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Postnatal iron overload destroys NA-DA functional interactions

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Summary C57/BL6 mice were administered either postnatal iron (Fe^{2+} 7.5 mg/kg, on postnatal days 10–12) or vehicle, followed by administration of either DSP4 (50 mg/kg, s.c., 30 min after injection of zimeldine, 20 mg/kg, s.c.) or vehicle (saline) at 63 days of age. Three weeks later, iron/vehicle treated, DSP4/vehicle treated mice were injected with either a low dose of MPTP (2×20 mg/kg, with a 24-hr interval between injections) or vehicle. Behaviour testing took place a further three weeks (spontaneous behaviour and L-Dopa induced) and two weeks (clonidine-L-Dopa induced) later. Postnatal iron administration exacerbated the bradykinesia induced by MPTP and virtually abolished all spontaneous motor activity in NA-denervated mice that were MPTP-treated. Postnatal iron administration reduced markedly the restoration of motor activity by suprathreshold L-Dopa (20 mg/kg) following a 60-min habituation to the test chambers. Pretreatment with DSP4 effectively eliminated the restorative effect of L-Dopa in the MPTP mice. The synergistic effects of co-administration of clinidine (1 mg/kg) with a subthreshold dose of L-Dopa (5 mg/kg) in elevating the motor activity of MPTP mice were reduced markedly by postnatal iron administration, as well as by pretreatment with DSP4. NA-denervation by DSP4, after postnatal iron treatment, totally abolished the activity-elevating effects of the α-adrenoceptor agonist + DA-precursor combination in MPTP mice, and virtually eliminated these effects in saline (non-MPTP) mice.

Postnatal iron administration caused enduring higher levels of total iron content in all the groups with an increased level in mice treated with DSP4 followed by MPTP. These divergent findings confirm the direct influence of NA innervation upon dopaminergic functional expression and indicate a permanent vulnerability both in the noradrenergic and dopaminergic pathways following the postnatal infliction of an iron overload.

Keywords: Postnatal iron, DSP4, MPTP, vehicle, motor deficits, locomotion, rearing, total activity, suprathreshold L-Dopa, restoration, clonidine, subthreshold L-Dopa, denervation, DA, NA, C57/BL6 mice, PD

Introduction

The selective denervating effects of DSP4 (N-[2-chloroethyl]-N-ethyl-2-bromobenzylamine), for noradrenaline (NA) neurons in the regions of the forebrain, midbrain, cerebellum, brain stem and spinal cord with concurrent changes in function, have been documented amply (Archer and Fredriksson, 2000, 2001; Archer et al., 1982, 1983, 1984, 1986a; Dooley et al., 1983a; Fredriksson and Archer, 2000; Jonsson and Hallman, 1982; Jonsson et al., 1981, 1982; Ponzio et al., 1981; Ross, 1976; Ross and Renyi, 1976). Systemic administration of higher doses of DSP4 (50 mg/kg, i.p. and above) induced marked reductions dopamine-β-hydroxylase activity (Archer et al., 1984; Ross, 1976; Ross and Renyi, 1976), as well as profound and permanent depletion of endogenous NA in the cerebral and cerebellar cortex, hippocampus and spinal cord, leaving dopamine (DA) and serotonin (5-HT) neurons apparently unaffected (Jonsson et al., 1981). Lower doses of DSP4 (3 and 6 mg/kg, i.p.) produced small, yet significant, decreases in NA concentrations in certain brain areas, e.g. cerebral cortex, hippocampus, olfactory bulb and spinal cord (Archer et al., 1984). Transient peripheral depletions of NA in DSP4-treated rats generally disappeared 14 days following injections of the 50 mg/kg dose (e.g. Archer et al., 1982). Systemic administration of DSP4 (50 mg/kg) has induced, although not always, a small depletion of cortical 5-HT levels (e.g. Archer et al., 1984, 1985). However, pretreatment with the selective 5-HT reuptake inhibitor, zimeldine (20 mg/kg, i.p.), prevents any loss of 5-HT, without affecting the actions of DSP4 upon NA neurons (Archer, 1982, 1986b; Heal et al., 1993; Post et al., 1987). Functional studies with receptor agonists of the DA and 5-HT systems have shown that these neurotransmitter systems were neither altered in DSP4-treated rats (Dooley et al., 1983b), nor did the DSP4 treatment induce any

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noticeable effect on the normal functioning of the hypothalamic-pituitary-adrenal axis. A comprehensive analysis of the neurotoxic actions of DSP4, by evaluating different patterns of monoamine depletion in different brain regions of two different strains of rats and mice, 3, 7, and 14 days after DSP4 administration has been performed (Fornai et al., 1996). Evident species and strain differences were obtained, particularly with regard to the effects on 5-HT which was fully preserved in DSP4-treated mice, but significantly depleted in specific brain regions following administration of the same dose to rats.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism in human and nonhuman primates (Langston, 1985), resulting in the loss of substantia nigra cells in the pars compacta of adult animals. Systemic administration of MPTP (2×40 mg/kg, s.c.) to C57 BL/6 mice caused L-Dopa reversible hypoactivity (Fredriksson et al., 1990; Sundström et al., 1990). A less rigorous dose treatment, e.g. 2×20, or 25 or 30 mg/kg, of MPTP has been found not to reduce motility in the C57 black mice although DA concentrations may indicate upto 50–80% reductions (Heikkila et al., 1989; Sonsalla and Heikkila, 1986). The parameters of MPTP-treatment neurotoxicity are long-lasting (upto and beyond 52 weeks after treatment) with a good correlation between the functional deficits, particularly hypokinesia, the neurochemical concomitant, severe depletions of DA, and a dose- and time-dependent recovery of several parameters of motor behaviour following treatment with the DA precursor, L-Dopa (Archer and Fredriksson, 2003; Fredriksson and Archer, 1994; Fredriksson et al., 1999, Sundström et al., 1990). Neonatal administration with iron (Fe^{2+}, at doses of 7.5 or 15 mg/kg) potentiated both the functional and neurochemical deficits induced by both a lower (2×20 mg/kg) and a higher (2×40 mg/kg) dose of MPTP (Fredriksson and Archer, 2003; Fredriksson et al., 2001).

In a recent study (Archer and Fredriksson, in press), mice were administered either DSP4 (50 mg/kg) or vehicle (saline) at 63 days of age. Three weeks later, DSP4 and Vehicle treated mice were administered either a high dose of MPTP (2×40 mg/kg), a low dose (2×20 mg/kg) or vehicle. Three weeks later, all six groups were tested for spontaneous motor behaviour, followed by injections of L-Dopa (20 mg/kg, s.c.), and then tested over a further 360 min in the activity test chambers. It was found that pretreatment with the selective NA neurotoxin, DSP4, deteriorated markedly the dose-dependent motor activity deficits observed in the vehicle pretreated MPTP treated mice. These 'ultra-deficits' in the spontaneous motor behaviour of MPTP-treated mice were observed over all three parameters: locomotion, rearing and total activity, and were restricted to the 1st and 2nd 20-min periods. Administration of L-Dopa (20 mg/kg) following the 60-min testing of spontaneous behaviour restored the motor activity of Vehicle + MPTP treated mice (neither the Vehicle + MPTP-Low nor the Vehicle + MPTP-High groups differed from the Vehicle-Vehicle group, here) but failed to do so in the DSP4 pretreated mice. Here, a dose-dependent deficit of L-Dopa-induced motor activity (over all three parameters) was obtained thereby offering further evidence of an 'ultra-deficit' of function due to previous denervation of the NA terminals.

The purpose of the present study was to examine further the implications of denervating central noradrenergic pathways, using DSP4 (50 mg/kg), following postnatal iron administration, for the surviving integrity of the nigrostriatal dopaminergic system following a low (2×20 mg/kg) dose administrations of MPTP by assessment of spontaneous motor and L-Dopa-induced behaviour. As previously demonstrated (Archer and Fredriksson, 2002), α2-adrenoceptor agonists, e.g. clonidine, may induce a synergistic restoration of motor activity in MPTP-treated, L-Dopa-tolerant mice. A further purpose was to examine whether or not NA-denervation would eliminate the propensity for co-administration of clonidine with a subthreshold dose of L-Dopa to restore motor activity.

Material and methods

Animals

Male C57 Bl/6 mice were purchased from B&K, Sollentuna, Sweden, and were maintained, five-to-a-cage, in plastic cages in a room at temperature of 22 ± 1°C and a 12/12 hrs constant light/dark cycle (lights on between 06.00 and 18.00 hrs) for one month, prior to treatment and testing. Male mice, postnatal days 10–12, were administered Fe^{2+} (see below) or saline. At 63 days of age, these mice, weighing 21–25 g, were administered either DSP4 (50 mg/kg, s.c., 30 min after injection of zimeldine, 20 mg/kg, s.c.) or vehicle (saline). Three weeks later, two groups (n = 10) of DSP4-treated and two groups of vehicle-treated mice were administered MPTP (2×20 mg/kg, s.c., 24 hrs between injections; Low dose administration), and two groups (n = 10) of DSP4-treated and two groups of vehicle-treated mice were administered vehicle. They were housed in groups of 5 animals, throughout, and tested only during the hours of light (08.00–15.00 hrs). Behavioural testing was initiated a further three weeks following the start of treatment with MPTP or vehicle. All testing was performed in a normally lighted room. This test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. motor activity test cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels and a small double-glass window to allow observation; each box had a dimmed lighting.

Experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC) after approval from the local ethical committee (Uppsala University and Agricultural Research Council), and by the Swedish Committee

for Ethical Experiments on Laboratory Animals (license S93/92 and S77/94, Stockholm, Sweden).

Drugs

Both DSP4 (synthesized by AstraZeneca, Södertälje, Sweden, 1×50 mg/kg, s.c.), MPTP (Research Biomedical Inc., MA, USA, 2×20 mg/kg or 2×40 mg/kg, s.c., with a 24-hr interval between injections in each case) and L-Dopa (Hässle, AstraZeneca, Mölndal, Sweden, 20 mg/kg, s.c.) were all dissolved in saline and administered s.c. in a volume of 2 ml/kg body weight. Saline was used as vehicle in each case. Ferromyn® (Iron succinate: 3.7 mg Fe^{2+}/ml, AB Hässle, Göteborg, Sweden). Dosages, expressed as mg Fe^{2+}/kg b.w., was administered orally via a metallic gastric tube in a volume of 10 ml/kg body weight. Saline was used as vehicle and to prepare the dose of Fe^{2+}. Ferromyn S is applied to the treatment of anemia and as a prophylactic measure for blood donors and pregnant women. Clonidine (gift from Astra Arcus AB) was dissolved in physiological saline (0.9%).

Behavioural measurements and apparatus

Activity test chambers: An automated device, consisting of macrolon rodent test cages ($40 \times 25 \times 15$ cm) each placed within two series of infra-red beams (at two different heights, one low and one high, 2 and 8 cm, respectively, above the surface of the sawdust, 1 cm deep), was used to measure spontaneous motor activity (RAT-O-MATIC, ADEA Elektronic AB, Uppsala, Sweden). The distance between the infra-red beams was as follows: the low levels beams were 73 mm apart lengthwise and 58 mm apart breadthwise in relation to the test chamber; the high level beams, placed only along each longside of the test chamber were 28 mm apart. According to the procedures described previously (Archer et al., 1986), the following parameters were measured: LOCOMOTION was measured by the low grid of infra-red beams. Counts were registered only when the mouse in the horizontal plane, ambulating around the test-cage. REARING was registered throughout the time when at least one high level beam was interrupted, i.e. the number of counts registered was proportional to the amount of time spent rearing. TOTAL ACTIVITY was measured by a sensor (a pick-up similar to a gramophone needle, mounted on a lever with a counterweight) with which the test cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced both by locomotion and rearing as well as shaking, tremors, scratching and grooming. All three behavioural parameters were measured over three consecutive 20-min. periods. The motor activity test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. activity cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels, and day-lighting. Motor activity parameters were tested on one occasion only, over three consecutive 20-min periods, at the age of three to four months.

Design and treatment

Eight treatment groups ($n = 10$) were derived from the postnatal administration of either iron (7.5 mg/kg Fe^{2+}, four groups) or vehicle (0.9% physiological saline, four groups, Veh) on postnatal days 10–12. At 63 days of age, two groups of Fe^{2+}-treated mice were administered DSP4 (50 mg/kg, s.c., 30 min after injection of zimeldine, 20 mg/kg) and two groups received Vehicle (saline). Concurrently, two groups of Veh-treated mice were administered DSP4 (50 mg/kg, s.c., 30 min after injection of zimeldine, 20 mg/kg) and two groups received Vehicle (saline). Three weeks later, two DSP4-treated groups [one Fe and one Veh] were administered MPTP (2×20 mg/kg, s.c., 24-hr interval between injections)[Fe-DSP4-MPTP and Veh-DSP4-MPTP] and two DSP4-treated groups [one Fe and one Veh] were administered Vehicle (saline) [Fe-DSP4-Veh and Veh-DSP4-Veh]. Concurrently, two Vehicle-treated groups were administered MPTP (2×20 mg/kg, s.c., 24-hr interval between injections)[Fe-Veh-MPTP and Veh-Veh-MPTP] and two Vehicle-treated groups were administered Vehicle (saline) [Fe-Veh-Veh and Veh-Veh-Veh], thereby providing the eight treatment groups.

A further three weeks later, all eighty mice were tested, singly, in the motor activity test chambers over a 60-min period measuring spontaneous motor activity. Following this, each mouse was removed, injected L-Dopa (20 mg/kg, s.c.) and then replaced in the same test chamber. Motor activity was then measured over a further 240 min. Two weeks later, each mouse was placed in the activity test chambers for a 60-min habituation period, and then removed, injected clonidine (1 mg/kg, s.c.) followed by L-Dopa (5 mg/kg), and then replaced in the same test chamber. Motor activity was then measured over a further 240 min.

Neurochemical analysis

Mice were killed by cervical dislocation within two weeks after completion of behavioural testing. Brain regions from 4 mice or 6 rats in each group (DSP4 and Vehicle/MPTP and Vehicle) were dissected (Glowinski and Iversen, 1966) and stored in minus 70°C until analysis. Frontal cortex, hippocampus and striatal regions were rapidly dissected out and stored at −80°C until analysis. Frozen tissue samples were weighed and homogenized in 1 ml of 0.1 M Perchloric acid, α-methyl-5-hydroxytrytophan added as internal standard. After centrifugation (12 000 rpm, i.e. 18600 *g*, 4°C, 10 min) and filtration, 20 µl of the supernatant was injected into the HPLC-EC to assay DA and NA. The HPLC system consisted of a PM-48 pump (Bioanalytical Systems, BAS) with a CMA/240 autoinjector (injection volume: 20 µl), a precolumn (15×3.2 mm^2, RP-18 Newguard, 7 µm), a column (100×4.6 mm^2, SPHERI-5, RP-18, 5 µm), and an amperometric detector LC-4B, BAS, equipped with an Ag/AgCl reference electrode and a MF-2000 cell) operating at a potential of +0.85 V. The mobile phase, pH 2.69, consisted of K_2HPO_3 and citric acid buffer (pH 2.5), 10% methanol, Na octyl sulphate (40 mg/L), and EDTA. The flow rate was 1 ml/min, and the temperature of the mobile phase was 35°C.

Statistical analysis

The locomotion, rearing and total activity data over three consecutive 20-min. periods in the activity test chambers from the spontaneous motor activity data were submitted to a split-plot ANOVA design (Kirk, 1995). Motor activity parameters over the total 240-min period following injections of L-Dopa (20 mg/kg) or L-Dopa (5 mg/kg) + clonidine (1 mg/kg) were submitted to one-way ANOVA based on a completely randomised design (Kirk, 1995). Pairwise testing between the different treatment groups was performed with the Tukey HSD test (Kirk, 1995). Correlation coefficients were analysed between the variables total iron content in the basal ganglia, apomorphine-induced locomotion, rearing and total activity over all 60 mice studied. The 1% level of significance was maintained throughout unless where otherwise stated.

Results

Spontaneous motor behaviour

Postnatal iron treatment induced a marked hypokinesia over the whole 40-min test period. Pretreatment with DSP4, at adult age, exacerbated the hypokinesia induced by MPTP, low dose (20 mg/kg), in mice treated postnatally with vehicle. In mice that received postnatal iron treatment, pretreatment with DSP4 abolished motor behaviour in MPTP-treated mice. Postnatal iron treatment, exacerbated

the hypokinesia induced by MPTP, low dose (20 mg/kg) in the vehicle (non-DSP4) treated mice. DSP4 treatment, by itself, did not affect the outcome of either postnatal iron or vehicle for motor activity.

Split-plot ANOVA indicated significant Treatment × Time period interactions for Locomotion: $F(14, 144) = 65.60$), Rearing: $F(14, 144) = 61.29$, and Total Activity: $F(14, 144) = 36.19$. Figure 1 presents the mean locomotion, rearing and total activity counts by postnatal iron-treated or vehicle-treated mice that were administered either DSP4 or vehicle at 63 days of age, followed by either MPTP or vehicle three weeks later.

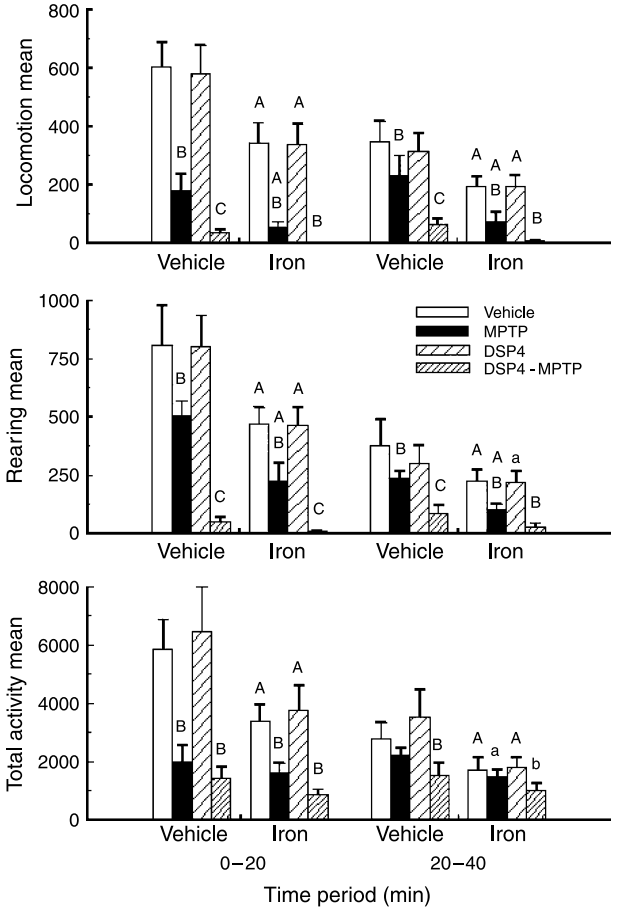

Fig. 1. Spontaneous motor behaviour by C57 Bl6 mice, administered either postnatal iron (Fe^{2+} 7.5 mg/kg, on postnatal days 10–12) or vehicle, followed by administration of DSP4 (50 mg/kg, 30 min after injection of zimeldine, 20 mg/kg) at 63 days of age, followed by a low dose of MPTP (2 × 20 mg/kg, with a 24-hr interval between injections) three weeks later. Behavioural testing occurred a further three weeks later. Mean locomotion, rearing and total activity counts by vehicle- and DSP4-treated C57/BL6 mice administered MPTP or vehicle. Mice were placed singly in the activity test chambers and motor activity parameters were measured over 60 min. Values represent means ± SD of 10 mice. $^{A}p < 0.01$, $^{a}p < 0.05$, compared to respective postnatal Vehicle groups, Tukey HSD-testing, $^{B}p < 0.01$, $^{b}p < 0.05$ compared to respective Vehicle/DSP4 groups, Tukey HSD-testing, $^{C}p < 0.01$, compared to respective MPTP group, Tukey HSD-testing

Tukey HSD-testing indicated the following pairwise differences:

Locomotion and Rearing over both 20-min periods:
Vehicle: Veh-Veh, DSP4-Veh > Veh-MPTP > DSP4-MPTP
Iron: Veh-Veh, DSP4-Veh > Veh-MPTP > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

Total activity:
0–20 min:
Vehicle: DSP4-Veh, Veh-Veh > Veh-MPTP, DSP4-MPTP
Iron: DSP4-Veh, Veh-Veh > Veh-MPTP > DSP4-MPTP
20–40 min:
Vehicle: DSP4-Veh, Veh-Veh, Veh-MPTP > DSP4-MPTP
Iron: DSP4-Veh, Veh-Veh, Veh-MPTP > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe, at 20–40 min only.

L-Dopa-induced activity

Acute administration of L-Dopa (20 mg/kg) restored the motor activity of MPTP-treated mice over all three parameters in the vehicle-treated animals. Pretreatment with DSP4 caused a deficit in the restorative effects upon motor behaviour, induced by the L-dopa treatment, in the MPTP-treated mice. Postnatal iron administration also caused a deficit in the restorative effects upon motor behaviour, induced by the L-dopa treatment, in the MPTP-treated mice. Notably, postnatal iron followed at adult age by DSP4 pretreatment virtually abolished the L-Dopa restorative effects. DSP4, by itself, did not alter the response to the DA precursor, neither in postnatal iron nor vehicle treated mice.

Thus, one-way ANOVA indicated significant Groups effects for both Locomotion: $F(7, 72) = 110.17$, Rearing: $F(7, 72) = 30.28$, and Total activity: $F(7, 72) = 41.76$. Figure 2 presents the mean locomotion, rearing and total activity counts by postnatal iron-treated or vehicle-treated mice that were administered either DSP4 or vehicle at 63 days of age, followed by either MPTP or vehicle three weeks later; all the mice were administered suprathreshold L-Dopa acutely, following a 60-min habituation to the test chambers.

Tukey HSD-testing indicated the following pairwise differences:

Locomotion:
Vehicle: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
Iron: Veh-Veh, DSP4-Veh > Veh-MPTP > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

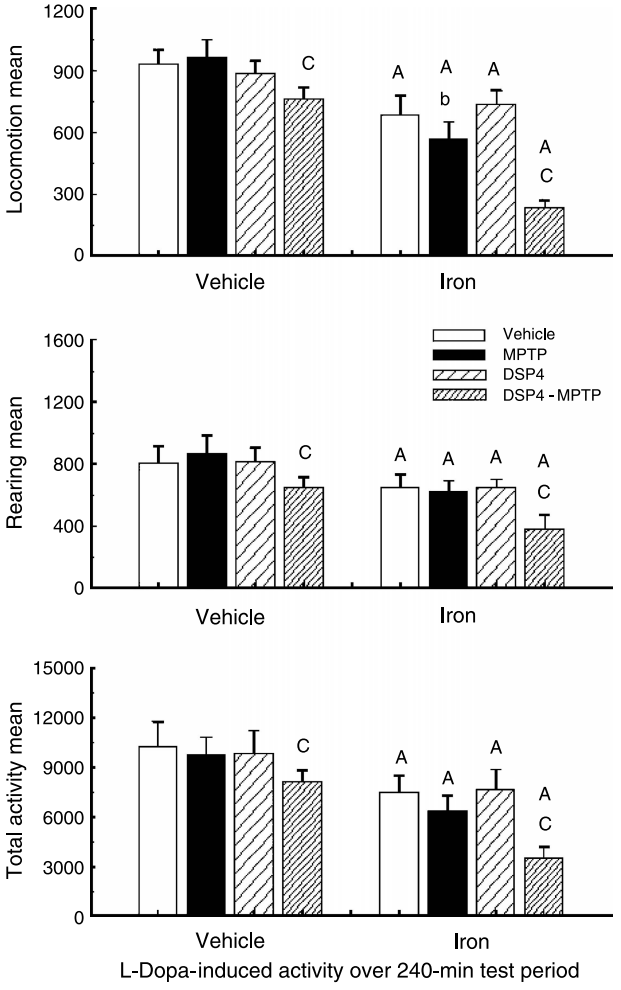

Fig. 2. L-Dopa-induced motor behaviour by C57 Bl6 mice, administered either postnatal iron (Fe^{2+} 7.5 mg/kg, on postnatal days 10–12) or vehicle, followed by administration of DSP4 (50 mg/kg, 30 min after injection of zimeldine, 20 mg/kg) at 63 days of age, followed by a low dose of MPTP (2×20 mg/kg, with a 24-hr interval between injections) three weeks later. Behavioural testing occurred a further three weeks later. Mean locomotion, rearing and total activity counts by vehicle- and DSP4-treated C57/BL6 mice administered MPTP or vehicle. Mice were administered L-Dopa (20 mg/kg) 60 min after placement in the activity test chambers and motor activity parameters were measured over a further 240 min. Values represent means ± SD of 10 mice. $^{A}p < 0.01$, $^{a}p < 0.05$, compared to respective postnatal Vehicle groups, Tukey HSD-testing, $^{b}p < 0.05$ compared to respective MPTP group, Tukey HSD-testing, $^{C}p < 0.01$, compared to respective DSP4 or MPTP group, Tukey HSD-testing

Rearing:
Vehicle: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
Iron: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

Total activity:
Vehicle: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
Iron: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

Clonidine + subthreshold L-Dopa-induced activity

The co-administration of clonidine (1 mg/kg) with a subthreshold dose (5 mg/kg) of L-Dopa caused a marked, but not complete, restoration of motor behaviour in MPTP-treated mice; this restorative effect of the combination was

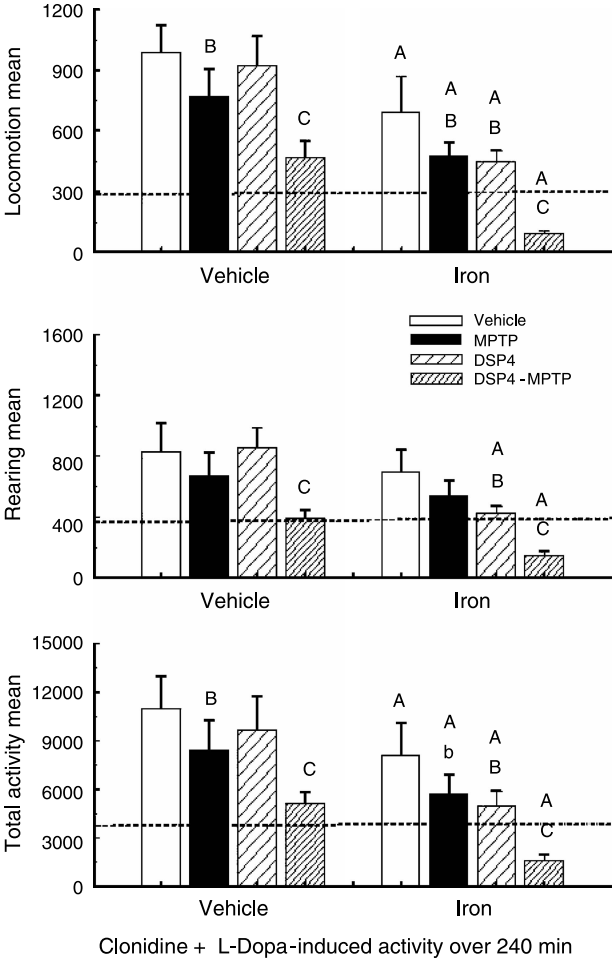

Fig. 3. Reinstatement of motor activity after acute co-administration of clonidine (1 mg/kg) with a subthreshold dose of L-Dopa (5 mg/kg) in C57 Bl6 mice, administered either postnatal iron (Fe^{2+} 7.5 mg/kg, on postnatal days 10–12) or vehicle, followed by administration of DSP4 (50 mg/kg, 30 min after injection of zimeldine, 20 mg/kg) at 63 days of age, followed by a low dose of MPTP (2×20 mg/kg, with a 24-hr interval between injections) three weeks later. Mean locomotion, rearing and total activity counts by vehicle- and DSP4-treated C57/BL6 mice administered MPTP or vehicle. Mice were administered clonidine + L-Dopa 60 min after placement in the activity test chambers and motor activity parameters were measured over a further 240 min. These test were carried out two weeks after the spontaneous motor activity and suprathreshold L-Dopa tests. Values represent means ± SD of 10 mice. $^{A}p < 0.01$, compared to respective postnatal Vehicle groups, Tukey HSD-testing, $^{B}p < 0.01$, $^{b}p < 0.05$ compared to respective acute Vehicle group, Tukey HSD-testing, $^{C}p < 0.01$, compared to respective DSP4 or MPTP group, Tukey HSD-testing

reduced significantly in mice administered postnatal iron. Pretreatment with DSP4 reduced markedly the restorative effects of the clonidine + L-Dopa combination in mice treated postnatal with vehicle, and abolished completely the restorative effect in mice treated postnatal with iron. Postnatal iron administration reduced the motor activity of DSP4-treated mice, following clonidine + L-Dopa, in comparison with both the Vehicle-DSP4 group and the Iron-Vehicle group strongly implicating noradrenergic modulation of DA functioning and DA-precursor mobilization.

Thus, one-way ANOVA indicated significant Groups effects for both Locomotion: $F(7, 72) = 67.38$, Rearing: $F(7, 72) = 40.73$, and Total activity: $F(7, 72) = 39.64$. Figure 3 presents the mean locomotion, rearing and total activity counts by postnatal iron-treated or vehicle-treated mice that were administered either DSP4 or vehicle at 63 days of age, followed by either MPTP or vehicle three weeks later; all the mice were co-administered clonidine with a subthreshold dose of L-Dopa acutely, following a 60-min habituation to the test chambers.

Tukey HSD-testing indicated the following pairwise differences:

Locomotion:
Vehicle: Veh-Veh, DSP4-Veh > Veh-MPTP > DSP4-MPTP
Iron: Veh-Veh > Veh-MPTP, DSP4-Veh > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

Rearing:
Vehicle: Veh-Veh, Veh-MPTP, DSP4-Veh > DSP4-MPTP
Iron: Veh-Veh, Veh-MPTP > DSP4-Veh > DSP4-MPTP
For DSP4-Veh and DSP4-MPTP: Veh > Fe

Total activity:
Vehicle: Veh-Veh, DSP4-Veh > Veh-MPTP > DSP4-MPTP
Iron: Veh-Veh > Veh-MPTP, DSP4-Veh > DSP4-MPTP
For Veh-Veh, DSP4-Veh, Veh-MPTP and DSP4-MPTP: Veh > Fe

Table 1. *Total brain iron in the frontal cortex and basal ganglia (μg/g wet weight) of C57 Bl6 mice following postnatal exposure to 7.5 mg Fe^{2+}/kg on days 10–12 after birth, followed by pretreatment with DSP4 (50 mg/kg, 30 min after injection of zimeldine, 20 mg/kg) or vehicle at 63 days of age, followed thereafter by MPTP (2 × 20 mg/kg, with a 24-hr interval between injections) or vehicle three weeks later. The mice were sacrificed one week after behavioural testing at about 126 days of age*

Groups	n	Frontal cortex	Basal ganglia
Postnatal vehicle			
Veh-Veh	10	25.14 ± 2.29	34.66 ± 4.98
Veh-MPTP	10	26.74 ± 2.77	38.05 ± 8.89
(%)		(106)	(108)
DSP4-Veh	10	25.92 ± 6.04	37.01 ± 5.27
(%)		(103)	(107)
DSP4-MPTP	10	26.97 ± 4.96	39.79 ± 7.11
(%)		(107)	(115)
Postnatal iron			
Veh-Veh	10	28.18 ± 2.66	59.27 ± 8.75*
(%)		(112)	(171)
Veh-MPTP	10	28.55 ± 6.43	64.17 ± 9.91*
(%)		(113)	(185)
DSP4-Veh	10	26.95 ± 6.83	60.93 ± 9.67•
(%)		(107)	(176)
DSP4-MPTP	10	30.01 ± 7.88	70.82 ± 8.24*,▲
(%)		(119)	(204)

*p < 0.01, versus postnatal vehicle Veh-Veh, Tukey HSD-testing.
▲p < 0.05, versus postnatal iron Veh-Veh, Tukey HSD-testing.
(%) = percent of postnatal vehicle Veh-Veh (control).

Analysis of total brain iron content

Postnatal administration of Fe^{2+} (7.5 mg/kg) increased significantly the levels of iron in the basal ganglia, but not frontal cortex, of the mice that were tested. Pretreatment with DSP4 followed by MPTP elevated the basal ganglia iron content of mice administered iron postnatal. Thus, one-way ANOVA indicated significant Groups effects for

Table 2. *Frontal cortex NA and Striatal DA concentrations in C57 BL6 mice that were administered postnatal iron (7.5 mg Fe^{2+}/kg on days 10–12 after birth) or vehicle, followed by pretreatment with DSP4 (50 mg/kg, 30 min after injection of zimeldine, 20 mg/kg) or vehicle at 63 days of age, followed thereafter by MPTP (2 × 20 mg/kg, with a 24-hr interval between injections) or vehicle three weeks later. The mice were sacrificed about 126 days of age in a separate experiment. Values are expressed as ng/g wet weight of tissue and represent means ± SD of 10 mice*

Groups	n	Frontal cortex Noradrenaline	Striatum Dopamine
Postnatal vehicle			
Veh-Veh	10	508 ± 102	14539 ± 1326
Veh-MPTP	10	498 ± 202	7541 ± 1297*
(%)		(98)	(52)
DSP4-Veh	10	48 ± 45	13996 ± 1574
(%)		(9)	(96)
DSP4-MPTP	10	65 ± 29	3655 ± 1711•
(%)		(13)	(25)
Postnatal iron			
Veh-Veh	10	531 ± 212	13795 ± 1433
(%)		(105)	(95)
Veh-MPTP	10	528 ± 196	5108 ± 1316▲
(%)		(104)	(39)
DSP4-Veh	10	56 ± 36	14603 ± 1487
(%)		(11)	(100)
DSP4-MPTP	10	51 ± 32	1636 ± 547■
(%)		(10)	(11)

*p < 0.01, versus postnatal vehicle Veh-Veh, Tukey HSD-testing.
•p < 0.01, versus postnatal vehicle Veh-MPTP, Tukey HSD-testing.
▲p < 0.05, versus postnatal iron Veh-Veh, Tukey HSD-testing.
■p < 0.05, versus postnatal iron Veh-MPTP, Tukey HSD-testing.
(%) = percent of postnatal vehicle Veh-Veh (control).

total iron content in the basal ganglia: $F(7, 72) = 11.46$. Table 1 presents the iron content in frontal cortex and basal ganglia of mice treated postnatal with either vehicle or iron, followed by DSP4 at adult age and then MPTP.

Neurochemical analysis

Administration of DSP4, at 63 days of age, induced marked depletions of NA in the frontal cortex (see Table 2). The administration of MPTP (2×20 or 2×40 mg/kg), three weeks later, induced marked DA depletions in the striatum. In mice that were injected previous with DSP4, the loss of DA in the striatum was more marked. Postnatal administration of iron exacerbated the MPTP-induced DA depletion, both in Veh and DSP4 pretreated animals.

Discussion

The results described above may be summarized as follows:

(1) Postnatal iron treatment induced a marked hypokinesia over the whole 40-min test period measuring spontaneous motor behaviour. Pretreatment with DSP4, at adult age, exacerbated the hypokinesia induced by MPTP, low dose (20 mg/kg), in mice treated postnatally with vehicle. In mice that received postnatal iron treatment, pretreatment with DSP4 abolished motor behaviour in MPTP-treated mice. Postnatal iron treatment, exacerbated the hypokinesia induced by MPTP, low dose (20 mg/kg), in the vehicle (non-DSP4) treated mice. (2) Following a 60-min habituation to the test chambers, acute administration of suprathreshold L-Dopa (20 mg/kg) restored the motor activity of MPTP-treated mice over all three parameters in the vehicle-treated animals. Pretreatment with DSP4 caused a deficit in the restorative effects upon motor behaviour, induced by the L-dopa treatment, in the MPTP-treated mice. Postnatal iron administration also caused a deficit in the restorative effects upon motor behaviour, induced by the L-dopa treatment, in the MPTP-treated mice. Notably, postnatal iron followed at adult age by DSP4 pretreatment virtually abolished the L-Dopa restorative effects. (3) Later, following the 60-min habituation to the test chambers once again, co-administration of clonidine (1 mg/kg) with a subthreshold dose (5 mg/kg) of L-Dopa caused a marked, but not complete, restoration of motor behaviour in MPTP-treated mice; this restorative effect of the combination was reduced significantly in mice administered postnatal iron. Pretreatment with DSP4 reduced markedly the restorative effects of the clonidine + L-Dopa combination in mice treated postnatal with vehicle, and abolished completely the restorative effect in mice treated postnatal with iron. Postnatal iron administration reduced the motor activity of DSP4-treated mice, following clonidine + L-Dopa, in comparison with both the Vehicle-DSP4 group and the Iron-Vehicle group, strongly implicating noradrenergic modulation of DA functioning and DA-precursor mobilization. (4) With regard to the restorative effects of clonidine + L-Dopa, postnatal iron disrupted significantly this effect for rearing behaviour in DSP4-treated, but not MPTP-treated, mice which both implicates further the noradrenergic role in iron-dopaminergic interactions and provides a valuable indication of denervation selectivity of action. (5) DSP4 treatment, by itself, did not affect the outcome of either postnatal iron or vehicle for motor activity, nor did DSP4, by itself, alter the response to the DA precursor, neither in postnatal iron nor vehicle treated mice. (6) Postnatal administration of Fe^{2+} (7.5 mg/kg) increased significantly the levels of iron in the basal ganglia, but not frontal cortex, of the mice that were tested. Pretreatment with DSP4 followed by MPTP elevated the basal ganglia iron content of mice administered iron postnatal.

Pretreatment with DSP4 before inducing MPTP neurotoxicity has been found to exacerbate the effects of the DA neurotoxin (Fornai et al., 1997; Marien et al., 1993). Marien et al. (1993) obtained a severe exacerbation of MPTP-induced striatal DA depletion. They pretreated MPTP (4×10 mg/kg, over 8 hrs) C57 Bl/6 mice with DSP4 (40 mg/kg); it was found that the MPTP treatment induced partial (40%) striatal DA loss at 7 days post-drug and DSP4 pretreatment, while not affecting DA levels by itself, exacerbated the DA deficit to 60%. Fornai et al. (1997) demonstrated that the lesioning of NA terminals with DSP4 (50 mg/kg) must occur before MPTP (30 mg/kg) administration in order to enhance the MPTP toxicity. Both the Marien et al. (1993) and the Fornai et al. (1997) studies identify a protective role for NA neurons upon the nigrostriatal pathway. By the former notion, damage to the locus coeruleus noradrenergic system, by removing a facilitatory influence on the nigrostriatal DA system, interferes with the ability of the nigrostriatal pathway to compensate for or recover from injury. In the latter, it is suggested that in NA-depleted mice a more pronounced neuronal sensitivity of DA neurons to MPTP has occurred, thereby underlining the acute protective influence of NA neurons. The present findings reinforce these notions at three levels: DSP4 pretreatment (i) exacerbated the MPTP-induced spontaneous behaviour deficits, (ii) interfered with the restorative effects of acute L-Dopa, and (iii) increased DA neuron sensitivity to MPTP, at both concentrations of the neurotoxin. In the recent study (Archer and Fredriksson, under review), it was found that DSP4 pretreatment

(50 mg/kg) enhanced the depletion of striatal DA at both the low (2×20 mg/kg) and the high (2×40 mg/kg) dose regimes of MPTP in C57 Bl6 mice. This enhancement of DA-loss was accompanied by an enhancement of the bradykinesic effect of MPTP, at both dose levels, as expressed by spontaneous motor behaviour over all three parameters of activity. In the present study utilising the lower (2×20 mg/kg) dose of MPTP, the enhanced bradykinesia, due to the DSP4 pretreatment, was confirmed in mice administered vehicle postnatal; the postnatal administration of iron reduced markedly, not only motor activity in Veh-Veh and DSP4-Veh mice, but that of Veh-MPTP mice even more severely, and abolished virtually all behavior of the DSP4-MPTP mice during 40 min of testing.

Nishi et al. (1991) treated 7-weekold C57 black mice, in four groups, as follows: (i) MPTP (30 mg/kg/day over 10 consecutive days), (ii) MPTP (as for the 1st group) + DSP4 (50 mg/kg, administered on the final day of MPTP treatment), (iii) DSP4 (50 mg/kg, administered once of the 10th day, and (iv) Vehicle (administered over 10 consecutive days). Spontaneous and L-Dopa induced locomotor activity was measured 7–10 days after the 10-consecutive-day treatment with MPTP-DSP4, MPTP, DSP4, vehicle. Although they obtained a marked reduction in spontaneous locomotor activity within 1–2 days after cessation of neurotoxin treatment by 7–10 days there were no significant differences between the groups. From about 30 min after L-Dopa (200 mg/kg) + DCI (25 mg/kg) treatment, there was a marked rise in the activity of MPTP-treated mice; this effect of the DA precursors was severely attenuated in the mice administered MPTP + DSP4, with a much lower peak effect (about 35% of the MPTP peak) and much lesser duration (about 60% of the MPTP duration). Loss of striatal dopamine was 82% in the case of the MPTP mice and 85% in the case of the MPTP + DSP4 mice. In the recent study (Archer and Fredriksson, under review), it was found that DSP4 pretreatment (50 mg/kg) disrupted markedly the restorative effects of a suprathreshold dose of L-Dopa (20 mg/kg) upon locomotion and locomotion at the low (2×20 mg/kg) dose of MPTP; DSP4 severely disrupted the restorative of L-Dopa upon all three parameters of activity at the high (2×40 mg/kg) dose of MPTP. In the present study with the lower MPTP dose, the postnatal administration of iron produced enduring effect in this respect: (i) suprathreshold L-Dopa failed to restore the motor activity of the MPTP mice (see Fig. 2, Iron-Veh-MPTP versus Vehicle-Veh-MPTP, and (ii) the effects of suprathreshold L-Dopa were severely disrupted, both in comparison with mice administered vehicle postnatal (see Fig. 2, Iron-DSP4-MPTP versus Vehicle-DSP4-MPTP) and with iron administered mice that only received MPTP (see Fig. 2, Iron-DSP4-MPTP versus Iron-Veh-MPTP).

Recently, it was argued that deficits in the noradrenergic system would contribute critically to the progression of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease (Marien et al., 2004). Thus, alterations of LC-noradrenergic functioning were shown to affect electrophysiological, neurochemical and behavioural expressions of neurotransmission in the nigrostriatal DA pathway and to influence the effects of experimental lesions (ibid).

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