Dependent Mechanism lacking in the dorsal striatum in wild-type mice and two mouse lines the effect of MPD on DA neurotransmission. The effect was studied in the dorsal striatum in wild-type mice and two mouse lines lacking α-syn by using in vivo voltammetry and microdialysis. MPD (1 mg/kg) attenuated evoked DA overflow only in mice lacking α-syn but produced a similar increase in the extracellular DA levels in all three lines. A kinetic analysis showed that MPD decreased DA release per stimulus pulse in α-syn-deficient mice but increased in wild-type mice. MPD blocked DA reuptake and produced a similar increase in the apparent affinity (Km) for DA reuptake in all three lines. Repeated-burst stimulation redistributes vesicular storage pools and facilitates DA overflow, and this form of facilitation is significantly enhanced in α-syn knockout mice. The DA reuptake inhibitor 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine (GBR12909) (10 mg/kg) completely blocked the facilitation of DA overflow in all three lines, whereas MPD (1 mg/kg) selectively decreased it only in mice lacking α-syn. MPD (5 mg/kg) and GBR12909 (10 mg/kg) produced equipotent inhibition of DA reuptake (in terms of Km), indicating that reuptake inhibition does not explain the MPD selectivity. Our data indicate that MPD decreases DA release probability in the absence of α-syn and increases it in control animals, whereas the effect of MPD on DA reuptake is independent of α-syn. We suggest that this selectivity is based on α-syn-dependent compartmentalization of presynaptic DA.

**Introduction**

Mutations in an abundantly expressed presynaptic protein, α-synuclein (α-syn), have been implicated in familial Parkinson’s disease and other neurodegenerative diseases (Lee and Trojanowski, 2006). α-Syn has also been shown to regulate dopamine (DA) neurotransmission (for a review, see Venda et al., 2010). The absence of α-syn does not alter DA overflow after short, relatively weak electrical stimulation (Nemani et al., 2010) but increases it after paired-pulse stimulation (Abeliovich et al., 2000) or repeated-burst stimulation (Yavich et al., 2004). A specific form of the plasticity of evoked DA overflow in the striatum, facilitation after repeated-burst stimulation, is believed to result from the mobilization of DA storage pools in presynaptic terminals (Yavich, 1996). In this case, the enhanced facilitation of DA overflow after repeated-burst stimulation in α-syn knockout mice is evidence of the alterations in the vesicular DA storage pools. Indeed, it has been shown that α-syn is differentially expressed in subpopulations of vesicles in the storage pools (Lee et al., 2008), and α-syn gets redistributed in vesicles after repetitive stimulation (for a review, see Fortin et al., 2010). The absence of α-syn in knockout mice alters the distribution of vesicles in the presynaptic terminals (Cabin et al., 2002). Increased expression of α-syn reduces synaptic vesicle density at the active zones and inhibits vesicles recoupling after endocytosis (Nemani et al., 2010). Furthermore, α-syn participates in mobilizing vesicles and regulating the size of vesicle pools by interacting with several presynaptic proteins mediating vesicle mobilization and exocytosis (Fortin et al., 2005; Tao-Cheng, 2006; for a review, see Bellani et al., 2010). α-Syn also

**ABSTRACT**

Methylphenidate (MPD) modulates dopamine (DA) overflow in part by redistributing vesicle pools, a function shared by the presynaptic protein α-synuclein (α-syn). We suggest that α-syn modifies the effect of MPD on DA neurotransmission. The effect was studied in the dorsal striatum in wild-type mice and two mouse lines lacking α-syn by using in vivo voltammetry and microdialysis. MPD (1 mg/kg) attenuated evoked DA overflow only in mice lacking α-syn but produced a similar increase in the extracellular DA levels in all three lines. A kinetic analysis showed that MPD decreased DA release per stimulus pulse in α-syn-deficient mice but increased in wild-type mice. MPD blocked DA reuptake and produced a similar increase in the apparent affinity (Km) for DA reuptake in all three lines. Repeated-burst stimulation redistributes vesicular storage pools and facilitates DA overflow, and this form of facilitation is significantly enhanced in α-syn knockout mice. The DA reuptake inhibitor 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine (GBR12909) (10 mg/kg) completely blocked the facilitation of DA overflow in all three lines, whereas MPD (1 mg/kg) selectively decreased it only in mice lacking α-syn. MPD (5 mg/kg) and GBR12909 (10 mg/kg) produced equipotent inhibition of DA reuptake (in terms of Km), indicating that reuptake inhibition does not explain the MPD selectivity. Our data indicate that MPD decreases DA release probability in the absence of α-syn and increases it in control animals, whereas the effect of MPD on DA reuptake is independent of α-syn. We suggest that this selectivity is based on α-syn-dependent compartmentalization of presynaptic DA.
promotes the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly, thereby enabling the neurons to release neurotransmitter during increased synaptic activity (Burré et al., 2010). These experimental data point to a role of α-syn in the organization of vesicular storage, trafficking, and exocytosis. Furthermore, maintenance of the vesicular DA storage and proper compartmentalization of DA requires reuptake via the DA transporter (DAT) and vesicular monoamine transporter-2 (VMAT2) (Bönisch and Eiden, 1998; Jones et al., 1998). Reuptake, in turn, can be affected by the absence of α-syn (Chadchankar et al., 2011).

Methylphenidate (MPD) is the drug of choice for the treatment of attention-deficit hyperactivity disorder (Volkow et al., 2001). MPD is known to inhibit DA reuptake (Pan et al., 1994) and redistribute synaptic vesicles within the reserve and readily releasable pools (RRPs) (Sandoval et al., 2002; Volz et al., 2008). It also up-regulates VMAT2, thereby increasing the rate of transport of cytosolic DA into the synaptic vesicles (Sandoval et al., 2002; Volz et al., 2008). However, the mechanisms of action of MPD in the treatment of attention-deficit hyperactivity disorder are still poorly understood.

We propose that at least some of the effects of MPD on DA neurotransmission are based on α-syn-dependent presynaptic machinery of DA compartmentalization and release. This study investigated the effects of MPD on extracellular DA levels by in vivo microdialysis and electrically evoked DA overflow by in vivo voltammetry at the doses considered therapeutically relevant (Volkow et al., 2001). We used various regimens of electrical stimulation of the ascending DA pathways and in vivo voltammetry to evaluate different aspects of DA neurotransmission including release, reuptake, and compartmentalization of presynaptic terminals of the dorsal striatum in wild-type and two mouse lines lacking α-syn. Our data show that MPD decreases the probability of DA release in the absence of α-syn and increases it in wild-type animals, whereas the effect of MPD on DA reuptake is independent of α-syn. We suggest that this selectivity is based on α-syn-dependent compartmentalization of vesicular storage pools.

Materials and Methods

Animals

We used three mouse lines in this study. The C57BL/6J subpopulation originating from Charles River Wiga (Sulzfeld, Germany) was used as the wild-type controls (referred to as b6+/+). The first α-syn-deficient line was the C57BL/6JOlahsd subpopulation of mice (referred to as b6−/−), which carries a spontaneous chromosomal deletion of α-syn and multimerin-1 loci (Harlan Olac, Bicester, UK) (Specht and Schoepfer, 2001, 2004). The second α-syn-deficient line was B6;129X1-SneαmΔHou/J, a gene-targeted α-syn knockout line (Abeliovich et al., 2000). A pair of these mice (referred to as b6−/−ros and originally produced by Dr. A. Rosenthal, Rinat Neuroscience Corporation, San Francisco, CA) was obtained from The Jackson Laboratory (Bar Harbor, ME), cleaned by embryo transfer, and bred together with the other two lines in the Laboratory Animal Centre at Kuopio, Finland. Animals were housed in controlled conditions at 21°C, 50 to 60% humidity, and 12-h day/night cycle. Food and water were provided ad libitum. The age of the animals used in this study was between 3 and 5 months. All experiments were conducted according to the guidelines of the Council of Europe (Directive 86/609) and the Finnish guidelines approved by the State Provincial Office of Eastern Finland.

In Vivo Voltammetry

Surgery. Six mice from each group were anesthetized with intraperitoneal administration of 450 mg/kg chloral hydrate (Sigma-Aldrich, St. Louis, MO) in a volume of 10 ml/kg. Anesthesia was maintained by a supplementary dose of 100 mg/kg every 45 to 60 min. Rectal temperature was constantly monitored and maintained at 37°C by using a heating lamp. Animal was fixed in a stereotaxic frame. Openings were made in the skull, and a carbon fiber working electrode was lowered to the following coordinates: AP, 1.42 mm; ML, 2.0 mm; dorsoventral, 3.3 mm in reference to bregma according to the brain atlas of Franklin and Paxinos (2007). A bipolar stimulating electrode (diameter 0.35 mm) was inserted into the medial forebrain bundle (MFb; AP, 1.6 mm, ML, 1.1–1.2 mm, ventral 5.1–5.3 mm from bregma). The exact depth of the stimulating electrode was adjusted for maximal DA release. An Ag/AgCl reference electrode was placed on the skull via a saline bridge. A stainless-steel screw fixed in the skull served as the auxiliary electrode.

Electrochemical Technique. A carbon fiber working electrode (Invilog Research, Kuopio, Finland), insulated with epoxy glue in a pulled glass capillary, was used to measure evoked DA release. The diameter of the carbon fiber was 30 μm, and the exposed length was in the range of 300 to 350 μm. Electrodes were postcalibrated after experiments with increasing concentrations of DA (1 μM in each step) in phosphate-buffered saline containing 400 μM ascorbic acid. The constant potential amperometry approach was used, because it is known to be a reliable method for measuring stimulated DA overflow (Dugast et al., 1994; Yavich, 1996). It is also faster and less sensitive to local pH shifts than fast-scan cyclic voltammetry (Kawago and Wightman, 1994). A potentiotstat (Invilog Voltammetry System; Invilog Research) maintained the working electrode at 0.4 V versus Ag/AgCl reference electrode. Data from the potentiotstat were digitized and analyzed offline by using Invilog Voltammetry System software.

Experimental Protocol. The MFB was stimulated by using a bipolar stimulating electrode and a computer-controlled interface of the Invilog Voltammetry System. Constant current was delivered by the same system or the stimulus isolation unit (World Precision Instruments, Aston, UK) and maintained at 200 to 250 μA. Two-second bursts of 1-ms bipolar pulses at 10 to 50 Hz were delivered to the MFB with a 3- to 4-min interval. Bursts at 10 to 30 Hz were delivered randomly, whereas the 50-Hz stimulation was always delivered after delivering the stimulation at lower frequencies. Repeated-burst stimulation consisted of six 2-s bursts at 50 Hz, delivered at an interstimulus interval of 5 s. High-frequency stimulation at intervals of a few seconds reveals a specific form of the short-term plasticity of neurotransmitter release (for review, see Rizzoli and Betz, 2005). This form of plasticity is attributed to the interaction between synaptic vesicle pools and has been demonstrated in ex vivo studies (Richards et al., 2003) and in vivo studies (Yavich, 1996). Low-frequency stimulation (2–20 Hz) releases only docked vesicles or the vesicles from the RRP (Richards et al., 2003). Much longer stimulation (e.g., 30-Hz, 40-s stimulation; Richards et al., 2003) or stronger high-frequency stimulation (e.g., 60 Hz, 900 pulses; Venton et al., 2006 or repeated 50-Hz, 2-s stimuli at short intervals; Yavich, 1996) is required to mobilize or deplete the reserve pool (Rizzoli and Betz, 2005). Therefore, high-frequency stimulation recruits the reserve pool and reveals an interaction between the RRP and the reserve pool. α-Syn is closely linked with the organization of vesicle pools (for review, see Venda et al., 2010), and high-frequency stimulation has demonstrated a distinct form of plasticity in mice lacking α-syn (Yavich et al., 2004). Therefore, we used repetitive high-frequency stimulation to investigate the effects of MPD in mice lacking α-syn.

Methylphenidate hydrochloride (Sigma-Aldrich) was dissolved in saline and injected intraperitoneally at doses of 1 or 5 mg/kg in a volume of 10 ml/kg. A specific dopamine reuptake inhibitor, 1-[2-[[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine (GBR12909)
Kinetic Analysis of DA Overflow and Reuptake

The extracellular DA levels in the terminal fields after brief electrical stimulation are a result of the balance between release and reuptake and can be described by the Michaelis-Menten equation (Wightman and Zimmerman, 1990):

\[ \frac{d[DA]}{dt} = \frac{[DA]_p \times f}{V_{max} / [DA] + 1} \]  

(1)

where \([DA]\) represents instantaneous DA concentration, \([DA]_p\) indicates the rate of reuptake, \([DA]_p\) indicates DA release per stimulus pulse, \(f\) is the frequency of stimulation, and \(V_{max}\) and \(K_m\) indicate the maximal rate of reuptake and apparent affinity, respectively.

After the end of stimulation, the change in DA concentration can be described by

\[ \frac{d[DA]}{dt} = -\frac{V_{max}}{K_m / [DA]} + 1 \]  

(2)

because the release component is absent. Michaelis-Menten-based analysis is a well-established method for estimating the parameters of DA release and reuptake (Kawagoe et al., 1992; Wu et al., 2001). We used a custom-written algorithm using LabView 5.5 (National Instruments, Austin, TX) to solve eqs. 1 and 2 with Euler’s approach and to find the best fit between experimental data and the data simulated by the model. The validity of the model was verified by delivering stimulation at different frequencies and by pharmacological means (see Supplemental Material). The validation proved that Michaelis-Menten-based model accurately simulated DA signals and showed a high level of correlation (\(r^2 = 0.97\)) with the real recordings, especially at higher frequencies. The best-fit parameters of \([DA]_p\), \(K_m\), and \(V_{max}\) for wild-type mice were in close agreement with the values reported in other studies (Wu et al., 2001).

In Vivo Microdialysis: Cannula Implantation

Eight male mice from each group were used in the experiments. Mice were anesthetized with a 1.5:1 mixture of ketamine (50 mg/ml; Intervet International B.V., Boxmeer, The Netherlands) and medetomidine (1 mg/ml; Orion Pharma, Turku, Finland; 2 ml/kg i.p.). In addition, lidocaine (10 mg/ml; Orion Corporation, Oulu, Finland) was applied to the scalp. A custom-made microdialysis cannula with an active membrane length of 2 mm (Hospal Industrie, Mezieres, France) was implanted into the right dorsal striatum (AP, +0.7 mm; ML, −1.8 mm; ventral, −3.9 mm from bregma) and secured by cranial screws and dental cement. Atipamezole hydrochloride (0.5 mg/kg s.c.; Orion Pharma) was used as an antioxidative agent, and cariprofen (5 mg/kg s.c.; Vericore Ltd, Dundee, UK) was used for postoperative pain relief. Saline (1 ml i.p.) was injected after surgery to maintain fluid balance.

In Vivo Microdialysis: Protocol and Analytical Procedure

The microdialysis cannula was connected to a rotating liquid swivel (Instech Laboratories, Plymouth Meeting, PA) 3 days after implantation. Artificial cerebrospinal fluid (145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, and 1.0 mM MgCl₂) was perfused at the rate of 2.2 µl/min (CMA/100 Microinjection Pump; CMA Microdialysis, Solna, Sweden). Perfusion was continued for 2 h, and four baseline samples were collected. After collection of the baseline samples, the mouse was injected first with saline (10 ml/kg i.p.). After 1.5 h, the mouse was injected a second time with MPD (1 mg/kg as described above), and samples were collected for the next 3 h. The dialysate was introduced online into the high-performance liquid chromatography injection loop and automatically injected every 15 min. Dopamine concentrations were measured from microdialysis samples by using high-performance liquid chromatography with electrochemical detection as described previously (Ihalainen and Tanila, 2004). After the experiment, the brain was removed from the skull and immersed in 4% paraformaldehyde. Coronal sections (50 µm) were cut and stained with cresyl violet for verification of the cannula placement.

Data Analysis and Statistical Procedures

Stimulated dopamine overflow was measured in molar concentrations based on the postcalibration of carbon fiber electrodes. For comparison of the effects of MPD and GBR12909 on peak DA overflow after single-burst stimulation at different frequencies, data were expressed as a percentage of the predrug levels. For analysis of the drug effects on the facilitation of DA overflow after repeated-burst stimulation, DA overflow after the first burst stimulation was expressed as 100%, regardless of whether the stimulation was delivered before or after drug. Peak amplitudes of stimulated DA overflow were measured by using Invilog Voltammetry System software. Changes in peak DA overflow (see Fig. 1) and extracellular DA levels (see Fig. 6) before and after the treatment were analyzed by two-way analysis of variance for repeated measures (RM-ANOVA) with two within-subjects (treatments) and three between-subjects (genotypes) factors. The effects of MPD and GBR12909 on DA overflow after repeated-burst stimulation were compared by RM-ANOVA with 12 within-subjects (two treatments × six bursts) and 3 between-subjects (genotypes) factors (see Figs. 2 and 3). Percentage increase in DA overflow at different frequencies of stimulation was analyzed by two-way RM-ANOVA with Bonferroni post hoc test (see Table 1). The effects of MPD (5 mg/kg) and GBR12909 (10 mg/kg) treatments were analyzed by two-way RM-ANOVA with Bonferroni tests for multiple comparisons (see Fig. 4). The effects of MPD (1 mg/kg) on \(K_m\), and \([DA]_p\) (see Fig. 5) were analyzed by two-way RM-ANOVA followed by Bonferroni post hoc tests. Data are presented as mean ± S.E.M.

Results

α-Synuclein-deficient Mice Exhibit a Blunted Response to Methylphenidate.

MPD at the dose of 1 mg/kg significantly increased DA overflow in both mice with (b6+) and without (b6−, b6−;ro) α-syn (Fig. 1). The main effect of the treatment in the three genotypes was significant (\(F_{1,15} = 7.1; p = 0.017\)); however, the genotype × treatment interaction was not significant (\(F_{2,28} = 0.8; p = 0.57\)). The effect of MPD was frequency dependent, with the maximal increase seen at 30-Hz stimulation (two-way RM-ANOVA; \(F_{2,15} = 51; p = 0.0001; n = 6\) per group). Table 1 shows the results from Fig. 1 as a percentage of the predrug levels. At the 10-Hz stimulation (Fig. 1; Table 1), the increase in DA overflow was significantly lower in b6− (\(p = 0.017\)) and b6−;ro (\(P = 0.011\)) mice than in b6+ mice (two-way RM-ANOVA, Bonferroni post hoc test).

Effects of Methylphenidate on DA Overflow after Repeated-Burst Stimulation Depend on the Presence of α-Synuclein.

Repeated-burst stimulation of the MFB at frequencies of 20 to 50 Hz facilitates DA overflow in the dorsal striatum in mice and rats in such a way that more DA is released after subsequent stimuli than that observed after the first stimulus (Yavich, 1996; Yavich and MacDonald, 2000; Kita et al., 2007). A working model shown in Fig. 7 (1a, 1b, 2a, and 2b) illustrates a simplified version of the mechanism underlying the facilitation of DA overflow after single and repeated-burst stimulation. Fig. 7, 1a shows that single-burst stimulation releases DA primarily from the RRP, whereas the second burst (Fig. 7, 1b) recruits also the recycling/reserve pool, contributing to increased DA overflow after repeated-burst stimulation. Consistent with an earlier
study (Yavich et al., 2004; Chadchankar and Yavich, 2011), b6− and b6–ros mice lacking α-syn demonstrated enhanced facilitation of DA overflow after repeated-burst stimulation. Figure 2, A and B shows the examples of this facilitation in a b6− mouse before and after MPD treatment, respectively. A comparison of mean peak DA overflow values in molar concentrations before and after MPD treatment for each genotype is shown in Fig. 2, C to E. The effects of MPD at the dose of 1 mg/kg on the facilitation of DA overflow were different in the three mouse lines (two-way RM-ANOVA; \(F_{2,15} = 4.5; p = 0.019; n = 6\) per group), such that MPD significantly decreased the facilitation of DA overflow in the two α-syn deficient lines while having no effect in b6+ mice. Bonferroni post hoc test revealed a significantly different effect of the treatment in b6− (\(p = 0.024\)) and b6–ros (\(p = 0.026\)) mice in comparison with b6+ mice. However, after MPD treatment the facilitation of DA overflow in α-syn deficient mice was “normalized,” i.e., it became comparable with that observed in control mice before MPD treatment (Fig. 2, C-E; no statistical difference was observed between untreated control mice and MPD-treated α-syn-deficient mice; two-way RM-ANOVA; \(F_{2,15} = 8.3; p = 0.74\)).

### Reuptake Dependent- and -Independent Mechanisms of Action of Methylphenidate

The facilitation of DA overflow during repeated-burst stimulation depends on the status of the RRP (Yavich, 1996; Yavich and MacDonald, 2000; Yavich et al., 2004), which is replenished by reuptake (Stevens and Wesseling, 1998; Richards et al., 2003). Newly synthesized DA also replenishes the RRP (Jones et al., 1998), although the contribution of synthesis is small in the 5-s time scale associated with repeated-burst stimulation. This led us to speculate that the differential effects of MPD on the burst-induced facilitation of DA overflow in mice with and without α-syn can be derived from the differences in the effects of

![Fig. 1. Effect of methylphenidate (1 mg/kg) on evoked dopamine overflow in control and α-syn-deficient mice. A, amperometric recordings of evoked DA overflow after stimulation at different frequencies in b6− mice. The bold line shows the recording made before drug, and the dotted line shows the recording made after MPD treatment. A black line below each recording indicates the duration of electrical stimulation. The recordings show that MPD administration increased evoked DA overflow. B to D, peak DA overflow (mean ± S.E.M.) in b6− (B), b6− (C), and b6–ros (D) mice before and after MPD administration at varying frequencies of stimulation. Data are shown in molar concentrations (logarithmic scale) based on the postcalibration of working electrodes. The increase in DA overflow after MPD administration was significantly lower in b6− and b6–ros mice than in b6+ mice after 10-Hz stimulation of the MFB (A). For statistics, see Results and Table 1.](image-url)

### Table 1

<table>
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<th>b6+</th>
<th>b6−</th>
<th>b6–ros</th>
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<tr>
<td><strong>Frequency of Stimulation</strong></td>
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<tr>
<td>10 Hz</td>
<td>217 ± 21*</td>
<td>127 ± 16#</td>
<td>121 ± 13#</td>
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<td>20 Hz</td>
<td>231 ± 38*</td>
<td>214 ± 51*</td>
<td>201 ± 40*</td>
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<td>30 Hz</td>
<td>297 ± 41*</td>
<td>243 ± 59*</td>
<td>312 ± 72*</td>
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<td>50 Hz</td>
<td>166 ± 27*</td>
<td>111 ± 17</td>
<td>132 ± 21</td>
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* \(p < 0.05\), significant difference from predrug levels within genotype.
# \(p < 0.05\), significant difference from wild-type mice at the same frequency of stimulation.
MPD on reuptake. If this is the case, other reuptake inhibitors should demonstrate the effects similar to that of MPD.

We first compared the effects of GBR12909 (10 mg/kg), a specific DA reuptake inhibitor, and MPD (1 mg/kg) on the dynamics of DA overflow during repeated-burst stimulation. The normalization approach used for this comparison is illustrated in Fig. 3 by the examples of the original recording of DA overflow after repeated-burst stimulation in b6+ mice before treatment (A), after MPD treatment (B), and after GBR12909 treatment (C). These examples show that both drugs increased DA overflow after the first stimulation burst; however, their effects after the subsequent bursts were different. GBR12909 completely eliminated the facilitation of DA overflow, and the effect of GBR12909 was not different in the three lines (Fig. 3D; two-way RM-ANOVA; the main effect of treatment, \( F_{2,15} = 15.04, p = 0.6 \)). In contrast, MPD

**Fig. 2.** Stimulated dopamine overflow after repeated-burst stimulation before and after MPD (1 mg/kg) in mice with and without \( \alpha \)-syn. A and B, representative amperometric recordings of DA overflow before (A) and after (B) MPD treatment from a b6− mouse. Six 2-s stimulus bursts at 50 Hz were delivered to the MFB at 5-s intervals. C to E, DA overflow (mean ± S.E.M) in molar concentrations after six repeated bursts of stimulation before and after MPD in b6+ (C), b6− (D), and b6−ros (E) mice. Before drug treatment, both mouse lines lacking \( \alpha \)-syn displayed enhanced facilitation of dopamine overflow. MPD significantly decreased the facilitation of DA overflow in mice lacking \( \alpha \)-syn (see Results for statistics) but did not change it in b6+ mice.

**Fig. 3.** Effects of MPD (1 mg/kg) and GBR12909 (10 mg/kg) on dopamine overflow during repeated-burst stimulation in mice with and without \( \alpha \)-syn. A to C, representative amperometric recordings of DA overflow in both molar concentrations and percentages after repeated-burst stimulation from b6+ mice before any drug (A), after 1 mg/kg MPD (B), and after 10 mg/kg GBR12909 (C). In each case, evoked DA overflow after the first stimulation burst is expressed as 100%. D, mean data (mean ± S.E.M.) on the effects of the drugs on DA overflow after repeated-burst stimulation as a percentage of DA overflow produced by the first burst stimulation. Shown is change in DA overflow in the three lines before any drug and after 1 mg/kg MPD and 10 mg/kg GBR12909. Note that MPD preserved the facilitation of DA overflow, which was abolished by GBR12909 in all three lines (see Results for statistics).
preserved the facilitation of DA overflow in b6+ mice (see Fig. 3D for statistical mean; also see Fig. 3, A-C for the examples of the original recordings) and decreased but did not disrupt the facilitation of DA overflow in α-syn-deficient mice. The drug effects were significantly different from each other in all three lines (two-way RM-ANOVA; $F_{2,15} = 25.0; p = 0.0001$).

Thus, reuptake is a crucial factor in the facilitation of DA overflow after repeated-burst stimulation, because reuptake inhibition blocks the facilitation (Chadchankar and Yavich, 2011). The specific reuptake inhibitor GBR12909 disrupted the facilitation of DA overflow similarly in all three lines, whereas MPD decreased the facilitation only in mice without α-syn. However, this ostensible selectivity of MPD in α-syn-deficient mice may be dose-dependent so that equipotent doses of these two drugs (in terms of their effects on reuptake) would similarly block the facilitation of DA overflow.

To test this possibility, we increased the dose of MPD to 5 mg/kg to produce a stronger reuptake inhibition. This dose was chosen based on pilot studies showing that 5 mg/kg of MPD and 10 mg/kg of GBR12909 similarly increased evoked DA overflow (Fig. 4A; two-way RM-ANOVA, Bonferroni tests for the effect of each drug on evoked DA overflow; MPD 5 mg/kg, $F_{1,7} = 29, p = 0.001$ and GBR12909 10 mg/kg, $F_{1,7} = 12, p = 0.017$). However, the effects of the two drugs on evoked DA overflow were not different (two-way RM-ANOVA; $F_{1,6} = 0.7; p = 0.49$; $n = 4–5$ per group) and produced the same degree of reuptake inhibition in terms of increasing the extracellular half-life of DA (data not shown).

Kinetic analysis of DA reuptake verified that indeed both drugs produced an approximately equivalent, 20-fold increase in the apparent affinity ($K_m$) for DA reuptake (Fig. 4B; two-way RM-ANOVA; $F_{1,6} = 88; p = 0.0001$). MPD (5 mg/kg) increased the $K_m$ from 0.25 ± 0.064 to 5.57 ± 0.96 μM, whereas GBR12909 (10 mg/kg) increased the $K_m$ from 0.33 ± 0.17 to 6.24 ± 0.78 μM. However, despite a comparable inhibition of reuptake, the effects of these two drugs on the facilitation of DA overflow were different (Fig. 4C; two-way RM-ANOVA; $F_{2,15} = 25.0; p = 0.0001$).

To understand the driving force preserving the facilitation of DA overflow after MPD treatment during reuptake inhibition (which disrupts facilitation), we analyzed the effect of DA reuptake inhibitor on DA release per stimulus pulse ([DA]$_p$). Kinetic analysis supported the most probable explanation for the above results: MPD (5 mg/kg) in control mice significantly increased [DA]$_p$ (two-way RM-ANOVA; Bonferroni test for the effect of MPD on [DA]$_p$; $F_{1,3} = 4.14; p = 0.025$), whereas GBR12909 did not change this parameter ($p = 0.29$) (Fig. 4D). We extended these calculations to the data obtained by MPD administration at the dose of 1 mg/kg in the three mouse lines. MPD, at this lower dose, also significantly increased [DA]$_p$ in control mice (two-way ANOVA; Bonferroni post hoc test; $F_{1,5} = 26.3; p = 0.004$). However, the treatment significantly decreased [DA]$_p$ in b6–ros mice ($F_{1,5} = 9.8; p = 0.011$) and approached significance in b6– mice ($F_{1,5} = 5.6; p = 0.064$) (Fig. 5A). A significant
Genotype \times treatment interaction was observed for the effect of MPD on [DA]p in the three lines (two-way RM-ANOVA; \( F_{2,15} = 15.4; p = 0.009 \)). However, MPD produced a similar unidirectional increase in \( K_m \) in all genotypes (two-way RM-ANOVA; the main effect of treatment; \( F_{2,1,13} = 39; p = 0.001 \); Fig. 5B). No genotype \times treatment interaction was observed for the effect of MPD (1 mg/kg) on \( K_m \) in the three lines (two-way RM-ANOVA; \( F_{2,1,15} = 12.1; p = 0.14 \)).

### Effects of Methylphenidate on Basal Extracellular Dopamine Levels

Next, we tested the effect of MPD (1 mg/kg) on the extracellular DA levels in the dorsal striatum by in vivo microdialysis. Consistent with our earlier study (Chadchankar et al., 2011), the basal DA levels differed between control and α-syn-deficient mouse lines (Fig. 6; two-way RM-ANOVA; the main effect of genotype; \( F_{2,22} = 51.42; p = 0.001 \)). A saline injection produced no significant change in the extracellular DA levels (Fig. 6). In contrast, MPD injected 90 min after saline significantly elevated the extracellular DA concentration in the dorsal striatum in all three mouse lines (two-way RM-ANOVA; the main effect of treatment; \( F_{1,1,21} = 14; p = 0.001; n = 7–8 \) per group), with the peak effect 30 min after drug injection. The percentage increase from the baseline (aggregated from the four baseline measurements) immediately before the drug injection in the extracellular DA levels was not different between the three lines (two-way RM ANOVA; genotype \times treatment interaction; \( F_{2,1,1} = 3.03; p = 0.74 \)) (Fig. 6, inset).

### Discussion

In this study, we demonstrate for the first time the α-syn-dependent selectivity of the effect of MPD on dopaminergic neurotransmission. MPD, at a therapeutically relevant dose of 1 mg/kg (Volkow et al., 2001), increased evoked DA overflow after single-burst stimulation in all three mouse lines. This effect was expected considering that MPD increases DA release (Volk et al., 2008) and inhibits reuptake (Pan et al., 1994). However, α-syn-deficient mice demonstrated an attenuated response to MPD, especially at the 10-Hz stimulation frequency. Previous studies have shown that stimulation at 2 to 10 Hz releases vesicles only from the RRP (Richards et al., 2003). In mice lacking α-syn, the balance between the RRP (the compartment most sensitive to reuptake inhibition) and reserve pool is altered (Cabin et al., 2002; Yavich et al., 2004; Lee et al., 2008; Fig. 7, 2a), which may in part explain the lower response to MPD. Another reason for the weaker effect of MPD in α-syn-deficient mice is the selective decrease of [DA]p (see Figs. 5 and 7, 4a). DA overflow is determined by the balance between reuptake and release (Wightman and Zimmerman, 1990). Because MPD inhibits reuptake similarly in all genotypes, decreased [DA]p may determine the lower peak DA overflow. In agreement with this, we did not find a significant effect of MPD on DA overflow at the 50-Hz stimulation in α-syn-deficient mice (Table 1). A recent study revealed that α-syn is directly involved in maintaining the SNARE-complex assembly during synaptic activity (Burre et al., 2010). The SNARE complex is important in mediating exocytotic DA release. Because MPD increases exocytotic DA release (Volz et al., 2008), deficits in α-syn-SNARE-complex interaction may play a role in the reduced DA overflow after MPD treatment, especially after high-frequency stimulation during which a small decrease in [DA]p leads to a significant overall decline in peak DA overflow (Table 1). In addition, MPD redistributes VMAT2 and the associated vesicles within the nerve terminal away from the synaptosomal membranes and into the cytoplasm (Volz et al., 2008). These data further support our finding that MPD decreased [DA]p in mice lacking α-syn. We speculate that the different effect of MPD on [DA]p, which is a reflection of DA release probability, can be a result of the altered compartmentalization of cytosolic DA in α-syn-deficient mice.

Repeated-burst stimulation of the ascending DA pathways at frequencies of 20 Hz or higher (Yavich and MacDonald, 2000) mobilizes DA vesicles from the recycling and reserve pool in presynaptic terminals in the striatum and facilitates DA overflow (Fig. 7, 1a and 1b). We showed previously that this form of facilitation relies on DA reuptake (Yavich et al., 2004; Chadchankar et al., 2011), which replenishes the RRP. Re-
uptake inhibition by GBR12909 disrupts the facilitation of DA overflow, MPD at both low (1 mg/kg) and high (5 mg/kg) doses did not influence the facilitation in b6+ mice. We have suggested that the mobilization of DA reserve pool and an increased refilling rate of the RRP contribute to the facilitation of DA overflow (Yavich and MacDonald, 2000). In line with this, when refilling of the RRP was inhibited by GBR12909, the facilitation of DA overflow was eliminated as well (Fig. 3, C and D). However, one may argue that the differential effects of the two drugs may be caused by their different potency as reuptake inhibitors at the doses used in this study. To eliminate this possibility, we performed kinetic analysis of DA reuptake. It revealed that both 5 mg/kg MPD and 10 mg/kg GBR12909 produced a comparable increase in $K_m$ (Fig. 4), but only MPD maintained the facilitation of DA overflow. Similar to other reuptake inhibitors (Wu et al., 2001), GBR12909 increased $K_m$ but not $V_{max}$. However, MPD increased DA release probability in wild-type mice. These results are consistent with an earlier finding showing that MPD increased the amplitude of DA release at a high dose of 40 mg/kg without an increase in the extracellular half-life of DA (Volz et al., 2007) and the suggestion that MPD enhanced DA release by increasing the vesicular DA content available for release (Volz et al., 2008).

We explain the effects of MPD on the facilitation of DA overflow as an interplay between increased $[DA]_p$ (see Figs. 4 and 7, 3a) and decreased reuptake. There was no net effect of MPD on the facilitation of DA overflow in wild-type animals (Fig. 7, 3b). Indeed, the facilitation of DA overflow after MPD tended to be lower at the higher dose of 5 mg/kg than at 1 mg/kg. We suggest that with increasing doses reuptake inhibition starts to dominate and weakens the action of MPD on the facilitation of DA release. However, MPD decreased reuptake as well as $[DA]_p$ in mice lacking $\alpha$-syn (Fig. 5); consequently, the facilitation of DA overflow decreased significantly in these animals (Fig. 7, 4b).

There are several possible explanations for this effect of MPD. Yavich and MacDonald (2000) showed that when VMAT2 is blocked by reserpine, no facilitation of DA overflow was observed. $\alpha$-Syn is known to directly interact with and modulate the activity of VMAT2 (Guo et al., 2008). Therefore, MPD may have different effects on VMAT2 function in the two genotypes. Although the molecular mechanism of MPD-mediated redistribution of vesicles and VMAT2 is unknown, indirect D2 receptor activation has been shown to reorganize DA vesicles after MPD treatment (Sandoval et al., 2002). Organization of vesicles depends on the cytoskeletal tethering of vesicles and presynaptic proteins, which modulate vesicle trafficking in the nerve terminal. $\alpha$-Syn plays an important role in vesicle trafficking, and changes in the presynaptic proteins regulating vesicle trafficking have been reported in mice lacking $\alpha$-syn (Murphy et al., 2000). This may be another cause of the different effect of MPD on vesicle redistribution in these mice.

An earlier study (Chadhankar et al., 2011) reported a modest reduction in $V_{max}$ in b6–ros mice. Reuptake is the main
factor contributing to the extracellular DA levels measured by microdialysis (Jones et al., 1998). Despite the elevated basal extracellular DA levels in α-syn-deficient mouse lines, MPD produced a similar (~40%) increase in the extracellular DA levels in all three lines (see Fig. 6, inset). This suggests that the difference in reuptake does not explain the blunted effect of MPD on DA overflow in α-syn-deficient mice. These data further support our conclusion, based on the results of in vivo voltammetry experiments, that MPD affects DA release but not reuptake in an α-syn-dependent manner. Intriguingly, an imaging study (Volkow et al., 2002) in humans showed that for the same level of DAT occupancy by MPD, increases in DA release of 40% occurred in aging study (Volkow et al., 2002) in humans showed that for the same level of DAT occupancy by MPD, increases in DA release.

Because the protocol of repeated-burst stimulation was not the same for all three lines (see Fig. 6, inset). This suggests that the difference in reuptake does not explain the blunted effect of MPD on DA overflow in α-syn-deficient mice. These data further support our conclusion, based on the results of in vivo voltammetry experiments, that MPD affects DA release but not reuptake in an α-syn-dependent manner. Intriguingly, an imaging study (Volkow et al., 2002) in humans showed that for the same level of DAT occupancy by MPD, increases in DA release.

Conclusions

We show that α-syn is a protein that modulates the action of MPD via a presynaptic mechanism of compartmentalization of DA, possibly by interacting with the vesicles comprising the reserve pool of DA. There are two components in the pharmacological spectrum of MPD in relation to DA neurotransmission: the first one is an α-syn-dependent action on DA release, and the second is an α-syn-independent action on DA reuptake.

Authorship Contributions

Participated in research design: Chadchankar and Yavic. Conducted experiments: Chadchankar and Ihalainen. Performed data analysis: Chadchankar and Yavic. Wrote or contributed to the writing of the manuscript: Chadchankar, Ihalainen, Tanila, and Yavic.

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Methylphenidate modifies overflow and pre-synaptic compartmentalization of dopamine via an alpha-synuclein dependent mechanism

Heramb Chadchankar, Jouni Ihalainen, Heikki Tanila, Leonid Yavich

Supplemental Data

Validation of the Michaelis-Menten based model for analysis of the kinetic parameters of dopamine neurotransmission

Michaelis-Menten based analysis of the kinetic parameters of dopamine (DA) release and re-uptake has been described in detail previously (Kawagoe et al., 1992; Wu et al., 2001). In this study, we reproduced the model. The mathematical basis for the model is explained in the manuscript.

We measured stimulated DA overflow in wild-type C57BL/6J mice (b6+, control) by stimulating the medial forebrain bundle (see Methods). The values reported here were obtained from the dorsal striatum of anesthetised b6+ mice.

Figure 1

Figure 1. Fit of the simulated DA signal to the actual, in vivo amperometric recording from the dorsal striatum. The black line indicates simulated DA signal and the dashed line show real amperometric recording of evoked DA overflow. Consistent with earlier studies (Kawagoe et al., 1992), simulated DA signal showed higher correlation ($r^2$) with the experimentally recorded DA signal with increasing frequency of stimulation. Real amperometric recording and its fit are shown for varying stimulation frequencies. The length of the stimulation burst was 2 s at all frequencies and is indicated by a bar below each recording. Note that the y-axis scale for DA concentrations is different for each recording.
Table 1

<table>
<thead>
<tr>
<th>Frequency of stimulation (Hz)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA release per pulse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[\text{DA}]_p$ (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>193 ± 23</td>
<td>142 ± 24</td>
<td>121 ± 26</td>
<td>103 ± 12</td>
<td>83 ± 14</td>
</tr>
</tbody>
</table>

Table 1. Change in DA release per pulse ($[\text{DA}]_p$) in response to increasing frequency of stimulation. Consistent with earlier studies (Kawagoe et al., 1992; Garris and Wightman, 1994), $[\text{DA}]_p$ at frequency of 10 Hz was significantly greater than $[\text{DA}]_p$ at all other frequencies.

Earlier studies have shown that re-uptake inhibitors increase extracellular DA concentration by decreasing the affinity of the DAT for DA, i.e., by increasing $K_m$. On the other hand, D2 autoreceptor antagonists are known to increase DA release per pulse without changing the affinity for DA re-uptake. We administered the DA re-uptake blocker GBR 12909 (10 mg/kg, as described in the Methods) and recorded stimulated DA overflow 45 min after the treatment. The effect of intraperitoneally injected D2 antagonist, haloperidol (0.5 mg/kg) was analysed 15 min after the treatment. The values of kinetic parameters obtained by this analysis under control and treatment conditions are shown below in Table 2.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ µM/s</th>
<th>$K_m$ µM</th>
<th>$[\text{DA}]_p$ nM</th>
<th>Number of animals (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.4 ± 0.84</td>
<td>0.31 ± 0.08</td>
<td>103 ± 12</td>
<td>15</td>
</tr>
<tr>
<td>GBR12909 (10 mg/kg)</td>
<td>5.9 ± 1.04</td>
<td>4.69 ± 0.47</td>
<td>108 ± 6.2</td>
<td>6</td>
</tr>
<tr>
<td>Haloperidol (0.5 mg/kg)</td>
<td>2.8 ± 1.63*</td>
<td>0.45 ± 0.12</td>
<td>113 ± 10</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2. The effect of drug treatments on the kinetic parameters of DA release and re-uptake in control mice.

The best fit parameters for GBR12909 show that it increased the $K_m$ for DA re-uptake similar to other re-uptake inhibitors (Wu et al., 2001). GBR 12909 had no effect on $[\text{DA}]_p$, consistent with an earlier study showing that the drug increases $K_m$ but does not have a dopamine releasing action (Budygin et al., 1999). The D2 antagonist haloperidol produced no change in $K_m$. However, haloperidol decreased $V_{\text{max}}$ in the dorsal striatum and showed a slight increase in $[\text{DA}]_p$. A similar phenomenon has been reported in rat dorsal striatum (Wu et al., 2002).
These data indicate that our model for analysis of kinetic parameters of DA release and re-uptake was able to estimate these parameters in agreement with the values reported in literature.

References:


