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ORIGINAL INVESTIGATION

Dissociations between cognitive and motor effects of psychostimulants and atomoxetine in hyperactive DAT-KO mice

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Abstract
Rationale Psychostimulants such as amphetamine and methylphenidate, which target the dopamine transporter (DAT), are the most frequently used drugs for the treatment of hyperactivity and cognitive deficits in humans with attention deficit hyperactivity disorder (ADHD). While psychostimulants can increase activity in healthy subjects, they exert a "paradoxical" calming effect in humans with ADHD as well as in hyperactive mice lacking the dopamine transporter (DAT-KO mice). However, the mechanism of action of these drugs and their impact on cognition in the absence of DAT remain poorly understood.
Objectives This study was conducted to investigate the effects of psychostimulants and noradrenergic and serotonergic drugs on cognition in DAT-KO mice and normal (WT) littermates.
Methods We used a recently developed behavioral apparatus, the automated H-maze. The H-maze involves the consecutive learning of three different rules: delayed alternation, nonalternation, and reversal tasks.
Results Treatment of WT animals with the psychostimulants replicated the behavior observed in untreated DAT-KO mice while "paradoxically" restoring cognitive performances in DAT-KO mice. Further investigation of the potential involvement of other monoamine systems in the regulation of cognitive functions showed that the norepinephrine transporter blocker atomoxetine restored cognitive performances in DAT-KO mice without affecting hyperactivity. In contrast, the nonselective serotonin receptor agonist 5CT, which antagonizes hyperactivity in DAT-KO mice, had no effect on cognitive functions.
Conclusions Taken together, these data allow dissociation of the locomotor and cognitive effects of ADHD drugs and suggest that the combination of DAT-KO mice with the automated H-maze can constitute a powerful experimental paradigm for the preclinical development of therapeutic approaches for ADHD.

Keywords DAT-KO mouse · H-maze · Psychostimulants · Atomoxetine

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Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most common psychiatric illnesses that affect about 3–7 % of the childhood population worldwide and may persist in adult life (Balint et al. 2008; Nair et al. 2006). The core symptoms of ADHD are hyperactivity, impulsivity, and inattentiveness (Barkley 1998; Chess 1960; Douglas et al. 1995). Accordingly, this disorder can be classified as inattentive, hyperactive–impulsive, or combined type, depending on which symptoms are strongest in a given individual. Possibly as a consequence of

these core symptoms, ADHD also involves a range of cognitive dysfunctions including learning deficits, lower cognitive flexibility, and impaired neurocognitive profile (Bedard et al. 2003; Cubillo et al. 2013; McLean et al. 2004). In line with this, ADHD patients are reported to experience poor academic performances, professional success, and social–emotional development (Chess 1960; Douglas et al. 1995).

The psychostimulants amphetamine (AMPH) and methylphenidate (MPH) are the most commonly used drugs for the treatment of ADHD (Wilens 2008). Interestingly, if these drugs induce hyperactivity and inattentiveness in healthy subjects (Amara and Kuhar 1993; Barkley 1998; Gingerich et al. 1998; Hechtman 1994), AMPH and MPH also mediate a reduction of hyperactivity and a partial restoration of cognitive functions in ADHD patients (for review, see Chamberlain et al. 2007). To date, the main known mechanism of action for these compounds is the targeting of the dopamine transporter (DAT), which is expressed by dopamine (DA) neurons to reuptake DA from the synaptic cleft (Giros and Caron 1993; Giros et al. 1996; Jones et al. 1998; Volkow et al. 1996). Furthermore, it is now well established that ADHD symptomatology could be influenced by alterations in other monoaminergic systems (Arime et al. 2011; Bradley et al. 2010; Contini et al. 2013; Gainetdinov et al. 1999). Indeed, AMPH and MPH have affinity for the norepinephrine (NE) transporter (NET) that is in the same order of magnitude than their affinity for the DAT (Han and Gu 2006). Furthermore, the average clinical maintenance dose of MPH has been shown to result in a 70 to 80 % occupancy of NET in humans (Hannestad et al. 2010). Finally, both MPH and AMPH can also exert their effect on the serotonin (5-HT) transporter (SERT), albeit with lower affinity than for the DAT and NET (Han and Gu 2006). That being said, the specific role of monoamines in the therapeutic effects of these drugs remains poorly understood (Gainetdinov et al. 1999; Moron et al. 2002; Oades 2008; Sora et al. 2009; Wilens 2008).

Several rodent models, like spontaneously hypertensive rats and mice lacking the DAT (DAT-KO), have been proposed to investigate the neurobiological basis of ADHD (Russell et al. 2005). DAT-KO mice display behavioral phenotypes that are reminiscent of the hyperlocomotion, impulsivity, attention deficits, and impaired cognitive functions observed in ADHD patients (Gainetdinov and Caron 2001; Gainetdinov et al. 1999; Hironaka et al. 2004). Acute treatments with AMPH and MPH reduce hyperactivity in DAT-KO mice while inducing it in wild-type (WT) mice (Beaulieu et al. 2006; Gainetdinov et al. 1999). While the construct validity of DAT-KO mice to the pathology of ADHD remains unclear due to different adaptive changes related to the complete absence of DAT (Bossé et al. 1997; Cyr et al. 2003), these mice represent a valuable animal model with predictive validity for the development of ADHD drugs (Revel et al. 2011). Given that DAT is the main target for psychostimulants and that this transporter is missing in DAT-KO mice, MPH and AMPH both fail to increase extracellular DA levels in these mice as they do in control animals (Gainetdinov et al. 1999; Jones et al. 1998). In line with this, it has been hypothesized that MPH and AMPH may induce behavioral outcomes in DAT-KO mice by acting on other neurotransmitter systems such as NE and 5-HT (Barnes et al. 2011; Beaulieu et al. 2006; Bymaster et al. 2002; Gainetdinov et al. 1999; Koda et al. 2010). Interestingly, administration of 5-HT reuptake inhibitors (SSRIs), the 5-HT precursor tryptophan (5HTP), or 5-HT receptor agonists such as 5-carboxamidotryptamine (5CT) to DAT-KO mice results in a reduction of hyperactivity (Beaulieu et al. 2006; Gainetdinov et al. 1999). However, the putative contribution of 5-HT neurotransmission in ADHD therapy remains controversial since SSRIs have shown no efficacy in reducing symptoms in ADHD patients (Newcorn 2008; Spencer et al. 2002; Verbeeck et al. 2009). In contrast, the selective NE reuptake inhibitor atomoxetine (ATX) and the tricyclic antidepressants desipramine and nortriptyline, which both display higher affinity for the NET than for the SERT, are effective in the treatment of ADHD (Bari et al. 2009; Newcorn 2008; Niederhofer 2008; Spencer et al. 2002; Tsutsui-Kimura et al. 2009; Verbeeck et al. 2009).

In this study, we used a recently developed behavioral apparatus, the automated H-maze (Del'Guidice et al. 2009), to investigate the effects of psychostimulants, 5-HT, and NE drugs on rule learning and cognitive flexibility in DAT-KO mice. The automated H-maze involved a delayed reaction paradigm, consisting in the consecutive discovery of three different rules, a delayed alternation task, a nonalternation task, and a delayed reversal task (Del'Guidice et al. 2009). Our results indicated that DAT-KO mice display profound cognitive deficits in this test. Furthermore, psychostimulants and ATX restored cognitive performances in these mutant mice, while a nonselective 5-HT receptor agonist reduced hyperactivity without affecting cognitive performances.

Material and methods

Animals

C57BL/6 J DAT-KO and WT mice were described previously (Beaulieu et al. 2006; Giros et al. 1996). DAT-KO mice (Giros et al. 1996) were backcrossed with C57BL/6 J obtained from the Jackson Laboratory (Bar Harbor, ME) for more than 20 generations. Since homozygous mice cannot be produced using WT C57BL/6 J mice, heterozygous backcrossed littermates were used to produce experimental cohorts. Three- to 4-month-old mice were used for all experiments and the respective WT littermates were used as controls for mutant mice. Animals were housed three to four per cage and maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) in a humidity-

controlled room at 23 °C. Mice were kept with food and water available ad libitum throughout the experiments, except for habituation and test periods. Four days before the H-maze test, they were intermittently water-deprived in order to develop a sufficient level of motivation to look for reward. This deprivation paradigm is aimed at maintaining body weight at approximately 85 % of its initial value throughout testing. All procedures were conducted in accordance with the policy of the Laval University Animal Care Committee, which is responsible for the application and enforcement of the rules of the Canadian Council on Animal Care.

Drug administration

All drugs were prepared in saline and administered intraperitoneally (i.p. 10 mL/kg) 30 min before the test session in the H-maze and the open field. MPH (Tocris-1812) was injected at 30 mg/kg as described previously (Beaulieu et al. 2006; Gainetdinov et al. 1999). AMPH (Tocris-2813) was injected at 0.2, 1, or 2 mg/kg. 5CT (Sigma-C117) was administered at 0.1 mg/kg as described (Beaulieu et al. 2006) and ATX has been injected acutely at 1 mg/kg as described (Koda et al. 2010). CP-809,101 (Tocris Cookson, Ballwin, MI) was administered at 1 mg/kg as described (Siuciak et al. 2007). Different groups of randomly selected WT and KO mice were used for each drug and compared with vehicle with respect to genotypes.

Automated H-maze

The automated H-maze (Fig. 1a) is an H-shaped apparatus consisting of two testing chambers (C1 and C2) connected by a straight plastic tube 5 cm in diameter and 23 cm in length. Each testing chamber is made of two plastic tubes 5 cm in diameter connected to an empty plastic cube. Cube sides are 6 cm. All tubes are made by joining two half tubes (a top half laid on a bottom half). The bottom tubes are joined together to form the floor of the maze. The top tubes are independent and can be removed easily to clean the maze. On top of the cubes, inverted fans eject neutral or scented air from the outside extremities of the tubes connected to the cubes. At each lateral extremity of the testing chambers, a water port in the shape of a well is located above the air and odor port. The activity of a mouse is detected by photoelectric cells located 5 cm from each tube extremity and at 5 cm from both extremities of the connecting straight tube. In this experiment, the odor was injected through all lateral extremities of the testing chambers at a flow rate of 5 L/min by forcing clean air (0.7 bar) through two Erlenmeyer flasks that contained 200 mL of water mixed with 5 g/L of isoamyl acetate (Sigma-Aldrich). The neutral air (1,000 mL Erlenmeyer flask containing 200 mL of water) was injected at the same flow rate on both sides of the two testing chambers. The olfactory H-maze was set on a square table, 160 cm above the floor. Inside the maze, the mice could move freely and all procedures and recordings were controlled by a computer (located in an adjacent room) via a program developed in our laboratory using LabVIEW software (National Instruments, Nanterre, France). Mouse behavior was observed directly in the testing room.

Procedure for the automated H-maze

Habituation session

On each daily habituation session, each mouse was weighed and then placed in the automated H-maze for 10 min. On the first day, the mouse could run freely throughout the maze with water and odor delivered ad libitum. The odor is an olfactive cue allowing to keep the same olfactory environment for each session, thus decreasing the anxiogenic effect of novelty. Then, the mouse was returned to its cage and water-deprived. Since 24 h without water would kill the mice and to avoid a weight loss superior to 15 %, access to water was given to the animals for 5 min twice a day ad libitum. On the second day, the odor was injected from each extremity from only one testing chamber. When the mouse crossed one of the two photocells in this testing chamber, 10 μL of water was delivered. The same odor was injected into the second testing chamber until the mouse crossed one of the two photocells to get the same reward. This automated odor injection was maintained for 10 min. On the third day, once the mouse was inside the maze, the same odor was injected randomly from only one of the testing chambers of the maze. When the mouse ran into this chamber, 10 μL of water was distributed in one extremity but not in the other. When the mouse went to the randomly chosen extremity, 10 μL of water was distributed again and so on for 10 min. After 3 days of habituation, the odor was associated with the presence of the reward and contributed, with hydric deprivation, to maintain the necessary motivation to look for water.

Test session

Before the test session, mice were weighed again to verify that they reached 85 % of their initial weight. At the beginning of the session, the top half of the connecting straight tube was lifted to introduce the tested mouse into the apparatus and then replaced. When the mouse was in the middle of the tube, the odor was injected from the right and from the left tubes of one randomly chosen testing chamber. During all the sessions, the scented air guided the mouse to the testing chamber where the reward could be distributed but did not indicate in which way to turn into the chamber to obtain the reward. At this time, only one side was associated with the reward (a drop of water ~10 μL). The response was given by crossing one of the two photocells at an extremity. The reward was distributed if the

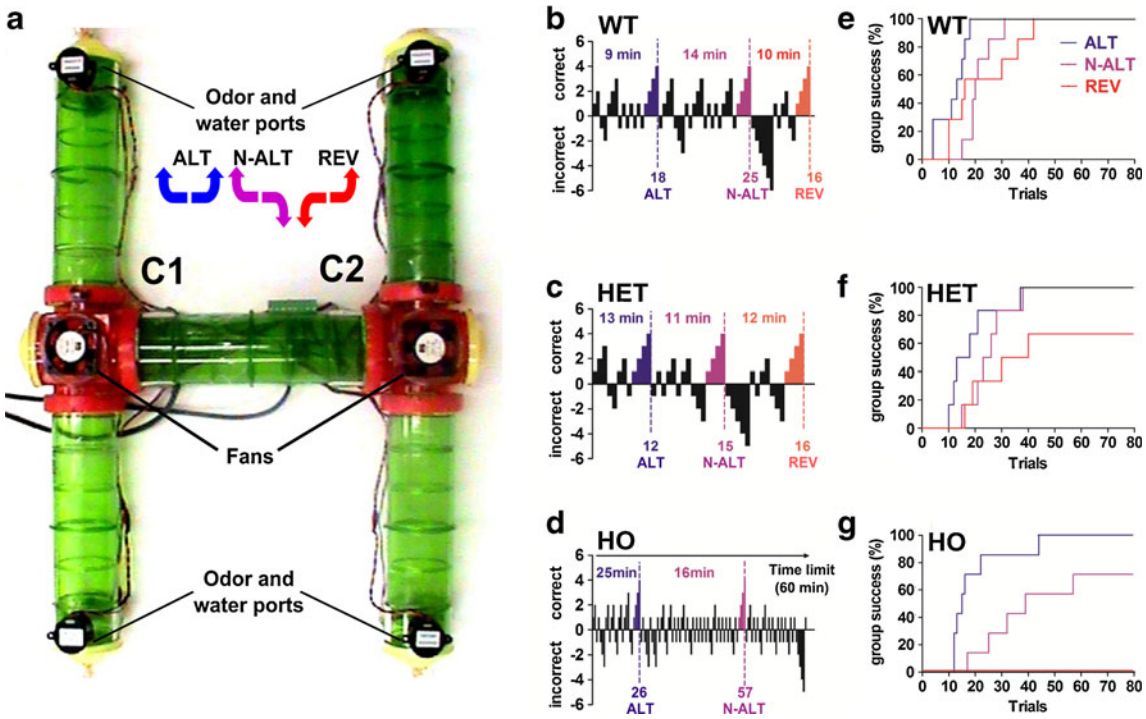

Fig. 1 The H-maze: a new automated apparatus to investigate learning and memory in mice. **a** Photograph of the H-maze with its two testing chambers (C1 and C2). The three sequential learning patterns (ALT: *blue*; N-ALT: *violet*; REV: *red*) present each moving task that animals had to perform to get the reward. **b** Example of the representative performances of one WT mouse that have completed the three tasks. Each positive (*top*) and negative (*bottom*) *bar* corresponds to a rewarded or a failed trial, respectively. *Dotted lines* indicate that the mouse has reached the criterion of four consecutive successful trials for the task (ALT: *blue*; N-ALT: *violet*; REV: *red*). *Numbers on top* of each task indicate the time taken by the mouse to complete the task. *Numbers on the bottom part* indicate the number of trials taken for each task. **c** Example of the representative performances of a HET mouse that has reached the three criteria of four consecutive correct trials for each rule. **d** Performances of a representative HO mouse that has failed in learning the three tasks. **e**–**g** Proportion (across trials) of each task that WT, HET, and KO mice successfully completed

mouse chose the correct side and was not distributed on the other side. After that, when the mouse crossed the photocell located at the extremity of the connecting tube closer to the other testing chamber, the same odor was injected at both extremities of the second testing chamber. The mouse had to go to one extremity of the second testing chamber to get the reward from the designated side and not to other end. Between trials, neutral air was distributed in both testing chambers. Once again, the mouse had to make a choice between the right and the left side of the testing chamber to get the reward. This continued until the criterion was reached. There was no fixed delay between trials. However, the minimal delay between the response in one testing chamber and the entrance to the opposite testing chamber was measured. This minimal intertrial delay is 4 s.

Three different rules had to be discovered: delayed alternation task (ALT), a delayed nonalternation task (N-ALT), and a reversal task (REV). The three tasks implementing the three rules were performed in succession. The rule switched automatically once the criterion was met, 1 h had passed, or 80 trials had been completed, whichever came first. From a previous study (Del'Guidice et al. 2009), the success criterion was set at four consecutive trials, which reduced to 1/16 the probability of success due to chance. At the end of the session, the testing chamber was opened by lifting up the half tubes and the mouse was removed and returned to the vivarium with water (ad libitum).

In the first task (ALT), mice had to learn to alternately move between both chambers of the H-maze to get reinforcement (turn left in C1, turn right in C2 in alternation). A mouse responded correctly by going to the side where the reinforcement was distributed and incorrectly by going to the other side. On the next trial, the reinforcement was distributed on the opposite side and so on (Fig. 1a, blue arrows). Once the mouse had succeeded in the first task, one of the sides (right or left) was randomly chosen to be the one to get the reinforcement on the N-ALT (Fig. 1a, violet arrows). Mice had to perform a response inhibition of the first task in order to learn the second rule (N-ALT). The N-ALT task consisted in always turning to the same side to enter each chamber. Maintenance of the ALT strategy under N-ALT conditions resulted in 50 % reward, while full reward can only be achieved by extinguishing the ALT strategy and learning the N-ALT rule. Once the criterion of the second task was met, the third task (REV) started. The mouse had to perform the complete opposite strategy learned in the N-ALT task and received the

reward on the opposite side to that assigned in the N-ALT (Fig. 1a, red arrows). Consequently, a mouse had to find out, from its previous stimuli, where the reward (left or right side of the testing chamber) will be distributed in regard to the rule assigned. Thus, this paradigm requires mental flexibility and attentional set. At the end of the session, comparisons between mutant and control mice, both treated with vehicle or different drugs, were made.

Olfactory perception in novelty-related test

Olfactory perception test was performed as described (Breton-Provencher et al. 2009). Before habituation and test sessions, the olfactory performances of DAT-KO mice, compared to WT littermates, were assessed to make sure that the mutation and consequent lack of DAT did not affect olfactory perception. Similar to the behavioral paradigm in the automated H-maze, mice were water-deprived for 3 days before olfactory perception testing. After two habituation days (5 min, twice daily) in the apparatus, we assessed the odor detection threshold of dehydrated WT and DAT-KO mice. Testing was run in the mouse's home cage.

The apparatus was a clean, unused cover grid resting on top of the cage and from which two Pasteur pipettes were hanging. The first pipette contained a saturated Whatman paper with nonodorant mineral oil, whereas the second one contained the odor, with different concentrations of isoamyl acetate or octanol. Time spent sniffing odors was measured for four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} μL/L), tested in four different 5 min sessions from the least to the most concentrated. We considered mice to have normal olfactory perception when time spent sniffing the odor was significantly greater in comparison with time spent investigating the mineral oil-containing pipette.

Locomotion in a novel environment

Novelty-induced locomotion test was assessed under illuminated conditions with an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH) open field as described in Beaulieu et al. (2004) and Xi et al. (2005). Mice were injected 30 min before the test and then placed into the activity monitor chamber for 30 min. Locomotor activity was expressed as the total distance covered.

Statistical analysis

Analysis of variance (ANOVAs) followed by Newman–Keuls post hoc comparisons was processed using the Prism statistic software (GraphPad) to compare performances of the three genotypes in the H-maze experiments. To compare the effects of drugs on cognition in the H-maze and in the olfactory perception test, we proceeded with two-way ANOVAs followed by Bonferroni post hoc tests. Two-tailed *t* tests were also used to compare the performances between vehicle WT and vehicle HO mice used as control for drug treatments and to analyze locomotor activity. Population success rates reported in Kaplan–Meier survival curves were analyzed with Mantel–Cox log-rank test. Comparisons were made either between genotypes (Fig. 1) or between drug treatments within mice of the same genotype (Fig. 4). The *p* values ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$) in the figures are denoted with asterisks (*, **, and ***, respectively). For each analysis of data from the automated H-maze, statistical analysis related to mutant and/or drug-treated mice has only included animals that completed the task. Only significant statistical analysis is reported.

Results

DAT-KO mice display impaired global performances in the H-maze

Performances of DAT-KO mice were assessed with three delayed response tasks in the H-maze. In the ALT task, mice had to learn to move in alternation from one chamber to another to get the hydric reward (see blue arrows in Fig. 1a). In the N-ALT task, mice had to always turn to the same side to enter each chamber (see violet arrows in Fig. 1a). In the REV task, mice had to completely inhibit the previous rule and perform the opposite strategy to obtain the reward (see red arrows in Fig. 1a).

Individual performances of each mouse were analyzed for each consecutive trial and for each task in the H-maze. There were no differences between a wild-type (WT) and a heterozygous (HET) mouse after learning the three rules (Fig. 1b, c). In contrast, representative homozygous (HO) mice (Fig. 1d) performed several more trials to complete the ALT and the N-ALT task and failed to learn the third rule before the time limit (60 min, black arrow).

The entire WT cohort learned the three rules (Fig. 1e). All HET animals completed the ALT and the N-ALT tasks (Fig. 1f) but only 66 % of this group learned the last REV task (log-rank test: $\chi(1)=11.61$, $p \leq 0.05$). All HO animals learned the ALT task (Fig. 1g), 70 % of the group completed the second N-ALT task (log-rank test: $\chi(1)=9.18$, $p \leq 0.01$), and none of the HO mice succeeded on the third REV task (log-rank test: $\chi(1)=26.77$, $p \leq 0.001$).

Performances of DAT-KO mice in the alternation task (ALT)

Mice from all genotypes (WT, HET, and HO) were able to reach the criterion of four consecutive successful trials. However, there were statistically significant differences between these three groups (ANOVA: $F(2, 17)=27.68$, $p \leq 0.001$). Compared to the performances of WT and HET

mice, HO animals required more trials to learn the first rule (Fig. 2a, Newman–Keuls test: $p \leq 0.001$ and $p \leq 0.01$, respectively). Accordingly, the time needed to learn the ALT rule was correlated to the number of trials performed by mice from each genotype (Fig. 2b). A significant difference also existed between the time spent by the mice from the three groups (ANOVA: $F(2, 17)=5.16$, $p \leq 0.05$). HO mice required more time than WT to complete the first task (Newman–Keuls test: $p \leq 0.05$).

Performances of DAT-KO mice in the non-alternation task (N-ALT)

A significant difference was found between the three genotypes in the N-ALT task (Fig. 2c—ANOVA: $F(2, 16)=6.97$, $p \leq 0.01$). All the WT and HET animals completed the N-ALT task with similar performances. In contrast, ~30 % of the HO group failed to complete this task. A significant difference in the number of trials taken to complete the task was found between the HO mice and animals from the other groups

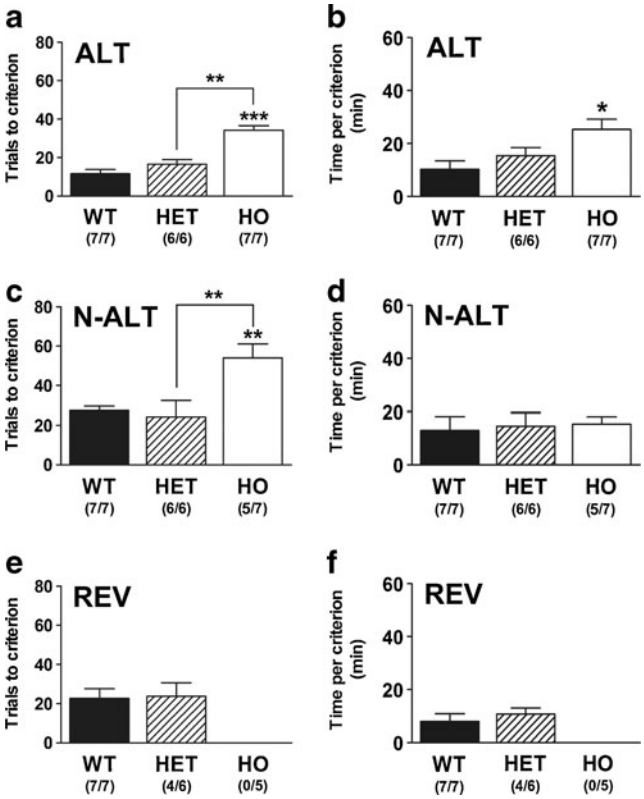

Fig. 2 DAT-KO mice display cognitive deficits in the learning of each rule in the H-maze. **a**, **b** Trial-to-criterion and time performed by each genotype in the alternation (ALT) task. **c**, **d** Trial-to-criterion and time performed by each genotype in the nonalternation (N-ALT) task. **e**, **f** Trial-to-criterion and time performed by each genotype in the reversal (REV) task. For each task, analyses do not take into account the animals that failed to reach a criterion. Data are presented as means ± SEM. Fractions below each bar indicate the proportion of mice that completed each task. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. One-way ANOVA test with Newman–Keuls tests

(Newman–Keuls test: $p \leq 0.01$). No significant difference was found between the time required by mice from the three genotypes to learn the rule (Fig. 2d). However, HO mice required more trials in comparison with WT and HET mice.

Performances of DAT-KO mice in the reversal task (REV)

Analysis of the third rule (REV) has been conducted only in mice that completed the two other tasks. Although all WT and 70 % of HET mice learned the third rule, none of the HO completed the REV task (Fig. 2e). No significant difference was found between the WT and the HET animals in either number of trials or time taken to complete the task (Fig. 2f).

DAT-KO mice do not display olfactory deficits

Since the automated H-maze relies on olfactory-assisted learning, we also evaluated olfactory perception in WT and HO mice to rule out a possible contribution of olfaction in the behavioral outcome observed in the H-maze. These tests showed that WT and HO mice displayed similar levels of odor detection in the olfactory sensitivity test (Fig. S1A, B). Two-way ANOVA confirmed that impaired performances in HO mice were not provoked by a loss of olfactory abilities in DAT-KO animals (Fig. S1A, B).

"Paradoxical" effect of psychostimulants on cognitive performances in WT and DAT-KO mice

Administration of psychostimulants to WT mice resulted in a dramatic reduction of cognitive performances in the automated H-maze while "paradoxically" improving the same performances in DAT-KO animals. Figure 3 shows a representative example of a vehicle-treated WT mouse that learned the three rules (Fig. 3a) and one of a vehicle-treated HO animal that failed to understand the third rule before the time limit (Fig. 3b). Typically, MPH- or AMPH-treated WT mice did not complete the REV tasks and failed more trials before completing the ALT and N-ALT tasks (Fig. 3c, e). In contrast, administration of either MPH or AMPH improved cognitive performances of HO mice, allowing the learning of the three rules (Fig. 3d, f).

All vehicle, MPH-, and AMPH-treated WT and HO mice completed the ALT task (Fig. 4a, b). Significant interactions were found in the number of trials taken to complete the task (trial-to-criterion) and time performances between genotypes and drug treatments in the ALT task (Fig. 5a, b—a: two-way ANOVA: $F(4, 66)=14.63$, $p \leq 0.001$ and b: two-way ANOVA: $F(4, 66)=8.45$, $p \leq 0.001$). Psychostimulants increased the number of trials and the time taken by WT animals to complete the task (Fig. 5a—Bonferroni post hoc test, MPH: $p \leq 0.05$, AMPH: $p \leq 0.01$ and Fig. 5b—Bonferroni post hoc test, MPH: $p \leq 0.01$, AMPH: $p \leq 0.01$). Furthermore, psychostimulants reduced the

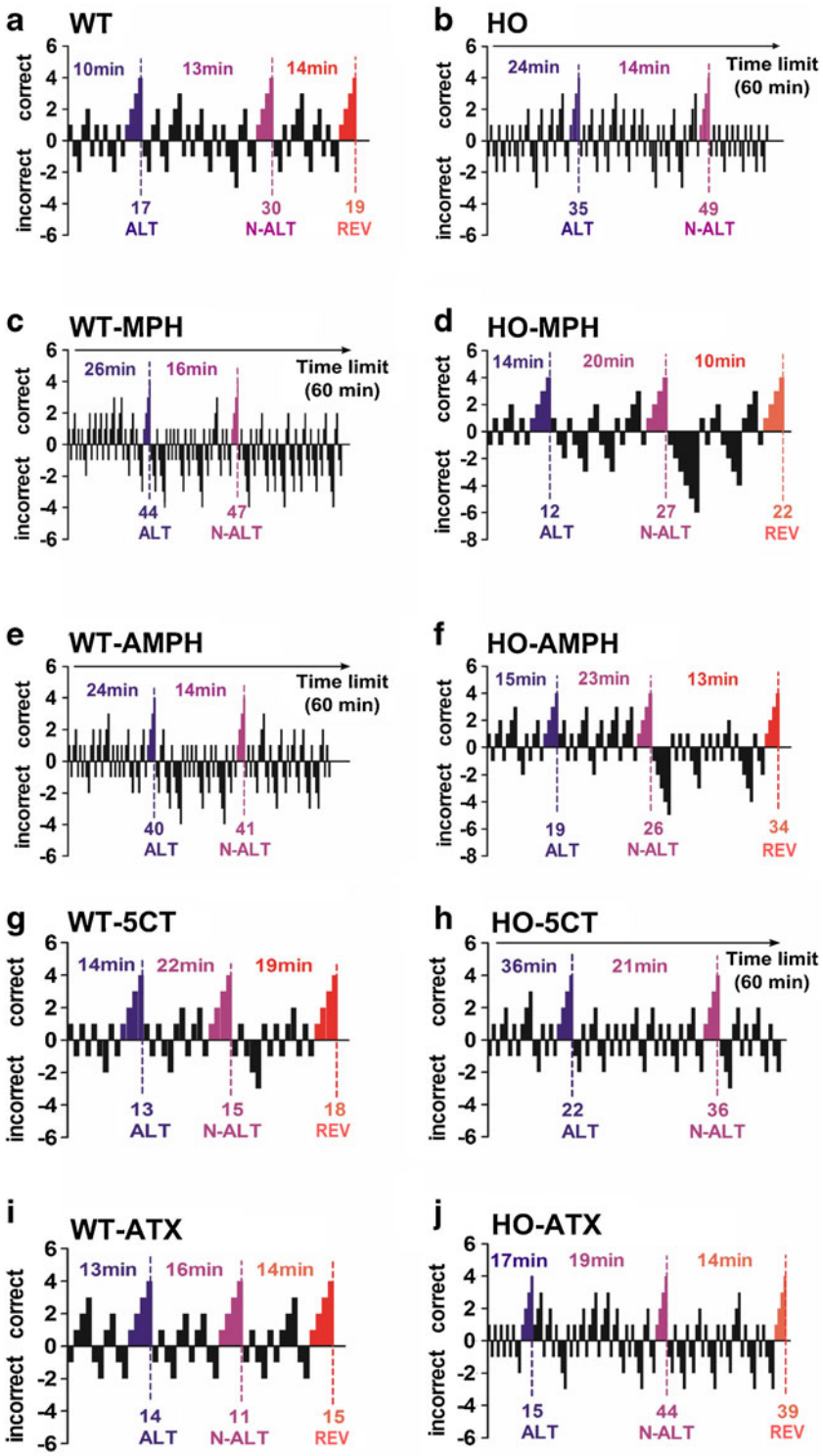

Fig. 3 Impact of psychostimulants, 5CT, and ATX on spatial learning and cognitive flexibility in individual DAT-KO mice. **a** Example of the representative performances of one WT mouse that has reached the three criteria. **b** Example of the representative performances of one HO mouse that failed to reach the three criteria before the time limit. **c**, **d** Representative examples of MPH-treated WT and HO mice. **e**, **f** Representative examples of AMPH-treated WT and HO mice. **g**, **h** Representative examples of 5CT-treated WT and HO mice. **i**, **j** Representative examples of ATX-treated WT and HO mice. Each positive (*top*) and negative (*bottom*) *bar* corresponds to a rewarded or a failed trial, respectively. *Dotted lines* indicate that the mouse has reached the criterion of four consecutive successful trials for the task (ALT: *blue*; N-ALT: *violet*; REV: *red*). *Numbers on top* of each task indicate the time taken by the mouse to complete the task. *Numbers on the bottom part* indicate the number of trials taken for each task. *Black arrows* indicate that the mouse reached the time limit before finding the three rules

number of trials but not the time taken by DAT-KO mice to complete the task (Fig. 5a—Bonferroni post hoc test, MPH: $p \leq 0.001$, AMPH: $p \leq 0.001$). Vehicle-treated HO animals required significantly more trials (two-tailed *t* test: $p \leq 0.001$) and more time (two-tailed *t* test: $p \leq 0.05$) to reach the ALT criterion as compared to vehicle-treated WT mice.

In the N-ALT task, all vehicle-treated WT animals learned the rule, while only 70 and 55 % of MPH- and AMPH-treated WT animals completed the task, respectively (Fig. 4c—log-rank test, MPH: $\chi(1)=14.42$, $p \leq 0.001$; AMPH: $\chi(1)=13.87$, $p \leq 0.001$). In contrast, all MPH- and AMPH-treated HO mice completed the N-ALT task, while only 60 % of vehicle-treated

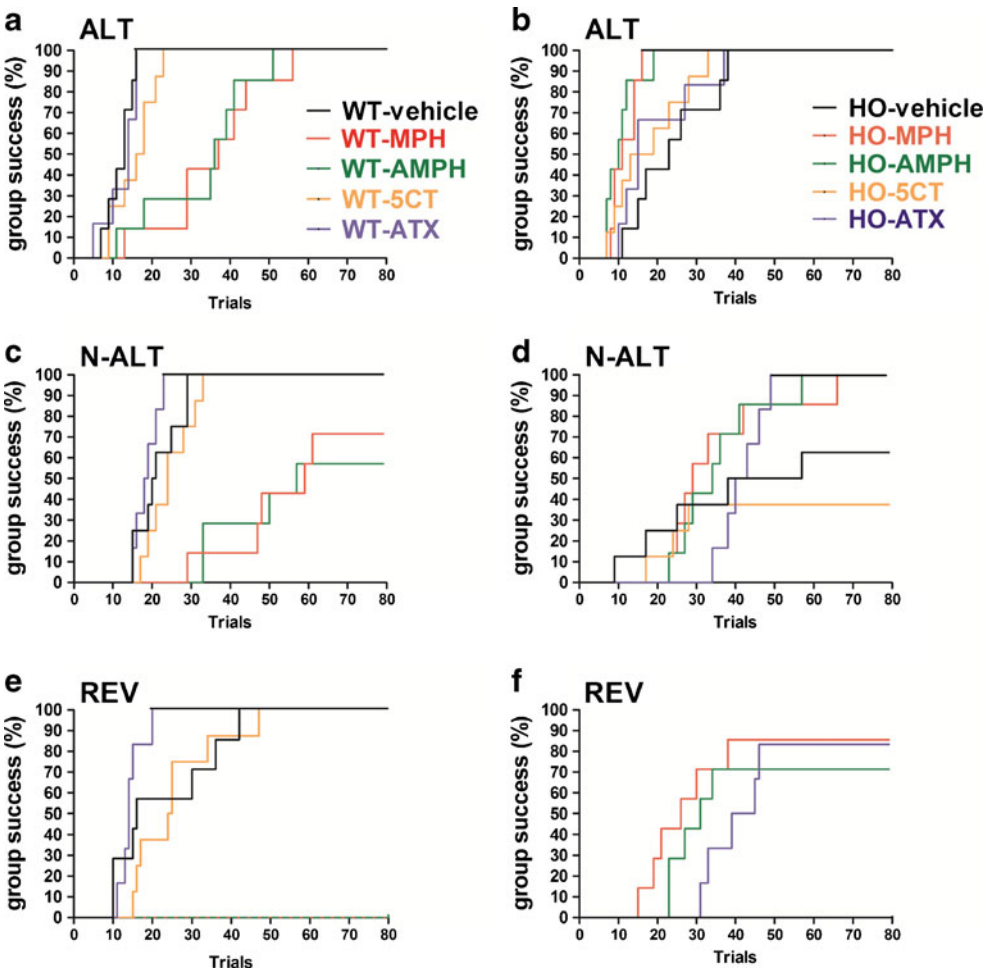

Fig. 4 Psychostimulants and ATX, but not 5CT, improved cognitive functions in HO mice in the H-maze. **a**, **b** Proportion (across trials) of successful WT and HO animals in the alternation (ALT) task for each drug treatment. **c**, **d** Proportion (across trials) of successful WT and HO animals in the nonalternation (N-ALT) task for each drug treatment. **e**, **f** Proportion (across trials) of successful WT and HO animals in the reversal (REV) task for each drug treatment

HO animals completed this task (Fig. 4d—log-rank test, MPH: $\chi(1)=7.63$, $p\leq0.01$; AMPH: $\chi(1)=6.15$, $p\leq0.05$). Interactions between genotypes and treatments were significant in trial-to-criterion performances (two-way ANOVA: $F(4, 50)=11.18$, $p\leq0.001$) and for the time used to reach the N-ALT task (two-way ANOVA: $F(2, 31)=6.04$, $p\leq0.01$). Treatment with psychostimulants increased the number of trials and the time taken by WT animals to complete the N-ALT task (Fig. 5c—Bonferroni post hoc test: $p\leq0.001$ and Fig. 5d—Bonferroni post hoc test: $p\leq0.05$). As for the previous task, MPH and AMPH treatment improved the number of trials, but not the time, taken by HO animals to complete the N-ALT task (Fig. 5c—Bonferroni post hoc test, MPH: $p\leq0.01$; AMPH: $p\leq0.05$). Vehicle HO animals required significantly more trials (two-tailed t test: $p\leq0.01$) and more time (two-tailed t test: $p\leq0.01$) to reach the N-ALT criterion compared to vehicle WT mice.

In the REV task, all vehicle-treated WT mice but none of the MPH- or AMPH-treated WT mice completed the task (Fig. 4e—log-rank test, MPH: $\chi(1)=14.88$, $p\leq0.001$; AMPH: $\chi(1)=13.71$, $p\leq0.001$). In contrast, all vehicle-treated HO animals failed to complete this task, while 85 % of MPH- and 70 % of AMPH-treated HO mice learned the rule (Fig. 4f—log-rank test, MPH: $\chi(1)=10.29$, $p\leq0.01$; AMPH: $\chi(1)=9.51$, $p\leq0.01$).

Finally, we performed a dose response with AMPH in WT mice to examine if lower doses of psychostimulants would enhance cognitive performances in the test. Two additional doses (0.2 and 1 mg/kg) were compared to existing datasets (Fig. S2). In the ALT task, a significant interaction was found between the three doses used (ANOVA: $F(3, 31)=8.86$, $p\leq0.001$). However, only the two highest doses of AMPH (1 and 2 mg/kg) impaired trial-to-criterion performances of WT mice (Fig. S2A—Newman–Keuls test: $p\leq0.01$ and $p\leq0.05$, respectively), while the lower (0.2 mg/kg) dose was without effect. A significant interaction between the three doses of AMPH also existed in the N-ALT task (ANOVA: $F(3, 24)=9.54$, $p\leq0.001$). As in the ALT task, only the two highest doses of AMPH (1 and 2 mg/kg) affected trial-to-criterion performances (Fig. S2C—Newman–Keuls test: $p\leq0.01$) and overall task completion (Fig. S2D—log-rank test, 1 mg/kg: $\chi(1)=19.73$; 2 mg/kg: $\chi(1)=18.69$, $p\leq0.001$) of WT mice. No WT animals treated with AMPH at 1 and 2 mg/kg completed the REV task (Fig. S2F—log-rank test,

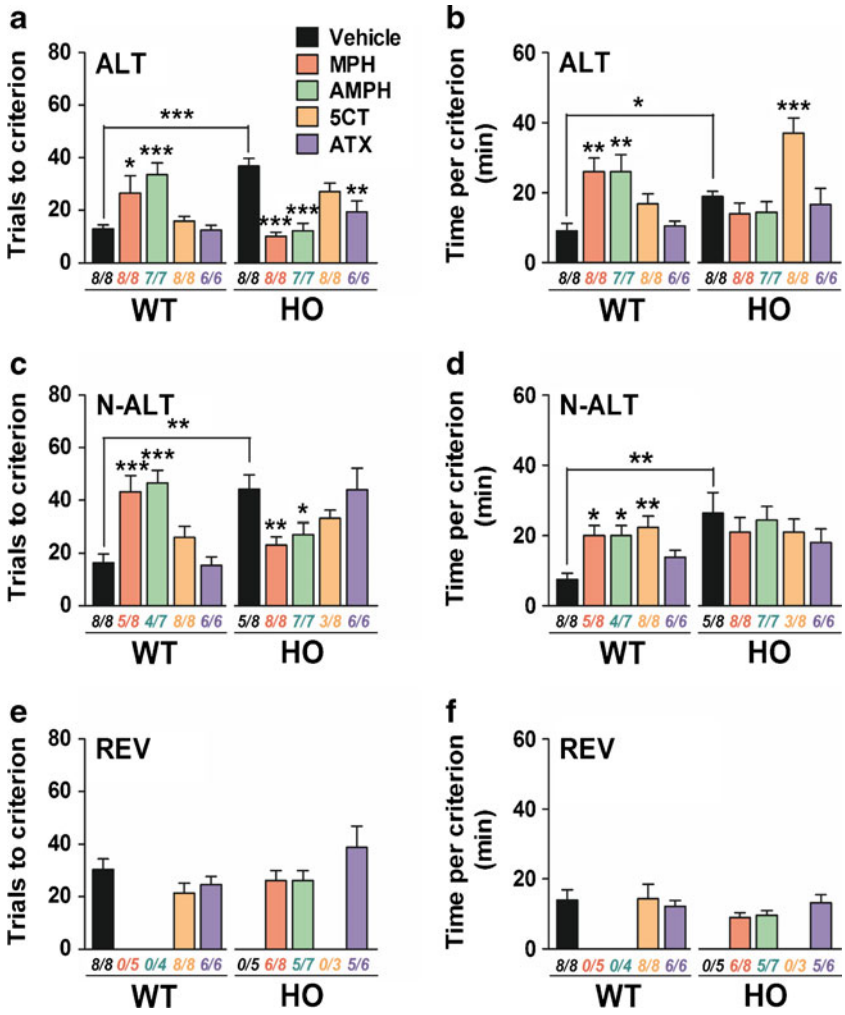

Fig. 5 Psychostimulants rescued spatial learning, cognitive flexibility, and reversal learning of DAT-KO mice in the H-maze. **a**, **b** Significant interactions in trial-to-criterion and time performances between genotypes and treatments in the ALT task. Vehicle HO animals required significantly more trials and more time to reach the ALT criterion compared to vehicle WT mice. **c**, **d** Interactions between genotypes and treatments were significant in trial-to-criterion performances and for the time used to reach the N-ALT task. Vehicle HO animals required significantly more trials and more time to reach the N-ALT criterion compared to vehicle WT mice. **e**, **f** Trial-to-criterion and time performed by both genotypes in the reversal (REV) task for each drug treatment. Data are presented as means ± SEM. Fractions below each bar indicate the proportion of mice that completed each task. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Two-way ANOVA followed by Bonferroni post hoc tests

1 mg/kg: $\chi(1)=27.57$; 2 mg/kg: $\chi(1)=25.29$, $p \leq 0.001$), while the overall performances of mice treated with 0.2 mg/kg were similar to those of vehicle treated animals (Fig. S2E, F).

Atomoxetine and serotonergic drugs have different effects on cognition in DAT-KO mice

Since NE and 5-HT neurotransmission can also be affected by psychostimulants, we used the H-maze as a drug-validating test to rescue cognitive functions of DAT-KO mice with the NET blocker atomoxetine (Bymaster et al. 2002) and the nonselective 5-HT receptor agonist 5CT that was previously shown to inhibit hyperlocomotion in DAT-KO mice (Beaulieu et al. 2006).

In a representative example (Fig. 3g), a 5CT-treated WT mouse completed the three tasks with similar trial-to-criterion performances as compared to vehicle-treated WT mice. In a representative 5CT-treated HO mouse, this compound did not improve cognitive performances in the automated H-maze (Fig. 3h). Treatment with ATX did not change the cognitive abilities of a representative WT mouse (Fig. 3i). In contrast, ATX treatment improved cognitive performances and led to the successful learning of the three rules (Fig. 3j) when administered to a representative HO mouse.

In the ALT task, all vehicle and 5CT- and ATX-treated WT and HO mice learned the rule (Fig. 4a, b). WT animals did similar trials and time performances in comparison with vehicle-treated animals (Figs. 4a and 5a, b). In HO mice, ATX treatment reduced the number of trials but not the time taken to learn the rule as compared to vehicle-treated animals of this same genotype (Fig. 5a, b—a: Bonferroni post hoc test, ATX: $p \leq 0.01$). In contrast, 5CT had no effect on the number of trials performed by HO mice compared to vehicle, but severely increased the time needed by mice from this genotype to learn the rule (Fig. 5a, b—b: Bonferroni post hoc test, 5CT: $p \leq 0.001$), possibly as a consequence of the reduction of locomotor activity induced by this drug in DAT-KO mice (Beaulieu et al. 2006).

In the N-ALT task, all vehicle-, 5CT-, and ATX-treated WT mice learned the rule with comparable success rates (Fig. 4c) and trial-to-criterion performances (Fig. 5c). However, 5CT increased the time taken by WT mice to complete this task

(Fig. 5d—Bonferroni post hoc test: $p \leq 0.01$). In HO mice, all ATX, 60 % of vehicle, and 35 % of 5CT-treated animals completed the N-ALT task (Fig. 4d). Among mice that completed the task, 5CT- and ATX-treated HO mice reached the N-ALT criterion with similar trials and time performances as compared to vehicle-treated HO mice (Fig. 5c, d).

All vehicle-, 5CT-, and ATX-treated WT mice completed the REV task (Fig. 4e). In contrast, no vehicle- and 5CT-treated HO mice learned the REV rule, but 85 % of ATX-treated HO animals did (Fig. 4f—log-rank test, ATX: $\chi(1)=11.29$, $p \leq 0.001$). Finally, treatment with the selective $5HT_{2c}$ receptors agonists, CP-809,101, which has been reported to improve cognitive functions in mice (Siuciak et al. 2007), also failed to affect the cognitive performances of WT and DAT-KO mice in the H-maze. Indeed, CP-809,101 did not change the representative profiles of WT and HO mice (Fig. S3A, B) and did not improve trials and time performances (Fig. S3C, D) nor overall success rates (Fig. S3E, F).

Atomoxetine treatment does not affect locomotor hyperactivity in DAT-KO mice

Since psychostimulants and 5CT reduce locomotor activity in DAT-KO mice (Beaulieu et al. 2006), we measured the possible locomotor effects of ATX in these mutant mice. Importantly, ATX had no effect on locomotor hyperactivity in DAT-KO animals (Fig. S4), therefore indicating that the cognitive enhancing action of this drug in DAT-KO mice is not related to an effect on locomotion.

Discussion

Data presented in this study indicate that DAT-KO mice display a cognitive deficit that is detectable in the automated H-maze. Furthermore, this deficit can be rectified, at least in part, by drugs used to treat ADHD in humans.

Cognitive deficit found in untreated DAT-KO was replicated in WT mice treated with psychostimulants that enhance DA tone acutely, therefore suggesting a direct contribution of exacerbated DA neurotransmission in contrast to long-term adaptive changes. In addition to their effects on ADHD, psychostimulants have also been reported to enhance cognitive ability in non-ADHD humans, therefore leading to illicit use of these drugs as cognitive enhancers (McCabe et al. 2005; Lucke et al. 2011). However, the overall benefits of psychostimulants on cognitive performances in control subjects remain controversial (Advokat and Scheithauer 2013). For instance, MPH has been shown to affect brain activity patterns without cognitive enhancement in healthy male subjects (Tomasi et al. 2010). In line with the result reported here, studies conducted in rats reported a negative effect of acute low dose (0.5 mg/kg AMPH) or chronic psychostimulant treatments on reversal learning (Idris et al. 2005; Kosheleff et al. 2012). Therefore, a better understanding of the specific executive cognitive domains that are affected by psychostimulants in healthy humans may be necessary to explain the lack of procognitive effects of these drugs on WT mice in the automated H-maze.

Interestingly, DAT-KO mice displayed deficiencies in all three tasks of the test and did not show specific repetitive/perseverative strategies in these tasks. This is in contrast to a previous study using the automated H-maze in mice with prefrontal cortex lesions (Del'Guidice et al. 2009) that have shown the occurrence of a type of perseverative behaviors consisting in the repetition of an unrewarded strategy following the first change of rule between the ALT and the N-ALT task. The occurrence of a different behavioral pattern in DAT-KO mice indicates that the automated H-maze can be used to identify different types of cognitive deficits and suggests that DAT-KO mice may suffer from different learning deficiencies in comparison with mice with simple prefrontal cortex lesions. Indeed, DAT-KO mice have been shown to exhibit mild impairments in spatial memory and deficits in cognitive flexibility, mainly in reversal learning and cued-associated memory tasks in the Morris water maze (Morice et al. 2007). Furthermore, impaired response inhibition has also been reported in these mutant mice, notably some perseverative error trials in the eight arms radial maze (Gainetdinov et al. 1999), and repetitive locomotor patterns in open field (Ralph et al. 2001). However, our observations indicate that in the H-maze, DAT-KO mice do not engage in perseverative failed problem solving strategies similar to those exhibited by mice with frontal cortex lesions. Taken together, this suggests that DAT-KO mice may suffer from several functional deficits involving not only the frontal cortex but also other brain regions such as the hippocampus, amygdala, and striatum (Gainetdinov et al. 1999; Morice et al. 2007; Weiss et al. 2007).

The precise mechanism by which psychostimulants exert their behavioral effects in ADHD patients or DAT-KO mice is not fully understood. It is important to note that even if DAT is the main target of AMPH and MPH, these psychostimulants still induce behavioral effects in mice lacking that transporter. Interestingly, an overexpression of DAT in bacterial artificial chromosome (BAC) transgenic mice (DAT-BACtg), which leads to decreased DA extracellular levels in subcortical areas, did not affect MPH-induced hyperlocomotion (Salahpour et al. 2008). Several investigations in DAT-KO mice have shown that psychostimulants have no effect on clearance and extracellular DA levels in subcortical areas involved in the regulation of locomotion by DA (Gainetdinov 2008). Furthermore, AMPH and fluoxetine both decrease hyperlocomotion in DAT-KO mice (Gainetdinov et al. 1999; Spielewoy et al. 2001; Beaulieu et al. 2006). These evidences suggest that the behavioral effects of psychostimulants on

ADHD-like symptoms may not involve only the DA system, but also other neurotransmitters, such as 5-HT and NE (Bymaster et al. 2002; Koda et al. 2010; Oades 2008).

In humans, the role of 5-HT in ADHD is controversial since drugs targeting specifically 5-HT neurotransmission are ineffective in treating ADHD symptoms (Newcorn 2008; Spencer et al. 2002; Verbeeck et al. 2009). However, previous studies have shown that drugs enhancing 5-HT tones and nonselective 5-HT receptor agonists, like the 5CT, are efficient to reduce hyperactivity in DAT-KO mice (Beaulieu et al. 2006; Gainetdinov et al. 1999; Spielewoy et al. 2001). Furthermore, modulation of 5-HT receptors has been proposed to be involved in the procognitive actions of ADHD drugs (Arnsten and Pliszka 2011; Robbins and Arnsten 2009). More specifically, previous studies have suggested a role for several 5-HT receptors in the regulation of reversal learning and cognitive flexibility by psychoactive drugs (Homberg 2012). The main putative targets of such processes are the $5HT_2$ receptors that are highly expressed in the prefrontal cortex (Clemett et al. 2000; Jensen et al. 2010). In order to address the role of 5-HT in cognitive functions, DAT-KO mice were treated with the nonselective 5-HT receptor agonist 5CT that is known to decrease hyperlocomotion in these mice (Beaulieu et al. 2006). Our findings showed that stimulation of 5-HT receptors by 5CT did not rescue the cognitive deficits of these mutant mice. Furthermore, selective stimulation of $5HT_{2c}$ receptors with CP-809,101, a compound that has been reported to improve cognitive functions in rodents (Siuciak et al. 2007), also failed to affect the performances of DAT-KO in the H-maze, suggesting that improvement of cognitive performances by psychostimulants in these mice is not associated with increased 5-HT neurotransmission. That being said, the marked innervations of subcortical regions by 5-HT projections and the clear involvement of 5-HT systems in the control of locomotion in animals strongly support that 5-HT neurotransmission may play a key role in motor dysfunctions associated with ADHD symptoms (Oades 2008).

Like psychostimulants, tricyclic antidepressants, which have a strong affinity for the NET as compared to other monoamine transporters, have been extensively prescribed for the treatment of ADHD (Bolden-Watson and Richelson 1993; Newcorn 2008; Niederhofer 2008; Verbeeck et al. 2009). In line with this, ATX has also been shown to improve core ADHD symptoms in several clinical trials (Bushe and Savill 2013), while some evidence point toward an effect of this drug on the amelioration of higher order neuropsychological deficits (Maziade et al. 2009). Furthermore, it has been suggested that blockade of NET can mediate procognitive effects and behavioral flexibility in both human and animals (Hammerness et al. 2009; Seu et al. 2009). In contrast to 5CT, ATX restored the cognitive abilities without attenuating locomotor hyperactivity in DAT-KO mice. Indeed, the dissociation between locomotion and cognition in this paradigm revealed that the hyperactivity of DAT-KO mice did not affect dramatically their cognitive abilities (Table 1). Recent investigations in mice have shown that ATX specifically increases extracellular DA and NE levels in the prefrontal cortex (Bymaster et al. 2002; Koda et al. 2010). Therefore, the topographic selectivity of ATX could explain, at least in part, its specific cognitive impact and its lack of subcortical-related locomotor effects in DAT-KO mice. The possible contribution of different mechanism to the regulation of locomotion and cognition is also compatible with the existence of different subtypes of ADHD with stronger hyperactive or inattentive components. However, further investigations using experimental systems that selectively target cortical catecholamine systems are required to better characterize the cognitive impact of DA, 5-HT, and NE in ADHD.

In conclusion, our findings showed that the "paradoxical" effects of psychostimulants on hyperactivity and cognition can be replicated in DAT-KO mice and measured using the automated H-maze. Therefore, data presented here support the predictive validity of a combination of DAT-KO mice with a complex behavioral paradigm like the H-maze to perform the

Table 1 Effects of different treatments on behavioral performances in WT and DAT-KO mice

	Vehicle	Psychostimulants (AMPH and MPH)	5CT	Atomoxetine	CP-809,101
WT	– Three rules learned	– Two rules learned – Spatial learning, cognitive flexibility, and reversal learning deficits – Hyperlocomotion	– Three rules learned – Lack of cognitive effects – Reduced locomotion	– Three rules learned – Lack of cognitive effects	– Three rules learned
HO	– Two rules learned – Spatial learning, cognitive flexibility, and reversal learning deficits – Hyperlocomotion	– Three rules learned – Cognitive improvements – Hyperlocomotion abolished	– Two rules learned – Lack of cognitive effects – Reduced locomotion	– Three rules learned – Cognitive improvements – Persistent hyperlocomotion	– Two rules learned – Lack of cognitive effects – Persistent hyperlocomotion

preclinical screening of ADHD drugs. Furthermore, we showed that drugs acting selectively on NE or 5-HT neurotransmission display distinct effects on cognition and locomotion in DAT-KO mice. This can provide a potential explanation for discrepancies between human clinical data and observation made solely on the basis of behavioral activity in these mice. These findings may contribute to a better understanding of the mechanisms underlying pharmacological therapies in ADHD and to the development of procognitive agents targeting catecholamine neurotransmission.

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Conflict of interest The authors declare no competing interests.

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