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Role of Serotonin in the Paradoxical Calming Effect of Psychostimulants on Hyperactivity

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The mechanism by which psychostimulants act as calming agents in humans with attention-deficit hyperactivity disorder (ADHD) or hyperkinetic disorder is currently unknown. Mice lacking the gene encoding the plasma membrane dopamine transporter (DAT) have elevated dopaminergic tone and are hyperactive. This activity was exacerbated by exposure to a novel environment. Additionally, these mice were impaired in spatial cognitive function, and they showed a decrease in locomotion in response to psychostimulants. This paradoxical calming effect of psychostimulants depended on serotonergic neurotransmission. The parallels between the DAT knockout mice and individuals with ADHD suggest that common mechanisms may underlie some of their behaviors and responses to psychostimulants.

The catecholamine dopamine (DA) is present in both the central and peripheral nervous systems, where it controls a variety of different physiological processes (*1*). Perhaps one of the most important regulators of dopaminergic function is the DA transporter (DAT) (*2*). This transporter is located on the plasma membrane of DA neurons, where it controls the concentrations of DA by rapidly removing the transmitter from the extracellular space and localizing it into the cytoplasm (*3*). A long-term interest in this transporter derives from its role as an endogenous target for psychostimulants, antidepressants, and several neurotoxins (*3*). Disruption of the DAT gene in mice (*4*) results in a phenotype that includes behavioral abnormalities, neuroendocrine dysfunction, dwarfism, and altered sensitivities to certain drugs (*2*, *5*). A prominent characteristic of these mice is their marked hyperactivity (*4*). This hyperkinetic behavior is consistent with the loss of transporter function and the resultant high concentrations of extracellular DA in the striata of these mice (*2*).

Recently, an association between polymorphisms in the noncoding regions of the human DAT and ADHD has been suggested (*6*). ADHD is a condition that is manifested by impulsivity, hyperactivity, and inattention (*7*). These symptoms are also defined as hyperkinetic disorder (HKD) (*8*). It is estimated that 3 to 6% of school-aged children are affected by this condition (*9*). Since the 1930s, treatment has involved the use of psychostimulant compounds that paradoxically serve to attenuate the hyperactivity and often improve cognitive performance (*9*, *10*). Although psychostimulants preferentially inhibit the DAT, they also block the action of other monoamine transporters (*3*, *5*, *11*). As a result, extracellular concentrations of DA, norepinephrine (NE), and serotonin (5-HT) can be elevated. Although dysregulation of each of these monoamine systems has been postulated to be involved in ADHD-HKD, it is commonly believed that the DA system is preferentially implicated in the etiology and pharmacotherapy of these disorders (*9*, *12*).

Drugs that block the DAT result in a pronounced increase in extracellular DA in brain regions that mediate enhanced locomotion and stereotypic behavior (*2–4*, *11*). DAT knockout (KO) mice were placed into a novel environment, and locomotor, rearing, and stereotypic activities were monitored (*13*). DAT-KO animals exhibited substantially higher levels of activity than wild-type mice (Fig. 1, A to C). Whereas the DAT-KO mice showed minimal habituation to the novel environment over 240 min of observation, habituation occurred within the first 30 to 40 min for the wild-type animals. Hyperactivity in the DAT-KO animals appeared to be novelty driven because locomotor activity was about 12-fold higher in the novel environment (Fig. 1D). Moreover, repetitive exposure to the open field augmented the activity of the DAT-KO mice over seven consecutive days of testing, whereas locomotor responses of the wild-type mice showed habituation (*14*). These results suggest that the DAT-KO animals might be less able to adapt to novel stimuli than the wild-type controls.

Extracellular DA concentrations were sampled by microdialysis in freely moving mice in both the familiar and novel environments (*15*). Despite the fact that DAT-KO mice have fivefold higher concentrations of extracellular DA (*2*), no differences were discerned as a function of time when samples were taken from mice in either environment (Fig. 1E). Dopaminergic neurotransmission, however, was required for the presence of locomotor activity because haloperidol, a DA receptor antagonist, suppressed activity in both the wild-type (*14*) and DAT-KO mice (Fig. 1F). These data suggest that although the enhanced spontaneous locomotor activity in the DAT-deficient animals was not due to additional augmentation of dopaminergic neurotransmission in striatum, it was dependent on dopaminergic tone (see also Fig. 4C). These findings indicate that additional mechanisms may be responsible for the novelty-induced hyperactivity of the DAT-KO mice.

An aspect of ADHD-HKD that may accompany or occur independently of the hyperactivity involves cognition. Animals were tested in an eight-arm radial maze with a win-shift paradigm over 21 consecutive days (*16*). In this procedure, only the first entry in each arm was rewarded. Initially, performance was very poor for both genotypes (Fig. 2A). As training progressed, wild-type mice attained high levels of performance within the first several sessions. As a group, the DAT-KO mice were significantly impaired. These differences in performance did not appear to be due to possible motivational differences because animals from both groups consumed practically identical amounts of food in the maze (*17*).

Another manner of evaluating performance in the radial maze is to examine the response patterns that are made while acquiring the task. One type of response is that of perseveration (*18*). DAT-KO mice made significantly more

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perseverative errors than the wild-type animals, and these mutants showed very little reduction in these types of errors over all sessions of training (Fig. 2B). Activity in the maze was also assessed. During any given session, most of the DAT-KO mice were unable to solve the maze within the allotted 300 s. By the fifth through the seventh test blocks, wild-type animals were reliably solving the maze within 120 to 160 s (*17*). When the time to complete the task was divided by the total number of arm entries, DAT-KO mice exhibited longer latencies to enter a given arm than their wild-type controls (Fig. 2C). In contrast to the open-field tests where the DAT-KO mice were hyperactive, these maze data imply that the mutants were less active than the wild-type mice in this setting. In fact, mutants were often observed to spend more time engaged in other extraneous activities (for example, sniffing, rearing, and so forth) than the controls (*17*). These results suggest that the DAT-KO mice are deficient in spatial learning and that they might have more difficulty than wild-type animals in suppressing inappropriate responses.

Conventional treatment of ADHD-HKD generally involves the use of psychostimulants. Although these drugs serve to enhance activity in normal individuals, they exert a calming effect in ADHD-HKD patients. Psychostimulant effects on the activities of wild-type and DAT-KO animals were evaluated in the open-field environment (*13*). Methylphenidate, dextroamphetamine, and cocaine enhanced locomotor activity in wild-type mice (Fig. 3, A to C). By contrast, activity in the DAT-KO animals was substantially attenuated (Fig. 3, E to G) (*19*). Similar effects on rearing and stereotypic activities were observed (*14*). The effects of methylphenidate on locomotion in wild-type mice were almost immediate, whereas those in the DAT-KO mice were much more delayed and long-lasting. Moreover, additional experiments revealed that the inhibitory effect of methylphenidate on the hyperactivity of the DAT-KO animals was dose-dependent [20 to 60 mg/kg, intraperitoneal (ip)] (*14*). The same doses in wild-type controls were excitatory (*14*), with the enhancement in activity following an inverted U-shaped function (*20*).

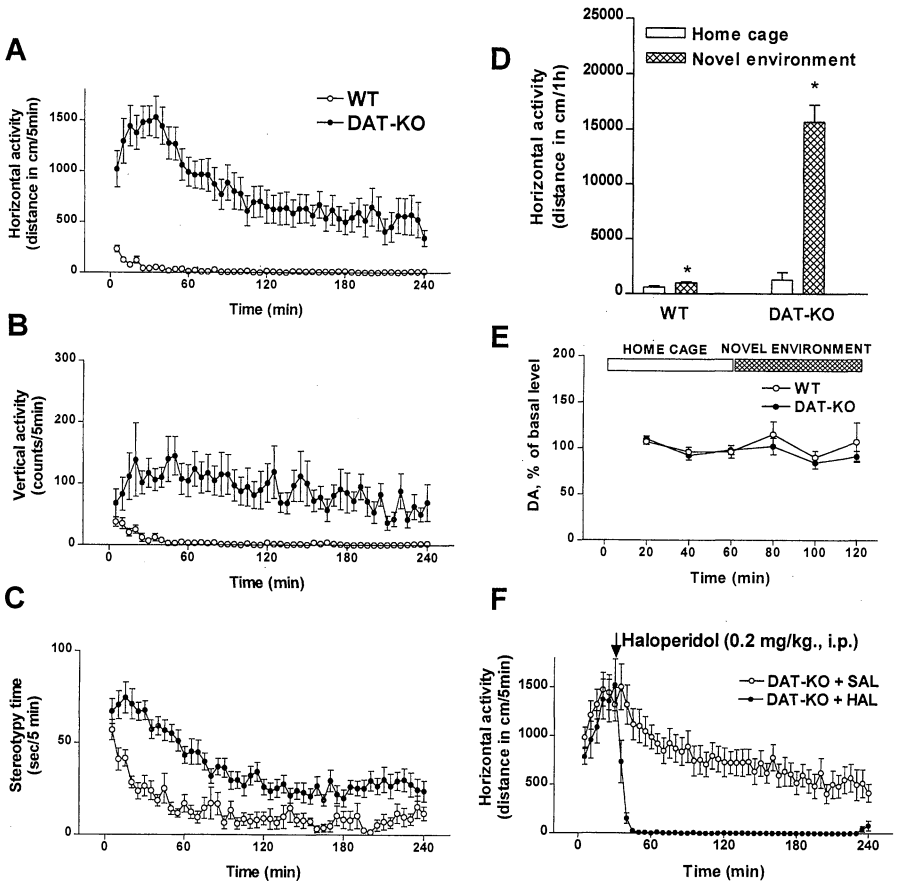

Fig. 1. Parameters of locomotor activity in an open-field environment and accompanying extracellular striatal DA concentrations in wild-type (WT) and DAT-KO mice. (**A** to **C**) Locomotion (horizontal activity), rearings (vertical activity), and stereotypy in a novel environment for wild-type and DAT-KO mice. Activity was recorded every 5 min over a 4-hour period; $n = 10$ for wild-type mice and $n = 16$ for DAT-KO animals. (**D**) Locomotor activity in either a familiar or a novel environment for wild-type and DAT-KO mice. Activity was monitored for 1 hour; $n = 10$ for wild-type and $n = 10$ for DAT-KO animals. *, $P < 0.05$—activity in familiar versus novel environment. No significant differences between the activities of DAT-KO and wild-type mice were discerned in their home cages. (**E**) Extracellular DA concentrations monitored by microdialysis in striatum of freely moving mice in home cages and the novel, open-field environment. Samples were taken every 20 min; $n = 4$ for wild-type and $n = 5$ for DAT-KO mice. (**F**) Effects of haloperidol on locomotor activity of the DAT-KO mice in a novel environment. After a 30-min exposure to the open field, DAT-KO mice and wild-type controls (*14*) were injected with either saline (SAL) or haloperidol (HAL); $n = 14$ for the DAT-KO mice given saline and $n = 6$ for the DAT-KO mice given haloperidol. Haloperidol effectively inhibited locomotion in both genotypes [total distance traveled by wild-type mice for 210 min was 219 ± 52 cm ($n = 6$, haloperidol-treated) compared with 640 ± 98 cm ($n = 10$, saline-treated); *, $P < 0.05$ (*14*)].

Extracellular DA concentrations in striatum were measured by microdialysis in freely moving animals after administration of methylphenidate (*15*). DA concentrations were markedly augmented in wild-type mice, whereas those for the DAT-KO animals were unperturbed by the methylphenidate (see Fig. 4A), amphetamine, or cocaine treatments (*5*). The time courses for the increase and decline in striatal DA concentrations in wild-type mice approximated the changes in locomotor activity (see Fig. 3A). By contrast, the DA concentrations in mutants, which were already increased, were unchanged in response to methylphenidate (see Fig. 4A) despite the attenuation in locomotor activity (see Fig. 3E). This association of striatal DA concentrations with activity in the wild-type mice and the dissociation of these two parameters in the DAT-KO animals suggests that different mechanisms may be involved in these responses to psychostimulants.

Psychostimulants have also been reported to interact with the NE (NET) and 5-HT transporters (SERT) (*3*, *5*, *11*). To test whether NE or 5-HT neurons could be involved in the control of hyperactivity of the DAT-KO mice, we selectively activated each of these systems. Nisoxetine, a selective inhibitor of the NET (*21*), administered at 4 mg/kg (*14*) and 10 mg/kg, exerted no effect on locomotion in wild-type (Fig. 3D) or DAT-KO mice (Fig. 3H). Similarly, no significant effects on rearing or stereotypic activity were noted (*14*). These findings indicate that the NET is unlikely to play an important role in the activating or calming effects of psychostimulants in these mice.

Administration of the SERT inhibitor, fluoxetine, markedly attenuated the activity of the DAT-KO mice (Fig. 3I), whereas it had no effect on wild-type animals (*14*). These actions were presumably mediated by increased extracellular concentrations of 5-HT due to blockade of the transporter because administration of quipazine, a nonselective 5-HT receptor agonist, also reduced hyperlocomotion in the DAT-KO mice (Fig. 3J). Another method for potentiating central 5-HT neurotransmission is through the

increased availability of precursor substrates (*22*). When mice were treated with 5-hydroxytryptophan or with the dietary 5-HT precursor (L-tryptophan), hyperlocomotion in the DAT-KO mice was again profoundly reduced (Fig. 3, K and L). Similar reductions in other activity parameters (rearing and stereotypic responses) were also observed (*14*). By comparison, the wild-type animals either did not respond to these serotonergic pharmacological treatments or responded with only marginal and transient reductions in locomotion (*14*). These pharmacological data emphasize the importance of 5-HT in the regulation of activity levels in the DAT-KO mice.

To determine whether serotonergic agents affect activity through modulation of striatal DA release, we measured extracellular concentrations of DA by microdialysis after fluoxetine treatment. Repeated 20-min samplings after drug administration revealed no significant alterations in striatal DA concentrations in either genotype (Fig. 4B). These data demonstrate that serotonergic neurotransmission can modulate hyperactivity without producing concurrent changes in extracellular striatal DA concentrations.

To examine the mechanism of 5-HT action, we performed additional experiments under conditions in which dopaminergic tone was provided by a direct DA receptor agonist. DAT-KO animals were treated with the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (αMT) for 40 min, which effectively depleted extracellular DA concentrations by greater than 80% (*14*) and completely abolished locomotion within 20 min (Fig. 4C). Locomotion was restored when these mice were treated with a direct D1/D2 receptor agonist (apomorphine) to replace the endogenous DA. When fluoxetine was given before apomorphine administration to enhance serotonergic neurotransmission, the locomotor response to apomorphine was completely absent. These data suggest that the 5-HT effects on hyperactivity are localized downstream of dopaminergic neurotransmission.

It is commonly believed that changes in dopaminergic tone are highly related to alterations in locomotor activity (*1*, *3*, *4*, *11*). DAT-KO mice display marked hyperactivity with respect to locomotor, rearing, and stereotypic responses over that of wild-type animals. These responses are most evident when the mice are placed into a novel environment. Hyperlocomotion of DAT-KO mice is similar to the behavior of ADHD-HKD children where many of these individuals have greater difficulty in suppressing their hyperactivity in novel surroundings (*9*, *23*).

In the present study, global processes associated with spatial learning and memory were impaired in the DAT-KO mice. Analysis of perseverative errors in the radial maze suggested that these mice might be unable to

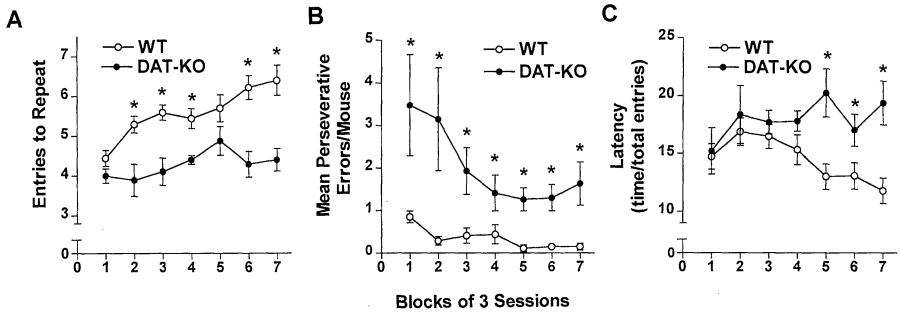

Fig. 2. Assessment of spatial cognitive performance in wild-type and DAT-KO mice. Spatial learning and memory were assessed with an eight-arm radial maze during win-shift testing over 21 sessions. (**A**) Performance as assessed by the number of entries made into each of the eight arms of the apparatus before making an error (entries to repeat). (**B**) Number of perseverative errors made per animal across the seven session blocks of testing. (**C**) Latency to enter a given arm (the time it took to enter all eight arms or 300 s divided by the total number of arms entered during this time). Wild-type (n = 9) (○) and DAT-KO mice (n = 9) (●); *, $P < 0.05$.

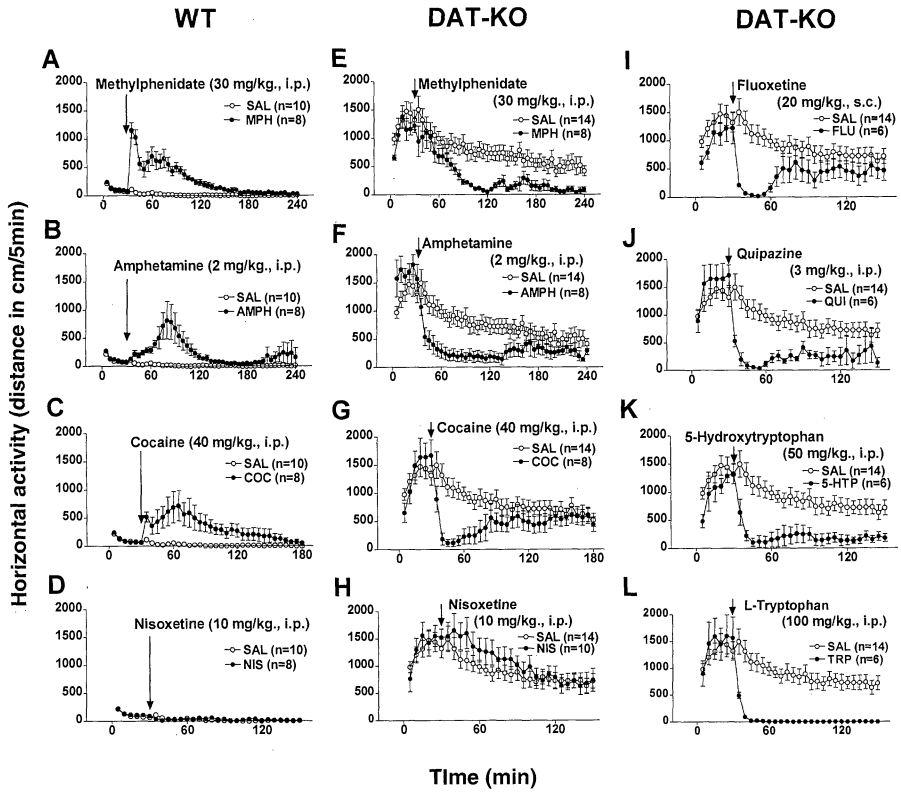

Fig. 3. Effects of various drugs on locomotor activity in a novel environment in wild-type and DAT-KO mice. (**A** to **D**) Wild-type and (**E** to **L**) DAT-KO mice were placed in the open-field apparatus for an initial period of 30 min and then were injected with the psychostimulants: methylphenidate (MPH), *d*-amphetamine (AMPH), and cocaine (COC); the NET inhibitor [nisoxetine (NIS)]; or various serotonergic agents: a SERT inhibitor [fluoxetine (FLU)], a mixed serotonin receptor agonist [quipazine (QUI)], or serotonin precursors [5-hydroxytryptophan (5-HTP) and L-tryptophan (TRP)]; controls were given saline (SAL; 10 ml/kg, ip). Mice were returned to the apparatus and activity was measured. Drug-naïve animals were used for each drug protocol and n represents the number of animals used. ○, control; ●, treated mice. All serotonergic drugs (I to L) potently inhibited the activity of DAT-KO mice, whereas in wild-type mice (*14*) only 5-HTP produced a significant decrease in the activity [total distance traveled by wild-type mice for 120 min was 221 ± 52 cm (n = 6, 5-HTP–treated) compared with 525 ± 79 cm (n = 10, saline-treated), $P < 0.05$]. In an additional set of experiments, lower doses of *d*-amphetamine (0.75 mg/kg, ip), quipazine (0.5 mg/kg, ip), 5-hydroxytryptophan (10 mg/kg, ip), and L-tryptophan (10 mg/kg, ip) were all able to significantly decrease the activity of DAT-KO mice. With the exception of amphetamine, no effects of these treatments on the activity of wild-type mice were observed (*14*). s.c., subcutaneous.

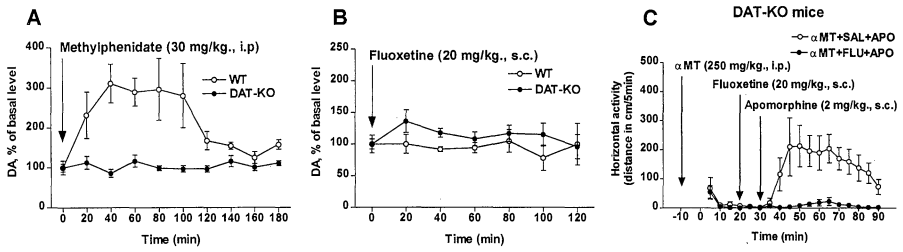

Fig. 4. Analyses of changes in extracellular DA concentrations in striatum and locomotor activity of DAT-KO and wild-type mice after administration of dopaminergic and serotonergic drugs. (**A** and **B**) Effects of methylphenidate or fluoxetine on extracellular DA concentrations in the striatum of freely moving mice measured in home cage environment. At least three samples were collected before each drug treatment (and the mean value was set to 100%). Samples were taken every 20 min for a period of 2 to 3 hours after drug administration; $n = 4$ for wild-type and $n = 5$ for DAT-KO mice. In wild-type mice, all points, except for the effect of methylphenidate 160 and 180 min after administration, were significantly different ($P < 0.05$) from wild-type saline-treated controls (*14*). There was no significant effect of methylphenidate in DAT-KO mice. Fluoxetine had no significant effect in either genotype. (**C**) DAT-KO mice were given αMT to block DA synthesis 10 min before exposure to the open-field apparatus. Forty minutes later, mice were administered a mixed D1/D2 dopamine receptor agonist [apomorphine (APO)] to restore locomotor activity ($n = 6$). A second group of DAT-KO animals were given the αMT, as described above, followed 30 min later by a SERT inhibitor [fluoxetine (FLU)], to enhance extracellular 5-HT concentrations, followed by apomorhine ($n = 6$).

inhibit inappropriate responses or that they might be inattentive to relevant cues in their environment (*18*). Interestingly, Barkley (*24*) has recently proposed that ADHD-HKD patients suffer primarily from an inability to inhibit their behavioral responses such that they are hyperresponsive to various stimuli. Therefore, impairments of cognitive function would be secondary to the hyperkinesis. Similar mechanisms may exist in the DAT-KO mice.

Psychostimulants exerted a calming effect in DAT-KO mice. The studies with nisoxetine suggested that noradrenergic transmission was unlikely to play an important role in this response. By contrast, agents that increased serotonergic neurotransmission were all observed to substantially reduce hyperactivity in the DAT-KO mice. Only minor effects were seen in wild-type mice. The relative magnitude of these effects suggests that levels of existing dopaminergic tone in wild-type and DAT-KO mice may determine the potency of the serotonergic inhibitory effect. Altogether, these data indicate that the primary calming effect of psychostimulants in DAT-KO mice is mediated by the 5-HT system (*25*).

Psychostimulant therapy in ADHD-HKD patients is somewhat controversial because these drugs have long-term sensitizing effects and a potential for abuse and they may be neurotoxic (*26*). The findings with the DAT-KO mice provide the tantalizing possibility that hyperkinetic behaviors may be controlled through the precise targeting of 5-HT receptors or even through enhanced availability of 5-HT precursors. 5-HT has been considered as inhibitory to behavioral activation (*27*) and impulsive behavior (*28*). Several investigators (*29*) have suggested that 5-HT may reduce psychostimulant-induced hyperactivity; however, the neuronal circuitry and mechanisms involved in this DA–5-HT interaction have remained elusive (*27*, *30*). This situation has become much more complicated with the identification of multiple 5-HT receptor subtypes that may activate, inhibit, or exert no effects on locomotion (*27*). Our results reaffirm in a genetic model that 5-HT can constrain hyperactivity and that this effect is localized downstream of DA neurons.

DAT-KO mice are hyperactive, show an impairment in spatial cognitive function, and exhibit paradoxical responses to psychostimulants. Despite these similarities between the mutant mice and humans with ADHD-HKD, it is unlikely that their phenotypes are completely identical. Nonetheless, the results with the DAT-KO mice emphasize the importance of a relative balance of the 5-HT and DA systems for normal motor activity. Therefore, alterations in any of the parameters that control this delicate homeostatic situation might underlie hyperactive states. Despite these reservations, the preponderance of common symptomatologies between the DAT-KO mice and individuals with ADHD-HKD suggests that these mice may not only serve as a useful animal model and as a resource to test new therapies but that they may also provide insights into the basic mechanisms that underlie the etiology of this and other hyperkinetic disorders.

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13. All experiments were conducted in accordance with NIH guidelines for the care and use of animals and with an approved animal protocol from the Duke University Animal Care and Use Committee. Wild-type and homozygous DAT-KO mice were derived from crossing (over seven to eight generations) heterozygous DAT C57BL6/129SvJ animals. Mice were housed four or five to a cage, maintained under standard lab conditions (12-hour light/dark cycle) with food and water provided ad libitum, and tested at 12 to 15 weeks of age. Locomotion was evaluated in an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH) under illuminated conditions. Animals were observed individually at 5-min intervals for a maximum of 4 hours. Locomotor activity was measured in terms of the total distance covered (horizontal activity), rearings were expressed in terms of the number of vertical beam breaks (vertical activity), and the stereotypy time refers to the total time that stereotypic behaviors (repetitive beam breaks of a given beam or beams with intervals less than 1 s) were observed. In the "home" cage condition, mice were housed individually in cages for 24 hours, and then the cage and mouse were placed in the Omnitech Digiscan apparatus and activity was monitored over 1 hour. The data are expressed as the total distance covered in 1 hour for the novel and home cage environments. For drug studies, mice were initially placed into the open field for 30 min, then injected with saline (10 ml/kg, ip) or the drug, and returned to the open-field apparatus. All data are presented as means and SEMs. Statistical significance of all data presented in this paper was analyzed by two-way analysis of variance followed by Duncan's tests. A $P < 0.05$ was considered significant.
14. R. R. Gainetdinov, unpublished data.
15. Microdialysate samples were collected from the right striatum 24 hours after surgery, separated, and quantitated by high-performance liquid chromatography as described earlier for freely moving mice (*2*, *5*). In

accordance with previous reports (*2*, *5*), basal extracellular DA concentrations in the DAT-KO mice were higher than those in the wild-type controls: Concentrations of DA in dialysates were 57.3 ± 13.4 fmol/20 μl in the wild-type mice (n = 12) and 354.5 ± 45.9 fmol/20 μl in the DAT-KO mice (n = 15). The data are presented as means and SEMs of the percentage of change from baseline (100%) from a mean of three samples from each mouse before exposure to the novel environment or before drug or saline administration.

16. All assessments of learning and memory processes were conducted in an eight-arm radial maze [E. D. Levin *et al.*, *Environ. Health Perspect.* **105**, 1320 (1997)]. The mice were food-deprived for about 5 hours before testing, and they were tested daily for 21 sessions with a win-shift paradigm. Before a given session, each of the eight arms was baited with slightly less than one-eighth of a piece of Fruit Loops breakfast cereal. At the start of each session, the mouse was placed into a cylinder in the center of the maze. After 10 s, the cylinder was removed and the mouse had full access to all arms of the maze. Only one entry in each arm was reinforced. The session continued until the mouse had entered all eight arms or 300 s had elapsed. Performance was assessed in terms of the numbers of arms entered (out of eight) before making an error (repeating an entry); these data were expressed as the entries to repeat. In addition, the numbers of perseverative errors were calculated. These errors refer to the tendency of a mouse to leave one arm and enter the arm where it had just been previously. Activity in the maze was also measured and expressed in terms of latency (total time in the maze divided by the total number of entries).
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Unresponsiveness to Cannabinoids and Reduced Addictive Effects of Opiates in CB_1 Receptor Knockout Mice

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The function of the central cannabinoid receptor (CB_1) was investigated by invalidating its gene. Mutant mice did not respond to cannabinoid drugs, demonstrating the exclusive role of the CB_1 receptor in mediating analgesia, reinforcement, hypothermia, hypolocomotion, and hypotension. The acute effects of opiates were unaffected, but the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced. These observations suggest that the CB_1 receptor is involved in the motivational properties of opiates and in the development of physical dependence and extend the concept of an interconnected role of CB_1 and opiate receptors in the brain areas mediating addictive behavior.

Marijuana and other derivatives of *Cannabis sativa* have been used for centuries for their therapeutic and mood-altering properties and are the most widely used recreational drugs today (*1*). The active compounds of *Cannabis*, including Δ^9-tetrahydrocannabinol (Δ^9-THC), as well as the endogenous cannabinoid anandamide, act through two G protein–coupled receptor subtypes. The CB_1 receptor is abundant in the central and peripheral nervous systems but is also expressed in several peripheral organs, whereas CB_2 receptor expression is essentially restricted to lymphoid organs (*2*). We investigated the in vivo function of the CB_1 receptor by invalidating its gene in a mouse model (*3*). Northern (RNA) blotting demonstrated the absence of CB_1 transcripts in brain and testis from knockout ($CB_1^{-/-}$) mice, and binding assays confirmed the absence of binding sites for cannabinoid ligands (*4*). Histology of brain and other organs, body weight monitored over a 6-month period, and blood ionogram and cell count appeared to be unaffected by CB_1 gene inactivation.

The consequences of CB_1 receptor inactivation on spontaneous behavior were analyzed. A moderate increase in locomotor activity (*5*) was observed in $CB_1^{-/-}$ mice when

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