The effects of methylphenidate on knock-in mice with a methylphenidate resistant dopamine transporter

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Abstract

Methylphenidate (Ritalin™) is one of the most commonly abused prescription drugs. It is a psychostimulant that inhibits the dopamine and norepinephrine transporters with high affinity. In mice, methylphenidate stimulates locomotor activity, is self-administered, and produces conditioned place preference - typical properties of an addictive drug. We have generated a knock-in mouse line bearing a mutant dopamine transporter that is approximately 80-fold less sensitive to cocaine-inhibition than wild type. Interestingly, this mutant is also almost 50-fold less sensitive to methylphenidate-inhibition, suggesting similarities in the binding site for cocaine and methylphenidate. Since methylphenidate is not effective at inhibiting the mutant dopamine transporter, we hypothesized that it would not stimulate locomotor activity or produce reward in the knock-in mice. In these knock-in mice, doses up to 40 mg/kg methylphenidate either inhibit or fail to stimulate locomotor activity, and do not produce conditioned place preference. Doses up to 40 mg/kg methylphenidate also fail to produce stereotypy in the knock-in mice. Nisoxetine and desipramine, selective norepinephrine transporter inhibitors, also reduce locomotor activity in wild type and knock-in mice. These results indicate that enhanced dopaminergic neurotransmission is required for methylphenidate’s stimulating and rewarding effects. In addition, we observed that drugs enhancing noradrenergic neurotransmission inhibit locomotor activity in mice, which is consistent with the notion that methylphenidate’s ability to inhibit the norepinephrine transporter may contribute to its efficacy in treating attention deficit hyperactivity disorder.
Introduction

Low doses of stimulants such as amphetamine and methylphenidate (Ritalin™) reduce activity levels in hyperactive individuals, and may increase their ability to focus. Therefore, methylphenidate is commonly used to treat attention-deficit/hyperactive disorder (ADHD). However, prescription psychostimulants, such as methylphenidate, are commonly abused among high school students and young adults (Teter et al., 2006) (Kroutil et al., 2006). Of these, methylphenidate is the most commonly abused, with up to 25% of college students reporting having used the drug at least once for nonprescription purposes (Teter et al., 2006) (Kroutil et al., 2006). While the most common nonprescription use is to improve academic performance or to improve concentration, 31% of abusers report using the drug to achieve a high (Teter et al., 2006) and over-use may result in dependence (Kollins et al., 2001). Therefore, a better understanding of how methylphenidate produces both its therapeutic and detrimental effects is crucial.

The mechanism by which low doses of methylphenidate reduce activity and increase the ability to focus is unknown. Mechanistically, methylphenidate is a high affinity inhibitor of the dopamine (DA) and norepinephrine (NE) reuptake transporters (DAT and NET, respectively) (Han and Gu, 2006). Studies have shown that methylphenidate is not effective at inhibiting the serotonin transporter (SERT) (Han and Gu, 2006) and that it does not affect serotonergic tone (Kuczenski and Segal, 1997). Therefore, methylphenidate’s beneficial effects are likely due to its ability to elevate dopaminergic and/or noradrenergic signaling. (Berridge et al., 2006) (Goodman et al., 2006) (Volkow et al., 2002b) (Volkow et al., 2002a).
There is a great deal of evidence that, of these two neurotransmitter systems, the dopaminergic system plays an important role in drug-induced locomotor stimulation and reinforcement. Most, if not all, addictive drugs elevate dopaminergic signaling (Wise and Bozarth, 1987) (Di Chiara and Imperato, 1988). Studies using mouse models with DAT deleted or mutated also show the importance of DAT in mediating the effects of psychostimulants (Giros et al., 1996) (Sora et al., 1998) (Rocha et al., 1998) (Chen et al., 2006). However, there is evidence that the noradrenergic system is an important modulator of drug effects as well. For example, antagonists to the alpha 1-adrenergic receptor reduce locomotor stimulation in response to amphetamine (Zhang and Kosten, 2005). Knockout mice lacking the alpha-1b-adrenoreceptor are less sensitive to the effects of addictive drugs, including the ability of amphetamine to trigger the release of DA in the nucleus accumbens (Drouin et al., 2002b) (Drouin et al., 2002a) (Auclair et al., 2002). Noradrenergic neurons can stimulate release of DA (Jones et al., 1977) (Liprando et al., 2004) and may exert some of their effects through modulating DA release. Norepinephrine in the frontal cortex has been shown to play an important role in the locomotor and rewarding effects of amphetamine and cocaine (Auclair et al., 2004) (Ventura et al., 2003) (Ventura et al., 2007). In addition, NET knockout mice show increased cocaine CPP (Xu et al., 2000). Also, NE has been shown to play roles in psychostimulant reinstatement (Davis et al., 1975) (Lee et al., 2004) (Leri et al., 2002).

Previously, we have reported the creation of a triple mutant of mouse DAT (DAT\textsuperscript{vcv}) that is resistant to cocaine inhibition (Chen et al., 2005). We then generated DAT-CI mice carrying the cocaine resistant DAT (Chen et al., 2006). We have shown that DAT-CI mice no longer display cocaine-induced conditioned place preference and that cocaine inhibits locomotor activity (Chen et al., 2006). Interestingly, the mutant
DAT\textsubscript{cv}v is also less sensitive to methylphenidate. Therefore, DAT-CI mice also provide a unique mouse model in which methylphenidate has markedly reduced potency on DAT. We will be able to separate the methylphenidate effects mediated through DAT inhibition from those mediated through actions on NET and other possible targets. Our data suggest that methylphenidate inhibition of DAT is necessary for its rewarding and stimulating effects, while its inhibition of NET is necessary for its suppression of locomotor activity. This finding gives important insight into the mechanism of methylphenidate treatment of ADHD.

Methods:

Animals

The DAT-CI mice were generated by homologous recombination in 129/SvJ embryonic stem cells as previously described (Chen et al., 2006). The mutant mice have been backcrossed to C57BL/6J mice for 10 or more generations and thus they are generally considered to be in the C57BL/6J background. The heterozygous male and female DAT-CI mice from the 10th backcrossing were bred to produce littermates of homozygous DAT-CI mice and wild type mice. From this population, male and female homozygous DAT-CI mice were bred to produce the mutant mice used in the experiments and wild type male and female mice were bred to produce the control mice. Only male mice were used in the experiments. Animals were provided food and water \textit{ad libitum}. Housing and all protocols and procedures were approved by the Ohio State University Laboratory Animals Resource. All animals were drug naïve and used only once in one experiment and with a single drug dose.
Drugs

Methylphenidate, desipramine and nisoxetine were purchased from Sigma (St. Louis, MO). All drugs were dissolved in 0.9% NaCl and delivered by interperitoneal injection (i.p.).

Synaptosome assays

Synaptosomes were prepared from fresh mouse striata by homogenization in Krebs-Ringers buffer containing 0.32 M sucrose. The samples were centrifuged for 5 minutes at 1,000 X g and the pellet was discarded. The remaining supernatant was centrifuged for 10 minutes at 12,000 X g and the pellet was resuspended in 0.32 M sucrose. Aliquots of the synaptosomes were added to Krebs-Ringers solution containing 1-2 µM [3H] labeled DA (PerkinElmer). DA uptake in the presence of increasing concentrations of methylphenidate was performed using methods described in (Chen et al., 2006). IC50 values were determined by nonlinear regression using Prism (Graphpad, LaJolla, California).

Locomotor Activity

The ability of methylphenidate to stimulate locomotor activity in DAT-CI mice was tested using an open field protocol in acrylic boxes 25 cm (W) x 25 cm (L) X 25 cm (H). Mouse activity was monitored by Lime Light (Evanston, IL), a video recording system. Mice were allowed to habituate to the locomotor chambers for one hour. Following habituation, the mice were injected (i.p.) with either drug or vehicle (saline), returned to the chamber, and their activity was recorded for 30 minutes.
Stereotypy

Vertical and horizontal activity was measured by beam-breaks using the Versamax system (Columbus, OH). Animals were habituated to the chambers for 30 minutes prior to testing. Animals were then injected with drug or vehicle and behavior was monitored for 30 minutes. The Versamax system produced the scores of stereotypy, stereotypy counts, number of stereotypy sessions, stereotypy time, number of rearing, and number of revolutions mice circled. Mice were carefully observed after they were placed into the behavior test chambers. Licking or gnawing times (sec) were scored manually and analyzed separately.

Conditioned Place Preference

The ability of methylphenidate to produce reward was assessed by its ability to produce CPP. For each round of experiments, about an equal number of DAT-CI mice and wild type mice were tested at the same time. The CPP boxes consist of 3 chambers with a gray middle chamber and two end chambers with different visual (wavy lines vs. thick gridlines), tactile (fine-mesh vs. thick-mesh floor mat) and olfactory (tea vs. coffee) cues. The cues were arranged into two groups – one with wavy lines, fine-mesh flooring, and tea and the other with thick gridlines, thick-mesh flooring, and coffee. Stocks were made by soaking 5 grams of either tea or coffee in 100 ml of hot (95 °C) water for 10 minutes, while agitating. The olfactory cues were produced by soaking the floor mats in a 1:100 dilution of either coffee or tea and allowing the liquid to dry on the mat. The treated mats were covered with an untreated mat to prevent the animals from coming into direct contact with the olfactory cue. On day 1 between 9 to 11 a.m., the mice were placed into the box and allowed free-access to all chambers for 30 minutes.
as a pre-conditioning test. The time spent in each chamber was recorded using Lime Light.

The mice were then assigned to either an experimental group that received alternating injections of vehicle and methylphenidate or to a control group that received vehicle for both injections. The drug-paired chamber was randomly assigned, and conditioning was achieved by associating drug with this cue set, and saline with the opposite cue set. During the conditioning phase, two injections were given each day. The first injection (saline) occurred in the morning between 9 a.m. and 11 a.m. and the second injection (methylphenidate, or saline for controls) occurred about in the afternoon between 2 p.m. and 4 p.m. Immediately after drug or vehicle injection, mice were confined to their assigned chamber for 30 minutes and then returned to their home cages. The conditioning was performed for 3 consecutive days (day 2 to day 4). On the test day (day 5) between 9 to 11 a.m., the mice were placed into the middle chamber and allowed free-access to all chambers for 30 minutes. The post-conditioning time spent in each chamber was recorded.

Statistic Analysis

SPSS software (SPSS Inc. Chicago, Illinois) was used to perform statistical analyses of the data. For each behavior measurement, an overall two-way ANOVA was performed first and when significant differences were observed (p < 0.5), one-way ANOVAs were performed to examine the drug effects within each genotype.

Results

Synaptosomes
Previous studies had indicated that DAT\textsubscript{vcv}, the mutant DAT in DAT-CI mice, was 50-fold less sensitive to methylphenidate inhibition \textit{in vitro}. In order to assess if DAT\textsubscript{vcv} retained its reduced sensitivity to methylphenidate \textit{in vivo}, we tested the ability of methylphenidate to inhibit DA uptake in DAT-CI mice. Figure 1 shows representative curves for methylphenidate inhibition of DA uptake into striatal synaptosomes made from wild type and DAT-CI mice. There was an approximate 20-fold shift in the K\textsubscript{i} of methylphenidate between wild type and DAT-CI mouse synaptosomes (p < 0.01, T-test, n = 8 each group). In wild type mice, the K\textsubscript{i} was 210 nM while in DAT-CI mice the K\textsubscript{i} was 3800 nM.

**Locomotion**

The ability of psychostimulants to elevate dopaminergic tone is believed to be critical for their stimulant properties. We tested the effect of methylphenidate on the locomotor activity of DAT-CI mice. We first analyzed the overall effect by two-way ANOVA and found the main effect to be by genotype (F\textsubscript{1, 67} = 83.8, p < 0.001), drug (F\textsubscript{4, 67} = 16.783, p < 0.001) and a significant effect of a drug X genotype interaction (F\textsubscript{4, 67} = 36.7, p < 0.001), thus indicating that there are significant differences between the genotypes, drug doses and how the genotypes respond to the drug. Next, one-way ANOVAs were used to look for within-genotype effects. As seen in Figure 2A, methylphenidate does not stimulate locomotor activity in DAT-CI mice; instead it has no effect or reduces locomotor activity, depending on the dose (F\textsubscript{4, 33} = 16.008, p < 0.001). Post hoc Bonferroni test revealed that in DAT-CI mice, 2 mg/kg methylphenidate and 40 mg/kg methylphenidate seemed to have little to no effect on locomotor activity (p > 0.05). However at the dose of 10 mg/kg, methylphenidate significantly reduced
locomotor activity (p < 0.01), while the reduction in response to the 20 mg/kg dose was not significant (p = 0.12). In contrast, methylphenidate is a strong stimulator of locomotor activity in wild type mice (F 4, 32 = 27.93, p < 0.001). Post hoc Bonferroni test revealed a significant increase in locomotor activity in wild-type mice at all doses of methylphenidate except the 2 mg/kg dose, with the greatest stimulation occurring at the 20 mg/kg dose (p > 0.05 for the dose of 2 mg/kg and p < 0.001 for the doses of 10 mg/kg, 20 mg/kg and 40 mg/kg). To test for a possible role for the noradrenergic system in methylphenidate’s sedative-like properties we used the selective NET inhibitors nisoxetine and desipramine. As seen in Figure 2B, low to moderate doses of desipramine and nisoxetine, both selective NET inhibitors, also inhibited locomotor activity in DAT-CI mice and wild type mice. Two-way ANOVA revealed significant effects for genotype (F 1, 38 = 88.044, p < 0.001), drug (F 2, 38 = 43.435, p < 0.001) and drug X genotype (F 2, 38 = 8.912, p < 0.01). One way ANOVA within the genotypes revealed that both desipramine and nisoxetine reduced locomotor activity in DAT-CI mice (F 2, 15 = 29.705, p < 0.001) and in wild type mice (F 2, 20 = 17.559, p < 0.001). Bonferroni post hoc test revealed significant effects for both drugs on both genotypes (p < 0.001). As previously reported, DAT-CI mice are hyperactive and have higher locomotor activity during the habituation period than WT mice. This is reflected by the higher activity after saline injection (Figure 2A and 2B).

Stereotypy

There is strong evidence that inhibition of the dopamine transporter is required for the stereotypy-inducing properties of psychostimulants. Stereotypy is considered a measure of the psychoactive properties of a drug (Carrera et al., 2004; Tang et al.,
To test the effects of methylphenidate on stereotypy we used the Versamax system to automatically monitor repetitive movements, circling, rearing, etc. The system records several types of stereotypy as well as locomotor activities simultaneously. The effect of methylphenidate on locomotor activities recorded by Versamax were similar to those recorded by Lime Light (data not shown). In Figure 3A we show the effects of methylphenidate on stereotypy counts. Stereotypy counts are the number of times a mouse breaks the same beam without breaking an adjacent beam. A two way ANOVA was performed and significant effects for genotype (F\(_{1,\,43}\) = 21.943, p < 0.001), drug (F\(_{2,\,43}\) = 28.631, p < 0.001) and drug X genotype interaction (F\(_{2,\,43}\) = 17.547, p < 0.001) were found. One way ANOVA revealed significant drug effect (F\(_{2,\,21}\) = 32.077, p < 0.001) and post hoc Bonferroni test indicated that both 5 mg/kg and 40 mg/kg methylphenidate significantly increased stereotypy counts in wild type mice (p < 0.001 and p < 0.01, respectively). However, no dose of methylphenidate affected stereotypy counts in DAT-CI mice. Figure 3B shows the comparison of the number of sessions of stereotyped activity. Mice often perform consecutive stereotyped movements interspersed between nonstereotyped actions. Each of these bouts of stereotyped movements is defined and counted as a stereotypy session by Versamax. Two way ANOVA revealed significant effects for genotype (F\(_{1,\,43}\) = 4.687, p = 0.0347), drug (F\(_{2,\,43}\) = 4.800, p = 0.014) and genotype X drug interaction (F\(_{2,\,43}\) = 5.343, p < 0.001). One way ANOVA indicated significant drug effect in the wild type mice (F\(_{2,\,21}\) = 5.235) and Bonferroni post hoc tests showed that 40 mg/kg methylphenidate greatly increased the number of stereotypy sessions (p < 0.05). However, none of the doses affected this measure in DAT-CI mice. In Figure 3C, we compared the two genotypes of mice in stereotypy time which is the time (in seconds) the mouse performed stereotyped movements. Two way ANOVA
revealed significant effects for genotype (F_{1,43} = 9.664, p < 0.001), drug (F_{2,43} = 18.458, p < 0.001) and genotype X drug interaction (F_{2,43} = 6.702, p < 0.01).

Independent ANOVAs indicated that methylphenidate significantly increased stereotypy time in wild type mice (F_{2,21} = 21.289, p < 0.001) and 5 and 40 mg/kg methylphenidate both significantly increased stereotypy time (p < 0.001 and p < 0.05, respectively, Bonferroni test) but neither doses had any effect on stereotypy time in DAT-CI mice. In Figure 3D we show the effects of methylphenidate on rearing activity. Two way ANOVA revealed no significant effect for genotype (F_{1,43} = 0.123, p > 0.05), but significant effects for drug (F_{2,43} = 4.451, p = 0.018) and genotype X drug interaction (F_{2,43} = 4.912, p = 0.013). One way ANOVA revealed a significant (F_{2,21} = 7.109, p < 0.01) in wild type mice, 5 mg/kg methylphenidate but not 40 mg/kg increased rearing activity (p < 0.05 and p > 0.05, respectively, Bonferroni test). However, none of the doses affected rearing activity in DAT-CI mice. Next, we examined circling behavior which is known to be stimulated by psychostimulants (Figure E). Two way ANOVA revealed significant effects for genotype (F_{1,43} = 22.351, p < 0.001), drug (F_{2,43} = 8.858, p < 0.01), and genotype X drug interaction (F_{2,43} = 7.660 < 0.01).

Methylphenidate greatly increased circling in wild type mice as revealed by one way ANOVA (F_{2,21} = 8.461, p < 0.01) at doses of 5 and 40 mg/kg (Bonferroni analysis, p < 0.01 and p < 0.05, respectively), however neither dose affected the circling behavior of DAT-CI mice. Finally, we measured the amount of time that the mice spent licking the floor. The results are shown in Figure 3F. This behavior was seen only in the wild-type mice at the 40 mg/kg dose of methylphenidate. Similar to the data on locomotor activity, DAT-CI mice also have higher basal levels of stereotypy counts.
Conditioned Place Preference

Psychostimulants, including methylphenidate, are known to produce conditioned place-preference in wild-type mice. The ability of methylphenidate to produce CPP in DAT-CI mice was determined in order to test the importance of DAT inhibition in methylphenidate’s rewarding effects. A two way ANOVA revealed significant effects for genotype ($F_{1, 50} = 14.128, p < 0.01$), drug ($F_{3, 50} = 11.197$) and drug X genotype interaction ($F_{3, 50} = 20.368, p < 0.001$). In our test, as shown in Figure 4, methylphenidate produced significant CPP ($F_{3, 28} = 29.26, p < 0.001$, one way ANOVA) at doses of 10 mg/kg and 20 mg/kg ($p < 0.01$ for both doses, Bonferroni test) in wild type mice. Methylphenidate did not produce CPP in DAT-CI mice at any dose ($F_{3, 28} = 5.15, p > 0.05$). None of the doses produced a significant aversion.

Discussion

In this study we investigated the effects of methylphenidate in a knock in mouse line that bears a DAT mutant (DAT$_{vcv}$) with reduced sensitivity to cocaine and methylphenidate (Chen et al., 2005). When tested in cell culture, DAT$_{vcv}$ was approximately 50-fold less sensitive to methylphenidate inhibition (Chen et al., 2005). To test if DAT$_{vcv}$ retained its reduced sensitivity to methylphenidate in vivo, we examined methylphenidate’s ability to inhibit DAT$_{vcv}$ in synaptosomes prepared from homozygous DAT-CI mice. We found the IC$_{50}$ of methylphenidate inhibition of DA uptake in DAT-CI mice was approximately 20-fold greater than wild type mice (Fig. 1), indicating that low doses of methylphenidate are not likely to strongly impact DAT. The reason for the difference between synaptosomes and cell culture measurements is not clear. It is known that many proteins interact with DAT in vivo (Torres, 2006). It is possible that a
different complement of proteins may be present in cultured cells, which may account for this difference.

We previously reported that cocaine inhibits locomotor activity in DAT-CI mice in a dose dependent manner (Chen et al., 2006), which is in sharp contrast to locomotor stimulation by cocaine in wild type mice. A reasonable explanation is that cocaine blockade of DAT and the resulting elevation of extracellular DA is stimulatory, while cocaine blockade of NET and SERT is inhibitory with respect to locomotion. Therefore, in wild type mice the stimulating effect of cocaine is dominant, whereas in DAT-CI mice the inhibitory effects of NET and SERT are unmasked due to the absence of the stimulatory DAT blockade. A similar, but not identical situation occurs with methylphenidate. A dose of 2mg/kg did not significantly change locomotor activity in either genotypes, while 10 mg/kg strongly stimulated locomotor activity in wild type mice and strongly suppressed locomotor activity in DAT-CI mice (Fig. 2A). The 10 mg/kg dose may have been very effective at inhibiting DAT in wild type mice, but ineffective at inhibiting DAT\textsubscript{vcv} in DAT-CI mice. However, since this dose of methylphenidate would have the same effect in both wild type and DAT-CI mice at other targets, such as NET, there is an overall suppression in locomotor activity. Higher doses of methylphenidate may begin to significantly inhibit DAT\textsubscript{vcv} in DAT-CI mice and may therefore start to have stimulating effects which counter the locomotor suppression. This is supported by the fact that there is a gradual rise in locomotor activity as the methylphenidate dose increased from 10 to 40 mg/kg (Fig. 2A).

The condition ADHD is characterized by hyperactivity, the inability to attend to a task, and increased impulsivity. Many drugs that are effective for the treatment of ADHD are known to be selective NET inhibitors, such as desipramine and atomoxetine. Others
are effective DAT and NET inhibitors. There is considerable disagreement over whether ADHD is the result of unbalanced noradrenergic signaling or unbalanced dopaminergic signaling (Biederman and Spencer, 1999) (van der Kooij and Glennon, 2007). It is noteworthy that atomoxetine is effective at both reducing hyperactivity (often associated with hyperdopaminergic activity) and increasing attentiveness (an attribute more likely due to NE). The DAT-CI mice are hyperactive and thus model at least some aspects of ADHD.

Cocaine and methylphenidate are psychostimulants and they stimulate locomotion in wild type mice. In contrast, these drugs suppress locomotion in DAT-CI mice because DAT is not effectively blocked by these drugs. In DAT-CI mice, cocaine still inhibit the wild type NET and SERT equally well while methylphenidate is over 500 time more potent at inhibiting NET than SERT (Han and Gu, 2006). Therefore, it is possible that the calming effect of methylphenidate observed in DAT-CI mice is due to its ability to inhibit NET. This is supported by the fact that the NET selective inhibitors nisoxetine and desipramine are capable of reducing the locomotor activities of both wild-type and DAT-CI mice (Fig. 2B). Therefore, NET inhibition may be an important mechanism for effective treatment of ADHD to reduce hyperactivity.

The above hypothesis would be further supported if it can be shown that NE receptor blockers reverse the locomotor suppression by the NET inhibitors. We tried a beta blocker alprenolol. We pretreated the mice with 0.5 mg/kg alprenolol and then tested the methylphenidate effects. DAT-CI mice treated with alprenolol and then methylphenidate had similar locomotor activity compared to the mice treated with saline and then methylphenidate (data not shown). However, it cannot be definitively concluded that alprenolol does not reverse the methylphenidate effect because the
relative low dose of alprenolol itself reduced locomotor activity (data not shown). This result is consistent with other studies showing that adrenergic receptor blockers alter locomotor activity (Saitoh et al., 2002) (Jimenez-Rivera et al., 2006) (Bruno and Hess, 2006). It is likely that in the absence of any drugs, the basal levels of NE and epinephrine interact with adrenergic receptors and some of these signalings may contribute to maintaining certain levels of locomotor activity. An adrenergic receptor blocker interferes with a subset of these interactions and thus may alter locomotor activity. In addition, these blockers affect neurotransmission mediated not only by NE but also by epinephrine. Therefore, major efforts are required to determine how exactly methylphenidate inhibition of NET might contribute to locomotor reduction.

Previously we reported that cocaine was effective at reducing many types of stereotyped movements in DAT-CI mice (Tilley and Gu, 2008). In this study we report the effects of methylphenidate on stereotypy in the absence of DAT inhibition (Fig. 3). Methylphenidate produces strong stereotypy in wild type mice; in fact it was a more potent producer of stereotypy than cocaine. In addition, methylphenidate increased the stereotypy time measure, which is the amount of time the animals spent performing stereotypy. The 40 mg/kg dose of methylphenidate produced a very severe licking and gnawing behavior in wild type mice. This behavior was observed starting approximately 5-minutes post injection and continued to the end of the monitoring period. This behavior was likely the reason many of the stereotypy measures decreased at the 40 mg/kg dose. Cocaine at doses up to 40 mg/kg was not capable of increasing this measure. However, methylphenidate had no effect on any of the measures of stereotypy in DAT-CI mice. The reason for the difference between cocaine and methylphenidate are unclear. One possible explanation is that the differences in behavior are due to cocaine
and methylphenidate affecting different targets. A technical limitation should be noted that the beam-break monitoring used in the VersaMax system is not sensitive to all psychostimulant-induced stereotypic behaviors that can be scored manually (Nally et al., 2003). Therefore, we were not able to evaluate all stereotypic behaviors.

DAT-CI mice did not develop CPP in response to methylphenidate (Fig. 4). Although this result is not surprising in the light that cocaine does not produce CPP in these mice, the results are still notable because they show that DAT inhibition is necessary for methylphenidate’s rewarding effects. This is consistent with the hypothesis that increased dopaminergic signalling plays a critical role in the rewarding effects of addictive psychostimulants. In addition, previously it was shown that mice lacking NET showed stronger cocaine CPP than wild type mice, thus indicating that NE may play a role in cocaine’s aversive effects. In the absence of DAT inhibition (at lower doses) methylphenidate inhibition of NET does not significantly impact CPP. It is neither rewarding nor aversive. However, cocaine is also a potent inhibitor of SERT while methylphenidate is not and the chronically elevated synaptic NE may alter how NET knockout mice respond to psychostimulants.

As previously reported, the mutated DAT in DAT-CI mice has reduced uptake function resulting in a higher basal DA tone and higher basal locomotor activity compared to wild type mice (Chen et al., 2006). It is possible that the lack of methylphenidate effects in DAT-CI mice is due to adaptive changes caused by the increased dopaminergic tone in DAT-CI mice. However, this is unlikely, because amphetamine and morphine both stimulate locomotor activities in these mice and amphetamine still produce reward (Chen et al., 2006). In addition, cocaine retains its rewarding and stimulating effects in DAT knockdown mice which have only 10% of DAT
expression level and a markedly elevated DA tone (Tilley et al., 2007). Therefore, increased dopaminergic tone is not likely to be responsible for the altered responses by DAT-CI mice to cocaine or methylphenidate.

In conclusion, methylphenidate does not produce reward or stimulate locomotor activity in mice carrying a DAT mutant with reduced methylphenidate sensitivity and it has no effect on several measures of stereotypy in these mice. These results are consistent with what is observed when DAT-CI mice are treated with cocaine, and they support the hypothesis that DAT inhibition is critical for the rewarding and stimulating effects of psychostimulants. Our data also support the notion that drugs inhibiting NET may have a calming effect. Methylphenidate is known to reduce activity in children with ADHD, which may be mediated at least partially through its ability to alter noradrenergic neurotransmission.
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Figure 1. Synaptosomal DA uptake was measured in the presence of increasing methylphenidate concentrations. Data are presented as fractions of uptake in the absence of methylphenidate. A significant shift was observed in the Ki values for methylphenidate inhibition of DA uptake into synaptosomes (p < 0.01, T-test, 8 samples from each genotype). In wild type mice, the Ki was 210 nM, while in DAT-CI mice; the Ki was shifted 18-fold to 3780 nM.

Figure 2. Methylphenidate effects on locomotor Activity. (A) Locomotor activities are presented as the total horizontal distance traveled in 30 minutes (cm, mean +/- SEM). Methylphenidate failed to stimulate locomotor activity at any dose in DAT-CI mice but significantly suppressed locomotor activity at a dose of 10 mg/kg. In wild type mice, 10 mg/kg, 20 mg/kg and 40 mg/kg methylphenidate significantly stimulated locomotor activity. Six to 8 wild type or DAT-CI mice were examined for each drug dose. (B) Both desipramine and nisoxetine also decrease locomotor activity in both DAT-CI and wild type mice at a dose of 10 mg/kg. Six to 8 wild type or DAT-CI mice were examined for each drug group. (* p < 0.05, ** p < 0.01, *** p < 0.001 compared to saline).

Figure 3. The effects of methylphenidate on stereotypy. For each mouse, 6 stereotypic behaviors were simultaneously monitored for 30 min after the injection of saline, 5 mg/kg, or 40 mg/kg methylphenidate. Six to 8 wild type or DAT-CI mice were studied at each drug dose. (A) Stereotypy Counts, the number of movements scored as stereotypy during the test period. Methylphenidate increased stereotypy counts in wild
type mice, but did not affect stereotypy counts in DAT-CI mice. (B) Number of stereotypy sessions. Methylphenidate increased the number of sessions of stereotyped activity in wild type mice but not in DAT-CI mice. (C) Stereotypy Time in seconds. Methylphenidate increased the time wild type mice spent in doing stereotyped movements but did not affect the time DAT-CI mice spent performing stereotyped movements. Methylphenidate also increased rearing behaviors (D), circling (E), and licking and gnawing activities (F) in wild type mice but not in DAT-CI mice. (* p < 0.05, ** p < 0.01, *** p < 0.001 compared to saline).

Figure 4. Conditioned Place Preference. Data represents the postconditioning time minus preconditioning time in the paired chamber with 6 – 8 mice examined at each drug dose. While 10 and 20 mg/kg methylphenidate produced significant CPP in wild type mice (** p < 0.01 compared to saline), none of the tested doses of methylphenidate produced significant CPP in DAT-CI mice.
Figure 1

Relative Uptake

Log \[\text{Methylphenidate} \text{, } \mu \text{M}\]

- **DAT-CI**
- **Wild type**

![Graph showing relative uptake vs. log of methylphenidate concentration for DAT-CI and wild type.](image-url)
Figure 2

A

Distance traveled

wild type    DAT-CI

saline  2 mg/kg  10 mg/kg  20 mg/kg  40 mg/kg

B

Distance traveled

wild type    DAT-CI

Saline  10 mg/kg des  10 mg/kg nis

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Figure 3
Figure 4

Wild type DAT-CI

Time Difference (s)

-100 0 100 200 300 400

Saline

2 mg/kg

10 mg/kg

20 mg/kg

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