

Research report

# Olfactory discrimination deficits in mice lacking the dopamine transporter or the D2 dopamine receptor

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## Abstract

Previous pharmacological studies have implicated dopamine as a modulator of olfactory bulb processing. Several disorders characterized by altered dopamine homeostasis in olfaction-related brain regions display olfactory deficits. To further characterize the role of dopamine in olfactory processing, we subjected dopamine transporter knockout mice (DAT  $-/-$ ) and dopamine receptor 2 knockout mice (D2  $-/-$ ) to a battery of olfactory tests. In addition to behavioral characterization, several neurochemical markers of olfactory bulb integrity and function were examined. DAT  $-/-$  mice displayed an olfactory discrimination deficit, but did not differ detectably from DAT wildtype (DAT  $+/+$ ) mice in odor habituation, olfactory sensitivity, or odor recognition memory. Neurochemically, DAT  $-/-$  mice have decreased D2 receptor staining in the periglomerular layer of the olfactory bulb and increased tyrosine hydroxylase immunoreactivity compared to DAT  $+/+$  controls. D2  $-/-$  mice exhibited the same olfactory deficit as the DAT  $-/-$  mice, further supporting the role of dopamine at the D2 synapse in olfactory discrimination processing. The findings presented in this paper reinforce the functional significance of dopamine and more specifically the D2 receptor in olfactory discrimination and may help explain the behavioral phenotype in the DAT and D2 knockout mice.

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## 1. Introduction

The interaction of dopamine (DA) at the glomerular (D2 receptor) and granule (D1 receptor) layers of the olfactory bulb suggests a role for dopamine in odor processing [6]. More specifically the D2 receptor has been implicated in odor discrimination. The interaction between DA and the D2 receptor provides an inhibitory influence on the input from the olfactory receptor neurons and also results in lateral inhibition of the mitral/tufted output cells in the glomerular layer through facilitation of GABAergic synaptic activ-

ity [4,7,12,25,32,45,49]. Depletion of olfactory bulb dopamine impairs olfactory discrimination in rodents and this effect is replicated by D2 receptor antagonist treatment [27,28,37,38,49]. In addition, an association between olfactory dysfunction and altered dopamine neurotransmission is observed in several neurological disorders, such as Parkinson's disease [3,6,11,26].

One of the major regulators of DA function is the plasma membrane dopamine transporter (DAT). High levels of DAT expression are found in the dopaminergic cells of the substantia nigra and ventral tegmental area, and in projection areas of the basal ganglia [8–10,34]. DAT is also expressed in the retina, olfactory bulb and the hypothalamus [8]. Deletion of the DAT gene results in increased extracellular DA and enkephalin expression, decreased tissue content of DA, reduced levels of D1 and D2 receptors, as well as loss of D2 autoreceptor function within the striatum [19,29,30].

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Several social anomalies are observed in dopamine transporter knockout (DAT  $-/-$ ) mice, including increased social interaction and aggression [48]. In addition, DAT  $-/-$  mice have well-defined behavioral abnormalities: hyperactivity, irritability, decreased habituation to a novel testing environment, and deficits in learning and maternal behavior [16,17,19,44]. These behavioral differences are reminiscent of the behavior seen in rodents with bilateral removal of the olfactory bulb. Bulbectomized rodents display hyperactivity, decreased habituation, increased aggression, altered maternal behavior, and learning and memory deficits [33,47]. Based on the striking similarities between DAT  $-/-$  mice and bulbectomized rodents and the pharmacological evidence supporting a role for DA in olfactory processing, we subjected DAT  $-/-$  mice to a battery of tests to determine whether they had olfactory deficits. In addition, based on the alterations in D2 receptor immunoreactivity that we found in the olfactory bulbs of DAT  $-/-$  mice and previous research implicating the D2 receptor in olfactory discrimination, we tested the hypothesis that D2  $-/-$  mice also have olfactory deficits. These findings are novel in that they are the first to demonstrate an inherent DA-mediated olfactory deficit in intact and drug-naïve animals.

## 2. Methods

### 2.1. Animals

Adult DAT  $-/-$  mice were obtained through genetic manipulation on a mixed C57/129SvJ background [19]. Dopamine transporter knockout mice and their wildtype (+/+) littermates were generated by crossing DAT heterozygote mice for seven to nine generations over approximately 2 years. Adult D2  $-/-$  mice were obtained through genetic manipulation [31]. Heterozygous D2  $-/-$  mice (derived from an N6 near congenic line in C57BL/6 background) were bred to generate D2  $-/-$  and their wildtype (+/+) littermates. Mice were individually housed in clear plastic cages, maintained on a 12-h light:12-h dark cycle, and given food and water *ad libitum*. All behavioral testing was performed between 7 and 9 a.m. in the animals' home cages. Cages were cleaned the afternoon prior to testing.

### 2.2. Behavioral tests

#### 2.2.1. Olfactory discrimination

**2.2.1.1. Experiment 1.** A glass plate was placed in the animal's home cage. Solution concentrations (100 ng/ml) were freshly prepared before each experiment and presented in 25  $\mu$ l aliquot solutions consisting of paprika, cinnamon, or sterilized water alone [14,18]. In a habituation–dishabituation paradigm, each mouse was presented with the first odor (either paprika or cinnamon) on one side of the plate and water (control) on the other side for five successive 3-min trials, separated by 15-min inter-trial intervals to ensure that the sensory adaptation did not influence information processing and later memory testing. The time spent investigating both the water and scented solution was recorded. Habituation response was measured on the first five trials by examining investigation time across trials. On the sixth trial, the mouse was presented with the alternative odor (cinnamon or paprika) for 3 min along with the water control. Discrimination was defined as the relation between time spent sniffing the familiar odor (fifth trial) and time spent sniffing the novel odor on the sixth trial. Sniffing was defined when the animal's nose was located 1 cm or less from the odor.

**2.2.1.2. Experiment 2.** Wooden blocks (1.8 cm<sup>3</sup>) were placed individually for 12 h in 50 ml conical tubes containing 1 g of animal bedding from test animals' cages. The animal was presented with a block scented with its own bedding and a block scented with another mouse's bedding (of the same sex). The time spent in contact with each block was recorded for a 2-min trial.

#### 2.2.2. Sensitivity

Olfactory sensitivity was assessed by comparing the time spent sniffing a paprika solution versus time spent sniffing a water-only solution, as described in Section 3.1.1 [18]. Three different concentrations of paprika were used (100, 10, and 1 ng/ml) in a step-down fashion, on separate sessions. Lack of detection was defined by equal investigation between the odor solution and the water control (50%). Sessions were separated by at least 2 days for DAT  $-/-$  mice and 4 h for D2  $-/-$  mice. Differences in inter-session intervals were a result of time restrictions on D2 mouse testing.

#### 2.2.3. Long-term habituation or memory for a single odor

Olfactory memory was tested by exposing mice twice to the same odor with varied intervals between odor presentation (20, 40, 60, 80, or 100 min) [18]. Intervals were examined in separated sessions at least 2 days apart, and the order of inter-trial intervals across sessions was random, as were odors (paprika or cinnamon: 100 ng/ml). Animals were considered to remember/recognize when a significant decrease in investigatory time of the presented odor on the second presentation was observed.

#### 2.2.4. Non-olfactory sensory tests

To assess non-olfactory sensory function in DAT  $-/-$  mice, we examined the responses of these animals in several sensory tests.

1. Responsiveness to tactile stimulation was measured as a latency to contact/remove a 113.1 mm<sup>2</sup> (1.3 cm in diameter) adhesive dot (Avery Office International) [40]. Animals were removed from their home cage, and the dot placed between the ears, on top of the head. Animals were then put back into their home cage, and cage returned to its normal position on the rack to reduce external distractions. Latencies to contact/remove dots were recorded. Recording was stopped at 2 min.
2. Quinine is often used in taste avoidance/rejection paradigms, due to its highly unpalatable bitterness. The aversive reaction to quinine was assessed. The tip of a cotton swab was placed into an aliquot of 2 mg/ml of quinine. Animals were removed from their home cage, and the tip of the cotton swab was inserted into their mouths. They were then placed back in their home cage and put into their normal resting position. Latency to groom and/or drag the jaw along the ground was recorded [21,43]. To control for the stimulation of the cotton swab alone, animals were exposed to a clean cotton swab as presented above, and latency to groom and/or drag the jaw was recorded. Presentation of clean versus quinine stimuli was counterbalanced between animals. The cut-off time for response in this test was 60 s.
3. The trigeminal nerve innervates areas of the olfactory epithelium and nasal mucosa and is responsible for non-odor sensations such as mild irritation and burning sensation. In this test, we assessed the function of the trigeminal nerve by exposing the mice to either ammonia, known to exert a trigeminal response, or water. A glass plate with 25  $\mu$ l of water or ammonia (counterbalanced between animals) was placed in the animal's cage. Time sniffing (as defined above) was recorded for a 2-min session.

### 2.3. BrdU labeling, tissue processing and histochemistry

Adult DAT  $+/+$  and DAT  $-/-$  mice ( $n = 6$  per group) received two intraperitoneal injections of BrdU (50 mg/kg) in phosphate-buffered saline (PBS) and 11 days later were perfused with 4% paraformaldehyde. Brains were removed, cryoprotected in 25% sucrose in PBS and then frozen in powdered dry ice. Forty microns coronal sections through the rostral subventricular zone (SVZ) (extending rostrally +0.2 mm from Bregma), rostral migratory stream and olfactory bulb were cut with a sliding microtome and every sixth section was used for Nissl or immunohistochemical stains. Diaminobenzidine peroxidase immunohistochemistry was performed on free-floating sections as described previously [36] using antibodies to BrdU (1:1000 dilution; mouse monoclonal; Boehringer Mannheim, Indianapolis, IN), doublecortin (1:5000; rabbit polyclonal; a gift of Chris Walsh, Harvard University, Boston, MA), GFAP (1:500; rabbit polyclonal; Sigma), TH (1:500; rabbit polyclonal; Chemicon, Temecula, CA), and D2 receptor (1:200; rabbit polyclonal; Chemicon, Temecula, CA). The only modification was incubation of sections in D2 receptor antibody for 24 h at room temperature instead of overnight at 4 °C. For Nissl staining, sections were mounted on slides

(Superfrost-plus, Fisher Scientific, Pittsburgh, PA), dehydrated and rehydrated in graded ethanols and xylenes, incubated in 1% cresyl violet (Sigma, St. Louis, MO) for 30 s, decolorized in acetic acid, and then dehydrated and coverslipped with Permount (Fisher Scientific).

2.4. Data analysis

Statistical analyses were run using SPSS Software. Data in Section 3.1.1 were analyzed using repeated-measures ANOVA (group × odor). Post hoc analyses examined each group response to the control (trial 5) and discriminative odor (trial 6). In addition, habituation to the odor for trials 1–5 was analyzed using one-way ANOVAs (odor/water × presentation) for each group. In Section 3.1.2, individual one-way ANOVAs per group were used to analyze the time spent investigating each block for the two paradigms. Similarly, each group was separately analyzed for odor sensitivity. One-way ANOVAs comparing time spent investigating the odor versus time spent investigating water were run for each concentration. Finally, a repeated measures ANOVA (group × inter-trial interval) was conducted in the long-term habituation/memory task.

Tactile and quinine sensitivity were analyzed with a one-way ANOVA for each group. Comparisons between water control and ammonia were tested using a one-way ANOVA for both wild type and knockout animals. In addition, the responses of each group to water control and ammonia were compared. When appropriate, post hoc analyses were run with a Bonferroni correction. Levels of significance were set at 0.05. Immunostained sections were examined by light microscopy and analyzed by a blinded observer. The intensity of olfactory bulb and striatal TH and D2 receptor immunostaining in DAT +/+ and -/- mice was scored as minimal (+/-), mild (+), moderate (++), or strong (+++) under 200× magnification (see Table 1).

3. Results

3.1. Behavioral manifestations of olfactory discrimination deficit in DAT and D2 -/- mice

3.1.1. Discrimination experiment 1

The first olfactory discrimination experiment examined the ability of the animal to discriminate between a familiar (habituated) odor and a novel odor. During the initial trials of the

Table 1

Summary table of immunostaining in olfactory bulbs of DAT +/+ and -/- mice

	DAT +/+	DAT -/-
Striatal TH	+++	+
Olfactory TH	++	+++
Striatal D2	+++	+
Olfactory D2	++	+/-

The intensity of olfactory bulb and striatal TH and D2 receptor immunostaining in DAT +/+ and -/- mice was scored as minimal (+/-), mild (+), moderate (++), or strong (+++) under 200× magnification.

habituation phase of this task, both DAT +/+ and DAT -/- animals clearly detected the paprika or cinnamon odor, demonstrated by a greater amount of time investigating these solutions over water only. Both groups of animals also demonstrated similar habituation to either the paprika or cinnamon odor across the first five presentations in this task (water:  $F(4,36) = 11.16$ ,  $p < .01$ ; odor:  $F(4,36) = 22.09$ ,  $p < .01$ ). No significant group differences were seen for either water ( $F(1,9) = 2.82$ ) or odor ( $F(1,9) = .52$ ) habituation measures (Fig. 1A and B).

A significant group by trial interaction was found in the first discrimination task comparing investigatory time between the familiar (habituated) odor and a novel odor ( $F(1,9) = 37.26$ ). Further examination compared each group on response to the habituated odor (trial 5) and response to the novel odor (trial 6). A significant increase in time spent investigating the novel odor was found for the DAT +/+ group ( $F(1,8) = 30.21$ ,  $p < .01$ ). In contrast, DAT -/- animals showed no significant change in investigatory time when presented with a novel odor ( $F(1,10) = .49$ ). DAT -/- animals performed in a similar fashion to DAT +/+ animals during the habituation phase, demonstrating that they are both capable of performing the task and show behavioral interest in novel odors. In contrast, when presented with a novel

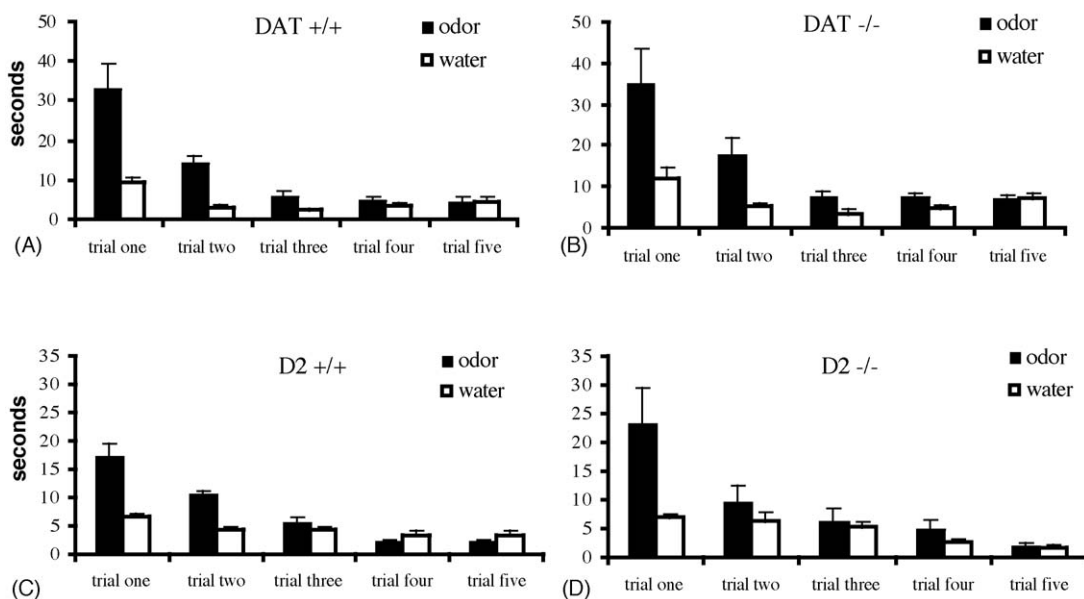


Fig. 1. Habituation to novel odor. (A) DAT +/+ shows initial preference for the novel odor vs. water, but habituate to both odors by the fifth trial. (B) DAT -/- mice show a similar habituation pattern as DAT +/+ control. (C) D2 +/+ show initial preference to the novel odor vs. water, but habituate to the odorant by the fifth trial. (D) D2 -/- mice habituate to a novel odor over trials in a similar manner as D2 +/+ controls.

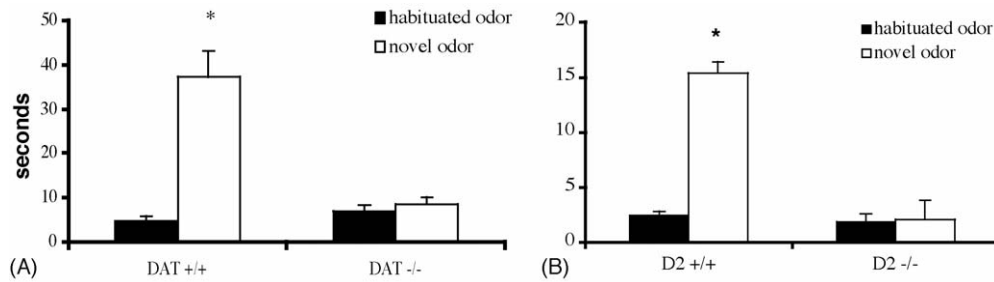


Fig. 2. Lack of olfactory discrimination in DAT  $-/-$  and D2  $-/-$  mice. (A) DAT  $+/+$  animals showed an increase in investigatory time following presentation of a novel odor on trial 6 vs. a habituated odor on trial 5. DAT  $-/-$  mice did not show increased investigation towards the novel odor. (B) As seen in DAT  $-/-$  mice, D2  $-/-$  animals displayed a significant deficit in discrimination upon presentation of a novel odor following a habituation/dishabituation paradigm. In contrast, D2  $+/+$  animals showed an increase in investigatory time following presentation of a novel odor on trial 6.

odor, DAT  $-/-$  mice show significant behavioral performance consistent with olfactory discrimination impairment (Fig. 2A).

As was seen with the DAT mice, both D2  $+/+$  and D2  $-/-$  animals showed similar habituation across presentation (water:  $F(4,32) = 8.59$ ,  $p < .01$ ; odor:  $F(4,32) = 9.31$ ,  $p < .01$ ) (Fig. 1C and D). No significant group differences were found for water ( $F(1,8) = .07$ ) or odor ( $F(1,8) = .85$ ) habituation performance, suggesting a normal ability of D2  $-/-$  animals to perform both the motor and memory tasks required for the habituation and discrimination tasks.

In time spent investigating the habituated odor versus time spent in contact with the novel odor, D2  $-/-$  animals showed a significant group  $\times$  trial interaction ( $F(1,8) = 42.00$ ,  $p < .01$ ). Post hoc analyses revealed a significant increase in investigatory time of the novel odor for D2  $+/+$  mice ( $F(1,8) = 156.99$ ,  $p < .01$ ), whereas D2  $-/-$  mice did not show any change in investigatory time of the novel odor compared to the habituated odor ( $F(1,8) = .01$ ). Again, these data are consistent with an olfactory discrimination deficit in the D2  $-/-$  mice that is not the result of an inability to perform the task or lack of motivation, as observed in the reactivity and habituation phase of this task (Fig. 2B).

### 3.1.2. Discrimination experiment 2

In this discrimination task, we tested the ability of the animal to discriminate between a block scented with its own bedding and a block scented with a novel animal's bedding. No group differences between initial contact times were observed between

the DAT  $+/+$  and DAT  $-/-$  mice ( $F(1,8) = .39$ ). However, the DAT  $+/+$  mice spent significantly more time investigating the block scented with another animal's odor versus the self-scented block ( $F(1,8) = 13.43$ ,  $p < .01$ ). In contrast, DAT  $-/-$  mice did not display a preference for exploration of either block ( $F(1,8) = .40$ ). The lack of preference was not due to decreased exploration; rather, the animals switched back and forth between the two blocks during the testing trial (Fig. 3A).

Mirroring the DAT  $+/+$  mice, D2  $+/+$  mice derived from the D2  $-/-$  colony spent significantly more time in contact with the block scented with another animal's bedding versus the self-scented (with bedding) block ( $F(1,8) = 15.80$ ,  $p < .01$ ). Unlike the D2  $+/+$  animals, D2  $-/-$  mice did not show preferential exploration of either odor ( $F(1,8) = 1.17$ ) (Fig. 3B). As was seen with the DAT  $-/-$  mice, this lack of preference was not due to a decrease in exploration, indeed, most D2  $-/-$  mice spent all their time exploring whichever block they contacted first. Wild-type animals from both groups made contact with both blocks and then returned to the "preferred" block for the majority of the time. It is possible, given the limited capacity of this task, to allow for habituation that D2  $-/-$  mice fail to disengage from the block initially contacted, similar to animals with moderate 6-hydroxydopamine induced degeneration of nigrostriatal DA terminals, which orient to sensory stimuli presented to either side of the body normally, but fail to respond to sensory stimuli presented to the affected side when engaged in eating, drinking or grooming [39].

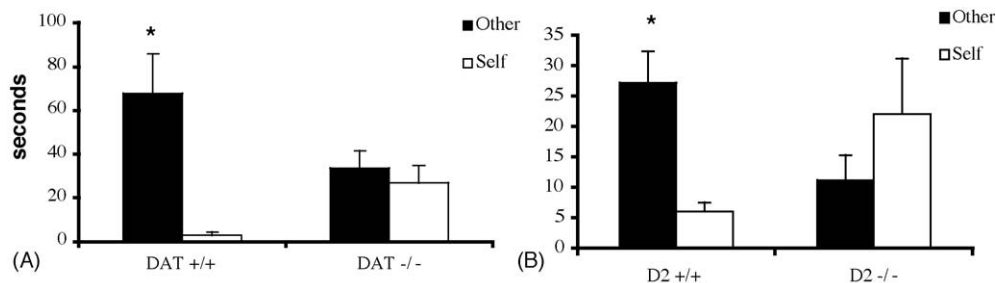


Fig. 3. Lack of between animal odor discrimination in DAT  $-/-$  and D2  $-/-$  mice. (A) When given a choice between a block scented with another animal's bedding and a block with the animal's own bedding, DAT  $+/+$  mice show preferential exploration of the block scented with a different animal's bedding. In contrast, DAT  $-/-$  mice do not show preferential exploration of either block. (B) Similar to DAT  $+/+$  mice, D2  $+/+$  mice prefer to explore the block scented with a different animal's bedding, whereas D2  $-/-$  animals did not show preferential exploration of either block.

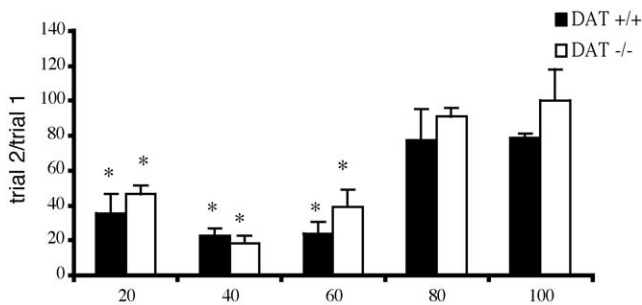


Fig. 4. Normal olfactory memory in DAT  $-/-$  mice. Both DAT  $+/+$  and DAT  $-/-$  mice show similar olfactory memory/long-term habituation performance. Both groups display memory for a previous odor up to inter-trial intervals of 60 min.

### 3.1.3. Sensitivity

To determine if the differences in olfactory discrimination seen in paradigm one were due to altered sensitivity, we estimated relative olfactory sensitivity across different concentrations of odorant. Significant odor detection was found in both the DAT  $+/+$  ( $F(1,8) = 12.18, p < .01$ ) and DAT  $-/-$  ( $F(1,10) = 6.43, p < .05$ ) groups at the 100 ng/ml odor concentration used in the discrimination task. Olfactory sensitivity was lost for both groups at the 10 ng/ml concentration (DAT  $+/+$ : ( $F(1,8) = 1.16$ ); DAT  $-/-$ : ( $F(1, 10) = .91$ ). These data demonstrate no difference in sensitivity at the concentration used in the first olfactory discrimination task.

Both D2  $+/+$  and D2  $-/-$  mice exhibited a significant sensitivity at the 100 ng/ml paprika concentration (D2  $+/+$ :  $F(1,8) = 10.30, p < .02$ ; D2  $-/-$ :  $F(1,8) = 30.70, p < .01$ ). As was seen in the DAT animals, sensitivity was lost for both D2  $-/-$  ( $F(1,8) = 3.32$ ) and  $+/+$  ( $F(1,8) = 3.28$ ) mice at the 10 ng/ml concentration.

### 3.1.4. Long-term habituation or memory for a single odor

In addition to discrimination and sensitivity, we measured olfactory memory in the different animals to determine whether there were any obvious group differences in olfactory memory performance. Neither a significant group  $\times$  time interaction ( $F(4, 36) = .47$ ), nor a significant group effect were detected ( $F(1,9) = 3.07$ ) in the olfactory memory task. Therefore, no significant differences between DAT  $-/-$  mice and DAT  $+/+$  mice were detected for long-term memory capacity (Fig. 4).

### 3.1.5. Non-olfactory sensory performance

Tactile, taste aversion, and trigeminal nerve responses were also measured to test for gross non-olfactory sensory deficits in DAT  $-/-$  mice (Fig. 5A–C). No significant performance differences were detected in tactile ( $F(1,9) = .69$ ) and quinine (negative taste reactivity) ( $F(1,9) = 1.96$ ) reactions between DAT  $+/+$  and DAT  $-/-$  mice. In addition, both DAT  $+/+$  and DAT  $-/-$  mice showed significant decreases in time spent investigating ammonia compared to water (DAT  $+/+$ :  $F(1,8) = 66.25, p < .01$ ; DAT  $-/-$ :  $F(1,10) = 135.75, p < .01$ ), but no significant difference between groups was seen in response to water ( $F(1,9) = .69$ ) or ammonia ( $F(1,9) = .19$ ). Thus, DAT  $-/-$  do not

differ from DAT  $+/+$  mice in measures of general tactile responsiveness, trigeminal nerve response, or aversive taste reactivity.

No significant differences were found in measures of tactile sensitivity ( $F(1,8) = 2.95$ ) or initiation of grooming or chin rubbing in response to quinine ( $F(1,8) = 3.43$ ). In addition, as with both DAT  $-/-$  and DAT  $+/+$  mice, both D2  $+/+$  and D2  $-/-$  mice showed significant decreases in investigatory time upon presentation of ammonia versus water (D2  $+/+$ :  $F(1,8) = 42.06, p < .01$ ; D2  $-/-$ :  $F(1,8) = 34.51, p < .01$ ), and no group differences were found in time spent investigating the water ( $F(1,8) = .59$ ), or time spent investigating the ammonia ( $F(1,8) = .02$ ) (Fig. 5D–F).

### 3.2. DAT $-/-$ mice do not display altered olfactory bulb architecture, nor neurogenesis

Nissl staining of olfactory bulb sections (40  $\mu$ m) in DAT  $+/+$  and DAT  $-/-$  mice showed the six characterized olfactory bulb layers. No obvious differences between DAT  $+/+$  and DAT  $-/-$  mice were found in the appearance of these structures (Fig. 6A and B). In addition to anatomical architecture, we also used BrdU labeling and immunostaining of migrating neuroblasts to investigate whether alterations in adult subventricular zone (SVZ)-olfactory bulb neurogenesis were apparent in DAT  $-/-$  mice. Impaired olfactory bulb neurogenesis in other genetically deficient mouse lines has been associated with olfactory discrimination defects [13,17]. BrdU was administered to label proliferating cells and mice were sacrificed 11 days later, a duration sufficient for labeled SVZ neuronal precursors to migrate to the olfactory bulb. No differences in BrdU-labeled cells in the SVZ, rostral migratory stream, or olfactory bulb were observed between DAT  $+/+$  and DAT  $-/-$  groups (data not shown). Immunostaining for doublecortin, a protein expressed by migrating neuroblasts in the SVZ-olfactory bulb pathway [20], and glial fibrillary acidic protein (GFAP) was also unchanged (data not shown). These findings suggest that persistent neurogenesis in the olfactory bulb is not disrupted in adult DAT  $-/-$  mice.

### 3.3. DAT $-/-$ mice display increased TH concentrations and a down-regulation of D2 receptors in the olfactory bulb

To determine whether dopamine regulation was disturbed in the glomerular layer of the olfactory bulb, we examined tyrosine hydroxylase (TH) expression in DAT  $+/+$  and DAT  $-/-$  mice by immunohistochemistry. TH immunostaining within the glomerular layer was substantially increased in DAT  $-/-$  mice compared to control animals (Fig. 6C and D; Table 1). Previous work has shown down-regulation of TH staining in the striatum of DAT  $-/-$  mice, and immunostaining of the striatum in our mice replicated this finding (Fig. 6E and F), reflecting differential regulation of TH in dopamine systems following DAT deletion. In addition to changes in TH staining, we found decreased D2 receptor staining in the olfactory receptor neuron layer and the glomerular layer in DAT  $-/-$  mice (Fig. 6G and H; Table 1). These findings demonstrate abnormal dopamine transmission within the olfactory bulb and specifically implicate the abnormal regulation of the D2 receptor as a cause of olfactory dysfunction in the DAT  $-/-$  mice.

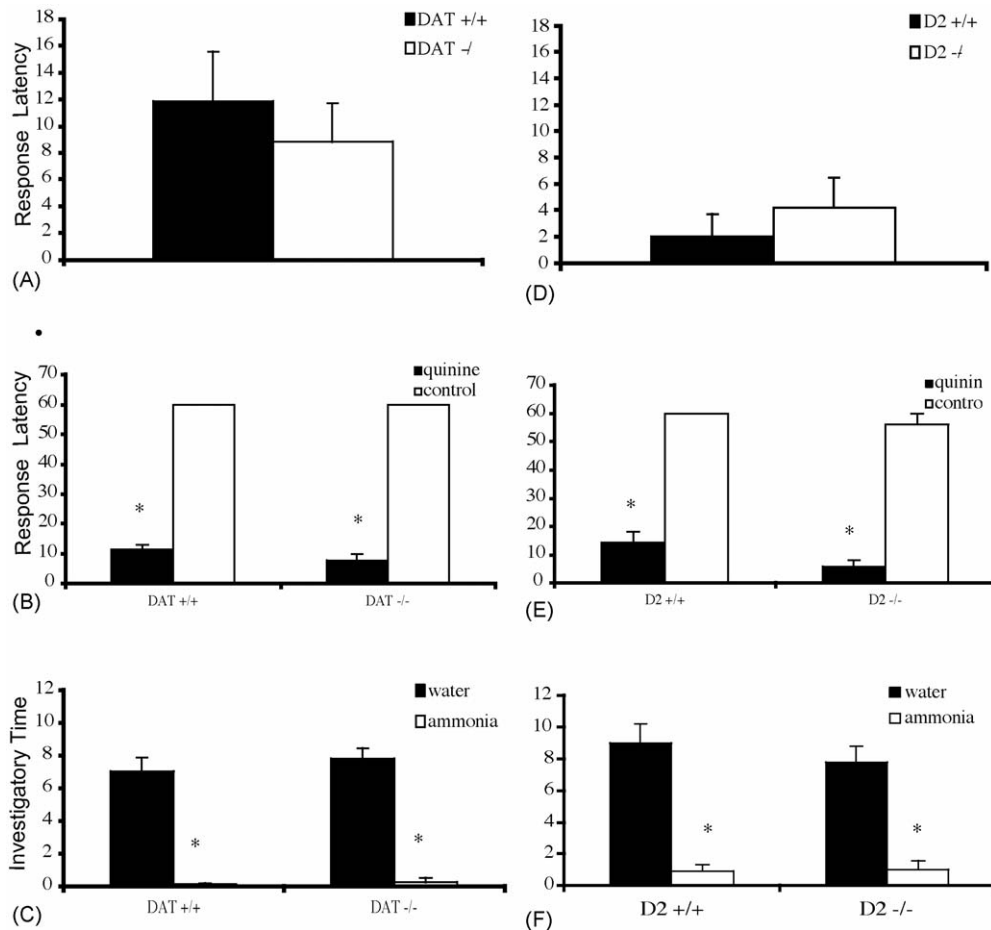


Fig. 5. Both DAT  $-/-$  and D2  $-/-$  mice perform similar to control mice on non-olfactory sensory tests. (A and D) Response to tactile stimulation was similar between both DAT and D2  $-/-$  groups compared to DAT and D2  $+/+$  controls. (B and E) Response latency for quinine was similar for both DAT and D2  $-/-$  groups compared to DAT and D2  $+/+$  controls. (C and F) Investigatory time for water vs. ammonia (aversive trigeminal stimulation) was similar between both DAT and D2  $-/-$  animals and DAT and D2  $+/+$  controls.

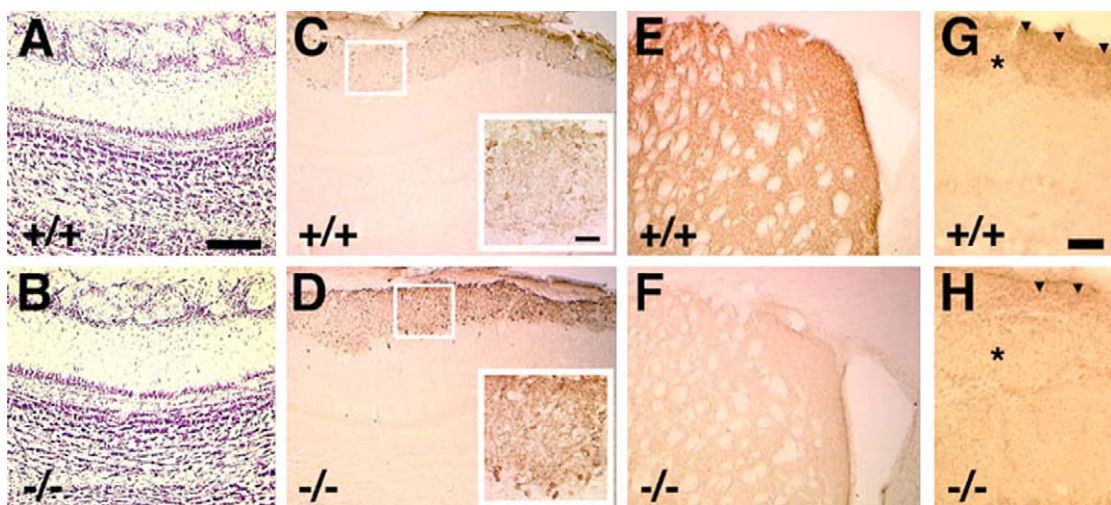


Fig. 6. Immunocytochemical analysis of olfactory bulb in DAT  $+/+$  and DAT  $-/-$  mice. Nissl staining of coronal sections through the olfactory bulb of DAT  $+/+$  (A) and DAT  $-/-$  (B) mice. Olfactory bulb structure was similar between the two groups (glomerular layer is at top). (C and D) Olfactory bulb TH immunostaining was increased in the glomerular layer of DAT  $-/-$  mice (D) compared to DAT  $+/+$  littermates (C). The boxed regions shown at higher magnification in the insets are single glomeruli. (E and F) TH immunoreactivity was decreased in the striatum of DAT  $-/-$  mice (F) compared to DAT  $+/+$  animals (E). (G and H) D2 receptor immunostaining of coronal olfactory bulb. D2 immunoreactivity was decreased in DAT  $-/-$  animals (H) compared to DAT  $+/+$  (G) in both the glomerular (asterisks) and olfactory nerve (arrowheads) layers. Scale bars: A–F (shown in A), 100  $\mu\text{m}$ ; insets in C and D (shown in C), 10  $\mu\text{m}$ ; G and H (shown in G), 40  $\mu\text{m}$ .

#### 4. Discussion

Several findings suggest that DA plays a role in olfactory bulb processing: DA and DAT localization in the periglomerular interneurons, D2 receptors on olfactory receptor neurons (ORN) and mitral/tufted cells, and D1 receptors within the granule layer [7]. More specifically, the interaction of DA at the D2 synapse has been implicated in odor discrimination [4,7,12,25,32,45,49]. While the majority of studies have utilized pharmacological methods to determine the role of DA in olfaction, we sought to further contribute to this knowledge by examining the role of DA in odor discrimination using mice, in which, either, DAT or the D2 receptor had been genetically deleted.

We first tested the hypothesis that deletion of DAT results in olfactory deficits. Animals lacking expression of DAT are unable to discriminate between a previously habituated odor and a novel odor (Fig. 2A). This effect was not based on an inability to perform the task, as both DAT  $-/-$  and DAT  $+/+$  animals explored odors for the same amount of time. Nor was this effect due to overall anosmia in the animals, as DAT  $-/-$  mice did not differ from DAT  $+/+$  animals in odor reactivity/habituation (Fig. 1A and B). To further support that DAT  $-/-$  mice suffer from an olfactory discrimination deficit, the animal's ability to discriminate between wooden blocks scented with the animal's own bedding and one scented with a novel animal's bedding was examined. As was seen in the first discrimination experiment, DAT  $-/-$  mice did not behaviorally display olfactory discrimination in this paradigm (Fig. 3A). No differences in olfactory sensitivity at the odor concentrations used in the first discrimination paradigms, olfactory memory, or non-olfactory sensory responses were detected between DAT  $-/-$  and DAT  $+/+$  mice (Figs. 4 and 5A). We suggest that the odor discrimination failure characterized by these data is a probable factor in many of the behavioral dysfunctions described in DAT  $-/-$  mice.

Following behavioral phenotyping of the olfactory deficit in DAT  $-/-$  mice, we examined the olfactory bulbs. The olfactory bulbs of DAT  $-/-$  mice did not differ from DAT  $+/+$  controls in basic architecture or neurogenesis patterns. Immunostaining of several DA markers within the olfactory bulb revealed increased TH immunoreactivity and decreased D2 immunoreactivity in the glomerular layer of DAT  $-/-$  mice (Fig. 6; Table 1). These findings are consistent with abnormal dopamine transmission within the olfactory bulb, and specifically implicate deregulation of the D2 receptor as a contributor to, or at least as a marker of olfactory dysfunction of DAT  $-/-$  mice.

Finally, based on the alterations in TH and D2 immunoreactivity in DAT  $-/-$  mice, we next examined olfactory function in D2  $-/-$  mice. As was found in DAT  $-/-$  mice, D2  $-/-$  mice show a specific olfactory discrimination deficit (Figs. 2B and 3B), but no alterations in habituation, sensitivity, or non-olfactory sensory responses (Figs. 1 and 5). The near identical behavioral olfactory phenotype between DAT and D2  $-/-$  mice further supports a role for the D2 receptor in olfactory discrimination. However, it should be noted that the behavioral performance of D2  $-/-$  mice on the second discrimination task could be interpreted as a disengage deficit similar to what is found in animals with neonatal or adult depletions of DA [41,42],

though this interpretation is not in opposition to an olfactory dysfunction.

The increase in olfactory bulb TH expression in DAT  $-/-$  mice may be derived from multiple effects of DAT deletion. Absence of DAT prevents recycling of DA, may impair D2 autoreceptor function [29], and results in increased demand on the periglomerular cells due to increased excitation of ORN and mitral/tufted cells. Although TH immunoreactivity was increased in the olfactory bulb of DAT  $-/-$  mice, we found decreased TH expression in the striatum, as has been described previously [30]. This disparity suggests that DA-containing neurons in local circuits of the olfactory bulb have a differential regulation of TH than do long-distance dopaminergic projection neurons in the striatum.

Although ORN show some specificity of response, less-specific binding does occur at receptor sites with a similar molecular structure [13,15]. The DA that binds the ORN originates from the periglomerular interneuron. DA binds D2 receptors on the ORN, creating a threshold of inhibition [2,22,23]. We hypothesize that the down-regulation of D2 receptors in DAT  $-/-$  mice and the deletion in D2  $-/-$  mice leads to a decrease in modulatory inhibition, resulting in a more generalized response of ORN to odorants, similar to what is seen following olfactory stimulation after a prolonged period of olfactory deprivation and D2 antagonist application [27,28,37,38,49].

Dopamine has been hypothesized to play a role in novelty-seeking [24,35]. Thus, one potential limitation to our study is that the primary deficits that we observed depend upon the animal discriminating a novel odor from a habituated odor. We believe that two lines of argument may discount this possibility. First, other studies have suggested that dopamine alterations in DAT or D2 receptor may lead to increases in novelty responses [46]. In addition, both D1 and D2/D3 antagonists have been shown to have no effect on visual object recognition tests at doses that affect conditioned place preference [5]. Second, our data demonstrate that initial odor exploration to either paprika or cinnamon is significantly greater than water exploration (Fig. 1). Together, these data suggest that our results are not due to a deficit in novelty-induced behavior. Rather, it suggests that our results using a novel odor recognition task are distinctly different from the novel visual object recognition task as reported by Besheer and colleagues [5]. Thus, the most parsimonious explanation for our findings is a specific deficit in olfactory discrimination.

Many disorders with hyperdopaminergic tone and/or D2 receptor gene alterations, such as Parkinson's disease (PD) show olfactory dysfunction [3,6,11,26]. In addition, Parkinson's disease patients exhibit olfactory deficits and abnormalities in the olfaction-related structures years before diagnosis of the disorder, including an increase in TH positive neurons in the olfactory bulb [3,6,11,26]. Sandyk [39] recently reported several case studies in which these characteristic PD olfactory deficits were improved following an intervention of pulsed electromagnetic fields, but only when it coincided with D2 receptor activation. In fact, D2  $-/-$  mice have been proposed to be an animal model of PD, displaying several hallmark characteristics of the disorder [1]. The findings presented in this paper reinforce the functional

significance of dopamine and more specifically the D2 receptor in olfactory discrimination. Moreover, the deficits in olfactory discrimination may explain some of the behavioral phenotype in the DAT and D2 knockout mice.

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