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Attenuation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity by the novel selective dopamine D_3-receptor partial agonist FAUC 329 predominantly in the nucleus accumbens of mice

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Abstract

We previously synthesised a novel dopamine (DA) partial agonist FAUC 329 with high affinity and selectivity for the DA D_3 receptor. This is the first *in vivo* study to investigate the protective effects of FAUC 329 in a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease. Adult male C57bl/6 mice were injected with FAUC 329 (0, 0.1, 0.5, 0.75, or 1 mg/kg) 30 min before MPTP (2 × 30 mg/kg, 4 hr apart). One week later, accumbal and striatal tissue was processed for DA and metabolite HPLC determination as well as immunohistochemical analysis of DA transporter positive neurons in the substantia nigra pars compacta and ventral tegmental area was carried out. FAUC 329 showed a significant attenuation of MPTP-induced DA reduction in the nucleus accumbens (0.5, 0.75 and 1 mg/kg) in a dose-dependent manner. FAUC 329 (0.75 mg/kg) partly protected against DA depletion in the dorsal striatum as well as protected against loss of DA transporter immunoreactivity in the substantia nigra pars compacta. The highest dose of FAUC 329 (1 mg/kg), however, showed a non-significant tendency to augment the MPTP-induced striatal DA reduction. The protective effect of FAUC 329 against MPTP-induced DA depletion was most pronounced in the nucleus accumbens and appears to be linked to the preferential abundance of D_3 receptors in this region. Targeting the mesolimbic DA system may have implications for improvement of impaired motor behaviour and particularly non-motor functions related to the nucleus accumbens.

Keywords: Dopamine D_3 receptor; MPTP; Parkinson's disease; FAUC 329; Neuroprotection; Nucleus accumbens

1. Introduction

Since the discovery of the DA D_3 receptor [1], D_3 agonists and antagonists have received attention as new targets for the treatment of pathophysiological conditions in which the expression of D_3 receptors is abnormal, such as schizophrenia [2,3], drug addiction [4,5], and PD [6].

In the rat brain, all mesencephalic DA neurons express D_3 receptors and the distribution pattern markedly differs between various brain regions. The largest D_3 receptor expression with dense signals was obtained in the islands of Calleja, in mammillary bodies and the medium-spiny neurons of the nucleus accumbens (shell). Lower expression was found in the frontoparietal cortex, substantia nigra, ventral tegmental area (VTA) and lobules 9 and 10 of the cerebellum, and the lowest or no expression was seen in the dorsal striatum and other brain areas [7–9]. Among the D_2 like DA receptor family the more abundant D_2 receptors, as well as the D_3 receptors, have autoreceptor function and are involved in the regulation of synaptic DA levels [1,10]. Thereby, D_3 autoreceptors have been reported

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Abbreviations: DA, dopamine; DAT, dopamine transporter; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area; ROS, reactive oxygen species.

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to play a small but significant function in partially regulating the secretion, but not the synthesis, of DA [11,12].

The lack of DA receptor stimulation in DA terminal areas is a main feature of PD. Several directly acting DA agonists have beneficial effects on PD symptoms (for review see [13,14]), and second generation non-ergoline DA receptor agonists with selectivity for D_3 over D_2 receptors, such as ropinirole and pramipexole, have been successfully established for adjunctive and monotherapy in PD [15]. However, most of the currently used D_3 agonists are not selective but rather D_3/D_2 agonists. Therefore, selective pharmacological targeting of D_3 receptors remains difficult. Based on interactive structure–activity relationship (SAR) studies, we discovered and synthesised a novel partial agonist FAUC 329 (Fig. 2) with high affinity and selectivity for the D_3 receptor [16]. We selected the MPTP mouse model to investigate the effects of FAUC 329 because the selective dopaminergic neurotoxin MPTP mimics the characteristic loss of dopaminergic innervation also in humans [17,18] and demonstrates a moderate to high degree of DA depletion in the striatum and nucleus accumbens depending on the dose regimen used (for review see [19,20]).

D_3 receptor density was found to be reduced after MPTP treatment in experimental primates [21,22], although in other studies it was not affected [23]. However, in brains from PD patients who were positively diagnosed with PD for more than 9 years, a clear reduction of the number of D_3 receptors was determined in the nucleus accumbens and putamen [6]. In addition, the reduction of D_3, but not of D_2, receptors was associated with the loss of response to L-dihydroxyphenylalanine (L-DOPA) [24]. Furthermore, the reduction of D_3 receptors seems to be related to the presence of dementia [24]. The development of novel D_3 agonists as anti-parkinsonian drugs may have implications for the treatment of motor impairment and may also have beneficial effects in the mesolimbic DA system of PD patients.

This is the first *in vivo* study to investigate the effects of FAUC 329 on its potential anti-parkinsonian properties, in particular on DA depletion and loss of DAT immunoreactivity, in the MPTP mouse model of PD. Since MPTP acts as a protoxin and becomes toxic after its conversion to its metabolite MPP^+, we investigated the effects of FAUC 329 on accumbal and striatal MPP^+ levels in a separate experiment.

2. Materials and methods

2.1. Animals

The present studies were conducted in adult male C57bl/6 mice. All mice were bred at the Research Unit Schwerzenbach and weighed 25–28 g at the beginning of the experiment. Mice were maintained under standard conditions, in temperature ($21 \pm 1.0^\circ$) and humidity ($55 \pm 5\%$) controlled rooms, on a 12–12 hr light/dark cycle (lights on at 7:00 h) with free access to standard diet (Nafag 9431, Nafag Ecossan) and water *ad libitum*. All animal studies were carried out in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the appropriate institutional governmental agency (Kantonales Veterinäramt).

2.2. MPTP and FAUC 329 treatment

To investigate the potential neuroprotective effects of FAUC 329, mice were subcutaneously injected twice with MPTP (30 mg/kg, calculated as free base and dissolved in saline) or saline (10 mL/kg) at 4 hr intervals. FAUC 329 (0.1, 0.5, 0.75 and 1.0 mg/kg dissolved in vehicle) or vehicle was intraperitoneally injected 30 min before the first and second MPTP administration.

2.3. Tissue preparation

One week after the MPTP or saline administration, mice were sacrificed by cervical dislocation. The brains were rapidly removed and placed on an ice-cooled plate for dissection of the ventral striatum (nucleus accumbens) and the dorsal striatum (Fig. 1) in a 1 mm thick section (AP 1.5–0.5 mm, mouse brain atlas [25]). Micropunches were dissected out with a punch needle of diameter 1 mm for the nucleus accumbens (including shell and core region) and diameter 1.5 mm for the dorsal striatum. Immediately afterwards, the tissue was ejected by a slight air pressure, placed in pre-weighed 1.5 mL plastic tubes and weighed. Ice-cooled perchloric acid (0.4 M) (300 μL for accumbal tissue and 500 μL for the dorsal striatum) was added and the tissue was homogenised for 10 s using ultrasound and centrifuged for 20 min at 15,000 *g* at 4°. The supernatant was passed through a 0.2 μm filter and kept at 4° in a refrigerated autosampler until HPLC analysis.

2.4. HPLC determination of striatal DA and metabolites

DA and DA metabolites (3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)) were analysed

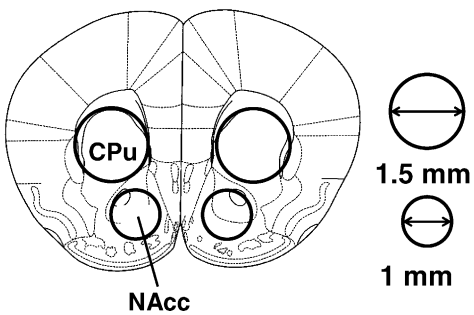

Fig. 1. Localisation of the micropunch area in the striatum (CPu) and nucleus accumbens (NAcc). The displayed section corresponds to 1.54 mm anterior to bregma [25].

using reversed-phase ion-pair chromatography combined with electrochemical detection under isocratic conditions [26]. The detector potential was set at +750 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase (0.6 mM 1-octanesulfonic acid, 0.27 mM Na_2EDTA, 0.043 M triethylamine and 5% acetonitrile, adjusted to pH 2.95 with H_3PO_4) was delivered at a flow rate of 0.5 mL/min at 22° onto the reversed-phase column (125 mm × 3 mm with pre-column 5 mm × 3 mm, both filled with Nucleosil 120-3 C18, Knauer). Ten microliter aliquots were injected by an autosampler with cooling module set at 4°. Data were calculated by an external standard calibration.

2.5. HPLC measurement of MPP^+

In order to measure the effects on MPP^+ formation, FAUC 329 (Fig. 2) (1 mg/kg) or vehicle was administered 30 min before MPTP (30 mg/kg). Sixty minutes after the MPTP administration accumbal and striatal tissue was analysed for MPP^+ levels using HPLC and UV detection at 295 nm [27]. In brief, the mobile phase (0.02 M potassium phosphate, acetonitrile (40%) adjusted to pH 3.5 with H_3PO_4) was delivered at a flow rate of 0.5 mL/min at 25° onto the reversed phase column (125 mm × 3 mm with pre-column 5 mm × 3 mm, both filled with Nucleosil 120-C18, Knauer). One hundred microliter aliquots were analysed using an autosampler with a cooling module set at 4°. Data were calculated by an external standard calibration.

2.6. Tissue fixation

One week after the last MPTP or saline injection, mice were decapitated and the remaining part of the brain containing the SNpc and the VTA, which was not used for HPLC DA determination, was post-fixed for 2–3 days in cold fixative with 4% *para*-formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). They were then transferred to a 30% sucrose solution and kept at 4° until they sunk. Subsequently, the tissue was cut with a freezing microtome and coronal sections (25 μm thick) were collected throughout the rostro-caudal extent of the SNpc and stored in a cryoprotectant solution.

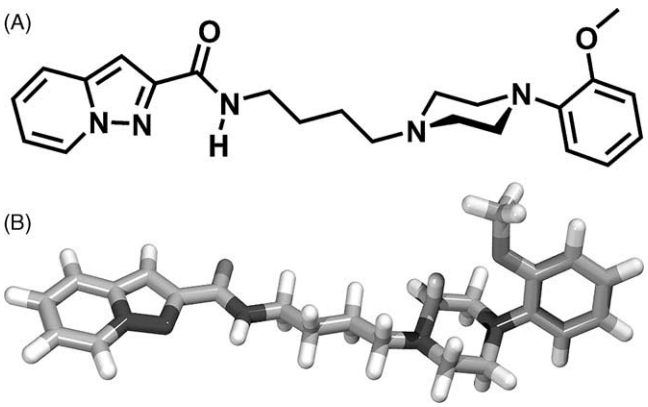

Fig. 2. Structure formula (A) and 3D representation (B) of the DA D_3 partial agonist 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-*a*]-pyridine (FAUC 329).

2.7. DAT immunolabeling

Midbrain sections were processed for the DAT using the standard peroxidase–antiperoxidase method. After 3 × 5 min rinses in PBS, free floating sections were blocked for 1 hr in PBS containing 5% normal goat serum plus 0.3% Triton X-100. Sections were then incubated in a solution of PBS and 2% normal goat serum plus 0.15% Triton X-100 containing the primary antibody rat anti-DAT (1:2000, Chemicon) for 2 days at 4°. Following this, the sections were rinsed and incubated for 1 hr in biotinylated secondary antibodies (goat–anti-rat, 1:300, Jackson Immuno Research) in a solution of 2% goat serum plus 0.15% Triton X-100 at room temperature. Subsequently, the sections were treated with avidin–biotin–horseradish peroxidase complex (Vectastain Elite, Vector Laboratories) for 1 hr at room temperature followed by 3 × 5 min rinses in 0.1 M Tris buffer (TB; pH 7.4). Immunoreactivity was visualised with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) and 0.004% H_2O_2 in TB for 3–5 min. Nickel chloride (0.08%) was added to the DAB solution to intensify the staining. Sections were then rinsed 3 × 5 min in TB and mounted on slides, air dried, dehydrated through an alcohol series, cleared in xylene and coverslipped. Dilution series were run to establish the optimum staining. Controls for non-specific staining were performed in which either the first or secondary antibody was omitted. These controls did not produce specific staining.

2.8. Microscopic quantification and analysis

DAT is a specific marker for dopaminergic cells and fibers and was therefore used to estimate the extension of the MPTP lesions in the SNpc and VTA [28]. The total numbers of DAT positive cells were counted using the image analysis computer software Neurolucida (version 4.0, MicroBrightField). DAT cell counting started at a random position and an average of six sections per animal was analysed (AP coordinates between 2.92 and −3.64 mm according to the mouse brain atlas [25]). Using this method, the contours of the SNpc and VTA on the left and right side of the brain were drawn using a 5× lens and the cells were counted at a magnification of 63× oil lens (numeric aperture 1.40). DAT positive cells were included in the measurements only when the DAT immunoreactivity could be unequivocally identified in the soma and at least in the proximal part of its processes. No distinction was made between the size and the shape of the cells. Digitised bright-field images were captured using a Zeiss Axiophot

microscope in combination with a video-camera (Kodak Megaplus, Eastman Kodak) and the image-based analysis computer software described above. The average of the counted sections per animal was used for statistical analysis.

2.9. Drugs and chemicals

MPTP hydrochloride, ethylenediaminetetraacetic acid disodium salt, 1-octanesulfonic acid sodium salt, triethylamine, phosphoric acid and HPLC grade acetonitrile were obtained from Fluka Chemie GmbH. MPTP–HCl was dissolved in 0.9% NaCl (saline) and FAUC 329 was dissolved in polyethylene glycol 400/propanediole/sodiumacetat buffer (20:20:60) pH 5.5 (vehicle). The injection volume was 10 mL/kg.

2.10. Statistical analysis

All values are expressed as mean ± SEM. One-way ANOVA was followed by Fisher's least significant difference test (PLSD) for *post hoc* comparisons of MPTP-treated groups. *P*-value <0.05 was considered as statistically significant.

3. Results

3.1. Accumbal and striatal DA levels and DA turnover

The absolute DA levels in the nucleus accumbens of the control group were lower (6.96 ± 0.56 ng/mg) than in the dorsal part of the striatum (13.61 ± 0.56 ng/mg). Figure 3A summarises the results of the effects of FAUC 329 on accumbal DA levels one week after MPTP treatment. ANOVA yielded a significant effect of treatment [$F(4,35) = 4.11$, $P < 0.01$]. *Post hoc* tests revealed that FAUC 329 treatment (0.5, 0.75, 1.0 mg/kg) led to a significant attenuation of the MPTP-induced accumbal DA depletion in a dose-dependent manner. Expressed as percentage of the non-MPTP-treated control group, MPTP produced a DA depletion of 56 and 94% in the nucleus accumbens and in the dorsal striatum, respectively (Figs. 3A and 4A). The accumbal DA turnover expressed as ratio (DOPAC + HVA)/DA was significantly reduced by FAUC 329 (1.0 mg/kg) (Fig. 3B).

Figure 4A summarises the effect of FAUC 329 on DA levels of the dorsal striatum. Again, ANOVA yielded a significant effect of treatment [$F(4,35) = 13.39$, $P < 0.001$]. *Post hoc* tests revealed that FAUC 329 (0.75 mg/kg) treatment led to a significant attenuation of the MPTP-induced striatal DA depletion. The highest dose of FAUC 329 (1.0 mg/kg) showed a tendency to augment the toxic effect of MPTP, although this was not significant. The enhanced striatal DA turnover was normalised by FAUC 329 (0.75 mg/kg) (Fig. 4B).

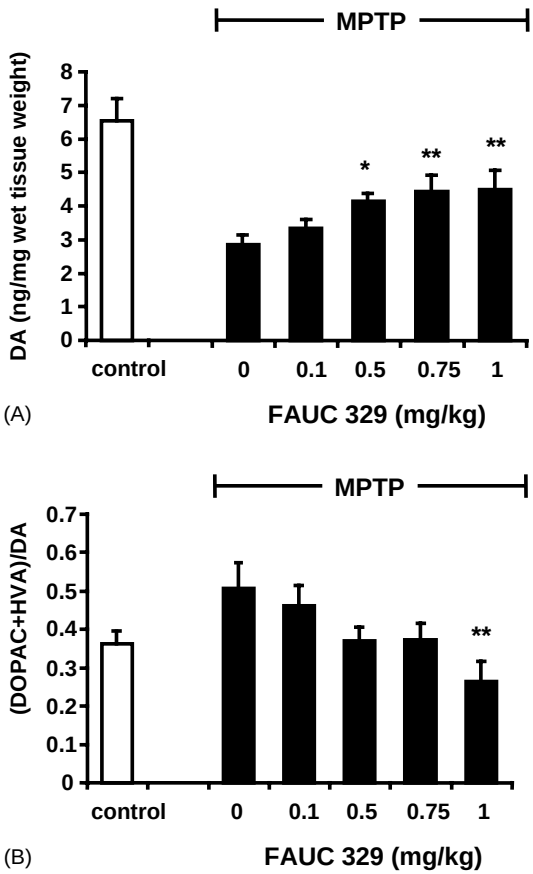

Fig. 3. Effects of FAUC 329 on MPTP-induced dopamine (DA) depletion (A) and DA turnover (B) in the nucleus accumbens. Mice were treated with MPTP (2 × 30 mg/kg, 4 hr interval) or saline. FAUC 329 (0.1, 0.5, 0.75 and 1.0 mg/kg) or vehicle was injected 30 min prior to MPTP administration. Control animals received vehicle and saline instead of FAUC 329 and MPTP, respectively. DA levels of the nucleus accumbens were analysed one week after MPTP treatment using HPLC. DA turnover is expressed as ratio (DOPAC + HVA)/DA. Data are mean ± SEM of N = 7–11 mice. ANOVA with *post hoc* Fisher's PLSD test for multiple comparisons ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle + MPTP group).

3.2. DAT immunoreactivity in the SNpc and VTA

Immunostaining of the SNpc and VTA using DAT antibodies shows the striking effect of MPTP treatment reducing the number of DAT positive neurons predominantly in the SNpc and to a lesser extent in the VTA (Fig. 5). For quantification of the effects of FAUC 329 we selected the dose 0.75 mg/kg as this was most effective in protecting against DA depletion. FAUC 329 (0.75 mg/kg) demonstrated a significant attenuation of MPTP-induced loss of DAT immunoreactivity in the SNpc [$F(1,10) = 25.23$, $P < 0.001$] (Fig. 6). In the VTA the following mean ± SEM values for DAT positive neurons were counted: saline + vehicle (N = 5) 65.2 ± 9.9, vehicle + MPTP (N = 5) 29.6 ± 5.4, FAUC 329 + MPTP (N = 7) 31.4± 3.6. There was no statistical difference between FAUC 329 and vehicle-treated MPTP groups in the VTA.

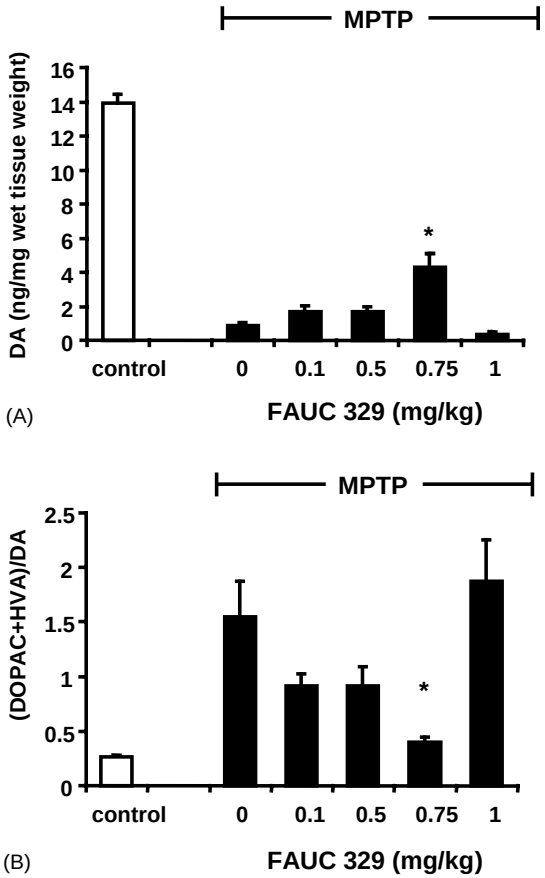

Fig. 4. Effects of FAUC 329 on MPTP-induced dopamine (DA) depletion (A) and DA turnover (B) in the dorsal striatum. Mice were treated with MPTP (2 × 30 mg/kg, 4 hr interval) or saline. FAUC 329 (0.1, 0.5, 0.75 and 1.0 mg/kg) or vehicle was injected 30 min prior to MPTP administration. Control animals received vehicle and saline instead of FAUC 329 and MPTP, respectively. DA levels of the dorsal striatum were analysed one week after MPTP treatment using HPLC. DA turnover is expressed as ratio (DOPAC + HVA)/DA. Data are mean ± SEM of N = 7–11 mice. ANOVA with *post hoc* Fisher's PLSD test for multiple comparisons ($^{*}P < 0.05$ vs. vehicle + MPTP group).

3.3. MPP^+ levels in the striatum

In order to measure the effects of FAUC 329 on striatal MPP^+ levels, the highest dose of FAUC (1 mg/kg) was selected to rule out the possibility that the protective effect of FAUC 329 is mediated by a reduction of the availability of MPP^+ in the striatum. Sixty minutes after MPTP injection, FAUC 329 (N = 7) and vehicle (N = 7) pretreatment resulted in the following accumbal MPP^+ levels in ng/mg (mean ± SEM): 30.05 ± 2.28 and 29.31 ± 4.27 and striatal MPP^+ levels: 26.38 ± 3.48 and 25.90 ± 2.99, respectively, which were not statistically different.

4. Discussion

In the present study, a novel partial DA D_3 agonist FAUC 329 demonstrated a protective effect against MPTP-induced DA depletion, which was most pronounced in the nucleus accumbens, and also reduced the MPTP-induced loss of DAT immunoreactivity in the SNpc.

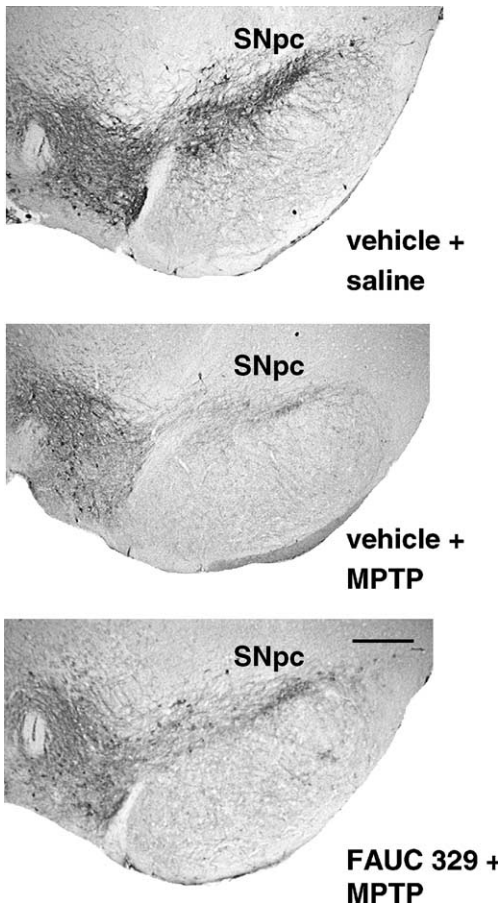

Fig. 5. Photomicrographs of DAT positive neurons in the SNpc of vehicle + saline (A), vehicle + MPTP (B) and FAUC 329 + MPTP (0.75 mg/kg) (C) treated mice. The brains were analysed for DAT immunoreactivity one week after MPTP or saline injection. Scale bar = 100 μm.

The MPTP model in mice is widely used to study neuroprotection because it replicates the key pathobiochemical

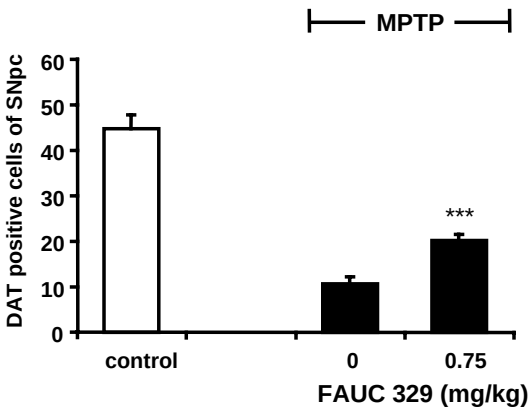

Fig. 6. Effects of FAUC 329 on MPTP-induced loss of DAT immunoreactivity in the SNpc. Mice were treated with MPTP (2 × 30 mg/kg, 4 hr interval) or saline. FAUC 329 (0.75 mg/kg) or vehicle was injected 30 min prior to MPTP administration. DAT immunoreactivity in the SNpc was assessed one week after MPTP treatment. Data are mean ± SEM of N = 5–7 mice. ANOVA with *post hoc* Fisher's PLSD test ($^{***}P < 0.001$ vs. vehicle + MPTP group).

features of PD such as oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammatory processes and apoptosis [19,20]. Drugs that are able to reduce the neurotoxicity may prove to be neuroprotective. However, interference with the biotoxification of MPTP by inhibiting its uptake into the brain, or by blocking its conversion via monoamine oxidase B (MAO-B) to MPP^+, will also result in reduced toxicity by decreasing the concentration of the ultimate neurotoxin MPP^+, rather than neuroprotection. MPP^+ levels are positively correlated with MPTP toxicity [29] and, because we did not find a reduction of accumbal and striatal MPP^+ levels by FAUC 329 treatment, a toxicokinetic effect can be ruled out as contributing to the protective effect of FAUC 329.

A less pronounced DA depletion (56%) was obtained in the nucleus accumbens than in the dorsal part of the striatum (94%), which is in agreement with other earlier reports [30–32]. The mechanism of reduced MPTP toxicity in the mesolimbic system is not fully understood. Brain-derived neurotrophic factor mRNA levels are more abundant in the mesolimbic pathway than in the nigrostriatal pathway. Furthermore, the endogenous antioxidative defense system is more effective in the mesolimbic system. These factors may contribute to the reduced vulnerability of the nucleus accumbens in comparison to the dorsal striatum against MPTP-induced toxicity [33,34].

Moreover, in the first years of the clinical course of PD the mesolimbic part of the DA system is obviously more resistant against neurodegenerative processes. In the final stage of PD, however, the further loss of mesolimbic DA neurons goes along with a permanent reduction of D_3 receptors in this region [6]. Impairment of goal-directed behaviour and locomotor activity, as well as loss of response to L-DOPA, has been suggested to involve a reduction of D_3 receptors in the later stages of PD [24]. Furthermore, positron emission tomography (PET) scans indicated a reduction in reward processing in the brains of PD patients which was discussed as being related to anhedonic behaviour in PD [35], and may also be influenced by a reduced D_3 receptor stimulation.

Another DA D_3 preferring agonist pramipexole exhibited protective effects that were attributed to its ability to reduce oxidative stress [36–38]. *In vivo* microdialysis measurement of hydroxyl radical formation induced by the dopaminergic neurotoxin 6-hydroxydopamine [39] or MPP^+ [37] demonstrated that pramipexole reduced the levels of hydroxyl radicals. FAUC 329 also may reduce ROS formation by an indirect mechanism that is in common with other DA agonists which stimulate DA autoreceptors and reduce the extracellular levels of DA, DA turnover and ROS formation [40]. Indeed, the oxidative DA metabolism via MAO-A (primary isoenzyme for DA metabolism in mice) and MAO-B not only produces DOPAC but also hydrogen peroxide which reacts with iron ions to form hydroxyl radicals. Thus, a D_3 receptor mediated attenuation of the MPTP-induced compensatory increase in DA turnover (Figs. 3B and 4B) may lead to less ROS formation.

Recently, an alternative mechanism for the neuroprotective effects of D_3 receptor agonists has been established suggesting that the induction of a 35 kDa protein, which was described as a constitutively produced DA autotrophic factor, contributes to the neuroprotective effects of various DA D_3 preferring agonists [41,42].

The partial D_3 agonist BP 897, which has some structural similarities with FAUC 329 [16], prevented cocaine seeking behaviour and was not self administered. This was discussed as being the advantage of a partial over a full agonist [5,43]. Also, BP 897 has recently been positively tested in MPTP-treated monkeys to reduce dyskinesias by preserving the anti-parkinsonian action of L-DOPA, whereas D_3 antagonists also reduced the severity of dyskinesias at the cost of a decreased anti-parkinsonian efficacy of L-DOPA [44]. FAUC 329, as a partial DA D_3 agonist, has similar advantages on dyskinesias which, however, have to be tested in an appropriate animal model. The partial agonism may lead to adverse effects in higher doses of FAUC 329 (above 1 mg/kg) when antagonistic properties become manifested and may have caused the trend to the aggravation of the MPTP-induced DA depletion in the striatum observed in the highest dose of FAUC 329 (1 mg/kg).

The protective effect of the partial D_3 agonist FAUC 329 against MPTP-induced DA depletion was most pronounced in the nucleus accumbens. The mechanism of this effect may involve a normalisation of the MPTP-induced increase of DA turnover, but other possibilities were also discussed. Targeting the limbic system in PD using selective D_3 agonists may improve deficits, in particular in motor behaviour such as goal-directed behaviour, initiation of movement and locomotor activity. Further studies may elucidate whether FAUC 329 or other D_3 agonists are able to slow down the decline of L-DOPA efficacy and reduce dyskinesias in a later stage of PD, and whether they may positively influence non-motor complications such as anhedonia which are frequently associated with PD and are related to the mesolimbic DA system.

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