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# Neurochemical and behavioural changes in zebrafish *Danio rerio* after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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**Abstract**

Dopaminergic deficiency in the brain of zebrafish was produced by systemic administration of two catecholaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and the neurochemical and behavioural changes were characterized. The levels of dopamine and noradrenaline decreased significantly after the injection of MPTP and 6-OHDA. Corresponding to these changes, fish exhibited characteristic changes in locomotor behaviour, i.e. the total distance moved and velocity decreased after both neurotoxins. Tyrosine hydroxylase and caspase 3 protein levels were not altered after MPTP or 6-OHDA injections, as studied by immunohistochemistry and western blotting. The catecholaminergic cell clusters suggested to correspond to the mammalian nigrostriatal cell group displayed normal tyrosine hydroxylase immunoreactivity after the toxin treatment and did not show signs of DNA fragmentation that would indicate activation of cascades that lead to cell death. The results show that single systemic injections of MPTP and 6-OHDA induce both biochemical and behavioural changes in zebrafish, albeit failing to produce any significant morphological alteration in catecholaminergic cell clusters at the tested doses. This approach may be used for the screening of chemicals affecting the dopaminergic system. The model may be especially useful for evaluation of the role of novel genes in neurotoxicity, as a large number of zebrafish mutants are becoming available.

**Keywords:** behaviour, dopamine, histamine, noradrenaline.

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Dopaminergic deficiency may be produced in brains of experimental animals by central or systemic administration of specific dopaminergic neurotoxins, among which 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are most commonly used (reviewed recently in Beal 2001). Administration of 6-OHDA produces severe damage of mesencephalic catecholaminergic neurones in the brain of rats (Perese *et al*. 1989), while MPTP most efficiently affects the central dopaminergic system of mice (Heikkila and Sonsalla 1987). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is used to produce a syndrome mimicking Parkinson's disease in primates (Burns *et al*. 1983). In humans, both occupational (Langston and Ballard 1983) and accidental (Langston *et al*. 1983) introduction of MPTP have resulted in clinical and pathological characteristics of Parkinson's disease. There are also reports of an MPTP-induced parkinsonian–like syndrome in goldfish (Poli *et al*. 1990; Pollard *et al*. 1992).

The mechanisms of action of the two neurotoxins are different. An oxidative metabolite of MPTP, 1-methyl-4-phenylpyridine, created by monoamine oxidase B (MAO-B), is taken up via the dopamine (DA) transporter (DAT) into DA neurones where it leads to the increased generation of reactive oxygen species (Tipton and Singer 1993; Chan *et al*.

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*Abbreviations used*: DA, dopamine; DAT, DA transporter; MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; RT, room temperature; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

 

1994) causing destruction of dopaminergic cells in the A9 and A10 cellular groups in mice (Przedborski and Jackson-Lewis 1998). The reactive structural analogue of DA, 6-OHDA, accumulates within catecholaminergic terminals after specific uptake by the DA (or noradrenaline) transporter and causes selective damage of all catecholaminergic neurones (Reader and Dewar 1999).

In this study, we introduced MPTP and 6-OHDA to zebrafish (*Danio rerio*). Zebrafish provides an excellent system to study the development of different neurotransmitter systems as it is easy to visualize the development of the transparent embryos. Also, large-scale methods of mutation screening are available, allowing the identification of the genes crucially important for development (Driever *et al*. 1996; Haffter *et al*. 1996). The cost efficiency of zebrafish breeding and maintenance also makes this model organism lucrative for large-scale drug testing.

The catecholaminergic systems in zebrafish brain have been recently described (Ma 1994, 1997; Kaslin and Panula 2001; Rink and Wullimann 2001). The dopaminergic system in the zebrafish brain interacts extensively with other neurotransmitter systems, especially the serotoninergic and histaminergic (Kaslin and Panula 2001) systems. Goldfish (*Carassius auratus*) have been used to induce a parkinsonian–like syndrome by MPTP injections (Pollard *et al*. 1992). So far, adult zebrafish have not been utilized extensively for neurotoxicological studies and little is known about the effects of neurotoxins on brain neurochemistry and fish behaviour.

The aim of this study was to examine whether zebrafish exhibit the neurochemical and behavioural alterations seen in rodents and humans after introduction of MPTP and 6-OHDA, associated with acute DA deficiency. As brain histamine was reported earlier to be elevated in Parkinson's disease (Anichtchik *et al*. 2000; Rinne *et al*. 2002), we also examined the histaminergic system of zebrafish brain after MPTP or 6-OHDA administration.

## Materials and methods

### Animals

Both outbred and AB strains of zebrafish (*D. rerio*) were used in this study. The fish of both sexes were kept in aged tap water at 28°C under a 14 : 10 h light : dark cycle. Feeding and maintenance of fish were done according to Westerfield (1995). Appropriate permits for the experiments were obtained from Åbo Akademi University committee for animal experiments and the Office of the Regional Government of western Finland in agreement with the ethical guidelines of the European Convention.

Experimental groups of fish were selected and kept in an aquarium at ambient temperature for at 3–7 days prior to injections.

### Injection of dopaminergic neurotoxins

A minimum of four fish for each time-point and dose were used. Before injection, fish were anaesthetized in water containing benzocaine (0.05–0.1 mg/mL) until calm. In initial pilot experiments fish were injected with different doses of MPTP hydrochloride (20, 40 and 80 mg/kg; RBI, Natick, MA, USA) or 6-OHDA hydrobromide (25, 50 and 100 mg/kg; Sigma, St Louis, MO, USA) i.m. using Hamilton syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland). Control fish were injected with similar volumes of 0.9% NaCl. The volume of injections was 4–7 μL, depending on the weight of the fish (0.5–0.7 g). After injections, fish were kept in an extensively aerated water tank until they recovered from the anaesthesia. All fish were then kept under equal conditions.

We used four time-points (1, 3, 6 and 9 days after injections) for HPLC measurement and consecutive behavioural analysis for 6-OHDA. Five time-points (1, 2, 5, 7 and 12 days after injection) were used for HPLC measurements of histamine and catecholamines after MPTP injections. These time-points were selected on the basis of pilot studies.

### Behavioural analysis

Before injections and at various time-points after the injection, fish behaviour was analysed using a digital video tracking system. The system consists of a digital video camera connected to a computer system running the analysis software EthoVision Pro 2.3 (Noldus Information Technology, Wageningen, the Netherlands). The system allowed individual simultaneous tracking of the swimming performance of six fish located in separate round water tanks with an inner diameter of 22 cm and a water depth of 8 cm (volume 3 L). The water depth of 8 cm was chosen as a compromise between restrictions of the vertical movement and maintenance of a water level best tolerated by the fish (low stress). The experiments were performed in a calm sealed area and the variation between temperatures in the aquaria and the test tanks was less than 2°C.

Before the injections, the locomotor activity of the fish was analysed for 5 min and about 15% of the fish were discarded due to signs of an excessive stress reaction to the handling and monitoring of the behaviour (such as rapid and disorganized swimming or immobility for 5 min or more).

After injections, locomotor activity was studied on the first, third, sixth and ninth day of the experiment (24, 72, 144 and 216 h after injections, respectively). Fish were allowed to accommodate to the environment of the tank for about 1 min before the start of the recording.

The swimming pattern of each fish was recorded for 10 min using a charge coupled device (CCD) camera at a frame rate of 8.33 samples/s. The total distance moved, mean velocity and turning angle were calculated from the acquired coordinates. The total distance moved was defined as the distance that the fish moved during one session (10 min) in centimetres. The mean velocity was calculated by dividing the total distance (cm) by the total session time (s). These two values characterize the general locomotor activity of the fish. Additionally, we analysed locomotor path and pattern complexity parameters, such as the mean turning angle and place preference in the tank. The turning angle measures the change in the direction of movement between two samples of the recording. For place preference in the tank, the tracking arena was divided digitally into two zones, an inner zone (IN zone) placed in the centre of the tank (16 cm diameter) and an outer zone (OUT zone) consisting of the area between the walls of the tank and the IN zone (radius 3 cm). The total time spent by each fish in these zones was recorded.

### HPLC measurements

Dopamine and noradrenaline concentrations in individual zebrafish brains were analysed using HPLC with coulometric electrochemical detection. Authentic reference standards and the internal standard used in the analysis, dihydroxybenzylamine, were from Sigma. Brains were homogenized in 200 μL of 0.1 M perchloric acid containing 0.05% $Na_2EDTA$ and 0.1% sodium metabisulphite. After extraction with activated alumina, sample components were separated on a reversed-phase C18 column (Ultrasphere ODS, 4.6 × 250 mm; Beckman Instruments, Fullerton, CA, USA) using a mixture of methanol (5%, v/v) and phosphate buffer [pH approx. 3.15 at room temperature (RT)] as mobile phase and heptane sulphonate (Fluka, Buchs SG, Switzerland) as ion-pairing reagent. Detection was accomplished using the oxidation–reduction mode of a high-sensitivity analytical cell coupled to a Coulochem II detector (ESA Inc., Bedford, MA, USA). The detection limit was less than 0.1 nmol/g of original tissue.

To analyse the histamine content, dissected brains were weighed and homogenized in 10 volumes of 2% perchloric acid and analysed using HPLC with post-column derivatization and fluorimetric detection according to the method of Yamatodani *et al*. (1985), as described in detail elsewhere (Eriksson *et al*. 1998). The detection limit was about 10 pmol/g of original tissue.

### Immunohistochemical procedures

For immunohistochemical procedures, fish were killed in ice-cold water, the brains were removed under a preparation microscope and immersed in 2% paraformaldehyde in 0.1 M phosphate buffer or, for histamine (HA) immunohistochemistry, in 2% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (Sigma) and 0.4% *n*-hydroxy-succinimide (Sigma) dissolved in 0.1 M phosphate buffer (pH 7.4) for up to 24 h at 4°C. Cryoprotection was achieved by transferring the brains to 20% sucrose in 0.1 M phosphate buffer at 4°C for about 15 h, until the pieces sank. Subsequently, brains were quickly frozen, embedded in M-1 embedding matrix (Lipshaw, Pittsburg, PA, USA) and cut in a cryostat at 20 μm. Sections were collected on gelatine-coated slides, air-dried for 1 h and stored at −20°C until use.

For the staining procedures, slides with zebrafish brain sections were thawed and left at RT for about 30 min. They were then washed in phosphate-buffered saline (PBS), pH 7.4, containing 0.25% Triton X-100 (pH 7.4) and incubated with primary antisera and 2% normal goat serum for 12 h at 4°C. They were then washed twice with PBS containing 0.25% Triton X-100 and incubated with secondary antibodies, goat anti-mouse or goat anti-rabbit Alexa-conjugated (488, 568) IgG, according to primary antibody specificity for 1 h at RT. Slides were then washed with PBS and mounted with PBS-glycerol (1 : 1).

Mouse monoclonal anti-tyrosine hydroxylase (TH) antibodies were from Diasorin (Stillwater, MN, USA) and rabbit anti-caspase 3 antibodies were from Sigma. The specificity and preparation of anti-histamine antibodies were described previously (Panula *et al*. 1984, 1990). The specificity of the histamine and TH antisera in zebrafish brain has been described previously (Kaslin and Panula 2001). The histamine antiserum does not detect L-histidine or histidine-containing peptides. Controls of the immunohistochemical staining in this study consisted of incubations of consecutive sections in solutions without the primary or secondary antibodies or replacing the primary antibodies with respective normal sera from the same species. No immunostaining was detected in these cases.

### TUNEL staining

In order to detect fragmented DNA as a sign of cell damage, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining on fish brains taken 1 and 3 days after saline or toxin injections (four individuals/group). The TUNEL staining was performed according to the manufacturer's protocol with minor modifications (*In situ* Cell Death Detection Kit, POD; Roche Diagnostics, GmbH, Mannheim, Germany). Briefly, fixed brains (2% paraformaldehyde) from three fish treated with 6-OHDA (20 mg/kg) 1 day after injection and three fish 3 days after injection, three fish 1 day after MPTP injection (25 mg/kg) and three similar fish 3 days after injection were cryosectioned at 20 μm thickness and the sections were collected on gelatin-coated slides. The slides were briefly washed in PBS. Endogenous peroxidase activity was blocked by incubation in methanol and 0.3% $H_2O_2$ for 15 min at RT, followed by brief washes in PBS and permeabilization with ice-cold buffer (freshly prepared) containing 0.1% Triton X-100 and 0.1% sodium citrate in distilled water for 3 min. The slides were washed in PBS and then incubated for 90 min at 37°C with the TUNEL reaction mix containing the terminal deoxynucleotide transferase (TdT) enzyme and fluorescein-labelled dUTPs. The slides were washed in PBS several times and incubated with peroxidase-labelled sheep anti-fluorescein Fab fragments for 2 h at RT. The slides were washed in PBS and the colour reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (2.5 mg/mL), $NiSO_4$ (3 mg/mL) and 0.3% $H_2O_2$ in 0.1 M Tris-buffered saline (pH 7.5). After the TUNEL staining, the slides were further processed for TH immunohistochemistry according to the protocol described above, but this time using an ABC-kit (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine tetrahydrochloride colour reaction for detection. The sections were dehydrated and mounted with Permount (Fisher Chemical, Fair Lawn, NJ, USA). Positive and negative controls were always included and processed in parallel with the samples. In the negative control the sections were treated normally except for the omission of the TdT enzyme from the labelling step. No staining was detected in the negative controls. Positive controls were done by induction of DNA fragmentation with Dnase. The positive control slides were incubated with 6 U/mL Dnase I (grade I, RNase free; Roche Diagnostics) and bovine serum albumin (1 mg/mL, high grade) in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ for 10 min at RT. In the positive controls virtually all Dnase-exposed cells showed DNA fragmentation and intense nuclear staining.

### Western blotting

Brains were homogenized in 10 mM $K_2HPO_4$ buffer, containing 1 mM EDTA (pH 7.4) and a cocktail of protease inhibitors (Complete Mini; Roche Diagnostics), according to the manufacturer's instructions. Homogenates were diluted in sample buffer (1 : 1 with 3× sodium dodecyl sulphate, 200 mM Tris, 6% sodium dodecyl sulphate, 15% glycerol and bromophenol blue). Proteins were separated by 9% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, pre-activated with methanol, using a Transblot cell (Bio-Rad, Richmond, CA, USA). Membranes were saturated with 2% skimmed milk in PBS,

containing 0.3% Triton X-100 and 0.05% Tween, for about 1 h at RT under constant agitation to prevent non-specific binding. Primary antibodies, dissolved in PBS containing 0.3% Triton X-100 and 0.05% Tween (1 : 1000), were applied for 24 h at 4°C followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 5000; DAKO, Glostrup, A/S Denmark). The results of polyacrylamide gel electrophoresis were visualized using the ECL Western Blotting detection system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's recommendations.

The amount of the protein in the brain homogenates was determined by the Bradford method (Bio-Rad).

### Image analysis and statistical procedures

The results of immunohistochemical staining were examined with a DM RXA epifluorescence microscope and TCS SP confocal system (both from Leica Microsystems, Wetzlar, Germany). To minimize cross-talk and overlapping between the green (Alexa 488) and red (Alexa 568) channels in double-staining experiments, images were acquired separately. The fluorophores were excited with the 488- and 568-nm lines from an argon–krypton laser (Omnichrome; Melles Griot, Carlsbad, CA, USA).

Where statistical evaluation was required, the differences between groups were analysed using a two-tailed Mann–Whitney statistical test using GraphPad Prism 2.01 software (GraphPad Software, Inc., San Diego, CA, USA).

## Results

### Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxydopamine on brain catecholamines and histamine

The concentrations of DA and noradrenaline in zebrafish brain were analysed after systemic (i.m.) administration of the dopaminergic neurotoxins MPTP and 6-OHDA (Fig. 1). In pilot experiments, several doses were tested. The lowest dose used (MPTP, 20 mg/mg and 6-OHDA, 25 mg/mg) induced almost maximal decreases in brain catecholamines (data not shown) and all subsequent studies were done with these low doses.

The concentration of DA in control zebrafish brains in our study was 2.09 ± 0.42 nmol/g and the corresponding concentration of noradrenaline was 4.53 ± 0.97 nmol/g. Twenty-four hours after injection of 20 mg/kg of MPTP, the concentration of DA was decreased by about 40%, as compared with saline-treated fish, while the concentration of noradrenaline was decreased by 30% (Fig. 1a). The maximal reduction in DA concentrations was observed 2 days after MPTP injections, when only 28% of the control DA level remained in the brains of lesioned fish. As late as 12 days after the MPTP injection, we still observed a 30% decrease in DA concentrations. Levels of noradrenaline changed more dramatically, being as low as 15% of the control levels after 2 days and 30% on the 12th day after MPTP injection (Fig. 1a).

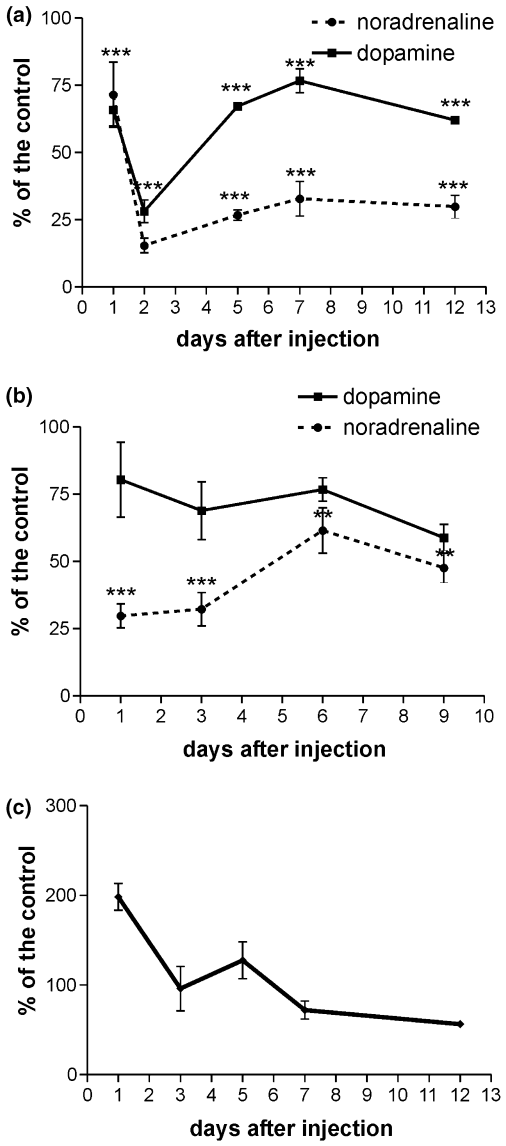


**Fig. 1** Dopamine and noradrenaline concentrations in the brains of zebrafish, injected with (a) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20 mg/kg) and (b) 6-hydroxydopamine (25 mg/kg), at different time-points after the injection. (c) The concentrations of histamine, as measured from the whole brain homogenates after different time-points after MPTP injections (20 mg/kg). All values are presented as a percentage of the control value; the control values are considered as 100% and not shown. $^{**}p < 0.01$, $^{***}p < 0.001$

The 6-OHDA injections also produced marked decreases in catecholamine concentrations. At 9 days after 6-OHDA administration (25 mg/kg), the concentration of DA was decreased to 59% of control values and noradrenaline was decreased to 47% (Fig. 1b). Again, levels of noradrenaline decreased more, being only 30% of the control level on the first and third days after the injection and 50% of the control level on the ninth day after injection.

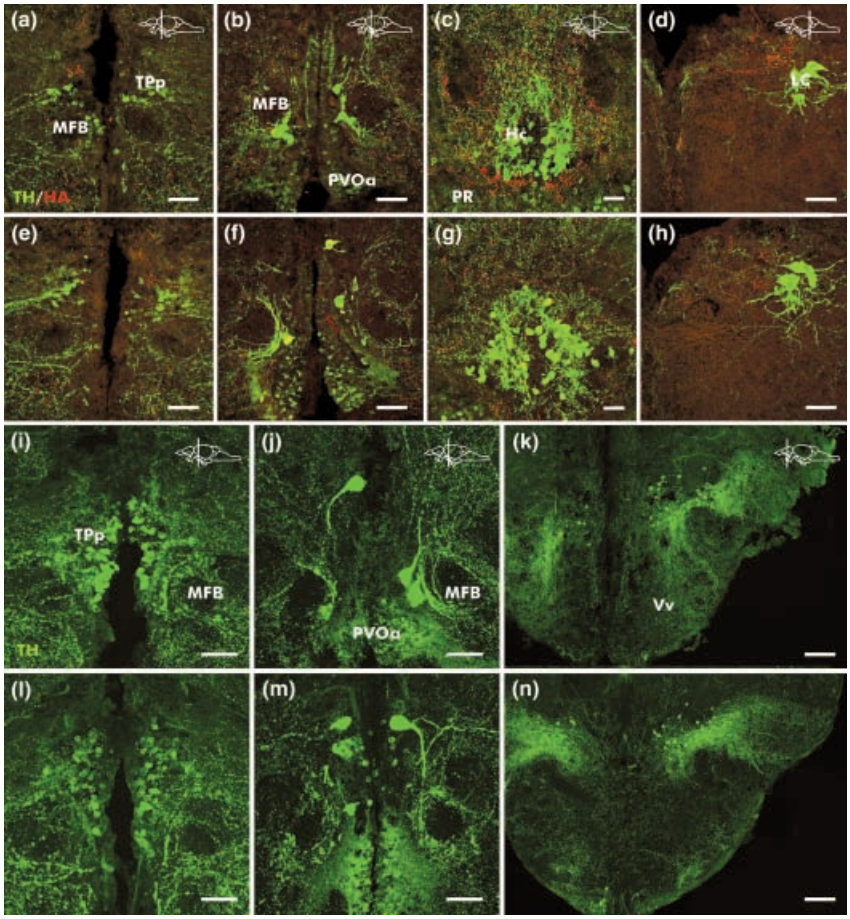


**Fig. 2** The distribution of tyrosine hydroxylase (TH) and HA in neurones and fibres in some of the proposed ascending dopaminergic clusters in saline-injected and toxin-treated fish. Coronal sections taken from different rostro-caudal levels showing the localization of TH immunoreactivity (green) and HA immunoreactivity (red) in saline-injected fish (a–d) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-injected fish (e–h; 3 days after injection of 20 mg/kg). No significant differences were seen in the distribution of TH or HA immunoreactivity between saline- and MPTP-treated fish. Coronal sections from saline-injected fish (i–k) and 6-hydroxydopamine (6-OHDA)-injected fish (l–n; 3 days after injection of 50 mg/kg) showing the localization of TH immunoreactivity (green) in different brain areas. No major differences were seen in the distribution of TH immunoreactivity in saline- or 6-OHDA-injected fish. Scale bars, 50 μm in a–j, l and m and 100 μm in k and n. MFB, medial forebrain bundle; TPp, periventricular nucleus of posterior tuberculum; PVOa, anterior part of the paraventricular organ; PR, posterior recess of diencephalic ventricle; Vv, ventral nucleus of the ventral telencephalic area.

The concentration of histamine was increased at 24 h after the MPTP injections but the subsequent decreasing trend at the other time-points studied was not significantly different from control values (Fig. 1c). Immunohistochemically, histamine-containing fibres and nerve cells were similarly distributed in hypothalamic and extrahypothalamic areas and no significant morphological alterations were detected after MPTP (Fig. 2).

### Effects of toxins on tyrosine hydroxylase levels

We examined the localization of the catecholamine-producing enzyme TH by immunohistochemistry. Tyrosine hydroxylase-positive neurones were distributed throughout the brain in distinct clusters, proposed to correspond to the mammalian nigro-striatal system (diencephalic clusters within or in the vicinity of the posterior tuberculum and telencephalic clusters), as recently described (Kaslin and Panula 2001; Rink and Wullimann 2001). Unexpectedly, we did not detect any significant changes in the distribution and/or shape of TH-positive neurones and fibres in the brains of lesioned zebrafish (Fig. 2). Occasionally, a slight decrease of immunoreactivity was detected in the diencephalic clusters and the locus coeruleus of 6-OHDA-treated fish. No significant changes were seen in any of the other catecholaminergic clusters. In accordance with the immunohistochemical data, we did not detect any significant change in total TH protein levels with western blotting of the proteins from MPTP/6-OHDA-lesioned brains and saline-treated fish (Figs 3a and b).

### TUNEL staining and levels of caspase 3 in lesioned zebrafish brains

We performed TUNEL staining to detect fragmented DNA, a process known to occur in injured cells that undergo apoptosis, necrosis or DNA repair. TUNEL staining in combination with immunohistochemistry of TH was done on brain sections from 1 and 3 days after saline and MPTP or 6-OHDA injections. No staining was detected in the negative controls and non-injected fish displayed a similar staining pattern to the saline-injected groups. In the positive controls virtually all Dnase-exposed cells showed DNA fragmentation and intense nuclear staining. Very little or no TUNEL staining of nuclei was detected in the saline-injected groups (1 and 3 days after injection). Cells associated with the brain membranes and in close proximity to the ventricular walls showed TUNEL-positive staining

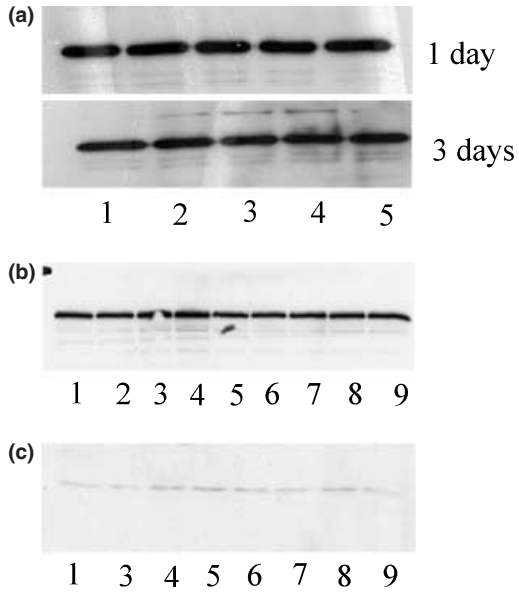


**Fig. 3** (a) Western blotting of zebrafish brain homogenates, probed with anti-tyrosine hydroxylase (TH) antibodies on first and third day after the injections of 40 mg/kg (2) and 80 mg/kg (3) of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 50 mg/kg (4) and 100 mg/kg (5) of 6-hydroxydopamine (6-OHDA). Lane 1 is the saline control-injected fish. Western blotting of zebrafish brain homogenates, probed with anti-TH antibodies (b) and with total caspase 3 antibodies (c) after injection of 25 mg/kg of 6-OHDA on the first day (1–3), third day (4–6) and sixth day (7–9) after the injection. 1, 4 and 7 are saline control-injected fish and 2, 3, 5, 6, 8 and 9 are 6-OHDA-injected fish. Each lane contains 15 mg/mL of the protein.

(Fig. 4a). There was no difference in distribution of the TUNEL-positive cells in the toxin-injected compared with the saline-injected fish groups 1 or 3 days after injection and we did not find TUNEL-stained nuclei in any TH-immunopositive cells (Figs 4b–g).

We examined levels of caspase 3 in the blots of MPTP/6-OHDA-lesioned zebrafish brains. The antibody, raised against total rabbit caspase 3, detected a single band of the expected molecular weight (about 30 kDa) in samples from both lesioned and control zebrafish brains (Fig. 3c). The size and intensity of the bands were not different between the groups. Similarly, there were no significant differences in immunohistochemical localization of caspase 3 in brains of lesioned and control fish (data not shown).

### Changes in locomotor activity after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxydopamine

Intramuscular injections of the dopaminergic neurotoxins MPTP and 6-OHDA produced marked alterations in the zebrafish swimming behaviour (Fig. 5). The total distance moved by lesioned fish was already decreased by 40% by 1 day after MPTP injections and fish swam 20% shorter total distance 9 days after injection. The mean velocity of MPTP-injected zebrafish was also decreased by 30% 24 h after the lesion and remained as low as 80% of control values on the ninth day after the lesion. The turn angle, calculated as a difference in degrees in direction changes, was almost doubled 1 day after MPTP injections, was increased by about

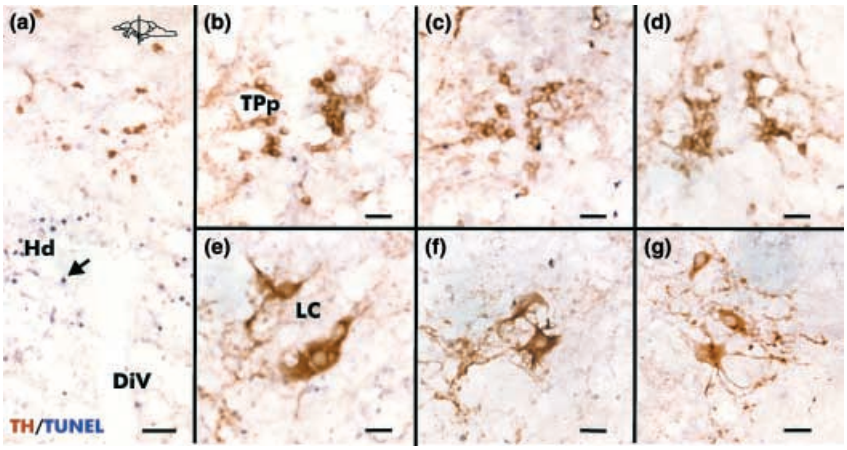


**Fig. 4** (a) Cross-section through hypothalamus from a saline-injected fish (3 days after injection) showing tyrosine hydroxylase (TH)-immunoreactive neurones (brown) and some TUNEL-positive nuclei (blue, arrow) in the hypothalamic periventricular cell layer. (b–d) Coronal sections through the rostral hypothalamus, showing TH-immunoreactive neurones in the periventricular nucleus of the posterior tuberculum. No TUNEL-positive nuclei were found among the TH-immunoreactive neurones in any of the injected groups 3 days after injection of saline (b), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (c) and 6-hydroxydopamine (6-OHDA) (d). (e and f) Coronal sections showing TH immunoreactivity of the noradrenergic locus coeruleus and lack of TUNEL-positive nuclei. (e) Saline-injected, (f) MPTP-injected and (g) 6-OHDA-injected fish (all 3 days after injection). Scale bar, 20 μm. Hd, dorsal zone of paraventricular hypothalamus; LC, locus coeruleus; TPp, periventricular nucleus of posterior tuberculum diencephalic ventricle (Div).

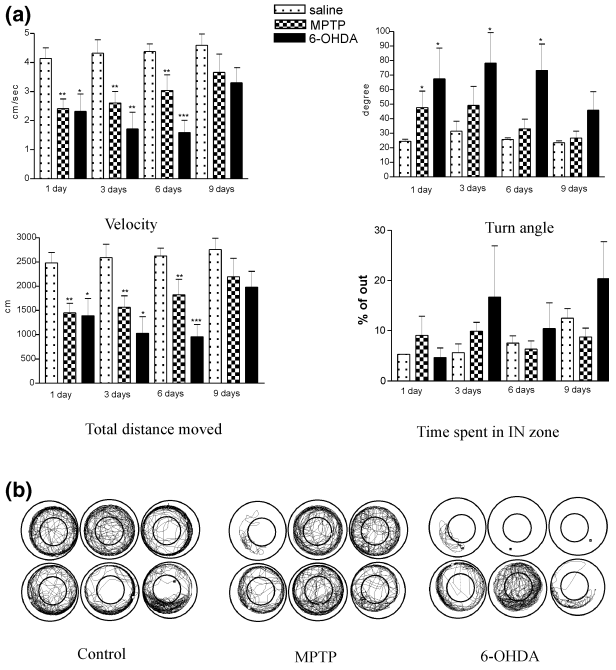


**Fig. 5** (a) Quantification of the swimming parameters of zebrafish, injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20 mg/kg) and 6-hydroxydopamine (6-OHDA) (25 mg/kg), at different time-points after the injection. (b) Graphical representation of the swimming patterns of the lesioned and control zebrafish. Large grey borders outline the edges of observation tanks and small grey borders separate IN zone from OUT zone. The black line represents the path of fish swimming during 10 min of the recording 3 days after the injections.

50% on the third day and was not significantly changed 9 days after the injection (Fig. 5a).

As with the catecholamine concentrations, 6-OHDA produced more pronounced changes in the studied locomotor parameters than MPTP. The total distance moved and velocity were decreased at all studied time-points after injections of 6-OHDA, being maximally decreased (up to 40% as compared with control fish) 6 days after the lesion. The turn angle was increased at all time-points.

After both MPTP and 6-OHDA injections, we observed a tendency to recovery of the behavioural alterations at the later time-points after the lesions.

Neurotoxin-injected zebrafish exhibited marked changes in their swimming patterns (Fig. 5b). While normal fish had a tendency to follow the walls of the observation tank and relatively seldom crossed the open area in the middle, lesioned fish appeared 'disoriented', often crossing the IN zone of the tank. They showed a tendency towards preference of the IN zone (Fig. 5a). However, there was considerable variability in this behaviour among the individual fish. Some fish displayed a special pattern, characterized by turning of the body or movement of a very short distance (two fish in Fig. 5b), resulting in short distances covered during the observation period. These movements did not leave a visible trace as the fish stayed on the same spot spatially but it was not immobile during the time course. The movements were easily seen in animations of the same fish swimming. This behaviour was clearly different from the immobility 'freezing' occasionally shown by stressed fish (not included in the experiments) without any drug treatment.

The administration of MPTP and especially 6-OHDA elicited a change in pigmentation that was readily detectable 1 day after the injections. The 6-OHDA-injected fish were much darker than the control and MPTP-injected fish. Fish started to regain their normal colour by day 6 after the injection.

## Discussion

In this study we report the development of catecholamine deficiency in the brain of zebrafish, obtained after single i.m. injections of the dopaminergic neurotoxins MPTP and 6-OHDA, which have been widely used for similar purposes in other experimental animals.

There are several earlier reports on effects of MPTP in goldfish (Pollard *et al.* 1992, 1996; Youdim *et al.* 1992). Pollard *et al.* (1992, 1996) reported a 72% decline in noradrenaline and a 57% decline in DA levels in goldfish brain on the fifth day after injection of 20 mg/kg of MPTP. They also reported the recovery of brain DA content after MPTP treatment. Recovery of catecholamine levels has also been reported in other species (Mori *et al.* 1988). In this study, we observed similar patterns of catecholamine changes in the zebrafish brain. Noradrenaline levels were affected more severely than DA after administration of MPTP or 6-OHDA. We detected the most profound decrease in DA and noradrenaline levels by 2 days after MPTP injection. It seems that the catecholamine systems of zebrafish are more susceptible to toxin-induced damage than those of goldfish at the doses used. The catecholamine levels also had a tendency to recover towards control values in our study and the same effect was reported in goldfish after 6-OHDA treatment (Contestabile *et al.* 1979)

Intramuscular injections of 6-OHDA produced significant decreases in the levels of brain catecholamines, indicating that, unlike in mammals, 6-OHDA penetrates the blood–brain barrier in zebrafish. This observation is in line with several studies indicating higher permeability of the blood–brain barrier in fish (Murray *et al.* 1975) as compared with mammals.

Differences in neuronal entry and metabolism could account for the differences in the effects of the neurotoxins that we observed. The crucial step for MPTP neurotoxicity is the conversion of MPTP into 1-methyl-4-phenylpyridine, which is considered to be carried out by MAO-B of proximate glia or neurones. The 1-methyl-4-phenylpyridine, actively or passively released from glia, enters DA neurones actively via the DAT (mechanisms reviewed in Speciale 2002). Both MPTP

and 1-methyl-4-phenylpyridine are rapidly metabolized in mice and, after a few hours, the concentration of both agents is below detectable levels (Markey *et al*. 1984). 6-Hydroxydopamine, in contrast to MPTP, directly enters the neuronal processes via the catecholaminergic transporters, employing its structural similarity with DA. Upon entry into the cell, both neurotoxins cause alteration of the cellular metabolism by participating in generation of reactive oxygen species and inhibition of the electron transport system of mitochondria (e.g. complex I) that leads to cell death either via apoptotic or necrotic mechanisms (Tipton and Singer 1993; Chan *et al*. 1994).

Very little is known about the function of DAT and MAO in zebrafish. The zebrafish DAT has been cloned and its distribution has been studied during development (Holzschuh *et al*. 2001). However, nothing is known about its distribution and function in the brain of adult fish. A MAO-related mechanism of MPTP metabolism was suggested for goldfish in previous studies (Poli *et al*. 1990). The zebrafish brain has a functionally active MAO enzyme but the subtype specificity is not yet clear (our unpublished data). Based on molecular and pharmacological data from several other fish species it seems likely that teleosts have only one form of MAO that is functionally similar to both of the mammalian isoforms, MAO-A and MAO-B (Nicotra and Senatori 1989; Obata *et al*. 1990; Adeyemo *et al*. 1993; Chen *et al*. 1994; Kumazawa *et al*. 1998).

Surprisingly, we were not able to detect any significant changes in the distribution or expression level of the catecholaminergic key enzyme TH, the rate-limiting enzyme in catecholamine synthesis. We have previously identified all of the aminergic cell clusters in zebrafish brain (Kaslin and Panula 2001). No major changes in intensity of immunoreactivity, cell number or morphology were seen in toxin-treated fish in any of the clusters proposed to be similar to the mammalian ascending dopaminergic system (Kaslin and Panula 2001; Rink and Wullimann 2001). In teleosts, several different diencephalic and even telencephalic clusters of catecholaminergic neurones have been proposed to correspond to the mammalian substantia nigra and ventral tegmental area (Poli *et al*. 1990; Pollard *et al*. 1992; Reiner and Northcutt 1992; Rink and Wullimann 2001). These neuroanatomical studies have quite clearly shown that teleosts lack a mesencephalic/midbrain population of catecholaminergic neurones but, based on neuro-hodological characteristics and putative prosomeric models, it is still likely that catecholaminergic cell groups in the posterior tuberculum and/or caudal hypothalamus would be functionally equivalent to the mammalian ascending dopaminergic systems (substantia nigra/ventral tegmental area; Reiner and Northcutt 1992; Kaslin and Panula 2001; Rink and Wullimann 2001, 2002). In goldfish treated with MPTP, telencephalic catecholaminergic neurones were most affected by the toxin and other diencephalic clusters were also affected (Goping *et al*. 1995; Pollard *et al*. 1996). We did not observe similar changes in the telencephalon of zebrafish with either one of the toxins used. Furthermore, we were unable to detect DNA fragmentation using the TUNEL method in TH-positive neurones after the MPTP or 6-OHDA doses used in this study, which induced significant biochemical and behavioural effects. This does not exclude the possibility that larger doses might induce specific or non-specific damage as has been shown in goldfish (Pollard *et al*. 1996).

However, we cannot exclude the possibility of a change in the activity of TH after neurotoxin administration in our study. Indeed, in mice the activity of TH was decreased by 80% after a similar dose of MPTP (Serra *et al*. 2002). Except for the activity and expression of TH, the catecholamine content in brain is also highly dependent on the active uptake via DAT, packing of amines into vesicles by the vesicular monoamine transporter and inactivation by the catabolic pathways via MAO or catechol-o-methyltransferase (COMT). It has been shown in mice and cats that the expression of TH, DAT and vesicular monoamine transporter is spatiotemporally highly variable after MPTP injection (Rothblat *et al*. 2001). If the same processes are relevant for zebrafish, they could explain the absence of difference in TH levels, studied by western blotting, at the same time as a clear decrease in the concentration of DA, studied by HPLC. The more extensive decrease in noradrenaline levels than those of DA after MPTP and even more after 6-OHDA administration, observed in our study, could indicate different properties of the zebrafish catecholaminergic transporters. This question requires further examination. Also, single injections of neurotoxins apparently do not induce a clear decrease in the dopaminergic cell population, as we did not detect any differences in TH, TUNEL staining, caspase 3 immunohistochemistry and western blotting.

Repeated administration of the toxins and/or higher doses may be needed to demonstrate the cellular impairment. We also cannot exclude the possibility of caspase 3-independent apoptosis or other forms of cell death.

Our previous studies in human post-mortem Parkinson's disease brain revealed an increase in histamine concentrations and histaminergic innervation in affected areas (Anichtchik *et al*. 2000; Rinne *et al*. 2002). In this study, injections of MPTP did not produce apparent changes in the histaminergic system, as examined by immunohistochemistry, in spite of a transient increase in histamine levels 1 day after the injections. It is possible that the more detailed study of the histaminergic system at the very early time-points after the MPTP injections is needed to reveal subtle changes in the innervation and/or transport of histamine.

We employed a video tracking system to detect changes in the swimming behaviour of lesioned zebrafish. Video tracking has previously been used to record, track and analyse the behaviour of vertebrates and invertebrates (Noldus *et al*. 2001). Similar methods to ours have been

previously successfully used to measure spontaneous locomotor activity in the crucian carp and goldfish (Nilsson *et al*. 1993; Kato *et al*. 1996) and behavioural dysfunctions in trout after exposure to cholinesterase inhibitors or heavy metals (Brewer *et al*. 2001; Beauvais *et al*. 2001).

Both MPTP and 6-OHDA produced marked alterations in the swimming behaviour of zebrafish. The general locomotor activity (distance moved and velocity) was markedly decreased and the fish displayed altered complexity of the swimming path (turn angle and time spent in IN and OUT zones). The lower swimming rate observed in toxin-treated zebrafish was not surprising as dyskinesia and bradykinesia are observed in other DA-deficient conditions and goldfish injected with MPTP show a lower swimming rate and an increased resting rate (Pollard *et al*. 1992). The recovery of behavioural alterations was well correlated with recovery of the levels of brain catecholamines. Furthermore, the activation of the dopaminergic system with apomorphine, a non-specific DA D1/D2 receptor agonist, significantly increased the locomotor activity in another teleost (Mok and Munro 1998).

We observed that the general pattern of swimming behaviour of saline-injected fish was different from that of neurotoxin-lesioned fish. The control fish tended to swim in a circular pattern along the walls of the observation tank, infrequently changing direction. We interpreted that this indicates a normal pattern of swimming, with a tendency to follow the wall. Similar behavioural patterns have been reported in goldfish and salmon (Clements *et al*. 2002). The tendency to follow the wall could perhaps be partly explained by initial exploratory behaviour and active avoidance of the IN zone, which offers the least amount of shelter. However, it is not solely an initial exploratory behaviour as this tendency can still be observed in fish kept for prolonged swimming bouts (several hours) in the tanks. The lesioned fish exhibited disorganized patterns of swimming in addition to the decreased speed and distance. They changed direction more frequently (reflected as an increase of the absolute turn angle values) and spent more time in the middle of the observation tank.

In both goldfish and zebrafish, the depletion of DA from the retinal cells leads to increased sensitivity to light (Yazulla *et al*. 1996; Li and Dowling 2000), impaired and tilting behaviour (dorsal light reaction) to the side of the lesioned eye (Lin and Yazulla 1994). It is, therefore, possible that impairments of the visual system might influence the swimming pattern of the toxin-treated fish. It is also possible that other visual structures are involved, as mild changes in the optic tectum have been reported in goldfish after MPTP injections (Pollard *et al*. 1996). Even though a visual impairment at any level might influence the swimming pattern, it is unlikely that the decrease in the total distance moved and velocity are solely attributable to this. We also measured the DA content in whole eyes from toxin- and saline-injected fish. We did not detect any change in the DA content of eyes from toxin-injected compared with saline-injected fish (data not shown), which is in agreement with a previous study on goldfish (Youdim *et al*. 1992).

It is well known that fish have the ability to change colour, either through a fast or a slow adaptation process. The slow colour adaptation is due to a change in morphology and density of chromophores (Sugimoto 2002). The rapid colour change, also known as the physiological colour change, is neuronally and hormonally controlled and is attributed to the rapid aggregation or dispersion of pigment granules in the chromophores. It has been shown that exogenous catecholamines induce dispersion of pigments and thus darkening of skin in fish (Grove 1969a,b). Darkening of skin has been reported in the trout and frog after peripheral injections of 6-OHDA (Tonosaki *et al*. 1995; Ryan *et al*. 2002). The darkening of skin in trout has been attributed to degeneration of noradrenergic nerve connections to melanophores as it was pharmacologically reversed by treatment with noradrenaline. Surprisingly, MPTP injection showed no effect on darkening of the skin in trout and, in contrast, they showed a paling effect (Ryan *et al*. 2002). The darkening of 6-OHDA-injected zebrafish was very obvious and easy to detect visually. However, although we did not detect any paling of MPTP-treated fish, it may still occur as we did not perform any tests that could have detected this. The different effects of the toxins on colouration of zebrafish could be explained by a higher susceptibility to 6-OHDA of peripheral catecholaminergic terminals that control the melanophores. As both toxins induced marked decreases of catecholamine contents in the brain, it seems most likely that the change in colouration is a peripheral effect.

Taken together, our data indicate that single systemic injections of MPTP (20 mg/kg) or 6-OHDA (25 mg/kg) produce significant behavioural alterations and decreases in the concentrations of catecholamines in the brain of zebrafish but no significant degeneration of any catecholaminergic neuronal cell clusters. Further studies are needed to evaluate the mechanisms of these alterations. However, this approach could be useful in studying the early changes caused by the decrease of cerebral catecholamines. The possibilities of using many of the available zebrafish mutants may provide new tools to investigate the mechanisms of selective neurotoxicity.

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