DOI 10.1007/s00702-005-0334-0
J Neural Transm (2006) 113: 313–329

Journal of Neural Transmission
Printed in Austria

Norharman-induced motoric impairment in mice: neurodegeneration and glial activation in substantia nigra

A. Östergren[1], **A. Fredriksson**[2], and **E. B. Brittebo**[1]

[1] Department of Pharmaceutical Biosciences, and
[2] Department of Neuroscience, Psychiatry Ulleraker, Uppsala University, Uppsala, Sweden

Received March 8, 2005; accepted May 21, 2005
Published online August 3, 2005;

Summary. The β-carboline norharman is present in cooked food and tobacco smoke and show structural resemblance to the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. C57BL/6 mice were injected subcutaneously with norharman (3 and 10 mg/kg) twice per day for five consecutive days. Eighteen hours after the last dose an increased expression of glial fibrillary acidic protein and fluoro-jade staining were demonstrated whereas the number of tyrosine hydroxylase positive cells were unchanged in the substantia nigra. Two weeks after the last treatment a decreased motor activity was observed whereas cognitive functions remained intact. In cultured PC12 cells norharman treatment induced mitochondrial dysfunction and increased the number of caspase-3 and TUNEL-positive cells. The results demonstrate that norharman induced apoptosis in cultured cells as well as early neurodegeneration, glial activation and sustained motor deficits in mice and suggest that exposure to norharman may contribute to idiopathic Parkinson's disease.

Keywords: Apoptosis, behavior, β-carbolines, caspase-3, fluoro-jade, GFAP, MTT-assay, necrosis, nigrostriatal degeneration, norharman, parkinsonism, PC12 cells, spontaneous motor activity, TUNEL.

Introduction

The development of Parkinson's disease appears to be a complex interaction between genetic and environmental factors. Many classes of heterocyclic molecules, structurally resembling the parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been suggested to be possible neurotoxicants involved in the pathogenesis of idiopathic Parkinson's disease. Neither MPTP, nor its neurotoxic metabolite 1-methyl-4-phenylpyridinium ion (MPP^+), are normally present in the environment. In contrast, various MPTP-like compounds such as β-carboline derivatives, are abundant in cooked food, tobacco smoke condensate, brewed coffee, alcoholic beverages, and in some plants (Rommelspacher et al., 1994; Totsuka et al., 1999; Herraiz, 2002). The β-carboline norharman (9H-pyrido[3,4-*b*]-indole) derives from tryptophan-containing sources and is methylated in vivo to a cationic metabolite, which structurally resembles MPP^+ (Matsubara et al., 1992; Collins et al., 1992; Gearhart et al., 1997). Norharman has

been detected in the human brain, where the level is 28 times higher in substantia nigra than in cortex (Matsubara et al., 1993), and in cerebrospinal fluids where increased levels of norharman are detected in patients with Parkinson's disease (Kuhn et al., 1996). We have recently suggested that the sequestering of norharman in substantia nigra is related to retention of β-carbolines in brain neuromelanin (Ostergren et al., 2004). Both norharman and harman are bound to synthetic dopamine-melanin in vitro. They are also bound to and retained in melanin-containing neurons in vivo.

High affinity binding sites for norharman have been detected in several brain regions such as hypothalamic, thalamic, accumbens, amygdaloid nuclei, hippocampal-, neocortical- and olfactory-related structures (Pawlik et al., 1990). Norharman is a potent ligand for benzodiazepine binding sites (Müller et al., 1981). The compound is also a weak inhibitor of MAO-B (May et al., 1991). A single iv or ip injection of norharman (20–25 mg/kg) in rats rapidly induces catalepsy and clonic convulsions (Fuentes and Longo, 1971; Rommelspacher et al., 1981; Pranzatelli and Snodgrass, 1987). Matsubara and co-workers (1998) have reported that repeated ip injections of norharman (84 mg/kg, twice per day for seven days) in pigmented C57BL/6 mice induce bradykinesia two days after the last dose. Reduced levels of dopamine were also noted in the striatum and midbrain. Most behavioral studies have focused on acute effects of norharman and the late effects are not known. Furthermore, the effects of norharman on learning ability and memory have not been reported.

In cultured cells norharman has been reported to induce a decreased cell viability and DNA-damage (Storch et al., 2004; Uezono et al., 2001). Furthermore, methylated norharman metabolites such as 2,9-methylated norharman induce an increased lactate dehydrogenase release in cultured PC12-cells and decreased cell viability in HEK-293 cells (Cobuzzi et al., 1994; Collins et al., 1992; Storch et al., 2004).

The aim of this study was to characterize norharman-induced early onset changes in the substantia nigra and late effects on motor and cognitive functions in C57BL/6 mice. This strain is relatively susceptible to MPTP-induced behavioral changes, although the results are conflicting; some studies report a MPTP-reduced motor activity whereas other report hyperactivity or no deficits (Ogawa et al., 1985; Sundstrom et al., 1990; Arai et al., 1990; Fredriksson and Archer, 1994; Rousselet et al., 2003). Our pilot studies revealed that a single sc dose of 84 mg/kg norharman in C57BL/6 mice caused symptoms of acute toxicity. The effects of repeated sc doses of 3 and 10 mg/kg norharman were therefore examined. The effects of norharman on the number of tyrosine hydroxylase-positive neurons, glial fibrillary acidic protein (GFAP) and on neurodegeneration in substantia nigra pars compacta were determined 18 hours after the last treatment. Starting two weeks after the last treatment behavioral studies (spontaneous motor activity, Morris water maze and radial arm maze) were performed. Finally, we examined the effects of norharman on cell viability and cell death in cultured PC12 cells. Cultured PC12 cells are known to produce dopamine and express dopamine transporters (Kadota et al., 1995) and are extensively used for studies on various aspects of dopaminergic neurons. The results demonstrate that repeated norharman-treatment induced early neurodegeneration, glial activation and sustained motor deficits in C56BL/6 mice whereas cognitive functions remained intact. Furthermore, norharman induced mitochondrial dysfunction, caspase-3 activation and DNA-fragmentation in cultured PC12 cells.

Material and methods

Chemicals and antibodies

Norharman (9H-Pyrido[3,4-*b*]-indole) hydrochloride and 3-(4,5-dimethylthioazol-2-yl)2,5-diphenyl-

tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MA, USA), polyclonal rabbit anti-tyrosine hydroxylase antibody from Chemicon (Temecula, CA, USA), biotin-goat anti-rabbit IgG from Zymed Laboratories Inc (San Fransisco, CA, USA), cleaved caspase-3 antibody from Cell Signaling technology (Beverly, MA, USA), rabbit anti-cow glial fibrillary acidic protein (GFAP) and avidin-biotin-horseradish peroxidase complex (ABComplex/HRP) from Dako (Glostrup, Denmark), Vector VIP substrate kit for peroxidase from Vector Laboratories (Burlingame, CA, USA), In Situ Cell Death Detection Kit, AP from Roche Molecular Biochemicals (Mannheim, Germany), and Fluoro-Jade from Histo-Chem (Jeffersson, AR, USA). RPMI1640, fetal bovine serum, horse serum and penicillin were obtained from the National Veterinary Institute (Uppsala, Sweden).

Animals and treatment

Adult male C57BL/6J mice (30 g) were purchased from B&K Universal, Sollentuna, Sweden. The animals were allowed ten days acclimatization. Ten animals per macrolone cage were kept at 22°C and light-controlled environment with a 12-hour light/dark cycle and were given a standard pellet diet (Ewos AB, Södertälje) and tap water ad libitum. The animal studies were conducted in accordance with the guidelines of the Swedish National Board for Laboratory Animals (CFN) policy LSFS 1988:534. The local Ethics Committee for Animal Research approved the studies.

The mice were injected subcutaneously twice a day for five consecutive days with norharman (3 or 10 mg/kg) dissolved in dimethylsulfoxide (DMSO) and diluted in sodium chloride (0.9%, pH 7.4). Some animals were used for fluoro-jade staining or immunohistochemistry. These animals were killed by cervical dislocation 18 hours after the last injection, and the brains were dissected and fixed in formaldehyde (4%) in phosphate buffer (pH 7.4). The fixed samples were sectioned (4 μm) on a microtome (Microm Heidelberg HM350) and mounted in distilled water on gelatin-coated slides and dried over night at 30°C. The remaining mice were used for behavioral studies two weeks after the last injection.

Fluoro-jade staining

Fluoro-jade is an anionic fluorescein derivative useful for localization of neuronal degeneration in brain tissue sections. Detection of neuronal damage using fluoro-jade has been validated by a variety of well-characterized neurotoxicants, such as MPTP (Schmued et al., 1997). Degenerating neurons and fibers stained with fluoro-jade appear bright yellow-green against a dark background (Schmued et al., 1997; Schmued and Hopkins, 2000). Fluoro-jade staining was accomplished according to the method described by Schmued and coworkers (1997) with minor adjustments. Sections were deparaffinized in xylene (2 × 15 min), rehydrated in graded ethanol solutions (5 min/solution) and transferred to potassium permanganate (0.06%) and gently shaken on a rotating platform. Sections were rinsed with distilled water (1 min), before and after incubation with fluoro-jade (0.001%). Subsequently the sections were dehydrated in graded ethanol solutions, immersed in xylene and mounted with Pertex. Images from substantia nigra were taken with epifluorescence microscope with a fluoroscein filter system. Cells and fibers emitting intense green fluorescence that distinctly exceeded the background were marked in PhotoShop. The size of the marked area was subsequently measured in Scion Image.

Tyrosine hydroxylase immunohistochemistry

For tyrosine hydroxylase-immunostaining, a polyclonal anti-tyrosine hydroxylase antibody, a biotinylated goat anti rabbit conjugate and a Vectastain elite ABC kit were used. The tissue sections were deparaffinized in xylene and rehydrated in solutions of decreasing concentrations of ethanol (2 × 5 min in 99.5%; 95%; 70%), washed in phosphate buffered saline (PBS) and PBS with 0.3% triton (PBST), and treated with one percent hydrogen peroxide in PBST (30 min). The sections were then washed three times in PBS followed by one hour pre-incubation with four percent bovine serum albumin (BSA) for one hour. The brain sections were then incubated with anti-tyrosine hydroxylase antibody (diluted 1:200 in 4% BSA) overnight (at 4°C). After 10 min rinsing in changes of PBS, the sections were incubated with biotinylated second antibody (diluted 1:300 in 4% BSA, 30 min) and then with ABComplex/HRP (30 min) at room temperature. Immunoreactions were visualized using Vector VIP substrate kit for peroxidase. Negative control sections were treated in the same way as described above except that the antibody against anti-tyrosine hydroxylase was omitted. Cell count analyses (5 animals/treatment) were performed on images. Each tissue-section ($n = 5$ sections from each animal) was examined independently by two persons.

GFAP immunohistochemistry

GFAP-immunostaining was performed as described above with a rabbit anti cow GFAP-antibody (diluted 1:500 in 4% BSA). Computerized analyzes of stained area was made with Adobe Photoshop and Scion Image.

Behavioral measurements and apparatus

Procedures and apparatus used for behavioral measurements have been described previously (Carlsson et al., 2003). In short, spontaneous motor activity, locomotion and rearing were measured in a macrolone rodent test cage with two series of infrared beams and a sensor registering all types of vibrations received from the test cage.

The radial eight-arm maze procedure is sensitive to deficits in spatial learning performance. Before testing, all mice were totally deprived of food for 24 hours. Each arm contained a pellet and photocells registered every time a mouse approached to within 3.0 cm of the food cup, thus monitoring every arm entrance from six to 30 cm the depth. Each mouse was tested for one trial only, on each of three consecutive days. Each animal was observed carefully to ensure that it consumed each pellet.

The circular swim maze learning was of Morris water maze type and gives a measurement of the spatial learning ability. Before the first trial, the mouse was placed on the submerged platform for 30 seconds. The mouse was then put in the south position with its head towards the wall of the tube and was then allowed 30 seconds to locate the platform. After three trials the platform was moved to the opposite side of the tube for the fourth trial.

Cell culture

PC12 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (5%), inactivated horse serum (10%) and penicillin (100 units/ml). Cells were grown at 37°C in 5% CO_2/95% air with 100% relative humidity. Cells were subcultured every four to six days. In a preliminary study cells were differentiated with nerve growth factor (NGF) (0.5 μg/ml) for 18 hours before exposure to norharman or vehicle. We observed, however, that NGF-differentiated cells were more sensitive to the vehicle than undifferentiated cells (data not shown). In continued studies, only undifferentiated cells were used.

MTT assay

After exposure to norharman, mitochondrial function was measured by using the MTT assay, based on the conversion of MTT to formazan crystals by mitochondrial succinate dehydrogenases. All experiments were performed on the cells plated at a density of 10000 cells/well on 96-well plates. The day after plating the cells were exposed to norharman for 24 or 96 hours. After exposure, the wells were decanted and fresh medium (100 μl/well) with MTT (10 μl/well, 5 mg/ml in PBS) was added. Four hours later, extraction buffer (100 μl/well, 50% dimethyl formamide, 20% sodium dodecyl sulphate, pH 4.8) was added. The solution was incubated for 24 hours. Absorbance was measured at 570 nm in a plate-reader (POLARstar OPTIMA, BMG Labtech). Cell viability was expressed as a percentage of the control culture value.

Trypan blue exclusion assay

PC12 cells (with a confluence of 95%) were exposed to norharman (5, 50 or 500 μM) or vehicle for 24 or 96 hours. The cells were trypsinated, resuspended and 50 μl was added to 50 μl of trypan blue solution (0.25% in PBS). The number of trypan blue-excluding (live) cells and trypan blue-stained (necrotic) cells were counted using a Bürker chamber.

Caspase-3 immunocytochemistry and TUNEL staining

PC12 cells with a confluence of 95% were exposed to norharman (5, 50 or 500 μM) or vehicle for 24 hours (n = 2/treatment). After exposure the cells were washed with pre-warmed PBS and fixed in formalin (4%, pH 7.4) for 10 minutes, scraped into one tube per treatment, and pelleted by centrifugation (1200 rpm, 5 min). The cells were washed one time with PBS and re-centrifuged. The cells were incubated with agarose (3% in PBS), one hour, at 50°C. Subsequently, the cells were put on ice and dehydrated in ethanol (70% over night, 95% for 4 h, 99.5% for 3 h and xylene for 30 min) and embedded in paraffin. The cells were sectioned (4 μm) on a microtome (Microm Heidelberg HM350). The immunocytochemistry against activated caspase-3 was performed as described above, but with microwave antigen retrieval. The number of stained and unstained cells was counted per arbitrary unit area.

For TUNEL staining the sections were washed with PBS and PBST, and treated with one percent hydrogen peroxide in PBST (5 min). The sections were then washed with PBST and PBS and incubated with TUNEL reaction mixture in 37°C for 60 minutes, and thereafter washed three times with TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA). After incubation with converter-AP (1:4 in dilution buffer) over night (at 4°C) the sections were washed with NTMT (100 mM NaCl, 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 1% Tween in distilled water) and incubated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphatase for one hour in room temperature according to instructions from the manufacturer. The sections were mounted in glycerine-gelatin.

Statistical analysis

Statistical analysis of the fluoro-jade, immunohistochemistry and immunocytochemistry was performed using a one-way ANOVA (GraphPad Prism). Post-hoc testing (GraphPad QuickCalcs) was used to compare the treatment groups with their respective control.

Statistical analysis of the variables in each behavioural test was performed with a split-plot factorial ANOVA design (Kirk, 1995). The use of a split-plot factorial design enables us to isolate the effects of nuisance variables, such as individual differences. The fixed main effects were the level of the dose and the time-periods or days. Interaction effects were the level of the dose $\times$ time-period. Pair-wise testing between the different treatment groups were performed with the Tukey HSD test (Kirk, 1995).

Results

No clinical signs of illness such as convulsions, tremors or lost of weight were observed in animals during exposure to norharman. The behavioral studies were performed two weeks after the last injection, while the animals used for immunohistochemistry and fluoro-jade were killed 18 hours after the last injection.

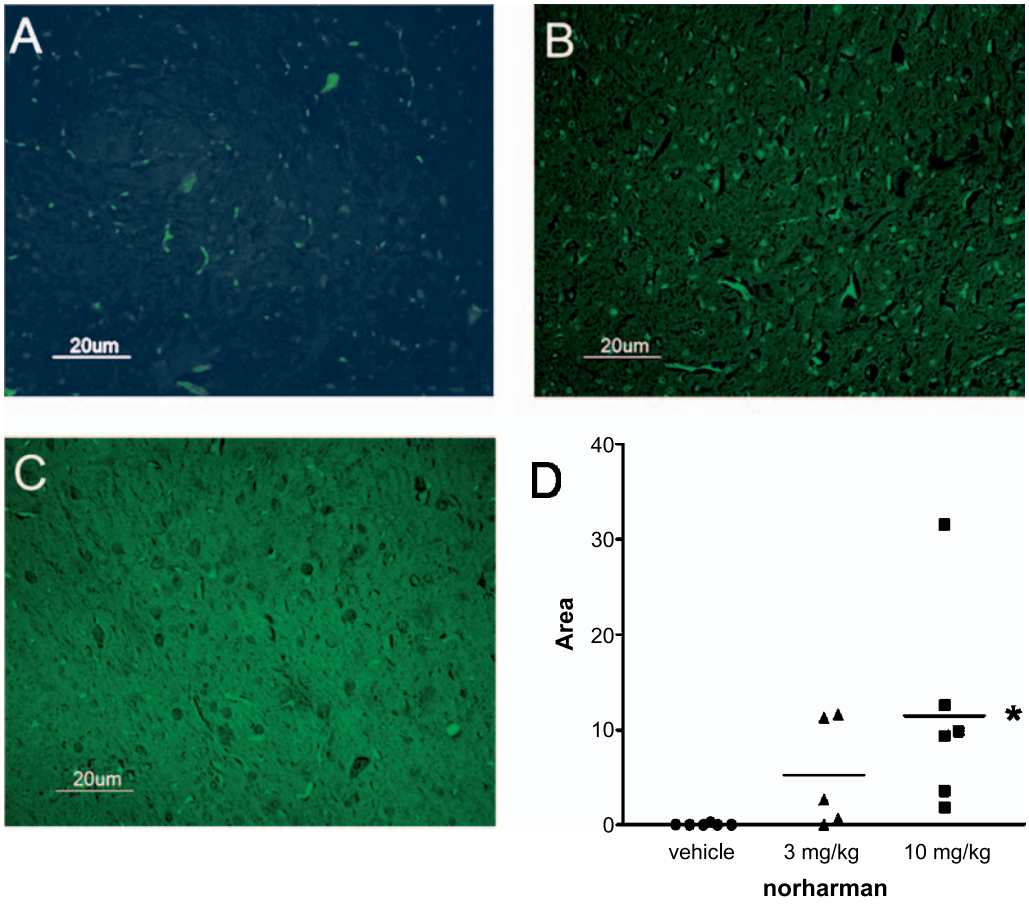

Fig. 1. Fluoro-jade staining showing degenerative changes in substantia nigra of C57BL/6 mice injected sc with norharman (3 or 10 mg/kg) or vehicle twice daily for five consecutive days. Eighteen hours after the last injection there is no staining in control (**A**), whereas there is a moderate staining in a mouse given 3 mg norharman/kg (**B**), and an intense staining in a mouse given 10 mg norharman/kg (**C**). Semiquantitative measurement of fluoro-jade stained areas were estimated using image analysis (**D**). The mean-value for each treatment is presented as a straight line, $n = 5$ animals for the 3 mg/kg norharman, $n = 6$ animals for the groups receiving vehicle or 10 mg/kg norharman (3 sections/mouse). Statistical analysis (ANOVA) indicated a significant difference between the groups, $p < 0.05$. Post-hoc testing indicated a higher value for norharman-treated animals compared to vehicle-treated animals, $^{*}p < 0.05$

Histological studies

Fluoro-jade staining

There was a dose-dependent increase of the fluoro-jade stained area in substantia nigra pars compacta of mice 18 hours after the last dose of norharman. Representative photomicrographs are shown in Fig. 1A–C, and semiquantitative analysis of stained area is shown Fig. 1D. One-way ANOVA showed a significant difference between the groups, $p < 0.05$, and post-hoc testing indicated that there was a significant larger fluoro-jade stained area in the substantia nigra of animals receiving 10 mg/kg norharman, compared to vehicle-treated controls ($p < 0.05$).

Tyrosine hydroxylase immunohistochemistry

The pattern and extent of immunoreactive neurons in the substantia nigra pars compacta were similar in norharman- and vehicle-treated mice. The number of tyrosine hydroxylase-positive neurons was 95.6% ± 28.4 (3 mg/kg), and 99.0% ± 42.3 (10 mg/kg)

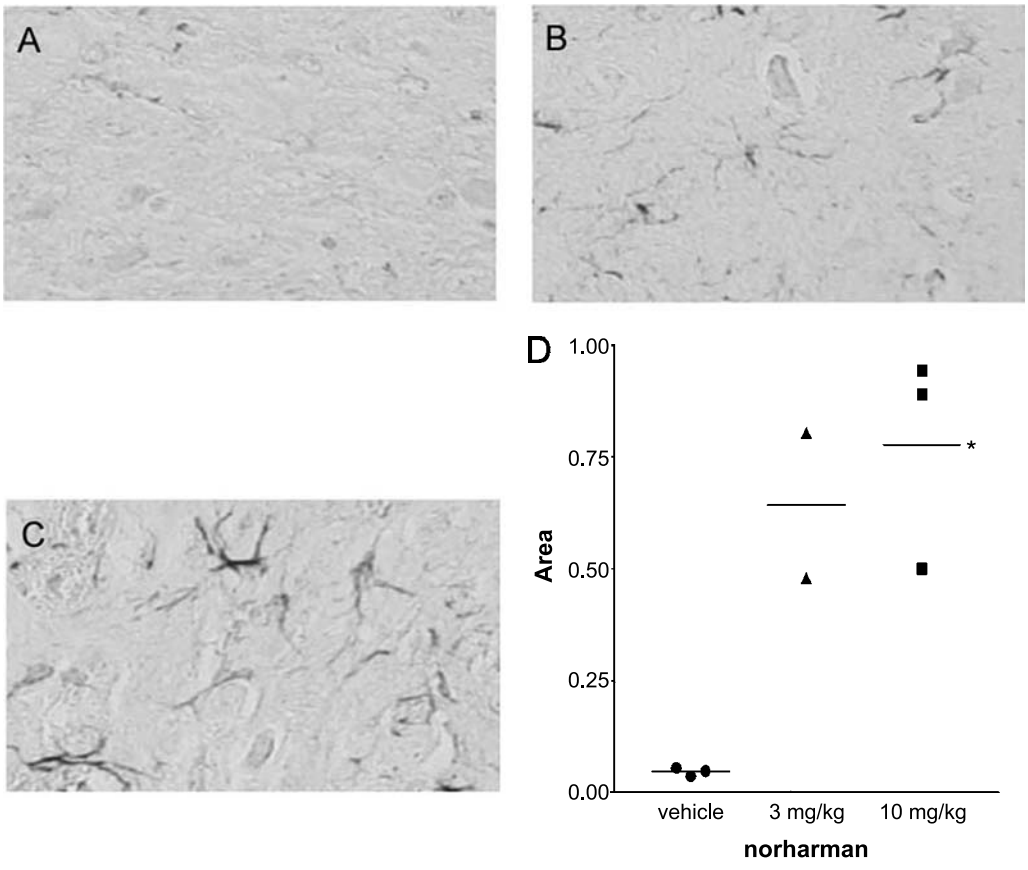

Fig. 2. Areas of GFAP staining in substantia nigra of C57BL/6 mice injected sc with norharman (3 or 10 mg/kg) or vehicle twice daily for five consecutive days. Eighteen hours after the last injection of norharman, there is only a low staining in control (**A**) whereas there is an increased staining in mice given 3 mg/kg (**B**) or 10 mg/kg (**C**). Semiquantitative measurement of GFAP stained areas were performed using image analysis (**D**). The mean-value for each treatment is presented as a straight line, $n = 2$ animals for the 3 mg/kg, $n = 3$ animals for the groups receiving vehicle or 10 mg/kg norharman (2–6 sections/mouse). Statistical analysis (ANOVA) indicated a significant difference between the groups, $p < 0.05$. Post-hoc testing indicated a higher value for the high dose-treated animals compared to vehicle-treated animals, $^{*}p < 0.05$

of the vehicle-treated control. There were no statistically significant treatment-related changes in the number of tyrosine hydroxylase-positive neurons in the substantia nigra (ANOVA, including post-hoc testing).

GFAP immunohistochemistry

Typical star-shaped astrocytes with numerous processes appeared in sections of substantia nigra pars compacta of mice 18 hours after

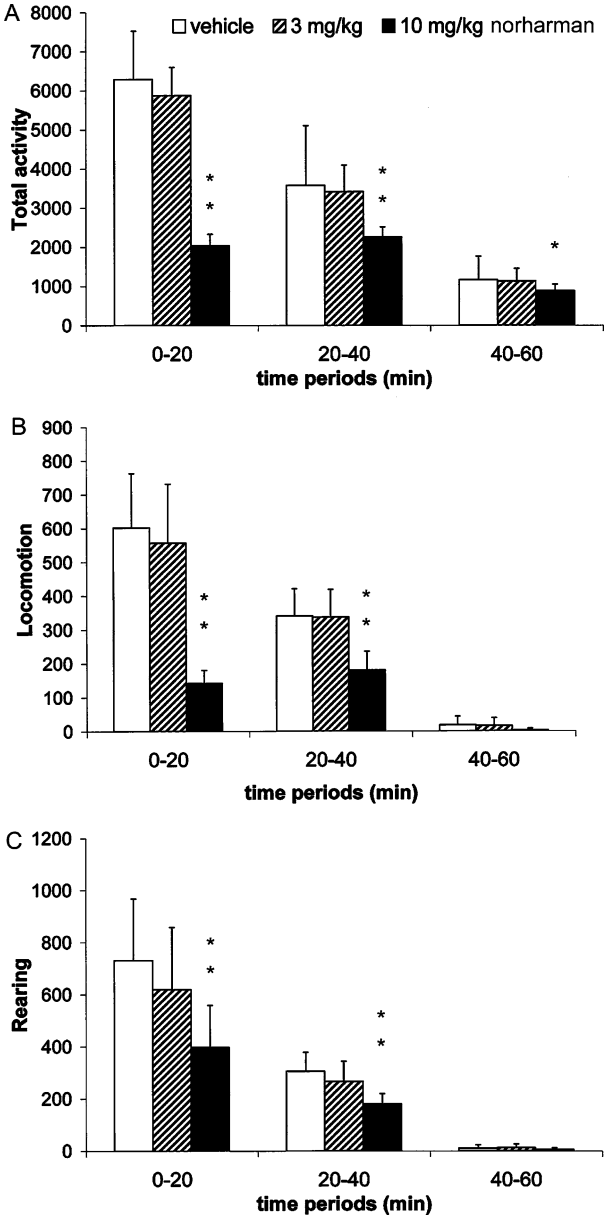

Fig. 3. Spontaneous motor behaviour, represented by total activity (**A**), locomotion (**B**) and rearing (**C**), in C57BL/6 mice two weeks after sc injections of norharman (3 or 10 mg/kg) or vehicle twice daily for five consecutive days. Values represent mean ± SD, n = 12 animals/treatment. Statistical analysis (ANOVA) indicated a significant difference between the groups, $p < 0.001$ for total activity, $p < 0.001$ for locomotion, and $p < 0.01$ for rearing. Post-hoc testing indicated a reduced level of activity in norharman-treated animals compared to vehicle-treated animals, $^{**}p < 0.01$, $^{*}p < 0.05$

the last dose of norharman. Representative photomicrographs are shown in Fig. 2A–C, and semi-quantitative analysis of stained area is shown Fig. 2D. One-way ANOVA showed a significant difference between the groups, $p < 0.05$, and post-hoc testing indicated that there was a significant larger GFAP-stained area in the substantia nigra of animals receiving 10 mg/kg norharman, compared to vehicle-treated controls ($p < 0.05$).

Behavioral studies

Spontaneous motor activity

Compared to vehicle-treated mice, the highest dose group (10 mg norharman/kg) had a time-

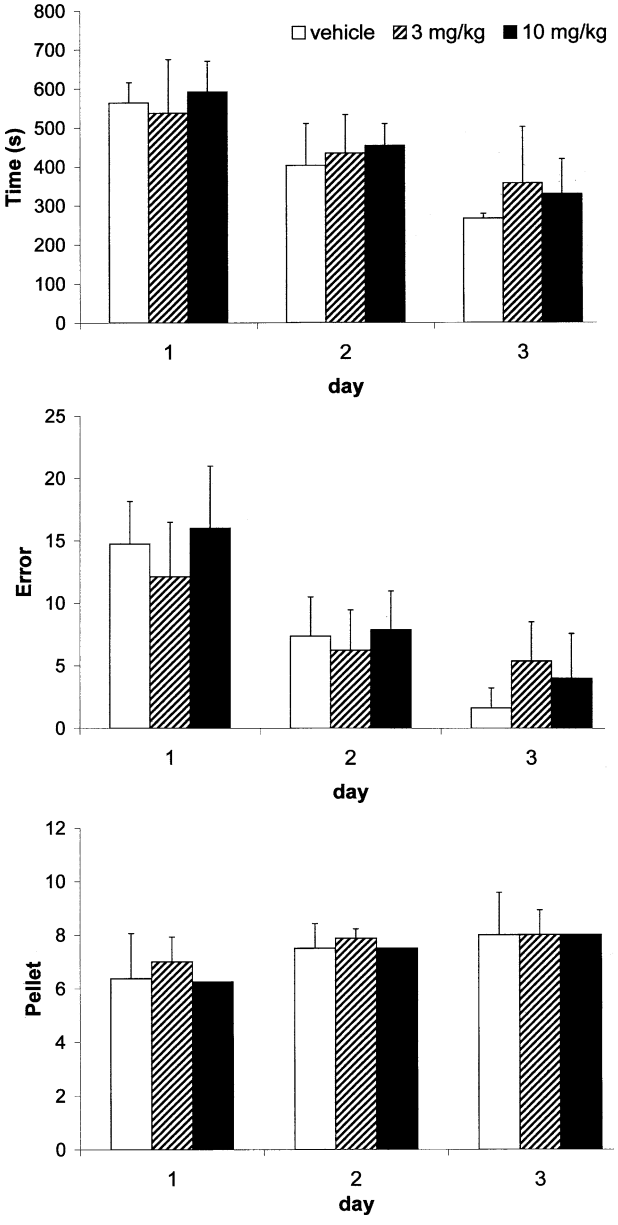

Fig. 4. Radial arm maze learning, measuring time (**A**), errors (**B**), pellet consuming (**C**), in C57BL/6 mice two weeks after sc injections of norharman (3 or 10 mg/kg) or vehicle twice daily for five consecutive days. Values represent mean ± SD, n = 12 animals/treatment. Statistical analysis (ANOVA) indicated no significant difference between the groups

dependent decrease in motor activity measured as total activity, reduced locomotion and rearing (Fig. 3A–C). The decrease was most pronounced during the initial high spontaneous activity phase within the first 20 minutes, but remained in the following steady-state phase. Split-plot ANOVA showed a significant difference between the groups, ($F = 54, 4) = 19.50$ ($p < 0.001$) for total activity, ($F = 54, 4) = 19.20$ ($p < 0.001$) for locomotion, and ($F = 54, 4) = 37.71$ ($p < 0.01$) for rearing. Post-hoc testing with Tukey HSD-testing indicated a significant decreased total activity, locomotion and rearing during the first 40 minutes for the group of mice receiving 10 mg/kg norharman ($p < 0.01$), and a significant decrease of total activity for the last 20 minutes period tested, after 40 minutes ($p < 0.05$).

Radial arm maze learning

All groups of mice had a relatively linear improvement in the radial arm maze acquisition performance, and no significant effect of treatment was observed for time (Fig. 4A), number of errors (Fig. 4B), or number of pellets taken (Fig. 4C). Post-hoc testing with Tukey HSD-testing indicated however that the 3 mg/kg dose group made more errors than the vehicle group on the third day.

Circular swim maze learning

The design of the swim maze was a three day long acquisition period followed by a reversal trial on the fourth day when the platform was relocated. When comparing the time for each mouse to reach the platform no significant difference between the groups was observed (Fig. 5).

In vitro studies

MTT assay

There was a dose-dependent decrease of mitochondrial activity in PC12 cells exposed to norharman (50, 100, 250, and 500 μM) for 24 or 96 hours (Fig. 6). One-way ANOVA showed a significant difference between the groups, $p < 0.0001$, for both exposure times. Post-hoc testing indicated a reduced mitochondrial activity for cells exposed to 500 μM norharman for 24 hours ($p < 0.001$), and for cells exposed to 50, 100, 250 and 500 μM norharman for 96 hours ($p < 0.001$), compared to vehicle-treated cells.

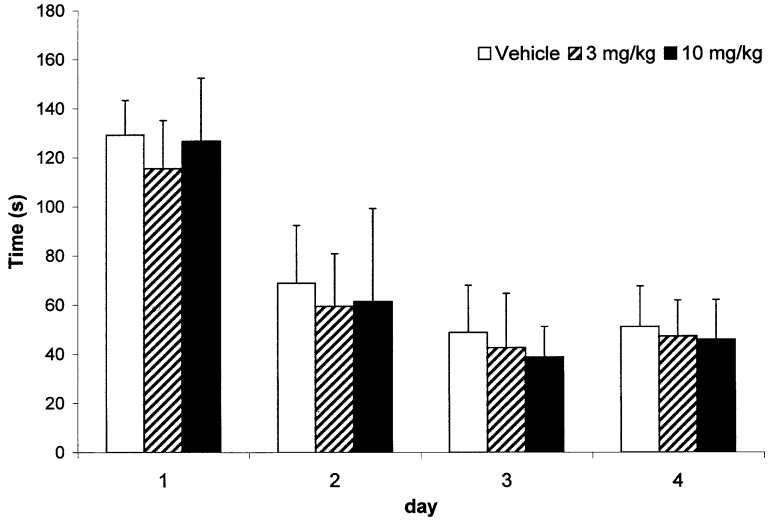

Fig. 5. Swim maze performance of C57BL/6 mice two weeks after sc injections of norharman (3 or 10 mg/kg) or vehicle twice daily for five consecutive days. Latencies to reach the platform were measured on three consecutive days. On the fourth day the platform was moved to the opposite side of the tube. Values represent mean ± SD, n = 12 animals/treatment. Statistical analysis (ANOVA) indicated no significant difference between the groups

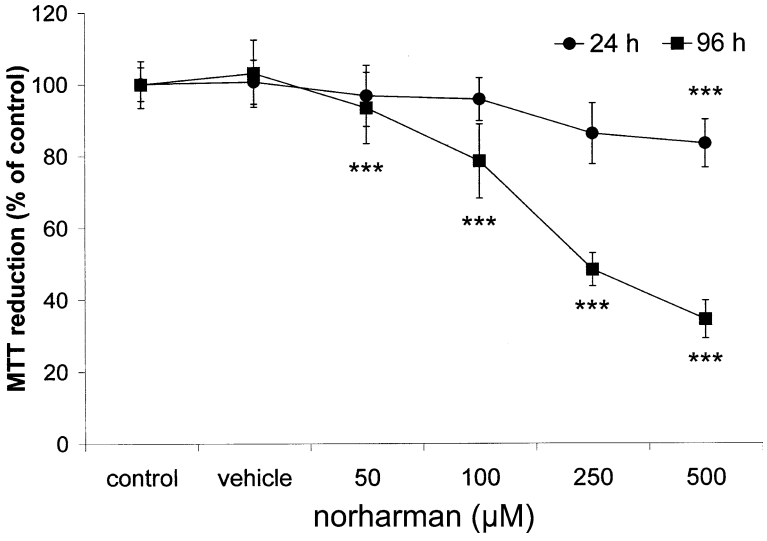

Fig. 6. Mitochondrial function as measured by the MTT-assay in PC12 cells after exposure to norharman for 24 or 96 hours. Values for cells without any exposure to vehicle or norharman were set to 100%. Values represent mean ± SD, n = 3 (6–14 wells/dose) experiments with 24 hours of exposure time, and n = 4 (17–28 wells/dose) experiments with 96 hours exposure time. Statistical analysis (ANOVA) indicated a significant difference between the different concentrations, $p < 0.0001$ for both 24 and 96 hours exposure time. Post-hoc testing indicated a reduced mitochondrial activity, ***$p < 0.001$ for norharman-treated cells compared to vehicle-treated cells

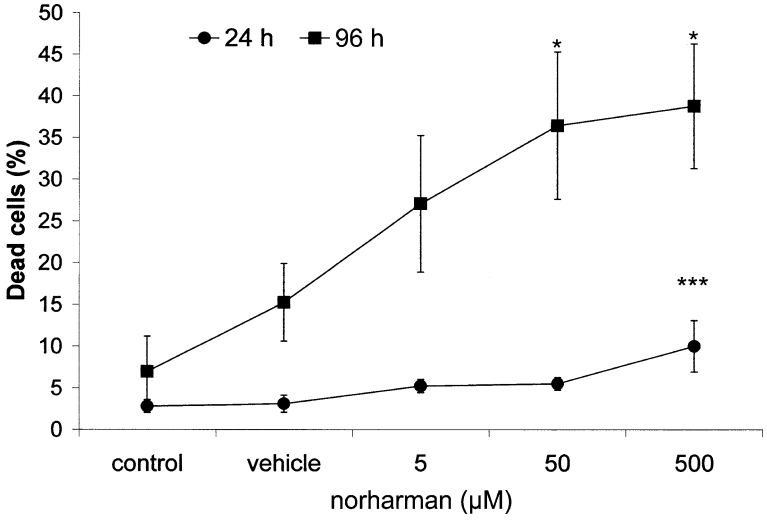

Fig. 7. Necrosis as measured by the trypanblue exclusion assay, in PC12 cells after exposure to norharman for 24 or 96 hours. Values represent mean ± SD, n = 5 experiments (including ≥1300 cells/dose) for 24 hours exposure time, n = 4 experiments (including ≥100 cells/dose) for 96 hours exposure time. Statistical analysis (ANOVA) indicated a significant difference between the different concentrations, $p < 0.001$ for 24 hours exposure time, and $p < 0.05$ for 96 hours exposure time. Post-hoc testing indicated an increased number of necrotic cells, ***$p < 0.001$ and *$p < 0.05$ for norharman-treated cells compared to vehicle-treated cells. In cells without exposure to vehicle or norharman 2.8% ± 0.8 was necrotic after 24 hours exposure, and 7.0% ± 4.2 after 96 hours

Trypanblue-exclusion assay

There was a dose-dependent increase of necrosis in PC12 cells exposed to norharman (5, 50, 500 μM) for 24 or 96 hours (Fig. 7). One-way ANOVA showed a significant difference between the groups, $p < 0.001$ for 24 hours exposure time, and $p < 0.05$ for 96 hours exposure time. Post-hoc testing

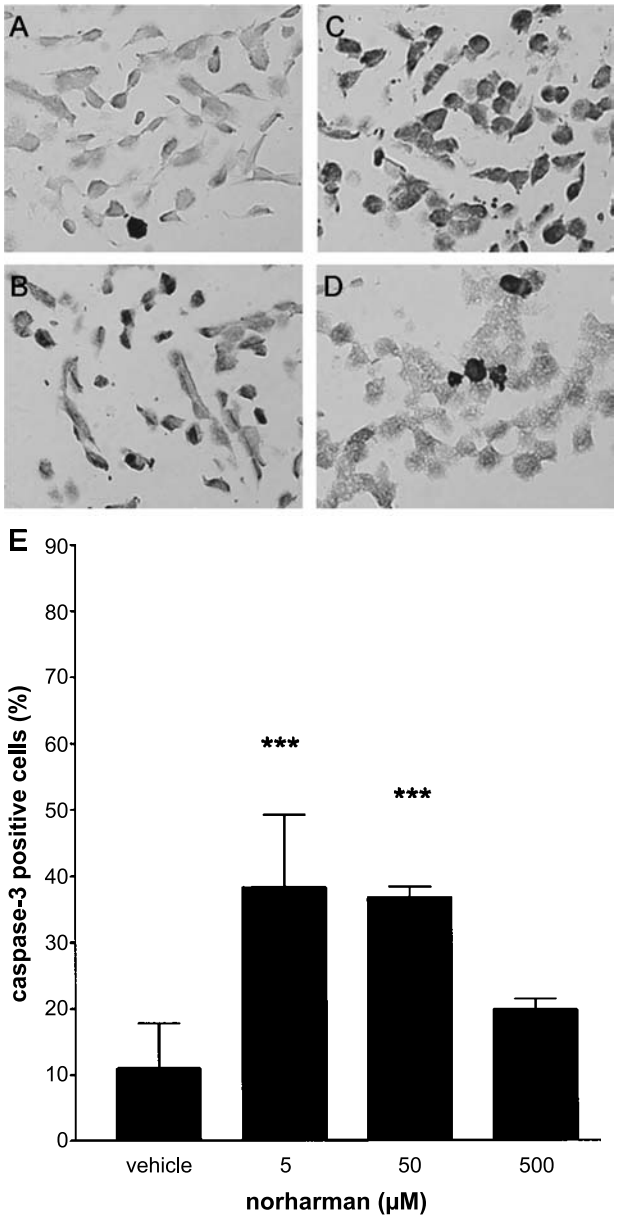

Fig. 8. Apoptosis as determined by caspase-3 activation, in PC12 cells after exposure to vehicle (**A**) or norharman 5 (**B**), 50 (**C**) or 500 μM (**D**) for 24 hours. The number of caspase-3 positive cells was evaluated by immunocytochemistry (**E**). Values represent mean ± SD, n = 4 stainings (including ≥800 cells/dose). Statistical analysis (ANOVA) indicated a significant difference between the different concentrations. Post-hoc testing indicated an increased number of apoptotic cells, ***$p < 0.001$ for norharman-treated cells compared to vehicle-treated cells

indicated a increased number of necrosis for cells exposed to 500 μM norharman for 24 hours ($p < 0.001$), and for cells exposed to 50 μM and 500 μM norharman for 96 hours ($p < 0.05$) compared to vehicle-treated cells.

Caspase-3 immunocytochemistry and TUNEL

Norharman exposure for 24 hours increased the number of cleaved caspase-3 positive PC12 cells. One-way ANOVA showed a

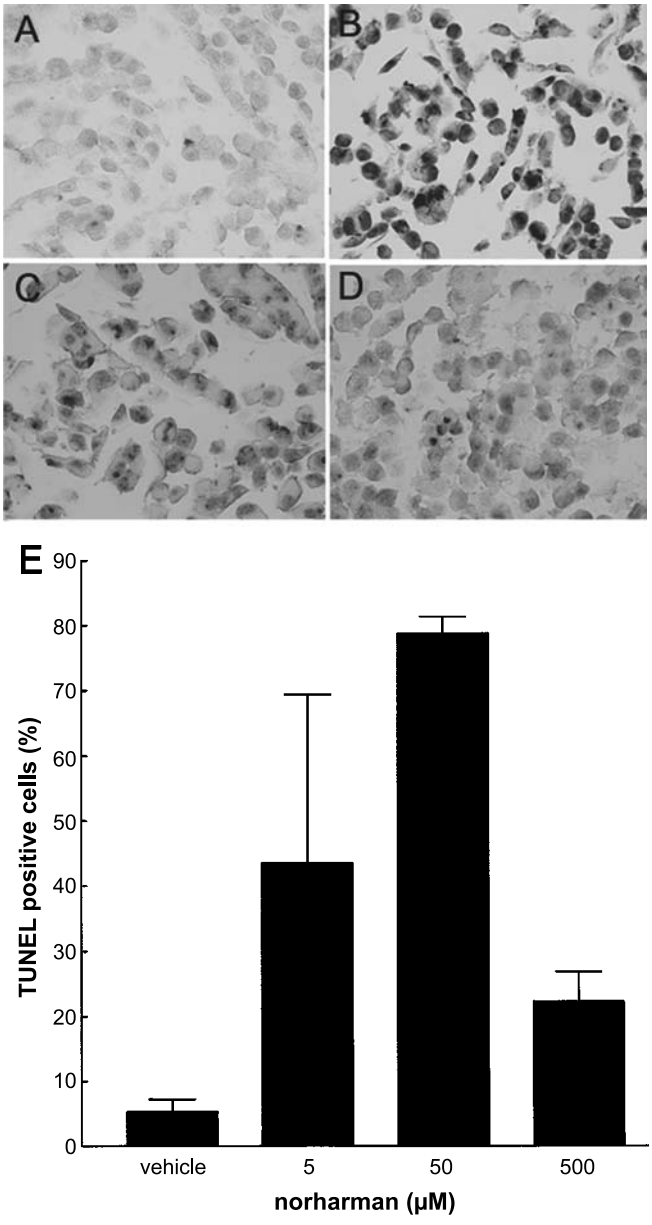

Fig. 9. DNA-fragmentation as determined by TUNEL-staining of PC12 cells after exposure to vehicle (**A**) or norharman 5 (**B**), 50 (**C**) or 500 μM (**D**) for 24 hours. Number of TUNEL-positive stained cells (**E**). Values represent mean ± SD, n = 2–3 sections (including >450 cells/dose)

significant difference between the groups, $p < 0.0001$. Post-hoc testing indicated an increased number of caspase-3-positive cells treated with 5 μM and 50 μM norharman ($p < 0.001$). At the highest concentration (500 μM) the number of cleaved caspase-3-positive cells decreased and was not significant different from vehicle-treated cells. Most cells were swollen with ruptured cell membranes (as shown in Fig. 8D).

The effect of norharman on DNA-fragmentation was similar to caspase-3 staining (as shown in Fig. 9).

Discussion

β-Carbolines such as norharman are present in cooked food and tobacco smoke and show structural resemblance to the parkinsonism-inducing neurotoxicant MPTP. The present subacute study revealed that repeated sc injections of norharman (10 mg/kg) during five days in mice caused an early onset neurodegenerative changes, glial activation in substantia nigra pars compacta and a significant decrease in spontaneous motor activity two weeks after the last dose. In contrast, no norharman-induced changes in learning and memory were observed. Although high doses of norharman are known to induce convulsions and acute behavioural changes this compound has not previously been observed to induce any late motor deficits. Matsubara and co-workers (1998) have reported an insignificant reduction of spontaneous motor activity and a increased bradykinesia in C57BL/6 mice two days after high ip doses of norharman. Temporal inactivity observed shortly after treatment may, however, be difficult to evaluate since also the handling may be stressful for the animals.

The highest dose of norharman caused a significant decrease of spontaneous motor activity in all periods of measurement, even though a normal habituation was observed. The habituation in a motor activity paradigm represents a complex behavioural endpoint, and may under some circumstances serve as an index of a simple, non-associative type of learning. Therefore the three sequential periods of measurement (i.e. 0–20, 20–40 and 40–60 min) were performed. The results suggest that the norharman-induced changes in mice were caused by motor impairment rather than alteration of learning. Neurotoxicant-induced dysfunction in the cholinergic system has been reported to affect learning and memory and several studies have indicated that water swim maze performance test is associated with muscarinic cholinergic function (as reviewed by Pepeu and Giovannini, 2004). In the present study the design of the swim maze was a three day long acquisition period followed by a reversal trial on the fourth day. The latencies to find the platform decreased in all treatment groups. Furthermore, the lack of changes in the spatial memory tasks is in concordance with previous reports demonstrating that norharman is only a weak inhibitor at muscarinic cholinergic receptors and in acetylcholinesterase assays (Muller et al., 1981; Skup et al., 1983). No changes in learning ability and coordination have been observed in MPTP-treated rodents suggesting that these tasks are not dependent on dopaminergic neurons of substantia nigra (Da Cunha et al., 2003).

The repeated treatment with norharman did not induce a significant early loss of tyrosine hydroxylase-positive neurons in the substantia nigra. This is in accordance with reports on subacute and acute treatment with MPTP (Petroske et al., 2001). The number of tyrosine hydroxylase-positive neurons at this site is not reported to change during the first month post-MPTP lesioning (Jakowec et al., 2004). The fluoro-jade staining revealed, however, that repeated norharman treatment induced early neurodegenerative changes in the substantia nigra. Fluoro-jade stains, however, all types of degenerating neurons and glial cells. Since we also detected an in-

creased amount of GFAP-staining in substantia nigra, the early changes detected by fluoro-jade staining is more likely to be due to glial activation. Glial cells are known to play a neuroprotective role and to maintain dopaminergic cell function by supply of trophic factors. Activated glial cells may have a decreased protective ability (Hirsch et al., 2003), and are reported to stimulate cytokines that activate caspases and eventually lead to apoptosis in dopaminergic neurons (Hunot and Hirsch, 2003). This is in accordance with reports demonstrating that MPTP-induced activation of glial cells may be involved in MPTP-induced dopaminergic cell death (Wu et al., 2002; Serra et al., 2002). The late depression of spontaneous motor activity is suggested to be due to early activation of the glial cells leading to degeneration of neurons.

Selective transport of neurotoxicants by use of the dopamine-transporter is often suggested to be the mechanism for accumulation of harmful substances in substantia nigra. However, our previous autoradiographic studies revealed no selective uptake and retention of norharman at this site in C57BL/6 mice (Ostergren et al., 2004). This is in analogy with studies demonstrating that there is only a limited uptake of norharman and monomethylated β-carbolines by dopamine transporters (Drucker et al., 1990). Recent reports have indicated, however, that there is a carrier-mediated uptake of 2-methylated β-carbolines in cells transfected with the human dopamine transporter (Storch et al., 2004). 2,9-Dimethylated norharman will not pass the blood-brain barrier but a single intranigral injection of this metabolite has been reported to cause marked nigrostriatal lesions (Neafsey et al., 1995).

The 2,9-dimethylated norharman is a potent mitochondrial toxicant in cultured PC12 cells (Fields et al., 1992; Collins et al., 1992; Cobuzzi et al., 1994). The present study demonstrated that norharman caused mitochondrial dysfunction as well as an increased number of caspase-3 positive cells, TUNEL- and trypanblue stained PC12 cells. The increase of caspase-3 positive and TUNEL-stained cells was most evident at the low concentrations (5 and 50 μM) suggesting that norharman preferentially induced apoptotic cell death. These observations are in accordance with studies reporting that norharman (250 μM) can induce DNA damage and morphological changes corresponding to apoptosis in cultured human neuroblastoma cells (Uezono et al., 2001). At the highest concentration (500 μM) the number of caspase-3 positive and TUNEL-stained PC12 cells decreased whereas the number of necrotic cells increased markedly. Also MPTP and MPP^+ are reported (McNaught et al., 1996; Kohda et al., 1998; Quigney et al., 2003) to cause apoptosis at low concentrations and necrosis at high concentrations (Hartley et al., 1994; Soldner et al., 1999). Norharman, norharman-metabolites and MPTP/MPP^+ can inhibit complex I in the mitochondrial respiratory chain, although with different potency (Albores et al., 1990; McNaught et al., 1996). Such inhibition may increase production of the superoxide anion (Hasegawa et al., 1990); consequently, radicals may play a role in norharman induced apoptosis at low concentrations when ATP synthesis is not severely compromised. Higher concentrations of these mitochondrial toxicants may severely decrease rates of ATP synthesis and result in inability of the cells to maintain normal cellular function and so result in necrosis (Hartley et al., 1994).

In conclusion, the present results demonstrated that repeated exposure to the food-derived toxicant norharman resulted in early onset degenerative changes and glial activation in the substantia nigra, and a late depression of spontaneous motor activity in C57BL/6 mice. In cultured PC12 cells norharman caused mitochondrial dysfunction, and apoptosis as detected by immunocytochemical analysis of activated caspase-3 and the TUNEL assay. Our previous studies have

demonstrated that norharman is selectively localized and retained in melanin-containing neurons in vivo (Ostergren et al., 2004). The presence of neuromelanin in substantia nigra has also been suggested to play a role for toxicant-induced damage at this site (Zecca et al., 2003; Ostergren et al., 2004). However, common laboratory animals such as the pigmented C57BL/6 mice lack neuromelanin in the substantia nigra (Barden and Levine, 1983). The observed depression in spontaneous motor function of norharman-treated C57BL/6 mice is therefore not related to a selective accumulation of the compound at this site but is suggested to be due to an early glial activation in the substantia nigra nigra and subsequent neurodegenerative changes. The role of exposure to norharman in idiopathic Parkinson's disease should be further investigated.

Acknowledgements

The authors like to thank Ms. R. Engdahl and L. Norgren for technical assistance. We would also like to thank Dr. J. Tallkvist for the help with the fluorescence microscope, and Dr. A.-L. Svensson for introducing us to the MTT-assay. Financial support was provided by the research council FORMAS and by Facias grant 2000-A-6.

References

Albores R, Neafsey EJ, Drucker G, Fields JZ, Collins MA (1990) Mitochondrial respiratory inhibition by N-methylated beta-carboline derivatives structurally resembling N-methyl-4-phenylpyridine. Proc Natl Acad Sci USA 87: 9368–9372

Arai N, Misugi K, Goshima Y, Misu Y (1990) Evaluation of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated C57 black mouse model for parkinsonism. Brain Res 515: 57–63

Barden H, Levine S (1983) Histochemical observations on rodent brain melanin. Brain Res Bull 10: 847–851

Carlsson C, Fredriksson A, Brandt I (2003) 2,6-dichlorophenyl methylsulphone induced behavioural impairments in rats and mice in relation to olfactory mucosal metaplasia. Pharmacol Toxicol 93: 156–168

Cobuzzi RJ Jr, Neafsey EJ, Collins MA (1994) Differential cytotoxicities of N-methyl-beta-carbolinium analogues of MPP^+ in PC12 cells: insights into potential neurotoxicants in Parkinson's disease. J Neurochem 62: 1503–1510

Collins MA, Neafsey EJ, Matsubara K, Cobuzzi RJ Jr, Rollema H (1992) Indole-N-methylated beta-carbolinium ions as potential brain-bioactivated neurotoxins. Brain Res 570: 154–160

Da Cunha C, Wietzikoski S, Wietzikoski EC, Miyoshi E, Ferro MM, Anselmo-Franci JA, Canteras NS (2003) Evidence for the substantia nigra pars compacta as an essential component of a memory system independent of the hippocampal memory system. Neurobiol Learn Mem 79: 236–242

Drucker G, Raikoff K, Neafsey EJ, Collins MA (1990) Dopamine uptake inhibitory capacities of beta-carboline and 3,4-dihydro-beta-carboline analogs of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) oxidation products. Brain Res 509: 125–133

Fields JZ, Albores R, Neafsey EJ, Collins MA (1992) Similar inhibition of mitochondrial respiration by 1-methyl-4-phenyl-pyridinium (MPP+) and by a unique N-methylated beta-carboline analogue, 2,9-dimethyl-norharman (2,9Me2NH). Ann NY Acad Sci 648: 272–274

Fredriksson A, Archer T (1994) MPTP-induced behavioural and biochemical deficits: a parametric analysis. J Neural Transm (PD Sect) 7: 123–132

Fuentes JA, Longo VG (1971) An investigation on the central effects of harmine, harmaline and related beta-carbolines. Neuropharmacology 10: 15–23

Gearhart DA, Neafsey EJ, Collins MA (1997) Characterization of brain beta-carboline-2-N-methyltransferase, an enzyme that may play a role in idiopathic Parkinson's disease. Neurochem Res 22: 113–121

Hartley A, Stone JM, Heron C, Cooper JM, Schapira AH (1994) Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. J Neurochem 63: 1987–1990

Hasegawa E, Takeshige K, Oishi T, Murai Y, Minakami S (1990) 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem Biophys Res Commun 170: 1049–1055

Herraiz T (2002) Identification and occurrence of the bioactive beta-carbolines norharman and harman in coffee brews. Food Addit Contam 19: 748–754

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A, Michel PP (2003) The role of glial reaction and inflammation in Parkinson's disease. Ann NY Acad Sci 991: 214–228

Hunot S, Hirsch EC (2003) Neuroinflammatory processes in Parkinson's disease. Ann Neurol 53 [Suppl 3]: 49–60

Jakowec MW, Nixon K, Hogg E, McNeill T, Petzinger GM (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J Neurosci Res 76: 539–550

Kadota T, Yamaai T, Saitoh Y, Kawashima S, Moroi K, Kadota K (1995) Expression of a dopamine transporter in PC12h cells: an immunohistochemical study with an antipeptide antibody. J Histochem Cytochem 44: 989–996

Kirk RE (1995) Experimental design, procedures for the behavioral science. Brooks/Cole Publ, Pacific Grove, CA

Kohda K, Noda Y, Aoyama S, Umeda M, Sumino T, Kaiya T, Maruyama W, Naoi M (1998) Cytotoxicity of 1-amino-4-phenyl-1,2,3,6-tetrahydropyridine and 1-amino-4-phenylpyridinium ion, 1-amino analogues of MPTP and MPP+, to clonal pheochromocytoma PC12 cells. Chem Res Toxicol 11: 1249–1253

Kuhn W, Muller T, Grosse H, Rommelspacher H (1996) Elevated levels of harman and norharman in cerebrospinal fluid of parkinsonian patients. J Neural Transm 103: 1435–1440

Marey-Semper I, Gelman M, Levi-Strauss M (1995) A selective toxicity toward cultured mesencephalic dopaminergic neurons is induced by the synergistic effects of energetic metabolism impairment and NMDA receptor activation. J Neurosci 15: 5912–5918

Matsubara K, Neafsey EJ, Collins MA (1992) Novel S-adenosylmethionine-dependent indole-N-methylation of beta-carbolines in brain particulate fractions. J Neurochem 59: 511–518

Matsubara K, Collins MA, Akane A, Ikebuchi J, Neafsey EJ, Kagawa M, Shiono H (1993) Potential bioactivated neurotoxicants, N-methylated beta-carbolinium ions, are present in human brain. Brain Res 610: 90–96

Matsubara K, Gonda T, Sawada H, Uezono T, Kobayashi Y, Kawamura T, Ohtaki K, Kimura K, Akaike A (1998) Endogenously occurring beta-carboline induces parkinsonism in nonprimate animals: a possible causative protoxin in idiopathic Parkinson's disease. J Neurochem 70: 727–735

May T, Pawlik M, Rommelspacher H (1991) [3H]harman binding experiments. II. Regional and subcellular distribution of specific [3H]harman binding and monoamine oxidase subtypes A and B activity in marmoset and rat. J Neurochem 56: 500–508

McNaught KS, Thull U, Carrupt PA, Altomare C, Cellamare S, Carotti A, Testa B, Jenner P, Marsden CD (1996) Toxicity to PC12 cells of isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurosci Lett 206: 37–40

Muller WE, Fehske KJ, Borbe HO, Wollert U, Nanz C, Rommelspacher H (1981) On the neuropharmacology of harmane and other beta-carbolines. Pharmacol Biochem Behav 14: 693–699

Neafsey EJ, Albores R, Gearhart D, Kindel G, Raikoff K, Tamayo F, Collins MA (1995) Methyl-beta-carbolinium analogs of MPP+ cause nigrostriatal toxicity after substantia nigra injections in rats. Brain Res 675: 279–288

Ogawa N, Hirose Y, Ohara S, Ono T, Watanabe Y (1985) A simple quantitative bradykinesia test in MPTP-treated mice. Res Commun Chem Pathol Pharmacol 50: 435–441

Ostergren A, Annas A, Skog K, Lindquist NG, Brittebo EB (2004) Long-term retention of neurotoxic beta-carbolines in brain neuromelanin. J Neural Transm 111: 141–157

Pawlik M, Kaulen P, Baumgarten HG, Rommelspacher H (1990) Quantitative autoradiography of [3H]norharman [(3H]beta-carboline) binding sites in the rat brain. J Chem Neuroanat 3: 19–24

Pepeu G, Giovannini MG (2004) Changes in acetylcholine extracellular levels during cognitive processes. Learn Mem 11: 21–27

Petroske E, Meredith GE, Callen S, Totterdell S, Lau YS (2001) Mouse model of Parkinsonism: a comparison between subacute MPTP and chronic MPTP/probenecid treatment. Neuroscience 106: 589–601

Pranzatelli MR, Snodgrass SR (1987) Harmala alkaloids and related beta-carbolines: a myoclonic model and antimyoclonic drugs. Exp Neurol 96: 703–719

Quigney DJ, Gorman AM, Samali A (2003) Heat shock protects PC12 cells against MPP+ toxicity. Brain Res 993: 133–139

Rommelspacher H, Nanz C, Borbe HO, Fehske KJ, Muller WE, Wollert U (1981) Benzodiazepine antagonism by harmane and other beta-carbolines in vitro and in vivo. Eur J Pharmacol 70: 409–416

Rommelspacher H, May T, Salewski B (1994) Harman (1-methyl-beta-carboline) is a natural inhibitor of monoamine oxidase type A in rats. Eur J Pharmacol 252: 51–59

Rousselet E, Joubert C, Callebert J, Parain K, Tremblay L, Orieux G, Launay JM, Cohen-Salmon C, Hirsch EC (2003) Behavioral changes are not directly related to striatal monoamine levels, number of nigral neurons, or dose of parkinsonian toxin MPTP in mice. Neurobiol Dis 14: 218–228

Schmued LC, Hopkins KJ (2000) Fluoro-Jade: novel fluorochromes for detecting toxicant-induced neuronal degeneration. Toxicol Pathol 28: 91–99

Schmued LC, Albertson C, Slikker W Jr (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751: 37–46

Serra PA, Sciola L, Delogu MR, Spano A, Monaco G, Miele E, Rocchitta G, Miele M, Migheli R, Desole MS (2002) The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induces apoptosis in mouse nigrostriatal glia. Relevance to nigral neuronal death and striatal neurochemical changes. J Biol Chem 277: 34451–34461

Skup M, Oderfeld-Nowak B, Rommelspacher H (1983) In vitro studies on the effect of beta-carbolines on the activities of acetylcholinesterase and choline acetyltransferase and on the muscarinic receptor binding of the rat brain. J Neurochem 41: 62–68

Soldner F, Weller M, Haid S, Beinroth S, Miller SW, Wullner U, Davis RE, Dichgans J, Klockgether T, Schulz JB (1999) MPP+ inhibits proliferation of PC12 cells by a p21(WAF1/Cip1)-dependent pathway and induces cell death in cells lacking p21(WAF1/Cip1). Exp Cell Res 250: 75–85

Storch A, Hwang YI, Gearhart DA, Beach JW, Neafsey EJ, Collins MA, Schwarz J (2004) Dopamine transporter-mediated cytotoxicity of beta-carbolinium derivatives related to Parkinson's disease: relationship to transporter-dependent uptake. J Neurochem 89: 685–694

Sundstrom E, Fredriksson A, Archer T (1990) Chronic neurochemical and behavioral changes in MPTP-lesioned C57BL/6 mice: a model for Parkinson's disease. Brain Res 528: 181–188

Totsuka Y, Ushiyama H, Ishihara J, Sinha R, Goto S, Sugimura T, Wakabayashi K (1999) Quantification of the co-mutagenic beta-carbolines, norharman and harman, in cigarette smoke condensates and cooked foods. Cancer Lett 143: 139–143

Uezono T, Maruyama W, Matsubara K, Naoi M, Shimizu K, Saito O, Ogawa K, Mizukami H, Hayase N, Shiono H (2001) Norharman, an indoleamine-derived beta-carboline, but not Trp-P-2, a gamma-carboline, induces apoptotic cell death in human neuroblastoma SH-SY5Y cells. J Neural Transm 108: 943–953

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 22: 1763–1771

Zecca L, Zucca FA, Wilms H, Sulzer D (2003) Neuromelanin of the substantia nigra: a neuronal black hole with protective and toxic characteristics. Trends Neurosci 26: 578–580

Authors' address: Prof. E. Brittebo, Department of Pharmaceutical Biosciences, Uppsala University, Box 594, SE-751 24 Uppsala, Sweden, e-mail: Eva.Brittebo@farmbio.uu.se