loop-helix transcription factors family implicated in

growth and differentiation of neurons and is expressed in postmitotic cells (Lee et al., 1995). A suitable model for PD should use cells that exhibit as many of these markers as possible.

Encinas et al. (2000) established a differentiation protocol for SH-SY5Y cells using RA and BDNF. They obtained homogenous populations of fully differentiated neuronal cells and thoroughly analyzed the differentiated cells by different methods. This is one of the few studies (Rebhan et al., 1994; Encinas et al., 2000; Edsjo et al., 2003) in which the cells were differentiated up to 12 days. Also, it is one of the rare examples where differentiated cells were extensively characterized by analyzing different neuronal markers. However, the system they developed would not have been suited for the long-term, perfusion, culture system we aimed to develop. A perfusion culture system is characterized by constant, slow addition of fresh media and removal, at the same rate, of the used media. This procedure has the advantage that, especially for long-term culture, the cells are kept in a quasi-constant environment, avoiding both sudden changes in the concentrations of nutrients and accumulation of toxic metabolites (Minuth et al., 1999). Since we planned to cultivate the cells for weeks, under constant renewal of medium, the cost of BDNF to be added to the culture medium would have been very high. This required the establishment of a different protocol for differentiation, which would not rely on expensive growth factors.

The cell culture presented in this paper yields differentiated cells that are very close to primary dopaminergic neurons. These differentiated cells present many neuronal markers at both mRNA and protein levels. Furthermore, we show that, as a consequence of differentiation, these cells exhibit a decrease of the mitotic active, proliferating population. Thus, such a culture is best suited for a long-term chronic intoxication and treatment strategy as would be the case for a PD model.

Materials and methods

Cell culture

SH-SY5Y cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were grown to confluence in T-25 flasks (Nunc) in Dulbecco's Modified Eagles Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, 1000 mg/l D-glucose and aminoacids (Gibco/Invitrogen #31885) to which were added 20% heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and Hepes (10 mM), in a 5% CO_2 humidified incubator at 37°C. Cultures were split twice a week and cells were seeded at 2.5×10^4 cells/cm^2.

Cells were plated at 2×10^5 cells/coverslip in 1 ml medium on 12 mm glass coverslips precoated with poly-D-lysine (PDL) (Beckton-Dickison). Plated coverslips were maintained in 4-well dishes (Nunc), in DMEM supplemented with 20% FBS, in a 5% CO_2 incubator for two days at 37°C in order to allow the cells to better adhere to coverslips and multiply them to the necessary density. Primary rodent cultures were kindly provided by G. Gille's group and were prepared according to Gille et al. (2002). After two days in these conditions, the coverslips were transferred into the perfusion culture system (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany). The system was connected to a peristaltic pump (Ismatec), which was set to 1 ml/h, equivalent to a total medium exchange within 3.5 h for the 6 coverslips perfusion container. Differentiation was started in L-15 medium (Invitrogen) supplemented with 10% FBS and all-*trans* retinoic acid (RA, 10 µM final concentration) for 14 days. After this, RA was removed from media and mitotic inhibitors (10 µM FdUr, 10 µM Ur and 1 µM araC) were added for the following 10 days. After these 10 days, the medium was supplemented only with FdUr and Ur for the rest of the time in culture. The protocol was modeled after Pleasure et al. (1992). Treatment with these mitotic inhibitors was typically performed for a total of 16 days.

Table 1. *List of antibodies used in the present work*

Antibody (reported specificity)	Protein accession number for the human counterpart	Supplier	Fold dilution	
			WB	IF
Tau (rabbit)	P27348	Chemicon	1000	100
TH (mouse)	P07101	Chemicon	1000	1000
MAP2 (rabbit)	P11137	Chemicon – Boehringer Mannheim	2000	1000
βIII tubulin (mouse)	Q13509	Sigma	1000	1000
Nestin (mouse), human specific	P04179	Chemicon	1000	1000
DAT (rat)	Q01959	Advanced Targeting Systems, San Diego, CA	500	NT
Synaptophysin (mouse)	P08247	Chemicon	500	100
NeuN (mouse)	Antigen identity unknown	Chemicon	1000	500
α tubulin (DM1α mouse)	NA	Sigma	NA	500
BrdU (rat) BU1/75	NA	abcam, Cambridge, UK	NA	200
Laminin B2 chain (rat)	P55268	Chemicon	1000	1000
Donkey anti mouse, donkey anti rabbit, TexasRed coupled	NA	BioRad	NA	100
Alexa Fluor 594 donkey anti rat	NA	Molecular Probes	NA	500
Anti actin (mouse), monoclonal AC-40	P68032	MPI-CBG Dresden, Antibody Facility	2500	NA
HRP-coupled secondary	NA	BioRad	3000	NA

NA not applicable, *NT* not tried, *WB* Western blotting, *IF* immunofluorescence.

Table 2. *List of primers used in the present work*

Gene symbol/name	Primer sequence 5′–3′ forward/reverse	Product length (bp)
DAT	Fw 5′-GAC TTT CTC CTG TCC GTC ATT GGC T-3′	278
	Rv 5′-GAG AAG AGA TAG TGC AGC GCC CAG-3′	
Tau	Fw 5′-GCG GCA GTG TGC AAA TAG TCT ACA A-3′	203
	Rv 5′-GGA AGG TCA GCT TGT GGG TTT CAA T-3′	
MAP2	Fw 5′-CAT GGG TCA CAG GGC ACC TAT TC-3′	209
	Rv 5′-GGT GGA GAA GGA GGC AGA TTA GCT G-3′	
DRD2	Fw 5′-TGC AGA CCA CCA CCA ACT ACC TGA T-3′	224
	Rv 5′-GAG CTG TAG CGC GTA TTG TAC AGC AT-3′	
Synaptophysin	Fw 5′-ATT GTG CCA ACA AGA CCG AGA GT-3′	195
	Rv 5′-CAG GAA GAT GTA GGT GGC CAG AG-3′	
Laminin	Fw 5′-GTT TAA CGA TCC CAA AGT TCT CAA GTC C-3′	208
	Rv 5′-GCA GGC ATT CAC TGG CAC TTT CC-3′	
HMBS	Fw 5′-TCG GGG AAA CCT CAA CAC C-3′	155
	Rv 5′-CCT GGC CCA CAG CAT ACA T-3′	
Nestin	Fw 5′-TGG CTC AGA GGA AGA GTC TGA-3′	148
	Rv 5′-TCC CCC ATT TAC ATG CTG TGA-3′	
βIII tubulin	Fw 5′-GGC CTC TTC TCA CAA GTA CG-3′	317
	Rv 5′-CCA CTC TGA CCA AAG ATG AAA-3′	
Neurogenin1	Fw 5′-GCC TAC AAC TAC ATC TGG GCT CTG-3′	173
	Rv 5′-GGC TGG GCT ACT GGG GTC A-3′	
NeuroD1	Fw 5′-CCG TCC GCC GAG TTT G-3′	118
	Rv 5′-GCG GTG CCT GAG AAG ATT G-3′	
TH	Fw 5′-GCC CTAC CAA GAC CAG ACG TA -3′	90
	Rv 5′-CGT GAG GCA TAG CTC CTG A-3′	

Quantitative real-time RT-PCR analysis

Total cellular RNA was extracted from undifferentiated and differentiated cells using the RNeasy total RNA purification mini kit (Qiagen) followed by treatment with RNAse-free DNAse. The reverse transcription was performed with SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen) and the obtained cDNA was used for the real time PCR reaction at 1 μg DNA/reaction. The DNA was amplified in a MX3000P thermocycler (Stratagene) using Brillant SYBR Green QPCR Master Mix (Stratagene) with primers at 1 μM final concentration using an annealing temperature of 60°C. Primer sequences (forward, reverse) and expected lengths of the amplified products are listed in the Table 2. Results are expressed relative to the housekeeping gene hydroxymethylbilane synthase (HMBS) that is considered to be the unity.

Western blotting analysis

Differentiated cells were removed from coverslips and undifferentiated cells were removed from flasks with trypsin/EDTA, washed with PBS and incubated with hot Laemmli sample buffer supplemented with Complete protease inhibitors (Roche) for 10 min. The protein concentration was determined using the BCA protein assay kit (Pierce). Ten micrograms of protein were loaded per minigel lane and separated on a 4–20% SDS-polyacrylamide gradient gel (Invitrogen), then electroblotted onto the nitrocellulose membrane (Schleicher and Schuell, 0.22 μm). Blocking was performed with a PBS/5% skimmed milk/0.5% Tween-20 solution. Membrane strips were incubated with the primary antibody (see Table 1) at 1 μg/mL, washed and incubated with the secondary, horseradish peroxidase (HRP)-coupled antibodies. Protein bands were revealed with the Enhanced chemiluminescence kit (Amersham) and recorded on Amersham Hyperfilm. Gels were scanned and lane densitometry analysis was performed using the ImageJ software (Rasband, 2006). Molecular weight was estimated using MagicMark (Invitrogen) and Prestained Protein Marker, Broad Range (New England Biolabs).

Immunofluorescence characterization of differentiated cells

Undifferentiated and differentiated SH-SY5Y cells and rodent primary dopaminergic neurons, all cultivated on glass coverslips precoated with PDL, were fixed in 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated in blocking buffer (2% BSA in PBS) for 20 min and then incubated with various primary antibodies for 30 min. Subsequently, the cells were incubated with the secondary, Texas Red coupled antibody, and, after a brief wash, with the FITC-coupled anti-tubulin antibody DM1α, in order to counterstain for the cytoskeleton. Finally, the coverslips were mounted on microscope slides in mounting medium containing *p*-phenylenediamine as antifade and DAPI for DNA staining. Fluorescence images were acquired using two microscopes: a Leica DMIRE2 inverted microscope equipped with a Leica DC350FX camera and a Zeiss Axioplan 2 equipped with a Hamamatsu C4742-95 camera.

BrdU incorporation

Undifferentiated and differentiated SH-SY5Y cells were incubated for 72 h in media supplemented with 10 μM BrdU (differentiated cells in the absence of mitotic inhibitors). For immunofluorescence, cells were treated as above, with the exception that the permeabilization step was followed by a DNA denaturation using 4N HCl at room temperature for 15 min in order to make the DNA accessible to the antibody.

Statistics

Results were obtained, in general, from at least three independent experiments (six for the RT-PCR analysis). Results are presented as mean values and error bars represent SEM. For assessing difference, two-tailed Student's *t*-test or Mann–Whitney test for unpaired samples were performed using the program InStat, with p values <0.05 considered significant (*).

Results

Differentiation of the SH-SY5Y cell line

The differentiation process was performed with RA in a perfusion system that requires the cells to be grown on coverslips. We used PDL precoated coverslips, since cells adhered poorly on plain glass and plastic coverslips were not suitable for fluorescence microscopy.

Morphological changes were seen for most cells after just 3–5 days, consistent with other reports (Encinas et al., 2000). Many cells elongated and extended neuritic processes (Fig. 1B). However, the original SH-SY5Y culture is comprised of two types of cell populations, which can actively interconvert: the substrate-adherent, differentiation resistant 'S' subtype and the neuronal-like, RA-sensitive, 'N' subtype (Ross et al., 1983). Due to the incapability of RA to induce differentiation (and thus growth arrest) of the 'S' subtype, this population would have overtaken the 'N' population. In order to filter out the undifferentiated cells, we used mitotic inhibitors (araC, Ur and FdUr), in the absence of RA for another 16 days. After this step, cells formed clusters connected via long processes that resembled axons. In order to make a valid comparison, the differentiated cells were compared not only with undiffer-

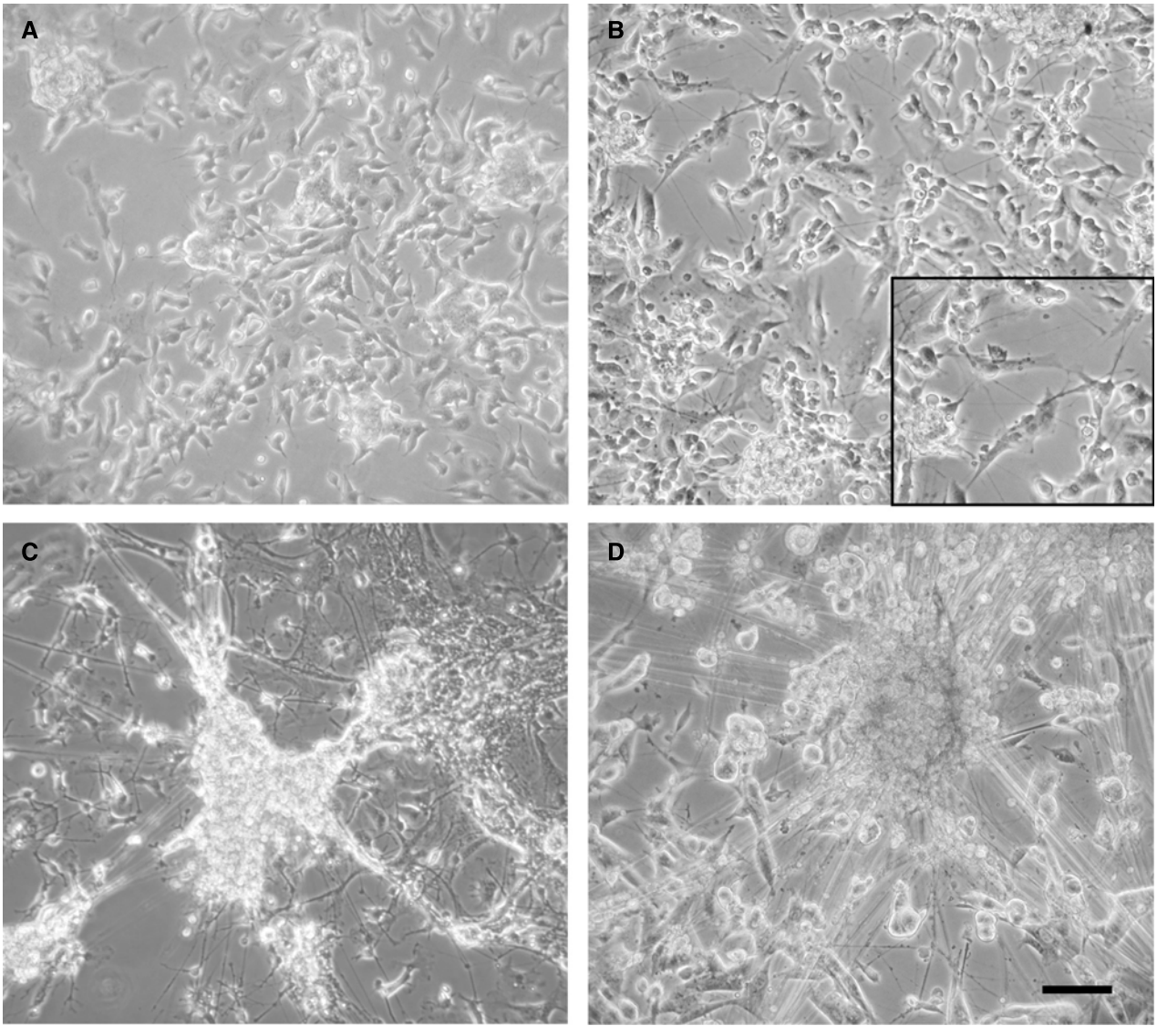

Fig. 1. Morphological comparison between undifferentiated and differentiated SH-SY5Y cells and primary dopaminergic neurons. **A** SH-SY5Y undifferentiated. **B** Eight days RA treatment. **C** Primary rodent dopaminergic neurons cultivated on the same type of coverslips. **D** 14 days RA and 15 days mitotic inhibitors treatment. Inset in **B** emphasizes neurite-like process formation; magnification twice as in the other panels. Scale bar 100 µm

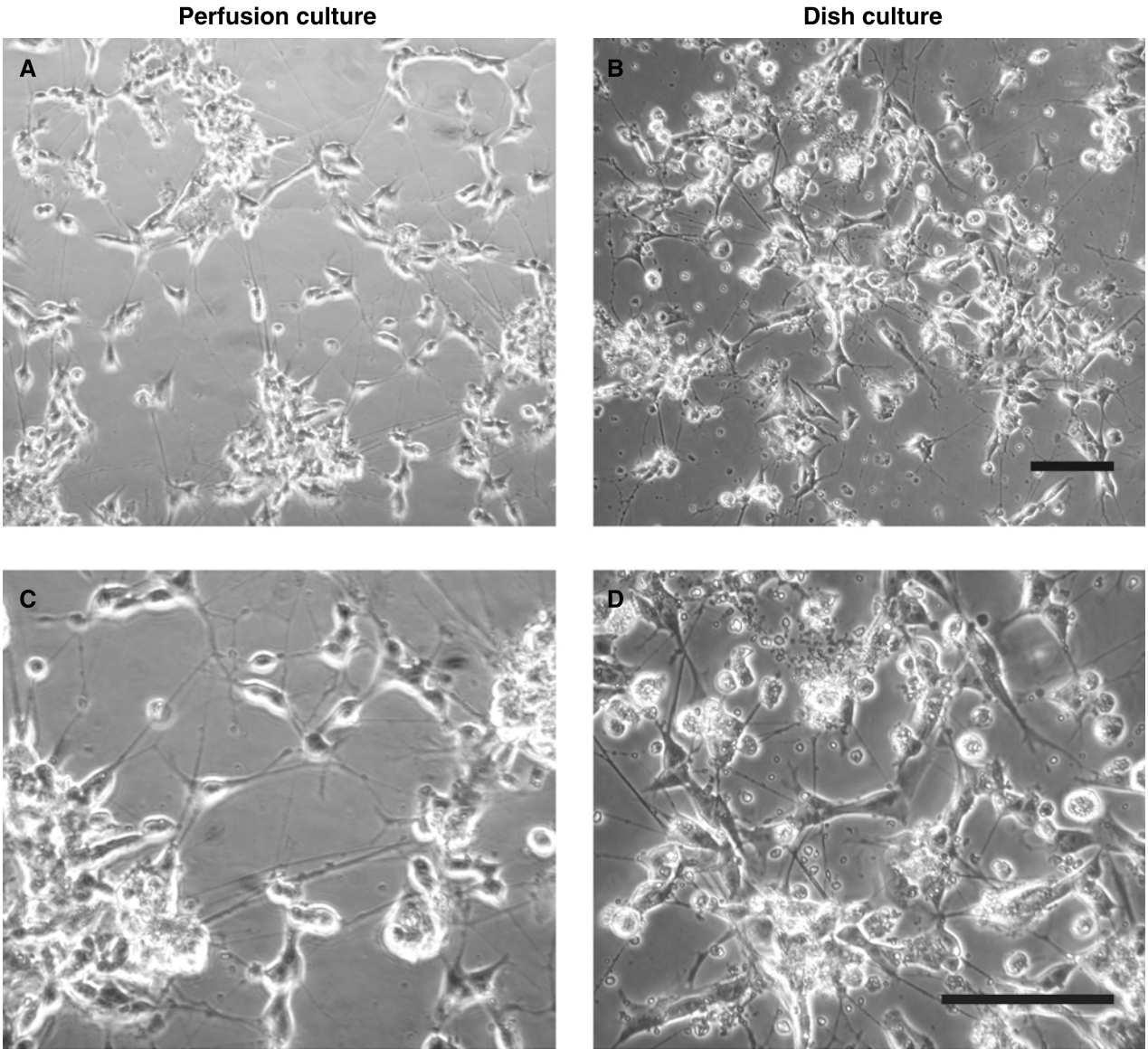

Fig. 2. Comparison between perfusion and plate cultivation during differentiation of cells (14 days RA and seven days mitotic inhibitors). **A** and **C** Perfusion. **B** and **D** dish. Note in **B** and **D** many apoptotic cells (round, bright floating cells) and many cells with a fibroblast-like morphology. Scale bar 100 µm. Same magnification for **A** and **B**, respectively **C** and **D**

entiated SH-SY5Y cells (cultivated in DMEM-20% FBS medium) but also with rodent primary dopaminergic neurons cultivated on identical coverslips (Fig. 1C and D). The differentiated cells had a morphology similar to rodent primary dopaminergic neurons. The differentiation process seemed more successful in the perfusion system compared with the classic cell culture method. As it can be seen in Fig. 2, in the dish culture there are more apoptotic, round cells, compared to perfusion culture (panels B and A, respectively). Moreover, in panel D (dish culture), many more fibroblast-like, undifferentiated cells can be observed compared to panel C.

BrdU staining for proliferation control

It is widely accepted that most of the neuronal cells in the adult brain cease dividing (Cajal, 1928; Gage, 2002). There is evidence for new neurons in the adult mammalian brain. However, proliferation is confined to the olfactory bulb and dentate gyrus (Rakic, 2002). Since the cells seemed to develop a neuronal morphology, and in order to test the efficiency of the mitotic inhibitors treatment, cell duplication was assessed by BrdU incorporation into cellular DNA.

Both types of cells (undifferentiated and differentiated, the latter in the absence of mitotic inhibitors) were incubat-

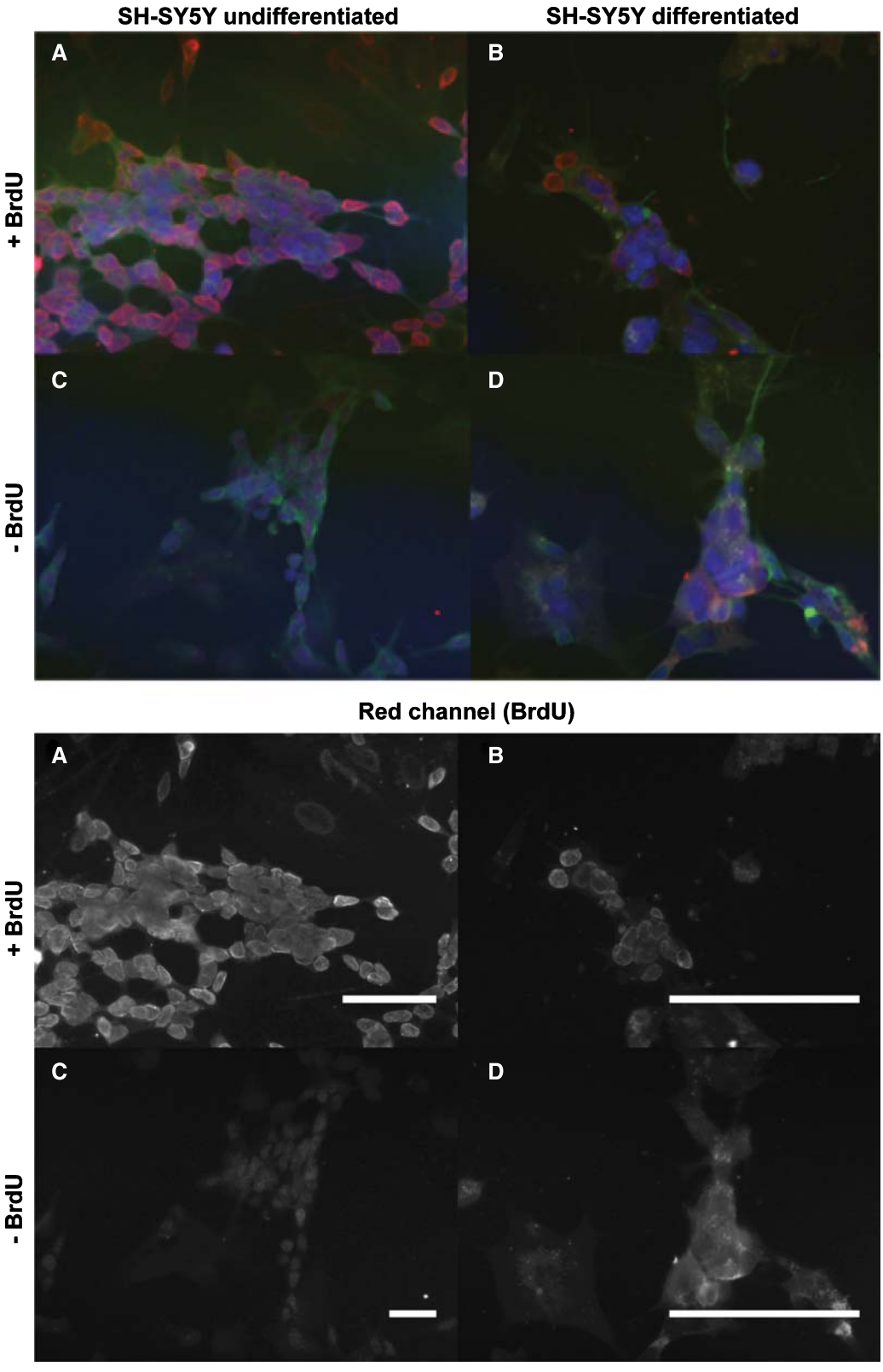

Fig. 3. BrdU incorporation as a control for cell cycle arrest. Top panel: **A** undifferentiated SH-SY5Y cells + BrdU. **B** Differentiated SH-SY5Y cells + BrdU. **C** and **D** No BrdU added (negative control). **C** Undifferentiated SH-SY5Y cells. **D** Differentiated SH-SY5Y cells. Lower panel: the BrdU channel from the top panel (cells have autofluorescence that increases with differentiation). Red: BrdU, Blue: DAPI, Green: tubulin. Scale bar 100 µm

ed with medium containing BrdU for 72 h and subsequently stained for BrdU incorporation. The negative control (undifferentiated and differentiated cells not treated with BrdU, but stained as the other ones) showed that the cells exhibit autofluorescence, which increases after differentiation (Fig. 3, the red channel). The BrdU signal in the differentiated cells is very close to the background (compare panels B and D), whereas the undifferentiated cells incorporated BrdU and led to a strong signal (in panel A) compared to their corresponding control (panel C).

Thus, we concluded that the cell divisions markedly slowed down after the mitotic inhibitors treatment and the differentiated cells are closer to "real" (slow dividing) neurons.

RT-PCR results confirm the differentiation of the cells

To confirm differentiation, we examined several neuronal markers. Mature neurons express specific markers that identify their specialized role in the nervous system. From various known neuronal markers, the twelve presented in the introduction were chosen for this study with the rationale that an increase in their expression (with the exception of nestin) would indicate that the cells are progressing towards a more neuronal phenotype.

As expected, RT-PCR results showed that the mRNA of many neuronal markers increased after differentiation (Fig. 4). For example, a significant change ($p < 0.05$) was observed for Neurogenin, tau, laminin and DRD2, while the message for other proteins (such as MAP2 and DAT) was increased, even if not at a statistically significant level.

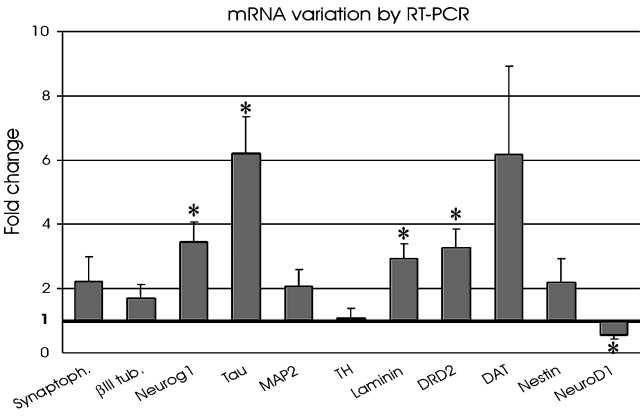

Fig. 4. Variation of neuronal markers after differentiation. Marker mRNA level quantification by QPCR, normalized to undifferentiated cells and HMBS. Reference level is one (mRNA level of marker in undifferentiated cells). The mRNA level decreases after differentiation for NeuroD1 and increases for all the other markers analysed. Asterisk mark statistically significant changes, i.e. $p < 0.05$

Thus, the RT-PCR results suggest that the treatment with RA and mitotic inhibitors led to an increase of the message for many neuronal markers.

Western blotting analysis

To confirm that changes in mRNA level resulted in changes in protein levels, we examined candidate markers by Western blotting. The bands corresponding to the proteins of interest (Fig. 5) were quantified using ImageJ and the β-actin band as a reference (Fig. 6).

Since not all proteins have a commercial antibody available, some antibodies are better than others and several large proteins are difficult to transfer, only a subset of the

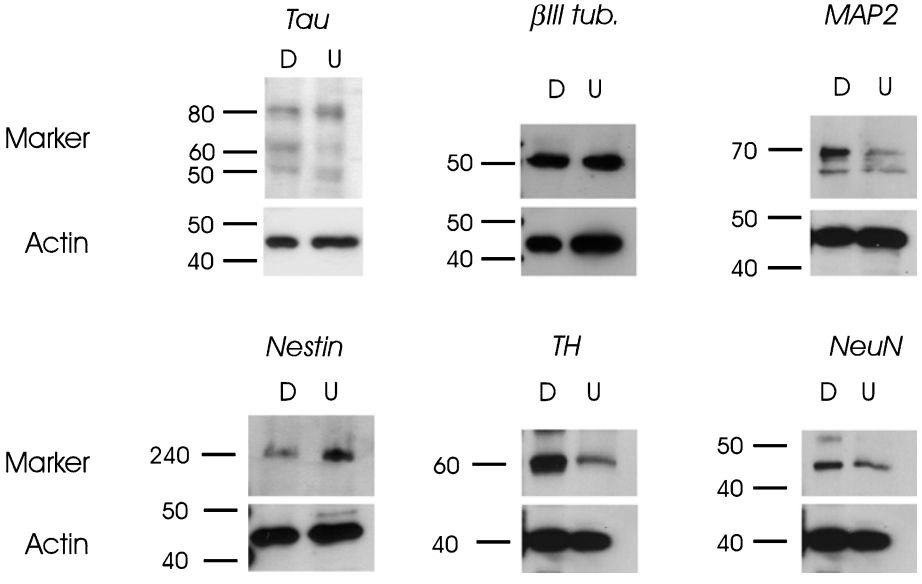

Fig. 5. Western blot analysis of marker proteins. D means differentiated SH-SY5Y cells. U means undifferentiated SH-SY5Y cells. Actin was used as loading control. Numbers on the left represent the molecular weight in kDa

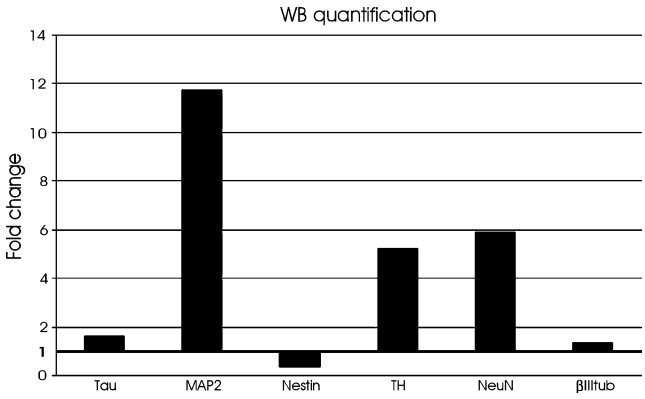

Fig. 6. Quantification of Western blot results for marker proteins from Fig. 5 and two other independent experiments. Comparison between undifferentiated and differentiated SH-SY5Y cells. A level of one means marker protein level unchanged with respect to undifferentiated cells (reference level). Nestin protein levels decrease, as is the case in neurons. Other markers show an increase in protein amount after differentiation

markers tested by RT-PCR could be assessed by Western blotting. Based on the results of Western blotting, MAP2, TH and NeuN increased following the differentiation protocol. Moreover, nestin, a marker for neuronal progenitor cells that decreases during differentiation, was decreased in SH-SY5Y differentiated cells (see Figs. 5 and 6), compared to undifferentiated SH-SY5Y. We concluded that the mRNA of the upregulated genes was indeed translated into increased protein amounts in the cell.

Immunostaining of the cells

We wanted to investigate whether the marker proteins are not only expressed differently in undifferentiated and differentiated cells, but also whether these proteins are localized where they are normally found in neurons. Thus, we have performed immunolabeling for eight different neuronal markers. To have a better comparison, we stained in parallel primary cell cultures of mouse dopaminergic neurons, cultivated on the same type of coverslips (glass, PDL precoated). However, the attachment of the differentiated cells to the glass coverslips was poor; during differentiation, the cells form a network that is very fragile and prone to detaching and, therefore, the number of cells recovered after the staining procedure was usually low. Nevertheless, the staining results were reproducible and consistent.

As it can be seen in Fig. 7, the red signal for the various markers is stronger in the differentiated cells than in the undifferentiated ones. TH, synaptophysin, βIII tubulin (to a lesser extent), MAP2 and laminin showed an increase upon differentiation, consistent with the RT-PCR and Western blotting results. The staining pattern of differentiated cells is close to that of rodent primary dopaminergic neurons and different from undifferentiated SH-SY5Y cells. Thus, the localization of the proteins agreed with our expectations and previous reports (Hashemi et al., 2003).

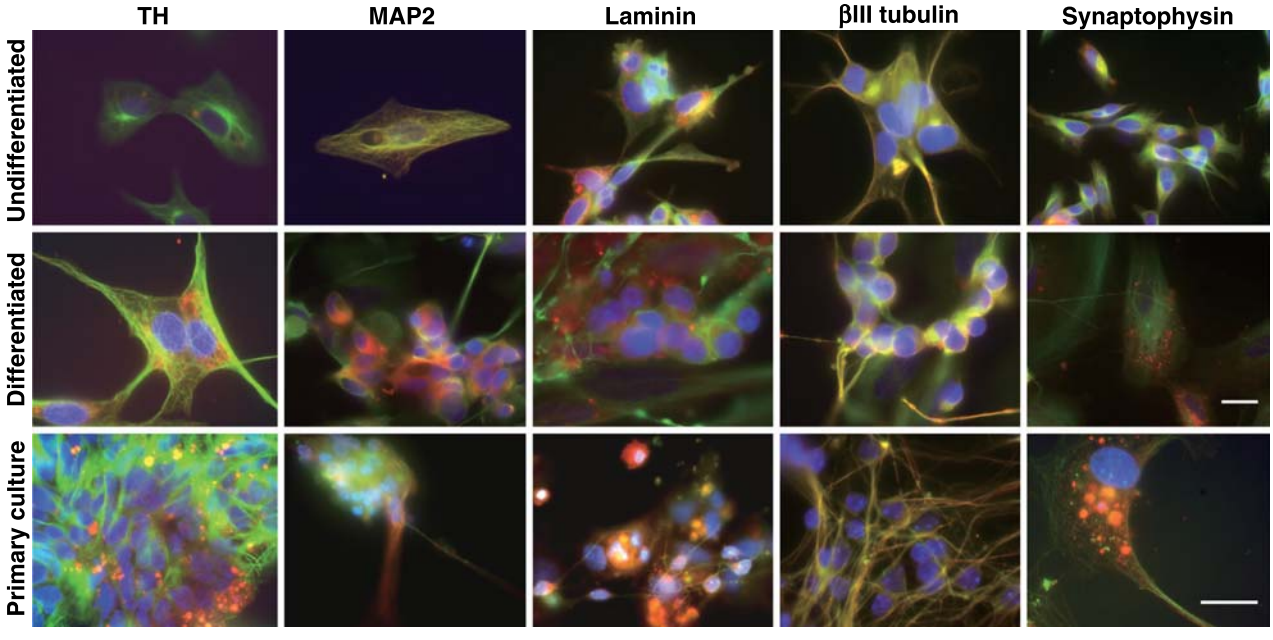

Fig. 7. Immunofluorescence staining for neuronal markers. The comparison was made between undifferentiated, differentiated SH-SY5Y cells and primary dopaminergic neurons derived from mouse embryos. Blue: DAPI, Red: antibody against the respective neuronal marker (Texas Red coupled), Green: DM1α (anti-tubulin) antibody coupled with FITC, Yellow: colocalization of red and green. Scale bar 20 μm for all images. The bottom-right panel is at a different magnification than the remainder of pictures

Taken together, these results suggest that the neuronal markers are expressed and localized as in neuronal cells.

Discussion

In the present work we show that the human dopaminergic neuroblastoma cell line SH-SY5Y can be differentiated to dopaminergic neurons using a specific protocol and a perfusion culture system. The results presented here show that these cells can be differentiated further than has been reported up to now (Nicolini et al., 1998; Maruyama et al., 1997). We have also performed a thorough characterization of the differentiated cells and have shown that many neuronal characteristics can be attained using this protocol.

While animal models probably mimic more accurately aspects of a disease, there are several distinct disadvantages, most obviously, time and cost. In a live animal, many variables can perturb the study of different mechanisms. Cell culture models present the advantage that they are more easily to perform and repeat, whilst being time- and cost-saving. This makes them a good candidate for preliminary studies on the efficiency of various substances, especially when a more controlled setting is required.

In order to have the basic cellular system for developing new oxidative stress models of PD, a human derived cell line was used, which is easier to cultivate than primary neurons, relatively homogenous in composition and closely resembling the cells affected in PD. For this purpose, the human dopaminergic neuroblastoma cell line SH-SY5Y was chosen as a starting point.

The SH-SY5Y cells are often used in cell culture models of PD because they possess many of the qualities of human neurons (Sherer et al., 2001). These cells have neuronal origin, express TH and dopamine-β-hydroxylase, which are specific to catecholaminergic neurons (Ross et al., 1983) and express receptors and transporters for DA and acetylcholine (Biedler et al., 1978; Willets et al., 1995). These cells also express genes associated with neuronal differentiation, including neurofilament proteins and neuron specific enolase among others. Despite expressing all these markers, they are considered immature neuroblasts at different stages of neuronal differentiation (Biedler et al., 1973) and have been shown to maintain the stem cell characteristics and to proliferate in culture for a long time with no contamination (Ross et al., 1983). This is important in the neuroscience and neurotoxicology fields, where the contaminating presence of glial cells, astrocytes and other types of cells can lead to unwanted effects. The SH-SY5Y cell line presents also the advantage that it can be grown and differentiated in the absence of growth factors (Nicolini et al., 1998). The effects of neurotrophic factors used in differentiation are confusing, especially if the cells are further used to study drug-induced neurotoxicity (for example antineoplastic drugs) and the effect of similar trophic factors (Nicolini et al., 1998).

Despite these advantages, there are several differences with respect to neurons, most notably a different expression level of neuronal cell markers (Farooqui, 1994) and confirmed cell proliferation (Pahlman et al., 1995). In particular, undifferentiated SH-SY5Y cells are not an ideal model for dopaminergic neurons as they have a low expression of DAT (Presgraves et al., 2004). Toxicity by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a neurotoxin widely used in PD pharmacological models) requires the presence of DAT to enter the cells and to be converted to the toxic ion MPP+ (Presgraves et al., 2004). This implies that undifferentiated SH-SY5Y cells are more resistant to MPTP than normal dopaminergic neurons, and are thus not a good starting point for an MPTP-based model of PD (Presgraves et al., 2004). Similarly, the relatively high oxidative stress imposed by DA synthesis makes dopaminergic neurons more susceptible to intoxication by Complex I inhibitors compared to other cells (Barzilai et al., 2001). This was our main reason to generate differentiated cells in order to be further used for a chronic PD model. Another reason to use differentiated cells is to have a constant, non-dividing cell population in order to establish a chronic intoxication model. This would avoid problems stemming from variations in cell numbers and the constant renewal of the cell population. In this respect, primary cells have the disadvantage that they cannot be maintained in culture for very long time whereas immortalized cell lines multiply too much.

We cultivated the cells plated on PDL precoated glass coverslips in a long-term perfusion culture system. This perfusion system is more convenient to use than a normal cell culture dish and the cells can be cultivated for a longer time and under better conditions (Minuth et al., 1999). The perfusion system is characterized by the continuous addition of fresh medium with nutrients and the concomitant withdrawal of the used medium with toxic metabolites. In this way, it is possible to cultivate the cells/tissues in conditions closer to the *in vivo* situation (Minuth et al., 1999, 2000).

The differentiation protocol started with the treatment of cells with RA for 14 days. After 8 days of treatment, cells elongated and exhibited branching similar to neurons (Fig. 1B), as described by several other authors for a shorter treatment (Nicolini et al., 1998; Maruyama et al., 1997). After about two weeks of treatment with RA and another two with mitotic inhibitors to eliminate the proliferating subpopulation, the cells resembled morphologically

the primary rodent dopaminergic neurons cultivated on the same type of coverslips. A BrdU incorporation assay showed that the cells, indeed, stopped proliferating, while RT-PCR, Western blotting and immunofluorescence were used to show that several neuronal markers were upregulated as a consequence of the differentiation protocol.

Quantification of immunofluorescence pictures is prone to many pitfalls; in this particular case, where cells aggregate, it is impossible to do a proper quantification over the entire volume, so the results are only qualitative. Even if the results from RT-PCR and Western blotting were not always in perfect agreement at the quantitative level, both methods, as well as the immunofluorescence suggested that most of the markers tested increased following the differentiation protocol. The immunofluorescence results also show that the proteins localized as expected for a neuronal cell. However, one has to keep in mind that SH-SY5Y cells have neuronal origin, so it is not surprising that, even before differentiation, they already express – albeit at lower levels – proteins that are considered markers for a neuronal cell. Still, there is an obvious signal increase for the above-mentioned markers (Fig. 7). An overview of the neuronal markers variation after differentiation is presented in Table 3.

Patch-clamp would be the ultimate way to prove that the cells are differentiated. However, the differentiated cells are fragile and entangled in a complicated network. Moreover, many cells are packed in large clusters which means patch-clamp would be very difficult (if not impossible) to perform.

Table 3. *Summary of the neuronal markers variation after differentiation*

Neuronal marker	Variation after differentiation			Expected from literature
	RT-PCR	WB	IF	
TH	±	+	+	+ (Hashemi et al., 2003)
MAP2	+	+	+	+ (Binder et al., 1985)
βIII tubulin	+	±	±	+ (Lee et al., 1990)
Tau	+	+	NO	+ (Wood et al., 1986)
Nestin	+	−	NO	− (Duggal and Hammond, 2002)
Laminin	+	NO	+	+ (Timpl and Brown, 1994)
NeuN	NA	+	NO	+ (Mullen et al., 1992)
Synaptophysin	+	NO	+	+ (Gaardsvoll et al., 1988)
Neurogenin1	+	NT	NT	+ (Ma et al., 1996)
DRD2	+	NT	NT	+ (Nestler and Aghajanian, 1997)
DAT	+	NO	NT	+ (Storch et al., 2004)
NeuroD1	−	NT	NT	+ (Lee et al., 1995)

+ = increase, − = decrease.
± no or very small variation, *NO* means no optimal result (problems with the antibody or the protocol, e.g. the transfer on the nitrocellulose membrane in Western blotting).
NT not tried (did not find a working antibody), *NA* not applicable (there is no possibility to design primers for NeuN, as the antigen is not known).
WB Western blotting, *IF* immunofluorescence.

In conclusion, in the present work we have developed a new cell culture system using human neuroblastoma cells differentiated in perfusion, which allows to better control vital parameters and to maintain the culture for longer time (i.e., weeks instead of just days) (Minuth et al., 2000).

The differentiation protocol presented here has several advantages. Much more time is allowed for the cells to differentiate and ''filter out'' many of the cells that do not undergo differentiation. Other cell culture models utilized short-term (a few days) treatment with RA with/without neurotrophins, tetradecanoylphorbol acetate or norepinephrine (Singh et al., 2003; Laifenfeld et al., 2002). In the present work, the differentiated cells were thoroughly characterized at both the morphological and molecular levels. The results presented suggest that the differentiation protocol was successful and the differentiated cells have a good similarity with primary neurons.

The low division rate of the cells, taken together with our own observations during cell handling, suggests that the population is relatively constant for a long time. A classical culture using cell lines would require splitting the culture every few days, which would skew the results of any viability testing. This new model gives the opportunity to try various neurotoxins in low dose and long time in culture. This way, the differentiated cells can be further used to model PD and other neurodegenerative disorders affecting the dopaminergic system of the brain. Moreover, in these models new potential therapies can be tested for their long-term effect. We are presently developing such a chronic model.

Acknowledgements

We thank G. Gille and her students for neuronal rodent primary cells, T. Rohrmeier, Neuroprofile GmbH, Regensburg for advice regarding the differentiation protocol, the A. Storch group for several RT-PCR primers and antibodies, T. Hyman, MPI-CBG Dresden for access to his laboratory equipment, the MPI-CBG Dresden Antibody facility for providing the actin antibody and Nathan Goehring for very useful comments on this manuscript.

The present work was supported by a MeDDrive 2004 grant from the Faculty of Medicine of Dresden University of Technology awarded to R. Constantinescu.

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J Neural Transm (2007) [Suppl 72]: 29–34

Printed in Austria

Journal of Neural Transmission

Endogenous oxidized indoles share inhibitory potency against [^{3}H]isatin binding in rat brain

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Summary Isatin is an endogenous oxidized indole that influences a range of processes *in vivo* and *in vitro*. It has a distinct and discontinuous distribution in the brain and [^{3}H]isatin binding sites are widely distributed in rat brain sections. The highest labelling is found in hypothalamic nuclei and in the cortex, hippocampus, and cerebellum (Crumeyrolle-Arias et al., 2003). However, the properties of most isatin binding sites and their physiological ligands remain unknown. In the present study the effects of three endogenous oxidized indoles (oxindole, 5-hyxdoxyoxindole, and isatin) on [^{3}H]isatin binding were investigated in rat brain sections. In most regions cold isatin (0.2 mM) significantly reduced [^{3}H]isatin binding. In addition to isatin, the other endogenous oxidized indoles, 5-hydroxyoxindole and oxindole were effective in displacing [^{3}H]isatin.

Total irreversible inhibition of monoamine oxidases caused inhibition of specific [^{3}H]isatin binding in 7 of 10 brain region studied. This was accompanied by altered sensitivity of [^{3}H]isatin binding to these indoles, including regions where a decrease of specific binding was not detected. The combinations of the three oxidized indoles produced two clear effects: augmentation (potentiation) and attenuation (blockade) of inhibitory activity compared with the independent effects of these compounds.

The different effects of oxidized indoles and their combinations (isatin + 5-hydroxyoxindole and isatin + oxindole) in various brain regions therefore suggest an interaction of [^{3}H]isatin with different and multiple isatin-binding sites, which exhibit different sensitivity to endogenous oxidizing indoles.

Keywords: Isatin, brain, isatin binding sites, oxindole, 5-hydroxyoxindole

Introduction

Isatin (indole-2,3-dione) is an endogenous compound, widely distributed in mammalian tissues and body fluids (Medvedev et al., 1996, 2005b; Sandler, 2000). It has a range of (neuro)physiological and behavioural effects (Medvedev et al., 1996, 2005b; Glover et al., 1998; Sandler, 2000). In the brain the highest levels have been found in the hippocampus (0.1 μg/g) (Watkins et al., 1990) and immunocytochemical staining revealed specific localisation within particular cells (Medvedev et al., 1996). The best studied isatin target (*in vivo* and *in vitro*) is monoamine oxidase (Glover et al., 1988, 1998; Medvedev et al., 1996, 2005b; Sandler et al., 2000). There is also evidence that isatin antagonizes A-type of natriuretic peptide receptors and nitric oxide-stimulated guanylate cyclase *in vitro* (Glover et al., 1995; Medvedev et al., 1996–2005).

Recently, using a real time β-imager and labelled [^{3}H]isatin, we demonstrated a wide distribution of isatin binding sites in the rat brain sections (Crumeyrole-Arias et al., 2003). Treatment of rats with a high dose of the mechanism-based monoamine oxidase inhibitor, pargyline, reduced [^{3}H]isatin binding in most brains regions but did not abolished it (Crumeyrole-Arias et al., 2003). This suggests the existence of isatin-binding sites others than monoamine oxidase. However, their nature remains unknown. In some brain structures atrial natriuretic peptide and C-type natriuretic peptide inhibited [^{3}H]isatin binding which, however, failed to reach 50% level at the highest concentrations of natriuretic peptides used (Medvedev et al., 2005a).

There is increasing evidence that other endogenous oxidized indoles, oxindole and 5-hydroxyoxindole may play a regulatory role in the brain and peripheral tissues, however, the mechanisms underlying their effects remain poorly understood. They possess evident structural similarities and share some regulatory effects. For example, oxindole causes sedation (Moroni, 1999), the same effect was

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also observed after administration of a large dose of isatin (Medvedev et al., 1996; Glover et al., 1998). It seems unlikely that the isatin-induced sedation may be attributed to inhibition of monoamine oxidase (Glover et al., 1998). On the other hand, 5-hydroxyoxindole and isatin, but not oxindole, share some effects in cell cultures (Cane et al., 2000).

In the present study we have investigated the effects of oxindole, 5-hyxdoxyoxindole, and isatin on [^{3}H]isatin binding in rat brain sections. Data obtained suggest that these oxidized indoles share inhibitory potency against [^{3}H]isatin binding in rat brain, and their effectiveness varies in different brain regions.

Materials and methods

Materials

Three month-old male Wistar rats (300–350 g) obtained from Janvier Breeding (Le Genest St. Isle, France) were used in experiments. Animals were treated with the mechanism-based monoamine oxidase inhibitor pargyline (80 mg/kg, sc) or vehicle (saline) of the same volume (5 ml/kg). After two hours rats were decapitated. The dose of pargyline and time interval after its administration and decapitation of animals were sufficient for total inhibition of both types of brain monoamine oxidases (Panova et al., 2000; Crumeyrolle-Arias et al., 2003). Immediately after decapitation brains were frozen on dry ice and stored at −70°C until sectioning. Brains were sectioned at −18°C with a microtome cryostat (Leica CM3050). Sections of 20 µm thickness mounted on gelatin coated slides and stored at −20°C until use in the binding experiments.

Binding experiments were carried out on the sagittal (lateral −0.1 to 0.4 mm Paxinos and Watson, 1982) and the para-sagittal sections (lateral 1.9–2.4 mm) as previously described (Crumeyrolle-Arias et al., 1994, 1996, 2003) using 20 nM [^{3}H]isatin. [^{3}H]Isatin (26 Ci/mmol) was prepared by Amersham (England, a custom made order). 5-Hydroxyoxindole was synthesized by Pr. Muriel Duflos (Laboratoire de Chimie Organique et de Chimie Therapeutique, Universite de Nantes, Nantes, France). Cold isatin and other chemicals were from Sigma (France). Incubations were performed in DMEM medium (Gibco, low glucose), at 4°C (1 h). Non-specific labelling was evaluated on adjacent sections in the presence of 0.2 mM non-radioactive ligands isatin, oxindole, or 5-hydroxyoxindole or their combinations.

β-Imager

The digitalized autoradiograms were obtained from the beta imager (Biospace, Paris, France). This real-time imager provides very fast cartography of tritium labelling (in dpm/mm^2) on tissue sections (Charpak et al., 1989; Dubois-Dauphin et al., 1991). The distribution of tritiated isatin was measured in various structures of the brain as well as in choroids plexus and pineal gland.

Statistics

The statistical analysis was performed using ANOVA analysis and t-test from GraphPadPrism 2.0 (GraphPad Software Inc, San Diego, CAL).

Results

In agreement with the previous study (Crumeyrolle-Arias et al., 2003) β-imaging showed the wide distribution of [^{3}H]isatin binding sites in the rat brain (Fig. 1a).

Pretreatment of rats with the mechanism-based monoamine oxidase inhibitor, pargyline, reduced [^{3}H]isatin binding in most of brain structures (Fig. 2). Two-way ANOVA

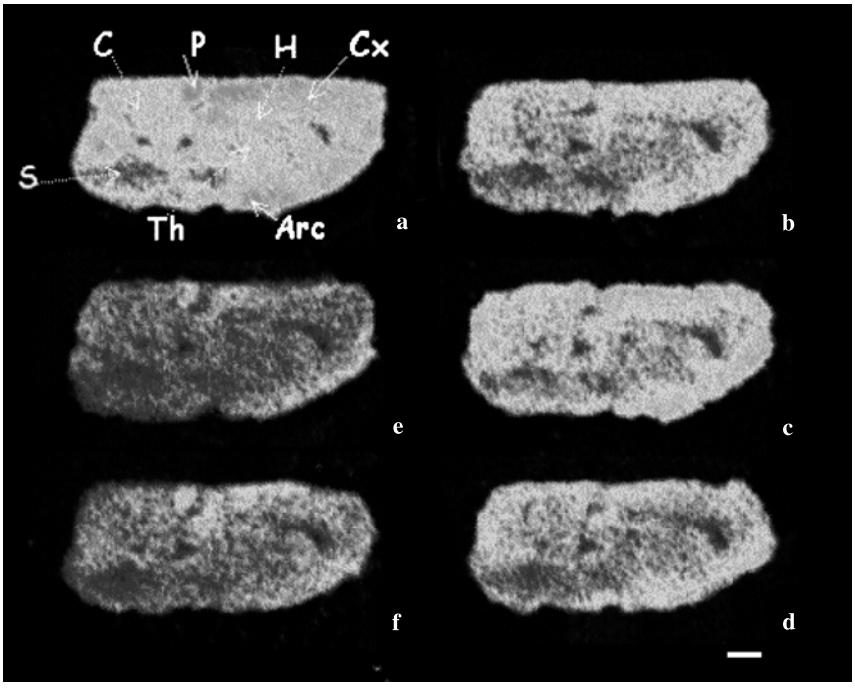

Fig. 1. The effect of oxidized indoles on the imaging of rat [^{3}H]isatin binding sites. Serial sections (lateral 0.4 mm) were incubated with 20 nM [^{3}H]isatin during the same experiment. Total binding was determined without addition of unlabelled oxidized indoles (**a**). Non specific binding was determined by co-incubation of 20 nM [^{3}H]isatin with an excess (200 µM) of cold isatin (**b**), oxindole (**c**), 5-hydroxyoxindole (**d**), isatin + oxindole (**e**), Isatin + 5-hydroxyoxindole (**f**). Images are digital autoradiograms obtained from beta-imager. Exposure time was 15 h. Scale bar = 2.0 mm. *Cx* Cortex; *H* hippocampus; *P* pineal gland; *C* cerebellum; *S* brain stem; *Th* thalamus; *Arc* arcuate nucleus of the hypothalamus

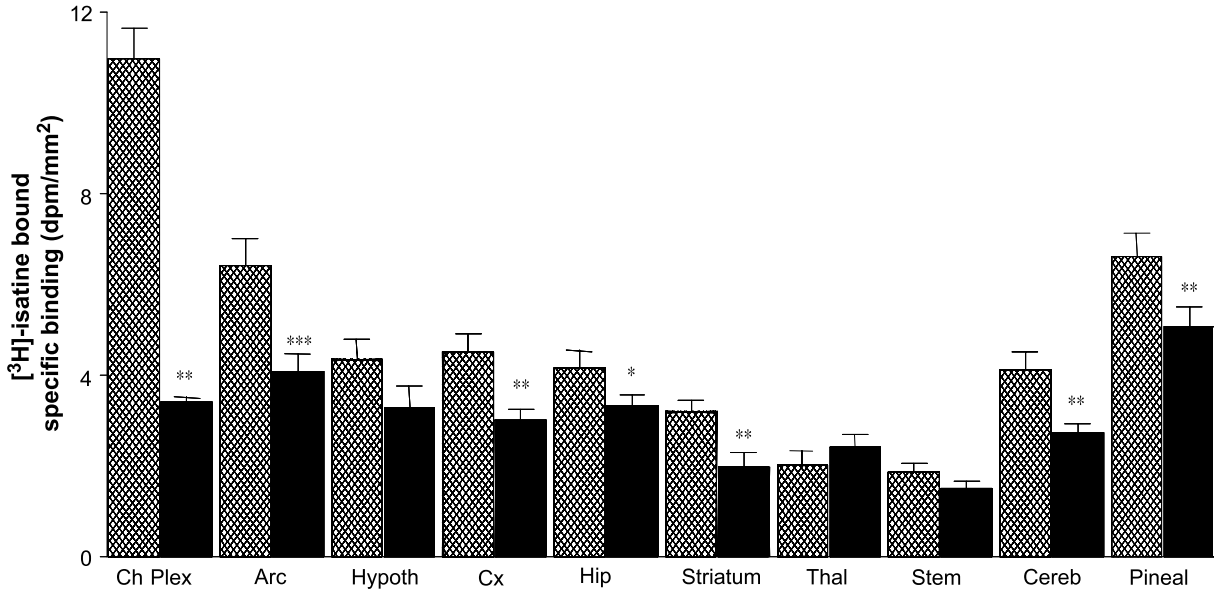

Fig. 2. The effect of pargyline administration to rats on the *in situ* specific binding of [^{3}H]isatin to brain sections. Data represent mean ± SEM from independent experiments performed using 4 sections from each brain (▨-control), (■-pargyline). For each structure, statistical comparisons between control and pargyline-treated rats were calculated using unpaired *t*-test ($^{***}p \leq 0.0004$, $^{**}0.001 \leq p \leq 0.006$, $^{*}p = 0.05$). *Ch Plex* Choroids plexus of the 3rd ventricle (lateral −0.1 mm), *Arc* arcuate nucleus of the hypothalamus (lateral 0.4 mm), *Hypoth* hypothalamus (lateral 1.9 mm), *Cx* whole cortex: frontal to striate, *Hip* whole hippocampus, *Striatum* (lateral 1.9–2.4 mm), *Thal* whole thalamus (lateral −0.1 to 0.4 mm, 1.9–2.4 mm), *Stem* brain stem (lateral −0.1 to 0.4 mm, 1.9–2.4 mm), *Cereb* cerebellum (lateral −0.1 to 0.4 mm, 1.9–2.4 mm), *Pineal* ((lateral −0.1 to 0.4 mm)

analysis shows highly significant effects of the pargyline treatment ($F1,201 = 82.51$ $p < 0.0001$) as well as between structures ($F9,201 = 34.96$ $p < 0.0001$). Moreover, the concerted changes in all structures, induced by pargyline treatment, were highly significant ($F9,201 > 16.32$ $p < 0.001$). The effect of pargyline treatment was especially prominent in choroids plexus, cortex, arcuate nucleus and striatum (−62, −49, −33%, respectively) while no significant changes were observed in thalamus and brain stem.

In most regions cold isatin (200 μM) significantly reduced [^{3}H]isatin binding. This suggests a relatively high level of specific binding of [^{3}H]isatin in the brain (about 50%). However, in cortex and brain stem cold isatin was less effective in displacing [^{3}H]isatin (Figs. 1b and 3).

Besides isatin other endogenous oxidized indoles, oxindole and 5-hydroxyoxindole were also effective in displacing [^{3}H]isatin. In the arcuate nucleus, cortex, brain stem, cerebellum and pineal gland there were insignificant differences between displacing potency of isatin and 5-hydroxyoxindole. In the hypothalamus, striatum and thalamus 5-hydroxyoxindole was a weaker competitor of [^{3}H]isatin than the unlabeled isatin (Fig. 3a). In most brain regions from control rats oxindole was as effective as unlabeled isatin in displacing [^{3}H]isatin and in thalamus oxindole was even more potent competitor than cold isatin for [^{3}H]isatin binding sites (Fig. 3b).

A study of the effect of two combinations of these oxidized indoles, isatin + 5-hydroxyoxindole and isatin + oxindole demonstrated that in brain sections of saline-treated rats displacing potency of isatin + 5-hydroxyoxindole was either the same as the effect of isatin alone (cortex, striatun, brain stem, cerebellum, pineal gland) or weaker than isatin (hippocampus, thalamus). The combination of isatin + oxindole exhibited the same displacing potency as isatin in most regions of control brain sections, however, in the thalamus, where oxindole was more potent than isatin, the combination of isatin + oxindole caused less potent inhibition of [^{3}H]binding than each indole added separately (Fig. 3).

Administration of pargyline caused not only a decrease in the level of specific binding in certain brain regions (Fig. 2), but also influenced the inhibitory potency of three indoles as inhibitors of [^{3}H]isatin binding (Fig. 3b). In the cerebellum and pineal gland 5-hydroxyoxindole was a more potent inhibitor of [^{3}H]isatin binding than isatin, whereas the inhibitory potency of oxindole corresponded to that of unlabeled isatin in all brain region of pargyline treated rats. The only exception was found in the pineal gland where oxindole was more effective than isatin. The combination of isatin + 5-hydroxyoxindole was more effective than isatin itself in actuate nucleus, whereas in all other regions it did not exceed the inhibitory potency of either isatin (cortex, hippocampus, striatum, thalamus,

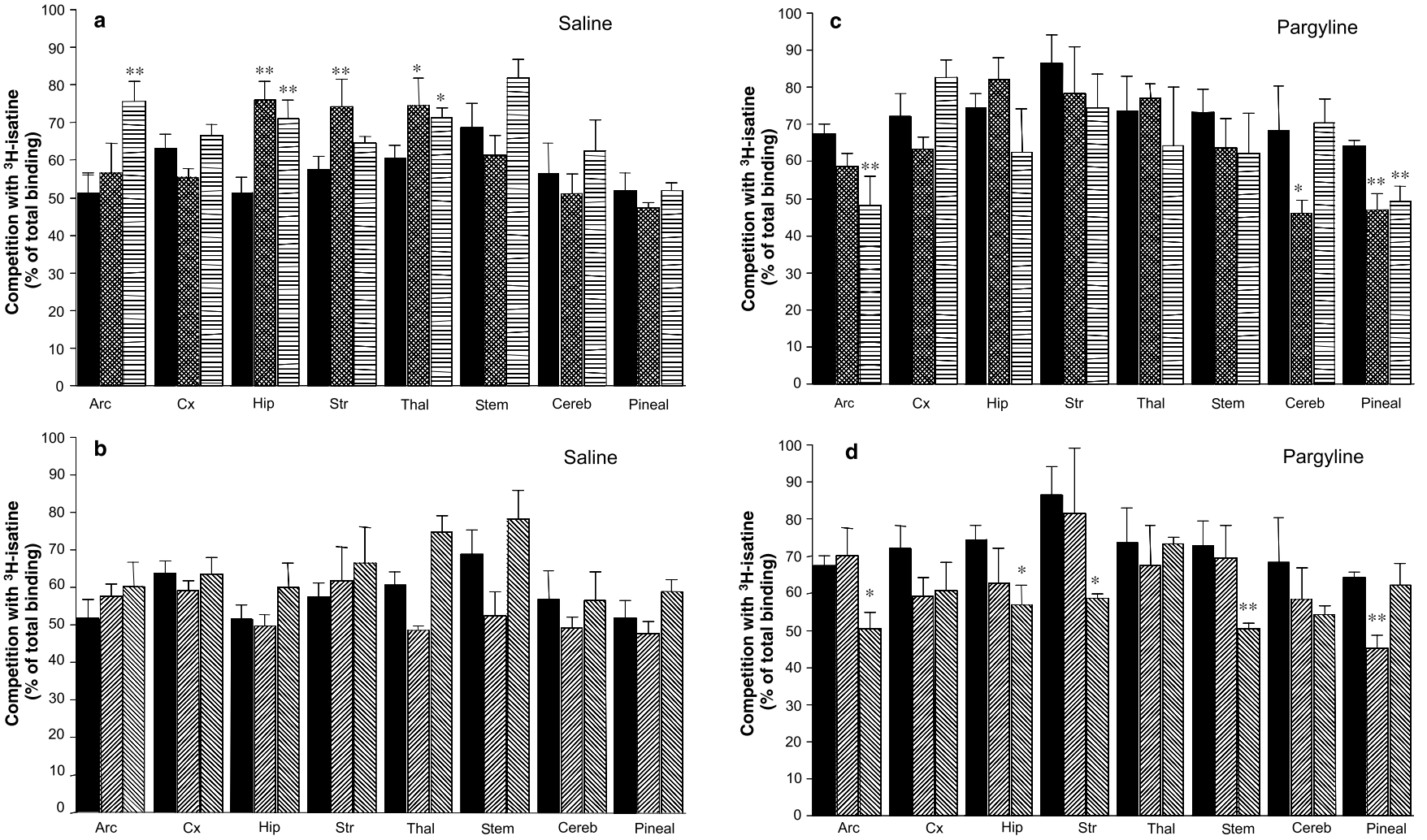

Fig. 3. Competition of 5-hydroxyoxindole (**a**, **c**) and oxindole (**b**, **d**) with [^{3}H]-isatin binding on brain sections from saline (n = 4) and pargyline (n = 4)-treated rats. Sections (lateral 0.4–1.9 mm) were treated as described in Fig. 1. Efficiency of an excess (200 μM) of cold isatin, 5-hydroxyoxindole and oxindole to compete with [^{3}H]-isatin is expressed in % of [^{3}H]-isatin total binding, defined as 100%. Data represent mean ± SEM obtained from 3 independent experiments. Asterisks show significant differences ($^*P < 0.05$; $^{**}P < 0.01$) of cold 5-hydroxyoxindole and oxindole efficiencies compare to cold isatin (Dunnett's test used as post-test of one-way ANOVA analysis performed for each structure). In each triplicate of columns **a**, **c**: 1) isatin, 2) 5-hydroxyoxindole, 3) isatin + 5-hydroxyoxindole; **b**, **d**: 1) isatin, 2) oxindole, 3) isatin + oxindole

brain stem, cerebellum) or 5-hydroxyoxindole (pineal gland). In the arcuate nucleus this combination a caused more potent inhibition than isatin whereas in cerebellum it basically blocked the effect of 5-hydroxyoxindole. Oxindole shared inhibitory potency with isatin in most brain regions. In pineal gland oxindole, as well as 5-hydroxyoxindole, were more potent inhibitors of [^{3}H]isatin binding than unlabeled isatin. However, in contrast to 5-hydroxyoxindole combination of isatin + oxindole was less active than oxindole in this brain region. In other regions (except cortex, thalamus, and cerebellum) combination of isatin + oxindole was more potent than each indole added separately (Fig. 3).

Discussion

The present work confirms that isatin binds to brain regions in a specific pattern. In all regions studied the level of specific binding of [^{3}H]isatin exceeded that for 8-arginine [^{3}H]vasopressin binding in hamster hypothalamus (0.5–1.6 dpm/mm^2) previously found using the same gaseous detection of β-particles (Dubois-Dauphin et al., 1991). This is consistent with isatin receptors playing a regulatory role in the brain (and possibly in peripheral tissues as well).

The nature of [^{3}H]isatin binding sites has not been fully characterized. Previous studies have shown that some [^{3}H]isatin binding was displaced by atrial and C-type natriuretic peptides (Medvedev et al., 2005a) and also that isatin effectively inhibited binding of [^{125}I]ANP (Glover et al., 1995). Both ANP and isatin exhibited comparable inhibitory potency with respect to [^{3}H]isatin and [^{125}I]ANP binding (IC_{50} 0.2 and 0.4 μM), respectively. This suggests that at least in certain brain regions [^{3}H]isatin binding sites may be the natriuretic peptide receptors type A and type C (Medvedev et al., 2005a). On the other hand no correlation was observed with the distribution of binding of [^{3}H]lazabemide, a highly selective tight binding reversible inhibitor of MAO B (Saura et al., 1992). This may suggest that in different brain regions isatin binds to some molecular targets other than natriuretic peptide receptors and MAO B.

The results of the present study demonstrate different displacing potency of three oxidized indoles and their combinations on [^{3}H]isatin binding in the brain. In brain sections from saline-treated rats isatin shared inhibitory potency with 5-hydroxyoxindole and oxindole in most regions studied (arcuate nucleus, cortex, brain stem, cerebellum, pineal gland). In other regions 5-hydroxyoxindole was weaker than isatin and oxindole was a more potent inhibitor in the thalamus.

Administration of a single dose of pargyline sufficient for total inhibition of brain monoamine oxidases (Panova et al., 2000; Crumeyrolle-Arias et al., 2003) reduced specific [^{3}H]isatin binding in 7 of 10 brain regions studied (Fig. 2). This was accompanied by some changes in the potency of these three indoles as inhibitors [^{3}H]isatin binding including the brain regions (hypothalamus, thalamus, brain stem), which did not exhibit any decrease in [^{3}H]isatin binding after treatment of rats with pargyline. The lack of pargyline effect on [^{3}H]isatin binding in thalamus is inconsistent with the results of our previous study (Crumeyrolle-Arias et al., 2003). The major difference in the experimental conditions used in this and the previous study (Crumeyrolle-Arias et al., 2003) is the concentration of [^{3}H]isatin (20 and 130 nM, respectively). It is possible that this discrepancy reflects different kinetic pattern of [^{3}H]isatin binding and thus subsequent study of the pargyline effect on [^{3}H]isatin binding to different brain structure may solve this problem.

The combinations isatin + 5-hydroxyoxindole and isatin + oxindole as the displacing agents of [^{3}H]isatin binding produced two clear effects: augmentation (potentiation) and attenuation (blockade) of inhibitory activity compared with the independent effects of these compounds. Clear augmentation of inhibition of [^{3}H]isatin binding was observed only in the brain regions from pargyline-treated rats: arcuate nucleus (isatin + 5-hydroxyoxindole, isatin + oxindole) striatum and brain stem (isatin + oxindole). 5-hydroxyoxindole blocked isatin effects in arcuate nucleus, hippocampus, striatum and thalamus of control rats, whereas oxindole blocked the isatin effect only in the thalamus of control rats. In brain section from pargyline-treated rats, 5-hydroxyoxindole blocked the isatin effect in the pineal gland, whereas the latter did the same to the 5-hydroxyoxindole effect in the cerebellum.

These different effects in various brain regions suggest an interaction of [^{3}H]isatin with different isatin-binding sites, which exhibit different sensitivity to endogenous oxidizing indoles. Although the nature of [^{3}H]isatin-binding sites remains unclear, certain evidence exists that the oxidized indoles share some (neuro)physiological effects. For example administration of either oxindole or a large dose of isatin causes sedation (Medvedev et al., 1996; Moroni, 1999). It is possible that this effect may be attributed to the interaction of oxidized indoles with oxindole/isatin sensitive [^{3}H]isatin-binding sites.

Acknowledgements

This work was initiated at CNRS FRE2412. MC-A is a CNRS researcher. This work was partially supported by grants from The Wellcome Trust (072381) and RFBR (06-04-48355).

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J Neural Transm (2007) [Suppl 72]: 35–38

Printed in Austria

Journal of Neural Transmission

Lipid content determines aggregation of neuromelanin granules *in vitro*

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Summary The neuromelanin pigment of the substantia nigra of the human brain is closely associated with lipids and other non-melanogenic compounds which appear to contribute to the unique and complex morphology of neuromelanin pigment granules. In this work we show that insoluble granules isolated from the human substantia nigra associate *in vitro* to form pigment aggregates similar to those present in the human brain. Extraction of neuromelanin-associated polar lipids by methanol and/or hexane significantly enhanced melanin aggregate size. A marked (10-fold) increase in granule size was seen after methanol treatment, whereas the application of hexane after methanol reduced this pro-aggregation effect. We have previously reported that hexane and methanol remove the neuromelanin-associated polyisoprenoids dolichol and cholesterol respectively. Thus, the current data suggests that pigment-associated lipids may be a factor regulating pigment aggregation and neuromelanin granule size *in vivo*.

Keywords: Neuromelanin, substantia nigra, aggregation, lipids, cholesterol, dolichol

Introduction

Neuromelanin (NM) is a dark pigment polymer which occupies a large proportion of the cytosol within certain catecholaminergic neurons in the human brain (Fedorow et al., 2005a). Melanised neurons are most abundant in the human substantia nigra and the pigment has attracted the interest of the broad scientific community because of the relationship between NM pigmentation and neurodegeneration in Parkinson's disease (Hirsch et al., 1988; Kastner et al., 1992). NM also accumulates α-synuclein, a protein central to Parkinson's disease pathogenesis (Fasano et al., 2003; Halliday et al., 2005), further linking this pigment with the disease process. The physiological function of NM is unclear but, like melanins in the periphery (Fedorow et al., 2005a), NM is suggested to play a protective role within the neurons which produce this pigment (Zecca et al., 2003; Li et al., 2005). The structure of NM is incompletely understood. Significant structural differences have been demonstrated between synthetic melanins, peripheral melanins and NM (Fedorow et al., 2005a). In addition to the pigment polymer, likely to be derived from dopamine oxidation, NM contains peptides (Double et al., 2000) possibly of lysosomal origin (Tribl et al., 2005). Early electron microscopy studies of NM demonstrated that the pigment is made up of individual units, termed granules, closely associated within the cytoplasm. Further, each granule exhibits a unique triphasic appearance, consisting of closely associated electron-dense and electron-lucent components, as well as a component of intermediate electron density (D'Agostino and Luse, 1964; Duffy and Tennyson, 1965; Moses et al., 1966; Hirosawa, 1968; Roy and Wolman, 1969; Schwyn et al., 1970). The electron-lucent component makes up 35% of the NM granule volume (Fedorow et al., 2006) and contains neutral isoprenoid lipids (Fedorow et al., 2005b). Dolichol accounts for approximately 12% of total NM granule mass, while lower levels of additional lipophilic compounds, such as cholesterol, ubiquinone-10 and α-tocopherol, are also present (Fedorow et al., 2006).

The complex appearance and irregular shape of the mature NM granule *in vivo* differentiates NM from other melanins. For example in the eye the mature melanosome is a smooth membrane-bound organelle with little or no heterogeneity at the ultrastructural level (Fedorow et al., 2005a). Clearly the mature NM granule contains non-melanogenic material, including lipids (Fedorow et al., 2005b), proteins and possibly membranous elements (Tribl et al., 2005). Therefore, it is feasible that these components may play a role in the NM granule similar to that of the proteinaceous matrix within melanosomes upon which peripheral melanin is deposited. In our laboratory the isolation of NM from the human brain for neurochemical studies routinely utilizes a series of digestive procedures designed to

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remove the majority of tissue- and NM-associated proteins and lipids from the polymer backbone (Double et al., 2000; Li et al., 2005). The isolated pigment polymer is similar in appearance to NM *in vivo* but comprises of pigment aggregates of differing sizes, including aggregates significantly larger than NM granules *in vivo*. This suggests the polymer has the ability to combine into larger aggregates than those present in the brain but it is unclear how granule size is regulated *in vivo*. Given the close association between pigment polymer and lipids in the mature NM granule *in vivo* we investigated whether NM-associated lipids might influence aggregation of the pigment.

Materials and methods

Materials

All buffer components, sodium dodecyl sulfate (SDS), proteinase-K, EDTA, and the cholesterol standard were purchased from Sigma (St Louis, MO, USA). Methanol, hexane, isopropanol and acetonitrile were HPLC grade and purchased from Merck (Darmstadt, Germany). Dolichol was purchased from American Radiolabelled Chemicals, (St Louis, MO, USA).

Neuromelanin isolation

NM was isolated from the entire SN of subjects with no history of neurological or neurodegenerative disease from Australia, with appropriate ethics approval, using our established method (Li et al., 2005). The program is approved by the Human Ethics Committee of the University of New South Wales under the Human Tissue Act of the State of New South Wales (Australia) and prospective consent for brain donation was obtained from all cases and their next of kin. Briefly, 1–3 g of the SN were dissected from the brain within 36 h of death on a cool plate and pooled in a glass-Teflon homogenizing vessel. The samples were homogenized in 20 mL water and centrifuged at 12,000 *g* for 10 min. The resulting pellets were washed twice with 50 mM phosphate buffer (pH 7.4), then incubated in 50 mM Tris buffer (pH 7.4) containing 0.5 mg/mL sodium dodecyl sulfate (SDS) at 37°C for 3 h, followed by a further 3 h incubation with the addition of 0.2 mg/mL proteinase K (Sigma) in the same buffer. The resulting pellets were pooled and consecutively washed with saline, water, then methanol and hexane to extract the NM-associated lipids.

Microscopy and image analysis

Images were collected using bright-field optics and UV 360–370 nm excitation/420 nm emission filter and Image Pro Plus software (Media Cybernetics, MD). Fractions of NM were collected at different stages of the isolation procedure, vortexed in distilled water for 2 min and 10 µl of solution was placed on glass slides and coverslipped for observation under a BH-2 Olympus microscope fitted with a Spot Digital Cooled Camera (Diagnostic Instruments, MI) and a deuterium light source. NM granule size was quantified by measurement of the diameter of all aggregates within three randomly chosen fields in 4–5 different slide views using Image Pro Plus and Scion Image software. Aggregate size was analysed using one-way analysis of variance followed by Bonferroni's *t*-test.

Spectrophotometry

Absorbance spectroscopy was used as an additional index of aggregate size in the differing NM samples as melanins absorb both ultra-violet and visible light (Ou-Yang et al., 2004). After treatment described in the text, samples of the NM preparations at the same concentrations were suspended in 1 ml distilled water and vortexed for 2 min. The concentrations of NM

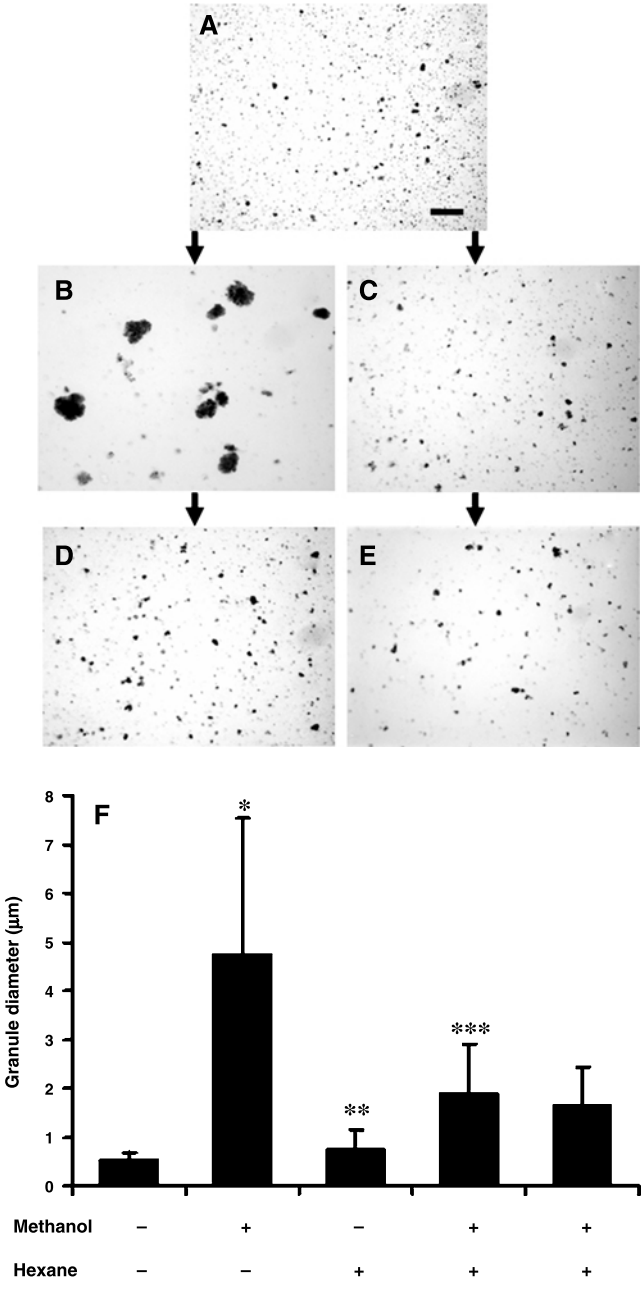

Fig. 1. NM granule size during isolation from the human brain. **A** Prior to solvent-based lipid extraction. **B** Following methanol extraction. **C** Following extraction with hexane. **D** Following methanol, then hexane extraction. **E** Following hexane, then methanol extraction. Scale bar represents 5 µm and is equivalent for a–e. **F** Mean size of NM aggregates (± S.E.), corresponding to panels A–E. *Significant difference between granule size before application of solvents and after treatment with methanol ($p < 0.0001$). **Significant difference between granule size before application of solvents and after treatment with hexane ($p = 0.01$). ***Significant difference between granule size; methanol treatment alone and methanol/hexane treatment ($p < 0.0001$). Other statistical cross-correlations between granule sizes are described in the text

varied depending on initial amount of SN tissue (1–3 g). Absorbance spectra were measured from 190 to 900 nm using a UV-1700 spectrophotometer (Shimadzu Oceania, NSW, Australia) in 10 mm quartz cuvettes (Starna, NSW, Australia) at room temperature.

Results

Following treatment of the nigral tissue homogenate with SDS and proteinase K NM appeared as insoluble granules of varying size (Fig. 1A). Quantification of the granules demonstrated a mean granule size of 0.51 ± 0.18 µm (range 0.25–1µm Fig. 1F). Application of methanol resulted in formation of aggregates of a ten-fold larger size (mean granule size 4.75 ± 2.80 µm, range 1.5–9.75µm, $p < 0.0001$, Fig. 1B and F) while application of hexane resulted in a significant, but much smaller, increase in granule size (mean granule size 0.75 ± 0.39 µm, range 0.5–2 µm, $p = 0.01$, Fig. 1C and F). Application of hexane following methanol treatment reduced granule size (granule size 1.88 ± 1.03 µm, range 1–5µm, $P < 0.0001$, Fig. 1D and F) although the aggregates remained significantly larger than lipidated granules ($p < 0.0001$). Similarly, application of methanol after hexane resulted in similarly sized aggregates ($p < 0.0001$, granule size 1.65 ± 0.8 µm, range 1–2.75 µm, Fig. 1E and F). Using HPLC analysis we have previously investigated the lipid content of the solvent fractions from our NM isolation procedure and have demonstrated the presence of cholesterol in the methanol, and dolichol in hexane, fractions respectively (Fedorow et al., 2005b), thus it is reasonable to expect these same lipids were removed from the lipidated NM granules during the current experiments.

Absorbance spectroscopy demonstrated that the lowest absorbance values for the 200–700 nm spectrum was exhibited by the methanol-treated NM suspension, indicative of a greater aggregate size, while the highest absorbance was exhibited after extraction with hexane, indicative of a small aggregate size (Fig. 2). Subsequent treatment with the opposing solvent, regardless of order of application, resulted in absorbance spectra similar to that produced by a NM suspension prior to lipid extraction (Fig. 2).

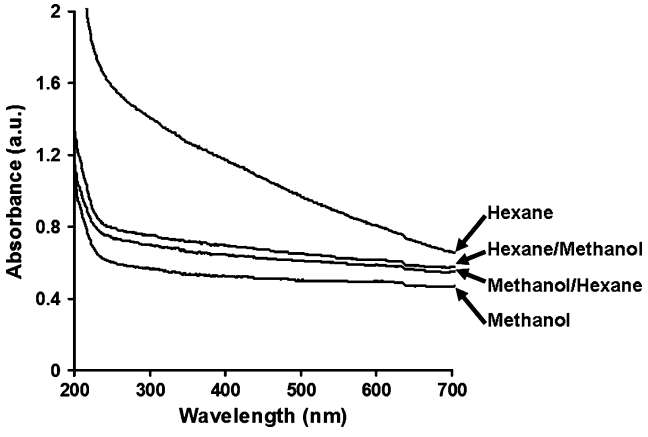

Fig. 2. Optical density of NM (isolated from 3 g of wet SN) in water after hexane extraction (trace Hexane), after extraction with methanol (trace Methanol), after hexane and methanol extraction (trace Hexane/Methanol), and after methanol and hexane extraction (trace Methanol/Hexane)

Discussion

A physiological role for NM-associated lipids has not been identified. Our current results demonstrate that lipids present in the NM granules influence aggregation of the pigment polymer *in vitro*. *In vivo* NM granules contain both variably sized aggregates of the pigment polymer and closely associated lipidic bulbs (Fedorow et al., 2006). Therefore, the polymer aggregates may be suggested to form within a matrix of lipids and proteins which form an intrinsic part of the mature granule. Indeed, in this work extraction of cholesterol by methanol resulted in an eight- to ten-fold enhancement in pigment granule size, whereas the pro-aggregation effect of dolichol extraction using hexane was significantly smaller. Nevertheless, removal of both cholesterol and dolichol resulted in significantly larger aggregates than those present prior to lipid removal. These data suggest that these lipids prevent the formation of large pigment aggregates and that cholesterol may be particularly effective in this role, although it accounts for only a small proportion of the total lipid mass in NM (Fedorow et al., 2005b). NM lipidic bulbs have only been partially characterised and it is possible that in addition to the lipids identified that they also contain other lipid species such as phospholipids and glycosphingolipids (GSL) that would not be resolved by the reversed phase HPLC method used here. Interestingly, NM granules are also reported to contain proteins involved in phospholipid and GSL metabolism (Tribl et al., 2005). It is unknown if these polar lipids are present in NM or play a role in NM formation but recent evidence has pointed to a role for sphingolipids in peripheral melaninogenesis (Saha et al., 2006).

We have previously described increasing NM granule size during pigment development and maturation in the child brain (Fedorow et al., 2006). The mechanisms regulating in NM pigment granule formation and development in the infant and mature human brain have not yet been described. It is feasible however, that NM pigment granule size may depend upon cellular factors that influence the environment in which the pigment is formed. Based on the data presented here, we suggest that pigment-associated lipids may be a factor regulating NM granule size *in vivo*.

Acknowledgements

We would like to thank the volunteers and their family for the donation of brain tissue. We also thank Ms. Heather McCann and Ms. Christine Song for assistance with tissue processing, Mr. Colin Yeo for advice on microscopy and image processing, and Ms. Heidi Cartwright for figure preparation. This work was supported by the National Health and Medical Research Council of Australia.

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J Neural Transm (2007) [Suppl 72]: 39–41

Printed in Austria

Journal of Neural Transmission

Short Communication

Hydrogen peroxide is a true first messenger

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Summary Hydrogen peroxide has been shown to act as a second messenger mediating intracellular redox-sensitive signal transduction. Here we show that hydrogen peroxide is also able to transmit pro-inflammatory signals from one cell to the other and that this action can be inhibited by extracellularly added catalase. If these data can be further substantiated, hydrogen peroxide might become as important as nitric oxide as a small molecule intercellular (first) messenger.

Keywords: Inflammation, lipopolysaccharide, advanced glycation endproducts, nitric oxide, hydrogen peroxide, redox-sensitive signaling

Introduction

The physiological role of nitric oxide (NO) signaling as an extracellular signaling molecule is now widely appreciated. NO, synthesized by NO synthase, typically acts in a paracrine fashion, where NO synthesized in one cell diffuses through the extracellular space and acts on a target in an adjacent cell. However, NO's mode of action appears not to be unique. Here, we present evidence that hydrogen peroxide (H_2O_2) acts in a very similar fashion – it mediates cell-to-cell communication and can thus be classified as a true first messenger.

Our hypothesis (and the comparison with NO) is based on the following facts:

1. H_2O_2 is generated in response to a pro-inflammatory stimulus through the action of NADPH oxidase (''respiratory burst'') and superoxide dismutase (Decoursey and Ligeti, 2005).
2. H_2O_2 can diffuse freely through cell membranes (Ohno and Gallin, 1985).
3. H_2O_2 can react with target molecules in the target cell – particularly reactive cysteine residues (Rhee et al., 2005), thereby activating specific signalling molecules e.g. leading to the activation of transcription factors such as NFκB and subsequent expression of NFκB-regulated cytokines or nitric oxide synthase (Pantano et al., 2006). This ability of H_2O_2 is well known and termed ''redox-sensitive'' signal transduction, and it can be downregulated by various types of ''anti-inflammatory'' antioxidants (Wong et al., 2001). However, H_2O_2 has been always regarded strictly as a second messenger mediating only intracellular signaling, e.g. from NADPH-oxidase down to redox-sensitive signaling proteins.

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Material and methods

Activation of N-11 microglia with LPS and BSA-AGE

N-11 microglia were grown in 175 cm^2 cell culture flasks on Dulbecco's Modified Eagle's Medium (DMEM) containing 5% foetal calf serum (FCS), supplemented with penicillin (200 U/ml), streptomycin (200 µg/ml) and Fungizone (2.6 µg/ml) at 37°C in a humid environment containing 5% CO_2. After cells had grown to confluence, they were removed using a rubber cell scraper. Cells were concentrated by centrifugation for 3 min at 900 rpm, re-suspended in a small volume of fresh DMEM (5% FCS). Cell densities were then estimated using a Neubauer counting chamber and adjusted to 10^6 cells/ml. 100 µl of cells were seeded into a 96 well plate and incubated at 37°C for 24 h with 5% FCS DMEM to allow growth to confluence. The media was removed by aspiration and replaced with media with 0.1% FCS for 18 h to minimize the effect of growth factors on cellular activation and differentiation during the experiments. All solutions subsequently added to wells were made up in serum-free DMEM. Cells were activated by 10 µg/ml LPS or 500 µg/ml glycated albumin with or without in the presence of varying concentrations of catalase at 37°C for 24 h, after which nitrite concentration in medium was determined.

Nitrite determination

NO production was monitored by measuring the concentration of nitrite in the media using the 'Griess reagent' as described previously (Dukic-Stefanovic et al., 2003). Briefly, conditioned media (50 µl) from each well was transferred to a fresh 96-well plate and 25 µl of Reagent 1 (1% *w/v* sulfanilamide in ddH_2O) and 25 µl of Reagent 2 (0.1% *w/v* naphthyethylene-diamine in 5% HCl) were added and the absorbance at 540 nm determined using a plate reader (Multiskan *Ascent* with Ascent software v2.4, Labsystems).

Cell viability assay

Cell viability (CV) was assessed using Neutral Red uptake. Following removal of media of 50 µl of DMEM containing 25 µg/ml Neutral Red was added to the wells and incubated for 1 h at 37°C (5% CO_2). The media was then removed and 100 µl of the cell lysis solution (50% ethanol, 10% acetic acid) added to each well. Plates were then shaken for 30 min and the absorbance at 540 nm was measures with a 96 well plate reader.

Results

With the following experiment, we have demonstrated that H_2O_2 is able to travel between cells and induce a signal in an adjacent cell, and that it thereby qualifies as a true first messenger. Activation of cells – here murine N-11 microglia – was performed with two pro-inflammatory stimuli – Lipopolysaccharide (LPS) and glycated albumin (BSA-AGE). AGEs are carbonyl-derived posttranslational modifications of long-lived proteins such as the amyloid plaques in the Alzheimer's disease brain (Münch et al., 1997, 1999).

These stimuli lead to the expression of the inducible form of nitric oxide synthase (iNOS) and subsequent production of NO (Münch et al., 1998; Wong et al., 2001). Our hypothesis was as follows: If H_2O_2 activates adjacent cells, extracellularly added catalase would convert it to water and oxygen in the extracellular space and the pro-inflammatory signal would not reach the next cell, leading to an overall reduction of the readout, NO.

In detail, N-11 microglia were activated with 10 µg/ml LPS or 500 µg/ml BSA-AGE (produced by the incubation of BSA with glucose at 65°C for 5 days), as these concentrations generated similar concentrations of nitrite, in the presence of increasing concentrations of catalase. The experiment clearly showed a concentration-dependent inhibition of NO production by catalase in response to both LPS and BSA-AGE, with an IC_{50} of 1000 U/ml and 800 U/ml, respectively (Fig. 1). The maximum inhibition of LPS-induced NO production was approximately 85 and 95% for BSA-AGE induced NO production, respectively. The decrease in NO production was not caused by cell death, since catalase had no significant effect on cell viability (Fig. 1).

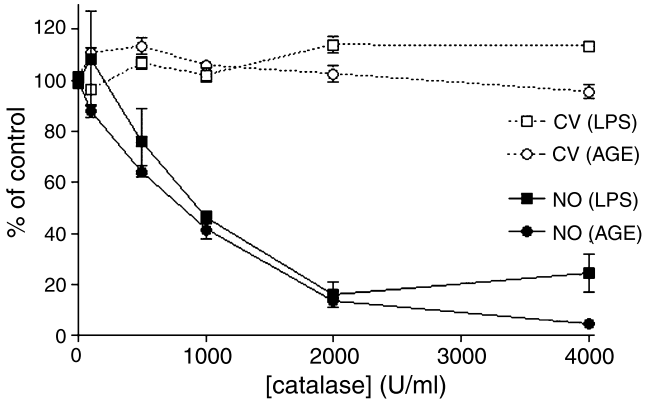

Fig. 1. Nitric oxide production by N-11 microglia activated with LPS or BSA-AGE in the presence of catalase. Nitric oxide (*NO*) was measured as nitrite present in the media after 24 h of incubation with LPS or BSA-AGE ($n = 3$) and increasing concentrations of catalase. Viability of cells (CV) was measured by Neutral Red uptake (*NR*) ($n = 3$). Data are presented as mean ± SEM

Discussion

In summary, we believe that H_2O_2 can be regarded as true first (extracellular) messenger, similar to NO. This concept can revolutionize drug development because H_2O_2 scavengers do not necessarily have to penetrate the cell membrane to exert their action – and even extreme hydrophilic molecules would be beneficial in diseases with chronic pro-inflammatory conditions such in Alzheimer's disease (Huber et al., 2006). In addition, our results suggest that nature's concept to use small membrane permeable signaling molecules for cell to cell signaling is not limited to NO but appears to be a general, widespread and important principle.

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J Neural Transm (2007) [Suppl 72]: 43–49

Printed in Austria

Journal of Neural Transmission

The therapeutic potential of siRNA in gene therapy of neurodegenerative disorders

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Summary RNA interference using small inhibitory RNA (siRNA) has become a powerful tool to downregulate mRNA levels by cellular nucleases that become activated when a sequence homology between the siRNA and a respective mRNA molecule is detected. Therefore siRNA can be used to silence genes involved in the pathogenesis of various diseases associated with a known genetic background. As for many neurodegenerative disorders a causative therapy is unavailable, siRNA holds a promising option for the development of novel therapeutic strategies. Here we discuss different siRNA target strategies aiming for an allele-specific degradation of disease-inducing mRNA and we review the literature in the field of siRNA and its application in animal models of neurodegenerative diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and spinocerebellar ataxia (SCA1).

Keywords: Gene therapy, siRNA, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), spinocerebellar ataxia type 1 (SCA1)

Abbreviations

AAV	adeno-associated virus
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
BACE1	β-secretase
EIAV	equine infectious anemia virus
HD	Huntington's disease
SCA1	spinocerebellar ataxia
shRNA	small hairpin RNA
siRNA	small interfering RNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
SCA1	spinocerebellar ataxia type 1
SNP	single nucleotide polymorphism
SOD1	Cu–Zn-superoxide dismutase

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Introduction

RNA interference (RNAi) was discovered in 1998 and represents a natural mechanism found in a wide range of eukaryotic species to regulate gene expression on a post-transcriptional level (Fire et al., 1998). The cellular endonuclease Dicer processes double stranded RNA into short RNA molecules called small interfering RNA (siRNA) (Bernstein et al., 2001). Thereafter, siRNA duplexes are assembled into an RNA-induced silencing complex (RISC) (Hammond et al., 2000), that guides a sequence-specific recognition of cellular mRNA followed by endonucleolytic degradation of the mRNA target (Elbashir et al., 2001; Hammond et al., 2001; Martinez et al., 2002; Yang et al., 2000) (Fig. 1). Thus, siRNA inhibits gene expression by triggering degradation of those mRNA molecules that share sequence homology with the siRNA.

In mammalian cells, siRNAs with a length of 21 nucleotides have the highest knockdown activity (Elbashir et al., 2001) and 19 of these nucleotides are directly involved in the recognition of the mRNA target sequence (Fig. 2). The sequence specificity of siRNAs to recognize an mRNA as a target is very high (Semizarov et al., 2003) and even a single nucleotide mismatch between the siRNA and the corresponding target region in the mRNA can cause a complete loss of siRNA activity (Schwarz et al., 2006). This makes siRNA an ideal tool for the selective downregulation of mutated genes without affecting wildtype allele expression.

The activity of an siRNA is not only dependent on a high sequence homology with the target mRNA but also remarkably varies with the exact position of the target sequence within the mRNA molecule (Reynolds et al., 2004). As the mechanism for these differences is still not fully understood, a functional screening of different candidate

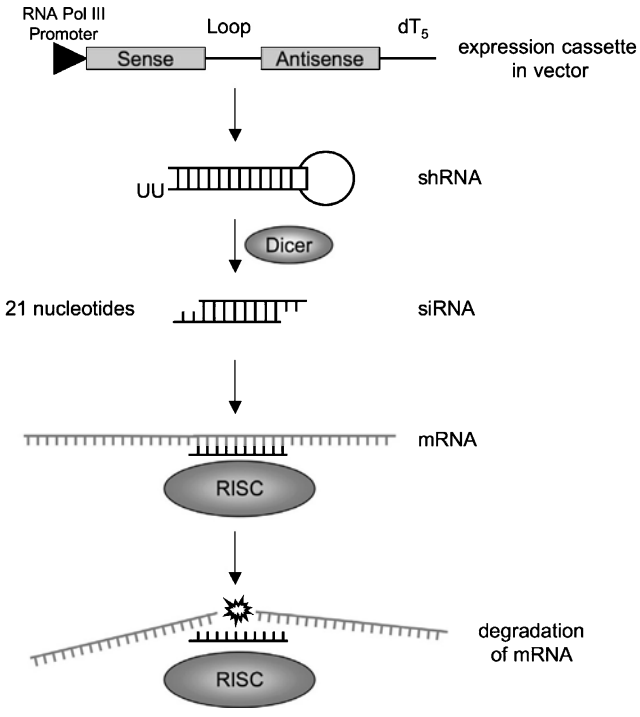

Fig. 1. The mechanism of shRNA-mediated mRNA knockdown. Small hairpin RNA (shRNA) can be delivered *in vivo* by expression vectors. shRNA is processed by the cellular nuclease Dicer into siRNA which in turn marks its target mRNA for a RISC-guided degradation

sequences is needed to identify an siRNA with the desired knockdown activity.

To achieve a transient knockdown of gene expression, synthetic siRNAs can be transfected, causing a temporary decline of the target gene expression (Harborth et al., 2001). For many potential therapeutic applications, however, long-term silencing of gene expression is desired, requiring the use of vector-based siRNA technology. This can be achieved by expression of small hairpin RNA (shRNA) that is subsequently processed into active siRNA by the cellular enzyme Dicer (Abbas-Terki et al., 2002) (Fig. 1).

siRNA strategies

As vector-delivered siRNA allows the specific and stable knockdown of genes involved in diseases, siRNA technology could be successfully exploited for gene therapy. Depending on the genetic background of a disease, different strategies may be required, including:

Knockdown of both alleles

As depicted in Fig. 3A, a siRNA designed to target a sequence present in both alleles of a gene (i.e. the wildtype sequence) can – depending on the characteristics of the siRNA – completely abolish expression of the gene product. This approach is usually being performed as an investigative tool to determine the function of a gene product within cell culture or animal experiments and it is therefore comparable to the generation of a gene knockout. The therapeutic potential of this strategy is restricted to gene targets that are dispensable for the organism (e.g. knockdown of the chemokine receptor CCR5 that serves as a coreceptor for HIV infection as an antiviral strategy (Qin et al., 2003)). By using siRNAs with an intended low knockdown activity that reduces but not abolishes target gene expression, this strategy could probably be extended to a broader variety of disease-related genes.

Allele specific knockdown by targeting the disease-inducing mutation

Diseases caused by dominant gain-of function mutations represent one promising target for the application of siRNA technology as a future treatment option. Since siRNAs can distinguish between target sequences that differ in only one nucleotide, the specific knockdown of the disease-inducing allele may be feasible (Fig. 3B) (Schwarz et al., 2006). This approach requires the identification of an siRNA with both high specificity and activity towards the mutated allele from a pool of 19 different candidates. Most of the siRNAs have a 19 nucleotides long antisense strand that pairs with the mRNA target. Therefore, 19 different siRNAs can be designed to target a point mutation within a mRNA. An example of a set of potential siRNAs targeting the ''Indian'' point mutation within the amyloid precursor protein (APP) gene is given in Fig. 2B.

Allele specific knockdown by targeting a SNP in the disease-inducing allele

If none of the 19 possible siRNAs that cover the mutation site exhibits the desired activity, single nucleotide polymorphisms (SNPs) that are allele specific (but not disease related) may be exploited as an alternative site for siRNA knockdown (Fig. 3C). This strategy is especially relevant for triplet repeat disorders as the extended repeats are neither sensitive to siRNA-mediated knockdown (Xia et al., 2004) nor specific for the disease-inducing allele (even the wildtype allele bears several triplet repeats that by far outnumber the 19 nucleotide window that can be covered by siRNAs). As the probability to find SNPs increases with the gene size, large genes such as the gene involved in Huntington's disease (Huntingtin, mRNA size 10.5 kb) could be ideal candidates for this strategy.

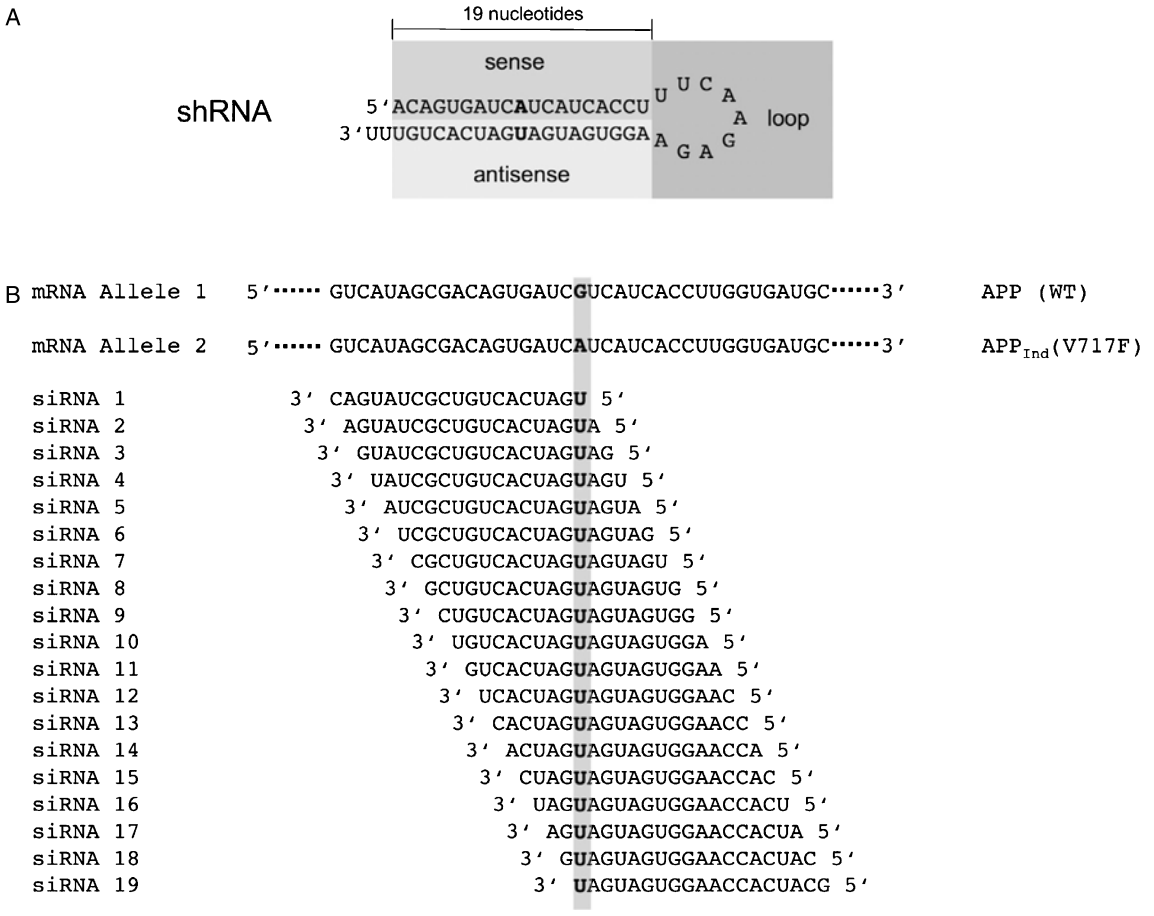

Fig. 2. The architecture of small hairpin RNA (shRNA). **A** shRNA forms a stem-loop RNA structure with a sense strand corresponding to the mRNA target sequence, a loop and an antisense strand that eventually pairs with the mRNA once the shRNA is being processed into siRNA. The picture shows the shRNA corresponding to siRNA 10 from **B**. **B** Using shRNAs with a sense strand of 19 nucleotides, 19 different target sequences can be designed that cover a point mutation within an mRNA. The picture shows the amyloid precursor protein (APP) gene bearing the "Indian" point mutation (V717F, referred to as APP_{Ind}) associated with early onset of AD

Knockdown of both alleles combined with the expression of a codon-altered transgene

In some cases, neither a mutation-directed nor an SNP-directed knockdown may be feasible. An alternative then could be to knockdown both alleles (i.e. the mutated *and* the wildtype mRNA) and to restore the wildtype gene expression by delivering a transgene with altered codon usage within the target area of the siRNA to make it resistant to the siRNA attack (Fig. 3D).

siRNA in Alzheimer's disease

Alzheimer's disease (AD) is a predominantly sporadic disease that is characterized by the formation of senile plaques and neurofibrillary tangles in the neocortex, hippocampus and amygdala in the AD brain. Plaque formation involves the proteolytic degradation of the APP by different secretases to form Aβ and alterations in processing APP plays a key role in the pathogenesis of the disease (Kamenetz et al., 2003). Several mutations in APP have been identified that correlate with early onset of the disease. Transgenic mice that express mutated APP (e.g. a double mutant containing the London V717I and Swedish K670M/N671L mutation) develop plaques in the frontal cortex at 3–4 months of age, followed by plaque formation in the hippocampus at 5–7 months (Rockenstein et al., 2001). Plaque formation is caused by increased Aβ-production. BACE1 (β-secretase) is involved in the proteolytic degradation of APP to the plaque forming amyloid-β peptide (Haass, 2004) and BACE1 expression in the brain cortex of AD patients is increased by factor 2.7 (Holsinger et al., 2002), making BACE1 an excellent therapeutic target for the treatment of AD.

Lentiviral expression of anti-BACE1 shRNA (targeting nucleotides 1785–1802) after intracranial injection into the

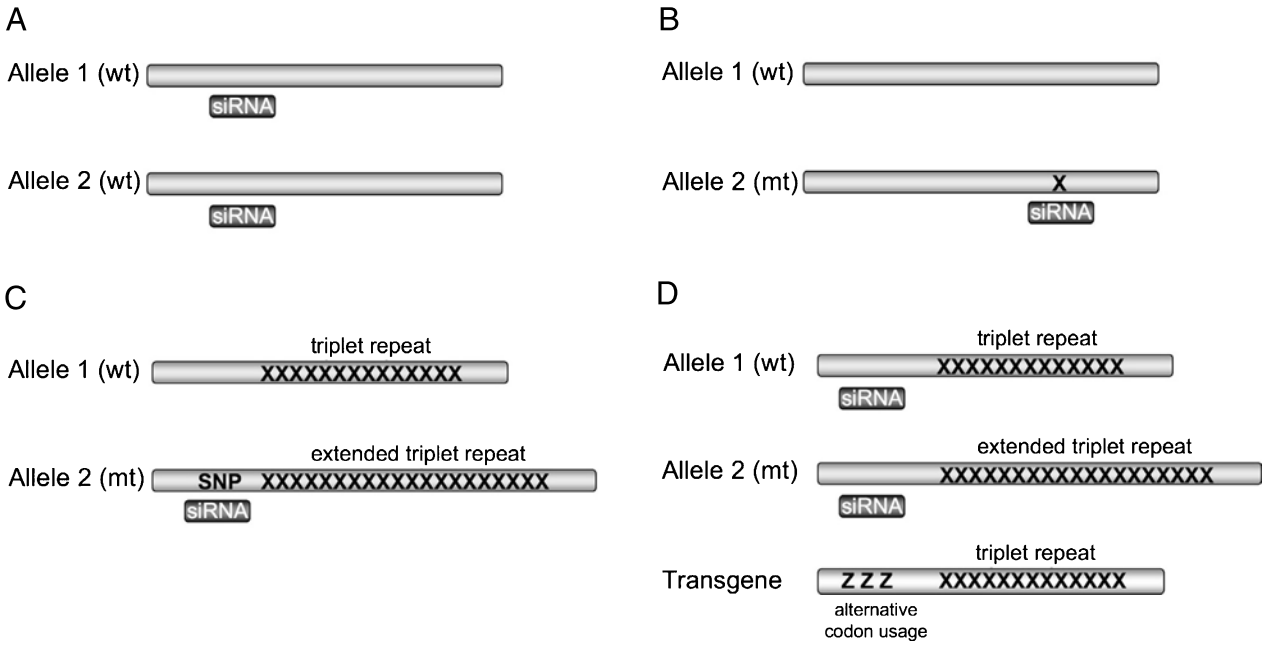

Fig. 3. Different strategies of small hairpin RNA (siRNA) knockdown. **A** siRNAs directed at a wildtype sequence has the potential to knock down both alleles if the two alleles share sequence homology within the siRNA target sequence. **B** siRNA directed at a mutated sequence has the potential to selectively downregulate expression of the mutated (disease-inducing) allele. **C** siRNA directed at a single nucleotide polymorphism (SNP) outside of the disease-inducing mutation may have the potential to selectively downregulate the disease inducing allele, if the SNP is restricted to the mutated allele. This strategy may be applied to selectively knockdown the disease-inducing allele of triplet repeat disorders, since siRNAs are too small to be able to distinguish between short (wt) or extended (disease inducing) repeats. **D** siRNAs directed at a wildtype sequence has the potential to knock down both (wt and mutated) alleles. Expression of the wildtype gene product is restored by expression of a wildtype transgene with altered codon usage within the target region of the siRNA

hippocampus of APP-transgenic mice reduced BACE1 expression in the hippocampus by about 50% (Singer et al., 2005). As a result, amyloid production decreased significantly and neurodegeneration as well as behavioral deficits were reduced. Studies in BACE-1 knockout mice did not reveal any differences to wildtype animals (including brain histochemistry), suggesting that BACE1 activity is not needed for proper brain function (Roberds et al., 2001). However, these findings became challenged by a recent study, demonstrating that BACE1 controls peripheral nerve myelination (Willem et al., 2006). Future studies aiming to treat experimental AD by targeting BACE1 must therefore carefully monitor the effects on myelination of peripheral nerves.

siRNA in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a progressive loss of motor neurons, skeletal muscle atrophy and paralysis, and it is associated with a mortality of 90% within 5 years after diagnosis (Rowland and Shneider, 2001). Although ALS is usually sporadic, about 10% of all cases have a familiar background, of which about 20% are caused by gain of function mutations within the Cu–Zn-superoxide dismutase (SOD1) gene (Rosen et al., 1993).

Transgenic mice expressing a human SOD1 gene with the disease-inducing point mutation G93A (GGT to GCT at nucleotide 428) develop a denervating, paralytic disease that resembles ALS (Gurney et al., 1994). These SOD_{G93A} transgenic mice become paralyzed in one or more limbs as a result of motor neuron loss from the spinal cord and die by 5–6 months of age.

This mouse model has been used by several groups to study the potential of siRNA as a therapeutic tool. Lentiviral vector-mediated expression of shRNAs targeting the human SOD_{G93A} gene (nucleotides 371–392) reduced SOD protein expression by 52% after delivery into the lumbar spinal cord of 40-day-old mice (Raoul et al., 2005). Motoneuron and motofiber losses were reduced and neuromuscular function improved. Onset of disease was delayed (120 days compared to 100 days) (Raoul et al., 2005). Similar observations were made when lentiviral (EIAV) vectors expressing shRNA directed at nucleotides 252–270 of the SOD_{G93A} gene were injected into multiple muscle groups of 7-day-old mice (Ralph et al., 2005). In both

studies SOD_{G93A} expression was not completely abolished, demonstrating that even a reduction in SOD-expression may positively influence the disease course.

siRNA in Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive development of motor abnormalities and cognitive impairments starting in midlife. HD is caused by an expanded (more than 35) polyglutamine-coding (CAG)-repeat within the huntingtin gene (Snell et al., 1993). Pathologically, HD is characterized by selective loss of brain neurons and the formation of intracellular aggregates (inclusion bodies). A causative therapy is not available.

Several mouse models exist for HD, including knock-out, transgene and knock-in animals. Knock-out and conditional knock-out mice demonstrated that huntingtin is required during embryonic development (Duyao et al., 1995; Nasir et al., 1995; White et al., 1997; Zeitlin et al., 1995) as well as for neuronal function and survival in adult animals (Dragatsis et al., 2000). Transgenic mice that express a fragment (R6/2 mice, Mangiarini et al., 1996) or a full-length copy (Reddy et al., 1998) of mutant human huntingtin in addition to the two endogenous copies of the mouse huntingtin gene exhibit typical phenotypes associated with HD, such as choreiform-like movements, involuntary stereotypic movements, tremor, epileptic seizures and movement disorders. Knock-in animals carry the poly CAG-repeat region of the mutated human HD gene inserted into the mouse huntingtin gene. Although the HD-phenotype in these animals is less pronounced than in transgenic animals (Wheeler et al., 1999), they offer the advantage to study the mutation in its appropriate genomic background (reviewed in Menalled, 2005).

Several studies investigating the effects of siRNA on disease progression in transgenic animals have been reported. In a non-viral delivery approach, siRNA was intraventricularly injected into brains of R6/2 mice using lipofectamine as transfection reagent. Mice were treated at an early postnatal period and phenotype analysis was performed at 8–15 weeks of age. The siRNA targeted a sequence in close proximity upstream of the CAG repeat within the first exon of the HD gene (nucleotides 343–363). Treated mice exhibited prolonged longevity, improved motor function and a slow down in the loss of body weight (Wang et al., 2005).

Stable, viral vector-mediated delivery of shRNAs was performed using AAV vectors in several transgenic mouse models, including HD-N171-82Q (Harper et al., 2005), R6/1 (Rodriguez-Lebron et al., 2005) and HD190QG mice (Machida et al., 2006). Anti-htt siRNA directed at nucleotides 416–436 induced a 85% knockdown in RNA levels and a 55% knockdown in protein levels after striatal injection and significantly improved some of the HD phenotype-associated symptoms, including behavioural and neuropathological abnormalities (Harper et al., 2005). No improvements were observed in stride length, decline in rotarod performances over time and weight loss of the animals (Harper et al., 2005). siRNA expression in the brain was not associated with any negative impact on motor behavior (Harper et al., 2005). In another study, shRNA targeting a region immediately upstream of the CAG repeats (nucleotides 343–363) or a region upstream the start codon (262–280, Rodriguez-Lebron et al., 2005) reduced mRNA levels by 78% and protein expression by 28% after receiving intrastriatal injections of AAV vectors (Rodriguez-Lebron et al., 2005). The size and numbers of neuronal intranuclear inclusions were significantly reduced and steady-state levels of preproenkephalin, dopamine- and cAMP-responsive phosphoprotein mRNA were normalized. Bilateral expression of the shRNA resulted in a delayed onset of the rear paw clapsing phenotype (Rodriguez-Lebron et al., 2005).

These studies demonstrate the potential of siRNA as a treatment option in HD. Further studies will have to reveal whether treatment of HD in its natural genetic background requires an allele-specific siRNA approach (Fig. 3C, D) or a mere reduction of htt-expression (both alleles) as performed in the studies cited above.

siRNA in spinocerebellar ataxia

Spinocerebellar ataxia type 1 (SCA1) represents another neurodegenerative triplet-repeat disorder. SCA1 is a dominantly-inherited progressive disease, caused by extended (44–82) glutamine-coding (CAG)-repeats that are located within the ataxin-1 gene (Orr et al., 1993). SCA1 is characterized by progressive ataxia, caused by cerebellar atrophy and a loss of cerebellar Purkinje cells and brainstem neurons.

Transgenic mice that express the human disease allele (ataxin-1-Q82) under the control of the Purkinje-cells specific promoter PCP-2 develop ataxia and a loss of Purkinje cells (Burright et al., 1995; Clark et al., 1997). In contrast to transgenic models, knock-in mice that carry an extended human SCA-1 repeat within the mouse SCA-1 locus did not show any of the neuropathological changes observed in other transgenic models and did not develop ataxia (Lorenzetti et al., 2000).

Using AAV vector-driven shRNA expression directed at various sequences of the ataxin-1 gene, transgenic SCA1 mice improved motor coordination and exhibited restored cerebellar morphology as well as a reduction of ataxin-1 inclusion bodies in Purkinje cells following intracerebellar inoculation (Xia et al., 2004). Ataxin-1 mRNA levels were reduced by 80% and protein levels by 50–60%. Interestingly, shRNA sequences directed at the CAG-repeat failed to downregulate mRNA levels (Xia et al., 2004).

Concluding remarks

We have learned from *in vitro* and animal studies that siRNA is a highly effective tool to downregulate different target genes involved in the pathogenesis of neurodegenerative diseases. Since many of these diseases cannot be treated with traditional drug therapy, they represent ideal candidates for therapeutic approaches with siRNA. The next steps in the development of siRNA-based treatment strategies for these diseases will have to include the analysis of gene silencing in their respective natural genetic background (e.g. by using knock-in models) and to address the problem of an allele-specific knockdown in diseases caused by dominant-negative mutations if wildtype allele expression is required. Moreover, effective siRNA delivery strategies will have to be developed in order to translate the high transduction efficiency obtained in small mouse brains into the considerably larger human brains.

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J Neural Transm (2007) [Suppl 72]: 51–55

Printed in Austria

Journal of Neural Transmission

Tyrosinase is not detected in human catecholaminergic neurons by immunohistochemistry and Western blot analysis

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Summary Catecholaminergic neurons of the primate substantia nigra (SN) pars compacta (SNc) and the locus coeruleus contain neuromelanin (NM) granules as characteristic structures underlying the pigmentation of these brain areas. Due to a phylogenetic appearance NM granules are absent in the rodent brain, but gradually become present in primates until they reach a maximal expression in humans. Although a possible mechanism of pigment formation may be autoxidation of the NM precursors dopamine or noradrenalin, several groups have suggested an enzymatic formation of NM mediated by tyrosinase or a related enzyme. Since tyrosinase mRNA is suggested to be expressed in the SN of mice and humans, we reinvestigated the expression of tyrosinase in the human SNc and the locus coeruleus at the protein level by immunohistochemistry and Western blot analysis, but could not detect tyrosinase in these brain regions.

Keywords: Neuromelanin synthesis, melanoma, substantia nigra, locus coeruleus

Introduction

Neuromelanin (NM) is a characteristic hallmark of dopaminergic neurons of the human substantia nigra (SN) pars compacta (SNc) and noradrenergic neurons of the locus coeruleus (LC) (Fedorow et al., 2005). While the SN of rodents is devoid of pigmentation, NM is increasingly expressed in primates and is found in highest amounts in the human SN (Marsden, 1961a). NM is localised in cellular organelles termed NM granules, which exhibit associated lipid bulbs and a protein matrix embedding the pigment (Duffy and Tennyson, 1965; Hirosawa, 1968). A recent proteomic analysis of isolated NM granules demonstrated lysosomal features of these organelles and suggested an origin from the endosomal system (Tribl et al., 2005, 2006b).

The formation schedule of NM granules has been mapped for humans starting with the first occurrence of slightly pigmented NM granules approximately at the age of three to five years, which are then rapidly established until the age of 20 (Fedorow et al., 2006). While the number of NM granules remains constant beyond this time point, the pigmentation is suggested to be continuously intensified within the organelles. The process of pigment formation, however, is still unclear (Fedorow et al., 2005), but a genetic program underlying NM biogenesis in the human brain was suggested (Tribl et al., 2005; Fedorow et al., 2006; Halliday et al., 2006).

Two major hypotheses suggest either an autoxidative polymerisation of dopamine (DA) or noradrenalin to NM, or a directly regulated biogenesis upon enzymatic catalysis. Autoxidation of DA is thought to be an ubiquitous process, and thus the specific regional confinement of NM to the DA neurons of the human SN is difficult to understand. One explanation thus suggests a higher concentration of cytosolic DA in the nigral neurons compared to other dopaminergic neurons (Liang et al., 2004). On the other hand, several groups provide evidence of an enzymatic activity present in the SN suggesting peroxidases (Okun et al., 1971), the phenylpyruvate tautomerase activity of the macrophage migration inhibitory factor (MIF) (Matsunaga et al., 1999), prostaglandin H synthase (Hastings, 1995), or

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tyrosinase (Marsden, 1961b; Miranda et al., 1984) to be involved in NM biogenesis, but until today, there is no clear-cut evidence that these enzymes are involved in the NM formation.

Tyrosinase is a copper-dependent monophenol monooxygenase (EC 1.14.18.1) and the key enzyme in the formation of oculocutaneous melanin (Barton et al., 1988). Thereby, tyrosinase mediates the hydroxylation of *L*-tyrosine and the subsequent oxidation of *L*-DOPA to quinone-intermediates that form the insoluble pigment melanin (Cooksey et al., 1997). DA and noradrenaline have been also shown *in vitro* to serve as substrates for tyrosinase (Prota, 2000). Tyrosinase is a highly glycosylated single-pass type I transmembrane protein located in the membrane of cellular pigmented lysosome-related organelles termed melanosomes that are underlying the pigmentation of the skin, hair, and the retina. Several mutations of the tyrosinase gene have been mapped and result either in the total loss of function and thus in the incapability to form melanin (oculaocutaneous albinism I A) or in a reduced enzymatic activity (oculaocutaneous albinism I B) (Giebel et al., 1991a, b; Tripathi et al., 1992; Oetting and King, 1999). Since pigmented melanocytes and neuronal cells are both of neuroectodermal origin, tyrosinase is suggested to be present not only in the skin, but also in neurons (Camacho-Hubner and Beermann, 2001).

The expression of tyrosinase especially in the pigmented neurons of the human SN, however, is still a matter of controversy. Tyrosinase mRNA is suggested to be expressed in the SN of mice and humans (Tief et al., 1998), but a role for tyrosinase in the biogenesis of NM remains to be established. The presence of the corresponding protein, however, has so far not been localized and visualized in the human SN by using immunohistochemical staining (Ikemoto et al., 1998).

The aim of this study was to detect tyrosinase protein in human brain tissue by using immunohistochemical staining and Western blot analysis.

Material and methods

Tissue

Frozen, unfixed human brain tissue and paraffin embedded tissue was obtained from the Brain Bank Würzburg, Department of Neuropathology, at the University of Würzburg. The SNc and the locus coeruleus specimens were dissected from a brain of a 32-year-old male individual with an idiopathic giant-cell myocarditis and hemorrhagic pulmonary infarction, who died from global heart failure. The *post mortem* delay was 12 h.

To provide a positive control for the Western blot analysis, 0.5 g of SN tissue was disrupted by pottering in 'Lysis Buffer' containing protease inhibitor cocktail (0.01% v/v; Sigma, Deisenhofen, Germany), proteins were extracted with 16 mM 3-[(3-cholamidopropyl)dimethylamino-1-propanesulfonate] (Calbiochem, Darmstadt, Germany), as reported previously (Tribl et al., 2005).

A mouse B16 melanoma cell line were cultured as a monolayer in Dulbecco's modified Eagle's medium (PAN Biotech, Aidenbach, Germany) and 8% fetal calf serum supplemented with *L*-glutamine, penicillin and streptomycin at 37°C and 5% CO_2. The cells were subcultured every three days at 70–80% confluence. The cells were harvested by 0.25% trypsin and 0.2% EDTA in PBS for five minutes and collected by centrifugation at 4000 *g* and 4°C.

Antibodies

For immunohistochemical staining of paraffin tissue sections the murine anti-human tyrosinase monoclonal antibody (clone T311, MoBiTec, Göttingen, Germany) and for Western blot analysis a polyclonal anti-human tyrosinase antibody was used (H109; Santa Cruz Biotechnology Inc., Heidelberg, Germany) and horseradish peroxidase-linked secondary anti-rabbit antibody was obtained from Cell Signalling (Cell Signalling, Frankfurt/Main, Germany).

Immunohistochemistry

Paraffin embedded tissue sections (8 µm) were prepared with the microtome. The sections were deparaffinized in xylene and rehydrated in graded ethanol. The sections were microwaved for 5 min in citrate-buffer (0.01 M, pH 6) and endogenous peroxidases were blocked by 0.5% H_2O_2. Non-specific binding sites were blocked using the blocking serum of the manufacturer. Sections were incubated with the primary antibody in 5% blocking serum over night at 4°C. The sections were shortly rinsed with phosphate-buffered saline (PBS; 0.01 M, pH 7.3) and incubated with a biotinylated secondary antibody, rinsed with PBS and incubated with a streptavidin-HRP (horseradish peroxidase) complex (biogenex). H_2O_2 was used as the substrate for HRP, 3-amino-9-ethylcarbazole (AEC) as chromogen. Finally the sections were counterstained with hematoxlyin, cleared and mounted.

Western blot analysis

The protein samples were separated electrophoretically on a 10–20% tricine gel (Novex, San Diego, CA, USA) in an XCell II™ Mini Cell (Novex, San Diego, CA, USA) using tricine-SDS running buffer. Following electrophoresis the gel was further processed for Western blotting.

The separated proteins were transferred electrophoretically onto a nitrocellulose membrane (Invitrogen GmbH, Karlsruhe, Germany) employing the XCell II™ blot module. Nonspecific binding was blocked with 5% w/v nonfat dried milk in Tris-buffered saline, pH 7.3 for 1 h at 20°C. Immunoblots were probed with primary antibody at the appropriate dilution at 4°C overnight. Membranes were washed in Tris-buffered saline containing 0.1% v/v Tween 20 (3 × 10 min) followed by incubation with the secondary antibody at 20°C for 1 h. Additional washing was performed with Tris-buffered saline containing 0.1% v/v Tween 20 (3 × 10 min) and the immunocomplexes were visualized by enhanced chemiluminescence (ECL-system™, Roche Diagnostics, Mannheim, Germany).

Results

Since tyrosinase is expressed in cells of melanomas, which are malignant tumors usually derived from skin melanocytes, a melanoma was thus used as a positive control for the detection of tyrosinase expression by using immunhistochemical staining. Indeed, the incubation with the well

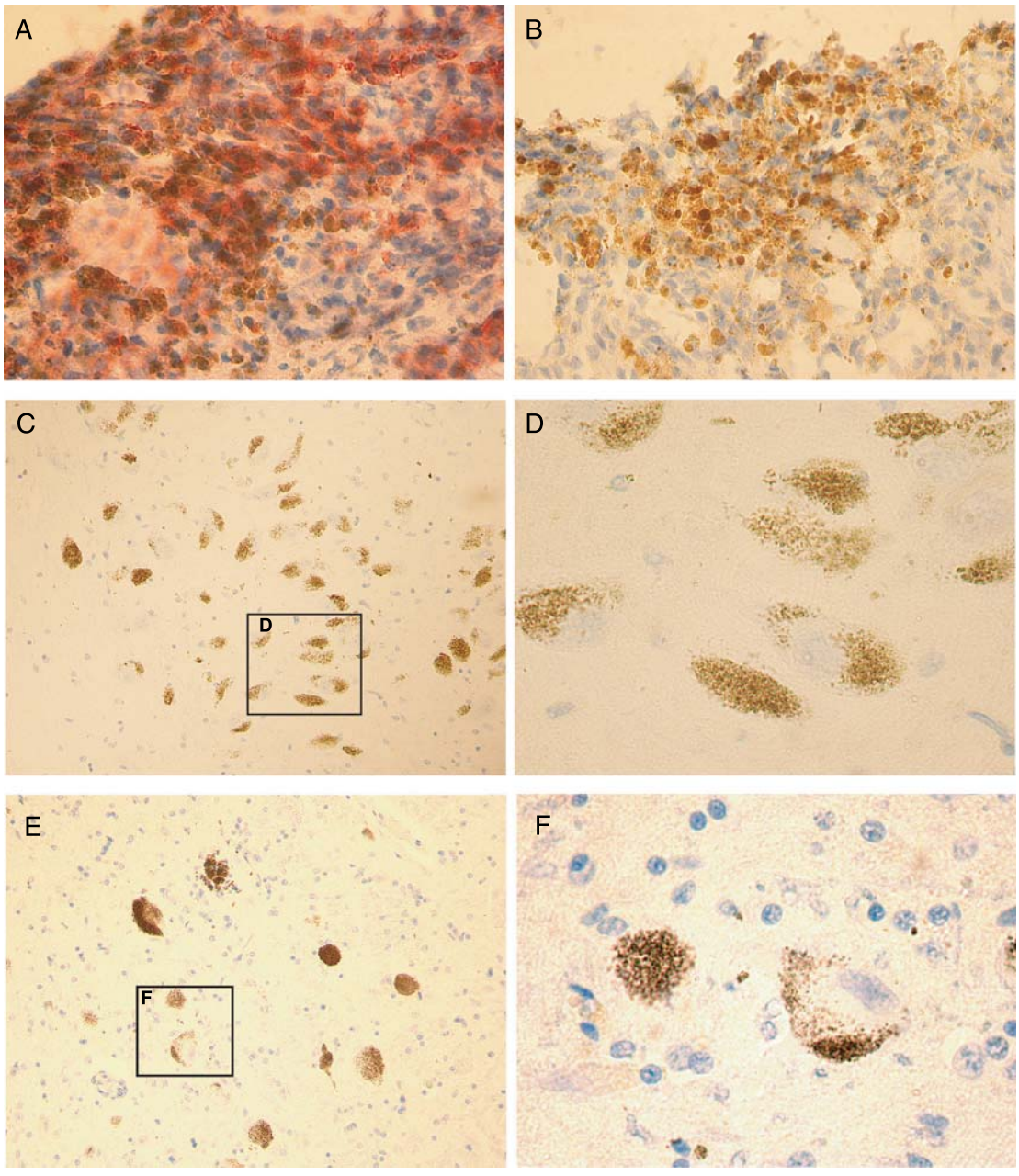

Fig. 1. Tyrosinase is expressed in pigmented melanoma cells, but not in neurons of human substantia nigra pars compacta and locus coeruleus. **A** Classical immunohistochemical analysis shows the expression of tyrosinase in pigmented cells of a human melanoma (magnification ×100). **B** Negative control. Omitting the anti-tyrosinase antibody no staining deriving from the secondary antibody or the avidin-biotin-HRP complex is visible (magnification ×100). **C** Both, neuromelanin-containing neurons and neurons devoid of neuromelanin of the substantia nigra pars compacta of a 32-year-old individual do not stain for tyrosinase (magnification ×50). **D** Detail of **C** (magnification ×200). **E** Neuromelanin containing neurons of the locus coeruleus of a 32-year-old individual do not stain for tyrosinase (magnification ×50), **F** Detail of **E** (magnification ×200)

established antibody T311 that is applied for routine diagnosis of melanoma gives an intense signal visible by red staining (Fig. 1A). Tyrosinase is especially stained in those cells, which exhibit the brownish melanin. We detected tyrosinase in homogenates of murine B16 melanoma cells by using Western blot analysis (Fig. 2).

The experimental setup successfully applied on melanoma tissue and murine B16 melanoma cells was subsequently transferred to the human brain tissue. However, we find no evidence for the expression of tyrosinase in the SNc and LC of the human brain neither by immunohistochemistry nor by Western blot analysis (Figs. 1C–F, 2).

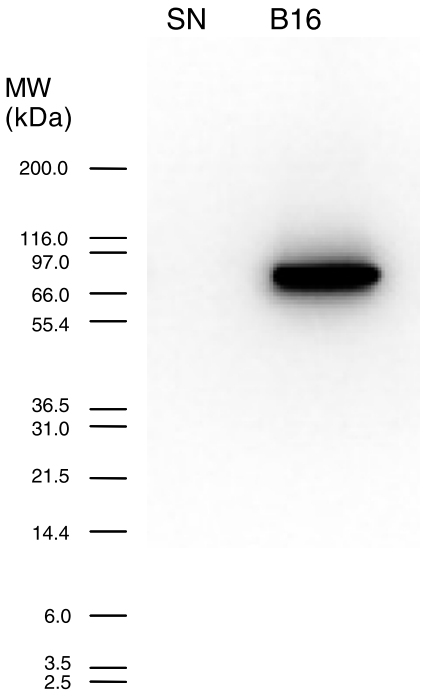

Fig. 2. Western blot analysis of murine melanoma cells and human brain homogenate. Applying a polyclonal antibody to tyrosinase (H109) the expression of tyrosinase was monitored in a homogenate of murine B16 melanoma cells and is visualised in a mass area between 66 and 90 kDa (B16). In contrast, tyrosinase is not detected in a human substantia nigra pars compacta homogenate (*SN*)

Discussion

By applying standard immunological approaches we detected tyrosinase expression in highly pigmented melanoma cells, but not in the pigmented neurons of the human SN and the LC. This result suggests that tyrosinase is not involved in the main pathway of NM synthesis. Similar results were reported by Ikemoto et al. (1998), showing no tyrosinase-immunoreactivity in the human pigmented midbrain, whereas neurons of these brain regions were strongly immunoreactive to tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis.

Using a reporter gene assay, the expression of tyrosinase in the brain of mice was detected during early developmental stages, including the SN (Tief et al., 1996, 1998). Expression of tyrosinase in murine forebrain and midbrain areas was also preserved in adult mice, but since no pigment biogenesis takes place in these areas an unknown function different from melanin formation has been suggested for tyrosinase. In the human brain tyrosinase mRNA was detected as an approximately 1600-bp long PCR fragment amplified from human brain cDNA and corresponding to the full-length transcript of human tyrosinase (Greggio et al., 2005). Thereby, tyrosinase mRNA was demonstrated in the human SN (Xu et al., 1997; Greggio et al., 2005), but also in regions, which do not produce NM, e.g. the cortex, the caudate nucleus, the globus pallidus and the putamen (Greggio et al., 2005). Nevertheless, since these PCR experiments required a high number of 40 amplifying cycles, the authors emphasize the very low expression level of the tyrosinase gene in these brain regions.

Therefore, the amount of tyrosinase protein is probably very low in the human brain, so it is rather difficult to unambiguously identify this protein by using immunohistochemical staining and Western blot analysis. Both methods are known to be rather insensitive. Nevertheless, rather than probing whole tissue homogenates, the enrichment of the target structure, e.g. a cellular compartment, prior to analysis may allow a more reliable detection (Tribl et al., 2006a). Tyrosinase is a transmembrane protein usually localised in the melanin-containing melanosomes in the oculocutaneous system. In cultured cells tyrosinase is sorted to lysosomal organelles (Hasegawa et al., 2003) since this glycoprotein disposes of a di-leucine-based lysosomal sorting motif (Honing et al., 1998; Calvo et al., 1999) and a tyrosine-based signal at the cytoplasmic *C*-terminus (Simmen et al., 1999). If expressed in the human brain, tyrosinase would thus be expected to be found in lysosomal compartments and most obviously in the pigment granules of the SN and the LC. Recently, the enrichment of the NM granules from the human brain was successfully accomplished, and in consistence with the data presented here, the investigation of the protein composition by mass spectrometric peptide sequencing, which facilitates a direct detection and identification of a protein, did not uncover the presence of tyrosinase therein (Tribl et al., 2005). Conversely, applying organelle enrichment and a mass spectrometric protein identification, tyrosinase was detected in oculocutaneous melanosomes (Basrur et al., 2003).

Based on our immunological data presented here and our previous work on the NM granule proteome we conclude that tyrosinase is unlikely to mediate the formation of NM in the human brain.

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J Neural Transm (2007) [Suppl 72]: 57–67

Printed in Austria

Journal of Neural Transmission

Luteolin protects rat PC12 and C6 cells against MPP$^+$ induced toxicity via an ERK dependent Keap1-Nrf2-ARE pathway

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Summary Oxidative stress is central to neuronal damage in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. In consequence, activation of the cerebral oxidative stress defence is considered as a promising strategy of therapeutic intervention. Here we demonstrate that the flavone luteolin confers neuroprotection against oxidative stress via activation of the *nuclear factor erythroid-2-related factor 2* (Nrf2), a transcription factor central to the maintenance of the cellular redox homeostasis. Luteolin protects rat neural PC12 and glial C6 cells from N-methyl-4-phenyl-pyridinium (MPP$^+$) induced toxicity *in vitro* and effectively activates Nrf2 as shown by ARE-reporter gene assays. This protection critically depends on the activation of Nrf2 since downregulation of Nrf2 by shRNA completely abrogates the protection of luteolin *in vitro*. Furthermore, the neuroprotective effect of luteolin is abolished by the inhibition of the luteolin-induced ERK1/2-activation. Our results highlight the relevance of Nrf2 for neural cell survival conferred by flavones. In particular, we identified luteolin as a promising lead for the search of orally available, blood brain barrier permeable compounds to support the therapy of neurodegenerative disorders.

Keywords: Luteolin, flavonoids, MPP$^+$, Nrf2, nuclear factor, Parkinson's disease

Nonstandard abbreviations

ARE	Antioxidant response element
C6	rat glioblastoma cells
GCS	gamma-glutamyl-cysteine-synthase
EpRE	electrophile response element
Keap1	Kelch-like ECH associating protein
MPP$^+$	1-methyl-4-phenyl-pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NQO1	NAD(P)H-quinone-oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PC12	rat phaeochromocytoma cells
PI3K	phosphoinositol-3-kinase
shRNA	short hairpin RNA
siRNA	short interference RNA
tBHQ	tert-butyl hydroquinone

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Introduction

Oxidative stress and mitochondrial dysfunction are considered to be central factors for neuronal degeneration in aging as well as Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis or Parkinson's disease (PD) (Andersen, 2004; Götz et al., 1994; Manfredi and Xu, 2005; Mariani et al., 2005; Wright et al., 2004). Multiple lines of evidence implicate the increased formation of reactive biological intermediates including reactive oxygen species, reactive nitrogen species, and electrophiles as aggravating factors in disease progression (Moore et al., 2005). In PD defects in complex I of the mitochondrial respiratory chain (Lestienne et al., 1990; Reichmann et al., 1990; Schapira et al., 1990) and increased levels of biomarkers of oxidative stress such as increased concentrations of iron and lipid peroxidation products in the substantia nigra were discussed (Dexter et al., 1989; Riederer et al., 1989; Götz et al., 2004; Zecca et al., 2004). PD is also associated with exposure to pesticides, many of which are either oxidants or mitochondrial toxicants (Tanner, 1989; Tanner et al., 1999). Rapid onset of parkinsonism in man, primate, and mouse following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983; Gerlach and Riederer, 1996) occurs through its active metabolite, 1-methyl-4-phenylpyridinium (MPP$^+$) (Langston et al., 1984), a reversible inhibitor of mitochondrial complex I, which leads to the depletion of energy stores and the induction of oxidative stress *in vivo* (Nicklas et al., 1987; Przedborski et al., 2004; Przedborski and Ischiropoulos, 2005). Therapeutic intervention with antioxidants, however, have failed to attenuate disease progression in PD and other neurodegenerative disorders (Shoulson, 1998).

An alternative strategy is the administration of compounds that enable the upregulation of endogenous antioxidative defence systems in the brain such as the *nuclear factor erythroid 2-related factor 2* (Nrf2). The activation of the Nrf2 transcription factor regulates the transcription of phase-II detoxifying enzymes and subsequently the redox homeostasis in numerous cell types including glia and neurons (Nguyen et al., 2000, 2003; Lee et al., 2003a, b). Nrf2 activity renders neural cells more resistant to oxidative and electrophilic stress particularly with regard to MPTP (Lee et al., 2005a; Burton et al., 2006). Binding of Nrf2 to the antioxidant response element (ARE) initiates the transcription of cytoprotective enzymes such as glutathione transferases (GST-α4, -μ1 and -μ3, π2), NADPH-quinone-oxidoreductase 1 (NQO1) as well as γ-glutamyl-cysteine-synthetase (GCS). Although the precise mechanisms of Nrf2 activation are controversially discussed, it is generally accepted that electrophiles disrupt the inhibitory Nrf2-Keap1 interaction, and stabilise Nrf2 which in turn activates the transcription of *ARE* responsive genes (Wakabayashi et al., 2004; Nguyen et al., 2005).

Flavonoids can attenuate death of neural cells (Datla et al., 2001; Dajas et al., 2003; Abdel-Wahab, 2005; Burton et al., 2006), but the underlying mechanisms of protection are not clarified and cannot be solely attributed to their intrinsic antioxidative properties. Here we have investigated the hypothesis that flavonoids that bear an electrophilic α-β-unsaturated carbonyl moiety may confer neuroprotection via activation of Nrf2. Our analysis included two flavones (luteolin and baicalein) and two isoflavones (genistein and daidzein) with intermediate polarity and solubility in DMSO. Luteolin is a natural flavone from esculent plants such as celery (Manach et al., 2004). The flavone baicalein purified from *Scutellaria baicalensis Georgi* is used as a traditional chinese herbal medicine and can protect from 6-hydroxydopamine-induced neurotoxicity (Im et al., 2005; Lee et al., 2005b). Genistein and daidzein are the aglycones of two isoflavones originating from leguminous plants such as soya and are considered as cancer chemopreventive agents (Kawanishi et al., 2005).

Here we show that selected flavonoids are potent activators of Nrf2, and that Nrf2 mediates the neuroprotection of flavonoids *in vitro*. These findings further elucidate the neuroprotective potential of Nrf2 activation.

Material and methods

Reagents

Luteolin, genistein, baicalein, daidzein, resveratrol, tBHQ, trolox, sulforaphane, PD98059, SP600125, SB203580, UO126 and wortmannin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and cell viability assay

Rat phaeochromocytoma cells (PC12) and rat glioblastoma cells (C6) were grown in DMEM-Ham's F12 1:1 medium with 2 mM glutamine (PAA-Laboratories, Pasching, Austria) and N2-supplement containing putrescine, IGF-1, transferrin, progesterone and selenite (Invitrogen). 5000 PC12 cells per well were plated on BIOCOAT Collagen I 96-well-plates (VWR-International, Hamburg, Germany) in 100 μl serum-free medium and allowed to attach for 24 h.

For 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST) assay, 10 μl/well WST were added to the media (Roche Diagnostics GmbH, Penzberg, Germany) 2 h before spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (Model680, Bio-Rad, Hercules, CA) and this reaction reflects the reductive capacity of the cell.

Plasmid construction

Both strands of *ARE1* of the rat NQO1 gene

5′-CAGTCTAGAGTCACAGTGACTTGGCAAAATCG-3′
5′ CTAGCGATTTTGCCAAGTCACTGTGACTCTAGACTGGTAC

with *Kpn*I and *Nhe*I ends were synthesized, annealed, and cloned at the *Kpn*I and *Nhe*I site of the pGL3-Promoter (Promega) to produce the reporter construct pNQO1-rARE.

Luciferase assays

1.5 μg of the NQO1-ARE reporter plasmids containing the firefly luciferase reporter gene, and 0.5 μg of the pRL-TK plasmid, containing the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase promoter as an internal control, were cotransfected into cells in a 10 cm plate by the lipotransfection method (Lipofectamine 2000, Invitrogen) according to the manufacturer's recommendation. 24 h after transfection, the cells were transferred into a 96-well plate. The activities of both, *Firefly* and *Renilla* luciferases were determined 48 h after transfection with the dual luciferase reporter assay system (Promega, Madison, Wis.). The luciferase activities were normalized to the corresponding *Renilla* luciferase activities.

Small interference RNA (siRNA)

The mammalian expression vector pGE1 (Stratagene) was used for the expression of siRNA in PC12 cells. The gene-specific insert which is specified by a 29-nucleotide sequence 5′-GTCTTCAGCATGTTACGTGAT GAGGATGG-3′ of the rat Nrf2 was separated by a 8-nucleotide non-complementary spacer (GAAGCTTG) from the reverse complement of the same 29-nucleotide sequence. This construct was inserted into the pGE1 using *Bam*HI and *Xba*I restriction sides, and referred to as pGE1-rNrf2. A control vector (pGE1-negative) served as a non-silencing control (Stratagene).

Protein analysis

PC12 cells were washed in ice cold PBS, harvested in 200 μl TNE lysis buffer containing 50 mM TRIS, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA (pH 8), and centrifuged for 15 min at 4°C at 14.000 *g*. The cytosols were deep frozen at −20°C. The nuclear pellets were dissolved in 50 μl buffer containing 10 mM HEPES, 400 mM NaCl, 1 mM DTT, and 0.2 mM EDTA by sonication with 10 pulses at 10% performance using a Bandelin electronic sonicator (Berlin, Germany) and stored at −20°C. Twenty microgram of total proteins were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes (Immobilin-P, Millipore Bedford MA, U.S.A.). The membranes were blocked for 1 h with

4% non-fat dry milk and incubated with the primary antibodies against ERK1/2 and phosphorylated ERK1/2 (each 1:2000, rabbit polyclonal IgG, Cell Signaling Technology, Beverly, U.S.A.), Nrf2 (1:1000, rabbit polyclonal IgG, Santa Cruz CA, U.S.A.).

After three washing steps with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody for 30 min. The membranes were developed using the ECL chemiluminescence system and Hyperfilm ECL (Amersham, Piscataway, U.S.A.). For reprobing, blots were stripped in 2% SDS, 62.5 mM Tris and 100 mM mercaptoethanol for 30 min at 50°C, washed with TBST, and blocked again. All measurements of dual-phosphorylated kinase (p-ERK1/2) levels were normalized by hybridization with antibodies against total kinase protein (total ERK1/2).

Results

Cytotoxicity of flavonoids

Initially we defined the dose-dependent cytotoxicity of the selected flavonoids in WST-assays. In naive PC12 cells, the threshold dose for cytotoxicity is 10 μM for tBHQ, sulforaphane, luteolin, baicalein and genistein. Interestingly, 5 μM luteolin as well as 1–5 μM tBHQ significantly increase the viability of PC12 cells compared with solvent controls (DMSO 0.5%; Fig. 1) suggesting a stabilizing mode of energy metabolism even in the absence of stressful stimuli. As oxidative stressor we used the toxicant MPP^+ that provoked a dose-dependent death of PC12 cells (Figs. 5 and 6). If not otherwise mentioned, flavonoids were used at 5 μM, the highest non-toxic concentration; MPP^+ was used at 100 μM, a dose which reduced cell viablility by around 50% (Fig. 6); the final concentration of the solvent DMSO was limited to 0.5% (64 mM), a dose which did not affect cell viability (data not shown).

Activation of Nrf2 through the measurement of NQO1-ARE response

For the investigation of the flavonoids' potency to activate Nrf2 we conducted dual luciferase reporter gene assays. The Nrf2-reporter gene contained a classical binding site for Nrf2, the *cis*-acting antioxidant response element (*ARE*) of the NQO1-gene, and an increase in the firefly luciferase expression indicates the binding of Nrf2 to the NQO1-*ARE* element. Sulforaphane, an isothiocyanate from broccoli, and the synthetic tBHQ are well defined activators of Nrf2 and therefore were used as positive controls.

All the flavonoids investigated as well as sulforaphane and tBHQ activated the luciferase gene expression in the absence of any intentional stimulus (Fig. 2A). The activation potency differed between the compounds and the rank order of significant NQO1-*ARE* response compared with the solvent 0.5% DMSO was luteolin > sulforaphane > genistein = tBHQ = baicalein = daidzein (ANOVA and post hoc Duncan's multiple range test). To prove the direct activation of Nrf2, we investigated the nuclear presence of Nrf2 following luteolin on Western blots. Indeed, Nrf2

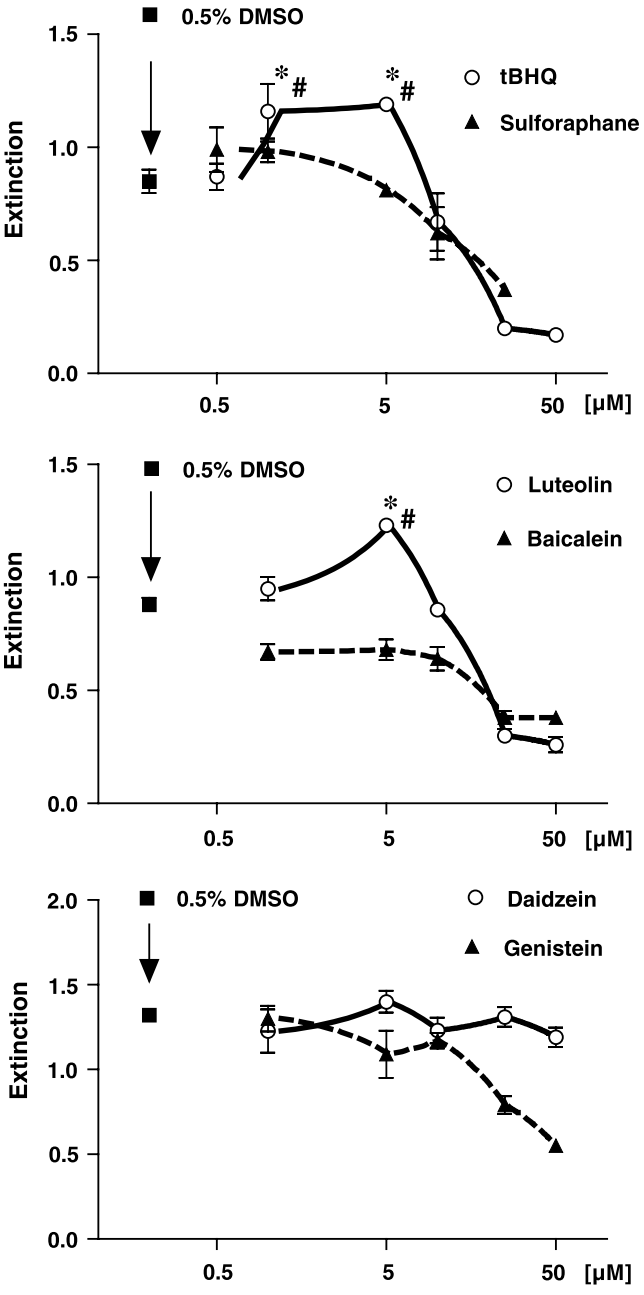


Fig. 1. Viability of rat PC12 cells as determined by the WST assay (Extinction). tBHQ, sulforaphane, luteolin, baicalein, daidzein, and genistein were administered at all doses with 0.5% DMSO as the solvent. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control, #significant difference versus 1 and 5 μM sulforaphane or versus 5 μM baicalein

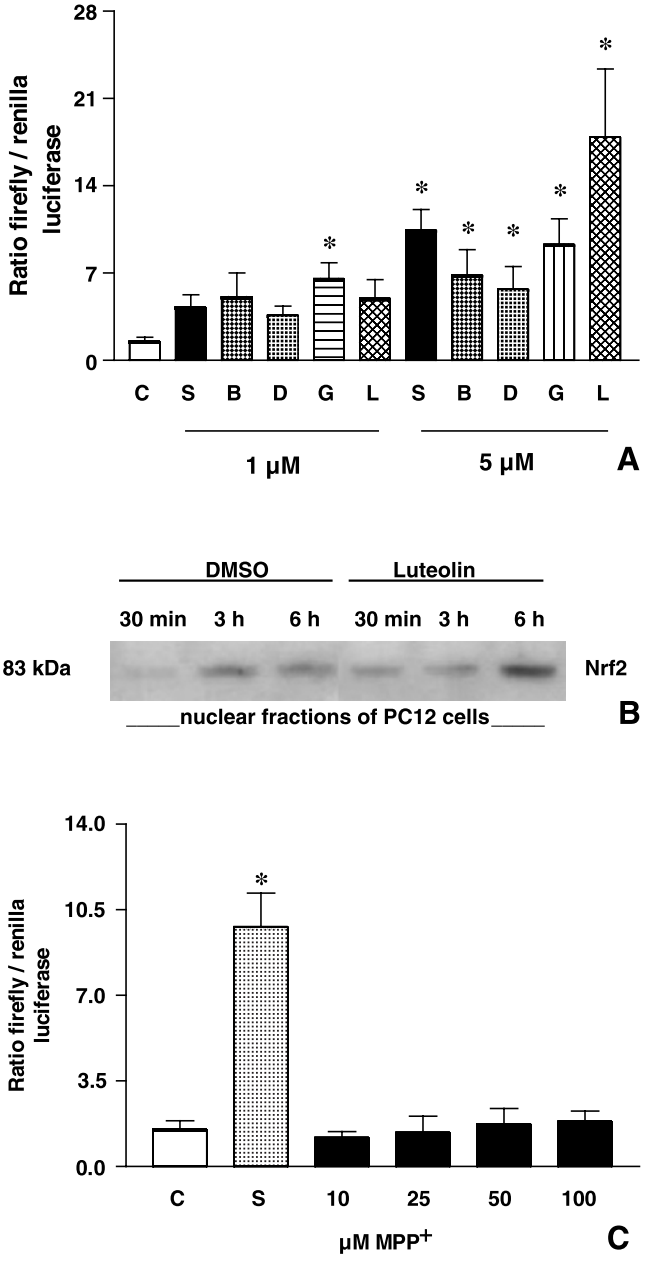


Fig. 2 **A** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in rat PC12 cells. Sulforaphane (*S*), baicalein (*B*), daidzein (*D*), genistein (*G*), and luteolin (*L*) activate NQO1-*ARE* response in a dose dependent manner. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences $p < 0.05$ between groups were evaluated using ANOVA and multiple range test; *significant difference versus control (*C*). **B** Western blots of PC12 cell extracts following DMSO or luteolin. Nrf2 was detected in the nuclear fractions following incubation times indicated. Each lane was loaded with 20 µg protein. **C** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in PC12 cells in the presence of sulforaphane (S) or 1-methyl-4-phenylpyridinium iodide (MPP^+). Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test: *significant difference versus control (C = 0.5% DMSO)

increased in the nuclear compartment within 6 h after exposure to luteolin (Fig. 2B).

Furthermore, we analysed the impact of MPP^+, the pathogenic stimulus used for the following experiments, on NQO1-*ARE* response. The neurotoxicant MPP^+ alone did not affect the expression of firefly luciferase (Fig. 2C).

Finally we wanted to know whether the induction of ARE merely depends on the antioxidative properties or requires defined structural properties e.g. electrophilic α,β-unsaturated carbonyls. We investigated the NQO1-*ARE* response following exposure to the well known and

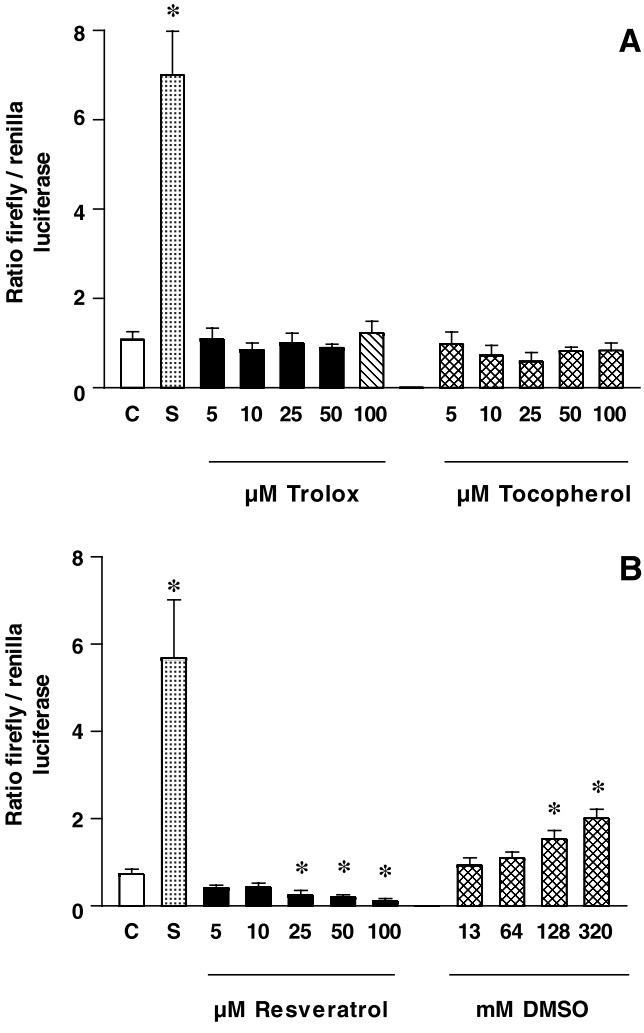
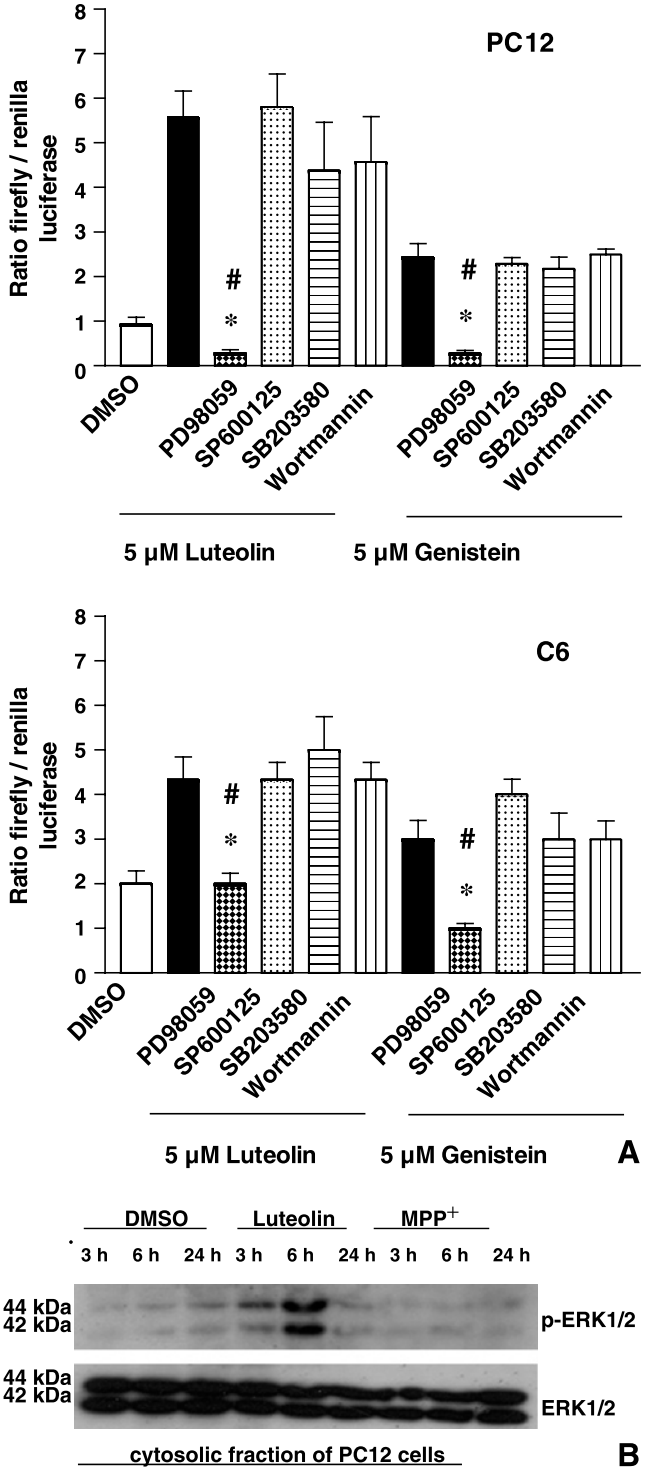


Fig. 3. Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in rat PC12 cells. **A** Cells were dose dependently stimulated for 24 h with trolox or tocopherolacetate in 0.5% DMSO. **B** Cells were dose dependently stimulated for 24 h with resveratrol in 0.5% DMSO or with different concentrations of solvent DMSO (0.1% = 12.8 mM; 0.5% = 64 mM; 1% = 128 mM; 2.5% = 320 mM). Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control group (*C* = 0.5% DMSO = 64 mM); (*S*) sulforaphane 5 µM

potent phenolic antioxidants resveratrol and trolox that do not belong to the flavonoid family but bear antioxidative phenolic moieties resembling that of flavonoids. Interestingly, neither resveratrol nor α-tocopherol nor trolox, a water soluble analogue to tocopherol, activate the NQO1-*ARE*-response in PC12 cells (Fig. 3). This finding suggests



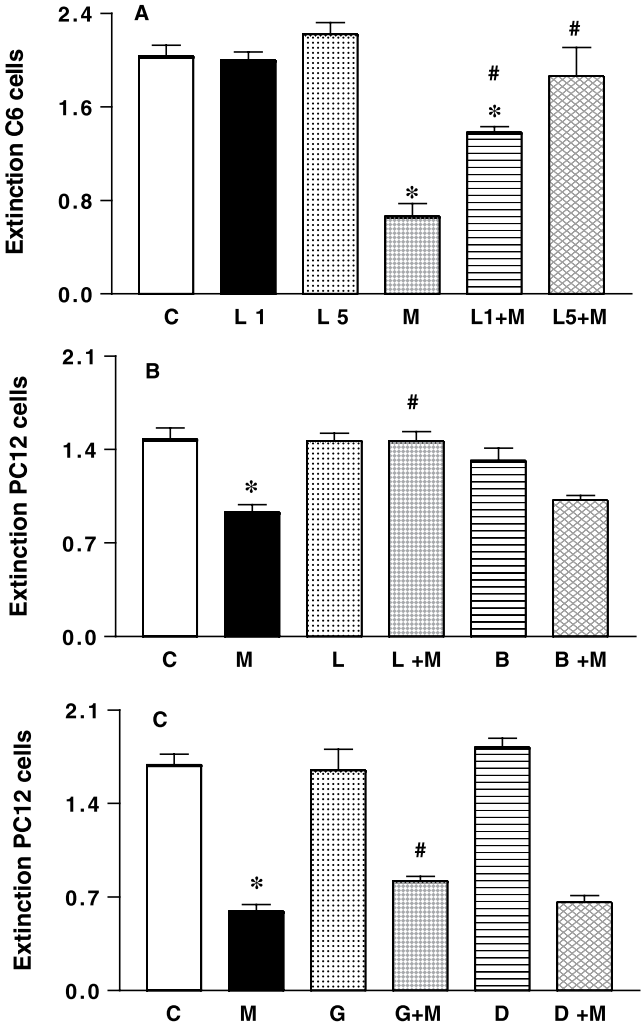


Fig. 5. Viability of PC12 cells and C6 cells as determined by the WST assay 24 h following 100 µM MPP^+ (*M*). **A** C6 glioblastoma cells were preincubated for 16 h with 1 µM luteolin (*L1*) or 5 µM luteolin (*L5*). PC12 cells were incubated with **B** 5 µM luteolin (*L*), or 5 µM baicalein (*B*); or **C** 5 µM genistein (*G*) or 5 µM daidzein (*D*). Values were collected 24 h following the exposure to 100 µM MPP^+ (*M*) and are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control (*C*), #significant difference versus MPP^+ (*M*)

Fig. 4. **A** Ratio of firefly luciferase expression to renilla luciferase expression in a dual luciferase reporter gene assay for the determination of the NQO1-*ARE* response in the absence or presence of kinases inhibitors in rat PC12 cells and in rat C6 cells. Values are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus control (0.5% DMSO), #significant difference versus luteolin and genistein without kinase inhibitor (solid bars). ERK1/2 inhibitor PD98059 (20 µM), JNK inhibitor SP600125 (2 µM), p38 inhibitor SB203580 (5 µM), PI3K inhibitor wortmannin (1 µM). **B** Western blots of PC12 cell extracts following 3, 6, or 24 h incubation with 5 µM luteolin or 100 µM MPP^+. Phospho-ERK1/2 and total ERK1/2 were determined in the cytosolic fractions. Phospho-ERK1/2 signal appears selectively increased 6 h following 5 µM luteolin. Each lane contains 20 µg of protein

that the NQO1-*ARE* response is not activated by the mere antioxidant action of flavonoids.

Activation of NQO1-ARE-response by luteolin involves the ERK1/2 pathway

The following experiments addressed the signaling pathway underlying the activation of Nrf2 by flavonoids. As visualised by the dual luciferase assay, flavonoid-mediated Nrf2 activation was almost completely abolished in PC12 and C6 cells by PD98059, an inhibitor of MEK1, the upstream kinase of ERK1/2 (Fig. 4A). In striking contrast, inhibition of c-Jun N-terminal kinases by SP600125, inhibition of p38 by SB203580 and inhibition of phospho-inositol-3-kinase (PI3K) by wortmannin did not affect the NQO1-*ARE* response in PC12 or in C6 cells. ERK1/2 are also directly activated by flavonoids (Fig. 4B). Between 3 and 6 h after exposure, luteolin evoked a strong phosphorylation of ERK1/2 which vanished after 24 h, whereas neither DMSO nor MPP^+ showed any effect on ERK1/2 phosphorylation. The pool of total ERK1/2 did not change in all specimens.

Cytoprotection by flavonoids

In the next experiments we investigated whether flavonoids protect PC12 cells from toxicity exerted by the mitochondrial complex I inhibitor MPP^+. Non-differentiated, i.e. mitotic C6 and PC12 cells were incubated with the flavonoids 16 h before the exposure to 100 μM MPP^+. Cell viability was measured after further 24 h incubation without medium exchange. 5 μM luteolin significantly enhanced viability of C6 cells following MPP^+ mediated toxicity (Fig. 5A). In PC12 cells, luteolin completely reversed the MPP^+ induced death, whereas genistein showed only moderate effect. Neither baicalein nor daidzein counteracted the MPP^+ toxicity (Fig. 5B, C).

Does luteolin also confer protection from MPP^+ toxicity in post-mitotic neuron-like PC12 cells which were differentiated for 6 days with NGF (50 ng/ml) prior to exposure? The complete protection of luteolin was similar to the

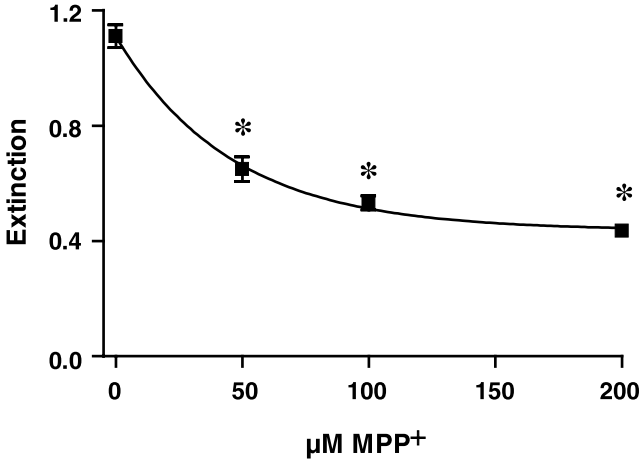


Fig. 6. PC12 cells differentiated with 50 ng/ml NGF for 6 days were exposed to various concentrations of MPP^+. Cell viability is quantified by the measurement of WST. Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of six independent determinations ($n = 6$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus 0.2% DMSO as control

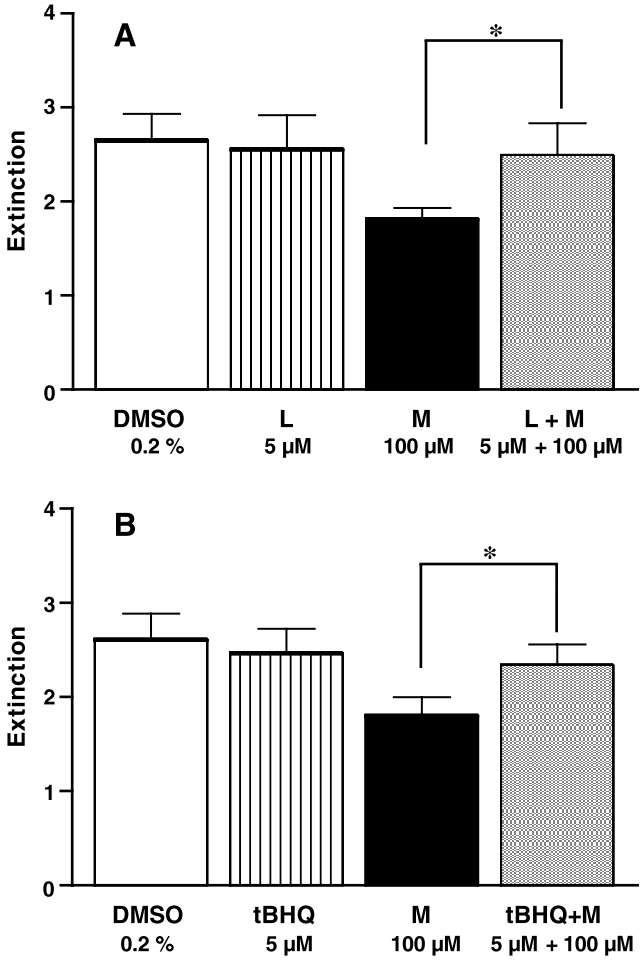


Fig. 7. **A** Luteolin and **B** tBHQ maintain the chemical reductive capacity of differentiated PC12 cells in the presence of 100 μM MPP^+. 5 μM luteolin or 5 μM tBHQ were incubated for 16 h prior to the addition of 100 μM MPP^+ and further incubation for 24 h without a medium exchange at a final concentration of 0.2% DMSO. Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of six independent determinations ($n = 6$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus MPP^+

protection of tBHQ (Fig. 7A, B), a strong synthetic activator of Nrf2, which was used as gold standard.

Inhibition of Nrf2 prevents neuroprotection by luteolin

So far we have shown that flavonoids – in particular luteolin – enhance the reportergene transcription driven by NQO1-*ARE* activation, increase the nuclear amount of Nrf2, activate ERK1/2 and protect against MPP^+ triggered neural death. The crucial question arose whether Nrf2 mediates the neuroprotection of luteolin. To clarify this issue, PC12 cells were stably transfected with a vector construct that expresses short hairpin RNA (*sh*RNA) against *Nrf2* mRNA which prevents the expression of Nrf2. In PC12 cells transfected with Nrf2-*sh*RNA, 5 μM luteolin did not increase the viability of untreated PC12 cells, and the oxidative stressor MPP^+ enhanced the death of Nrf2-*sh*RNA transfected PC12 cells (Fig. 8) compared to control-transfected PC12 cells. Importantly, 5 μM luteolin lost all its neuroprotective effect in MPP^+ stressed PC12 cells transfected with Nrf2-*sh*RNA, whereas 5 μM luteolin was perfectly protective in control-transfected PC12 cells (Fig. 8).

Intracellular signaling of luteolin involved in cytoprotection

As shown in the preceding experiments, luteolin induced the phosphorylation of ERK1/2 (Fig. 2B), and the Nrf2 activation by luteolin was antagonised by the MEK1 inhibitor PD98059 (Fig. 4). These findings imply that ERK1/2 triggers the cytoprotection of luteolin. Indeed, cytoprotection by luteolin was significantly attenuated in the presence of UO126, a direct inhibitor of MEK1/2 and indirect inhibitor of ERK1/2 (Fig. 9). Thus, the MEK1/2-ERK1/2 signaling also mediates the luteolin-induced cytoprotection against MPP^+.

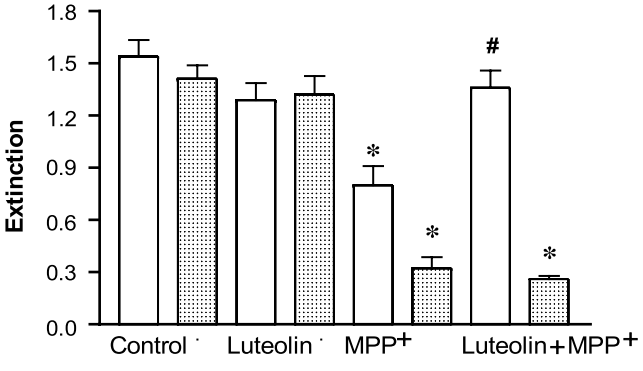


Fig. 8. Stable expression of shRNA against Nrf2 abrogates protection from toxicity of MPP^+ by luteolin. Luteolin was preincubated for 16 h with PC12 cells stably transfected with a control vector (open bars), or with cells stably expressing shRNA against Nrf2 thus downregulating Nrf2 expression (hatched bars). Values were collected 24 h following the exposure to MPP^+ and are expressed as means ± SD of eight independent determinations ($n = 8$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus DMSO or luteolin controls, respectively; #significant difference versus MPP^+

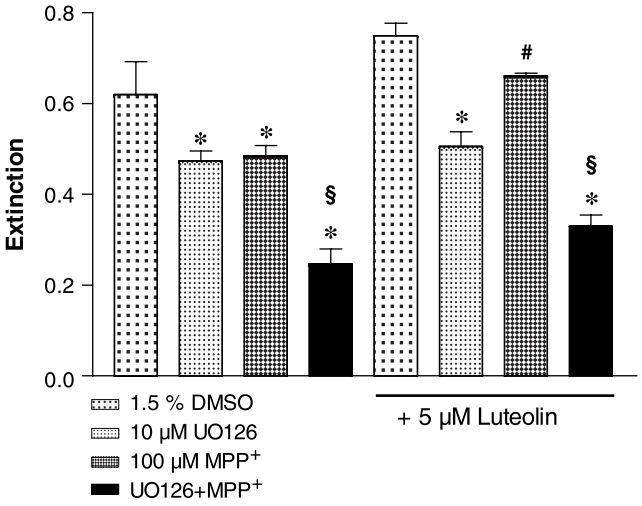


Fig. 9. Luteolin maintains the reductive capacity of differentiated PC12 cells in the presence of 100 μM MPP^+, but not in the presence of the MEK inhibitor UO126 (10 μM) that was given 1 h before luteolin. Luteolin was incubated for 16 h prior to the addition of 100 μM MPP^+ and further incubation for 24 h without a medium exchange at a final concentration of 1.5% DMSO given as three times 0.5% at the respective time points. Values are expressed as means ± SD of three independent determinations ($n = 3$). Statistical differences ($p < 0.05$) between groups were evaluated using ANOVA and multiple range test. *Significant difference versus DMSO and DMSO + luteolin; #significant difference versus MPP^+; §significant difference versus UO126

Discussion

In the present study, we have provided novel insights into the mode of neuroprotection of flavonoids. The flavones luteolin and baicalein as well as the isoflavones genistein and daidzein activate the NQO1-*ARE*, a classical consensus element of the Nrf2 transcription factor. This implicates that these flavonoids enhance the neuronal defence via Nrf2 activation. Of these flavonoids only genistein and luteolin elicit protection from MPP^+ toxicity; in particular luteolin, that shows the strongest Nrf2-activation, completely protects from MPP^+ toxicity. Indeed, inhibition of Nrf2 by shRNA abrogates the neuroprotection of luteolin which triggers neuroprotection in a MEK-ERK1/2 dependent manner.

Activation of Nrf2

Oxidative stress and mitochondrial dysfunction are considered as central factors for neuronal damage in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease, and amyotrophic lateral sclerosis (ALS). Consequently, activation of the endogenous cerebral oxidative stress defence mechanisms appears as a valid strategy for therapeutic intervention (Lee and Johnson, 2004; van Muiswinkel and Kuiperij, 2005). *In vivo* and *in vitro* studies have demonstrated that polyphenolic flavonoids have neuroprotective potential (for a review, see Mandel et al., 2004), but the underlying mechanisms are still under intense investigation. We have demonstrated that the flavonoids luteolin, genistein, baicalein and daidzein potently activate Nrf2 at a concentration of 5 μM in PC12 and C6 cells. This activation depends on ERK1/2 signaling which is blocked by the MEK1/2 inhibitors PD98059 as well as UO126. This strongly indicates that ERK1/2 activation is a prerequisite for Nrf2 activation by the flavonoids investigated (Fig. 10). However, Nrf2 might not directly be a substrate of ERK1/2. Instead, it is discussed that ERK1/2 phosphorylates the nuclear transcription coactivator CREB-binding protein (CBP), and that CBP enhances Nrf2 transcriptional response (Shen et al., 2004).

Cytotoxicity of MPP^+ and flavonoids

Rapid onset of parkinsonism in man, primate, and mouse, following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Langston et al., 1983; Gerlach and Riederer, 1996) occurs through its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Langston et al., 1984), a reversible inhibitor of mitochondrial complex I. MPP^+ leads to the depletion of energy stores and the induction of oxidative stress *in vivo* (Nicklas et al., 1987; Przedborski et al., 2004). The flavones luteolin and baicalein, as well as the isoflavones genistein and daidzein are characterized by their intermediate lipophilicity and solubility in DMSO as well as by their electrophilic α,β-unsaturated carbonyl groups. This structural property is in contrast to many other flavones, isoflavones, flavonols, flavanols, flavanones,

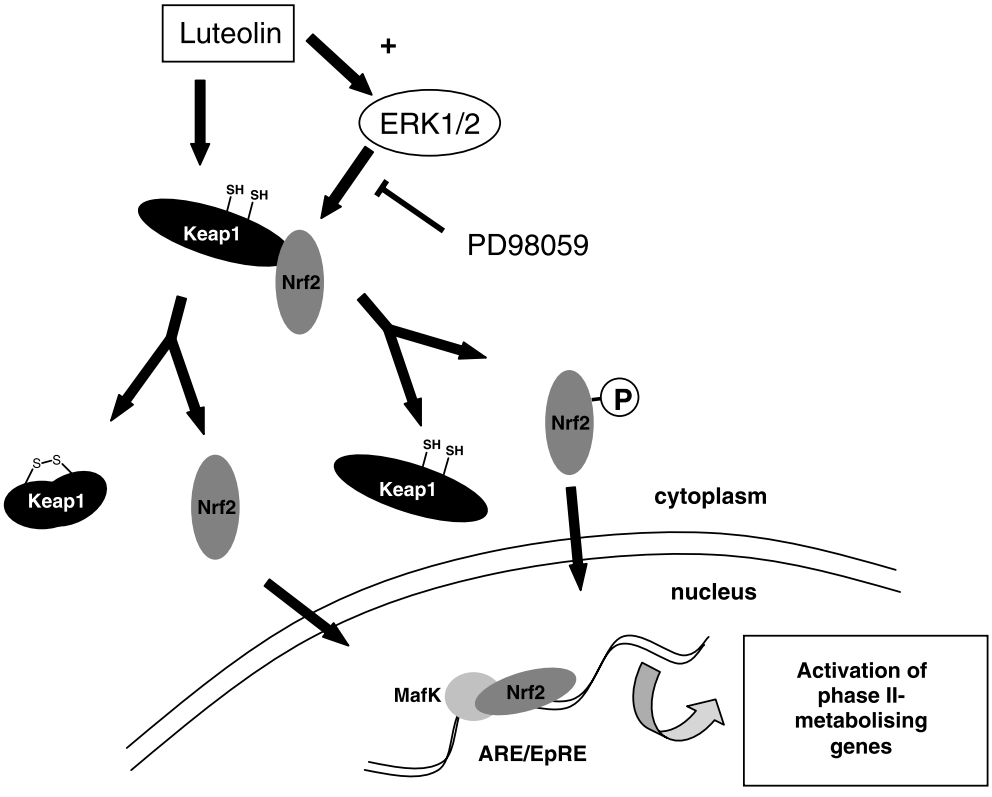


Fig. 10. A model of the mechanism postulated to be involved in the protection of PC12 cells from MPP^+ toxicity is schematically presented. ERK1/2 phosphorylation probably leads to Nrf2 phosphorylation that is consequently liberated from Keap1 to enable nuclear transcription of cytoprotective genes with the help of small Maf proteins. Alternatively, a direct interaction with nucleophilic thiol groups of Keap1 with luteolin or luteolin oxidation products such as o-quinones from catechol moieties may be postulated, but remains to be experimentally proven. PD98059 is a MEK1/ERK1/2 inhibitor

flavanonols, anthocyanins and chalcones (Nagao et al., 1999). Up to 5 μM, genistein, baicalein, and luteolin are not cytotoxic in PC12 cells. Daidzein is not cytotoxic up to 50 μM in PC12 cell culture, since above 10 μM daidzein starts to precipitate and cannot passively diffuse into cells anymore.

Interestingly, 5 μM luteolin, but not baicalein, significantly increases PC12 cell viability. This bell-shaped dose response curve holds also true for tBHQ, but not for sulforaphane, two well described Nrf2-activators, pinpointing a specific mode of action of luteolin and tBHQ within a narrow concentration range and highlighting the necessity to establish narrow dose-toxicity relationships prior to cytoprotection studies.

Cytoprotection by flavonoids

Some of the first studies on cytoprotection with Nrf2 activators at low micro Molar concentrations were undertaken with tBHQ which easily crosses lipid bilayers. tBHQ protects from 6-OHDA-induced oxidative stress in neural cell lines (Lee et al., 2001; Hara et al., 2003; Jakel et al., 2005). In addition to tBHQ, triterpene electrophiles isolated from acacia victoriae (Haridas et al., 2004) activate the innate stress response of Hep G2 cells by redox regulation of a set of target genes driven by the activation of Nrf2.

Oral administration of tBHQ (100 mg/kg in mice) prior to 2′CH3-MPTP for 7 days normalized the GSH content and SOD activity, and ameliorated several indices of lipid peroxidation (Abdel-Wahab, 2005). In a very recent second *in vivo* study, Burton et al., 2006 demonstrated that MPTP-induced neurotoxicity is diminished by oral administration of 3H-1,2-dithiole-3-thione depending on Nrf2 activation. On the other hand, tBHQ attenuates the neuronal death following stroke in $Nrf2^{+/+}$, but not in $Nrf2^{-/-}$ mice (Shih et al., 2005), demonstrating Nrf2-specific actions of tBHQ mediated neuroprotection in the nervous system.

As well in our hands, tBHQ activates Nrf2 in PC12 cells and protects from MPP^+ toxicity. Thus, we used tBHQ as the gold standard in screening experiments that aimed to identify novel Nrf2 activating compounds with neuroprotective potential.

We identified luteolin as one of the most effective compounds in protecting PC12 cells from MPP^+ toxicity in PC12 and C6 cells. Genistein was modestly cytoprotective, whereas baicalein and daidzein failed to do so. The Nrf2-activation by luteolin, is a precondition of protection since luteolin does no longer protect against MPP^+ in PC12 cells stably transfected with *sh*RNA targeting Nrf2.

The Nrf2 activation by luteolin is mediated by ERK1/2 with MEK1 as upstream activator. Since the MEK1/2 inhibnhibitors PD98059 and UO126 potently inhibit Nrf2 activation, ERK1/2 must be involved in the maintenance of Nrf2 signaling.

The pronounced protection by luteolin may be based on its superior free radical scavenging and metal chelating properties (Arora et al., 1998; Ishige et al., 2001). Luteolin is the only compound that we investigated bearing a catechol moiety in the phenyl group and therefore may be oxidized to an ortho-quinone. This electrophilic quinone could directly react with thiol groups in Keap1 with consequent liberation of Nrf2. Very recently Lee-Hilz et al. (2006) identified planar flavones that have a high intrinsic potential to generate oxidative stress and for redox cycling as potent activators of hNQO1-ARE response in Hepa1c1c7 cells. This result favours the scientific view that the pro-oxidant activity of flavonoids-induces ARE-mediated gene expression. PKC was not involved in flavonoid-induced *ARE*-mediated gene transcription in Hepa1c1c7 cells. We instead identified ERK1/2 pathway as an important additional element in Nrf2 activation in neural cells.

In conclusion we demonstrate that the flavones luteolin and baicalein and the isoflavones genistein and daidzein activate NQO1-*ARE* in a dose dependent manner. Further, dose dependent cytoprotection against MPP^+ is best conferred by luteolin and is critically depending on ERK1/2 activation, and on the subsequent activation of Nrf2. In addition to antioxidative capacity, luteolin exhibits another very important protective property, i.e. the induction of the phase 2 response. The mere antioxidant action of these compounds, however, is apparently not sufficient for neuroprotection. Based on our results we extend previous models established for the explanation of the cytoprotective effects of the green tea polyphenol epigallocatechingallate and other flavonoids (Mandel et al., 2004; Boerboom et al., 2006; Lee-Hilz et al., 2006), in which we conclude that the flavone luteolin, and the synthetic compound tBHQ activate the MAPK pathway via an electrophilic-mediated stress response, leading to the transcription activation by Nrf2/Maf heterodimers on ARE enhancers. This may induce the expression of cellular defence/detoxifying genes including conjugating enzymes which protect the cells from toxic environmental insults and thereby prolong cell survival (Fig. 10).

Thus, it is tempting to assume that the long-term activation of Nrf2 in vulnerable neural cells might become a promising therapeutic strategy to halt the progression of neuronal demise in aging and neurodegenerative diseases.

Acknowledgments

We acknowledge the fundings from the University of Kiel and the Deutsche Forschungsgemeinschaft (SFB 415, A12).

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J Neural Transm (2007) [Suppl 72]: 69–85

Printed in Austria

Journal of Neural Transmission

Behavioural and expressional phenotyping of nitric oxide synthase-I knockdown animals

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Summary The gaseous messenger nitric oxide (NO) has been implicated in a wide range of behaviors, including aggression, anxiety, depression, and cognitive functioning. To further elucidate the physiological role of NO and its down-stream mechanisms, we conducted behavioral and expressional phenotyping of mice lacking the neuronal isoform of nitric oxide synthase (NOS-I), the major source of NO in the central nervous system. No differences were observed in activity-related parameters; in contrast to the a priori hypothesis, derived from pharmacological treatments, depression-related tests (Forced Swim Test, Learned Helplessness) also yielded no significantly different results. A subtle anxiolytic phenotype however was present, with knockdown mice displaying a higher open arm time as compared to their respective wildtypes, yet all other investigated anxiety-related parameters were unchanged. The most prominent feature however was gender-independent cognitive impairment in spatial learning and memory, as assessed by the Water Maze test and an automatized holeboard paradigm. No significant dysregulation of monoamine transporters was evidenced by qRT PCR. To further examine the underlying molecular mechanisms, the transcriptome of knockdown animals was thus examined in the hippocampus, striatum and cerebellum by microarray analysis. A set of >120 differentially expressed genes was identified, whereat the hippocampus and the striatum showed similar expressional profiles as compared to the cerebellum in hierarchical clustering. Among the most significantly up-regulated genes were *Peroxiredoxon 3, Atonal homologue 1, Kcnj1, Kcnj8, CCAAT/enhancer binding protein (C/EBP), alpha*, 3 genes involved in GABA(B) signalling and, intriguingly, the glucocorticoid receptor *GR*. While GABAergic genes might underlie reduced anxiety, dysregulation of the glucocorticoid receptor can well contribute to a blunted stress response as found in *NOS1* knockdown mice. Furthermore, by CREB inhibition, glucocorticoid receptor up-regulation could at least partially explain cognitive deficits in these animals. Taken together, *NOS1* knockdown mice display a characteristic behavioural profile consisting of reduced anxiety and impaired learning and memory, paralleled by differential expression of the glucocorticoid receptor and GABAergic genes. Further research has to assess the value of these mice as animal models e.g. for Alzheimer's disease or attention deficit disorder, in order to clarify a possible pathophysiological role of NO therein.

Keywords: Knockout, mouse, NO, NOS-I, microarray, gene chip, ADHD, depression

Abbreviations

BDNF	brain derived neurotrophic factor
CREB	cyclic AMP response element-binding protein
DAT	dopamine transporter
GABA	γ-amino-butyric acid
GR	glucocorticoid receptor
5-HT	serotonin
5-HTT	serotonin transporter
LTP	long-term potentiation
NO	nitric oxide
NOS	nitric oxide synthase
PCP	phenylcyclidine
SSRI	selective serotonin reuptake inhibitor

Introduction

The gaseous messenger molecule nitric oxide (NO) is synthesized from its precursor L-arginine by a family of three NO synthases (NOS), designated as ''neuronal'' NOS-I, ''inducible'' NOS-II and ''endothelial'' NOS-III. In the adult brain, the inducible isoform NOS-II is present only at very low levels in microglia and immune cells, while ''endothelial'' NOS-III is expressed predominantly in the vasculature. Whether or not this isoform is also expressed in neural cells, is still a matter of debate but data arguing

Dedicated to Prof. Dr. Peter Riederer on occasion of his 65th birthday.

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for this are only sparse. The quantitatively major source for NO in the CNS thus is the ''neuronal'' isoform NOS-I present in approximately 1% of all neurons. Nitrinergic transmission is especially important in limbic structures, in the basal ganglia – where NO regulates striatal output – and in the cerebellum (Snyder and Ferris, 2000). NO exerts multiple actions in the CNS and from animal studies, it has been suggested that it is involved in behavioral processes such as learning and memory formation. Pathologies of the NO pathway have been implicated in almost every major neu-

Table 1. *Summarized behavioural phenotype of NOS1 knockdown and NOS3 knockout animals. Findings of the present study are printed in bold*

Test	NOS-I knockdown	NOS-III knockout
General		
Sensorimotor screening/ observation	Increased touch-escape reaction, body position, locomotion, elevation and reduced vocalization, increased grooming → ''anxiety like behaviour'' (Weitzdoerfer et al., 2004)	Increased forelimb strength, otherwise no difference (Demas et al., 1999)
Pole/plank test (balance/ coordination)	Nocturnal impairment (Kriegsfeld et al., 1999); no difference (not shown; (Nelson et al., 1995))	No difference (Demas et al., 1999; Dere et al., 2002)
Rotarod	No difference (Chiavegatto et al., 2001); no difference (Kirchner et al., 2004); **No difference**	Not published
Hotplate (pain sensitivity)	Increased sensitivity to pain ((Nelson et al., 2006), unpublished). **No difference**	No difference (Reif et al., 2004)
Activity & Novelty		
Open field	More active during the active cycle, more time spent in center of the open field (Bilbo et al., 2003); no difference (not shown; (Nelson et al., 1995)); more center crossings and more center entries (Weitzdoerfer et al., 2004); normal (Chiavegatto et al., 2001); Higher center time/crossings (Kirchner et al., 2004); No difference (Salchner et al., 2004); **No difference**	Reduced exploratory activity with no habituation; more time spent in center of open field (Dere et al., 2002); no difference (not shown; (Demas et al., 1999)); less activity, more time spent in corners (Frisch et al., 2000); no difference (Reif et al., 2004)
Novelty seeking	**No difference**	Not published
Emotionality & Depression		
Light-Dark-Box	**No difference**	No difference (Reif et al., 2004)
Porsolt	Reduced immobility time (Salchner et al., 2004); fewer depression-like responses ((Nelson et al., 2006), unpublished); **No difference**	No difference (Reif et al., 2004)
Learned helplessness	**No difference**	Better & faster learning (Reif et al., 2004)
Tail suspension	n.p.	Not published
Novel cage	**No difference**	No difference (Reif et al., 2004)
Elevated plus maze	No difference (Bilbo et al., 2003); more time spent in closed arm (Weitzdoerfer et al., 2004), more entries in closed arm (Kirchner et al., 2004); **Higher open arm time**	Less locomotion in open arms, otherwise no difference (Dere et al., 2002); no difference (Demas et al., 1999); less time in open arms, more time in closed arms (Frisch et al., 2000)
Mazes & Learning		
COGITAT/holeboards	**Impaired spatial learning**	Not published
8-Arm radial maze	n.p.	No difference (Dere et al., 2001)
Morris water maze	Worse performance (Weitzdoerfer et al., 2004); worse performance (Kirchner et al., 2004); **Impaired spatial learning**	Superior performance (Frisch et al., 2000)
T-Maze	Better performance (Weitzdoerfer et al., 2004)	Not published
Aggression & Maternality		
Resident-intruder aggression	Males – increase (Nelson et al., 1995), testosterone-dependent (Kriegsfeld et al., 1997); not in BL/6 back-crossed mice (Le Roy et al., 2000)	Male animals – no aggression (Demas et al., 1999)
Neutral aggression	Males – increase (Nelson et al., 1995), testosterone-dependent (Kriegsfeld et al., 1997); not in BL/6 back-crossed mice (Le Roy et al., 2000)	Male animals – no aggression (Demas et al., 1999)
Maternal aggression	Absent (Gammie and Nelson, 1999)	No difference (Gammie et al., 2000)
Maternal behavior	Otherwise no difference (Gammie and Nelson, 1999)	No difference (Gammie et al., 2000)
Stress		
Baseline corticosterone	Higher in knockout mice (Bilbo et al., 2003)	n.p.
Stress-induced increase	Dampened corticosterone response in knockout animals (Bilbo et al., 2003)	n.p.

ropsychiatric disorder including schizophrenia (Bernstein et al., 2005), affective disorders (van Amsterdam and Opperhuizen, 1999), alcoholism (Gerlach et al., 2001), Alzheimer's dementia (Law et al., 2001), Parkinson's and Huntington's disease (Hunot et al., 1996). For some of these disorders, NOS-I has also been identified as a risk gene in human case-control association studies (Galimberti et al., 2007; Reif et al., 2006a, b). The role of NO in the regulation of normal human brain functioning however is still unclear, although first genetic studies argue for a function of NOS-I in the regulation of impulsive behaviors.

Knockout animals are valuable tools to identify both the behavioral impact of a given gene, as well as subsequent changes of the transcriptome to correlate behavior to molecular pathways. With respect to NOS-I, two genetically modified mouse strains have been described in the literature. While in the recently generated KOex6 knockout, disruption of NOS-I exon 6 results in the complete absence of catalytically active NOS-I (Packer et al., 2003), previously generated animals harbor a targeted deletion of exon 1 (Nelson et al., 1995). The latter results in a loss of the PDZ binding domain and thus residual NOS-I expression of up to 7%, rendering these mice actually NOS-I knockdown animals. This situation may more closely mirror human genetic variation of NOS-I, since a complete disruption of the gene has not yet been described in man. There are several studies on the behavioral phenotype of these animals (as summarized in Table 1); however, they are in part contradictory and lack dedicated investigations of depression-like behaviors. Thus, we aimed to perform detailed behavioral phenotyping of *NOS1* knockdown animals with special emphasis on depression- and ADHD-relevant tests. To reveal molecular mechanisms underlying the behavioral phenotype, we also performed a microarray study using a custom made gene chip featuring almost 1.000 genes which have been a priori selected for their relevance to CNS functioning.

Materials and methods

Animals

For behavioural experiments, wildtype control (+/+) and homozygous NOS-I knockdown (−/−) mice aged between 2 and 6 months were used. In all experiments, the respective controls were wildtype littermates. For the micorarray study, an additional set of 7 knockdown (−/−) and 7 wildtype controls (+/+) were examined, which were also littermates. All animals had the same genetic background (C57BL/6, for review see Huang et al., 1995) and were housed under identical conditions. Genotype was confirmed in each animal by PCR, and also immunohistochemistry showed complete loss of NOS-I protein in the hippocampus, striatum and the cortex (data not shown). All animal protocols have been reviewed and approved by the review board of the Government of Lower Franconia and the University of Würzburg and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Behavioural analyses

All experiments were preceded by an acclimatization period of approximately 30 min (Forced Swim Test, Hotplate, Learned Helplessness, Novel Cage, Rotarod) or 24 h (Elevated Plus Maze, Open Field, Water Maze, COGITAT holeboard) to the experimental room. During all experiments, the experimenter was blind to the genotype.

Barrier test

The Barrier test was performed in a type II macrolon cage, which was divided into two sections by a 1 cm high hurdle. Observation was conducted with red light illumination assessing the latency to cross the barrier within a maximum duration of 300 sec.

Open field test

The open field consisted of a PVC plastic box ($82 \times 82 \times 25$ cm).

Activity monitoring was conducted using the computer-based video tracking software VideoMot 2 (TSE, Bad Homburg). Illumination at floor level was 200 lux. The area of the open field was divided into a 70×70 cm central zone and the surrounding border zone. Mice were individually placed in a corner of the arena. The time spent in the central zone, the number of entries into the central zone and the overall distance travelled by the mice were recorded during a period of 5 min.

Novel cage test

The novel cage test is used to investigate exploratory behavior in a new environment by measuring vertical activity. Animals were placed into a new standard macrolon cage and rearings were counted for 5 min.

Light–Dark Box

The Light–Dark Box consisted of a square box divided into a black and a white compartment, connected by a small tunnel; the white compartment was brightly illuminated with a 600 lux light source. Light intensity in the black compartment, covered by a black top, was 1 lux. Latency to first exit, total number of exits, and time in the light compartment were recorded for 5 min.

Porsolt's Forced swim test

Mice were placed twice, at 24 h interval, into a glass cylinder (23 cm height, 13 cm diameter) which was filled with water (23°C) up to a height of 10 cm, which prevented the mice from touching the bottom of the beaker with their paws or the tail. Mice were tested for 5 min and their behavioural activity was scored by a well-trained observer. The times spent on climbing, swimming, and immobility were recorded to determine active vs. passive stress-coping performance. Mice were considered immobile when floating passively in the water, performing only those movements required to keep their heads above the water level (Cryan et al., 2002). In addition, duration of immobility was automatically assessed using the ''mobility'' feature of the Noldus software, EthoVison 1.96 (Noldus Information Technology, Wagingen, NL).

Morris water maze

The water maze consisted of a dark-gray circular basin (120 cm diameter) filled with water (24–26°C, 31 cm deep) made opaque by the addition of

non-toxic white tempera paint. A circular platform (8 cm diameter) was placed 1 cm below the water surface in the centre of the goal quadrant, 30 cm from the wall of the pool. Distant visual cues for navigation were provided by the environment of the laboratory; proximal visual cues consisted of four different black and white posters placed on the inside walls of the pool. Animals were transferred from their cages to the pool in an opaque cup and were released from eight symmetrically placed positions on the pool perimeter in a predetermined but not sequential order. Mice were allowed to swim until they found the platform or until 180 sec had elapsed. In this last case, animals were guided to the platform and allowed to rest for 20 sec. The animals were submitted to six trials per day for five days using a hidden platform at a fixed position (south-east) during the first three days (18 trials, acquisition phase) and in the opposite quadrant (north-west) for the last two days (12 trials, reversal phase). Trials 19 and 20 were defined as probe trials to analyze the precision of spatial learning.

Elevated plus maze

A plus-shaped maze made of grey PVC plastic was used. The device comprised two opposing open arms (30×5 cm) and two opposing closed arms (30×5 cm) that had 15 cm high, nontransparent walls. The four arms were connected by a central platform (5×5 cm). The maze was elevated 500 mm above the floor. The open arms were illuminated with an intensity of 200 lux, the central area with 150 lux and the closed arms with 100 lux. Mice were initially placed in the centre of the maze facing one of the open arms and then were allowed to investigate the area for 5 min. Their behaviour was recorded by video-tracking (VideoMot2, TSE Systems, Bad Homburg, Germany). Entry into an arm was defined when the mouse placed its four paws into the arm. The time spent in, and the number of entries made into the open and closed arms as well as the centre time were measured, and the total distance travelled during the test session was recorded.

Hot plate test

Each mouse was placed on a metal surface maintained at 53.0 ± 0.2°C (ATLab, Montpellier, France). The response to the heat stimulus was measured by assessing the latency to first reaction, i.e. hindpaw lick or jump, which are considered as typical nociceptive responses (Hammond and Ruda, 1989). Animals were removed from the plate immediately after responding or after a maximum of 45 s (cut-off) to prevent tissue damage.

Learned helplessness

This experiment was performed as previously described (Chourbaji et al., 2005; Reif et al., 2004; Ridder et al., 2005). Briefly, animals were exposed to a transparent plexiglass shock chamber equipped with a stainless steel grid floor (Coulborn precision regulated animal shocker, Coulborn Instruments, Düsseldorf, Germany), through which they received 360 footshocks (0.150 mA) on two consecutive days. Footshocks were unpredictable with varying shock- and interval-episodes (1–12 s), up to a total duration of 52 min. 24 h after the second day of the shock procedure, learned helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf, Germany). The shuttle box consisted of two equal-sized compartments, separated by a gate, and was equipped with a grid floor, through which the current was applied. Spontaneous initial shuttles were counted during the first two minutes by infrared beams. Performance was analyzed during 30 shuttle escape trials (light stimulus: 5 s, footshock: 10 s, intertrial interval: 30 s). Avoidance was defined as the adequate reaction to a cuing light stimulus by changing to the other compartment; escapes were defined as shuttling to the other compartment as reaction to the electric shock; when no attempt to escape was made, a failure was denoted. Total time of testing for helplessness was about 20 min depending on the animals' individual performance.

Modified holeboard paradigm

To assess attention and spatial memory, a modified 5×5 holeboard system equipped with 3-level infrared beams was used (COGITAT, Cogitron, Goettingen, Germany), which was connected to a videotracking software system (VideoMot 2, TSE Systems, Bad Homburg, Germany) as described by Wultsch et al. (submitted). During this test, animals were trained to learn a pattern of baited holes, in which odor-free pellets were hidden. The ground below the feeding plate and the cylindrical tubes were covered with vanilla powder to prevent animals from working out the pattern of the distribution of the pellets by using olfactory stimuli. By the combined use of the IR grid and the videotracking software, a number of measures including erroneous visited holes, eaten pellets, time to learn the pattern, total activity and distance travelled was taken.

Microarray studies

Hippocampus, striatum and cerebellum of 7 wildtype (+/+) and 7 NOS-I knockdown (−/−) mice were prepared. Total RNA was thereafter isolated using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and the RNase-free DNase Set (Qiagen). Mean RNA concentration was 154 ng/ml. RNA samples of each structure have been randomly assigned to 3 pools for both wildtypes as well as knockdown animals. 3 µg of each RNA sample were labeled, wildtype total RNA in Cyanine 3 and knockout total RNA in Cyanine 5. The incubation took place over night at 42°C. Purification of the samples was performed with a QIAGEN kit (Protocol GP4). At this step, purified samples were labeled blue (Cy5) or pink (Cy3). Each eluate was quantified on a Nanodrop ND-1000 device (PeqLab, Erlangen, Germany) to determine cDNA quantity. Samples were pooled two by two, but some volumes were readjusted to obtain the same quantity of cDNA. Purified samples were evaporated in a Speedvacuum (for 30 min), and then resuspended in the specific hybridization mix. Thereafter, 9 slides were hybridized over night at 42°C with a total of 18 samples (3 structures, 2 phenotypes); for each sample, 15 µl were used. The chips have been washed with decreasing stringency bathes and scanned on a Scanarray Scanner. Signal quantification was performed with Imagene 4.1 (BioDiscovery, Inc., El Segundo, CA) and data were normalized with VARAN (http://www.bionet.espci.fr/; Golfier et al., 2004). Three hybridization types have been statistically analyzed and compared (cerebellum, striatum and hippocampus experiments) using the SAM software (http://www-stat.stanford.edu/~tibs/SAM/). The purpose of this tool is to allow the selection of genes associated to significant variations between the conditions analyzed based on biologically independent experimental replicates. SAM is based on the computation of a statistic called d (for difference). A gene will be selected as significantly differentially expressed if the observed d is significantly higher than an expected value, computed using the whole set of experimental data. A threshold (δ-value) is defined by the user and corresponds to the minimum absolute value of δ(observed)- δ(expected) that will be associated to a significant variations. This value can be defined regarding measurements provided by SAM such as false discovery rate, the number of selected significant genes and the number of false positives. For determination of global knockout effects, δ has been set such as the number of false positive is lower than one. For all other analyses, δ has been set in order to select a number of significant genes similar to the number selected for the whole analysis of the 9 experiments.

Real-time PCR

After the preparation of the striatum and the brainstem, containing the raphe nuclei, total RNA was isolated as described above. 0.5 µg of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit® (BIO-RAD, München, Germany). Real-time PCR was performed using an iCycler iQ™ Real-Time Detection System (BIO-RAD Laboratories, Hercules, USA) in the presence of SYBR-green. The optimization of the real-time PCR re-

action was performed according to the manufacturer's instructions but scaled down to 25 µl per reaction. Standard PCR conditions were used following the manufacturers protocol (serotonin transporter, 5-HTT: iQTM SYBR® Green Supermix protocol, BIO-RAD; dopamine transporter, DAT: QuantiTectTM SYBR® Green PCR protocol, Qiagen, Hilden, Germany). Three series of experiments were performed with similar results; PCR reactions of each series were run in duplicate. Ribosomal 18 s and GAPDH were used to normalize each template using the GeNorm normalization program (Vandesompele et al., 2002). Standard curves for each amplification product were generated from 10-fold dilutions of pooled cDNA amplicons isolated from Agrarose gel electrophoresis. The primer sequences were as follows: 5-HTT forward, 5′ – GAC AGC CAC CTT CCC TTA CA – 3′; 5-HTT reverse, 5′ – CTA GCA AAC GCC AGG AGA AC – 3′; GAPDH forward, 5′ – AAC GAC CCC TTC ATT GAC – 3′; GAPDH reverse, 5′ – TCC ACG ACA TAC TCA GCA C – 3′; 18S forward, 5′ – GAA ACT GCG AAT GGC TCA TTA AA – 3′; and 18S reverse, 5′ – CCA CAG TTA TCC AAG TAG GAG AGG A – 3′.

Results

Behavioral assessment

As a number of pharmacological studies argue for an involvement of NOS in the pathophysiology of depression and anxiety, we examined whether *NOS1* knockdown mice display behavioral traits related to such phenotypes. However, no significant differences could be observed in the Forced Swim Test (Fig. 1A) or any of the most relevant parameters of the Learned Helplessness paradigm (Fig. 1B, C), strongly arguing against a depression-like phenotype of *NOS1* knockdown animals. In the Hotplate test there were no changes in pain sensitivity, which could have interacted with the unpleasing stress procedures, supporting the results obtained in the Learned Helplessness procedure (Fig. 1D). Further potential influences by alterations of activity-, or motoric-dependent traits could also be excluded, since no significant alterations were detected in the Novel Cage (Fig. 1E), the Rotarod (Fig. 1F), Barrier- (data not shown), or Open Field test (see below). In the Elevated Plus Maze, mutant mice spent significantly more time in the open arm of the Plus Maze (Fig. 2A, B), indicating less anxiety. In another anxiety-related paradigm, i.e. the Light–Dark Box, no significant differences however emerged (data not shown). Differences in the Elevated Plus Maze could not

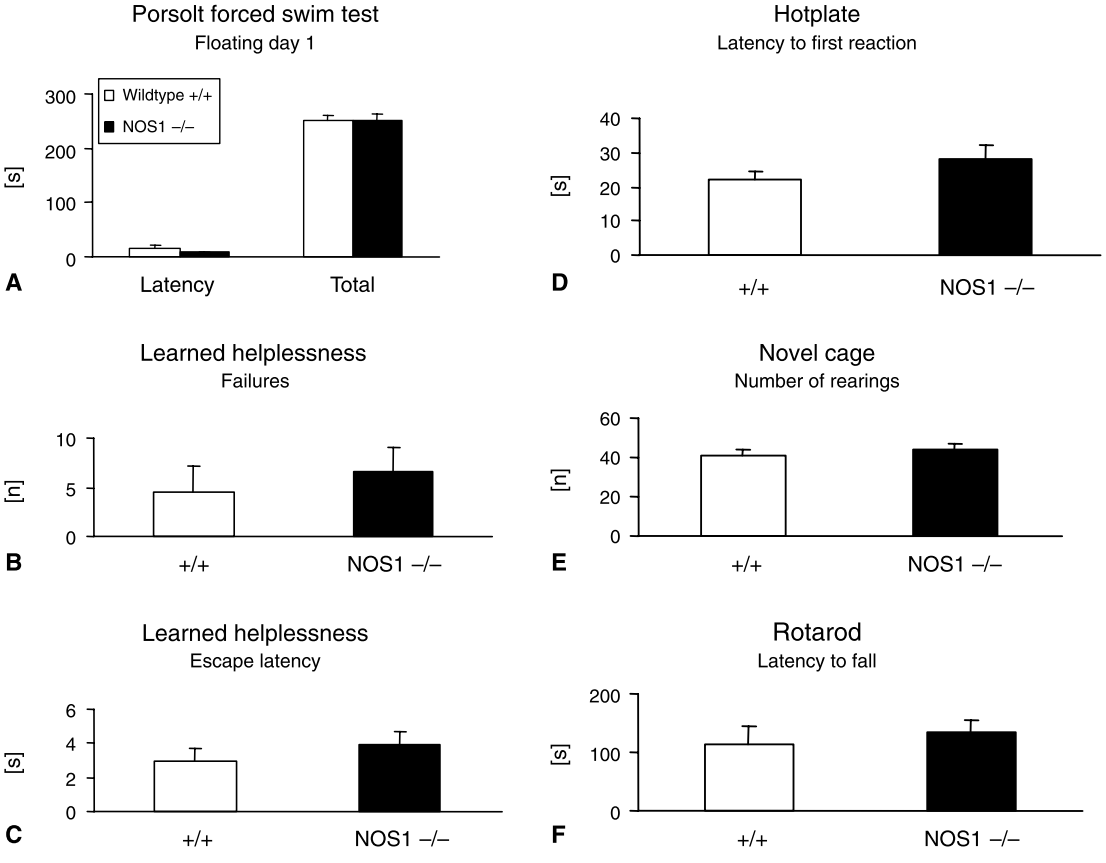


Fig. 1. Depression-related behaviors of male *NOS1* knockdown mice. Neither in the Forced Swim Test (**A**), nor in the Learned Helplessness Paradigm (**B**, **C**), *NOS1* knockdown mice displayed depressive-like behavior. Also in control experiments testing for pain sensitivity (**D**), novelty-related emotionality (**E**) and overall locomotor activity (**F**), *NOS1* (−/−) mice showed no significantly different behavior. Closed bars, knockdown mice (n = 13); open bars, wildtype littermates (n = 13)

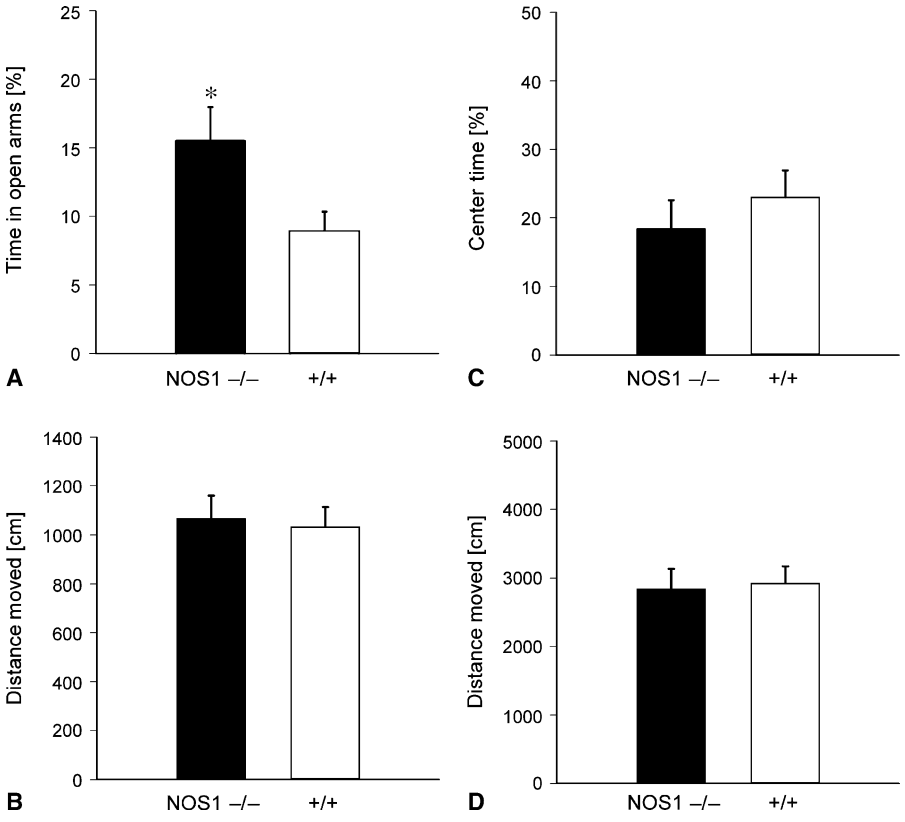


Fig. 2. Anxiety- and activity-related behaviors of male *NOS1* knockdown mice. While *NOS1* knockdown (−/−) mice had a higher open arm time in the Elevated Plus Maze as compared to wildtype (+/+) animals (**A**, **B**), arguing for reduced anxiety, activity-related parameters in the Open Field (**C**, **D**) were not significantly different. Closed bars, knockdown mice (n = 10); open bars, wildtype littermates (n = 10); *, significant difference ($p > 0.05$, Student's *t*-test)

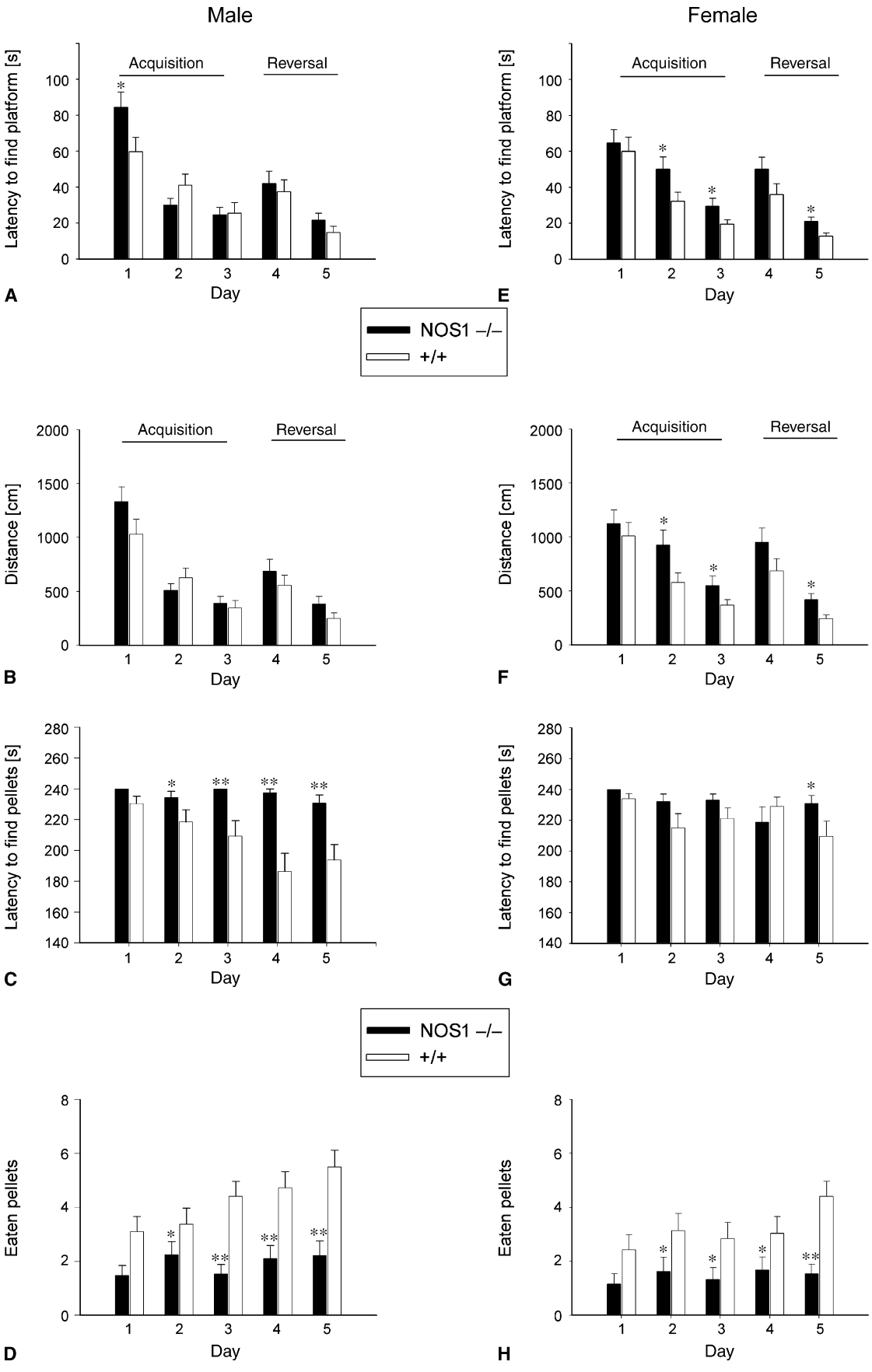
be attributed to alterations in locomotor activity, as performance in the Rotarod test (Fig. 1F) and all measured parameters in the Open Field test (center time, distance to walls, total distance moved, velocity; Fig. 2C, D) were unaltered in knockdown (−/−) animals.

In a second series of experiments, we investigated whether *NOS1* knockdown animals have cognitive deficits. Therefore, two different paradigms were employed: the Morris Water Maze, the standard test for hippocampus-dependent spatial memory, and a modified holeboard paradigm. In the Morris Water Maze, knockdown (−/−) mice had a higher latency to find the hidden platform (Fig. 3A) in acquisition, but not reversal trials. Correspondingly, in the holeboard test, knockdown (−/−) animals did not show a decreasing latency to find pellets during the trials, indicating that spatial learning was impaired (Fig. 3C). Furthermore, the number of found pellets was significantly lower in knockdown (−/−) as compared to wildtype (+/+) mice (Fig. 3D). As cognitive deficits were the most pronounced behavioral phenotype, we also tested a cohort of female animals to examine whether this is a gender-specific effect. As depicted in Fig. 3E–H, also females were cognitively impaired in both paradigms with the Water Maze results being even more pronounced in females, as they were impaired both in acquisition as well as in reversal trials.

Quantitative PCR of 5-HTT and DAT transcripts

Because tight interactions of NO and both the serotonergic as well as the dopaminergic systems have been suggested, we speculated that disruption of the *NOS1* might lead to a modified expression of the DAT or the 5-HTT, which are key molecules in the regulation of serotonin (5-HT) and dopamine circuitries. For 5-HTT, RNA was extracted from the brainstem containing the raphe nuclei, where 5-HTT RNA is almost exclusively detectable. However, no significant differences in 5-HTT expression were observed (Fig. 4A). Quantification of DAT transcripts in the

Fig. 3. *NOS1* knockdown mice are cognitively impaired. Male *NOS1* knockdown mice had a higher latency to find the platform in the Water Maze in acquisition, but not in reversal trials (**A**) while the total distance moved was not different in males (**B**), but females (**F**). In the COGITAT holeboard test, knockdown animals had a longer latency to find the hidden pellets (**C**) and found less pellets (**D**). Comparable results were obtained with female animals (**E–H**). Closed bars, knockdown mice (n = 10 males and 11 females); open bars, wildtype littermates (n = 10 males and 9 females); *, significant difference ($p > 0.05$, ANOVA for repeated measures. Significant differences between genotypes were identified with the Holm-Sidak method). Together, these results indicate that both male as well as female *NOS1* knockdown mice are cognitively impaired


Male
Female
Acquisition
Reversal
Latency to find platform [s]
Distance [cm]
Latency to find pellets [s]
Eaten pellets
Day
NOS1 –/–
+/+
A
B
C
D
E
F
G
H

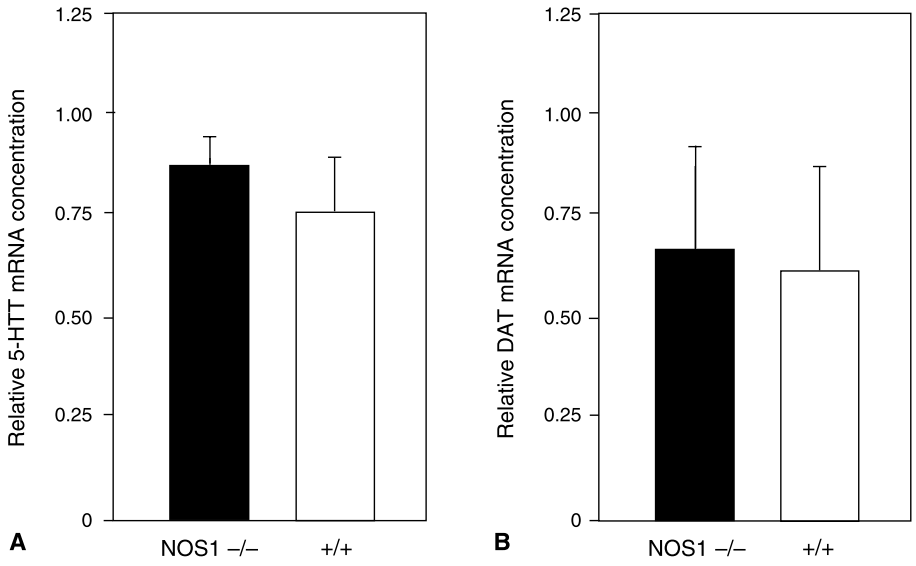


Fig. 4. Serotonin (5-HTT) and dopamine transporter (DAT) mRNA levels in *NOS1* knockdown mice. By means of qRT PCR, no significant differences in the expression of 5-HTT (brainstem, containing the raphe nuclei; **A**) or DAT (striatum; **B**) were observed in *NOS1* knockdown mice as compared to their wildtype littermates (Student's *t*-test, $p > 0.1$). Absolute cDNA values have been normalized against the housekeeping genes 18S and GAPDH. Closed bars, *NOS1* knockdown mice; open bars, wildtype controls. Data are given as means ± SEM; 7 animals have been investigated in each group

striatum (n = 7 knockdown and 7 wildtype control animals) by quantitative real-time PCR also revealed no significant changes in its relative expression (Fig. 4B).

Microarray experiments

To examine whether changes in the transcriptome of *NOS1* knockdown animals parallel behavioral changes, a gene chip microarray study was conducted. The hippocampus, being the prime region responsible for spatial learning, was investigated along with the NOS-I rich structures striatum and cerebellum. For each of these structures, 7 knockdown $(-/-)$ and 7 wildtype $(+/+)$ mice were examined; pooled structures were hybridized in triplicate. For the global knockout effect, each experiment has been considered as identical in order to reveal knockout induced global or

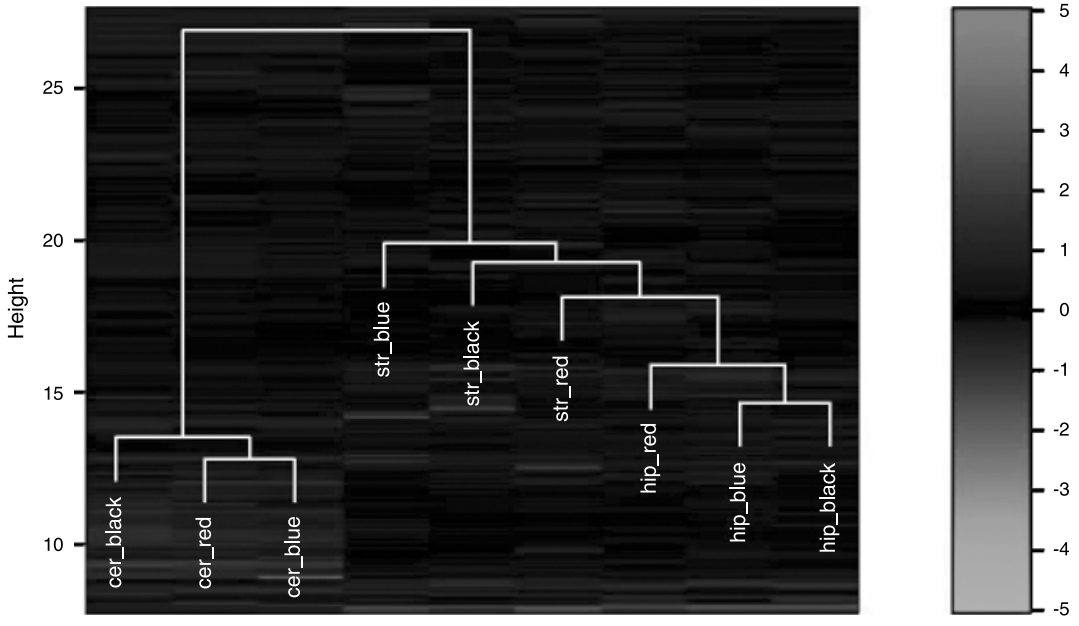


Fig. 5. Hierarchical clustering of hybridization profiles. The white dendrogram tree summarizes the results of the experiments clustering. Each column of the matrices corresponds to an experiment and each row to a probe (gene). The color scale ranges from wildtype over-expressed genes to *NOS1* knockdown over-expressed genes and is given in the Scaled_fold value as computed by VARAN. Genes have been clusterized based on their similarity expression profiles among the experiments. Probes associated to Scaled_fold ranging from −1 to 1 are localized in the VARAN error areas, where a differentially expressed gene cannot be distinguished from an invariant gene at the single hybridization level due to experimental variability. The dendrogram shows that the hybridization profile of the hippocampus and the striatum are very similar yet different to the profile of the cerebellum. Colour figure available on request from the communicating author

Table 2. *Significantly and meaningfully (>two-fold) up-regulated genes. Region denotes the structure, in which a significantly up-regulated gene was detected: either the striatum, the hippocampus, the cerebellum, both the hippocampus and the striatum combined, or a global knockdown effect, i.e. each experiment has been considered as identical in order to reveal knockdown induced global or common effects between all cerebral structures. d, the observed d absolute-value as computed by the SAM software (see Material and Methods). A gene will be selected as significantly differentially expressed if it is significantly higher than an expected value, computed using the whole set of experimental data. SD, standard deviation of d; p, the p-value of the classical t-test; q, the q-value of the statistical test performed by SAM, which is a modified t-test. Both p and q values correspond to the probability of an error when a gene is selected as significantly differentially expressed between the conditions knockout and wildtype. Fold-change denotes the degree of up-regulation (e.g., fold-change of 2 corresponds to a doubled expression). Name, official gene symbol; Full name, official gene full name. Rows of identical genes are shaded for the sake of clarity. Genes which are >3-fold up-regulated are printed in bold*

Region	*d*	SD	p	q	Fold-change	Name	Full name
Hippocampus	8.5489	0.0809	0.0001	0.1435	6.3038	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Global	5.9819	0.3065	<0.0001	0.0023	4.3674	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Striatum	6.2980	0.2169	0.0007	0.1649	5.0813	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Str. and Hip.	9.3724	0.1587	0.0001	0.0188	6.5111	DrosophilaAtoh1	**atonal homolog 1 (Drosophila)**
Cerebellum	1.4162	0.0732	0.0010	0.0985	2.2376	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha
Hippocampus	4.1162	0.3313	0.0018	0.1435	4.9580	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Global	6.4689	0.2675	<0.0001	0.0022	4.1344	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Striatum	5.2895	0.3497	0.0013	0.1649	6.3739	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Str. and Hip.	6.9230	0.2302	0.0002	0.0188	5.6226	Cebpa	**CCAAT/enhancer binding protein (C/EBP), alpha**
Hippocampus	4.3416	0.2068	0.0017	0.1435	3.7209	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Global	4.9821	0.2468	0.0001	0.0025	2.7777	Gabbr2	gamma-aminobutyric acid (GABA) B receptor 2
Striatum	4.8852	0.2460	0.0019	0.1649	3.8945	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Str. and Hip.	7.0349	0.1445	0.0001	0.0188	3.8075	Gabbr2	**gamma-aminobutyric acid (GABA) B receptor 2**
Hippocampus	2.2189	0.2371	0.0093	0.1453	2.0506	Nr3c1	glucocorticoid receptor 1
Striatum	4.8402	0.1037	0.0021	0.1649	2.3857	Nr3c1	glucocorticoid receptor 1
Str. and Hip.	4.4865	0.1256	0.0004	0.0188	2.2120	Nr3c1	glucocorticoid receptor 1
Striatum	4.4538	0.1587	0.0025	0.1649	2.6379	Hes7	hairy and enhancer of split 7 (Drosophila)
Striatum	4.8394	0.0585	0.0022	0.1649	2.0499	Hgf	hepatocyte growth factor
Hippocampus	2.2442	0.3419	0.0089	0.1453	2.4333	Hoxb7	homeo box B7
Global	5.6997	0.1690	<0.0001	0.0023	2.3658	Hoxb7	homeo box B7
Striatum	3.0286	0.3456	0.0053	0.1649	2.8634	Hoxb7	homeo box B7
Str. and Hip.	3.9634	0.2237	0.0005	0.0188	2.6399	Hoxb7	homeo box B7
Cerebellum	1.5900	0.0171	0.0003	0.0985	2.3222	Hoxb9	homeo box B9
Hippocampus	8.2754	0.0867	0.0003	0.1435	6.1435	Hoxb9	**homeo box B9**
Global	7.7041	0.2192	<0.0001	0.0022	4.1888	Hoxb9	**homeo box B9**
Striatum	9.4005	0.0962	0.0004	0.1649	5.1558	Hoxb9	**homeo box B9**
Str. and Hip.	11.8297	0.0811	<0.0001	0.0188	5.6299	Hoxb9	**homeo box B9**
Cerebellum	1.3302	0.3164	0.0018	0.0985	2.6664	Hoxc6	homeo box C6
Hippocampus	3.8073	0.1879	0.0024	0.1435	3.0111	Hoxc6	**homeo box C6**
Global	8.2376	0.1426	<0.0001	0.0022	2.9856	Hoxc6	homeo box C6
Striatum	3.8905	0.2891	0.0029	0.1649	3.3169	Hoxc6	**homeo box C6**
Str. and Hip.	5.7845	0.1573	0.0002	0.0188	3.1607	Hoxc6	**homeo box C6**
Hippocampus	3.4955	0.1932	0.0026	0.1435	2.7868	Hoxd13	homeo box D13
Str. and Hip.	4.7135	0.2061	0.0003	0.0188	2.9957	Hoxd13	homeo box D13
Global	6.1236	0.1741	<0.0001	0.0023	2.5773	Hoxd13	homeo box D13
Striatum	3.0077	0.4053	0.0055	0.1649	3.2194	Hoxd13	**homeo box D13**
Hippocampus	3.0883	0.1630	0.0035	0.1435	2.3184	DrosophilaIrx1	iroquois related homeobox 1 (Drosophila)
Str. and Hip.	3.8519	0.1438	0.0005	0.0188	2.0757	DrosophilaIrx1	iroquois related homeobox 1 (Drosophila)
Hippocampus	2.7801	0.1711	0.0046	0.1435	2.1654	DrosophilaIrx5	iroquois related homeobox 5 (Drosophila)
Cerebellum	1.3750	0.1484	0.0011	0.0985	2.3482	Junb	jun-B oncogene
Cerebellum	1.0937	0.6522	0.0062	0.0990	2.8891	Lamr1	laminin receptor-like 1/ribosomal protein SA
Hippocampus	2.9641	0.1879	0.0039	0.1435	2.3590	Lhx1	LIM homeobox protein 1
Striatum	5.2604	0.0448	0.0014	0.1649	2.0759	Lhx1	LIM homeobox protein 1
Str. and Hip.	5.0831	0.0958	0.0003	0.0188	2.2131	Lhx1	LIM homeobox protein 1
Hippocampus	5.9673	0.0928	0.0010	0.1435	3.7976	Lyl1	**lymphoblastomic leukemia**
Global	4.8840	0.2247	0.0001	0.0026	2.5255	Lyl1	lymphoblastomic leukemia
Striatum	4.5387	0.1917	0.0024	0.1649	2.9812	Lyl1	lymphoblastomic leukemia
Str. and Hip.	6.9224	0.1232	0.0002	0.0188	3.3655	Lyl1	**lymphoblastomic leukemia**
Cerebellum	1.4702	0.4801	0.0008	0.0985	3.4930	Prdx3	**peroxiredoxin 3**
Hippocampus	4.8584	0.3102	0.0013	0.1435	6.1614	Prdx3	**peroxiredoxin 3**
Global	8.2297	0.2159	<0.0001	0.0022	4.5309	Prdx3	**peroxiredoxin 3**

(*continued*)

Table 2 (*continued*)

Region	*d*	SD	p	q	Fold-change	Name	Full name
Striatum	5.2374	0.2479	0.0015	0.1649	4.3255	Prdx3	**peroxiredoxin 3**
Str. and Hip.	6.9488	0.2111	0.0002	0.0188	5.1635	Prdx3	**peroxiredoxin 3**
Cerebellum	1.5877	0.0957	0.0004	0.0985	2.5287	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Hippocampus	4.0897	0.0872	0.0019	0.1435	2.4561	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Global	10.3064	0.0853	<0.0001	0.0022	2.6098	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Striatum	3.8589	0.2380	0.0032	0.1649	2.8648	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Str. and Hip.	5.5545	0.1237	0.0002	0.0188	2.6530	Kcnj1	potassium inwardly-rectifying channel, subfamily J, member 1
Hippocampus	3.5613	0.3241	0.0025	0.1435	3.9246	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Global	5.1275	0.2924	0.0001	0.0025	3.3650	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Striatum	3.5077	0.5326	0.0037	0.1649	5.3279	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Str. and Hip.	5.1555	0.2957	0.0003	0.0188	4.5735	Kcnj8	**potassium inwardly-rectifying channel, subfamily J, member 8**
Hippocampus	4.6003	0.3238	0.0015	0.1435	5.8424	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Global	4.8389	0.3619	0.0001	0.0026	3.9680	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Striatum	11.7943	0.0833	0.0003	0.1649	7.0450	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Str. and Hip.	9.2194	0.1612	0.0001	0.0188	6.4167	Gm1357	**similar to GABA type B receptor, subunit 2 precursor**
Hippocampus	1.9702	0.4041	0.0119	0.1461	2.3765	Slc6a12	slc6 (neurotransmitter transporter, betaine/GABA) 12
Hippocampus	5.3764	0.0655	0.0011	0.1435	3.0057	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Global	3.2615	0.2949	0.0006	0.0083	2.1759	Tle2	transducin-like enhancer of split 2, hom. of Drosophila E(spl)
Striatum	5.5345	0.1709	0.0008	0.1649	3.4983	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Str. and Hip.	7.5427	0.0954	0.0001	0.0188	3.2434	Tle2	**transducin-like enhancer of split 2, hom. of Drosophila E(spl)**
Hippocampus	7.6808	0.1550	0.0004	0.1435	7.7578	Vax2	**ventral anterior homeobox containing gene 2**
Global	5.5139	0.3774	<0.0001	0.0025	5.1017	Vax2	**ventral anterior homeobox containing gene 2**
Striatum	13.9215	0.0759	0.0001	0.1649	9.3307	Vax2	**ventral anterior homeobox containing gene 2**
Str. and Hip.	13.6041	0.0974	<0.0001	0.0188	8.5103	Vax2	**ventral anterior homeobox containing gene 2**
Cerebellum	1.2929	0.0400	0.0022	0.0985	2.0250	IkarosZnfn1a1	zinc finger protein, subfamily 1A, 1 (Ikaros)
Striatum	3.2333	0.1762	0.0047	0.1649	2.1031	IkarosZnfn1a1	zinc finger protein, subfamily 1A, 1 (Ikaros)

common effects between all the cerebral structures. By doing so, 54 genes were found to be significantly over-expressed, while 12 genes were under-expressed. Hierarchical clustering (Fig. 5) revealed that the set of differentially regulated genes were closer together for the striatum and the hippocampus as compared to the cerebellum, suggesting that the mechanisms of expressional control due to NOS-I are similar for the further two structures. Accord-

Table 3. *Significantly and meaningfully (<0.5-fold) down-regulated genes. For further explanations see legend to Table 2*

Region	*d*	SD	p	q	Fold-change	Name	Full name
Cerebellum	−1.2559	0.0661	0.0025	0.0985	0.4926	OxyR	cold shock domain protein A
Cerebellum	−1.5239	0.2859	0.0006	0.0985	0.3358	Gna14	guanine nucleotide binding protein, alpha 14
Cerebellum	−1.1987	0.0679	0.0037	0.0985	0.5080	Grid1	glutamate receptor, ionotropic, delta 1
Cerebellum	−1.3333	0.0433	0.0017	0.0985	0.4816	Mef2b	myocyte enhancer factor 2B
Cerebellum	−1.0405	0.7473	0.0087	0.0990	0.3403	Ppox	protoporphyrinogen oxidase
Cerebellum	−1.1994	0.0805	0.0036	0.0985	0.5025	v-reloncogenerelatedB(Relb)	avian reticuloendotheliosis viral (v-rel) oncogene related B
Cerebellum	−1.2255	0.1186	0.0029	0.0985	0.4792	Sstr4	somatostatin receptor 4
Cerebellum	−1.6838	0.1020	0.0001	0.0985	0.3711	Lamr1	laminin receptor-like 1/ribosomal protein SA
Global	−5.6208	0.1723	<0.0001	0.0025	0.4223	Lamr1	laminin receptor-like 1/ribosomal protein SA
Str. and Hip.	−3.9597	0.2290	0.0005	0.0188	0.3736	Lamr1	laminin receptor-like 1/ribosomal protein SA
Cerebellum	−1.3209	0.1710	0.0021	0.0985	0.4314	Chrna6	cholinergic receptor, nicotinic, alpha polypeptide 6
Cerebellum	−1.3429	0.0653	0.0015	0.0985	0.4694	DrosophilaNkx2-4	NK2 transcription factor related, locus 2 (Drosophila)
Cerebellum	−1.2559	0.0661	0.0025	0.0985	0.4926	OxyR	cold shock domain protein A
Cerebellum	−1.5239	0.2859	0.0006	0.0985	0.3358	Gna14	guanine nucleotide binding protein, alpha 14

ingly, hybridization profiles were very similar for the striatum and the hippocampus with 53 over- and 13 under-expressed genes in both structures combined. In the striatum alone, only 25 over-expressed genes were detected, while in the hippocampus alone, 64 genes were up- and one gene was down-regulated. In the cerebellum on the other hand, 13 genes were over- and 53 genes were under-expressed. When the cerebellum was compared against the striatum plus the hippocampus, 65 genes were found to be over-expressed in the striatum and hippocampus of knockdown (−/−) animals but not modulated or under-expressed in the cerebellum. Table 2 presents all genes which were significant and up-regulated at least two-fold. Printed in bold are the most meaningful genes (up-regulation >3-fold); those include *Peroxiredoxin 3, Atonal homolog 1, CCAAT/enhancer binding protein, Kcnj8, Vax2, HoxB9, GABA-B receptor 2* and *Similar to GABA-B receptor subunit 2 precursor.* In Table 3, all significant genes which were down-regulated by at least 50% are denoted.

Discussion

The animal model investigated in this study harbors a targeted deletion in exon 1 of the *NOS1* gene resulting in a loss of the PDZ binding domain. Thus, residual NOS-I enzyme and activity (up to 7% of the wildtype) has been reported in these animals which therefore actually constitute knockdown mice. In contrast, complete knockout animals have been engineered in which exon 6, coding for a part of the catalytic center of the enzyme, has been disrupted (Packer et al., 2003). Unfortunately no behavioral data exist for these animals, and likewise, behavioral studies are lacking for *NOS1*-overexpression transgenics (Packer et al., 2005). The *NOS1* knockdown used in the present (and all other, except for the aforementioned Packer et al. study; Packer et al., 2003) investigations might however more closely resemble human genetic variation in *NOS1*, as this likely will result in dysregulation of the gene but not in a complete knockout. In our hands, these animals are thus still highly valuable tools in NOS research.

The present investigation attempted to correlate the behavioral phenotype of *NOS1* knockdown mice to their gene expression profile as assessed by a custom made chip encompassing >1.000 brain-specific genes. *NOS1* knockdown (−/−) animals displayed a specific behavioral phenotype with cognitive deficits and decreased anxiety, while no depression-related behavior was evident. This was paralleled by a set of up-regulated genes, while only one gene (laminin-receptor like 1) was meaningfully down-regulated.

Cognitive deficits in NOS1 knockdown mice

The most consisting finding in the present study was that *NOS1* knockdown animals were cognitively impaired in two different tasks (the Morris Water Maze and the Cogitat Holeboard). The latter allows scrutinizing spatial learning and re-learning parallel to activity measures. In the Water Maze, knockdown animals had a higher latency to find the hidden platform in acquisition and, in females, in reversal trials. This was paralleled by the holeboard results: knockdowns had a higher latency to find all hidden pellets, and accordingly ate less food pellets. No significant activity changes related to these data ensuring that indeed a disturbance of spatial learning, a hippocampus-dependent task, was observed. Likewise, a previous set of studies also demonstrated an increased latency in finding the hidden platform (Kirchner et al., 2004). However, in this set of experiments, learning in the multiple T-maze was not negatively affected. As this is considered a less stressful task as compared to the Water Maze, it was argued that NOS-I inhibition selectively impairs learning under stressful, aversive conditions. The underlying connections between stressful learning conditions and the differential effects of nitric oxide knockdown are however unclear. Involved mechanisms might include dysregulation of the hypothalamus-pituitary-adrenal axis, as *NOS1* knockdowns feature higher baseline corticosterone levels and a dampened stress-induced corticosterone response (Bilbo et al., 2003) and, on the other hand, stressful situations activate nitrinergic neurons (Beijamini and Guimaraes, 2006). Nevertheless, in addition to the Water Maze, we used a less stressful learning paradigm and thereby replicated spatial learning deficits of the *NOS1* knockdown mice. Yet not only hippocampus-dependent learning tasks, but also amygdala-dependent fear conditioning requires nitric oxide signalling (Schafe et al., 2005) arguing that NO-mediated retrograde messaging is a prerequisite for long-term potentiation(LTP)-dependent learning mechanisms. Taken together, decreased nitrinergic tone as found in *NOS1* knockdown animals as well as in animals treated with NOS inhibitors (Koylu et al., 2005; Majlessi et al., 2003; Prendergast et al., 1997) clearly impairs spatial learning.

Phenylcyclidine (PCP) administration represents an accepted rodent model of schizophrenia (Javitt and Zukin, 1991) as it mimics several key symptoms of schizophrenia such as impairment of prepulse inhibition, deficits in social behavior and cognitive dysfunctioning. A functional NO system has however to be present to obtain these effects (Bird et al., 2001). Most interestingly, spatial learning in the Water Maze was shown to be impaired upon PCP ad-

ministration, and this was normalized upon NOS inhibition (Wass et al., 2006a, b). Administration of the inhibitor alone however had no effect on Water Maze performance. This, together with our study, suggests that NO-mediated learning processes are fine-tuned and that either decreased (knockdown) as well as increased (PCP administration) nitrinergic tone leads to impaired learning mechanisms. Given that NO in the PCP model acts as the second messenger of the NDMA receptor, and that it thereby acts as a retrograde messenger mediating LTP, increased levels of NO may result in neuronal noise leading to dysfunctional memory traces and impaired learning and memory. Furthermore, highly elevated NO concentrations are neurotoxic, which can further contribute to cognitive dysfunctioning. Grossly decreased NO production on the other hand, as found in *NOS1* knockdown animals, most likely will result in impaired LTP (Hawkins et al., 1998) and consecutive behavioral abnormalities. Thus, the effect of NO on cognitive functioning seems to follow an U-shaped curve with either too less or too much NO causing cognitive impairment.

NOS1 knockdown mice and anxiety and depression-like behavior

The role of NO in the regulation of mood and anxiety is less clear than its involvement in cognition. From pharmacological experiments, there is numerous data from rats and mice, which however are highly inconsistent, pointing towards a complex role of NO in these behaviors. Several studies using systemic administration argued for an anxiogenic effect of NOS inhibition (Czech et al., 2003; De Oliveira et al., 1997; Pokk and Vali, 2002b; Vale et al., 1998), which was also the case when the inhibitor L-NAME was injected directly into the amygdala or the hippocampus (Monzon et al., 2001). At the same time, NOS inhibition, either intra-amygdalar (Forestiero et al., 2006) or systemical (Del Bel et al., 2005; Dunn et al., 1998; Faria et al., 1997; Pokk and Vali, 2002a, b; Volke et al., 1995, 1997, 2003; Yildiz et al., 2000), resulted in anxiolytic effects in several paradigms. These discrepant results were obtained using similar experimental protocols, comparable animal strains, similar inhibitor compounds (in most cases, N^{ω}-nitro-L-Arginine, L-NAME oder 7-nitroindazole) and partially by the same working group, so that these differences cannot be readily explained. Again, the baseline stress level of the animals and the according set of the hypothalamus-pituitary-adrenal axis might be crucial, as NOS inhibition was shown to counteract anxiolytic effects of corticotrophin (Reddy and Kulkarni, 1998). Furthermore, it was shown that NOS inhibition does not follow a linear cause – effect relationship but rather an inverse U-shaped curve with respect to anxiolytic behaviors (Volke et al., 1995). Considering that, in some paradigms, NOS inhibition also accomplished decreased locomotor activity especially in higher doses (Del Bel et al., 2005; Yildiz et al., 2000), motor side effects of NOS inhibitors might affect anxiety measures in several tests (like the elevated plus maze and the Light–Dark Box) as well, which again highlights the necessity of comprehensive behavioral phenotyping.

Finally, with respect to anxiety-related parameters, *NOS1* knockdown animals (Table 1) displayed a unchanged (Bilbo et al., 2003; Kirchner et al., 2004) or more anxious (Weitzdoerfer et al., 2004) phenotype in previous studies. The latter finding however is doubtful, as the same group in a parallel paper failed to replicate this data (Kirchner et al., 2004), and in our set of experiments, NOS knockdown clearly resulted in a less anxious phenotype independent of locomotor impairment. The lux value is a crucial factor which has to be taken into account due to the anxiogenic effect of bright light, but, however, is not given in these papers. Taken all this data together, the effect of NO on anxiety-related behaviors seems to be complex and state-dependent; most of the studies published to date however argue, if at all, to a anxiogenic effect of NO.

The role of NO in depression-like behaviors is equally controversial. Treatment with NOS inhibitors results in a reduced immobility time in the Forced Swim Test to the same extend as imipramine (either systemically; Harkin et al., 1999; Karolewicz et al., 2001; Volke et al., 2003; or by direct application into the hippocampus; Joca and Guimaraes, 2006), i.e., NOS inhibitors can be regarded as antidepressant-like in these tests. This effect however occurs only in lower, but not high doses of the inhibitors (da Silva et al., 2000; Ergun and Ergun, 2007) so that again a U-shaped curve was suggested. Paradoxically, not only NOS inhibitors, but also its substrate L-arginine has biphasic anti- as well as prodepressant properties (da Silva et al., 2000; Ergun and Ergun, 2007; Inan et al., 2004). However, only the pro-, but not the antidepressant effect of L-arginine seems to be due to NO mediated pathways (Ergun and Ergun, 2007).

A number of pharmacological studies have assessed the connection between the serotonergic system, its pharmacology and the NO pathway. Indeed it was shown that selective 5-HT reuptake inhibitors (SSRIs) as well as tricyclic anti-depressants decrease the activity of hippocampal NOS (Wegener et al., 2003). On the other hand, NOS inhibitors exerted their anti-depressant effect only in the presence of 5-HT, as 5-HT depletion abolished it completely (Harkin

et al., 2003). These compounds decrease 5-HT turnover in frontal cortex, similar to imipramine, and in low doses cause an increase in frontal cortical 5-HT concentrations (Karolewicz et al., 2001). This is similar to findings in *NOS1* knockdown mice, where 5-HT turnover in the frontal cortex is reduced along with a concomitant increase in frontal 5-HT as well as 5-HT_{1A} and 5-HT_{1B} receptor hypofunctioning (Chiavegatto et al., 2001). Furthermore, either local or systemic administration of NOS inhibitors increased the extracellular concentration of 5-HT and dopamine in the hippocampus, while L-arginine had the opposite effect (Wegener et al., 2000). One possible mechanism might be the direct nitrosylation of the monoamine reuptake transporters (Kiss and Vizi, 2001). Endogenous NO thereby is able to stimulate monoamine reuptake (Kilic et al., 2003), which is prevented by NOS inhibition. Accordingly, treatment with NOS inhibitors significantly enhanced the antidepressant properties of SSRIs (Harkin et al., 2004), while L-arginine treatment counteracted it (Inan et al., 2004).

Given the above considerations, we expected to find an antidepressive phenotype in *NOS1* knockdown animals which however was not the case. Though neither in the Forces Swim Test nor the Learned Helplessness paradigm, the animals behaved different to their littermates strongly arguing against an ''affective'' phenotype of these mice. This is however in conflict with two previous studies, one of which is only presented in a review, demonstrating reduced immobility time in the Forced Swim Test (Nelson et al., 2006; Salchner et al., 2004). The reasons for this are unclear, it has however to be noted that the highly aggressive phenotype described earlier in *NOS1* knockdown animals (Chiavegatto et al., 2001; Chiavegatto and Nelson, 2003; Nelson et al., 1995) was also not observed in our animals. As it was shown that backcrossing of the mice onto a C57BL/6J background, as it was done in our strain, results in a less aggressive phenotype (Le Roy et al., 2000), genetic background effects might well account for these discrepancies. The unexpected lack of an antidepressant phenotype in the *NOS1* knockdown mice however might be due to developmental effects of the knockdown. Likewise, 5-HTT knockout mice, initially reasoned to mirror the effects of SSRIs, do not display an according behavioral phenotype (Holmes et al., 2002); the development of conditional knockout models thus is a desiderate.

The expressional profile of NOS1 knockdown mice

As NO directly nitrosylates monoamine transporters, we hypothesized that this also feeds back to the expressional levels of these molecules. This however was not the case, as both 5-HTT as well as DAT mRNA levels were unchanged in knockdown mice. We therefore chose to apply a less hypothesis-driven approach by conducting a genechip microarray study, which yielded a set of dysregulated genes (Tables 2 and 3). Some of the significantly and meaningfully up-regulated genes deserve a further look.

Peroxiredoxin 3

Peroxiredoxin 3 (*Prdx3*) was up-regulated in all examined structures by at least four-fold. It is localized in the mitochondria and considered an important intracellular antioxidant, regulating the level of H_2O_2 (Nonn et al., 2003). Prx-3 protects against reactive oxidative species and especially protein nitration, thereby protecting hippocampal neurons from excitotoxic cell death (Hattori et al., 2003), and it is down-regulated upon chlorpromazine treatment (La et al., 2006) and in spinal motor neurons of patients with motor neuron disease (Wood-Allum et al., 2006). As *NOS1* is up-regulated in the latter condition (Anneser et al., 2001), this further argues for a connection between the expressional control of both enzymes. The up-regulation of *Prdx3* might be a counter-regulatory detoxifying mechanism, as *NOS1* knockdown results in elevated xanthine oxidoreductase activity leading to a significant increase in superoxide production (Khan et al., 2004).

Atonal homolog 1

The transcription factor atonal homolog 1 (*Atoh1*; synonymous: *Math1*) is crucial for axial guidance and neuronal development, especially for cerebellar granule cells (Ben-Arie et al., 1997), where *NOS1* has an important developmental role as well (Schilling et al., 1994). Thus, *Atoh1* might a potential mediator of the neurodevelopmental roles of nitric oxide. The differential regulation of homeo box genes with largely unknown functions in tissue development (*HoxA3, B4, B7, B9, C6, D11, D13, iroquois related hox* genes as well as further homeodomain transcription factors) and *CCAAT/enhancer binding protein (C/EBP), alpha* (which however has not yet been convincingly been shown to affect neuronal development) further point to the important function of NO in the developing organism. The latter protein however interestingly binds to the promoter and subsequently regulates the L-arginine synthesizing enzyme arginosuccinate lyase (Chiang et al., 2007). The NOS1 promoter itself also harbors C/EBP binding sites.

Ion channels

While the potassium channel *Kcnj8* seems to have a predominant role in the cardiac conduction system, with a knockout of the gene resulting in Prinzmetal angina, a role in the central nervous system has not yet been established. Likewise, *Kcnj1* is involved in renal potassium absorption and, at least in some cases, in the pathogenesis of Bartter's syndrome, yet no function in excitable tissue has been described.

Glucocorticoid receptor 1

Most notably, the *glucocorticoid receptor 1* (*GR*) was up-regulated two-fold both in the striatum and in the hippocampus. At the same time *NOS1* knockdown mice also demonstrate higher baseline corticosterone levels and a blunted stress response (Bilbo et al., 2003), which is counter-intuitive to up-regulated *GR* levels. However, the up-regulation of *GR* may be compensatory to a primary dysregulated hypothalamus-pituitary-adrenal axis in *NOS1* knockdown mice. Intriguingly, mice which over-express *GR* are less susceptible to develop depressive-like behaviour, i.e., they feature an ''antidepressive'' phenotype (Ridder et al., 2005) along with increases in hippocampal brain derived neurotrophic factor (BDNF) content (Schulte-Herbruggen et al., 2006). Thus, an increase in *GR* expression might also underlie the antidepressive phenotype of *NOS1* knockdown animals in previous studies (Nelson et al., 2006; Salchner et al., 2004). As in our mice no increase in neither of BDNF mRNA nor protein was observed (Fritzen et al., in press), a differential effect of the BDNF response might explain the aforementioned discrepancies in their behavioural profile. Chronic GR activation was shown to inhibit the transcriptional activity of cyclic AMP response element-binding protein (CREB, Focking et al., 2003). As this condition might be mimicked by GR over-expression in *NOS1* knockdown mice, this mechanism might contribute to the observed learning and memory defects as CREB has a crucial role therein (Silva et al., 1998).

GABAergic genes

A whole set of genes impacting on the GABAergic systems were identified to be up-regulated in *NOS1* knockdown mice. Those include the GABA transporter *GAT2* (*Slc6a12*), *GABA-B receptor 2* and the gene *Similar to Gamma-aminobutyric acid type B receptor, subunit 2 precursor.* The heteromeric GABA(B) receptor complex also acts as a heteroreceptor at hippocampal glutamatergic neurons, and was shown to be implicated in anxiety and depression (Cryan and Kaupmann, 2005). In the hippocampus, all NOS-I-positive non-pyramidal cells are GABAergic local circuit neurons (Valtschanoff et al., 1993a, b), as it is the case in the prefrontal cortex (Gabbott and Bacon, 1995). Also in the striatum, GABAergic interneurons were shown to be positive for NOS-I (Kubota et al., 1993); there, NO negatively regulates extracellular GABA (Semba et al., 1995) arguing for a close connection between both systems as previously suggested (Fedele et al., 1997a). It is well established that NO is a downstream mediator of the behavioural effects of benzodiazepines and $GABA_A$ receptor agonists (Elfline et al., 2004), also arguing for a role of NO in the regulation of anxiety. Whether or not it is also involved in $GABA_B$ signalling is less clear, however, it was demonstrated that $GABA_B$ inhibitors increase hippocampal NO production (Fedele et al., 1997b). Most interestingly however, the $GABA_B$ antagonist baclofen caused marked memory deficits which were reversed upon treatment with a NO donor (Pitsikas et al., 2003) suggesting that $GABA_B$ has a role in learning and memory, which is mediated by NOS. Thus, $GABA_B$ up-regulation might be counter-regulatory; the $GABA_B$ – NO signalling cascade therefore might be implicated in the cognitive deficits of *NOS1* knockdown animals.

Conclusions

Taken together, in this study we demonstrate that *NOS1* knockdown mice feature a distinct behavioral phenotype including reduced anxiety and cognitive impairment. This was not paralleled by expressional changes in DAT or 5-HTT, but by a set of differentially regulated genes in the hippocampus and striatum. These genes included, amongst others, $GABA_B$ receptor subunits and the glucocorticoid receptor who may also be implicated in cognitive (dys-)functioning. These findings aid in the identification of nitrinergic signalling cascades and their role in memory formation; furthermore, *NOS1* knockdown animals might therefore be considered as rodent models of Alzheimer's dementia and/or attention deficit disorder, warranting further investigations.

Acknowledgements

We thank H. Wetzstein and C. Brandwein for excellent technical assistance and Dr. B. Holtmann as well as H. Brünner, T. Töpner and H. Klüpfel for their kind help in animal housing and genotyping. This study was supported by the Deutsche Forschungsgemeinschaft (Grant RE1632/1-1 and 1-3 to A.R., KFO 125/1-1 to M.G., E.G., A.R. and K.P.L., SFB581 to K.-P.L., SFB636-B3 to P.G.), Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (IZKF Würzburg, 01KS9603, to K.P.L) and the European Commission (NEWMOOD LSHM-CT-2003-503474, to K.P.L.).

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J Neural Transm (2007) [Suppl 72]: 87–90

Printed in Austria

Journal of Neural Transmission

Animal models in neurodegenerative diseases

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Summary Ideally, animal models of neurodegenerative diseases should reproduce the clinical manifestation of the disease and a selective neuronal loss. In this review we will take as an example Parkinson's disease because its pathophysiology is well known and the neuronal loss well characterized. Indeed, Parkinson's disease is characterized by 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 two ways to develop animal models of PD 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. In this review we discuss the advantages and the drawbacks in term of neuroproction of the currently used models.

Keywords: Cell death, symptoms, cellular aggregation

Major characteristics of animal models of neurodegenerative disorders

Neurodegenerative disorders are characterized by a slow and progressive degeneration of neurons in specific locations of the central nervous system. It is this topographical arrangement of neuronal death that accounts for the specific clinical manifestations of each disease. Indeed, a major consequence of this neuronal loss is to induce changes in the functioning of the neuronal circuits downstream to the lesions and these changes are responsible for the clinical manifestation of the disease. In this context, changes in neuronal activity in motor systems will induce motor symptoms such as those seen in Parkinson's disease (PD), Huntington disease (HD), hemiballism, etc. This concept may be extended to all kinds of symptoms and neurological disorders. Yet, most of the neurodegenerative disorders are also characterized by changes in non-neuronal cells. For instance, both an astroglial and a microglial reaction have been reported in Alzheimer's disease (AD), PD, multiple sclerosis, etc. Last but not least, several neurogenerative disorders also display protein aggregation within neurons or glial cells. Schematically, four types of protein aggregations have been described: a) α-synuclein accumulation in PD and other neurological disorders called synucleinopathies; b) accumulation of Tau proteins in the form of neurofibrillary tangles in neurons or glial cells in AD, progressive supranuclear palsy (PSP) and other tauopathies; c) Aβ peptide in extracellular plaques in AD; and d) intranuclear inclusions made up of mutated proteins in inherited diseases characterized by an increased number of nucleotide triplets in the affected gene (for review, see Ross and Poirier, 2005).

Ideally animal models of neurodegenerative disorders should reproduce all the changes specific to a given disease. Unfortunately, most of the existing models do not reproduce the full spectrum of the lesions and symptoms. This is probably why animal models of neurodegenerative disorders are poorly indicative of the efficacy of neuroprotective substances in humans. Since the neuronal lesions in PD are relatively specific to dopaminergic systems, this disease will be taken as an example to discuss the development of animal models of neurodegenerative disorders.

Animal models of Parkinson's disease

Parkinson's disease (PD) is characterized by a triad of symptoms, namely akinesia, rigidity and tremor. These symptoms are due to the loss of dopaminergic neurons projecting to the striatum. Yet, other dopaminergic neurons are also affected in PD, albeit to a lesser extent. Furthermore, despite the fact that degeneration of non dopaminergic neurons has been reported for almost 30 years, considerable attention has only recently come to be focussed on non

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dopaminergic lesions, which still represent a major limitation of current therapies (Hirsch et al., 2003). Furthermore, this dopaminergic and non-dopaminergic neuronal degeneration is accompanied by the production of intraneuronal inclusions called Lewy bodies. There are at least three ways to develop animal models of PD reproducing all these characteristics. The first two are based on the etiology of the disease and consist either of reproducing in animals the mutations seen in inherited forms of the disease or of 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 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 (for review, see Michel et al., 2002).

How can genetics help to develop animal models of Parkinson's disease?

Several genes and loci involved in inherited forms of PD have been identified during the past ten years (for review, see Gasser, 2005). Yet, none of the models based on these mutations reproduces all the characteristics of the disease. Interestingly, a model based on α-synuclein over-expression in the fly has been developed by Feany and Bender (2000). They reported reduced climbing activity in flies over-expressing the wild type or the mutated form of α-synuclein, the presence of intracellular inclusions and neuronal degeneration. Yet, similar motor changes have been seen with mutations in genes unrelated to PD, such as those involved in an inherited form of AD that provokes similar behavioural changes. Furthermore, there is no definite proof that the disappearance of tyrosine hydroxylase-positive neurons is due to neuronal death as it could be the consequence of a reduced tyrosine hydroxylase expression. Transgenic mice have also been developed for α-synuclein and other mutated genes but, so far, most of them lack the loss of dopaminergic neurons seen in PD. This has led to strategies based on gene transfer methodology using a strong promoter (prion promoter) which results in the death of dopaminergic neurons in the substantia nigra due to α-synuclein over-expression (Kirik et al., 2002). Thus, further developments are still needed in order to develop suitable genetically based animal models of PD.

Table 1. *Animal models of Parkinson's disease*

Type of model	Dopaminergic neuronal loss	Non-dopaminergic neuronal loss	Protein accumulation or inclusion	Symptoms	Some references
MPTP acute mice/monkeys	yes	no	no	yes but different from PD	Dauer and Przedborski (2003) and Rousselet et al. (2003)
MPTP chronic osmotic pumps mice	yes	no	α-synuclein		Fornai et al. (2005)
Rotenone rat	yes but variable	yes but discussed	α-synuclein and tau	yes	Betarbet et al. (2000) and Höglinger et al. (2003, 2005)
Annonacine rat	yes	yes	tau	yes	Champy et al. (2004)
Paraquat rat	yes	no	?	?	McCormack et al. (2002)
6OHDA intracerebral rat or mouse	yes	no	no	yes	Pycock (1980) and Sauer and Oertel (1994)
Proteasome inhibition by peripheral injection	yes but has not been reproduced	no	yes but has not been reproduced	yes but has not been reproduced	McNaught et al. (2004) and Bové et al. (2006)
LPS intra nigral injection	yes	?	no	yes	Herrera et al. (2000)
Prenatal exposure to LPS	yes	?	yes	yes, in aged animals	Ling et al. (2004)
α-synuclein over expression or mutation in fly	yes	?	yes	yes	Feany and Bender (2000)
α-synuclein mouse	no	no	yes	no	Rockenstein et al. (2002), Song et al. (2004) and Fleming et al. (2004)
DJ1 KO mouse	no	no	no	no	Goldberg et al. (2005) and Kim et al. (2005)
Parkin KO mouse	no	no	no	yes	Itier et al. (2003), Goldberg et al. (2003), Palacino et al. (2004) and Von Coelln et al. (2004)
Pink1 drosophila	yes	no	?	yes	Park et al. (2006)

How to develop animal models of Parkinson's disease based on the use of neurotoxins

Most of the models of PD are based on the use of a neurotoxin that mimics the effect of environmental toxins or reproduces the biochemical changes seen in PD. In line with this, animal models have been developed on the basis of oxidative stress (paraquat, 6-hydroxydopamine), mitochondrial complex-1 inhibition (MPTP, rotenone, annonacine), proteasome inhibition (PSI) and proinflammatory compounds (lipopolysaccaride) (for review, see Hirsch et al., 2006 and Table 1). Among these potential models, complex-1 inhibition with MPTP being administered continuously in mice by an osmotic pump has recently been shown not only to induce a degeneration of dopaminergic neurons but also the formation of α-synuclein and ubiquitin-positive inclusions (Fornai et al., 2005). Yet, with the exception of the MPTP model controversial results have been published. For instance, non-dopaminergic lesions have been reported in the rotenone model (Höglinger et al., 2005), whereas the model induced by proteasome inhibition (McNaught et al., 2004) could not be reproduced by other investigators (Bové et al., 2006). Furthermore, these models still lack the non-dopaminergic lesions seen in PD.

Conclusions

In summary, several animal models have been developed to test neuroprotective strategies in neurodegenerative disorders. However, most of them are poorly predictive of an effect in patients. There are several reasons for these poor outcomes. First, as indicated in this review, the models have to be improved so as to reproduce the full spectrum of the disease. Second, for a given disease, the etiology (inherited vs. sporadic cases) and the clinical manifestation may differ from one patient to another. Consequently, neuroprotective strategies will probably have to be adapted to the different etiologies or mechanisms of cell death for a given clinical entity. This calls for patients enrolled in neuroprotective trials to be subdivided into groups according to individual differences. Such a methodology may help to improve neuroprotective strategies, which may be effective for some patients but not others.

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J Neural Transm (2007) [Suppl 72]: 91–104

Printed in Austria

Journal of Neural Transmission

Morphological substrates of parkinsonism with and without dementia: a retrospective clinico-pathological study*

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Summary A retrospective study of a 50-year autopsy series of 900 patients with the clinical diagnosis of parkinsonism (31.2% with dementia) revealed pure Lewy body disease (LBD) in 84.9%, but only 44.7% with idiopathic Parkinson disease (PD); 16% were associated with cerebrovascular lesions, 14.8% with Alzheimer pathology; 8.9% were classified dementia with Lewy bodies (DLB), 9.4% showed other degenerative disorders, and 5.6% other/secondary parkinsonian syndromes. The frequency of LBD during different periods was fairly stable, with increase of DLB and PD plus Alzheimer changes, but decrease of associated cerebrovascular lesions during the last decades. Using variable clinical diagnostic criteria not only by specified neurologists, the misdiagnosis rate ranged from 11.5 to 23% and was similar to that in most previous clinico-pathological studies. The majority of cases with false clinical diagnosis of PD had a final pathological diagnosis of DLB with or without Alzheimer lesions. A postmortem series of 330 elderly patients clinically diagnosed as parkinsonism with (37.6%) and without dementia showed that IPD, Braak stages 3–5 were rarely associated with cognitive impairment, which was frequently seen in IPD with associated Alzheimer pathology (35.5%), DLB (33.9%), and in Alzheimer disease (AD) or mixed dementia (17%), whereas it almost never was associated with minor cerebrovascular lesions. Clinico-pathological studies in DBL, demented and non-demented PD, and AD cases showed a negative relation between cognitive impairment and Alzheimer changes, suggesting that these either alone or in combination with cortical Lewy body pathologies are major causes of cognitive dysfunction. Further prospective clinico-pathological studies are needed to validate the currently used clinical criteria for PD, to increase the diagnostic accuracy until effective biomarkers are available, and to clarify the impact of structural and functional changes on cognitive function in parkinsonism as an ultimate goal of early disease detection and effective treatment.

Keywords: Parkinsonism, Parkinson disease with and without dementia, diagnostic accuracy, clinico-pathological study, neuropathological evaluation

Introduction

The correct diagnosis of Parkinson disease (PD) is important for prognostic and therapeutic reasons and is essential for clinical, epidemiological and genetic research. A large group of neurodegenerative disorders with parkinsonian signs and symptoms are pathobiologically featured by the deposition of characteristic protein aggregates. Synucleinopathies share the deposition of α-synuclein (AS) either within nerve cells (e.g. PD, dementia with Lewy bodies/DLB) or within oligodendrocytes (e.g. multiple system atrophy/MSA) (Lantos, 1998; Spillantini et al., 1997; Wakabayashi et al., 1998). Clinically, movement disorders referred to as synucleinopathies are often difficult to differentiate from other causes of parkinsonism and dementia, thereby highlighting the need for disease-specific, sensitive surrogates and biomarkers (Michell et al., 2004). Since until recently, there were no biological markers for the antemortem diagnosis of parkinsonian disorders available; the diagnosis relied mainly upon the presence and progression of clinical features, and diagnostic confirmation depended on neuropathology. Genetic testing (see Fung et al., 2006; Hardy et al., 2006; Klein and Schlossmacher, 2006; Mizuta et al., 2006; Toft and Farrer, 2005) and other sensitive surrogate tests, such as olfactory testing (Doty et al., 1992; McShane et al., 2001; Mesholam et al., 1998; Olichney et al., 2005), (functional) magnetic resonance imaging (MRI) (Burton et al., 2005; Junque et al., 2005), dopamin-transporter single-photon-emission computer tomography (SPECT) or positron emission tomography (PET) imaging (Bohnen et al., 2006; Brooks and Piccini, 2006; Chou et al., 2004; Dagher, 2005; Dhawan and Eidelberg, 2007; Eckert et al., 2005; Eerola et al., 2005; Im et al., 2006; Ravina et al., 2005; Shinotoh, 2006), transcranial sonography (see Berg, 2006a, b; Prestel et al., 2006; Sasaki et al., 2006; Zecca et al., 2005), myocardial 6-F-18-fluorodopamine radioactivity (Tipre and Goldstein, 2005), 123I-metaiodobenzylguanidine myocardial scintigraphy (Hanyu et al., 2006; Kim et al., 2006) or

* Dedicated to my friend Peter's special anniversary with best wishes for many more years of good health and prosperity

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detection of AS pathology in peripheral autonomic neurons (Bloch et al., 2006; Minguez-Castellanos et al., 2006), considerably help with clinical diagnostic decisions. Only very recently methods to directly quantify AS in cerebrospinal fluid (CSF) (Mollenhauer et al., 2007; Tokuda et al., 2006), plasma (El-Agnaf et al., 2003, 2006; Lee et al., 2006), and blood platelets (Shults et al., 2006) have been developed, but need further validation. The same holds true for increased CSF levels of DJ-1, an antioxidant protein whose loss of function by gene mutation has been linked to familial PD (Sun et al., 2006; Waragai et al., 2006).

Several sets of clinical diagnostic criteria for PD have been proposed (Gelb et al., 1999; Geser et al., 2005; Gibb and Lees, 1988; Hughes et al., 1992a, b; Suchowersky et al., 2006; Ward and Gibb, 1990), UK Parkinson's Disease Society Brain Bank (UK PDSBB) clinical diagnostic criteria (Daniel and Lees, 1993), and have shown highly variable accuracy with sensitivity and specificity values ranging from 66 to 93% and 14 to 86%, respectively (Litvan et al., 2003; Suchowersky et al., 2006). This is, at least in part, related to the clinical and pathological heterogeneity of idiopathic Parkinson disease (IPD) (Foltynie et al., 2002).

Validity and reliability of clinical studies

Several clinico-pathological studies have shown significant false-positive and false-negative rates for diagnosing parkinsonian disorders, misdiagnosis being especially common during the early stages of these diseases, even among movement disorder specialists (Litvan et al., 1996; Rajput et al., 1991; Schrag et al., 2002). This limitation, despite several currently used clinical diagnostic consensus criteria strongly affects epidemiological studies and clinical trials.

In a community-based study of 402 patients with presumed PD using recommended clinical diagnostic criteria, parkinsonism was confirmed in 74% and probable PD in 53%. The most common causes of misdiagnosis were essential tremor, Alzheimer disease (AD), and vascular pseudoparkinsonism; over 25% of these patients did not benefit from anti-parkinsonian medication (Meara et al., 1999).

In a population based study on the prevalence of PD in London, Schrag et al. (2002) reported on the data of a long-term clinical evaluation of 202 patients. The initial diagnosis of probable PD was later confirmed in 83% plus 2% each with atypical features and possible PD. In 15% the initial diagnosis was later rejected, while 19% of patients not diagnosed as PD were later found to have the disorder. Their conclusion was that in 15% of the cases the clinical criteria of PD were not followed, in accordance with previous retrospective clinico-pathological studies of parkinsonism, in which the rate of false positive diagnosis ranged between 22–24% (Hughes et al., 1992a, b; Rajput et al., 1991) and 15–18% (Ansorge et al., 1997; Jellinger, 2001). Using more strict diagnostic criteria by movement disorder experts, this figure recently was reduced further to around 10%, with a surprisingly high positive predictive value (PPV) for IPD of 98.6% and for other parkinsonian syndromes of 71.4%, e.g. 85.7% for MSA and 78–80% for progressive supranuclear palsy (PSP) (Hughes et al., 2001; Osaki et al., 2004).

Referring to these data, Schrag et al. (2002) suggested that at least 10% of the patients with the final clinical diagnosis of PD may have other disorders. In pathological series, the incidence of atypical parkinsonism is substantial. For example, PSP is found in 6–22% of autopsy cases, MSA in 5–11.4%, vascular pseudoparkinsonism in 2–4%, and AD in demented PD patients in 2–6% (Hughes et al., 2001; Jellinger, 2001, 2003b). Epidemiological studies suggest a 3–5% mean prevalence of vascular parkinsonism (see Sibon and Tison, 2004).

A prospective survey of 1000 consecutive patients presenting to two European tertiary referral centres for movement disorders (London, UK and Barcelona, Spain) showed that 4–5% of parkinsonian patients could not be categorized using currently available clinical diagnostic criteria for parkinsonian syndromes (Jain et al., 2006; Katzenschlager et al., 2003).

In all these studies, the most common reason for misdiagnosis were essential tremor, vascular parkinsonism, and atypical parkinsonian syndromes, less frequent AD, DLB, and drug-induced parkinsonism (Litvan, 2005; Tolosa et al., 2006). Clinical ''red flags'' to assist the identification of atypical parkinsonism have been published recently (Abdo et al., 2006).

Clinico-pathological studies

Most clinico-pathological studies have been retrospective, limiting the conclusions. In general, the number of cases in published series have been small, clinical evaluations were not standardized, and diagnostic determinations were performed by different clinicians who were not necessarily trained in movement disorders (Hughes et al., 1992a, b; Jellinger, 1987; Litvan et al., 1996; Wenning et al., 2000). There are several epidemiological and methodological limitations in the statistical validity measures of these studies.

A review of 2351 autopsy cases in Tokyo in the 1970's selected 40 cases (1.7%), who received a clinical diagnosis of PD (24 men and 16 women, with a mean age at death of 76.3 ± 7.2 years). Clinical informations were retrieved

from the hospital charts, and histological examination including immunohistochemistry revealed 25 Lewy body diseases (LBD), 5 pure nigral degeneration without LBs, two PSP, one CBD, 3 vascular parkinsonism, and 4 cases of non-specific pathology. Among the LBD cases, two also fulfilled the diagnostic criteria of PSP and one of CBD, while two others were complicated by mild PSP-like tau-pathology, and one case of LBD with PSP had been clinically diagnosed as PD, while a pure CBD case had been regarded as typical PD with good response to levodopa (Sengoku et al., 2006).

An early clinico-pathological study (Ward and Gibb, 1990) found that only 69–75% of the patients with autopsy-confirmed PD had at least two of the three cardinal manifestations of PD: tremor, rigidity, and bradykinesia. While 20–25% of patients who showed two of these cardial features had a pathological diagnosis other than PD, 13–19% of patients who had demonstrated all three cardinal symptoms typically associated with a clinical diagnosis of IPD had another pathological diagnosis.

Rajput et al. (1991) reported autopsy results in 59 patients with parkinsonian syndromes. All had been examined longitudinally by a single neurologist who had based the clinical diagnosis of PD on the presence of two of the three cardinal manifestations mentioned above, excluding postural instability, because it is usually not present in early PD. They also excluded any identifiable cause of parkinsonism or other central nervous system (CNS) lesions. After a long-term follow-up period, the clinical diagnosis of PD was retained in 41–59 patients, but only 75% of them showed histopathological signs of IPD at autopsy.

In another series of 100 patients with a clinical diagnosis of PD, who had been examined during life by different neurologists using poorly defined diagnostic criteria, autopsy performed after a mean interval between symptom onset and death of 11.9 years, found PD in 76%. Using the UK PDSBB clinical criteria for PD, 89 of the 100 patients were considered to have IPD, but only 73 (82%) were confirmed at autopsy (Hughes et al., 1992b). The same group has since studied the accuracy of the clinical diagnosis of PD in 100 consecutive patients, of which 90% fulfilled pathological criteria for PD. Ten were misdiagnosed: MSA (6), PSP (2), postencephalitic parkinsonism and vascular pseudoparkinsonism (one each) (Hughes et al., 2001). They next examined the accuracy of diagnosis of parkinsonian disorders in a specialist movement disorder service. Reviewing the clinical and pathological features of 143 cases of parkinsonism, they found a surprisingly high positive PPV (98.6%), and only one of 73 patients diagnosed with PD during life was found to have an alternative diagnosis. This study demonstrated that the clinical diagnostic accuracy of PD could be improved by using stringent criteria (Hughes et al., 2002).

A large retrospective series of 580 patients with the diagnosis of parkinsonism using various clinical criteria showed confirmation at autopsy in 85% (Jellinger, 1987).

Correlating clinical features with pathological findings in 39 autopsy cases (17%) among 364 patients with parkinsonism in Olmsted County, MN, USA, 16 cases (41%) pathologically showed LBD, 7 (18%) MSA, 5 had vascular disease, and of 8 cases given the clinical diagnosis of drug-induced parkinsonism, 6 had basal ganglia pathology. Despite the selection bias of more frequent atypical cases, the small sample size, and the retrospective data collection, these data illustrated the difficulty in achieving an accurate ante mortem diagnosis of parkinsonism (Bower et al., 2002).

The accuracy of the differentiation between PD and DLB was examined by an international study group in 105 clinical movement disorder cases including 15 cases of PD and 90 with other diseases; all diagnoses had been established by autopsy (Litvan et al., 1998). The inter-rater sensibility for the diagnosis of PD was moderate at the first visit ($\kappa = 0.54$) and substantial at the last visit ($\kappa = 0.64$). Median sensitivity for PD rose from 73 to 80% and median specificity increased from 85.6 to 92.2%. Closer examination of misdiagnosed PD cases at first visit revealed that they were complicated cases; false-positive misdiagnoses primarily involved DLB, MSA, and PSP.

Cognitive impairment in parkinsonism

Mental dysfunction including dementia in PD with a reported prevalence between 24 and 41% and 0.5% of subjects aged 65 plus years, an incidence rate of 4.2–9.5% per year, and a 70% cumulative incidence of dementia, is suggested to have an about six-fold lifetime risk compared with age-matched controls (Aarsland et al., 2005b; Bosboom et al., 2004; Emre, 2003; Hobson and Meara, 2004; Levy and Marder, 2003). CNS lesions contributing to cognitive impairment in PD are multifold, including dysfunction of subcortico-cortical striato-frontal, cholinergic forebrain and other neuromodulator networks due to neuron loss in brainstem and limbic systems. They cause cholinergic deficits in various cortical regions and thalamus, decreased striatal dopaminergic function (Ziabreva et al., 2006), widespread decrease of nicotinic acetylcholine receptors (Fujita et al., 2006), limbic and cortical Lewy body (LB) and Alzheimer pathologies with loss of synapses and neurons, and variable combinations of these lesions

(Braak et al., 2005; Hilker et al., 2005; Jellinger, 2004, 2006c; Nagano-Saito et al., 2005; Summerfield et al., 2005; Zgaljardic et al., 2004). The pathogenesis and morphological basis of PD plus dementia (PDD), the impact of the variable pathologies on cognitive impairment in parkinsonism, and the relationship between the onset of dementia and key morphological and neurochemical characteristics are under discussion (Ballard et al., 2006). In particular the correlation between dementia and cortical LB and Alzheimer-related pathologies is a matter of dispute. While some authors showed increasing cognitive decline with increasing LB stages (Braak et al., 2005; Brooks and Piccini, 2006), others have not found such an association (Colosimo et al., 2003; Harding and Halliday, 2001; Jellinger, 2006c). Diffuse DLB with mild to moderate Alzheimer type pathology has been suggested as a major pathological substrate of PDD (Aarsland et al., 2005a; Apaydin et al., 2002), others reported significant correlations between cognitive impairment and widespread AD pathology in both PD and DLB (Jellinger, 2006c; Papapetropoulos et al., 2005). In a recent longitudinal study of 103 elderly patients (mean age 74 years; 10 controls, 42 PD, 20 DLB, 31 AD), 83% of subjects with PD developed dementia. Their clinical features were identical with those observed in DLB and differed from AD, visual hallucinations predicting dementia in PD. The morphologic substrates of PDD included DLB (38%), AD (32%), and nigral LBs alone (24%) (Galvin et al., 2006).

Samples from brain banks and specialized institutions are generally considered to overrepresent atypical disorders owing to the referral bias inherent in such samples (Hughes et al., 2002). Therefore, we present data from a large consecutive autopsy series of patients with the clinical diagnosis of parkinsonism observed during the last 50 years in Vienna, Austria, and the morphological substrates of parkinsonian syndromes with and without dementia in a more recent part of this cohort. Although the clinical data were collected retrospectively, this study is hoped to contribute to the question about the validity of the clinical diagnosis of parkinsonism and the neuropathology of parkinsonism with dementia.

Material and methods

A total of 900 patients (458 females, 442 males, aged 47–98 years) with the clinical diagnosis of parkinsonism from a consecutive autopsy series (1957–2006) from three large hospitals in Vienna, Austria, all with acute and chronic care facilities, underwent neuropathological examination. Clinical information was retrieved from the hospital charts; diagnosis of PD was performed according to the UK PDDSBB clinical diagnostic criteria (Daniel and Lees, 1993), that of DLB according to McKeith et al. (1996), for PSP (Litvan et al., 1996; Osaki et al., 2004), for MSA (Gilman et al., 1999), etc. Moderate to severe dementia (with Mini-Mental examination/MMSE/ scores 0–20) were reported in 281 patients (31.2% of the total cohort). Neuropathological studies were performed according to an established protocol (see Jellinger, 2003a, 2004, 2006b, c): multiple formalin-fixed sections from neocortex, limbic system, basal ganglia, various levels of the brain stem, and cerebellum were examined using routine stains, modified Bileschowsky silver impregnation, Gallyas-Braak stain, and since the 1990's, immunohistochemistry for phosphorylated tau protein (antibody AT-8, Innogenetics, Ghent, Belgium), Aβ amyloid peptide (clone 4G8, mouse monoclonal antibody, Signet Labs, Dedham, MA, USA), GFAP (Daco, Gilstrup, Denmark); α-synuclein (rabbit and polyclonal antibody, Chemicon, Temecula, CA, USA, dilution 1:1000). Many of the earlier cases were re-examined using immunochemistry. Neuropathology diagnoses were performed unaware of any demographic and clinical information, according to published pathological criteria (see Braak and Braak, 1991; Braak et al., 2003, 2004; Dickson, 1999; Dickson et al., 2002; Hauw et al., 1994; Hyman and Trojanowski, 1997; Jellinger and Mizuno, 2003; Jellinger, 2006a; Lantos, 1998; Lowe, 1998; McKeith et al., 2004; Mirra et al., 1991; Munoz et al., 2003; Trojanowski and Dickson, 2001). The different types of parkinsonism found by neuropathological examination in different time periods, all performed in the same neuropathological department, were compared among each other and with those by Hughes et al. (1992a, b, 2001) and the clinical classification by Schrag et al. (2002), as well as the rates of misdiagnoses in various studies.

In a group of 345 elderly patients with the clinical diagnosis of parkinsonism (159 males, 186 females, aged at death 60–98, mean 80.9 ± 7.9 SD years) coming to autopsy between 1988 and 2005, all included in the total autopsy series, morphological classification was performed for non-demented (62.3%) and demented patients (37.7%), and the pathological substrates of PDD in this cohort were evaluated. Part of these cases have been reported recently (Jellinger, 2006c).

Finally, major clinical changes (cognitive impairment) and Alzheimer-related pathologies in 117 cases of LB-related disorders, AD, and age-matched controls from are compared.

Results

Morphological classification and diagnostic accuracy

The neuropathological findings of the total series of 900 autopsy cases with a clinical diagnosis of parkinsonism are summarized in Table 1. Pure LBD was seen in 84.9%, but only 44.7% showed the classical picture of IPD of the LB type or brainstem form of LBD, while 16.1% were associated with cerebrovascular lesions (lacunar state, small old infarcts or hemorrhages), and 14.8% with Alzheimer-type pathology, 12.5% showing neuritic Braak stages 5 and 6. DLB was diagnosed in 8.9% (5.8% diffuse DLB without severe AD lesions, and 3.1% corresponding to the Lewy body variant of AD (LBV/AD) (Brown et al., 1998; Hansen et al., 1990). Other degenerative parkinsonian syndromes were observed in 9.4%, including AD (3.6%), MSA (2.7%), PSP (2.4%) and CBD or Pick disease (0.6%), while other/secondary parkinsonian syndromes, associated with vascular encephalopathies (3.7%), other disorders (hydrocephalus, Creutzfeldt-Jakob disease/CJD, etc.), unclassified nigral lesions or nothing abnormal related to clinical diagnosis of essential tremor, were seen in 5.6%.

Table 1. *Pathology findings in 880 autopsy cases with clinical diagnosis of parkinsonism (1957–2006)*

Neuropathology	Total	%
Primary Lewy body disease	764	84.9
Idiopathic Parkinson disease (IPD)	402	44.7
IPD + CVD (status lacunaris/infarct/MIE 115/9/20)	144	16.0
IPD + AD/DAT (Braak stage 5 and 6)	112	12.5
IPD + AD pathology (limbic AD)	21	2.3
Lewy body variant AD	28	3.1
DLB (without AD)	52	5.8
IPD + other pathology	4	0.5
Other Parkinson syndromes	136	15.1
Other degenerative parkinsonism	*86*	*9.8*
AD/DAT	32	3.6
PSP	22	2.4
MSA	25	2.8
CBD, Pick's disease	5	0.6
Postencephalitic parkinsonism	3	0.3
Secondary parkinsonism	*49*	*5.5*
MIE/SAE/MIX 3/28/2	33	3.7
Nigral lesion unclassified	6	0.7
Others (hydrocephalus, JCD)	3	0.3
Nothing abnormal (essential tremor?)	7	0.8
Total	900	100.0

IPD, Idiopathic Parkinson disease; *CVD*, cerebrovascular disease; *AD*, Alzheimer disease; *DAT*, dementia of the Alzheimer type; *DLB*, dementia with Lewy bodies; *MIE*, multi-infarct encephalopathy; *SAE*, subcortical arteriosclerotic encephalopathy; *MIX*, Alzheimer's disease plus vascular encephalopathy; *PSP*, progressive supranuclear palsy; *MSA*, multiple system atrophy; *CBD*, corticobasal degeneration; *JCD*, Jakob-Creutzfeldt disease.

The incidence of different types of parkinsonian syndromes observed in different periods between 1957 and 2006 are listed in Table 2, and compared with the final clinical classification (Schrag et al., 2002) and the autopsy series reported by Hughes et al. (1992b, 2001). The results of autopsy studies of the Vienna cohort in different periods showed a fairly stable incidence of LBDs ranging between 73 and 82.4%, with slight decrease in morphologically ''pure'' IPD without other pathologies, a considerable decrease of associated cerebrovascular lesions but increase of concomitant Alzheimer-type pathology, probably related to increasing age at death. Since the 1980's, an increased number of DLB cases was diagnosed, owing to improved histological techniques (use of AS immunohistochemistry). DLB was not mentioned in previous studies (Hughes et al., 1992a, b, 2001; Schrag et al., 2002). In the two recent studies (1989–2000; 2001–2006), the frequency of ''pure'' IPD was 6 and 67% compared to IPD plus AD pathology (6 and 20%) and cerebrovascular lesions (6 and 12.2%, respectively), compared to 16 and 22.6% LBD. There was also a mild but steady increase in the frequency of other degenerative parkinsonian syndromes (13.6 and 16%, respectively) compared to 33% reported by Hughes et al. (1992a, b, 2001), with a fairly stable frequency of secondary parkinsonism (7.7–11%, respectively) which was also less frequent than in the study by Hughes et al. Since 1988, there was a complete absence of postencephalitic pakinsonism, also not mentioned in the British series.

The diagnostic accuracy in the different study periods ranged from 77% during the last five years, 84.7% between 1971 and 1988, and 88.5% between 1989 and 2001, thus being similar as in previous clinico-pathological studies (see Table 3). The variations in misdiagnosis (11.5–23%) were due to the fact that the clinical examinations were performed in different departments; those in 1989–2001 in specialized departments by neurologists experienced in movement disorders, while previous and later clinical data came from less specialized neurological, intern and geriatric departments, using poorly defined diagnostic criteria.

Morphological diagnoses in non-demented and demented parkinsonism

The clinical and pathological diagnoses in 330 patients with parkinsonism with (37.6%) and without dementia (151 males, 179 females, aged 65–98 years over a 17-year period – 1988–1006) are given in Table 4. All these cases had been included in the total series. The mean age at onset in the demented PD (PDD) group was higher than in the non-demented one (65.8 ± 7.9 vs. 76.6 ± 8.7 years), whereas the survival was significantly shorter in the demented group (mean 7.4 ± 3.0 vs. 12.8 ± 7.8 years, $p < 0.01$). Kaplan–Meyer curves showed significant decrease of survival time in patients with high neuritic AD stages (CERAD C, Braak stages 5 and 6) compared to those with no or less severe AD pathology (CERAD 0–1, Braak stages 0–3) and, thus, confirmed increased mortality risk in patients with PDD (de Lau et al., 2005; Hughes et al., 2004; Jellinger, 2005, 2006c).

Because 61 cases (18.5% of the total cohort displayed other pathological syndromes (AD 6%, subcortical arteriosclerotic encephalopathy/SAE 3.6%, other degenerative disorders – PSP and MSA, 3.3 and 2.1%, respectively) only 81.5% were confirmed as primary LBD, including IPD with PD stages 3–5 (35.2%) and DLB-LBV/AD cases (17.5%), all corresponding to PD stages 5 or 6 (Braak et al., 2003, 2004). The majority of non-demented IPD cases was classified PD stages 3–5, whereas only two such patients without other concomitant lesions had been reported as being demented (0.6% of the total, and 1.6% of demented cases). Additional cerebrovascular lesions (lacunar state, small old

Table 2. *Incidence of different types of parkinsonism in autopsy series (percentage)*

Type of lesion	Schrag et al. (2002) (Clin. PD)	Hughes et al. (1990–1999)	Jellinger (1957–1970)	Jellinger (1971–1988)	Jellinger (1989–2000)	Jellinger (2001–2006)
Lewy body disease (total)			81.8	82.4	78.7	73.0
Idiopathic Parkinson disease (IPD)	61.4	50.0	79.2	77.9	56.1	67.0
IPD + cerebrovascular pathology			25.5	19.6	12.2	6.0
IPD + Alzheimer pathology			5.5	17.4	6.3	20.0
Lewy body dementia (DDLB + LBV/AD)			2.6	4.5	22.6	16.0
Other degenerative parkinsonism	4.5	33.0	9.1	10.8	13.6	16.0
MSA	1.5	22.0	4.5	2.9	3.1	4.0
PSP	3.0	11.0	2.8	2.6	2.7	4.0
Pick disease, corticobasal degeneration		?	0.9	0.8	0.6	0
Alzheimer disease		?	0.9	4.5	7.2	8.0
Secondary parkinsonism	31.8	17.0	9.1	6.8	7.7	11.0
Vascular parkinsonism (MIE, SAE, MIX)	5.5	?	3.7	4.2	4.0	6.0
Postencephalitic parkinsonism		?	2.8	0.8	0	0
Symptom parkinsonism (JCD, tumors, etc.)	3.5	?	0.9	0.3	0.5	2.0
Toxic/drug induced parkinsonism		?	0.9	0.3	0.5	0
Posttraumatic/boxer dementia		?	0	0.3	0.5	0
Unclassified/no lesion (“tremor”)	22.8	?	0.9	1.0	2.2	3.0
Total *n*	202	143	110	380	221	100

DDLB, Diffuse dementia with Lewy bodies; *LBV/AD*, Lewy body variant of AD; *MSA*, multiple system atrophy; *PSP*, progressive supranuclear palsy; *MIE*, multi-infarct encephalopathy; *SAE*, subcortical arteriosclerotic encephalopathy; *MIX*, Alzheimer’s disease plus vascular encephalopathy; *JCD*, Jakob-Creutzfeldt disease.

Table 3. *Misdiagnosis in necropsy series of clinical Parkinson’s disease (with or without dementia)*

Pathology	Hughes et al. (1992b) ($n = 100$) $n = \%$	Rajput et al. (1992) ($n = 41$) %	Jellinger (2001) (1971–1988) ($n = 380$) %	Jellinger (1989–2001) ($n = 260$)		Hughes et al. (2001) $n = 143$ %	Jellinger (2001–2006) ($n = 100$) %
				n	%		
Alzheimer disease (AD)	6	2.0	2.6	5	1.9	?	4.0
Vascular encephalopathy (VaE)	0	2.0	3.5	2	0.8	?	4.0
Progressive supranuclear palsy	8	10.0	1.8	3	1.1	3.5	3.0
Multiple system atrophy	5	2.0	2.2	3	1.1	3.0	1.0
Nigral atrophy (unclassified)	2	0	0.5	1	0.4		0
MIX encephalopathy (AD + VaE)	0	0	0.5	1	0.4		1.0
Lewy body dementia	1	0	3.6	12	4.6		8.0
Pick’s disease, corticobasal degeneration	0	0	0.2	0	0		0
Unclassified, no lesions (essential tremor?)	1	0	0.3	2	0.8	8.7	1.0
Others (pallido-nigral degeneration, toxic, etc.)	0	2.0	0.3	1	0.4		1.0
Postencephalitic parkinsonism	1	4.0	0	0	0		0
Total	24	22.0	15.3	30	11.5	15.2	23.0

infarcts or hemorrhages) did not contribute to cognitive impairment in PD, except in rare cases with additional SAE or mixed dementia (PD + SAE). On the other hand, SAE without degenerative changes, diagnosed pathologically in 3.6% of the total cohort, was mainly seen in non-demented patients. Dementia in other degenerative parkinsonian disorders (PSP; MSA) usually was associated with considerable Alzheimer-type pathology.

The pathology of demented parkinsonian cases is given in Table 5. Around 35.5% of demented PD cases displayed morphological LB stages 4 or 5 with superimposed neuritic AD pathology (stages 4–6). More than half of them showed strong relationship between the severity of AS and tau pathologies, particularly in the limbic system (data not shown). DLB with low or high-grade AD lesions was seen in almost 40% of demented parkinsonian patients, but

Table 4. *Neuropathology of clinical parkinsonism patients without and with dementia (1988–2006) (n = 330, 65–98 years, 151 males, 179 females)*

	Non-demented PD		Demented PD		Total cohort		Dementia group %
	n	Total %	*n*	Total %	*n*	%	
Primary Lewy body disorders	*175*	*53.0*	*94*	*28.5*	*269*	*81.5*	*75.8*
IPD Braak stages 2 + 3	4	1.2	0	0.0	4	1.2	0.0
IPD Braak stages 3 + 4	88	26.7	1	0.3	89	27.0	0.8
IPD Braak stage 5	22	6.7	1	0.3	23	7.0	0.8
IPD + cerebrovascular lesions	27	8.2	1	0.3	28	8.5	0.8
IPD Braak stage 4 + AD B/B 5	0	0.0	13	3.9	13	3.9	10.5
IPD Braak stage 5 + AD B/B 5	0	0.0	16	4.8	16	4.8	12.9
IPD Braak stage 4 + AD B/B 4	11	3.3	8	2.4	19	5.8	6.5
IPD Braak stage 5 + AD B/B 4	1	0.3	7	2.1	8	2.4	5.6
IPD + vascular encephalopathy	4	1.2	3	0.9	7	2.1	2.4
IPD + mixed dementia (AD + SAE)	0	0.0	2	0.6	2	0.6	1.6
Lewy body variant of AD	0	0.0	15	4.5	15	4.5	12.1
Dementia with Lewy bodies	16	4.8	27	8.2	43	13.0	21.8
IPD + other pathology	2	0.6	0	0.0	2	0.6	0.0
Other/secondary parkinsonism	*31*	*9.4*	*30*	*9.1*	*61*	*18.5*	*24.2*
AD (+ nigral lesion*)	0	0.0	20 (*8)	6.1	20	6.1	16.1
Mixed dementia (AD + SAE)	0	0.0	1	0.3	1	0.3	0.8
SAE	10	3.0	2	0.6	12	3.6	1.6
PSP (+ AD*)	9	2.7	*2	0.6	11	3.3	1.6
Multisystem degeneration (+ AD*)	4	1.2	*3	0.9	7	2.1	2.4
Corticobasal degeneration	0	0.0	1	0.3	1	0.3	0.8
Other disorders (hydrocephalus, JCD)	3	0.9	1	0.3	4	1.2	0.8
Nothing abnormal (essential tremor?)	5	1.5	0	0.0	5	1.5	0.0
Total	206	62.4	124	37.6	330	100.0	100.0

PD, Parkinson disease; *AD*, Alzheimer disease; *VaE*, vascular encephalopathy; *SAE*, subcortical arteriosclerotic encephalopathy; *PSP*, progressive supranuclear palsy; *JCD*, Jakob-Creutzfeldt disease.

Table 5. *Pathology of parkinsonism with dementia (1988–2006)*

Neuropathology	*n*	%
IPD Braak stages 3–5	2	1.6
IPD + cerebrovascular lesions (lacunar state, small infarcts)	1	0.8
IPD + AD (B/B 4–6)	44	35.5
IPD + vasc. encephal. (VaE)	3	2.4
IPD + MIX (AD + VaE)	2	1.6
LB variant of AD	15	12.1
Dementia with LB, diffuse	27	21.8
IPD + other pathologies	0	0.0
AD/MIX (AD + VaE)	21	16.9
Subcort. vasc. encephalopathy (SAE)	2	1.6
PSP (+AD), CBD	3	2.4
MSA + AD	3	2.4
Other disorders (hydrocephalus)	1	0.8
Total	124	100.0

almost one-third of diffuse DDLB cases, i.e. those with mild AD lesions restricted to amyloid plaques or tau pathology in the limbic system (Ince and McKeith, 2003) did not show considerable dementia. Other degenerative parkinsonian disorders with superimposed Alzheimer-pathology accounted for 4.8% of all demented cases.

A comparison of major clinical/cognitive and Alzheimer-related morphological changes in 117 autopsy cases of LB-related disorders, AD (without other pathologies), and age-matched controls is given in Table 6. The age at death and the duration of illness did not significantly differ among the groups. Brain weight in AD, DDLB, PDD and LBV/AD did not differ significantly, and was higher in both non-demented PD cases and aged controls ($p > 0.01$). MMSE scores were lowest in AD and LBV/AD, non-significantly higher in PDD, much higher in DDLB (without severe AD-lesions), similar in non-demented PD cases, and highest in aged controls. Neuritic Braak stages, being highest in AD and LBV/AD, similar in PDD and DDLB, and lowest in non-demented PD and controls, correlated well with the level of cognitive impairment. These data also correlate with progressive hippocampal atrophy in PD > PDD > AD (Burton et al., 2004; Nagano-Saito et al., 2005), and the severity of involvement of the cholinergic system with neuronal loss in the nucleus basalis of Meynert, being more severe in LBV/AD than in pure AD and non-demented PD (Jellinger, 2006c), and widespread cholinergic losses differentiate DLB and LBV/Ad from classical AD (Camicioli

Table 6. *Major clinical and Alzheimer-related changes in Lewy body-related disorders, Alzheimer disease, and age-matched controls*

	LBV/AD ($n=26$)	DDLB ($n=31$)	PDD (+AD) ($n=11$)	AD ($n=30$)	PD non-dem. ($n=13$)	Controls ($n=7$)
Age (years)	79.8 ± 4.9	76.0 ± 6.1	77.1 ± 5.1	79.0 ± 5.3	74.3 ± 5.4	77.7 ± 3.2
Sex (M/F)	8/18	9/22	3/8	25/5	5/8	5/2
Duration (years)	5.9 ± 2.3	7.4 ± 2.5	7.3 ± 3.2	6.8 ± 3.1	9.5 ± 4.2	–
MMSE ($n=12/8$)	2.0 ± 1.0	15.1 ± 5.2	4.9 ± 3.2	0.5 ± 0	24.7 ± 1.0	28.0 ± 0.5
Brain weight (g)	1182 ± 112	1206 ± 92	1188 ± 86	1081 ± 48	1246 ± 51	1337 ± 118
CERAD 0	0	19	1	0	11	7
A	0	9	3	0	1	0
B	11	3	3	1	1	0
C	15	0	4	29	0	0
Braak stage	4.76 ± 0.2	2.61 ± 0.3	4.1 ± 0.5	5.5 ± 0.2	2.1 ± 0.3	1.3 ± 0.2

AD, Alzheimer disease; *LBV/AD*, Lewy body variant of Alzheimer disease; *DDLB*, diffuse dementia with Lewy bodies; *PD*, Parkinson disease; *PDD*, Parkinson disease with dementia.

et al., 2003; Perry, 2004; Tam et al., 2005; Tiraboschi et al., 2002).

Discussion

General clinico-pathological relations

Clinico-pathological studies are needed to validate the proposed clinical diagnostic criteria for PD, but such studies are difficult to conduct since there are no universally accepted neuropathological criteria for PD (see Jellinger and Mizuno, 2003). The Braak et al. (2003, 2004) PD staging scheme, although being a useful construction to evaluate LBD (Muller et al., 2005) and to show the potential progress of the disease (Dickson et al., 2006; Halliday et al., 2006), does not fit for a certain proportion of cases (Braak et al., 2006; Ding et al., 2006; Jellinger, 2003a, 2004; Parkkinen et al., 2003; Saito et al., 2003; Uchikado et al., 2006a).

While in some clinico-pathological studies of rather small numbers of cases, 50–61.4% were pathologically diagnosed LBDs, in the present 50 years autopsy series of 900 patients with the clinical diagnosis of parkinsonism, 84.9% were classified as primary LBD. The frequency of autopsy-proven IPD cases ranged from 50 to 79% with slight decrease during the last two decades, while that of DLB – not presented in previous studies – increased significantly to about 20% of the total, probably due to modern histological techniques, in particular AS immunohistochemistry, which has been shown to be definitely more specific and sensitive in detecting LBs than other methods (Gomez-Tortosa et al., 2000). Moreover, the percentage of other degenerative parkinsonian syndromes, e.g. PSP, MSA, etc., accounting for 33% in the autopsy series by Hughes et al. (1992b, 2001), steadily increased in the present series from 9 to 16%, as did the incidence of AD clinically often misdiagnosed as parkinsonism, probably due to the increasing age of the patients. The same was true for IPD plus superimposed AD pathology that increased significantly from 5.5 to 20%, whereas the percentage of IPD cases with additional mild cerebrovascular lesions decreased significantly from 25.5 to 6%, the reasons of which are obscure (Jellinger and Attems, 2006). On the other hand, parkinsonism related to vascular encephalopathies, in particular SAE, remained fairly stable during the periods, its frequency ranging from 3.7 to 6% being comparable to that in other clinico-pathological series (Sibon and Tison, 2004).

The diagnostic accuracy and positive PPV for IPD that has been significantly increased from 76–84.8 to 98.6% during the last years, using more strict diagnostic criteria by movement disorder experts (Hughes et al., 2002), in the present series ranged from 77 to 88.5%, depending on the expertise of clinicians with regard to movement disorders.

It is remarkable that a large part of cases with a clinical diagnosis of IPD with or without dementia in more recent years had a final neuropathological diagnosis of DLB (increasing from 2.6 to 16–22.6%). In the autopsy cohort 1988–2006, DDLB without severe Alzheimer-type pathology which often clinically presents with initial parkinsonism (McKeith et al., 1996; Seppi et al., 2000) was much more frequent than LBV/AD, i.e. DLB with severe neuritic AD changes (8.2 vs. 4.5%). While more than one-third of DDLB cases showed no considerable cognitive decrease, virtually all cases of LBD/AD had been demented.

The morphology and immunohistochemistry of cortical and subcortical LBs, the pattern and severity of synuclein pathology and neuronal loss in brainstem nuclei did not differ significantly between DLB and classical IPD, except

for occasionally more severe involvement of the dorsolateral versus the ventral part of substantia nigra compacta and significantly more frequent affection of the hippocampal CA 2/3 subareas in DLB (79 vs. 36%). Another difference was the more severe load with diffuses amyloid plaques and less severe tau pathology in the striatum in DLB cases, irrespective of the severity of cortical neuritic Braak stages (Jellinger and Attems, 2006). Progressed stages of AD without or with nigral lesions, i.e. neuronal loss mainly related to nigral tau pathology and less to α-synuclein deposits, as recently shown by Attems et al. (2007) were present in 12% of the total cohort of patients with the clinical diagnosis of parkinsonism, all being severely demented, accounting for 16% of demented parkinsonian patients (see Table 5).

Morphological substrates of cognitive impairment

Although a few cortical LBs are found in virtually all cases of IPD, the impact of cortical LB and AD pathologies on cognitive impairment is a matter of discussion. Recent studies have demonstrated that the number of LBs in the frontal gyri is the most statistically significant predictor of cognitive status in PD (Mattila et al., 2000), and that LBs densities in the limbic cortex are a better predictor of dementia in PD than Alzheimer-type pathology (Apaydin et al., 2002; Hurtig et al., 2000; Kovari et al., 2003). Some authors demonstrated increasing cognitive decline (with decreasing MMSE scores) with increasing LB stages from 3 to 6 (Braak et al., 2005, 2006), while others have not found such an association (Colosimo et al., 2003; Harding and Halliday, 2001; Jellinger et al., 2002; Jellinger, 2006c). In the present cohort only single cases of IPD stage 4 or 5 without accompanying pathologies had been reported to be demented, and diffuse DLB with mild to moderate Alzheimer-like pathology have been proposed as major pathological substrate of PDD (Aarsland et al., 2005a; Apaydin et al., 2002), whereas others reported significant correlations between cognitive decline and widespread Alzheimer-pathology in both IPD and DLB (Jellinger, 2006c; Papapetropoulos et al., 2005). In the present cohort II, more than one-third of PDD cases showed severe coexistent neuritic AD pathology, as was seen in almost 22% of DLB cases, all corresponding to LB scores 5 or 6 (Braak et al., 2003, 2004).

These and other data emphasize the necessity of proper neuropathological methods, in particular of specific immunohistochemistry for tau and AS lesions in the diagnostic evaluation of parkinsonian syndromes with and without cognitive impairment.

Synuclein and tau interactions

The presence of AS-positive lesions in 7–71% of sporadic and familial AD even in the absence of subcortical LBs (Arai et al., 2001; Hamilton, 2000; Marui et al., 2000; Popescu et al., 2004; Trembath et al., 2003; Uchikado et al., 2006b), and the involvement of other brain areas (Braak et al., 2006; Jellinger, 2003a, 2004; Parkkinen et al., 2003; Saito et al., 2003), the co-localization of tau and AS epitopes in LBs (Ishizawa et al., 2003) as well as clinical biochemical and morphological overlap between IPD, DLB and AD with and without amygdala LBs (Uchikado et al., 2006b) suggest that the process of LB formation is triggered, at least in part, by AD pathology (Iseki et al., 2003; Saito et al., 2004). This collision of two processes may occur in the same brain region or within a single cell in the human brain (Arai et al., 2001; Iseki et al., 1999, 2003; Marui et al., 2000; Schmidt et al., 1996) and in transgenic mice (Maries et al., 2003). The upregulation of the PD-associated protein DJ-1 in tau neuronal inclusions in Pick disease, PSP, CBD, and AD and in glial inclusions in PSP, CBD and MSA (Neumann et al., 2004), and the association of dopaminergic neuronal degeneration, accumulation of AS and tau or both proteins in LRRK2 mutations (Adler et al., 2006; Giasson et al., 2006; Zimprich et al., 2004), induction of hyperphosphorylation of tau by AS in the MPTP-model of parkinsonism (Duka et al., 2006), and the association of atypical protein kinase C (aPKC) and phospho-tau or AS in NFTs and LBs (Shao et al., 2006), as well as the *in vitro* promotion of tau aggregation (fibrillation) by AS and *vice versa* (Giasson et al., 2003) highlight the interface between the two proteins (Galpern and Lang, 2006; Geddes, 2005). The frequent relationship between the intensity of both LB and AD lesions suggest that both pathologies independently or synergistically contribute to both movement disorders and cognitive impairment or may have common origin with mutual triggering, but their pathogenic relationship and clinical impact need further clarification.

On the other hand, no direct interaction between tau and AS has been observed in PSP, a 4-repeat tauopathy (Uchikado et al., 2006a), suggesting that LBs and some types of tauopathies represent independent disease processes that are unrelated to tau pathology. Others have suggested that amyloid rather than tau leads to increased frequency of AS pathology, since it has been shown to increase with the increased density of neuritic plaques (Mikolaenko et al., 2005) and that Aβ amyloid enhanced the development of AS pathology in PD (Pletnikova et al., 2005). Support for this hypothesis also comes from studies in transgenic mice

that develop enhanced AS (or tau) pathology when they are engineered to also deposit Aβ-amyloid (Lewis et al., 2001; Masliah et al., 2001). Interactions between AD and AS may be a molecular mechanism in overlapping pathology of AD and PD in DLB (Mandal et al., 2006). However, it is unclear whether there is a common underlying pathological mechanism inducing both neurodegeneration and fibrillary protein aggregates that are typical of two or more different disease processes (double or triple amyloidosis) or if these lesions represent a common final pathology leading to neuronal degeneration causing both movement disorders and dementia.

In addition to morphological similarities between IPD, PDD, DLB and AD with and without amygdala LBs (AD/ALB) and with other LB pathologies (AD/DLB or LBV/AD), showing both cortical LBs and Alzheimer-related changes, various subcortical transmitter systems may contribute to both movement disorders and cognitive dysfunction in parkinsonian and related syndromes, in particular the cholinergic forebrain system. This in fact, illustrated by severe neuronal loss in the nucleus basalis Meynert and affected cholinergic dysfunctions, being more severe in PDD/DLB than in IPD and "pure" AD (Bohnen et al., 2003; Jellinger, 2006c; Perry, 2004; Tiraboschi et al., 2002), appears of special clinical and therapeutic interest. On the other hand, cerebrovascular risk factors and small CVLs were not associated with incident dementia in IPD (Haugarvoll et al., 2005; Jellinger, 2006c), and the concomitant presence of white matter lesions/hyperintensities in patients with PDD had no significant effect on cortical AChE activity (Marshall et al., 2006). In conclusion, the pathogenesis, mutual relationship and functional impact of morphological lesions contributing to movement disorders and cognitive dysfunctions in parkinsonism and related disorders await further elucidation, possibly by further prospective-pathological studies.

Note added in proof

In a group of 57 prospectively assessed patients confirmed at autopsy (29 DLB, 28 PDD) Ballard et al. (2006) reported an association between longer duration of parkinsonism prior to dementia and less severe cortical AS pathology, lower CERAD plaque scores, but not Braak & Braak staging, as well as more pronounced cortical cholinergic deficits. These data do not support an arbitrary cut-off between PDD and DLB.

Acknowledgements

The study was supported by the Society for the Support of Research in Experimental Neurology, Vienna, Austria. The author thanks the medical staff of many Vienna hospitals for clinical data and the Departments of Pathology, Lainz Hospital and Otto Wagner Hospital, Vienna, Austria, for brain material. Special thanks are due to Mrs. V. Rappelsberger for excellent laboratory work and Mr. E. Mitter-Ferstl, PhD, for secretarial and statistical work.

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J Neural Transm (2007) [Suppl 72]: 105–112

Printed in Austria

Journal of Neural Transmission

Ginsenoside Rd attenuates neuroinflammation of dopaminergic cells in culture

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Summary In Parkinson's disease clinical and experimental evidence suggest that neuroinflammatory changes in cytokines caused by microglial activation contribute to neuronal death. Experimentally, neuroinflammation of dopaminergic neurons can be evoked by lipopolysaccharide (LPS) exposure. In mesencephalic primary cultures LPS (100 μg/ml) resulted in 30–50% loss of dendritic processes, changes in the perikarya, cellular atrophy and neuronal cell loss of TH-immunoreactive (TH^+) cells. iNOS activity was increased dose dependently as well as prostaglandin E_2 concentrations. Ginsenosides, as the active compounds responsible for ginseng action, are reported to have antioxidant and anti-inflammatory effects. Here ginsenoside Rd was used to counteract LPS neurodegeneration. Partial reduction of LPS neurotoxic action was seen in dopaminergic neurons. Cell death by LPS as well as neuroprotective action by ginsenoside Rd was not selective for dopaminergic neurons. Neuronal losses as well as cytoprotective effects were similar when counting NeuN identified neurons. The anti-inflammatory effect of ginsenoside Rd could equally be demonstrated by a reduction of NO-formation and PGE_2 synthesis. Thus, protective mechanisms of ginsenoside Rd may involve interference with iNOS and COX-2 expression.

Keywords: Dopaminergic, lipopolysaccharide, ginsenoside Rd, neuroinflammation

Introduction

Parkinson's disease (PD) as an age-related, progressive neurodegenerative disorder is characterized by massive depletion of striatal dopamine as a result of the degeneration of dopaminergic neurons in the substantia nigra. Clinical and neuropathological data indicate that chronic inflammatory processes could be responsible for this cell death. Particularly, activation of microglia seems to be of importance, as their proliferation and invasion into neuronal tissue involve the release of inflammatory cytokines. The formation of neurotoxic factors such as nitric oxide (NO) and reactive oxygen species equally participates (Ruano et al., 2006). Therefore, the term neuroinflammation, referring to changes characteristic of immune activation, regardless of their origin, regulation or consequence has been applied by Hunot and Hirsch (2003).

Inside nervous tissue, as well as experimentally in neuronal culture systems lipopolysaccharides (LPS) as the active components of the cell wall of Gram negative bacteria trigger the synthesis and release of cytokines and NO. Primary cell cultures of mesencephalic neurons, when exposed to LPS undergo molecular inflammatory processes that have been well documented (Chock and Giffard, 2005; Minghetti et al., 2005). NO is a diatomic free radical generated from conversion of L-arginine to L-citrulline by inducible nitric oxide synthase (iNOS). NO plays important roles as an immune regulator, vasodilator and neurotransmitter in a variety of tissues, and as a mediator under inflammatory conditions. Exceedingly high levels of NO produced by iNOS, however, are considered to be cytotoxic in inflammation and endotoxemia. Here, nitric oxide can react with superoxide to produce peroxynitrite and other oxygen radicals (Beckman and Crow, 1993). These reactive agents, in turn, can produce extensive cellular damage by oxidizing DNA, proteins and lipids.

Therefore, an effective anti-inflammatory therapy should not only alleviate the disease-associated symptoms, but also interfere with glial reactions, an increase in inflammatory factors and progressive dopaminergic cell death. The neuroprotection conferred by preventing iNOS expression may thus come from a reduction in oxygen free radicals and related NO reaction products. Anti-inflammatory compounds have been tested for their ability to counteract these processes. Non steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, the non-selective cyclooxygenase (COX-1/COX-2) inhibitor indomethacin and the selective COX-2

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inhibitor NS-398 may reduce inflammatory mediators and neurotoxicity by inhibiting COX induction (Kyrkanides et al., 2002), decreasing iNOS mRNA expression (Heneka et al., 1999), or down-regulation of NF-κB activation (Yin et al., 1998).

However, in clinical practice the long term use of NSAIDs may cause gastrointestinal complications and even result in potentially fatal peptic ulceration and bleeding, next to liver and kidney damage as long term effects (Langford, 2006). Ginsenosides, as the active ingredients of ginseng root (Panax ginseng) with a saponin structure, are known for their antioxidant, anti-inflammatory and anti-apoptotic properties. Min et al. (2006) found that ginsenoside Rg3 prevented human endothelial cells apoptosis via inhibition of the mitochondrial apoptotic signaling pathway. Experimentally ginsenosides scavenge free radicals, and counteract glutamate excitotoxicity in dopaminergic neuronal cultures (Radad et al., 2004). Ginsenosides Rb_1 and Rg_1 decreased tumour necrosis factor-α (TNF-α) production by macrophages (Cho et al., 1998), pre-treatment with ginsenoside Rg_3 abrogated COX-2 expression in response to 12-O-tetradecanoylphorbol-13-acetate in mouse skin (Keum et al., 2003), and ginsenoside Rh_2 inhibited the expression of COX-2, pro-inflammatory TNF-α and interleukin-1β (IL-1β) in BV-2 cells induced by LPS/IFN-γ (Bae et al., 2006). However ginsenosides appear to exert different potencies of their anti-inflammatory action. Particularly the minor ginsenoside Rd, formed by hydrolyzing and removing a sugar moiety from the major ginsenosides Rb_1, Rb_2 and Rc, has been shown to protect neural systems by attenuating NO overproduction (Choi et al., 2003). The pharmaceutical property of ginseng in protecting neurons from neurotoxic kainic acid is attributed mostly to ginsenoside Rd (Lee et al., 2003). Therefore, the potential of ginsenoside Rd to interfere with LPS neurotoxicity was tested in mesencephalic dopaminergic cultures.

Materials and methods

Materials

Pregnant OF1/SPF mice were purchased from the Institute for Laboratory Zoology and Veterinary Genetics in Himberg (Austria). Dulbecco's Modified Eagles Medium (DMEM), fetal calf serum (FCS), diaminobenzidine, L-glutamic acid (monosodium salt), sulfanilamide, N-1-naphthylyethylenediamine dihydrochloride, paraformaldehyde and LPS (*E. coli*, L8274) were obtained from Sigma (Germany). Penicillin-streptomycin, anti-tyrosine hydroxylase antibody (anti-TH antibody) and DNase I were obtained from Roche Molecular Biochemicals (Germany). The Vectastain ABC Elite Kit (Mouse IgG) was purchased from Vector Laboratories (USA). B-27 (without antioxidants), trypsin-EDTA, soybean trypsin inhibitor, Dulbecco's PBS (w/o Ca^{2+}, Mg^{2+} and Na^{+} bicarbonate) and Hanks' balanced salt solution (w/o Ca^{2+} and Mg^{2+}, HBSS) were ordered from Invitrogen (Germany).

Preparation of mouse primary neuron-glia cultures

Primary mesencephalic neuron-glia cultures were prepared from embryonic mouse brains at gestation day 14. Embryos were carefully removed under aseptic conditions and collected in buffered saline (DPBS) at room temperature. Brains were dissected under a stereoscope (Nikon SMZ-1B, 10× magnification) and the ventral mesencephala excised. Primary cultures were prepared according to Radad et al. (2006). Briefly, after careful removal of the meninges, tissues were mechanically cut into small pieces in DPBS and transferred into a sterile test tube containing 2 ml 0.1% trypsin and 2 ml 0.02% DNase I in DPBS. The tubes were incubated in a water bath at 37°C for 7 min. Then, 2 ml of trypsin inhibitor (0.125 mg/ml in DPBS) were added, the tissue was centrifuged (Hettich, ROTIXA/AP, Germany) at 800 rpm for 4 min and the supernatant was aspirated. The tissue pellets were triturated with a fire-polished Pasteur pipette in DMEM containing 0.02% DNase I. Dissociated cells were collected in DMEM supplemented with HEPES buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 0.1 mg/ml, respectively) and heat-inactivated fetal calf serum (FCS, 10%). The cell suspension was plated into 4-well multi-dishes (Nunclon, Germany) pre-coated with poly-D-lysine (50 μg/ml). Cultures were grown at 37°C in an atmosphere of 5% CO_2/95% air and 100% relative humidity.

Treatment with LPS and ginsenoside Rd

LPS was diluted to the desired final concentration in medium, containing 2% FCS, HEPES buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 0.1 mg/ml, respectively) and 2% B-27 medium. Ginsenoside Rd was freshly prepared in DMEM before diluting to the desired final concentrations (1, 10, 50 μM) with medium. The seven-day-old cultures were pretreated with vehicle or ginsenoside Rd for 2 h at 37°C before treatment with LPS (100 μg/ml). The culture medium was changed every 3 days containing the above compounds. Experiments were run at least in triplicate with three wells for each treatment condition.

Identification of TH^+ neurons

On the 13th DIV cultures were rinsed carefully with PBS (pH 7.2) and fixed in 4% paraformaldehyde for 45 min at 4°C. Fixed cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Kit) for 90 min to block non-specific binding sites. Cells were sequentially incubated with anti-TH antibody overnight at 4°C, biotinylated secondary antibody (Vectastain) and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature and washed with PBS between incubations. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide. The numbers of TH^+ neurons were counted at 100× magnification with a Nikon inverted microscope. To measure length changes of neuronal processes in different groups, the pictures of 10 randomly selected neurons per well were taken and lengths of the processes measured with Scion® Image software.

Nitrite assay

The NO levels in the supernatants were indirectly assayed by quantitating the nitrite concentrations by the Griess reaction. Briefly, Griess reagent [0.5% sulfanilamide, 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride, and 2.5% phosphoric acid] was added to an equal volume of supernatant and incubated at room temperature for 10 min. The absorbance at 550 nm was measured with a semi-auto biochemical analyzer (SBA-860, Sunostik, China), and the nitrite concentrations were calculated using sodium nitrite in culture medium as a standard (1–100 μM). Fresh culture medium served as a blank.

PGE_2 assay

PGE_2 concentrations were quantified using a commercial PGE_2 assay kit (R&D Systems, USA) on 96 well polystyrene microplates coated with a goat anti-mouse polyclonal antibody following the manufacturer instructions. One-hundred microlitres of supernatants (3- or 10-fold dilution) and standards were added to the wells. The optical density of each well was determined within 30 min, using a microplate reader set to 450 nm.

Statistical analyses

Data were expressed as mean ± standard error of mean (SEM). Significant differences between different groups were calculated by using One-way ANOVA assay and subsequent Student-Newman-Keuls test. Differences with $p < 0.05$ were considered statistically significant.

Results

LPS had dose dependent toxicity to TH^+ neurons

Dopaminergic neurons in primary culture undergo degeneration when exposed to LPS. Cultures were treated for 6 days with different concentrations of LPS (50–400 μg/ml) from the 7th DIV. Cell numbers of dopaminergic cells and other cell populations were decreased dose dependently. Changes in morphology were manifested in losses of dendritic processes and branching and deterioration of cell shape and nuclei (Fig. 1). Our results indicated that LPS destroyed TH^+ neurons in a dose-dependent manner. LD_{50} values in such culture systems were in the range of 100–200 μg LPS/ml medium (Fig. 2).

LPS equally affects other neuronal populations

Neuron-specific nuclear protein (NeuN) is expressed in the nuclei and cell bodies of most neuronal cells. It has been used successfully as a neuronal marker in cell cultures. Dopaminergic cultures exposed to LPS were stained by NeuN immunocytochemistry and the numbers of $NeuN^+$ cells were counted. A high amount of cell loss is evident with 400 μg/ml LPS (Fig. 3). This decrease was dose-dependent. The numbers of $NeuN^+$ cells were reduced by about 24, 41, 65, 92% in the 50, 100, 200, 400 μg/ml LPS-treated groups, respectively (Fig. 4).

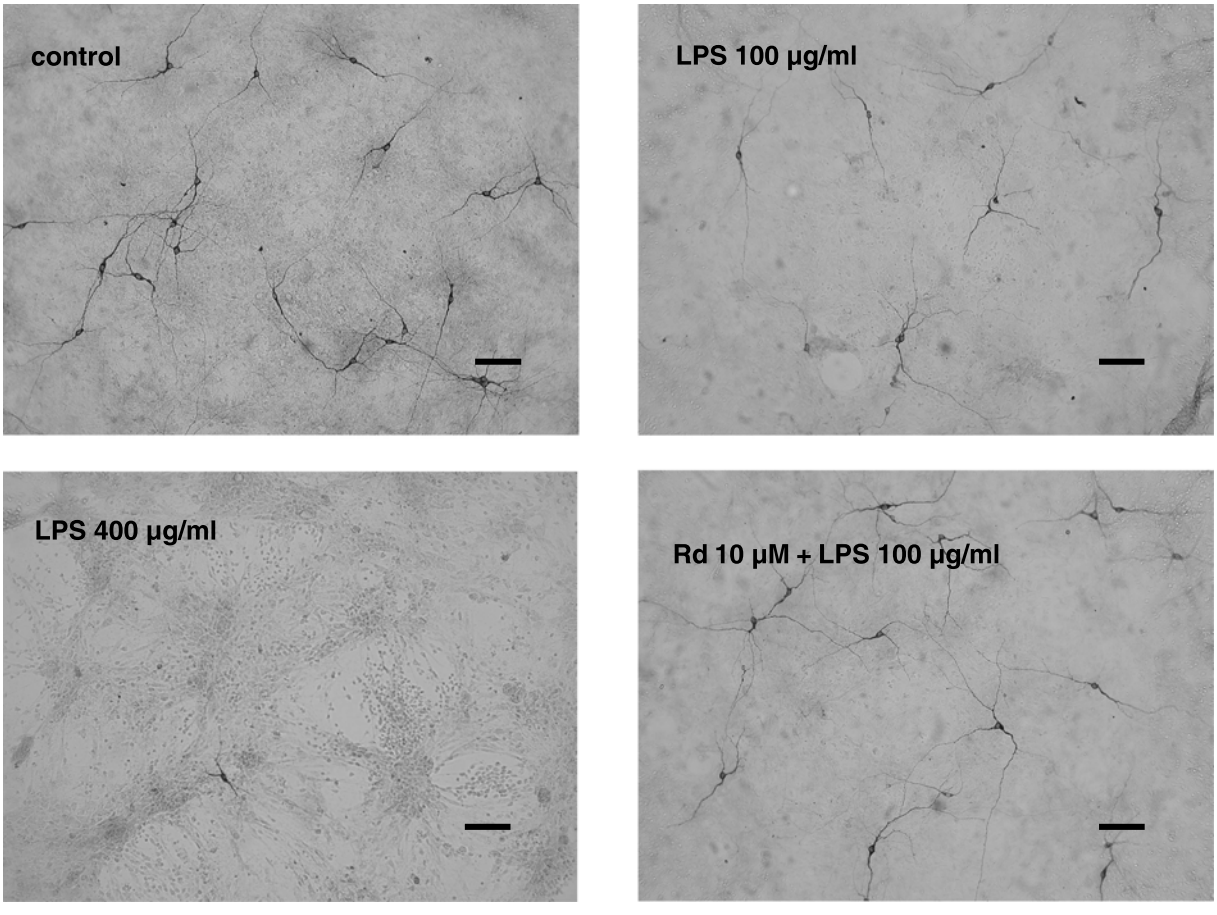

Fig. 1. Degenerative changes in TH^+ cells by LPS. Note the shrinkage and loss of dendritic processes and changes in the perikarya in the LPS (100 μg/ml) group. A high extent of cell loss is evident at 400 μg/ml LPS (bar = 100 μm)

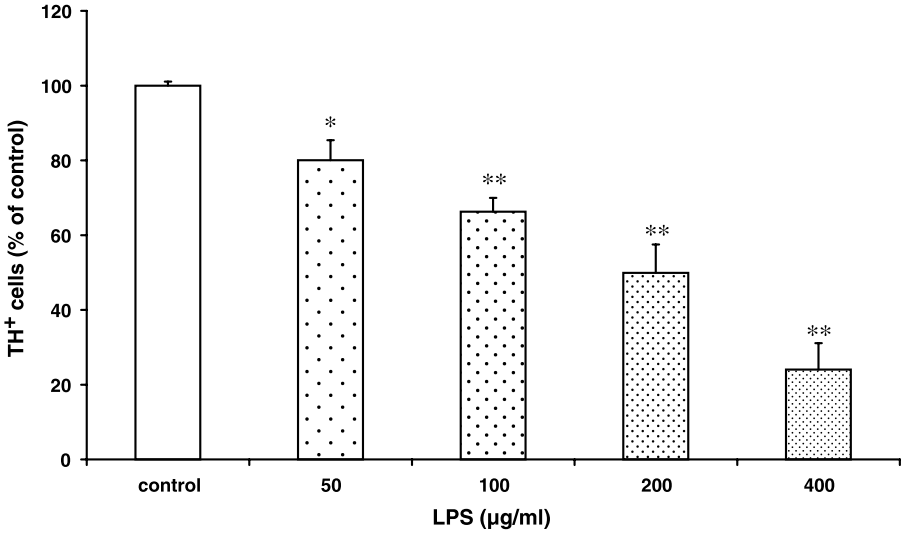

Fig. 2. Effects of LPS on TH^+ cells in the primary neuron-glia cultures. The cultures were treated with different concentration of LPS for 6 days and stained for TH. The results are the mean ± SEM of three experiments. $^*p < 0.05$, $^{**}p < 0.01$ versus control (without LPS)

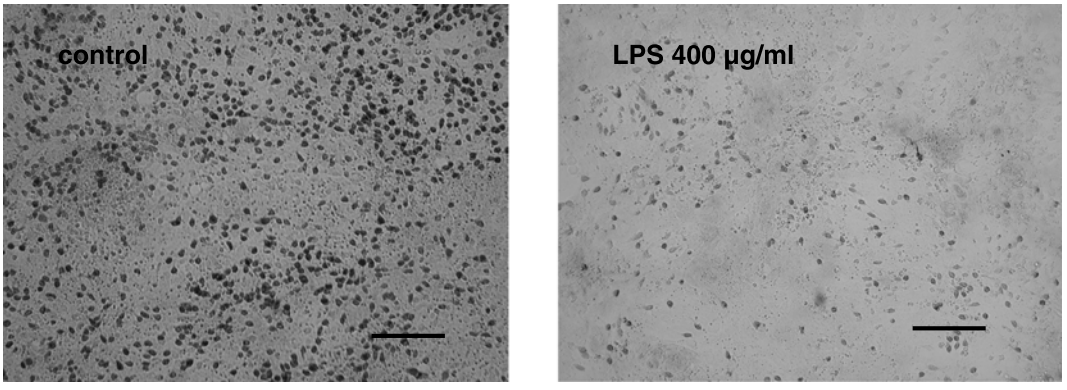

Fig. 3. LPS leads to cell loss of $NeuN^+$ cells in mesencephalic cultures. Dramatic loss of $NeuN^+$ cells and decreased staining intensity is evident at 400 μg/ml (bar = 100 μm)

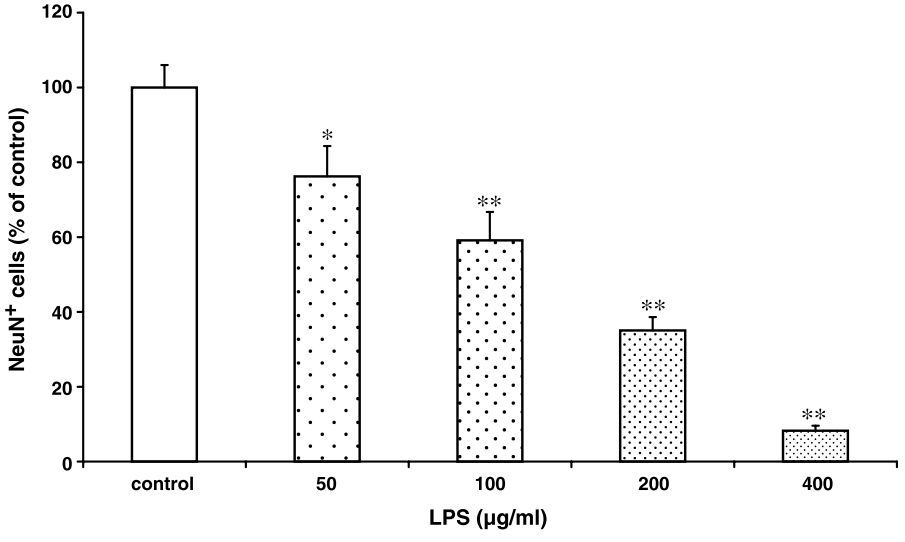

Fig. 4. Effect of LPS on $NeuN^+$ cells in mesencephalic cultures. The cells were treated with different concentration of LPS for 6 days and stained for NeuN. The results are the mean ± SEM of three experiments. $^*p < 0.05$, $^{**}p < 0.01$ versus control (without LPS)

LPS increased NO formation

Supernatants from cultures were collected to assay NO formation following incubation with different concentrations of LPS. Basal NO formation was about 5 μM in control cultures. There was a dose-dependent increase in NO formation with LPS concentrations ranging from 6.25 to

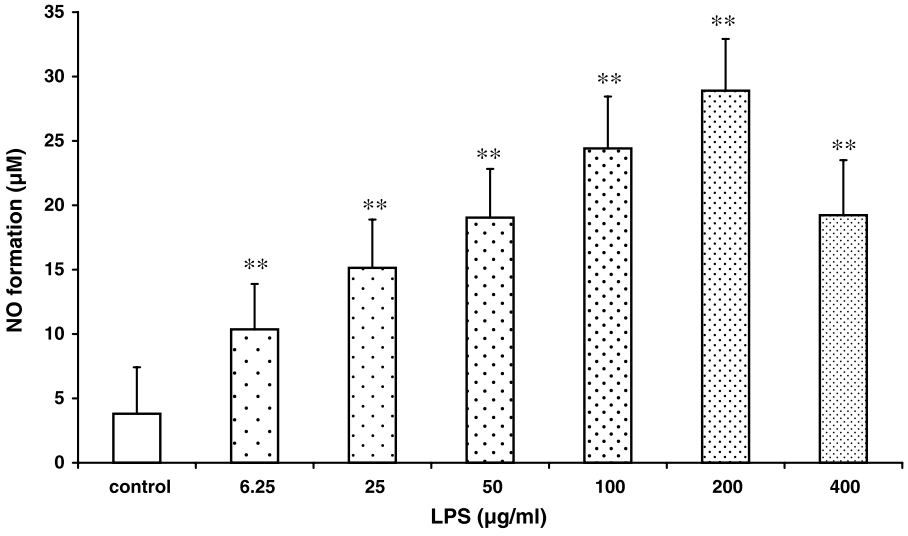

Fig. 5. LPS induced NO formation. Mesencephalic cultures were treated with different concentrations of LPS on the 7th DIV, incubated for 3 days, supernatants were assayed by the Griess reaction. Data represent the mean ± SEM of three experiments. $^{**}p < 0.01$ versus control (without LPS)

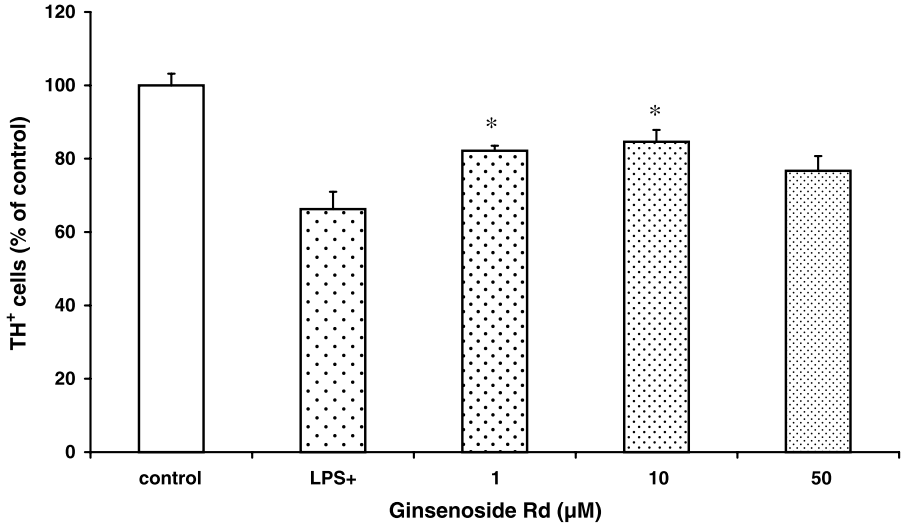

Fig. 6. Effect of ginsenoside Rd on TH^+ cells after exposure to LPS. The cultures were pre-treated with ginsenoside Rd for 2 h before adding 100 μg/ml LPS, and stained for TH after six days incubation. The results are the mean ± SEM of three experiments. $^{*}p < 0.05$ in comparison with the cultures treated with LPS alone

200 μg/ml that reached a peak with 200 μg/ml of LPS (8-fold above basal level, Fig. 5). Above that reduced NO formation at 400 μg/ml possibly reflected the generalized toxic effect of LPS on all cells.

Ginsenoside Rd protected dopaminergic neurons against LPS-neurotoxicity

Significant dopaminergic cell loss underlies as the primary pathology of PD. Therefore, we tested the effects of ginsenoside Rd on LPS-induced dopaminergic cell loss. The mesencephalic cultures were pretreated with ginsenoside Rd (1–50 μM) before including LPS (100 μg/ml, approx. LD_{50}). At the end of the 6 day treatment period, TH-immunostained neurons were determined. As shown in Fig. 6, ginsenoside Rd (1–50 μM) exerted a significant neuroprotective effect on dopaminergic neurons (123.1, 127.7% with 1 and 10 μM, respectively) compared to cultures treated with LPS.

Effect of ginsenoside Rd on LPS-induced $NeuN^+$ cytotoxicity

Mesencephalic cultures exposed to 100 μg/ml LPS for 6 days were stained for NeuN immunoreactivity. As shown in the Fig. 7, the results demonstrate that 41% NeuN immunoreactive ($NeuN^+$) cells were lost. Ginsenoside Rd attenuated the LPS-induced reduction in the number of $NeuN^+$ cells similar to TH^+ cells (Fig. 7). In the 1 μM ginsenoside Rd-treated group, the $NeuN^+$ cells were increased by 16% ($p < 0.05$). In the 10 μM ginsenoside Rd-treated group, the cell counts of $NeuN^+$ cells were higher by 24% compared to the LPS group ($p < 0.05$).

Inhibition of LPS-induced NO formation by ginsenoside Rd

To test whether ginsenoside Rd has inhibitory effects on LPS-induced NO formation, we assayed nitrite production

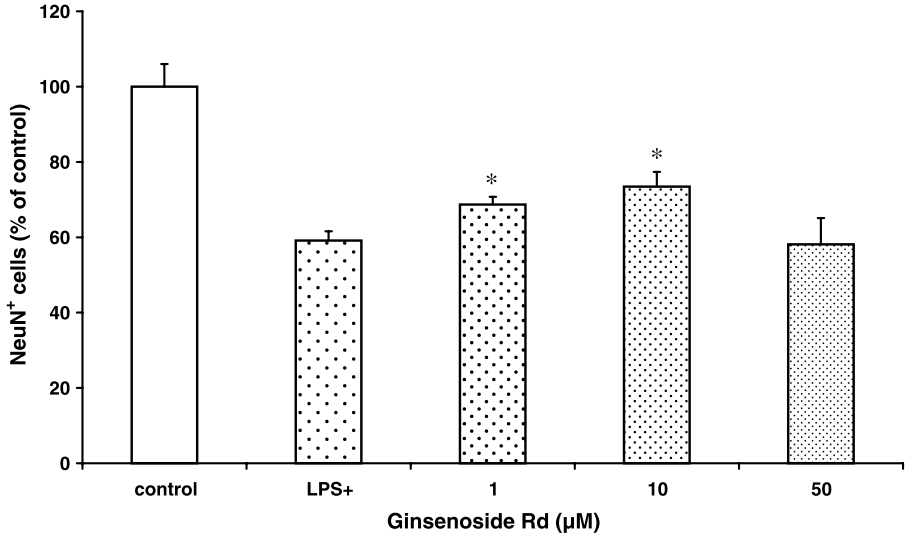

Fig. 7. Effect of ginsenoside Rd on $NeuN^+$ cells of LPS-induced cytotoxicity. The results are the mean ± SEM of 10 counted fields for each condition, given as percentage of controls. $^*p < 0.05$ in comparison with the cultures treated with LPS

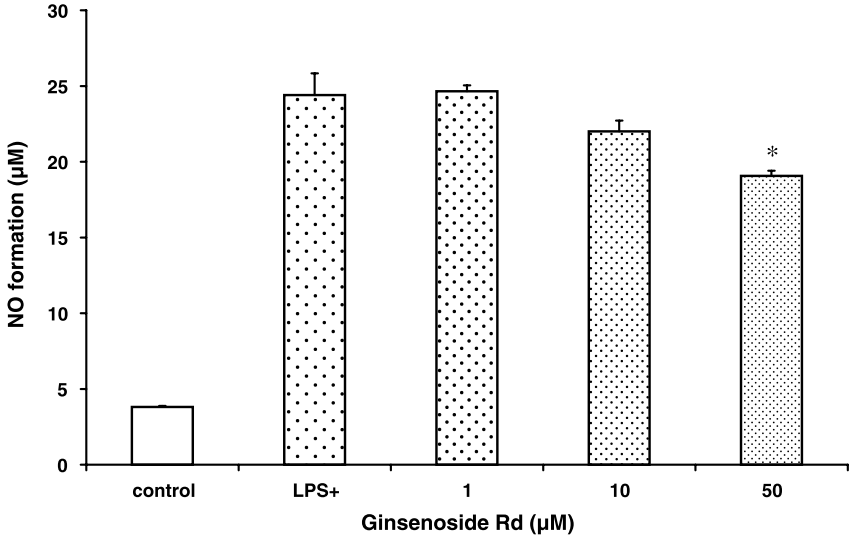

Fig. 8. Inhibition of LPS-induced NO formation by ginsenoside Rd. The cultures were pre-treated with Rd for 2 h before treated with 100 μg/ml LPS, supernatants were collected at 3 days and assayed by the Griess reaction. The results are the mean ± SEM of three experiments. $^*p < 0.05$ in comparison with the cultures treated with LPS

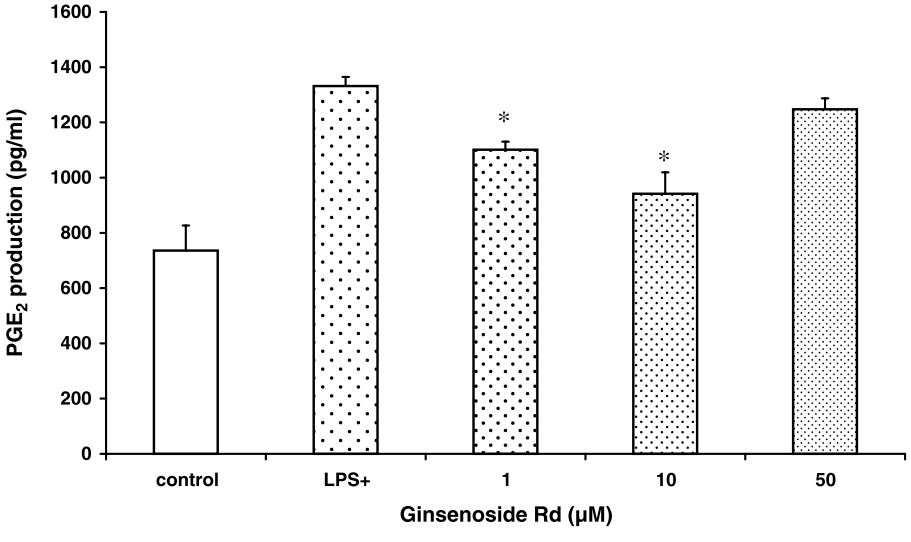

Fig. 9. Effect of ginsenoside Rd on LPS-induced PGE_2 production. Data represent the mean ± SEM of three independent determinations. $^*p < 0.05$ in comparison with the cultures treated with LPS

in the culture medium of neuron-glia cultures. As shown in Fig. 8, co-incubation of ginsenoside Rd with LPS could inhibit the production of NO at 50 μM ginsenoside Rd by about 20%.

Effect of ginsenoside Rd on LPS-induced PGE_2 production

The influence of different concentrations of ginsenoside Rd on PGE_2 production was investigated. Basal conditions are characterized by PGE_2 of about 750 pg/ml (Fig. 9). LPS caused a 2-fold increase in the biosynthesis of PGE_2 as compared to controls. However, when challenged with LPS in the presence of ginsenoside Rd significant reductions at 1 and 10 μM ginsenoside Rd were observed.

Discussion

Over the last years, LPS has been used experimentally either in vivo or in neuronal cultures as this model compound provides a tentative mechanistic link between the occurrence of inflammation in the brain and dopaminergic neurodegeneration. LPS significantly reduced the number of TH^+ neurons compared to control cultures and affected cells had fewer dendrites, shorter or even truncated axons (Li et al., 2005). In our study 100 μg/ml LPS (approx. LD_{50}) caused neuronal loss in primary mesencephalic cultures. LPS led to death of TH^+ cells in a dose dependent manner, however fairly high concentrations of LPS were required. Similar findings have been shown by Gayle et al. (2001) with different approaches. From their data TH^+ cells were progressively decreased by LPS concentrations ranging from 10 to 320 μg/ml. Other authors showed that much lower LPS concentrations (10 ng/ml) induced the same extent of TH^+ cell loss (Wang et al., 2004). This disparity is likely due to various sources of LPS used or different cell culture condition applied, as e.g. LPS from Salmonella typhimurium displayed similar higher toxicities in our hands (data not shown).

Cytotoxicity of LPS however was not selective for dopaminergic neurons, as other neurons were similarly affected. NeuN is expressed in nuclei and cell bodies of most neuronal cell types (Mullen et al., 1992) but not by glial cells. In our hands LPS equally affected $NeuN^+$ cells and the loss of TH^+ neurons correlated with the disappearance of $NeuN^+$ cells. Increases in NO and PGE_2 levels may both contribute to elevated TH^+ cell death. Our results are supportive to previous studies where massive neuronal death was induced by excessive NO formation in LPS-treated cultures (Jeong et al., 2003). In addition, NO has been shown to inhibit mitochondrial respiration and react with superoxide to produce peroxynitrite ($ONOO^-$), which is a highly toxic oxidant (Bal-Price et al., 2002). 200 μg/ml LPS led to the accumulation of about 30 μM NO and there was a dramatic decrease in the number of TH^+ cells when compared with controls. A similar aspect holds true for affecting PGE_2 increases which are derived from the metabolism of arachidonic acid by COX and PGE synthesizing enzymes. Our study demonstrated that stimulation of the neural cultures with LPS increased the synthesis of PGE_2. Takadera and Oyashiki (2006) showed that PGE_2 directly stimulated several signaling pathways via EP receptors, resulting in changes in cAMP and cellular levels of phosphoinositides (Narumiya et al., 1999) that induced apoptosis in hippocampal neurons. LPS treatment also induced production of other pro-inflammatory and neurotoxic factors, such as TNF-α, IL-1β and reactive oxygen species (ROS) (Liu and Hong, 2003). The precise mechanisms of LPS-induced neurodegeneration in mesencephalic dopaminergic culture thus may be even more complex.

Ginsenoside Rd could partially prevent the toxic action of LPS. This has not been shown for dopaminergic cells so far, however anti-inflammatory action of ginsenosides has been observed in different non-neuronal systems. For example, ginsenoside Rb_1 can inhibit LPS-induced expression of the proinflammatory cytokine TNF-α in vitro (Cho et al., 1998), ginsenoside Rb_1 inhibited LPS-induced IL-6 and/or TNF-α production in murine macrophages (Smolinski and Pestka, 2003) and compound K, the Rb_1 metabolite by intestinal bacteria, potently inhibited the production of NO and PGE_2, reduced the expression levels of the iNOS, COX-2 proteins, and prevented the activation of NF-κB (Park et al., 2005). Our study indicates that the neuroprotective effect of ginsenoside Rd against LPS toxicity involves anti-inflammatory mechanisms. Ginsenoside Rd (10 μM) significantly increased the survival rate of dopaminergic cells in the primary neuron-glia cultures and caused a (though small) reduction of NO levels (at 50 μM). In this context, ginsenoside Rd also exerted inhibitory action against NO production induced by LPS plus TNF-α in C6 glioma cells (Choi et al., 2003). However, also other protective mechanisms should be considered. The neuroprotective effect of ginsenoside Rd on survival of dopaminergic cells may be mediated through improving energy metabolism and preserving the structural integrity of neurons (Jiang and Qian, 1995). Induction of antioxidant enzymes by ginsenosides which are important for maintaining cell viability may equally contribute by lowering free radicals (Nishiyama et al., 1994). On the other hand, ginsenoside Rd could inhibit lipid peroxidation. That was explained by its intervention with the GSH/GSSG redox status (Yokozawa et al.,

2004). Additional modes of action of ginsenoside as an interference with NMDA-receptors and calcium metabolism (Kim and Rhim, 2004) as well as stabilizing cellular membrance fluidity (Li and Zhang, 1997) could as well contribute to its neuroprotective action.

Conclusions

Mesencephalic dopaminergic cultures exposed to LPS undergo degeneration caused by microglial activation and a concomitant increase of NO and PGE_2. A partial reversal of dopaminergic cell death by ginsenoside Rd indicates that anti-inflammatory actions of this compound are of relevance. Beyond the pharmacological action of this natural compound in cell culture, chronic treatment in animal models of experimental inflammation should help to validate its actual therapeutic value for degenerative aging diseases.

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J Neural Transm (2007) [Suppl 72]: 113–120

Printed in Austria

Journal of Neural Transmission

Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects

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Summary Biochemical studies on postmortem brains of patients with Parkinson's disease (PD) have greatly contributed to our understanding of the molecular pathogenesis of this disease. The discovery by 1960 of a dopamine deficiency in the nigro-striatal dopamine region of the PD brain was a landmark in research on PD. At that time we collaborated with Hirotaro Narabayashi and his colleagues in Japan and with Peter Riederer in Germany on the biochemistry of PD by using postmortem brain samples in their brain banks. We found that the activity, mRNA level, and protein content of tyrosine hydroxylase (TH), as well as the levels of the tetrahydrobiopterin (BH4) cofactor of TH and the activity of the BH4-synthesizing enzyme, GTP cyclohydrolase I (GCH1), were markedly decreased in the substantia nigra and striatum in the PD brain. In contrast, the molecular activity (enzyme activity/enzyme protein) of TH was increased, suggesting a compensatory increase in the enzyme activity. The mRNA levels of all four isoforms of human TH (hTH1–hTH4), produced by alternative mRNA splicing, were also markedly decreased. This finding is in contrast to a completely parallel decrease in the activity and protein content of dopamine β-hydroxylase (DBH) without changes in its molecular activity in cerebrospinal fluid (CSF) in PD. We also found that the activities and/or the levels of the mRNA and protein of aromatic L-amino acid decarboxylase (AADC, DOPA decarboxylase), DBH, phenylethanolamine N-methyltransferase (PNMT), which synthesize dopamine, noradrenaline, and adrenaline, respectively, were also decreased in PD brains, indicating that all catecholamine systems were widely impaired in PD brains. Programmed cell death of the nigro-striatal dopamine neurons in PD has been suggested from the following findings on postmortem brains: (1) increased levels of pro-inflammatory cytokines such as TNF-α and IL-6; (2) increased levels of apoptosis-related factors such as TNF-α receptor R1 (p 55), soluble Fas and bcl-2, and increased activities of caspases 1 and 3; and (3) decreased levels of neurotrophins such as brain-derived nerve growth factor (BDNF). Immunohistochemical data and the mRNA levels of the above molecules in PD brains supported these biochemical data. We confirmed by double immunofluorescence staining the production of TNF-α and IL-6 in activated microglia in the putamen of PD patients. Owing to the recent development of highly sensitive and wide-range analytical methods for quantifying mRNAs and proteins, future assays of the levels of various mRNAs and proteins not only in micro-dissected brain tissues containing neurons and glial cells, but also in single cells from frozen brain slices isolated by laser capture micro-dissection, coupled with toluidine blue, Nissl staining or immunohistochemical staining, should further contribute to the elucidation of the molecular pathogenesis of PD and other neurodegenerative or neuropsychiatric diseases.

Keywords: Parkinson's disease, postmortem brain, laser micro-dissection, biochemistry, enzymes, cytokines, neurotrophins

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Introduction

The main symptoms of movement disorder, i.e., akinesia, muscle rigidity, and resting tremor, in Parkinson's disease (PD) are caused by a deficiency in the level of the neurotransmitter dopamine at the nerve terminals in the striatum of the nigro-striatal dopamine neurons as the result of selective neurodegeneration of dopamine neurons in the substantia nigra. Most PD is aging-related and sporadic without any hereditary history. Familial PD (PARK) is estimated to represent only ∼5% of PD cases. The presence of intracelluar inclusions called Lewy bodies, which are mainly composed of α-synuclein (*α-synuclein* is the causative gene of PARK1), is another feature of sporadic PD. The molecular mechanism of neural degeneration in sporadic PD is speculated to be multiple (Riederer et al., 2001; Nagatsu and Sawada, 2006), involving environmental and/or endogenous potential neurotoxins, oxidative stress, mitochondrial dysfunction, altered iron homeostasis, immune-mediated mechanisms, and susceptibility genes that might be related to the causative genes in familial PD (Mizuno et al., 2006) such as *α-synuclein* or *parkin*. Noradrenaline deficiency in noradrenaline neurons is also observed in the locus coeruleus. These dopamine and noradrenaline deficiencies in the brain of PD patients were first observed by Ehringer and Hornykiewicz (1960). As Foley et al. (2000)

pointed out, Sano et al. (1960, 2000) also observed greatly reduced dopamine levels in the substantia nigra and striatum in one case of postmortem PD brain. This discovery of a dopamine deficiency in the nigro-striatum was a landmark finding of biochemical studies on PD, and led to the development of L-DOPA therapy to supplement the deficient dopamine. L-DOPA was the first neurotransmitter supplementation therapy, and it is still the gold standard of drug therapy for PD.

Up to 1960, even after development of sensitive spectrofluorometric assays, biochemical studies on such unstable compounds as dopamine and noradrenaline had been thought to be difficult to conduct on human postmortem brains. However, after the successful confirmation of the dopamine deficiency in the nigro-striatal region in postmortem PD brains in 1960, biochemical studies on postmortem brains were expanded from various small molecules such as catecholamine neurotransmitters to mRNAs and proteins of enzymes and cytokines related to PD, Alzheimer's diseases (AD), and other neurodegenerative or neuropsychiatric diseases, and have greatly contributed to elucidation of their molecular pathogenesis. This review focuses on the historical development and future prospects of biochemical studies on postmortem brains from PD patients.

Problems in the biochemistry of postmortem brain samples

Biochemical quantitative analyses of human postmortem brain samples have intricate problems, because there are many uncontrollable factors in such samples. The following considerations are generally required in biochemical studies using postmortem brain tissues. (1) Approval of the local ethics committee is essential. (2) Precise clinical information on the patient is required, as drugs administered to the patient may affect primarily or secondarily the level of the compound to be assayed. Most PD patients are administered L-DOPA or dopamine receptor agonists. (3) The condition before death such as the cause of death and the duration of coma may affect the objective compound. No consuming diseases and a short agony stage are necessary conditions to obtain reliable biochemical data. (4) Postmortem time may affect the results. Such compounds as dopamine or noradrenaline are unstable and easily degraded non-enzymatically or enzymatically by monoamine oxidase (MAO). mRNAs and proteins are also unstable. Therefore, the postmortem delay must be as short as possible (preferably within 12 h). (5) Age and postmortem time of PD patients must be similar to those of the control patients. (6) The brain regions to be dissected and the methods of brain dissection should be the same between PD brains and control ones. Punching-out of the micro brain regions from tissue slices ($\sim$1–2 cm) is generally used, and the brain location to be dissected out must be the same in each brain sample. As described below, single cell analysis by laser micro-dissection (Hashida et al., 2002; Kawahara et al., 2003) will be a new and valuable method to further our knowledge of the biochemistry of the postmortem brain. (7) Dissected samples should be frozen immediately on dry ice, completely packed and sealed, and stocked at -80° in a deep freezer. (8) Since large numbers of samples are required for proper statistical analysis, a brain bank should be established.

Figure 1 shows schematically the brain bank system in Germany (Riederer P, personal communication).

Changes in catecholamine neurotransmitters and related enzymes in postmortem PD brains

After the discovery of the dopamine deficiency in the nigro-striatum in PD, various neurotransmitters and their related enzymes were measured in postmortem PD brains by us and by other workers. Nagatsu's group first collaborated with Hirotaro Narabayashi (Juntendo University School of Medicine, Tokyo, Japan), who supplied the brain samples from his own brain bank (established by Hirotaro Narabayashi and Reiji Iizuka), and further collaborated with Peter Riederer who established a brain bank at Würzburg University (Würzburg, Germany; Fig. 1).

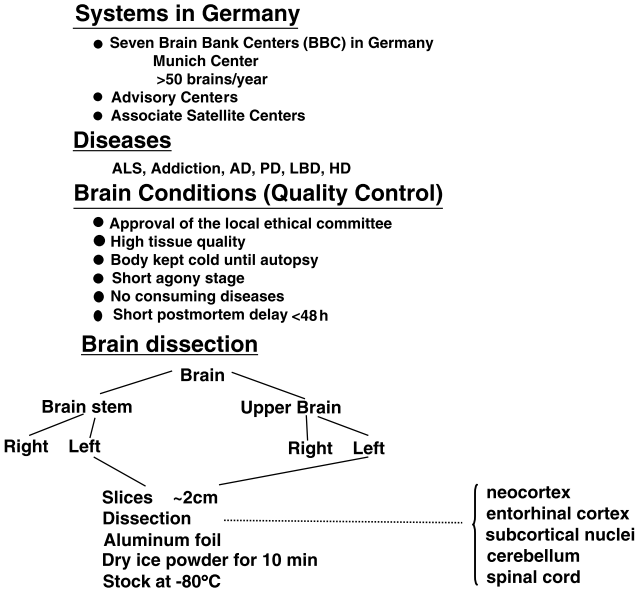

Fig. 1. The brain bank system in Germany (P. Riederer, personal communication)

Table 1. *Changes reported in catecholamine-related enzymes in Parkinson's disease*

Enzymes	Sample source	mRNA	Protein	Activity	Molecular activity (activity/protein)
TH					
Total	striatum		decreased	decreased	increased
Total	SN	decreased	decreased	decreased	increased
hTH1	SN	decreased			
hTH2	SN	decreased			
hTH3	SN	decreased			
hTH4	SN	decreased			
AADC	SN	decreased		decreased	
DBH	CSF		decreased	decreased	normal
	hypothalamus			decreased	
GCH1	striatum			decreased	
PNMT	hypothalamus			decreased	

AADC, aromatic L-amino acid decarboxylase; *CSF*, cerebrospinal fluid; *DBH*, dopamine β-hydroxylase; *GCH1*, GTP cyclohydrolase I; *LC*, locus coeruleus; *PNMT*, phenylethanolamine N-methyltransferase; *SN*, substantia nigra; *TH*, tyrosine hydroxylase.
From Nagatsu et al. (1977, 1981, 1984, 1986), Mogi et al. (1988a, b) and Ichinose et al. (1994).

The results are summarized in Table 1. In our early studies we measured the activities and protein contents of the enzymes related to catecholamine metabolism. We found the presence of phenylethanolamine N-methyltransferase (PNMT) in the control and PD brains, supporting the presence of adrenaline neurons in the human brain (Nagatsu et al., 1977; Trocewicz et al., 1982). We (Nagatsu et al., 1977, 1984) also found a marked decrease (to ∼10–20% of controls) in the activity of tyrosine hydroxylase (TH) in the nigro-striatum in PD, in agreement with the results of other workers (Lloyd et al., 1975; McGeer and McGeer, 1976). Riederer et al. (1978) found TH activity to be decreased also in the adrenal medulla in PD, indicating the general impairment of the catecholamine system. DOPA decarboxylase (aromatic L-amino acid decarboxylase, AADC) activity was found to be decreased in the nigro-striatum in PD (Lloyd and Hornykiewicz, 1970). We also found decreased activities in dopamine β-hydroxylase (DBH) for noradrenaline synthesis and PNMT for adrenaline synthesis in PD brains (Nagatsu et al., 1977, 1984). Furthermore, the level of the tetrahydrobiopterin (BH4) cofactor of TH and the activity of the BH4-synthesizing enzyme GTP cyclohydrolase I (GCH1) were found to be decreased in PD brains (Nagatsu et al., 1981, 1986). These results indicate that not only the nigro-striatal dopamine neurons but also all catecholamine neurons are generally affected in PD. Braak et al. (2006) recently proposed, based on the pathology of Lewy bodies, that PD may start in the pre-symptomatic phase from the medulla oblongata where noradrenaline and adrenaline neurons are localized.

The activity of the serotonin-synthesizing enzyme tryptophan hydroxylase (TPH2) was also moderately decreased in the substantia nigra in PD (Sawada et al., 1985). In contrast to PD, in Alzheimer's disease (AD) the activities of TPH2 and TH, and the contents of the biopterin cofactor in the AD brain were found to be moderately decreased in various brain regions, indicating a reduction in the numbers of both serotonin and catecholamine neurons in wide monoamine regions in AD (Sawada et al., 1987).

We examined not only the enzyme activity, but also the protein content measured by enzyme immunoassay, of TH in PD brains. Although both TH protein and TH activity in the nigro-striatum were markedly decreased in parallel in PD brains as compared with those of the control brains, the molecular activity (activity per enzyme protein, also called homo-specific activity) was significantly increased in PD brains. The increase in the molecular activity of residual TH in PD brains suggests that such molecular changes in TH molecules represent a compensatory increase in TH activity (Mogi et al., 1988a). We also measured in cerebrospinal fluid (CSF) of control and PD patients the protein contents and activities of DBH, which synthesizes noradrenaline and adrenaline and is secreted from noradrenaline and adrenaline neurons in the brain into the CSF. In contrast to TH, both DBH activity and protein content in the CSF of PD patients were reduced in parallel ($r = 0.79$) to ∼20% of control values without changes in the molecular activity, suggesting only a decreased content in DBH without molecular changes in DBH protein in the noradrenaline and adrenaline neurons in PD (Mogi et al., 1988b). Human TH is markedly activated by the cofactor Fe^{2+}. There are no significant changes in the stimulation of TH activity in the human caudate nucleus by Fe^{2+} in PD, whereas such differences are noted between PD and control brains when exogenous protein kinase is used as a stimulant (Rausch et al., 1988).

Four isoform proteins of human TH (hTH1–hTH4) are expressed by alternative mRNA splicing from a single gene in the brain (Haycock, 2002; Grima et al., 1987; Kaneda et al., 1987; Kobayashi et al., 1988). In human AADC, a single protein is produced by a tissue-specific alternative promoter from neuronal and non-neuronal mRNAs encoded by a single gene (Ichinose et al., 1992). We quantified all four types of human TH mRNAs and AADC mRNA in human brains (substantia nigra) from control, PD, and schizophrenia patients by using the quantitative reverse transcription-polymerase chain reaction (RT-PCR;

Ichinose et al., 1994). All four types of TH mRNAs were detected in the substantia nigra in the control brains examined; and the ratio of hTH1, hTH2, hTH3, and hTH4 mRNAs to the total amount of TH mRNAs was 45, 52, 1.4, and 2.1%, respectively, in the substantia nigra. The levels of TH and AADC mRNAs were highly correlated in the control cases. We found that PD brains had very low levels of all four TH isoform mRNAs and AADC mRNA in the substantia nigra compared with control brains, whereas no significant differences were found between schizophrenic brains and normal ones. We found that monkeys [Japanese monkeys (*Macaca irus* and *Macaca fuscata*), gibbon, orangutan, gorilla, and chimpanzee] have two TH isoforms corresponding to hTH1 and hTH2 (Ichikawa et al., 1990; Ichinose et al., 1993). Monkeys, like humans, are highly susceptible to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a chemical that produces PD in humans (Langston et al., 1983). Thus, we also measured the levels of the two types of TH mRNAs in PD monkeys produced by use of MPTP and compared these levels with those for normal monkeys (Ohye et al., 1995). The levels of both monkey TH mRNAs were significantly decreased specifically in the substantia nigra, which results are similar to those in human PD. All these results indicate that catacholamine-synthesizing enzyme systems are generally decreased in all catecholamine neurons especially in the nigro-striatal dopamine neurons. These decreases may be caused by neuronal degeneration. However, it is not still clear yet when such changes in catecholamine-synthesizing enzymes start in catecholamine neurons in relation to neurodegeneration in sporadic PD. We found that in MPTP-produced animal PD models the changes in the TH system occur soon after MPTP treatment, as evidenced first by a decrease in TH activity, then inactivation followed by a decrease in the protein levels (Nagatsu, 1990).

Presence of MPTP-like neurotoxins in postmortem brains in PD

MPTP inhibits complex I in mitochondria, produces reactive oxygen species, and causes apoptotic cell death in MPTP-induced PD in animals. Dopamine cell death in sporadic PD is also thought to be caused by apoptosis (Hirsch et al., 1999). Since MPTP is a chemically synthesized PD-producing neurotoxin in humans, efforts have been made to find MPTP-like neurotoxins in postmortem PD brains by us and by other workers (Nagatsu et al., 1997, 2002a). Two groups of MPTP-like compounds, isoquinolines (IQs) and β-carbolines, were identified in postmortem human PD brain and in CSF by gas chromatography-mass spectrometry. Similar to MPTP, these IQs and β-carbolines generally inhibit mitochondrial complex I, and cause apoptotic death of catecholamine-producing cells in cultures. Like MPTP, which is converted to toxic 1-methyl-4-phenyl-pyridinium (MPP+) by MAO B, IQs and β-carbolines are also generally N-methylated by N-methyltransferase and then oxidized by MAO B to isoquinolinium ions or carbolinium ions to produce neurotoxicity in animals *in vivo*. Some probable neurotoxins such as (R)-N-Me-salsolinol are assumed to be endogenously synthesized from dopamine in the brain. When (R)-N-Me-salsolinol is administered directly into the striatum in rats, it produces Parkinson-like movement disorders (Naoi et al., 1996). These properties are similar to those of MPP+. The following IQs have been identified in the brain of patients with PD and also of control patients (Nagatsu, 1997; 2002a): tetrahydroisoquinoline (TIQ), 1-Me-TIQ, N-Me-TIQ, N-Me-6,7-(OH)2-TIQ (N-Me-norsalsolinol), 1, N-(Me)2-6,7-(OH)2-TIQ (N-Me-salsolinol), 1-phenyl-TIQ, N-Me-1-phenyl-TIQ, and 1-benzyl-TIQ (1-Bn-TIQ). Among these IQ compounds, 1-Bn-TIQ (Kotake et al., 1995) and (R)-N-methyl-salsolinol (Naoi et al., 1996) are the most potent in producing PD in animals. Among β-carbolines, norharman, harman, 2-Me-norharmanium, and 2,9- (Me)2-norharmanium have been identified in the brain and CSF in normal controls and PD (Collins and Neafsey, 2000; Matsubara, 2000). 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) is another neurotoxic β-carboline (Bringmann et al., 2000). Some of these neurotoxins are increased in the brain and/or CSF in PD. However, their distributions in the brain are not specific to the nigrostriatal pathway, and none of them, except MPTP, have been proved to produce PD in humans. Therefore, the significance of these neurotoxins with respect to PD remains unknown.

Changes in cytokines and neurotrophins in postmortem brains in PD

The brain is generally considered to be a ‘‘privileged’’ site, i.e., one free from immune reactions, since it is protected by being behind the blood-brain barrier. However, recent findings revealed that immune responses do, in fact, occur in the brain in PD or in other neurodegenerative diseases, probably by microglia activation that produces pro-inflammatory cytokines (Hayley and Anisman, 2005; Hirsch et al., 2003; McGeer and McGeer, 1995; McGeer et al., 1988; Nagatsu and Sawada, 2005; Nagatsu et al., 1999; Sawada et al., 2006). As described below, PD animals produced by

Table 2. *Changes reported in various cytokines, growth factors, and apoptosis-related factors in Parkinson's disease*

Cytokines, growth factors, or apoptosis-related factors	Tissue studied			
	Substantia nigra	Striatum	Ventricular CSF	Lumbar CSF
TNF-α		increased		increased
IL-1β		increased	increased	increased
IL-2		increased	increased	
IL-4			increased	
IL-6		increased	increased	increased
EGF		increased		
TGF-α		increased	increased	
TGF-β1		increased	increased	
TGF-β2			increased	
NGF	decreased			
BDNF	decreased			
GDNF	no change			
bFGF		no change		
TNF R1 (p55)	increased			
caspase 1 (activity)	increased			
caspase 3 (activity)	increased			
β2-microglobulin		increased		
bcl-2		increased		
solubles Fas		increased		

From Nagatsu et al. (1999) and Nagatsu (2002).

MPTP or 6-hydroxydopamine showed apoptotic death of the nigro-striatal dopamine neurons with increased levels of pro-inflammatory cytokines and decreased levels of neurotrophins. Therefore, we examined changes in the levels of pro-apoptotic cytokines, neurotrophins, and other apoptosis-related factors in the nigrostriatal pathway in postmortem PD brains initially by using the enzyme-linked immunosorbent assay (ELISA; Mogi and Nagatsu, 1999; Mogi et al., 2000; Nagatsu, 2002b; Nagatsu et al., 1999, 2000a, b). Our results are shown in Table 2. We further measured mRNA levels by RT-PCR, and also identified cytokine production by immunohistochemistry at the cellular level (Imamura et al., 2003, 2005; Sawada et al., 2006). We obtained the first ELISA evidence for a marked increase in the level of TNF-α in the brain (striatum) and lumbar CSF (Mogi et al., 1994). This finding was supported by the result of an immunohistochemical study by Boka et al. (1994).

We found that the levels of the following cytokines and apoptosis-related factors in the nigrostriatal pathway, and/or in ventricular and lumbar CSF were elevated: TNF-α, IL-1β, IL-2, IL-4, IL-6, EGF, TGF-α, bFGF, TGF-β1, TNF-β2, Bcl-2, soluble FAS, TNF-α receptor R1 (p55), caspases 1, and 3. We also found decreased levels of neuroprotective neurotrophins, BDNF and NGF, in the substantia nigra. These data on changes in the levels of cytokines in human PD brains were also supported by the results obtained from animal models of PD such as MPTP-treated mice (Mogi et al., 1998) and PD rats produced by injecting 6-hydoxydopamine (Mogi et al., 1999).

Studies on cytokines at the cellular level in the postmortem PD brain: immunohistochemistry and mRNA levels measured by RT-PCR

Inflammatory changes called neuroinflammation, most probably induced by activated microglia, in PD brains have been reported by us and other workers (Angrade et al., 1997; Hirsch et al., 1999, 2003; Jellinger, 2000; McGeer et al., 1988; McGeer and McGeer, 1995; Mogi and Nagatsu, 1999; Nagatsu et al., 1999; Nagatsu and Sawada, 2005; Rogers and Kovelowski, 2003; Sawada et al., 2006). We assume that activated microglia are present in the PD brain to produce pro-inflammatory cytokines and neuroinflammation, ultimately promoting death of dopamine neurons in the substantia nigra. Imamura et al. (2003) of our group identified by Western blot analysis TNF-α and IL-6 proteins in the PD brain. By double immunofluorescence staining, they also proved that ICAM-I- and LFA-1-positive MHC class II-bearing activated microglia in the putamen from sporadic PD patients had produced TNF-α and IL-6 proteins.

Activated microglia and neuro-infammation are observed not only in postmortem brains of patients with sporadic PD, but also in brains of patients with PD caused by MPTP (Langston, 1999) and in MPTP-PD monkeys years after MPTP exposure (McGeer et al., 2003). The question is whether these activated microglia are neuroprotective or neurotoxic toward the nigro-striatal dopamine neurons. Based on the *in vitro* finding of a toxic change from a neuroprotective microglial clone to a toxic one by transduction with HIV-1 Nef protein, resulting in increased NADPH oxidase activity (Vilhardt et al., 2002) and on neuropathological findings of the presence of neurotoxic and neuroprotective subsets of activated microglia in the brains of PD and Lewy body disease (LBD) patients by Imamura et al. (2003, 2005), Sawada has hypothesized that activated microglia may be neuroprotective at least in the initial early stage and may later become neurotoxic by a toxic change during the progression of PD, AD, or other neurorodegenerative diseases (Sawada et al., 2006). This microglia-toxic change hypothesis, if correct, would be expected to be useful for developing drugs against PD. Anti-inflammatory drugs, which are speculated to be useful for the treatment of PD, may inhibit

the toxic change in microglia or act only on the toxic subtypes of microglia.

Future prospects of studies of postmortem PD brains

Biochemical and molecular biological studies of postmortem brains in PD have greatly contributed to our understanding of PD pathogenesis at the molecular level, though interpretation of the data must be made with caution due to the complexity of factors in the postmortem human brain.

First, establishment of brain banks including control brains are the most important to obtain an adequate number of samples. It is desired to establish the same and common system of brain banks in order to exchange brain samples among many brain banks.

Second, precise clinical records of the patients including drug administration are essential.

Third, one must consider that punched-out brain tissues, however small the sample is, contain various neurons and glial cells. Therefore, the precise brain location for punching-out tissues becomes highly critical. Various micropunching techniques have been developed, owing to the increased sensitivity of analytical systems, e.g., high-performance liquid chromatography (HPLC) with micro-bore columns (internal diameter <1–2 mm) for various neurotransmitters, and RT-PCR for the assay of mRNA contents. For example, the detection limit for catecholamines by using micro-bore HPLC is $\sim$50 fmol (Nagatsu and Kokjima, 1988). Thus, even tissues of $\sim$mg order can be analyzed.

Kanazawa's group for the first time performed single-cell analysis of CAG repeats in brains of two patients with dentatorubral-pallidoluysian atrophy (DRPLA) by using a newly developed excimer laser microdissection system to analyze somatic mosaicism in their brains (Hashida et al., 2001). They also provided the first quantitative measurements of the mRNA expression profile of AMPA receptor subunits in human single neurons from patients with amyotrophic lateral sclerosis (ALS) by means of quantitative RT-PCR with a laser micro-dissector (Kawahara et al., 2003).

Sawada's group has also established a method for single-cell analysis by laser capture micro-dissection for identification of cells by immunohistochemistry. Analysis of the effects of various biologically active compounds can now be carried out on a single cell or the same group of cells, which are isolated by laser capture microdissection and identified by immunohistochemical staining. Biochemical studies on postmortem human brains at the cellular level will further contribute to elucidation of the molecular pathology of PD, AD, and other neurodegenerative and neuropsychiatric diseases.

Acknowledgements

We dedicate this paper to Prof. Dr. Peter Riederer in commemorate his great contributions to basic research on PD. We have collaborated with Dr. Peter Riederer for many years on biochemical studies of postmortem human brains from the brain bank established by him at Würzburg University. We are very grateful to Dr. Riederer for his friendship and to Professors Dr. Hirotaro Narabyashi and Dr. Mitsuo Yoshida of Japan for having introduced us to the biochemical study of postmortem brains. We thank all of our collaborators: Drs. M. Mogi, K. Imamura, K. Ono, H. Suzuki, and Y. Hashizume for their participation in our recent work; and Drs. R. Iizuka, T. Kondo, Y. Mizuno, I. Kanazawa, and S. Kuno for supplying us postmortem brain samples from their brain banks in Japan.

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J Neural Transm (2007) [Suppl 72]: 121–131

Printed in Austria

Journal of Neural Transmission

Neuroprotection by propargylamines in Parkinson's disease: intracellular mechanism underlying the anti-apoptotic function and search for clinical markers

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Summary In Parkinson's and other neurodegenerative diseases, a therapeutic strategy has been proposed to halt progressive cell death. Propargylamine derivatives, rasagiline and (−)deprenyl (selegiline), have been confirmed to protect neurons against cell death induced by various insults in cellular and animal models of neurodegenerative disorders. In this paper, the mechanism and the markers of the neuroprotection are reviewed. Propargylamines prevent the mitochondrial permeabilization, membrane potential decline, cytochrome c release, caspase activation and nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase. At the same time, rasagiline induces anti-apoptotic pro-survival proteins, Bcl-2 and glial cell-line derived neurotrophic factor, which is mediated by activated ERK-NF-κB signal pathway. DNA array studies indicate that rasagiline increases the expression of the genes coding mitochondrial energy synthesis, inhibitors of apoptosis, transcription factors, kinases and ubiquitin-proteasome system, sequentially in a time-dependent way. Products of cell survival-related gene induced by propargylamines may be applied as markers of neuroprotection in clinical samples.

Keywords: Apoptosis, propargylamine, rasagiline, mitochondria, permeability transition pore, GDNF, Bcl-2, nuclear transcription factor

Abbreviations

ANT	adenine nucleotide translocator
BDNF	brain-derived neurotrophic factor
BPAP	1-(benzofuran-2-yl)-2-propylaminopentane
CyP-D	cyclophilin-D
CsA	cyclosporin A
$\Delta\Psi m$	mitochondrial membrane potential
FACS	fluorescence-augmented flow cytometry
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDNF	glial cell-line derived neurotrophic factor
R-2HMP	*N*(*R*)-(2-heptyl)-*N*-methyl-propargylamine
IL	interleukin
MAO-A and *MAO-B*	type A and B monoamine oxidase
MAP	mitogen-activated protein
MEM	Hanks' minimum essential medium
mPT	mitochondrial permeability transition
NM(R)Sal	*N*-methyl(*R*)salsolinol
PD	Parkinson's disease
PI	propidium iodide
TNF	tumor necrosis factor
VDAC	voltage-dependent anion channel

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease and affects 1–2% of the aged population. PD is pathologically characterized by selective cell death of dopamine neurons in the substantia nigra pars compacta, and biochemically by depletion of dopamine neurotransmitter in the striatum. The etiology for the sporadic form of PD remains enigmatic, whereas a growing understanding of responsible genes for familiar forms of PD suggests that the processes leading to neuronal loss may be common with those in the sporadic form of PD (Eriksen et al., 2005; Vila and Przedborski, 2004). The loss of nigral dopamine neurons in PD is hypothesized as the mutations in genes detected in the familiar form sensitizes the neurons to intrinsic and extrinsic insults. Increased oxidative stress, mitochondrial dysfunction, impaired ubiquitine-proteasome system, abnormal inflammatory cytokines, and excitotoxicity are considered to cause cell death in dopaminergic neurons, in which dopamine itself should be involved by not fully clarified mechanisms. At present, available therapies for patients with PD are limited to ameliorate the symptoms. Dopamine replacement relieves the major symptoms at least for the beginning several years. However,

progressive loss of dopamine neurons results in motor fluctuation and cognitive dysfunction, hallucinations, depression and dementia. A therapy intervening the disease progress itself is now seriously required, and ''neuroprotective'' therapy to rescue neurons from cell death and ''neurorestrorative'' therapy to restore deteriorated neurons to a normal state have been proposed (Dawson and Dawson, 2002). The therapy should target intracellular death cascade, which is activated rather slowly for decades to the end point showing the clinical signs and regulated by well-conserved and -regulated cell death system (Riederer, 2004). Using cellular and animal PD models, the molecular mechanisms behind neuronal loss have been intensively studied, and several agents have been confirmed to prevent the cell death processing. In order to ameliorate the pathogenic factors, neuroprotective agents have been proposed, including antioxidants, neurotrophic factors, anti-inflammatory drugs, mitochondria supplement, inhibitors of monoamine oxidase (MAO), and drugs interfering glutamate excitotoxicity. Since signal proteins for apoptosis increase in the nigral neurons of Parkinsonian brains, anti-apoptotic agents altering apoptotic signal pathway have been gathering attention (Maruyama et al., 2002a; Mandel et al., 2003; Simpkins and Jankovic, 2003; Youdim et al., 2006). The anti-apoptotic function is confirmed in inhibitors of type B MAO (MAO-B) and caspase inhibitors, immuno-modulators, Co-Q10, NMDA receptor antagonists and neurotrophic factors in cellular and animal model systems. Recently, several clinical trials were reported to examine effects of propargylamine MAO-B inhibitors, rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Youdim et al., 2001) and (−)deprenyl [selegiline, *N*, α-dimethyl-*N*-2-propynylbenzene-ethanolamine], in Parkinsonian patients, and beneficial effects were confirmed to slow the progression of the symptoms (Parkinson Study Group, 2004, 2006; Pälhagen et al., 2006). However, the final conclusion about the neuroprotective efficiency remains to be clarified (Riederer et al., 2004; Schapira and Olanow, 2004; Suchowersky et al., 2006).

Rasagiline and (−)deprenyl were applied in PD to increase dopamine availability through inhibiting the oxidative deamination by MAO (Birkmayer et al., 1977). In addition, MAO-B inhibitors inhibit the oxidation of protoxicants to toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-phemylpyridinium ion (MPP^+), scavenge reactive oxygen species, and prevent the lipid peroxidation and the formation of toxic dopamine quinone. Later clinical observations suggest that they may protect neurons against cell loss in PD, AD and other neurodegenerative disorders. We studied the mechanism behind protection of rasagiline against apoptotic or necrotic cell death induced in human neuroblastoma SH-SY5Y cells by oxidative stress (Maruyama et al., 2002c) and neurotoxins, such as *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] (Naoi et al., 2002a) and 6-hydroxydopamine (6-OHDA) (Maruyama et al., 2001b, 2002b). *N*M(*R*)Sal binds to type A MAO (MAO-A) in mitochondrial outer membrane, opens a mega-channel called mitochondrial permeability transition (mPT) pore, initiates rapid reduction of mitochondrial membrane potential, $\Delta\Psi m$, and swelling of mitochondria (Akao et al., 2002a; Maruyama et al., 2002a; Naoi et al., 2006; Yi et al., 2006a). Induction of mPT results in the cytochrome c release signaling subsequent apoptosis, or the loss of ATP production leading to necrosis. Bcl-2 protein family in mitochondria directly regulates the apoptotic pathway, and intracellular signaling strictly regulates the synthesis and posttranslational modification. Neuroprotective agents intervene these apoptotic processes, either by suppressing apoptogenic factors or increasing pro-survival, anti-apoptotic factors in cells.

In this paper, our recent understanding on the mechanism underlying anti-apoptotic function of propargylamines is reviewed. The effects of propargylamine derivatives were examined in relation to the regulation of mPT and the induction of pro-survival proteins, Bcl-2 and neurotrophic factors. To confirm the involvement of cell signaling, gene expression by the propargylamines was studied by cDNA array analyses. Hitherto clinical studies indicate that the more quantitative, biochemical and molecular evaluation is required to confirm the neuroprotection in Parkinsonian patients. Our recent results by use of primate suggest that gene products increased by rasagiline in the CSF and serum may be used as clinical markers to quantify the potency of putative neuroprotective drugs in clinical samples. The expected future development of neuroprotective therapy is discussed.

Materials and methods

Materials

Rasagiline and related compounds were kindly donated by Teva Pharmaceutical (Netanya, Israel). *N*-Propargylamiine and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA); JC-1, Hoechst33342, MitoTracker Orange and Green, and Rhodamine 123 from Molecular Probes (Eugene, OR, USA). Anti-Bcl-2 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); anti-β-actin antibody from Oncogene (Boston, MA, USA); mouse monoclonal anti-GAPDH antibody from Chemicon International (Temecyla, CA, USA). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO_2. Bcl-2 was overexpressed in SH-SY5Y cells as reported previously (Akao et al., 2002a). Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999).

Determination of apoptosis

Apoptotic and necrotic cell death were assessed quantitatively using fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA) (Yi et al., 2006a). To determine apoptotic cells, the cells were stained with PI solution in phosphate-buffered saline (PBS) containing 1% Triton X-100 and subjected to FACS analysis. Cells with a lower DNA content showing less PI staining than G1were defined to be apoptotic (subG1 peak) according to Eckert et al. (2001).

Measurement of mitochondrial membrane potential, $\Delta\Psi m$

The $\Delta\Psi m$ in isolated mitochondria was quantified by FACS using MitoTracker Orange and Green. The mitochondria were treated with agents at 37°C for 3 h, and stained with 100 nM MitoTracker Orange and Green, then subjected to FACS. The laser emission at 560–640 nm (FL-2) and at shorter than 560 nm (FL-1) with excitation at 488 nm were used for the detection of MitoTracker Orange and Green fluorescence, respectively. In other experiments, mitochondria were prepared from male Donryu rat liver or transgenic mice expressing human Bcl-2 in the liver, as previously described (Shimizu et al., 1998). $\Delta\Psi m$ was assessed also by measurement of reduction in Rhodamine 123 fluorescence, which was ascribed to $\Delta\Psi m$-dependent uptake of Rhodamine 123 into the mitochondria (Narita et al., 1998).

Measurement of mRNA and protein of Bcl-2 family proteins

SH-SY5Y cells were cultured in the presence of various concentrations (10 μM–1 pM) of rasagiline for 24 h or for a various incubation time with 100 nM rasagiline. The whole cells were gathered and the total RNA was extracted by the phenol/guanidinium thiocyanate method. The cDNA was generated by reverse transcription of the total RNA, and the cDNA fragments were amplified using the PCR primers (Akao et al., 2002b). PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin cDNA was used as an internal standard.

Quantitative measurement of mRNA and protein of GDNF

SH-SY5Y cells were treated with rasagiline in 96 well plates with Hanks' minimum essential medium (MEM). The effect of sulfasalazine (100 μM), an inhibitor of IκB, was examined by adding the inhibitor 30 min before the treatment with rasagiline. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). Samples or standard were added to GDNF antibody-coated wells, and incubated for 12–18 h at 4°C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boehringer Mannheim) for 1 h. The enzyme activity in each well was measured by incubation with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. The fluorescence intensity of produced 4-methylumbelliferone was measured at 360 nm with excitation at 448 nm. The mRNA of GDNF was measured by reverse transcription-polymerase chain reaction (RT-PCR), as reported (Maruyama et al., 2004a).

Quantitation of activated NF-κB

Activation of NF-κB was determined by NF-κB binding to κB sites using NF-κB p65 transcription assay kit (Active Motif, Carlsbad, CA, USA) (Maruyama et al., 2004a). Five μg of the extract of Hela cells stimulated with TNF-α for 30 min was used as a positive control. The activation of NF-κB was expressed as % of the positive control.

cDNA array for gene expression in apoptosis

The cells were incubated with 100 nM rasagiline for 6, 12, and 24 h, and the total RNA was extracted. Using AMV reverse transcriptase, total RNA isolated from the sample and control was labeled with Cy3- or Cy5-dUTP. The levels of gene expression were quantitatively analyzed by cDNA expression array using TaKaRa IntelliGene Human Apoptosis CHIP (Takara Biomedicals, Ohtsu, Japan).

Statistics

Experiments were repeated at least 4 times and the results were expressed as mean and SD. Difference was statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's F-test. A p-value less than 0.05 was considered to be statistically significant.

Results

Stabilization of mitochondrial contact sites by propargylamines

A series of propargylamines, rasagiline, (−)deprenyl, aliphatic (*R*)*N*-(2-heptyl)-*N*-methylpropargylamine (*R*-2HMP) and free *N*-propargylamine, prevent the activation of apoptotic cascade and protect SH-SY5Y cells against apoptosis induced by neurotoxins, *N*M(*R*)Sal and 6-OHDA, and oxidative stress caused by dopamine oxidation and a peroxynitrite-generating agent, SIN-1 (Akao et al., 2002a; Maruyama et al., 2002a, b, c; Yi et al., 2006b). Figure 1 shows the chemical structure of examined propargylamines. An endogenous neurotoxin *N*M(*R*)Sal induces the mPT and apoptosis (Naoi et al., 2002b, 2006). As summarized in Fig. 2, these propargylamines completely suppress opening of mPT pore caused by neurotoxins and oxidative stress. Rasagiline inhibits mitochondrial swelling and $\Delta\Psi m$ reduction (Akao et al., 2002a), and prevents release of cytochrome c, caspase 3 processing and nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Maruyama et al., 2002a). Rasagiline protected MAO-A-expressing SH-SY5Y cells from apoptosis and transfection-enforced expression of MAO-B did not increase the sensitivity to rasagiline, indicating that neuroprotective function does not depend on the MAO-B inhibition (Yi et al., 2006a). On the other hand, clorgyline [*N*-methyl-*N*-propargyl-3(2,4-diclorophenpxy)-propylamine] did not prevent, but induced mPT. Table 1 shows the results on the structure-activity relationship for direct stabilization of mPT among propargylamine derivatives with different hydrophobic structure, indanyl (rasagiline), phenyl (deprenyl), aliphatic (2-HMP) and benzofuranyl groups [1-(benzofuran-2-yl)-2-propylaminopentane, BPAP]. The aminoindan derivatives are the most active followed by the phenyl derivatives, and the derivatives with aliphatic and benzofuranyl structures require rather high concentrations for preventing mPT. The modification of aminoindan ring does not affect the potency to stabilize mPT pore, as shown

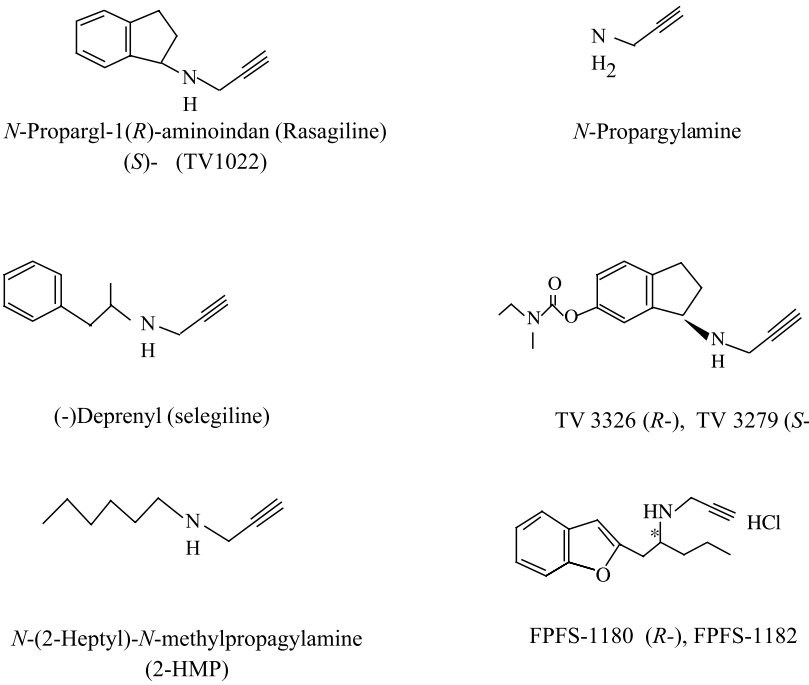

Fig. 1. Chemical structures of propargylamines with neuroprotective potency

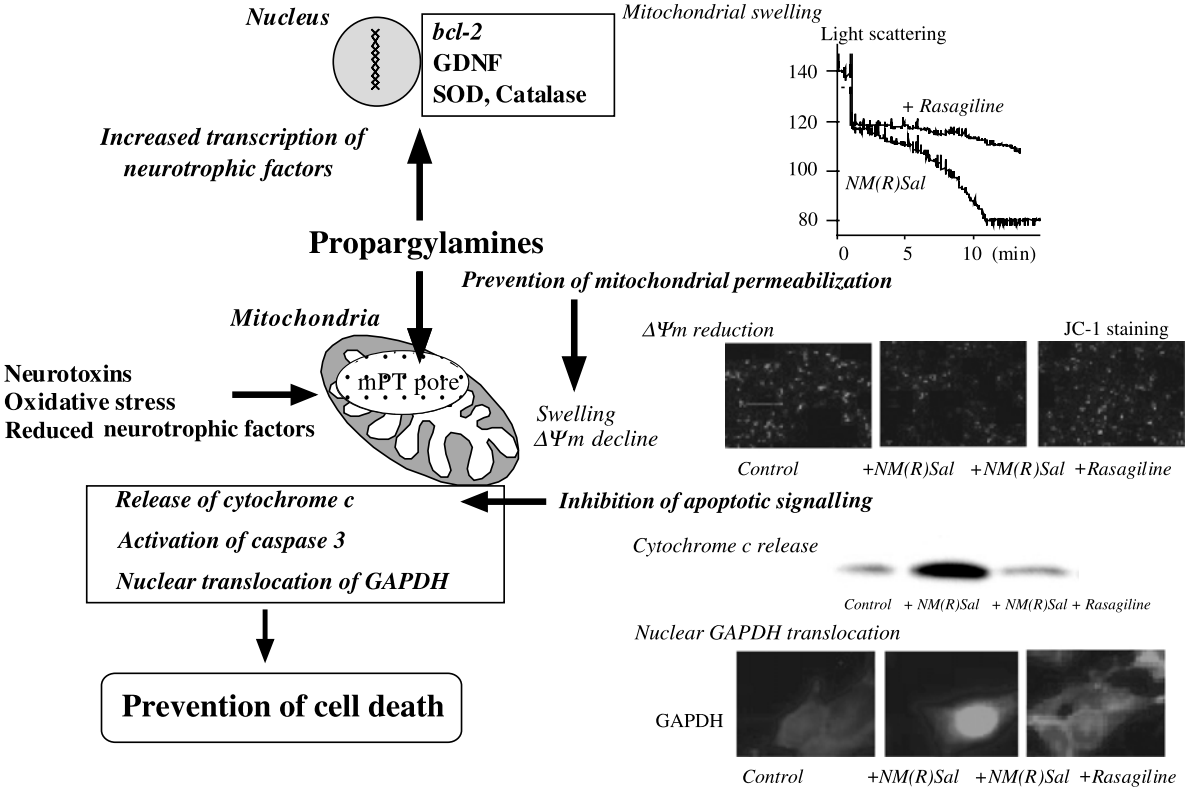

Fig. 2. Target sites of neuroprotective propargylamines in apoptosis cascade. Rasagiline and related compounds suppress mPT, as shown by prevention of mitochondrial swelling and ΔΨm reduction. They inhibit also cytochrome c release, caspase 3 activation and nuclear GAPDH translocation. In addition, the propargylamines increase the expression of anti-apoptotic Bcl-2 family protein, neurotrophic factors (GDNF, BDNF), and antioxidant enzymes (SOD, catalase)

with TV 3326 [(*N*-propargyl)-(3*R*)-aminoindan-5-yl]-ethymethyl carbamate and its hydroxyl metabolite, TV 3294 (Maruyama et al., 2003). In general, the *R*-enantiomers are more potent to prevent the mPT than the *S*-enantiomers (Maruyama et al., 2001a, b). The *S*-enantiomer of rasagiline, TV1022, lacks the MAO inhibiting function, but it still

Table 1. *Structure and neuroprotective characteristics of propargylamines*

Name [Structure]	Prevention of mPT	Induction of Bcl-2	Induction of GDNF
Rasagiline [*R*(+)-*N*-propyl-1aminoindan]	10 μM–1 nM	10 μM–1 nM, 10–1 pM	1 μM–100 pM
TV1022 [*S*(−)-*N*-propyl-1-aminoindan]	1 μM–100 nM	–*	–
Aminoindan	–	–	–
N-Propargylamine	1 μM–10 nM	100–1 nM	N.D.**
N-Methylpropargylamine	–	–	N.D.
Propiolaldehyde	–	–	N.D.
(−)Deprenyl	1 μM–100 nM		1 μM–10 nM
(+)Deprenyl	10 μM	–	–
Desmethyldeprenyl	10–1 nM		1 μM–10 nM
TV3326 [5-ethyl ethyl carbamate-rasagiline]	100–10 nM	–	–
TV3294 [5-hydroxyl-rasagiline]	100–10 nM	–	–
R-*N*-(2-Heptyl)-*N*-methylproparylamine	1 μM–100 nM	N.D.	N.D.
S-*N*-(2-Heptyl)-*N*-methylproparylamine	10 μM	N.D.	N.D.
R-*N*-(2-Heptyl)-propargylamine	1 μM–100 nM	N.D.	N.D.
R-3-(2-Heptylamine)-*N*-methylpropionic acid	–	N.D.	N.D.
R-(−)-BPAP	–	100–1 nM	1 nM***
S-(+)-BPAP	1 μM–10 nM	–	N.D.
R-(+)-*N*-(2-propynyl)-BPAP	1 μM–10 nM	100–1 nM	N.D.
S-(−)-*N*-(2-propynl)-BPAP	–	–	N.D.

* Not affective, ** not determined, *** Hirai et al. (2005).

prevents mPT, suggesting again that the anti-apoptotic function is not related to the MAO inhibition. In the case of the benzylfuranyl derivatives, the stabilization of mPT pore depends on the absolute structure of propargylamines. The compounds with dextro-rotation prevented $\Delta\Psi m$ decline by neurotoxins, whereas the corresponding enantiomer with levo-rotation did not (Maruyama et al., 2004b). The propargylamine group is essentially required for the activity as in the case with free *N*-propargylamine itself, whereas the analogues without a propargyl residue, aminoindan and *R*-3-(2-heptylamino)-propionic acid, did not prevent mPT. The methylation of the amino residue in *N*-propargylamine abolished the activity to prevent $\Delta\Psi m$ reduction (Yi et al., 2006b).

The precise mechanism leading to the permeabilization of mitochondria is still unclear, even though several models have been proposed. The mPT pore is primarily composed of adenine nucleotide translocator (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane, which binds to ANT at the contact sites between the inner and outer membrane. In addition, peripheral benzodiazepine receptor (PBR) and MAO in outer membrane and hexokinase at the contact site are associated with the mPT pore. Cyclophilin-D (CyP-D) binds to the matrix site of ANT and induces conformation change to form a non-specific pore leading to release of any molecules of less than 1.5 kDa, and metabolic gradients across the inner membrane are dissipated, with accumulation of Ca^{2+}. Opening of the mPT pores results in swelling of the matrix and rupture of the outer membrane, which leads to the release of apoptogenic factors (cytochrome c, apoptosis-inducing factor, Smac/DIABLO, Omi/HtrA2) resulting in activation of caspase system. Oxidative stress and other insults facilitate the mPT pore opening though cross-linking of thiol groups of cysteine residues in ANT and increases the binding of CyP-D to the ADP binding site (McStay et al., 2002). Neurotoxins, PBR ligands (PK11195, protophorphirin IX), bax and other pro-apoptotic Bcl-2 protein family, heavy metals, inorganic phosphate, fatty aids, quinones and uncouplers of mitochondrial oxidative phosphorylation system induce mPT. On the other hand, viral proteins, such as HIV viral protein R (Jacotot et al., 2001) and myxoma poxvirus protein, M11L (Everett et al., 2002), bind to the CyP-D binding site and prevent the pore formation. Another model of mPT is that Bcl-2 interacts directly with VDAC and regulates ANT activity, which was proved in a model system composed of VDAC in liposomes (Shimizu et al., 1999; Tsujimoto and Shimizu, 2000). According to this model, VDAC interacts with apoptogenic Bax and Bak, functions as ''VDAC modulators'', changes its conformation leading to formation of a megachannel to allow cytochrome c to pass through, whereas anti-apoptotic Bcl-xL closes the channel. In this case, the outer membrane might be intact without rupture. More recently, lipid bilayer was proposed to play an important role in mPT by interacting with ANT or other mitochondrial components (Lucken-Ardjomande and Martinou, 2005).

*N*M(*R*)Sal binds to MAO-A in the outer membrane and opens mPT pore, which CsA and bongkrekic acid antagonize through binding to CyP-D and ANT. *N*M(*R*)Sal, dopamine and its oxidation product quinone, neuromelanin, and peroxynitrite modify sulfhydryl (SH) groups in mitochondria

and induce mPT (Yi et al., in preparation). Rasagiline prevents the reduction of free SH residues in mitochondria and the mPT, regardless of the types of insults leading to mPT (toxins, PBR ligands and oxidative stress). Rasagiline is bound to MAO-B, MAO-A, or other components in mPT pore, stabilizes the contact site and prevents the conversion of ANT into a pro-apoptotic pore. The study is under way whether rasagiline can bind directly to ANT or CyP-D. In addition, propargylamines bind to several other proteins in cells. (−)Deprenyl and its analogue TCH346 [CGP3466, dibenzo(*b*,*f*)oxepin-10-yl-methyl-methyl-prop-2-ynyl-amine], bind to GAPDH, and prevent the *S*-nitrosylation of GAPDH, the binding to Siah and its nuclear translocation (Hara et al., 2006). Another candidate binding site is poly(ADP-ribose)-polymerase-1 (Brabeck et al., 2003). However, in apoptotic processes these putative binding sites are downstream of mPT and our results demonstrate that the binding of rasagiline to mitochondrial protein and the regulation of mPT are the primary events in preventing apoptosis.

Induction of neuroprotective Bcl-2 family proteins

It is well known that some kinds of protein, Bcl-2 family protein, anti-oxidants and neurotrophic factors, alleviate neuronal loss through suppression of oxidative stress, prevention of apoptotic signal transduction and promotion of cell survival. Rasagiline, and (−)deprenyl increase the activity of anti-oxidative enzymes, superoxide dismutase (SOD) and catalase, in the rat brain after the systemic administration (Carrillo et al., 2000, Kitani et al., 2000). (−)Deprenyl and desmethyldeprenyl increase mRNA level of SOD 1 and 2, Bcl-2 and Bcl-xL, nitric oxide synthase, c-JUN, and NAD dehydrogenase in PC12 cells (Tatton et al., 2002). Our and Youdim's group have clarified the detailed mechanism underlying the induction of anti-apoptotic proteins by rasagiline analogues.

The family of Bcl-2-related proteins constitutes one of biologically most relevant regulatory gene products against apoptosis through controlling mitochondrial permeabilization (Kroemer, 1997). Bcl-2 family proteins are subdivided into three groups on the basis of the pro- and anti-apoptotic function and the Bcl-2-homology (BH) domains (BH1 to BH4). Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) have 4 BH domains, whereas pro-apoptotic multidomain protein (Bax, Bak, Bok/mtd) lacks BH4. BH3 only proteins (Bid, Bim/Bod, Bad, Bmf) are also pro-apoptotic and link specific apoptotic stimuli to mPT. Bcl-2 is mainly localized in the mitochondrial inner membrane, and the family proteins form homo- or hetero-dimers between anti- and pro-apoptotic members and determine cellular sensitivity to apoptotic stimuli by titrating one another's function. Anti-apoptotic Bcl-2 family proteins prevent apoptosis either by inhibiting pro-apoptotic Bcl-2 members directly, controlling endoplasmic reticulum and mitochondrial homeostasis, or defending against oxidative stress. On the other hand, pro-apoptotic Bcl-2 family proteins induce mPT and trigger the release of mitochondrial apoptogenic factors into the cytosol, as discussed above.

Overexpression of Bcl-2 protects various neuron paradigms *in vivo* and *in vitro* from death induced by neurotoxins and other insults. Bcl-2-overexpression in SH-SY5Y cells prevented apoptosis induced by *N*M(*R*)Sal, which is relevant with the results that $\Delta\Psi$m decline induced by *N*M(*R*)Sal was suppressed in mitochondria prepared from Bcl-2 overexpressed mouse liver (Akao et al., 2002a; Maruyama et al., 2002a). These results suggest that rasagiline may induce Bcl-2 protein, in addition to the direct stabilization of the mPT pore. We found that rasagiline increases the mRNA and protein levels of *bcl-2* and *bcl-xL* in SH-SY5Y cells, as shown in Fig. 3 (Akao et al., 2002b). Rasagiline showed a reverse-bell shape curve of the concentration-activity relationship and the increase of Bcl-2 was detected at 10 μM–10 nM, and also at 10–1 pM. Bcl-2 protein level increased from 6 to 24 h of the treatment. Rasagiline induced mRNA levels of anti-apoptotic *bcl-2* and *bcl-xL*, but not those of pro-apoptotic *bax* and *mcl-l*. Other MAO-A and -B inhibitors, clorgyline and pargyline, did not affect the mRNA level at the concentrations examined.

The results of structure-activity relationship of propargylamine derivatives to Bcl-2 induction are summarized in Table 1. Rasagiline and *N*-propargylamine increased Bcl-2 mRNA and protein, whereas aminoindan and *N*-methylpropargylamine did not (Maruyama et al., 2002b; Yi et al., 2006b). The structure required for Bcl-2 induction is the propragylamine group, as in the case for preventing mPT. Also among BPAP derivatives, *R*(−)-*N*-propynyl compound, FDFS-1180, induced Bcl-2, more than FDFS-11169 without proynyl group (Maruyama et al., 2004b). For Bcl-2 induction, *R*-propargylamines are more potent than the *S*-enantiomers.

Induction of neurotrophic factors by propargylamines

Neurotrophic factors, including nerve growth factor, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor, prevent cell death in specified type neurons. GDNF is a member of the transforming growth factor-β superfamily and effectively protects dopaminergic neurons against

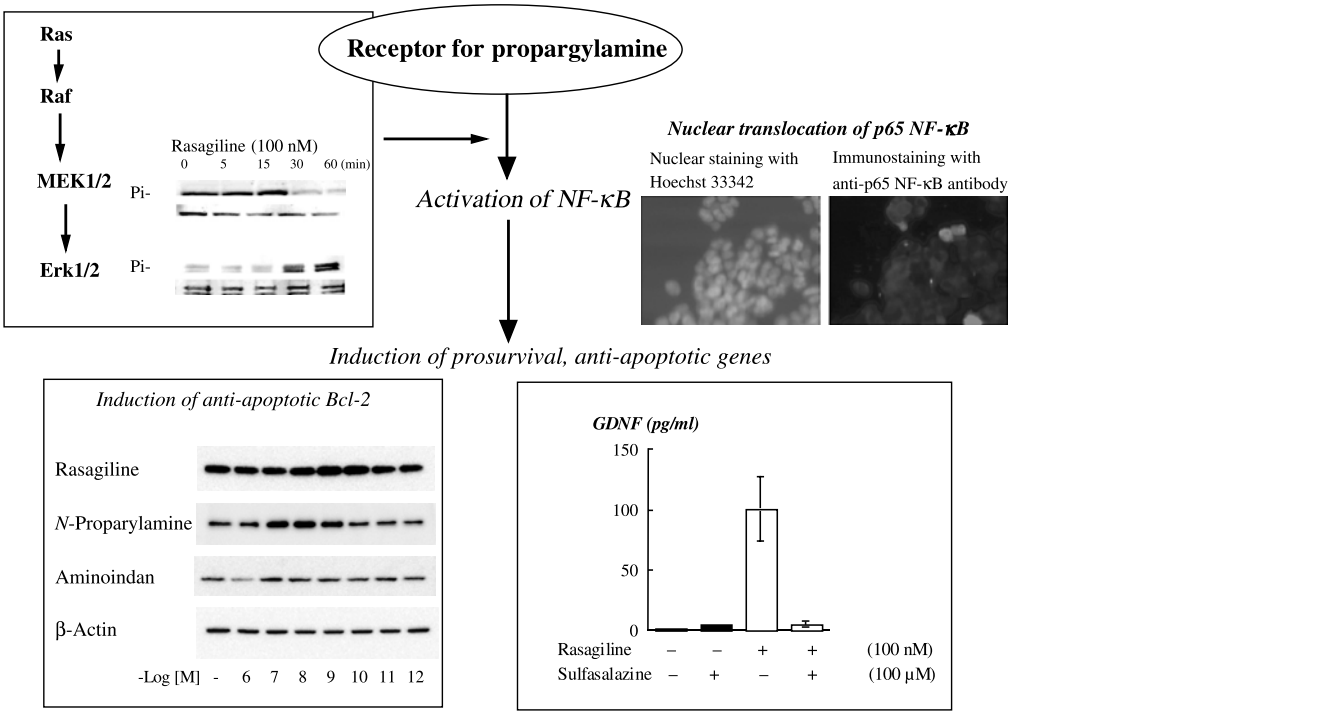

Fig. 3. Rasagiline increases anti-apoptotic Bcl-2 family and GDNF, a dopamine neuron-specific neurotrophic factor, through activation of ERK-NF-κB pathway. Anti-apoptotic propargylamines bind to the putative receptor on the membrane and activate the MEK1/2-ERK1/ERK2 pathway. The activated phosphorylated forms of ERK1/2 were detected after 30 min incubation with 100 μM rasagiline. After 3 h treatment with rasagiline, NF-κB was activated and p65 subunit was translocated into nuclei, as shown by staining using anti-p65 antibody for GAPDH and Hoechst 33342 for nuclei. The involvement of NF-κB in the induction of GDNF and Bcl-2 was also confirmed by use of an inhibitor of IκB kinase, sulfasalazine, which inhibited the increase of GDNF protein in SH-SY5Y cells treated with 100 nM rasagiline. The structure required for the Bcl-2 induction is a propargylamine structure, since aminoindan without a propargyl residue did not increase Bcl-2 levels

cell death in various animal PD models prepared with 6-hydroxydopamine and MPTP. Since GDNF and other neurotrophic factors cannot penetrate into the brain though the blood-brain barrier, several trials have been reported, delivering GDNF in the substantia nigra by direct administration, gene therapy, and cell implant (Bauer et al., 2000; Gill et al., 2003).

As shown in Fig. 3, rasagiline increases GDNF in SH-SY5Y cells. GDNF mRNA was virtually not detectable in SH-SY5Y cells, but after the treatment with 100 nM rasagiline for 3 h considerable amount of GDNF mRNA was detected. GDNF protein level in the control cells was less than 1 pg/ml and increased to be more than 100 pg/ml after rasagiline treatment. Induction of neurotrophic factors, GDNF, BDNF, NGF and neurotrophin-3 (NT-3), by propargylamines was examined in SH-SY5Y cells. Depending on the type of propargylamines, different neurotrophic factors were induced; rasagiline induced GDNF, and (−)deprenyl BDNF (Maruyama et al., in preparation). This result suggests that a specified propargylamine compound can induce a definite neurotrophic factor beneficial for selective type of neurons.

Signal transduction and gene expression by rasagiline for neuroprotection

These results on Bcl-2 and GDNF induction suggest that rasagiline may activate intracellular signals for induction of genes coding these anti-apoptotic proteins. NF-κB is the common transcription factor to induce anti-apoptotic *bcl-2*, neurotrophic GDNF and anti-oxidative SOD, all of which were increased by rasagiline (Carrillo et al., 2000; Akao et al., 2002b; Maruyama et al., 2004a). NF-κB consists of 2 subunits of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52), and is sequestered in the cytoplasm as an inactive complex with NF-κB inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, dissociated from the complex and degraded by the ubiquitin-proteasome system. This reaction allows translocation of free, active NF-κB complex into nuclei, where it binds to specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription, as shown by the p65 binding assay. The translocation of activated p65 subunit into nuclei by rasagiline was confirmed by Western blot analysis of the subcelluar fractions and also by immunohistochemical

observation using the p65 antibody and Hoechst 33342 for nuclear staining (Fig. 3) (Maruyama et al., 2004a). The involvement of phosphorylation of inhibitory IκB subunit on the activation of NF-κB, was demonstrated by use of sulfasalazine, an inhibitor of by IκB kinase (Fig. 3). Sulfasalazine inhibited also the increase of mRNA of *bcl-2* and *bcl-xL* as in the case with GDNF, suggesting the involvement of NF-κB transcription factor in the induction of neuroprotective proteins in common.

Rasagiline and related propargylamines protect cellular and animal models of neurodegenerative disorders, including PD, AD and ischemia (Mandel et al., 2003, 2005). By screening the signal factors activated rasagiline, we found that extracellular-regulated kinase-1/2 (ERK1/ERK2) was activated as an upper signal of NF-*k*B activation (Maruyama et al., 2004a) (Fig. 3). After treatment with 100 nM rasagiline, phosphorylated ERK1/ERK2 was increased in a time-dependent way, which PD98059, an inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase-1 (MEK 1/2), inhibited. CF10923x and Calphosin, inhibitors for protein kinase C (PKC), suppressed the increase of Bcl-2 and activated NF-κB by rasagiline, suggesting the involvement of the pathway through activation of PKC, Ras/Raf and MEK 1/2 in the induction of these proteins. Youdim and his group reported detailed data concerning the activation PKC system by rasagiline, which up-regulates MAP kinase/ERK cascades (Youdim et al., 2003a; Mandel et al., 2005; Weinreb et al., 2004). Recently, in mice treated with MPTP rasagiline was reported to activate signal pathway from neurotrophic factor responsive-tyrosine kinase receptor to phosphatidylinositol 3 kinase protein (Sagi et al., 2007). However, as shown later in DNA array studies, kinases may be activated not only primarily by rasagiline itself, but also secondarily by the following death-regulating processes. At present, it requires further studies to identify the initial signal to induce anti-apoptotic genes.

To screen the gene induction by rasagiline, we examine the time-dependent expression of genes by rasagiline. SH-SY5Y cells were treated with 100 nM rasagiline for 6, 12 and 24 h and mRNA was extracted and reverse-transcribed with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, and comparing to the control values obtained by the cells without treatment of rasagiline (Table 2). Rasagiline increased 108, 57 and 82 genes (>1.5 compared to control) and reduces 37, 54 and 104 genes (<0.5) after 6, 12 and 24 h treatment, respectively. Rasagiline affected genes with different cellular function in a time-dependent way. After 6 h treatment, mRNA of *bcl-2*, and genes related to NF-κB related transcription factors, cytokines and the receptors [interleukin (IL) receptors], mitochondrial ATP synthesis (cytochrome c oxidase, NADH-coenzyme Q reductase, ATP synthase, aconitase) and the ubiquitin-proteasome system were increased. In addition, genes of mPT pore components (ANT, VDAC and MAO-A) were also increased. On the other hand, genes coding growth factor (BDNF, transforming growth factor), cytokines and receptors [tumor necrosis factors (TNF), IL, fibroblast growth factor] were reduced. At 12 h of the treatment, most marked increase was observed in MAP-KK and cytokine receptors. In addition, rasagiline increased mRNA for ANT, VDAC and mitochondrial proteins (complex I–IV, mitochondrial transcription factor A). On the other hand, kinases associated with death signal (MAP kinase activating death domain, MAPKKK 4, TNF receptor associated factor 5, death-associated protein kinase-1), growth factors (NGF), and cytokines decreased. It is interesting that mRNA of *bcl-2*, MAO-B and also transcription factors were reduced significantly at this point. Rasagiline treatment for 24 h enhanced significantly the genes for *bcl-2*, apoptosis inhibitors (apoptosis inhibitors 1, 2 and 4, neuronal apoptosis inhibitory protein) and cell signals, including kinases (MAPK, MAPKK, cyclin-dependent kinase), cytokines and the receptors, and the transcription factors. It may be hypothesized that rasagiline sequentially increases ATP-dependent activation of kinases and transcription factors, the ubiquitine-proteasome system, which degrades the cleaved phosphorylated inhibitors of kinases and transcription factor, increases cytokines and the receptors, and finally induces pro-survival genes.

Table 2. *Gene induction in SH-SY5Y cells by rasagiline*

Rasagiline (100 nM) treatment for		
6 h	12 h	24 h
Increased genes	Increased genes	Increased genes
ATP-synthesis-related mitochondrial mPT pore related	Kinases	Bcl-2
Cytokine receptors	Cytokine and IL receptors	Apoptosis inhibitors
NF-κB related transcription factors	Mitochondrial complex I–IV	TNF and receptors
Ubiquitin-proteasome system	mPT pore related	Growth factors
Reduced genes	Reduced genes	
IL and TNF	Bcl-2	
Cytokin-related transcription factors	Kinases	
Growth factors	IL and TNF	
	Transcription factors	
	Growth factors	

Discussion

The clinical trials to prove the neuroprotective function of rasagiline and (−)deprenyl were reported, but the results are still contradicting, and biomarkers to estimate the progression of neuronal loss should be invented (Michell et al., 2004). The markers for the disease progression and treatment efficiency are based on clinical evaluation of symptoms, PET and SPECT imaging, transcranial ultrasound and some biochemical tests. However, blood tests for PD progression are limited to monitor the pathogenic factors, such as increased oxidative stress (malondialdehyde, superoxide radicals, 8-hydrox-2′-deoxyguanosine), or the reduced complex I (Schapira et al., 1990) and increased MAO-B activity in platelets (Zhou et al., 2001). (−)Deprenyl may reverse the increase in MAO-B and the subsequent reduction of β-phenylethylamine in plasma, but these markers represent MAO inhibitory function of (−)deprenyl, but not the neuroprotective activity. α-Synuclein and its phosphorylated proteins were proposed as the markers, but the recent results did not support this view. In CSF, increased levels of 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-guanosine and malondialdehyde were detected (Abe et al., 2003). However, these markers do not present information for progression of selective neuronal loss in PD.

At present, mechanistic makers for factors intervening the disease progress may be the only available markers to assess the neuroprotective potency. As described above, rasagiline induces GDNF in cultured cells, suggesting that the levels of neurotrophic factors specific for dopamine neurons may be used as markers. Indeed, we examined the change in neurotrophic factors in monkey CSF after systemic treatment of rasagiline (Maruyama et al., in preparation). The results proved the validity of our view, which was supported further by the analyses of the CSF from Parkinsonian patients before and after treatment of (−)deprenyl, even the limited number of the samples (Maruyama et al., in preparation). We are now examining the candidates of the biomarkers for the neuroprotective function in serum and CSF from Parkinsonian patients and primate models.

Recently, an increasing number of evidences indicate that rasagiline and related compounds can ameliorate pathogenic processes in AD and other neurodegenerative disorders. Rasagiline analogues with inhibitor potency to cholinesterase, TV 3326, and its *S* enantiomer TV 3279 were reported to regulate the processing of amyloid precursor protein (APP) and increase the soluble APP secretion through activation of α-secretase activity and the reduction of holo-APP protein (Youdim et al., 2003b; Yogev-Falach et al., 2006). Their results suggest that propargylamines intervene the pathogenic processes in neurodegenerative disorders in general and ameliorate the disease process.

The stereo-chemical and enantiomeric specificity of the propargylamine for their neuroprotective activity suggests the occurrence of the target protein in mitochondria and other cell components. The identification of the binding site of neuroprotective propargylamines may give us a clue to find the most adequate chemical structure for the function, and develop new drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

Acknowledgement

This work was supported by a Grant-in-Aid on Scientific Research for Young Researcher (B) (M. S-N) and Comprehensive Research on Aging and Health from the Ministry of Health, Labor and Welfare (W. M. and M. N.), the Promotion of Fundamental Studies in Health Sciences of National Institute of Biomedical Innovation (W. M), Japan. Rasagiline was kindly donated by TEVA Pharmaceutical Co. (Netanya, Israel), and aliphatic propargylamines from Professor A. Boulton, and *R*-(−)-BPAP and related derivatives from Fujimoto Pharmaceutical Co. (Matsubara, Japan).

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J Neural Transm (2007) [Suppl 72]: 133–143

Printed in Austria

Journal of Neural Transmission

Intrastriatal transplantation of mouse bone marrow-derived stem cells improves motor behavior in a mouse model of Parkinson's disease

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Summary Strategies of cell therapy for the treatment of Parkinson's disease (PD) are focused on replacing damaged neurons with cells to restore or improve function that is impaired due to cell population damage. In our studies, we used mesenchymal stromal cells (MSCs) from mouse bone marrow. Following our novel neuronal differentiation method, we found that the basic cellular phenotype changed to cells with neural morphology that express specific markers including those characteristic for dopaminergic neurons, such as tyrosine hydroxylase (TH). Intrastriatal transplantation of the differentiated MSCs in 6-hydroxydopamine-lesioned mice led to marked reduction in the amphetamine-induced rotations. Immunohistological analysis of the mice brains four months post transplantation, demonstrated that most of the transplanted cells survived in the striatum and expressed TH. Some of the TH positive cells migrated toward the substantia nigra. In conclusion, transplantation of bone marrow derived stem cells differentiated to dopaminergic-like cells, successfully improved behavior in an animal model of PD suggesting an accessible source of cells that may be used for auto-transplantation in patient with PD.

Keywords: Multipotent mesenchymal stromal cells (MSCs), dopamine, dopaminergic neurons, Parkinson's disease (PD), stem cells

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of over 50–60% of the dopaminergic neurons in the substantia nigra causing resting tremor, rigidity, bradykinesia and postural instability (Kish et al., 1988). Current drug therapy of PD by administration of dopamine (DA) precursors and agonists has many limitations and therefore the option of cell replacement therapy is constantly appealing (Lindval and Bjorklund, 2004; Winkler et al., 2005). However, largely negative results from previous controlled transplantation trials with fetal mesencephalic neurons in PD patients raise doubts about both the therapeutic benefit and disabling of such an approach in addition to safety and ethical concerns (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003).

Embryonic stem cells (ESc) may overcome the limitations of fetal donor tissue by offering both extensive cell proliferation and controlled differentiation to DA neurons (Kawasaki et al., 2000; Lee et al., 2000). Studies on ESc encouraged researchers to generate dopaminergic cells as an alternative source for transplantation in PD. Indeed, several groups reported that mouse and non-human ESc demonstrate dopaminergic characteristics following induced differentiation. Moreover, in subsequent experiments, transplantation in an animal model of PD demonstrated integration as well as behavioral recovery (Björklund et al., 2002; Kim et al., 2002; Barberi et al., 2003; Sanchez-Pernaute et al., 2005; Takagi et al., 2005; Kim et al., 2006). Recent work shows that human ESc might also differentiate into DA neurons, but the function of these cells has not yet been fully established (Ben-Hur et al., 2004; Zeng et al., 2004; Park et al., 2005; Yan et al., 2005; Brederlau et al., 2006; Roy et al., 2006). However, the poor survival of the ESc-derived TH positive cells following transplantation, in addition to the ethical and the safety issues, including teratoma formation, restrict the clinical usefulness of this type of stem cell (Carson et al., 2006, Roy at al., 2006).

We therefore focused on the induction of adult stem cells as an alternative. Multipotent mesenchymal stromal cells (MSCs) were previously reported to be multipotent and they can be induced *in vitro* to differentiate into a variety of tissues including osteoblasts, adipocytes, and chondrocytes (Prockop, 1997; Pittenger et al., 1999; Bianco and Robey, 2000; Bianco et al., 2001; Colter et al., 2000; Deans

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and Moseley, 2000; Krause, 2002). Additionally, recent findings including those from our laboratory indicate that mouse, rat and human MSCs can also be induced to differentiate into neuron-like cells (Sancez-Ramos et al., 2000; Woodbury et al., 2000; Levy et al., 2003; Blondheim, 2006). Moreover MSCs have the potential to migrate into injured neural tissues and differentiate into neurons (Mahmood et al., 2001; Munoz-Elias et al., 2004; Kan et al., 2005; Helman et al., 2006). Li et al. (2001) demonstrated that naïve mouse MSCs grafted into the striatum of mouse model of PD, promote some functional recovery at 28 days after transplantation. However, only about 0.8% of the grafted cells expressed tyrosine hydroxylase (TH).

In our previous studies, we demonstrated that mouse MSCs (mMSCs), exposed to an inducing cocktail, activated the neuron specific enolase (NSE) promoter and expressed typical neuronal markers (Levy et al., 2003). Moreover, most of the key gene for neuro-dopaminergic function are expressed in human MSCs (Blondheim, 2006). To examine whether bone marrow might be used for autologous cell replacement in PD, we isolated MSCs from enhanced green fluorescent protein (EGFP) transgenic mice, induced dopaminergic differentiation and transplanted the cells into a mouse model of PD. Our data indicate that differentiated mMSCs transplanted into the striatum of 6-OHDA-lesioned mice survive for several months, continually express neuronal markers and improve the amphetamine-induced rotational behavior.

Material and methods

Animals

Primary culture of mMSCs were obtained from adult B5/EGFP transgenic (Tg) mice bearing the *EGFP* gene and expressing the EGFP protein in all the tissues (Hadjantonakis et al., 1998). The behavior of mutant cells can be followed with simple ultra violet (UV) microscopic observation. The Tg mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). C57/b1 male mice (~30 gr) (Harlan Lab, Israel) were used for 6-OHDA lesions. All animals were housed in standard conditions: constant temperature ($22 \pm 1^{\circ}C$), humidity (relative, 50%), 12-h light, 12-h dark cycle and free access to food and water. All the animal experiments were performed under the supervision of the Animal Care Committee and Experimentation of The Faculty of Medicine at Tel Aviv University and at the Rabin Medical Center, Israel.

Isolation and culture of mMSCs

Mouse MSCs were isolated and cultured as described in our previous report (Levy et al., 2003). Briefly, cells were extracted from tibia and femur bones and placed in Hank's balanced salt solution (HBSS; Biological Industries, Bet-Haemek, Israel), centrifuged and plated in growth medium containing Dulbecco's Modified Eagle's Medium (DMEM; Biological Industries) supplemented with 15% fetal calf serum (FCS; Biological Industries), 5% horse serum (HS; Biological Industries), 1× nonessential amino acid (Biological Industries), 0.001% β-mercaptoethanol (Sigam, St. Louis, MO, USA), 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 12.5 units/ml nystatin (SPN; Biological Industries, Israel), in polystyrene plastic tissue cultures 75 cm^2 flask (Corning Incorporated, Corning, NY, USA), maintained at 37°C in an humidified 5% CO_2 incubator. Cells were incubated for 48 h then non-adherent cells were removed. The tightly adhered mMSCs cells were washed twice with Dulbecco's phosphate buffered saline (PBS; Biological Industries, Israel) and fresh growth medium was added. The medium was replaced every 3 or 4 days and when cells reached 70–90% confluency, cultures were harvested with trypsin-EDTA solution (0.25% trypsin and EDTA 1:2000 in puck's saline; Biological Industries, Israel) for 5 min at 37°C.

Flow cytometry analysis (FACS)

Following thirty days in culture, the isolated mMSCs were harvested from the tissue culture flasks. The cells (0.5×10^6) were stained for 45 min at 4°C with anti CD45 (1:200, eBioscience, San Diego, USA), anti CD90 (1:20, Miltenyi Biotec, Auburn, CA, USA), anti CD106 (VCAM-1) (1:400, BioLegend, San Diego, USA) conjugated to FITC or PE. Isotype control staining was performed with IgG2b-FITC (1:200, eBioscience) and IgG2b-PE (1:200, eBioscience). The labeled cells were thoroughly washed twice in flow-buffer (5% FCS, 0.1% sodium-azid in PBS).

The intracellular detection was performed as described previously (Hamann et al., 1997). Cells were fixed with 4% paraformaldehyde (Sigam) in PBS. Fixation was followed by permeabilization with 0.1% saponin (Sigma/Fluka), 10% goat serum (Biological Industries, Israel) in PBS at 4°C for 10 min. Washing buffer containing 0.1% saponin, 0.5% bovine serum albumin (Sigma) in PBS was used for all subsequent incubation and washing steps. Cells were stained with anti neurofilament heavy 200 (NF-200; Sigma, 1:100) primary antibody. They were incubated for 30 min at room temperature (RT) and followed by second antibody conjugated with Alexa 488 (Molecular-Probes, Oregon, USA, 1:500). Control staining was performed only with the secondary antibody.

Cells were resuspended in 0.5 ml PBS and studied by a FACSCalibur™ flow cytometer using an argon ion laser, adjusted to an excitation wavelength of 488 nm (FACS; Becton Dickinson Immunocytometry System, San Jose, CA, USA). Data was acquired and analyzed by CELLQuest™ version 3.0 software (Becton Dickinson). A minimum of 10,000 events were examined per sample. A non-specific isotype control was included in each experiment, and specific staining was measured from the cross point of the isotype with the specific antibody graph. Each value is the mean ± S.E. if more than two independent experiments were involved.

Differentiation to adipocytes

Adipogenic differentiation was induced and cells were stained following detailed protocols by Peister et al. (2004). Briefly, the mMSCs were incubated in DMEM that was supplemented with 10% FCS, 10% HS, SPN, 12 mM L-glutamine, 5μg/mL insulin (Sigma), 50 μM indomethacin (Sigma), 1 μM dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). The medium was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 min at RT and stained with 0.5% Oil Red O (Sigma) in methanol (Sigma) for 20 min at RT. Adipogenic differentiation was identified by Oil Red O staining of lipid vacuoles, seen as bright red inclusions within the cells.

Differentiation to neuron-like cells

mMSCs were cultured for at least 14 days, as described above. To induce differentiation to neuron-like cells we used our previously described protocol with some modifications (Levy et al., 2003; Blondheim et al., 2006).

Growth medium was replaced with Differentiation Medium I consisting of DMEM supplemented with 10% FCS, 2 mM glutamine, SPN, 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA), 10 ng/ml epidermal growth factor (EGF; R&D Systems), and N2 supplement (5μg/ml insulin; 20 nM progesterone; 100 μM putrescine; 30 nM selenium; 100 μg/ml transferring; all from Sigma) (Bottenstein, 1985), for 24–48 h. Differentiation media-I was then removed and cells were washed with PBS and transferred to Differentiation Medium II, composed of DMEM supplement with 2 mM glutamine, SPN, N2 supplement, 200 μM butylated hydroxyanisole (BHA; Sigma, Israel), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), and 1 μM all-trans-retinoic acid (RA; Sigma) for 48–72 h.

Western blot analysis

Protein extracts from mMSCs and neuron-like cells from mMSCs were prepared in 50 μl of cold buffer containing 105 mM Tris (Sigma), 5 mM EDTA (BDH Laboratory Supplies, Poole, UK), 140 mM NaCl (BioLab, Jerusalem, Israel), 10 mM sodium fluoride (Sigma), 0.5% NP-40 (United States Biochemical Corporation, Cleveland, OH, USA), 1 μM PMSF (Sigma). Homogenates were centrifuged at $13000 \times g$ for 20 min at 4°C, and supernatants were collected. Protein concentration was determined and 50 μg samples diluted 1:5 with sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% Glycerol, 2% sodium dodecyl sulfate, 5% 2-β-mercaptoethanol, 0.0025% bromophenol blue, Sigma) and boiled for 5 min heated prior to loading. Proteins were size fractionated on 12.5% SDS-polyacrylamide gels and electroblot transferred to polyvinylidene difluride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with primary antibodies mouse anti TH (1:10000, Sigma), and actin (1:1000, Chemicon, Temecula, CA, USA) was used to evaluate and quantify the changes during the induction. Membranes were then exposed to horseradish-peroxidase conjugated goat anti-rabbit IgG diluted at 1:25000, or anti-mouse IgG diluted at 1:20000 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), for 30 min at room temperature. The membranes were then stained using the enhanced SuperSignal® chemiluminescent detection kit (Pierce, Rockford, IL, USA) and exposed to medical X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometry of the specific proteins bands was preformed by VersaDoc® imaging system and Quantity One® software (Bio-Rad Laboratories).

Immunocytochemistry

mMSCS were plated and treated in slides chamber (Nalge Nunc International, Napervilee, IL, USA) previously treated with poly-L-lysine (Sigma). Cells were fixed with 4% paraformaldehyde (Sigma) and blocked with 0.1% Triton X-100 (Sigma) and 10% goat serum (Biological Industries, Israel) in PBS. The differentiated mMSCs were stained with the mouse antibodies against TH (1:2000 Sigma), neuronal nuclei antigen: Neu-N (1:40, Chemicon). Appropriate Cy3-labeled secondary antibodies (1:400 v/v; Jackson ImmunoResearch Laboratories) were used for visualization.

6-Hydroxydopamine lesion in mice

c57/bl male mice (30 gr) were anesthetized with chloral hydrate 350 mg/kg intra-peritoneally (i.p.) and secured in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Mice were unilaterally injected with 6-OHDA hydrobromide (4 μg in 2 μl saline with 0.01% ascorbate, Sigma) using a Hamilton 10 μl syringe with a 26-gauge needle (Hamilton, Reno, NV, USA), into the right striatum, at rate of 1 μl/min. The coordinates of the injections into the striatum were as follows: anterior 1.1 mm, lateral 2.3 mm, dorsa ventral 4.2 mm, with respect to bregma, based on the mice Stereotaxis Atlas (Paxinos and Franklin, 2001). At the completion of the injection, the needle was left in place for another 3-min period and then withdrawn at 1 mm/min in order to prevent a vacuum. Lesioned mice were tested for ipsiversive rotational behavior induced by an intraperitoneal (i.p.) injection of amphetamine (10 mg/kg; Sigma) 14 days after the 6-OHDA lesion. This test is widely used as a reliable index of dopamine depletion in the striatum (Hefti et al., 1982; Carman et al., 1991; Hudson et al., 1993; Thomas et al., 1994; Pavon et al., 1998). The clockwise turnings of each animal were measured visually, in turn, in a round tool for a period of 30 min. Only mice with a rotation rate of above 160 turns per 30 min were considered to be an established PD model and were used later for the grafting and control experiments.

Cell transplantation

Three weeks after the 6-OHDA lesion, the mice were divided into four experimental groups: saline, fibroblasts, mMSCs and differentiated mMSCs ($n = 5$). Saline or 2×10^5 vital cells/2μl were stereotactically injected into the lesioned striatum using a stereotaxic frame (anterior 1.1 mm, lateral 2.3 mm, dorsa ventral 4.2 mm, with respect to bregma). The rotational behavior was measured for 30 min, 30 min following amphetamine injection (i.p. 10 mg/kg).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Jackson-Lewis and Liberatore, 2000) with some modification. Briefly, at the end of treatment, mice were anaesthetized with chloral hydrate (350 mg/kg), then perfused transcardially with 20 mL of saline for 3 min followed by 80 mL of 4% paraformaldehyde in 0.1 M sodium-phosphate buffer (pH 7.1). Brains were removed, fixed for 72 h at 4°C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 2 days at 4°C. The brains were frozen by immersion in dry ice-cooled 2-methylbutane and stored at −70°C until sectioned. For each mouse, cryostat-cut sections (20 μm) throughout the entire ventral midbrain were collected free floating and adjacent sections were stained for TH. Briefly, sections, were first rinsed (3×5 min) with 0.1 m PBS (pH 7.4), followed by incubation with 5% normal goat serum (NGS; Biological Industries) for 60 min. Sections were then incubated on a shaker with the primary antibody rat anti-TH (1:2000, v/v; Calbiochem, San Diego, CA) in PBS, containing 2% NGS and 0.3% Triton X-100, for 48 h at 4°C. After rinsing in PBS, secondary antibody donkey anti rabbit conjugated to AMCA or Cy3 (1:100 v/v; Jackson ImmunoResearch Laboratories) in PBS, pH 7.4, containing 2% NGS was added and the sections were incubated for 60 min at room temperature. Transplanted cells were identified by immunostaining using goat anti-EGFP antibodies (1:2000 v/v; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by second antibody donkey anti goat conjugated to Cy2 (1:100 v/v; Jackson ImmunoResearch Laboratories). All sections were then washed for 3×5 min in PBS, Sections were coverslipped with fluorescence mounting medium (DAKO, Denmark).

Microscopy and image analysis technique

An Olympus BX52TF microscope was used to analyzed slides for histopathology. ViewfinderLite™ software, with a DP50 microscope digital camera system attached to the microscopes, was used to acquire images and the StudioLite™ software was used to edit and analyze recorded images (Olympus, Tokyo, Japan).

Image analysis was performed on four representative areas of each slide using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD, USA). The analysis of brain slices used to quantify the entire area of the striatum, ventricle, thalamic nucleus nigrostriatal bundle, ventral tegmental, medial globus pallidus, medial forbrain bundle, internal capsule and

substantia nigra, was photographed in a series of nine frames (a total of 45 pictures for each brain).

Dopamine content analysis

Hemispheric dopamine (DA) levels were determined in the 6-OHDA lesioned mice. Each hemisphere was homogenized in 1 ml of 0.1 N perchloric acid (Sigma) and centrifuged at $15000 \times g$ for 15 min at 4°C. The supernatant was filtered through a nylon filter (COSTAR, Spin X HPLC, 0.22 µm; Corning, NY, USA). An aliquot of the filtrate was injected into a high performance liquid chromatography system with an electrochemical detector (HPLC-ECD) (LC-4B and TL-5A, Bioanalytical Systems, West Lafayette, IN, USA) equipped with a C18, reverse phase column (125 mm × 4.6 mm) (Hichrom, Berkshire, UK). The sample was eluted by a mobile phase made of a 150 mM monochloroacetate buffer (pH 3) containing 10% methanol, 30 mg/L sodium 1-octanesulfonate, and 2 mM EDTA at flow rate of 1.2 ml/min. DA peak was determined by electrochemical detection at a potential of 650–700 mV. DA was identified by retention time and was validated by co-elution with catecholamine standards under varying buffer conditions and detector settings.

Statistical analysis

All data presented as means ± standard error of the mean (SEM). Significance of the differences between the rotational behavioral data following amphetamine administration was analyzed by the one-way Anova (SPSS, version 11.5) in order to analyze the data presented in Fig. 5. All the *in vitro* experiments were performed at least twice, in triplicate, and a representative figure is shown. In all tests, significance was assigned when $P < 0.05$.

Results

Isolation and culture of EGFP-Tg mice MSCs

Mouse bone marrow cells were isolated from tibias and femurs bones of EGFP-Tg mice and were plated in a growth medium. The cells were incubated for two days and non-adherent cells were removed. The plastic-adherent cells from EGFP-Tg mice bone marrow, divided and grew to 80–90% confluency within about 20 days. During this culture period, the cells expanded from a few cells in the flask to 1.5×10^6, demonstrating the homogeneous morphology of mesenchymal cells, a typical spindle-like cell morphology (Fig. 1A).

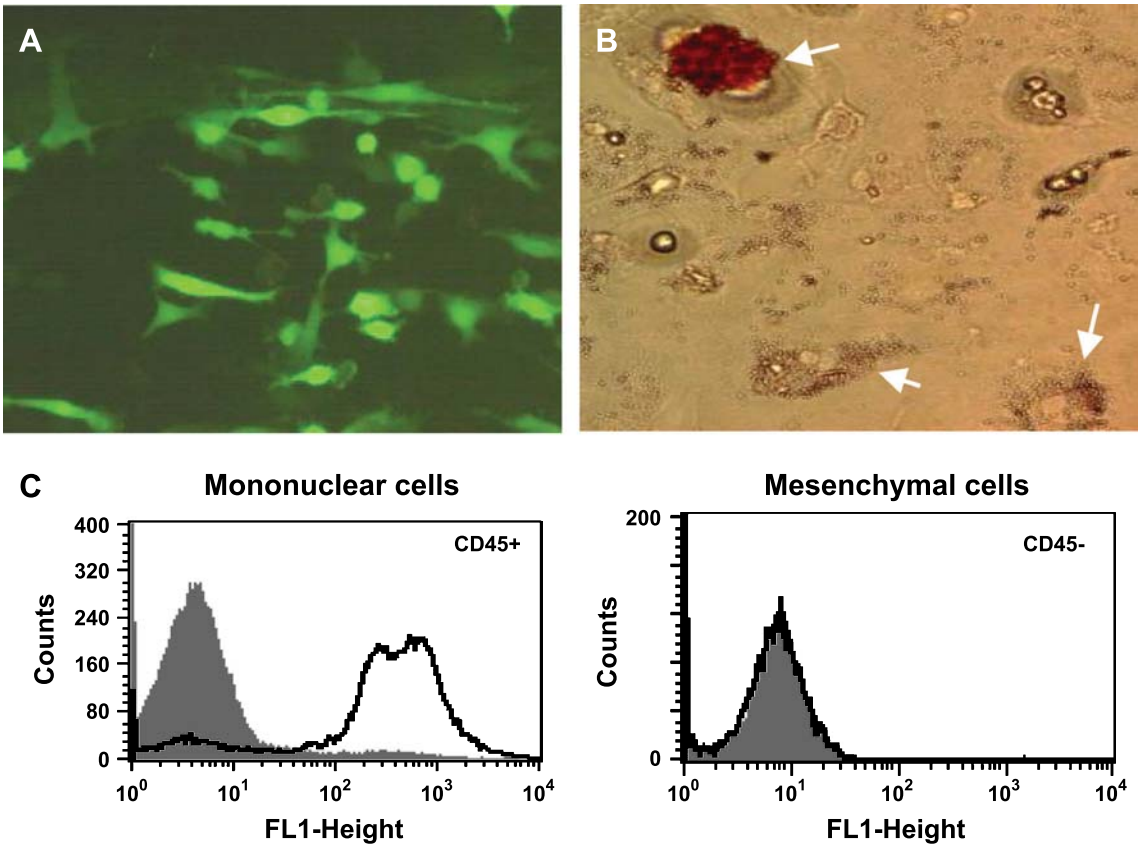

Fig. 1. Characterization of mouse multipotent mesenchymal stromal cells (mMSCs). **A** Isolated mouse mMSCs from EGFP transgenic mice. **B** mMSCs following adipogenic differentiation were stained by Oil Red O to detect lipid vacuole production by the cells (bright red inclusions within the cells). **C** Comparison of FACS analysis for CD45 of bone marrow derived mononuclear cells (left panel), with the plastic adherent cell population cultured *in vitro* over two weeks (right panel). After two weeks *in vitro*, a significant area of staining was found for hematopoietic markers CD45. Emphasized lines represent staining for non-specific IgG isotype fluorescence used as a control. Quantitative measurements were made from the cross point of the IgG isotype graph with the specific antibody graph

The cultured cells displayed further traits of MSCs, including the capability of readily differentiating into adipocytes as indicated by Oil-Red O staining when exposed to appropriate differentiation conditions (Fig. 1B).

The percentage of cells expressing mesenchymal markers significantly increased from day 0 to 20 and onward indicated by flow cytometry analysis. The cultured cells were positive for CD106 (27%) and CD90 (20%), typical mouse mesenchymal markers, and negative (<5%) for the hematopoetic marker CD45. In contrast, mononuclear cells in the bone marrow aspirate were positive to CD45 (80%, Fig. 1C). Although the mesenchymal cells isolated from different mice strains differ in their profile of surface markers, they share the same capacity to adhere to the plastic, show typical morphology and have a similar differentiation potential which indicate their identity as mesenchymal stromal cells (Peister et al., 2004; Dominici et al., 2006).

Differentiation of mMSCs to neuron-like cells

Following the 20 day of culture we induced neuronal differentiation by a two-step procedure. Firstly, cells were grown for 48 h in medium supplied with bFGF, EGF and N2 reagents. Secondly, cells were incubated with the serum free medium that contained BHA, RA, N2 supplement, and elevated cAMP for another 48 h, similar to the protocol we previously described for human MSCs (Levy et al., 2003; Blondheim et al., 2006; Hellmann et al., 2006). Microscope analysis indicated that during the induction of differentiation cells developed typical neural-like structures resembling dipolar cells, retractile cell bodies, neurites and axons, and long branching processes with growth cone-like terminal structures. The changes could be detected 24 h following the addition of the differentiation media and were sustained for three days (Fig. 2A). In contrast, cells grown in the expanded growth medium, exhibited the typical flat fibroblast-like morphology.

The neuronal-like morphological changes were accompanied by positive immunoreactivity for a typical neuronal marker. As indicated by FACS analysis, most cells exhibiting neuronal morphologies expressed neurofilament-200 (NF-200), a filament protein present in the axons and the synaptic terminals (Fig. 2B); neuron specific enolase (NSE), an isozyme of the glycolytic enzyme enolase expressed in

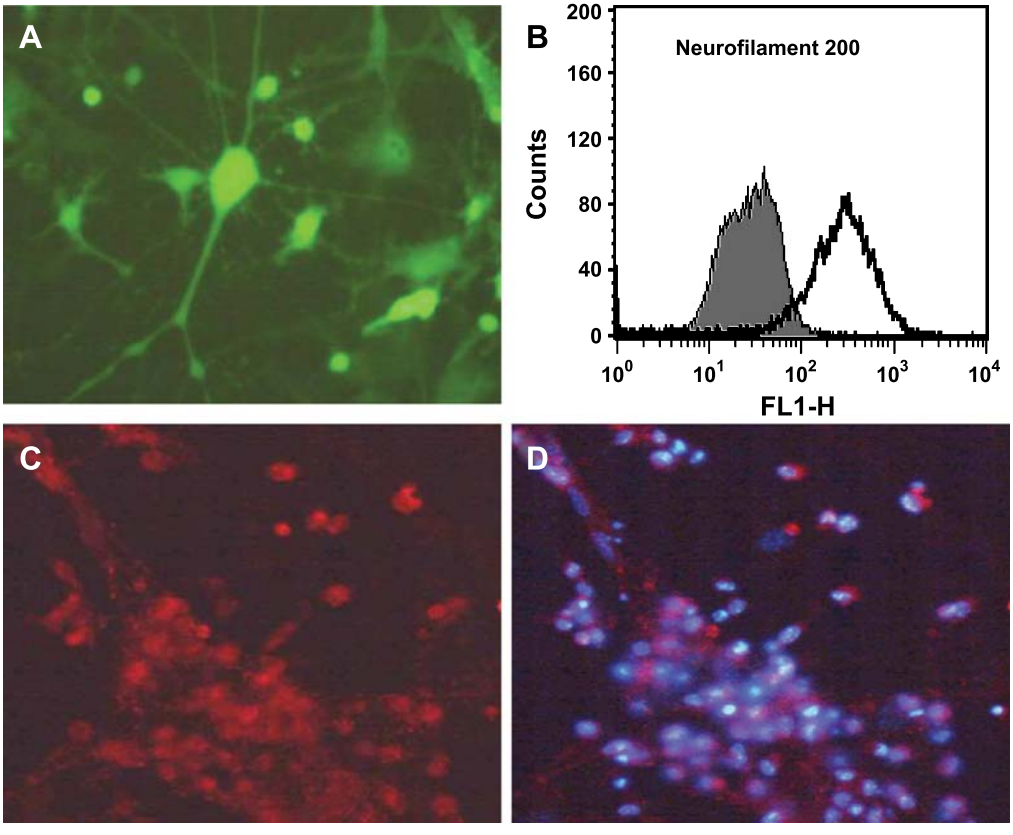

Fig. 2. Mouse multipotent mesenchymal stromal cells differentiate into neuron-like cells. Bone marrow cells were isolated from the femur and tibia bones of EGFP-Tg and differentiated to neuron-like cells. **A** Adherent cells after induction of differentiation. **B** FACS analysis for neurofilament-200. **C** Immunocytochemistry analysis with anti-NeuN antibodies and **D** nuclear DNA staining with DAPI

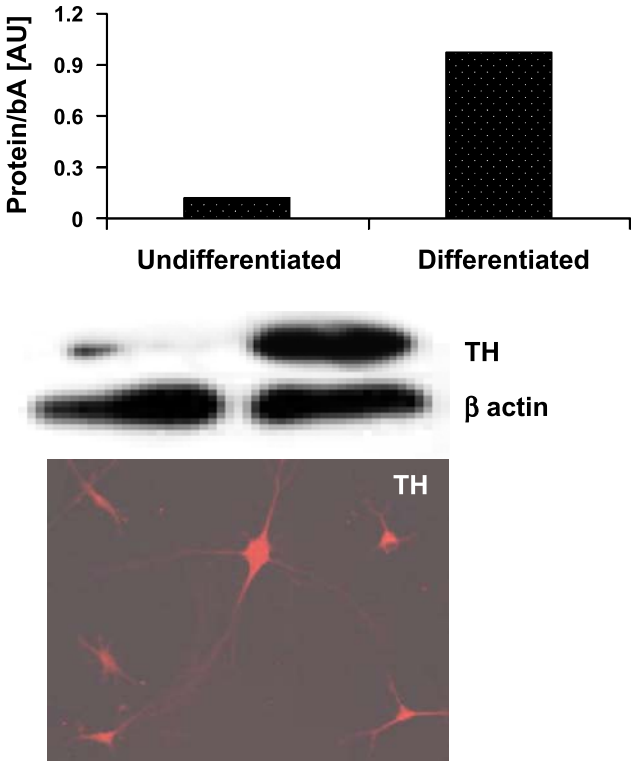

Fig. 3. Differentiated mouse multipotent mesenchymal stromal cells express tyrosine hydroxylase (*TH*). After 48 h of differentiation anti-TH were used for immunocytochemistry and Western blot analysis. The TH immunoreactivity and densitometry was normalized to the reaction with anti-beta-actin antibodies

all neuronal cell types (data not shown). In addition, the expression of the neuronal nuclei antigen Neu-N protein was markedly increased following the differentiation induction, as seen by immunohistochemistry (Fig. 2C, D).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of dopamine and a marker of ventral midbrain neurons. Furthermore, the differentiation induced a dramatic increase in tyrosine hydroxylase (TH) protein expression, as indicated by immunocytochemistry and Western blot analysis (Fig. 3).

Intrastriatal cell transplantation in a mouse model of PD model

To test whether the differentiated mMSCs might be beneficial following brain transplantation, we generated mice with DA-neuronal damage as an animal model of PD. As seen in Fig. 4, intrastriatal injection of 6-OHDA induced lesion and decreased the number of the TH positive cells in the substantia nigra. Accordingly, dopamine concentration was reduced by 50%, which indicates relatively moderate damage. The 6-OHDA lesioned mice emphasized the typical rotational-behavior induced by amphetamine.

Three weeks post 6-OHDA injection, MSCs and DA-differentiated cells, taken from EGFP-Tg mouse, were injected into the lesion striatum. The control groups included transplantation with non-differentiated mMSCs cartilage-derived ear fibroblasts and saline. During the three month experiment, amphetamine-induced rotations were compared to the number of rotations before the saline injection or cell transplantation of each mouse. The rotational behavioral was measured 2, 4, 6, 8, 12 weeks post engraftment for fibroblast cells and mMSCs group, or 3, 5, 8, 10, 11 weeks post engraftment for saline and differentiated mMSCs group. In the saline-injected group ($n = 5$), there were no changes and the mice demonstrated $92–106 \pm 8.7\%$, of the initial rotations during the experimental period (Fig. 5). The group of mice with engrafted fibroblast cells ($n = 5$) demonstrated stable rotational behavior for 12 weeks with no significant change (Fig. 5). By contrast, a moderate reduction of about 50% ($p < 0.05$) was seen after transplantation of EGFP non-differentiated mMSCs ($53 \pm 15\%$) (Fig. 5). However, transplantation of the DA-differentiated mMSCs cells demonstrated a marked reduction in the rotational behavior which peaked after 11 weeks by which time mice did not rotate at all following the amphetamine challenge ($13.5 \pm 8.5\%$, $p < 0.001$ vs. saline, Fig. 5).

Histological analysis was performed on the sacrificed mice at the end of the experiment (12 weeks). Most of the EGFP-positive transplanted cells were located in the striatum, around the injected area (Fig. 6). Surprisingly, few of the transplanted cells migrated to the neighboring areas along the dopaminergic track, whereas cells were observed in the nigrostriatal bundle, ventral tegmental, medial globus pallidus, medial forbrain bundle and internal capsule. Double immunostaining in the striatum revealed that some of the EGFP-positive transplanted cells were also TH positive, indicating a continuous stable expression of the dopaminergic marker 12 weeks post-transplantation (Fig. 6). Indeed, we found that low, but significant amount of cells migrated toward the substantia nigra indicated the presence of TH positive bone marrow derived EGFP cells adjacent to the endogenous dopaminergic cells.

Discussion

In the present study we further examined the differentiation induction of mMSCs into neuron-like cells. The mouse multipotent mesenchymal stromal cells underwent similar neural differentiation processes with minor modification to those noted in our previous studies using human MSCs

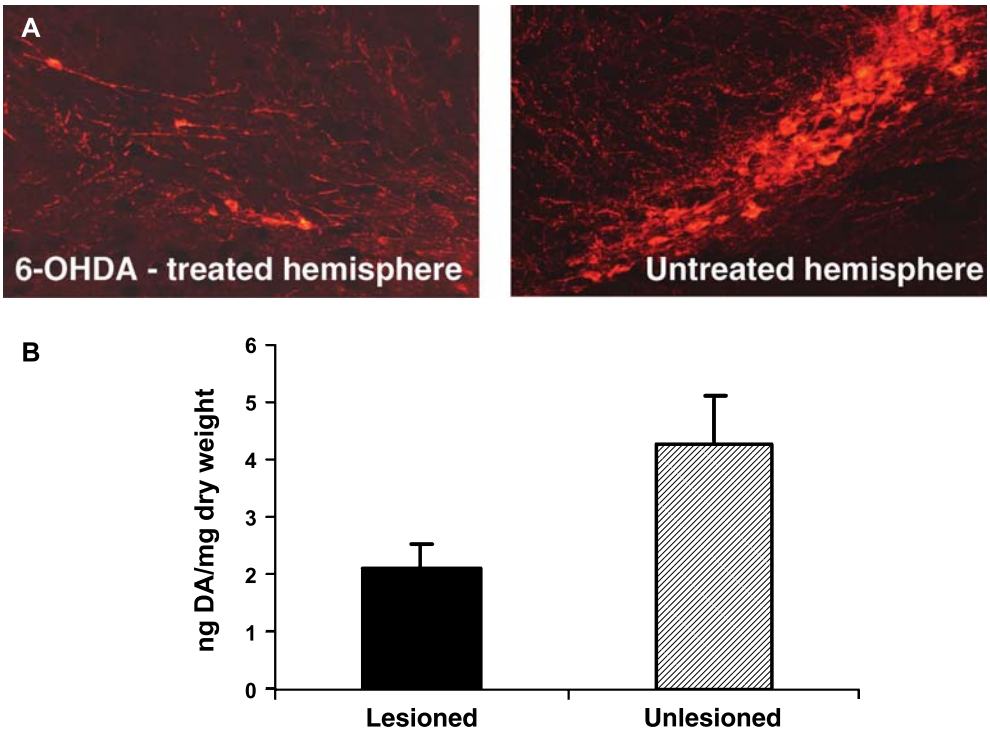

Fig. 4. Intrastriatal injection of 6-OHDA reduces the number of tyrosine hydroxylase (TH) positive neurons in the ipsilateral substantia nigra (SN). **A** Loss of dopaminergic cell bodies in the SN was achieved by injection of 6-OHDA to the mouse striatum as indicated by anti-TH staining. **B** The dopamine level in the hemisphere extract as measured by HPLC

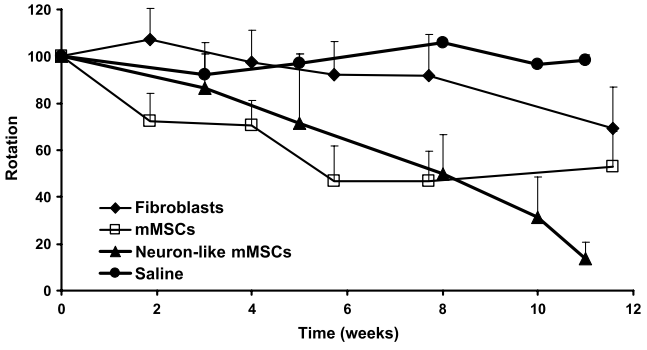

Fig. 5. Intrastriatal transplantation of differentiated multipotent mesenchymal stromal cells in 6-OHDA lesioned mice reduces amphetamine-induced rotational behavior. Three weeks after 6-OHDA injection, saline, fibroblasts, mMSCs and differentiated mMSCs (2×10^5 cells, n = 5). Rotational behavior was measured 30 min following amphetamine challenge, for a period of 30 min

(Levy et al., 2003, 2004; Blondheim et al., 2006; Kan et al., 2007). After three weeks in culture, the plastic adherent mMSCs expressed CD106 and CD90, which are known as mouse markers for mesenchymal cells (Baddoo et al., 2003; Peister et al., 2004). Although most of the fresh bone marrow contained a high percent of hematopoietic stem cells, the mMSCs subpopulation did not express the hematopoietic marker CD45. In addition, the cells were capable of differentiating into adipocytes, and demonstrating their characteristics.

Following neural differentiation protocol, cells changed their morphology from fibrocystic-like cells to a neuronal-like morphology including bipolar and long process formation. We also followed the expression of several neuronal specific markers such as Neu-N, NSE, NF-200 and found a marked increase in their presence during the three days of differentiation induction. Most interestingly, TH, the key enzyme for dopamine synthesis, was dramatically elevated, indicating that the cells differentiated into the dopaminergic pathway.

The latter assumption was further examined *in vivo*, using the unilateral instrastriatal 6-OHDA mouse model of PD. We have chosen to use mice to allow allogenic engraftment with $EGFP^+$ mMSCs. Unilateral 6-OHDA injections into the striatum caused a marked decrease in striatal TH^+ immunostaining terminals and also in the dopamine levels, as indicated by HPLC. The lesions also induced a rotational behavior after amphetamine challenge. This mouse 6-OHDA model has been used in several studies and it was shown that both the terminals and the cell bodies were damaged although not all of the nigral dopminergic cells

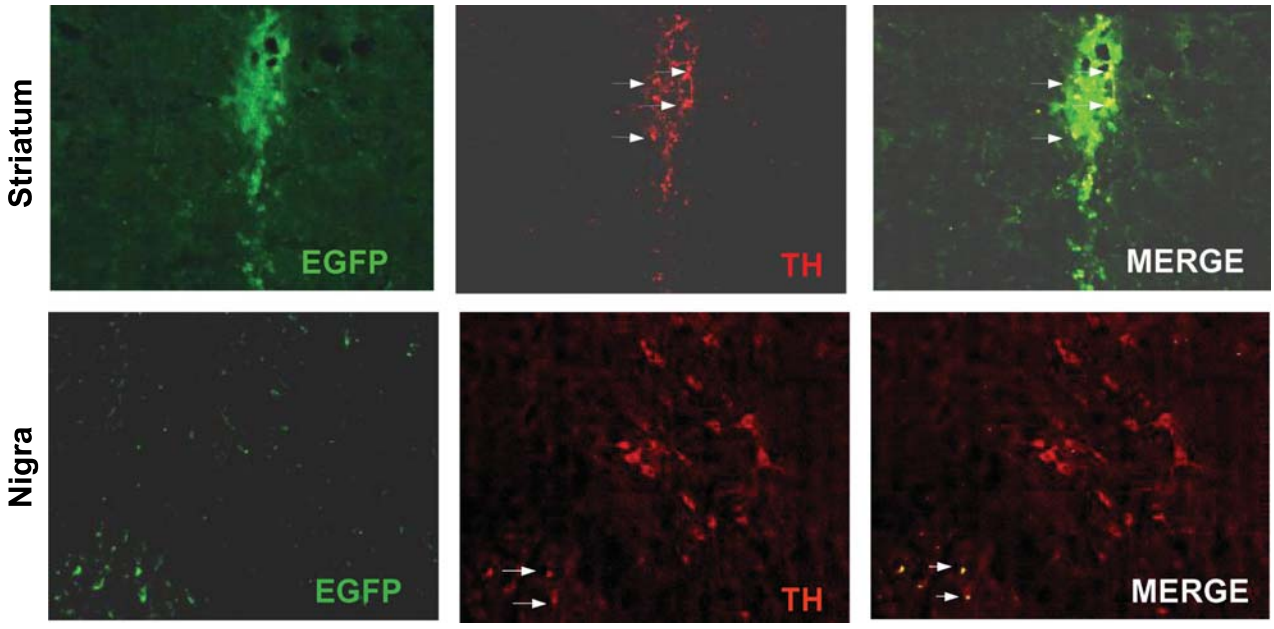

Fig. 6. Survival and migration of engrafted differentiated multipotent mesenchymal stromal cells in the injected hemisphere. Immunohistochemistry analysis using anti-tyrosine hydroxylase (TH) and anti-EGFP antibodies revealed the presence of TH-expressing cells among the transplanted EGFP-positive cells in the injection site (superior panel). TH^+-EGFP cells are also seen in the substantia nigra, probably after migration (lower panel)

disappear (Akerud et al., 2001). Therefore, recovery of the dopaminergic system should include replacement of the dopaminergic neuronal loss.

As we described previously (Levy et al., 2004; Blondheim et al., 2006), exposure to N2 supplement, BHA, RA, and an elevated of intracellular cAMP level, direct the MSCs to neuronal differentiation in human MSCs. In the present study we used mouse MSCs for transplantation in mouse model of PD. We observed a significant improvement two weeks following transplantation of the DA-differentiated MSCs, which constantly increase during the experiment and show almost full recovery after 11 weeks. The (non-differentiated) MSCs also show benefit six weeks post transplantation, than the rotational behavior was stable demonstrating no further improvement.

The capability of MSCs to protect and even regenerate affected neurons have been reported in various animal models of neurodegenerative diseases, such as multiple sclerosis (Zhang et al., 2005, 2006), amyotrophic lateral sclerosis (Mazzini et al., 2006) and stroke (Mahmood et al., 2005; Seyfried et al., 2006; Shen et al., 2007).

Two previous studies demonstrated some improvement of MSCs grafted into model of PD. The first, Li et al. (2001) grafted undifferentiated mMSCs into the striatum of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. The grafted MPTP-treated mice exhibited significant improvement on the rotarod test at 35 days after transplant, compared to nongrafted controls. However, only ~0.8% of the implanted cells expressed TH immunoreactivity. The second, Dezawa et al. (2004) showed motor improvement in a rat model of PD following intrastriatal implantation of MSCs transfected with Notch intracellular domain (NICD).

Indeed, it has already been shown in rodents and monkeys and even in some parkinsonian patients that replacement and significant reduction in the symptoms can be achieved with a relatively low number of engrafted dopaminergic cells (Studer et al., 1998; Takagi et al., 2005; Piccini et al., 1999). However, our study is the first time that neuronal-like cells differentiated from MSCs, without artificial gene overexertion, exhibited a long period of viable engraftment, cell survival and demonstrated improvement in the PD model. Further investigation is required to understand the mechanism of recovery. It is not known whether the grafted cells increase production of DA or whether other processes, such as the secretion of neurotrophic factors by the marrow-derived cells, mediate the improvement in motor function (Arnhold et al., 2006; Chen et al., 2005) and neurogenesis (Chen et al., 2003; Mahmood et al., 2005). Furthermore, we cannot exclude the possibility that a small number of MSCs differentiated into dopaminergic cells in the transplanted environment as we, and others, found that MSCs express low levels of neuronal markers and the show predisposition to differentiated into mature neurons (Blondheim et al., 2006). In contrast to the MSCs, the differentiated cells demonstrated a constant

reduction in the rotational behavior until almost full recovery after 12 weeks. The improvement was associated with the survival of a considerable amount of the engrafted (GFP^+) cells. Moreover, most of the cells remained in the striatum and a significant percent of them expressed TH. Notably, the engrafted cells could be detected in the striatum-nigra track and even a few GFP-TH^+ cells integrated into the nigra. Since we have no data on the changes in the dopamine level following transplantation we cannot conclude that the engrafted cells replaced the original cells. However, we can assume that the improvement may have been due only partially to the undifferentiated MSCs and mainly to TH^+ cells. The migration of the TH^+ cells to the substantia nigra may also indicate their relevance to the relief of symptoms. Our data is similar to the reported benefits of embryonic stem cells with neuronal and dopaminergic characteristics in rodent models of PD (Ben-Hur et al., 2004; Brederlau et al., 2006; Roy et al., 2006). However, this is the first report on the symptomatic amelioration following allogeneic transplantation of TH^+-differentiated bone marrow derived cells.

In conclusion, we have shown that differentiated mouse MSCs express neuronal markers including TH and when transplanted into the striatum, can improve motor behavior in a mouse model of PD. Our findings suggest that differentiated dopaminergic neurons, generated from adult bone marrow-derived stem cells, may be used in autologous transplantation for neurorestoration in parkinsonian patients.

Acknowledgment

This study was partially supported by The Norma and Alan Aufzien Chair for Research in Parkinson's disease (EM) and Brainstorm Cell Therapeutics Ltd.

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J Neural Transm (2007) [Suppl 72]: 145–148

Printed in Austria

Journal of Neural Transmission

Low dose methylphenidate improves freezing in advanced Parkinson's disease during off-state

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Summary Five men with advanced idiopathic Parkinson's disease (PD) were examined to assess the effect of low dose methylphenidate (MPD) on gait. The patients were tested during "off" state before and two hours after the intake of 10 mg MPD while walking an "8 trajectory". The total walking time, total freezing time, number of freezing episodes and the non-freezing walking time were assessed. The obtained data were compared by the Wilcoxon Signed Rank test with a type I error rate of 0.05. The results showed a statistically significant improvement in all gait parameters after MPD intake. Moreover, a good correlation in the grade of improvement for each individual gait characteristic was found. The study demonstrates that low dose of MPD may improve gait, and especially freezing, in patients with severe PD, without the need for exogenous L-dopa. The mechanism of MPD action in patients with advanced PD is further discussed.

Keywords: Parkinson's disease, freezing, methylphenidate

Introduction

One of the common and incapacitating features of parkinsonian gait is freezing (gait freezing-GF). GF is a usually short period of immobility, despite the efforts of the patient to overcome the motor block (Panisset, 2004). It can appear spontaneously during walking or in certain situations, such as when the gait is initiated, on turning, when obstacles are encountered or when the patient is approaching a target (Fahn, 1995). GF is not associated with plasma dopamine levels and can occur during "on" as well as during "off" states (Barbeau, 1976). It tends to appear later in the course of the disease and is a common cause of falls and related complications in patients with Parkinson's disease (PD) (Balash et al., 2005).

The anatomical and functional basis for GF is not yet understood. It was suggested that basal ganglia disease in PD leads to dysfunction of automatic locomotion at the level of the spinal cord (Hashimoto, 2006). Consequently, frontal cortical locomotor centers become responsible for gait regulation, turning thus the gait to a voluntary action and explaining some of the features of GF (Honey et al., 2003; Amsten, 1997).

Several studies have identified decreased levels of some neurotransmitters in the cerebrospinal liquor, brain and serum in PD patients with GF. For example, GF has been associated with low dopamine, norepinephrine, serotonin, homovanillic acid and 5-hydroxyindoleacetic acid (Toghi et al., 1993; Finnegan, 1993). Consequently, administration of drugs with dopaminergic activity (selegiline, rasagiline) or norepinephrine precursors (L-threo-3,4, dihydroxyphenylserin) has been tried with varying results (Toghi et al., 1993b; Shoulson et al., 2002; Rascol et al., 2003; Toghi et al., 1993c). Lately, several studies examined the effect of methylphenidate (MPD) – a dopamine transporter (DAT) inhibitor – on motor functions, and particularly on gait in PD (Auriel et al., 2006; Camicioli et al., 2001). Most results showed an enhancing effect of MPD when administered together with L-dopa leading to a significant improvement in gait and freezing episodes, while MPD alone caused no improvement. However, these studies differed by their methodology such as MPD dosage, route of L-dopa administration and outcome measures as well as the severity of PD in tested patients.

In the present study we examined the effect of low dose of MPD on gait freezing in patients with severe PD during off state.

Methods

Subjects

Five men with idiopathic PD were examined. Their characteristics are summarized in Table 1.

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Table 1. *Data of patients with PD*

Patient	1	2	3	4	5
Stage	IV	III	IV	IV	III
MMSE	27	24	26	24	19
Hamilton Depression scale	13	21	19	15	19
L-dopa (mg/day)	1162	400	1150	375	1000
Pergolide (mg/day)	3	2	–	–	–
Entacapone (mg/day)	1000	–	600	400	800
Amantadine (mg/day)	–	–	–	200	100
Fluvoxamine (mg/day)	–	50	–	–	100
Clozapine (mg/day)	–	–	–	25	–
Trihexyphenidyl (mg/day)	–	–	–	–	6.25

The mean age was 71.8 ± 7.9 years (range 64–82).The severity of the disease, according to the Hoehn and Yahr classification, was stage III in three and stage IV in two patients.

The Mini Mental State Exam scores (MMSE) were more than 24 in all but one patient whose MMSE was 19, indicating dementia. When tested for depression by the Hamilton test, two patients who were treated with antidepressants, exceed the score of 17.

All patients were L-dopa responsive. The mean therapeutic dose of L-dopa was 817.4 ± 397.7 mg daily (range 375–1162 mg daily).

Four out of five patients were treated with the COMT-inhibitor entacapone, two with the dopamine-agonist pergolide and further two patients received amantadine. One patient had a history of L-dopa induced psychosis which was managed by low doses of clozapine.

Protocol

The study was approved by the local committee of the Helsinki conference and the patients gave an informed written consent before taking part in the study.

Twelve hours before examination, all antiparkinson medication was stopped. The gait of patients was tested by the same physiotherapist at two conditions: before and two hours after a single oral administration of 10 mg MPD.

The patients were instructed to walk, as quickly as they can, on trajectory that passes around two chairs placed at 3 and 6 m from the starting sitting position. The patient had to get up and to walk towards the first chair, bypass it on the left side, then continue to the second chair, surround it on the right and return to the starting position while passing the first chair on the left before sitting down again. This recently induced test in our clinic, called the "8 trajectory", proved to be a simple clinical testing method which can be easily applied in every outpatient clinic.

The measured gait parameters consisted of the total walking time (TWT), total freezing time (TFT) i.e. the sum of duration of each single freezing episode of the patient, number of freezing episodes (TFE) and the total non-freezing walking time (TNWT) i.e. the time of walking the trajectory disregarding freezing. Each patient was tested three times and the results were averaged for each patient.

Statistical methods

In view of the non-continuous distribution of the individual test results, the nonparametric Wilcoxon Signed Rank test was applied to compare the averages of the different gait parameters before and after MPD administration. All tests were two-tailed and used a type I error rate of 0.05.

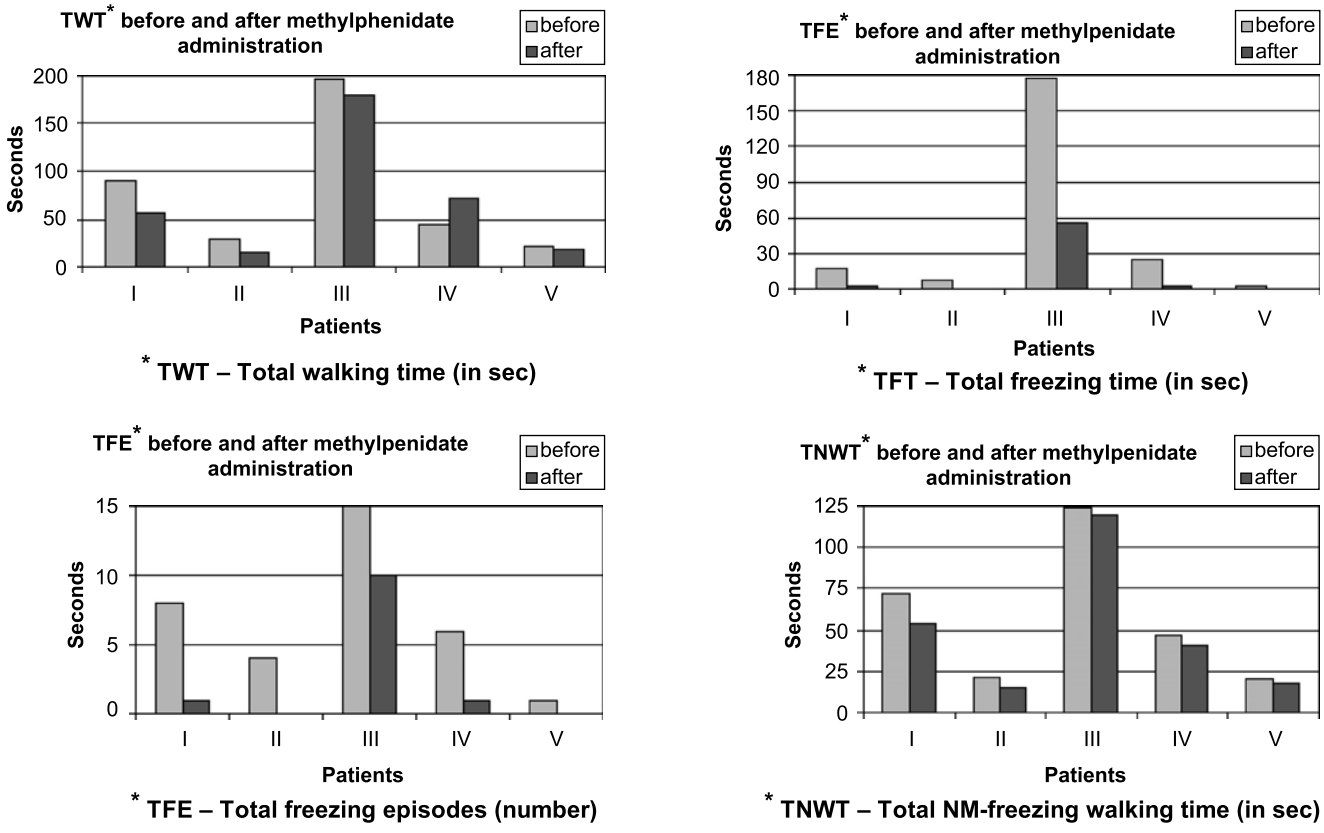

Fig. 1. Histograms showing changes in gait parameters before and after methylphenidate administrated in individual patients

Table 2. *Comparison of gait parameters of PD patients before and after methylphenidate administration*

Gait parameter	Before*		After*		Significance**	Correlation
	Mean	SD	Mean	SD		
TWT	76.4	72.4	68.4	67.06	0.013	0.950
TFT	46	73.7	12.4	24.4	0.000	0.990
TFE	6.8	5.2	2.4	4.2	0.029	0.915
TNWT	56.8	43.2	49.6	42.5	0.001	0.989

* Before and after methylphenidate administration.
** Wilcoxon Signed Ranks test.
TWT Total walking time (in sec), *TFT* total freezing time (in sec), *TFE* total freezing episodes (number), *TNWT* total non-freezing walking time (in sec).

Results

The effects of MPD on distinct gait parameters are illustrated in the histograms (Fig. 1).

Table 2 demonstrates the differences in gait variables measured before and after MPD intake. The results show that there was a statistically significant improvement in all tested gait characteristics with MPD. There was also a good and consistent correlation in the grade of improvement for each individual gait parameter.

Discussion

MPD raises the extracellular dopamine level by blocking the DAT and preventing the reuptake of dopamine into the neuron (Nutt et al., 2004). During normal nerve activity, extracellular dopamine levels rise transiently after stimulation due to pulsatile release of dopamine from the nerve terminal (Seeman and Madras, 1998). At low therapeutic doses (0.2–0.5 mg/kg), MPD elevates the resting dopamine level in the synaptic cleft by blocking DAT. However, the raised levels of dopamine in the synapse bind to the presynaptic dopamine D2 receptors located on the nerve terminal which in turn inhibit the pulsatile release of dopamine following stimulation (Meador-Woodruff et al., 1994). The final effect of the low dose of the drug is thus decreasing the relative rise in the pulsatile release of dopamine. This mechanism possibly explains how MPD reduces psychomotor activity in hyperactive children (Seeman and Madras, 1998).

On the contrary, higher doses of MPD cause a marked elevation of the resting extracellular dopamine but also increase its pulsatile release (Seeman and Madras, 1998). This results in a big outflow of dopamine that cannot be overcome by presynaptic inhibition and leads to a generalized stimulation of the nervous system, including enhanced motor activity.

The question rises how does MPD at low doses improve gait in PD patients?

In PD, dopamine nerve terminals and consequently DAT are reduced 30–50% in early disease and more in severe PD (Ichise et al., 1999; Ribeiro et al., 2002, Seibyl et al., 1995). Administration of low dose MPD to patients with early disease would have little or no effect after blockage of DAT, due to the relative preservation of dopamine striatal terminals. In advanced PD the presynaptic dopamine D2 receptors are substantially reduced and even a low dose of MPD that increases the extracellular dopamine level, may overcome the inhibitory action of the reduced pool of presynaptic receptors and result in net stimulation of the postsynaptic dopamine receptors by dopamine.

A further question relates to the effect of MPD on motor activity in the absence of exogenous L-dopa, i.e. when the patients were without antiparkinson medication overnight and actually in an ''off state''. Most studies reported that MPD was pharmacologically active only when administered with L-dopa. One proposed explanation was that MPD has less effect on extracellular dopamine concentration in subjects with low dopamine neuronal activity and dopamine release. However, our patients showed a good motor response to MPD without concomitant L-dopa treatment. This again can by explained by the fact that we tested only patients with advanced disease (and severe reduction of the striatal terminals) where also low levels of endogenous dopamine, without the need of exogenous dopamine, precluded the inhibitory action of presynaptic receptors (Seibyl et al.,1995; Benamer et al., 2000).

In addition to dopamine enhancement, other mechanisms may play a role in MPD induced gait improvement (Panisset, 2004). The effect of the norepinephrine precursor L-threo-3,4-dihydroxyphenylserine (L-DOPS) on freezing in PD patients was repeatedly tested with conflicting results (Panisset, 2004; Toghi et al., 1993a). Altogether, the gait improvement after L-DOPS is incomplete and inconsistent and might be due to pharmacological properties of the drug. Despite its relative specificity for DAT in the striatum, MPD is known to inhibit also the norepinephrine transporter (Seeman and Madras, 1998; Nutt et al., 2004). In the state of dopamine depletion, as in advanced PD, administration of MPD could lead to an increase of norepinephrine levels and contribute to gait improvement by stimulating the brainstem-, norepinephrine-dependent locomotor centers that project directly on the spinal cord and modify gait pacing.

In summary, our study shows that MPD may improve gait and freezing in advanced PD already at low doses, without the need for exogenous L-dopa. However, GF occurs during the ''off'' as well as during the ''on'' state and the

patients in our study were tested while "off". In the future more extensive examinations of MPD influence on freezing during these two functional states would thus be certainly justified as GF while "on" is still a challenge for treatment.

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J Neural Transm (2007) [Suppl 72]: 149–154

Printed in Austria

Journal of Neural Transmission

The long-term effects of the neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) on cognitive performance in rats

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Summary The neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) has been reported, both in vitro and in vivo models, to produce neurodegeneration and parkinsonian symptoms after prolonged exposure in rats. The aim of the present study was to investigate the effects of TaClo on the cognitive performance of rats. We used the COGITAT hole board system where rats can find hidden pellets by exploring the board. TaClo-treated rats found as many pellets as control rats treated with saline. Furthermore, their search was as efficient as that of control animals since there were no differences between the groups regarding explorative activity, visits to non-baited holes and time needed to find the pellets. These results suggest that there is no deficit in spatial memory following the chronic administration of TaClo to rats.

Keywords: 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline, TaClo, cognition, spatial memory, COGITAT, rat, Parkinson's disease, animal models

Introduction

The neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) bears striking similarities to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that is widely being used to produce an animal model of Parkinson's disease (Riederer et al., 2002). Both TaClo and MPTP are complex I inhibitors; TaClo is in this regard about 10 times more potent than MPTP (Bringmann et al., 1995; Janetzky et al., 1995). Furthermore, TaClo induces an increase in hydroxyl radicals (Gerlach et al., 1998) and neuronal degeneration in cell culture experiments. In line with this degeneration, a reduction of dopamine uptake by 66% has been found (Rausch et al., 1995). Furthermore, Bringmann et al. (2002) have found a reduction in tyrosine hydroxylase (TH) activity and a reduced formation of L-Dopa (L-3,4-Dihydroxyphenylalanin) in the nucleus accumbens of rats (Bringmann et al., 2002). Four to nine days after chronic TaClo administration the spontaneous locomotor activity in TaClo-treated rats was increased compared to saline-treated rats indicating an increased dopaminergic activity in TaClo rats. Chronic administration of TaClo induces a behavioural supersensitivity to the dopaminergic agonist apomorphine and the behavioural response to apomorphine changes over time (Sontag et al., 1995). After apomorphine challenge, the increase in locomotor activity in TaClo-treated rats was smaller than in saline-treated rats. This finding indicates either a reduced sensitivity to apomorphine or an exhausted dopaminergic system in TaClo-treated rats. However, 12 weeks after TaClo administration, when TaClo is completely metabolized, the apomorphine-induced locomotor activity in TaClo-treated rats was lower than after four to seven days. These findings suggest a slowly progressive functional lesion of the nigrostriatal dopaminergic system. In contrast to these observations, the effects of MPTP can be seen shortly after its administration. TaClo appears therefore to mimic the slow progressive neurodegeneration of Parkinson's disease better than MPTP. Furthermore, TaClo can be formed endogenously by the condensation of tryptamine and trichloroacetaldehyde. One source of trichloroacetaldehyd could be volatile chlorinated organic solvents found in the environment. A possible connection between idiopathic Parkinson's disease in humans and the exposure to chlorinated organic

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solvents has been shown by Kochen et al. (2003). TaClo may therefore serve as a useful animal model of Parkinson's disease.

A good animal model should mimic most if not all symptoms of a disease. The most prominent symptoms of Parkinson's disease are motor disturbances. In addition, cognitive deficits are present in most patients with Parkinson's disease (Brown and Marsden, 1988; Zgaljardic et al., 2003; Pahwa et al., 1998; Taylor and Saint-Cyr, 1995). These deficits include impairments in executive functions, attention, memory, visual-spatial abilities and speech. While the importance of dopaminergic dysfunction in the brain in connection with the motor symptoms in Parkinson's disease is well established, the involvement of central dopaminergic systems in the frontal lobe-like deficits of executive functions is less clear. Dopaminergic dysfunction may contribute to impaired ''frontal'' cognitive performance in several ways. First, there is a direct mesencephalic dopaminergic projection to the prefrontal cortex (Bjorklund and Lindvall, 1984), which is affected in Parkinson's disease (Javoy-Agid and Agid, 1980). In the rhesus monkey, depletion of frontal dopamine causes impairments in delayed alternation which are reversible by administration of L-Dopa (Brozoski et al., 1979). In patients with Parkinson's disease, frontal lobe-like deficits have been shown to be improved following treatment with L-Dopa (e.g. Lange et al., 1992, 1995). Second, there is a close association between the frontal lobes and the striatum via cortico-striato-thalamo-cortical loop systems (Alexander et al., 1986) and lesions of different parts of the striatum can produce cognitive alterations similar to those caused by frontal lobe damage (Divac et al., 1967).

So far only the effects of TaClo on motor behaviour have been assessed in rats. The aim of the present study was to investigate the long-term effects of chronic treatment with TaClo on the cognitive performance of rats. We used the COGITAT hole board system where the rats can find hidden pellets by exploring the board. The hole board used allows the investigation of various cognitive functions including working memory and spatial memory (for details see Heim et al., 2000).

Material and methods

Animals

Male Wistar rats aged two to three months (weight 200–260 g) were used. The animals were kept on a 12:12 light/dark circle (room temperature 21°C, humidity 55%). The rats were divided in two groups. The rats of the TaClo group ($N = 12$) received a daily injection of 0.2 mg/kg TaClo for seven weeks and the control animals ($N = 13$) were injected daily with saline for seven weeks. Both TaClo and saline were administered intraperitoneally. All experiments were performed in accordance with national laws concerning animal experiments.

The COGITAT Hole Board

The learning behaviour of the rats was tested with the COGITAT Hole Board System (Cogitron GmbH, Göttingen, Germany) This system consists of a board (size 825 × 825 mm inner surface) with 25 holes (Fig. 1) and is surrounded by an acrylic glass wall (height 270 mm). Each hole (diameter 60 mm; distance apart center to center, 165 mm) consists of a cylindrical tube which is closed at its lower end by an adjustable feeding plate (60, 75, 90 mm below the upper surface) with a depression for a food pellet (sucrose pellets 0.0451 g, Bio-Serv Frenchtown, NJ, USA). Feeding plate and food pellets were of the same colour. The ground below the feeding plate was covered with the same pellets as those used in the cylindrical tubes, in order to prevent the animals from finding the pattern of the pellet distribution by using olfactory stimuli. Each hole was fitted with infrared light beams at different levels of the hole to measure activity. The first level was 10 mm beneath the surface to record head dips at the upper level of the tube (inspections of the holes). The second level was about 20 mm above the

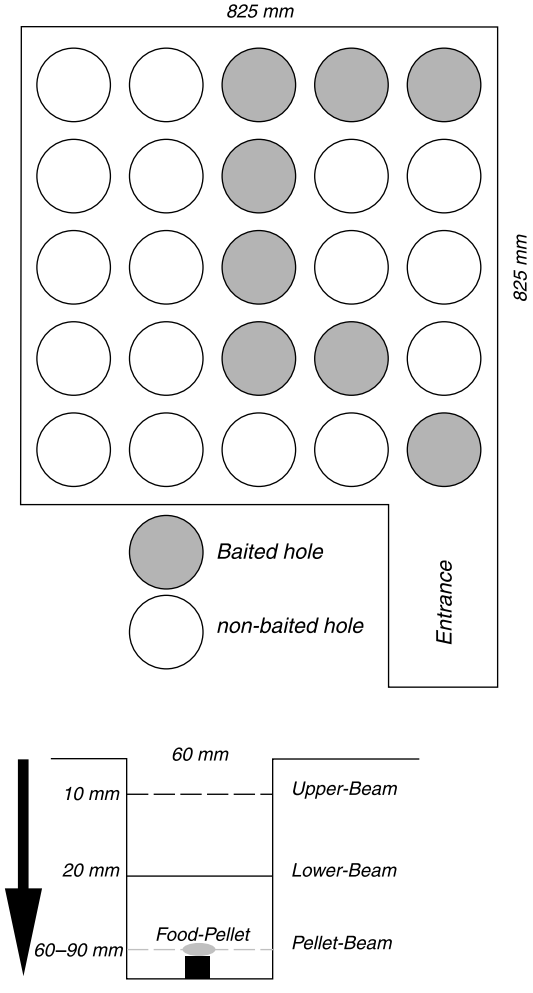

Fig. 1. Schematic drawing of the COGITAT-System

pellet to record deep exploration into a hole (visits to the hole). Finally, there was an infrared beam at the feeding plate measuring the collection of the food pellet. A more detailed description of the COGITAT Hole Board System can be found elsewhere (Heim et al., 2000).

Feeding procedure

The rats were put on starvation rations during the week prior to testing with the COGITAT System and throughout the subsequent test periods. The animals' weight was carefully controlled and a weight reduction of more than 10–15% was avoided in order to prevent stress and subsequent changes in the dopaminergic system. The rats were fed one hour prior to the start of testing. This procedure was chosen for two reasons: (1) rats awaiting their daily feeding after testing may not search properly during the trials; (2) feeding shortly before testing avoids the decrease in dopamine release associated with chronic food deprivation (Pothos et al., 1995).

Cognitive performance tests

The testing of the rats started approximately eight months after the end of the injections of TaClo or saline, respectively. The rats were adapted to the laboratory in their home cages for one hour and fed with 12–15 g of laboratory chow. In this experiment, eight of the 25 holes were baited (Fig. 1). The rats were tested in random order. At the start of testing each animal was allowed an adaptation period of 10 s in the start box at the entrance of the board. A trial was completed when a rat had collected all pellets within a given period of time or when the given time span had elapsed. After the end of each trial the rats were placed into their home cages.

The whole experiment was divided into two parts. In the first part, the rats were allowed five trials (one per day over five days) of 180 s each to explore and find the pellets. In the second part, there were 10 trials (one per day over 10 days) of 60 s each to find the pellets.

Statistics

Statistical analysis was performed using non-parametric tests. The comparison within trials was performed using the Friedman test for each group separately. The comparisons between groups of each single trial were performed using the Mann–Whitney *U*-test. For statistical analysis an alpha level of 0.05 was applied. All statistical analyses were carried out using the Statistical Package for Social Sciences 12.0 for Windows.

Results

Overall explorative activity (the sum of inspected and visited holes, with and without food collection Fig. 2)

180 s

Both groups showed an increase in explorative behaviour (Friedman test: NaCl, $p < 0.001$; TaClo, $p < 0.001$). Between the groups there were no significant differences regarding single trials.

60 s

The two groups did not differ with regard to explorative behaviour and showed no increase in this variable (Friedman test: NaCl, $p = 0.93$; TaClo, $p = 0.28$). No significant differences regarding single trials were observed.

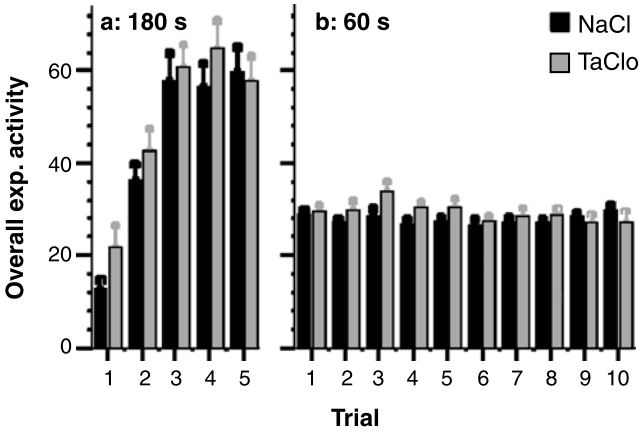

Fig. 2. Overall explore activity

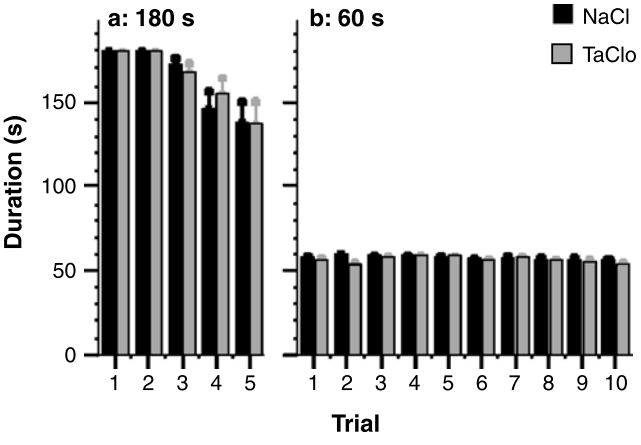

Fig. 3. Duration of the single trials

Time to find the pellets (duration, Fig. 3)

180 s

In both groups, there was a significant reduction in the time needed to find the pellets (Friedman test: NaCl, $p < 0.001$; TaClo, $p < 0.001$). There was no group difference between single trials. Both groups needed less than 180 s to find all the hidden pellets. There were no differences between the groups.

60 s

The two groups were not able to reduce the time needed to find all pellets to less than 60 s (Friedman test: NaCl, $p = 0.60$; TaClo, $p = 0.33$). This indicates that the minimum time needed by these rats to find the pellets is 60 s. There were no group differences regarding single trials.

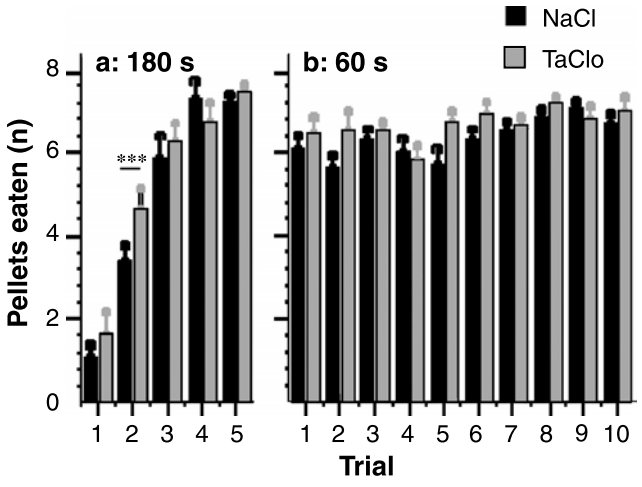

Fig. 4. Number of pellets eaten

Number of pellets eaten (Fig. 4)

180 s

In both groups, there was a significant increase in the number of pellets eaten (Friedman test: NaCl, $p<0.001$; TaClo, $p<0.001$); the NaCl group reached the maximum in trial 4 and the TaClo group in trial 5. There was a significant difference between the groups in trial 2 (NaCl versus TaClo, $p=0.041$); there were no significant differences between the groups in any other trial.

60 s

The saline-treated group showed a significant increase in the number of pellets eaten (Friedman test: NaCl, $p=0.037$), the maximum was reached in trial 9. An increase in the TaClo-treated rats was not observed (Friedman test: TaClo, $p=0.183$). The comparison of the overall means showed that the TaClo-treated rats found significantly more pellets than the NaCl group (NaCl versus TaClo, $p=0.015$). No group differences were found in any single trial.

Number of visits of baited holes (Fig. 5)

180 s

Both groups showed a significant increase in the number of visits of baited holes (Friedman test: NaCl, $p<0.001$; TaClo, $p<0.001$). The NaCl group reached its maximum in trial 4 and the TaClo group in trial 5. The two groups did not differ in any single trial. In addition, the mean of all trials did not differ between the groups.

60 s

The NaCl group showed a significant increase in the number of visits of baited holes (Friedman test: NaCl, $p<0.001$), this was not the case in the TaClo group (Friedman test: TaClo, $p=0.070$). There were no significant differences between the two groups regarding the overall mean or any single trial.

Number of visits of non-baited holes (Fig. 6)

180 s

Both groups showed an increase followed by a decrease in the number of visits of non-baited holes (Friedman test: NaCl, $p<0.001$; TaClo, $p=0.002$). There was no group difference in any of the single trials.

60 s

Both groups showed a reduction in the number of visits of non-baited holes (Friedmann test: NaCl, $p<0.001$; TaClo,

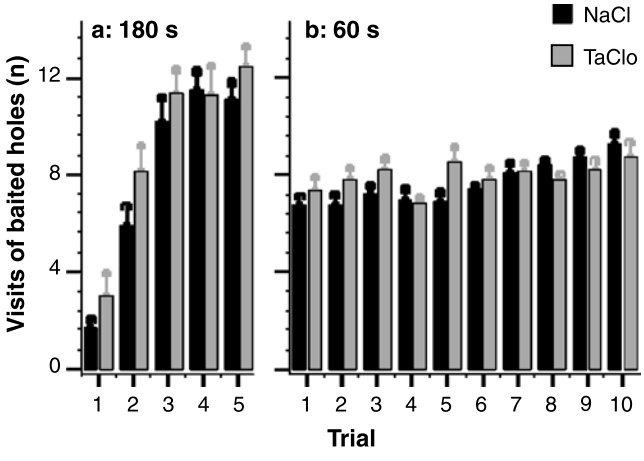

Fig. 5. Visits of baited holes

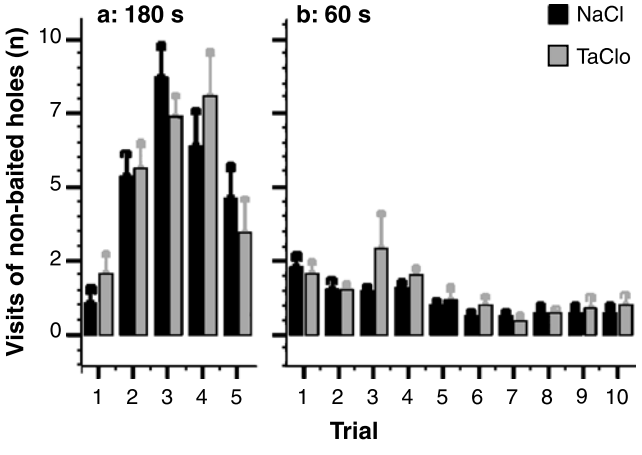

Fig. 6. Visits of non-baited holes

$p = 0.006$). None of the single trials showed any group difference.

Discussion

Chronic administration of TaClo to rats can lead to pathological changes similar to those observed in patients with idiopathic Parkinson's disease (Riederer et al., 2002). The aim of the present study was to investigate the effects of the neurotoxin TaClo on the cognitive performance of rats.

The present results showed that rats treated with TaClo do not show any learning deficits. In the 180-s trials, TaClo-treated rats found as many pellets as control rats (Fig. 4a). Furthermore, their search was as efficient as that of controls since there were no differences between the groups regarding explorative activity (Fig. 2a), visits to non-baited holes (Fig. 6a) and time needed to find the pellets (Fig. 3a). These findings show that the TaClo-treated rats were able to learn the given pattern and do not suggest a deficit in spatial memory in these animals.

Neither of the two groups was able to reduce the time to find the hidden pellets to less than 60 s, which appears to be the minimum period of time needed (Fig. 3b). However, significant differences regarding the number of eaten pellets could be observed. Rats treated with TaClo ate more pellets than saline-treated animals. Since the TaClo-treated rats found more pellets than the control animals within the same timespan, it is unlikely that TaClo produced any retrieval deficits. In the 60 s condition, the number of pellets eaten was variable in the saline-treated rats while the animals treated with TaClo showed a constant performance (see Fig. 3b). The change in condition, i.e. a trial time of 180 s versus 60 s, has different effects on the groups, with the control animals being disturbed by the change while the TaClo-treated rats seem to ignore it. The behavioural adaptation to changing conditions requires additional mental resources. One may speculate that the control rats notice the change in condition, need time and resources to adapt and show variable behaviour over time. In contrast, the TaClo-treated animals may show stable behaviour because they continue using their acquired behavioural routines. Whether or not this behaviour following the administration of TaClo represents a kind of mental rigidity remains to be established in further studies.

The present study has attempted to assess the effects of the neurotoxin TaClo on the cognitive performance of rats. TaClo can cause neuropathological changes similar to those seen in Parkinson's disease (Kochen et al., 2003). The results of the present study suggest that, under the conditions used, the chronic administration of TaClo does not affect spatial learning and memory in rats. The same regime of TaClo administration has been shown to affect motor functions in rats (Sontag et al., 1995). A possible explanation for the differing results concerning motor and cognitive abilities following TaClo administration is that TaClo may differentially affect the fronto-striatal loops underlying movement and cognition (Alexander et al., 1986; DeLong, 2000; Middleton and Strick, 2000) or that a higher dosage of TaClo is needed to produce cognitive deficits in rats.

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J Neural Transm (2007) [Suppl 72]: 155–158

Printed in Austria

Journal of Neural Transmission

Observations on the cortical silent period in Parkinson's disease

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Summary Transcranial magnetic stimulation is a tool in the neurosciences to study motor functions and nervous disorders, amongst others. Single pulses of TMS applied over the primary motor cortex lead to a so-called cortical silent period in the recording from the corresponding muscle, i.e. a period of ~100 ms with no muscle activity. We here show that in Parkinson's disease (PD), this cortical silent period in some cases is interrupted by short bursts of EMG activity. We describe in detail these interruptions in two patients with PD. These interruptions may number up to 3 per cortical silent period and show a consistent frequency across trials and hemispheres within a given patient; the two patients described here do differ, however, in the time-delay of the interruptions and hence the induced frequency. For one patient, the frequency of the interruptions proved to be around 13 Hz, the other patient showed a frequency of around 17 Hz. The results corroborate earlier findings of cortical oscillations elicited by pulses of TMS and may be related to abnormal oscillatory activity found in the cortical-subcortical motor system in PD.

Keywords: TMS, oscillations, EMG, beta frequency, motor cortex

Introduction

Transcranial magnetic stimulation (TMS), is a non-invasive and painless technique to stimulate nervous tissue, including the brain (Kobayashi and Pascual-Leone, 2004). This is shown most easily by placing the stimulating coil over the primary motor cortex: when the intensity of stimulation is above the individual threshold, the stimulation will result in a motor evoked potential (MEP) in a muscle, most commonly in the hand or arm.

TMS can be used for diagnostic, scientific and possibly therapeutic purposes in Parkinson's disease (Wassermann and Lisanby, 2001; Cantello et al., 2002).

We have recently shown that pulses of TMS applied over the primary motor cortex result in an oscillation in the beta frequency range (13–30 Hz), measured using EEG (Paus et al., 2001; Van der Werf and Paus, 2006). In addition, we have shown that this induced beta oscillatory response has a higher amplitude in patients with PD than in healthy subjects; when the pulses of TMS were applied to the hemisphere in which the thalamus was lesioned for relief of the parkinsonian tremor, the amplitude of the beta oscillatory response was at the level of that seen in healthy control subjects (Van der Werf et al., 2006).

We have argued that these induced oscillations reflect the propensity of the stimulated neurons to oscillate in the beta frequency range. The oscillations arise from phase resetting rather than the induction of new oscillations; the phase-locking of the single-trial beta responses is highly significant, whereas the amplitude modulation is not. This indicates that the strong beta oscillation results from synchronizing pre-existing and ongoing oscillations rather than from eliciting new neural responses.

We here present additional evidence for resetting of oscillatory activity using pulses of TMS over the primary motor cortex in Parkinson's Disease patients.

Materials and methods

As part of a larger study, of repetitive TMS as a treatment for Parkinson's disease, we have obtained EMG recordings of so-called cortical silent periods (CSP) in 18 subjects aged 55–80 y. Two of these patients were analyzed in detail for the current investigation (Table 1). Both patients were on their normal medication at the time of testing. The CSP is a period of relatively low-voltage activity in the EMG recording of a given muscle, generally a hand muscle such as the first dorsal interosseus (FDI) muscle. The patient is asked to contract the muscle by pressing the tips of the thumb and index finger together using moderate and sub-maximal force that they can maintain throughout the recording, i.e. approximately one minute. While they contract, pulses of TMS are delivered over the representation of the hand in the primary motor cortex. TMS consists of monophasic pulses using a MagPro stimulator (Medtronic) at an intensity of 120% of the threshold for eliciting a motor evoked potential during contraction. Twenty

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Table 1. *Patient characteristics. UPDRS was measured on regular medication*

	Age	Sex	Disease duration	Dexterity	Most affected side	Medication	UPDRS
Patient 1	67	M	7	R	L	pramipexol levodopa	22
Patient 2	64	M	10	R	R	levodopa ropinirol propranolol	10

pulses are delivered over both hemispheres at intervals of 4, 5, 6 or 7 s, distributed randomly. EMG is recorded throughout, using a bipolar belly-tendon montage of the FDI and 5 kHz sampling with a Keypoint EMG amplifier (Medtronic).

Results

Visual inspection of the recordings showed that in several, but not all, cases the CSPs were interrupted by short bouts of EMG activity. These short EMG activations could occur alone, or in a repeated fashion. Examples of interrupted and uninterrupted CSPs are given in Fig. 1. We investigated in detail two representative patients who showed such interruptions.

When the interruptions occurred, they proved very consistent from trial to trial within a given patient and consistent between recordings from the left and right hand, corresponding to stimulations applied over the contralateral hemispheres. In addition, when the interruptions recurred within a single CSP, the intervals were highly consistent. In some cases, the number of interruptions numbered up to 3, allowing to measure a maximum of 3 cycles. We measured the time intervals preceding each interruption.

In patient 1, of 20 stimulations applied over the primary motor cortex of the right hemisphere, 5 resulted in interrupted CSPs, of which two were doubly interrupted (Table 2). The average duration of the interval preceding these interruptions was 59.3 ms (standard deviation 12.2), i.e. a mean frequency of 16.9 Hz.

Of the stimulations delivered over the left hemisphere in the same patient, 7 showed interruptions of which two double. The average interval was 57.2 ms (SD 14.6), corresponding to a frequency of 17.5 Hz.

In patient 2, of the CSPs recorded in response to pulses delivered over the right hemisphere, 12 showed interruptions (6 single, 4 double, 2 triple). The average interval measured 80.2 ms (SD 11.5), resulting in a mean frequency of 12.5 Hz. Of the 20 pulses administered over the left hemisphere, 16 showed interruptions (6 single, 7 double, 3 triple). The average interval duration was 74.8 ms (SD 14.4), i.e. a mean frequency of 13.4 Hz.

Discussion

We here show that the CSP in Parkinson's Disease patients can present as a period of low-amplitude activity in the EMG interrupted by short activations. These interruptions seem to be timelocked to the MEP induced by the TMS pulse. The induced frequency of the interruptions within subjects appears very consistent within subjects from trial to trial and between hemispheres. The consistency of the induced frequency between the two patients is smaller.

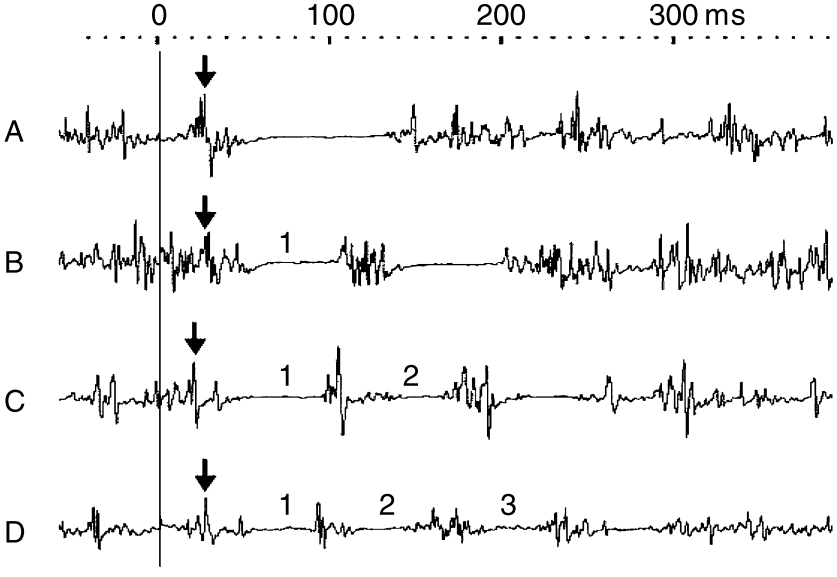

Fig. 1. Examples of cortical silent periods (CSPs) induced in our patients. The TMS pulse is delivered at time point 0, the motor evoked potential is indicated by the arrow. In all cases the intervals preceding the interruption, marked by numbers 1–3, were used for calculation of the interval duration and frequency. Examples are shown of uninterrupted CSPs (**A**, patient 1), single interruptions (**B**, patient 1), double interruptions (**C**, patient 2) and triple interruptions (**D**, patient 2)

Table 2. *Durations of the intervals preceding the interruptions in the CSPs obtained from both hands in response to 20 pulses of TMS applied over the contralateral hemispheres of two PD patients. Of the 20 pulses, a total of 5, 7, 12 and 16 CSPs showed interruptions for patients 1 and 2, right and left hemisphere, respectively*

Interval	1st	2nd	3rd	Average interval duration (standard deviation) and frequency
Patient 1 right hemisphere	64.8	56.2		59.3 (12.2) ms
	39.0			16.9 Hz
	63.4			
	56.6	55.6		
	79.4			
Patient 1 left hemisphere	46.6	68.0		57.2 (14.6) ms
	36.0			17.5 Hz
	58			
	57.8			
	60.4	86		
	43.8			
	58.4			
Patient 2 right hemisphere	76.0			80.2 (11.5) ms
	81.0			12.5 Hz
	83.0	84.6		
	62.4	101.6	91.2	
	58.6			
	87.8			
	82.8			
	83.0	75.4		
	78.6	83.4		
	94.8	63.4		
	75.0	97.4	68.4	
	75.6			
Patient 2 left hemisphere	49.8			74.8 (14.4) ms
	53.6			13.4 Hz
	56.8	74.0		
	63.8	71.6		
	77.6	70.4		
	64.8	68.8	78.8	
	68.0	88.8		
	65.2	101.4		
	57.8	100.0		
	102.0			
	63.4	85.2	101.8	
	80.6			
	84.8			
	67.0	72.2	76.4	
	70.6			
	79.6			

It remains to be investigated: a) how stably these interruptions occur in patients across time, b) how large the variability is between patients in terms of the number and induced frequency of the interruptions, c) whether the occurrence of the interruptions relates to the degree of tremor or other clinical characteristics of the patients and d) whether the interruptions respond to dopaminergic or other types of treatment.

We take these induced oscillations in the EMG to reflect a central oscillator, following findings that both pathological tremors and voluntary phasic movements are driven by a central oscillator in the contralateral primary motor cortex (Marsden et al., 2000; Timmermann et al., 2002). Interestingly, the oscillation that we show here has a frequency different from that of the resting tremor that is so characteristic of PD. In patient 1, the induced oscillation had a mean frequency of ~17 Hz oscillations in the beta frequency range, similar to that of the induced oscillation that we observed in the EEG of healthy subjects and PD patients upon pulses of TMS applied over the primary motor cortex. Such TMS-induced oscillations in the EEG fall in the low-beta frequency range, with a mean frequency of 17 Hz (Van Der Werf and Paus, 2006). Low beta frequencies are prevalent in the parkinsonian motor system, for example in the subthalamic nucleus and internal segment of the globus pallidus and in the coherence between these structures (Brown, 2003; Brown and Williams, 2005).

In patient 2, however, the induced oscillation had a lower frequency of ~13 Hz. This oscillation is reminiscent of the 12–15 Hz oscillation found in the projections of the internal segment of the globus pallidus to the ventrolateral nucleus of the thalamus (Llinás and Paré, 1995). In the parkinsonian state, this low-beta oscillatory input from the GPi is in an as yet poorly understood fashion transformed into an oscillation at tremor frequency, imposing a strong thalamocortical rhythmical input that drives the tremor (McAuley, 2003; Llinas et al., 1999). The CSP might reflect a temporary release from this thalamocortical drive, allowing other oscillations to come to the surface.

Interpretations of the nature of this oscillation remain necessarily speculative and await further study. An approach to investigate further these hypotheses and the underlying mechanism of the observed oscillatory phenomena would be to perform intracerebral recordings during TMS-induced CSPs.

The findings corroborate further our interpretation of resetting intrinsic oscillations by single pulses of TMS. A pulse of TMS would act to synchronize neural ensembles, firing at the same frequency but out of phase, and bring them transiently into synchrony, allowing the oscillation to be measured in the form of cortical waveforms (Paus et al., 2001; Van der Werf and Paus, 2006). Such an interpretation of the effect of TMS on cerebral oscillations is in accord with findings reported using TMS-induced oscillations in the EEG (Schürmann et al., 2001; Fuggeta et al., 2005) and findings of tremor resetting by single pulses of TMS in both healthy subjects and PD patients (Britton et al., 1993; Pascual-Leone et al., 1994).

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J Neural Transm (2007) [Suppl 72]: 159–163

Printed in Austria

Journal of Neural Transmission

CYP 2E1 mutant mice are resistant to DDC-induced enhancement of MPTP toxicity

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Summary In order to reach a deeper insight into the mechanism of diethyldithiocarbamate (DDC)-induced enhancement of MPTP toxicity in mice, we showed that CYP450 (2E1) inhibitors, such as diallyl sulfide (DAS) or phenylethylisothiocyanate (PIC), also potentiate the selective DA neuron degeneration in C57/bl mice. Furthermore we showed that CYP 2E1 is present in the brain and in the basal ganglia of mice (Vaglini et al., 2004). However, because DAS and PIC are not selective CYP 2E1 inhibitors and in order to provide direct evidence for CYP 2E1 involvement in the enhancement of MPTP toxicity, CYP 2E1 knockout mice (GONZ) and wild type animals (SVI) of the same genetic background were treated with MPTP or the combined DDC + MPTP treatment. In CYP 2E1 knockout mice, DDC pretreatment completely fails to enhance MPTP toxicity, although enhancement of MPTP toxicity was regularly present in the SVI control animals. The immunohistochemical study confirms our results and suggests that CYP 2E1 may have a detoxifying role.

Keywords: MPTP, neurotoxicity, CYP 2E1, DDC, neurodegeneration, Parkinson's disease

Abbrevations: *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, *SNpc* substantia nigra pars compacta, *VTA* ventral tegmental area, *TH* tyrosine hydroxylase, *MAO* monoaminoxydase, *SVI* Cyp 2e1+/+ (129S1/SvImJ); *GONZ* Cyp 2e1−/− (129/SV-Cyp $2e1^{tm1Gonz}$)

Introduction

Parkinson's disease (PD) is a progressive, age-related, neurodegenerative disease characterized by bradykinesia, resting tremor, rigidity and gait disturbance. The disease is also characterized by a massive progressive distruction of dopaminergic neurons in the substantia nigra (SN). In human and rodents, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is well know to produce clinical, biochemical and neurochemical changes similar to those seen in PD (Heikkila et al., 1984; Thuski et al., 1991). This neurotoxin also leads to a marked depletion in dopamine (DA) contents in the striatum and a decrease in the number of nigrostriatal dopaminergic neurons in several species (Burns et al., 1983; Johannessen, 1991; Ricaurte et al., 1986; Schneider and Markham, 1986). Elucidation of the biochemical steps leading to the MPTP-induced selective degeneration of the nigrostriatal DA pathway has provided new clues to DA neurones vulnerability (Kopin, 1992). Furthermore, marked species differences in MPTP toxicity have been described, and this differential sensivity to the neurotoxin has provided further information about the genetic factors determining cell susceptibility to xenobiotic insults (Corsini, et al., 2002). The neurotoxic effects of MPTP are thought to be initiated by 1-methyl-4-phenyl-piridinium ion (MPP+) a major metabolite of MPTP formed by monoamine oxidase (MAO) B-mediated oxidation (Chiba et al., 1984; Markey et al., 1984). MPP+ is taken up by high-affinity dopamine uptake system and is subsequently accumulated within mitochondria of nigrostriatal dopaminergic cells, directly inhibiting complex I (Nicklas et al., 1985; Ramsay and Singer, 1986; Sonsalla and Nicklas, 1992).

This can lead to a number of deleterious effects on cellular function, resulting in neuronal cell death. Therefore, MPTP-treated animals, including nonhuman primates and rodents, are widely used as models for PD (Gerlach and Riederer, 1996; Heikkila et al., 1989; Zuddas et al., 1992). Induction of parkinsonism by MPTP in mice has generated a wealth of neurochemical, pharmacological and anatomical findings. In 1985, Corsini et al. reported unexpected data on MPTP toxicity by demostrating, for the first time,

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that a compound, diethyldithiocarbamate (DDC), potentiates MPTP toxicity in this mouse model. In order to understand the mechanism responsible for this effect and after testing several compounds, only a few other enhancers MPTP (acetaldehyde and ethanol) were found (Corsini et al., 1985). DDC, ethanol and acetaldehyde have recently been reported to be specific inhibitors of CYP 2E1 when acutely administered (Stott et al., 1997). 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 C57/bl brain (Vaglini et al., 2004), in DA neurons of the SN and in caudate nucleus. More recently we demonstrated that, similar to DDC, CYP 2E1 inhibitors such as DAS and PIC markedly enhance MPTP toxicity, as measured by a dramatic fall in striatal DA content of C57/bl mice. In this study, in order to provide direct evidence for CYP 2E1 involvement in the DDC-induced enhancement of MPTP toxicity, CYP 2E1 knockout mice (GONZ) and their wild type counterparts (SVI) were challenged with MPTP or the combined DDC + MPTP treatment. Subsequently, we performed tyrosine-hydroxylase (TH) immunoreactivity analyses in brain section from our treated mice.

Materials and methods

Knockout mice

Male *Cyp2e1* knockout mice (*129/SV-Cyp2e1^{tm1Gonz}*) (*Cyp2e1−/− 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 with a 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. Animals 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.* Assessment of the *Cyp2e1−/−* genotype in adult animals was confirmed by the absence of CYP 2E1, by liver DNA PCR genotyping by Charles River Laboratories. 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.

Experimental protocol

Eight weeks old mice, twelve per group, received i.p. injection of either MPTP hydrochloride (single dose, 36 mg/kg) or distilled water. Animals were pretreated i.p. with DDC (400 mg/kg) or the vehicle 1 hr before MPTP administration. As DDC was readily soluble in distilled water, the vehicle consisted of distilled water. Seven days after treatment, eight animals were killed by cervical dislocation, and their brains were removed and dissected. Dissection was performed as described by Glowinski and Iversen (Glowinski et al., 1966) with minor modifications. Immediately after dissection, the striatum was frozen on dry-ice until assayed. The remaining four animals were deeply anesthetized by i.p. injection of chloral hydrate (400 mg/kg), perfused through the left ventricle with 50 ml of saline solution and fixed with 200 ml 4% paraformaldehyde in 0.1 M PBS. The brains were subsequently removed from the skulls and then cryoprotected at 4°C in 0.1 M PBS containing 30% sucrose for further sectioning. Striatal modification of DA content and TH immunohistochemistry in SN was performed according to Vaglini et al. (2004). For statistical evaluation ANOVA with Sheffe-F analysis was used.

The slides were photographed using an Olympus DP-50 digital camera mounted on an Olympus BX60 light microscope (Olympus Optical Co., Hamburg, Germany) at 4× magnification, interfaced with a personal computer. Quantification of the cell number was performed in the region corresponding to bregma −2.80/−3.52 mm as indicated in the atlas of Paxinos and Franklin (2001), counts were performed in this area with a dedicated software (MetaMorph 1.07b).

Results

Striatal modification of DA in CYP 2E1−/− mice

The effect of the acute administration of CYP 2E1 inhibitor DDC, administered 1hour before a single exposure to MPTP, on the striatal DA content in the *CYP 2E1−/−* (GONZ) and wild type (SVI) mice is shown in Fig. 1. Seven days after combined treatment with DDC and MPTP in SVI mice, striatal DA content dropped to 57% of untreated controls (48.7 ± 5.1 and 114.1 ± 8.3 ng/mg protein, respectively). The animals treated with DDC alone did not show any change compared with control values, whereas the administration of a single dose of MPTP alone, induced a 29% decrease in striatal DA content compared with untreated controls. Thus, pre-treatment with DDC causes an enhancement of the MPTP toxicity of about 40%. These results

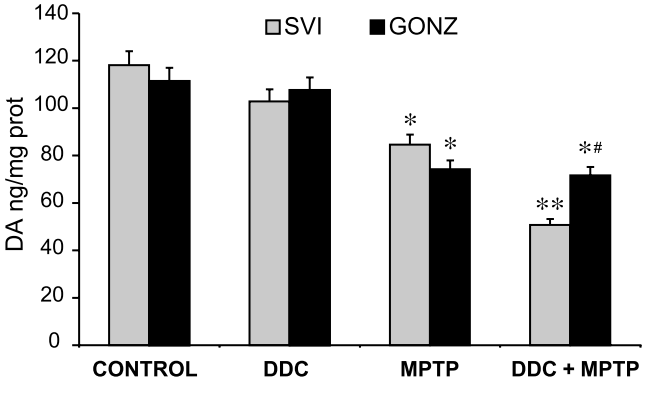

Fig. 1. Effect of DDC on striatal tissue levels of dopamine (*DA*) in SVI (Cyp2e1+/+) and GONZ (Cyp2e1−/−) male mice treated with MPTP. The results are the mean ± s.e. of n(10–20) animals for each group. SVI and GONZ were treated with MPTP (30 mg/kg i.p.) or saline solution 60 min after DDC at the dose of 400 mg/kg i.p. The animals were sacrified 7 days later. $^{*}p < 0.05$ in comparison with control mice $^{**}p < 0.05$ in comparison with MPTP-treated animals. $^{\#}$Not significant in comparison with MPTP-treated animals

confirm previous studies in C57/Bl mice, and demonstrate that the combined treatment of DDC and MPTP significantly enhanced MPTP toxicity ($p < 0.05$).

For GONZ mice a single treatment of MPTP caused a reduction of 30% in the DA content compared to untreated controls, however, the pretreatment of DDC did not reduce the striatal DA content compared to MPTP alone (control values were 109.4 ± 9.8 ng/mg protein; 76.5 ± 6.3 and 73.7 ± 5.7 ng/mg protein represent DA content of MPTP alone and DDC + MPTP, respectively). Thus the DDC pretreatment, in accordance with the usual time and dose schedule, completely failed to potentiate the DA fall in CYP 2E1 mutant mice (30 and 32% reduction in DA content compared to untreated mice, for MPTP and DDC + MPTP, respectively). These results demonstrate that the combined treatment of DDC + MPTP did not significantly enhance MPTP toxicity ($p < 0.05$).

TH immunohistochemistry in SN

Figure 2 shows the TH-IR DA neurons in midbrain coronal sections of wild-type and CYP 2E1 −/− after treatment with DDC + MPTP and MPTP alone. Nigral TH-immunoreactive neurons and fibers were easily detectable in groups of control SVI and GONZ in normal conditions (untreated mice). Dopaminergic cell bodies and fibers were intensely stained with evident TH-immunopositive processes. According to previous results, eight days after acute MPTP administration, SVI mice show a decrease in TH-immunoreactive fibers in the SN (Fig. 2B). IN SVI mice, pretreat-

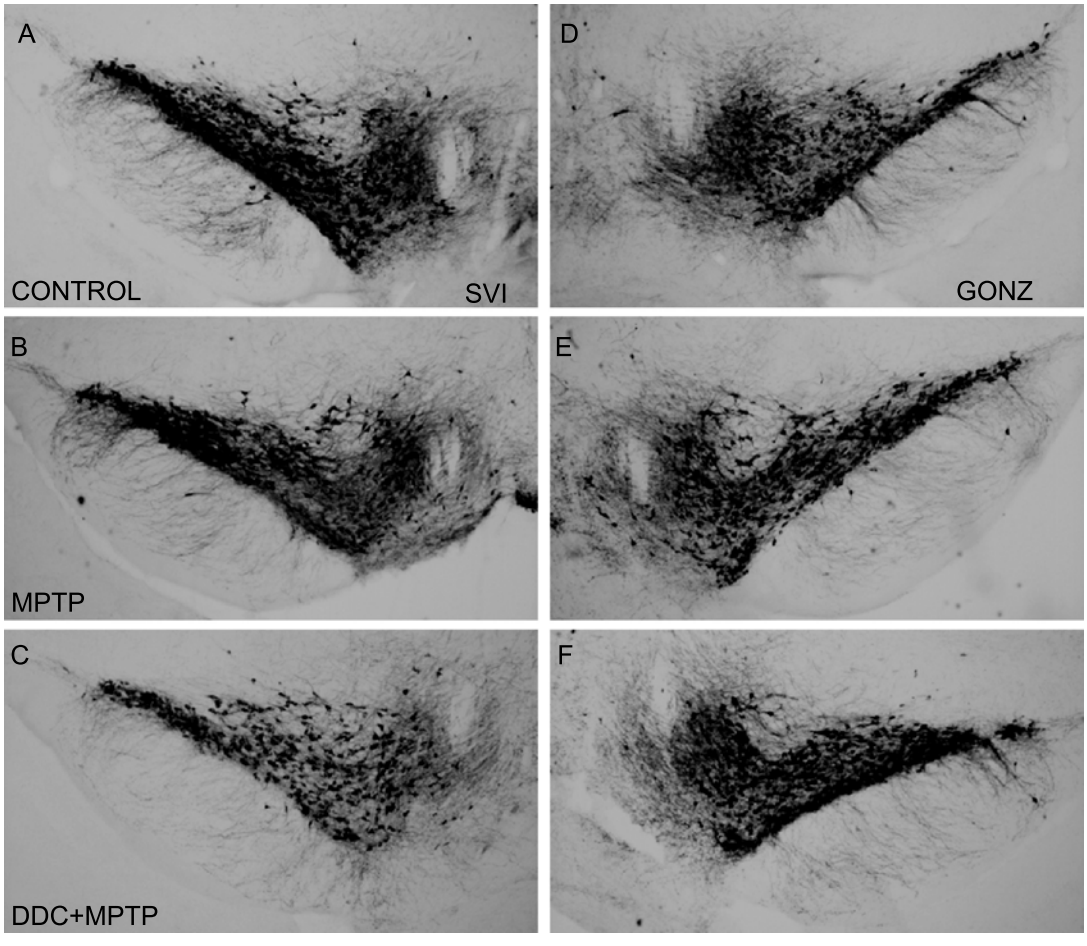

Fig. 2. Tyrosine hydroxylase immunoreactivity (TH-IR) in midbrain coronal sections of SVI (Cyp2e1+/+; A–C) and GONZ (Cyp2e1−/−; D–F) mice. Micrograph **A** illustrates TH-IR cells and dendrites in the substantia nigra (SN) and ventral tegmental area (VTA) of untreated control mice. Micrographs **B** and **C** illustrate SN and VTA TH-IR in MPTP- and CYP 2E1 inhibitor-treated mice, respectively: (**B**) vehicle + MPTP, (**C**) DDC + MPTP. The pretreatment with CYP 2E1 inhibitor increases MPTP toxicity on the dopaminergic neurons in SNpc. **D–F** Tyrosine hydroxylase immunoreactivity (TH-IR) in midbrain coronal sections of GONZ mice. Micrograph **D** illustrates TH-IR cells and dendrites in the substantia nigra (SN) and ventral tegmental area (VTA) of untreated GONZ mice. **E** and **F** illustrate SN and VTA TH-IR in MPTP- and CYP 2E1 inhibitor-treated mice, respectively: (**E**) vehicle + MPTP, (**F**) DDC + MPTP. The pretreatment with CYP 2E1 inhibitors does not increase MPTP toxicity on SNpc dopaminergic neurons

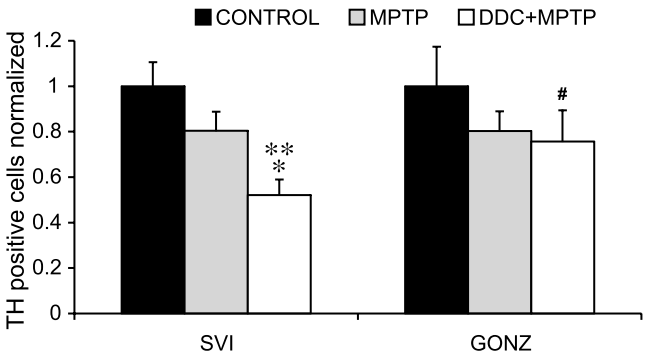

Fig. 3. Effect of DDC on the number of TH positive cells in SVI (Cyp2e1+/+) and GONZ (Cyp2e1−/−) male mice treated with MPTP. The results are the mean ± s.e. of $n \geq 5$ animals for each group. SVI and GONZ were treated with MPTP (30 mg/kg i.p.) or saline solution 60 min after DDC at the dose of 400 mg/kg i.p. The animals were sacrified 7 days later. $^{*}p < 0.05$ in comparison with untreated mice $^{**}p < 0.05$ in comparison with MPTP-treated animals. $^{\#}$Not significant in comparison with MPTP-treated animals

ment with DDC, together with administration of MPTP, resulted in an extensive loss of TH-positive cells and TH-positive fibres in comparison to untreated controls and animals treated with MPTP alone (Fig. 2C).

These images indicate that the MPTP treatment alone, slightly affects DA neurons of the SNpc, whereas MPTP administration after CYP 2E1 inhibition affects this area considerably. In the SNpc of CYP 2E1−/− animals, a decrease in the number of TH-immunopositive fibers, comparable to the wild-type mice, was observed eight days after MPTP treatment (Fig. 2E). In contrast, following DDC + MPTP treatments TH-immunoreactivity in SNpc is almost similar to that of control mice, indicating that no further cell damage occurred (Fig. 2F).

The immunoistochemically processed brains were then counted to quantify the loss of DA cells caused by the MPTP treatment in combination with DDC in both genotypes (Fig. 3). The number of cells in the SNpc of untreated SVI and GONZ were similar. Treatment with MPTP produced a decrease in the number of DA cells of around 13.1 and 19.7% in SVI and GONZ, respectively. However, pretreatment with DDC, one hour before the neurotoxin, exerted a loss of DA cells of 47.9 and 24.4% in the SVI and GONZ, respectively. These data indicate that the previously described enhancement of the MPTP toxicity by DDC occurs in SVI mice but completely failed in GONZ (Cyp2e1−/−) mice.

Discussion

Cytochrome P450 (CYP) are a superfamily of hemecontaining monoxygenases that metabolize a large number of compounds or xenobiotics including drugs, toxicants and chemical carcinogens (Gonzalez, 2005). CYPs were originally reported to contribute to only a small extent of brain protein content, however many of these early reports considered the brain as a homogenous organ, without taking into account single neuronal populations. Brain regions differ tremendously in their cellular composition, cell density and function and the expression pattern of brain CYPs is also extremely heterogeneous. Therefore, the levels of CYPs and their functions in specific neuronal populations can be comparable to those in hepatocytes (Miksys and Tyndale, 2002).

CYP 2E1 is best known for its role in chemical detoxification/activation, fatty acid metabolism, metabolism of acetone to gluconeogenic intermediates, and free radical production. The expression of CYP 2E1 may vary as a result of polymorphisms in CYP 2E1 promoters; consequently, the levels of this enzyme are by no means constant among individuals, yet they do not exhibit the marked interindividual variation characteristic of other P450 enzymes (Parkinson, 1996). CYP 2E1 was first identified as the microsomal ethanol oxidizing system (MEOS) (Lieber, 1990). This enzyme is inhibited by several compounds including DDC, aldehydes, DAS and PIC (Lieber, 1997). More recently, the discovery that DAS and PIC, markedly enhance MPTP toxicity in C57/bl brain suggested an involvement of CYP 2E1 in DDC-induced enhancement of MPTP toxicity (Vaglini et al., 2004). However, because DAS and PIC, as well as DDC, are not selective CYP 2E1 inhibitors (Nissbrandt et al., 2001), direct evidence of the participation of CYP 2E1 in this mechanism was still missing. Thus, in order to assess a direct participation of CYP 2E1 in DDC-mediated enhancement of MPTP toxicity, CYP 2E1 knockout mice (GONZ) and their wild type counterparts (SVI) were challenged with MPTP and with the combined DDC + MPTP treatment. The lack of effect of the DDC treatment, administered one hour before MPTP, in the CYP 2E1 mutant mice demonstrate that the presence of CYP 2E1 is necessary for DDC-induced enhancement of MPTP toxicity.

GONZ mice revealed a sensitivity to MPTP neurotoxicity similar to that of SVI animals, but significantly lower than C57/bl strain, indicating that, at least in this strain, the absence of CYP 2E1 expression does not significantly alter MPTP neurotoxicity. It is likely that transgenic mice compensate the lack of CYP 2E1 with some other isozyme, as compensation among different P450 enzymes, in this strain, was already observed for acetaminophen toxicity (Lee et al., 1996).

More importantly, in these knockout mice, DDC completely failed to enhance MPTP toxicity; this effect was

instead regularly observed in the wild type animals. Our data indicate that the presence of CYP 2E1 is essential to mediate this process, directly demonstrating the involvement of CYP 2E1 in the DDC-induced enhancement of MPTP toxicity.

In order to confirm the data obtained with DA content quantification, we performed tyrosine-hydroxylase immunoreactivity in brain slices and quantified the loss of DA cell bodies.

Our results clearly indicate that MPTP treatment produced, in wildtype mice, a loss of DA perikaria in the SNpc (about 13%), whereas the combined treatment of DDC and MPTP, induced at least 47% damage of the DA neurons, as previously observed with DDC in C57/bl mice. In contrast, in CYP 2E1 knockout mice, the single dose of MPTP produced a similar loss in the SNpc (19%), however the combined treatments did not induce a significant further loss of DA neurons compared with neurotoxin alone (24%).

In conclusion, we provide data, using neurochemical and immunoistochemical analysis, confirming that DDC strongly potentiate MPTP toxicity in wild-type mice but not in CYP 2E1 mutants, thereby demonstrating that CYP 2E1 mediates the DDC-induced enhancement of MPTP toxicity.

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J Neural Transm (2007) [Suppl 72]: 165–173

Printed in Austria

Journal of Neural Transmission

Pharmacokinetic studies of (−)-deprenyl and some of its metabolites in mouse

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Summary (−)-Deprenyl is a selective irreversible inhibitor of MAO-B. The parent compound is responsible for the enzyme inhibitory effect, but its metabolites are also playing a role in the complex pharmacological activity of the substance. In the present studies male NMRI mice were treated orally, subcutaneously, intraperitoneally and intravenously with 5 mg/kg of (−)-deprenyl. The time related changes of the plasma concentrations of the parent compound and its main metabolites (methamphetamine, desmethyl-deprenyl and amphetamine) were determined by GC/MSD technique. The main pharmacokinetic parameters (C_{max}, t_{max}, $t_{1/2}{}^{\beta}$, AUC_{0-6}, $AUC_{0-\infty}$) have been calculated. (−)-Deprenyl is well absorbed after oral and parental treatment. The peak concentrations (C_{max}) were reached at 15 min after treatment and the absorption was followed by a fast elimination ($t_{1/2}{}^{\beta} \leq 2$ h). (−)-Deprenyl has an intensive ''first pass'' metabolism after oral treatment; only 25% of the parent compound reaches the systemic circulation. Increased bioavailability was detected after subcutaneous (87.1%) and intraperitoneal (78.7%) administration. The main metabolic pathway of (−)-deprenyl is the *N*-depropargylation, leading to the formation of methamphetamine. *N*-demethylation of (−)-deprenyl leads to formation of desmethyl-deprenyl. Amphetamine is produced from both former metabolites. After oral treatment the plasma concentrations of methamphetamine are higher during the first 6 h than that of (−)-deprenyl, while the opposite was found after parental treatment. The results indicate, that (−)-deprenyl, a potent MAO-B inhibitor, might induce a different spectrum of activity (e.g. antidepressant), when it is administered parenterally (transdermally). The new spectrum can be due to the special pharmacokinetic behaviour of the inhibitor.

Keywords: (−)-Deprenyl, pharmacokinetics, deprenyl metabolites, transdermal application

Introduction

Many studies have been reported on the metabolism of (−)-deprenyl in man and rodents (Reynolds et al., 1978; Heinonen et al., 1989; Szökő and Magyar, 1995; Lengyel et al., 1997). It was concluded that the metabolites have pharmacological activities different from the parent compound (Magyar, 1994; 1997; Magyar et al., 1998). (−)-Deprenyl is a selective irreversible inhibitor of MAO-B, while the metabolites lack the enzyme inhibitory potency. Nevertheless, they inhibit the reuptake or induce the release of biogenic amines to or from the nerve endings (Knoll and Magyar, 1972; Magyar et al., 1996). In addition, pharmacokinetic studies with ^{14}C-labelled compounds indicated an intensive ''first pass'' metabolism of (−)-deprenyl (Szökő and Magyar, 1996). The metabolic pattern is highly influenced by the routes of drug administration (Magyar et al., 1995). It was also proved that racemisation did not occur during metabolic conversion of the enantiomers (Schächter et al., 1980; Szökő and Magyar, 1996). In spite of many studies, to the best of our knowledge, experiments dealing with the quantitative changes of the plasma concentrations of (−)-deprenyl and its main metabolites have not yet been reported. For the quantitative determination of (−)-deprenyl and its main metabolites (amphetamine, methamphetamine, *N*-desmethyl-deprenyl) in mouse plasma a highly sensitive assay method has been developed. A gas chromatography/mass spectrometric detector (GC/MSD) was used to determine the plasma concentrations of (−)-deprenyl and the *N*-pentafluorobenzoyl derivatives of its three metabolites. Quantitation was performed by stable isotope dilution, by using deuterated (−)-deprenyl and methamphetamine as internal standards. Selective ion monitoring of the characteristic fragment ions of both analytes and internal standard resulted in attaining lower quantitation limit of 0.5 ng/ml for (−)-deprenyl and 1.0 ng/ml for metabolites.

The objective of the present pharmacokinetic analysis was to determine the exact rate of the ''first pass'' metabo-

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lism of (−)-deprenyl, following different ways of administration, by the supposition that parenteral application might basically change the spectrum of pharmacological activity of the drug. At the early pre-clinical studies (−)-deprenyl was administered subcutaneously, while in human therapy it was almost exclusively given orally (Knoll et al., 1965; Knoll and Magyar, 1972). This discrepancy substantiated our present experimental efforts. We suppose that a new spectrum of activity, as a result of different kind of parenteral applications of the drug, cannot be explained simply by the raised blood concentrations of the substance, but a new pattern of distribution cannot be neglected.

Materials and methods

Animals and treatments

Male, NMRI mice (BR, SPF) weighing 22–25 g were used in these studies. The animals were housed at room temperature (22 ± 2°C) and fed on standard laboratory mice food (LATI, Gödöllő, Hungary). Oral treatment was preceded by 12 h starvation. Water was supplied ad libidum. The study was approved by the Regional Ethical Committee of Semmelweis University of Medicine.

The animals (three mice in a group) were treated orally (via a gastric probe), subcutaneously, intraperitoneally or intravenously with 5 mg/kg of (−)-deprenyl dissolved in physiological saline in a volume of 0.2 ml/20 g animal.

Blood samples were taken at zero time and after decapitation at:

0.08; 0.25; 0.5; 1.0; 1.5; 2.0; 4.0; 6.0 h after *oral* and *subcutaneous* treatments
0.0016; 0.08; 0.25; 0.5; 1.0; 1.5; 2.0; 4.0; 6.0 h after *intraperitoneal* and *intravenous* administrations.

The blood samples were collected in Eppendorf tubes containing 50 IU of heparin, which was followed by centrifugation (3000 rpm for 15 min) and the supernatant was used for determinations. Plasma samples were stored at −20°C pending assay.

Materials

Standard materials (±)-amphetamine HCl, (±)-methamphetamine HCl, (±)-*N*-desmethyl-deprenyl HCl and (−)-deprenyl HCl were synthesised in the Chinoin Co. Ltd. (Budapest, Hungary). The deuterated chromatographic internal standards (±)-[$^2H_4$]-deprenyl HCl (d_4-D) and (±)-[$^2H_4$]-methamphetamine HCl (d_4-MA) were prepared at the Institute of Drug Research (Budapest, Hungary).

All chemicals and reagents used in these studies were of high purity under the guaranty of manufacturer. They were purchased from various commercial sources: sodium hydroxide (NaOH), sodium chloride (NaCl), toluene and n-hexane were purchased from Chemolab Ltd. (Budapest, Hungary), *N*-pentafluorobenzoyl chloride (acylating agent) was obtained from Fluka Chemie AG (Buchs, Switzerland). The water used for solution and sample preparations were of Milli Q-quality (Millipore, USA).

For derivatization of the metabolites a 5% v/v solution of *N*-pentafluorobenzoyl chloride in dichloromethane (acylating mixture) was prepared.

Sample processing

For the determination of (−)-deprenyl 0.1 ml water, 0.04 ml internal standard solution and 0.1 ml mouse plasma were pipetted into 0.5 ml polyethylene microcentrifuge tubes. The mixture was salted out by the addition of 0.05 ml of saturated NaCl solution and alkalinized by 0.02 ml of a 10 N NaOH solution. Finally, it was extracted by 0.1 ml toluene using GlaCol® rotator equipment for 40 min. After the separation of organic phase, 3.0 µl aliquots were injected into the gas chromatograph.

For the determination of the three metabolites, the toluene fraction, remaining after (−)-deprenyl determination, was transferred to a 5 ml conical test tube and 1.0 ml of n-hexane and 0.1 ml of acylating mixture were admixed. The *N*-pentafluorobenzoyl derivatives of the metabolites by a 1 h reaction time at room temperature, were obtained.

After evaporation of the reaction mixture to dryness under reduced pressure at room temperature in a SpeedVac® system, the evaporation residue was dissolved in a 0.04 ml of toluene and 3.0 µl aliquots were injected into the gas chromatograph.

Gas chromatography and mass spectrometry conditions

GC/MSD analysis was performed by using an HP5890 series II gas chromatograph (GC, Hewlett-Packard, USA), coupled to an HP5971A mass selective detector (MSD) set in EI operation mode. Samples were introduced by a split-less injection (0.8 min) delaying time from an HP9673 auto sample onto a fused silica HP-5MS capillary column (30 m × 0.2 mm; 0.25 µm film thickness; Hewlett-Packard, USA).

For chromatography of deprenyl the column temperature was raised by automatic programming from an initial value of 100°C, held for 0.8 min, to 210°C at 70°C min^{-1}, held for 3 min, then raised from 210 to 300°C at 70°C min^{-1}, finally held for 8 min. For chromatography of pentafluorobenzoylated metabolites column temperature was raised by automatic programming from an initial value of 90°C, held for 1 min to 110°C at 70°C min^{-1}, then raised from 110 to 280°C at 15°C min^{-1}, finally held for 8 min.

Helium ultra pure (6.0; Messer, Austria) was used at a column head pressure of 25 psi. The injector and transfer line (interface) temperatures were maintained at 290 and 300°C, respectively.

Quantitation was performed by selected ion monitoring (SIM) of the characteristic fragment ions $[M-C_6H_5-CH_2^{\bullet}]^+$ for deprenyl (m/z = 90), d_4-deprenyl (m/z = 100), pentafluorobenzoylated amphetamine, methamphetamine, desmethyl-deprenyl and d_4-methamphetamine (m/z = 238, 252, 276 and 256, respectively) (Fig. 1).

Tuning (calibrating) of the MSD was executed using PFTBA mass fragments m/z = 100, 219 and 414 for deprenyl assay as well as m/z = 131, 219 and 414 for metabolites assay.

Data acquisition and processing were made by ChemStation software (Version B.01.00; Hewlett-Packard, USA). The retention times for deprenyl, pentafluorobenzoylated amphetamine, methamphetamine and desmethyl-deprenyl from the mass spectrometer were 3.7, 5.04, 5.07 and 5.3 min, respectively, (Fig. 2). For calibration curve fitting linear regression forced through origin based on analyte/internal standard peak area ratio values were selected.

Based on the analysis of calibration and quality control samples, the method was proved to be accurate and precise for the determination of deprenyl between 0.5 and 2000 ng/ml, *N*-desmethyldeprenyl, methamphetamine and amphetamine between 1 and 1000 ng/ml concentrations.

Pharmacokinetic and statistical analysis

The pharmacokinetic parameters were calculated from plasma concentration-time data with ChemStation software (Version B01.00; Hewlett-Packard, USA) pharmacokinetic package. In case of maximal plasma concentration (C_{max}) and the corresponding time value (t_{max}), the measured concentration values and the respective time points were presented. The area under the plasma concentration-time curves was calculated by trapezoidal rule (AUC_{0-t}) with extrapolation to infinity ($AUC_{0-\infty}$). The terminal elimination rate constant (β) was estimated from the linear least-squares regression of the terminal phase of the log concentration-time profile. The apparent biological half life ($t_{1/2}{}^{\beta}$) was calculated as 0.693/β.

Table 1. *Time related changes in the plasma concentrations of (−)-deprenyl and its metabolites after 5 mg/kg (−)-deprenyl treatment (ng/ml; mean ± S.D.; n = 3)*

Time (h)	po				sc			
	D	MA	DMD	A	D	MA	DMD	A
0.017	–	–	–	–	–	–	–	–
0.08	119.9 ± 39.7	202.1	95.3 ± 14.6	27.8	438.6 ± 136.0	33.3 ± 8.7	51.6 ± 10.5	7.5 ± 0.8
0.25	136.7 ± 70.7	205.4 ± 30.5	158.0 ± 27.8	41.5 ± 16.9	472.0 ± 58.7	57.2	64.3	10.9
0.5	115.6 ± 41.6	204.0 ± 42.4	66.8 ± 11.4	132.4 ± 17.3	447.5 ± 45.7	131.4 ± 1.8	104.4 ± 6.1	36.7 ± 1.4
1	90.6 ± 33.7	191.2	57.4	79.5	171.9 ± 49.2	109.8 ± 42.1	35.8	50.4
1.5	26.2 ± 22.7	93.6	20.2 ± 1.6	53.2 ± 7.3	113.8 ± 31.8	83.5 ± 20.4	14.9 ± 4.3	29.2 ± 14.2
2	9.5 ± 3.5	85.0 ± 10.6	12.3 ± 2.8	31.9 ± 7.3	96.7 ± 26.2	69.4	8.4	19.8
4	5.5 ± 1.7	60.2 ± 19.2	3.0	15.4 ± 0.9	14.9 ± 4.6	45.9 ± 6.3	1.1 ± 1.1	9.2 ± 4.0
6	2.0 ± 0.2	33.6 ± 12.0	1.0 ± 1.0	6.5 ± 2.2	3.8 ± 1.5	17.1 ± 2.6	0.2 ± 0.1	3.0 ± 0.3
–								
	ip				iv			
	D	MA	DMD	A	D	MA	DMD	A
–								
0.017	580.2 ± 96.9	71.9 ± 42.0	138.3 ± 83.1	5.3 ± 3.4	2436.1 ± 102.3	208.8 ± 37.7	355.3 ± 10.7	3.6 ± 2.4
0.08	635.8 ± 74.2	259.6 ± 56.3	305.6	191.6	1720.6 ± 612.2	223.4	267.8	31.3
0.25	506.0 ± 277.6	190.9 ± 35.2	110.6 ± 33.0	99.8 ± 13.4	623.2 ± 90.9	341.5 ± 83.4	208.5 ± 57.4	84.8 ± 23.4
0.5	385.2 ± 53.1	182.9 ± 80.4	58.2 ± 16.2	386.7 ± 35.2	324.9 ± 63.4	197.1	120.1 ± 42.0	108.0 ± 41.6
1	263.0 ± 109.3	142.3 ± 45.5	28.6 ± 16.7	60.5 ± 19.6	172.4 ± 16.7	156.1 ± 40.1	39.1 ± 26.5	66.6 ± 9.9
1.5	61.9 ± 15.7	108.5 ± 24.7	5.4 ± 2.2	49.7 ± 28.8	23.7 ± 3.7	96.9 ± 15.0	8.4 ± 1.0	32.6 ± 27.8
2	29.1 ± 7.9	88.1 ± 19.6	0.5 ± 0.8	33.6 ± 6.4	19.3 ± 0.7	81.9 ± 9.1	6.2 ± 1.6	11.6
4	4.0 ± 2.0	28.5 ± 4.7	0.0 ± 0.0	8.8 ± 7.2	7.7 ± 1.7	53.5 ± 18.4	0.7 ± 0.4	5.0 ± 4.0
6	3.2 ± 1.2	15.0	0.0 ± 0.0	0.3 ± 0.5	2.1 ± 0.2	19.8 ± 2.7	0.0 ± 0.0	1.6 ± 0.8

D (−)-Deprenyl; *MA* methamphetamine; *DMD* desmethyl-deprenyl; *A* amphetamine.

Results

The time related changes of plasma concentration (ng/ml) of (−)-deprenyl and some of its metabolites (methamphetamine, desmethyl-deprenyl, amphetamine) following oral, subcutaneous, intraperitoneal and intravenous administration of 5 mg/kg substance, are presented in Table 1. From these data the main pharmacokinetic parameters (C_{max}, t_{max}, $t_{1/2}{}^{\beta}$, AUC_{0-6} and $AUC_{0-\infty}$) were calculated and the results are shown in Table 2. (−)-Deprenyl is well absorbed from the gastrointestinal tract after oral treatment and fast absorption was also experienced following subcutaneous or intraperitoneal administration of the drug. The time related changes of the blood concentrations of (−)-deprenyl and its metabolites within 6 h after oral and parenteral administration are presented in Fig. 3. The peak concentrations (C_{max}) were reached at around 15 min, following any routes of treatment. The absorption is followed by a fast elimination e.g. the $t_{1/2}{}^{\beta}$ values were less than 2 h (Table 2). Big difference was detected between the AUC values of the parent compound, following different routes of drug administration. The smallest $AUC_{0-\infty}$ was obtained after oral treatment, (F = bioavailability) which was 24.5%, that of the intravenous treatment. Higher values of bioavailability were obtained after subcutaneous (87.1%) and intraperitoneal (78.7%) adminis-

Table 2. *Pharmacokinetic parameters of (−)-deprenyl and of its metabolites*

Pharmacokinetic	Parameters	po	sc	ip	iv
(−)-Deprenyl					
C_{max}	ng/ml	137.00	472.00	636.00	–
t_{max}	h	0.25	0.25	0.08	–
$t_{1/2}{}^{\beta}$	h	1.78	0.86	1.26	1.25
AUC_{0-6}	ng · h/ml	170.30	619.10	558.00	712.60
$AUC_{0-\infty}$	ng · h/ml	175.50	623.80	563.80	716.40
f_{abs}	%	24.50	87.10	78.70	100.00
Methamphetamine					
C_{max}	ng/ml	205.00	131.00	260.00	342.00
t_{max}	h	0.25	0.50	0.08	0.25
$t_{1/2}{}^{\beta}$	h	2.99	1.98	1.57	1.95
AUC_{0-6}	ng · h/ml	547.50	357.80	449.30	535.70
$AUC_{0-\infty}$	ng · h/ml	692.40	406.60	483.20	591.5
Desmethyl-deprenyl					
C_{max}	ng/ml	158.00	104.00	306.00	355.00
t_{max}	h	0.25	0.50	0.08	0.02
$t_{1/2}{}^{\beta}$	h	1.10	1.11	0.17	0.55
AUC_{0-6}	ng · h/ml	131.20	97.30	103.30	166.10
$AUC_{0-\infty}$	ng · h/ml	132.80	97.30	103.40	166.50
Amphetamine					
C_{max}	ng/ml	132.00	50.40	192.00	108.00
t_{max}	h	0.50	1.00	0.08	0.50
$t_{1/2}{}^{\beta}$	h	1.74	1.47	1.18	1.40
AUC_{0-6}	ng · h/ml	205.40	102.90	191.00	137.80
$AUC_{0-\infty}$	ng · h/ml	221.70	109.30	191.30	141.00

f_{abs} Bioavailability in % ($AUC_{0-\infty}/AUC_{0-\infty iv} \cdot 100$).

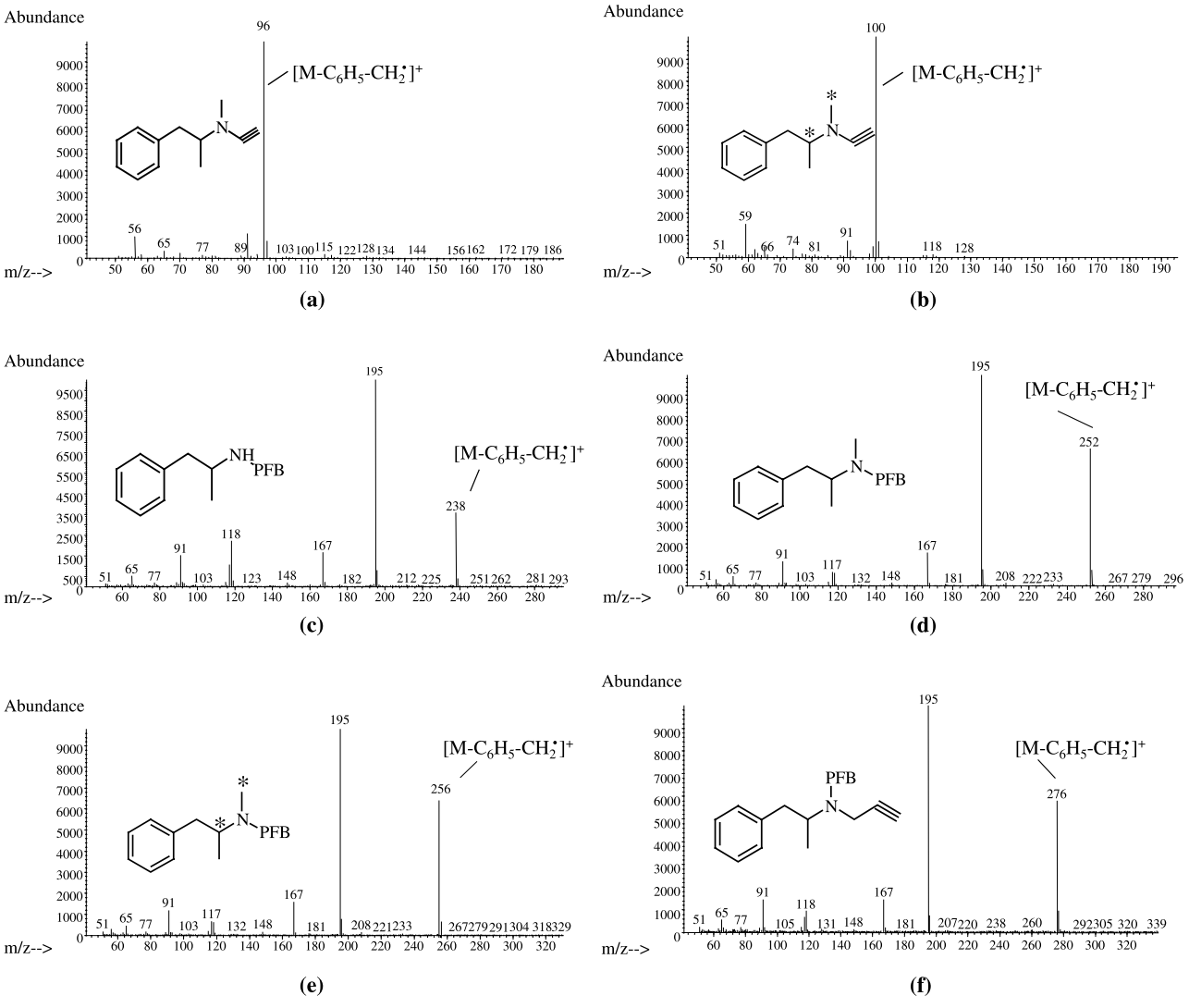

Fig. 1. Electron impact (EI) mass spectra of (**a**) (−)-deprenyl, (**b**) d_4-deprenyl, (**c**) *N*-pentafluorobenzoyl-amphetamine, (**d**) *N*-pentafluorobenzoyl-methamphetamine, (**e**) *N*-pentafluorobenzoyl-d_4-metamphetamine and (**f**) *N*-pentafluorobenzoyl-desmethyl-deprenyl. Asterisk indicates site of deuterium label and PFB indicate pentafluorobenzoyl group

tration of the drug (Table 2). These results indicate an intensive ''first pass'' metabolism of the substance.

(−)-Deprenyl is rapidly metabolized in vivo mainly by the liver microsomal enzymes. The main route of metabolism is *N*-depropargylation, leading to the formation of methamphetamine. *N*-demethylation of (−)-deprenyl was also detected, which produced desmethyl-deprenyl. Both from methamphetamine and from desmethyl-deprenyl, amphetamine is formed by *N*-demethylation or *N*-depropargylation, respectively.

The quantity of the metabolites formed from (−)-deprenyl is highly influenced by the routes of administration. This is well documented in Fig. 4, which presents the $AUC_{0-\infty}$ values of (−)-deprenyl and its metabolites following oral, subcutaneous, intraperitoneal and intravenous treatments. The plasma concentration of the main metabolite (e.g. methamphetamine) proved to be higher than that of the parent compound at every time of sampling after oral treatment (Fig. 5), while the opposite was found after parenteral administration of the drug (Fig. 6).

In spite of the short experimental period (6 h) the difference between the $AUC_{0-\infty}$ and AUC_{0-6} (AUC_{0-Rest}) is less than 3% at every type of administration, which reflects the fast elimination of the drug.

Discussion

(−)-Deprenyl is a selective irreversible inhibitor of MAO-B (Knoll and Magyar, 1972, Magyar et al., 1967), which is frequently used alone or in combination with other anti-

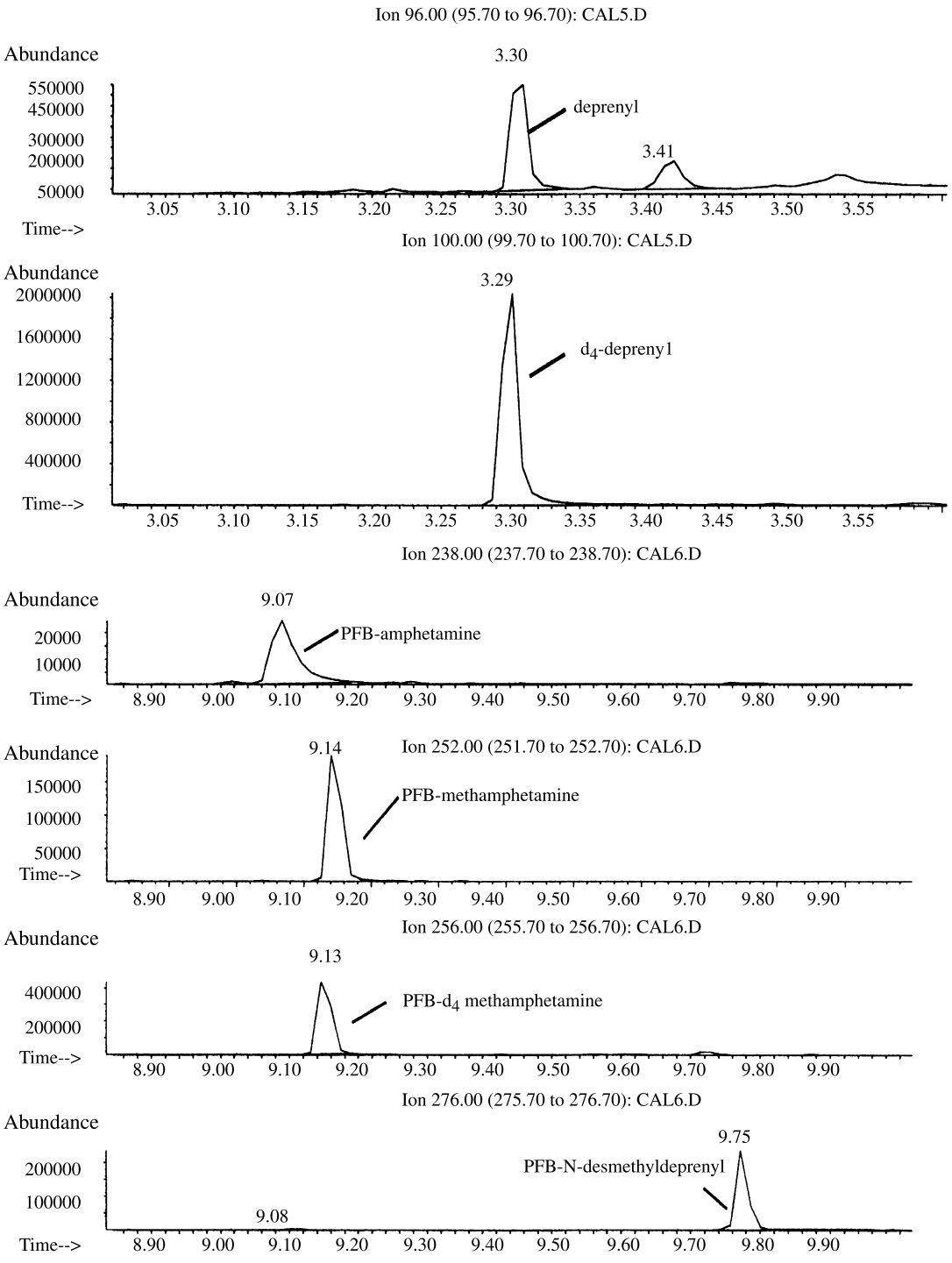

Fig. 2. Selective ion-chromatogram of a calibration standard (10 ng/ml of (−)-deprenyl, amphetamine, methamphetamine and desmethyl-deprenyl) for determination of the content of (−)-deprenyl and its metabolites

parkinsonian drugs in the treatment of Parkinson's disease (Birkmayer et al., 1985; The Parkinson Study Group: DATATOP, 1993). The enzyme inhibitory effect is due to the parent compound, but regarding the complex pharmacological activity of the substance, the role of the metabolites cannot be neglected (Magyar, 1994). Patients are treated almost exclusively orally and the usual daily dose of the inhibitor is 5–10 mg (0.07–0.14 mg/kg; Olanow, 1996). In our studies mice were treated with 5 mg/kg of (−)-deprenyl at every routes of administration, which

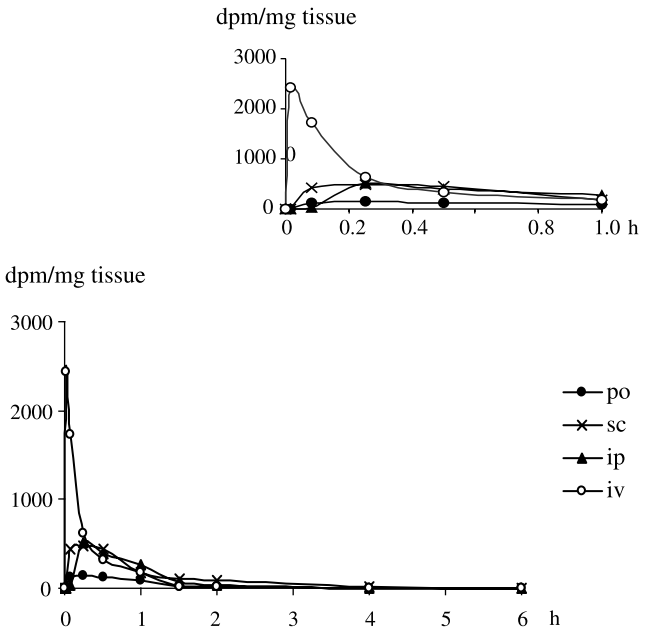

Fig. 3. The time related changes of plasma concentrations of (−)-deprenyl, following different routes of administration. Dose: 5 mg/kg; administrations: oral (*po*), subcutaneous (*sc*), intraperitoneal (*ip*), intravenous (*iv*). Values obtained within 1 h are magnified and presented up on the right corner

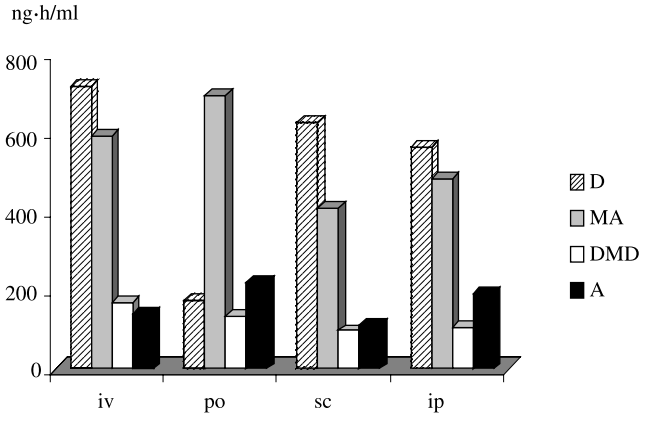

Fig. 4. The $AUC_{0-\infty}$ values of (−)-deprenyl (*D*) and of its metabolites methamphetamine (*MA*), desmethyl-deprenyl (*DMD*) and amphetamine (*A*)

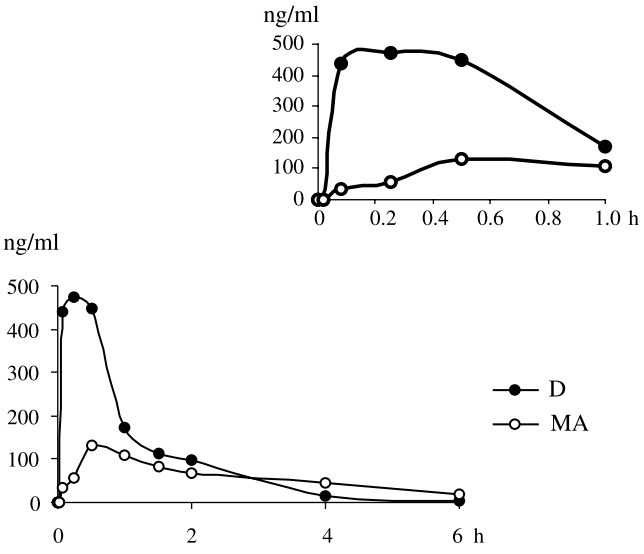

Fig. 5. The time related changes of plasma concentrations of (−)-deprenyl (*D*) and methamphetamine (*MA*) after oral treatment. Experimental conditions and the presentation of the results are as at Fig. 3

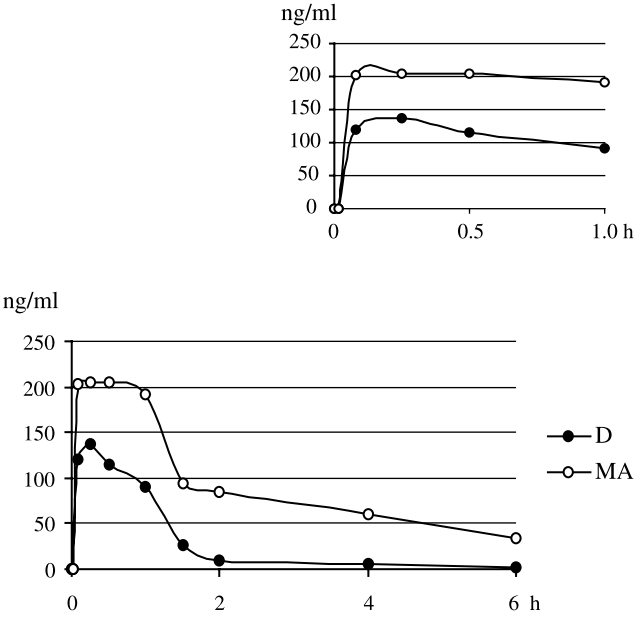

Fig. 6. The time related changes of plasma concentrations of (−)-deprenyl (*D*) and methamphetamine (*MA*) after subcutaneous treatment. Experimental conditions and the presentation of the results are as at Fig. 3

amount is 70 times higher, than the therapeutic dose. It is clearly demonstrated, that about 25% of the oral dose can reach the systemic circulation as a parent compound (Table 2). The poor bioavailability of (−)-deprenyl cannot be due to the incomplete absorption of the drug. Many studies have been carried out in our laboratory in rats and beagle dogs with radioactive labeled (−)-deprenyl (Magyar et al., 1995). These studies with side chain labeled ^{14}C-deprenyl unequivocally proved, that the oral absorption is complete. The pharmacokinetic parameters obtained in our studies showed a fast absorption ($t_{max} = 15$ min), which is followed by a rapid elimination of the drug ($t_{1/2}{}^{\beta}$ is less than 2 h). In spite of the short experimental period (6 h) the differences between the AUC_{0-6} and the extrapolated $AUC_{0-\infty}$ values were small (AUC_{Rest}), due to the fast drop in plasma concentrations, observed after every routes of administration (Fig. 3 and Table 2). In our studies the AUC_{Rest} is less than 3% at all kinds of treatment. Never-

theless, in bioequivalence studies AUC_{Rest} are acceptable, when its value is below 20%.

MAO-B activity is playing a substantial role in dopamine metabolism; the inhibition of the enzyme leads to dopamine potentiation (for review, see Magyar, 1993; Magyar and Haberle, 1999). Consequently, MAO-B inhibition decreases the level of oxygen radical species (ORS) formed from hydrogen peroxide, which is the normal product of the enzyme reaction, using dopamine or other mainly primary, but secondary and tertiary amines as substrates. Oral administration of (−)-deprenyl decreases the bioavailability of the drug, e.g. less amount of the parent compound reaches the systemic circulation. Parenteral administration, especially the subcutaneous treatment, increases the bioavailability of the drug. These findings indicate a better bioavailability of the parent compound after all of the parenteral, such as transdermal application of the inhibitor.

Regarding the complex pharmacological activity of (−)-deprenyl the metabolites are also playing role in the potentiation of dopaminergic tone. Methamphetamine and amphetamine are more potent inhibitors of the reuptake of dopamine to the nerve endings (Magyar, 1994; Magyar et al., 1996; Magyar and Haberle, 1999; Haberle et al., 2002). According to our present studies depropargylation of the molecule is the main metabolic pathway of the drug to form methamphetamine. *N*-demethylation is only a secondary pathway, which can form desmethyl-deprenyl and amphetamine from deprenyl and methamphetamine, respectively. Our results presented strong evidence that after oral administration the blood level of methamphetamine during 6 h is higher than that of the parent compound at any time of sampling (Fig. 5). It can reach nearly two times higher concentration in the blood than (−)-deprenyl. An opposite finding was obtained when (−)-deprenyl was administered parenterally (Fig. 6). It should be noted, that MAO-B inhibition remarkably increases the endogenous substrate of β-phenylethylamine (PEA) concentration. PEA and methamphetamine, due to their dopamine releasing activity, can also lead to dopamine potentiation. Chronic treatment with (−)-deprenyl might result in antioxidant activity, which does not related totally to MAO-B inhibition. Indirect antioxidant effects are also elicited due to the induction of SOD1 and SOD2 activities in tissues (Carillo et al., 1991).

In addition to the dopamine potentiating and antioxidant activity, pre-treatment with (−)-deprenyl can protect neurons against selective neurotoxins, such as MPTP (dopaminergic toxin; Langston et al., 1980), 6-OH-dopamine (dopaminergic toxin; Knoll, 1989), DSP-4 (noradrenergic toxin; Finnegan et al., 1990), AF64A (cholinergic toxin; Ricci et al., 1992), but does not protect the effect of serotonergic neurotoxins (5,6-dihydroxy-tryptamine; Magyar and Szende, 2000). The uptake inhibitory effect of (−)-deprenyl and mainly of its metabolites can play a role in the protection, consequently (−)-deprenyl pre-treatment can protect the toxicity of MPP^+ as well. Experiments carried out in the tissue cultures proved that (−)-deprenyl in rather low concentrations (10^{-9}–10^{-13} M) inhibits the apoptosis of PC12 and human melanoma cell lines induced by serum deprivation (Tatton et al., 1994; Magyar and Szende, 2000). These concentrations are too low to inhibit MAO-B activity. The antiapoptotic effect of (−)-deprenyl can be prevented by inhibiting the metabolism of the drug by SKF-525A (Magyar et al., 2004 for review). This finding indicates that some of the metabolites are responsible for the antiapoptotic activity of the drug.

Meanwhile, it has been published that a compound CGP 3466 has shown antiapoptotic activity without any effect on MAO-B (Waldmeier et al., 2000). CGP 3466 is a propargylamine-derivate, which group seems to be essential for antiapoptotic activity. Following this line it is a good reason to believe, that the metabolites, having antiapoptotic activity, should contain propargyl-group. On the analogy, it has been suggested by Tatton, that desmethyl-deprenyl could be responsible for the antiapoptotic activity (Tatton et al., 1994; Tatton and Chalmers-Redman, 1996). Nevertheless, we were not able to confirm this supposition on melanoma cell line (Magyar and Szende, 2000). Deprenyl-*N*-oxide is the other metabolite, which contains propargyl group and it might be susceptible, that it has antiapoptotic properties. Deprenyl-*N*-oxide is under examination. These results also underline the importance of the metabolic studies, but the metabolite responsible for the antiapoptotic activity of the drug is still unknown.

Former studies carried out in our laboratory, using capillary electrophoresis method proved that during metabolism from the parent compound racemisation did not occur. From (−)-deprenyl (−)-optical isomers are formed, which have less psychostimulant activity than the (+)-enantiomer.

(−)-Deprenyl is applied orally in human therapy in the treatment of Parkinson's disease. Based on our results it could be concluded, that only 25% of the dose reaches the systemic circulation, as a parent compound, due to the intensive "first pass" metabolism of the drug. The parent drug is responsible for the inhibition of MAO-B. In addition, parenteral administration of the inhibitor (subcutaneous, transdermal) improves the bioavailability of the unchanged compound being responsible for MAO-B inhibition.

In our earlier experiments, we studied the transdermal absorption of radio-labeled (−)-deprenyl from liposome on

guinea pigs. After decapitation of the animals beside the concentration of radioactivity, MAO activity was also determined in the brain and some other tissues. MAO-B activity was totally inhibited, whereas MAO-A was only partly reduced. Domestic pigs were also treated with liposome, containing (−)-deprenyl and the absorption and distribution of the inhibitor was followed by measuring the level of platelet MAO-B and tissue MAO-B and MAO-A inhibition (Gaál et al., 2000). The results coincided with our present studies. The absorption of (−)-deprenyl from liposome was complete; its rate was constant both in guinea pigs and domestic pigs.

A new transdermal preparation (transdermal patch, called Emsam (selegiline); (Bristol-Mayers Squibb); New York) has been developed and used for treating major depression. It contains 6, 9 or 12 mg of selegiline over 24 hrs; it is the same therapeutic dose which is used in the case of oral application. Using this preparation MAO inhibition is more expressed, because of the higher blood concentration of the parent compound. Within this circumstances (−)-deprenyl looses its selectivity and MAO-A, beside MAO-B blockade, is significantly inhibited. Nevertheless, MAO-A activity of the gastrointestinal tract is only slightly influenced. The dosage used for oral application in clinic, proved to be an effective antidepressant transdermally, which in spite of MAO-A inhibition, can be used without dietary restriction (Patkar et al., 2006). It has been published also, that transdermal patch was used for 7 days in rats and the effect was measured by behavioral test (forced-swim test). In this case, the rise of blood pressure (cheese effect) could not be provoked by tyramine administration, but positive results were detected on the behavioral forced-swim test, indicating effective antidepressive properties of the drug (Gordon et al., 1999).

The intensive ''first pass'' metabolism of (−)-deprenyl, which was considered to be a handicap of the drug, now it is its intrinsic value. Deprenyl was developed as an antidepressant in the early sixties, but because of the cheese effect together with the other inhibitors has fallen into disrepute. After 40 years the ''boomerang'' arrived back to the starting point; (−)-deprenyl became a potent antidepressant transdermally, in an orally used therapeutic dose, due simply to its pharmacokinetic properties.

Acknowledgements

The authors gratefully acknowledge for the financial support of the Hungarian Ministry of Health (ETT 141/2003), Hungarian Academy of Sciences Neurochemical Research Unit and Hungarian Scientific Research Found (OTKA T042595; OTKA K63415).

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J Neural Transm (2007) [Suppl 72]: 175–179

Printed in Austria

Journal of Neural Transmission

Serum lipoprotein profile and APOE genotype in Alzheimer's disease

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Summary Alterations in cholesterol homeostasis are associated with Alzheimer's disease (AD). The role played by specific fractions of serum lipoproteins in modifying the risk of AD, and the interaction with APOE genotype has not yet been investigated. We studied serum lipoprotein profiles using a gradient-density ultracentrifugation method in a cohort of late-onset sporadic AD patients without cerebrovascular lesions and in healthy elderly subjects.

In the AD group the lipoprotein cholesterol distribution showed an increase in LDL cholesterol, reaching a significant difference with respect to controls in the LDL sub-fractions representing the transition between small dense-LDL (fraction 11, $p = 0.04$) and normal-density LDL particles (fraction 12, $p = 0.03$). APOE genotype and LDL cholesterol were independently associated with AD. The mean concentration of LDL in fractions 11 and 12 increased the risk of developing AD ($p = 0.01$ and $p = 0.025$, respectively).

These results confirm that an alteration of cholesterol homeostasis is associated with AD and that serum concentrations of LDL cholesterol are higher in AD patients without cerebrovascular pathology than in elderly normal subjects. The presence of the APOE ε4+ allele is a risk factor for AD independent of increased serum cholesterol or a modification of other vascular risk factors. Increased levels of specific sub-fractions of LDL cholesterol may be associated with increased risk of AD.

Keywords: Alzheimer's disease, lipid profile, cholesterol, LDL, APOE

Introduction

Sporadic late-onset Alzheimer's disease (AD) is a progressive neurodegenerative disease with a multi-factorial aetiology in which genetic and environmental factors modulate the risk and the expression of the pathological process. In the last decade epidemiological research has demonstrated that several cardiovascular risk factors may modify the risk to develop AD (Hayden et al., 2006; Luchsinger et al., 2005; Stampfer, 2006). Although the mechanism by which vascular brain lesions interplay with neurodegenerative pathology in AD is not fully elucidated, several studies showed that cerebral small vessel alterations, namely arteriolosclerosis, endothelial proliferation and neo-vascularization, with disrupted blood–brain barrier and amyloid angiopathy, are early events in AD (De la Torre, 2004; Zlokovic, 2005). It is still controversial whether AD-like pathology and cerebrovascular lesions are coexisting but unrelated pathologies or whether they result from different synergistic pathological mechanisms.

A growing amount of evidence points to an association between alterations in cholesterol homeostasis and AD (Evans et al., 2000; Lesser et al., 2001). A longitudinal population-based long-term study has shown that high plasma concentrations of cholesterol in mid-life may determine the risk of developing late onset AD (Kivipelto et al., 2006). In contrast, studies of mean serum cholesterol levels in elderly people affected by AD at different stages of the disease report conflicting results (Kivipelto and Solomon, 2006; Panza et al., 2006; Romas et al., 1999; Tan et al., 2003). However, the presence of alterations in specific fractions of serum lipoproteins and the putative role of such changes of the lipid profile in AD is unknown.

The objective of this study was to assess modifications of serum lipoprotein profile in a homogeneous cohort of sporadic AD patients without cerebrovascular lesions, and to investigate any interaction with Apolipoprotein E (APOE) genotype.

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Patients and methods

Subjects

Patients with clinical diagnosis of probable AD according to National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's disease and related disorders Association (NINCDS-ADRDA) criteria (McKahn et al., 1984) were invited to take part in the study.

Fifty-four AD patients (78% female; mean [±SD] age: 76.8 [±6.1] years, range 63–94 years) and 62 healthy elderly volunteers (76% female; mean [±SD] age: 73.8 [±7.7] years, range 60–94 years) were enrolled. Exclusion criteria were the presence of cerebrovascular disease, psychiatric or other neurological diseases associated with cognitive deficits, other than AD. Patients and controls on statins or other lipid-lowering treatments were excluded since the therapeutic intervention could influence the serum lipoprotein profile.

Each subject underwent a full clinical history, neurological evaluation and blood analysis (glycaemia, total cholesterol, triglycerides, homocysteine, folate, vitamin B_{12}). Plasma total homocysteine levels were measured using an IMx analyzer (Abbott). Folate levels were measured by ACS:180 automated chemiluminescence's analyzer (Bayer). A blood sample for the study of lipid profile and for APOE genotyping was obtained. ECG and ultrasonography of both carotid arteries were also performed. The presence of cognitive impairment was assessed using the Mini Mental State Examination (MMSE) test. AD patients were further investigated with a full neuropsychological assessment. A brain CT or MRI scan was performed in each subject to assess the degree of vascular brain changes. The Wahlund's Age-Related White Matter Changes (ARWMC) scale, applicable to both CT and MRI images, was used to quantify the degree of cerebrovascular lesion load (Wahlund et al., 2001). The scale was applied to each scan film by two independent readers (G.Z. and D.M.) blinded to patient diagnosis. The study conformed to the guidelines set out in the Declaration of Helsinki of 1975 and all the subjects provided written informed consent.

Methods

Lipoprotein profile

Serum concentrations of lipoproteins were measured by using a density-gradient ultracentrifugation method for apolipoprotein B-containing lipoproteins, as previously described (Chung et al., 1980, 1986). After creating a discontinuous salt density gradient in an ultracentrifuge tube, samples were centrifuged at 65,000 rpm for 90 min (total $\omega r2 = 2.36 \times 1011$) at 10°C in a Sorvall TV-865B vertical rotor. Thirty-seven 0.34 ml fractions were then collected from the bottom of the centrifuge tube. Cholesterol was measured in each fraction and a lipoprotein profile across a density range was therefore obtained.

APOE genotyping

Genomic DNA was isolated using a standard DNA extraction. The APOE genotype was evaluated by the method of Hixson and Vernier (1990). All genotyping results were read by two reviewers blinded to the clinical status of the patient.

Statistical analysis

Demographic characteristics were compared for the AD and control groups using *t*-tests for variables with normal distribution, Mann-Whitney *U*-tests for ordinal qualitative variables, χ^2 and Fisher's exact tests for nominal variables. Multivariate analysis was performed using factorial Anova in order to test the dependence of serum lipoprotein fractions from APOE genotype, group (AD vs. Controls) and eventually the first order interaction effect. The logistic regression model was used to reveal independent variables related to presence of AD. Results were considered statistically significant when the p value were equal to, or less than, 0.05.

Results

AD patients were significantly older ($p = 0.02$) and had completed a lower level of education ($p = 0.01$) than the control subjects. The AD and normal controls did not differ with respect to gender. The mean value of the MMSE score was 17.9 ± 5.8 in AD patients and 29.1 ± 1.3 in healthy controls ($p < 0.001$).

The prevalence of vascular risk factors (systolic and diastolic hypertension, coronary heart disease, cardiac arrhythmia, carotid atherosclerosis and diabetes) was similar in the two groups, as shown in Table 1. Cerebrovascular lesions were absent or minimal in both groups as demonstrated by comparing the indexes obtained with the application of the ARWMC scale (Wahlund index: 1 ± 2.2 in AD patients and 0.5 ± 1.1 in controls; $p = 0.2$). No difference in serum levels of glucose, homocysteine and folate was found among the two groups.

The serum lipid profile showed a similar level of triglycerides and an increase of total cholesterol level in AD patients compared to controls (total chol: AD 5.8 ± 1.4 mmol/l; controls 5.3 ± 1.3 mmol/l; $p = 0.05$). A detailed analysis of the 37 fractions obtained using the density-gradient ultracentrifugation method demonstrated a trend towards an increase of LDL cholesterol fractions in the AD group compared to controls, with a significant difference for fractions 11 ($p = 0.04$) and 12 ($p = 0.03$) representing the LDL compound of transition between small-dense-LDL (sd-LDL, fraction 11) and average-density LDL particles (fraction 12) (Fig. 1). A modest increase of the other fractions of sd-LDL, average-density LDL and HDL in the AD group did not reach significance. The increased levels of serum total cholesterol and LDL cholesterol (fraction 11 and 12) in the AD group were not age-related.

Table 1. *Demographic and clinical characteristics of patients with AD and controls*

Demographic and clinical variables	AD ($n = 54$)	Controls ($n = 62$)	p value
Age (years) ± sd	76.8 ± 6	73.8 ± 8	*0.02*
Female (%)	77.8	75.8	0.8
MMSE ± sd	17.9 ± 5.8	29.1 ± 1.3	*<0.001*
Smoking (%)	8.2	13.5	0.39
CHD (%)	7.5	6.4	0.82
Arrhythmia (%)	17.3	11.3	0.36
Carotid atherosclerosis (%)	50.0	27.0	*0.04*
Systolic hypertension (%)	46.3	51.6	0.58
Diastolic hypertension (%)	42.6	51.6	0.33

CHD Coronary heart disease. Hypertension was considered present when systolic blood pressure was >140 mmHg and diastolic pressure >90 mmHg at more than three different measurements. Carotid atherosclerosis defined by the presence of arterial plaque >15–20%

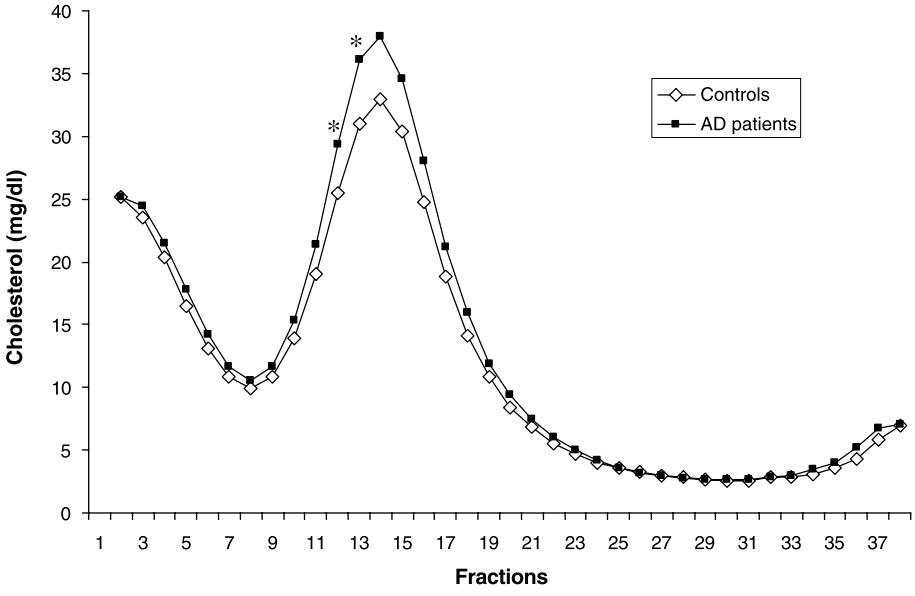

Fig. 1. Lipid profile in controls (open squares) and AD (solid squares) obtained using a density gradient ultracentrifugation method. We obtained 37 fractions with high density lipoproteins (HDL) at one extreme (fractions 1–6) and very low density lipoproteins (VLDL) at the other extreme (fractions 29–37). Fractions 7–11 represent small/dense low-density lipoproteins (sd-LDL); fractions 11–18 contain large/buoyant LDL (lb-LDL) and fractions 19–28 intermediate LDL (IDL). AD patients showed an increase of sub-fractions of LDL which reaches significance for fractions 11 and 12 ($p<0.05$)

Table 2. *APOE genotype distribution in patients with AD and controls*

APOE genotype	AD ($n=53$) %	Controls ($n=60$) %	p value
			<0.001
			df = 2
ε4−/ε4−	50.9	89.7	
ε4+/ε4−	43.4	10.3	
ε4+/ε4+	5.7	0	

Genotypic distribution did not differ from those predicted by Hardy-Weinberg equilibrium in the whole sample. The distribution of APOE genotypes (ε4−/ε4−; ε4−/ε4+; ε4+/ε4+) was significantly different among AD patients and controls ($\chi^2=20.7$, df: 2, $p<0.001$) (Table 2).

The logistic regression model showed that APOE genotype and serum level of LDL fractions 11 or 12 were independent variables associated with AD.

Grouping the AD patients by APOE gene status in APOE ε4− and ε4+, we found a significant increase of total sd-LDL particles (sum of fractions 7, 8, 9, 10 and 11) in those without the ε4 allele compared to those with the ε4+ (AD ε4−: 97.7 ± 33 mg/dl, AD ε4+: 80.1 ± 19 mg/dl; $p=0.02$). A comparison of mean total cholesterol and levels of lipoprotein fractions among AD and controls grouped by APOE ε4 genotypes was limited by the small number of normal controls with APOE ε4+ genotypes.

Discussion

To our knowledge this is the first study on the plasma lipoprotein profile, using a density-gradient ultracentrifugation method, performed in patients affected by sporadic late-onset AD without concomitant vascular brain lesions. The results of this study confirm that alterations of cholesterol homeostasis are associated with AD even in the absence of concomitant cerebrovascular pathology. A specific fraction of LDL cholesterol, represented by the LDL compound of transition between oxidized sd-LDL and normal density-LDL, was higher in AD patients. In the last two decades, several studies have shown an association between increased total serum cholesterol (Evans et al., 2000; Lesser et al., 2001) and LDL cholesterol (Bonarek et al., 2000; Kuo et al., 1998; Lesser et al., 2001) concentrations in sporadic late-onset AD patients compared to healthy elderly controls. These data were not confirmed by other studies that observed decreased total cholesterol and LDL cholesterol in AD patients (Panza et al., 2006; Romas et al., 1999). It has been shown that total cholesterol may decline with increasing age and with the full clinical manifestation of AD brain pathology (Notkola et al., 1998). Differences in demographic characteristics and in the degree of disease severity may at least partially explain some discrepancies in the results obtained studying populations of late-onset elderly AD patients. Recently, it has been confirmed that high total serum cholesterol during middle age or early old age confers an increased risk for developing AD in older age (Kivipelto et al., 2006; Notkola et al., 1998).

The epidemiological observations of a link between cholesterol and AD are strengthen by cell biology studies demonstrating that the processing of the amyloid precursor protein and the production of $A\beta_{1-42}$ are strongly modulated by cholesterol (Shie et al., 2002; Shobab et al., 2005; Simons et al., 1998). Furthermore, alterations of cerebral cholesterol metabolism may influence tau phosphorylation and the assembly of neurofibrillary tangles indirectly

through the up-regulation of $A\beta_{1-42}$ or through a direct modulation of tau phosphorylation (Ohm and Meske, 2006). Nonetheless, we have to keep in mind that cerebral metabolism of cholesterol is independent and separated from that of plasma cholesterol (Dietschy and Turley, 2004). Thus plasma levels of total cholesterol or LDL fractions are not a direct reflection of intra-cerebral cholesterol metabolism. In addition, hypercholesterolemia can indirectly influence the AD risk via stimulation of cerebrovascular damage, thus inducing a defective brain oxygenation (De la Torre, 2004) and a neurovascular dysfunction (Zlokovic, 2005). Our results support an association between hypercholesterolemia, particularly in the LDL fraction, and AD, but do not clarify whether increased LDL cholesterol represents a risk factor or, instead, reflects a possible altered mechanism shared by cholesterol metabolism and AD pathogenesis.

Apolipoprotein E is one of the major cholesterol transport proteins in the blood and in the brain and the APOE ε4 status is the only consistently recognized genetic risk factor for AD. The nature of the relationship between cholesterol and APOE ε4 in increasing the susceptibility to AD is far from elucidated. Several studies have demonstrated that APOE ε4+ does not increase the risk of AD by modulating cholesterol metabolism (Borroni et al., 2006; Evans et al., 2000; Wolozin et al., 2006). The results of our study confirm and further support these previous findings. In fact, total LDL cholesterol levels were associated with AD independently of APOE ε4 status. Interestingly, small/dense lipoproteins were higher in those AD patients with no ε4 allele. These data suggest that dense lipoproteins, usually associated with carotid atherosclerosis and coronary heart disease, may have a potential role in AD even without imaging evidence of concurrent cerebrovascular lesions and that this effect may be masked by the presence of APOE ε4 allele.

In conclusion, high levels of serum total cholesterol and LDL cholesterol were associated with sporadic, late-onset AD. Whether serum hypercholesterolemia is a risk factor for AD or is a consequence of shared patho-mechanisms involved in the neurodegenerative processes and cholesterol homeostasis remain to be established. APOE genotype influences the risk of AD through a mechanism independent of cholesterol metabolism. Interestingly, the lipoprotein profile differs in AD patients depending on the APOE ε4 status with increased sd-LDL in those AD with no ε4. A better understanding of the role of small, dense and oxidized LDL in the pathogenesis of AD, with and without concomitant cerebrovascular lesions, and its relationship with APOE genotypes and carotid atherosclerosis warrants further research.

Acknowledgements

The work was supported by a grant to A.C., A.L. and A.Z. from the Italian Ministry of Health within the Finalized Research Projects 2006–2007 (type II, no 107).

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J Neural Transm (2007) [Suppl 72]: 181–188

Printed in Austria

Journal of Neural Transmission

VITA study: white matter hyperintensities of vascular and degenerative origin in the elderly

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Summary The etiology of white matter hyperintensities (WMH) seen on T2-weighted cranial magnetic resonance images is a matter of debate. We investigated deep and periventricular WMH in the brains of a community-based cohort of 532 subjects aged 75–76 years. The objective of this study was to determine whether WMH at age of 75 years were associated rather with vascular factors than with degenerative factors.

Arterial hypertension treated with antihypertensive drugs favored WMH, and WMH were found more frequently in subjects with focal vascular lesions. Additionally, we found significant associations between both, deep white matter and periventricular hyperintensities, and focal atrophy of medial temporal lobe structures. The odds ratio for deep WMH in subjects with more severe medial temporal atrophy was 4.4 (95%-CI: 1.9–9.8) that for periventricular hyperintensities was 3.9 (95%-CI: 1.7–8.8).

These findings might indicate that not only vascular factors alone but also degenerative factors favor the occurrence of WMH after the age of 75 years.

Keywords: White matter hyperintensities, Alzheimer dementia, vascular lesions, medial temporal lobe atrophy

Introduction

White matter hyperintensities (WMH) on T2-weighted cranial magnetic resonance images (MRI) are common in the brains of elderly people, and their frequency increases strongly with age (Fazekas et al., 1987; Christiansen et al., 1994; Longstreth et al., 1996). The etiology of WMH is still a matter of debate. They are interpreted as a consequence of chronic cerebral hypoperfusion, favor a clinical diagnosis of ''subcortical vascular encephalopathy'', and they are seen as a significant part of the pathophysiology of vascular cognitive impairment (Breteler et al., 1994b; Liao et al., 1996; Longstreth et al., 1996; Pantoni et al., 1996; Pantoni, 2002; De Leeuw et al., 1999; O'Brien et al., 2003). Patients with such abnormalities are described as being more likely exposed to vascular risk factors and infarcts (Schmidt et al., 1992, 1999; Breteler et al., 1994b; Lindgren et al., 1994; Fukuda and Kitani, 1995; Ylikoski et al., 1995; Liao et al., 1996; Longstreth et al., 1996; De Leeuw et al., 1999; Vermeer et al., 2002; Streifler et al., 2003).

WMH were found to be more severe in subjects with a diagnosis of Alzheimer dementia (AD) where these lesions are not necessarily associated with hypertensive vascular changes (Brun and Englund, 1986; Breteler et al., 1994b; De Groot et al., 2000). The few existing clinico-pathological case-reports describe a non-ischemic nature of a certain proportion of WMH, but most of these clinico-pathological cases had died at an age younger than 75 years (Janota et al., 1989; Chimowitz et al., 1992; Fazekas et al., 1993; Thomas et al., 2002; Ukada et al., 2002).

We investigated WMH in the brains of a large community-based population of 75–76 years of age. We report the frequency of both, deep white matter hyperintensities (DWMH) and periventricular hyperintensities (PVH). We calculated the impact of various vascular risk factors, and of cerebral infarcts on both, DWMH and PVH, and compared

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these effects with the impact of medial temporal lobe atrophy. The question posed was whether DWMH and PVH at an age of 75 years were associated rather with vascular factors than with degenerative factors, as medial temporal lobe atrophy has been shown to characterize AD (Scheltens et al., 1992, 1995b, 1997; Wahlund et al., 2000; Petersen et al., 2000).

Subjects and methods

The VITA study is a prospective longitudinal study on mental aging. The study design, recruitment strategy and participation rate have been described: at baseline the study tried to investigate every 75-year old inhabitant of a geographically defined area of Vienna, which lies on the left shore of the Danube (''Vienna Transdanube''), born between May 1925 and June 1926 (Fischer et al., 2002). This area consists of two districts (21st and 22nd districts of Vienna) with 264.672 inhabitants. We invited all individuals of this geographical birth-cohort to participate in the study (institutionalized and non-institutionalized).

The main aim of the VITA is the prediction of incident cases of dementia after 30 months and after 60 months. Because age is the strongest predictor of cognitive decline in the elderly, the variance of age was minimized in the VITA and subjects were invited to participate in the sequence of their birth, which means that older subjects of the cohort were seen earlier. With the help of a liberal recruitment strategy, which had been accepted by the local ethics commission, 1505 inhabitants were contacted. The participation rate was 46% (telephone interview or short investigation or complete investigation). The standard deviation of age in the 606 subjects (40.3% of the 1505) who underwent the complete investigation was only 0.4 years. The complete investigation comprised medical and psychosocial interviews, psychological tests, psychiatric and neurological scales, blood characteristic, and a cranial MRI (Fischer et al., 2002). It lasted about 9 hours per patient (contact session including informed consent; some days later: main session including MRI; 2 weeks later: discussion of results with the participant).

The basal population seen between 2000 and 2002 consisted of 606 individuals, who underwent all investigations including blood sampling. Exclusion criteria for MRI were conventional: 1) presence of a cardiac pacemaker, some types of valvular prothesis, or other internal electrical device; 2) history of neurosurgery or aneurysm before 1985; and 3) presence of metal fragments in the eyes, brain, or spinal cord. Cranial MRI could be carried out in 532 of the 606 participants. VITA participants without MRI did not differ from those with MRI as far as vascular risk factors, history of stroke, history of cardiovascular disease, and cognitive scores are concerned. The MRI investigation was performed using a 1.0 Tesla unit (Siemens Impact Expert) with a circular polarized skull coil. The following sequences were obtained: transverse PD and T2-weighted TSE, coronary T1-weighted gradient echo sequence (MPRAGE) and a thin-section IR sequence in the olfactory region. Lacunes (i.e. small infarcts) were defined as cyst-like lesions with signal-intensity isotense to cerebrospinal fluid on both, T1 and T2 measurements, and a maximum diameter of 15 mm. Lesions of more than 15 mm in size that follow a vascular territory regardless of grey or white matter were rated as infarcts. For the rating of medial temporal lobe atrophy (MTA) the hippocampal area along the longitudinal axis of the hippocampus was reconstructed.

The presence and severity of WMH was determined by the Fazekas rating including a four point scale to assess PVH (0, absent; 1, caps or thin lining; 2, smooth halo; and 3, irregular areas extending into the deep white matter) and a four point scale to assess DWMH (0, absent; 1, large punctate foci; 2, beginning confluence of foci; and 3, large confluent areas) (Fazekas et al., 1987). Evaluation of various rating systems for WMH showed the superiority of this rating method concerning interrater reliability and also showed high validity compared with quantitative volumetric measurement of white matter changes (Kapeller et al., 2003). The MRIs of the first consecutive 105 subjects were assessed independently by two experienced radiologists. Inter-rater reliability for PVH and DWMH was high (PVH: Spearman $\rho = 0.621$; $p < 0.000$; DWMH: $\rho = 0.648$; $p < 0.000$). For statistical calculations only ratings of radiologist 1 (W.K.) were taken, who rated all 532 MRIs blind to all demographic, medical, neurological, psychiatric, and neuropsychological data.

Local atrophy of the medial temporal lobe (MTA) was assessed qualitatively on a 0–4 scale according to Scheltens et al. (1992, 1995b, 1997): scores range from 0 (no atrophy) to 4 (very severe atrophy), but 4 was not found in our community-based age-cohort. The rating scale was based on a visual estimation of both the volume of the medial temporal lobe, including the hippocampus proper, dentate gyrus, subiculum, and parahippocampal gyrus, and the volume of the surrounding cerebrospinal fluid spaces, in particular, the temporal horn of the lateral ventricle and the choroid fissure on both sides. This visual method of scoring correlates well with linear and volumetric measurements and has reasonably good inter- and intrarater reliability (Scheltens et al., 1995b, 1997; Kapeller et al., 2003). Comparisons between volumetric methods and visual rating

of MTA have shown no advantage for volumetry (Wahlund et al., 2000).

Vascular risk factors were described in the population of 532 subjects who completed the cranial MRI. We included the following vascular risk factors in orienting analyses of associations to WMH: HbA1c in serum; serum levels of low-density lipoprotein cholesterol (LDL), of high-density lipoprotein cholesterol (HDL), and of triglyzceride; lipoprotein (a) plasma level, homocysteine serum level; fibrinogen plasma level; C-reactive protein; body mass index; years of smoking; therapy with lipid-lowering drugs (no/yes); therapy with blood pressure lowering drugs (no/yes), systolic blood pressure (sBP), diastolic blood pressure (dBP). High blood pressure was defined as systolic blood pressure >135 mmHg or diastolic blood pressure >85 mmHg according to the seventh report of the joint national committee on high blood pressure (Chobanian et al., 2003). Subjects with high blood pressure after 5 min rest or those taking antihypertensive medication were considered ''hypertensives''. A tendency of orthostatic hypotension after standing motionless for 1 min following resting position (sitting or whenever possible supine for at least 10 min) was characterized by the difference of systolic blood pressure during rest minus the systolic blood pressure after 1-minute standing (Polinsky and Martin, 1994).

Statistical analyses (except ordinal regressions) were performed using the SPSS-11.5 Statistical Package for the Social Sciences. Group differences in categorical variables were analyzed by χ^2 tests. Spearman nonparametric correlations were calculated between each vascular risk factor on the one hand and DWMH or PVH on the other hand. Only correlations with a *p*-value lower than 0.15 were considered for further regression analysis. Stepwise ordinal regressions were performed to search for DWMH- and PVH-inducing risk factors using the DWMH scores (no–mild–moderate–severe) and PVH scores (no–mild–moderate–severe) as dependent variables and gender, BMI, vascular risk factors, and vascular or degenerative MRI findings as predictors. Vascular MRI findings were lacunes (i.e. small infarcts) or infarcts. The MRI finding possibly indicating degeneration was the visual rating of MTA. Vascular risk factors in the ordinal regressions were: HbA1c, LDL-cholesterol, HDL-cholesterol, triglyzcerides, fibrinogen, homocysteine, C-reactive protein, lipoprotein (a), sBP, dBP, smoking, lipid-lowering drugs (no/yes), and antihypertensives (no/yes). Then again, a stepwise ordinal regression was performed with all significant variables and their interactions. To reduce the number of missing observations, a fixed, non-stepwise model was then calculated with all significant variables. The probability of being in a higher category of white-matter lesions was modeled. The probability to enter or to stay in the model was set to 0.05. Ordinal regression analyses were done using SAS 8.

Results

Prevalence of DWMH and PVH in the VITA participants are shown in Table 1a.

DWMH were not rated because of a possible artifact in one male subject. A total of 31% (167 of 531) were free of any DWMH, 55% (290 of 532) did not show any PVH. WMH of any type or severity were found in 72% of subjects

Table 1a. *Vascular risk factors in patients with various degrees of deep WMH or periventricular hyperintensities*

Characteristics	DWMH				PVH			
	0	1	2	3	0	1	2	3
Total, *n*	167	194	111	59	290	167	17	58
Gender, m/f	74/93	81/113	35/76	23/36	116/174	63/104	9/8	26/32
Body mass index	27 (4)	27 (4)	27 (4)	28 (4)	27 (4)	27 (4)	29 (5)	27 (4)
HbA1c	5.8 (1)	6.0 (4)	6.0 (1)	6.0 (1)	6.0 (3)	6.0 (1)	6.0 (1)	6.0 (1)
LDL-cholesterol	148 (40)	141 (44)	143 (36)	143 (37)	149 (40)	146 (42)	160 (40)	140 (36)
HDL-cholesterol	58 (15)	60 (15)	61 (17)	56 (14)	59 (15)	59 (14)	60 (17)	59 (17)
Triglyzcerides	129 (59)	130 (64)	138 (63)	148 (78)	130 (62)	137 (71)	126 (42)	143 (59)
Fibrinogen	390 (82)	391 (91)	387 (87)	386 (91)	393 (91)	385 (79)	380 (69)	389 (94)
Homocysteine	14 (5)	14 (7)	14 (5)	15 (4)	114 (6)	14 (4)	17 (8)	15 (5)
C-reactive protein	5.1 (8.8)	4.8 (8.5)	3.6 (4.9)	4.6 (7.6)	5.2 (9.1)	3.5 (5.0)	3.2 (4.2)	4.7 (6.1)
Lipoprotein, Lp(a)	0.18 (0.17)	0.18 (0.18)	0.18 (0.17)	0.17 (0.12)	0.18 (0.18)	0.17 (0.18)	0.10 (0.02)	0.20 (0.12)
BP diastol, mmHg	81 (9)	83 (11)	81 (10)	84 (10)	82 (10)	82 (11)	87 (11)	81 (9)
BP systol, mmHg	145 (21)	148 (21)	145 (19)	147 (23)	146 (20)	147 (22)	155 (22)	142 (19)
Orthostatic hypotension	7.2 (16)	7.0 (18)	8.2 (15)	8.2 (17)	7.8 (17)	7.0 (17)	8.1 (23)	6.7 (14)
Smoking, % positive history	42	44	35	30	42	42	41	45
% on antihypertensive drugs	62	59	65	76	61	62	82	69
% on lipid-lowering drugs	22	19	23	25	19	23	24	26

Table 1b. *Vascular risk factors in patients without severe WMH compared to patients with both, severe DWMH and severe PVH (SD in brackets; p-values refer to t-tests or χ^2 test)*

	Neither severe DWMH nor severe PVH $n=351$	Both, severe DWMH and severe PVH $n=65$	p-Value
Gender, m/f*	150/201	30/35	0.609
Body Mass Index	27 (4)	28 (5)	0.203
HbA1c	6.0 (3.2)	5.8 (0.9)	0.619
LDL-cholesterol	150 (42)	143 (37)	0.208
HDL-cholesterol	59 (15)	59 (17)	0.850
Triglyzcerides	130 (61)	139 (54)	0.233
Fibrinogen	390 (88)	385 (93)	0.636
Homocysteine	14 (5.6)	15 (4.9)	0.143
C-reactive protein	5.0 (8.7)	4.4 (5.4)	0.637
Lipoprotein, Lp(a)	0.18 (0.18)	0.18 (0.12)	0.941
BP diastol, mmHg	82 (10)	82 (9)	0.835
BP systol, mmHg	147 (20)	144 (18)	0.270
Orthostatic hypotension	7.1 (17.2)	7.1 (16.5)	0.993
Smoking history *	43%	43%	0.993
Antihypertensives *	60%	74%	0.036
Lipid-lowering drugs*	19%	23%	0.501

* χ^2 test.

and 42% had both, DWMH and PVH of any severity. DWMH and PVH were highly intercorrelated (Spearman $\rho=0.658$; $p<0.0001$; $n=531$). No single subject had only confluent DWMH without PVH and no single subject had only irregular PVH without DWMH.

A comparison of the 351 patients with neither confluent DWMH nor confluent PVH with the 65 patients with both, confluent DWMH and PVH, concerning vascular risk factors showed only one significant comparison: patients with confluent white matter changes took antihypertensive drugs more frequently (Table 1b).

Each vascular risk factor was found in a high percentage of these 532 subjects: 68.6% had arterial hypertension at the time of the investigation measured after 10 min rest in sitting position; 62.8% are currently taking antihypertensives. We found elevated BP (>135/85) in 238 subjects, i.e. 71% of the 334 subjects who were taking antihypertensives. Taken together, 86.7% had either antihypertensive treatment and/or high blood pressure and could be labeled as ''hypertensives''.

A tendency to orthostatic hypotension is described by a postural fall of systolic blood pressure: 16.4% of the probands had a drop of systolic blood pressure of more than 20 mmHg. The drop in systolic blood pressure correlated significantly with a) systolic blood pressure ($\rho=0.305$; $p<0.0001$), b) diastolic blood pressure ($\rho=0.099$; $p=0.024$), and c) with the amplitude of blood pressure after 1 min standing motionless ($\rho=-0.372$; $p<0.0001$). The postural fall of systolic blood pressure was not associated with other vascular risk factors or any findings on MRI.

A positive history of smoking was found in 226 participants: 34.3% of the population had a history of at least 15 years of smoking, 10.7% had a history of smoking of at least 45 years. Diabetes mellitus was present in 80 subjects. Mean HbA1c of the entire cohort was 5.9% (sd = 2.6): 14.8% had values higher than 6.5 and 9.5% had values higher than 7.0%. Mean HDL-cholesterol was 59.1 mg/dl (sd = 15.2); 28.9% had HDL-cholesterol lower than 50 mg/dl. Mean LDL-cholesterol was 147.6 mg/dl (sd = 40.5); LDL-cholesterol was higher than 170 mg/dl in 28% of the cohort and higher than 200 mg/dl in 9.8% of the patients. Statines were given to 17.5% of the subjects (93 patients), other lipid-lowering drugs to 25 subjects (4.7%): 5 subjects took both – statines and other lipid-lowering drugs. Mean homocysteine blood level was 14.1 μmol/l (sd = 5.4): 30.6% had homocysteine levels higher than 15.0 μmol/l, 19.1% higher than 17.5 μmol/l. Homocysteine levels were highly correlated with both low folic acid serum levels ($r=-0.26$; $p=0.000$; $n=529$) and low serum vitamin B12 levels ($r=-0.24$; $p=0.000$; $n=528$). Lipoprotein (a) levels (mean 0.18 g/l, sd = 0.17) showed weak correlation with LDL-cholesterol levels ($\rho=0.183$; $p=0.021$).

MTA was found to be mild in 13%, moderate in 3.3% (20 subjects) and severe in 0.3% (2 subjects) of the probands. MTA could not be rated due to artifacts in 8 subjects. Lacunes or infarcts (diameter on MRI smaller or greater than 1.5 cm) were found in 100 out of 532 probands. MRI lacunes correlated significantly with MRI infarcts ($\rho=0.178$; $p<0.0001$). Lacunes without infarcts were found in 64 subjects, and 13 subjects showed lacunes together with greater infarcts.

Associations between vascular risk factors, WMH and MTA are shown in Table 2. Both – DWMH and PVH – were associated only very weakly with vascular risk factors. Without correction for multiple testing, DWMH and PVH were both associated with MTA and focal vascular lesions on MRI.

Ordinal regressions are shown in Table 3. Concerning DWMH, 9 out of 532 patients were deleted due to missing observations (artifacts in MRI in 1, missing vascular risk factor in 8 subjects). As the probability of being in a higher category is modeled, the values of DWMH are placed in descending order. The $R2=0.03$ is very small. A stepwise ordinal regression enters the categorical variable of ''taking antihypertensives'' ($p=0.043$) and the rating of MTA ($p=0.0011$). Because the third group of MTA has only

Table 2. *Associations between deep WMH (DWMH) and periventricular hyperintensities (PVH), MRI parameters, and vascular risk factors (non-parametric correlations (Spearman's ρ, p-values) in case of quantitative variables; Mann–Whitney U-tests (Z, p-value) concerning dichotomised variables labelled with**

Risk factor	DWMH		PVH	
	ρ, Z	p-Value	ρ, Z	p-Value
Gender*	−1.740	0.082	−0.445	0.657
Body Mass Index	0.037	0.395	0.060	0.167
HbA1c	0.016	0.712	0.054	0.217
LDL-cholesterol	−0.044	0.311	−0.058	0.185
HDL-cholesterol	0.033	0.455	−0.017	0.700
Triglyzcerides	0.073	0.095	0.077	0.075
Fibrinogen	−0.023	0.606	−0.035	0.430
Homocysteine	0.070	0.109	0.054	0.211
C-reactive protein	−0.060	0.172	−0.082	0.059
Lipoprotein-a, Lp(a)	0.049	0.556	0.021	0.801
Blood pressure >135/85*	−1.439	0.150	−0.396	0.692
Diastolic BP	0.061	0.162	0.007	0.879
Systolic BP	0.027	0.537	0.000	0.994
Orthostatic hypotension	0.016	0.714	−0.025	0.565
Smoking history*	−0.060	0.952	−0.127	0.899
Antihypertensives*	−1.581	0.114	−1.254	0.210
Lipid-lowering drugs*	−0.623	0.533	−1.452	0.146
Medial temporal lobe atrophy	0.112	0.010	0.142	0.001
Focal vascular lesions on MRI*	−2.239	0.025	−3.043	0.002

Table 3. *Analyses of maximum likelihood estimates of ordinal regressions on DWMH (a) and PVH (b) with vascular risk factors, focal vascular MRI findings, and MTA as predictors*

Parameter	DF	Estimate	Standard error	Wald χ^2	$Pr > \chi^2$
a) Dependent variable DWMH					
Intercept 3	1	−2.4276	0.1868	168.9785	<0.0001
Intercept 2	1	−1.0536	0.1503	49.1066	<0.0001
Intercept 1	1	0.4976	0.1442	11.9040	0.0006
On antihypertensives	1	0.3357	0.1659	4.0940	0.0430
MTA moderate–severe	1	1.4791	0.4096	13.0387	0.0003
MTA mild	1	0.2558	0.2248	1.2944	0.2552
b) Dependent variable PVH					
Intercept 3	1	−2.2943	0.1688	184.8100	<0.0001
Intercept 2	1	−1.9835	0.1552	163.3083	<0.0001
Intercept 1	1	−0.2883	0.1192	5.8500	0.0156
C-reactive protein	1	−0.0324	0.0146	4.9012	0.0268
MTA moderate–severe	1	1.3614	0.4169	10.6668	0.0011
MTA mild	1	0.4274	0.2347	3.3168	0.0686
Focal MRI lesions	1	0.6272	0.2143	8.5652	0.0034

one observation, it is united with group 2. The odds of subjects on antihypertensives being in a higher category of DMWH is 1.4 (95%-CI: 1.01–1.94) times the odds of subjects not taking antihypertensives. That means, people on antihypertensive drugs have a higher risk of DWMH. Comparing patients with mild-MTA versus no-MTA, the odds of being in a higher category of DMWH is 1.3 (95%-CI: 0.83–2.01), comparing moderate–severe MTA versus no-MTA, the odds increase to 4.4 (95%-CI: 1.97–9.80), showing that patients with a higher degree of atrophy of medial temporal structures often have more severe DWMH.

Concerning PVH, 11 out of 532 patients were deleted due to missing observations (vascular risk factor). Again the probability of being in a higher category of PVH was modeled and resulted in a low $R2 = 0.05$. A stepwise ordinal regression enters the categorical variables of MTA ($p = 0.0016$) and focal lesions on MRI ($p = 0.0034$) and the blood parameter C-reactive protein ($p = 0.0016$). People with higher degree of MTA, with focal vascular lesions on MRI, and with lower C-reactive protein level had more PVH. People with focal vascular lesions on MRI were likely to be in a higher category of PVH (1.9; 95%-CI: 1.23–2.85).

Comparing patients with mild-MTA versus no-MTA, the odds of being in a higher category of PVH was 1.5 (95%-CI: 0.97–2.43), comparing moderate–severe MTA versus no-MTA, the odds increased to 3.9 (95%-CI: 1.72–8.83), showing that patients with a higher MTA often have higher PVH. The variable C-reactive protein had a negative estimate. That means, that a high C-reactive protein indicates a low rating of PVH. If C-reactive protein increased by one unit, the odds of being in a higher rather than a lower category of PVH was 0.97 (95%-CI: 0.941–0.996).

Discussion

We found associations between WMH and vascular factors in the community-based age-cohort of the VITA study. Arterial hypertension treated with antihypertensive drugs significantly favored DWMH, and PVH were more frequently found in subjects with focal vascular lesions on MRI. A higher rate of WMH in stroke patients had already been described in many individuals of a rather young age and although in older stroke patients (Inzitari et al., 1987; Schmidt et al., 1992; Breteler et al., 1994a, b; Ylikoski et al., 1995; Henon et al., 1996; Longstreth et al., 1996; Streifler et al., 2003). The importance of arterial hypertension and sBP, especially, for WMH had been established in various samples (Inzitari et al., 1987; Lindgren et al., 1994; Fukuda and Kitani, 1995; Jorgensen et al., 1995; Liao et al., 1996; Longstreth et al., 1996; Coskun et al., 2003; Dijk et al., 2004a; Heijer et al., 2005) and only some had failed to find this relation (Schmidt et al., 1992; Ylikoski et al., 1995; Henon et al., 1996) at least in older subjects (Breteler et al., 1994b). In some studies the successful treatment of hypertension had resulted in less

WMH in patients with controlled hypertension (Fukuda and Kitani, 1995; Liao et al., 1996; Dijk et al., 2004a).

In addition to this relation between arterial hypertension, focal vascular lesions and WMH we found significant associations between both – DWMH and PVH – and focal atrophy of medial temporal lobe structures. Although hippocampal volume loss is not a specific feature of AD, both, the entorhinal cortex and hippocampus have been shown to be less affected by subcortical ischemic vascular dementia than by AD (Du et al., 2002). Thus, our findings might be interpreted in such a way that a degenerative process leading to MTA, as observed in AD, might have favored the occurrence of WMH. The effect was small but significant over the whole spectrum of severity of white matter lesions and severity of MTA. However, in our cross-sectional investigation it was impossible to determine whether MTA was a cause or rather a consequence of WMH (Leeuw et al., 2004; Heijer et al., 2005). The longitudinal part of the VITA will help to elucidate the relation between MTA, AD, vascular risk, and WMH. One clinico-pathological study described vascular changes possibly associated with cerebral amyloid angiopathy in brains of patients with Alzheimer dementia (Janota et al., 1989). That could mean that a vascular factor of degenerative brain disease might favor WMH (Dijk et al., 2004a, b).

Clinico-neuropathological investigations of WMH already indicated that these changes are not of pure vascular-ischemic origin. Studies described vascular changes in some, especially younger patients but also categorized these MRI lesions of the white matter as myelin pallor (Chimowitz et al., 1992), diffuse areas of demyelination (Ferrer et al., 1990), reduced or absent myelin staining, and enlarged perivascular spaces (Van Swieten et al., 1991), loss of myelinated axons (Scheltens et al., 1995a), perivenous damage, and gliosis (Fazekas et al., 1993) or myelin loss, axonal loss, astrogliosis, and dilatation of perivascular space (Ukada et al., 2002). Dilated perivascular spaces were described as associated with brain atrophy (Van Swieten et al., 1991). Clinico-pathological studies also showed scattered microinfarcts or other vascular-type lesions of the white matter in many patients with WMH. Only a few of these autopsied patients belonged to the age group of 75–76 years investigated in the VITA.

Except arterial hypertension, all known classical and novel vascular risk factors were unrelated to DWMH and PVH in the age-cohort of the VITA. Diabetes mellitus, represented by HbA1c levels, did not favor leukoaraiosis. Orthostatic hypotension did not correlate with either type of WMH. Also the lipid status, including total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyzceride and lipoprotein (a) level was not associated with WMH in our birth-cohort. Fibrinogen and homocysteine serum levels were independent from DWMH and PVH. Higher levels of C-reactive protein, discussed as a novel vascular risk factor, were even associated with significantly fewer PVH at age 75. We did not find any relation between smoking or years of smoking and any type of WMH as reported in one stroke sample (Longstreth et al., 1996), but not in others (Jorgensen et al., 1995; Coskun et al., 2003). That diabetes mellitus predicts WMH had been shown in some individuals with strokes at a young age (Schmidt et al., 1992; Ylikoski et al., 1995; Streifler et al., 2003) others did not find an influence of diabetes mellitus on WMH (Henon et al., 1996; Coskun et al., 2003; Schmidt et al., 2004). A relation between cholesterol level and WMH was only described in subjects younger than 75 years (Breteler et al., 1994b). Fibrinogen levels were related to these changes, irrespective of age, in one sample (Breteler et al., 1994b). One population-based study found a significant association between plasma homocysteine levels and both, DWMH and PVH (Vermeer et al., 2002), another one could not replicate this finding (Longstreth et al., 2004).

The weak relation between vascular factors and WMH in the VITA population were not explained by low frequencies of DWMH and PVH. As we investigated an age-cohort at a mean age of 75.6 years with a standard deviation of age of only 0.4 years, our findings could also not be explained by the covariate age. Another explanation might be that we investigated survivors. Patients with vascular risk factors without protective factors might not have survived to the age of 75 or may not be healthy enough to participate in such an epidemiological study. But we could prove that non-participants did not differ from participants with regard to the intake of antihypertensives or antidiabetics.

The weak association between vascular risk factors and WMH together with the high correlation between MTA and these hyperintensities allow us to presume that any pathological process of preclinical Alzheimer dementia might favor the occurrence or severity of both, DWMH and PVH. However, the cross-sectional design of this study does not allow the establishment of a causal relationship. It is possible that brain shrinkage with loss of interconnectivity and consecutive loss of myelin and widening of perivascular spaces explains some variation of WMH in old age. Such an association between brain atrophy and WMH has already been described clinically (Ylikoski et al., 1995; Henon et al., 1996; Capizzano et al., 2004; Leeuw et al., 2004) and neuropathologically (Van Swieten et al., 1991).

A strong relation between brain atrophy and white matter changes may be valid for the very elderly investigated in

this study but may not necessarily apply to younger patients, i.e., with Binswanger's disease following malignant arterial hypertension. At a younger age, leukoencephalopathy and focal subcortical lesions may even coexist with a determined genetic origin called CADASIL. Moreover, infarcts were significantly associated with the ratings of hyperintensities in our study as also described by others (Inzitari et al., 1987; Breteler et al., 1994b; Fukuda and Kitani, 1995; Ylikoski et al., 1995; Longstreth et al., 1996; Coskun et al., 2003). We, thus, do not claim that WMH are mainly caused by brain shrinkage at every age group. Moreover, we think it possible that WMH in the very elderly are causally related not only to vascular factors, e.g. hypertension, but also to cerebral atrophy and degenerative brain disease, such as AD.

Acknowledgement

This work was supported by the Ludwig Boltzmann Society and carried out at the Ludwig Boltzmann Institute of Aging Research.

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J Neural Transm (2007) [Suppl 72]: 189–193

Printed in Austria

Journal of Neural Transmission

α-Lipoic acid as a new treatment option for Alzheimer's disease – a 48 months follow-up analysis

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Summary Oxidative stress and neuronal energy depletion are characteristic biochemical hallmarks of Alzheimer's disease (AD). It is therefore conceivable that pro-energetic and antioxidant drugs such as α-lipoic acid might delay the onset or slow down the progression of the disease. In a previous study, 600 mg α-lipoic acid was given daily to nine patients with AD (receiving a standard treatment with choline-esterase inhibitors) in an open-label study over an observation period of 12 months. The treatment led to a stabilization of cognitive functions in the study group, demonstrated by constant scores in two neuropsychological tests (the mini mental state exam, MMSE and the Alzheimer's disease assessment score cognitive subscale, ADAScog). In this report, we have extended the analysis to 43 patients over an observation period of up to 48 months. In patients with mild dementia (ADAScog < 15), the disease progressed extremely slowly (ADAScog: $+1.2$ points/year, MMSE: -0.6 points/year), in patients with moderate dementia at approximately twice the rate. However, the progression appears dramatically lower than data reported for untreated patients or patients on choline-esterase inhibitors in the second year of long-term studies. Despite the fact that this study was not double-blinded, placebo-controlled and randomized, our data suggest that treatment with α-lipoic acid might be a successful 'neuroprotective' therapy option for AD. However, a state-of-the-art phase II trial is needed urgently.

Keywords: Dementia, Alzheimer's disease, lipoic acid, neuroprotection, open clinical trial

Introduction

Peter Riederer has proposed for more than 20 years that oxidative stress is a major cause of cell death in Parkinson's and Alzheimer's disease (AD). He and his co-workers proposed that a gradual impairment of cellular defense mechanisms leads to cell damage including accumulation of advanced glyction endproducts because of toxic substances e.g. superoxide from mitochondrial respiration being increasingly formed during normal cellular metabolism. This point of view brings into consideration the possibility that, besides exogenous factors, the pathogenetic process of neurodegeration is triggered by endogenous mechanisms, either by an endogenous toxin or by inherited metabolic disorders, which become progressively more evident with aging (Frölich and Riederer, 1995; Götz et al., 1994; Retz et al., 1998; Rösler et al., 1998). AD is on of the most likely diseases involving oxidative stress as a causative pathogenic factor which occurs earlier than the pathological hallmarks of the disease, amyloid plaques and neurofibrillary tangles (Perry et al., 1998). Besides oxidative stress, neuronal energy depletion is a second characteristic biochemical hallmarks of AD (Münch et al., 1998). It is therefore conceivable that pro-energetic and antioxidants such as α-lipoic acid might delay the onset or slow down the progression of the disease (Holmquist et al., 2006).

We have previously conducted a small pilot study with 9 patients over a period of nine months showing some indication that α-lipoic acid may fulfil this therapeutic need (Hager et al., 2001). A naturally-occurring precursor of an essential cofactor for mitochondrial enzymes, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, α-lipoic acid has been shown to have a variety of properties which can interfere with pathogenic principles of AD. For example, α-lipoic acid increases acetylcholine production by activation of choline acetyltransferase and increases glucose uptake, thus supplying more acetyl-CoA for the production of acetylcholine. α-Lipoic acid chelates

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redox-active transition metals, thus inhibiting the formation of hydroxyl radicals and also scavenges reactive oxygen species (ROS), thereby increasing the levels of reduced glutathione (Packer et al., 1995). Via the same mechanisms, downregulation redox-sensitive inflammatory processes can also be achieved (Wong et al., 2001). Furthermore, α-lipoic acid can scavenge lipid peroxidation products such as hydroxynonenal and acrolein. The reduced form of α-lipoic acid, hydrolipoic acid (DHLA), is the active compound responsible for most of these beneficial effects. R-α-lipoic acid can be applied instead of DHLA, as it is reduced by mitochondrial lipoamide dehydrogenase, a part of the pyruvate dehydrogenase complex (Biewenga et al., 1997). In this study, cognitive functions of 43 AD patients treated with α-lipoic acid for periods up to 4 years were analyzed.

Patients and methods

The study was designed as an open, non-randomized investigation of outpatients presented at the memory clinic with an initial diagnosis of probable Alzheimer's disease. Subjects underwent an evaluation using clinical interview, mental status assessment, physical and neurological examinations. All participants met the criteria of DSM-III-R (APA, 1987) for probable AD. Subjects were required to be aged 45 years or older upon the first signs of memory complaint, and have a closely related caregiver (spouse, parent or child). Patients with a history suggesting a familial form of AD were excluded. Informed consent was obtained from each subject, the caregiver or the legal guardian. The study was approved by the institutional review board. Patients received the standard treatment of an choline-esterase inhibitor at least 3 months prior to starting the α-lipoic acid treatment, which was given once daily in a dose of 600 mg, administered in the morning, 1 h before breakfast. For assessing cognitive performance, the mini-mental state examination (MMSE) and the cognitive subscale of the AD assessment scale (ADAS-cog) were applied (Storey et al., 2002). Between 1998 and 2004, 43 patients – divided in three groups according to the severity of their dementia – were included in the study (Table 1).

Results

The patients included in our study were tested by means of MMSE and ADAScog prior to and several times (in most cases every 6 months) after the start of the treatment with 600 mg daily of α-lipoic acid up to a total of 48 months. Before starting treatment with α-lipoic acid, despite cognitive training as well as treatment with acetylcholinesterase inhibitors, the test results showed a constant decline. Test results for the moderate-advanced group could only be obtained for 2.5 years because the patients increasingly were admitted to nursing homes where medication and testing was discontinued after that period. As expected for an irreversible disease as AD, all three patient groups showed a steady decline of the cognitive functions but the decline in the α-lipoic acid treated patients appears to be much slower compared to may other studies published in the current literature. Similar to other observations in the literature, cognitive decline was slower in the early stages of the disease, as the mild AD group showed the slowest degree of decline (MMSE: −0.6 points per year) compared to the other groups (MMSE: −1.4 points per year) (Table 2, Fig 1). It has to be noticed that for the mild and the early moderate patient group, the progression rate slows down after 3 years. However, this is rather caused by the selection of the ''slow decliners'' than an overall slowdown of disease progression. In summary, α-lipoic acid showed some promising effects in this larger study supporting our previous data but we are cautious with a too positive interpretation of the data because of the open design of the study.

Table 1. *Characteristics of the study population*

Patients group	Number of patients on LS treatment	Age (years)	Type of choline-esterase inhibitor	Time between start of choline-esterase inhibitor and LS treatment (days)	Period of data collection (years)
Mild dementia	Start: 12 1 yr: 12 2 yrs: 11 3 yrs: 7 4 yrs: 7	65.0 ± 12.6	Aricept: 7 Reminyl: 3 no choline-esterase inhibitors: 2	411 ± 341	4
Moderate-early dementia	Start: 19 1 yr: 19 2 yrs: 16 3 yrs: 13 4 yrs: 9	63.0 ± 7.4	Aricept: 15 Reminyl: 4	436 ± 406	4
Moderate-advanced dementia	Start: 12 1 yr: 12 2 yrs: 5 2 yrs: 3	69.5 ± 8.7	Aricept: 10 Reminyl: 2	638 ± 509	2.5

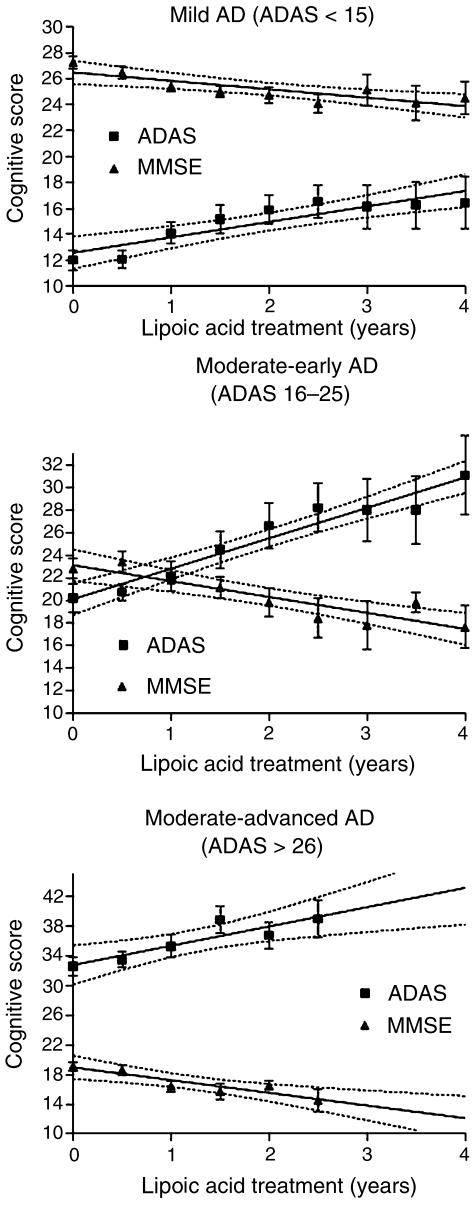

Fig. 1. Time-dependent changes in MMSE (triangles) and ADAScog scores (squares) in patients (divided into subgroups according to severity) treated with α-lipoic acid over an observation period of up to 4 years. Data are presented as mean ± SEM

Discussion

The natural history of Alzheimer's disease is one of progressive decline; cognitive, physical, and social functions gradually deteriorate. Thus, ''improvement'' from an intervention for Alzheimer's disease means slowing the rate of decline. The rate of decline in Alzheimer's disease is not linear, however. People with mild dementia (ADAScog < 15) experience an average rate of decline of 5 or fewer ADAScog points (2 or fewer MMSE points) per year. By contrast, those individuals with moderate dementia (ADAScog >15 but <55) experience an average decline in cognition of 7–11 ADAScog points (2–4 MMSE points) annually (Stern et al., 1994). With a decrease of less that two points in the MMSE and an increase of less than three points in the ADAScog per year, the decline of the lipoic acid treated patients was relatively small compared to data from the literature (Table 3), indicating that lipoic acid slows down the progression of dementia. The slow decline is unlikely the consequence of the choline-esterase inhibitors treatment for two reasons: a) the majority of patients started choline-esterase inhibitors treatment several months before entering the LS study and b) the slower decline continued beyond the first year of the study where usually the positive effects of choline-esterase inhibitors level off (AD2000 Collaborative Group, 2004). However, our open trial is open to a biased selection of patients. It is conceivable that patients or caregivers willing to try novel therapies are more likely to try other beneficial lifestyle changes such as nutritional approaches and physical and mental exercise as well. On the other hand, patients whose disease progresses rapidly despite the standard therapies with choline-esterase inhibitors might particularly ask for α-lipoic acid as the ''drug of last resort'' and our study would particularly attract the more rapid decliners. In summary, our data suggest that a pro-energetic and antioxidant drug such as α-lipoic acid might delay the onset or slow down the progression of the disease, and we are confident that our results will encou-

Table 2. *Time-dependent changes in cognitive scores of α-lipoic acid treated patients*

Patient group	ADAScog scores (at start LS treatment)	Increase in ADAScog scores per year	MMSE scores (at start LS treatment)	Decrease in MMSE scores (per year)
Mild dementia (ADAS 0–15)	12.0 ± 2.7	1.2 ± 0.2 (2.0 ± 0.2 in the first 30 months)	27.3 ± 1.7	0.6 ± 0.2 (1.2 ± 0.1 in the first 30 months)
Moderate-early dementia (ADAS 16–25)	20.2 ± 5.5	2.7 ± 0.2 (3.4 ± 0.3 in the first 30 months)	22.8 ± 3.4	1.4 ± 0.2 (1.6 ± 0.2 in the first 30 months)
Moderate-advanced dementia (ADAS > 26)	32.6 ± 4.3	2.6 ± 0.2	19.1 ± 2.1	1.4 ± 0.4

Table 3. *Time-dependent decline in cognitive scores – comparison with published studies*

ADAS				
ADAScog scores of our patient group (at start LS treatment)	Change in ADAScog in our patient group per year	ADAScog scores of comparative patient group at start	Change in ADAScog in comparative patient group per year	Study information
12.0 (mild)	$+1.2 \pm 0.2$ (2.0 in the first 30 months)	<15	approx. 5	untreated AD patients (Stern et al., 1994)
		18 (9.4) hydroxychloroquine and 17.6 (9.1) placebo	estimated 5.6 (5.9) and 5.4 (6.8), respectively	randomised double-blind trial with 168 AD patients for 18-month (Van Gool, 2001)
20.2 (moderate-early)	$+2.7 \pm 0.2$ (3.4 ± 0.3 in the first 30 months)	21.8	4.4 (0–3 years) 6.5 (1st–3rd years)	open label trial with 4–6 mg rivastigmine for up to 5 years (Farlow et al., 2005)
		20	6	Mohs (1996)
32.6 (moderate-advanced)	$+2.6 \pm 0.2$	40	13	Mohs (1996)
		60	7	Mohs (1996)
MMSE				
MMSE scores of our patient group (at start LS treatment)	Change in MMSE in our patient group per year	MMSE scores of comparative patient group at start	Change in MMSE in comparative patient group per year	Study information
27.3 (mild)	-0.6 ± 0.2	n/a (i.e. many clinical trials in AD restrict MMSE entry scores to 10–25)	n/a	MMSE is less sensitive for patients with mild cognitive impairment (Galasko et al., 2000)
		approx. 26	approx. −4.4	course of decline was studied in 16 AD patients for up to 5 years (Haxby et al., 1992)
		23.7 ± 2.4	-3.9 ± 4.1	1 year, multicenter (27) evaluation, in an AD cooperative study/USA (Ferris et al., 1997)
22.8 (moderate-early)	-1.4 ± 0.2	19.26 ± 4.54, 19.37 ± 4.37 (range 10–26)	−2.3 without or −0.5 with donezepil	randomised double-blind trial with 286 AD patients for 1 year (Winblad et al., 2001)
		23 ± 3.9	−2.3	Study investigating the rate of progression, 54 AD patients (Rascovsky et al., 2005)
		mean ranged from 7.2 to 26 (median: 18.4)	−3.3	meta-analysis of 37 studies, 3492 AD patients, over an average of 2 years (Han et al., 2000)
19.3 (moderate-advanced)	-1.4 ± 0.4	19.0	−3 without or −2.4 with donezepil	randomised double-blind trial with 565 AD patients for 2 years (AD2000 Collaborative Group, 2004)
		mean ranged from 7.2 to 26 (median: 18.4)	−3.3 (95% CI: 2.9–3.7)	meta-analysis of 37 studies, 3492 AD patients, over an average of 2 years (Han et al., 2000)
		18.5 ± 4.6	approx. −2.81	longitudinal evaluation of 3 mental status examinations, 92 AD patients (Salmon et al., 1990)

rage the initiation of a state-of-the-art phase II clinical trial.

Acknowledgements

This manuscript is dedicated to Peter Riederer for his lifetime work and particularly his inspirational work on oxidative stress in neurodegenerative diseases. This work was supported by the Alzheimer Initiative e.V. (AFI). The clinical trial was not supported by manufacturers of lipoic acid such as Degussa, Asta Medica or Viatris. Limited support was provided by Degussa for data analysis (to GM).

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J Neural Transm (2007) [Suppl 72]: 195–202

Printed in Austria

Journal of Neural Transmission

Long-term abnormalities in brain glucose/energy metabolism after inhibition of the neuronal insulin receptor: implication of tau-protein

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Summary The triplicate intracerebroventricular (icv) application of the diabetogenic compound streptozotocin (STZ) in low dosage was used in 1-year-old male Wistar rats to induce a damage of the neuronal insulin signal transduction (IST) system and to investigate the activities of hexokinase (HK), phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GDH), pyruvate kinase (PK), lactate dehydrogenase (LDH) and α-ketoglutarate dehydrogenase (α-KGDH) in frontoparietotemporal brain cortex (ct) and hippocampus (h) 9 weeks after damage. In parallel, the concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP) and creatine phosphate (CrP) were determined. We found reductions of HK to 53% (ct) and 60% (h) of control, PFK to 63/64% (ct/h); GDH to 56/61% (ct/h), PFK to 57/59% (ct/h), α-KGDH to 37/35% (ct/h) and an increase of LDH to 300/240% (ct/h). ATP decreased to 82/87% (ct/h) of control, GTP to 69/81% (ct/h), CrP to 82/81% (ct/h), ∼P to 82/82% (ct/h), whereas ADP increased to 189/154% (ct/h). The fall of the activities of the glycolytic enzymes HK, PFK, GDH and PK was found to be more marked after 9 weeks of damage when compared with 3- and 6-week damage whereas the diminution in the concentration of energy rich compound was stably reduced by between 20 and 10% relative to control. The abnormalities in glucose/energy metabolism were discussed in relation to tau-protein mismetabolism of experimental animals, and of sporadic AD.

Keywords: Brain, glucose/energy metabolism, enzyme activities, brain cortex, hippocampus, tau-protein, streptozotocin, sporadic Alzheimer disease

Introduction

It is well documented that the glucose metabolism in the brain is of pivotal significance to maintain brain function (for review, Maurer and Hoyer, 2006; Hoyer and Frölich, 2007). The glycolytically formed compounds 1) fructose-6-phosphate is the source of the hexosamine biosynthetic pathway synthesizing UDP-N-acetylglucosamine (UDP-GlcNAc) for protein O-glycosylation (for review, Gong et al., 2006), and 2) pyruvate yields the energy-rich compound acetyl-CoA which is used a) for further oxidation in the tricarboxylic acid cycle (TCAC) to ATP (more than 95% of acetyl-CoA), b) for the formation of the neurotransmitter acetylcholine (1–2% of acetyl-CoA) (Gibson et al., 1975; Perry et al., 1980), and c) for the formation of cholesterol in the 3-hydroxy-3-methyl-glutaryl-CoA cycle (Michikawa and Yanagisawa, 1999).

Acetyl-CoA is the starting metabolite from which free fatty acids can be formed or which is formed from fatty acids by beta-oxidation (Singh et al., 1989). Also, from acetyl-CoA, glucose-derived carbon is rapidly transferred into amino acids via the GABA shunt (Sacks, 1957). In the TCAC, glutamate, glutamine, aspartate and gamma-aminobutyric acid are formed most abundantly (Wong and Tyce, 1983).

It has been also well established that the mammalian brain is an insulin-sensitive organ (Schulingkamp et al., 2000; Park, 2001; Porte Jr et al., 2005), and that the brain itself synthesizes insulin (Schechter et al., 1992, 1996; Devaskar et al., 1994). However, total brain insulin consists of two different sources: a smaller proportion derives from de novo synthesis, but the larger proportion is transcytosed from the circulation (Banks, 2004). Insulin receptor mRNA has been shown to be present in the brain from the last third of pregnancy throughout the whole life (Hill et al., 1986; Werther et al., 1987; Raizada et al., 1988; Unger et al., 1989; Schechter et al., 1996).

The functional role of the CNS insulin signal transduction (IST) system is multifold and summarized elsewhere (Gerozissis, 2003; Porte Jr et al., 2005). Gathering evidence indicates that the IST system plays an important role in the maintenance of brain glucose homeostasis, in the regulation of the neuronal glucose metabolism and energy generation, and in the maintenance of learning and memory processes (Schulingkamp et al., 2000; Park, 2001; Gerozissis,

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2003; Hoyer, 2004; for review Hoyer and Frölich, 2007). Beside its effects on brain glucose/energy metabolism, the IST system has been demonstrated to control the activity of several enzymes involved in the generation of the amyloid precursor protein (APP) and the phosphorylation of tau-protein (for details, Maurer and Hoyer, 2006).

Numerous studies have documented that the experimental inhibition of the neuronal IST system by means of the diabetogenic compound streptozotocin (STZ) applied intracerebroventricularly (icv) in very low doses (1–3 mg/kg) induced marked abnormalities in cerebral glucose metabolism (Nitsch et al., 1990; Plaschke and Hoyer, 1993; Duelli et al., 1994), in cholinergic neurotransmission (Hellweg et al., 1992; Prickaerts et al., 1999), monoaminergic neurotransmission (Lackovic and Salkovic, 1999; Ding et al., 1992), energy formation (Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998), in the composition of membrane phospholipids (Müller et al., 1998), and in learning and memory capacities in adult rats (Mayer et al., 1990; Blokland and Jolles, 1993; Lannert and Hoyer, 1998; Prickaerts et al., 1999). In this model no systemic diabetes mellitus was found.

Recent findings regarding the gene expression level clearly demonstrated that icv STZ caused different changes. Downregulations were found in the mRNAS of insulin-1 and 2-mRNA, insulin receptor and IGF-1 receptor, immediate-early-gene-transcription factor NGF-OB and metallothionein-1/2, whereas gene expression related to potassium channels, GABA receptors and glutamate receptors was upregulated up to 3 months after icv STZ application (Grünblatt et al., 2004, 2006, 2007). Intracerebral application of STZ in 3-day old pups confirmed the decrease in both insulin and insulin receptor gene expression 4 weeks after damage (de la Monte et al., 2006). The STZ icv-induced damage to the IST system caused time-dependent changes in the protein kinase B/glycogen synthase kinase-3 pathway in that the level of phosphorylated glycogen synthase kinase-3α/β protein was increased 1 month after icv STZ, whereas 3 months after icv STZ, both phosphorylated glycogen synthase kinase-3α/β and protein kinase B tended to decrease. These time-dependent metabolic abnormalities were found to be accompanied with increased tau-protein concentration 1 month after icv STZ (Salkovic-Petrisic et al., 2006) and increased hyperphosphorylated tau-protein 3 months after icv STZ (Grünblatt et al., 2007).

The aim of the present study was to investigate whether or not time-dependent disturbances in glucose/energy metabolism run in parallel with the time-dependent abnormalities in tau-protein metabolism what may indicate a functional relationship in the pathophysiology of these two pathways. We found a marked deterioration of the enzyme activities working in glycolytic chain and TCA cycle 9 weeks after icv STZ, accompanied by a permanent reduction in energy rich phosphates. These data are discussed with respect to 3- and 6-week changes in both glucose metabolism and tau-protein metabolism.

Material and methods

Animals

One-year-old (adult) male Wistar rats weighing between 430 and 540 g (breeder: Zentralinstitut für Versuchstierzucht, Hannover, Germany) were used throughout the study: they were housed in individual cages in a temperature controlled animal room with a reversed 12:12 h light–dark cycle (lights on at 19.00 h). Experiments were conducted during the dark period of the cycle. Food pellets from Altromin (standard, no. 1324) were used. Water was freely available throughout the experiment. By psychometric testing, well performing and poorly performing animals were discriminated (Hoyer et al., 2004). Only good performers were included in the study ($n = 40$; 10 animals per group).

Operation procedure

The animals were anesthetized with chloral hydrate (240 mg/kg b.w. in a 4% solution ip). Burr holes were bilaterally drilled into the skull and icv application was performed with either STZ or artificial cerebral spinal fluid (2 µl volume/injection site) (for details, see Lannert and Hoyer, 1998). The injections were repeated on day 3 and day 20 under chloral hydrate anesthesia (see above). The final experiment was performed 9 weeks after the first icv injection. After general anesthesia with chloral hydrate, the femoral artery and vein were exposed and cannulated to measure blood pressure and to sample blood for analyses. Controlled intubation anesthesia was induced: 1.5 vol% halothane (initial) to 0.5 vol% halothane (final) in N_2O/O_2 (70:30). The animals were monitored over a 20-min steady-state period of normal arterial blood pressure, normoxemic and normocapnic arterial blood gases, normal acid-base parameters and normothermia at 37°C. After the 20-min steady-state, the brains were frozen at −80°C. Cerebral fronto-parietotemporal cortex and hippocampus were prepared at approximately −20°× in a cryostat (Brandau, Darmstadt, Germany) and stored at −80°C until analysis.

Treatment procedure

Artificial CSF contained 120 mM NaCl, 3 mM KCl, 1.15 mM $CaCl_2$, 27 mM $NaHCO_3$ and 0.33 mM NaH_2PO_4 adjusted to pH 7.2 by CO_2. Only freshly prepared solutions were used.

STZ: This compound was injected in a subdiabetogenic dosage (0.50 mg/kg injection site/treatment dissolved in 2 µl artificial CSF. Only freshly prepared solutions were used.

In general, STZ is a cytotoxic agent acting selectively on pancreatic β cells by generating its destruction by participation of the insulin-responsive glucose transporter GLUT2 (Weiss, 1982; Takasu et al., 1991; Kröncke et al., 1995; Szudelski, 2001; Grünblatt et al., 2007). When applied systematically, either iv or ip, STZ causes a fall-out of insulin production and, thus, a stable diabetes mellitus at STZ dosages >40 mg/kg. Lower dosages of STZ induced a transient diabetes mellitus characterized by decreased autophosphorylation of the insulin receptor in acute experiments (Kadowaki et al., 1984); and increased insulin receptor density (Giorgino et al., 1992; Sechi et al., 1992). When applied intracerebroventricularly, STZ did not affect non-nervous tissue, i.e. no peripheral diabetes mellitus was found (Nitsch and Hoyer, 1991). A single icv STZ application of a subdiabetogenic dosage stably reduced the levels of ATP and CrP and stably enhanced lactate

concentration as markers of glucose/energy metabolism over 3 weeks. However, these data partly recovered at 6 and 9 weeks duration (data not shown). In contrast, a triplicate low-dose icv STZ injection of STZ caused a long-lasting depletion of glucose/energy metabolism over at least 3 months (Lannert and Hoyer, 1998). It may, thus, be assumed that the triplicate low-dose icv STZ application causes a permanent and stable reduction in the parameters of brain glucose/energy metabolism.

Biochemical analysis

Enzyme activities

The brain areas were homogenized in 0.02 M Tris–HCl buffer (1:10 w/v) containing 0.1 mM DTT, 0.25 M sucrose and 100 µl of 10% Triton X-100 at pH 7.5 by an ice-cold Potter homogenizer. The homogenates were centrifuged in a Beckman microfuge at 100,000 rpm for 15 min at 2°C. The supernatants were stored at −80°C. The enzymatic activities were determined by continuous optical tests at 340 nm and 30°C by use of microcuvettes with a final volume of 1 ml. All assays were performed in triplicate.

Enzyme activities were determined according to the following protocols: hexokinase (HK) (Lai et al., 1985), phosphofruktokinase (PFK) (Sorbi et al., 1983), pyruvate kinase (PK) (Leong et al., 1981), glyceraldehyde-3-phosphate dehydrogenase (GDH) and lactate dehydrogenase (LDH) (Bergmeyer, 1974), and α-ketoglutarate dehydrogenase (α-KGDH) (Mastrogiacomo et al., 1993).

Protein concentration was determined by the method of Lowry et al. (1951).

Energy rich phosphates

After preparation, tissue was weighed and homogenized in 10 vol $CHCl_3$ at approximately −25°C with an Ultraturrax. Proteins were precipitated with 1.97% $HClO_4$ (0.8 M), and homogenates were centrifuged at 10,000 *g* for 10 min. Supernatants were neutralized to pH 7.2 + 0.4 with KOH and filtered through a 45-µm Millipore membrane filter after which 100 µl of each filtrate was analyzed by high performance liquid chromatography (HPLC; Harmson et al., 1982). For further analytical details see Lannert and Hoyer, 1998). "∼P" is defined as the sum of available phosphates from adenosinetriphosphate (ATP) and creatine phosphate (CrP).

Statistics

The significance of group differences was tested by Kruskal–Wallis ANOVA median test, followed by Mann–Whitney *U*-test. Statistically significant differences were accepted at $p \leq 0.05$.

Results

The enzyme activities determined in frontoparieotemporal brain cortex and hippocampus under the experimental conditions are shown in Table 1. We found statistically significant reductions between 50 and 60% of the activities hexokinase, phosphofructokinase glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase relative to control in both areas studied where the fall in the activity of α-ketoglutarate dehydrogenase to 37/36% of normal was more marked. In contrast, the activity of lactate dehydrogenase increased to 300% (cortex) and 240% (hippocampus) as compared to normal.

The concentrations of energy rich phosphates in cerebral fronto-parietotemporal cortex and hippocampus 9 weeks

Table 1. *Enzyme activities in frontoparietotemporal brain cortex and hippocampus in control rats and 9 weeks after a triplicate icv SZT application*

Enzyme	Cortex		Hippocampus	
	Control	STZ	Control	STZ
Hexokinase	0.23 ± 0.02	0.12 ± 0.02*	0.21 ± 0.02	0.13 ± 0.02*
Phosphofructokinase	1.45 ± 0.09	0.91 ± 0.07*	1.29 ± 0.09	0.83 ± 0.06*
Glyceraldehyde-3-phosphate dehydrogenase	1.45 ± 0.08	0.80 ± 0.09*	1.25 ± 0.06	0.76 ± 0.13*
Pyruvate kinase	2.25 ± 0.19	1.29 ± 0.12*	2.09 ± 0.11	1.24 ± 0.14*
Lactate dehydrogenase	4.60 ± 0.56	13.80 ± 1.26*	4.58 ± 0.59	11.01 ± 0.92*
α-Ketoglutarate dehydrogenase	9.00 ± 0.60	3.30 ± 0.90*	8.60 ± 0.80	3.10 ± 0.70*

Values are expressed as µmol/mg protein/min at 30°C and represent means ± SD, $n = 10$ per group.
* $p \leq 0.05$ vs. control.

Table 2. *Mean values ± SD expressed as nmol/mg wet weight (n = 10/group) of energy state in cerebral frontoparietotemporal cortex and hippocampus in controls and 9 weeks icv STZ*

Substrate	Cortex		Hippocampus	
	Control	STZ	Control	STZ
Adenosine triphosphate (ATP)	2.74 ± 0.11	2.24 ± 0.23*	2.49 ± 0.22	2.16 ± 0.13*
Adenosine diphosphate (ADP)	0.39 ± 0.02	0.73 ± 0.08*	0.44 ± 0.03	0.67 ± 0.06*
Guanosine triphosphate (GTP)	0.70 ± 0.03	0.48 ± 0.03*	0.61 ± 0.03	0.49 ± 0.06*
Creatine phosphate (CrP)	4.97 ± 0.23	4.10 ± 0.34*	6.05 ± 0.41	4.88 ± 0.49*
ATP/ADP (ATP turnover)	7.21 ± 0.21	3.12 ± 0.32*	5.94 ± 0.39	3.47 ± 0.42*
∼P	1.24 ± 0.07	1.02 ± 0.06*	1.36 ± 0.08	1.12 ± 0.05*

* $p \leq 0.05$ vs. control.

after icv STZ-application are listed in Table 2. The inhibition of the neuronal insulin receptor caused significant changes in all parameters measured in both areas. ATP, CrP, GTP ∼P, and the turnover of ATP were significantly reduced whereas ADP increased.

Discussion

The data of the present study may indicate a severe abnormality in the function of the glycolytic breakdown of glucose and its oxidation and in energy formation, in both frontoparietotemporal brain cortex and hippocampus after experimentally induced inhibition of the neuronal insulin receptor 9 weeks after the damage. These disturbances may be assumed to have impacts 1) on tau-protein glycosylation, 2) tau-protein (hyper)phosphorylation and 3) the intracellular pH secondarily shifting it to the acidic site.

Ad1) As was pointed above, the glycolytic compound fructose-6-phosphate is the source from which UDP-N-acetylglucosamine derives. Tau-protein has been demonstrated to be modified by 0-Glc NA cylation and the latter process inversely regulates tau-protein phosphorylation (Hart, 1997; Liu et al., 2004; Gong et al., 2006). The capacity of glycolytic enzyme activities after inhibition of the neuronal insulin receptor has been found to be diminished by between 10 and 30% relative to normal in frontoparietal cerebral cortex and hippocampus 3 and 6 weeks after damage (Plaschke and Hoyer, 1993). However, this study demonstrates, that the same experimental procedure induced a decrease of the same enzyme activities by 40% and more compared to normal 9 weeks after the damage in both areas studied (Fig. 1). An increased production of total tau-protein has been shown to occur 1 month after experimental neuronal insulin receptor inhibition (Salkovic-Petrisic et al., 2006). Three months after that damage, the increased production of total tau-protein could be confirmed. However, as an additional finding, hyperphosphorylated tau-protein was formed (Grünblatt et al., 2007). Together, the reduced capacity in the glycolytic breakdown of glucose may run in parallel a) with an overproduction of tau-protein and b) with hyperphosphorylation of the latter.

In this respect, in may be worth to consider another aspect, too. In cell culture studies, the increase in tau-protein concentration caused an inhibition of axonal transport including the anterograde trafficking of the amyloid precursor protein (APP). Axons and dendrites were found to be nearly completely devoid of APP which accumulated in the cell body. The disturbed flux of APP down

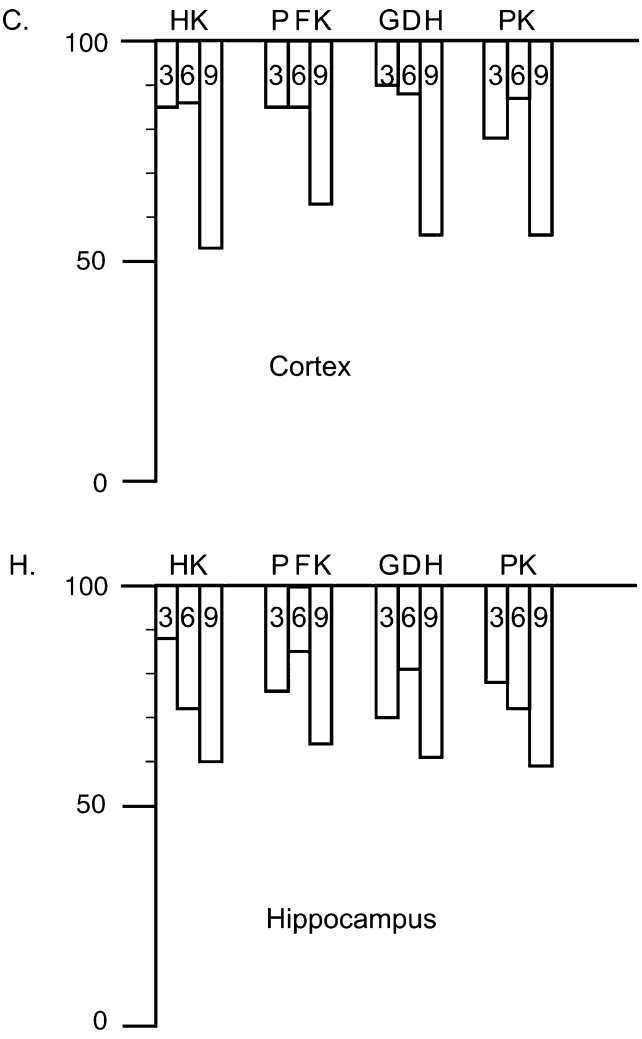

Fig. 1. Mean percent changes (100 ∼ control level) of activities of the enzymes hexokinase (*HK*), phosphofructokinase (*PFK*), glyceraldehyde-3-phosphate dehydrogenase (*GDH*) and pyruvate kinase (*PK*) in frontotemporoparietal brain cortex (*C*) and hippocampus (*H*) of 1-year-old male Wistar rats, 3, 6, and 9 weeks after a triplicate intracerebroventricular application of streptozotocin. The 3- and 6-week data are taken from Plaschke and Hoyer (1993). They were gained under identical experimental conditions as were the 9-week results of this study

the axon has been found to degenerate both axons and synapses (Stamer et al., 2002; Mandelkow et al., 2004). It is tempting to assume that the inhibition of the insulin signal transduction pathway caused the mismetabolism in tau-protein and finally of APP via the impaired of both the glucose metabolism (this study) and insulin signalling (Salkovic-Petrisic et al., 2006), running in parallel.

Ad2) The changes found in the concentrations of energy rich phosphates after experimental inhibition of the neuronal insulin receptor may be mainly due to the reduced activity of dehydrogenating enzyme complexes in the TCA cycle as was exemplified by α-ketoglutarate dehydrogenase

activity. Three weeks after insulin receptor inhibition, the fall was to 50% of normal (Terwel et al., 1995), whereas it was 37/36% of normal 9 weeks after damage (this study). However, the growing deterioration in oxidative metabolism was not mirrored in energy production which was characterized by a 15–20% reduction compared to control in both short-term experiments (3 weeks) (Nitsch and Hoyer, 1991), and long-term experiments (9 weeks, this study) and 3-month investigations (Lannert and Hoyer, 1998). As an alternative pathway to form energy in emergency conditions (e.g. lack of glucose), an anaplerotic reaction (Goldberg et al., 1966; Lewis et al., 1974; Norberg and Siesjö, 1976; Hoyer and Krier, 1986) may have to be taken into account. When glucose is lacking as substrate for oxidation, succinate can be generated from fatty acids derived from membrane phospholipid catabolism (Müller et al., 1998) via β-oxidation, acetyl CoA, oxaloacetate, malate and fumarate. Succinate accumulates as an end product of anaerobic catabolism of glucose derivatives for nonoxidative energy production.

The long-lasting stable energy deficit may be assumed to disturb the hierarchy of ATP-consuming/dependent processes (Buttgereit and Brand, 1995) among which are ATP-dependent protein kinases PK^{erk36} and PK^{erk40} which have been demonstrated to control normal tau-protein phosphorylation (Röder and Ingram, 1991). It is however, presently not known in which way the permanent shortage of ATP contributes directly to tau-protein hyperphosphorylation.

Both, protein levels and activity were not found to necessarily correlate to mRNA levels (Gygi et al., 1999). A recent study demonstrated a statistically significant downregulation of the gene expression profiles of 15 out of 51 members of the metabolic enzyme transcripts working in glycolytic and oxidative metabolism in postmortem AD brain among which is the glucose-6-phosphate isomerase gene (Brooks et al., 2007). Its enzyme generates fructose-6-phosphate being the source of UDP-N-acetylglucosamine for protein cylation (see above).

Ad3) The extreme increase in the activity of lactate dehydrogenase after experimental inhibition of the neuronal insulin receptor may be assumed to mirror increased lactate production in brain tissue also found as increased release of lactate from tissue into the venous blood (Nitsch et al., 1990). In brain tissue, lactate may be buffered by the tissue bicarbonate reserve to avoid severe tissue acidification (Weyne et al., 1968a, b). Pathological conditions accompanied by increased tissue lactate production were found to reduce the tissue bicarbonate concentration (Kaasik et al., 1970; Zwetnow, 1970). As a result, CO_2 may be generated in the tissue (Hoyer et al., 1973), dysregulating cellular pH (Kjällquist et al., 1969) and, thus, cellular metabolism (e.g. Km values, Bmax values). As yet it is not known which cascade of abnormal reactions is set into motion when cellular pH is dysregulated.

Both cell cultures and transgenic animals carrying genetically modified APP were generally used for Alzheimer disease (AD) research. However, these models represent a minority of hereditary AD cases only and may not be assumed to be valid for the vast majority of sporadic AD. Instead, it has been proposed that an early damage of the neuronal IST system plays the major role in the etiopathogenesis of this neurodegenerative disease (Frölich et al., 1998; Hoyer, 1998, 2004; Grünblatt et al., 2004, 2006, 2007; Rivera et al., 2005; Steen et al., 2005; de la Monte et al., 2006; Lester-Coll et al., 2006; Salkovic-Petrisic et al., 2006). The results of a former investigation (Plaschke and Hoyer, 1999) and of this study corresponded to abnormalities of enzyme activities found in Alzheimer brain re phosphofructokinase and other glycolytic enzymes (Bigl et al., 1996, 1999, 2000), pyruvate dehydrogenase (Perry et al., 1980; Sorbi et al., 1983; Butterworth and Besnard, 1990), and α-ketoglutarate dehydrogenase (Mastrogiacomo et al., 1993; Bubber et al., 2005). The reduction of the capacity of dehydrogenating enzymes may be assumed to cause diminished ATP generation (Sims et al., 1983; Brown et al., 1989; Hoyer, 1992).

As discussed above in relation to the data from animal experiments, the diminished glucose/energy metabolism may initiate the mismetabolism of tau-protein in SAD patients, too. However, direct evidence is missing as yet. Admittedly, early and severe abnormalities were found in cerebral glucose utilization paralleling the worsening of clinical dementia symptoms (Mielke et al., 1994; Minoshima et al., 1997). There is ample evidence that tau-protein is increased in cerebrospinal fluid (CSF) in sporadic AD patients, and some evidence may point to a relationship between severity of dementia, tau-protein in CSF and age of onset of AD (Hock et al., 1995; Skoog et al., 1995; Schönknecht et al., 2003). Also, first results point to a relationship between elevated tracer binding to plaques and tangles and lower values of glucose utilization in the posterior cingulate and parietal brain regions in mild cognitive impairment (Small et al., 2006), and between elevated p-tau levels and decreased glucose metabolism in temporal, parietal and cingulate brain regions (Fellgiebel et al., 2004). However, a clear correlation between severity of dementia, glucose utilization and CSF tau-protein/phospho tau-protein in the course of mild cognitive impairment and its conversion to SAD is lacking as yet.

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J Neural Transm (2007) [Suppl 72]: 203–206

Printed in Austria

Journal of Neural Transmission

Long-term tetrahydroaminoacridine treatment and quantitative EEG in Alzheimer's disease

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Summary The development of therapies for Alzheimer's disease (AD) has focused on drugs designed to correct the loss of cholinergic function within the central nervous system. Quantitative EEG (qEEG) changes associated with AD consist of background slowing. One way to study the effects of cholinergic drugs may be through assessment of their qEEG effects. The aim of the current work was to evaluate the effect of long-term treatment with tetrahydroaminoacridine (THA) on qEEG in AD patients.

Keywords: Dementia, Alzheimer's disease, qEEG, tetrahydroaminoacridine, cholinesterase inhibitors, therapy

Introduction

Alzheimer's disease (AD) is the most prevalent dementing illness in the western world. Currently available drugs, which are indicated for dementia, do not share a common mechanism of action. A growing body of evidence supports the importance of cholinergic dysfunction in the symptomatology of AD (Bartus et al., 1982; Perry, 1986). Neuropsychological and neurophysiological studies on the influences of cholinergic and anticholinergic drugs in AD patients found that the acute administration of some cholinergic drugs improved memory and attention and exhibited a tendency to shift the EEG into more normal patterns (Agnoli et al., 1983; Alhainen and Riekkinen, 1993), whereas anticholinergic drugs induced opposite effects (Agnoli et al., 1983; Neufeld et al., 1994). The first widely used cholinesterase inhibitor, tetrahydroaminoacridine (THA), reduces EEG slowing characteristic of AD, but to date the effects have only been reported following acute administration or short-term (7 weeks) treatment (Alhainen and Riekkinen, 1993). Because patients with AD are likely to be treated for extended periods of time, we studied the long-term effects of THA on EEG, using our established quantitative evaluation (qEEG) method (Neufeld et al., 1999). Although THA is now rarely used, the effects on the EEG with othercholinergic agents would be of interest.

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Methods

Patients and drug protocol

Sixteen patients (9 males and 7 females) with age 75 ± 9.6 years (range 58–89 years) and clinically diagnosed with AD according to DSM-IV (American Psychiatric Association 1994) and NINCDS-ADRDA (McKhann et al., 1984) criteria participated in an open label study of THA (Tacrine, USA). The patients were subdivided into two groups; mildly demented ($n = 10$) and moderately/severely demented ($n = 6$), based on the Clinical Dementia Rating (CDR) examinations (Hughes et al., 1982).

THA treatment began with 40 mg daily which was increased every 6 weeks, depending on patients tolerability, to a maximum dose of 80 mg daily (in 5 mildly demented patients and 1 severely demented) or 120 mg daily (5 mildly demented and 5 moderately/severely demented patients).

EEG procedure

Baseline qEEGs were recorded before the initiation of treatment with THA and repeated after 5.4 ± 1.5 months (mean $\pm$ SD) of treatment. Recordings were carried out on an 18-channel Grass machine model 8 which was connected to a Bio-logic Brain Atlas commercial computer system with data acquisition and field mapping capabilities for 21 electrodes as previously described (Neufeld et al., 1994, 1999). Linked mandibles were used as reference. The patients were instructed to remain alert and relaxed with the eyes closed.

The EEG was recorded for 4 min on computer system that allows visual inspection of the EEG tracing in real time. The low-frequency filter was set at 1 Hz and the high-frequency filter at 35 Hz, and a 50 Hz notch filter was

used. We recomputed all EEG leads based on the digital computer averaged reference with eyes closed. Sixteen samples of 2-sec EEG epochs were digitized at 128 samples per second.

A fast Fourier transformation was applied on the 16 samples, which were then averaged. Frequency spectra were calculated from 18 electrode values, whereas values under Fp1, Fp2, and Fpz electrodes were extrapolated. This transformation yielded a value representing the amplitude in the different frequencies.

The EEG variables of interest were the delta (0.0–3.5 Hz), theta (4–7.5 Hz), alpha (8–11.5 Hz), and beta1 (12–15.5 Hz) bands. Logarithmic transformations of the mean absolute amplitude (log a) and the mean relative amplitude [$\log(x/1-x)$] were performed to approach gaussian distributions, where "a" represents the mean average amplitude and "x" represents the fraction of averaged amplitude in each frequency band. The EEG bands which were averaged and compared before and following treatment were those derived from electrodes of three areas: the frontal (F = Fp1, Fz1, Fp2, F7, F3, Fz, F4, F8), temporo-parietal area (TP = T3, T4, T5, T6, P3, Pz, P4), occipital area (O = O1, Oz, O2).

Comparisons were performed by means of a 2-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni post-hoc analyses. The following factors were used to compare the baseline qEEG in mild as opposed to moderate/severe AD patients: 1) "state" as "between" factor with two levels (mildly demented AD patients versus moderately/severely demented ones) and 2) "areas" as "within" factor with three (F, TP, O) levels.

Comparisons of qEEG before and during treatment were performed using two "within" factors: 1) "treatment" factor with two levels (before and during treatment), and 2) "area" factor with three (F, TP, O) levels.

We calculated the qEEG differences before and during treatment (main treatment effect); between different cortical areas (qEEG before and during treatment combined for each area; main regional effect); the different effect of treatment in different cortical areas (the regional-treatment interactions) in the group as a whole and separately in mildly demented patients with low and high dose of THA, and in moderately/severely demented patients with high dose.

When ANOVA analysis was performed, the Bonferroni correction was applied to determine in detail the effect of treatment on each brain area of interest, resulting in a significance level of $p < 0.0166$.

We studied the effects of treatment of low (80 mg/day) and high (120 mg/day) doses of THA on the qEEG in 16 patients (age 75 ± 9.6 years, range 58–89 years) clinically diagnosed as having AD according to DSM-IV and NINCDS-ADRDA criteria. The patients were subdivided into mildly ($n = 10$) and moderately/severely demented ($n = 6$) cases, based on the Clinical Dementia Rating (CDR) scale. The qEEG of the mean absolute and relative amplitudes of delta, theta, alpha and beta1 activities were obtained at baseline and during long-term THA treatment (5.4 ± 1.5 months). Comparisons were performed by a 2-way ANOVA with repeated measures.

Results

There were no statistically significant qEEG changes for the whole group during treatment. Treatment with low dose (80 mg daily) THA in mildly demented patients significantly reduced the mean absolute delta activity. No significant changes were observed in the qEEG during THA treatment in moderately/severely demented patients.

When the AD patients were divided into mildly and moderately/severely affected groups, a comparison of the baseline (before treatment) qEEG showed regional differences between mildly and moderately/severely demented patients (ANOVA: $p = 0.004$). Following Bonferroni correction analysis there was a significant decrease in mean absolute beta 1 activity in TP ($p < 0.0010$) and O ($p < 0.001$) areas in the more severely demented patients.

Table 1. *Differences in the qEEG activity before and during treatment with THA in various cortical areas of Alzheimer's disease patients*

	Frequency	Treatment-related difference*		
		Frontal	Temporo-parietal	Occipital
A	Delta	0.06 ± 0.93	0.05 ± 0.95	0.16 ± 0.78
	Theta	-0.03 ± 1.14	-0.03 ± 1.32	0.09 ± 1.39
	Alpha	-0.28 ± 0.75	-0.41 ± 1.00	-0.27 ± 1.14
	Beta 1	-0.09 ± 0.22	-0.13 ± 0.30	-0.06 ± 0.27
B	Delta	-0.74 ± 0.46**	-0.80 ± 0.54**	-0.50 ± 0.47**
	Theta	-0.82 ± 1.09	-0.97 ± 1.34	-0.73 ± 1.11
	Alpha	-0.62 ± 0.91	-0.81 ± 1.31	-0.61 ± 1.36
	Beta 1	-0.13 ± 0.21	-0.18 ± 0.37	-0.06 ± 0.32
C	Delta	0.40 ± 0.67	0.46 ± 0.65	0.49 ± 0.37
	Theta	-0.05 ± 0.27	0.03 ± 0.36	-0.09 ± 0.21
	Alpha	-0.47 ± 0.32	-0.68 ± 0.38	-0.35 ± 1.03
	Beta 1	-0.06 ± 0.14	-0.17 ± 0.17	-0.06 ± 0.10
D	Delta	0.71 ± 0.93	0.67 ± 0.90	0.63 ± 0.88
	Theta	0.94 ± 1.10	1.01 ± 1.20	1.26 ± 1.67
	Alpha	0.30 ± 0.54	0.34 ± 0.62	0.22 ± 0.99
	Beta 1	-0.09 ± 0.34	-0.03 ± 0.33	-0.06 ± 0.37

A, Whole group of Alzheimer's disease patients ($n = 16$); *B*, mildly demented Alzheimer's disease patients ($n = 6$) with low doses of THA; *C*, mildly demented Alzheimer's disease patients ($n = 5$) with high doses of THA; *D*, moderately/severely demented Alzheimer's disease patients ($n = 5$) with high doses of THA.

* Mean absolute amplitude during treatment minus mean absolute amplitude before treatment (μv), ± SD. Negative numbers – decrease amplitude of EEG during treatment; positive numbers – increase amplitude of EEG during treatment. Note that statistical comparison was performed after logarithmic transformation of the mean absolute amplitude. ** Significant difference (Bonferroni correction test).

THA treatment did not cause significant changes in the qEEG in the group as a whole ($n = 16$) (Table 1). However, treatment with low dose (80 mg daily) THA in six patients (five mildly demented and one severely demented) significantly reduced the mean absolute delta activity (main treatment effect) (ANOVA: $p = 0.011$). The results of Bonferroni correction analysis showed a significant decrease in the F ($p < 0.001$), TP ($p < 0.001$) and O ($p < 0.001$) areas (Table 1).

No significant changes were observed in the qEEG during treatment with higher THA doses (120 mg daily) in either mildly demented ($n = 5$; Table 1) or in those with moderately/severely demented patients ($n = 5$; Table 1).

Discussion

Many studies have shown that the levels of the acetylcholine-synthesizing enzyme, choline acetyltransferase, are reduced in the brains of patients with AD (Bartus et al.,

1982; Perry, 1986). Based on these abnormalities in the cholinergic system, a variety of pharmacological approaches have been undertaken in the treatment of AD, but the first drug to have shown clinical efficacy was THA (Alhainen and Riekkinen, 1993). This treatment produced varying degrees of clinical improvement in AD patients and contradictory neurophysiological effects (Alhainen and Riekkinen, 1993; Minthon et al., 1993; Shigeta et al., 1993; Gustafson, 1993; Gracon, 1996).

Previous studies of baseline EEG and qEEG in AD revealed an increase in the theta and delta bands and a decrease in the alpha and beta bands (Stigsby et al., 1981; Penttila et al., 1985). A gradual decrease in the percentage power of the alpha band and the ratio of powers in the alpha to delta bands was observed with progression of AD (Penttila et al., 1985). Analysis of the beta band has not been performed, because some AD patients used benzodiazepines, which may increase beta activity (Penttila et al., 1985). In our study, there were only two patients (one mildly demented, and the other severely demented) using benzodiazepines (brotizolam, oxazepam) but this is unlikely to have affected the results of the study since the dose of these drugs did not change throughout the investigation period. In our study more severe dementia was associated with significantly decreased baseline mean absolute beta1 activity only in TP and O areas. This may be related to the predominance of cortical degeneration and decreased regional cerebral blood flow and cholinergic metabolism in the temporolimbic and temporoparietal association cortex (Gustafson and Risberg, 1979; Brun and Englund, 1981; Friedland et al., 1983).

In the present study, long-term THA treatment of AD patients was not manifested by significant changes in the qEEG. There were, however, individual differences within the total sample, when comparing the pretreatment and the treatment conditions, and, accordingly, patients were subdivided into three groups (the mildly demented with lower or higher doses of THA and moderately/severely demented with higher doses of THA) for closer examination of the treatment effects.

In the mildly demented AD patients who received low (80 mg/day) dose of THA there was evidence of an improvement of the qEEG (decrease in absolute delta activity diffusely in all research areas). Indeed decreased slowing and increased mean frequency in qEEG during THA treatment has been reported in other studies (Alhainen and Riekkinen, 1993; Shigeta et al., 1993). A single dose of THA (50 mg) also increased alpha/theta and alpha/delta ratios in AD patients who were later classified as responders to THA following 7 weeks of treatment with 100 mg/day (Alhainen and Riekkinen, 1993). Responders showed significant increases in the absolute alpha power and in alpha/delta and alpha/theta ratios (Alhainen and Riekkinen, 1993). A comparison of short- and long-term treatment (Shigeta et al., 1993) revealed an improvement in frontal EEG (increase of the mean frequency) only after short-term low-dose treatment (3 weeks of 80 mg/day THA) but, consistent with our results, EEG reverted to the pretreatment level following long-term treatment (6–25 months).

Long-term treatment with higher doses (120 mg/day) of THA produced variable changes in the mean absolute delta activity that was manifested by the large values of their standard deviations (Table 1), contributing to a lack of significant changes in mean absolute delta activity (main treatment effect). Gracon (1996) reported that the mean values of cognitive scores of THA- and placebo-treated groups of AD patients declined over 24–30 weeks.

Thus, the tendency towards normalization of qEEG was seen only in cases of long-term treatment with low doses of THA mostly in mildly demented patients. Such differences in the qEEG with THA treatment in patients with different stages of dementia may be related to different levels of compensatory capacities. Presumably, patients with more advanced disease suffered more extensive degeneration, and therefore, the effect of THA may have been negligible.

The number of cases we studied was too small to differentiate among those in whom THA was clinically effective from those lacking a therapeutic response, but it would be interesting to note in future studies whether the EEG effects reflect clinical improvement. So far, such a correlation has not been seen with any drug used to treat dementia.

Conclusion

qEEG normalization during THA treatment in patients with different stages of dementia may be related to levels of compensatory capacities in brain.

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J Neural Transm (2007) [Suppl 72]: 207–215

Printed in Austria

Journal of Neural Transmission

Enhanced apoptosis, oxidative stress and mitochondrial dysfunction in lymphocytes as potential biomarkers for Alzheimer's disease

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Summary Alzheimer's disease (AD) is the most common progressive neurodegenerative disease. Today, AD affects millions of people worldwide and the number of AD cases will increase with increased life expectancy. The AD brain is marked by severe neurodegeneration like the loss of synapses and neurons, atrophy and depletion of neurotransmitter systems in the hippocampus and cerebral cortex. Recent findings suggest that these pathological changes are causally induced by mitochondrial dysfunction, increased oxidative stress and elevated apoptosis. Until now, AD cannot be diagnosed by a valid clinical method or a biomarker before the disease has progressed so far that dementia is present. Furthermore, no valid method is available to determine which patient with mild cognitive impairment (MCI) will progress to AD. Therefore, a correct diagnosis in the early stage of AD is not only of importance considering that early drug treatment is more effective but also that the psychological burden of the patients and relatives could be decreased. In this review, we discuss the potential role of elevated apoptosis, increased oxidative stress and mitochondrial dysfunction as biomarker for AD in a peripheral cell model, the lymphocytes.

Keywords: Lymphocytes, Alzheimer's disease, biomarker

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting more than 25 million people world wide (Wimo et al., 2003). AD manifests as gradual deterioration in memory and cognition, behavior and the ability to perform activities of daily living. The AD brain is marked by severe neurodegeneration like the loss of synapses and neurons, atrophy and depletion of neurotransmitter systems in the hippocampus and cerebral cortex. The majority of AD patients suffer from sporadic AD where ageing itself represents the main risk factor. The minority of AD patients are affected from rare genetic mutations in the amyloid precursor protein (APP) or in the presenilins PS1 and PS2. The clinical progress of these familiar forms is characterized by an early onset of cognitive symptoms. The sporadic and familiar forms share the same pathological hallmarks. They are both characterized by deposition of β-amyloid (Aβ) plaques, accumulation of intracellular neurofibrillary tangles, and pronounced neuronal cell loss. Altered proteolytic processing of APP resulting in the production and aggregation of neurotoxic forms of amyloid beta (Aβ1-40, Aβ1-42) is considered to be central for AD (Selkoe, 2004). Currently, the main hypothesis concerning the origin of AD is based on the neurotoxic effect of Aβ causing increased apoptosis in neurons, elevated oxidative stress, hindered energy metabolism, mitochondrial dysfunction, and consequently synaptic dysfunction (Malaplate-Armand et al., 2006; Kriem et al., 2005).

The diagnosis of AD is still largely based on exclusion criteria of secondary causes and other forms of dementia with similar clinical profile, thus the diagnostic accuracy is only suboptimal. In the United States a diagnostic accurancy of 50–60% and 80–90%, respectively at specialized centers is reached using the common criteria (NINCDS-ADRDA) (Ferris and Yan, 2003; Turner, 2006). Until now, no valid clinical method or biomarker is available to accurately identify AD in the very early phase and to determine which patient with mild cognitive impairment (MCI) will progress to AD (Frisoni et al., 2004; Borroni et al., 2006). This is of special relevance because drug treatment is more effective in the early stage of the disease. Therefore, a valid and easy accessible biomarker for AD or a combination of biomarkers representing the multiplicity of pathophysiological processes taking place in AD would

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simplify the diagnosis, increase the accuracy and enhance the efficacy of drug therapy. At the moment, two different types of biomarkers are discussed: cerebrospinal fluid (CSF) markers like total tau protein or Aβ1-42 and markers in plasma or peripheral cell types like lymphocytes, platelets or fibroblasts (Migliore et al., 2005a). In this review we focus on lymphocytes as a peripheral cell model for AD. Lymphocytes show similar defects like neurons in AD. We and others observed elevated apoptosis, increased oxidative stress and changes in mitochondrial function in lymphocytes. The strong advantage of lymphocytes as a peripheral model compared to CSF is the simple non-invasive, inexpensive and time-saving separation from blood of patients. Repeated samples from patients can be taken as the particular study requires. Therefore, lymphocytes could be an applicable cell model to find a valid and easy detectable biomarker for AD.

Similar effects of AD relevant stressors on mitochondrial dysfunction and apoptosis in human lymphocytes and neuronal cell lines

Lymphocytes show similar reactions to AD relevant stressor like the neuronal like cell line, PC12 cells. We investigated the effects of staurosporine, Aβ1-42, H_2O_2, sodium nitroprusside and complex inhibitors of the mitochondrial respiratory chain on apoptosis and mitochondrial membrane potential (MMP) in human lymphocytes. Staurosporine, which is widely used to induce apoptosis in a variety of cell types, leads to a significant increase in apoptotic cells in human lymphocytes as well as in PC12 cells (Table 1) (Leutz et al., 2002). Additionally, lymphocyte treatment with Aβ1-42 and H_2O_2 results in enhanced apoptosis (Eckert et al., 1998). Treatment of lymphocytes with relevant concentrations of sodium nitroprusside, a NO donor, induces a reduction of MMP in both cell types. Again, the different inhibitors of the respiratory chain initiate a decrease of MMP in human lymphocytes and PC12 cells. Therefore, different AD relevant stressors lead to similar effects like elevated apoptosis or decreased MMP in human lymphocytes and PC12 cells. These results suggest that lymphocytes are a suitable peripheral cell model to study AD relevant pathological changes like apoptosis, oxidative stress or mitochondrial dysfunction.

Table 1 *Comparison of the effects of different stressor in human lymphocytes and PC12 cells on MMP and apoptosis*

	Human lymphocytes	PC12 cells
MMP		
Stressors:		
SNP	↓	↓
Complex inhibitors		
Complex I Rotenone	↓	↓
Complex II Thenoyltrifluoroaceton	↓	↓
Complex III Antimycin	↓	↓
Complex IV Natriumazide	↓	↓
Complex V Oligomycin	↓	↓
Apoptosis:		
Aβ1-42	↑	↑
Staurosporin	↑	↑
H_2O_2	↑	↑

Data published in part (Eckert et al., 1998a; Leutz et al., 2002).

Elevated apoptosis in lymphocytes of AD patients

Despite the various genetic and environmental factors that may lead to AD, increasing evidence from AD brain tissue, transgenic animals, and cell lines suggest that the underlying neurodegeneration is associated with morphological and biochemical features of apoptosis (Culmsee and Landshamer, 2006; Mattson, 2004). Apoptotic hallmarks are DNA fragmentation, cytoplasmic shrinkage, chromatin condensation and caspase activation (Jellinger, 2006). Two major signaling pathways lead to apoptosis, the TNF-receptor-mediated (extrinsic) and the mitochondria-based (intrinsic) pathway. The extrinsic pathway is activated by the stimulation of death receptors, e.g. cytokine receptors of the TNF family like the Fas receptor (CD 95), whereas the intrinsic pathway is associated with perturbed mitochondrial function including a loss of MMP, increase in reactive oxygen species (ROS) and the release of cytochrome C followed by caspase 9 and caspase 3 activation. Evidence that many neurons undergo apoptosis in AD includes elevated neuronal DNA-fragmentation in AD postmortem brain tissue, and high levels of activated apoptotic proteins such as caspase 3 and BAX in neurons that exhibit neurofibrillary tangle pathology (Mattson, 2004; Eckert et al., 2003). APP and PS mutations are shown to be sufficient to trigger apoptosis in AD animal models (Keil et al., 2004; Marques et al., 2003). Furthermore, recent findings indicate that the expression of mutant PS1 or mutant APP in PC12 cells sensitizes cells to apoptosis (Eckert et al., 2001d; Guo et al., 1997). In addition to genetic evidence that Aβ induces neuron degradation *in vivo*, recent *in vitro* experiments suggest that oligomeric, intracellular Aβ and not aggregated Aβ like previously thought leads to apoptosis (Malaplate-Armand et al., 2006; Kriem et al., 2005; Deshpande et al., 2006).

Studies in lymphocytes from sporadic AD patients have provided evidence for elevated apoptosis in peripheral blood cells (Table 2). Aging itself induces an increase in

Table 2. *Elevated apoptosis in lymphocytes from AD patients and transgenic animals*

Author	Significant changes in sporadic AD patients compared to aged controls	Significant changes in transgenic animals
Eckert et al. (1998a)	– enhanced basal levels of DNA-fragmentation	
Eckert et al. (1998b)	– enhanced basal levels of DNA-fragmentation – enhanced spontaneous apoptosis – increased apoptosis after oxidative stress (d-ribose)	
Eckert et al. (2001b)		PS1 mutations – enhanced basal apoptosis – increased apoptosis after oxidative stress (d-ribose, H_2O_2)
Schindowski et al. (2003)	– enhanced basal apoptosis – enhanced spontaneous apoptosis – increased apoptosis after oxidative stress (d-ribose)	App and PS1 mutations – enhanced basal apoptosis – enhanced spontaneous apoptosis – increased apoptosis after oxidative stress (d-ribose)
Tacconi et al. (2004)	– significant increase in caspase-3, caspase-6, caspase-8 activity	
Lombardi et al. (2004)	– hyperexpression of Fas mRNA and surface Fas receptor	
Frey et al. (2006)	– enhanced basal apoptosis – enhanced spontaneous apoptosis – increased apoptosis after oxidative stress (d-ribose) – increased caspase 3-activity – increase in Fas expression	
Schindowski et al. (2006)	– enhanced basal apoptosis	

vulnerability to apoptosis (Schindowski et al., 2000). This enhanced susceptibility seems to be even more pronounced in lymphocytes from sporadic AD patients (Eckert et al., 2001a, 2003; Schindowski et al., 2006). Elevated DNA fragmentation was seen in freshly prepared AD lymphocytes compared to controls and spontaneous apoptotic cell death after 24 h was significantly elevated. Importantly, elevated basal apoptosis from AD patients correlated significantly with the Mini Mental State Examination (MMSE)

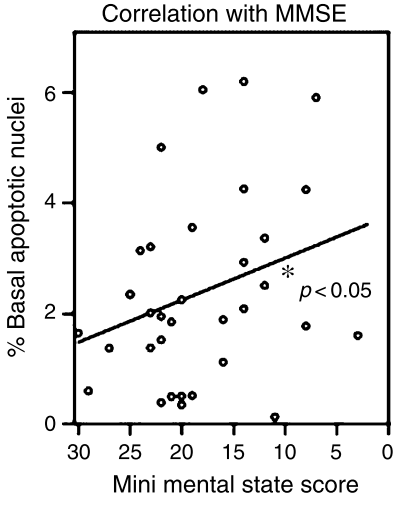

Fig. 1. Correlation of apoptosis in lymphocytes of AD patients with MMSE. Basal levels of apoptotic nuclei in lymphocytes of sporadic AD patients correlate significantly with cognitive decline determined with MMSE ($n = 34$, $^*p < 0.05$) (Schindowski et al., 2006)

of these AD patients (Fig. 1). Furthermore, lymphocytes from AD patients showed an increased vulnerability to pro-apoptotic stimuli like 2-desoxy-ribose (D-ribase) or staurosporine. Analysis of activated lymphocytes gave further evidence for elevated levels of apoptosis in these peripheral blood cells. Significantly elevated levels of DNA-fragmentation were found in activated AD lymphocytes undergoing spontaneous *in vitro* apoptosis or enhanced apoptosis after the treatment with D-ribose. These result point to a faster turnover of apoptotic pathways in AD patients (Eckert et al., 2001a). Importantly, a robust difference in cell death sensitivity between AD patients and patients suffering from vascular dementia was detected.

Moreover, increased CD 95 expression on the surface of T cells from sporadic AD patients and elevated caspase-3, caspase-8 and caspase-9 levels in comparison with non-demented controls refer to an enhanced proneness of AD lymphocytes to cell death (Lombardi et al., 2004; Tacconi et al., 2004; Frey et al., 2006). These findings suggest involvement of the extrinsic and intrinsic apoptotic pathway. CD 95/Fas leads via the extrinsic pathway to apoptotic cell death by the activation of the initiator caspase-8 and the effector caspase-3. Since the activation of the effector caspase-3 is shared by the intrinsic and extrinsic pathway and cytochrome c release from mitochondria is followed by caspase-9 activation, the intrinsic apoptotic pathway could be also important for the increased vulnerability of lymphocytes from AD patients.

The above illustrated findings cannot be explained by changes in the distribution of lymphocyte subsets. No changes in subset distribution of T, B or NK cells were found in AD patients compared to aged controls (Schindowski et al., 2003, 2006). In contrast, a significant decrease in T lymphocytes was determined in healthy persons >60 years compared to young persons <30 years (Schindowski et al., 2002). Again, no changes in the distribution of T lymphocyte population in AD patients compared to aged controls were found (Schindowski et al., 2003, 2006; Frey et al., 2006), but a significant loss of $CD3^+$, $CD4^+$ and $CD8^+$ occurred during aging. Interestingly, several recent findings indicate that mainly $CD4^+$ cells contribute to the increased apoptotic levels in peripheral lymphocytes of AD patients, whereas no changes in the susceptibility of $CD8^+$ T cells to apoptosis were determined (Fig. 2) (Schindowski et al., 2003, 2006; Frey et al., 2006).

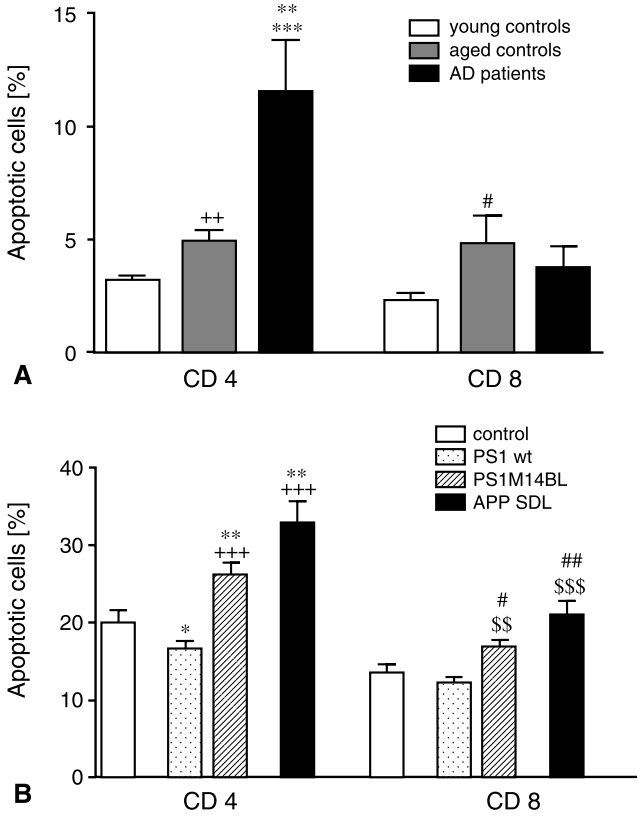

Fig. 2. Increased spontaneous apoptosis in $CD4^+$ lymphocytes compared to $CD8^+$lymphocytes of AD patients and PS1 and APP transgenic mice. **A** Spontaneous *in vitro* apoptosis in $CD4^+$ and $CD8^+$ T cells from young controls ($n = 11$), non-demented aged controls ($n = 12$), and AD patients ($n = 12$) after 24 h incubation, $^{**}p < 0.01$ vs $CD4^+$ from aged controls; $^{***}p < 0.001$ vs $CD4^+$ from young controls; $^{++}p < 0.01$ vs $CD4^+$ from young controls; $^{\#}p < 0.05$ vs $CD8^+$ from young controls. **B** Spontaneous *in vitro* apoptosis in $CD4^+$ and $CD8^+$ T cells from nontransgenic, controls and transgenic animals expressing either mutant human APP (APP695SL) or mutant human PS1 (PS1 M146L) or human wild-type PS1 (PS1 wt) ($n = 6$/group). $^{*}p < 0.05$, $^{**}p < 0.01$ vs $CD4^+$ from control; $^{++}p < 0.01$, $^{++}p < 0.001$ vs $CD4^+$ from PS1wt; $^{\#}p < 0.05$, $^{\#}p < 0.01$ vs $CD8^+$ from controls, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs $CD8^+$ from PS1wt; modified according to Schindowski et al. (2003)

Besides aging, apolipoprotein E (ApoE) genotype is the most important risk factor for sporadic AD. The three major human isoforms E2, E3 and E4 differ in two amino acids in the positions 112 and 158. The isoform ApoE4 is associated with an increased risk to develop AD. Different effects of ApoE 4 contributing to the pathophysiology of AD like the modulation of the deposition and clearance of Aβ, the impairment of the antioxidative defense system or an increased phosphorylation of Tau are currently discussed (Huang, 2006). Interestingly, lymphocytes from AD patients bearing one or two Apo ε 4 alleles (heterogen ε4/ε3 or homogen ε4/ε4) exhibit a higher rate of apoptotic cell death and caspase 3 activation than Apoε3/ε3 carrier (Frey et al., 2006; Schindowski et al., 2006).

Further, elevated apoptosis was also found in lymphocytes of familiar AD-patients and AD animal models bearing AD relevant APP or PS1 mutations (Fig. 2) (Parshad et al., 1996; Eckert et al., 2001b; Schindowski et al., 2003), supporting the idea that AD specific changes lead to elevated susceptibility of T lymphocytes.

Increased oxidative stress in lymphocytes of AD patients

A large body of evidence suggests that enhanced oxidative stress plays an important role in the dysfunction and apoptotic death of neurons in AD. Studies in post mortem brain tissue of AD patients provided evidence for increased levels of cellular oxidative stress, immunohistochemistry revealed increased protein oxidation, protein nitration, and lipid peroxidation in brain areas with neurofibrillary tangles and Aβ plaques (Perry et al., 2000; Mattson, 2002). Additionally, alteration in levels of antioxidant enzymes such as catalase, Cu/Zn-superoxide-dismutase, and Mn-superoxide-dismutase support the evidence for increased oxidative stress in AD post-mortem tissue and AD animal models (Aksenov et al., 1998; Schuessel et al., 2005, 2006). Membrane lipid oxidation, particularly toxic for neurons, leads to the generation of toxic aldehyds such as 4-Hydroxynonenal (HNE) or malondialdehyde (MDA). The mechanism how oxidative stress accumulates in AD is still unknown but several findings suggest a link between Aβ toxicity and generation of reactive oxygen species (Abdul et al., 2006). Lipid membrane damage is promoted by Aβ aggregates (Murray et al., 2005; Schuessel et al., 2006) and enhanced ROS were found as a consequence of

Table 3. *Increased oxidative stress in lymphocytes from AD patients and transgenic animals*

Author	Significant changes in sporadic AD patients compared to aged controls	Significant changes in transgenic animals
Mecocci et al. (1997)	– elevated basal levels of oxidative DNA damage	
De Leo et al. (1998)	– increased Mn-superoxide-dismutase mRNA levels	
Morocz et al. (2002)	– elevated basal levels of oxidative DNA damage – elevated levels of oxidative DNA damage after oxidative stress (H_2O_2)	
Mecocci et al. (2002)	– elevated basal levels of oxidative DNA damage	
Kadioglu et al. (2004)	– elevated basal levels of oxidative DNA damage	
Migliore et al. (2005)	– elevated basal levels of oxidative DNA damage	
Leutner et. al. (2006)	– enhanced basal ROS levels – elevated ROS levels after staurosporine	
Schüssel et al. (2006)		– elevated ROS levels – increased HNE levels

Aβ mediated mitochondrial dysfunction (Keil et al., 2004; Marques et al., 2003).

Elevated oxidative stress is again not only found in neurons of AD patients but also in peripheral cells like lymphocytes and fibroblasts (Table 3) (Drouet et al., 1999; Schindowski et al., 2003; Huang et al., 2005). The leakage of reactive oxygen species (ROS) from mitochondria, e.g. the superoxide anion radical is converted to H_2O_2 which can take part in the Fenton reaction resulting in the production of the reactive hydroxyl radical cumulating in DNA-oxidation. Here, a major product is 8-hydroxy-2-deoxyguanosine (8-OHdG). Our group showed that lymphocytes from AD patients handle oxidative stress differently than lymphocytes of aged-matched controls. Firstly, lymphocytes of AD patients have increased basal ROS levels and secondly they react differently to oxidative stressors like staurosporine. They show increased levels of ROS after the treatment with staurosporine (Leutner et al., 2005). Our findings are supported by results of altered levels and activities of antioxidant enzymes. De Leo et al. provided evidence that the activity of the Cu/Zn superoxide-dismutase in red blood cells is significantly elevated and mRNA levels of Mn-superoxide dismutase are significantly increased in lymphocytes, supporting the hypothesis of an increased level of ROS in AD (De Leo et al., 1998). These results are supported by different groups (Mecocci et al., 1997, 2002; Cecchi et al., 2002; Morocz et al., 2002; Kadioglu et al., 2004; Migliore et al., 2005b). They all found significantly higher concentrations of 8-OHdG in different peripheral cell models. Supporting the hypothesis of elevated peripheral oxidative stress in AD, significantly lower plasma levels of antioxidants were detected in blood from sporadic AD patients compared to aged controls (Mecocci et al., 2002; Straface et al., 2005). Another group reported significantly elevated levels of oxidative DNA damage at basal levels in lymphocytes of sporadic AD and after additional oxidative stress induced by H_2O_2 (Morocz et al., 2002). In addition, DNA-oxidation altered activity and expression of antioxidant enzymes were found in peripheral blood cells of sporadic AD patients (De Leo et al., 1998).

In lymphoblasts and fibroblasts from familial AD patients with PS and APP mutations a clear increase in lipid-peroxidation products, MDA and HNE was found (Cecchi et al., 2002). Furthermore, the anti-oxidant capacity in lymphoblasts from peripheral blood of familial AD patients was reduced (Cecchi et al., 1999). These results are confirmed by findings in transgenic animals. Elevated ROS levels were found in lymphocytes of PS1 mutant mice (Eckert et al., 2001b; Schuessel et al., 2006).

Mitochondrial dysfunction in lymphocytes as a potential biomarker for AD

The increased ROS levels and enhanced apoptosis found in AD brain and periphery can be explained by mitochondrial dysfunction taken place in AD. Mitochondria are essential for the maintenance of cell function and viability. Mitochondria are the major source of ROS. They are exposed to high concentrations of ROS and may therefore be particularly susceptible to oxidative stress. Analyses of AD brains provide substantial evidence for disturbed mitochondrial energy metabolism (Beal, 2000) and for decreased glucose metabolism (Hoyer, 2000; Blass et al., 2002). These metabolic changes are due to the dysfunction of the mitochondrial electron transport enzymes. The most consistent finding in AD is a deficiency in complex I (cytochrome C oxidase) of the respiratory chain (Parker Jr, 1991; Parker Jr et al., 1994; Maurer et al., 2000; Butterfield et al., 2001). Additionally, a reduction of the activities of pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate were found in AD brains (Bubber et al., 2005). Fur-

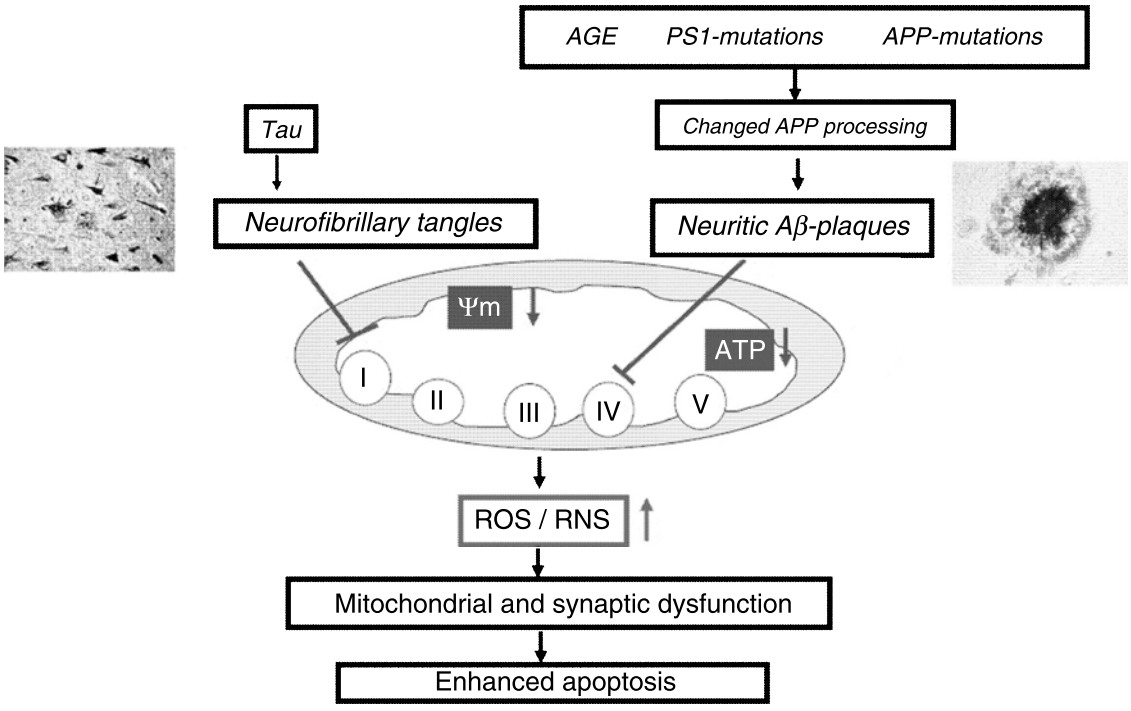

Fig. 3. Mitochondrial dysfunction as an early event in sporadic and familiar AD. Mitochondrial dysfunction as an early common pathway of aging, tau pathology and other unknown risk factors of sporadic AD as well as APP and PS1 mutations, modified according to Hauptmann et al., 2006

thermore, Hirai et al. found an increase in mitochondrial DNA in neurons of AD patients and ultrastructural changes of the mitochondria (Hirai et al., 2001; Rodriguez-Santiago and Nunes, 2005). These results are confirmed in AD animal and cell models (Anandatheerthavarada et al., 2003; Blanchard et al., 2003; Marques et al., 2003; Keil et al., 2004). Our group found decreased mitochondrial membrane potential and diminished enzymatic activity of respiratory chain complexes III and IV in 3 months old APP transgenic mice, which show no Aβ plaques at this age. We suggest that oligomeric Aβ induces mitochondrial dysfunction in these mice (Hauptmann et al., 2006). Therefore, we suggest that mitochondrial dysfunction is an early event in AD leading to several pathological features of this disease. In addition, we determined reduced complex I activity, impaired mitochondrial respiration and ATP synthesis in P301L tau transgenic mice (David et al., 2005). We propose the following hypothetical sequence of events linking to AD (see Fig. 3). Aβ as well as Tau pathology lead to mitochondrial dysfunction before Aβ plaques or Tau tangles can be detected. Consequently, ATP levels are reduced and ROS production is increased. We suggest that when the inhibition of mitochondrial function has reached a threshold and severe energy deprivation appears, mitochondrial and synaptic dysfunction can appear. Therefore, mitochondrial dysfunction could be an early marker for AD. Furthermore, the detection of mitochondrial dysfunction could become a tool to distinguish between MCI patients who develop AD or not.

Again, mitochondrial dysfunction was not only observed in brains of AD patients, but also in peripheral tissues such as platelets. Several studies showed a decreased cytochrome c activity in human platelets from AD patients (Bosetti et al., 2002; Mancuso et al., 2003; Cardoso et al., 2004). In accordance with these findings, platelets of AD patients show decreased ATP levels and increased levels of ROS (Cardoso et al., 2004).

In lymphocytes of sporadic AD patients only few studies were conducted referring to mitochondrial dysfunction. Our group investigated a protein factor that act upstream of mitochondrial dysfunction, Bcl2 (Schindowski et al., 2006). The antiapoptotic Bcl2 can form heteromers with the proapoptotic Bax and can therefore prevent its apoptogenic activity (Culmsee and Landshamer, 2006). We found a tendency of elevated Bcl2 in T cells of sporadic AD patients compared to aged controls. Again, $CD4^+$ cells were more sensitive to AD related changes. Bcl2 levels were significantly elevated in $CD4^+$ cells compared to $CD8^+$ cells. Interestingly, when splitting up AD-patients into mild (MMSE >20) and severe (MMSE <20) AD, a dual regulation was observed. Bcl2 is up-regulated in mild AD while further progression of the disease the Bcl2 content decreases with cognitive loss. We

Table 4. *Mitochondrial function in a preliminary set of patients with AD or MCI compared to aged controls*

	Mitochondrial membrane potential		ATP levels	
	AD	MCI	AD	MCI
Complex I (Rotenone)	$\downarrow^{*}$	ns	ns	ns
Complex II Thenoyltrifluoroaceton	ns	ns	$\downarrow^{**}$	ns
Complex III Antimycine	$\downarrow^{*}$	$\downarrow^{*}$	ns	ns
Complex IV Natriumazide	$\downarrow^{*}$	$\downarrow^{*}$	ns	ns
Complex V Oligomycine	$\downarrow^{*}$	$\downarrow^{*}$	ns	ns

Mitochondrial dysfunction was investigated in 8–12 AD patients, MCIs and aged controls. MMSE aged controls 29.88 ± 0.35, MCI 27.33 ± 3.04, AD patients 20.12 ± 5.78

ns not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $\downarrow$ reduction relating to control

suggest that in the early stage of AD, Bcl2 is up-regulated to protect cells against apoptosis. Furthermore, we found in a preliminary set of patients (Table 4) increased sensitivity of complex I–V in lymphocytes of AD patients compared to aged controls (Table 4). The mitochondrial membrane potential was significantly reduced after stressing lymphocytes of AD patients with complex I, III, IV, and V inhibitors of the respiratory chain. Importantly, here we found a graduation of susceptibility to complex inhibitors between AD patients, MCIs and aged controls. Additionally, there was a significant decrease in ATP-levels graduated from AD patients to MCI and aged controls after stimulation with the complex II inhibitor.

However, other groups investigated the basal activities of the complexes of the respiratory chain in lymphocytes of sporadic AD patients. They found no significant differences between aged controls and AD patients (Molina et al., 1997; Casademont et al., 2003).

Conclusion

In several studies, lymphocytes were shown to be a suitable cell model studying pathological changes in AD. This cell type shows similar vulnerability to AD relevant stressors like Aβ1-42 or nitrosative or oxidative stress *in vitro*. Increased basal apoptosis, elevated ROS levels, altered levels of antioxidant enzymes, elevated hydroxyl radical induced DNA-oxidation and increased mitochondrial susceptibility were found in AD patients compared to controls.

According to the proposal of a consensus group on molecular and biochemical markers for AD (Consensus report of the Working group on molecular and Biochemical Markers of Alzheimer Disease, 1998), an ideal biomarker should detect the essential feature of neuropathology of AD. Its sensitivity for detecting AD and its specificity for distinguishing other dementias should be more than 80%. Also, the biomarker should be reliable, reproducible, non-invasive, simple to perform and inexpensive. Keeping these requirements in mind, lymphocytes are an adequate biomarker model. Lymphocytes can be easily obtained from blood samples. Their separation is inexpensive and time-saving. Repeated samples from patients can be taken as the particular study requires.

Considering the applicability of the above discussed parameters, elevated apoptosis, increased oxidative stress and mitochondrial dysfunction are essential for the neuropathology of AD. Therefore, they meet one crucial criteria of the consensus group. Regarding the specificity, we detected robust differences in cell death susceptibility between AD and vascular dementia. For oxidative stress and mitochondrial dysfunction, studies comparing different forms of dementia need to be conducted. Furthermore considering the reliability, elevated apoptosis and increased oxidative stress were found in many studies. The measurement of oxidative stress as a biomarker has one disadvantage. Oxidative stress is also found in other neurodegenerative disease e.g. Parkinson disease (PD). Increased levels of MDA in serum, plasma and CSF for example were observed in PD patients (Ilic et al., 1999). Furthermore, elevated oxidative DNA-damage could be detected in lymphocytes of PD patients (Petrozzi et al., 2002) as well as significantly increased levels of 8-OHdG in serum and CSF of PD patients (Kikuchi et al., 2002).

From our point of view, mitochondrial dysfunction could be a promising concept as a biomarker for AD. Although in AD, like in PD no basal changes of complex activities of the respiratory chain in lymphocytes could be detected (Martin et al., 1996), we found enhanced susceptibility of complex I–V of the respiratory chain in a small sample of AD patients. Importantly, only here a graduation of susceptibility between AD patients, MCIs and aged controls could be detected. These results need to be confirmed in larger sample of patients.

Taken together, lymphocytes are a promising cell model for establishing biomarkers for AD, but further studies need to be conducted to evaluate which is the most adequate biomarker.

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J Neural Transm (2007) [Suppl 72]: 217–233

Printed in Austria

Journal of Neural Transmission

Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach

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Summary A growing body of evidence implicates impairments in brain insulin signaling in early sporadic Alzheimer disease (sAD) pathology. However, the most widely accepted hypothesis for AD aetiology stipulates that pathological aggregations of the amyloid β (Aβ) peptide are the cause of all forms of Alzheimer's disease. Streptozotocin-intracerebroventricularly (STZ-icv) treated rats are proposed as a probable experimental model of sAD. The current work reviews evidence obtained from this model indicating that central STZ administration induces brain pathology and behavioural alterations resembling those in sAD patients. Recently, alterations of the brain insulin system resembling those in sAD have been found in the STZ-icv rat model and are associated with tau protein hyperphosphorylation and Aβ-like aggregations in meningeal vessels. In line with these findings the hypothesis has been proposed that insulin resistance in the brain might be the primary event which precedes the Aβ pathology in sAD.

Keywords: Brain insulin, sporadic Alzheimer's disease, streptozotocin rat model

Introduction

Although neuropathologically Alzheimer's disease (AD) is characterized by the accumulation of extracellular plaques, consisting primarily of a low molecular weight amyloid-β (Aβ) peptide, and intracellular neurofibrillary tangles of aggregated hyperphosphorylated tau protein, it is well documented that AD is not a single entity. Currently, the leading hypothesis assumes that pathological assemblies of Aβ are the cause of all forms of AD, whereas other neuropathological changes, including tau hyperphosphorylation, are downstream consequences of pathological Aβ accumulation (Hardy and Selkoe, 2002). However, the amyloid cascade hypothesis is consistent with only a very small proportion of all AD cases, that is those caused by missense mutations in three chromosomes (http://www.molgen.ua.ac.be/ADMutations/) leading to autosomal dominant familial AD with an early onset. In the great majority of AD patients disease is sporadic in origin (millions worldwide) with age and several susceptibility genes as risk factors, and is of late onset (Hoyer and Frölich, 2006). A growing body of evidence implicates impairments in the brain insulin signaling pathway in sAD pathology (Frölich et al., 1998; Hoyer, 1998; Hoyer and Frölich, 2006; de la Monte and Wands, 2005). Since insulin has been shown to affect both Aβ levels and tau hyperphosphorylation in the brain, this issue has recently emerged as a novel field of sAD ethiopathogenesis and therapy research (de la Monte et al., 2006; Hoyer, 2004). Due to the developmentally specific nature of sAD, its early stages being clinically unrecognisable, and brain analysis being possible only post mortem (frequently in only the severe late stage cases), brain neurochemistry that characterizes the initiation of this disease in humans is mostly unknown. Experimental models of sAD may provide clues to early brain changes in this disorder. This review presents the information gained to date in the experimental rat model of sAD which has paved the way to the new hypothesis, the implications of which may provide novel ethiopathogenic and therapeutic approaches in sAD research.

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Insulin in the brain

Until the last three decades, the brain has not been thought of as an insulin-sensitive organ. The first evidence arguing against this hypothesis was the detection of immunoreactive insulin in dog cerebrospinal fluid (Margolis and

Altszuler, 1967), suggesting that circulating insulin could cross the blood–brain barrier. The discoveries of insulin and insulin receptors (IR) in the brain that followed (Havrankova et al., 1978a, b) raised further questions about the origin of insulin in the brain, as well as physiological and pathophysiological role(s) of insulin and IR in this organ. An extensive review of the current knowledge of insulin and IR and their roles in the brain has been published previously (Hoyer and Frölich, 2006), and will be presented briefly here for the purpose of comparison with the data from human and experimental models of sAD.

It is a common belief that in the mature adult brain the majority of insulin originates from the periphery; that is it is transported from the circulation after secretion from the pancreatic β-cells. The transport of insulin across the blood–brain barrier (BBB) is mediated via a saturable transport mechanism for which regional specificity in transporter distribution and kinetics has been reported (Banks, 2004). However, evidence has emerged that a smaller proportion of insulin is produced within the brain itself (Wozniak et al., 1993). Insulin gene expression and insulin synthesis have been demonstrated in both immature and mature mammalian neuronal cells (Schechter and Abboud, 2001; Schechter et al., 1992, 1996). In humans and in the chicken only one insulin gene is present, whereas in mice and rats insulin is produced by two independent genes that code for proinsulin I and II, both of which are localized to chromosome 1 (Todd et al., 1985). Insulin-1 and -2 mRNA were found to be distributed in a highly specific pattern with the highest density in the pyramidal cells of the hippocampus and high densities in medial prefrontal cortex, the entorhinal and perirhinal cortices, the thalamus and the granule layer of

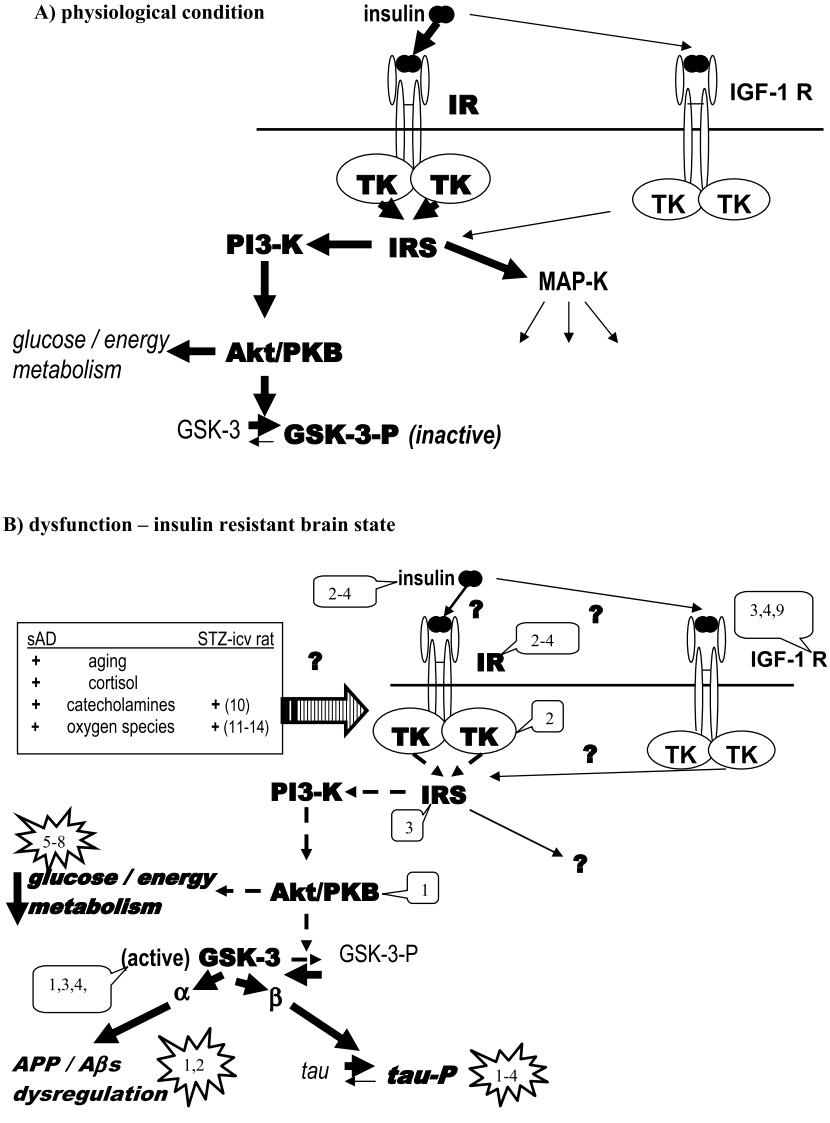

Fig. 1. Brain insulin receptor signaling cascade in physiological conditions (**A**) and in the insulin resistant brain state (**B**) induced by the streptozotocin-intracerebroventricular treatment. *IR* Insulin receptor; *IGF-1R* insulin-like growth factor-1 receptor; *TK* tyrosine kinase; *IRS* insulin receptor substrate; *MAP-K* mitogen activated protein kinase; *PI3-K* phosphatidylinositol-3 kinase; *Akt/PKB* protein kinase B; *GSK-3* glycogen synthase kinase-3; *GSK-3-P* phosphorylated glycogen synthase kinase-3; *APP* amyloid precursor protein; *A*β amyloid beta; *tau* tau protein; *tau-P* phosphorylated tau protein; *sAD* human sporadic Alzheimer's disease; *STZ-icv* streptozotocin-intraverebroventricularly treated rats. Number of reference in brackets: (1) Salkovic-Petrisic et al. (2006); (2) Grünblatt et al. (2006); (3) Lester-Coll et al. (2006); (4) de la Monte et al. (2006); (5) Plaschke and Hoyer (1993); (6) Duelli et al. (1994); (7) Lannert and Hoyer, (1998); (8) Pathan et al. (2006); (9) Grünblatt et al. (2004); (10) Lackovic and Salkovic (1990); (11) Sharma and Gupta (2001a); (12) Pathan et al. (2006); (13) Shoham et al. (2006); (14) Ishrat et al. (2006)

the olfactory bulb, as well as the hypothalamus (Devaskar et al., 1994; Grünblatt et al., 2006). Neither insulin mRNA nor synthesis of the hormone were observed in glial cells (Devaskar et al., 1994). The release of insulin from brain synaptosomes is stimulated by glucose (Santos et al., 1999).

Insulin signaling in the brain

Insulin in the brain binds to IRs which are abundantly but selectively distributed. Rodent studies have shown that the highest concentration of IRs is found in the nerve terminals of key brain regions, such as the olfactory bulb, hypothalamus, cerebral cortex, cerebellum and hippocampus (van Houten et al., 1979, 1980; Unger et al., 1989; Abbott et al., 1999). IR mRNA is abundantly present in neuronal somata (Schwartz et al., 1992). The neuronal IR binds insulin in a highly specific and rapid manner (Raizada et al., 1988). It has been hypothesized that the differing distribution patterns of insulin-1 and IRs in the brain may suggest that IRs in different brain regions may use insulin from different sources, either peripherally or locally synthesized, for cell-to-cell communication and neuronal signal transduction (Zhao et al., 2004). The IR is a tetramer composed of two extracellular α-subunits and two intracellular β-subunits. The neuronal (brain) IR differs from the peripheral IR in that both the α- and β-subunits have a slightly lower molecular weight, and the neuronal IR is not down-regulated by insulin, which otherwise activates a similar signalling cascade (Adamo et al., 1989; Heidenreich et al., 1983). Binding of insulin to the IR α-subunit induces autophosphorylation of the β-subunit by phosphorylation of its intrinsic tyrosine residues 1158, 1162 and 1163, thus triggering tyrosine kinase activity (Fig. 1A) (Combettes-Souverain and Issad, 1998). The location of phosphotyrosine-containing proteins corresponds to IR distribution (Moss et al., 1990). The receptor's activation state is regulated by its phosphorylation state. Deactivation may be induced by the action of both phosphotyrosine phosphatase causing dephosphorylation of the β-subunit (Goldstein, 1993) and by serine or threonine kinases causing phosphorylation at serine residues 1305 and 1306, and threonine residue 1348, respectively (Häring, 1991; Avruch, 1998). Insulin binding to the IR activates two parallel functional signal transduction cascades; one acting through the phosphatidylinositol-3 kinase (PI3K) pathway, and the other acting through the mitogen activated protein kinase (MAPK) pathway (Johnston et al., 2003). The former will be discussed later in the text. Briefly, tyrosine phosphorylation of IR β-subunits induces specific recruitment of proteins containing particular domains (SH2, PTB, etc.), amongst the most prominent of which are the proteins from the insulin receptor substrate family (IRS). It has been shown that IRS1 and the IR are co-expressed in particular brain regions, including the hippocampus (Baskin et al., 1994). Upon IR activation, the IRS becomes phosphorylated on tyrosine residues and capable of recruiting various specific (e.g. SH2) domain-containing signalling molecules; among them PI3K which becomes phosphorylated and consequently activated (Johnston et al., 2003). The activation of the PI3K pathway, in turn activates protein kinase B (Akt/PKB) (Fig. 1). The activated Akt/PKB triggers glucose transporter (such as GLUT4) translocation and consequently increases cellular glucose uptake (Vannucci et al., 1998; Johnston et al., 2003). Akt/PKB also phosphorylates (at the serine 9 residue) and consequently inactivates both α and β cytosolic forms of glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995). GSK-3 plays a key role in numerous cell functions, but only those that may be involved in sAD pathology will be briefly mentioned here. GSK-3α regulates the production of Aβ peptides, the amyloid precursor protein (APP) derivatives (Phiel et al., 2003). The promotion of APP secretion from the intracellular to the extracellular space and the inhibition of its degradation by insulin-degrading enzyme is mediated by insulin and the tyrosine kinase activity of the IR (Gasparini et al., 2001). Furthermore, insulin signaling via activation of PI3K regulates APP release into the extracellular space (Solano et al., 2000). GSK-3β isoform is involved in tau-protein phosphorylation (Ishiguro et al., 1993). Tau-proteins belong to a family of microtubule-associated proteins that stimulate the generation and stabilization of microtubules within cells, and control axonal transport of vesicles (Stamer et al., 2002). Accumulation of hyperphosphorylated tau protein leads to the formation of neurofibrillary tangles. The phosphorylation and dephosphorylation of the tau protein is regulated by several protein kinases, including GSK-3β, and by several protein phosphatases, including PTP-1, -2A, -2B (Ishiguro et al., 1992, 1993). Prolonged exposure to insulin has been shown to induce down-regulation of glycogen synthase kinase-3β activity and, thus, decreased phosphorylation of tau-protein (Cross et al., 1997; Hong and Lee, 1997).

Insulin's role in learning and memory

Evidence has been provided that brain insulin and the IR are functionally linked to improved cognition, in particular general and spatial memory, by up-regulation of insulin mRNA in the hippocampus and increased accumulation of the IR in hippocampal synaptic membranes (Zhao et al., 1999, 2004; for review Park, 2001). Recent *in vivo* evi-

dence has demonstrated that the effect of intrahippocampal microinjection of insulin on spatial learning and memory in rats is dose-dependent, that is cognitive function is impaired with low insulin doses, unchanged with intermediate doses, and improved with high insulin doses (Moosavi et al., 2006). Although the exact mechanism(s) by which insulin could affect learning and memory is unclear, several pathways have been suggested, for example those related to glucose metabolism and the modulation of neurotransmission by different neurotransmitters. The overlapping distributions of insulin, the IR and the insulin-sensitive glucose transporter (GLUT) isoforms support the hypothesis of insulin-stimulated glucose uptake in selective brain regions, the hippocampus in particular (Apelt et al., 1999; McEwen and Reagan, 2004). Since hippocampal glucoregulatory activities contribute to cognitive function (Reagan, 2002), insulin modulation of glucose metabolism in this structure appears to be one of the key components of hippocampal vulnerability. Additionally, insulin is likely to modulate memory via other molecular events, such as increasing the probability of inducing long-term amplification, a molecular model of learning, by promoting N-methyl-D-aspartate receptor conductance (Wang and Salter, 1994), as reviewed elsewhere (van der Heide et al., 2006). Insulin may also modulate cognitive functions via its effects on neurotransmission, e.g. low doses of insulin can reverse the amnestic effects of cholinergic blockade (Blanchard and Duncan, 1997), and high levels of insulin reduce neuronal norepinephrine reuptake (Figlewicz et al., 1993). Thus the data suggests that normal insulin and IR signaling is a prerequisite for normal learning and memory function.

An experimental rat model of sAD: streptozotocin-intracerebroventricularly treated rats

Given the complex nature of AD, it is difficult to establish an experimental animal model that would faithfully mimic the developmental pathology of this disease in humans. Frequently exploited are *transgenic Tg2576 mice* that over express the Swedish mutation of the human APP and demonstrate a progressive, age-related cortical and hippocampal deposition of Aβ plaques (Hsiao et al., 1996). Transgenic Tg2576 mice however, represent a model of AD induced by gene manipulation, and therefore, are unlikely to be representative of the sporadic type of this disease. Given the presence of the IR and insulin, as well as the possibility of its synthesis in the brain, and of disturbed insulin signal transduction in human sAD (Hoyer and Frölich, 2006), an experimental rat model was developed by using the drug streptozotocin (STZ).

STZ treatment

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose)) is a betacytotoxic drug which, following peripheral (parenteral) administration at high doses, selectively destroys insulin producing/secreting β cells in the pancreas, and causes type I diabetes mellitus in adult animals (Szkudelski, 2001). Type II diabetes can also be induced in rats by parenteral injections of STZ on the day of birth, resulting in a mild basal hyperglycemia, an impaired response to the glucose tolerance test, and a loss of β GSK-3 plays a key role in numerous cell functions, but only those that may be involved in cell sensitivity to glucose, 10 weeks post-injection (Szkudelski, 2001). Treatment with low to moderate doses of STZ in short-term experiments causes insulin resistance (Blondel and Portha, 1989) via a decrease in autophosphorylation (Kadowaki et al., 1984) and an increase in total number of IRs, but with little change in phosphorylated IR-β subunit (Giorgino et al., 1992), and maintained insulin-immunoreactive cells in the pancreas generating a transient diabetes mellitus (Rajab et al., 1989; Ar'Rajab and Ahren, 1993). Considering the presence of insulin (from both periphery and brain) and IRs in the brain, an experimental rat model was developed by using STZ applied intracerebroventricularly (icv) in doses of up to 100 times lower (per kg body weight/b.w./) than those used peripherally to induce an insulin resistant brain state (Nitsch and Hoyer, 1991; Duelli et al., 1994; Lannert and Hoyer, 1998). Central STZ administration caused neither systemic metabolic changes nor diabetes mellitus. In the past 17 years, since the first literature report of central STZ application, STZ has been administrated mostly in doses ranging from 1–3 mg/kg b.w., injected 1–3 times, either uni-or bi-laterally into the lateral cerebral ventricles (Table 1). Identical biochemical changes were found in the left and right striatum after administration of STZ to the right lateral cerebral ventricle only (Salkovic et al., 1995), suggesting that STZ-icv induced effects are not related to the direct non-specific toxic effect of STZ at the site of drug administration and that differing effects following uni- or bi-lateral application of STZ are not to be expected. In some of the experiments however, a wide variation in the susceptibility of individual animals has been demonstrated as a characteristic feature of STZ-icv treatment (Blokland and Joles, 1993, 1994; Prickaerts et al., 2000). Most experiments with STZ-icv applications used Wistar rats, with only a few utilising the Sprague-Dawley (Shoham et al., 2006) or Lewis strains (Prickaerts et al., 2000). Male animals were used in all experiments. Recently, bilateral intra-cortical administration of low STZ

Table 1. *Previous studies using central administration of streptozotocin in rats*

Reference	STZ dose	Age at STZ dose	Time after STZ treatment		Findings
			Cognitive tests (learning and memory)	Biochemical/histological analyses (brain tissue)	
Mayer et al. (1990)	1 × 1.5 mg/kg icv (unilat.)	1 year	11–13 days		↓ learning and memory
Lackovic and Salkovic (1990)	1 × ≤20 mg/kg icv (unilat.)	2 months		1 week DA, NA, 5-HT	↓ monoamine turnover rate ↑ monoamine content
Nitsch and Hoyer (1991)	1 × 1.25 mg/kg icv (unilat.)	1 year		3 weeks energy metabolism	↓ (CTX)
Ding et al. (1992)	1 × 1.5 mg/kg icv (unilat.)	1 year		3 weeks monoamine content and turnover	↓ content (enthorhinal CTX-5HT,NA; frontal CTX, S- NA) ↑ 5HT turnover rate
Hellweg et al. (1992)	1 × 1.5 mg/kg icv (unilat.)	1 year		1, 3 weeks NGF, ChAT	↓ NGF (1 w) – septum ↑ NGF (3 w) – HPC, CTX ↓ ChAT activity (HPC)
Blokland and Jolles (1993)	1 × 1.5 mg/kg icv (bilat.)	4 months	10–14 days	∼20 days ChAT	↓ learning and memory in middle-aged ↓ ChAT activity (HPC)
Plaschke and Hoyer (1993)	2 × 1.25 mg/kg icv (bilat.) (3 w) 3 × 1.25 mg/kg icv (bilat.) (6 w)	1 year		3 and 6 weeks glycolitic enzymes	↓ glycolytic enzyme activity (CTX, HPC)
Blokland and Jolles (1994)	1 × 1.5 mg/kg icv (bilat.)	2 years	2 weeks	∼20 days ChAT	no decline in learning and memory (active avoidance and open field test) ↓ ChAT activity (HPC)
Duelli et al. (1994)	3 × 1.5 mg/kg icv (bilat.)	1.5 years		6 weeks glucose utilization	↓ glucose utilization (CTX, HPC)
Terwel et al. (1995)	1 × 1.5 mg/kg icv (unilat.)	18 months		3 weeks peptidases, dehydrogenases, ChAT	↓ ChAT activity (HPC) ↓ peptidase and dehydrogenase activity (septum)
Salkovic et al. (1995)	1 × 0.5 mg/kg icv (unilat.)	2 months		1 week dopaminergic D1, D2 receptors, G-proteins	↓ D1 Bmax (striatum) ∼ D2 Bmax, ∼ Gs/Gi protein
Müller et al. (1998)	1 × 1.5 mg/kg icv (bilat.)	1 year		3 weeks free fatty acids phospholipids	↑ lipolysis (HPC, T-CTX)
Lannert and Hoyer (1998)	3 × 1 mg/kg icv (bilat.)	1 year	20, 40, 80 days	85 days energy metabolism	↓ learning and memory ↓ energy metabolism
Prickaerts et al. (1999)	1 × 1.25 mg/kg icv (bilat.)	20 months	3 weeks	3 weeks astrogliosis marker ChAT	↓ learning and memory astrogliosis (S, HPC, septum) ↓ ChAT activity (HPC)
Hoyer et al. (1999)	3 × ∼ 1 mg/kg icv (bilat.)	1 year	12 weeks	12 weeks energy metabolism	↓ learning and memory ↓ energy metabolism
Yun et al. (2000)	1 × 1 mg/kg icv (bilat.)	15 months		1 month energy metabolism	↓ energy metabolism
Prickaerts et al. (2000)	1 × 1.25 mg/kg icv (bilat.)	20 months	3 weeks	5 weeks astrogliosis NO/NOS (IHC)	partly ↓ learning and memory ↓ energy metabolism ↑ astrogliosis NOS is not involved in STZ induced toxicity
Sharma and Gupta (2001a)	2 × 3 mg/kg icv (bilat.)	∼4 months (320–350 g)	17 days	1, 7, 21 days oxidative stress	↓ learning and memory ↑ oxidative stress (progressive)
Weinstock et al. (2001)	1 × 1.5 mg/kg icv (unilat.) 3 × 0.5 mg/kg icv (bilat)	4 months	N.D.	N.D. morphology	↓ learning and memory ↑ microgliosis, axonal degeneration, distorted morphology of axonal fibres
Sharma and Gupta (2002)	2 × 3 mg/kg icv (bilat.)	4 months	2.5 weeks	3 weeks oxidative stress	↓ learning and memory ↑ oxidative stress
Salkovic-Petrisic and Lackovic (2003)	1 × 0.5 mg/kg icv (bilat.)	∼2–3 months (150–200 g)		1, 4 weeks monoamine transporter mRNA	↓ DAT 4 w (VMB); ↓ NAT 4 w (LC) ↑ NAT 1, 4 w (A1)

(*continued*)

Table 1 (*continued*)

Reference	STZ dose	Age at STZ dose	Time after STZ treatment		Findings
			Cognitive tests (learning and memory)	Biochemical/histological analyses (brain tissue)	
Shoham et al. (2003)	1 × ~1.8 mg/kg icv (bilat.)	3.5–4 months (270–290 g)	40 days	40 days morphology	↓ learning and memory neurotoxicity to myelin and axons in fornix and HPC
Veerendra Kumar and Gupta (2003)	1 × 3 mg/kg icv (bilat.)	3 months	2, 3 weeks	3 weeks oxidative stress	↓ learning and memory ↑ oxidative stress
Grünblatt et al. (2004)	3 × 1 mg/kg icv (bilat.)	1 year		6 weeks gene expression	altered gene expression (IGF-1R, GABA-R, glutamate transporter, potassium channels)
Grieb et al. (2004)	2 × ? (N.D.) icv (bilat.)	N.D.		3 weeks Golgi apparatus	↑ trans part of Golgi complex (F-CTX)
Sonkusare et al. (2005)	2 × 1.5 mg/kg icv (bilat.)	4 months	2.5 weeks	3 weeks AChE	↓ learning and memory ↑ AChE activity (whole brain)
Chu and Qian (2005)	1 × 3 mg/kg icv (bilat.)	N.D.		3 weeks morphology, IHC – tau protein and amyloid beta (1-40; 1-42)	↑ expression of tau and amyloid beta (CTX, HPC)
Salkovic-Petrisic et al. (2006)	1 × 1 mg/kg icv (bilat.)	3 months	1, 3 months	1, 3 months IR signaling cascade	↓ learning and memory at 1 and 3 m disturbed IR signaling pronounced at 3 m
Pathan et al. (2006)	2 × 3 mg/kg icv (bilat.)	~4 months (320–350 g)	14 days	21–25 days oxidative stress glucose utilization	↓ learning and memory ↑ oxidative stress ↓ glucose utilization
Shoham et al. (2006)	1 × 3 mg/kg icv (bilat.)	4 months (320–340 g)	2, 4 weeks	1, 8 weeks IHC – cholinergic markers, oxidative – nitrative stress	↓ learning and memory at 4 w gliosis at 1 w (CTX, HPC-CA1, septum, c. callosum); ↑ oxidative stress; unchanged ACh neurons (1, 8 w)
Ishrat et al. (2006)	1 × 1.5 mg/kg icv (bilat.)	1 year	2 weeks	3 weeks oxidative stress, ATP, ChAT-AChE	↓ learning and memory ↑ oxidative stress (HPC, CTX) ↓ ATP (HPC, CTX) ↓ ChAT-↑AChE activity (HPC)
De la Monte et al. (2006)	1 × 40 µg/kg (intracerebral – bilat.)	pups	1 month	1 month cholinergic markers NOS IR signaling	↓ learning and memory ↓ ChATmRNA (TL, CB); AChEmRNA ↓TL; ↑HPT ↑ NOS2, 3mRNA (TL) altered IR signaling
Grünblatt et al. (2006)	1 × 1 mg/kg 3 × 1 mg/kg icv (bilat.)	3–4 months	2 weeks, 2 months	3 months insulin and IR	↓ learning and memory at 2 w, 1 and 2 m altered insulin and IR

STZ Streptozotocin; *icv* intracerebroventricular; *unilat* unilateral; *bilat* bilateral; *w* week; *m* month; *DA* dopamine; *NA* noradrenaline; *5-HT* serotonin; *NGF* nerve growth factor; *ChAT* choline acethyltransferase; *AChE* acethyl cholinesterase; *NO* nitric oxide; *NOS* nitric oxide synthase; *IR* insulin receptor; *MAO* monoamine oxidase; *IHC* immunohistochemistry; *IGF-1R* insulin-like growth factor-1 receptor; *GABA-R* gamma aminobutiric acid receptor; *CTX* cerebral cortex; *F-CTX* frontoparietal cerebral cortex; *T-CTX* temporal cerebral cortex; *S* striatum; *HPC* hippocampus; *VMB* ventral medial bundle; *LC* locus coeruleous; *TL* temporal lobe; *CB* cerebellum; *HPT* hypothalamus; *N.D.* data not available; ↑ increase; ↓ decrease

doses (40 µg/kg) to three-day-old rat pups has been reported (Lester-Coll et al., 2006; de la Monte et al., 2006).

STZ mechanism of action

The mechanism of central STZ action and its target cells/molecules have not yet been clarified but a similar mechanism of action to that in the periphery has been recently suggested. In the periphery, STZ selective β cell toxicity results from the drug's chemical structure which allows it to enter the cell via the GLUT2 glucose transporter. The predominant site of GLUT2 localization is the pancreatic β cell membrane (Szkudelski, 2001). *In vitro* studies have also demonstrated that GLUT2 itself is a key target molecule for STZ as the drug reduces GLUT2 protein expression in a concentration-dependent manner (Gai et al., 2004). GLUT2 may also be responsible for the STZ induced effects in the brain as GLUT2 also is reported to

have regional specific distribution in the mammalian brain (Brant et al., 1993; Leloup et al., 1994; Ngarmukos et al., 2001; Arluison et al., 2004a, b). The neuronal localization of GLUT2 is relatively similar to that of glucokinase (GLUT2 coupled with glucokinase participate in the glucose sensing mechanism of β-cells), supporting the hypothesis that GLUT2 is expressed by brain neurons involved in glucose sensing (Arluison et al., 2004a, b). However, since GLUT2 localization in the brain does not entirely parallel that of glucokinase at the quantitative level (Li et al., 2003), participation of GLUT2 in functions other than glucose sensing in the brain has been suggested (Arluison et al., 2004a, b). Following peripheral administration STZ causes alkylation of β-cell DNA which triggers activation of poly ADP-ribosylation, leading to depletion of cellular NAD+ and ATP (Szkudelski, 2001). Decreased levels of ATP have also been reported following STZ-icv treatment (Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998). The chemical structure of STZ also suggests this compound may produce intracellular free radicals, nitric oxide (NO) and hydrogen peroxide (Szkudelski, 2001); indeed evidence of increased oxidative stress has been found in the brain of STZ-icv treated rats (Table 1). Possible STZ effects on insulin producing/secreting and insulin sensitive cells within the brain will be discussed later in the text. The exact intracellular effects stimulated by icv administration of STZ are likely to be elucidated only following identification of the brain cells targeted by this compound.

Neurochemical, structural and behavioral changes in the STZ-ICV rat model

Glucose/energy metabolism

Glucose is the principal source of energy production in the brain, and undisturbed glucose metabolism is critical for normal functioning of this organ. Brain glucose, and its metabolism, has been investigated from 3 weeks following STZ-icv administration (Table 1), where concentrations of glucose and ADP, as well as glycogen levels, were increased in the cerebral cortex (Nitsch and Hoyer, 1991), and glucose utilization was significantly decreased (44%) (Pathan et al., 2006). Further, 6 weeks following STZ-icv treatment, reduced glucose utilization (up to 30%) was found in 17 of 35 brain areas, particularly the frontal, parietal, sensory motor, auditory and entorhinal cortex and in all hippocampal subfields (Duelli et al., 1994). In addition, significant decreases in activities of glycolytic key enzymes were found in the brain cortex and hippocampus 3 and 6 weeks post- STZ-icv administration (Plaschke and Hoyer, 1993) resulting in diminished concentrations of the energy-rich compounds ATP and creatine phosphate (Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998). This fall in cerebral ATP, GTP and creatine phosphate levels was significantly improved by 40-day subcutaneous treatment with estradiol or intraperitoneal injection with the antioxidant coenzyme Q10, in parallel with the initial administration of STZ (Lannert et al., 1998; Ishrat et al., 2006). Decreased glucose utilization in hippocampal and cortical tissue was significantly, and dose-dependently, increased by 2-week long oral treatment with the peroxisome proliferator activated γ receptor (PPARγ) agonist, pioglitazone, applied from 5 days before to 9 days after the STZ-icv treatment (Pathan et al., 2006). PPARγ agonists are approved as oral hypoglycemic agents used in the treatment of insulin resistance in type 2 diabetes, but have also demonstrated some neuroprotective effects (Santos et al., 2005; Bordet et al., 2006).

Cholinergic transmission

Investigations of cholinergic transmission in STZ-icv treated rats are important as abnormalities in the central cholinergic system affect learning and memory (Spencer and Lal, 1983). A decrease in choline acetyltransferase (ChAT) activity has been consistently found in the hippocampus of STZ-icv treated rats as early as 1 week following drug treatment and is still present 3 weeks post-injection (Hellweg et al., 1992; Blokland and Jolles, 1993, 1994; Prickaerts et al., 1999; Terwel et al., 1995) (Table 1). This is followed by a significant increase in acetylcholinesterase (AChE) activity (Sokusare et al., 2005; Ishrat et al., 2006). A decrease in hippocampal ChAT activity was completely prevented by 2-weeks of orally administered acetyl-L-carnitine, which acts by enhancing the utilization of alternative energy sources (Prickaerts et al., 1995; Terwel et al., 1995). Chronic administration of cholinesterase inhibitor drugs reduced AChE activity in a dose dependent manner, in STZ-icv treated rats regardless of whether treatment began 1 week prior to, in parallel or 13 days after STZ-icv administration (Sonkusare et al., 2005; Shoham et al., 2006). AChE activity was inhibited in the cortex but not in the hippocampus (Shoham et al., 2006), and concomitant administration with the calcium channel blocker, lercanidipine, potientated a decrease in AChE activity (Sonkusare et al., 2005). Changes in both ChAT and AcheE in the hippocampus were prevented by chronic intraperitoneal treatment with the antioxidant coenzyme Q10 (Ishrat et al., 2006). Intra-cortical administration of STZ to rat pups was followed by reduced expression of ChAT and increased expression of the AChE gene, a response which could not

be prevented by any of the three subtypes of PPAR agonists (Lester-Coll et al., 2006; de la Monte et al., 2006).

Oxidative stress

Evidence of increased oxidative stress has been found in whole brain homogenates in particular brain regions of STZ-icv treated rats (Table 1). Estimations of oxidative stress induced by STZ-icv treatment commonly ultilise the measurement of malonaldehyde levels (MDA), a product of lipid peroxidation used as an indicator of free radical generation, and glutathione levels, an endogenous antioxidant that scavenges free radicals and protects against oxidative stress, or immunohistochemically for nitrative stress. Significant elevations of MDA levels and decreased glutathione levels have been found in the brain of STZ-icv treated rats (Sharma and Gupta, 2001a, 2002; Ishrat et al., 2006; Pathan et al., 2006). A progressive trend towards oxidative stress has been found 1, 7 and 21 days following STZ-icv administration (twice 3 mg/kg injections) to 4 month old rats (Sharma and Gupta, 2001a). Oxidative stress was found also in the brain of 1-year-old rats, 3 weeks following a lower single STZ-icv dose of 1.5 mg/kg (Ishrat et al., 2006). STZ-icv generates NO (Szkudelski, 2001), and oxidative-nitrative stress was found 1 and 8 weeks following a single 3 mg/kg STZ-icv dose (Shoham et al., 2006). Nevertheless it appears that generation of NO by NO synthase is not involved since inhibition of this enzyme has not prevented STZ-induced responses (Prickaerts et al., 2000). Chronic treatment with the antioxidant coenzyme Q10 starting from the day of STZ-icv treatment significantly reduced all parameters of oxidative stress (Ishrat et al., 2006). A similar effect has been reported for other antioxidants (Sharma and Gupta, 2001a, 2002).

Morphology

STZ-icv administration has been associated with certain brain morphological changes in the brain as early as 1 week following a single drug dose (Shoham et al., 2006), and in both $\geq$1 year and 4 month old rats (Terwel et al., 1995; Prickaerts et al., 2000; Shoham et al., 2003, 2006) (Table 1). Glial fibrillary acidic protein (GFAP), a marker of astrogliosis, a stereotypic reaction of astrocytes to neuronal damage (Prickaerts et al., 1999), has been found to be increased in both brain homogenates and tissue sections (Prickaerts et al., 1999, 2000). Increased GFAP immunocytochemical staining was mainly located in peri- and paraventricular regions including the septum, fornix and fimbria, striatum and hippocampus, suggesting that altered hippocampal function could result from an impaired innervation and direct damage to this region (Prickaerts et al., 2000; Shoham et al., 2003). Inflammatory processes and myelin and axonal neurotoxicity has been reported following STZ-icv treatment. Severely affected STZ-icv treated rats had not only astrogliosis, but also extensive cell loss, inferred from the increase in the volume of the ventricular system (Prickaerts et al., 2000; Shoham et al., 2003). Interestingly, one week after a single STZ-icv 3 mg/kg dose, no change in the number or morphology of cholinergic neurons was detected in the basal forebrain nuclei, medial septum, diagonal band or the nucleus basalis magnocellularis and there was no change in the density of cholinergic terminals in the hippocampus (Shoham et al., 2006). Not withstanding, AChE activity was increased, a phenomenon the authors explained as a reduction in synaptic function associated with increased GFAP expression in activated astrocytes. Astrogliosis was prevented by a chronic treatment with ladostigil, a cholinesterase and monoamine oxidase-B inhibitor with neuroprotective effects (Shoham et al., 2006). At the ultrastructural level, 3 weeks following STZ-icv administration a significant enlargement of the trans-Golgi segment in the rat cerebral cortex was found, which did not resemble the Golgi atrophy found in the brain of sAD patients, but the authors suggested that considering the proamyloidogenic processing of beta-amyloid precursor protein may occur preferentially in the trans-Golgi segment, the observed early response of neuronal ultrastructure to desensitisation of the IR may predispose cells to form Aβ-amyloid deposits (Grieb et al., 2004). Differences in morphological changes in the brain in human sAD and the STZ-icv rat model could be related to direct STZ actions.

Reduced expression of neuronal- and oligodendroglia-specific genes and increased expression of genes encoding GFAP and microglia-specific proteins were also found in rat pups administered with STZ intra-cortically (STZ-ic) (Lester-Coll et al., 2006). Some authors have suggested the hypoplasia and degeneration of the cerebellum found in STZ-ic treated rat pups, unlike findings in AD, are related to the early postnatal development of the cerebellum in rodents. PPAR agonists produced receptor subtype- and region-dependent positive therapeutic effects in STZ-ic treated pups with PPAR-δ agonists being the most effective (PPAR $\delta > \alpha > \gamma$ subtype effectiveness) in preservation of hippocampal and temporal lobes (de la Monte et al., 2006).

Learning and memory

STZ-icv treated rats consistently demonstrate deficits in learning, memory, and cognitive behaviour (Table 1).

Cognitive deficits are long-term and progressive, observed as early as 2 weeks after STZ-icv administration and are maintained up to 12 weeks post treatment (Lannert and Hoyer, 1998; Grünblatt et al., 2006; Salkovic-Petrisic et al., 2006; Shoham et al., 2006). They are found regardless of age in both 1–2 year and 3-month old rats, and also after either a single 1 or 3 mg/kg injection or multiple 1 mg/kg STZ-icv injections (Mayer et al., 1990; Lannert and Hoyer, 1998; Weinstock and Shoham, 2004; Grünblatt et al., 2006; Pathan et al., 2006; Salkovic-Petrisic et al., 2006; Shoham et al., 2006), although some STZ-icv dose-dependency has been suggested with lower STZ doses inducing less severe cognitive deficits (Blokland and Joles, 1994; Prickaerts et al., 2000; Grünblatt et al., 2006). The correlation between spatial discrimination performance in the Morris task and the decrease in hippocampal ChAT activity which resembles the relationship between cognitive and biochemical cholinergic changes observed in AD has been found in STZ-icv treated rats (Blokland and Jolles, 1993, 1994). However, results of the effectiveness of chronic acetyl-L-carnitine treatment in the prevention of hippocampal ChAT activity and abolishing memory deficits in the Morris water maze swimming (MWM) test, are inconsistent (Terwel et al., 1995) (Prickaerts et al., 1995). Interestingly, it has also been demonstrated that STZ-icv induced development of reactive gliosis and oxidative stress 1 week post-treatment, preceded the induction of memory deficits at 3 weeks post-treatment (Shoham et al., 2006; Sharma and Gupta, 2001a), where no signs of neuronal damage or any reduction in specific cholinergic markers were detected in the cortex or hippocampus (Shoham et al., 2006).

Although the exact mechanism by which STZ-icv treatment damages cognitive function remains unknown, all changes discussed above; energy deficits, reduced activity of choline acetyltransferase (cholinergic deafferentiation), induction of oxidative stress and direct neurotoxic damage found in the fornix, anterior hippocampus and periventricular structures, may form the biological basis for the marked reduction in learning and memory capacities. Concordingly, memory deficits were reported to be prevented by chronic treatment with several types of drugs with differing mechanisms of action (as reviewed by Weinstock and Shoham, 2004); (I) drugs generating alternative energy sources such as acetyl-L-carnitine (Prickaerts et al., 1995); (II) cholinesterase inhibitors such as donepezil and ladostigil (possessing also monoamine oxidase B inhibition and neuroprotective activity which also prevent gliosis and oxidative stress (Sonkusare et al., 2005; Shoham et al., 2006); (III) estradiol which prevents reduction in cerebral ATP (Lannert et al., 1998); (IV) antioxidants such as melatonin, resveratrol and coenzyme Q10 which prevent an increase in free radical generation (Sharma and Gupta, 2001b, 2002; Ishrat et al., 2006). Treatment with the NO synthase inhibitor L-NAME had no protective effect on cognitive deficits in STZ-icv treated rats (Prickaerts et al., 2000).

Brain insulin and the IR signaling cascade in STZ-ICV rat

Although alterations of the brain insulin system are the focus of human sAD research (Hoyer and Frölich, 2006), investigations in experimental models of this neurodegenerative disorder are rare, particularly those exploiting central STZ administration (Tables 2 and 3). We have previously reported changes in the brain insulin and tau/Aβ system following the bilateral application of a single or multiple 1 mg/kg STZ dose into the lateral cerebral ventricles of adult ≥3 month old rats (Salkovic-Petrisic et al., 2006; Grünblatt et al., 2004, 2006) and the group of de la Monte et al. (de la Monte et al., 2006; Lester-Coll et al., 2006) has reported changes in the brain insulin system following bilateral intra-cerebral 40 μg/kg STZ dose to three-day-old rat pups. Since only the abstract of a Chinese paper is available regarding brain immunohistochemical analysis of tau protein and Aβ expression, 3 weeks following STZ-icv treatment (Chu and Qian, 2005), there is little data for a reliable interpretation of their findings.

In STZ-icv treated adult rats region-specific alterations of the brain insulin system (including insulin, the IR and downstream IR signaling cascade) were identified, and these changes are progressive beyond the STZ-icv treatment period (Table 2) (Grünblatt et al., 2006; Salkovic-Petrisic et al., 2006). A decrease in the expression of the insulin gene 1 and 2, as well as the IR gene, was identified in the hippocampus and frontoparietal cortex 12 weeks following drug treatment. The IRβ protein was decreased in the frontoparietal cortex and hypothalamus, but the levels of phosphorylated IRβ (p-IRβ) were increased and tyrosine kinase activity was unchanged in these regions, whereas in the hippocampus IRβ protein levels were decreased, but p-IRβ levels, as well as tyrosine kinase activity, were increased. Downstream from the PI3-K signaling pathway, hippocampal Akt/PKB remained unchanged at 4 weeks and decreased by 12 weeks post-treatment, whereas in the frontoparietal cortex Akt/PKB expression was decreased 4 weeks and increased by 12 weeks post STZ-icv treatment. Total GSK-3 levels were unchanged whereas the p-GSK-3/GSK-3 ratio in the hippocampus was decreased 12 weeks following STZ-icv treatments, suggesting a change in GSK-3 activity. In line with this finding, as a downstream target

Table 2. *Comparison of brain insulin system alterations in sporadic Alzheimer's disease and in an experimental model of this disease, streptozotocin-intracerebroventricularly treated rats*

Brain insulin system	Human	STZ-icv rat		
		Time after treatment		Therapy improvement
		≤1 month	3 months	
Insulin				
mRNA	HPC&HPT&F-CTX&CB ↓ (1)		HPC↓ (Ins1) (4) F-CTX ↓ (Ins2) (4)	
		STZ-ic pups: TL&HPT&CB ↓ (2, 3)		STZ-ic pups: PPAR agonists: no (3)
protein	CTX↑/∼ (5)			
IGF-1				
mRNA	F-CTX&HPT ↓ (1)			
		STZ-ic pups: TL ↓; HPT&CB ∼ (3)		STZ-ic pups: PPAR agonists: yes (3)
IR				
mRNA	FHPC&HPT&F-CTX ↓ (1)		HPC↓ (4) F-CTX ↓ (4)	
		STZ-ic pups: TL&CB ↓; HPT ∼ (2, 3)		STZ-ic pups: PPAR agonists: yes (partly) (3)
Protein (IRβ)	HPC ↓ (1)		F-CTX ↓ (4) HPC ↑ (4) HPT ↓ (4)	
p-IRβ	HPC ↓ (1)		F-CTX ↑ (4) HPT ↑ (4)	
TK activity	↓ (5)		HPC ↑ (4)	
density (Bmax)	↑ (5)	STZ-ic pups: TL&HPT&CB ↓ (2, 3)		STZ-ic pups: PPAR agonists: yes (partly) (3)
IGF-1R				
mRNA	HPC&HPT&F-CTX ↓ (1)	CTX, S ↓ (6) STZ-icv pups: HPT&CB ↓; TL ∼ (2, 3)		STZ-ic pups: PPAR agonists: yes (partly) (3)
protein	HPC ↓ (1)			
density (Bmax)		STZ-ic pups: TL ↑ (3)		STZ-ic pups: PPAR agonists: no (3)
IRS				
mRNA	F-CTX&HPC&HPT ↓ (IRS1) (1) HPC ↓ (IRS2) (1)	STZ-ic pups: ↓ (2)		
p-IRS	HPC&HPT ↓ (IRS1) (1)			
Akt/PKB				
mRNA				
protein	HPC&HPT ∼ (1) CTX ↓ (cytosol) (7)	F-CTX ↓ (8)	HPC ↓ (8) F-CTX ↑ (8)	
p-protein	HPC&HPT ↓ (1) F-CTX ↑ (9)			
pAkt/Akt ratio				
activity	T-CTX ↑ (soluble fraction) (10)			
GSK-3				
mRNA				
protein		HPC&CTX ∼ (8) STZ-ic pups: TL&HPT ∼ (3)	HPC&F-CTX ∼ (8)	
p-protein		HPC ↑ (8) STZ-ic pups: TL&HPT ∼ (3)		
p-GSK-3/GSK-3 ratio		HPC ↑ (8) F-CTX ↓ (8) STZ-ic pups: TL&HPT ↓ (3)	HPC ↓ (8) F-CTX ↓ (8)	STZ-ic pups: PPAR agonists: no (3)

(*continued*)

Table 2 (*continued*)

Brain insulin system	Human	STZ-icv rat		
		Time after treatment		Therapy improvement
		≤1 month	3 months	
GSK-3α				
p-GSK-3α	F-CTX ↑ (11)			
GSK-3β	HPC&HPT ∼ (1)	STZ-ic pups: ↑ (2)		
p-GSK-3β	HPC&HPT ↓ (1) F-CTX ↑ (11)			
IDE				
mRNA	HPC&HPT ∼ (1)			
protein	HPC ↓ (12)			
activity (Aβ degrading)		↓ (13)		

STZ Streptozotocin; *icv* intracerebroventricular; *ic* intracerebral; *IGF-1* insulin-like growth factor 1; *IR* insulin receptor; *IGF-1R* insulin-like growth factor 1 receptor; *IRS* insulin receptor substrate; *Akt/PKB* protein kinase B; *GSK-3* glycogen synthase kinase 3; *IDE* insulin degrading enzyme; *p* phospho; *Aβ* amyloid beta; *HPC* hippocampus; *HPT* hypothalamus; *TL* temporal lobe; *CB* cerebellum; *F-CTX* frontoparietal cerebral cortex; *PPAR* peroxisome-proliferator activated receptor; ↓ decrease; ↑ increase. Number of reference in brackets: 1) Steen et al. (2005); 2) Lester-Coll et al. (2006); 3) de la Monte et al. (2006); 4) Grünblatt et al. (2006); 5) Frölich et al. (1998); 6) Grünblatt et al. (2004); 7) Griffin et al. (2005); 8) Salkovic-Petrisic et al. (2006); 9) Pei et al. (2003); 10) Rickle er al. (2004); 11) Pei et al. (1999); 12) Cook et al. (2003); 13) Perez et al. (2000).

Table 3. *Comparison of brain tau protein and amyloid beta alterations in sporadic Alzheimer's disease and in an experimental model of this disease, streptozotocin-intracerebroventricularly treated rats*

	Human	STZ-icv rat		
		Time after treatment		Therapy improvement
		≤1 month	3 months	
Tau protein				
mRNA	HPC&HPT ↓ (1)	STZ-ic pups: TL&HPT&CB ↓ (2, 3)		STZ-ic pups: PPAR agonists: yes (partly) (3)
protein	F-CTX ↑ (6)	HPC ↑ (4, 5) ↑ (7) STZ-ic pups: TL&HPT ∼ (3)	HPC ↑ 4, 5)	
p-protein	F-CTX ↑ (6)	HPC ∼ (4) STZ-ic pups: TL&HPT ↑ (2, 3)	HPC ↑ (4)	STZ-ic pups: PPAR agonists: yes (3)
p-tau/tau ratio		STZ-ic pups: TL&HPT ↑ (3)		STZ-ic pups: PPAR agonists: yes (3)
APP				
mRNA	HPC&HPT ↑ (1)	STZ-ic pups: TL&HPT ↑ (2, 3)		STZ-ic pups: PPAR agonists: yes (partly) (3)
protein				
Aβ				
Aβ 40		STZ-ic pups: (2)		
Aβ 42		↑ (7) STZ-ic pups: (2)		
aggregates		absent (5)	in meningeal capillaries (5)	

STZ Streptozotocin; *icv* intracerebroventricular; *ic* intracerebral; *p* phospho; *APP* amyloid precursor protein; *Aβ* amyloid beta; *HPC* hippocampus; *HPT* hypothalamus; *TL* temporal lobe; *CB* cerebellum; *F-CTX* frontoperietal cerebral cortex; *PPAR* peroxisome-proliferator activated receptor; ↑ increase; ↓ decrease. Number of reference in brackets: 1) Steen et al. (2005); 2) Lester-Coll et al. (2006); 3) de la Monte et al. (2006); 4) Grünblatt et al. (2006); 5) Salkovic-Petrisic et al. (2006); 6) Pei et al. (1999); 7) Chu and Qian (2005).

of the IR signaling cascade, increase in the expression of tau protein and the p-tau/tau ratio were found in the hippocampus 4 and 12 weeks following STZ-icv treatment, respectively, and Aβ-like aggregates were absent 4 weeks following drug treatment, but were found in leptomeningeal capillaries 12 weeks post STZ-icv treatment (Table 3).

Although the investigated parameters in the brain and the direction of changes identified following the intra-cortical (ic) application of STZ (decreased mRNA expression of insulin, the IR and IGF-1R, decreased pGSK-3/GSK-3 ratio) are similar to those induced by STZ-icv administration (Tables 2 and 3) (de la Monte et al., 2006; Lester-Coll et al., 2006), the results are not quite comparable for several reasons; (I) Intra-cortical administration of STZ in the rat pups could be expected to induce more non-specific localized tissue damage than administration of STZ into the cavity of the lateral cerebral ventricles of adult rats. (II) Sensitivity of brain neurons to STZ toxic effects could be expected to differ in the three-day-old pups and adult or old rats, at least due to differences in the DNA excision repair processes known to be important for STZ intracellular toxicity (Szkudelski, 2001). (III) Strain differences in the susceptibility to STZ-induced diabetes have been reported (Rodrigues et al., 1997), and two different rat strains were used in icv treatment of adult/old rats (Wistar strain, most frequently used in other STZ-icv treatment experiments) and ic treatment of rat pups (the Long Evans strain is generally used for the investigation of retinal complications of diabetes (Puro, 2002). (IV) Brain regions investigated were not completely comparable; frontoparietal cortex, hippocampus and hypothalamus in STZ-icv, and temporal lobe, cerebellum and hypothalamus in STZ-ic treatment. The region-specific STZ-induced changes in the brain, including the difference in changes observed in the hippocampus in comparison with the cerebral cortex (Salkovic-Petrisic et al., 2006), could be masked in STZ-ic treated pups where temporal lobe (including hippocampal formation) was biochemically analysed. (V) Time dependency of changes could not be concluded from experiments with STZ-ic treated rat pups as these were measured at only a single post-treatment period of 4 weeks (de la Monte et al., 2006) or three post-treatment periods were investigated (7, 14, and 21 days) but results were reported only for the 14-day period (Lester-Coll et al., 2006). Some changes were not observed at 4 weeks but appeared 12 weeks following STZ-icv treatment, like those of tau protein and Aβ (Salkovic-Petrisic et al., 2006), but were already present two or four weeks after STZ-ic treatment (Lester-Coll et al., 2006; de la Monte et al., 2006) (Table 3). Some additional parameters of tau protein and the Aβ system (tau and APP mRNA expression) were investigated in STZ-ic treated pups with a discrepancy between significantly decreased tau mRNA and unchanged tau protein expression observed in the hypothalamus (de la Monte et al., 2006).

Modest data has been reported regarding the therapeutic effects of drugs on brain insulin system alterations induced by central STZ application, i.e. only the effects of PPAR agonists in STZ-ic treated rat pups were investigated (de la Monte et al., 2006). These drugs demonstrated some therapeutic effects with respect to the insulin/IR signaling cascade dysfunction, but it is import to note that the effects were region- and PPAR subtype-specific. Not all PPAR-subtype agonists showed positive effects on STZ-induced damage and some of the alterations (insulin mRNA and p-GSK-3/GSK-3 ratio) did not respond to any of the PPAR-subtype agonists used (Tables 2 and 3). Although the PPAR-δ subtype agonists were the most effective (this subtype was found to be most abundant both in rat and human brain) (de la Monte et al., 2006), they were incapable of normalizing the changes to APP mRNA in the temporal lobe in contrast to the PPAR-α and -γ subtype agonists. Also, it has to be kept in mind that the positive therapeutic effects of PPAR agonists in STZ-ic treated pups on IR dysfunction (as well as on cholinergic transmission and neuronal damage), were obtained 4 weeks after a single intraperitoneal injection given on the same day as STZ. Evidence for the entry of these PPAR agonist substances through the blood–brain barrier, which is of vital importance for their effects within the brain after only a single intraperitoneal injection, has not been provided by authors. Further experimentation is needed to clarify the meaning of these findings in respect to the mechanism of PPAR agonists since the positive therapeutic effects of treatment with the chronic PPAR-γ agonist rosiglitazone (which probably does not pass the blood–brain barrier in a significant amount) (Pedersen and Flynn, 2004) on cognitive deficits in both humans with sAD and transgenic Tg2576 Alzheimer mice, are suggested to be related to an improvement of peripheral insulin resistance which positively affects cognition (Watson et al., 2005; Pedersen et al., 2006; Landreth, 2006). Such a mechanism does not seem likely in 3-day-old rat pups which do not develop peripheral insulin resistance since decreased blood glucose levels were found 14 days following the STZ-ic administration (Lester-Coll et al., 2006).

Insulin resistant brain state as a probable trigger for sAD pathology

Convincing evidence indicates that central STZ administration induces alterations resembling those found in sAD

patients (Wada et al., 2005; Qiu and Folstein, 2006; Hoyer and Frölich, 2006; Watson and Craft, 2006; Cole and Frautschy, 2007). Similarities between human sAD and the STZ-icv model have been noted at three different levels; (I) biochemically, in the region-specific decrease in glucose utilization, reduction in cholinergic transmission and activation of the markers of oxidative stress damage, (II) morphologically, in neuronal damage and loss in hippocampal volume and associated structures accompanied by astroglyosis and neuronal inflammation, and finally, (III) behaviourally, manifested as progressive learning and memory deficits. However, regardless of these similarities, none of these biochemical alterations were able to provide a missing link that could connect all these changes to give a clue to the primary event which could initiate the pathological hallmarks of sAD, hyperphosphorylated tau protein in neurofibrillary tangles and aggregated Aβ peptide in amyloid plaques. Region-specific and, for some parameters, time-progressive, changes of the rat brain insulin system following STZ-icv treatment seem to be the missing link. Disturbances in insulin action, IR function and downstream signaling pathways have been found post mortem in the brain of sAD patients (Wada et al., 2005; Qiu and Folstein, 2006; Hoyer and Frölich, 2006; Watson and Craft, 2006; Cole and Frautschy, 2007), suggesting a condition of brain insulin resistance, very similar to that found in STZ-icv and STZ-ic treated rats (de la Monte et al., 2006; Grünblatt et al., 2006; Salkovic-Petrisic et al., 2006).

The STZ-icv treated rat model has provided additional evidence that a progression of the brain insulin resistant state over time leads to Alzheimer-like tau protein and Aβ pathology. Three major questions arise from this hypothesis; (1) what could trigger the brain insulin resistant state in sAD and at which point in the insulin/IR/downstream signaling cascade; (2) how is the brain insulin resistant state connected to pathological changes in the brain; and (3) what are the implications of this hypothesis in relation to the drug treatment of sAD?

One of the main risks for sAD is aging, associated with increased cortisol action due to a shift in the hypothalamic pituitary-adrenal (HPA) axis to an increased basal tone (Cizza et al., 1994, for review Hoyer, 2004), frequently reported in AD patients (Raber, 1998). Cortisol may be a candidate for compromising the function of the neuronal IR via its dysregulation of the phosphorylation site of tyrosine residues in the receptor, and noradrenaline (found increased in cerebro-spinal liquid in sAD patients) may desensitise the neuronal IR by phosphorylation of serine/threonine residues (Fig. 1B) (Häring et al., 1986; Giorgino et al., 1993, reviewed by Hoyer, 2004). Desensitisation of the IR in sAD is suggested by findings of up-regulated IR density associated with reduced activity of IR tyrosine kinase (Frölich et al., 1998). This point seems to be the only major difference between sAD and the STZ-icv rat model regarding the brain insulin system. Human data contradicts the findings of decreased or unchanged expression of the IRβ subunit, and the increased or unchanged expression of the tyrosine-phosphorylated IRβ subunit and tyrosine kinase activity seen in the STZ-icv model (Grünblatt et al., 2006). However, this inconsistency could be related to the possible peripheral (e.g. glucocorticoid-induced) origin of the neuronal IR alterations in sAD and its lack in STZ-icv treated rats in which direct STZ-induced damage could be involved instead. On the other hand, data from STZ-icv model studies may point to an imbalance between the IR tyrosine phosphorylation and dephosphorylation under pathological conditions. This is in agreement with a generally known phenomenon of insulin receptor signaling dysfunction (i.e. an insulin resistant state) which may be caused when tyrosine phosphorylation, and/or when tyrosine dephosphorylation fails (Goldstein, 1993), and/or when serine/threonine phosphorylation is increased and maintained at a high level (Häring, 1991; Avruch, 1998) as induced by the cytokine, tumor necrosis factor-α (Hotamisligil et al., 1994). There is some evidence that failing receptor dephosphorylation may inhibit autophosphorylation activity (Lai et al., 1989) and that with time, tyrosine-phosphorylated IRs become inaccessible to phosphatases, therefore allowing persistence of tyrosine kinase activity (Paolini et al., 1996). The activity of the protein tyrosine phosphatase (PTP) subtype 1B known to negatively regulate the function of the IR has not been particularly investigated in AD, but the likelihood of a decrease in function cannot be ruled out. Namely, IR autophosphorylation/dephosphorylation has been reported to be mediated by reactive oxygen species; i.e. exposure to hydrogen peroxide could lead to oxidative activation of IR tyrosine kinase activity and oxidative inactivation of PTP (Droge, 2005). STZ generates reactive oxygen species (Szkudelski, 2001), and similarly aging and AD are associated with oxidative stress in the brain (Barja, 2004). However, increased IR tyrosine activity does not necessarily lead to increased IR signal transduction throughout the cell, if intracellular downstream pathway signaling elements are affected, which is the case both in the animal model (Fig. 1B) (Lester-Coll et al., 2006; Salkovic-Petrisic et al., 2006) and in humans (Hoyer and Frölich, 2006).

Interestingly, the activity of the PTP enzyme was found to be regulated by insulin (Kenner et al., 1993), and the activity of another protein phosphatase subtype, PP 2A, has been found largely decreased in the mouse brain 3 days fol-

lowing peripheral STZ administration (Clodfelder-Miller et al., 2006), but also in the human AD brain (Gong et al., 1995). PP 2A and 2B are involved in phoshorylation/dephosphorylation regulation of tau protein (Gong et al., 1994a, b). Thus, insulin resistant brain state, via IR signaling pathway dysfunction, may with time lead to the tau hyperphosphorylation directly through the PI3-GSK-3β pathway, or through the PP 2A pathway. Importantly, the PI3-GSK-3α pathway may also lead to Aβ pathology, as demonstrated in STZ-icv adult rats (Salkovic-Petrisic et al., 2006) and STZ-ic rat pups (Lester-Coll et al., 2006) (Fig. 1B), due to insulin-dependent production of APP derivatives, β-amyloid peptides (Phiel et al., 2003). The time-dependent development of Aβ pathology has been clearly shown in the STZ-icv rat model with no pathological signs visible 4 weeks following STZ-icv treatment, and Aβ-like intracellular aggregates are visible 12 weeks afterwards (Salkovic-Petrisic et al., 2006). This is in agreement with what has been recently proposed, namely that intracellular, rather than extracellular accumulation of β-amyloid is an initiating factor in the pathogenesis of AD (Oddo et al., 2003). Low brain insulin levels reduce the release of Aβ from intracellular compartments into extracellular compartments where clearance is believed to occur. On the other hand, once generated, both derivatives of APP, β-amlyoid (1-40) and β-amlyoid (1-42) decrease the affinity of insulin for its receptor, resulting in reduced receptor autophosphorylation (Xie et al., 2002), closing the circle back to IR signaling dysfunction. Although insulin protein concentration in the brain has not been measured directly in STZ-icv (ic) rats, it could be assumed that brain-derived insulin formation is reduced according to the downregulation of insulin mRNA expression both in animals (Grünblatt et al., 2006; Lester-Coll et al., 2006) and humans (Steen et al., 2005). However, this may be compensated by peripherally formed insulin, due to undisturbed pancreas function after central STZ administration (Lester-Coll et al., 2006), as the main source of brain insulin is the pancreas, assuming that the pathobiochemistry induced after STZ-icv injection, is mainly due to dysfunction of the IR and downstream signalling. Data from sAD post mortem studies have demonstrated stronger insulin-immunoreactivity in neocortical pyramidal neurons but unchanged insulin and c-peptide biochemical levels compared to the age-matched controls (Frölich et al., 1998).

Convincing evidence indicates that central STZ administration induces brain pathology and behavioural alterations resembling those found in sAD patients. Additionally, alterations of the brain insulin system found in this experimental model support the hypothesis that central insulin resistance might be the primary event which precedes Aβ pathology in sAD. Further studies are necessary to clarify this issue and its implications in relation to the drug treatment of sAD.

Acknowledgement

Supported by the Ministry of Science, Education and Sports, Republic of Croatia (No. 108-1080003-0020) and Deutscher Akademischer Austausch Dienst (No. A/04/20017).

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J Neural Transm (2007) [Suppl 72]: 235–240

Printed in Austria

Journal of Neural Transmission

Brain antioxidant capacity in rat models of betacytotoxic-induced experimental sporadic Alzheimer's disease and diabetes mellitus

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Summary It is believed that oxidative stress plays a central role in the pathogenesis of metabolic diseases like diabetes mellitus (DM) and its complications (like peripheral neuropathy) as well as in neurodegenerative disorders like sporadic Alzheimer's disease (sAD). Representative experimental models of these diseases are streptozotocin (STZ)-induced diabetic rats and STZ-intracerebroventricularly (STZ-icv) treated rats, in which antioxidant capacity against peroxyl ($ORAC_{-ROO^{\bullet}}$) and hydroxyl ($ORAC_{-OH^{\bullet}}$) free radical was measured in three different brain regions (hippocampus, cerebellum, and brain stem) by means of oxygen radical absorbance capacity (ORAC) assay. In the brain of both STZ-induced diabetic and STZ-icv treated rats decreased antioxidant capacity has been found demonstrating regionally specific distribution. In the diabetic rats these abnormalities were not associated with the development of peripheral diabetic neuropathy. Also, these abnormalities were not prevented by the icv pretreatment of glucose transport inhibitor 5-thio-D-glucose in the STZ-icv treated rats, suggesting different mechanism for STZ-induced central effects from those at the periphery. Similarities in the oxidative stress alterations in the brain of STZ-icv rats and humans with sAD could be useful in the search for new drugs in the treatment of sAD that have antioxidant activity.

Keywords: Rat, streptozotocin, diabetes mellitus, antioxidant capacity, oxidative stress

Introduction

The free radical (FR) hypothesis of metabolic diseases and neurodegeneration suggests that pathological disturbances occur as a result of an increasing inability to cope with oxidative stress (OS). Oxidative stressors induce an imbalance between oxidants and antioxidants in favor of the former leading to the oxidative damage of the molecules like DNA, lipids and proteins (Altan et al., 2006; Sofic et al., 1991, 2002). OS plays an important role in the pathogenesis of diabetes mellitus (DM) and its complications, microangiopathy and associated neuropathy in particular (Altan et al., 2006). Hyperglycemia induces both the disruption of mitochondrial membrane potential, and an increase in reactive oxygen species (ROS) which are capable of decreasing the antioxidant status in man (Sharma et al., 1999; Danova et al., 2005). In line with that, decreased antioxidant capacity (AC) has been found in the serum of patients with diabetic polyneuropathy in comparison to the control (non-diabetic) patients, as measured by oxygen radical absorbance capacity (ORAC) assay (Sofic et al., 2002; Prior et al., 2003). Additionally, it has been demonstrated that both in diabetic humans and experimentally diabetic rats, OS seems to play an important role in the brain damage (Biessels et al., 2002). Increased hydroxyl radical formation as well as increased levels of free fatty acids and malondialdehyde and decreased activities of antioxidative enzymes catalase and superoxide dismutase have been found in the brain of experimentally diabetic rats (Kumar and Menon, 1993; Ohkuwa et al., 1995). Investigation of the brain AC in relation to the peripheral diabetic neuropathy has not been reported.

Experimental DM in animals is usually produced by betacytotoxic drugs streptozotocin (STZ) and alloxan (AL) which given parentherally in high doses damage pancreatic beta cells and decrease insulin production/secretion

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(Szkudelski, 2001). Central, intracerebroventricular (icv) administration of low STZ and AL doses, respectively, does not produce diabetes mellitus in rats, but produces regionally specific brain neurochemical changes that are, in general, similar to those found in betacytotoxic-induced diabetes (Ding et al., 1992; Lacković and Šalković, 1990; Salkovic et al., 1995; Salkovic-Petrisic and Lackovic, 2003). Because of induction of the long-term and progressive cognitive deficits and decreased brain glucose and energy metabolism (Lannert and Hoyer, 1998; Prickaerts et al., 1999), STZ-icv treated rats have been proposed as an experimental model of sporadic Alzheimer's disease (AD) in which the existence of the brain type of non-insulin dependent diabetes (⟪cerebral diabetes⟫) has been suggested (Hoyer, 1998). In addition to that, Alzheimer's like alterations in the insulin receptor signaling cascade in the brain have been reported recently in the STZ-icv treated rats (Salkovic-Petrisic et al., 2006). This gives a further support to the hypothesis that this model is the representative experimental model of sporadic AD which, contrary to the transgenic Tg 2756 mice model of AD (Hsiao et al., 1996), is not based on the gene manipulations. STZ treatment has been reported to generate reactive oxygen species in addition to release of nitric oxide (Szkudelski, 2001). In line with that, significant elevation of malondialdehyde and decrement of glutathione levels have been found in the brain of STZ-icv treated rats, supporting the hypothesis of OS development (Sharma and Gupta, 2001, 2002). This is in agreement with the report of FR and OS involvement in the pathophysiology of AD (Mariani et al., 2005).

The present study was aimed to investigate the AC in the different rat brain regions following the central (icv) non-diabetogenic and peripheral diabetogenic treatment of betacytotoxic drugs in the conditions of present or absent peripheral diabetic neuropathy, as well as following the icv-treatment of glucose transport inhibitor alone or combined with STZ-icv treatment, by means of ORAC method involving two different FR generators (Cao et al., 1993, 1995, 1996, 1997; Prior et al., 2003; Sofic et al., 2002; Wang et al., 2004), modified by Sofic et al. (2006). The ORAC method has an advantage over other assays, because this method utilizes an area-under-curve technique and thus combines both inhibition time and inhibition degree of FR action by an antioxidant into a single quantity. Fluorescein (FL) was used as a target of FR attack, with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, and hydrogen peroxide and cupric sulphate penta hydrate as a hydroxyl radical generator. In original developed automated method β-phycoerythrin (β-PE), a protein from *Porphyridium cruentum*, was used as a target of FR attack (Cao et al., 1993, 1995, 1996, 1997; Prior et al., 2003; Sofic et al., 2002, Wang et al., 2004). An improved ORAC method has been developed and validated using FL as the photosensor. Ou et al. (2001) demonstrated that FL is superior to β-PE. The oxidized FL products were identified by LC/MS and the reaction mechanism was determined.

Materials and methods

Chemicals

Albumin (BSA), lyophilized powder was purchased from Sigma; Fluorescein, Standard Fluka for fluorescence – free acid was obtained from Fluka Chemie GmbH, Steinheim, Germany; 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), streptozotocin (STZ) and alloxan monohydrate (AL) were purchased from Sigma Aldrich Chemie GmbH Germany; 5-thio-D-glucose (TG) was purchased from BioChemika. Cupric sulphate penta hydrate and hydrogen peroxide were obtained from Kemika, Zagreb, Croatia.

Animals

Adult male, 2–4 months old Wistar rats (Department of Pharmacology, School of Medicine, University of Zagreb, Croatia) were used throughout the study. In all experiments including those with diabetes induction all animals were kept on standardized food pellets and water *ad libitum*.

Treatments

Intracerebroventricular (icv) drug administration

Rats were randomly divided in 4 groups (5–6 *per* group) and given general anaesthesia (chloralhydrate 300 mg/kg, ip), followed by injection of different drugs icv bilaterally into the lateral ventricle (2 μL/ventricle), according to the procedure described by Noble et al. (1967). The following drug treatments were applied in a single dose: (I) STZ (1 mg/kg, dissolved in 0.05 M citrate buffer pH 4.5); (II) 5-thio-D-glucose (TG) (375 μg/kg, dissolved in the same vehicle as STZ); (III) TG (375 μg/kg) + STZ (1 mg/kg); (IV) an equal volume of vehicle (controls). Animals were sacrificed three months after the drug icv treatment. Brains were quickly removed, hippocampus (HPC), cerebellum (CB) and brain stem (BS) cut out, immediately frozen and stored at −80°C. STZ-icv-treated animals had no symptoms of diabetes and steady-state blood glucose level did not differ in comparison with control animals.

Diabetes induction

Experimental diabetes was induced by a single intraperitoneal (ip) injection of streptozotocin (70 mg/kg, dissolved in 0.05 M citrate buffer pH 4.5) or a single subcutaneous (sc) injection of alloxan monohydrate (150 mg/kg, dissolved in saline) (Szkudelski, 2001). The corresponding control animals were treated with an equal volume of vehicle ip or sc, respectively. Animals which developed polydipsia, polyuria, and polyphagia, and had blood glucose levels >20 mmol/L after 1 week were used for the experiments. Streptozotocin- and alloxan-induced diabetic animals and corresponding controls were sacrificed 10 and 21 weeks following the diabetes induction, respectively.

Measurement of pain reactivity, i.e. peripheral diabetic neuropathy

In the streptozotocin-induced diabetic animals the existence of peripheral diabetic neuropathy was tested by the paw pressure test by (Randall and

Selitto, 1957; Bach-Rojecky and Lackovic, 2005) three weeks following the diabetes induction. Mechanical nociceptive thresholds were measured 3 times at 10-min intervals by applying increased pressure to the hind paw until the paw-withdrawal or overt struggling was elicited. Only those diabetic animals which demonstrated decreased withdrawal threshold values in comparison with the control non-diabetic animals were considered to have developed the peripheral diabetic neuropathy.

Sample preparation

The crude tissue extracts from the rat brain HPC, BS and CB were prepared by homogenizing the tissues in a 75 mM phosphate buffer pH = 7.3 (10 ml buffer per gram of tissue). The homogenates were spined down and separation of the soluble fractions was performed by two – step centrifugation (15 000 rpm 30 min, and 15 000 rpm 10 min at 4°C). The supernatant was ready for analysis after appropriate dilution with the buffer solution.

Oxygen radical absorbance capacity (ORAC) assay

ORAC analysis was performed on a Perkin Elmer spectrometer LS 55 with fluorescent filters (Ex: 485 nm; Em: 520 nm) (Sofic et al., 2002). Diluted supernatant (1:50) was used for the analysis. The final assay mixture (2 mL total volume) was prepared by adding of 50 μL 0.42 μM fluoresceine (10.5 nM final concentration, f.c.), 100 μL biological sample, and 1800 μL phosphate buffer pH 7.30. This mixture was thermostated at 37°C for 15 min after which the following was added: 50 μL 640 mM AAPH (16 mM f.c.) as a peroxyl radical generator ($ORAC_{-ROO^{\bullet}}$ assay) or 25 μL 7.2 mM Cu^{2+} ($CuSO_4 \times 5H_2O$-90 μM f.c.) and then 25 μL 9.06 M H_2O_2 (113.3 mM H_2O_2 f.c.) as mainly a hydroxyl radical generator ($ORAC_{-OH^{\bullet}}$ assay). The intensity of relative fluorescence was measured every 10 min up to 180 min. Standard solution was 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), a water-soluble vitamin E analogue (1 μM). Blank solution was only solution of fluoresceine in buffer and generator of FR.

The spectrofluorometer was programmed to record the fluorescence of fluoresceine every 10 min after AAPH and Cu^{2+}, H_2O_2 were added for as long as 180 min and the samples were thermostated at 37°C (KP 20-Lauda, Lauda Koenigshofen). All fluorescence measurements were expressed relative to the initial reading.

Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample and expressed as a μmol standard equivalents per g of fresh tissue or μmol per mg of protein:

$$\mathrm{ORAC}_{(TE,\ \mu\mathrm{mol/g})} = k \cdot \frac{(S_S - S_B)}{(S_{St} - S_B)}. \quad (1)$$

ORAC oxygen radical absorbance capacity expressed as trolox equivalents (TE, μmol/g)
k dilution factor
S_S integrated area under decay fluorescent curve for sample solution
S_B integrated area under decay fluorescent curve for blank solution
S_{St} integrated area under decay fluorescent curve for standard solution

Integrated area under decay fluorescent curve for sample, standard or blank is calculated by formula of trapeze area (Bronstein and Semendjajev, 1962) as follows:

$$S = \frac{1}{2}\left[(t_1 - t_0)\cdot(f_1 + f_0) + (t_2 - t_1)\cdot(f_2 + f_1) + \cdots + (t_{i+1} - t_i)\cdot(f_{i+1} + f_i)\right]. \quad (2)$$

S integrated area under decay fluorescent curve for sample, blank or standard
t incubation time
f relative fluorescence intensity

Protein assay

Total protein content was determined using the modified Biuret reaction. This method is based on measurements of the absorbance of Cu(II) – protein complex at 545 nm (Gernot, 1977).

Statistical analysis

Data was sent electronically from the Perkin-Elmer spectrofluorometer LS 55 to a PC system running fluoresceine WinLab software (Perkin-Elmer). Differences between the control and the treated groups were calculated by the Student's t-test.

Ethics

The animal treatments were carried out in Croatia, and were under the guidance of the Principles of Laboratory Animal care (NIH Publication No. 86-23, revised in 1985), according to the Croatian Act on Animal Welfare (NN19; 1999) and were approved by The Ethics Committee of the Zagreb University School of Medicine (No. 04-1343-2006).

Results

Measurements of the AC against hydroxyl- and peroxyl-radical in three different brain regions of the control rats treated with a vehicle only, demonstrated that both $ORAC_{-ROO^{\bullet}}$ and $ORAC_{-OH^{\bullet}}$ values were the highest in the cerebellum in comparison to the significantly lower levels found in the brain stem and the hippocampus (Table 1).

Decreased AC against hydroxyl- ($ORAC_{-OH^{\bullet}}$) and peroxyl- ($ORAC_{-ROO^{\bullet}}$) radical has been found in the brain of experimentally diabetic rats. Significantly decreased $ORAC_{-ROO^{\bullet}}$ values were found in the cerebellum of the streptozotocin-induced diabetic rats in comparison with the control group regardless whether the peripheral diabetic neuropathy had been manifested or not (Table 2). Interestingly, decreased $ORAC_{-ROO^{\bullet}}$ values in the cerebellum were found also in animals which were treated with the diabetogenic dose of streptozotocin but did not develop hyperglycaemia and diabetes (Table 2). A tendency of decreased AC against the hydroxyl-radical in the cerebellum was also observed in the streptozotocin-induced diabetic

Table 1. *Antioxidant capacity against hydroxyl- ($ORAC_{-OH^{\bullet}}$) and peroxyl-radical ($ORAC_{-ROO^{\bullet}}$) in different brain regions of the control rats*

Region	$ORAC_{-OH^{\bullet}}$ (μmol/g fresh tissue)	$ORAC_{-ROO^{\bullet}}$ (μmol/g fresh tissue)
CB (9)	1664.0 ± 343.5	4555.3 ± 249.2
BS (10)	455.6 ± 250.2*	3213.6 ± 293.9**
HPC (4)	660.5 ± 60.4*	1906.2 ± 212.3***

Data are expressed as mean ± SD; number of animals is given in parenthesis; *ORAC* oxygen radical absorbance capacity assay; *CB* cerebellum; *BS* brain stem; *HPC* hippocampus. *$p < 0.05$; **$p < 0.03$; ***$p = 0.04$ vs. cerebellum by Student's t-test.

Table 2. *Antioxidant capacity against hydroxyl- ($ORAC_{-OH}^{\bullet}$) and peroxyl-radical ($ORAC_{-ROO}^{\bullet}$) in different brain regions of the streptozotocin-induced diabetic (10 weeks) rats with or without manifested peripheral diabetic neuropathy*

Region	Treatment	$ORAC_{-OH}^{\bullet}$ (μmol/g fresh tissue)	$ORAC_{-ROO}^{\bullet}$ (μmol/g fresh tissue)
CB	C (4)	2474.6 ± 1747.6	6261.7 ± 2360.5
	STZ − D (4)	2740.3 ± 1705.0	3230.1 ± 1595.0*
	STZ + D (5)	1932.2 ± 592.9	3156.8 ± 383.7*
	STZ + Dn (5)	1675.0 ± 721.3	5605.9 ± 1840.4*
BS	C (4)	216.9 ± 162.7	3637.0 ± 826.6
	STZ − D (4)	494.4 ± 434.1	2866.0 ± 1156.8
	STZ + D (5)	360.4 ± 201.7	3402.3 ± 1136.8
	STZ + Dn (5)	341.6 ± 136.1	3376.6 ± 443.5

Data are expressed as mean ± SD; number of animals is given in parenthesis; *ORAC* oxygen radical absorbance capacity assay; *CB* cerebellum; *BS* brain stem; *HPC* hippocampus; *ND* no data; *C* control group; *STZ−D* group treated parentherally with a diabetogenic streptozotocin dose which did not become diabetic; *STZ + D* streptozotocin-induced diabetic group which did not develop peripheral neuropathy; *STZ + Dn* streptozotocin-induced diabetic group which developed peripheral neuropathy. $^{*}p < 0.05$ vs. control group by Student's *t*-test.

rats with or without manifested neuropathy but the difference was not statistically significant and no change in the AC against both radicals has been found in the brain stem as well (Table 2). In the alloxan-induced diabetic rats decrement in the brain AC was more pronounced against the hydroxyl- than the peroxyl-radical, the difference being statistically significant in the cerebellum, the brain stem and the hippocampus, respectively while significantly decreased AC against peroxyl-radical ($ORAC_{-ROO}^{\bullet}$ values) were found in the brain stem of the alloxan-induced diabetic rats only (Table 3).

In comparison to the controls, significantly decreased AC against the hydroxyl-radical ($ORAC_{-OH}^{\bullet}$ values) was found

Table 3. *Antioxidant capacity against hydroxyl- ($ORAC_{-OH}^{\bullet}$) and peroxyl-radical ($ORAC_{-ROO}^{\bullet}$) in different brain regions of the alloxan-induced diabetic (21 weeks) rats*

Region	Treatment	$ORAC_{-OH}^{\bullet}$ (μmol/g fresh tissue)	$ORAC_{-ROO}^{\bullet}$ (μmol/g fresh tissue)
CB	C (4)	510.3 ± 219.2	2061.3 ± 182.0
	AL + D (4)	432.4 ± 206.3***	2117.7 ± 265.3
BS	C (3)	584.4 ± 163.5	1737.4 ± 268.4
	AL + D (5)	435.3 ± 164.2**	1479.9 ± 129.9*
HPC	C (4)	660.5 ± 60.4	1906.2 ± 212.3
	AL + D (4)	595.3 ± 61.8#	2066.7 ± 347.0

Data are expressed as mean ± SD; number of animals is given in parenthesis; *ORAC* oxygen radical absorbance capacity assay; *CB* cerebellum; *BS* brain stem; *HPC* hippocampus; *C* control group; *AL + D* alloxan-induced diabetic group; $^{*}p < 0.05$; $^{**}p < 0.04$; $^{***}p < 0.03$; $^{\#}p = 0.02$ vs. control group by Student's *t*-test.

Table 4. *Antioxidant capacity against hydroxyl- ($ORAC_{-OH}^{\bullet}$) and peroxyl-radical ($ORAC_{-ROO}^{\bullet}$) in different regions of the rat brain three months following the intracerebroventricular treatment of streptozotocin, glucose transport inhibitor 5-thio-D-glucose and their combination, respectively*

Region	Treatment	$ORAC_{-OH}^{\bullet}$ (μmol/mg proteins)	$ORAC_{-ROO}^{\bullet}$ (μmol/mg proteins)
CB	C (5)	1015.5 ± 369.4	3651.4 ± 1467.4
	STZ (5)	634.2 ± 185.1*	2494.4 ± 477.2
	TG (6)	366.4 ± 171.4**	3108.2 ± 796.9
	TG + STZ (6)	420.8 ± 180.0**	3772.8 ± 871.1
BS	C (6)	614.7 ± 144.4	2931.3 ± 1062.6
	STZ (5)	420.4 ± 174.8*	2188.4 ± 486.2
	TG (6)	465.7 ± 126.6*	2216.1 ± 732.9
	TG + STZ (6)	280.7 ± 163.3**	2169.4 ± 384.7
HPC	C (7)	1265.7 ± 142.0	1473.4 ± 145.7
	STZ (7)	1111.7 ± 103.7	1383.4 ± 30.8
	TG (7)	868.9 ± 207.4**	1292.2 ± 84.7*
	TG + STZ (7)	910.9 ± 104.2**	1384.4 ± 107.8

Data are expressed as mean ± SD; number of animals is given in parenthesis; *ORAC* oxygen radical absorbance capacity assay; *HPC* hippocampus; *CB* cerebellum; *BS* brain stem; *C* control group; *STZ* streptozotocin-intracerebroventricularly (icv) treated group; *TG* 5-thio-D-glucose-icv treated group; *TG + STZ* group with combined TG and STZ icv treatment. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control group by Student's *t*-test.

in the cerebellum and the brain stem of the STZ-icv treated rats while AC against the peroxyl-radical ($ORAC_{-ROO}^{\bullet}$ values) remained unchanged (Table 4). In the TG-icv treated rats decrement of the AC was even more pronounced since significantly decreased $ORAC_{-ROO}^{\bullet}$ levels were found in the hippocampus and significantly decreased $ORAC_{-OH}^{\bullet}$ levels were fund in all three investigated brain regions in comparison to the control rats (Table 4). Interestingly, similar decrement of the brain AC was found following the combined TG + STZ-icv treatment, i.e. the values of $ORAC_{-OH}^{\bullet}$ were also significantly decreased in all three investigated brain regions in comparison to the control ones while those of $ORAC_{-ROO}^{\bullet}$ remained unchanged (Table 4).

Discussion

Diabetics and experimental animal models exhibit high oxidative stress (OS) due to persistent and chronic hyperglycemia (Biessels et al., 2002). A close relationship has been demonstrated between OS, diabetic macroangiopathy and microangiopathy including peripheral neuropathy (Kuyvenhoven and Meinders, 1999). Both micro- and macro-angiopathy and direct neuronal damage caused by chronically elevated intracellular glucose concentrations are implicated in the alterations of the central nervous system in DM. Enhanced intraneuronal glucose oxidation

leads to the ROS overproduction within the brain (Evans et al., 2002) which might overwhelm the antioxidant defense, as supported by the findings of decreased antioxidative enzymes activity in the brain of diabetic rats (Kumar and Menon, 1993; Ohkuwa et al., 1995), leading to cell damage. Our results of decreased AC, expressed as $ORAC_{-ROO^{\bullet}}$ and $ORAC_{-OH^{\bullet}}$ [trolox equivalents – TE, μmol/g fresh tissue or μmol/mg proteins], in the brain of rats made diabetic by two different betacytotoxic drugs are in agreement with that. The regional and FR specificity of the decreased AC in the brain of diabetic rats has been suggested in the present experiments. Both betacytotoxic drug (STZ or AL)- and diabetes duration (10 or 21 weeks)-dependency could account for these specificities. Decrement of AC against FR was more spread in alloxan-induced, longer lasting and metabolically considered more severe diabetes (Szkudelski, 2001), but on the other hand, AC decrement was more severe (50% of control vs. 74–90% of control) in the STZ- than in the AL-induced diabetes, respectively. Interestingly, significantly decreased AC against peroxyl radical was found in the cerebellum of all rats treated with a diabetogenic STZ dose regardless whether the diabetes has been developed or not. Contrary to that the AC against hydroxyl radical in cerebellum as well as in the brain stem did not change in similar conditions. It is important to mention that the AC in the control rat brain is region specific with the cerebellum demonstrated the highest AC values against both hydroxyl and peroxyl radical in comparison with the brain stem and hippocampus. Similar uneven distribution of AC was demonstrated in the rat brain cortex and cerebellum (Cao et al., 1996). Possible differences in the brain AC between the two models of diabetes are not likely to be related to the age of animals, as at the time of sacrification they were all approximately 6-months old and the AC in the brain has not been found significantly declined during aging in the adult and old rats (Cao et al., 1996). From the methodological point of view AC is dependent on the chemical composition and FR generator used in assay. In this study, AC of hydrophilic antioxidants was measured. Peripheral diabetic neuropathy, a part of long-term diabetic complications known as microangiopathy, has not been investigated in relation to the AC of the brain so far. It seems likely that the peripheral diabetic neuropathy does not influence the AC against peroxyl and hydroxyl radical within the investigated brain regions which may suggest that diabetic patients with such a neuropathy would have no additional risk of more extensive OS damage in the brain than those diabetic patients without peripheral neuropathy. However, because of the high individual variations expressed as a standard deviation a larger number of samples per a group and more extensive experiments would be needed before drawing a conclusion. Our results demonstrated that STZ-icv treatment although not inducing diabetes is capable of decreasing the AC against hydroxyl radical (for approximately 13–38%) in the investigated regions of rat brain three months following the drug administration while the antioxidative capacity against peroxyl radical remained unchanged. This observation is in line with the previous reports of increased OS in the brain of STZ-icv treated rats (Sharma and Gupta, 2001, 2002) and strongly supports the hypothesis of STZ-icv rats being a representative experimental model of sporadic AD (Hoyer, 1998) since the FR generation and OS involvement in the pathophysiology of AD have been recognized (Mariani et al., 2005). The mechanism of STZ action in the brain has not been explored so far. However, in line with the peripheral toxic effects of these drugs on the insulin producing/secreting cells (Szkudelski, 2001) and altered brain insulin receptor signaling in following the icv drug administration (Salkovic-Petrisic et al., 2006), similarity to the peripheral mechanism of action seems likely. STZ betacytotoxicity at the periphery is related to the STZ entering the beta cell through the glucose transporter GLUT2 (Szkudelski, 2001), the expression of which has been found also in the rat brain (McEwen and Reagan, 2004). STZ transport by GLUT2 and consequently diabetes induction is inhibited by glucose transport inhibitor TG (Szkudelski, 2001), but combined TG-icv + STZ-icv treatment has not prevented STZ-icv induced decreased in AC against the hydroxyl radical in none of the three investigated rat brain regions. Interestingly, TG-icv treatment alone also induced decrease in AC against hydroxyl radical. These data are in line with the similar cognitive deficits found in TG-icv and STZ-icv treated rats (Salkovic-Petrisic et al., 2006) and the fact the combined TG-icv + STZ-icv treatment did not abolished these cognitive deficits (Grünblatt et al., 2007).

Our results point to a decreased AC in the brain of rats with the experimental diabetes mellitus and rats with the cerebral diabetes representing an experimental model of sporadic AD. The latter model offers a possibility for searching for new antioxidant drugs in the treatment of a neurodegenerative disease such as sporadic AD.

Acknowledgements

Research was supported by the DAAD-German Academic Exchange Service, Stability Pact for South-Eastern Europe (project no. A/04/20017) and the Croatian Ministry of Science, Education and Sport (project no. 0108253). Mrs. Bozica Hrzan is thanked for the technical assistance.

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J Neural Transm (2007) [Suppl 72]: 241–247

Printed in Austria

Journal of Neural Transmission

Improving linear modeling of cognitive decline in patients with mild cognitive impairment: comparison of two methods

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Summary *Background.* High variability of estimates of cognitive decline in patients with Alzheimer's disease (AD) derived from unbalanced longitudinal designs may result as much from the applied statistical model as from true biological variability.

Objective. To compare the accuracy of two statistical models, serial subtraction score (SSA) and mixed-effects regression analysis (MEM), to estimate rates of cognitive decline in patients with amnestic mild cognitive impairment (MCI), a group at risk for AD.

Methods. We recorded serial mini mental state examination (MMSE) scores from 78 MCI patients. Additionally, we derived simulated trajectories of cognitive decline with unequally spaced observation intervals. Rates of change were assessed from clinical and simulated data using SSA and MEM models.

Results. MEM reduced variability of rates of change significantly compared to SSA. In a polynomial model, overall length of observation time explained a significant amount of variance of SSA, but not of MEM estimates. For simulated data, MEM was significantly more accurate in predicting true rates of change compared to SSA ($p < 0.001$).

Conclusion. MEM yields more accurate estimates of cognitive decline from unbalanced longitudinal data. Simulation studies may be useful to select the appropriate statistical model for a given set of clinical data.

Keywords: Longitudinal study, Alzheimer's disease, dementia severity, mixed effect regression, simulation study

Introduction

An accurate description of disease progression in Alzheimer's disease (AD) is a prerequisite for the identification of clinical prognostic markers and for the evaluation of treatment strategies designed to modify the course of AD (Wilson et al., 2000). Clinical studies exploring AD progression often are based on unbalanced longitudinal designs where the number and frequency of observations vary between subjects. It has been suggested that a considerable part of the high variability in estimated rates of disease progression found in earlier studies with unbalanced data (Galasko et al., 1991) results from the employed statistical approach and not from true biological variability between patients (Fig. 1) (Galasko et al., 1993).

The most widely employed statistical approach to determine progression of cognitive decline from clinical data are serial subtraction scores (and in the case of more than two observations per subject individual least square estimates of change), a type of summary statistics analysis (SSA). SSA can often be calculated even if data derive from an unbalanced design. However, the accuracy of SSA-derived individual rates of change depends upon the total length of time of observation and to a lesser degree upon the number of observations made and the interval of observations (Hand and Crowder, 1996; van Belle et al., 1990). Individuals with measurements with greater time spacing will have more accurate slope estimates than individuals with measurements at very close time intervals.

In contrast to SSA, mixed effects models (MEM) make full use of all available data and can handle unbalanced designs common to clinical longitudinal studies (Berg et al., 1992; Brown and Kempton, 1994; Mungas et al., 2001; Rasmusson et al., 1996; Teri et al., 1995). It is thought that they have higher power to detect significant correlations with predictor variables (Gould et al., 2001), and they are believed to lower the probability of detecting spurious results (Berg et al., 1992).

In the present study, we compared estimates of rates of cognitive decline between SSA and MEM, using serial

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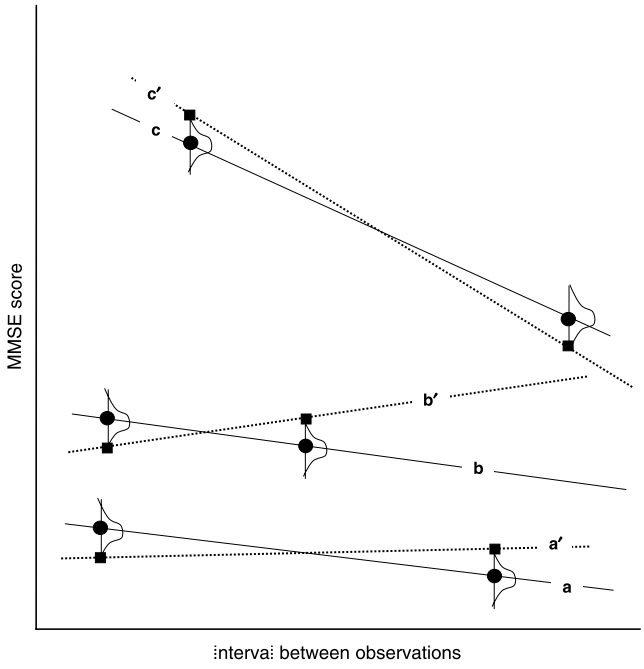

Fig. 1. Effect of random error on estimates of change from subtraction scores. Plot of two hypothetical trajectories of true mini mental state examination (MMSE) score decline (unbroken lines *a* and *c*) and of one trajectory parallel to *a* (line *b*), each being observed at two time points (filled circles). Observed MMSE scores (filled squares) can be thought of as being derived from true scores by the addition of a normally distributed source of random error (▷). Even if true MMSE scores decline, subtraction of observed serial scores can lead to an observed increase in the individual trajectory of MMSE score change (compare lines *a* and *a′*, and lines *b* and *b′*). With constant random error, this effect becomes stronger with shorter observation time (compare lines *a′* and *b′*). Averaging across all observed trajectories of decline (*a′* and *c′*), however, still may yield an estimate of mean rate of change that is close to its (in reality not observable) true value, if the deviation of observed scores from true scores is mainly due to random error

mini mental state examination (MMSE) (Folstein et al., 1975) scores from 78 patients with amnestic mild cognitive impairment, considered a predementia stage of AD (Almkvist et al., 1998). The interval between observations was highly variable, a characteristic common to many clinical studies. As we investigated a group of patients with a relatively homogeneous clinical stage of disease at baseline, we assumed that rates of cognitive decline would be approximate to a linear trajectory. We focused on the influence of the varying observation interval on the accuracy of estimates of change from both techniques. We modeled this effect with an inverse parabolic function, because we hypothesized that shorter observation times would lead to both an extreme increase and an extreme decrease in observed cognitive functions of patients. In the second part of the study we analyzed simulated trajectories of cognitive decline that paralleled essential features of the clinical data. The simulated data allowed us to compare the accuracy of estimated rates of change between both statistical models with a hypothetical 'true rate of change'.

Methods

Subjects

We studied 78 patients (mean age 71.6 years, SD 7.9 years; mean baseline MMSE score 28.0, SD 1.8, 38 females) with a clinical diagnosis of amnestic mild cognitive impairment according to the Mayo criteria (Petersen et al., 2001). Patients were followed over an average interval of 13 months (SD 14), ranging between 2 and 66 months, with serial MMSE examinations. 66 patients had two examinations, 4 patients had three, 6 patients had four and 2 patients had five examinations.

Neuropsychology measure

The MMSE is a widely used screening scale of global cognitive function that is often used to track the longitudinal course of cognitive impairment (Becker et al., 1988; Haxby et al., 1992). Numerous studies show that the rate of change in MMSE scores varies with severity of dementia (that is, the change is non-linear), yet in the early stages decline may be usefully modeled by linear models (Bélisle et al., 2002; Stern et al., 1996). We considered that the effect of non-linearity would be minimized in our sample, because we enrolled patients at one relatively homogeneous clinical stage of disease.

Statistics

Summary statistics analysis

Intra-individual rate of change was defined by the slope of the regression of time between subsequent assessments (in years) on observed MMSE scores within an individual. When there are only two subsequent observations per subject, this model reduces to the rate of change defined by the last MMSE score minus the first divided by the time, in years, between these two assessments. The Statistical Package for the Social Sciences release 10.0 (SPSS Inc., Chicago, IL, USA) was used for this analysis. See Appendix A for further details.

Mixed effects regression analysis (MEM)

The rate of change is conceptualized as a straight line represented by a slope and an intercept. Individual slopes and intercepts are regarded as a random sample of a normal population with unknown mean and variance. Individual slopes are often determined by iterative restricted maximum likelihood estimation (Laird and Ware, 1982), taking into account the information of the entire sample to determine individual rates of change. This estimation procedure leads to 'shrinkage' or empirical Bayes estimators and represents a compromise between estimates based only on an individual subject's data and estimates based only on the population mean. Variables on the subject or the observation level can be incorporated to model the effect of confounding variables or potential predictors of rate of cognitive decline.

MEM analysis was calculated with SAS 8.02 Proc Mixed software (SAS Institute Inc., Cary, NC, USA) (Littell et al., 1996). The models incorporated random-effects terms to account for subject and subject by time differences in MMSE scores, and for effects of length of observation time on initial MMSE scores and on individual rates of change of MMSE scores. As primary outcome variables of these models, we derived individual random regression coefficients of time related change in MMSE score. See Appendix B for further details.

Simulation study

We generated 100 baseline MMSE scores using random numbers from a normal distribution (mean 28, SD 2). The distribution parameters were chosen to resemble the distribution of baseline MMSE scores in the clinical data. Accordingly, scores were constrained to be ≤ 30 and ≥ 0, and to be integers. We generated 100 slopes (mean -1.5 points/year, SD 1 point/year) and 100 observation intervals (mean 10 months, SD 14 months) using random numbers from a normal distribution. Intervals were constrained to be ≥ 2 months. Each interval and slope was randomly assigned to a baseline MMSE score using pseudo random numbers from Excel 97 (Microsoft Corporation, USA), resulting in 100 triples of baseline MMSE scores, slopes and intervals. From these triples, we determined hypothetical *true MMSE scores* at follow up, as

$$\textit{true MMSE score} = \textit{baseline MMSE score} + \textit{slope} * \textit{interval}.$$

True MMSE scores then were overlaid with random error from a normal distribution (mean 0 points, SD 1 point) to generate *observed MMSE scores*. The standard-deviation was chosen, following earlier evidence for a test–retest variability of MMSE score in the range of 1 point (Folstein et al., 1975; Thal et al., 1986). Finally, estimated rates of change were determined from *observed MMSE scores* and intervals using SSA (Appendix A) and MEM (Appendix B, II). The MEM model included length of observation time as covariate. The simulation was programmed with Microsoft Visual Basic for Applications (Microsoft Corporation, USA).

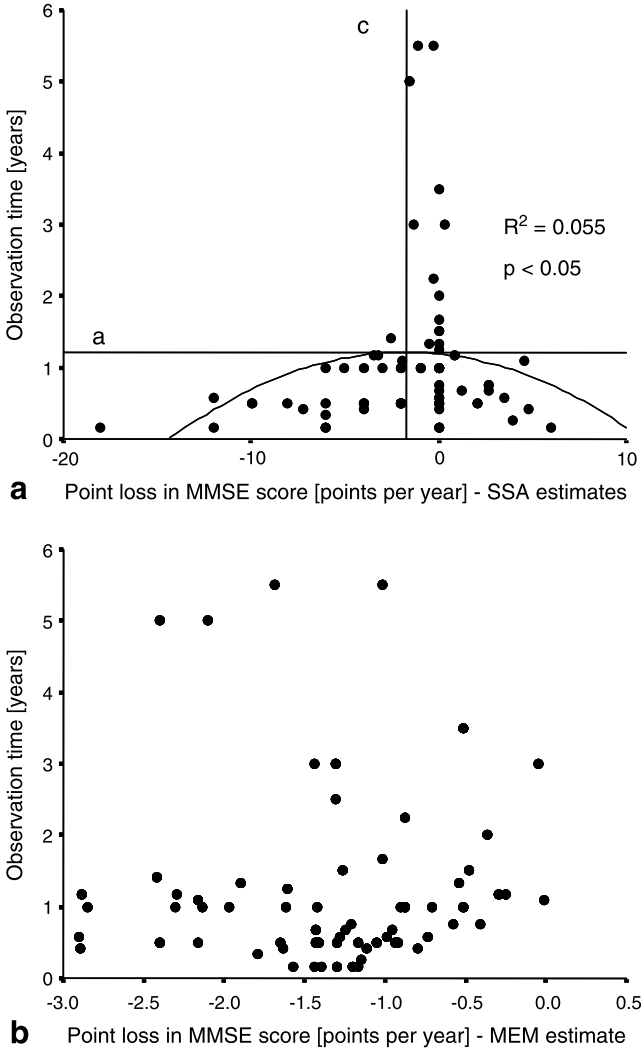

Fig. 2. Observation time and estimates of change – clinical data. Scatter plot of point loss in MMSE score derived from summary statistics analysis (SSA) estimates (**a**) and mixed effects model analysis (MEM) estimates (**b**) of clinical data and length of observation time. The polynomial regression (**a**) results from a least square fit of a predefined quadratic model (see text for details). Model parameters: a = intercept and c = lateral shift. No significant linear or quadratic model can be fitted to MEM estimates (**b**). Note differences in scaling of x-axes between **a** and **b**

Results

Clinical data

From the SSA, mean rate of MMSE change was -1.66 (SD 3.96) points per year, ranging between $+6$ and -18 points per year. From the MEM, mean rate of MMSE change was -1.31 (SD 0.69) points per year, ranging between -0.01 and -2.90 points per year (dependent sample t-test, 77 df, $T = 0.88$, $p = 0.38$). The coefficient of variation was lower for the MEM than for the SSA estimates (0.53 vs. 2.39).

We expected that extreme values in both directions (i.e. rapid increase or rapid decrease in MMSE scores) would predominantly occur with a short observation time. Mathematically, this assumption corresponds to an inverse parabolic function. Therefore, we fitted the following model to the data:

$$y = a + b * (x + c)^2,$$

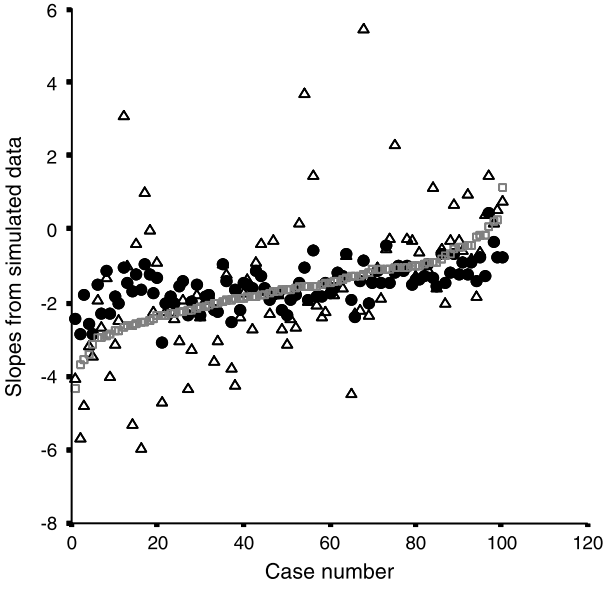

Fig. 3. Model fit for simulated data. Overlay scatter plot based on simulated data: true rates of point loss in MMSE (open squares), rates of point loss in MMSE estimated from summary statistics analysis (open triangles) and mixed effects model analysis (filled circles) by number of cases sorted according to the value of true slope in ascending order. Estimated slopes from mixed effects model analysis appear much closer to true slopes than estimated slopes from summary statistics analysis

with $y =$ length of observation time, $x =$ estimated rate of change of MMSE score per year, and $b < 0$.

For the SSA estimates of rates of change, a least square fitting procedure resulted in the following equation:

$$y = 1.21 - 0.0076 * (x + 1.75)^2.$$

The model accounted for a significant amount of variance of estimated rates of change (standardized $b = -0.23$, $R^2 = 0.055$, $F_{76}^1 = 4.38$, $p < 0.05$) (Fig. 2a). For comparison, a linear fit accounted for 2.0% of variance ($p = 0.22$).

For the MEM, observation time did not account for a significant amount of variance of estimated rates of change in a quadratic model nor a linear model ($R^2 < 0.01$ for linear and quadratic models) (Fig. 2b).

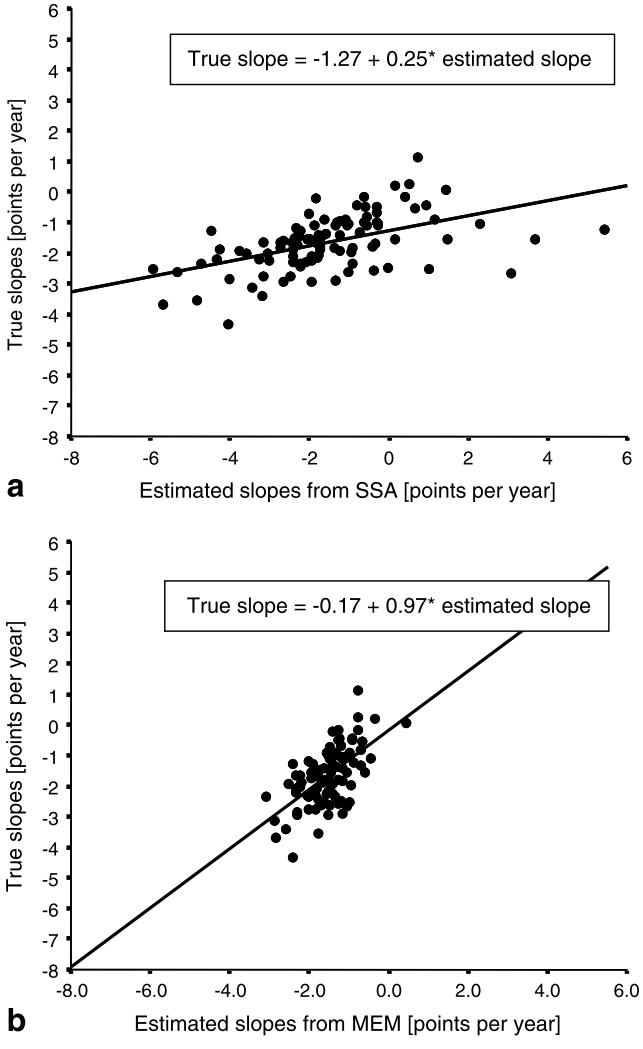

Fig. 4. True and estimated rates of point loss in MMSE – simulated data. Scatter plot of true rates of point loss in MMSE and rates of point loss in MMSE estimated from summary statistics analysis (SSA) (**a**) and mixed effects model analysis (MEM) (**b**) based on the simulated data

Simulation data

The average of estimated slopes was almost identical between SSA and MEM, with −1.53 and −1.54 points per year. The standard deviation of estimated slopes was higher for SSA compared to MEM (SD 1.88 and 0.58, respectively). Slopes ranged from 5.44 to −5.94 points per year for SSA estimates and from 0.44 to −3.07 points per year for MEM estimates. Simulated *true slopes* had a mean of −1.65, a standard deviation of 0.91 and a range from 1.15 to −4.35 points per year.

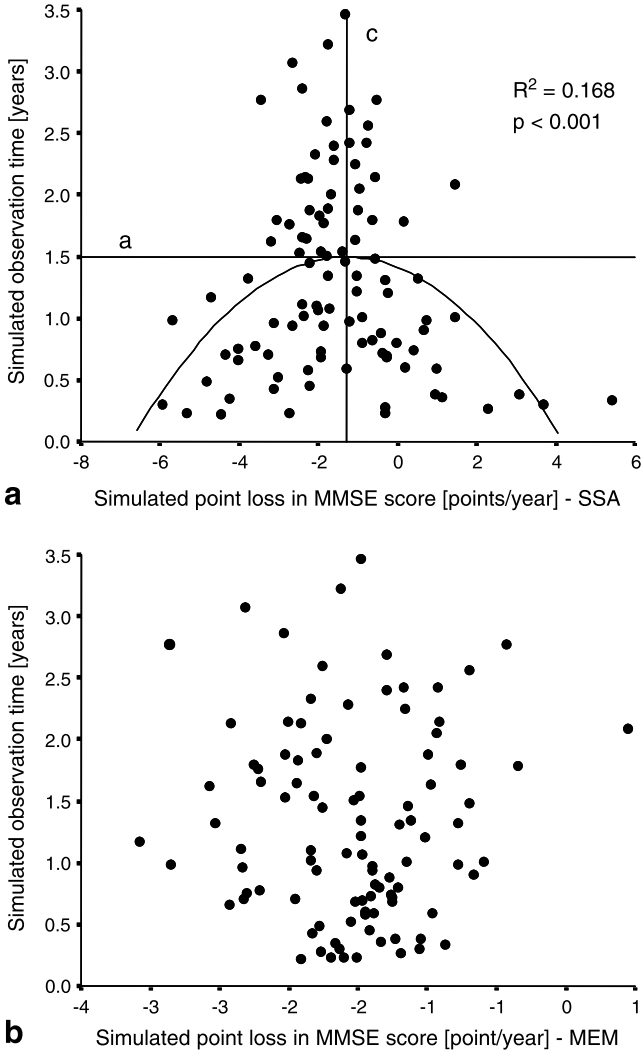

Fig. 5. Observation time and estimates of change – simulated data. Scatter plot of point loss in MMSE score derived from summary statistics analysis (SSA) (**a**) and mixed effects model analysis (MEM) (**b**) of simulated MMSE scores and observation intervals. The polynomial regression (**a**) results from a least square fit of a predefined quadratic model (see text). Model parameters: $a =$ intercept and $c =$ lateral shift. No significant linear or quadratic model can be fitted to MEM estimates (**b**). Note differences in scaling of x-axes between **a** and **b**

Residuals, i.e. absolute differences between estimated and true slopes, were significantly higher for SSA compared to MEM estimates (1.1 (SD 1.2) vs. 0.6 (SD 0.5), $t = -4.3$, 99 df, $p < 0.001$). Figure 3 illustrates the better fit of MEM estimates to true slopes compared to SSA estimates. Figure 4 shows the regression of true slopes on SSA (Fig. 4a) and MEM (Fig. 4b) estimates.

In the quadratic explanatory model, observation time accounted for about 17% of variance in the estimated slopes from the SSA ($R^2 = 0.17$, $F_1{}^{98} = 15.3$, $p < 0.001$) (Fig. 5a). In a linear model, there was no significant effect of observation time on SSA estimates of change ($R^2 < 0.01$). Observation time did not account for a significant amount of variance in a quadratic nor a linear model for MEM estimates ($R^2 < 0.01$ for linear and quadratic models) (Fig. 5b) or the true rates of change ($R^2 = 0.02$ for linear and quadratic models).

Discussion

In the present study, we compared rates of change in MMSE scores of patients with MCI derived from two different statistical models. Previous studies had compared statistical models to assess rates of cognitive change using either clinical data (Gould et al., 2001; Mendiondo et al., 2000) or simulated trajectories of cognitive decline (Milliken and Edland, 2000). In our study, we combined the analysis of clinical and simulated data. The clinical data allowed investigating two criteria for the goodness of fit of the two models: the variability of inter-individual rates of change, and the degree to which inter-individual differences in rates of change depend on differences in length of observation time. The simulated data allowed assessing the accuracy of both models to predict *hypothetical true rates of change*.

Average rates of change were not different between SSA and MEM for clinical and simulated data and agree with an average loss of 1.65 points per year in the MMSE in an independent study of MCI patients (Hodges et al., 2006). The coefficient of variation, however, was 3–5 times higher in the SSA than in the MEM estimates. Estimated rates of change from SSA ranged between two extremes which are unlikely to represent true biological effects (+6 and −18 points per year, compared to −0.01 and −2.90 points per year from MEM estimates). Coefficients of variation of 2.39 from the SSA analysis compare with a coefficient of variation of 1.25 for rates of decline of MMSE scores observed in a recent study on AD using SSA, where the range of follow up intervals was much more restricted than in our study (Kleiman et al., 2006). As illustrated in Fig. 1, with unbalanced data the SSA approach is expected to yield higher estimates of rates of change with shorter observation intervals. This corresponds to an inverse parabolic relationship between observation time and estimated rates of change. Based on the clinical data, length of observation time alone accounted for approximately 5.5% of the variance of the slopes estimated from SSA (explaining a rapid increase or a rapid decrease in MMSE scores) beyond the effect of any biological variables (Fig. 2a). In contrast, MEM derived estimates showed no correlation with length of observation time (Fig. 2b). For simulated data, the correlation between observation time and SSA estimated individual slopes was even higher than for clinical data (accounting for 17% of variance) and was low for MEM estimates and for *true rates of change* (Fig. 5). The polynomial model was clearly superior to the simpler linear model to describe the effect of observation time on SSA estimates of change. Using the simulated data, we also demonstrated that MEM yielded significantly smaller prediction residuals for *true rates of change* than SSA (Fig. 3). This was the case, even though *the true rates of change* had been determined according to a SSA type linear model. This finding supports the notion that MEM is more accurate than SSA in predicting rates of cognitive decline from unbalanced longitudinal data. The association between rates of decline and time of follow up has been illustrated by a recent study (Noda et al., 2006). Variability of percentage of rapid decliners (determined by SSA) between multiple sites was significantly reduced when time of follow up was restricted to be at least 11 months. These empirical data support the notion that SSA may inflate the variability of rates of change dependent on the variability of follow-up intervals.

The simulation was based on the assumption that MMSE scores would decline linearly during observation time. This assumption does not hold in clinical practice, since numerous studies suggest that speed of cognitive decline depends on dementia severity (Milliken and Edland, 2000; Morris et al., 1993; Teri et al., 1995). Our clinical and simulated data, however, were based on a group of patients in a homogeneous clinical stage of amnestic MCI so that, at least for a modest follow up period, there should be no major effect of stage of disease on rates of change.

With the above reservation, the simulation data support the interpretation of our clinical data. Results suggest that the average of the true rates of change in our clinical sample is close to the average values estimated from SSA and MEM. Furthermore, they suggest that SSA significantly overestimates variability of slopes, whereas MEM slightly underestimates the true variability. Thus the variability in true slopes is probably closer to the MEM derived estimate

than the SSA estimate (but may be slightly higher the MEM estimate). Finally, our simulation supports the notion that SSA estimates are biased by inter-individual differences in length of observation time.

In summary, using sampling from a narrow portion of the entire natural history of a disease can only approximate the ''true rate of cognitive change''. Our findings suggest that both the SSA and MEM yield reliable estimates of average rates of change in patients if applied at an early (homogeneous) stage of cognitive impairment. MEM yielded more accurate estimates of the variability of rates of change across subjects. As most clinical data will include varying inter-observational intervals, we suggest that MEM estimates may be more useful because results are less biased by differences in length of observation time than SSA estimates. Furthermore, our findings suggest that simulations can assist in selecting an appropriate model for disease progression and to test a priori assumptions underlying models of cognitive decline. The MMSE is used in this study as a convenient outcome measure, but our findings may relate to the analysis of other longitudinal data. Future studies are warranted that include patients at different clinical stages of dementia and also to consider the application of non-linear models.

Acknowledgments

Part of this work was supported by a grant of Janssen-CILAG (Neuss, Germany) to H.H. and S.J.T. and by a grant of the Medical Faculty of the Ludwig-Maximilian University, Munich, Germany, to S.J.T.

Appendix

A. Summary statistics analysis to determine individual rates of change

The rate of change is determined separately for each individual patient based on the observed MMSE scores according to the following model:

$$MMSE_i = a_0 + a_1 * t_i \quad \text{(I)}$$

with $MMSE_i = MMSE$ score at time i, $a_0 =$ intercept, $a_1 =$ rate of change, $t_i =$ observation at time i.

The coefficients a_0 and a_1, representing intercept and slope of the regression of *MMSE* on observation time (t_i), are determined separately for each individual patient using least square estimation. Subsequently, individual slopes can be regressed as dependent variables on potential predictor variables of inter-individual differences in rates of change.

In the case of only two observations per subject, the least square estimate of a_1 simplifies to

$$a_1 = \frac{MMSE_2 - MMSE_1}{t_2 - t_1} \quad \text{(II)}$$

with $MMSE_{1,2} = MMSE$ at times 1 and 2 and $t_{1,2} =$ observation times.

B. Mixed effects regression model to determine individual rates of change

The individual baseline values and rates of change are modeled as the group mean (fixed effect) and randomly distributed individual deviations from the group mean (random effect):

$$MMSE_{ij} = a_0 + a_1 * t_{ij} + u_{0j} + u_{1j} * t_{ij} \quad \text{(random trend model)} \quad \text{(I)}$$

with $a_0 =$ group intercept, $a_1 =$ group rate of change, $u_{0j} =$ individual random deviation for subject j from group intercept a_0 [$u_{0j} \sim N(0, \sigma_{uj})$ (normal distributed with expected value 0 and variance σ_{u0})], $u_{1j} =$ individual random deviation for subject j from group rate of change a_1[$u_{1j} \sim N(0, \sigma_{u1})$], $t_{ij} =$ observation at time i in subject j.

Mixed effects regression models include regression coefficients representing random effects (u_{0j}, u_{1j}) that vary between subjects around the group mean (a_0, a_1 – fixed effects). Random and fixed effect coefficients are determined using restricted maximum likelihood estimation.

In our study, to model the effect of different spacing of observations between subjects on individual rates of change, individual length of observation time (Δt_j) was incorporated into the mixed effects regression model:

$$MMSE_{ij} = a_0 + a_1 * t_{ij} + a_2 * \Delta t_j + a_3 * \Delta t_j * t_{ij} + u_{0j} * t_{ij} \quad \text{(random trend model with subject level covariate)} \quad \text{(II)}$$

with $\Delta t_j =$ observation time for subject j, a_2 and $a_3 =$ fixed effect regression coefficients to model the effect of Δt on the individual intercepts and slopes.

Introductions to mixed effects models theory can be found in (Brown and Prescott, 1999), with special emphasis on medical applications, and, in the terminology of multilevel modeling, in (Snijders and Bosker, 1999) and in (Goldstein, 1995). Software for fitting mixed effects models is available through dedicated programs (e.g. MLWin (Goldstein et al., 1998), MIXOR (Hedeker and Gibbons, 1996), HLM (Raudenbush and Bryk, 2002; Raudenbush et al., 2001)), but also through several standard statistical

packages (e.g. PROC MIXED in SAS (Littell et al., 1996), and more recently SPSS 11.0 and higher and SYSTAT 10.0).

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J Neural Transm (2007) [Suppl 72]: 249–259

Printed in Austria

Journal of Neural Transmission

Interaction of attention and graphomotor functions in children with attention deficit hyperactivity disorder

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Summary The present article provides a review of a series of studies in children with attention deficit hyperactivity disorder (ADHD) concerning (1) the effects of methylphenidate on various attentional functions, (2) the stimulant-induced changes of both qualitative and quantitative (i.e. kinematic) aspects of handwriting, (3) the interaction between conscious control of handwriting and fluency of handwriting movements, and (4) possible therapeutic approaches to graphomotor disturbances. Children with ADHD showed impairments in various aspects of attentional functioning. Pharmacological treatment of ADHD children with methylphenidate resulted in marked improvements of various components of attentional functioning. In comparison to the performance following the withdrawal of methylphenidate, children with ADHD on methylphenidate displayed a significant improvement in task accuracy in the areas of vigilance, divided attention, selective attention (inhibition, focused attention and integration of sensory information) and flexibility. However, the comparison with healthy children revealed considerable deficits regarding vigilance, divided attention, flexibility and selective attention (focused attention and integration of sensory information) in children with ADHD on methylphenidate. The comparison of writing movements of children on and off methylphenidate revealed that medication resulted in a better handwriting, but a deterioration in handwriting fluency as assessed by kinematic analysis. Children with ADHD may use their increased attentional capacities to focus on skills (e.g. handwriting) that are independent of conscious control or may even be disturbed by attention. The findings summarized in this paper indicate, therefore, that administration of methylphenidate alone is insufficient in the treatment of children with ADHD. Children with ADHD may benefit from instructions on how to best use their improved attentional capacities.

Keywords: Attention, hyperactivity, ADHD, graphomotor functions, handwriting, conscious control, methylphenidate

Introduction

The core symptoms of attention deficit hyperactivity disorder (ADHD), i.e. inattention, impulsiveness and motor restlessness, often result in academic failure or underachievement in children with ADHD (Hoza and Pelham, 1993). Difficulties at school may frequently lead to an initial referral for evaluation. The common pharmacological treatment of ADHD is stimulant drug therapy using methylphenidate, which is considered to be the most effective treatment (Hoza and Pelham, 1993; Peeples et al., 1995). The stimulant drug methylphenidate increases the concentration of dopamine and norepinephrine in the synaptic cleft by blocking the re-uptake of these neurotransmitters (Zametkin and Rapoport, 1987; Seeman and Madras, 1998). Methylphenidate is believed to affect predominantly the dopaminergic system (DuPaul et al., 1998). Neuroimaging studies using MRI or PET (Hynd et al., 1993; Semrud-Clikeman et al., 1994; Castellanos et al., 1996; Filipek et al., 1997) and genetic research using molecular genetic techniques (Cook et al., 1995; LaHoste et al., 1996) have shown that the neurobiological substrates of ADHD involve abnormalities of the dopaminergic and the frontostriatal systems. Both dopamine and norepinephrine are thought to be involved in the processing of attentional functions and inhibition (Heilman et al., 1991; Marrocco and Davidson, 1998; Riccio et al., 2001). Various studies have shown the effectiveness of methylphenidate in reducing symptoms of hyperactivity, impulsivity and inattentiveness in children with ADHD (Shaywitz and Shaywitz, 1991; Schachar and Tannock, 1993; Swanson et al., 1993). The administration of methylphenidate significantly improves social interactions, diminishes oppositional and aggressive behaviour, and improves classroom behaviour (Whalen et al., 1989; Barkley, 1991). In addition, methyl-

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phenidate has been shown to improve the performance of children with ADHD in tests measuring attention, memory or executive functioning (Losier et al., 1996; O'Toole et al., 1997; Kempton et al., 1999; Zeiner et al., 1999). With regard to fundamental academic skills, beneficial effects of methylphenidate on handwriting have been demonstrated (Barkley, 1998).

Good handwriting is one of the fundamental skills necessary for success at school. Children who have difficulty producing legible handwriting often experience frustration, lowered self-esteem and a decreased level of motivation (Kaminsky and Powers, 1981; Cornhill and Case-Smith, 1996). As Ackerman et al. (1986) have stated, automating basic academic skills such as handwriting is one of the major objectives of elementary school. The handwriting of children with ADHD has been shown to be markedly impaired (Barkley, 1998) and to improve significantly following treatment with stimulant medication. Improvements were seen in qualitative aspects of handwriting such as legibility, accuracy, spacing and uniformity (Lerer et al., 1979; Whalen et al., 1981). However, adverse effects of stimulant drug therapy on the kinematic aspects of handwriting, i.e. a disturbance of handwriting fluency, have been observed in children with ADHD (Tucha and Lange, 2001). These effects have been attributed to an increase in attentional control of handwriting movements (Tucha and Lange, 2004b). The improvement of attentional functions following stimulant drug therapy may interfere with fluent or automated handwriting processes.

The present article provides a review of a series of studies in children with ADHD concerning (1) the effects of methylphenidate on various attentional functions, (2) the stimulant-induced changes of both qualitative and quantitative (i.e. kinematic) aspects of handwriting, (3) the interaction between conscious control of handwriting and fluency of handwriting movements, and (4) possible therapeutic approaches to graphomotor disturbances.

Methylphenidate and attentional functions in children with ADHD

Although several clinical studies have been performed, our knowledge concerning the effect of methylphenidate on attentional functioning of children with ADHD is still limited. There is empirical evidence that attention represents a multi-dimensional construct including several specific functions which may be selectively impaired by brain pathology (Cohen, 1993; Van Zomeren and Brouwer, 1994). The multi-component model of attention devised by Van Zomeren and Brouwer (1994) is a theoretical framework of attentional functions referring to the component theory of Posner and colleagues (Posner and Boies, 1971; Posner and Rafal, 1987), the distinction between aspects of selectivity and intensity made by Kahneman (1973) and the concept of a supervisory attentional control system as devised by Shallice (1982). In their model the authors have subdivided attention into five interrelated components, including alertness (subdivided into tonic and phasic alertness), vigilance/sustained attention, selective attention, divided attention and strategy/flexibility. While tonic alertness refers to a relatively stable level of attention which changes slowly according to diurnal physiological variations of the organism, phasic alertness is the ability to enhance the activation level following a stimulus of high priority. Vigilance is a special type of sustained attention and describes the ability to maintain attention over a prolonged period of time during which infrequent response-demanding events occur. Selective attention is defined as the ability to focus attention in the face of distracting or competing stimuli. Divided attention is required to respond simultaneously to multiple tasks or multiple task demands. Flexibility refers to the ability to shift the focus of attention in order to control which information from competing sources will be selectively processed. A study by Tucha et al. (2006c) was based on the above-mentioned multi-component model of attention by Van Zomeren and Brouwer (1994) and examined several components of attention in children with ADHD. This study investigated the exact nature of the attention deficit of children with ADHD following withdrawal of methylphenidate. It also addressed the question of what effects individually tailored doses of methylphenidate have on different components of attention in these children and whether children with ADHD on methylphenidate reach a level of attentional functioning comparable to that of healthy children.

In the study by Tucha et al. (2006c), the attentional functioning of 58 children with ADHD according to the DSM-IV criteria without psychiatric comorbidity was assessed (49 boys, 9 girls; mean age $= 10.8 \pm 0.3$ years; mean IQ $= 98.1 \pm 1.5$). The study used a double-blind, placebo-controlled, crossover design in which children with ADHD were assessed both on their usual medication with methylphenidate and following withdrawal of the drug. At the time of the study, all children with ADHD were receiving individually tailored and clinically appropriate doses of methylphenidate and, according to parental and medical reports, were responding favourably to their medication. None of the children were taking concurrent medications known to affect the central nervous system. Furthermore, 58 healthy children (49 boys, 9 girls) who were matched to

Table 1. *Computerized neuropsychological test battery for attentional functions*

Function	Measure
Alertness	
tonic alertness	alertness task
phasic alertness	alertness task
Vigilance	vigilance task
Selective attention	
inhibition	visual scanning task
focused attention	incompatibility task
integration of sensory information	test of crossmodal integration
Divided attention	divided attention task
Flexibility	flexibility task

children with ADHD according to age, sex and handedness, participated in the study. No healthy child was taking medication known to affect the central nervous system. All participants were tested with a computerized test battery consisting of reaction time tasks (Table 1), including measures of alertness, vigilance, divided attention, flexibility and aspects of selective attention such as focused attention, inhibition and integration of sensory information (Zimmermann and Fimm, 1989, 2002). In comparison to the test performance of healthy sex-, handedness- and age-matched children, ADHD children displayed marked impairments of various aspects of attentional functioning (Tucha et al., 2006c). Attentional dysfunction was seen in an increased reaction time (divided attention, focused attention, integration of sensory information and flexibility), an enhanced variability of reaction time (vigilance, divided attention, inhibition, focused attention, integration of sensory information and flexibility) and in poorer task accuracy, as indicated by an increased number of omission and/or commission errors (vigilance, divided attention, inhibition, focused attention, integration of sensory information and flexibility). Statistical comparison of attentional functioning of ADHD children on and off methylphenidate revealed that the medication resulted in an improved task accuracy regarding vigilance, divided attention, inhibition, focused attention, integration of sensory information and flexibility (Tucha et al., 2006c).

The findings of Tucha et al. (2006c) confirm observations in the literature that children with ADHD off methylphenidate display a considerable deficit in various aspects of attentional functioning when compared to healthy children. These impairments were seen in aspects of arousal, selective attention, distractibility, shifting, vigilance and sustained attention (Sroufe et al., 1973; Trommer et al., 1988; Van der Meere and Sergeant, 1988; Kupietz, 1990; Shue and Douglas, 1992; Grodzinsky and Diamond, 1992; Corkum and Siegel, 1993; Oommen et al., 1993; Losier et al., 1996; Jonkman et al., 1999; Borger et al., 1999; Perugini et al., 2000; Tucha et al., 2006d). However, previous studies on unitary concepts of attention assessed only single components of attention. These studies did not account for current models of attention which suggest that attentional functioning involves multiple components (Sohlberg and Mateer, 1987; Mirsky et al., 1991; Cohen, 1993; Van Zomeren and Brouwer, 1994).

Individually tailored doses of methylphenidate led to improvements of attentional functioning of children with ADHD. On treatment with methylphenidate, children with ADHD displayed a significantly improved task accuracy with regard to vigilance, divided attention, inhibition, focused attention, integration of sensory information and flexibility. In previous studies examining single components of attention, drug treatment with methylphenidate was shown to improve tonic alertness (Reid and Borkowski, 1984), phasic alertness (Cohen et al., 1971), divided attention (Keith and Engineer, 1991), flexibility/shifting of attention (Kempton et al., 1999) and aspects of selective attention such as inhibition (Van der Meere et al., 1999) and focused attention (Musten et al., 1997) in children with ADHD. Although Tucha et al. (2006c) found a significantly decreased reaction time in a divided attention task, their results further confirm previous studies demonstrating that the number of omission errors and commission errors in attention tasks are particularly sensitive to medication effects (Losier et al., 1996; Riccio et al., 2001). In addition, the administration of methylphenidate resulted in no deterioration in any test variable measured in the study of Tucha et al. (2006c). Therefore, the positive effects of methylphenidate on various measures of attention may justify the use of methylphenidate in the treatment of attention deficits of children with ADHD.

Although methylphenidate induced improvements of attention were observed, children with ADHD did not reach an undisturbed level of attentional functioning. Not only did the comparison between the test performance of healthy children and ADHD children off methylphenidate reveal significant impairments of attention in ADHD children but also comparison between healthy children and ADHD children on methylphenidate. These impairments refer to vigilance, divided attention, focussed attention, integration of sensory information and flexibility. Although a positive effect of methylphenidate was observed, the impairments of ADHD children on methylphenidate were still considerable. Since children with ADHD on methylphenidate continued to display deficits in various components of attention seen in these children off medication,

additional treatment appears to be necessary. The effective treatment of attention deficits is clinically relevant and of special importance in children, since such deficits may cause considerable handicap including academic failure at school.

Methylphenidate and graphomotor functions in children with ADHD

Children with ADHD have been shown to be markedly impaired in graphomotor output (Barkley, 1998). This fundamental academic skill was found to show substantial improvements following the treatment with stimulant medication. Improvements were seen in qualitative aspects of handwriting such as legibility, accuracy, spacing and uniformity (Lerer et al., 1979; Whalen et al., 1981). As Ackerman et al. (1986) state, automating basic academic skills such as handwriting is one of the major objectives of elementary school. This involves not only automating the production of legible and accurate handwriting, but also the quality of handwriting movements.

The introduction of digitizing tablets for the assessment of writing and drawing has altered the approach to the examination of handwriting from product-oriented to process-oriented (Van Galen, 1991). Kinematic aspects of handwriting movements can be analyzed using these digitizers. The analysis of velocity and acceleration of handwriting provides evidence of the existence of simple motor programs. It has been suggested that free handwriting in healthy subjects is formed by the sequential activation of these motor programs which are probably stored in the form of a spatial code (Thomassen and Van Galen, 1992). Single letter-strokes, the smallest relevant units of the writing process, are formed by open loop movements which are characterized by velocity profiles with only one peak (inversion of the direction) and a bell shaped course. Single strokes of automated movements lead to a smooth and repetitive course (Fig. 1). On the basis of these findings, Mai and Marquardt (1992) revealed that automated and non-automated handwriting movements can be distinguished from one another by the profiles of velocity and acceleration. Only one inversion in velocity is expected, when the writing movement is an open loop (fully automated or fluent). More than one inversion of velocity per stroke points to a disturbance of handwriting fluency or automation (Tucha et al., 2000, 2004). This means that the more inversions produced by the subject, the more poorly the movement has been mastered. In addition, the maximum velocity and acceleration per stroke gives further information about handwriting movements (Mai and Marquardt, 1992). Several studies have revealed marked differences regarding automation and both maximum velocity and maximum acceleration per stroke between healthy participants and adult patients with neurological or psychiatric

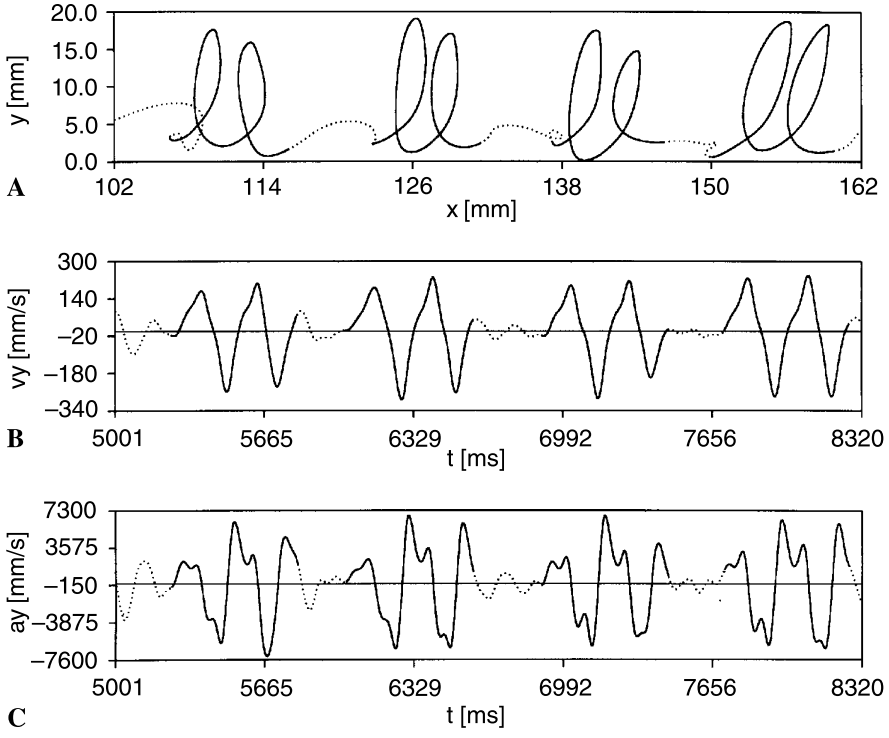

Fig. 1. Repetitive writing of the letter combination "ll" of a healthy participant with the corresponding velocity profile and acceleration profile. Solid lines represent handwriting movements on the paper and dotted lines movements with the pen raised. **A** Recording of handwriting. **B** Corresponding velocity profiles (v): The profiles are smooth, repetitive and single peaked. **C** Corresponding acceleration profiles (a): The profiles are smooth and repetitive

diseases. These alterations indicate disturbances of motor control which are clinically relevant since such disturbances may cause considerable handicap including loss of employment (Mai and Marquardt, 1994). The prevention of such handicaps is of special interest in children who may experience frustration and academic failure at school as a result of their impaired handwriting performance. The clinical relevance of the assessment of these measures is supported by the finding that disturbances of kinematic aspects of handwriting can be improved by pharmacological treatment or psycho-educational intervention (Mai and Marquardt, 1994; Tucha et al., 2002, 2006a, e; Tucha and Lange, 2004a; Lange et al., 2006). A study by Tucha and Lange (2001) examined the stimulant-induced changes of both qualitative and quantitative (i.e. kinematic) aspects of handwriting of boys with ADHD.

In the study by Tucha and Lange (2001), the writing performance of 21 boys with a diagnosed ADHD (combined type) according to the DSM-IV criteria, was assessed both on the usual methylphenidate treatment and following the withdrawal of the drug. Furthermore, 21 control boys underwent the same examination. Diagnosis of ADHD was based on clinical assessment (DSM-IV) including interviews with the boys and their parents. At the time of the study, all boys with ADHD were being treated with methylphenidate and according to parental reports were responding favorably to the medication. Boys with ADHD received individually tailored doses of methylphenidate. None of the children were taking concurrent psychotropic medications at the time of the study. In the drug withdrawal condition, boys with ADHD were not given methylphenidate for ten hours prior to the start of testing. Handwriting of children with ADHD on methylphenidate was assessed approximately one hour following administration of the usual medication dose. None of the control boys had a history of neurological or psychiatric disease or displayed signs of ADHD or learning disability. Control boys were matched to boys with ADHD according to age, grade and handedness.

A short text containing the letter combination ''ll'' a number of times was dictated to the participants. A copy of the text was also presented, in order to reduce disturbances of handwriting due to spelling mistakes. The text was suitable reading for second grade school children. Half of the boys with ADHD were tested first on methylphenidate and then following withdrawal of the drug and the remaining boys were examined in the reverse order. Boys were randomly assigned to each condition. The time-period between testing and retesting of boys with ADHD was seven days. All writing tasks were accomplished on unruled paper.

For the registration of handwriting movements, a digitizing tablet (WACOM IV) with a specific pen containing a normal ink refill was used. The position of the pen on the tablet, velocity and acceleration were measured continuously during writing. Data were stored on a personal computer which was connected to the tablet. It was possible to localise the tip of the pen with an accuracy of 0.2 mm in both directions (x/y) at a frequency of 100 Hz. Data processing was performed using a computational program for the analysis of handwriting movements (Mai and Marquardt, 1992). Writing conditions were nearly natural since the tablet was constructed to resemble a common desk pad. No restrictions of posture, speed or size of writing were given. Children were not instructed to write neatly, accurately or legibly. For the analysis of kinematic aspects of handwriting, only the letter combination ''ll'' was chosen since these letters represent a simple letter combination which is usually executed in script type. Furthermore, while writing the letter combination ''ll'', the pen is not lifted from the tablet. In the evaluation of kinematic data, the mean number of inversions of the direction of the velocity (NIV) and the acceleration profiles (NIA) of the letter combination ''ll'' were calculated. In addition, the maximum velocities of both ascending and descending strokes and both the maximum positive and negative acceleration (slowing down) were measured. Mean scores of maximum velocities and accelerations were calculated. Data analysis focused on the vertical component of the strokes as the examination of the ascending and descending strokes were of primary interest. The examiners were blinded to both treatment condition (on versus off methylphenidate) and group identity (ADHD versus control). In addition, every writing specimen was rated independently by four examiners in regard to form, alignment, spacing, legibility and uniformity of handwriting. The effect of methylphenidate on handwriting was assessed by comparing the performance of boys with ADHD during the usual methylphenidate treatment with their performance following withdrawal of the drug.

The results by Tucha and Lange (2001) confirm previous studies (Resta and Eliot, 1994) and show that ADHD children off methylphenidate display a poorer quality of handwriting than healthy control boys (Fig. 2). Marked improvements of form, alignment, spacing, legibility and uniformity of handwriting in children with ADHD on the usual treatment with methylphenidate were observed (Whalen et al., 1981). Since children with ADHD perform poorly in tasks measuring motor coordination and perceptual-motor skills, the improved quality of handwriting following stimulant drug therapy has been attributed to

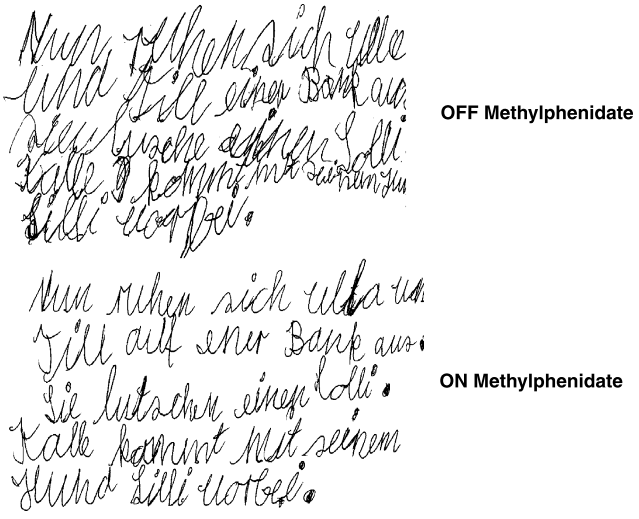

Fig. 2. Handwriting specimens of a boy with ADHD on and off methylphenidate treatment. The quality of handwriting is significantly improved on methylphenidate

stimulant-induced improvements of these functions (Lerer et al., 1979). However, the kinematic analysis of handwriting movements of the study by Tucha and Lange (2001) revealed that boys with ADHD on methylphenidate displayed both more inversions in the direction of velocity and acceleration profiles and lower maximum velocities and accelerations than following the withdrawal of the drug. These findings indicate that on methylphenidate, handwriting movements of boys with ADHD are less fluent than off methylphenidate (Mai and Marquardt, 1992). Boys with ADHD off methylphenidate did not differ from healthy control boys in the kinematic aspects of handwriting. Since medication with methylphenidate has been shown to improve the test performance of boys with ADHD when copying a complex drawing (Seidman et al., 1995), a reduction of handwriting fluency due to impairments of visuo-spatial or visuo-constructive abilities can be excluded. The findings by Tucha and Lange (2001) may be due to a secondary effect of stimulant drug treatment. Methylphenidate has often been shown to improve the ability to focus attention and to decrease both impulsiveness and distractibility in children with ADHD (Hoza and Pelham, 1993; Tucha et al., 2006c). Therefore, the findings of Tucha and Lange (2001) suggest that the positive effects of methylphenidate enable boys with ADHD to focus attention on their handwriting. The conscious control of handwriting which may result from the intention to write more neatly, interferes with fluent or automated handwriting processes. The desire to write neatly may be the result of the emphasis placed upon accurate handwriting by teachers. Whalen et al. (1981) remarked that ADHD children with poor handwriting are often misjudged as unmotivated by their teachers. When children with ADHD write neatly, stress and frustration can be diminished. These children are able to produce legible handwriting but they do not achieve automaticity of handwriting (Ackerman et al., 1986). Automated processes do not require conscious control (Näätänen, 1992). Therefore, when children have mastered automaticity of handwriting, more attentional capacity is available for comprehension and problem solving. These assumptions are supported by information-processing psychology.

The importance of automated handwriting movements should not be overemphasized since automaticity without legibility leads to fluent but illegible handwriting. A helpful approach could be to minimise the need to write by hand by encouraging the use of computers or pre-printed notes. However, in order to diminish the probability of school failure and frustration in children with ADHD caused by difficulties in handwriting, educational approaches concerning handwriting should consider the fluency to the same extent as the quality of handwriting.

Conscious control and graphomotor functions in healthy adults

In further studies, Tucha and Lange (2005) examined the hypothesis that children with ADHD on stimulant medication show an increase in attentional control during handwriting. Two experiments were performed regarding the effect of conscious control on handwriting in healthy adult participants and children with ADHD. The first experiment examined whether conscious control of handwriting impairs the fluent execution of handwriting movements. Therefore, the writing movements of healthy students were examined under different conditions regarding visual and attentional control during handwriting. Healthy students were chosen since handwriting in adults is a well-habituated motor skill consisting of automated movements and needs no conscious monitoring during execution (Tucha et al., 2006b). If attentional control of handwriting disturbs the movement execution in adult subjects with acquired automaticity of handwriting, the fluent execution of handwriting movements should be particularly disturbed in children who have not acquired automaticity. In the second experiment, handwriting movements of children with ADHD on medication were assessed under different instructions.

Twenty-six right-handed students (13 females, 13 males; mean age $= 23.2 \pm 1.7$ years; mean education $= 13.0 \pm 0.4$

years; mean IQ = 106.2 ± 7.5) participated in the experiment. In all experiments, a digitizing tablet (WACOM IV) was used for the registration of handwriting movements. The participants were asked to write the sentence ''Ein helles grelles Licht'' (''A bright and glaring light'') in cursive script under five different conditions. The conditions consisted of normal writing, writing with eyes closed, neat handwriting, writing while visually tracking the pen tip and writing with closed eyes while mentally tracking the highest position in each letter. The sequence of conditions was randomly assigned to the participants. The test sentence was written a total of five times by each participant per condition. For the analysis of kinematic aspects of handwriting, only the letter combination ''ll'' of the German words ''helles'' (bright) and ''grelles'' (glaring) were taken (for further experimental details see Tucha and Lange, 2005).

With regard to handwriting movements, no differences were found between normal handwriting and writing with closed eyes. In comparison with movements during normal handwriting, movements were less fluent when participants were requested to write neatly, to write while visually tracking the pen tip and when they were asked to write with closed eyes while mentally tracking the highest position in each letter. These results indicate that automated handwriting movements are independent from visual feedback. This finding corresponds with previous observations of Marquardt et al. (1996) who could also demonstrate that automated handwriting movements can be performed without vision. Furthermore, the results by Tucha and Lange (2005) suggest that conscious attention given to accuracy of handwriting (neatness), to visual feedback and also to mental control of graphomotor output results in less fluent handwriting movements. The participants' attention to handwriting hampered the generation of automated movements. Automated processes are therefore not just independent of conscious control as suggested by Näätänen (1992), they are indeed disturbed by conscious attention. These findings may explain the results of the previous study of Tucha and Lange (2001) in which improvements of qualitative aspects of handwriting, such as legibility, were observed in boys with ADHD following treatment with stimulant medication. However, kinematic analysis of handwriting movements revealed that these boys with ADHD on stimulant drug treatment performed less fluent writing movements than following the withdrawal of the drug or than control participants. The less fluent handwriting processes of children with ADHD on medication may therefore be the result of the intention to write more neatly.

Conscious control and graphomotor functions in children with ADHD

In a placebo-controlled examination (Tucha and Lange, 2005), the writing performance of 12 children (9 boys, 3 girls; mean age = 10.1 ± 1.2 years) with ADHD/com-combined type according to the DSM-IV criteria was assessed. At the time of the study, all children with ADHD were being treated with methylphenidate and were responding favourably to the medication. Children with ADHD received individually tailored doses of methylphenidate. The children were asked to write the sentence ''Ein helles grelles Licht'' (''A bright and glaring light'') both on the usual methylphenidate treatment and following the withdrawal of the drug. In the methylphenidate condition the mean dosage of methylphenidate at the time of testing was 11.7 mg (SD = 3.4 mg). In the placebo-condition the children received a placebo and the mean time to the last medication was approximately 12 hours. Half of the children were tested first on methylphenidate and then on placebo. The remaining children were examined in the reversed order. The time-period between testing and retesting was between five and seven days. In addition, children on methylphenidate were instructed to write the test sentence with eyes closed and to write faster than normal. The sequence of conditions was randomly assigned. A digitizing tablet (WACOM IV) as described was used for the registration of handwriting movements.

Kinematic analysis of children's normal handwriting revealed that, in comparison to placebo, medication with methylphenidate resulted in a reduced fluency of handwriting (Tucha and Lange, 2005). Automated handwriting movements could be elicited in children with ADHD on medication, when they were instructed to write faster than normal or to write with eyes closed, although these children under the same pharmacological condition were not able to produce automated movements during normal writing of a short sentence. These results suggest that both visual and mental control of handwriting movements affect the automation of these movements. According to these findings, the disturbed movements during normal handwriting in children with ADHD on stimulant medication cannot be attributed to medication-induced peripheral changes or alterations of brain states. The disturbances of automaticity of movements may be due to a secondary effect of stimulant drug treatment. It is probable that the positive effects of methylphenidate enable children with ADHD to focus attention on their handwriting. However, the intention to control handwriting movements in order to write more neatly is accompanied by an impair-

ment of fluent handwriting movements. When attentional control of writing is prevented by the use of simple verbal instructions, fluent writing movements could be performed. These findings may have considerable significance regarding educational approaches to handwriting in children with ADHD, in which not only aspects of form, size and legibility but also aspects of automaticity should be taken into consideration.

Handwriting fluency may not be the only area in ADHD which is subject to disturbing effects of methylphenidate as a result of increased attentional control. Children and adults with ADHD may have difficulties in skills whose acquisition starts as a labored and conscious learning process which becomes automatic following consistent and frequent practice. Skills that may be affected include reading, typing or driving a car.

Therapeutic implications

On the basis of the experiments described above, the potential value of a training procedure was assessed in children with ADHD.

The diagnosis of ADHD/combined type in four children (3 boys, 1 girl, mean age $= 9.3 \pm 1.0$ years) was based on clinical assessment (DSM-IV) including interviews with the children and their parents. The children had been receiving methylphenidate for 18–40 months. The current average dose per day was 23.8 mg (range 15–30 mg). According to a parental report, they were responding favourably to medication. The parents described disturbances in their children's handwriting which had resulted in considerable difficulties at school. In written tests, in particular in writing to dictation, they were not able to finish the test in time, even when given extra time by the teacher. The teachers reported that when writing, the children failed to understand the content of texts or lessons. However, the children's comprehension was not disturbed in general. When they were not asked to write, they were able to understand the contents of texts or lessons.

In the four children, the examination of handwriting on and off the usual methylphenidate treatment, the examination of basic writing movements and the writing under different instructions revealed the same pattern of results as reported for a group of children with ADHD (see above). Form, spacing, legibility and uniformity were better on treatment with methylphenidate than following withdrawal of the drug. Since treatment with methylphenidate had resulted in marked improvements in the children's attention, social behaviour and fundamental academic skills such as reading and arithmetic abilities, the training sessions were performed when they were on methylphenidate. The finding that the generation of fluent handwriting movements of children with ADHD can be aided by simple instructions was taken as the starting point of the training procedure. The aim of the training procedures was to direct attention (conscious control) away from the writing process, in particular from accuracy of handwriting. Therefore, at the start of each training session, it was emphasized that accuracy and legibility of handwriting were not important. In training sessions, the children were asked to copy short texts with an easy readability level sentence by sentence. The examiner instructed them to write faster than normal or to write as fast as possible. During writing the examiner gave additional auditory reminders to write ''fast'' or to write ''faster''. Furthermore, it was repeatedly emphasised that form, uniformity, spacing and legibility of handwriting are not important. The children were praised each time that automated handwriting movements were produced.

By the end of the first training sessions, fully automated handwriting movements were observed in the handwriting of all four children, even when they were asked to do some homework and when they were not aware that their handwriting was being recorded. Kinematic analysis of handwriting at the beginning of the second session revealed that the effect of the first session had not been maintained. However, using the same procedures, perfectly smooth handwriting profiles could be elicited after only a few sentences. The procedures were therefore also employed by the parents when the children were doing their homework. Over a period of up to four weeks, four or five further training sessions were performed. The external cues (instruction to write ''faster'') and the verbal feedback (reinforcement of automated movements) enabled the children to progress to a quick, fluent and effective handwriting. The speed of handwriting increased dramatically as reported by the parents and the teachers. The average movement time of simple sentences containing four words decreased from 42 seconds (range 27–56 seconds, first session) to 14 seconds (range 11–17 seconds, sixth session). Kinematic analysis revealed perfectly smooth (automated) handwriting profiles. The script produced by the children was legible but was accompanied by irregularities of alignment. The increased writing speed enabled the children to finish written tests within the given time. This resulted in better grades and a higher degree of motivation. The level of accuracy and legibility of handwriting was not stable but varied markedly during sessions, although the level of automaticity was unchanged. The latter finding could be potentially

very significant in the training of handwriting of children with ADHD. The results of a follow-up examination indicated that the improvements of handwriting movements were still present four to six weeks after the end of the training.

Therefore, the disturbance of automaticity in handwriting of children with ADHD cannot be attributed to an overall disturbance of the generation of automated movements. Our findings rather support the assumption that the positive effects of stimulant drug treatment of children with ADHD result in an increased focus of attention on handwriting. These findings may have considerable implications for the training of handwriting disturbances in children with ADHD. Despite all the limitations of studies examining small samples, the potential of simple training procedures focusing on automaticity and smoothness of movements could be demonstrated.

Conclusion

In conclusion, it has been demonstrated that children with ADHD suffer from an attention deficit comprising impairments of various aspects of attentional functioning. In comparison to control children, children with ADHD displayed an increased rate of both omission and commission errors as well as increased reaction times and an increased variability of reaction times. Pharmacological treatment of children with ADHD using individually tailored doses of methylphenidate resulted in marked improvements of various components of attentional functioning. In comparison to the performance following the withdrawal of methylphenidate, children with ADHD on methylphenidate displayed a significant improvement in task accuracy in the areas of vigilance, divided attention, selective attention (inhibition, focused attention and integration of sensory information) and flexibility. However, the comparison with healthy children revealed considerable deficits regarding vigilance, divided attention, flexibility and selective attention (focused attention and integration of sensory information) in children with ADHD on methylphenidate. In addition, our studies have shown that children with ADHD may use their increased attentional capacities to focus on skills (e.g. handwriting) that are independent of conscious control or may even be disturbed by attention. Therefore, the findings summarized in this paper indicate that administration of methylphenidate alone is insufficient in the treatment of children with ADHD. Children with ADHD may benefit from instructions on how to best use their improved attentional capacities.

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J Neural Transm (2007) [Suppl 72]: 261–267

Printed in Austria

Journal of Neural Transmission

Therapeutic drug monitoring of antidepressants – clinical aspects

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Summary Therapeutic Drug Monitoring (TDM) is a tool to optimise antidepressant pharmacotherapy improving efficacy and avoiding side effects. The Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP)-TDM group has worked out consensus guidelines to make progress in the use of TDM which in spite of its obvious advantages, is far from optimal in everyday clinical practice. Research-based levels of recommendation were defined with regard to routine monitoring of plasma concentrations for dose titration. Main indications of TDM compromise control of compliance, lack of clinical response or adverse effects at recommended doses, drug interactions, pharmacovigilance programs, presence of a genetic predisposition particularity concerning the drug metabolism, children, adolescents and elderly patients. Therapeutic ranges of plasma concentrations that are considered to be optimal for treatment are proposed, implications on pharmacoeconomics aspects are discussed. The need to improve the implementation of TDM in routine patient care is emphasized.

Keywords: Drug monitoring, antidepressants, pharmacovigilance, pharmacotherapy

Introduction

50 years after the discovery of the first antidepressant, imipramine, followed by at least 40 different antidepressants worldwide, adequate or optimal dosing still lacks the answer. So-called therapy-resistant depressions covering approximately 30% of the patients belong to the mean challenges in depression treatment until nowadays (Fava and Davidson, 1996). Main reasons for this comprise non-compliance (Johnson, 1996) as well as individual differences regarding pharmacokinetic (metabolism, interactions) and pharmacodynamic factors. Alexanderson et al. described as early as 1969 that nortriptyline plasma concentrations were, in part, genetically determined in twins receiving this antidepressant.

The first report on plasma concentration – clinical effectiveness relationship of nortriptyline has to be considered as the basis for therapeutic drug monitoring (TDM) in psychiatry (Asberg, 1971). The aim of TDM is to improve clinical efficacy and to minimize adverse drug effects. Bertilsson et al. (1981) were the first to provide proof of the high diagnostic efficacy of combining TDM with pharmacogenetic tests with immediate consequences for the patient: A patient presented with a genetic deficiency of debrisoquine hydroxylation (CYP2D6) deficiency displayed unusually high plasma concentrations of nortriptyline and severe adverse effects.

During the past years, TDM has been introduced for many drugs in psychiatry. Some authors recommend TDM of tricyclic antidepressants (TCAs) even as ''standard aspect of care'' (Burke and Preskorn, 1999). Increasingly, TDM is combined with pharmacogenetic tests (Kirchheiner et al., 2001). Its use in practice has been limited to few patients and few indications, however. Moreover, valid recommendations on how to use TDM adequately to improve psychopharmacotherapy are rare. With the excep-

* Dedicated to Peter Riederer as memory for 7 years working together at Wuerzburg and more than 20 years of friendship.

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tion of a report of the Task Force on the Use of Laboratory Tests in Psychiatry (Glassman et al., 1985) which was restricted on the TDM of tricyclic antidepressants and an update by Orsulak (1989), consensus guidelines on the use of TDM in psychiatry have not been published in this field.

In 1991 the first German consensus conference of TDM in psychiatry took place in Würzburg and a report on the state of the art of TDM in psychiatry was published (Laux and Riederer, 1992; Riederer and Laux, 1992). Linder and Keck (1997) have communicated aspects of TDM services for treatment with tricyclic antidepressants, but treatment guidelines like the World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for biological treatment of depressive disorders do not report how to use TDM (Bauer et al., 2002). The TDM group of the AGNP (Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie), an interdisciplinary expert group comprising of chemists, clinical biochemists, clinical pharmacologists and psychiatrists, therefore compiled information from the literature and worked out consensus guidelines to assist psychiatrists and laboratories involved in psychopharmacotherapy to optimise the use of TDM of psychotropic drugs (Baumann et al., 2004; Hiemke et al., 2005, see below). Additionally, the same group has published the section of TDM guidelines for antidepressants (Baumann et al., 2005).

Pharmacokinetics and pharmacogenetics

Most antidepressants are similar in chemical properties but differ in pharmacokinetic characteristics, e.g. half-life or metabolism. Since metabolites may be of importance in the overall effect of antidepressants, TDM must include active metabolites, for example in case of clomipramine (norclomipramine) or venlafaxine (desmethylvenlafaxine). Pharmacogenetic research of the last years showed that individual genetic disposition is fundamental for the activity of drug-metabolizing enzymes. The number of active alleles in a gene determines how much of the enzyme is expressed (phenotype) – patients with low, normal and high activity of certain enzymes can be characterised (''Poor metabolizer [PM], extensive metabolizer, ultra rapid metabolizer'' [UM]). Genetic polymorphism of drug-metabolising enzymes is clinically important because unexpected side effects and toxic reactions may occur in PM due to increased plasma concentrations, non-response in UM due to subtherapeutic plasma levels. From the cytochrome P450 (CYP) isoenzyme system CYP1A2, CYP2D6, CYP2C19 and CYP3A4/5 are the most important ones for psychotropic drugs. The genetic polymorphism of CYP2D6 catalyses TCAs and serotonin reuptake inhibitors (SSRIs) causing clinically relevant in differences individually (Goodnick, 1994).

Relationships between drug doses, its plasma concentrations and clinical variables

TDM is based on the assumption that there is a definable relationship between plasma concentration and clinical effects (therapeutic effect, adverse effects and toxicity), the so-called ''therapeutic window''. These relationships have been investigated mainly for lithium, tricyclic antidepressants and classical antipsychotic drugs with inconsistent results (for review see Balant-Gorgia and Balant, 1995). Methodological shortcomings of numerous studies are most likely responsible for the lack of an evident relationship. Systematic reviews and meta-analyses (Glassman et al., 1985; Perry et al., 1994) that were based on adequately designed studies led to convincing evidence of a relationship between clinical variables and plasma concentration for nortriptyline, imipramine and desipramine, the size of the effect being proportional to the plasma level of the drug for low to intermediate plasma levels, and no further increase – or even a decrease – in effectiveness for high to very high plasma levels. For amitriptyline a meta-analysis of 45 studies has shown almost identical results (Ulrich and Läuter, 2002).

Preliminary results of the subproject TDM of the Kompetenznetz Depression recently could show the benefit of TDM for TCAs:

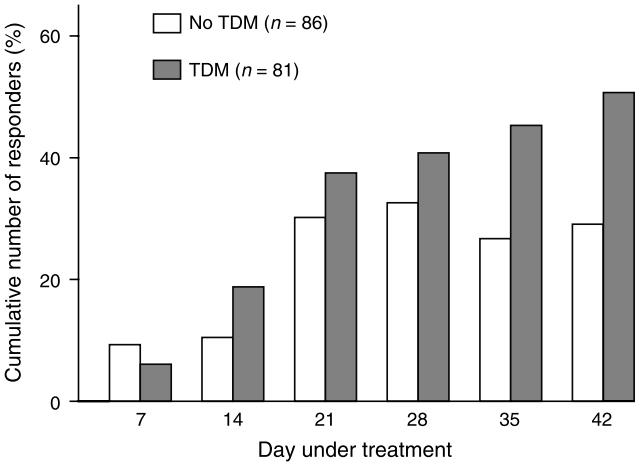

Fig. 1. Cumulative rate of response in depressed patients treated with antidepressant drugs with and without inclusion of TDM. Patients were treated with tricyclic antidepressants (14%), selective serotonin reuptake inhibitors (59%) or venlafaxine (27%). Patients were categorized as responders when initial HAMD-17 total score at day 0, which was at least 17 points, had decreased by $\geq$50% (Kompetenznetz Depression)

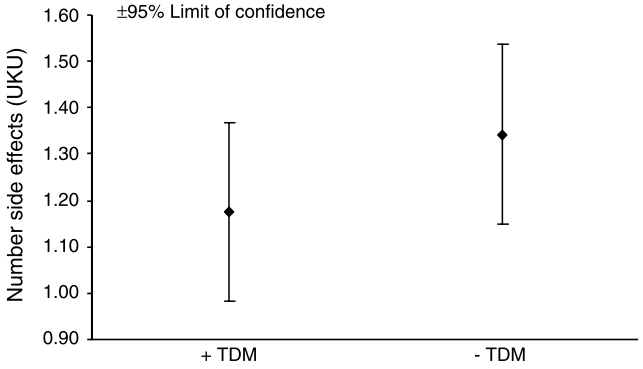

Fig. 2. Number of side effects (UKU Scale) under treatment with antidepressants with (+) or without (−) TDM (Kompetenznetz Depression)

As shown in Fig. 1 improvement in patients treated with antidepressants in optimal TDM range was superior compared with treatment without TDM. Rate of side effects was lower, additionally (Fig. 2). Regarding SSRIs routine monitoring of plasma levels is not recommended (Bauer et al., 2002). A study, however, revealed a beneficial effect of TDM: Patients were randomized to a group with either TDM or no TDM. At baseline, the mean Hamilton depression score on the HAMD-17 (17 items) scale of 167 depressed patients was 24.4 ± 6.3 in patients without TDM ($n = 86$) and 25.8 ± 5.0 in the group with TDM ($n = 81$). Preliminary analysis of the data revealed more responders ($\geq$50% improvement) in the group with TDM than in the group without TDM (Fig. 3). Moreover, drug discontinuation due to non-response or side effects occurred in only 9 patients with TDM wheras in the group without TDM the antidepressant medication was changed or discontinued in 24 patients (Hiemke et al., 2003).

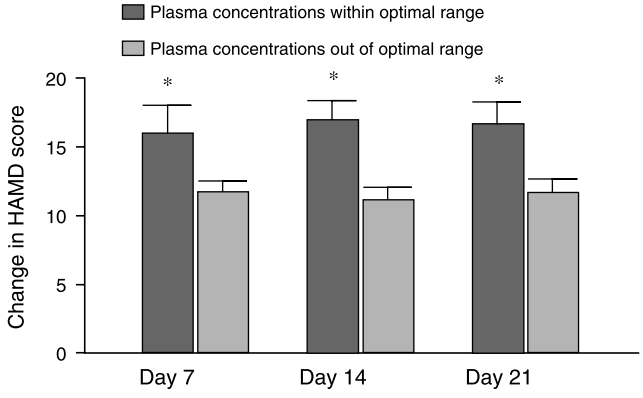

Fig. 3. Change in HAMD scores in depressed patients treated with antidepressant drugs with and without inclusion of TDM. Patients were treated with tricyclic antidepressants (14%), selective serotonin reuptake inhibitors (59%) or venlafaxine (27%). $^{*}p < 0.05$ (taken from Hiemke et al., 2005, with kind permission of Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, Germany)

The following items have to be considered for quality assessment of TDM studies dealing with the therapeutic window in clinical treatment settings (Ulrich and Läuter, 2002):

- valid chemical-analytical method
- adequate psychopathology rating scale and sufficient severity of the disorder at treatment onset, appropriate registration of change and exclusion of known non-responders
- reporting of patient selection criteria
- reporting of exclusion criteria
- concentration design, i.e. representative plasma used in the data analysis and adequate range of plasma concentrations investigated
- dose regimen, i.e., consideration of causality (false evidence of a relationship by flexible dose design)
- reporting of pre-medication, adequate washout period before randomisation
- reporting of co-medication
- adequate sample size

Indications for TDM, consensus guidelines

This chapter is a summarized version of the concerning part of the article in *Pharmacopsychiatry 2004; 37:243-265*. Table 1 presents a list of indications for TDM. Suspected non-compliance or under-dosing (''pseudo-therapy-resistance'') or intoxication is considered as essential, the validity of other indications has to be examined on an individual basis dependent on each case.

A comprehensive list of therapeutic and toxic plasma concentrations of most common drugs available including psychotropic agents was published, but unfortunately, the authors did not explain how they selected these ranges from the literature (Regenthal et al., 1999).

In 2004 the AGNP-TDM expert group presented consensus guidelines sreening the available literature and

Table 1. *Indications for therapeutic drug monitoring (TDM) of psychotropic drugs such as antidepressants (modified according to Baumann et al., 2004)*

Suspected non-compliance
Suspected intoxication
Lack of clinical response, or insufficient response even if doses considered as adequate
Adverse effects despite the use of generally recommended doses
Suspected drug interactions
Recurrence despite good compliance and adequate doses
Presence of a genetic particularity concerning the drug metabolism (genetic deficiency, gene multiplication)
Patients with pharmacokinetically relevant comorbidities (hepatic or renal insufficiency, cardiovascular disease)

selecting reports according to the following decreasing priority: 1) Guidelines, 2) meta-analyses (e.g. amitriptyline), 3) prospective studies on the clinical effectiveness of drugs in which drug plasma concentrations were reported, and 4) pharmacokinetic studies (Baumann et al., 2004; Hiemke et al., 2005).

Using this strategy and based on empirical evidence these guidelines defined 5 levels of recommendation for TDM, which range from ''strongly recommended'' to ''not recommended''. In a second step, a recommendation tailored to the individual drug was defined.

In patients suspected to be non-compliant, TDM is recommended for all drugs.

The following five research-based levels of certainty have been proposed:

1 = Strongly recommended

Established therapeutic range.

Level of evidence: Controlled clinical trials have shown benefit of TDM, reports on toxic effects at ''supratherapeutic'' plasma concentrations.

Clinical consequences: At therapeutic plasma concentrations highest probability of response, at ''subtherapeutic'' plasma concentrations response rate similar to placebo, at plasma concentrations higher than therapeutic concentrations increasing risk of adverse effects.

2 = Recommended

Suggested therapeutic ranges obtained from plasma concentrations at therapeutically effective doses (fixed dose studies).

Level of evidence: At least one well designed prospective study with well-defined outcome criteria reports on intoxications at ''supratherapeutic'' plasma concentrations.

Clinical consequences: TDM most probably will optimise response in non-responders: at ''subtherapeutic'' plasma concentrations; risk of poor response, at ''supratherapeutic'' plasma concentrations; risk of adverse effects and/or decreased response.

3 = Useful

Suggested therapeutic ranges are plasma concentrations at effective doses obtained from steady-state pharmacokinetic studies.

Level of evidence: Clinical data from retrospective analysis of TDM data, single case reports or non-systematic clinical experience.

Clinical consequences: TDM useful to control whether plasma concentrations are plausible for a given dose; optimising of clinical response in non-responders who display low concentrations is possible.

4 = Probably useful

Suggested therapeutic ranges from steady-state pharmacokinetic studies at therapeutically effective doses.

Level of evidence: Valid clinical data so far lacking or inconsistent results.

Clinical consequences: TDM useful to control whether plasma concentrations are plausible for a given dose.

5 = Not recommended

Unique pharmacology of the drug, e.g. irreversible blockade of an enzyme or flexible dosing according to clinical symptoms.

Level of evidence: Textbook knowledge, basic pharmacology.

Clinical consequences: TDM should not be used.

Table 2 presents the recommended therapeutic ranges for antidepressants.

TDM is established for TCAs, as for many of them a plasma concentration – clinical effectiveness relationship was shown, and TDM is recommended to avoid intoxications.

Evidence for a significant relationship between drug concentration and therapeutic outcome is lacking for tetracyclic antidepressants (maprotiline, mianserin and mirtazapine), trazodone and reboxetine, the monoamine oxidase inhibitors moclobemide and tranylcypromine (Goodnick, 1994), and also for SSRIs (Baumann, 1996; Rasmussen and Brosen, 2000), but recent data from Sweden revealed that TDM of SSRI is cost-effective (see below) since it helps to use minimum effective doses (Lundmark et al., 2000).

Some groups of patients, such as elderly patients, are typically treated with co-medications according to multimorbidity and frequently present age-related sensitivity to medication. In this cases TDM may help to distinguish between pharmacokinetic and pharmacodynamic factors in the occurrence of adverse effects.

It should be noticed, that antidepressants are also introduced for other treatments than depressions (e.g. anxiety states, obsessive-compulsive disorders). Little information is available on optimal plasma concentrations in these situations. Therefore, the therapeutic ranges listed in Table 2 are generally those for the ''main'' indication depression.

Regulatory authorities strongly recommend pharmacokinetic measurements and concentration-response relationship in Phase II and III studies as stated by the EMEA (European Agency for the Evaluation of Medicinal Products, www.emea.eu.int/pdfs/human/ich/037895en.pdf). TDM could gain much more attention and importance, if it would be required in Phase IV and drug surveillance studies, accordingly.

Table 2. *Recommended target plasma concentration ranges for antidepressants and levels of recommendation for routine monitoring (modified according to Baumann et al., 2004, 2005; Hiemke et al., 2005)*

Drug and active metabolite	Recommended therapeutic range (consensus) (ng/ml)[1]	Level of recommendation[2]
Antidepressants		
Amitriptyline plus nortriptyline	80–200	1
Citalopram	30–130	3
Clomipramine plus norclomipramine	175–450	1
Desipramine	100–300	2
Doxepin plus nordoxepin	50–150	3
Escitalopram	15–80	4
Fluoxetine plus norfluoxetine	120–300	3
Fluvoxamine	150–300	4
Imipramine plus desipramine	175–300	1
Maprotiline	125–200	3
Mianserin	15–70	3
Mirtazapine	40–80	3
Moclobemide	300–1000	4
Nortriptyline	70–170	1
Paroxetine	70–120	3
Reboxetine	10–100	4
Sertraline	10–50	3
Tranylcypromine	0–50	5
Trazodone	650–1500	3
Trimipramine	150–350	3
Venlafaxine plus O-desmethylvenlafaxine	195–400	2
Viloxazine	20–500	3

[1] Therapeutic ranges indicate trough concentrations of drugs in serum or plasma of patients under steady state medication.

[2] Level of recommendation:

1 Strongly recommended (for lithium TDM should be a standard of care): Established therapeutic range.

2 Recommended: Suggested therapeutic ranges obtained from plasma concentrations at therapeutically effective doses (fixed dose studies).

3 Useful: Suggested therapeutic ranges are plasma concentrations at therapeutically effective doses obtained from steady-state pharmacokinetic studies.

4 Probably useful: Suggested therapeutic ranges from steady-state pharmacokinetic studies at therapeutically effective doses.

5 Not recommended (explanations see text).

Economic aspects of TDM

Pharmacoeconomy has gained much attention in modern health systems. As for many other tests, cost-effectiveness studies are required for TDM.

Cost-effectiveness has been provided for nortriptyline in a setting that used prospective pharmacokinetic dosing prediction. The benefit of TDM was demonstrated, in that patients who underwent TDM were earlier discharged from the hospital and returned to work earlier than empirically dosed patients (Simmons et al., 1985). Preskorn and Fast (1991) calculated significant savings when using TDM for tricyclic antidepressants primarily by avoiding adverse events which brought about extra costs. With regard to new antidepressants, Lundmark et al. (2000) have shown that TDM is cost effective in elderly patients. TDM led to dose reduction with sustained clinical efficacy, which resulted in a 38% cost reduction considering costs for drugs and laboratory assays.

Practical aspects of TDM, recommendations

Retrospective as well as prospective analyses of routine TDM in psychiatric hospitals revealed considerable inappropriate use of TDM in many cases. Recently Mann et al. (2006) presented a retrospective analysis of routine TDM in a psychiatric hospital based on 748 plasma levels of antidepressants. Inappropriate use has been identified frequently. Overall, 30% of blood samples were taken too early, before steady state had been reached, a high number of repeat tests without preceding change of drug dose points to potentially redundant use. Frequent discrepancies were found between the laboratory's recommendations and actual clinical decision making, suggested dose changes were followed in only 30%. Additionally, a prospective investigation, which was conducted under naturalistic conditions, failed to find a clinically significant impact of the TDM of tricyclic antidepressants (Müller et al., 2003), but the study revealed that in many cases dose adjustment was inappropriate and did not take the results of the laboratory assays into consideration. A study on the clinical use of TDM of tricyclic antidepressants in a psychiatric university hospital showed that between 25 and 40% of the requests for TDM were inappropriate and the interpretation of the results led to about 20% of inappropriate therapeutic adjustments (Vuille et al., 1991). The criteria for inappropriate requests included an inappropriate indication for TDM, absence of steady-state conditions and transcription errors on the request form. Among the inappropriate interpretations of the TDM result was the lack of adjustment of the dose of the antidepressant. Therefore, some practical recommendations may be helpful for the optimal use of TDM, as illustrated in Fig. 4.

TDM is based on trough steady-state plasma concentrations. Blood should therefore be collected at least 5 drug half-lives after changes of dose and during the terminal β-elimination phase. In clinical practice, the appropriate sampling time is one week after stable daily dosing and immediately before ingestion of the morning dose, i.e.

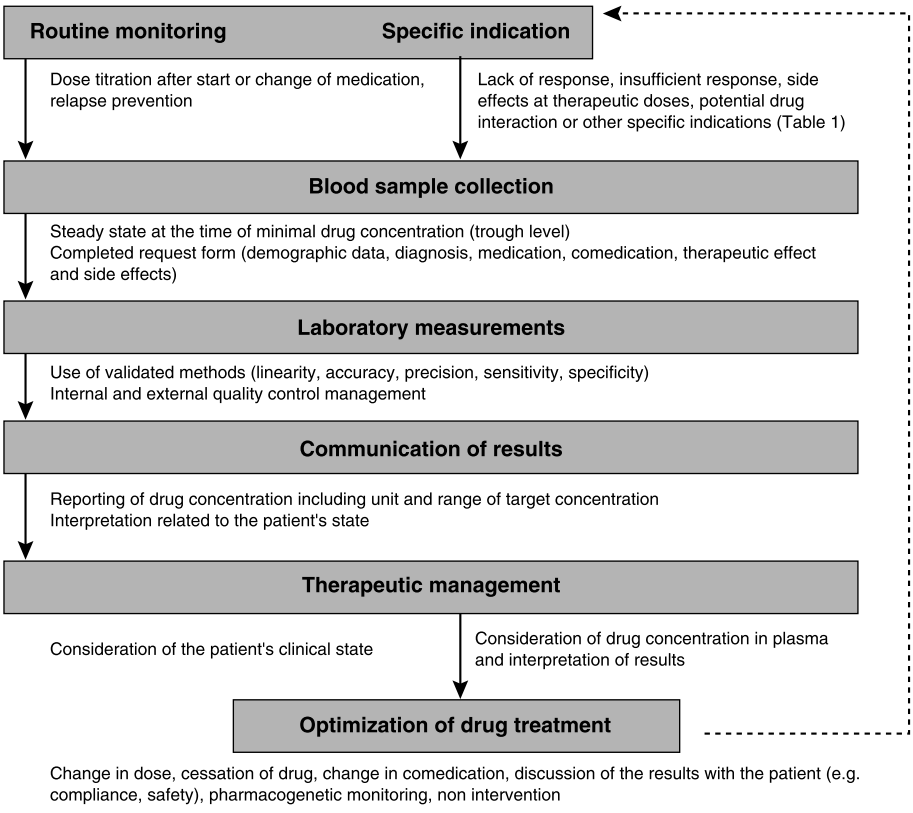

Fig. 4. Summary of TDM process (taken from Baumann et al., 2004, with kind permission of Georg Thieme Verlag KG, Stuttgart)

about 12–16 hours (or 24 hours if the drug is given once daily) after the last medication.

Both after a modification of the dose and after prescription of a comedication, which may inhibit or enhance the metabolism of the drug to be measured, TDM should be delayed until steady-state conditions are reached again. Of course, TDM should be carried out earlier if unexpected side effects are observed. The plasma level may be considerably influenced by comedications (and their metabolites!). Precise information on comedications may help the laboratory to avoid analytical problems (interferences with other drugs). It is absolutely necessary to fill out the request forms adequately and completely (diagnosis, comorbidities, comedications, treatment duration, doses, sex and age of the patient, reasons for the request).

Finally, it may be advantageous for the clinician to involve a laboratory for TDM, which offers pharmacological consultation. This is always recommended, when due to the result of TDM, a pharmacogenetic test is advised.

TDM-interpretation and patient treatment

It is certainly wise to take into account the level of recommendation for TDM of the particular drug. Evidently, the treating physician has to decide whether the treatment strategy is to be changed or not, as he alone knows his patient. On the other hand, when the advice given on the TDM report is not followed, the reason must be substantiated to evaluate the decision of the treating psychiatrist if the consequences for the patient retrospectively turned out to be unfavourable.

Reporting of results

The results should be available for clinical interpretation within a clinically meaningful time. A 48-hour TDM service is sufficient in most cases, but many laboratories may not be able to respond to this criterion. Shorter intervals may be required in case of intoxications. Interpretation and clinical-pharmacological advice should be provided with every report. Therefore, it is advantageous for the clinician to choose a laboratory that offers this service. Otherwise, a trained expert of the clinic should interpret the results. In any case, the interpretation must be undertaken in the light of sound clinical adjustment (Hiemke and Laux, 2002).

In sum, TDM will remain a valuable approach to optimise the often lifelong medication of psychiatric patients with antidepressants.

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J Neural Transm (2007) [Suppl 72]: 269–280

Printed in Austria

Journal of Neural Transmission

The immunological basis of glutamatergic disturbance in schizophrenia: towards an integrated view

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Summary This overview presents a hypothesis to bridge the gap between psychoneuroimmunological findings and recent results from pharmacological, neurochemical and genetic studies in schizophrenia. In schizophrenia, a glutamatergic hypofunction is discussed to be crucially involved in dopaminergic dysfunction. This view is supported by findings of the neuregulin- and dysbindin genes, which have functional impact on the glutamatergic system. Glutamatergic hypofunction is mediated by NMDA (N-methyl-D-aspartate) receptor antagonism. The only endogenous NMDA receptor antagonist identified up to now is kynurenic acid (KYN-A). KYN-A also blocks the nicotinergic acetycholine receptor, i.e. increased KYN-A levels can explain psychotic symptoms and cognitive deterioration. KYN-A levels are described to be higher in the CSF and in critical CNS regions of schizophrenics.

Another line of evidence suggests that of the immune system in schizophrenic patients is characterized by an imbalance between the type-1 and the type-2 immune responses with a partial inhibition of the type-1 response, while the type-2 response is relatively over-activated. This immune constellation is associated with the inhibition of the enzyme indoleamine 2,3-dioxygenase (IDO), because type-2 cytokines are potent inhibitors of IDO. Due to the inhibition of IDO, tryptophan is predominantly metabolized by tryptophan 2,3-dioxygenase (TDO), which is located in astrocytes, but not in microglia cells. As indicated by increased levels of S100B, astrocytes are activated in schizophrenia. On the other hand, the kynurenine metabolism in astrocytes is restricted to the dead-end arm of KYN-A production. Accordingly, an increased TDO activity and an accumulation of KYN-A in the CNS of schizophrenics have been described. Thus, the immune-mediated glutamatergic-dopaminergic dysregulation may lead to the clinical symptoms of schizophrenia. Therapeutic consequences, e.g. the use of anti-inflammatory cyclooxygenase-2 inhibitors, which also are able to directly decrease KYN-A, are discussed.

Keywords: Schizophrenia, immune system, glutamate, NMDA receptor, COX-2, PGE2

Abbreviations

CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
HLA	human leukocyte antigen
3-HK	3-hydroxykynurenine
ICAM-1	intercellular adhesion molecule-1
IDO	indoleamine 2,3-dioxygenase
Ig	Immunoglobulin
IFN	interferon
IL	interleukin
KMO	kynurenine-monoxygenase
KYN	kynurenine
KYN-A	kynurenic acid
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
PGE2	prostaglandin E2
TDO	tryptophan 2,3-dioxygenase
Th1/2	T-helper cell type 1 or 2, respectively

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Dopaminergic – glutamatergic imbalance in schizophrenia

There is no doubt that a disturbance in the dopaminergic neurotransmission plays a key-role in the pathogenesis of schizophrenia (Carlsson, 1988; Jentsch and Roth, 1999). This view is based on the evidence that most drugs ameliorating psychotic symptoms act as dopamine receptor blockers, in particular D2 receptor blockers. However, despite the fact that only a part of the patients respond to antipsychotic drugs and the long-term outcome of antipsychotic treatment is unsatisfactory in many cases, attempts to explain the disease solely in terms of dopaminergic dysfunction leave many aspects of schizophrenia unsolved.

The glutamate hypothesis of schizophrenia postulates an equilibrium between inhibiting dopaminergic and inhibiting glutamatergic neurons; the model of a cortico-striato-thalamo-cortical control loop integrates the glutamate hypothesis with neuroanatomical aspects on the pathophysiology of schizophrenia (Carlsson et al., 2001). A hypofunction of the glutamatergic cortico-striatal pathway – i.e.

an inhibition of the inhibitory GABA neurons by hyperactivated dopaminergic receptors as well as reduction of the glutamatergic input – is associated with opening of the thalamic filter, which leads to an uncontrolled flow of sensory information to the cortex and to psychotic symptoms (Carlsson, 2006).

Dopamine-release can be challenged by amphetamine, the challenge can be blocked by NMDA-receptor antagonists. In an animal experiment, the treatment with NMDA (N-methyl-D-aspartate) receptor antagonists leads to a marked, dose-dependent increase of amphetamine-induced dopamine release (Miller and Abercrombie, 1996). This observation has been confirmed in humans: Blocking of the NMDA-receptor by ketamine induced a significant enhancement of amphetamine-induced dopamine release in healthy controls (Kegeles et al., 2000). The magnitude of the increase was comparable to the exaggerated response patients with schizophrenia had to amphetamine alone (Laruelle et al., 2005); in schizophrenics, the amphetamine-induced dopamine release is much higher compared to healthy controls (Laruelle et al., 1996). This observation is in accordance with the view that the abnormally elevated dopamine release revealed by the amphetamine challenge in schizophrenia results from a disruption of glutamatergic neuronal systems regulating dopaminergic activity (Laruelle et al., 2005).

NMDA-receptor hypofunction and schizophrenia

Hypofunction of the glutamatergic neurotransmitter system as a causal mechanism in schizophrenia was first proposed due to the observation of low concentrations of glutamate in the cerebrospinal fluid (CSF) of schizophrenic patients (Kim et al., 1980).

Phencyclidine (PCP), ketamine, and MK-801 all block the NMDA receptor complex and are associated with schizophrenia-like symptoms through hypofunction of the glutamatergic neurotransmission (Krystal et al., 1994; Olney and Farber, 1995). Other substances, acting as NMDA antagonists, but not at the PCP site, have psychotogenic properties, too (CPP, CPP-ene, CGS 19755; 8). NMDA receptor hypofunction can explain schizophrenic positive and negative symptoms; cognitive deterioration and structural brain changes can be a consequence of NMDA receptor dysfunction (Olney and Farber, 1995). Recently, the first in vivo evidence for a NMDA receptor deficit has been reported in medication-free schizophrenic patients (Pilowsky et al., 2006).

Decreased plasma levels of the NMDA co-agonist glycine in schizophrenics and a correlation of glycine levels with schizophrenic negative symptoms were found (Sumiyoshi et al., 2004). Baseline glycine levels predicted the treatment outcome of clozapine in negative symptoms (Sumiyoshi et al., 2005). Clinical investigations targeted the glycine co-agonist site of the NMDA receptor by administering the amino acids glycine or D-serine, or a glycine pro-drug such as milacemide (Tamminga et al., 1992). Some of these studies have yielded positive results, particularly against the schizophrenic deficit syndrome (Heresco-Levy et al., 1999).

Genetics of the immune system and of the NMDA system

Schizophrenia is a complex genetic disorder. Given the hereditary component and the role of an inflammatory/immunological process in schizophrenia, immunologically relevant genes may shape up as susceptibility genes for schizophrenia.

Genetic factors influence acquiring infectious diseases, both with respect to susceptibility (Cook and Hill, 2001) and to resistance to infection (Hill, 1996). Mechanisms for genetically mediated responses to infection occur through genetic variations in immune mediators such as cytokines and HLA genes. The HLA region on chromosome 6 is located within or very near a region which has a high susceptibility risk for schizophrenia (Schwab et al., 2000). So far, associations of certain HLA-loci with schizophrenia were described (Laumbacher et al., 2003), but replication in larger, independent samples are still lacking. Studies of genetic polymorphisms of the pro-inflammatory cytokine TNF-alpha – also located in the HLA region of chromosome 6 – show divergent results, the difference in the outcome possibly being related to ethnic differences between the samples (Riedel et al., 2002; Meira-Lima et al., 2003). An analysis of polymorphisms of the type-1 cytokine IL-2 and the type-2 cytokine IL-4 revealed a possible genetic base for this imbalance (Schwarz et al., 2005). Several other cytokine genes and components of the immune system have been studied without conclusive results. Methodological problems of samples and diagnoses, the small genetic load of every individual gene, the pleiotropic function of the immune components, and their marked functional compensatory abilities may explain weak genetic associations.

There is consensus that genetic variations of dysbindin, located also on chromosome 6p22 (Numakawa et al., 2004) and neuregulin-1, located at chromosome 8p (Williams et al., 2003) are associated with an increased risk for schizophrenia. Both genes have been identified in large studies

over the last years. Although the functions are not yet fully elucidated and at least the neuregulin-1 has multiple functions, interestingly, both genes code for proteins which are involved in the glutamatergic neurotransmission (Collier and Li, 2003). Neuregulin-1 regulates the NMDA receptor expression/presence in glutamatergic synaptic vesicles and dysbindin, located in glutamatergic neurons, is reduced in schizophrenia (Talbot et al., 2004). These recent findings support the view that the glutamatergic neurotransmission plays a key role in schizophrenia.

Neurodevelopmental aspects of inflammation and NMDA receptor dysfunction

The discovery of environmental risk factors for schizophrenia, acting before, during, and shortly after birth has been central for the neurodevelopmental hypothesis of schizophrenia (Murray and Lewis, 1987). Genetic and environmental risk factors interact during the crucial phase of development of the central nervous system (CNS) causing subtle abnormalities, which leave the individual vulnerable to psychosis in later life. Established risk factors are obstetric complications, prenatal or postnatal infections. Cytokines, mediators of the immune response, are growth factors of the nervous system and of glial cells, therefore crucial for the development of the CNS. Obstetric complications such as hypoxia and injury of the CNS are associated with a change in cytokine release in the CNS (Dean and Murray, 2005).

On the other hand, it has been argued that the effect of obstetric complications might be mediated by glutamatergic excitotoxic damage in the foetal/neonatal brain (Fearon et al., 2000). This view is supported by an animal model showing that glutamatergic damage is not associated with functional impairment in early life, but regularly manifests itself during early adulthood (Farber et al., 1995). The sensitization of the CNS to glutamatergic toxicity seems to be a process of maturation, which becomes symptomatic during adulthood. Accordingly, the occurrence of psychotic symptoms following the use of the NMDA receptor antagonist ketamine in humans is age dependent, with psychotic symptoms occurring rarely, if ever, in prepubertal children, but manifesting in nearly 50% of young to middle-aged adults (Marshall and Longnecker, 1990).

Sensitization studies with pro-inflammatory cytokines show, that an increased production of pro-inflammatory cytokines such as IL-1 during an infectious or inflammatory process in the perinatal period can induce long-lasting, probably permanent, alterations in the CNS neurotransmitter systems. These results indicate that an increased production of IL-1 during an infectious or inflammatory process in the perinatal period can induce long-lasting, probably permanent, alterations in the central (and peripheral) neurotransmitter systems (Kabiersch et al., 1998).

Inflammation and schizophrenia

The role of infection in the aetiology of schizophrenia has gained more attention during the last years (Rapaport and Müller, 2001; Müller, 2004). Infection during pregnancy in mothers of off-springs later developing schizophrenia has been repeatedly described (Brown et al., 2004; Buka et al., 2001; Westergaard et al., 1999) and is discussed as an explanation for the seasonality of schizophrenic births between December and May (Torrey et al., 1997).

Results of a Finnish epidemiological study showed that infection of the CNS in childhood increases the risk of becoming psychotic later on five-fold (Koponen et al., 2004). A five-fold increased risk for developing psychoses later on was also observed in Brazil after a (bacterial) meningitis epidemic (Gattaz et al., 2004). Taking into account a sensitization process, an infection during early childhood is in accordance with the assumption that an infection-triggered disturbance in brain development might play a key role in the aetiology of schizophrenia.

On the other hand, a persistent (chronic) infection as aetiological factor in schizophrenia is discussed since many years. Signs of inflammation were observed in schizophrenic brains (Körschenhausen et al., 1996) and the term ‘mild localized chronic encephalitis’ was proposed (Bechter et al., 2003). Following the hypothesis of an ongoing immune process, it would be expected that the brain volume reductions, regularly found in schizophrenia, are not only due to a neurodevelopmental disturbance (Jakob and Beckmann, 1986), but also directly preceding the first episode of schizophrenia and, being further progressive. In fact, the Edinburgh High Risk Study, recently showed that a marked reduction of the inferior temporal gyrus over the time preceded the first onset of schizophrenia (Job et al., 2006) and the progressive loss of brain volume has repeatedly been demonstrated (e.g. Chakos et al., 2005).

Due to the characteristics of infectious agents, there are difficulties in proving a localized infection in the brain. A virus or other intracellular infectious agent may be silently hidden in cells of the lymphoid or the nervous system and exacerbate under certain conditions, such as stress. The estimation of serum-antibody-titers is a method with limited sensitivity for a localized mild infectious process in the CNS. Nevertheless, antibody titers against viruses have been examined in the sera of schizophrenic patients for

many years (Yolken and Torrey, 1995). The results, however, were inconsistent, beneath others due to the fact that interfering factors were not controlled. Antibody levels are associated with the medication state, this finding partly explaining earlier controversial results (Leweke et al., 2004).

There is major evidence supporting pre- or perinatal exposure to infection as a risk factor for developing schizophrenia, with main focal points on the influenza, rubella, measles, and herpes simplex viruses (Pearce, 2001). Moreover, viral infections during childhood (Koponen et al., 2004) and even preceding the onset of the illness have been associated with schizophrenia (Leweke et al., 2004). The levels of the pro-inflammatory cytokine IL-8 were increased during the second trimenon of pregnancy in the serum of those mothers, whose offsprings developed schizophrenia later, i.e. increased IL-8 levels were associated with an increased risk for schizophrenia in the offspring (Brown et al., 2004).

Recent research points out that not one single pathogen but the immune response of the mother is related to the increased risk for schizophrenia (Zuckerman and Weiner, 2005).

Polarized type-1 and type-2 immune responses

The cellular arm of the adaptive immune system is mainly activated by T-helper-1 (Th-1) cytokines like Interleukin-2 (IL-2) and Interferon-γ (IFN-γ). Since not only T-helper cells (CD^{4+} cells) but also monocytes/macrophages (M1) and other cell-types produce these cytokines, the immune response is called type-1 immune response. The humoral arm of the adaptive immune system is mainly activated via the type-2 immune response. T-helper-2 cells (Th-2) or monocytes/macrophages (M2) produce IL-4, IL-10, and IL-13 (Mills et al., 2000). Other pro-inflammatory cytokines such as tumor-necrosis-factor-α (TNF-α) and IL-6 are primarily secreted from monocytes and macrophages. While TNF-α is a ubiquitious cytokine, mainly activating the cellular immune response, IL-6 activates the type-2 response including the antibody production.

The type-1 system promotes the cell-mediated immune responses against intracellular pathogens, whereas the type-2 response helps B-cell maturation and promotes the humoral immune responses against extracellular pathogens. Type-1 and type-2 cytokines antagonize each other in promoting their own type of response, while suppressing the immune response of the other.

Table 1. *Overview of the cytokines of the polarized immune response*

	Type-1	Type-2
Cytokines	IL-2	
	IL-12	IL-4
	IFN-γ	IL-13
	IL-18	[IL-10]
	(TNF-α)	

Table 2. *Cellular sources of the polarized immune response. Type-1 cytokines are released from T-helper-1 cells, macrophages type-1, and microglia. Type-2 cytokines are released from T-helper-2 cells, monocytes/macrophages type-2 and from astrocytes*

Source	Type-1	Type-2
Blood and lymphatic organs	Monocytes/ Macrophages Type-1 (M1)	Monocytes/ Macrophages Type-2 (M2)
T-helper cells	CD^{4+}(Th-1)	CD^{4+} (Th-2)
CNS	[Microglia]	[Astrocytes]

Reduced type-1 immune response in schizophrenia

A well established finding in schizophrenia is the decreased in vitro production of IL-2 and IFN-γ (Wilke et al., 1996; Müller et al., 2000), indicating a blunted production of type-1 cytokines. Additionally, decreased levels of IFN-γ in the peripheral blood of schizophrenic patients have been described by several groups (Avgustin et al., 2005; Chiang et al., 2004). Although some authors argued that the blunted in vitro production of IL-2 and IFN-γ may indicate an exhaustion of the immune cells due to enhanced in vivo production of these cytokines (Rothermundt et al., 2001), several other immunological data point to a reduced activation of the cellular immune system in schizophrenia. The decreased response of lymphocytes after stimulation with specific antigens reflect a reduced capacity for a type-1

Table 3. *Findings of the type-1/type-2 immune response in schizophrenia (adapted from Schwarz et al., 2001). In schizophrenia, type-1 cytokine production is decreased and type-2 production relatively increased.* ↑ *increase* ↓ *decrease* ↔ *no change (*↑↑ ↓↓ *replicated)*

Site of cytokine expression	Type-1	Type-2
In vitro production	IFN-γ ↓↓	IL-10 ↑
	IL-2 ↓↓	IL-3 ↑
Peripheral levels	IFN-γ ↔↓	IgE ↑↑
	IL-2 ↔	antibodies against several
	sIL-2R ↑↑	antigens ↑↑
	sICAM-1 ↓	IL-6 ↑↑
	Neopterin ↓	After remission: IL-6 ↓↓
	TNF-Receptor ↓	
CSF levels	IL-2 ↓↑	IgG ↑
	IFN-γ↓	IL-4↑
	sICAM-1 ↓	TGF-β1 ↔
		TGF-β2 ↔

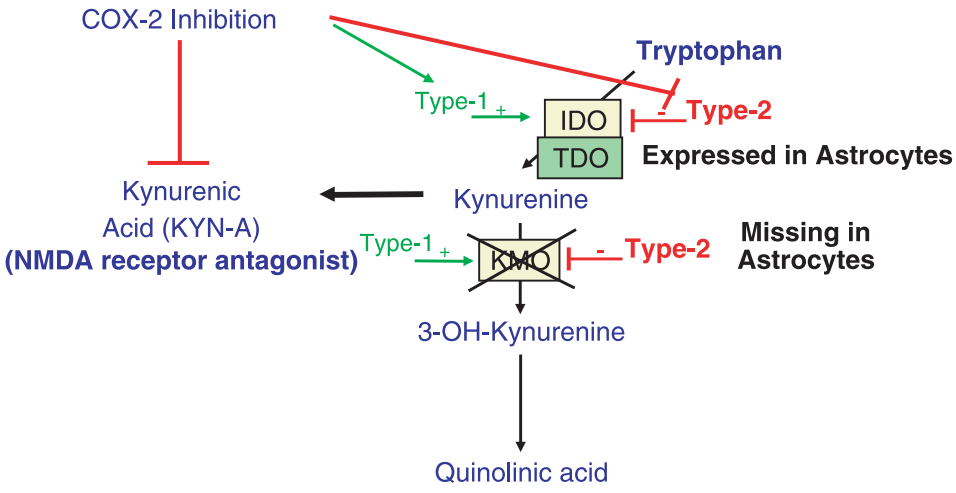

Fig. 1. Metabolization pathways form tryptophane/kynurenine to KYNA and quinolinic acid. IDO and KMO are activated by type-1 cytokines and inhibited by type-2 cytokines. TDO is expressed in astrocytes, KMO is missing in astrocytes. KYN-A decreases during COX-2 inhibition

immune response in schizophrenia, as well (Müller et al., 1991). Decreased levels of neopterin, a product of activated monocytes/macrophages (Sperner-Unterweger et al., 1999), as well as decreased levels of soluble(s) ICAM-1 represent an under-activation of the type-1 immune system (Schwarz et al., 2000).

A blunted response of the skin to different antigens in schizophrenia was observed before the era of antipsycho-

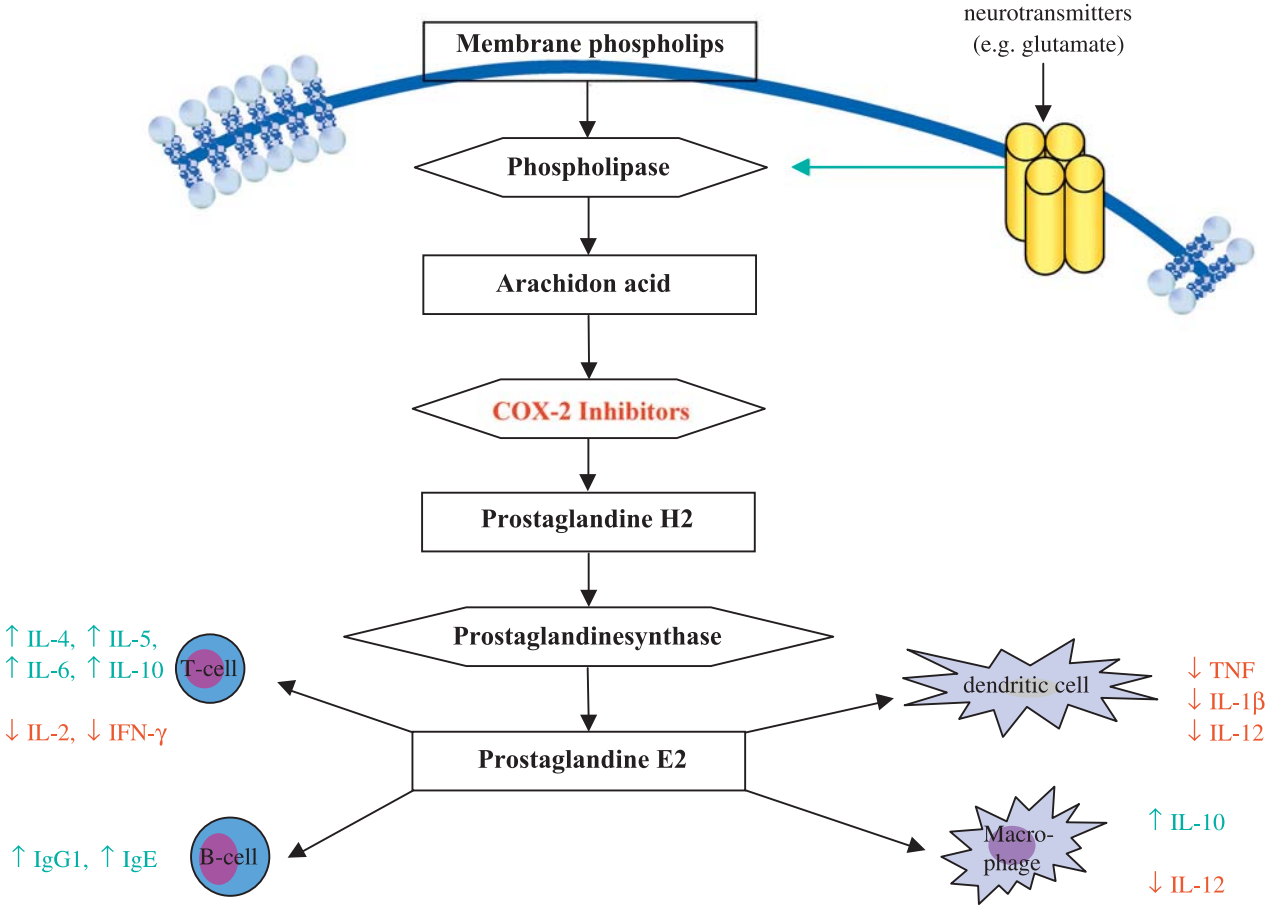

Fig. 2. Arachidon acid metabolism and relationship to cyclo-oxygenase-2, prostaglandine E2, and immune function

tics (Molholm, 1942). A study using a skin test of the cellular immune response (Multitest Merieux) in unmedicated schizophrenic patients also showed a decreased reaction (Riedel et al., 2006).

Increased type-2 immune response in schizophrenia

Several reports described increased serum IL-6 levels in schizophrenia. IL-6 serum levels might be especially high in patients with an unfavourable course of the disease (Müller et al., 2000). IL-6 is a product of activated monocytes and of the activation of the type-2 immune response. Moreover, several other signs of activation of the type-2 immune response are described in schizophrenia, including the increased production of IgE (Schwarz et al., 2001) and an increase of IL-10 serum levels (Cazzullo et al., 1998). IL-10 levels in the CSF are related to the severity of the psychosis (van Kammen et al., 1997).

A lot of studies described an increased antibody production – reflecting type-2 activation – in schizophrenic patients, those observations leading to the discussion of an autoimmune origin of schizophrenia (Ganguli et al., 1987). Although findings have repeatedly shown that about 20–35% of schizophrenic patients show features of an autoimmune process (Müller and Ackenheil, 1998), the role of actual or former therapy with neuroleptics may not have been enough taken into consideration. An increase of IgG-antibodies are mainly IgG-antibodies – in the CSF has been described especially in patients with predominant negative symptoms (Müller and Ackenheil, 1995). Increased antibodies against heat shock protein 60 are one of the recent interesting findings in schizophrenia, because it may reflect a mechanism of loss of neuronal protection (Schwarz et al., 1999; Kilidireas et al., 1992).

The key-cytokine for the type-2 immune response is IL-4. Increased levels of IL-4 in the CSF of juvenile schizophrenic patients have recently been reported (Mittleman et al., 1997). The CSF findings point out that the increased type-2 response in schizophrenia is not only a phenomenon observed in the peripheral immune system.

Given the heterogeneity of the schizophrenic syndrome, the heterogeneity and high variability of the immune system, methodological problems in the determination of immune variables, in particular of circulating cytokines, and the multiplicity of interfering variables, it is not astonishing that the results of immunological studies in schizophrenia in part also show diverging results (Rapaport and Müller, 2001; Kim et al., 2002). The crucial role of the interfering antipsychotic medication for the outcome of immune variables, which is not regarded in several studies, is discussed elsewhere (Müller et al., 2000; Schuld et al., 2004; Schwarz et al., 2001).

Anti-psychotic drugs rebalance the type-1/type-2 imbalance

In vitro studies show that the blunted IFN-γ production becomes normalized after therapy with neuroleptics (Wilke et al., 1996). An increase of $CD^{4+}CD45RO^{+}$ cells ('memory cells') – one of the main sources of IFN-γ production – during anti-psychotic therapy with neuroleptics was observed by different groups (Müller et al., 1997b). Additionally, an increase of soluble IL-2 receptors (sIL-2R) – the increase reflects an increase of activated, IL-2 bearing T-cells – during anti-psychotic treatment was described (Müller et al., 1997a). Reduced sICAM-1 levels show a significant increase during short term anti-psychotic therapy (Schwarz et al., 2000) and the leucocyte function antigen-1 (LFA-1), the ligand of ICAM-1, shows a significantly increased expression during anti-psychotic therapy (Müller et al., 1999). Moreover, the blunted reaction to vaccination with salmonella typhii was not observed in patients medicated with anti-psychotics (Ozek et al., 1971). Recently, an elevation of IL-18 serum levels was described in medicated schizophrenics (Tanaka et al., 2000). Since IL-18 plays a pivotal role in the type-1 immune response, this finding is consistent with other descriptions of type-1 activation during antipsychotic treatment.

Regarding the type-2 response, several studies point out that anti-psychotic therapy is accompanied by a functional decrease of the IL-6 system (Maes et al., 1997; Müller et al., 2000).

However, there are also several studies, indicating an anti-inflammatory effect of antipsychotic drugs (for review see Drzyzga et al. (2006).

Type-1–type-2 immune response imbalance in schizophrenia promote the production of the endogenous NMDA receptor antagonist kynurenic acid

The only known naturally occurring NMDA receptor antagonist in the human CNS is KYN-A (Stone, 1993). KYN-A is one of the three neuroactive intermediate products of the kynurenine pathway. Kynurenine (KYN) is the primary major degradation product of tryptophan (TRP). While the excitatory KYN metabolites 3-hydroxykynurenine (3HK) and quinolinic acid are synthesized from KYN en route to NAD, kynurenic acid (KYN-A) is formed in a dead end side arm of the pathway (Schwarcz and Pellicciari, 2002).

KYN-A acts both, as a blocker of the glycine coagonist site of the NMDA receptor (Kessler et al., 1989) and as a noncompetitive inhibitor of the α7 nicotinic acetylcholine receptor (Hilmas et al., 2001).

The production of KYN-A is regulated by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). Both enzymes catalyze the first step in the pathway, the degradation from tryptophan to kynurenine. Type-1 cytokines, such as IFN-γ and IL-2 stimulate the activity of IDO, while type-2 cytokines like IL-4 and IL-10 are IDO inhibitors (Grohmann et al., 2003).

There is a mutual inhibitory effect of TDO and IDO: a decrease in TDO activity occurs concomitantly with IDO induction, resulting in a coordinate shift in the site (and cell types) of tryptophan degradation (Takikawa et al., 1986). While it has been known for a long time that IDO is expressed in different types of CNS cells, TDO was thought to be restricted to liver tissue for many years. It is known today, however, that TDO is also expressed in CNS cells including neurons and astrocytes (Miller et al., 2004).

The type-2 or Th-2 shift in schizophrenia may result in two functional consequences:

The expression of IDO, normally increased by type-1 cytokines, in particular INF-γ, is down-regulated, while TDO is up-regulated. Thus, the type-1/type-2 imbalance is associated with the IDO/TDO imbalance. Second, the encymatic imbalance between IDO and TDO is related to the activation of astrocytes, which in turn are involved in the type-1/type-2 related astrocyte/microglial imbalance (Aloisi et al., 2000).

The functional overweight of astrocytes leads to further accumulation of the end product KYN-A. Indeed, a study referring to the expression of IDO and TDO in schizophrenia showed exactly these results. An increased expression of TDO compared to IDO was observed in schizophrenic patients and the increased TDO expression was found, as expected, in astrocytes, not in microglial cells (Miller et al., 2004, 2006).

Astrocytes, microglia, and type-1/type-2 response

The cellular sources for the polarized immune response in the CNS are astrocytes and microglia cells. Microglial cells, deriving from peripheral macrophages, secrete preferably type-1 cytokines such as IL-12, while astrocytes inhibit the production of IL-12 and ICAM-1 and secrete the type-2 cytokine IL-10 (Xiao and Link, 1999; Aloisi et al., 2000).

In the CNS, the type-1/type-2 imbalance seems to be represented by an imbalance in the activation of microglial cells and astrocytes. The view of an over-activation of astrocytes in schizophrenia is supported by the finding of increased levels of S100B – a marker of astrocyte activation of the medication state (Rothermundt et al., 2004a, b). Microglia activation, however, was only found in a small percentage of schizophrenics and is discussed to be a medication effect (Bayer et al., 1999). A type-1 immune activation as an effect of neuroleptic treatment has been observed repeatedly.

Cellular source of kynurenic acid in the CNS

Astrocytes play a key role in the production of kynurenic acid in the CNS, because astrocytes are the main source of KYN-A (Heyes et al., 1997). The cellular localization of the kynurenine metabolism is primarily in macrophages and microglial cells, but also in astrocytes (Speciale and Schwarcz, 1993; Kiss et al., 2003).

Interestingly, kynurenine 3-monooxygenase (KMO), a critical enzyme in the kynurenine metabolism, is absent in human astrocytes (Guillemin et al., 2001). Accordingly, it has been described that astrocytes cannot produce the intermediate 3HK but are able to produce large amounts of KYN and KYN-A (Guillemin et al., 2001). This supports the observation that inhibition of KMO leads to an increased KYN-A production in the CNS (Chiarugi et al., 1996). The complete metabolism of kynurenine to quinolinic acid is observed only in microglial cells, not in astrocytes. Due to the lack of KMO, KYN-A accumulates in astrocytes.

A second key-player in the metabolizing of 3-HK are monocytic cells infiltrating the CNS. Monocytes are responsible for the conversion of astrocytic produced KYN to quinolinic acid (Guillemin et al., 2001). However, the low levels of sICAM-1 (ICAM-1 is the molecule that mainly mediates the penetration of monocytes and lymphocytes into the CNS) in the serum and in the CSF of non-medicated schizophrenic patients (Schwarz et al., 1998, 2000) and the increase of adhesion molecules during antipsychotic therapy indicate that the penetration of monocytes may be reduced in nonmedicated schizophrenic patients (Müller et al., 1999).

The possible role of kynurenic acid in schizophrenia

The accumulation of KYN-A may lead to schizophrenic symptoms (Erhardt et al., 2003). Accordingly, increased levels of KYN-A have been observed in the CSF of schizophrenic patients (Erhardt et al., 2001a). Since most of the patients in this study were drug-naive first-episode patients,

this increase could not be caused by antipsychotic treatment. At any rate, chronic drug treatment with antipsychotics does not result in an increase, but rather in a decrease of KYN-A (Ceresoli-Borroni et al., 2006).

An investigation of CNS tissue specimens in different cortical regions revealed increased KYN-A levels in schizophrenics compared to a control sample, particularly in the prefrontal cortex (Schwarcz et al., 2001). In the amygdala, a small but not significant increase of KYN-A in medicated schizophrenics was observed (Miller et al., 2006). The prefrontal cortex is an area involved in the pathophysiology of schizophrenia (Andreasen et al., 1992).

In recent years, drugs acting as elevators of endogenous KYN-A in the CNS have been identified. One of these substances is PNU 156561A, an inhibitor of KMO. This substance enables studies of the effects of increased endogenous KYN-A levels in animals (Speciale et al., 1996). The effects were similar to the effects observed after administration of MK-801 or PCP: In particular, dopaminergic neurons in the midbrain showed an increased activity (Erhardt et al., 2001b).

Clozapine, however, has modulating, in higher doses inhibitory effects on the activity of dopaminergic neurons in the midbrain, which is mediated by the glycine-site of the NMDA receptor (Schwieler et al., 2004). This inhibitory effect of clozapine may account for its beneficial effects in ameliorating symptoms of schizophrenia.

Beside the effects on the NMDA-receptor, KYN-A is also a potent antagonist of the $\alpha 7$ nicotinic acetylcholine receptors (Hilmas et al., 2001). This antagonism is associated with cognitive impairment. Compared to other schizophrenic symptoms, cognitive decline is a basic disturbance in schizophrenia (Huber, 1983). The effect on acetylcholine receptors is effective already at lower concentrations of KYN-A compared to the antagonism to the NMDA receptor; the affinity of KYN-A to the $\alpha 7$ nicotinic acetylcholine receptor is about twice as high compared to the NMDA receptor (Hilmas et al., 2001). This finding indicates that the impairment of cognitive functions is induced by lower concentrations of KYN-A, while psychotic symptoms appear only at higher concentrations of KYN-A. This view fits with the earlier onset of cognitive disturbance in schizophrenia compared to the acute psychotic symptoms.

It was suggested that increased intracerebral KYN-A levels should be related to an enhanced dopaminergic neurotransmission. A recent study published by Robert Schwarcz's group, however, demonstrated that in the striatum, KYN-A significantly *inhibits* dopamine release (Wu et al., 2006). This effect is mediated through the $\alpha 7$ nicotinic ACh receptor antagonism, while the NMDA receptor was obviously not involved in this effect. The authors proved this effect for both, acute and chronic (knock-out mice) modulation of KYN-A levels. Since this effect has specifically been demonstrated for the striatum, it remains to be investigated if KYN-A has the same effect in other brain regions.

COX-2 inhibitors inhibit the production of kynurenic acid, rebalance the type-1/type-2 immune response, and have therapeutic effects in early stages of schizophrenia

Additionally to the above described immunological mechanism, selective cyclooxygenase-2 (COX-2) inhibitors reduce the KYN-A levels by a prostaglandin-mediated mechanism (Schwieler et al., 2005). COX-inhibition provokes differential effects on the kynurenine metabolism: while COX-1 inhibitors increase the levels of KYN-A, COX-2 inhibitors decrease them. Therefore, psychotic symptoms and cognitive dysfunctions, observed during therapy with COX-1 inhibitors, were assigned to the COX-1 mediated increase of KYN-A (Schwieler et al., 2005). The balance between KYN-A and quinolinic acid – as the balance between the type-1 and type-2 immune responses – seems to be crucial not only in schizophrenia, as indicated by a reduced KYN-A/quinolinic acid ratio in Huntington's disease (Stoy et al., 2005; Guidetti et al., 2004).

Recently, prostaglandin E2 (PGE2) has been shown to enhance the production of type-2 cytokines such as IL-4, IL-5, IL-6, and IL-10; PGE2 also drastically inhibits the production of the type-1 cytokines IFN-γ, IL-2, and IL-12 (Stolina et al., 2000). Therefore, inhibition of PGE2 synthesis is hypothesized to be beneficial in the treatment of disorders with dysregulated T-helper cell responses (Harris et al., 2002).

One class of modern drugs is well known to induce a shift from the type-1 like to a type-2 dominated immune response: the selective COX-2 inhibitors. Several studies demonstrated the type-2 inducing effect of PGE2 – the major product of COX-2, while inhibition of COX-2 is accompanied by inhibition of type-2 cytokines and induction of type-1 cytokines (Pyeon et al., 2000; Stolina et al., 2000). PGE2 levels in schizophrenia are not well studied; increased levels of PGE2, however, have been described (Kaiya et al., 1989). PGE2 induces the production of IL-6, a cytokine which is consistently described to be increased in schizophrenia. COX-2 inhibition seems to balance the type-1/type-2 immune response by inhibition of IL-6, PGE2, and by stimulating the type-1 immune response (Litherland et al., 1999). Moreover, an increased COX-2 expression was found

in schizophrenia (Das and Khan, 1998), although controversial data have recently been published (Yokota et al., 2004). Therefore COX-2 inhibition seems to be a promising approach in the therapy of schizophrenia.

In a prospective, randomized, double-blind study of therapy with the COX-2 inhibitor celecoxib add-on to risperidone in acute exacerbation of schizophrenia, a therapeutic effect of celecoxib was observed (Müller et al., 2002). Immunologically, an increase of the type-1 immune response was found in the celecoxib treatment group (Müller et al., 2004b). The clinical effect of COX-2 inhibition was especially pronounced regarding cognition in schizophrenia (Müller et al., 2005). The finding of a clinical advantage of COX-2 inhibition, however, could not be replicated in a second study. Further analysis of the data revealed that the outcome depends on the duration of the disease (Müller et al., 2004b). The efficacy of therapy with a COX-2 inhibitor seems most pronounced in the first years of the schizophrenic disease process. This observation is in accordance with results from animal studies showing that the effects of COX-2 inhibition on cytokines, hormones, and particularly on behavioural symptoms are dependent on the duration of the preceding changes and the time-point of application of the COX-2 inhibitor (Casolini et al., 2002). Thus, a point of no return for therapeutic effects regarding the pathological changes during an inflammatory process has to be postulated.

Regarding the role of the inflammatory process in schizophrenia and possibly other psychiatric disorders, anti-inflammatory therapy should be taken into the focus of further research (Müller et al., 2004a); COX-2 inhibition is one option among others. Therapeutic research, however, has to consider different levels and different mechanisms for therapeutic targets in the neuroimmune system and the dopaminergic-glutamatergic neurotransmission circuits including the kynurenine pathway of the tryptophan metabolism.

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J Neural Transm (2007) [Suppl 72]: 281–285

Printed in Austria

Journal of Neural Transmission

Deficits of neuronal glutamatergic markers in the caudate nucleus in schizophrenia

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Summary Abnormal glutamate neurotransmission has been implicated in the pathophysiology of schizophrenia. In the present study we investigated two potential neuronal glutamatergic markers, the Excitatory Amino Acid Transporter 3 (EAAT3) and the Vesicular Glutamate Transporter 1 (VGluT1), in post-mortem striatal tissue from control subjects and from subjects with schizophrenia ($n = 15$ per group). We also investigated the possible influence of chronic antipsychotic administration (typical and atypical) on striatal VGluT1 expression in the rat brain. We found deficits in EAAT3 in all striatal regions examined in schizophrenia when compared to controls. Following correction for confounding factors (post-mortem interval), these deficits only remained significant in the caudate nucleus ($p = 0.019$). We also found significant deficits in VGluT1 in the caudate nucleus ($p = 0.009$) in schizophrenia. There were no significant differences in VGluT1 in the striatum of antipsychotic treated rats when compared to their vehicle treated controls.

The data provides additional evidence for a glutamatergic synaptic pathology in the caudate nucleus in schizophrenia and may reflect a loss of glutamatergic cortico-striatal pathways. The absence of an effect of antipsychotic administration on VGluT1 indicates that the deficits in schizophrenia are unlikely to be a consequence of pharmacotherapy and thus likely to be a correlate of the disease process.

Keywords: Caudate nucleus, excitatory amino acid transporter, vesicular glutamate transporter, antipsychotics, schizophrenia

Introduction

Glutamate is the most abundant amino acid in the central nervous system, where it plays a role as the major excitatory neurotransmitter in the brain. Riederer et al. (1991) were among the first to develop a rational argument for a role for glutamatergic hypofunction in schizophrenia, based on the functional importance of the interaction between glutamatergic and dopaminergic innervation of the basal ganglia (Carlsson and Carlsson, 1990). Others have reviewed more recently the suggestion that a reduction of glutamatergic neurotransmission may be involved in the pathophysiology of schizophrenia (Goff and Coyle, 2001; Tamminga et al., 2003). This hypothesis arose in part from the observation that phencyclidine (PCP), a non-competitive antagonist at the glutamate N-methyl-D-aspartate (NMDA) receptor, can induce a schizophrenia-like psychosis including both the positive (e.g., hallucinations, paranoia) and negative (e.g., emotional withdrawal, motor retardation) symptoms in otherwise healthy individuals (Javitt and Zukin, 1991). PCP given either acutely or chronically to animals can also mimic certain aspects of the disorder, while chronic administration can result in enduring pathological features which has led to the suggestion that NMDA-receptor (glutamatergic) hypofunction might be implicated in schizophrenia and may contribute to the cognitive dysfunction which characterizes the disorder (Olney and Farber, 1995). These pathological changes include deficits in specific GABAergic markers (Abdul-Monim et al., 2007; Reynolds et al., 2004) and cognitive deficits with relevance to the disease (Abdul-Monim et al., 2006).

There are several observations implicating abnormalities of glutamate in the brain in schizophrenia. In a previous study we demonstrated an increase in the density of NMDA receptors in the putamen from schizophrenic patients when compared to controls (Aparicio-Legarza et al., 1998). Additionally, increased AMPA receptor ligand binding was found in the caudate nucleus in schizophrenia (Kornhuber et al., 1989). Such increases in glutamate receptor densities have been interpreted as compensatory and due to a glutamatergic hypofunction. Further evidence indicating a deficit of cortico-striatal glutamatergic innervation

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in subcortical regions comes from another study where we identified a deficit in the number of neuronal glutamate uptake sites in striatal regions in schizophrenia (Aparicio-Legarza et al., 1997). These alterations in indicators of glutamate systems in the striatum suggest that other components of the glutamatergic synapse may be abnormal in this region in schizophrenia. Two such components are the excitatory amino acid transporter 3 (EAAT3) and the vesicular glutamate transporter 1 (VGluT1).

Excitatory amino acid transporters (EAATs) are a group of molecules essential for normal glutamatergic neurotransmission. They are Na^+ -dependent glutamate transporters located on the plasma membranes of neurons and glial cells, where their function is to rapidly terminate the action of glutamate, thus maintaining its extracellular concentrations below excitotoxic levels. EAATs have specific patterns of cellular localization: EAAT1 and EAAT2 have been localized to astroglia, whereas EAAT3 and EAAT4 have been localized to neurons (Shigeri et al., 2004). EAAT3 is localized to both post- and presynaptic neuronal soma and is responsible for up to 40% of glutamate transport in the rat brain (Rothstein et al., 1994).

Due to their highly selective expression in excitatory presynaptic terminals, vesicular glutamate transporters (VGluTs) are potentially valuable markers of glutamatergic terminals in brain tissue. VGluTs are glutamate transporters localized on the membrane of synaptic vesicles in the presynaptic neuron. To date three subtypes of VGluTs have been identified. VGluT1 is distributed throughout the cerebral cortex, striatum and hippocampus while VGluT2 and VGluT3 are found in subcortical structures and in some serotonergic and cholinergic neurons respectively (Fremeau et al., 2004). The function of these transporters is to load glutamate into synaptic vesicles in the presynaptic terminal where it is stored until it is released into the synaptic cleft by exocytosis. VGluT1, in particular, is exclusive to glutamatergic terminals, and undetectable in other cell types and neuronal compartments (Bellocchio et al., 1998; Fremeau et al., 2001), and as such provides a very specific and selective marker for presynaptic glutamatergic terminals in the brain.

We have investigated glutamatergic innervation by determining EAAT3 and VGluT1 immunoreactivity in brain regions taken post mortem from psychiatric patients and control subjects. We report here the results of these studies in striatal regions (caudate nucleus, putamen and nucleus accumbens) from patients with schizophrenia and matched controls. We have also investigated the possible influence of chronic antipsychotic drug administration on striatal VGluT1 expression in the rat brain.

Material and methods

Subjects

Postmortem brains from the Stanley Foundation Neuropathology Consortium were studied. Two groups of 15 subjects, with a diagnoses of schizophrenia and a control group were used (tissue is part of a larger collection which also includes subjects with depression and bipolar disorder). A summary of the subjects characteristics are presented in Table 1 (for more details see Torrey et al., 2000). Cryostat-sectioned slides (14 μm) from fresh frozen brain hemispheres were stored at −70°C until used. Sections were processed simultaneously for both groups. Tissue specimen selection has been described previously (Zhang and Reynolds, 2002).

Animal study

Male Sprague-Dawley rats (275–330 g) were housed 2–4 per cage and handled daily for 1 week prior to treatment. Haloperidol and clozapine purchased from Sigma-RBI (Poole, Dorset, UK) were each dissolved in a minimal volume of 0.1 M tartaric acid, diluted in Dulbeccos phosphate buffered saline (DPBS) and buffered to pH 6 with 0.1 N NaOH before dilution to volume with DPBS. Three groups of six weight-matched rats received single daily intraperitoneal (i.p.) injections of haloperidol (1.5 mg/kg), clozapine (25 mg/kg) or an equivalent volume of vehicle (2.5 ml/kg), for 21 consecutive days. All treatment procedures were in accordance with the UK Home Office Animals Act (1996).

Tissue preparation and immunohistochemistry

Human striatal frozen sections

Frozen sections were fixed for 2 h by immersion in 4% formaldehyde in 90% ethanol at −20°C. Sections were incubated for 30 mins in a hydrogen peroxide solution to inhibit endogenous peroxidase activity. Non-specific binding was minimized by incubation for 1 h in 5% normal rabbit (EAAT3) or 2% normal goat (VGluT1) serum. Sections were incubated overnight at 4°C with a polyclonal antibody against the neuronal glutamate transporter (EAAC1/EAAT3) at a dilution of 1:1000; or a polyclonal antibody against vesicular glutamate transporter type 1 (VGluT1) at a dilution of 1:5000 (both Chemicon International Inc., Temecula, CA) in protein blocking solution. The sections were washed before incubation for 2 h at room temperature with the appropriate biotinylated secondary antibody (Vector Laboratories) diluted 1:200 in protein blocking solution. This was followed

Table 1. *Characteristics of subjects*

	Schizophrenia	Controls
n	15	15
Sex (M, F)	9, 6	9, 6
Side of brain (R, L)	6, 9	7, 8
Age (years)	44.5 (12.7)	48.1 (10.3)
Range	25–62	29–68
Post-mortem interval (h)	33.7 (14.1)	23.7 (9.6)
Range	12–61	8–42
Brain pH	6.16 (0.25)	6.27 (0.23)
Range	5.8–6.6	5.8–6.6
Onset of psychosis (years)	23.2 (7.7)	–
Range	13–42	–
Duration of illness (years)	21.3 (11.0)	–
Range	5–45	–
Antipsychotic medication[a]	12/15	0/15

[a] Treatment with psychotropic medication within 6 weeks of time of death.

by incubation for 2 h at room temperature with avidin-biotin-peroxidase complex (ABC kit) (Vector Laboratories). Protein immunoreactivity was visualised using the chromogen diaminobenzidine (DAB), intensified with nickel chloride (Vector Laboratories). The sections were dehydrated and mounted. Immunoreactivity was not present in control sections in which the primary antibody was omitted from the staining protocol. All slides were analyzed blind to diagnosis. Human striatum was defined as the caudate nucleus, putamen and nucleus accumbens. Identification of these human brain subfields was based on cresyl violet Nissl staining of adjacent sections for each slide.

Rat striatal paraffin-embedded sections

Rats were sacrificed by deep anaesthesia with pentobarbital followed by transcardial perfusion with 10% formalin/saline. Brains were removed and fixed in 10% phosphate buffered formalin for 3 days before being embedded in wax. Three sections (10 µm) from the striatum (bregma −1 mm), determined using a rat brain atlas (Paxinos and Watson, 1998) from vehicle and antipsychotic-treated rats were mounted onto slides coated with 3-aminopropyltriethoxysilane (APES) (Sigma) and stained for VGluT1. Briefly, following clearing in xylene and rehydration in graded alcohol, sections were incubated in hydrogen peroxide solution to inhibit endogenous peroxidase activity; following this the sections underwent the same procedure described above for the human striatal sections for VGluT1 immunohistochemistry.

Measurement of optical density and statistical analysis

Sections were scanned and analyzed with Scion Image Software based on NIH image (v. beta 3b; www.scioncorp.com; 1998). The densitometry measurements were used to quantify the optical density of EAAT3 and VGluT1 immunoreactivity in the three striatal regions (caudate, putamen, and nucleus accumbens). The optical measurements were made blind to the diagnostic category of the cases. Three to five regions of interest were measured in each of the caudate nucleus and putamen and two regions of interest were applied to nucleus accumbens. The software was used to obtain the integrated optical density of the region. The value is the sum of the optical densities of all pixels in the region divided by number of pixels. Background values were obtained from the sections processed omitting the primary antibody. The average of values from two sections for each subject was used for statistical analysis.

Results

EAAT3 in striatal regions

ANOVA showed significant deficits of EAAT3 optical density in the caudate ($p = 0.003$), putamen ($p = 0.048$) and nucleus accumbens ($p = 0.008$) in schizophrenia when compared to control subjects. Stepwise regression analyses was undertaken in order to investigate the effects of potential confounding variables i.e. age, post-mortem interval (PMI), brain pH, brain weight and storage time on the optical density of EAAT3 in the striatal regions of both subject groups. These analyses showed an effect of PMI on EAAT3 optical density in the caudate ($p = 0.026$), putamen ($p = 0.033$) and nucleus accumbens ($p = 0.018$). Univariate analysis of variance, with PMI as a covariate, showed that the deficits in EAAT3 in schizophrenia only remained significant in the caudate nucleus ($p = 0.019$) (Fig. 1).

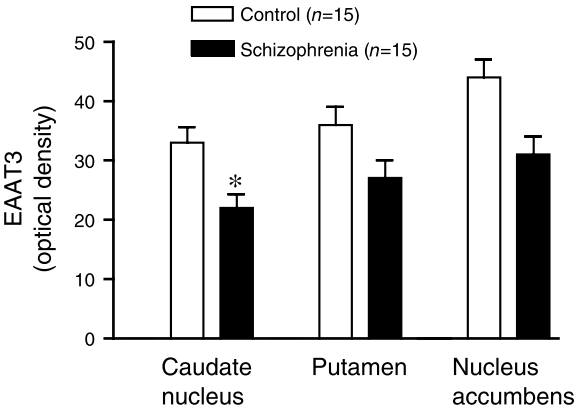

Fig. 1. EAAT3 optical density in the caudate nucleus, putamen and nucleus accumbens of schizophrenia and control subjects. Values represent Mean ± SEM (arbitrary units). * $p < 0.05$

VGluT1 in striatal regions

ANOVA showed significant deficits of VGluT1 optical density in the caudate nucleus ($p = 0.000$), with no significant change in the putamen ($p = 0.104$) or nucleus accumbens ($p = 0.110$), in schizophrenia when compared to control subjects. Stepwise regression analysis was again undertaken in order to assess the effects of potential confounding variables on the optical density of VGluT1 in the caudate nucleus of both subject groups. This showed an effect of storage time on VGluT1 optical density in the caudate nucleus ($p = 0.012$). Following univariate analysis of variance, with storage time as a covariate, these deficits remained significant ($p = 0.009$) (Fig. 2).

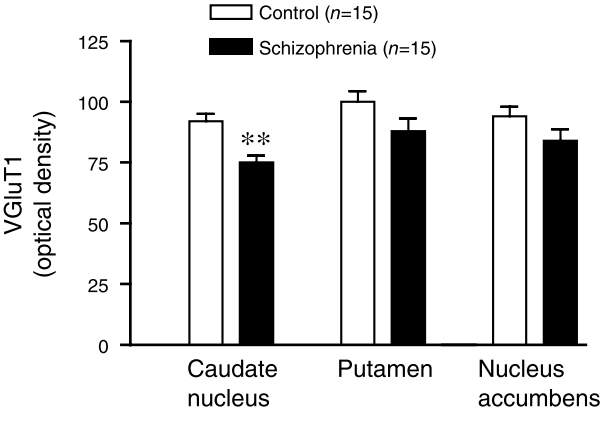

Fig. 2. VGluT1 optical density in the caudate nucleus, putamen and nucleus accumbens of schizophrenia and control subjects. Values represent Mean ± SEM (arbitrary units). ** $p < 0.01$

Table 2. *VGluT1 optical density in the dorsal and ventral striatum of rats following chronic administration of haloperidol or clozapine and in vehicle treated controls. Values represent Mean ± SEM (arbitrary units)*

	Dorsal striatum	Ventral striatum
Vehicle	87 ± 4	85 ± 3
Haloperidol	84 ± 3	83 ± 2
Clozapine	90 ± 3	87 ± 2

Furthermore, chronic administration of haloperidol or clozapine had no significant effect on VGluT1 optical density in the striatum, when compared to vehicle treated animals (Table 2).

Discussion

The main finding of the present study was a deficit in both EAAT3 and VGluT1 immunoreactivity in the caudate nucleus in schizophrenia, providing additional evidence for a glutamatergic synaptic pathology in this region in schizophrenia.

One confounding factor in post mortem studies of neuropsychiatric diseases is the influence of chronic drug treatment. In the present study there were no differences between drug-naïve and drug-treated subjects with schizophrenia. However, the small number (3) of unmedicated subjects at the time of death may be inadequate to draw clear conclusions. Decreased EAAT3 gene expression has been found in nucleus accumbens following haloperidol administration (Schmitt et al., 2003). Therefore, we cannot fully rule out the effect of antipsychotic drugs on the results of EAAT3 immunodensity deficits in schizophrenia. However, the findings from animals administered with haloperidol or clozapine suggest that such changes, in VGluT1 at least, are unlikely to be consequences of chronic treatment with antipsychotic drugs, and thus may relate directly to the disease process.

These findings are consistent with previous studies that have reported deficits in EAAT3 mRNA expression (McCullumsmith and Meador-Woodruff, 2002), as well as decreased saturable [^{3}H] D-aspartate binding (Aparicio-Legarza et al., 1997) in the striatum of subjects with schizophrenia. Further recent studies provide evidence for a disturbance of the presynaptic glutamatergic system in the brain in schizophrenia. Deficits in VGluT1 mRNA have been reported in both the hippocampal formation and in the dorsolateral prefrontal cortex in schizophrenic patients (Eastwood and Harrison, 2005). Deficits of another glutamatergic presynaptic protein, Dysbindin-1, have also been reported in glutamatergic terminals of the hippocampal formation in schizophrenia (Talbot et al., 2004), while cortical deficits in complexin-II expression have also been interpreted as reflecting presynaptic glutamatergic deficits (Eastwood and Harrison, 2005).

Taken together, our findings provide further support for a glutamatergic dysfunction in schizophrenia, with abnormalities involving molecules which are responsible for maintaining appropriate synaptic glutamatergic function in the caudate nucleus.

The striatum receives a large glutamatergic input from the cortex. Non-human primate studies have demonstrated that the motor and somatosensory cortices project massively to the putamen (Kunzle, 1975; Leichnetz, 1986; Alexander and Crutcher, 1990; Takada et al., 1998) while prefrontal areas project mainly to the caudate nucleus (Yeterian and Pandya, 1991; Eblen and Graybiel, 1995). This is in agreement with some human studies showing that prefrontal regions of the cortex send glutamatergic projections to the head of the caudate nucleus (Wiesendanger et al., 2004). Furthermore, the main vesicular glutamate transporter, VGluT1, is found in the terminals of these cortico-striatal projections (Kaneko et al., 2002). Thus the findings of the present study suggest deficits of this cortico-striatal pathway, possibly relating an underlying pathology of the prefrontal cortex. Previous studies have demonstrated structural and functional deficits of the prefrontal cortex in schizophrenic patients (For review see Weinberger et al., 2001). These deficits may produce cognitive impairment but also contribute to secondary dysfunction in subcortical dopaminergic transmission (Laruelle et al., 2003; West et al., 2003). Thus our findings provide further evidence for a disturbance of subcortical glutamatergic innervation in schizophrenia underlying an imbalance between glutamate and dopamine neurotransmission in the basal ganglia (Carlsson and Carlsson, 1990; Riederer et al., 1991).

Acknowledgements

This research was supported in part by a research grant from the Stanley Medical Research Institute to GPR. PP and SNT were supported by Thai Government Scholarships. Postmortem brain specimens were donated by the Stanley Foundation Brain Bank and Neuropathology Consortium.

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J Neural Transm (2007) [Suppl 72]: 287–296

Printed in Austria

Journal of Neural Transmission

Therapeutic drug monitoring of tricyclic antidepressants in everyday clinical practice

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Summary Data about therapeutic drug monitoring (TDM) of psychotropic medications are often obtained from samples of highly selected individuals, who may not be representative for the average psychiatric patient. These data therefore may have limitations with regard to their transferability to everyday clinical practice. Therefore studies under naturalistic conditions are important to clarify the full clinical relevance of TDM. We retrospectively evaluated all TDM-analyses of the tricyclic antidepressants (TCA) amitriptyline and clomipramine during a 12-month period in an unselected sample of patients in a standard clinical setting. We especially examined the relationship between serum levels on one hand and clinical response and adverse effects on the other hand. In patients with amitriptyline, responders showed a significantly higher serum level than non-responders, whereas in patients with clomipramine a serum level within the recommended therapeutic range was associated with clinical response. We also found significantly higher serum concentrations in patients with adverse effects compared to patients without adverse effects in the clomipramine group. No such relationship could be shown in patients treated with amitriptyline. Our results suggest that therapeutic ranges in naturalistic settings in some ways differ from those obtained in controlled clinical settings and that TDM studies in everyday clinical practice are necessary and beneficial.

Keywords: Therapeutic drug monitoring, gender differences, anti-depressants, pharmacokinetic variability, concentration-effect relationship

Introduction

The aim of therapeutic drug monitoring (TDM) of psychotropic drugs is in the first place to optimize the drug treatment of a ''standard'' psychiatric patient under the conditions of everyday clinical practice by controlling the influence of pharmacokinetic factors on the relationship between administered dose and the concentration of the drug at the site of action (Vuille et al., 1991; Laux and Riederer, 1992; Tonkin and Bochner, 1994; Balant-Georgia and Balant, 1995; Hiemke et al., 2000). Pharmacokinetic parameters like the interindividual variability of hepatic metabolism account for an under- or overdosage of psychotropic drugs in 30–50% of all patients even if the recommended oral dose is strictly maintained. TDM has therefore become a widely recommended procedure for many psychopharmacological interventions (Preskorn and Fast, 1991; Mitchell, 2001; Baumann et al., 2004), and could be more and more implemented in everyday clinical practice due to the availability of reliably and rapidly applicable analytical techniques (Hiemke et al., 2003). Especially for the treatment with tricyclic antidepressants (TCAs) several studies could demonstrate the usefulness of TDM resulting in a high level of recommendation for TDM of these drugs (Preskorn and Fast, 1991; Ulrich et al., 2001; Ulrich and Läuter, 2002; Baumann et al., 2004).

However, there are comparatively few data about TDM of these drugs in everyday clinical practice (Hollister, 1982; Rao et al., 1996; Müller et al., 2003), and evaluations of the quality of TDM in routine psychiatric inpatient care identified considerable inadequate use of TDM under these conditions (Mann et al., 2006). Most TDM-data are obtained under the particular conditions of a scientific study in patient samples comprised of highly selected individuals (Hiemke, 1995; Gross, 2001), which means that mostly moderately ill male patients of a younger age without relevant co-morbidity and co-medication are included (Bengtsson, 2006). In contrast, clinical reality demands the treatment of patients of both genders and various ages

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with multiple co-morbidity and co-medication. Therefore the particular conditions and the selected patient samples in a TDM-study often may not be considered as representative for the ''average'' patient in a standard psychiatric setting.

This might account for some limitations regarding existing data about TDM for psychotropic drugs. In particular, there are no conclusive data available on a positive relationship between serum concentration and treatment response under naturalistic conditions, and the association between serum levels and adverse effects also has to be clarified. As a consequence, more studies in a naturalistic clinical setting are needed to evaluate the full spectrum of benefits and problems of TDM which is in the first place an instrument to improve pharmacotherapy in daily practice (Bengtsson, 2006).

To obtain more information on TDM under naturalistic conditions we conducted a retrospective evaluation of all routine TDM-analyses of the TCAs amitriptyline and clomipramine performed in the psychiatric university hospital of Würzburg during a 12-month period. We chose these drugs because the literature shows a good evidence for the usefulness of TDM in these drugs and TCAs are in everyday clinical practice still widely used especially in severely ill hospitalised depressive patients. The aim of this evaluation was to examine the relationship between the serum concentrations of these drugs and their main metabolites, treatment response and adverse effects in a heterogeneous patient sample.

Subjects and methods

Study design, patients and treatment

All TDM-analyses for amitriptyline and clomipramine performed according to the guidelines of the AGNP expert group (Baumann et al., 2004) in the Psychiatric University Hospital of Würzburg during a 12-month period (July 2003 to June 2004) were retrospectively assessed (Table 1). In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered for evaluation to avoid the problem of multiple determinations. The target symptomatology in all cases was ''depression'' or ''anxiety with depression'' independent of the underlying diagnosis, which comprised the whole spectrum of psychiatric disorders from organic disorders (F0) to personality disorders (F6) according to ICD-10 (World Health Organization, 1991). All patients were treated during the entire study according to clinical decisions regarding choice of drug and dosage. No restriction was made with respect to co-medication or concomitant diseases.

A specially designed request form was used to obtain all relevant information regarding the patient, the drug and clinical parameters in a structured and standardised manner. Blood samples for TDM were collected at steady state in the morning before the first daily drug intake approximately 10–12 hours after the last dose. Serum monovettes (7.5 ml) without anticoagulants and additives were used. Serum was obtained by centrifugation at 1800 *g* for 10 min and stored at −20°C until analysis.

Table 1. *Sample characteristics. For the investigation of the relationship between administered dose and serum level all patients irrespective of the underlying diagnosis were included. Examining the relationship between serum levels and treatment response or adverse effects patients with organic, substance-induced and schizophrenic disorders were excluded*

	Amitriptyline group	Clomipramine group
Patients (*N*, total sample)	195	102
Gender (*N*)		
male	84	37
female	111	65
Age (years), mean ± S.D.	44.4 ± 13.96 (range 18–77)	43.4 ± 16.69 (range 18–81)
Dose (mg/day), mean ± S.D.	141 ± 52.9 (range 25–350)	129 ± 58.1 (range 25–300)
Dose-corrected serum concentration (ng/mL/mg), mean ± S.D.	1.57 ± 1.21 (range 0.11–8.02)	3.60 ± 2.26 (range 0.55–16.56)
Smokers (N)	81	41
CGI[a]-item 1, mean ± S.D.	4.7 ± 1.24 (range 2–7)	4.8 ± 1.10 (range 2–7)
Patients with organic (ICD-10 F0), substance induced (ICD-10 F1) or schizophrenic (ICD-10 F2) disorder (*N*)	37	23
Patients with affective (ICD-10 F3), adjustment (ICD-10 F4) or personality (ICD-10 F6) disorder (*N*)	158	79
Responders[b]		
yes (CGI ≤3)	106	30
no (CGI >3)	50	31
missing data	2	18
Adverse effects[b]		
yes (UKU ≥1)	77	22
no (UKU = 0)	66	39
missing data	15	18

[a] *CGI* Clinical global impression.
[b] Only patients with ICD-10-diagnoses F3, F4 or F6 are included.

Assessment of clinical response and side effects

The severity of illness was rated by the attending physician applying the 7-point scale of the item 1 of the "Clinical Global Impression Scale" (CGI; Guy, 1976). The efficacy of the treatment was estimated using the 7-point scale of the CGI-item 3.1. Adverse effects were rated by administering a 4-point global scale for severity of adverse effects as it is defined in the "Udvalg for Kliniske Undersøgelser" (UKU) rating scale (Lingjaerde et al., 1987).

Determination of amitripyline, clomipramine and metabolites

Serum concentrations of amitriptyline, clomipramine and their main metabolites nortriptyline and norclomipramine were analyzed by an isocratic reversed-phase high performance liquid chromatography (HPLC)-method with ultraviolet detection (Agilent LC Systems, Series 1100, Agilent Technologies Inc., Santa Clara, USA). Adsorption of the drugs and metabolites on the solid-phase extraction column (CN 20 µm; 20 × 4 mm; Machery-Nagel, Düren, Germany) was followed by washing unwanted material to waste and subsequent chromatographic separation on the analytical column (Nucleodur 3 µm; 200 × 4 mm; Machery-Nagel, Düren, Germany). After 6 min on the extraction column the sample was transferred to the analytical column by automated column switching. Twelve min later the switching valve was moved back. The column temperature was 40°C, the flow rate 1.25 ml/min. The mobile phases contained 10% acetonitrile + 90% aqua destillata (extraction phase) and 52.5% 10 mM potassium dihydrogenphosphate + 47.5% acetonitrile (analytical phase). A pH-value of 6.4 was adjusted using orthophosphoric-acid. Retention times were as follows: amitriptyline 20.46 min, nortriptyline 18.80 min, clomipramine 23.71 min, norclomipramine 21.37 min. The wavelength for UV-detection was set at 210 nm for the determination of amitriptyline and nortriptyline, and 229 nm for the determination of clomipramine and norclomipramine. Internal quality control samples were integrated in each analytical series.

The absolute extraction recovery for all analysed compounds was 96%. The intra-assay coefficient of variation determined from each 10 samples of 44, 132 and 264 ng/ml of all analysed compounds was 4%. The method was linear in a range of 5–1000 ng/ml ($r = 0.9998$), and the lower limit of detection was 3.0 ng/ml. External quality control samples were analysed monthly (Cardiff Bioanalytical Services, The Cardiff Medic Center, Cardiff, UK) without reject.

Statistical analysis

For the investigation of the relationship between administered dose and serum level all patients irrespective of the underlying diagnosis were included (Table 1). Examining the relationship between serum levels and clinical parameters like treatment response or adverse effects patients with organic, substance-induced and schizophrenic disorders were excluded, since a depressive symptomatology usually represents only a minor element within the full clinical presentation of these disorders and will have a rather small influence on the global estimation of clinical parameters like treatment response. Therefore only patients with the diagnosis of an affective disorder (ICD-10 F3x), neurotic or adjustment disorder (ICD-10 F4x) or personality disorder (ICD-10 F6x) were considered for this purpose.

For statistical analysis, continuous variables like daily doses and serum concentrations were compared between groups with *t*-tests. Correlation coefficients (Spearman-rho) were calculated for the relationship between applied daily doses and serum levels of parent compounds and metabolites. Additionally, the determined serum concentrations were classified dichotomically ("above the upper limit of the recommended therapeutic range" vs. "below the upper limit of the recommended therapeutic range" and "within the therapeutic range" vs. "outside the therapeutic range") on the basis of the recommended therapeutic ranges of the consensus paper of the AGNP-TDM expert group (Baumann et al., 2004). Similarly, the parameters for adverse effects and therapeutic efficacy were transformed into categorical variables. These categorical data were analyzed using chi-square tests. Statistical analysis was performed with the software SPSS, version 14.0. For all statistical analyses, a *P*-value <0.05 was considered statistically significant.

Results

Sample characteristics

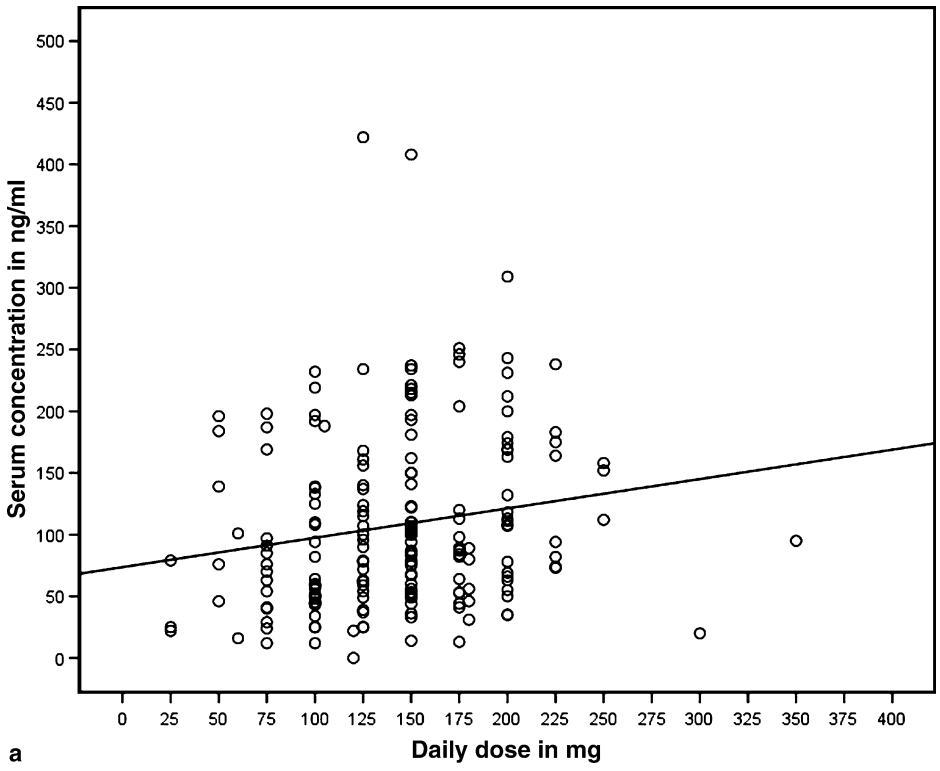
In total, 605 TDM-analyses were carried out in 195 patients treated with amitryptiline, and 313 TDM-analyses in 102 patients treated with clomipramine. A broad range from 1 to 14 TDM-analyses per patient was performed with a mean of 3.1 for both drugs.

The patients characteristics are summarized in Table 1. In both treatment groups more females than males were included (amitryptiline: 43% male, 57% female; clomipramine: 36% male, 64% female). The mean age of approximately 44 years was similar in both groups. Twenty-six (13.3%) of the patients treated with amitriptyline and 19 (18.6%) of the patients treated with clomipramine were older than 60 years. Roughly 41% of included patients were smokers in both treatment groups.

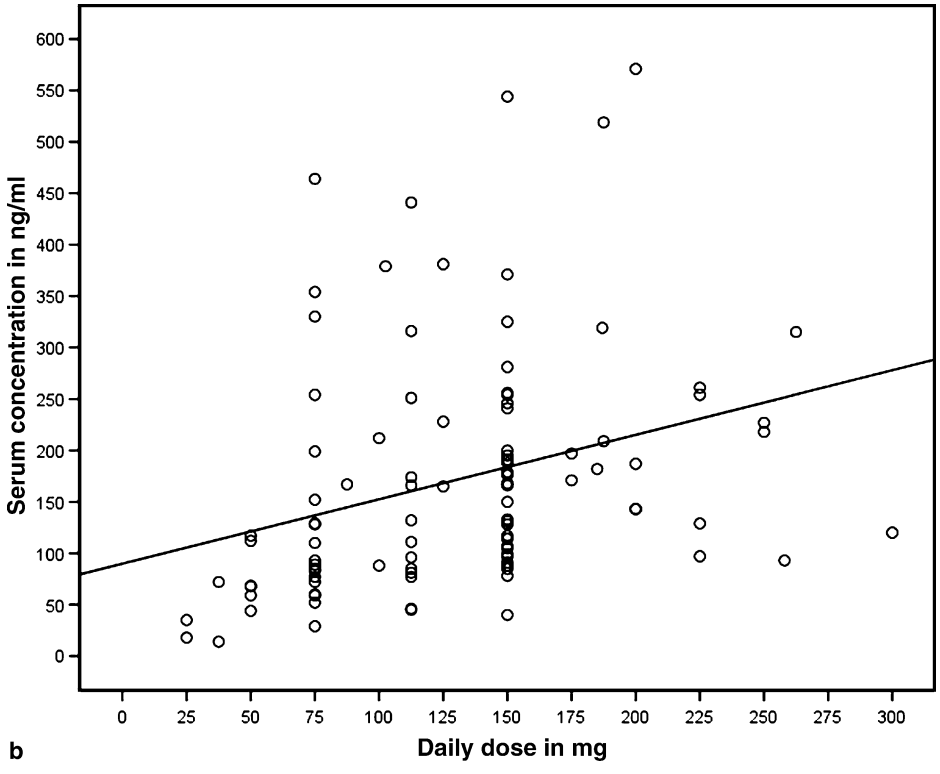
Our naturalistic approach with a sample of mostly severely ill patients entailed a wide variety of non-psychotropic and psychotropic co-medication in the great majority of cases. The psychotropic co-medication comprised mainly benzodiazepines (lorazepam, temazepam), but also various (mostly atypical) antipychotics. Due to the variety of concomitant drugs a separate evaluation with respect to different forms of co-medication was not carried out.

Relationship between dose and serum concentration

Serum concentrations and dose of amitriptyline and clomipramine were not normally distributed; thus the Spearman correlation coefficient was computed to assess their relationship. We found a weak correlation between dose and serum concentration for the parent compounds (Fig. 1; amitriptyline: $r_s = 0.240$; $p < 0.01$, clomipramine: $r_s = 0.462$; $p < 0.01$), the main metabolites (Fig. 2; nortriptyline: $r_s = 0.246$; $p < 0.01$, norclomipramine: $r_s = 0.483$; $p < 0.01$), and the sum of parent compounds and the respective main metabolite (Fig. 3; amitriptyline + nortriptyline: $r_s = 0.258$; $p < 0.01$, clomipramine + norclomipramine: $r_s = 0.509$; $p < 0.01$). There was a marked variability of the serum concentrations, ranging from 14–829 ng/ml (amitriptyline + nortriptyline) and 48–1588 ng/ml (clomipramine + norclomipramine; Fig. 3).

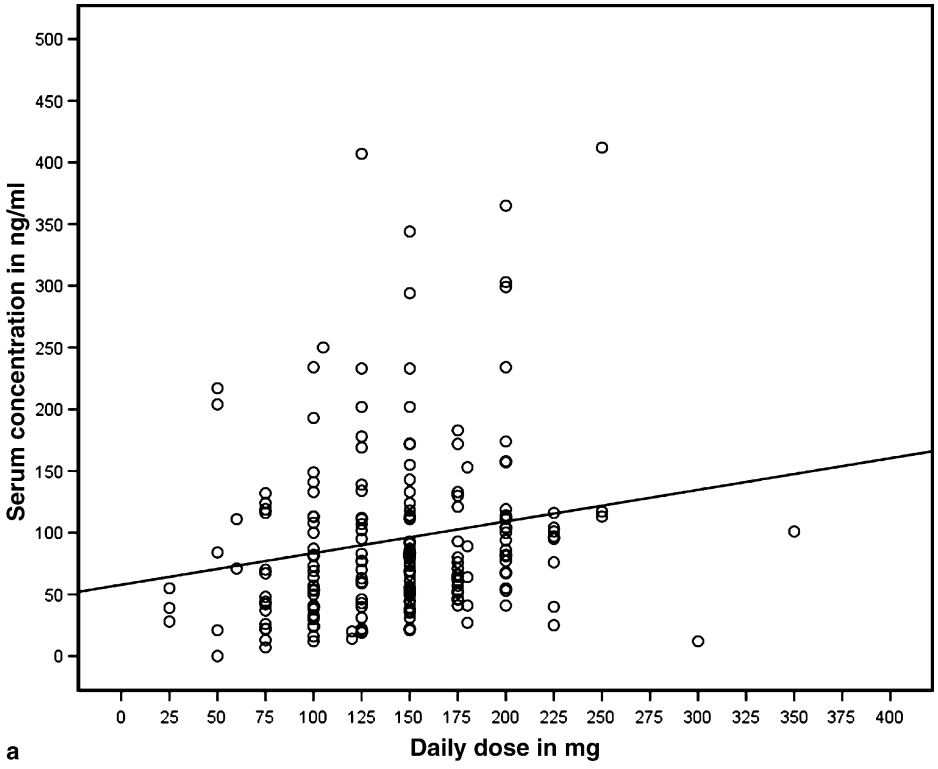


a

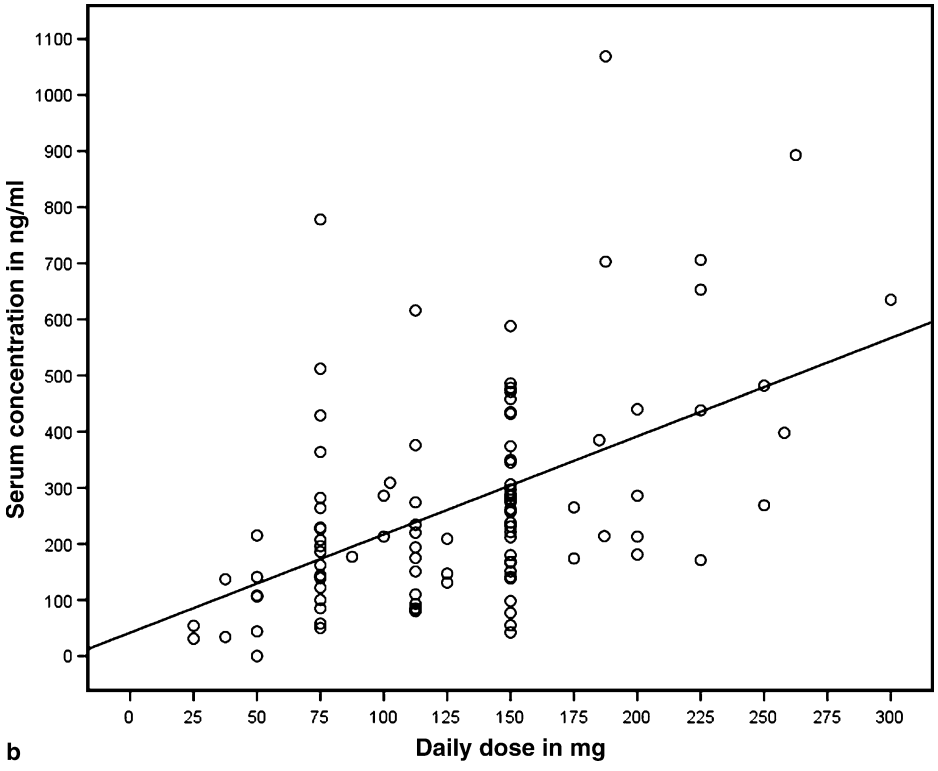


b

Fig. 1. Correlation (Spearman-rho) between daily doses of amitriptyline (**a**), clomipramine (**b**), and the respective trough serum concentrations of the parent compounds. All patients irrespective of the underlying diagnosis were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered



a



b

Fig. 2. Correlation (Spearman-rho) between daily doses of amitriptyline (**a**), clomipramine (**b**), and the respective trough serum concentrations of their metabolites nortriptyline and norclomipramine. All patients irrespective of the underlying diagnosis were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered

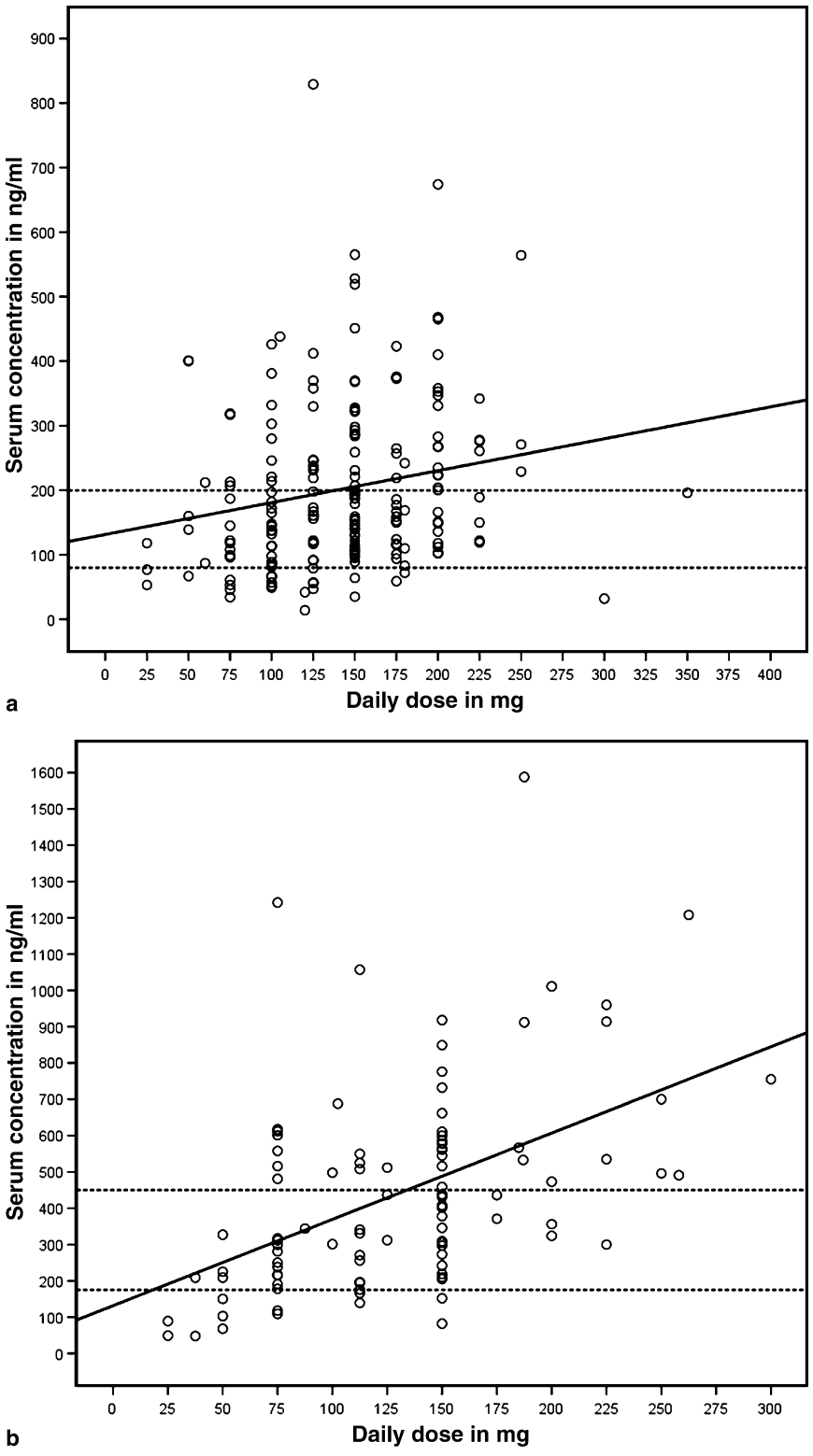


Fig. 3. Correlation (Spearman-rho) between daily doses of amitriptyline (**a**), clomipramine (**b**), and the respective trough serum concentrations of the sum of the parent compounds and their main metabolites nortriptyline and norclomipramine. All patients irrespective of the underlying diagnosis were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered. The dashed lines mark the lower and upper limit of the recommended therapeutic ranges

Forty-seven and 46 percent of the analysed serum concentrations were within the recommended therapeutic range (Baumann et al., 2004) for amitriptyline (80–200 ng/ml) and clomipramine (175–450 ng/ml), respectively. Roughly 40% of the determined serum concentrations of amitriptyline and clomipramine were above the recommended range.

Although female patients received a lower daily dose of amitriptyline than male patients ($p < 0.01$), they had higher

Table 2. *Age and sex dependent changes of serum concentrations in patients treated with amitriptyline and clomipramine. All patients irrespective of the underlying diagnosis were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered to avoid the problem of multiple determinations*

	Daily dose (mg): mean ± S.D.	Serum concentration (ng/ml): mean ± S.D.		
		Parent compound	Metabolite	Parent compound + Metabolite
Amitriptyline				
All patients ($N=195$)	141 ± 52.9	106 ± 72.0	93 ± 72.1	199 ± 130.2
Males ($N=84$)	155 ± 54.4	88 ± 56.8	80 ± 60.5	168 ± 102.0
Females ($N=111$)	130 ± 49.1[b]	119 ± 79.4[b]	103 ± 78.7[a]	222 ± 144.3[b]
≤60 years ($N=169$)	142 ± 52.9	106 ± 71.1	94 ± 71.5	200 ± 128.2
>60 years ($N=26$)	136 ± 53.4	106 ± 79.8	88 ± 77.5	194 ± 145.1
Smokers ($N=81$)	143 ± 55.2	103 ± 71.1	101 ± 79.6	204 ± 138.3
Non-smokers ($N=114$)	150 ± 50.5	113 ± 75.1	92 ± 67.6	205 ± 126.5
Clomipramine				
All patients ($N=102$)	129 ± 58.1	170 ± 116.2	268 ± 195.0	439 ± 277.3
Males ($N=37$)	140 ± 58.4	163 ± 106.0	279 ± 210.0	442 ± 294.4
Females ($N=65$)	122 ± 57.2	175 ± 122.2	262 ± 187.4	437 ± 269.4
≤60 years ($N=83$)	136 ± 57.4	172 ± 115.4	277 ± 206.6	449 ± 293.0
>60 years ($N=19$)	99 ± 53.7[c]	169 ± 112.7	244 ± 138.0	413 ± 201.9
Smokers ($N=41$)	137 ± 56.4	153 ± 91.2	261 ± 176.5	414 ± 243.0
Non-smokers ($N=61$)	121 ± 60.1	177 ± 128.6	269 ± 189.1	446 ± 273.9

[a] Significant difference ($p<0.05$) between male and female patients.
[b] Significant difference ($p<0.01$) between male and female patients.
[c] Significant difference ($p<0.05$) between patients younger and older than 60 years.

serum concentrations of amitriptyline ($p<0.01$) and its active metabolite nortriptyline ($p<0.05$; Table 2). The body-mass-index (BMI) did not differ between males and females (27.4 vs. 27.1) in this group. We found no differences in amitriptyline doses and serum levels between smokers and non-smokers as well as between patients aged over and below 60 years.

Interestingly, we analysed in patients aged over 60 years lower doses of clomipramine than in patients aged below 60 years ($p<0.05$; Table 2). The serum levels of clomipramine and its main metabolite, however, did not differ between both age groups. Males and females as well as smokers and non-smokers showed no differences with respect to daily dose and serum level of clomipramine and norclomipramine (Table 2).

Relationship between clinical response and serum concentration

Patients with the diagnosis of an organic (ICD-10 F0), substance-induced (ICD-10 F1) or schizophrenic (ICD-10 F2) disorder were excluded from the following analyses which are based upon the evaluation of 156 patients receiving amitriptyline and 61 patients receiving clomipramine, who were suffering from an affective (ICD-10 F3), neurotic/adjustment (ICD-10 F4) or personality disorder (ICD-10 F6) with prominent depressive symptoms (Table 1).

The patients were divided according to the CGI-rating of the attending physicians in responders (CGI ≤3) and non-responders (CGI >3). The mean concentration of the

Table 3. *Daily doses, serum concentrations and clinical response (CGI) in patients treated with amitriptyline and clomipramine. Only patients with the diagnosis of an affective disorder (ICD-10 F3x), neurotic or adjustment disorder (ICD-10 F4x) or personality disorder (ICD-10 F6x) were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered*

	Daily dose (mg): mean ± S.D.	Serum concentration (ng/ml): mean ± S.D.		
		Parent compound	Metabolite	Parent compound + Metabolite
Amitriptyline				
Responder ($N=106$)	147 ± 52.5	118 ± 75.8	100 ± 77.5	218 ± 138.0
Non-responder ($N=50$)	133 ± 51.1	96 ± 65.5	77 ± 60.9	173 ± 113.2[a]
Clomipramine				
Responder ($N=30$)	140 ± 53.0	172 ± 115.1	298 ± 184.9	471 ± 257.1
Non-responder ($N=31$)	140 ± 56.9	189 ± 117.4	292 ± 164.7	481 ± 244.0

[a] Significant difference ($p<0.05$) between responders and non-responders.

Table 4. *Daily doses, serum concentrations and adverse effects (UKU) in patients treated with amitriptyline and clomipramine. Only patients with the diagnosis of an affective disorder (ICD-10 F3x), neurotic or adjustment disorder (ICD-10 F4x) or personality disorder (ICD-10 F6x) were included. In the case of multiple TDM-analyses of the same drug in one patient only the last determination was considered*

	Daily dose (mg): mean ± S.D.	Serum concentration (ng/ml): mean ± S.D.		
		Parent compound	Metabolite	Parent compound + Metabolite
Amitriptyline				
Adverse effects ($N=77$)	149 ± 58.6	113 ± 74.1	99 ± 80.4	212 ± 140.2
No adverse effects ($N=66$)	140 ± 45.3	112 ± 72.8	85 ± 61.5	197 ± 118.5
Clomipramine				
Adverse effects ($N=22$)	140 ± 58.3	223 ± 112.0	348 ± 203.3	571 ± 271.1
No adverse effects ($N=39$)	141 ± 52.2	163 ± 110.8*	261 ± 155.5	424 ± 228.7[a]

[a] Significant difference ($p<0.05$) between patients with vs. without adverse effects.

sum of amitriptyline and its metabolite was higher in responders compared to non-responders ($p<0.05$; Table 3). Categorization into serum level ranges (''within recommended therapeutic range'' vs. ''outside recommended therapeutic range'') according to the recommendations of the AGNP-TDM-expert-group did not reveal any differences with regard to the frequency of responders and non-responders.

In the clomipramine group (Table 3), responders showed even a slightly lower mean concentration of the parent compound and the main metabolite compared to values of non-responders. However, the mean values of the serum levels in responders and non-responders were both slightly above the recommended therapeutic range for clomipramine and norclomipramine (Table 3). If we dichotomized the serum levels of clomipramine and norclomipramine into the categories ''within recommended therapeutic range'' vs. ''outside recommended therapeutic range'', we found more often responders if the serum level was within the therapeutic range ($\chi^2=4.841$, $p<0.05$).

Relationship between adverse effects and serum concentration

The patients were divided according to the UKU-rating of the attending physician in a group with adverse effects (UKU $\geq$1) and a group without adverse effects (UKU = 0). Information on adverse effects was missing in 15 patients with amitriptyline and 18 patients with clomipramine resulting in 143 and 61, respectively, included subjects (Table 1).

The mean serum concentration of clomipramine as well as the sum of the mean serum levels of clomipramine and norclomipramine were higher ($p<0.05$) in patients with adverse effects compared to patients without adverse effects (Table 4). In the amitriptyline group patients with adverse effects showed a slightly higher mean serum level of the metabolite nortriptyline than patients without adverse effects, but the difference did not reach statistical significance (Table 4).

Dichotomizing the sum of the serum levels of the parent compound and its main metabolite into the categories ''above the upper limit of the recommended therapeutic range'' vs. ''below the upper limit of the recommended therapeutic range'' and comparing the frequencies of adverse effects in both groups, we did not find any significant differences in amitriptyline- and clomipramine-treated patients (data not shown).

Discussion

The patient sample of the present study included adult patients of both genders and all ages suffering from rather severe forms of depressive syndromes and displaying a variety of co-morbidities. The data therefore may not be representative for outpatients with a less severe depressive symptomatology, but should reflect rather adequately TDM in a seriously ill inpatient sample.

Naturally, a retrospective analysis of data is subject to several limitations. It can provide plausible clues for possibly existing associations, but not prove any causal relationship. One obvious limitation of a study under naturalistic conditions is the multiplicity of different raters involved in the rating of therapeutic efficacy and adverse effects. The use of rather simple and global ratings that can be applied rapidly and easily by the attending physician should allow for this potential problem, but nevertheless our results have to be interpreted with the necessary caution.

Regarding the influence of age, sex and smoking on the relationship between dose and serum level, our most remarkable finding was that women treated with amitripty-

line had higher serum concentrations, although they received lower doses of the drug compared to male patients. There was no difference in the body mass index between both sexes and no observable gender-specific difference concerning the applied co-medication which could account for this finding. On the other hand low serum concentrations could be a result of a higher metabolism and/or a rapid clearance. However, we did not identify other pharmacokinetic factors which might explain our finding. Further studies using independent samples and a prospective design, and investigation of clinically relevant interactions with various co-medications are warranted to confirm and to explain this result.

In our sample, a rather high percentage of serum concentrations was above the recommended therapeutic range for both antidepressants. This may be due to the severity of the depressive syndromes and may reflect the fact that the recommended therapeutic ranges are usually derived from studies with moderately severe ill patients.

The evaluation of the relationship between clinical response and serum levels was complicated by several confounding factors. On the one hand, the highly variable dosing of the target drugs and the lack of restrictions regarding co-medication and co-morbidity may substantially impede the disclosure of such a relationship; on the other hand, patients with more severe depressions generally are less likely to respond to antidepressants (Dunner, 2001). Nevertheless we found in patients receiving amitriptyline a higher mean serum level of the sum of amitriptyline and nortriptyline in responders compared to non-responders. In responders, the mean serum level was even slightly above the recommended therapeutic range suggesting that in severely ill patients rather high serum levels may be necessary to reach a sufficient treatment response. In the clomipramine group, a mean serum level within the recommended therapeutic range was associated with clinical response; responders as well as non-responders showed a mean serum level somewhat above the recommended therapeutic range with responders having a slightly, but not significantly lower mean serum level closer to the upper limit of the therapeutic range.

Considering adverse effects, an association with a serum level above the therapeutic range could be verified neither in the amitriptyline nor in the clomipramine group. However, in both groups patients with adverse effects showed higher serum levels than patients without adverse effects, although only in the clomipramine group this difference reached statistical significance. Our findings regarding adverse effects differ in some aspects from results reported in the literature. For example, a rather strong relationship between adverse effects and serum levels or serum level ranges were found by Hodgkiss et al. (1995) and Müller et al. (2003). A possible explanation is that with respect to adverse effects the influence of the target drugs might be confounded by the influence of a psychotropic co-medication, which in our sample of severely ill patients was administered in almost every patient and may often exhibit similar adverse effects like those of the target drug.

In summary, the results of our study provide further evidence that for amitriptyline and clomipramine there exists a relationship between serum levels, but not drug doses, and adverse effects as well as clinical response also in everyday clinical practice in accordance with previous studies (Rao et al., 1996; Müller et al., 2003). In addition, our data suggest that in severely ill depressive patients a serum level near or even slightly above the upper limit of the recommended therapeutic range may be necessary to reach an adequate clinical response. As a consequence, we propose that TDM should be used regularly to optimise antidepressive treatment with amitriptyline and clomipramine, especially in severely ill patients. Further studies under naturalistic conditions are necessary also for other antidepressants to adapt recommended serum levels obtained from scientific studies with highly controlled conditions to the needs of everyday clinical practice.

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J Neural Transm (2007) [Suppl 72]: 297–301

Printed in Austria

Journal of Neural Transmission

FZD3 is not a risk gene for schizophrenia: a case-control study in a Caucasian sample*

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Summary *Background*. Polymorphisms in the human frizzeled-3 (FZD3) gene have been associated with schizophrenia in an Asian population sample. However, this finding could not be confirmed in subsequent studies investigating other populations. Here we attempted to replicate this finding in a sample of 192 German chronically ill schizophrenic subjects.

Methods. Three single nucleotide polymorphisms in the FZD3 gene have been genotyped by primer extension and MALDI-TOF measurement. Subsequently, associations for single markers as well as haplotypes were tested.

Results. In German patients, neither single markers nor haplotypes in FZD3 were associated with schizophrenia. Further exploratory analyses using a different diagnostic approach did also not yield significant results.

Conclusions. FZD3 is unlikely to play a role in the genetic predisposition towards schizophrenia in the Caucasian population.

Keywords: Haplotype analysis, polymorphism, schizophrenic psychoses, FZD3, schizophrenia, bipolar disorder

Introduction

Schizophrenia (SCZ) is a multifactorial disease with a substantial genetic contribution. Genetic linkage analyses for SCZ in various populations pointed to several positional candidate genes, like DTNBP1, RGS4, and neuregulin (Kirov et al., 2005). Interestingly, several genes also emerged as candidates for SCZ as well as bipolar disorder, indicating an at least partial common genetic background of these disorders. Genes which have been suggested to play a role in both bipolar disorder and SCZ are e.g., Akt, G72, DAAO, and MLC1 (Verma et al., 2005). For each of the candidate genes however both positive and negative findings have been published. While inter-ethnic variation might account for discrepant findings, genetic and clinical heterogeneity of schizophrenic and bipolar psychoses are possible reasons for the failure to detect significant association (Mirnics and Lewis, 2001).

Human frizzled-3 (FZD3) is an attractive candidate gene for SCZ due to its function and its localization on chromosome 8p21 (3 Mb upstream of neuregulin), a region which has been repeatedly shown to harbour at least one SCZ susceptibility locus (Gurling et al., 2001; Liu et al., 2005; Park et al., 2004). FZD3 encodes a receptor for Wnt glycoproteins and is highly expressed in the adult CNS (Sala et al., 2000). Wnt signaling has an important role in neural development and migration, neurogenesis and synaptogenesis. Knockout of frizzled-3 results in severe defects in major forebrain axon tracts (e.g., thalamocortical and nigrostriatal tract, anterior commissure, and variably in the corpus callosum) (Wang et al., 2006). Along this rationale, two independent groups from Asia (China (Yang et al., 2003) and Japan (Katsu et al., 2003)) proposed a role of the FZD3 gene in SCZ. The Chinese study genotyped a three marker haplotype in family trios, while the Japanese investigation used a case-control approach and two-marker haplotype analysis. In both cohorts association of FZD3 with SCZ was found, on the single marker level as well as on the haplotype level. These results were corroborated by a second study from China, employing approximately 250 cases and controls (Zhang et al., 2004). However, following studies failed to replicate these results: a large Japanese case-control and family-based investigation yielded negative results (Ide et al., 2004), as did a case-control study from South Korea (Ide et al., 2004) and a third study from Japan (Ide et al., 2004). In the latter study, also bipo-

* Dedicated to Prof. Dr. Peter Riederer on the occasion of his 65th birthday

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lar patients were examined ($n = 91$), with negative results. As yet only one study investigated a non-Asian population, namely 120 British family trios, and likewise failed to demonstrate association (Wei and Hemmings, 2004).

To further elucidate a possible role of FZD3 in the genetic predisposition towards SCZ in non-Asian populations, we investigated 3 SNPs and respective haplotypes in a genetically controlled case-control sample including 192 chronic schizophrenic subjects. To overcome the problem of clinical heterogeneity of SCZ, we have used an alternative diagnostic system by Leonhard (1999) additionally to the ICD-10.

Materials and methods

Subjects

The present study investigated a sample described earlier in detail (Reif et al., 2006). A total of 192 unrelated patients from the German Lower Franconia area, which were ascertained at the Department of Psychiatry and Psychotherapy, University of Würzburg, participated in this study. All patients were diagnosed by means of an extensive, semi-structured interview analogous to the AMDP interview (Arbeitsgemeinschaft für Methodik und Dokumentation in der Psychiatrie, 2000) performed by an experienced psychiatrist (A.R. or C.P.J.), along with chart reviews and further information from family informants and case records from other hospitals. Chart reviews of every patient were done by A.R. An ICD-10 diagnosis as well as a diagnosis according to Leonhard's diagnosis system (Leonhard, 1999) was made prior to genetic analysis. Patients suffered from the following schizophrenic disorders according to ICD-10 criteria: $n = 56$, paranoid type; $n = 39$, hebephrenic type; $n = 6$, catatonic type; $n = 9$, undifferentiated type; $n = 25$, residual type; $n = 7$, schizophrenia simplex; $n = 3$, schizophrenia not otherwise specified; $n = 13$, delusional disorder; $n = 37$, schizoaffective disorder. The entire sample consisted of chronic schizophrenic patients, as none of the subjects remitted completely. None of the subjects showed significant neurological comorbidity, epilepsy, mental retardation, or other somatic disorders suggesting organic psychosis. Patients with substance-induced disorders were excluded as well.

According to the Leonhard classification system, 55 patients suffered from affect-laden paraphrenia, 55 from periodic catatonia, and 36 from cataphasia (i.e., 146 suffered from type A schizophrenia; mean age of onset 28 ± 10 years). Thirty-three patients suffered from systematic hebephrenia, 7 from systematic paraphrenia and 6 from systematic catatonia (i.e., 46 suffered from type B schizophrenia; mean age of onset 23 ± 6 years; $p = 0.0003$, Student's t-test).

The control sample consisted of 284 subjects who were healthy blood donors coming from the same recruiting area as the patients. By means of genomic control, it was shown that patients and controls are genetically homogenous (Reif et al., 2006). Only patients and volunteers who gave written informed consent were enrolled in the study, which complied with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Würzburg.

Genotyping

Three SNPs, selected from previous studies, have been investigated in the present examinations: rs960914 (intronic, IVS3 + 258 T > C), rs2241802 (exon 5, 435 G > A) and rs352203 (intronic, IVS5 + 9020 T > C). Initially,

Table 1. *Genotype frequencies of FZD3 markers*

Marker		Controls $N = 284$	SCZ		
			Total $N = 189$	Type B $N = 46$	Type A $N = 143$
rs960914	A/A	81 (0.28)	60 (0.32)	15 (0.33)	45 (0.32)
	A/G	143 (0.50)	100 (0.53)	22 (0.48)	78 (0.55)
	G/G	60 (0.21)	29 (0.15)	9 (0.20)	20 (0.14)
			$\chi^2 = 2.56$, $P = 0.278$	$\chi^2 = 0.32$, $P = 0.850$	$\chi^2 = 3.19$, $P = 0.203$
	A	305 (0.54)	220 (0.58)	52 (0.56)	168 (0.59)
	G	263 (0.46)	158 (0.42)	40 (0.44)	118 (0.41)
			$\chi^2 = 1.86$, $P = 0.172$	$\chi^2 = 0.25$, $P = 0.614$	$\chi^2 = 1.96$, $P = 0.162$
rs2241802	A/A	62 (0.22)	33 (0.18)	8 (0.17)	25 (0.18)
	A/G	145 (0.51)	105 (0.56)	26 (0.56)	79 (0.55)
	G/G	77 (0.27)	51 (0.27)	12 (0.26)	39 (0.27)
			$\chi^2 = 1.52$, $P = 0.469$	$\chi^2 = 0.61$, $P = 0.737$	$\chi^2 = 1.20$, $P = 0.548$
	A	269 (0.47)	171 (0.45)	42 (0.46)	129 (0.45)
	G	299 (0.53)	207 (0.55)	50 (0.54)	157 (0.55)
			$\chi^2 = 0.41$, $P = 0.522$	$\chi^2 = 0.09$, $P = 0.761$	$\chi^2 = 0.39$, $P = 0.533$
rs352203	C/C	60 (0.21)	26 (0.14)	7 (0.15)	19 (0.13)
	C/T	146 (0.51)	102 (0.54)	25 (0.54)	77 (0.54)
	T/T	78 (0.28)	61 (0.32)	14 (0.30)	47 (0.33)
			$\chi^2 = 4.43$, $P = 0.109$	$\chi^2 = 0.87$, $P = 0.647$	$\chi^2 = 4.22$, $P = 0.121$
	C	266 (0.47)	154 (0.41)	39 (0.42)	115 (0.40)
	T	302 (0.53)	224 (0.59)	53 (0.58)	171 (0.60)
			$\chi^2 = 3.41$, $P = 0.065$	$\chi^2 = 0.63$, $P = 0.428$	$\chi^2 = 3.38$, $P = 0.066$

SCZ Schizophrenia; types A and B refer to the sub-classification of SCZ as defined in the Materials and methods section.

standard PCRs were performed with 40 ng genomic DNA. Primer sequences are available on request. The PCR products were treated with shrimp alkaline phosphatase before SNP-specific primer extension reaction. The primers were 5′-biotinylated and contained a photocleavable linker (BioTeZ GmbH, Berlin, Germany). For single nucelotide-extension, TERMIpol polymerase (Solis BioDyne) was used. Products were purified with the Genostrep 96 Kit (Bruker Daltonics, Bremen, Germany), the primers cleaved by UV and the mass of extended oligonucleotides was determined by MALDI-TOF.

Statistical and haplotype analysis

Single association tests were performed by means of chi-square tests using SPSS for Windows 9.0 (SPSS Inc., Chicago, USA). Pairwise linkage disequilibrium between the polymorphisms was assessed using 2LD (Zhao, 2004). Tests for global haplotype associations and for significance of differences between controls and patients in estimated frequencies of specific haplotypes were performed using the GENECOUNTING/PERMUTE utility of the GENECOUNTING software (Zhao, 2004). GENECOUNTING performs permutation tests for global association/significance of specific haplotypes by randomly reassigning case and control labels in the actual data. The resulting *P*-values reflect the proportion of replicates that produce values of statistics at least as large as the observed. In the current study, 10,000 permutations were performed. Power calculations were performed by using the GPOWER software package (Erdfelder et al., 1996).

Results

The genotype distributions for all diagnostic groups were in Hardy-Weinberg equilibrium (not shown). Power calculations using GPOWER indicated that the sample was adequate to detect an effect of 0.15 for the distinct genotypes and 0.10 for allele-wise tests, i.e., the sample was well sized to detect small effects according to Cohen's convention (not shown).

First, we tested for associations of single markers with SCZ; thereafter, in an exploratory analysis, we investigated whether they were associated with all cases combined, type A SCZ or type B SCZ alone, or with any of the specific subgroups. As shown in Table 1, neither marker was significantly associated with any disease entity, although there was a trend for an association of rs352203 with SCZ before applying Bonferroni's correction. As all SNPs were in significant LD ($D' = 0.92$, 0.94 and 0.63, respectively; all $P < 0.0001$), we computed a haplotype analysis (Table 2). A test for global haplotype association with SCZ did not yield a significant result ($P = 0.14$ and 0.27). As shown in Table 2, on the level of specific haplotypes there were some significant associations, but significances disappeared after correction for multiple testing. Furthermore, all of these haplotypes were rare (<3%).

Table 2. *Estimated FZD3 haplotype frequency differences between controls and patients using GENECOUNTING*

Marker			Controls	SCZ		
rs960914	rs2241802	rs352203		Total	Type B	Type A
A	A	C	0.02	0.02	0.01	0.02
A	A	T	0.01	0.03*	0.03	0.03*
A	G	C	0.01	0.01	0.00	0.01
A	G	T	0.49	0.53	0.52	0.53
G	A	C	0.43	0.39	0.41	0.38
G	A	T	0.02	0.02	0.00*	0.02
G	G	C	0.01	0.00	0.00	0.00
G	G	T	0.01	0.02	0.02	0.01
	global P			0.140	0.402	0.253

* $P < 0.05$ (uncorrected); *SCZ* schizophrenia; types A and B refer to the sub-classification of SCZ as defined in the Materials and methods section.

Discussion

Initial studies that were conducted in Asia demonstrated a genetic association of FZD3 with SCZ. In this study, we attempted to replicate these results in a Caucasian (German) population; furthermore, we tried to narrow the SCZ phenotype by applying an alternative diagnostic system. However, neither of the single markers that we tested nor the respective haplotype showed significant associations. This result is in accordance with a recent meta-analysis (Jeong et al., 2006), published while this manuscript was in preparation, which also yielded negative results for the combined data from the Asian studies. The SNPs included in the present study were scrutinized in the meta-analysis, and cover all haplotypic blocks (Fig. 1) hitherto described in different populations (http://www.hapmap.org). Two of the SNPs have been investigated in the British trio design study (Wei and Hemmings, 2004). These SNPs are carried mainly by two distinct ancient haplotypes, A–C and G–T, respectively (for rs2241802–rs352203). However, in the British study, only the second haplotype block (Fig. 1, Block 2) is covered while no haplotype tagging SNP of the Chinese haplotype Block 1 was included. In contrast, our experimental design also allows to rule out a contribution of this region. A further difference of the British to our study was the ascertaining method (trio vs. case-control). However, both European studies strongly argue against an association of FZD3 with SCZ in Caucasians. As our sample was controlled for stratification (Reif et al., 2006), ethnic admixture is unlikely to account for the negative finding. However, it has to be considered that our sample consisted of chronically ill patients, showing a varying yet considerable degree of negative symptoms. Thus, we cannot rule out a role of FZD3 in remitting psychosis, i.e., good-outcome schizoaffective disorder and polymorph psychotic disorder. Furthermore, there still remains the possibility that FZD3 is only operative in a given genetic environment, i.e., that genetic background effects

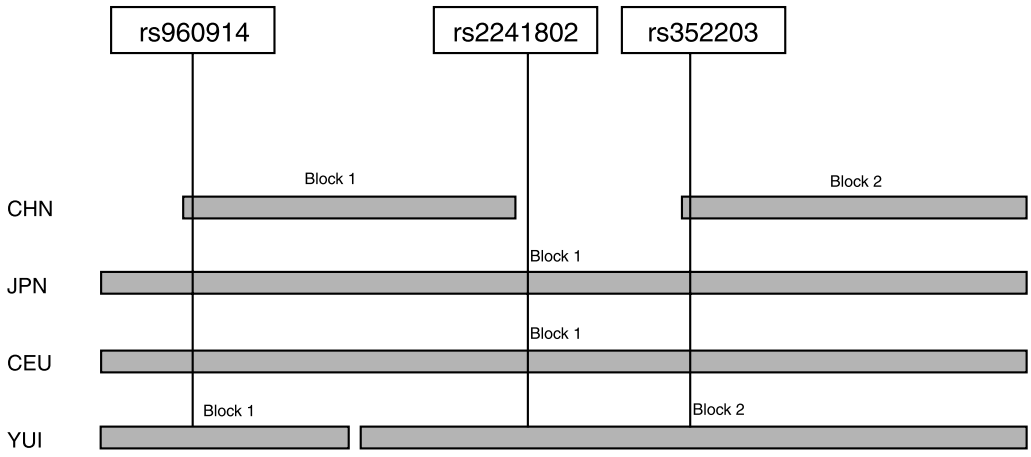

Fig. 1. Haplotype blocks of FZD3 (as modified from Jeong et al., 2006) and the SNPs investigated in the present study. *CHN* Han Chinese in Beijing, China. *JPN* Japanese in Tokyo, Japan. *CEU* Utah residents with ancestry from northern and western Europe, USA. *YRI* Yoruba in Ibadan, Nigeria

play a role thereby implicating FZD3 in the predisposition towards SCZ in Asia only which however was discarded by the meta-analysis (Jeong et al., 2006).

Intriguingly, the Chinese haplotype variation is more complex, showing two haplotype blocks and greater haplotype diversity. FZD3 thus is more conserved in the Caucasian population possibly explaining the failure to replicate the positive association studies as also pointed out by Wei and Hemmings (2004). Other causes for non-replication of association studies like ethnic admixture, clinical assessment and small sample sizes (Colhoun et al., 2003) have been addressed in the present study by investigating a reasonably sized population with sufficient power to detect meaningful effects, employing genomic control and the usage of two diagnostic systems. Thus, in line with the pooled data from Asian studies, as summarized by Jeong et al. (2006), and the British trio study (Wei and Hemmings, 2004), our data argue against the hypothesis that genetic variation of FZD3 predisposes towards SCZ. Rather than FZD3, another nearby gene in LD with FZD3 might thus explain the initial positive findings; the prepronociceptin gene (PNOC; Blaveri et al., 2001), zinc finger protein 395 gene (ZNF395) and exostosin-like 3 gene (EXTL3) are potential candidates in this respect and warrant further research.

Acknowledgement

We are indebted to T. Töpner and G. Kristjansson for excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (Grant RE1632/1-1 and 1-3 to A.R., KFO 125/1-1 to A.R. and K.P.L. and SFB581 to K.P.L.), and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (IZKF Würzburg, 01KS9603) and the European Commission (NEWMOOD LSHM-CT-2003–503474).

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J Neural Transm (2007) [Suppl 72]: 303–309

Printed in Austria

Journal of Neural Transmission

Impaired cortical inhibition in adult ADHD patients: a study with transcranial magnetic stimulation*

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Summary The aim of this study was to analyze motor inhibition and facilitation of adult ADHD patients using double pulse transcranial magnetic stimulation (TMS). Twenty-six right handed adult ADHD patients according to DSM-IV were investigated and compared to 26 age and sex-matched controls. In the left hemisphere, mean motor inhibition was 0.53 ± 0.33 (mean $\pm$ SD) in ADHD patients and 0.34 ± 0.16 (mean $\pm$ SD) in controls ($p = 0.012$). There were no significant differences in motor excitability concerning facilitation or in the right hemisphere. Decreased motor inhibition correlated with a higher symptom score derived from the Wender Reimherr Interview (WRI) ($\rho = 0.28$; $p = 0.04$) and also with self rated hyperactivity/impulsivity symptoms ($\rho = 0.30$; $p = 0.03$). In conclusion, decreased motor inhibition in adult ADHD corroborate similar findings in children with ADHD (Moll et al., 2000) and reflect disturbed impulsivity and hyperactivity on a neurophysiological level.

Keywords: Attention deficit/hyperactivity disorder (ADHD), adults, transcranial magnetic stimulation (TMS), inhibition, facilitation, double pulse stimulation

Introduction

The attention deficit/hyperactivity disorder (ADHD) is one of the most frequent disorders not only in child and adolescent psychiatry but also in general psychiatry. The prevalence in adulthood has been determined as 4% (Kessler et al., 2006). According to DSM-IV the core psychopathology in all ages comprises hyperactivity, impulsivity and attention deficits. These symptoms are significantly correlated with deficits in social functioning (Barkley et al., 2006). In comparison with healthy controls individuals with ADHD have lower levels of school and vocational education, more job changes, higher rates of divorces (Biederman et al., 2006) and are at increased risk of severe accidental injuries (Grützmacher, 2001). Persons with ADHD commit significantly more traffic violations compared with controls (Jerome et al., 2006). The prevalence of ADHD in forensic populations is remarkably high (Vermeiren, 2003), particularly in young male offender populations (Rösler et al., 2004a).

In this respect it is useful to question which abnormalities of the cerebral structures and functions may contribute to the emergence of psychopathology and social dysfunction. There is an overwhelming amount of evidence that ADHD is a disease with a profound genetic component (Faraone, 2004). From twin studies, heritability estimates of 0.7 and higher were derived. These are among the highest heritability estimates in psychiatric disorders besides autism spectrum disorders. In molecular genetic studies associations were found predominately with genes controlling for the dopaminergic and serotonergic transmitter system (Heiser et al., 2004). Both transmitter systems are involved in the function of the prefrontal cortex. Dopamine plays a significant role in the anterior attention system (Pliszka, 2005) and serotonin is a major component of the anterior cingulate cortex (Mantere et al., 2002), which seems to be affected in ADHD (Fallgatter et al., 2004; Seidman et al., 2006). In structural and functional imaging studies abnormalities of the prefrontal cortex are widely accepted findings, in addition to changes in the striatum and the cerebellum (Schneider et al., 2006).

Transcranial magnetic stimulation (TMS) is a non-invasive method to examine cortical function and to study the activation of brain regions based on the ability of a magnetic field to penetrate skull and brain meninges,

* Dedicated to Univ.-Prof., Univ.-Doz., Dipl.-Ing., Dr. Peter Franz Riederer, Head Clinical Neurochemistry, Department of Psychiatry, University Hospital of Würzburg (Germany) on the occasion of his 65th birthday.

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subsequently inducing neuronal depolarization and the generation of action potentials (Bailey et al., 2001). Single or paired pulse TMS and repetitive TMS (rTMS) is differentiated on the basis of stimulation type. The effects of single or paired pulse TMS do not outlast the period of stimulation, whereas rTMS can produce effects which outlast the stimulation period depending on the stimulation parameters (Ziemann, 2004). TMS is presently under investigation as a treatment for different psychiatric disorders, mainly affective disorders (Simons and Dierick, 2005). In addition to therapeutic approaches, TMS can also be used to examine cortical function in different brain areas. One of the main areas of TMS application is the investigation of cortical excitability, which is mediated by neurochemical and synaptic processes in neurons, which are affected by TMS. Strafella et al. (2003) demonstrated that rTMS of the prefrontal cortex induced focal dopamine release in the ipsilateral caudate nucleus and increased extracellular dopamine concentrations. TMS studies subsequently reported that excitability of the motor cortex was changed by stimulation of contralateral cortical areas (Baumer et al., 2006; Porro et al., 2007).

Only a limited number of TMS investigations have been performed in individuals with ADHD. In ADHD children, Moll et al. (2000) reported reduced intracortical inhibition (ICI) associated with normal intracortical facilitation (ICF). After application of 10 mg methylphenidate (MPH) ICI was enhanced in these children. Gilbert et al. (2005) reported that ADHD scores in a sample of children and adults with Tourette syndrome were inversely correlated with ICI measured with TMS. In their study, hyperactivity scores, but not inattention, accounted for this finding. Moreover, disturbed transcallosally mediated contralateral motor inhibition in children with ADHD (Buchmann et al., 2003; Garvey et al., 2005) and modulation of cortical excitability by MPH and atomoxetine have been described (Buchmann et al., 2006; Gilbert et al., 2006) in TMS studies.

In this study we hypothesized that impaired cortical inhibition found in children (Moll et al., 2000) is also present in adults with ADHD. We also hypothesized that reduced motor inhibition or increased facilitation is correlated with the severity of ADHD.

Methods

Subjects and instruments

The study was performed on 26 right-handed subjects with ADHD recruited from a specialized ambulance for ADHD-associated disorders. Each group consisted of 13 female and male subjects, respectively. All patients were without any DSM-IV axis 1 diagnosis, and were completely drug naïve. Further exclusion criteria were any history of neurological events, such as brain injury or any kind of vascular, inflammatory or degenerative brain disturbance (e.g. meningitis, encephalitis in childhood, developmental or degenerative disorder). Patients with low intelligence (IQ < 85) were not included in the study.

Table 1. *Descriptive statistics for cases and controls*

	ADHD ($N = 26$) mean (Std)	Controls ($N = 26$) mean (Std)	Z-value p-value
Age (SD)	32.4 (9.1)	32.2 (7.9)	$Z = -0.2$ 0.857
Gender	13 male 13 female	13 male 13 female	
WURS-k*	47.3 (13.6)	7.0 (9.2)	$Z = 6.1$ <0.0001
ADHD-SR* total score	36.8 (6.3)	6.0 (5.17)	$Z = 6.4$ <0.0001
ADHD-SR attention deficit subscore[a]	18.2 (4.6)	3.0 (2.5)	$Z = 6.3$ <0.0001
ADHD-SR hyperactivity/impulsivity subscore[a]	18.6 (4.1)	3.0 (3.3)	$Z = 6.4$ <0.0001
WRI score[a]	43.3 (5.4)	9.3 (8.2)	$Z = 6.4$ <0.0001

[a] Information missing on one individual with ADHD.
WURS-k Wender-Utah-Rating Scale, German short version (Retz-Junginger et al., 2002, 2003).
ADHD-SR ADHD self-rating (Rösler et al., 2004b).
WRI Wender-Reimherr Interview (Rösler et al., submitted).

The patients of the ADHD study group were initially diagnosed by experienced psychiatrists and fulfilled the diagnostic criteria of DSM-IV according to the ADHD-DC (ADHD Diagnostic Checklist, Rösler et al., 2004b). Twenty-four patients were classified as ADHD combined type and 2 as ADHD hyperactive/impulsive subtype. Control subjects were recruited from the staff of different university departments. They were matched with ADHD patients according to their sex and age.

Table 1 gives descriptive statistics for ADHD cases and controls with regard to age, sex distribution and ADHD symptoms.

All patients displayed a sum score of at least 30 points in the Wender-Utah Rating Scale (WURS-k, Retz-Junginger et al., 2002, 2003), which is highly indicative for childhood ADHD symptoms, obtained by self assessment. Diagnostic criteria of ADHD were first obtained from the ADHD self rating scale (ADHD-SR) for adults according to DSM-IV (Rösler et al., 2004b). Total scores ranged from 0 to 54 points. Maximum score for the attention deficit subscale is 27 and 27 for the hyperactivity/impulsivity subscale.

Second, individuals were interviewed by expert clinicians with the authorized German version of the Wender Reimherr Interview (WRI, Rösler et al., submitted) to obtain Utah criteria for adult ADHD. The WRI is also known as TAADDS (Wender, 1995). The WRI is a semi-standardized diagnostic interview for the assessment of adult ADHD. It comprises 28 psychopathological items on 7 subscales which are inattention, hyperactivity, temper, affective lability, emotional hyperreagibility, disorganisation and impulsivity. Each item is quantified on a Lickert Scale ranging from 0 to 2 resulting in a maximum total WRI score of 56.

Patients with the clinical diagnosis of ADHD were included in the study only if (1) the WURS-k score was at least 30 points, (2) at least 6 of 9 items of inattention and/or hyperactivity/impulsivity were rated as present

(score ≥ 1) on the ADHD-SR and (3) ADHD-SR attention deficit and/or hyperactivity/impulsivity subscores were at least 12 points.

Transcranial magnetic stimulation technique (TMS)

TMS utilizes the principle of electromagnetic induction by the discharge of very large currents over a short period which flow through a copper-wire coil. A rapid time-varying magnetic field is induced by an impulse of approximately 270 µsec. When the coil is held to the head of a subject, the magnetic field pulse induces a small current parallel to the plane of the coil in the adjacent second conductor, the brain. When the induced current flowing parallel to the brain surface is sufficient, depolarization of neuronal membranes occurs, and hence an action potential is generated. Thereby preferentially interneuronal elements are activated, which are oriented horizontally to the surface of the brain (Day et al., 1989). TMS is thought to predominantly activate the pyramidal cells transsynaptically through excitatory interneuronal elements (Amassian et al., 1990; Day et al., 1989; Di Lazzaro et al., 1998; Nakamura et al., 1996).

With this technique, two magnetic stimuli are delivered in close sequence to the same cortical spot through a single stimulation coil (Kujirai et al., 1993). The first, conditioning pulse (CP) is a subthreshold pulse and is considered to condition the response for the second, the test stimulus (TS) which is a suprathreshold stimulus and follows within a time period of several milliseconds. The magnetically evoked potential (MEP) obtained depends on the intensity of the CP, the interval between the stimuli (interstimulus interval, ISI), and the intensity of the TS (Pascual-Leone et al., 1998). Different circuits are recruited by varying intensities of CP and TS. At a ISI of 1–20 ms ICI and ICF may be studied. In our study, MEP's after an ISI of 1, 3, and 5 ms were accepted to indicate inhibition, and MEP's after an ISI of 7, 9, 11, 13, and 15 ms to indicate facilitation.

TMS was applied with a Medtronic MagPro $\times$ 100 Stimulator with MagOption (Medtronic, Denmark). A figure of eight coil with a diameter of 65 mm was placed at the skull above the supposed hand area of the motor cortex. Surface electromyography was recorded from the contralateral first dorsal interosseus muscle (FDI) with a standard electromyographic amplifier (Medtronic Keypoint 4; recording software Medtronic Keypoint V 5.01). The bandwidth of the filters was set to 1 Hz and 10 kHz, respectively. The optimal position of the coil was determined by moving the coil by 0.5 cm steps until an optimal MEP could be registered. Resting (RMT) and active motor thresholds (AMT) were determined according to the protocol of Kujirai et al. (1993). Briefly, RMT was the minimal stimulus intensity which was required to produce motor potentials of more than 50 µV peak to peak amplitude in 50% of the testpulses. AMT was determined in analogy to RMT, while the subject tonically distended a dynamometer with 10–20% of maximum power.

"In-Out"-curves were determined by increasing stimulus intensity in steps of 10% beginning from subthreshold intensities up to maximum stimulus output at 100%. Eight MEP's were averaged at each stimulus level.

Double-pulse stimulation was performed according to the technique of Kujirai et al. (1993). The subthreshold conditioning pulse (CP) was set at 80% of RMT and was followed by a suprathreshold test stimulus (TS). The TS was adjusted to produce a mean MEP of 0.5–1.2 mV peak to peak amplitude, when unconditioned (uMEP). The interstimulus intervals were set between 1 and 15 ms at distances of 2 ms. At each interstimulus interval 8 trials were averaged. The time between two measurements was at least 5 sec to avoid secondary effects, such as potentiation. Peak-to-peak amplitudes of these conditioned MEP's (cMEP) were set into relation to a MEP elicited by the mean of 8 testpulses without any conditioning prepulse (unconditioned MEP, uMEP). Data are presented as relative amplitudes of the uMEP (relative amplitude = cMEP/uMEP). Care was taken to relax the subjects and to avoid any movements of the extremities during recordings. Each MEP was checked optically to ensure that first dorsal interosseus muscle (FDI) was completely relaxed. Recordings were discarded if any evidence of electromyographic activity was detected and repeated.

Statistical analysis

Descriptive statistics to compare ADHD scores and mean MEP after different ISI in cases and controls were performed by the non-parametric Wilcoxon rank sum test. Spearman correlations were calculated to assess correlation of ADHD scores with mean inhibitory MEP, i.e. the averaged response after an ISI of 1, 3, and 5 ms.

To compare inhibition and facilitation between groups, multivariate statistical analysis of co-variance (MANCOVA) adjusted for age, was performed on four blocks of variables: log transformed MEP after the ISI 1, 3, and 5 ms on the (1) right and (2) left (inhibition) and log transformed MEP after the ISI 7, 9, 11, 13, and 15 ms on the (3) right and (4) left (facilitation). Due to this multivariate analysis, no further adjustment for multiple testing was made. To assess the influence of attention and hyperactivity/impulsivity on inhibition, exploratory linear regression analyses were performed with the log transformed mean MEP after ISI 1, 3, and 5 ms (inhibition) of the left hemisphere as the dependent, and the ADHD self assessment scores or the Wender-Reimherr-Interview score as the independent variables, adjusted for age. Residuals were normally distributed.

Statistical analyses were performed using SAS 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

In Table 1, descriptive data for ADHD patients and healthy controls are shown. There was no age difference in ADHD patients and controls. Groups were matched for sex. ADHD scores of the patients were significantly elevated compared to controls.

Mean relative amplitude of resting motor threshold (RMT) was 50.0 (SD 6.5) (left hemisphere – LH) and 51.9 (SD 8.9) (right hemisphere – RH) in the ADHD group and 47.7 (SD 5.8) (LH) and 49.6 (SD 9.96) (RH) in the control group. For active motor threshold (AMT) the mean relative amplitude was 43.6 (SD 6.2) (LH) and 42.9 (SD 7.5) (RH) in the ADHD group and 39.8 (SD 5.6) (LH) and 41.7 (SD 9.7) (RH) in the control group. Neither AMT nor RMT differed significantly between the groups on either hemisphere. There was also no correlation of thresholds with

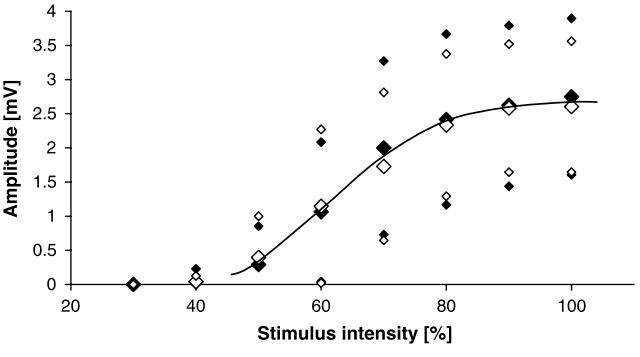

Fig. 1. Input-output eyefit curve (stimulation left hemisphere); stimulus intensity [%] of maximum stimulator output; mean absolute amplitudes of the MEP [mV]; filled diamonds represent mean (SD) MEP in ADHD; open diamonds represent mean (SD) MEP in controls

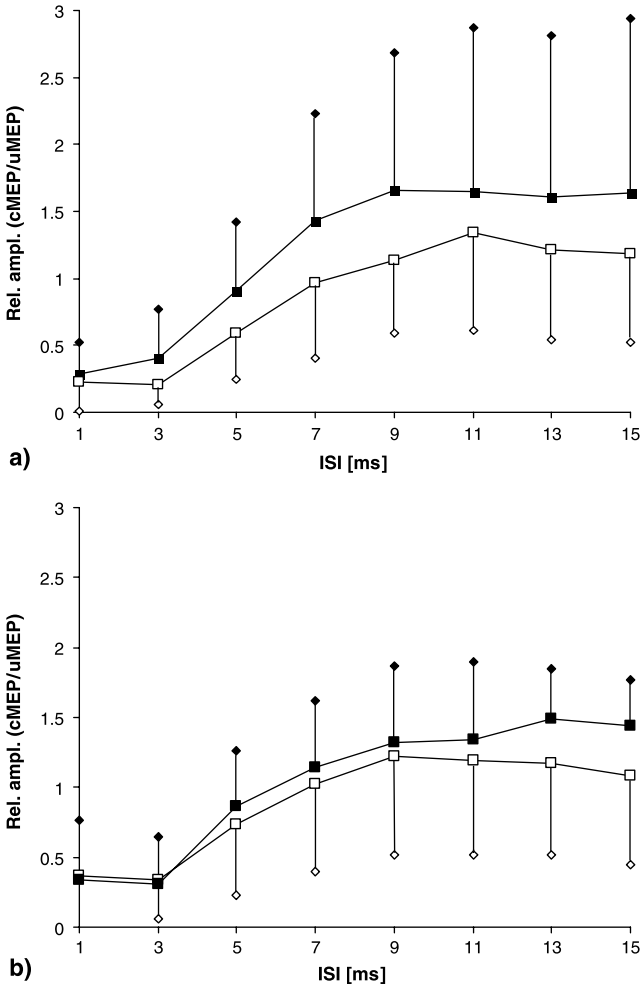

Fig. 2. Relative amplitudes (conditioned MEP (cMEP)/unconditioned MEP (uMEP)) after double pulse stimulation in ADHD patients and controls: **a**) left hemisphere; **b**) right hemisphere. Filled squares represent mean relative amplitudes in ADHD, filled diamonds represent standard deviation in ADHD; open squares represent mean relative amplitude in controls, open diamonds represent standard deviation in controls. *ISI* interstimulus interval

ADHD symptom rating scores. Means and standard deviations of input-output curves did not differ between patients and controls, indicating that excitatory mechanisms of motor systems did not differ between groups (Fig. 1).

Significant differences in motor excitability between ADHD patients and controls were found with the double pulse protocol. As shown in Fig. 2, the relative MEPs (cMEP/uMEP) increased with longer ISIs and displayed a sigmoidal curve in both groups. Lowest relative amplitudes (<1) were found in short ISI (1–5 ms) of paired-pulse curves, reflecting intracortical inhibition (ICI). Intracortical facilitation (ICF), corresponding to relative amplitudes >1 occurred at ISI of above 5 ms and reached maximum values at 15 ms ISI. ADHD patients showed generally higher mean relative amplitudes at each ISI compared to controls. There was also an increasing variability of the MEPs with increasing ISI in ADHD patients.

Multivariate analysis of co-variance (MANCOVA) performed on the four ISI blocks (ICI and ICF, left and right hemispheres) revealed significantly impaired inhibition of the left hemisphere in ADHD patients compared to controls (Wilk's Lambda = 0.84; $F = 3.23$; 3, 51 DF; $P = 0.030$; see Table 2). A similar change was found, when the mean of the MEP after an ICI of 1, 3, and 5 ms was compared between groups (mean motor inhibition in ADHD 0.53 ± 0.33 (mean $\pm$ SD); in controls 0.34 ± 0.16 (mean $\pm$ SD); $F = 6.85$; 1 DF; $P = 0.012$). There was no further statistically relevant difference concerning facilitation of the left hemisphere and in the inhibition or facilitation of the right hemisphere (Table 2).

Mean relative MEP significantly correlated with the WRI total score (β-estimate = 0.009; $P = 0.029$; $\rho = 0.30$) and the impulsivity/hyperactivity score of the ADHD-SR subscale (β-estimate = 0.019; $P = 0.037$; $\rho = 0.30$), but not

Table 2. *TMS paired pulse stimulation. The table shows mean relative amplitudes (cMEP (conditioned magnetically evoked potential)/uMEP (unconditioned magnetically evoked potential))*

Inter-stimulus interval		Left hemisphere mean (SD)		Statistic[a]	Right hemisphere[b] mean (SD)		Statistic[a]
		ADHD	Controls		ADHD	Controls	
Intracortical inhibition (ICI)	1 ms	0.29 (0.23)	0.23 (0.22)	Wilk's Lambda = 0.84; $F = 3.23$ (3, 51) $P = 0.030$	0.37 (0.40)	0.34 (0.44)	Wilk's Lambda = 0.95; $F = 0.72$ (3, 46) $P = 0.543$
	3 ms	0.40 (0.37)	0.21 (0.15)		0.34 (0.31)	0.31 (0.25)	
	5 ms	0.91 (0.51)	0.59 (0.34)		0.74 (0.52)	0.86 (0.63)	
Intracortical facilitation (ICF)	7 ms	1.44 (0.79)	0.97 (0.54)	Wilk's Lambda = 0.84; $F = 1.92$ (5, 49) $P = 0.108$	1.02 (0.60)	1.14 (0.74)	Wilk's Lambda = 0.90; $F = 1.01$ (5, 45) $P = 0.425$
	9 ms	1.66 (1.02)	1.13 (0.54)		1.22 (0.65)	1.32 (0.80)	
	11 ms	1.65 (1.22)	1.30 (0.79)		1.19 (0.71)	1.34 (0.82)	
	13 ms	1.61 (1.20)	1.21 (0.67)		1.17 (0.68)	1.49 (0.97)	
	15 ms	1.64 (1.30)	1.18 (0.66)		1.08 (0.69)	1.44 (0.99)	

[a] MANCOVA on log-transformed values.
[b] Measures missing on 3 ADHD and 2 control individuals.

Table 3. *Correlation of ADHD-scores with left hemisphere inhibition in the combined ADHD and control sample. Results of the linear regression analyses on the log-transformed mean of relative MEP after ISI 1, 3, and 5 ms as dependent variable*

Independent variable	β-estimate	Standard error	*t*-value	*P*
ADHD-SR total score	0.008	0.004	1.71	0.092
ADHD-SR attention deficit subscore	0.010	0.009	1.17	0.248
ADHD-SR hyperactivity/impulsivity subscore	0.019	0.009	2.14	0.037
WRI total score	0.009	0.004	2.23	0.029

ADHD-SR ADHD self-rating (Rösler et al., 2004b).
WRI Wender Reimherr Interview (Rösler et al., submitted).

with the attention deficit subscore or the ADHD-SR total score respectively (Table 3).

Discussion

In this study, we found reduced intracortical inhibition in a sample of adult ADHD patients compared to controls, using transcranial magnetic double pulse stimulation. The findings are in line with a previous study in ADHD children (Moll et al., 2000) and a preliminary report in adults with ADHD (Richter et al., 2006). In our adult ADHD sample we found no significant increase of cortical facilitation, in agreement with previously reported findings in ADHD children (Moll et al., 2000). Our findings therefore suggest that changes of motor excitability in adults with ADHD are similar to those in children with ADHD.

When the dimensional characteristics of ADHD were assessed, inhibitory deficits in our ADHD subjects were positively correlated with two different symptom scores, i.e. the less inhibition the more symptoms. Interestingly, we found a significant correlation with hyperactivity and impulsivity, but not with inattention scores. Similarly, Gilbert et al. (2005) reported an inverse correlation ($r = 0.53$) of ICI with ADHD symptom scores in Tourette patients. The strength of the association in the latter study was also greater with the hyperactivity/impulsivity subscore than with the inattention subscore, suggesting that hyperactive/impulsive behaviours are linked with cortical inhibitory dysfunction in Tourette patients. Compared to the study of Gilbert et al. (2005) however, the correlation between ICI and hyperactivity/impulsivity in our study was less pronounced. This difference could be due to a lack of motor tics or spontaneous motor activity in our study population without Tourette disorder or might be based on different pathophysiological mechanisms underlying these disorders.

Moreover, the WRI score that comprises not only ADHD core symptoms but also additional emotional components of adult ADHD according to the Utah criteria (Wender, 2005) was significantly related to inhibitory deficits. This finding suggests, that emotional problems in ADHD like affective lability, emotional overreagibility and hot temper might be related to the same neuropathological processes as impulsivity and hyperactivity, i.e. deficits of cortical inhibition.

Another result of our study was the identification of inter-hemispheric asymmetry of ICI deficits in ADHD patients, which was not present in healthy controls. In adults with ADHD, ICI deficits occurred only in the left hemisphere. Brain asymmetry in ADHD has been reported several times in the literature. Schrimsher et al. (2002) could predict the cumulative severity ratings of inattentive behaviours by measuring caudate volume asymmetry from serial sagittal magnetic resonance images from childhood to adolescence. Unilateral volume reductions of the pallidum have also been shown in several studies in children with ADHD (Aylward et al., 1996; Castellanos et al., 1996, 2002; Overmeyer et al., 2001). Most studies however, describe volume reductions within the right hemisphere. Therefore, the hypothetic mechanisms leading to reduced inhibition of the left side can only be deduced from unknown and complex mechanisms leading to contralateral effects.

In our study we did not find differences between ADHD subjects and healthy controls regarding resting motor (RMT) and active motor thresholds (AMT). Also simple stimulus response curves did not differ between ADHD patients and controls. This agrees with previously reported observations in children with ADHD (Moll et al., 2000). Thus, there is no evidence for motor hyperexcitability within the motor system in ADHD at the membrane level of cortical neurons (Ziemann et al., 1996d).

In general, our results suggest that TMS, and the paired-pulse technique in particular, is a useful tool for motor excitability research in ADHD. It allows the study of intracortical circuits (Ziemann, 1999; Kujirai et al., 1993; Valls-Solé et al., 1992; Ziemann et al., 1996d) and displays little inter- and intraindividual variability (Maeda et al., 2002). ICI deficits have been shown to have reliable test-retest stability in adults with ADHD (Richter et al., 2006). ICI and ICF appear to be due to activation of separate circuits (Ziemann et al., 1996d), represented by inhibitory interneurons or inhibitory connections between cortical output cells (Wassermann et al., 1996) and facilitatory interactions partially in the motor cortex, at or upstream from corticospinal neurons (Ziemann et al., 1996d). Conditioning

pulses in the double-pulse stimulation technique are thought to activate inhibitory interneurons within the motor system including projections onto corticospinal neurons (Kujirai et al., 1993). Several neural transmission systems like GABAergic, dopaminergic and glutamatergic systems appear to play an important role in these mechanisms. Medications that enhance GABAergic activity have been shown to increase the degree of ICI and decrease ICF evoked by paired TMS stimuli (Inghilleri et al., 1996; Werhahn et al., 1998; Ziemann et al., 1996b, c). Dopaminergic drugs have been shown to enhance ICI in normal subjects (Berardelli et al., 1996; Priori et al., 1994; Ridding et al., 1995; Ziemann et al., 1996a). Glutamatergic drugs have been shown to increase intracortical inhibition and decrease facilitation (Schwenkreis et al., 1999; Ziemann et al., 1998).

In conclusion, this paired-pulse magnetic stimulation study is the first to demonstrate that intracortical inhibition in adult ADHD is reduced compared to matched controls and occurs only in the left hemisphere. These deficits correlate with ADHD symptomatology, especially with hyperactivity and impulsivity behaviour. The findings therefore corroborate the hypothesis of inhibition deficits on a neuronal level. Compared to previous findings of double pulse TMS in ADHD children our results suggest that there is no fundamental difference between affected children and adults with respect to excitability of the frontal motor cortex. Further studies are needed to confirm and to further define these excitatory phenomena in ADHD to gain a greater insight into the etiology of ADHD and to generate useful endophenotypes of this disorder.

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J Neural Transm (2007) [Suppl 72]: 311–315

Printed in Austria

Journal of Neural Transmission

Association of Parkinson's disease with symptoms of attention deficit hyperactivity disorder in childhood

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Summary Methylphenidate (MPH) is a centrally acting (psycho)stimulant which reversibly blocks the dopamine re-uptake transporter. At present MPH is one of the most frequently prescribed drugs for the symptomatic treatment of attention deficit hyperactivity disorder (ADHD). Although MPH has been in use for about 50 years, there is no information available concerning the long-term benefits and risks of medication. Based on experiments in rats it has been suggested that MPH treatment may affect the maturation of central dopaminergic systems and may be a risk factor for the development of Parkinson's disease (PD). The aim of the present case-control study was to gain information about (1) ADHD-like symptoms that may precede PD motor symptoms, and (2) the exposure to psychostimulants in childhood. We used a German short version of the Wender Utah Rating Scale (WURS-k, Retz-Junginger et al., 2002) which is a reliable measure for the retrospective diagnosis of childhood ADHD, and another questionnaire including a rating scale for symptoms of ADHD in childhood (Q-ADHD-Child) according to DSM-IV and ICD-10 criteria.

A total of 92 patients with PD and 115 control subjects were enrolled in this study. Ninety-six percentage of PD patients ($N = 88$) completed the two rating scales. The data of these patients and of 88 randomly selected individuals of the controls were included for analysis. In the WURS-k, the PD group showed higher total scores compared to control subjects. In addition, we found increased scores in PD patients regarding the items attention deficit, hyperactivity and anxious and depressive symptoms, but not regarding impulsivity, oppositional behaviour and deficits in social adaptation. The results of the Q-ADHD-Child also showed increased scores in PD patients regarding attention deficit and hyperactivity. However, one cannot conclude that the PD patients enrolled in this study had suffered from childhood ADHD, since the average total WURS-k score of (14.4) was far below the cut-off score of 30 or higher which is considered to identify childhood ADHD. Finally, we found no evidence that PD patients had been exposed to psychostimulants such as MPH and amphetamine.

Keywords: Parkinson's disease, depression, attention deficit hyperactivity disorder, methylphenidate, stimulants, cognitive deficits

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Introduction

Idiopathic Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and affects approximately two percent of humans aged 65 and above (De Rijk et al., 1997). The cardinal clinical features of PD are resting tremor, rigidity, and bradykinesia. However, signs of postural instability, autonomic dysfunctions and psychiatric symptoms such as depression and dementia are also present in a large percentage of patients. PD is characterized by a preferential loss of neuromelanin-containing dopamine neurons in the pars compacta of the substantia nigra, with intracellular proteinaceous inclusions (Lewy bodies) and a reduction in striatal dopamine (for review see Jellinger, 1989; Sian et al., 1999). The progressive loss of nigral dopaminergic neurons leads to the clinical diagnosis due to the motor symptoms such as bradykinesia, rigidity and tremor, which result from a striatal dopamine reduction of about 70 percent or more (Bernheimer et al., 1973; Riederer and Wuketich, 1976). This is the rationale for dopamine-substitution therapies, including treatment with L-DOPA (L-3,4-dihydroxyphenylalanine, levodopa), peripheral aromatic amino acid decarboxylase- and catecholamine-O-methyltransferase (COMT) inhibitors, dopamine receptor agonists, selective monoamine oxidase (MAO) type B inhibitors and drugs that indirectly improve dopaminergic functions (e.g. glutamate antagonists).

Attention deficit hyperactivity disorder (ADHD) is one of the most common behavioural disorders in child and adolescent psychiatry. This disorder affects 8–12% of children worldwide and is characterized by impaired attentional functions, hyperactivity and increased impulsivity (Biederman and Faraone, 2005). Methylphenidate (MPH) is a centrally

acting (psycho)stimulant which reversibly blocks the dopamine re-uptake transporter (DAT). At present MPH is one of the most frequently prescribed drugs for the symptomatic treatment of ADHD. There is strong evidence in support of the use of stimulant medication in the management of inattention, impulsivity and hyperactivity in school-age children (Rappley, 2005). MPH has consistently been shown to be efficacious and safe when compared with placebo in randomized, controlled short-term trials. Although MPH has been in use for about 50 years, there is no information available concerning the long-term benefits and risks of medication. However, a recent report of a persistent reduction in DAT density following MPH administration in young rats raised questions about the long-term effects of MPH treatment (Moll et al., 2001). Based on this data it has been suggested that MPH treatment may affect the maturation of the dopaminergic system and may be a possible risk factor for the development of PD (Gerlach, 2003; Gerlach et al., 2003).

The aim of the present case-control study was to gain information about (1) ADHD-like symptoms that may precede PD motor symptoms, and (2) the exposure to psychostimulants in childhood. We used a German short version of the Wender Utah Rating Scale (WURS-k, Retz-Junginger et al., 2002) and another questionnaire including a retrospective self-report rating scale (Q-ADHD-Child) for symptoms of attention deficit and hyperactivity in childhood according to DSM-IV and ICD-10 criteria. Preliminary results of this paper have been published elsewhere (Walitza et al., 2005).

Patients and methods

Study sample

A total of 92 patients with PD and 115 control subjects were enrolled in the present study. Patients with PD were recruited by clinical consultants at meetings of self-help groups of patients with PD (Deutsche Parkinson Vereinigung, S.W.) and at the outpatient unit of the Department of Neurology of the University of Bochum (T.M.). Inclusion criterion was the presence of PD including early-onset PD, i.e. with an age of onset of less than 44 years. Any form of dementia was an exclusion criterion. Patients with early-onset PD were particularly included since MPH was more likely to be used during the last four or five decades than in patients aged 50 or above. In addition, young patients may have a better memory of childhood symptoms.

Ninety-two control subjects were patients of a general practitioner, 23 controls were spouses or partners but no relatives of the PD patients. The control subjects did not suffer from PD, any psychiatric disease or any form of dementia.

Following clinical assessment the subjects received the questionnaires and were requested to return the completed questionnaires to the University Department of Child and Adolescent Psychiatry in Würzburg. The questionnaires were provided with code numbers to which the investigators were blinded.

All participants gave written informed consent to participate in the study which was approved by the Ethic Committee of the University of Würzburg.

Questionnaires

The participants were provided with the WURS-k (Retz-Junginger et al., 2002; original version by Ward et al., 1993) which is used in adults for the retrospective diagnosis of ADHD in childhood. This scale comprises 21 items which are rated on a 5-point scale (0–4). Factor analysis of the WURS-k yielded 5 components, i.e. attention deficit/hyperactivity (8 items), impulsivity (4 items), anxious depressive symptoms (4 items), oppositional behaviour (3 items) and deficits in social adaptation (2 items). The total score ranges from 0–84. For the retrospective diagnosis of ADHD in childhood the authors recommended a cut-off score of 30 or higher (only for male subjects (Retz-Junginger et al., 2003). The internal consistency coefficients ranged from $\alpha = 0.19$ to 0.61, with a test-retest correlation of $r_{tt} = 0.9$.

In order to obtain additional information regarding childhood symptoms of ADHD, a second questionnaire (Q-ADHD-Child) was constructed. In this 46-item checklist, adult participants rated the presence of behavioural problems during childhood on 5-point scales (0–4). The items were based on the diagnostic criteria for ADHD according to DSM-IV (American Psychiatry Association, 1994) and ICD-10 (Remschmidt et al., 2006). The items comprised the factors attention (8 items, e.g. "How often did your exercises show slips of the pen?"), hyperactivity (6 items, e.g. "Did you often fidget or shift around on your chair?") and impulsivity (6 items, e.g. "Did you often blurt out before the question had been asked"). Furthermore, there were 19 items to detect other symptoms of psychiatric childhood disorders (e.g. anxiety or depressive disorders). All participants were asked to rate the frequency of the above mentioned symptoms present from early childhood to the end of elementary school.

In addition, there were 7 open-ended questions concerning neurological diseases, substance abuse during childhood, any long-time medication in childhood, past hospital stays, onset of PD and previous treatment with MPH.

Data analysis

The comparison between PD patients and control subjects regarding the retrospective assessment of ADHD symptoms in childhood and previous medication with MPH, was performed using the Mann-Whitney *U*-test. For statistical analysis an alpha level of 0.05 was applied. All statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS, version 14.0).

Results

A total of 96% of PD patients ($N = 88$) completed the two questionnaires. The data of these patients and of 88 randomly selected individuals out of the 115 enrolled controls were included for analysis. The two groups (Table 1) did not differ regarding age ($p = 0.22$) and gender distribution. In the patient group, there were more missing values regarding education level.

It is noteworthy that the group of PD patients comprised a subgroup of 43 patients with early-onset PD (age of onset <44 years). The results of this subgroup were compared to those of a randomly selected subgroup of control subjects ($N = 43$). There was no difference between the groups regarding age at investigation (PD patients: mean $\pm$ SD 48.2 ± 9.7 years; controls: 50.6 ± 8.9 years) and gender

Table 1. *Characteristics of patients with Parkinson's disease (PD) and healthy subjects (controls)*

	PD patients ($N = 88$)	Controls ($N = 88$)
Age: (mean ± SD, years)	55.6 ± 11.1	57.2 ± 9.5
Range	33–82	35–84
Gender: male/female	47/41	47/41
Education		
Not applicable	22	10
Primary school and secondary school with lower academic standards	24	22
Secondary school with average academic standards	20	18
Secondary school with higher academic standards	22	38

distribution (PD patients: 19 males, 22 females; controls: 22 males, 21 females).

The total score of the WURS-k ranged between 0 and 62 (mean ± SD 14.4 ± 13.0) in the PD group and between 0 and 42 points in the control group (mean ± SD 9.8 ± 10.03). There was a significant difference between the groups (Table 2). Differences were also found for the two items attention deficit/hyperactivity and anxious/depressive symptoms but not for the factors impulsivity, oppositional behaviour and deficits in social adaptation (Table 2).

The analysis using the Q-ADHD-Child score showed similar results (Table 3). Patients with PD had higher scores for the items attention deficit and hyperactivity compared to controls, but not for the factor impulsivity.

The early-onset PD subgroup showed similar differences in the WURS-k and Q-ADHD-Child scores and subscores as the total PD sample (data not shown).

The answers to the open-ended questions of the Q-ADHD-Child concerning substance abuse during childhood, long-time medication in childhood and treatment with MPH showed that no subject had been exposed to MPH in the past. Substance abuse was reported by three of the PD patients (tetrahydrocannabinol, LSD or alcohol) and one of the control subjects (tetrahydrocannabinol) reported substance abuse.

Table 2. *Retrospective diagnosis of childhood attention deficit hyperactivity disorder by using the German short version of the Wender Utah Rating Scale (WURS-k; mean ± SD)*

	Patients ($N = 88$)	Controls ($N = 88$)	p value*
Total score	14.4 ± 13.0	9.8 ± 10.0	0.01
Factors (each item was rated on a 5-point scale, 0–4)			
Attention deficit/hyperactivity	0.8 ± 0.8	0.6 ± 0.6	0.01
Impulsivity	0.6 ± 0.7	0.5 ± 0.6	0.09
Anxious depressive symptoms	0.8 ± 0.8	0.4 ± 0.6	0.00
Oppositional behaviour	0.5 ± 0.7	0.5 ± 0.7	0.6
Social adaptation	0.1 ± 0.5	0.01 ± 0.07	0.4

* Mann-Whitney *U*-test.

Table 3. *Retrospective diagnosis of childhood attention deficit hyperactivity disorder by using the novel Q-ADHD-Child scale (mean ± SD)*

	Patients ($N = 88$)	Controls ($N = 88$)	p values*
Q-ADHD-Child factors (the frequency of each item was rated on a 5-point scale, 0–4)			
Attention deficit	1.6 ± 0.5	1.3 ± 0.5	0.002
Hyperactivity	1.3 ± 0.6	1.0 ± 0.4	0.006
Impulsivity	1.4 ± 0.7	1.3 ± 0.6	0.3

* Mann-Whitney *U*-test.

Discussion

We used the WURS-k and the Q-ADHD-Child questionnaire to gain retrospective information about ADHD-like symptoms that may precede PD, and (2) the exposure to psychostimulants in childhood. The WURS-k is a German version of the Wender Utah Rating Scale, which is a reliable measure for the retrospective diagnosis of childhood ADHD (Ward et al., 1993; Retz-Junginger et al., 2002). In comparison to control subjects, a higher total score in the PD group was found using the WURS-k. In addition, statistical analysis of the WURS-k results revealed increased scores in PD patients regarding the items attention deficit and hyperactivity but not regarding impulsivity, oppositional behaviour and deficits in social adaptation. We also assessed ADHD symptoms in childhood according to ICD-10 and DSM-IV with the Q-ADHD-Child questionnaire, a 46-item scale constructed by the authors of the present study. In accord with the WURS-k results, patients with PD showed increased scores for the items attention deficit and hyperactivity but not for impulsivity. Finally, we found no evidence for exposure of PD patients to psychostimulants such as MPH and amphetamine.

However, one cannot conclude that the PD patients enrolled in this study had suffered from childhood ADHD. The WURS-k with 21 items was administered to German adults comprising 95 patients with a psychiatric diagnosis, 321 prison inmates and 287 control subjects. The authors found an average score of 24.4 ± 15.4 in psychiatric patients, 25.2 ± 13.7 in prisoners and 16.5 ± 9.8 in the control group (Retz-Junginger et al., 2002). In a population of 63 adult patients with ADHD (according to ICD-10 and DSM-IV) and 1303 male control subjects, the receiver-operating characteristic analysis indicated high sensitivity (85%) and specificity (76%) for diagnosis of childhood

ADHD in males when a cut-off point of 30 was used (Retz-Junginger et al., 2003). The authors found higher total WURS-k scores in males compared with females. This is in accordance with the higher incidence of ADHD in male children. In our study nine of our 88 patients with PD and six of 88 controls had a total score equal or higher than 30. This is still in accord with the expected prevalence of childhood ADHD (Biederman and Faraone, 2005).

The total WURS-k scores (mean $\pm$ SD) found in our study were 14.4 ± 13.0 in the PD group (range 0–62) and 9.8 ± 10.03 in the control group (range 0–42). Interestingly, these scores were lower compared to those reported for the German samples of Retz-Junginger et al. (2002, 2003). One reason for lower scores in our study groups could be the higher age of the enrolled subjects (mean $\pm$ SD; in our study 55.6 ± 11.1 years for PD patients and 57.2 ± 9.5 years for controls; in the study of Retz-Junginger et al., 29.8 ± 11.8 years for controls, 35.2 ± 10.7 years and 38.7 ± 12.6 years for prison inmates and psychiatric patients, respectively).

The present findings of increased scores of PD patients in the items attention deficit and hyperactivity suggest that these symptoms may precede parkinsonian motor deficits. In his autobiography, Michael J. Fox who suffers from early-onset PD, reported ADHD-like symptoms in childhood (Fox, 2002). In addition, some similarities have been noted between the behavioural deficits observed in patients with early PD, patients with ADHD and in an animal model of early parkinsonism, i.e. monkeys chronically treated with low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Roeltgen and Schneider, 1991). In a case report, a PD patient was described, many years prior to the manifestation of parkinsonian motor signs, as (1) looking vacant, unresponsive, and inattentive, (2) displaying restless, irritable, and impatient behaviours, and (3) showing a disinclination to continue motor tasks (Lees, 1992). Recent findings suggested that cognitive impairment is common in early stages of PD, with deficits being most prominent in the domains of memory, and attention and executive function (Berg et al., 1999; Muslimovic et al., 2005).

There are several limitations of the present study. As in every retrospective study there is the possibility of poor recall (underreporting or overreporting) and of recall bias. However, age-dependent recall problems seem to be unlikely since the findings in both the early-onset and the late-onset PD groups were similar. In addition, our results showed an increased score for the factor anxious and depressive symptoms in the PD group (Table 2). This result is in accord with previous reports showing that depression precedes the diagnosis of PD (Müller, 2004; Ishihara and Brayne, 2006; Lieberman, 2006).

Acknowledgements

The authors are grateful to all participants and Ms Eva Schmoeger (Deutsche Parkinson Vereinigung) for her support with recruiting patients with Parkinson's disease. The present study was supported in part by a grant of the „Wissenschaftliches Herausgeberkollegium der Münchener Medizinischen Wochenschrift e.V.“ (Munich, Germany) and by the KFO/1-1 (DFG).

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J Neural Transm (2007) [Suppl 72]: 317–322

Printed in Austria

Journal of Neural Transmission

Biostatistical analysis of gene microarrays reveals diverse expression clusters between macaque subspecies in brain SIV infection

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Summary In this study we investigated differences in the gene expression profiling of the brains of rhesus macaques that were uninfected or infected with SIV in the asymptomatic stage or AIDS. The main aim was to use biostatistical methods to classify brain gene expression following SIV infection, without consideration of the biological significance of the individual genes. We also used data from animals treated with different pharmacological substances such as dopaminergic drugs, N-methyl-D-aspartate (NMDA) antagonists or antioxidants during the early stage of infection as these animals exhibited an accelerated or attenuated neuropsychiatric disease progression.

We found macaque subspecies to be a more important factor for disease classification based on gene expression profiling than clinical symptoms or neuropathological findings. It is noteworthy that SIV-infected pharmacologically-treated. Chinese animals clustered near uninfected animals independent on the outcome of the treatment, whereas untreated SIV infected animals were clustered in a separate subtree. It is clear from this study that NeuroAIDS is a diverse disease entity and that SIV brain genes can be differentially regulated, depending on the disease type as well as changed dependent on the monkey subspecies.

Keywords: Gene profiling, Affymetrix, SIV, HIV, Chinese, Indian, array, CNS

Introduction

HIV penetrates the brain soon after the initial systemic infection, however, viral RNA and antigens are detected only infrequently during asymptomatic HIV infection (Albright et al., 2003). Although the period between acute HIV infection and overt AIDS may be asymptomatic, the CNS may undergo changes that could be progressive and cause additional damage as the disease develops, suggesting that investigations of pathogenesis and therapy should focus on the early stages of infection. Human brain tissue in this stage is rarely available, making determination of the basis for CNS involvement problematic. The simian immunodeficiency virus (SIV)-infected rhesus macaque provides an excellent model for mimicking CNS abnormalities induced by HIV in humans (Sopper et al., 2002). We found previously that SIV-infected macaques exhibit dramatic impairments in dopamine (Jenuwein et al., 2004; Koutsilieri et al., 1997; Scheller et al., 2005) and acetylcholine (Koutsilieri et al., 2000, 2001) neurotransmitter systems as early as 2–3 months of infection without presenting with overt clinical symptoms, detectable virus levels or pathological features. This suggests that indeed neuronal dysfunction is initiated very early in the course of infection and progresses with time resulting in changes in other neural circuits, e.g. glutamatergic (Koutsilieri et al., 1999) and finally in SIV encephalitis and neuropsychiatric disease.

In this study we investigated differences in the gene expression profiling of rhesus macaques that were uninfected or infected with SIV in the asymptomatic stage or AIDS with a gene microarray analysis. The main aim was to use biostatistical methods to classify brain gene expression following SIV infection, without consideration of the biological significance of the individual genes. We also included data from uninfected animals, as well as animals which were treated with different pharmacological substances during early stage of infection and exhibited either an accelerated or attenuated disease progression.

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We found that macaque subspecies is a more important factor for disease classification based on gene expression profiling than clinical symptoms or neuropathological findings. Moreover, it is obvious from this study that AIDS is a completely diverse disease entity and is not only associated with a greater differential regulation of the same genes in SIV infection.

Materials and methods

Animals

Twenty-five Juvenile rhesus monkeys (*Macaca mulatta*) of Chinese and Indian origin (12 and 13 subjects, respectively) were housed individually in indoor facilities on a 12:12 light:dark schedule at the German Primate Center (Göttingen, Germany). Dry food as well as bananas and apples as a dietary supplement was provided twice a day and water was available ad lib.

Prior to inoculation, animals were demonstrated to be seronegative for STLV-1, SRV-1 (type D retrovirus), herpes B virus, and SIV. Monkeys were infected under ketamine anesthesia (10 mg/kg) with 100 MID_{50} (50% monkey infective dose) of SIVmac251MPBMC. Ten of these animals were sacrificed at predetermined time points without signs of simian AIDS. A further five monkeys were killed when they became moribund. Ten uninfected animals served as controls. Animals were monitored clinically. Blood and CSF samplings were performed at regular intervals under ketamine anesthesia from experienced veterinarians in the German Primate Center Göttingen.

Animal experiments were approved by, and performed according to the guidelines set out by the ethics committee for animal experimentation of the Bezirksregierung Braunschweig (604.42502/08-02.95) and the revised Directive 86/609/EEC on the protection of Animals used for experimental and other scientific purposes.

Collection and dissection of monkey brain tissue

Necropsy for all animals was performed at the same time of the day. Anesthetized animals were sacrificed by exsanguination at different time points after infection ranging from 6.3–67.4 weeks post infection (wpi). Brains were thoroughly perfused with two liters of RPMI 1640 medium (Gibco, Eggenstein, Germany) containing 3% fetal calf serum. The brains were quickly removed, partly immersion-fixed (5% neutral buffered formaldehyde for light microscopy) and partly frozen at $-70^{\circ}C$ for biochemical analysis. Prior to neurochemical investigations, putamen was dissected on a Teflon plate at $-20^{\circ}C$ according to a stereotactic atlas using standardized procedures.

Animals with pharmacological treatments are not presented here in detail, as their only function for this article was to assist in the clustering of brain SIV infection.

Total RNA extraction

Total RNA was extracted from putamen of rhesus macaques using the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany). The samples were spectrophotometrically scanned from 220–320 nm; the A260/A280 of total RNA was typically >1.9. RNA quality was tested on the RNAStdsenseChip® using the Experion electrophoresis (BioRad, Germany). For all total RNAs extracted, the 28S/18S ratio was >1.5. Total RNAs were subjected to DNase-I (Qiagen GmbH, Hilden, Germany) digestion to remove genomic DNA residues and subsequently cleaned by the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany). No significant differences between the spectral purity, rate of degradation, molecular size, yield, or tissue pH between the groups were noted.

Preparation of labeled cRNA

The procedures described in detail in the Affymetrix Gene Chip® Expression Analysis Manual (Affymetrix Inc., Santa Clara, CA, USA) were essentially followed. Hundred nanogram of pooled RNA for each group was converted to double-stranded cDNA, using the Affymetrix Two-Cycle Eukaryotic Target Labeling procedure (Two-Cycle Target Labeling and Control Reagents Kit, Affymetrix Inc., Santa Clara, CA, USA). Briefly, after first cycle cDNA synthesis the cDNA was used for preparation of an unlabeled cRNA (MEGAscrpit® T7 Kit, Ambion Inc., Austin, TX, USA). The unlabeled cRNA template was reversed transcribed in single-stranded cDNA using random primers. Subsequently, a double stranded cDNA was synthesized utilizing oligo dT primer containing the T7 promotor. The cDNA was cleaned by the provided Affymetrix Sample Cleanup Module (Affymetrix Inc., Santa Clara, CA, USA) and used for biotinylated cRNA preparation (GeneChip IVT Labeling Kit, Affymetrix Inc., Santa Clara, CA, USA). The biotinylated cRNA was cleaned (Sample Cleanup Module, Affymetrix Inc., Santa Clara, CA, USA), quantitated and fragmented.

Array hybridization

Additional confirmation of total RNA quality was conducted by hybridizing a small portion of labeled cRNA target to Affymetrix Test3 Array (Affymetrix 900341). After confirming that all RNA samples were in good quality, labeled cRNA was hybridized to GeneChip Rhesus Macaque Genome Array (Affymetrix 900656) in an Affymetrix Fluidics Station 400. After hybridization, the solutions were removed, and the arrays were washed on the Affymetrix Fluidics Station 400. Hybridized arrays were stained with 10 µg/ml streptavidin-R phycoerythrin (Molecular Probes, Eugene, OR, USA), followed by staining with 3 µg/ml biotinylated goat anti-streptavidin antibody (Sigma Chemical, St. Louis, MO, USA). Arrays were then stained once again with streptavidin-R phycoerythrin and scanned using a GeneChip Scanner 3000.

The Gene Chip used contained several probe sets specific for rhesus macaque house-keeping genes such as beta-actin, elongation factor 1 and GAPDH which served as positive controls. Several murine and yeast probe sets on each chip served as negative controls, and externally spiked bacterial bioB, bioC, bioD and cre served as positive hybridization controls.

Data interpretation and analysis

For data analysis we used different R packages from the Bioconductor project (www.bioconductor.org). Resulting signal intensities were normalized by variance stabilization (Huber et al., 2002). Quality of all data sets was tested by density plot, RNA degradation plot and correspondence analysis. Statistical analysis to select differentially expressed genes was performed using the LIMMA (Linear Models for Microarray Analysis) package (Smyth et al., 2003). LIMMA is a library for the analysis of gene expression microarray data, especially the use of linear models for analyzing designed experiments and the assessment of differential expression. In a first step a gene-wise linear model was fitted to the data. Given a series of related parameter estimates and standard errors, moderated t-statistics and log-odds of differential expression were computed by empirical Bayes shrinkage of the standard errors towards a common value. This empirical Bayes linear modeling approach was implemented to enable stable analysis even for smaller sample sizes. As an output a table of the top-ranked genes from the linear model fit including a gene list, ratio on the natural log scale (M), average gene intensities (A), moderated *t*-statistic, adjusted *p*-value with fdr correction and log odds were provided with this analysis.

Results and discussion

This article reports an overview of gene expression profiling in the putamen of rhesus monkeys categorized into 5 groups: uninfected Chinese, uninfected Indian, SIV-infected Chinese, SIV-infected Indian and AIDS (both Chinese and Indian). We chose to analyze the putamen because of the particular involvement of this nucleus in brain dysfunction during SIV and HIV infection, the number of infected cells in this nucleus and the subcortical motor symptomatology associated with impairment of this region in HIV-infected patients (Arendt et al., 1990; Koutsilieri et al., 2002; Nath et al., 1987).

To avoid bias in our statistical analysis, we initially investigated comparability across cases based on the quality of RNA and gene intensity. In order to check whether RNA amplification with a two-cycle amplification was satisfactory, we plotted RNA integrity. We could show that the RNA was of comparable quality across groups (Fig. 1A). To further assess the comparability of the data sets, we plotted all data in a boxplot. All medians and interquartile ranges were in good concordance, indicating that data are comparable (Fig. 1B).

We then clustered the cases based on the similarities or dissimilarities between groups using an unsupervised hierarchical cluster algorithm (Fig. 2A). At first inspection uninfected Indian macaques, SIV-infected Indian macaques and monkeys with AIDS appear to be grouped separately (form a distinct subtree) from all other cases. Within this subtree, the AIDS group differed from the other two cases. The second major separation was within the Chinese ma-

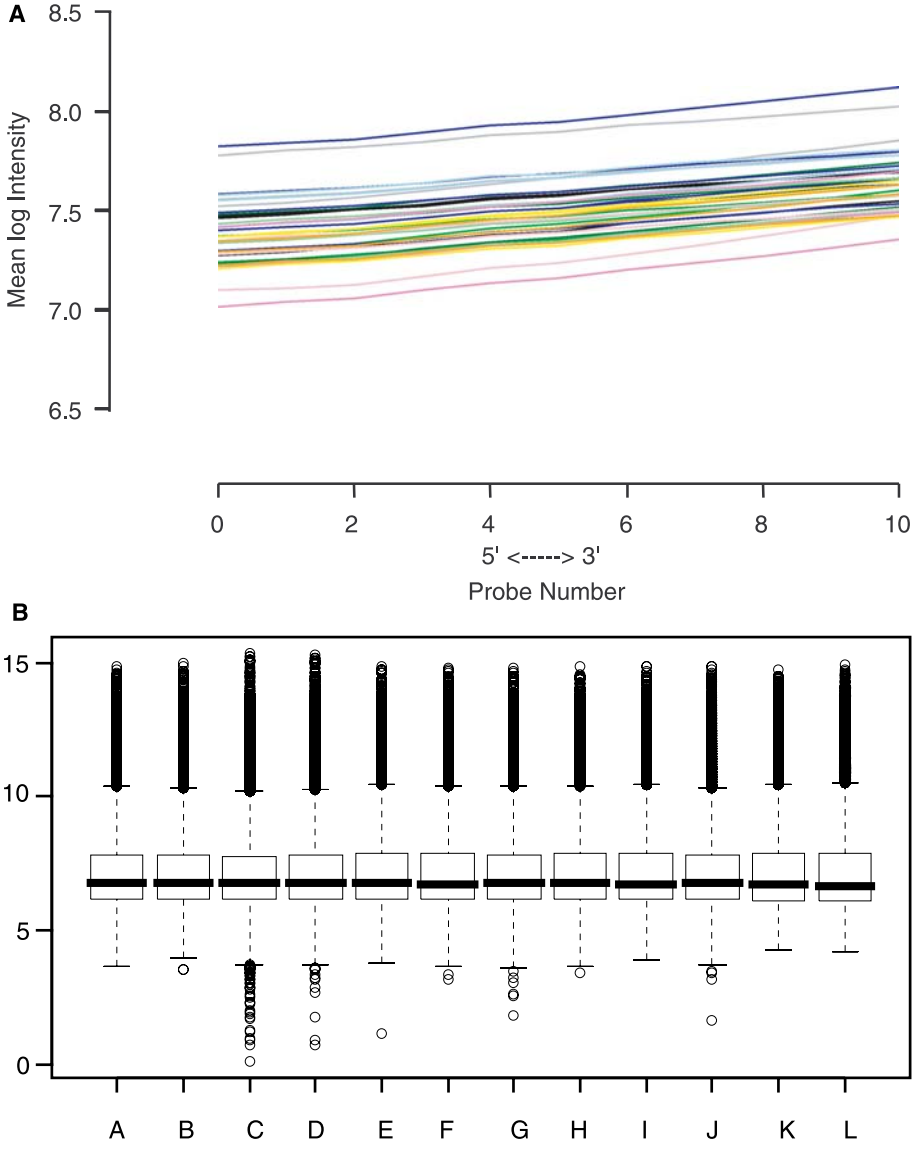

Fig. 1. **A** RNA digestion plot. Y axis represents log intensity prior to normalization averaged by probe number across all genes. Probes are numbered directionally from the 5′ end to the 3′ end (X axis). Different colours indicate different cases (groups). **B** Boxplots illustrating the distribution of log intensity of genes in each expression category. The centre line of each box represents the median; the box encompasses the first quartile above and below the median; the brackets extending from each box represent the range except for the outliers; other points are regarded as outliers. *A–L* represent cases (Uninf-China, SIV-China, Uninf-India, SIV-India, AIDS, SIV-infected/selegiline-treated, selegiline-treated, SIV-infected/ memantine-treated, SIV-infected/ selegiline-/meman-tine-treated, SIV-infected/selegiline-/L-DOPA-treated, SIV-infected/N-acetylcysteine-/melatonin-treated, SIV-infected/N-acetylcysteine-/selegiline-/melatonin-treated), respectively. Data are pooled replicates from all groups after variance stabilization normalization (VSN)

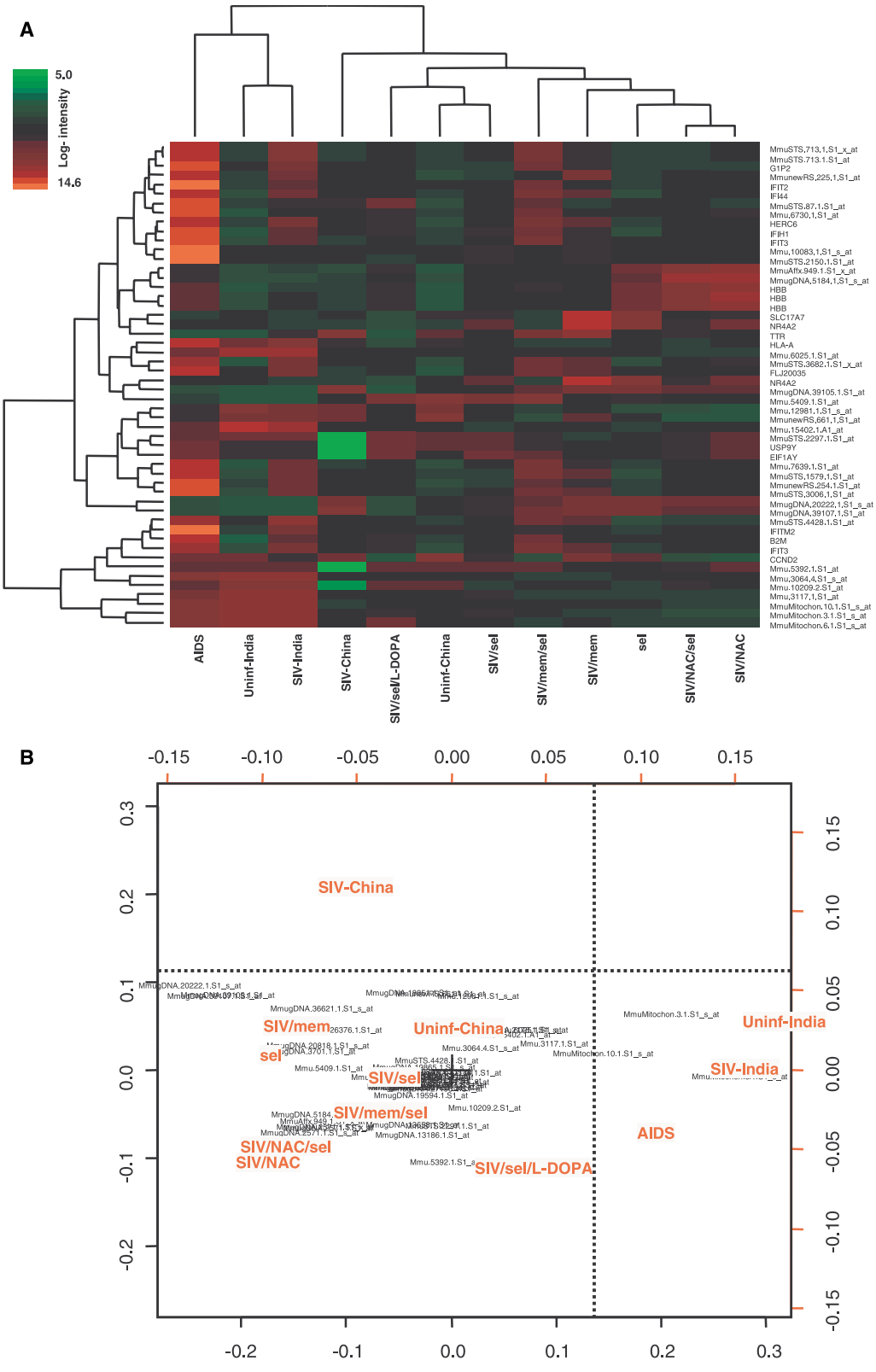

Fig. 2. **A** Heat map with dendrograms. Hierarchical cluster dendrograms of the cases using correlation similarity metric and average linkage clustering. The rows contain genes and the columns contain array samples (cases). Dendrogram above compares gene expression between columns; dendrogram side compares individual genes across cases. For clustering median expression levels of three replicates have been used. Genes with an SD $\geq$ 0.8 were used for the analysis. Only known genes are shown in gene symbols. **B** Correspondence analysis. Genes with an SD $\geq$ 0.8 were used for the analysis. Dotted lines for both X and Y axes assist the graphic presentation as a first site separation of the cases

caques. SIV infection itself was separated from other groups (Fig. 2A). It is interesting that the other cases of Chinese macaques included groups with medical treatment which exacerbated or attenuated neuropathology. However, all groups were clustered within another subtree separated from SIV infection alone, indicating that these treatments did not alter neuropathological outcome due to changes in differential gene expression. We further confirmed the clustering of the cases with correspondence analysis (Fig. 2B). We displayed associations between arrays (cases) and genes and found a similar separation or association of groups as that seen using the heat map and dendrograms. To our knowledge, only the group of Howard Fox has previously published gene profiling of brains in SIV-infected macaques (Roberts et al., 2004). They were concerned with the biological significance of individual gene regulation however, and did not assess clustering of SIV infection itself. Therefore, we cannot discuss our findings in relation to others.

We identified alterations in the expression of only 20 genes in the infected Chinese macaques comparing to the Indian animals with a differential gene expression of 237. None of the regulated genes were shared between these subspecies (Fig. 3). Genes regulated in AIDS were related more to infection of Indian macaques than to the infection of Chinese macaques (Fig. 3). It is obvious that gene expression differences between these geographically separated groups may confuse the interpretation of the differential gene profiling in SIV infection. Several lines of evidence suggest that these Chinese and Indian monkeys actually constitute two subspecies, which can reliably be differentiated through genetic markers (Penedo et al., 2005). This diversity in gene expression may explain the fact that these two subspecies have great differences in viral load and disease progression upon inoculation of various strains of SIV through different routes as reported in several studies (Demuth et al., 2000; Joag et al., 1994; Ling et al., 2002; Marthas et al., 2001; Trichel et al., 2002). Differences in the immune response to vaccination have also been observed (Stahl-Hennig, pers. communication). Although rapid disease progression can occur in both subspecies (Marthas et al., 2001), the proportion of animals succumbing to the infection within the first few months is much lower for Chinese animals. Typical signs of rapid disease progression (Sauermann et al., 2000), such as unrestricted viral replication, lack of substantial seroconversion, sustained loss of memory CD4+ T-cells in blood and disease were up to 2% in our Chinese macaque population compared to approximately 25% in Indian macaques.

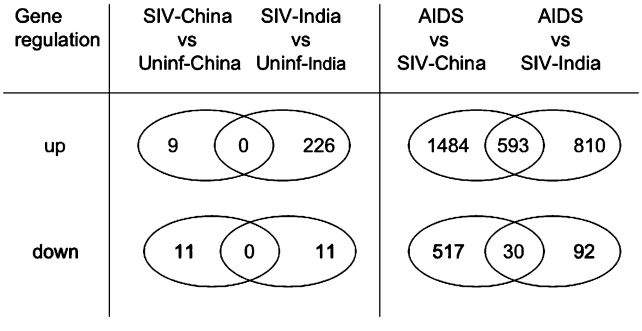

Fig. 3. Venn diagrams showing shared and distinct expression of statistically significant altered genes after SIV infection and AIDS in Chinese and Indian macaques

In our study we used an ''Indian'' viral strain, the SIVmac251, which replicates equally well in *in vitro* cultures of PBMCs isolated from monkeys of different origin (Marthas et al., 2001), suggesting that viral factors are not important for the reported differences in viral kinetics in vivo. This is consistent with the finding that viral load at peak viremia is not different between these subspecies (Marthas et al., 2001). However, peak viremia may indeed differ between these subspecies when rapid progressors are overrepresented in the Indian group (Ling et al., 2002). Immunological differences, leading to a better restriction of viral replication, are more significant if one considers that viral load in the postacute phase is reported to be significantly lower in Chinese monkeys in all studies to date (Joag et al., 1994; Ling et al., 2002; Marthas et al., 2001; Trichel et al., 2002).

After infection with neurotropic strains of SIV, encephalitis as well as overt neurological disease develops more frequently in rapid progressors, animals with a poor immune response, unrestricted viral replication and faster disease progression i.e. AIDS within the first 7 months of infection (Sopper et al., 1998). Thus, it is not surprising that severe SIV-encephalitis is a rare event in rhesus monkeys of Chinese origin. However it is not known whether the differences in the overall disease course are the sole explanation for the decreased susceptibility of Chinese macaques to the development of neurological disease. Likewise, it is possible that intrinsic CNS factors also contribute to the difference in neuronal vulnerability between the two subspecies.

Our results demonstrate marked differences in the gene expression profile between Chinese and Indian macaque subspecies, as well as a unique differential gene expression pattern in response to SIV infection in these two subgroups. Studies to investigate the biological importance of genes implicated in the identified clusters will help to improve our understanding of the pathophysiology of brain SIV infection in macaques.

Acknowledgements

We thank Margarete Göbel and Miryame Hofmann for technical assistance. The study was supported by the ''Gesellschaft für AIDS-Forschung'' and BMBF KI 0211 Competence Network HIV/AIDS.

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J Neural Transm (2007) [Suppl 72]: 323–329

Printed in Austria

Journal of Neural Transmission

Kynurenines, redox disturbances and neurodegeneration in multiple sclerosis

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Summary Multiple sclerosis (MS) is a chronic, demyelinating disease of unknown origin. Sophisticated analytical methods have made it possible to measure small biologically active molecules at low endogenous levels, and understand their role in the network of other biologically active compounds actively involved in inflammatory and neurodegenerative processes. Evidence is accumulating as concerns the disturbances of the kynurenine pathway and redox changes in MS. A new promising metabolite of the kynurenine pathway seems to beneficially influence experimental allergic encephalomyelitis. More clinical evidence is needed to prove the role of kynurenic acid analogues and/or enzyme inhibitors as potential medications in MS in the future. Various compounds have been shown to be important in the pathophysiological processes of the disease and are targets for pharmaceutical intervention.

Keywords: Antioxidants, experimental allergic encephalomyelitis, kynurenine, kynurenic acid, multiple sclerosis, redox disturbance

Introduction

Multiple sclerosis (MS) is a chronic, demyelinating disease of unknown origin that affects the central nervous system (CNS) in young adults. The measurement of biological compounds with precise analytical methods has provided a new insight into the network of molecules actively involved in inflammatory and neurodegenerative processes. Imbalance in the kynurenine pathway is proved in different neurological diseases, such as Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, stroke and epilepsy (for review see Németh et al., 2006). One of its metabolites quinolinic acid takes part in lipid peroxidation and produces free radicals. It also affects mitochondrial function by decreasing superoxide dismutase (SOD) activity (Santamaria et al., 2001). Due to the glutamate-mediated excitotoxic changes, decreased kynurenic acid (KYNA) concentration causes elevated reactive oxygen species (ROS) (Greene and Greenamyre, 1996). ROS are thought to be derived from activated inflammatory cells and to play role in demyelination and axonal damage in MS. Growing body of evidence support that these compounds play an active role in this disease of still unknown origin. The biochemical pathways involved in the metabolism of these molecules are related to pathophysiological processes of interest and are potential targets for new medication (Fig. 1) (Vécsei, 2005).

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Kynurenine pathway disturbances in MS

Tryptophan is predominantly metabolized in the kynurenine pathway in mammalian peripheral tissues (reviewed by Wolf, 1984), as well as in central nervous tissue as demonstrated in rat (Minatogawa et al., 1974). The activated immune system seems to enhance the degradation of tryptophan to formyl-kynurenine (Widner et al., 1997). While 3-hydroxykynurenine and quinolinic acid exert neurotoxic properties, KYNA antagonizes excitotoxic neuronal death (Fig. 2). KYNA exerts its neuroprotective effects binding at the excitatory glutamate receptors, particularly by strychnine insensitive glycin receptors of the N-methyl-D-aspartate (NMDA) receptor complex and alpha-7-subunit of nicotinic receptors (Vécsei, 2005). It is primarily derived from kynurenine aminotransferase (KAT) II enzyme activity in the glia. For observations with kynurenine and related compounds in animal experiments see the review by Németh et al. (2006). In this study we are focusing on the data derived from clinical studies.

Fluctuations in kynurenine concentrations have discrete effects on the nervous and immune systems (Schwarcz,

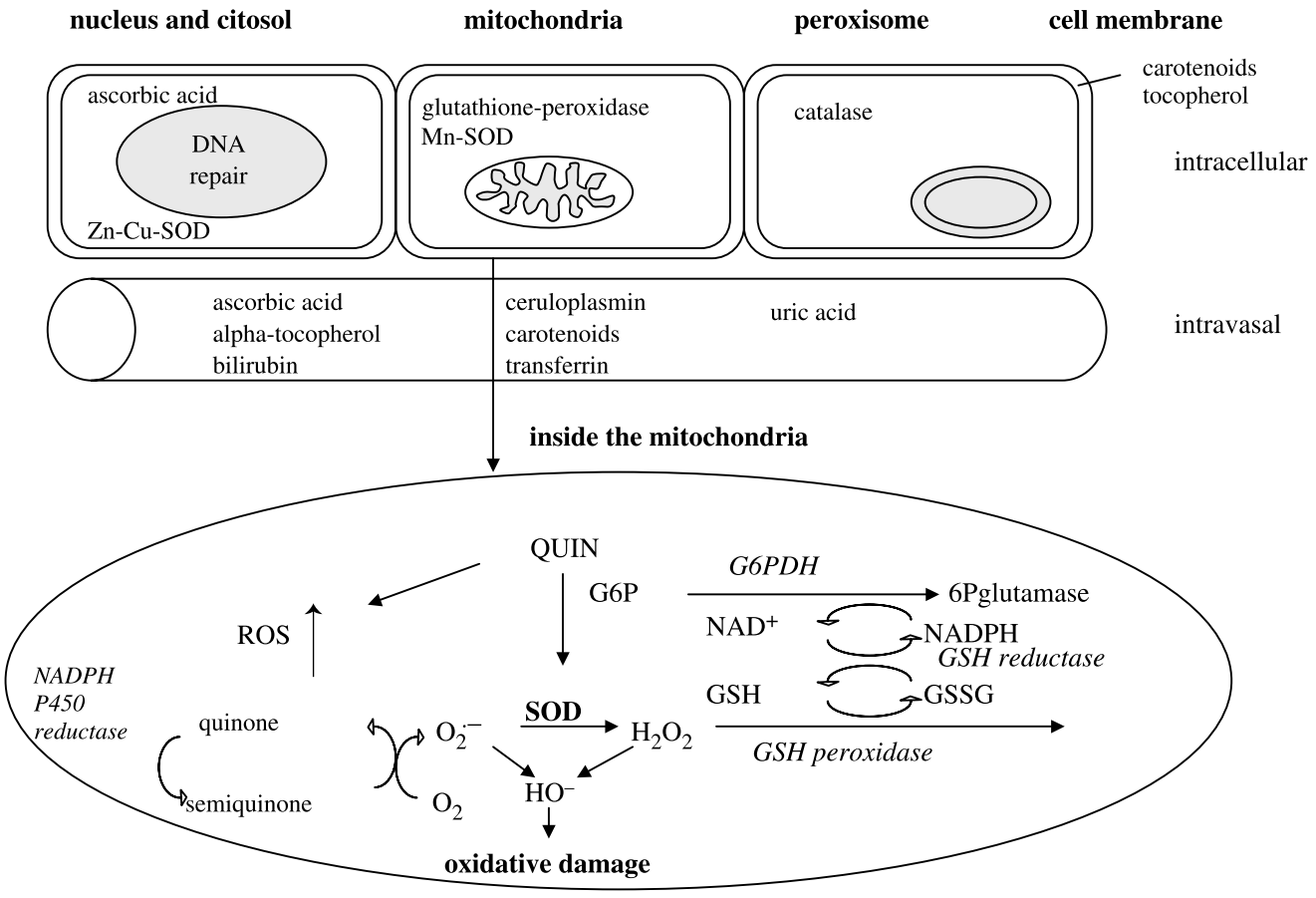

Fig. 1. Antioxidant systems (modified from the websites www.sigmaaldrich.com and qcom.etsu.edu). *G6P* Glucose-6-phosphate, *G6PDH* glucose-6-phosphate dehydrogenase, *6Pglutamase* 6-phospho-glutamase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *NAD* nicotinamide adenine dinucleotide, *NADP* nicotinamide adenine dinucleotide phosphate, *QUIN* quinolinic acid, *SOD* superoxide dismutase

2004). By shifting the metabolism of tryptophan towards KYNA, the astrocytes act in a neuroprotective manner (Guillemin et al., 2001). In stable MS patients, decreased cerebrospinal fluid (CSF) levels of KYNA have been reported (Rejdak et al., 2002). Another study described increased activities of the KYNA-synthesizing kynurenine aminotransferases in the erythrocytes, and elevated levels of KYNA in the plasma of MS patients (Hartai et al., 2005). This effect could be connected with the oxidative stress in blood, whereas in plasma of stable MS patients no significant changes of KYNA were found during 24 h of monitoring (Amirkhani et al., 2005). In long-term interferon-beta (IFN-β)-treated patients the levels of the tryptophan metabolites were stable; the increase of the kynurenine to tryptophan (K/T) ratio in MS patients receiving IFN-β for the first time during the study indicated the earlier described induction of indoleamine-2,3-dioxygenase by IFN-β (Amirkhani et al., 2005; Guillemin et al., 2001). KYNA is a potentially promising therapeutic approach, but its use limits the poor transport across the blood-brain barrier (BBB). The solution may be the development of KYNA analogues, which pass the BBB or the use of kynurenine derivates that ensure the increased KYNA concentration within the CNS (Németh et al., 2006). Lately, a catabolite of tryptophan, N-(3-4-dimethoxicinnamoyl) anthranilic acid proved to be a promising molecule for treating Th1-mediated autoimmune diseases such as MS (Platten et al., 2005).

Redox disturbances in MS

ROS are containing unpaired electrons making the molecule more reactive. If the level of ROS is enormously elevated or the mechanisms eliminating ROS are exhausted the antioxidant balance break up and oxidative stress occurs leading to cellular damage or death. Free radicals are produced via both enzymatic and non-enzymatic pathways. The free radicals are generated from oxygen and nitrogen free radicals, or by enzymatic or non-enzymatic antioxidant systems. The latter is consisted from a directly acting (such as ascorbic acid, lipoic acid, carotenoids, glutathione,

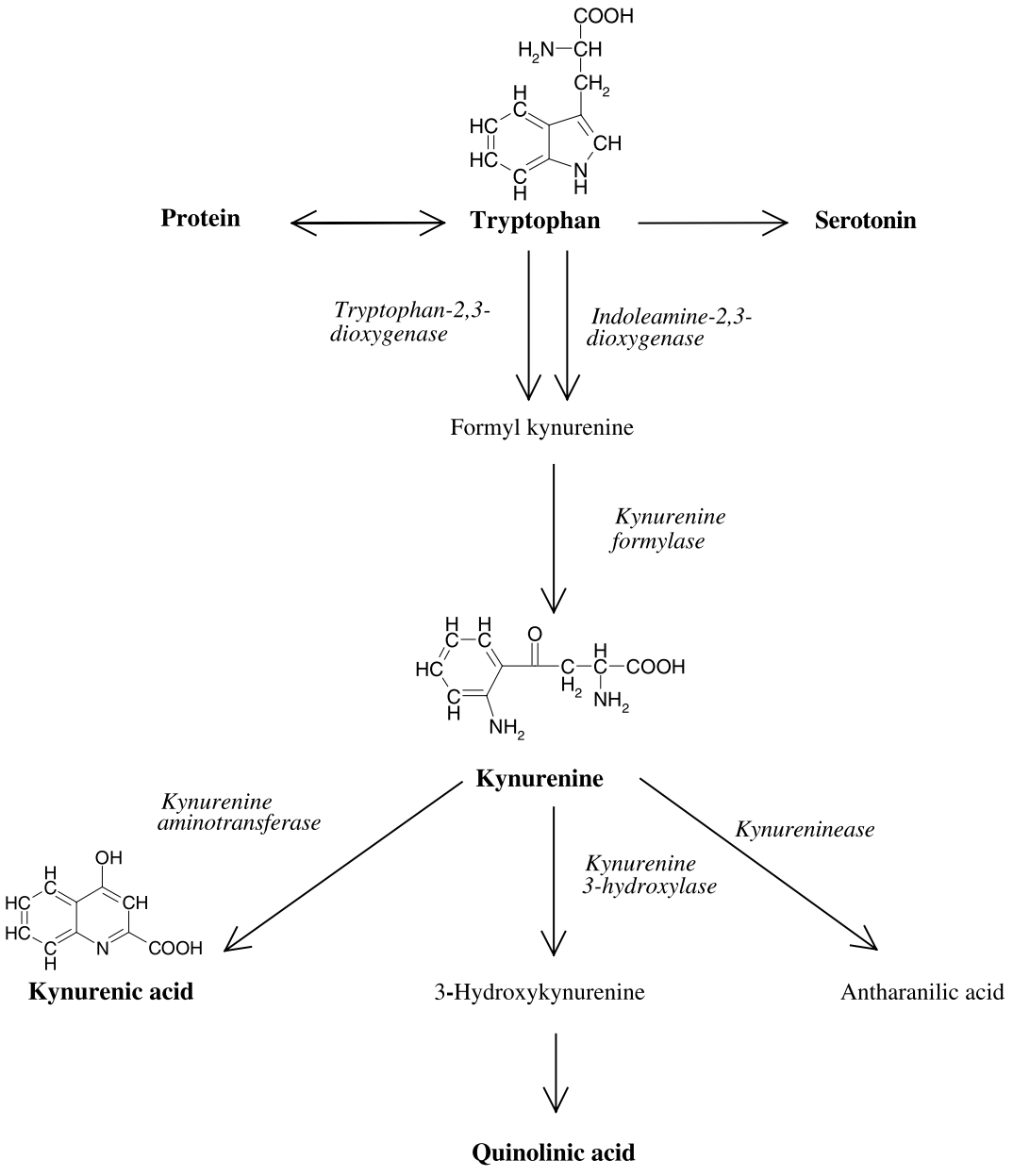

Fig. 2. Abbreviated pathway of tryptophan metabolism showing pathways to protein, serotonin and kynurenine (with permission from Ardeshir Amirkhani)

nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), etc.) and indirectly-acting antioxidants (ubiquinone, alpha-tocopherol, etc.) (Gilgun-Sherki et al., 2004). Oxidative stress is manifested by lipid peroxidation, protein oxidation and other markers, affecting many easily oxidized target molecules in the CNS such as catecholamines, unsaturated fatty acid and the DNA. For more detailed information on the observations of the role of antioxidant balance in animal models see the review by Gilgun-Sherki et al. (2004).

Reactive oxygen and nitric species

Accumulating data indicate that oxidative stress plays a major role in the pathogenesis of MS. The nervous system has a high metabolic rate and is very rich in oxidizable substrates, e.g. catecholamines and polysaturated lipids, in addition to DNA (Halliwell, 1999). ROS are generated primarily in macrophages, and have been implicated as mediators of demyelination and axonal damage in both MS and experimental allergic encephalomyelitis (EAE). ROS cause damage to cardinal cellular components such

Table 1. *Summary of the redox changes in MS*

Redox systems in MS	Tissue	Reference
1. Reactive oxygen (ROS) and nitric species		
↑ formation of ROS	leukocyte	Ferretti et al. (2006)
↑ lipid peroxide	erythrocyte	Polidoro et al. (1984)
↑ lipid peroxide	plasma + CSF	Hunter et al. (1985)
Administration of IFN-β:		
– ↓ formation of ROS and	serum	Lucas et al. (2003)
– ↑ of sulfhydryl groups	serum	Lucas et al. (2003)
– ↑ alpha tocopherol	erythrocyte	Karg et al. (2003)
– transient ↓ in NADPH	erythrocyte	Karg et al. (2003)
– transiently ↑ triglycerid	plasma	Karg et al. (2003)
– ↑ iNOS activity	CSF	Calabrese et al. (2003)
– ↑ GSH and GSH/GSSG ratio and ↓ NO reactive metabolites after treatment with acetylcarnitine	CSF	Calabrese et al. (2003)
– ↑ iNOS activity	CSF	Calabrese et al. (2003)
2. Enzymatic and nonenzymatic antioxidants		
– ↑ GSH and GSH/GSSG ratio and ↓ NO reactive metabolites after treatment with acetylcarnitine	CSF	Calabrese et al. (2003)
– ↑ glutathione peroxidase	lymphocyte	Sorensen et al. (1992)
– ↓ glutathione peroxidase and ↑ after antioxidant treatment	blood	Mai et al. (1990)
– n.d. glutathione peroxidase	blood	Ansari et al. (1986)
– ↑ GSH and GSSG, ↓ alpha-tocopherol ratio	plasma (relapse)	Karg et al. (1999)
– ↓ superoxide dismutase	erythrocyte	Ansari et al. (1986)
– ↑ MT-I – II	inactive MS lesion	Penkowa et al. (2003)
– autoantibodies to transaldolase	serum + CSF	Banki et al. (1994)
– ↓ ascorbic acid, betacarotene, retinol, alpha-tocopherol, ↑ lipid peroxidation products	serum (relapse)	Besler et al. (2002)
– steroid therapy:: ↓ lipid peroxidation	serum + CSF	Keles et al. (2001)
– ↑ lipid oxidation	plasma	Besler and Comuglu (2003)
– ↑ LDL-autoantibodies	serum	Besler and Comuglu (2003)
– ↑ UA	MS plaque	Langemann et al. (1992)
– ↓ glutathione	MS plaque	Langemann et al. (1992)
– ↓ alpha-tocopherol	MS plaque	Langemann et al. (1992)
– ↓ UA in MS	serum	Sotgiu et al. (2002)
– ↓ UA in optic neuritis	serum	Knapp et al. (2004)

CSF Cerebrospinal fluid, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *ic*. intracellular, *IFN-β* interferon-β, *iNOS* inducible nitric oxide synthase, *LDL* low density lipoprotein, *MS* multiple sclerosis, *MT* metallothionein, *NADPH* nicotinamide adenine dinucleotide phosphate hydrogenase, *n.d.* not detected, *NO* nitric oxide, *ROS* reactive oxygen species, *UA* uric acid.

as lipids, proteins and nucleic acids, resulting in cell death by necrosis or apoptosis (Gilgun-Sherki et al., 2004).

In the CSF and blood of MS patients, increased lipid peroxide levels have been observed (Hunter et al., 1985; Polidoro et al., 1984; Table 1). Oxidative stress occurs in all clinical forms of MS (Koch et al., 2006), and in leukocytes higher levels of spontaneous intracellular ROS formation have been detected (Ferretti et al., 2006). The administration of IFN-β-1a decreased the ROS production in MS patients and raised the serum levels of sulfhydryl groups to the values measured in the healthy controls (Lucas et al., 2003). IFN-β-1b treatment normalized the reduced level of alpha-tocopherol in the erythrocytes of MS patients. The transient fall in NADPH in parallel with the rise in plasma triglycerides suggests the stimulation of fatty acid synthesis by the medication (Karg et al., 2003).

Nitric oxide (NO) and other reactive nitric species play roles in neuromodulation, neurotransmission and synaptic plasticity, but they are also involved in neurodegeneration and neuroinflammation. The susceptibility of different brain cell types to NO and peroxynitrite exposure may be dependent on factors such as the intracellular reduced glutathione and cellular stress resistance signal pathways (Calabrese et al., 2003). Inducible NO synthase is upregulated in EAE and MS. Patients with active MS have significantly increased NO synthase activity, associated with the enhanced nitration of proteins in the CSF. Acetylcarnitine administration decreased the CSF levels of NO-reactive metabolites and protein nitration and increased the content of reduced glutathione and reduced/oxidized glutathione ratio (Calabrese et al., 2003).

Enzymatic and non-enzymatic antioxidant systems

The primary defense of the blood against ROS is the glutathione redox system of the erythrocytes. The protective mechanism of glutathione results in an increased formation of oxidized glutathione. After cytokine exposure, astrocytes increase nitric oxide (NO) generation, which may diffuse out to cause mitochondrial damage and cell death. Glutathione peroxidase is one of the major antioxidants in the human brain. Its activity in the peripheral blood of MS patients has been found to be increased (Sorensen et al., 1992) while in other studies the activity was found not to differ from the control group (Ansari et al., 1986) or even to be decreased (Mai et al., 1990). In our study in MS relapse, the oxidized and reduced glutathione level increased and the ratio of alpha-tocopherol decreased, suggesting increased free radical production and consumption of the scavenger molecules (Karg et al., 1999). The level of superoxide dismutase was lower in the MS group, and exposure to hyperbaric oxygen led to an increase in its concentration (Ansari et al., 1986). In an animal model of experimental optic neuritis, glutathione peroxidase and catalase decreased the vascular permeability of the BBB, suggesting a role of hydrogen peroxide and its reactive by-products in the pathogenesis of BBB disturbances (Guy et al., 1989).

Heme oxygenase-1 (HO-1) is confined to a small population of neurons and neuroglia; its gene is upregulated in glial cells within MS plaques. Glial HO-1 induction may be the final common pathway leading to pathological iron sequestration and mitochondrial insufficiency (Schipper, 2004). Bilirubin prevented and suppressed EAE, protected the BBB from free radical changes, and interfered with the invasion of inflammatory cells into the CNS (Liu et al., 2003).

The metallothioneins (MTs) are low-molecular weight metal-binding proteins that e.g. form ''vesicular'' pools of zinc, in the subclass of glutamatergic neurons (Ebadi et al., 1995). In the brain lesions of MS patients, the cells expressing MT-I + II proved to be mainly astrocytes and activated monocytes/macrophages. Slightly increased levels were detected in inactive lesions in comparison with the active lesions, suggesting that MTs may be important in disease remission (Penkowa and Hidalgo, 2003; Penkowa et al., 2003).

Other radical-scavenger antioxidants in blood include free sulfhydryl groups, alpha-tocopherol, retinol and uric acid. These scavenger molecules can act cooperatively and in a synergistic way to afford appropriate protection against oxidative attacks. Proinflammatory cytokines inhibit the expression of myelin genes in human primary oligodendrocytes through alteration of the cellular redox processes (Jana and Pahan, 2005). Transaldolase is a key enzyme of the nonoxidative pentose phosphate pathway, providing ribose-5-phosphate for nucleic acid synthesis and NADPH for lipid biosynthesis. It also maintains glutathione in a reduced state to protect sulfhydryl groups and cellular integrity from oxygen radicals. Autoantibodies to recombinant transaldolase have been detected in the serum and CSF of MS patients. The results suggesting that molecular mimicry between viral core protein and transaldolase may play a role in breaking immunological tolerance and leading to a selective destruction of oligodendrocytes in MS (Banki et al., 1994). The serum levels of ascorbic acid, betacarotene, retinol and alpha-tocopherol were significantly lower, while the lipid peroxidation products were elevated in MS relapse (Besler et al., 2002). Corticosteroid therapy reduced the lipid peroxidation in the serum and particularly in the CSF of MS patients (Keles et al., 2001). During relapse, significant increases were detected both in the in vitro lipid oxidizability in the plasma and in the levels of autoantibodies against oxidized low-density lipoproteins, with a strong decrease in the plasma total antioxidant capacity (Besler and Comuglu, 2003). Uric acid is a free-radical marker that selectively inhibits peroxynitrite-mediated reactions. Administration of the precursors of uric acid suppressed the appearance of the clinical signs of EAE and promoted recovery from the ongoing disease (Scott et al., 2002). Uric acid-treated animals had no BBB breakdown, and inducible NO synthase-positive cells most often failed to reach the CNS tissue in EAE (Kean et al., 2000). In the plaques of MS patients, the level of uric acid was increased and that of glutathione correspondingly decreased. The alpha-tocopherol concentration was lowest in the plaques. The ascorbic acid, cysteine, tyrosine and tryptophan levels were not changed significantly (Langemann et al., 1992). Uric acid is decreased in MS (Sotgiu et al., 2002) and optic neuritis, suggesting a reduced antioxidant reserve as an early pathogenic mechanism in inflammatory demyelination (Knapp et al., 2004). Alpha-lipoic acid is a neuroprotective metabolic antioxidant that has been shown to cross the BBB; its administration in EAE prevented progression, associated with a reduction in the CNS-infiltrating T cells and macrophages and decreased demyelination (Morini et al., 2004).

Discussion

An upregulated cellular antioxidant defense mechanism in the benign as well as in the primary progressive forms of MS suggests that ROS formation is not necessarily deleterious (Koch et al., 2006). ROS together with the activation of cytoprotective genes can suppress inflammatory responses (Chen and Kunsch, 2004; Lee and Johnson, 2004).

As oxidative damage is known to be involved in inflammatory and autoimmune-mediated tissue destruction, the modulation of oxygen free radical production comprises a new approach to the treatment of inflammatory and autoimmune diseases. The CNS tissue is particularly vulnerable to oxidative damage, suggesting that oxidation plays an important role in the pathogenesis of MS and EAE (Ilhan et al., 2004).

Neuroprotection against excitotoxic and oxidative stress-mediated neuronal damage is considered important for the progression of chronic neurodegenerative diseases. The synthetic tocopherol failed to be effective in clinical MS compared to the effectivenes in its animal model, EAE. One explanation might be the different bioavailability of the natural and synthetic tocopherol in humans and rodents (Acuff et al., 1994).

The peripheral cells may serve as a model to see what is going on inside the CNS. However, we still do not know if the observations made at the periphery correlate with the situation in the CNS. Well-designed, long-term studies are needed with large number of patients to explore the effectiveness of antioxidants and determine their role as therapeutical facility. A new promising metabolite's analogue of the kynurenine pathway seems to beneficially influence EAE (Platten et al., 2005). More clinical evidence is needed to prove the role of KYNA analogues and/or enzyme inhibitors as potential medications in MS in the future. Furthermore a continuous development of more sensitive and selective methods for the analysis of endogenous levels of the metabolites of the kynurenine pathway is needed. Some of us have recently developed a capillary electrophoresis method for separation and detection of these compounds in cerebrospinal fluid using time-of-flight mass spectrometry (Arvidsson et al., 2006). Compared to techniques like liquid chromatography, capillary electrophoresis is superior when it comes both to speed, simplified sample preparation and the analysis of minute sample volumes since injection volumes are in nanoliters instead of microliters. As compared to the existing liquid chromatography mass spectrometric method developed by our group (Amirkhani et al., 2002), the limits of detection and quantitation are 10 times higher in concentration, but calculated as an amount of moles injected, we are still 300 times lower in detection. These advantages could prove to be very valuable in the monitoring of rapid changes of endogenous levels during both biomarker screening and intervention studies.

Acknowledgement

This work was supported by grants 215/2006 of the Hungarian Ministry of Public Wellfare, DNT 08/2004, Jedlik Ányos Foundation, and the Swedish Research Council Grant (621-2002-5261, 629-2002-6821, 621-2005-5379) (J.B). Jonas Bergquist holds a senior research position at the Swedish Research Council (VR).

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J Neural Transm (2007) [Suppl 72]: 331–337

Printed in Austria

Journal of Neural Transmission

Neural stem cell transplantation in a model of fetal alcohol effects

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Summary Neural stem cell (NSC) transplantation has been investigated and developed in areas such as brain injury, stroke and neurodegenerative diseases. Recently, emerging evidence suggest that many of clinical symptoms observed in psychiatric disease are likely related to neural network disruptions including neurogenesis dysfunction. In the present study, we transplanted NSCs into a model of fetal alcohol effects (FAE) for the purpose of investigating the possibility of regenerative therapy for FAE. We labeled NSCs with fluorescent dye and radioisotope which were transplanted into FAE rats by intravenous injection. The transplanted cells were detected in wide areas of brain and were greater in number in the brains of the FAE group compared to the control group. Furthermore NSC transplantation attenuated behavioral abnormalities in FAE animals. These results suggest NSC transplantation as a potential new therapy for human FAE.

Keywords: Fetal alcohol effects, neural stem cell, transplantation, ethanol, fetal alcohol syndrome

Introduction

FAE is a cluster of abnormalities occurring in children born to mothers with histories of alcohol drinking during pregnancy. Fetal alcohol syndrome (FAS) is the most clinically recognizable form of FAE and is characterized by a pattern of minor facial anomalies, prenatal and postnatal growth retardation, and functional or structural central nervous system (CNS) abnormalities.

A growing number of mechanisms have been identified as potential candidates responsible for FAE and substantial evidence supporting several individual candidate mechanisms of neuroteratogenic effects of ethanol has emerged from experimental studies focused on specific molecular interactions of ethanol with target tissues.

Sari and Zhou (2004) reported that prenatal exposure to a moderate dose of ethanol induced a deficit of serotonergic neurons, and the reduction of serotonergic neurons was long-lasting continuing to the young adult stage. It has also been reported that abnormal 5-HT neuron development can be reversed by treatment with a 5-HT1A agonist in a prenatal alcohol exposure model (Kim et al., 1997). In addition, derivatives of molecules known as activity-dependent neuroprotective factor (ADNF) have recently been identified as potential therapeutic agents for ethanol-induced teratogenesis (Bearer et al., 1999; Ramanathan et al., 1996).

However, these treatments are prophylactic. Without early intervention, the consequences of FAE are permanent.

NSC transplantation has been investigated and developed for conditions such as injury to the CNS, cerebral infarction and Parkinson's disease. Recently it was reported that cerebral morphologic changes are the key mechanism of various clinical conditions of psychiatric disorders.

In a previous study, we investigated the action of ethanol on NSC differentiation. Ethanol inhibits NSC differentiation to neurons, and increases differentiation to glia. We also found that neurotrophic factors reduced ethanol inhibition of NSC differentiation to neuron. It was suggested that the neurotrophic factor signaling changes were involved in these effects. It is possible that the increase of astrocytes and oligodendrocytes after ethanol exposure is a compensatory mechanism to repair the impaired neural network by promoting neurite outgrowth and increasing newly generated neurons.

In this study, we transplanted NSCs into a FAE model to investigate the possibility of regenerative therapy for FAE.

Materials and methods

Materials

The following materials were purchased: Hank's balanced salt solution (HBSS), neurobasal medium (NBM), B27 and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) from Invitrogen (Carlsbad, CA, USA), recombinant human basic-FGF (FGF-2) from PeproTech (London, UK),

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L-[^{35}S]-methionine from GE Healthcare Biosciences (Buckinghamshire, England), and cyclosporine from Novartis Pharma (Tokyo, Japan).

Animals and prenatal treatment

Pregnant Wistar rats were purchased from Clea Japan, Inc. (Sapporo, Japan). Rats were administered ethanol (3 g/kg) or an equivalent volume of physiological saline via an intragastric catheter every 12 hr for 4 days on gestational days 10–13 (Endres et al., 2005). Each rat was housed in an individual cage until delivery and was allowed to give birth. The pups were fed by their biological lactating mothers and weaned at 21 days. Rats were housed at 22°C on a 12:12 hr light/dark cycle with free access to food and water. All experimental procedures were approved by the institutional animal care committee and conducted following the Sapporo Medical University Guidelines for the Care and Use of Laboratory Animals.

Experimental design

The experiment schedule of NSC transplantation to the FAE model rats is described in Fig. 1.

Four groups of rats were used for this study.

(1) Control group: age-matched rats ($n = 6$) were given an injection of saline at 1 month of age without prenatal ethanol exposure.
(2) FAE group: rats ($n = 8$) were given an injection of saline at 1 month of age after prenatal ethanol exposure.
(3) FAE with transplantation group: rats ($n = 8$) received NSC transplantation at 1 month of age after prenatal ethanol exposure.
(4) Control with transplantation group: age-matched rats ($n = 5$) received NSC transplantation at 1 month of age without prenatal ethanol exposure.

Cell preparation and labeling

NSCs were obtained from 13.5-day-old rat embryos and cultured in a monolayer as previously described (Tateno et al., 2004). Briefly, the telencephalic neuroepithelium was dissected and trimmed in ice-cold HBSS. Cells were dissociated by mechanical trituration and collected by centrifugation (300 *g* for 5 min at 4°C). The dissociated cells were plated in culture dishes coated with poly-L-ornithine/fibronectin in NBM supplemented with 2% B27, 0.5 mM L-glutamine and 20 ng/ml FGF-2 at the density of 3×10^5 cells per 100 mm dish. After 7 days of cell expansion culture under 5% CO_2 at 37°C, cells were stained with fluorescein-based dye to trace their migration by incubating the cells in PBS buffer containing 5 μM CFSE for 15 min, and labeled with [^{35}S]-methionine for quantification of transplanted NSCs migrating into the brain. Briefly, cells were labeled in culture with 8.25 μM of [^{35}S]-methionine for 24 hr, and then washed and centrifuged (300 *g* for 5 min at 4°C). Viability was determined using the trypan blue dye exclusion method, and the cell concentration was adjusted to 10^7 cells/ml.

Transplantation

Newborn rats with prenatal ethanol exposure were injected in the tail vein with a suspension of NSCs slowly for 1 min at 30 days of age. All rats received daily intraperitoneal injections of 10 mg/kg cyclosporine from 1 day before transplantation.

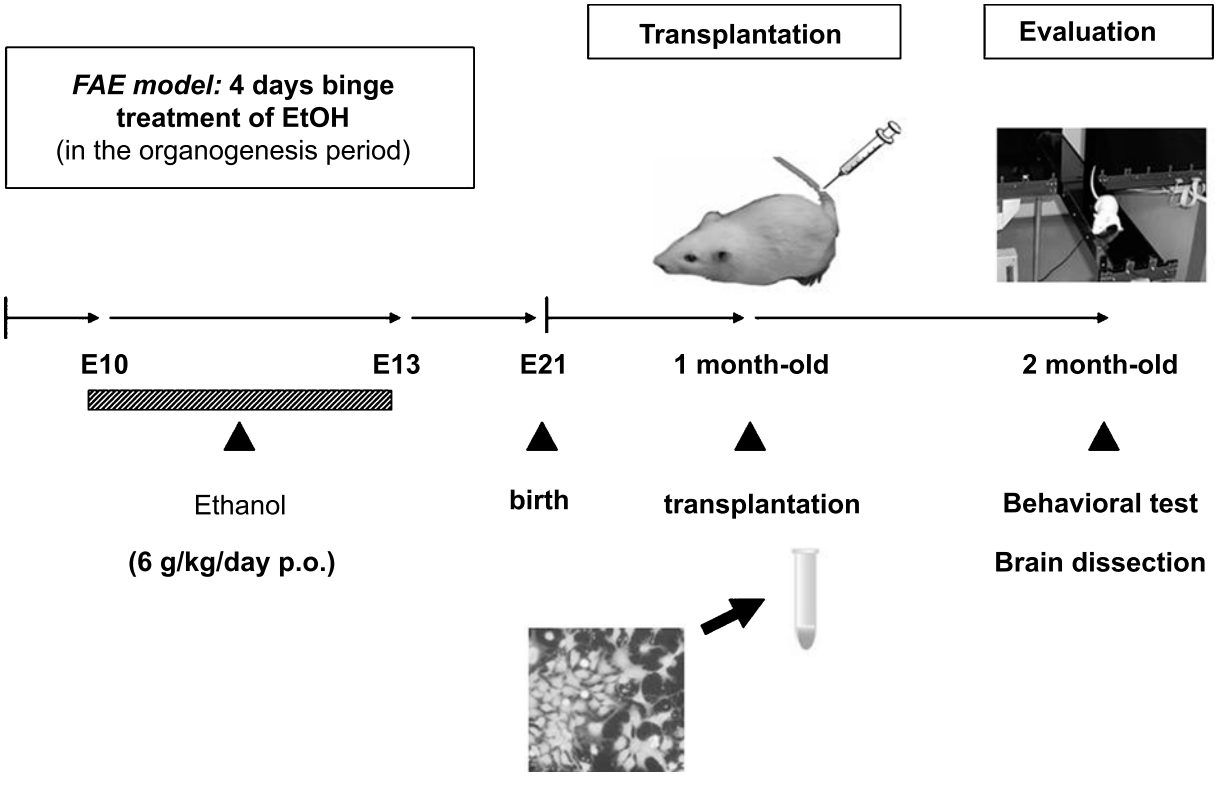

Fig. 1. Experimental schedule of intravenous NSC transplantation to the FAE model rat. The FAE model rat was obtained by treatment of ethanol (6 g/kg/day) in the pregnancy. The double labeled (fluorescence dye + RI) NSC solution was injected intravenously into 30-day-old newborn rats of control and FAE group. Behavioral performance was evaluated by elevated plus maze on 40th day after transplantation following quantitation and localization analysis of NSCs in the brain

Behavioral test

Rats were tested in an elevated plus maze on the 40th day after transplantation. The plus maze was elevated to 50 cm above the floor and consisted of two open and two closed (fenced on three sides) arms. The cage containing the rat was placed in a dark room for 5 min before the test and individuals were tested in the maze for 5 min. Animals were placed in the center of the maze with the nose pointing into a closed arm. This task relies on the conflict between a rat's natural aversion to open space and its exploratory behavior. Entries into the open arms and time in open arms are indicative of reduced anxiety or fear, while the total entries into all the arms correlate with the activity level. The maze was thoroughly cleaned and dried with clean tissues after each individual was tested.

Tissue preparation

After the completion of behavioral testing, rats were anaesthetized with an overdose of Nembutal and transcardially perfused with heparinized saline (0.5%). Part of the forebrain was fixed with 20% formaldehyde, paraffin embedded and cut serially at the coronal plane into 20 μm sections. The remaining brain was divided into the cortex, hippocampus, striatum and subventricular zone (SVZ) including the septal nucleus. The brain fractions were analyzed using a liquid scintillation counter. The results were expressed as counts per minute (cpm) of [^{35}S]-methionine incorporated per aliquot of each brain tissue.

Statistical analysis

Data are presented as the mean ± standard deviation of the mean. The statistical significance between experimental observations was determined by the one-way ANOVA, and post hoc comparisons were using Turkey's HSD test. In all cases, statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS 11.0 for Windows (SPSS Japan Inc. [Tokyo, Japan]).

Results

Characteristics of experimental FAE rats

Both the body weight and brain volume of FAE model rats were not significantly different when compared to control rats (body weight: 99 ± 2.79 (% of control, *t*-test, $p = 0.865$), brain volume: 94 ± 2.99 (% of control, *t*-test, $p = 0.256$)).

Intravenously transplanted NSCs enter the brain

To confirm that NSCs can migrate into the brain after intravenous injection, donor cells were double-labeled with

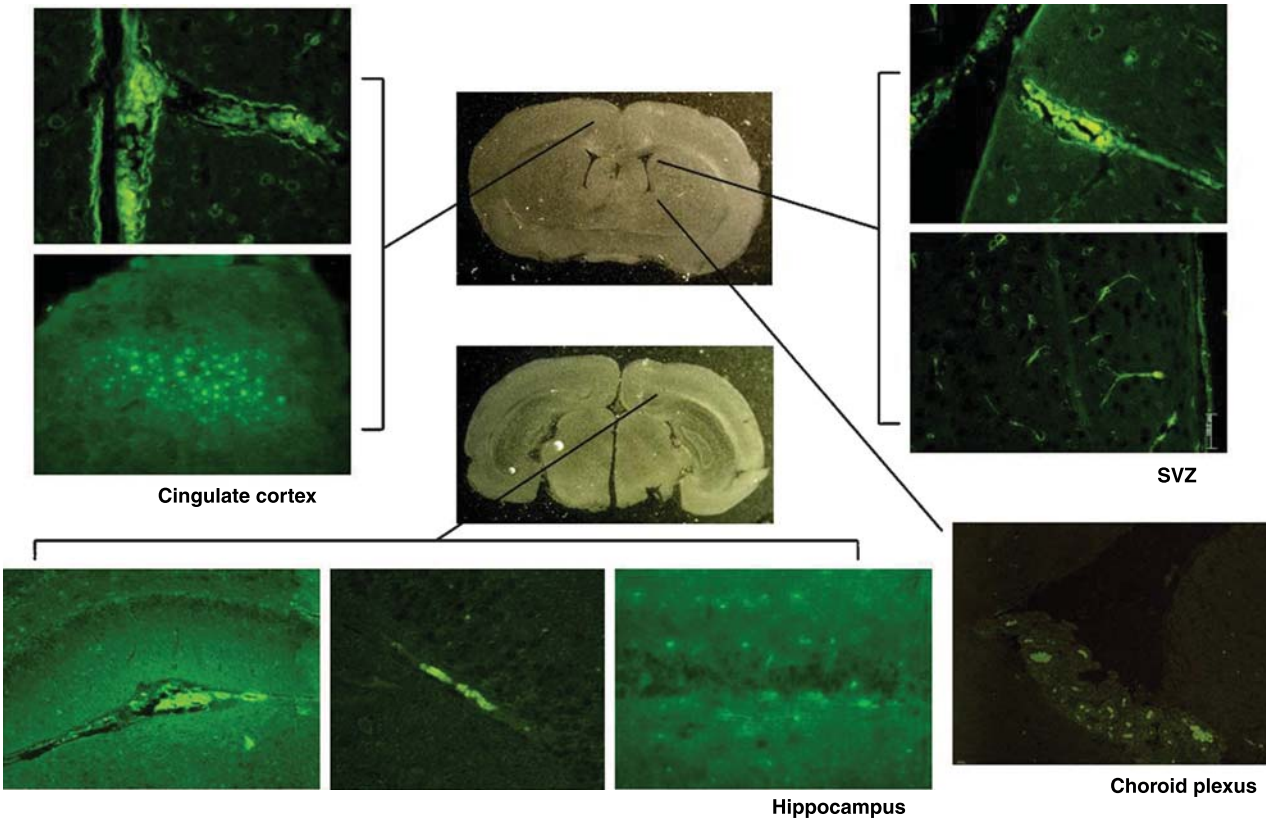

Fig. 2. Distribution of transplanted NSCs in the brain. Photomicrographs (magnifications; × 100–200) showed the transplanted NSCs in the indicated regions of FAE model rat brain, 40 days after intravenous injection labeled with fluorescence dye, CFSE (green). The labeled NSCs were detected in the wide areas of brain, especially in the cingulate cortex, hippocampus, SVZ, and in the choroid plexus

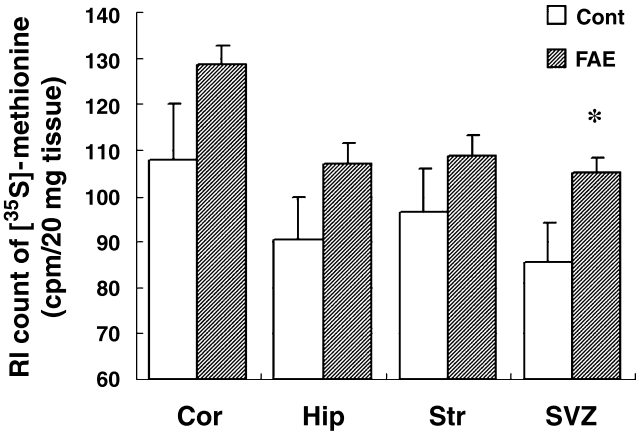

Fig. 3. Quantitation of transplanted-NSCs migrated into the brain. Radioactivities of various brain region tissues were counted in control and FAE rats, 40 days after transplantation of [^{35}S]-methionine labeled NSCs. The transplanted NSCs tended to migrate into the brain of FAE larger than control. *T*-test, control; $n=5$, FAE; $n=8$, $p=0.58$ in Cor, $P=0.082$ in Hip, $p=0.186$ in Str, $p=0.016$ in SVZ (significantly different from control), *Cor* Cortex, *Hip* Hippocampus, *Str* Striatum, *SVZ* subventricular zone including septal nucleus

CFSE fluorescent dye and [^{35}S]-methionine. No CFSE-positive cells were observed in the slides from Control group and FAE group. Within the brain tissue, NSCs labeled by CFSE survived and were distributed throughout the brains of FAE with transplantation group and Control with transplantation group (Fig. 2). Analysis with the liquid scintillation counter demonstrated greater radioactivity in all four areas of FAE with transplantation group compared with the Control with transplantation group (Fig. 3).

NSC transplantation ameliorates behavioral impairment in FAE

Rodents exposed to ethanol during early development show several behavioral and cognitive impairments as adults, and these impairments appear to correlate with ethanol-induced neuronal loss. We have shown here that NSC transplantation improves the abnormal behavior of newborn rats exposed to ethanol during prenatal development. Animals were exposed to either saline or ethanol on gestational days

Treatment	n	Mean	SE	* p-Value vs Control	# p-Value vs EAE
Total entries					
Control	6	6.00	0.68	0	**# 0.024**
FAE	8	13.38	2.33	* **0.024**	0
FAE + T	8	6.88	1.42	0.982	**# 0.033**
Control + T	5	4.80	0.80	0.969	**# 0.012**
Open arm entries					
Control	6	2.33	0.21	0	0.078
FAE	8	4.13	0.72	0.078	0
FAE + T	8	2.38	0.38	1.000	0.059
Control + T	5	2.20	0.20	0.998	0.071
% Time in open arms					
Control	6	2.06	0.06	0	**# 0.019**
FAE	8	3.20	0.34	* **0.019**	0
FAE + T	8	2.24	0.25	0.963	**# 0.034**
Control + T	5	2.09	0.07	1.000	**# 0.030**

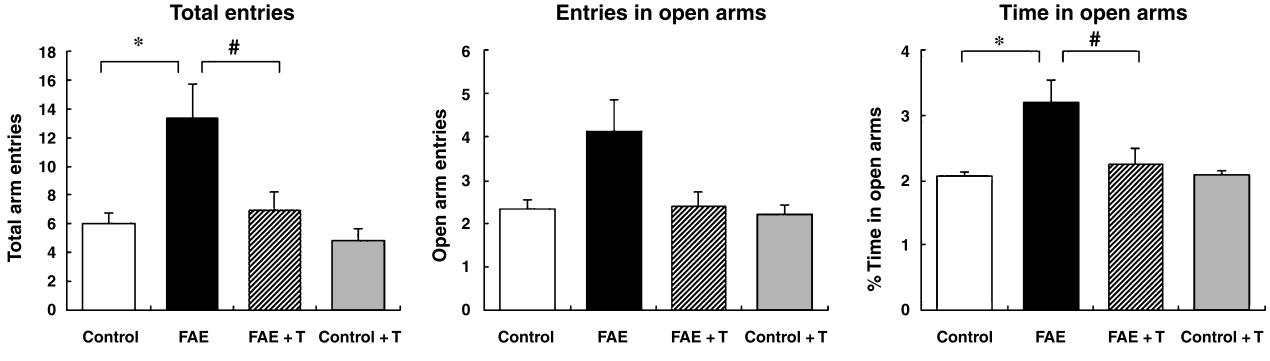

Fig. 4. Behavioral analysis of the effect of NSC transplantation for FAE model rat using elevated plus maze. FAE + T and Control + T groups received NSC transplantation. FAE group significantly showed an increase in the total number of entries and stayed in open arms for longer periods of time. In contrast, FAE + T group did not show any differences when compared with control group. $^{*}p$-value and $^{\#}p$-value from one-way ANOVA. Comparisons are considered significant with a p-value less than 0.05. *T* Transplantation

10–13. We transplanted NSCs to FAE model rats when they were 30 days old, and tested the rats in the elevated plus maze on the 40th day after transplantation. FAE model rats showed an increase in the total number of entries into both open and closed arms, indicating increased activity. Furthermore, FAE model rats tended to increase their numbers of entries into the open arms and also stayed in the open arms for longer periods of time. In contrast, FAE rats that received NSC transplantation did not show any different behaviors when compared with control rats (Fig. 4).

Discussion

In the present study, we demonstrated that NSC transplantation ameliorated behavioral abnormalities of rats with prenatal ethanol exposure. Intravenously transplanted NSCs were detected in the brain by visualizing a fluorescent marker, and the number of transplanted NSCs in brains of FAE model rats was greater than in the control rat brains in all four areas (cortex, hippocampus, striatum, SVZ), as demonstrated by RI labeling.

In the experimental treatments of FAE rats, some studies suggested that 5-HT1A agonists and ADNF prevent neuroteratogenic effects of alcohol exposure in rodents. 5-HT1A receptors have been proposed to be effectors mediating 5-HT signal transduction for the neurogenesis and differentiation of neurons bearing these receptors. The compromised development of 5-HT neurons by prenatal alcohol exposure in rats has been reported to be prevented by concurrent treatment with the 5-HT1A agonists (Sari and Zhou, 2004).

In addition, L1, which is a neural cell adhesion molecule, mediates functions such as adhesion, neurite extension, neuronal migration, and axon fasciculation. Physiologic concentrations of ethanol have been shown to inhibit L1-mediated neurite outgrowth. Two peptides associated with NAPVSIPQ (NAP) and SALLRSIPA (SAL) have been identified as having extremely potent neurotrophic functions. NAP and SAL prevent neuroteratogenic effects of alcohol exposure by antagonizing ethanol inhibition of L1 cell adhesion in an animal model (Bearer et al., 1999; Ramanathan et al., 1996). However, if these treatments are not started in parallel with alcohol exposure during pregnancy, effects are not obtained. NSC transplantation was performed in rat pups 1 month old, and ameliorated behavioral abnormalities, so we suggest that NSC transplantation has the possibility of being applied as a new therapy for human FAE.

The nervous system, unlike many other tissues, has a limited capacity for self-repair; mature nerve cells lack the ability to regenerate, and neural stem cells, although they exist even in the adult brain, have a limited ability to generate new functional neurons in response to injury. For this reason, there is great interest in the possibility of repairing the nervous system by transplanting cells that can replace those lost through damage or disease. At present, many studies of stem cell transplantation are performed for various conditions such as brain injury (Lu et al., 2001), cerebral infarction (Chen et al., 2003), intracerebral hemorrhage (Jeong et al., 2003), Parkinson's disease (Brederlau et al., 2006), Alzheimer's disease (Wang et al., 2006), Huntington's disease (Lee et al., 2005) and multiple sclerosis (Pluchino et al., 2003), so that the effectiveness of transplantation can be determined.

The mechanisms responsible for NSC migration into the brain remain unclear. In a rat stroke model, it has already been suggested that intravenously transplanted human NSCs can migrate to damaged areas as a result of blood brain barrier (BBB) disruption or in response to signals from cytokines and cell surface receptors and antigens (Lu et al., 2001). Haorah et al. (2005) demonstrated that ethanol (10–50 mM) impaired BBB permeability through tight junction modification using a monolayer of microvascular endothelial cells, and that ethanol effects were reversible after 2–4 hr withdrawal. Thus, in our study, the mechanism of NSC migration into the brain may have had little to do participation with disruption of the BBB.

The mechanism of the functional improvement caused by stem cell transplantation in the FAE model brain remains unknown. Increased neurogenesis, neuroprotection by neurotrophic and growth factors, and new synaptic formation with reorganization have been suggested by stroke models (Chu et al., 2004a). Increased brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) secretions were noted in a mesenchymal stem cell-transplanted group in a stroke model (Chen et al., 2003). NSCs may subserve constitutive and inducible plasticity.

The present study shows that intravenously transplanted NSCs can migrate to the brain in an FAE model and induce the functional recovery. There have been no reports concerning therapy for FAE by NSC transplantation. We demonstrated the possibility of regenerative therapy for FAE. However, to determine the mechanism responsible, further investigations are needed.

Acknowledgements

This work was supported by grant-in-aid for Scientific Research No. 18390322 and No. 18659335 (T. Saito) from the Japan Society for the Promotion of Science (JSPS).

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