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Novelty Seeking and Stereotypic Activation of Behavior in Mice with Disruption of the *Dat1* Gene

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Disruption of the dopamine (DA) transporter (*Dat1*) gene in mice leads to a 50% reduction or complete elimination of *Dat1* expression in striatum of respective heterozygous (HZ) and knockout (KO) mice. Compared to wild-type (WT) controls, extracellular DA is increased approximately two- and five-fold in the mutants. Although open field (OF) activity is similar for WT and HZ animals, it is enhanced for KO mice. The purpose of the present investigations was to study spontaneously emitted behaviors and to determine the behavioral and neurochemical mechanisms that may contribute to the hyperactivity of KO animals. Heterozygotes are less anxious than other genotypes and they engage in novelty-seeking behaviors that include increased time spent in the center of the OF, enhanced investigation of objects, and augmented free exploration of a novel environment. By comparison, KO mice display neophobia when initially exposed to novel conditions. Over time the anxiety-like response habituates and behaviors become activated and stereotyped; these responses are unrelated to exploration or novelty seeking. No alterations in extracellular DA levels or tissue contents from several brain regions are detected at the time of stereotypic activation of KO mice. By contrast, this behavior is accompanied by changes in serotonin metabolism in basal ganglia. This feature may contribute to the behavioral inflexibility of KO mice in different experimental contexts. Collectively, these findings suggest that disruption of the *Dat1* gene in mice leads to two different phenotypes; one related to anxiety-reducing and novelty seeking, while the other has some homology to disorders with a stereotypical-perseverative spectrum.
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INTRODUCTION

Dopamine (DA) is a well-recognized neurotransmitter within the central and peripheral nervous systems that exerts control over motor performance, emotion, motivation, cognition, and neuroendocrine responses (Le Moal and Simon, 1991; Jackson and Westlind-Danielsson, 1994). Alterations in dopaminergic function have been associated with several different neurological and psychiatric conditions that include Parkinson's and Huntington's diseases, schizophrenia, Tourette's syndrome, attention deficit-hyperactivity disorder (ADHD), and substance abuse (Carlsson, 1987). A primary mechanism for regulating the concentrations of extracellular DA is through control of catecholamine reuptake by the DA transporter (DAT) (Amara and Kuhar, 1993; Garris *et al*, 1994). The distribution of the DAT within the mammalian CNS has been described and it is positively associated with the density of DA innervation (Scheffel *et al*, 1991). Through imaging studies, investigators have related changes in density of the DAT in human striatum to severity of symptoms in Parkinson's disease (Seibyl *et al*, 1995), as well as, Tourette's syndrome and ADHD (Krause *et al*, 2002).

A strain of mice has been developed in which the *Dat1* gene has been disrupted and this effect reduces DAT expression by approximately 50% in heterozygotes and eliminates it in knockout (KO) mice (Giros *et al*, 1996). Although tissue levels of DA are reduced in heterozygous (HZ) animals, evoked release is depressed, clearance times are prolonged, and extracellular concentrations in striatum are increased; these same parameters are further exacerbated in the homozygous mutants (Jones *et al*, 1998). In KO mice, extracellular levels are comparable to those observed after psychostimulant administration, which is known to cause stereotyped activation of locomotion in rodents (Bradberry *et al*, 1991; Kuczenski and Segal, 1997). An examination of open field (OF) behavior reveals that the activities of untreated HZ animals are not distinguished

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from WT controls; however, KO animals display enhanced locomotor, rearing, and stereotypy as measured by photobeam interruptions (Giros *et al*, 1996; Gainetdinov *et al*, 1999; Spielewoy *et al*, 2000). The hyperactivity of KO mice is dependent upon DA tone since haloperidol, a DA receptor antagonist, readily attenuates this behavior (Gainetdinov *et al*, 1999).

In the OF, hyperactivity of KO mice is accompanied by thigmotaxis or 'wall-hugging' behavior (Ralph *et al*, 2001). The hyperactivity and thigmotaxis of KO mice could be due to several mechanisms that include enhanced exploration, increased emotional responses, or stereotypic activation of behavior. For example, rats that are more active in the OF appear to seek novelty, variety, and emotional stimulation (Dellu *et al*, 1996). In these more active animals, DA levels in ventral striatum are also higher under basal and stress-related conditions. The increased activity of KO mice can also be due to strong emotional responses. For instance, exposure to a novel environment can lead to increased anxiety and thigmotaxis (Treit and Fundytus, 1988). Benzodiazepines suppress the anxiety-like response. Alternatively, the hyperactivity and thigmotaxis may be indicative of stereotypical responses where the same locomotor paths are traversed repeatedly (see Ralph *et al*, 2001). In this case, stereotypy and perseveration can restrict the animal's behavioral repertoire and cause responses to become inflexible in a variety of different experimental settings. This characteristic is a feature of disorders as diverse as schizophrenia, obsessive-compulsive disorder, and autism where patients display an inability to adapt to novel conditions (Ridley, 1994). It is noteworthy that perseveration in KO mice is also observed in the radial arm maze (Gainetdinov *et al*, 1999) and in social interactions (Rodriguiz *et al*, 2004).

In the present investigations, behaviors of WT, HZ, and KO *Dat1* mice were examined in different experimental settings to address several issues. First, we wanted to evaluate whether changes in the levels or patterns of activity were accompanied by enhanced exploration. Second, we wished to ascertain whether alterations in activity were indicative of emotional responses to novel conditions. Third, we wanted to determine whether stereotypic activation of behavior was a characteristic feature of the *Dat1* mice. Finally, pharmacological and neurochemical procedures were used in an attempt to identify the neurotransmitter systems that may contribute to the abnormal behaviors in the KO mice. The pharmacological studies evaluated the effects of haloperidol and diazepam on behavior in the OF and zero maze, while tissue monoamine and microdialysis experiments related neurochemical changes to stereotyped behaviors of the KO mice.

MATERIALS AND METHODS

Animals

WT, HZ, and KO males (3–5 months of age) were obtained from C57BL/6J × 129 Sv/J hybrid *Dat1* mice that had been intercrossed for more than 15 generations as described (Giros *et al*, 1996). Animals were housed in groups of 3–5 animals/cage in a temperature (22°C) and humidity (45%)-controlled room with a 14 : 10 light–dark cycle (lights on at 0700 hours). Food and water were provided *ad libitum*. All experiments were conducted during the light cycle, except the home-cage emergence test that occurred in the dark. All studies were performed in accordance with NIH guidelines for the care and use of animals and with an approved animal protocol from the Duke University Institutional Animal Care and Use Committee.

OF and Exploratory Behavior

To ensure that initial spontaneous activity would be at its lowest level, animals were placed into the test room at least 2 h before testing. Locomotor (horizontal distance in cm), vertical (counts), and stereotypical (repetitive beam breaks <1 s apart) activities were measured with the VersaMax program (AccuScan Instruments, Columbus, OH). The large OF ($42 \times 42 \times 30$ cm) was illuminated at 340 lux. Horizontal activity was monitored by 16 photobeams, spaced 2.5 cm apart, positioned 2.25 cm from the floor, and located around the perimeter of the OF. Vertical activity monitors consisted of 16 photobeams, spaced 2.5 cm apart, positioned 8.25 cm from the floor, and located on opposite walls of the OF. With the VersaMap program (AccuScan Instruments), the total OF area could be divided into perimeter and center zones (each 21×21 cm). The data were collected in 1-min intervals for 30 min over 7 days to evaluate effects of repeated exposure to the novel environment. The pattern of activity was analyzed on the first day in the OF by the VersaMap program. On days 1–6 following the 30 min assessment of activity, three Lego objects (Enfield, CT) were introduced into the OF without disturbing the animals and behavior was videotaped for 10 min. The same objects were used for each mouse and they were wiped with 70% ethanol prior to testing each animal. Video recordings were digitized and the following behaviors were scored with the Noldus Observer program (Noldus, Sterling, VA): number of rears and jumps, duration of stereotyped rearing, jumping, climbing, and grooming behavior. Stereotyped behaviors refer to repeated responses occurring within 2 s of each other. The latency to first object contact, and number and duration of object contacts were also tabulated over this 10-min period. Only physical contacts were counted which included nose or forepaw contacts and when the mouse reared against an object. A single observer who was blind to the animal's genotype assignment coded behaviors.

During the first 3 days, one object was positioned in the center of the OF, while the other two were placed in opposite corners 8–9 cm from the walls to form a diagonal. Objects were secured by double-sided tape to the bottom of the OF. The center object ($1.5 \times 2.3 \times 4.0$ cm) was black and white in color, whereas the two corner objects ($1.5 \times 2.3 \times 6.0$ cm) were yellow and blue. After 3 days of testing, the spatial configuration was changed such that one of the corner objects was moved to another corner forming a triangle. This arrangement was maintained for the fourth and fifth test days. On day 6, the center object was replaced with a new one ($5.0 \times 6.0 \times 2.0$ cm) composed of the old object with a 'cap' made of colored Lego elements.

In a separate experiment run with naïve mice, the OF ($21 \times 21 \times 30$ cm) was reduced to one-half the size of the large OF to enhance the probability that animals would encounter and interact with the objects. Animals were

initially placed into the OF and 30 min later, two objects were introduced. One object was black and white in color, while the other was yellow and blue (see above). Objects were placed 5 cm from the walls, at positions that were opposite to each other and located along the midline of the chamber. After 10 days, the same animals were returned to the large OF and the procedure was repeated as a control trial where a larger ($1.5 \times 5.0 \times 4.0$ cm) black and white object in the same configuration was paired with a novel ($1.5 \times 2.3 \times 6.0$ cm) red and white object. The two objects were positioned as described. The OF and objects were always wiped with 70% ethanol in-between mice to eliminate possible odor cues.

Pharmacological responses to haloperidol (Sigma Chemical Company, St Louis, MO) were evaluated in the large OF. Naive animals were injected either with vehicle (saline containing 0.01% ethanol) or drug. One group (pre-exposed) was immediately placed into the OF. After 30min, the black and white, and yellow and blue objects were positioned 5 cm apart in the center of the OF. A second group (non pre-exposed) remained in the home cage for 30 min after drug injection, then were placed into the OF, and objects were introduced 1 min later. Both activity and object exploration were monitored for 5 min after object introduction.

Another pharmacological test was conducted in the large OF with diazepam (Sigma-Adrich, St Louis, MO) using mice that had participated in the zero maze experiment 2 weeks prior to this study (see below). Animals were injected with vehicle (saline containing 0.4% ethanol) or drug, immediately placed into the large OF, and activity was recorded for 2 h.

Home-Cage Emergence Test

Naïve mice were housed in cages identical to their home cages except one wall had a hole (4.2 cm in diameter) closed with a rubber stopper. The food/water hopper was removed, but the filter top was retained. Water was available in a small cup and food pellets were placed on the floor. Following 48 h of acclimatization, the cage and animal were placed into a corner of the large OF. After 1 h, the stopper was removed and the mouse could freely enter the new environment for 1 h. The latency to enter the OF, the number of entries/exits into the OF, and activity in the home cage and OF were recorded.

Zero Maze

The maze was comprised of a 5.5 cm-wide metal circular platform painted black. The inside diameter was 34 cm and it was 43 cm from the floor. Two opposite quadrants were enclosed by black metal walls 11 cm high on the inner and outer edges of the platform; the open and closed quadrants were equal in areas. The maze was illuminated at 50–60 lux and enclosed by black curtains. Behavior was recorded by video camera suspended 100 cm above the maze.

Naïve mice were placed in a closed quadrant and allowed to freely investigate the maze for 35 min. Anxiety-like behavior was video recorded at 0–5 min, while stereotyped behaviors were recorded at 30–35 min. The scored behaviors included percent of time in open quadrants and total number of transitions between quadrants. In a separate experiment, naïve mice were injected with diazepam or vehicle and placed into the apparatus 30 min later. Behavior was videotaped for the first 5 min to evaluate anxiety-like responses. Videos were analyzed with Noldus Observer (Noldus, Sterling, VA). Animals were tested for only one session.

Tissue Monoamines

Monoamine concentrations were compared between WT and KO mice that were immediately removed from their home cage or following 30-min in the OF. Mice were killed within 30–40 s by cervical dislocation and decapitation in another room. Brains were rapidly removed, placed ventral side up onto a cold metal block and cut with a razor blade. The olfactory bulbs and the most rostral part of the frontal cortex were discarded ($\sim +3.2$ to $\sim +2.7$ mm bregma; Franklin and Paxinos, 1997). The frontal cortex was dissected from a second (0.6–0.7 mm thick), third (1.0 mm), and fourth (0.9 mm) transections. The last section was made at the level of preoptic area where the characteristic 'wings' of the anterior commissure appear on the slice ($\sim +0.14$ mm bregma; Franklin and Paxinos, 1997). The ventral striatum, including the nucleus accumbens and olfactory tubercle, were removed from the third section; the striatum was dissected from the third and fourth sections. Samples were frozen immediately in liquid nitrogen and later sonicated in ice-cold 0.1 M HCl–0.1 mM sodium metabisulfate with 33–100 ng/ml 3,4-dihydroxybenzylamine (DHBA; Bioanalytical Systems Inc., West Lafayette, IN) as an internal standard. After centrifugation at 10 000*g* for 10 min at 4°C, samples were filtered with 0.22 μm filters (Millipore, Bedford, MA). In total, 10 μl of filtrate was injected onto the HPLC.

The HPLC system consisted of a Dionex gradient pump (Dionex Corporation, Sunnyvale, CA), Rheodyne (Cotati, CA) model 7125 injector with a 20 μl sample loop, model 5020 guard cell, model 5011 dual electrode analytical cell, model 5100A Coulometric electrochemical detector (EC), and model 501 data module (ESA Inc., Bedford, MA). The guard cell potential was set at $+0.35$ mV, and the first and the second (quantitating) working electrodes were at -0.15 and $+0.28$ mV, respectively. Monoamine separation was achieved at 0.5 ml/min on an ESA 150×3.2 mm 3 μm RP-C18 column with 75 mM sodium phosphate (pH 2.9), 1.7 mM 1-octanesulfonic acid, 25 μM EDTA, and 11% acetonitrile. DA, *L*-norepinephrine (NE), DHBA (Bioanalytical Systems Inc.), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) (Sigma, St Louis, MO, USA) standards were baseline separated from each other within 11 min.

Microdialysis

Animals were anesthetized with ketamine/xylazine and stereotaxically implanted with concentric microdialysis probes (1.0 mm membrane length, 6000 Da cut-off; CMA/Microdialysis, North Chelmsford, MA). Coordinates for the guide cannula to the right nucleus accumbens were: AP $+1.3$, L $+2.2$, V -5.8 (the tip of the microdialysis membrane was positioned within the nucleus accumbens/

tuberculum olfactorium). At 24 h after surgery, mice were transferred in individual cages to the experimental room and the dialysis probe was perfused with artificial cerebrospinal fluid (147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$) at 0.5 μl/min. Perfusate was collected every 20 min. After DA levels stabilized, three additional samples were collected (baseline) and the mice were placed in the small OF (21 × 21 × 30 cm) for 60 min. Next day, after three basal samples were collected in the home cage, 50 mM KCl was perfused through the probe for 40 min. At the end of experiments, toluidine blue was perfused through the probe to confirm its location. Changes in DA, DOPAC, and 5-HIAA release were measured by HPLC-EC in 5 μl perfusate and expressed as percent change from baseline. The HPLC-EC apparatus consisted of a model 582 pump and 5200A detector, model 501 data station, 5020 guard cell set at +0.18 V, and model 5041 analytical cell set at +0.15 V, and ESA 1 × 150 mm 3 μm microbore column. Monoamines were separated at 0.060 ml/min with a mobile phase consisting of 75 mM lithium acetate, 4 mM 1-heptane sulfonic acid, 100 μM EDTA, and 8% methanol (pH 4.7).

Statistical Analyses

Results are presented as means and standard errors of the mean. All analyses were conducted with the Statistical Package for the Social Sciences (SPSS), Version 11.0 (Chicago, IL). A χ^2-test was applied to the percent of mice that entered the OF in the home emergence test. Paired *t*-tests were used to determine differences in activity and object exploration for KO mice in the large and small OFs. Univariate analyses of variance (ANOVA) tests were used to examine haloperidol effects on object exploration and activity, activity and the latency to exit the home cage and enter the OF in the home-cage emergence tests, behavior in the zero maze at 5 min, activity in the perimeter or central zones of the OF following diazepam treatment, and tissue monoamine levels in brain. Repeated measures ANOVA (RMANOVA) for within-subjects effects were used to assess repeated observations in the same animals for tests of activity in the OF, interactions with objects over days, zero maze performance at 5 and 30 min, and the microdialysis data. When time-dependent effects for within-subjects tests in RMANOVA were not significant, between-subjects tests were reported and are noted in the text. In cases in RMANOVA where main effects of time were significant but the interaction with genotype and/or drug was not significant, time-based changes were evaluated within genotypes or drug treatment conditions by Bonferroni pairwise comparisons (see Ventura *et al*, 2004). *Posthoc* analyses of main effects in the ANOVA and RMANOVA tests and decomposition of significant interaction terms were conducted with Bonferroni corrected pair-wise tests to control for multiple comparisons (Winer, 1971). In all cases, a $p < 0.05$ was considered statistically significant.

RESULTS

Activity in the OF

Locomotor, rearing, and stereotypical (repeated photobeam interruptions) activities were analyzed during the first 30 min over 7 consecutive days of testing in the large OF. For locomotion on the first day of testing (Figure 1a), a RMANOVA for within-subjects effects revealed a significant time ($F(29,725) = 8.880$, $p < 0.001$) and time by genotype interaction ($F(58,725) = 15.064$, $p < 0.001$). Decomposition of the interaction with Bonferroni corrected pair-wise comparisons between genotypes at each time point demonstrated that activity was higher for KO mice than for WT and HZ animals beginning at 8 min ($ps < 0.035$) and this difference persisted to the end of testing ($ps < 0.001$). Although locomotion tended to be higher in HZ than WT mice over time, this difference did not achieve significance. An examination of locomotor activity patterns on day 1 revealed that KO animals displayed a strong thigmotaxis (Figure 1b). Moreover, the dense areas on the map represent repetitive horizontal back-and-forth movements

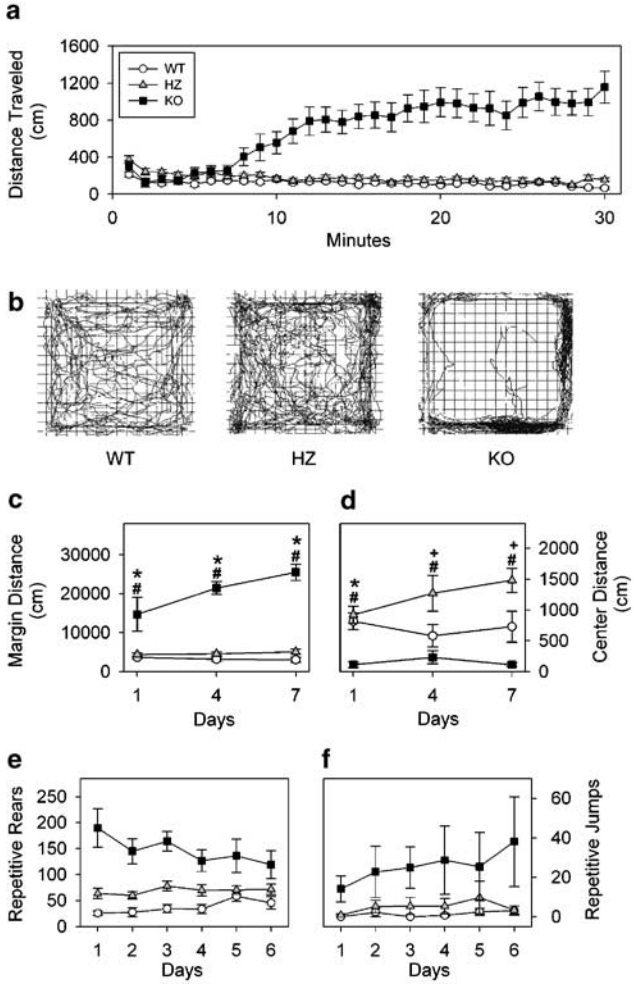

Figure 1 Horizontal activity in the large OF for WT, HZ, and KO mice for 30 min over 7 test days. (a) Locomotor activity in 1-min segments over 30 min on day 1 of testing. $N = 8$–16 mice/group. (b) Activity maps for WT (left), HZ (middle), and KO (right) mice on the first day of testing over the 30 min period. (c) Total distance traveled in the perimeter of the OF over 7 test days. (d) Total distance traversed in the center of the OF over the 7 days of testing. (e) Repetitive rears in the OF across test days. (f) Repetitive jumps in the OF across days. $N = 8$–10 mice/group. *, $p < 0.05$ KO from WT mice; #, $p < 0.05$, KO *vs* HZ mice; +, $p < 0.05$, WT *vs* HZ animals.

accompanied by stereotyped repetitive rearing against the walls. When locomotor activity in the perimeter of the OF was compared across days, a RMANOVA found the test day by genotype interaction to be significant ($F(4,38) = 2.775$, $p < 0.041$). Decomposition of the interaction revealed that perimeter locomotor activity of KO animals was significantly higher on days 1, 4, and 7 than for WT ($p < 0.010$) and HZ mice ($p < 0.013$) that did not differ from each other (Figure 1c). In addition, activity of KO mice in the perimeter of the OF was increased between test days 4–7 ($p < 0.031$). An examination of activity in the center of the OF by RMANOVA determined the test day by genotype interaction to be significant ($F(4,38) = 4.428$, $p < 0.005$). Bonferroni corrected pair-wise comparisons between genotypes demonstrated that locomotion in the center of the OF was significantly lower on day 1 for KO mice than WT or HZ animals ($ps < 0.001$). However, by test days 4 and 7, center activity of HZ mice was higher than that for WT ($ps < 0.035$) and KO animals ($ps < 0.001$) that did not differ from each other (Figure 1d). Interestingly, KO mice took much more time to enter the center of the OF on day 1 (153 ± 37.4 s) than the WT (43 ± 29.8 s) and HZ mice (10 ± 4.6 s) and this latency increased by almost five-fold (729 ± 195.2 s *vs* 106 ± 28.9 and 114 ± 40.1 s, respectively) by day 7. Relationships similar to those for locomotion were observed for rearing and stereotypical activities on the first test day and across test days in the perimeter and center zones of the OF for all three genotypes of mice (data not shown). Together, these findings show that after a brief delay, the KO mice become hyperactive in the perimeter of the OF and their horizontal, vertical, and stereotypical activities increase over days with thigmotaxis and stereotyped locomotor responses predominating. At the same time, heterozygotes spend more time in the center of the OF and their locomotion, rearing, and stereotypy increase over days in this location.

To further evaluate behavior in the OF, video recordings were made at 30–40 min over the first 6 days of testing. Special emphasis was placed upon analyses of stereotypical behavior (eg repetitive rearing, jumping, and stereotyped climbing). RMANOVA for within-subjects effects for repetitive rears or jumps failed to discern any time-based significant differences. Hence, the between-subjects test found significant genotype differences for repetitive rears ($F(2,19) = 57.072$, $p < 0.001$) and repetitive jumps ($F(2,19) = 3.663$, $p < 0.045$). Bonferroni corrected pairwise tests showed that the incidences of repetitive rearing were the highest for KO animals ($ps < 0.001$ from HZ and WT mice), and the incidences of these behaviors were also higher in HZ mice ($p < 0.019$) than in the WT controls (Figure 1e). The *post hoc* tests also demonstrated that the homozygous mutants engaged in more repetitive jumps ($ps < 0.05$) than the WT mice (Figure 1f). Besides these repetitive behaviors, there was a propensity for two of the KO animals to display stereotyped climbing lasting from 4 to 330 s across the six test sessions. This form of stereotypy involved continuous 'scratching' against the wall with the animal balancing on its tail. These data clearly indicate that the homozygous mutants display several different forms of stereotypical activity and that they may be highly individualized and persistent for a given animal over time.

Exploration in the OF

One reason why the KO animals are hyperactive in the OF is that they may have a propensity to explore their environment. To examine this possibility, objects were introduced into the OF after 30 min and exploration was evaluated. Initially, three objects were placed in a diagonal arrangement and the latencies for object interaction, as well as the numbers and duration of contacts were scored. Although no genotype differences in the latency to first object contact were noted, RMANOVA for within-subjects effects for the numbers of object contacts demonstrated a significant test day ($F(2,38) = 9.728$, $p < 0.001$) and a test day by genotype interaction ($F(4,38) = 2.787$, $p < 0.054$). Decomposition analyses revealed that HZ mice made more contacts with objects ($ps < 0.003$) than either the WT or KO animals on all 3 test days (Figure 2a). RMANOVA for the duration of object contacts also found test day to be significant ($F(2,38) = 4.035$, $p < 0.026$), but no significant test day by genotype interaction emerged. However, the between-subjects test was significant for genotype ($F(2,19) = 4.459$, $p < 0.026$) and Bonferroni *a posteriori* analyses showed that HZ animals interacted with objects for a longer duration ($p < 0.030$) than KO mice (Figure 2b). Collectively, these data suggest that the hyperactivity of the KO animals is not due to enhanced exploration. By contrast, the HZ mice explore novel stimuli for a longer period of time and to a greater extent than the other animals. Additionally, while HZ mice show a reduction in the number of contacts over days, they compensate for this behavior by spending more time interacting with objects.

Although initial results with the object test suggest that the hyperactivity of KO mice is not due to enhanced exploration, it is unclear whether the mutants can detect changes in their environment. This is an important point since their performance in the radial arm maze has been attributed to deficiencies in attention and spatial memory (Gainetdinov *et al*, 1999). To determine whether the KO mice were responsive to spatial change, the configuration of objects was altered on day 4 from a diagonal to a triangular arrangement (Figure 2c). It should be emphasized that the time spent with the to-be-displaced object across the first 3 days of testing was constant for WT, HZ, and KO mice (data not shown). As a result, changes in preference for the displaced object as percent contact time were compared on days 3 and 4. RMANOVA for within-subject effects revealed a significant test day effect ($F(1,19) = 19.349$, $p < 0.001$), but no significant test day by genotype interaction—indicating that the increase in exploration for the displaced object was similar across the three genotypes. Bonferroni corrected pairwise comparisons within genotype showed that the change in spatial preference from days 3 to 4 was significant for HZ ($p < 0.021$) and KO mice ($p < 0.001$). Hence, KO mice can recognize spatial change when memory constraints are minimal, but may experience difficulty when the task becomes more demanding as in the eight-arm radial maze (see Gainetdinov *et al*, 1999).

To examine further whether the KO mice attend to stimuli in their environment, the configuration of objects was maintained on day 5 and the center object was replaced with a novel one on day 6 (Figure 2d). RMANOVA for within-subjects effects for percent time spent with the novel object

revealed a significant test day effect ($F(1,19) = 18.273$, $p < 0.001$); no significant test day by genotype interaction was found. The Bonferroni corrected pairwise comparisons within genotype showed that preference for the novel object was increased on day 6 for WT ($p < 0.022$), HZ ($p < 0.011$), and KO animals ($p < 0.045$). All animals detect the novel object. In summary, although the hyperactivity of KO mice is not accompanied with enhanced exploration, they can attend and respond to simple changes in their environment.

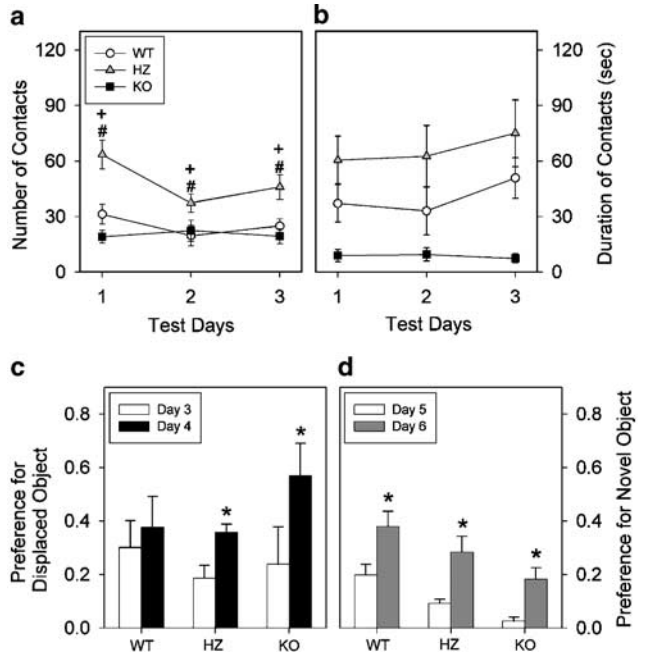

Figure 2 Object exploration in the large OF by WT, HZ, and KO mice over 6 days. On days 1–3, objects were arranged in a diagonal configuration, on days 4 and 5 the objects were in a triangular configuration, and on day 6 the center object was replaced with a new one. (a) The number of object contacts over a 10-min period during the first 3 days in the OF. (b) The total duration of object contacts over the same period. #, $p < 0.05$, KO *vs* HZ mice; +, $p < 0.05$, WT *vs* HZ animals. (c) On day 4, the spatial location of one of the three objects was changed. Preference prior to (day 3) and after displacement (day 4) are displayed for that object. *, $p < 0.05$, day 3 *vs* day 4. (d) On day 6, the center object was replaced with a new one. Preference for the familiar (day 5) and replaced objects (day 6) are shown for each genotype. In both tests, preference refers to the time interacting with the displaced or novel object divided by the total contact time for all three objects. These data were collected with the same mice as in Figure 1. $N = 7$–8 mice/group. *, $p < 0.05$, day 5 *vs* day 6.

Constraints on Hyperactivity and Exploration of KO Mice

One reason object exploration is reduced for KO mice may be due to an incompatibility between exploration and hyperactivity. To examine this possibility, the OF was reduced in size because a smaller arena should place spatial constraints upon the activity of the animals such that the speed of locomotion would be reduced and they would have a greater opportunity to explore the objects. To evaluate this parameter for KO mice, activity and object exploration were examined in the small OF and responses were re-evaluated in the large OF 10 days later (Table 1). Paired *t*-tests showed that in the small OF locomotion of the homozygous mutants was attenuated ($t(1,8) = 5.009$, $p < 0.001$), while rearing was enhanced ($t(1,8) = 2.218$, $p < 0.054$). Additionally, the number of object contacts in the small OF was augmented ($t(1,8) = 4.521$, $p < 0.002$) and the duration of contacts was increased ($t(1,8) = 3.071$, $p < 0.015$). Hence, spatial constraints on locomotion can influence object exploration of KO mice.

Another manner of imposing constraints on locomotion is by pharmacological intervention. Since stereotypical activation of behavior by dopaminomimetics can be diminished with DA receptor blockade (Randrup and Munkvad, 1969), this strategy was applied to KO mice. Following haloperidol administration, animals were assigned to two groups. One remained in the home cage for 30 min after haloperidol treatment and was then placed into the OF with objects (non pre-exposed group). The other group was placed into the large OF immediately and two objects were introduced 30 min later (pre-exposed group). Hence, object exploration was monitored from both groups 30–35 min after haloperidol administration. Parenthetically, in the pre-exposed condition, one may anticipate that the KO animals will be stereotypically activated, while in the other they should not be activated. The 0.01 mg/kg dose of haloperidol exerted no significant effects on WT or KO performance regardless of exposure condition (data not shown). A univariate ANOVA for the number of object contacts revealed significant effects of genotype ($F(1,72) = 6.419$, $p < 0.013$), exposure ($F(1,72) = 17.072$, $p < 0.001$), and drug ($F(1,72) = 13.838$, $p < 0.001$), and a significant three-way interaction ($F(1,72) = 5.690$, $p < 0.020$). Bonferroni corrected pairwise comparisons revealed no genotype or drug effects in the non-pre-exposed groups (Figure 3a). However, KO mice given 0.03 mg/kg haloperidol made more object contacts ($ps < 0.001$) than any other group

Table 1 Activity and Object Exploration of KO Mice in the Large and Small Open Fields

	Photocell results[a]		Video-recording results[a,b]	
OF size	**Total distance (cm)**	**Vertical activity (counts)**	**Number of contacts**	**Duration of contacts (s)**
Small	3006 ± 508	312 ± 88	30.9 ± 3.9	16.7 ± 3.9
Large	19 462 ± 3692[c]	198 ± 50[c]	12.4 ± 2.6[c]	5.0 ± 2.1[c]

[a]The data are presented as means and ± standard error of the mean, $N = 10$.
[b]Behavior was observed during 30–35 min in the OF.
[c]Paired *t*-test, $p < 0.05$.

(Figure 3a,b). The univariate ANOVA of contact duration demonstrated a significant genotype ($F(1,72) = 3.424$, $p < 0.030$), exposure ($F(1,72) = 6.920$, $p < 0.010$), and drug effect ($F(1,72) = 4.781$, $p < 0.029$). The *post hoc* tests revealed that the genotype and drug effects were present only for pre-exposed animals where the duration of contacts was increased by haloperidol for KO mice (Figure 3c,d). Thus, these data show that haloperidol does not influence the numbers or duration of contacts for WT animals, whereas the same drug enhances this response but only in KO mice pre-exposed to the OF.

Effects of haloperidol on locomotion in the large OF were also examined. Univariate ANOVA found effects of genotype ($F(1,72) = 27.650$, $p < 0.001$), exposure ($F(1,72) = 20.400$, $p < 0.001$), drug ($F(1,72) = 11.160$, $p < 0.001$), and the three-way interaction to be significant ($F(1,72) = 6.761$, $p < 0.011$). As anticipated, activity of untreated KO mice was higher ($p < 0.001$) than that for WT controls, but only when the mice were pre-exposed to the OF (Figure 3e,f). By comparison, haloperidol depressed activity in both the WT ($p < 0.040$) and KO ($p < 0.001$) pre-exposed groups. Together, these results demonstrate that activation of stereotypic locomotion in KO mice (eg pre-exposed group) is highly dependent upon DA receptor activation and this behavior interferes with object exploration.

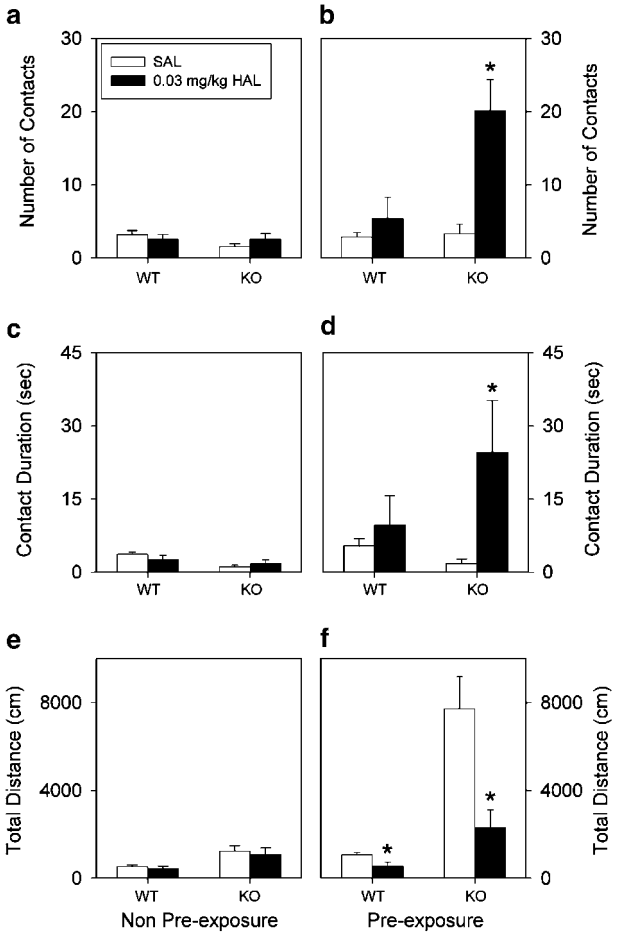

Figure 3 Effect of haloperidol on object exploration and motor activity of WT and KO mice. Animals were given haloperidol and returned to the home cage or placed immediately into the large OF; behavior was monitored 30 min later in the OF. (a) Number of object contacts (over 5 min) in the non-pre-exposed group. (b) Number of contacts following 30 min of pre-exposure to the OF. (c) Duration of object contacts (over 5 min) in the non-pre-exposed group. (d) Duration of contacts following 30 min of pre-exposure to the OF. (e) Horizontal activity (over 5 min) in the non pre-exposed group. (f) Locomotion following 30 min of pre-exposure to the OF. $N = 9$–13 mice/group. *, $p < 0.05$ saline *vs* haloperidol (HAL).

Free Exploration in a Novel Environment

In the previous experiments we show that exploration by KO mice can be manipulated by altering the size of the OF and by pharmacological intervention. In these paradigms mice were placed into a novel environment and, as such, were forced to explore their surroundings. Since this feature might have influenced their behavior, animals were tested in a free-choice situation. This procedure has been proposed to measure 'trait anxiety,' as in contrast to 'state anxiety' that is commonly associated with forced-choice tests (Griebel *et al*, 1993). According to this construct 'trait anxiety' is evident if the mouse remains in its home cage, whereby 'impulsivity' or novelty-seeking behavior is manifested as increased entries into the novel environment. In our experiment, locomotor and rearing activities in the home cage were comparable among the three genotypes (data not shown). By contrast, a univariate ANOVA ($F(2,49) = 3.181$, $p < 0.050$) and Bonferroni tests found the latency to exit the home cage to enter the OF was shorter for HZ ($p < 0.054$) than WT mice. Additionally, 70% of the HZ mice exited their home cage, whereas only 18% of the WT and 29% of the KO animals ($n = 17$–20 animals/genotype) engaged in this behavior ($\chi^2(2,51) = 11.75$, $p < 0.01$). Once the mice entered the OF no genotype differences in locomotor or rearing activities were observed. Hence, the KO mice did not become hyperactive when allowed free access to a novel environment, and they did not display the reduced anxiety or novelty-seeking phenotype that was evident in HZ animals.

Zero Maze Performance

We have shown previously that OF activity of KO mice is initially suppressed, but after the first 5–10 min locomotion is increased and it becomes highly stereotyped with thigmotaxis. The initial responses of the homozygous mutants suggest that they may have a strong neophobia or display anxiety-like behavior in the OF. To test this idea, naïve animals were placed in a zero maze where this behavior was assessed over first 5 min (see Shepherd *et al*, 1994). Since stereotyped behavior appears in the OF after a longer period of time, animals remained in the zero maze for an additional 30 min and their responses were examined for repetitive behaviors.

To evaluate animals for anxiety-like responses, behaviors were assessed from 0–5 min in the zero maze. Univariate ANOVA for the percent of time in the open arms revealed a significant genotype effect ($F(2,39) = 4.763$, $p < 0.014$), and Bonferroni analyses demonstrated that KO mice spent significantly ($ps < 0.04$) less time in the open areas than WT or HZ animals (Figure 4a, left). This diminution in open arm time was not due to enhanced freezing because WT mice froze for 4.5 ± 1.78 s, while KO animals froze for

7.2 ± 2.75 s during the 5-min test. When the numbers of transitions were considered over the first 5 min, no significant genotype effects were found (Figure 4b, left). Collectively, these findings collected over the first 5 min in the maze suggest that the KO mice may display an anxiety-like phenotype.

Since KO mice become hyperactive in the OF after 30 min of exposure, activity was also evaluated in the zero maze after 30 min exposure to determine whether hyperactivity was a common feature of KO mice as they adapt to their new surroundings. When the percent time spent in the open arms was examined by RMANOVA for within-subjects effects, significant time ($F(1,39) = 52.176$, $p < 0.001$) and a significant time by genotype interaction were obtained ($F(2,39) = 8.361$, $p < 0.001$). Bonferroni corrected pairwise comparisons showed that by 30 min, the HZ ($p < 0.002$) and KO animals ($p < 0.001$) spend more time in the open arms than the same mice at 5 min (Figure 4a). However, at 30 min, only the KO mice spend more time in the open arms ($p < 0.017$) than the WT controls (Figure 4a, right). RMANOVA of the numbers of transitions reveal a significant time ($F(1,39) = 18.916$, $p < 0.001$) and time by genotype interaction ($F(2,39) = 10.921$, $p < 0.001$). Decomposition analyses demonstrate that these differences are due to an enhancement in transitions at 5 *vs* 30 min for KO mice ($p < 0.001$) and the numbers of transitions are also higher for the homozygous mutants than the HZ ($p < 0.001$) or WT controls ($p < 0.001$) (Figure 4b, right). Together, these data show that the KO mice initially present an anxiety-like phenotype in the zero maze and, with time, these responses are displaced by hyperlocomotion and stereotyped behaviors.

To pharmacologically validate anxiety-like behaviors in the zero maze, different groups of naïve mice were treated with saline, or with 0.5 or 1.0 mg/kg diazepam, and placed into the maze for 5 min (Figure 4c,d). For the percent time spent in the open areas, a univariate ANOVA revealed significant drug effects ($F(2,118) = 5.375$, $p < 0.004$) and a genotype by drug interaction ($F(4,118) = 3.404$, $p < 0.030$). Bonferroni corrected pairwise comparisons demonstrated that, relative to the saline controls, the 0.5 and 1.0 mg/kg doses of diazepam were sufficient to increase time spent in the open arms ($ps < 0.010$) but only for KO mice (Figure 4c). Interestingly, all genotypes of mice responded similarly to the 0.5 mg/kg dose, whereas 1.0 mg/kg diazepam continued to have anxiolytic effects only for KO mice.

When the numbers of transitions from closed to open arms were evaluated, univariate ANOVA found main effects of genotype ($F(2,118) = 2.926$, $p < 0.054$), drug ($F(2,118) = 4.406$, $p < 0.014$), and the interaction to be significant ($F(4,118) = 2.966$, $p < 0.053$). Decompostion analyses showed that both 0.5 and 1.0 mg/kg diazepam increased the numbers of transitions ($ps < 0.004$) but only for KO animals. Together these findings show that diazepam treatment can reduce anxiety-like behaviors in KO mice and they suggest that the KO animals display anxiety-like behavior.

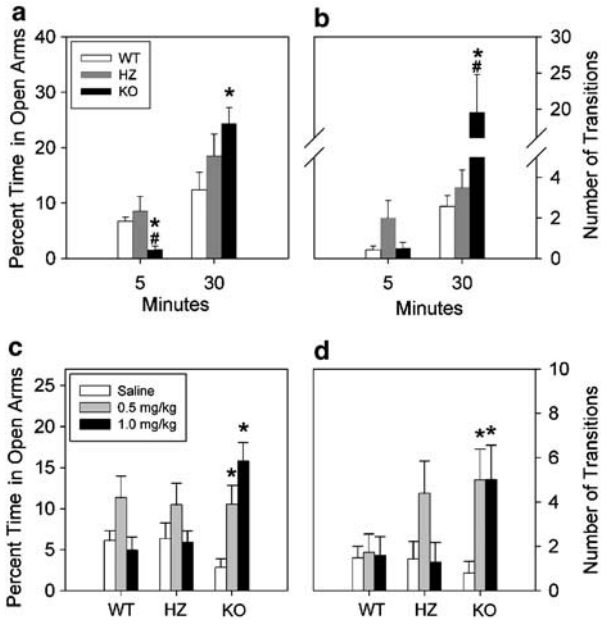

Figure 4 Zero maze performance of WT, HZ, and KO mice. (a) Percent time spent in the open arms of the maze at 0–5 or 30–35 min. (b) Number of transitions between the enclosed quadrants of the maze as a function of the same time intervals. *, $p < 0.05$ KO from WT mice; #, $p < 0.05$ KO from HZ animals. (c) Effect of diazepam on the percent time spent in the open arms of the maze during 0–5 min. (d) Effect of diazepam on number of transitions between the enclosed quadrants. $N = 10$–19 mice/group. *, $p < 0.05$ compared to saline controls within each genotype.

Effects of diazepam in the OF

The zero maze results suggest that the initial low activity levels of KO mice in the OF may be due to enhanced anxiety. To determine whether benzodiazepines could modify these behaviors, animals were given diazepam and immediately placed into the OF. The 0.5 mg/kg dose was chosen because it was the most effective in increasing time in the open areas of the zero maze for all three genotypes of mice (see above). An omnibus RMANOVA for within-subjects effects demonstrated a significant time ($F(23,736) = 25.557$, $p < 0.001$) and time by genotype interaction ($F(23,736) = 16.657$, $p < 0.001$). The decomposition analysis demonstrated that activity for KO animals was higher than that for WT controls at all time points ($ps < 0.033$). Since the absence of a significant drug effect was probably due to enhanced activity of the homozygous mutants, the RMANOVA was run for each genotype separately (Figure 5a). RMANOVA for WT animals demonstrated a significant time ($F(23,345) = 40.820$, $p < 0.001$) and time by drug interaction ($F(23,345) = 5.902$, $p < 0.028$). Bonferroni corrected pairwise comparisons found that diazepam increased activity ($ps < 0.050$) at 5,10, 20, 25, 30, 40, 50, and 60 min relative to the respective saline controls (Figure 5a). The KO mice also demonstrated significant time ($F(23,391) = 23.193$, $p < 0.001$) and time by drug effects ($F(23,391) = 3.635$, $p < 0.034$). The decomposition analysis revealed that diazepam augmented activity at all time points between 10 and 90 min compared to their saline controls ($ps < 0.054$). Together, these data show that diazepam increases activity for both genotypes of mice; however, the drug effects appear to be more potent in KO animals.

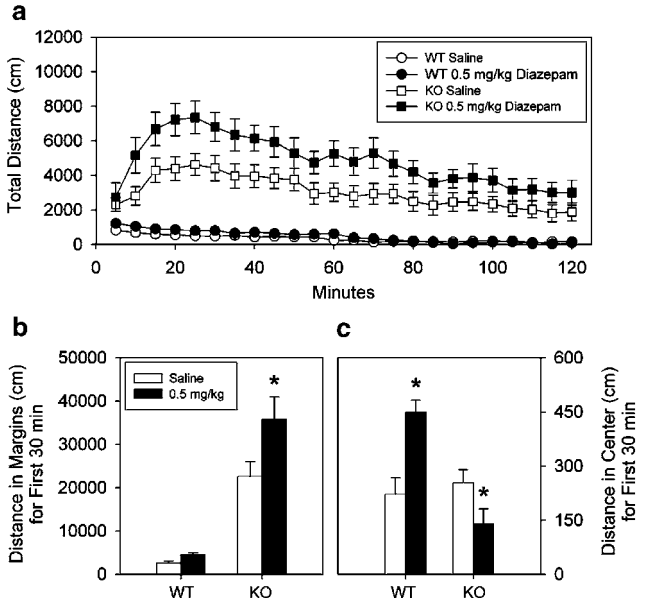

Figure 5 Effects of diazepam on activity in the large OF for WT and KO mice. (a) Total distance traveled in 5 min segments over 2 h. (b) Total distance traveled in the margins of the OF during the first 30 min. (c) Total distance traveled in the center of the OF during the first 30 min. $N = 8$–10 mice/group. *, $p < 0.05$, diazepam *vs* saline control.

Since under nondrug conditions activity of KO mice was primarily confined to the perimeter of the OF (recall Figure 1c), effects of diazepam on activity were examined in the marginal and central zones of the OF for both genotypes (Figure 5b,c). Univariate ANOVA in the margins of the OF revealed significant genotype ($F(1,32) = 64.473$, $p < 0.001$]) drug ($F(1,32) = 5.573$, $p < 0.024$), and interactive effects ($F(1,32) = 3.113$, $p < 0.054$). The Bonferroni corrected pairwise comparisons demonstrated that diazepam increased locomotion in the perimeter of the OF ($p < 0.001$), but only for KO mice (Figure 5b). An examination of activity in the center of the OF by univariate ANOVA showed a main effect of genotype ($F(1,32) = 12.390$, $p < 0.001$) and the genotype by drug interaction to be significant ($F(1,32) = 18.647$, $p < 0.001$). The decomposition analysis revealed that diazepam enhanced center time in the OF for WT animals ($p < 0.001$), while it depressed activity in the center zone ($p < 0.044$) for KO mice (Figure 5c). Together, these findings support the idea that the initial low levels of activity of KO mice in the OF (recall Figure 1a) may be due to anxiety-like behavior and relief of these responses allows the stereotyped behaviors to emerge.

Brain Monoamines

Experiments in the OF and zero maze show that the KO mice become stereotypically activated within 30 min after being placed into these novel environments. To investigate a possible neurochemical basis for the stereotypical activation, WT and KO animals were removed from their home cage (no activation) or the large OF after 30 min (behaviorally activated) and taken for monoamine analyses. This method allowed sampling of monoamine contents in several brain regions simultaneously.

In the frontal cortex, a univariate ANOVA revealed significant genotype effects for NE ($F(1,50) = 6.412$, $p < 0.014$), DA ($F(1,50) = 15.529$, $p < 0.001$), DOPAC ($F(1,50) = 4.492$, $p < 0.039$), and HVA ($F(1,50) = 19.765$, $p < 0.001$); significant main effects of exposure for NE ($F(1,50) = 3.489$, $p < 0.049$), DOPAC ($F(1,50) = 5.503$, $p < 0.023$), HVA ($F(1,50) = 6.332$, $p < 0.015$), 5-HT ($F(1,50) = 17.433$, $p < 0.001$), and 5-HIAA ($F(1,50) = 9.082$, $p < 0.003$); and a significant genotype by exposure interaction for DA ($F(1,50) = 3.702$, $p < 0.045$). Bonferroni corrected pairwise comparisons were used to analyze these results. While basal levels (eg home cage) of NE in frontal cortex are similar in WT and KO mice, only mutants respond with reductions in NE contents ($p < 0.045$) following 30 min of exposure to the OF (Table 2). Basal DA concentrations are also reduced in KO animals ($p < 0.001$) and only WT mice respond to OF exposure with a decrease in levels ($p < 0.053$). With respect to catecholamine metabolism, basal HVA concentrations are enhanced in KO mice ($p < 0.025$) and both DOPAC ($p < 0.007$) and HVA contents ($p < 0.006$) are augmented in these animals with exposure to the OF. For 5-HT, OF exposure increases levels of 5-HT in WT ($p < 0.001$) and KO ($p < 0.050$) mice, as well as 5-HIAA in both genotypes ($p < 0.022$ and 0.045, respectively). Hence, DA in frontal cortex of KO mice fails to respond to OF exposure and the magnitude of change for 5-HT and 5-HIAA concentrations is reduced relative to responses of the WT controls.

Univariate ANOVA for tissue monoamines in caudate-putamen demonstrated significant main effects of genotype for NE ($F(1,50) = 8.917$, $p < 0.004$), DA ($F(1,50) = 138.338$, $p < 0.001$), DOPAC ($F(1,50) = 6.382$, $p < 0.039$), HVA ($F(1,50) = 148.531$, $p < 0.001$), 5-HT ($F(1,50) = 16.027$, $p < 0.001$), and 5-HIAA ($F(1,50) = 3.596$, $p < 0.054$); significant effects of exposure for HVA ($F(1,50) = 4.531$, $p < 0.038$), 5-HT ($F(1,50) = 6.767$, $p < 0.012$), and 5-HIAA ($F(1,50) = 16.971$, $p < 0.001$); and a significant genotype by exposure interaction for DOPAC ($F(1,50) = 4.132$, $p < 0.047$). These results were analyzed by Bonferroni corrected pairwise comparisons. Levels of both NE and DA in dorsal striatum of KO mice under both exposure conditions are lower ($ps < 0.054$) than those of WT mice; neither genotype displays any changes in catecholamines upon placement into the OF (Table 2). Basal concentrations of DOPAC are similar between WT and KO animals; however, metabolism is increased only in WT mice with OF exposure ($p < 0.014$). Conversely, HVA contents are higher in KO than WT mice under basal conditions ($p < 0.025$) and with exposure to the OF ($p < 0.001$). Levels of 5-HT are higher in WT littermates than KO animals under both exposure conditions ($ps < 0.025$) and OF exposure increases concentrations only in WT mice ($p < 0.054$). By contrast, concentrations of 5-HIAA in dorsal striatum are enhanced by OF exposure in both WT ($p < 0.001$) and KO animals ($ps < 0.001$). Thus, these results show that basal concentrations of NE, DA, and 5-HT in caudate-putamen are depressed in KO animals, and that placement in the OF increases 5-HIAA contents in both genotypes of mice.

In the ventral striatum, univariate ANOVA revealed significant main effects of genotype for NE ($F(1,50) = 25.168$, $p < 0.001$), DA ($F(1,50) = 127.556$, $p < 0.001$), HVA ($F(1,50) = 113.406$, $p < 0.001$), and 5-HT ($F(1,50) = 28.117$,

Table 2 Tissue Levels of Monoamines and their Metabolites from Different Brain Regions in WT and KO Mice

Brain region	Condition	Genotype	NE[a]	DA	DOPAC	HVA	5-HT	5-HIAA
FC[b,c]	Home cage	WT	0.699 ± 0.029	0.072[d,e] ± 0.008	0.012 ± 0.004	0.039[d] ± 0.007	0.687[e] ± 0.029	0.147[e] ± 0.011
	30-min	WT	0.676 ± 0.025	0.055 ± 0.006	0.015 ± 0.004	0.053 ± 0.007	0.873 ± 0.026	0.214 ± 0.020
	Home cage	KO	0.653[e] ± 0.028	0.034 ± 0.004	0.014[e] ± 0.003	0.079[e] ± 0.018	0.738[e] ± 0.029	0.182[e] ± 0.018
	30-min	KO	0.564 ± 0.038	0.041 ± 0.006	0.032 ± 0.006	0.132 ± 0.012	0.822 ± 0.041	0.238 ± 0.028
CPU	Home cage	WT	0.246[d] ± 0.029	15.149[d] ± 1.636	1.101[e] ± 0.155	1.079[d] ± 0.061	0.579[d,e] ± 0.043	0.282[e] ± 0.022
	30-min	WT	0.266 ± 0.036	16.049 ± 0.236	1.660 ± 0.236	1.524 ± 0. 057	0.704 ± 0.044	0.429 ± 0.047
	Home cage	KO	0.178 ± 0.026	0.742 ± 0.037	1.026 ± 0.115	3.734 ± 0.272	0.397 ± 0.037	0.357[e] ± 0.021
	30-min	KO	0.162 ± 0.019	0.697 ± 0.064	0.969 ± 0.072	4.223 ± 0.311	0.514 ± 0.061	0.478 ± 0.042
VST	Home cage	WT	0.227[d] ± 0.020	6.991[d] ± 0.646	1.137 ± 0.191	0.605[d] ± 0.046	0.915[d] ± 0.073	0.276 ± 0.033
	30-min	WT	0.191 ± 0.019	6.756 ± 0.748	1.314 ± 0.184	0.828 ± 0.043	0.967 ± 0.069	0.376 ± 0.039
	Home cage	KO	0.071 ± 0.013	1.091 ± 0.143	0.932 ± 0.119	2.239 ± 0.196	0.529[e] ± 0.029	0.304[e] ± 0.035
	30-min	KO	0.102 ± 0.041	1.501 ± 0.222	0.973 ± 0.070	2.554 ± 0.219	0.727 ± 0.057	0.436 ± 0.053

[a]The abbreviations for neurochemistry are NE = norepinephrine, DA = dopamine, DOPAC = 3,4-dihydroxyphenylacetic acid, HVA = homovanillic acid, 5-HT = serotonin, 5-HIAA = 5-hydroxyindoleacetic acid. The concentrations are in ng/mg wet weight.
[b]The abbreviations for brain regions are FC = frontal cortex, CPU = caudate-putamen, VST = ventral striatum.
[c]The data are presented as means and standard error of the mean, $N = 11$–16 mice/genotype.
[d]ANOVA and Bonferroni corrected pairwise comparisons, $P < 0.05$, comparison between WT and KO for the home cage condition.
[e]ANOVA and Bonferroni corrected pairwise comparisons, $P < 0.05$, within genotype comparison between the home cage condition and after 30 min in the OF.

$p < 0.001$); significant effects of exposure for 5-HT ($F(1,50) = 4.504$, $p < 0.039$) and 5-HIAA ($F(1,50) = 8.462$, $p < 0.005$); none of the interactive terms were significant. Analyses were performed by Bonferroni corrected pairwise comparisons. Concentrations of catecholamines in ventral striatum of KO mice in both exposure conditions are lower ($ps < 0.020$) than those for WT animals and these levels are not significantly altered upon OF exposure (Table 2). Conversely, levels of HVA are higher under both exposure conditions in KO than WT mice (p's < 0.025). Interestingly, contents of 5-HT in KO animals are depressed under home cage ($p < 0.001$) and OF conditions ($p < 0.010$), and placement into the OF alters both 5-HT ($p < 0.019$) and 5-HIAA levels ($p < 0.012$) but only in KO mice. These data show that levels of NE, DA, and 5-HT are depressed in ventral striatum of the homozygous mutants and that exposure to the OF serves to perturb only 5-HT and further create an imbalance in 5-HT relative to catecholamines. Hence, in KO mice, only the 5-HT system appears responsive to environmental change at the time when stereotypic activation of behavior is most apparent.

Since DA release in the ventral striatum is well known to be involved in psychostimulant-induced hyperlocomotion (Pijnenburg *et al*, 1976), we used microdialysis to sample DA levels in the ventral striatum and compared these to WT and KO responses in the home cage and OF. RMANOVA for within-subjects effects demonstrated a significant effect of time ($F(4,44) = 4.430$, $p < 0.004$), but no significant time by genotype interaction. Bonferroni corrected pairwise comparisons within each genotype revealed that DA release for WT mice was significantly higher than baseline when they were first introduced to the OF at 20 min ($p < 0.024$) and at 80 min ($p < 0.004$) when the animals were returned to their home cage (Figure 6a). By contrast, no alterations in DA release were detected in KO animals, although there was a slight enhancement in DA levels upon return to the home cage. No significant changes in DOPAC levels were observed for either genotype in the OF (data not shown). These results suggest that microdialysis may not be sensitive enough to detect changes in DA release in ventral striatum of KO animals, probably because baseline levels are already very high under resting conditions and, consequently, only small relative changes may occur upon exposure to the OF. Gainetdinov *et al* (1999) reached similar conclusions when they sampled from striatum. Importantly, the present findings show that stereotyped activity in the OF is not accompanied by changes in DA levels in ventral striatum of KO mice.

The previous experiment showed that DA neurons in ventral striatum of KO mice were not very responsive to environmental change. To determine whether DA neurons respond to exogenous stimulation, 50 mM [K^+] was infused through the microdialysis tubing the next day (Figure 6b). RMANOVA for within-subject effects demonstrated a significant time ($F(4,40) = 9.660$, $p < 0.001$) and a time by genotype interaction ($F(4,40) = 9.195$, $p < 0.001$). Compared to baseline values, the Bonferroni corrected pairwise comparisons found that WT mice showed enhanced DA release to the [K^+] stimulus at 20 ($p < 0.005$) and 40 min ($p < 0.042$). By contrast, in KO mice, DA release was slightly increased at 20 min; however, it was dramatically and statistically depressed at 40 ($p < 0.001$) and 60 min ($p < 0.005$). In summary, the miniscule response and decrease in DA levels to [K^+] depolarization in KO mice supports the idea that tissue levels are low (see Table 2) and it suggests that the DA storage pool in ventral striatum is very small and easily depleted.

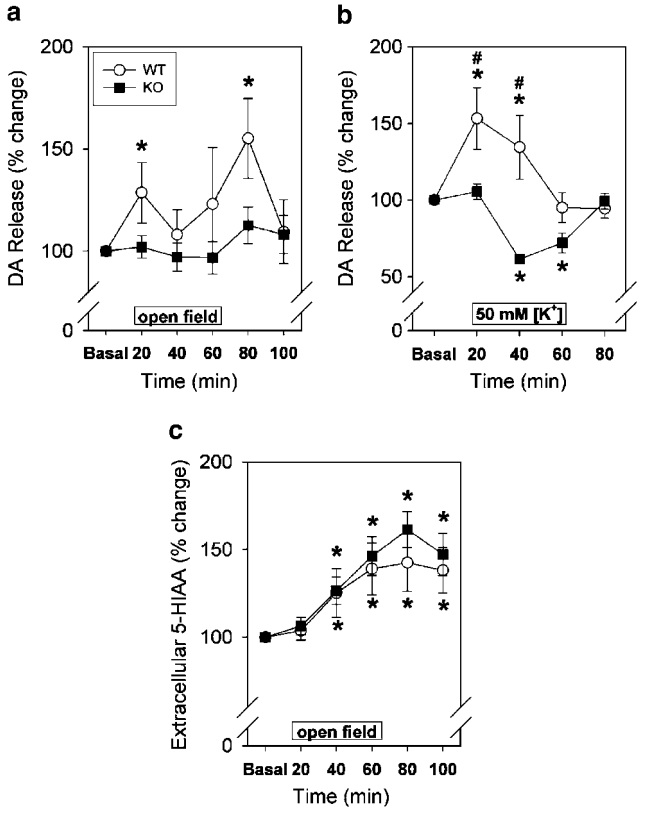

Figure 6 Microdialysis samples from the ventral striatum of WT and KO mice in the home cage and during exposure to the OF. (a) Effect of exposure to a novel environment on extracellular DA levels. Mice were removed from the home cage and placed into the OF for 1 h. Thereafter, they were returned to the home cage and sample collection was continued over the next 1 h. Basal DA values were 15.1 ± 5.2 fmol/sample for WT mice and 278.4 ± 58.0 fmol/sample for KO mice. (b) Effect of perfusion with 50 mM potassium chloride ([K^+]) on DA release. Baseline levels were collected and 50 mM [K^+] was infused into the probe for 40 min. Afterwards, artificial CSF was infused through the probe for the remaining 40 min. (c) Effect of the novel environment on extracellular concentrations of 5-HIAA. Basal 5-HIAA levels were 1301 ± 142 fmol/sample for WT mice and 836 ± 104 fmol/sample for KO mice. $N = 6$–7 mice/group. *, $p < 0.05$, KO or WT mice from basal secretion; #, $p < 0.05$, WT *vs* KO mice.

As tissue 5-HIAA levels in ventral striatum were responsive to environmental change in KO mice (see Table 2), alterations in extracellular 5-HIAA levels were examined in this brain region (Figure 6c). RMANOVA for within-subjects effects revealed significant time effects ($F(4,44) = 15.707$, $p < 0.001$), but no significant time by genotype interaction. Bonferroni corrected pairwise comparisons within genotype demonstrated significant increases in 5-HIAA for WT and KO mice at 40–100 min (WT: $ps < 0.047$; KO: $ps < 0.029$) relative to their respective baselines (Figure 6c). These findings suggest that locomotor activation of KO animals is accompanied by increased 5-HT metabolism in the ventral striatum.

DISCUSSION

In brain, DA systems are involved in fundamental mechanisms related to behavioral adaptation (Le Moal and Simon, 1991; Jackson and Westlind-Danielsson, 1994). Dysfunction in pathways coordinating different DA terminal fields can lead to behavioral deficits that may be reflected in a variety of different psychiatric conditions. Lesion and stimulation studies have shown that DA plays an important integrative role in motor performance, exploration, and cognition (Le Moal and Simon, 1991). Spatial and temporal signaling of DA neurotransmission is controlled by the plasma membrane DAT (Amara and Kuhar, 1993; Garris *et al*, 1994). In the present study, we have investigated behaviors in mice that have either one or both *Dat1* alleles disrupted. HZ and KO animals are deficient in clearance of DA where rates are approximately 2- or 300-fold longer than in WT animals (Jones *et al*, 1998). At the same time, differences in stimulated release from HZ and KO mice are much less apparent with reductions of 5–25 and 50%, respectively, compared to WT controls. These results clearly demonstrate that DA neurotransmission is more aberrant in KO than HZ mice and, as a consequence, one may anticipate that their behavioral phenotypes may be different.

In the OF, spontaneous activity of HZ mice is similar to that of WT controls. These findings confirm those of Giros *et al* (1996). However, several additional characteristics distinguish the two genotypes. First, HZ mice engage in more horizontal activity in the center and, presumably, more aversive area of the OF and the latency to enter this area for the first time is shorter than that for WT and KO mice. Second, the enhanced activity in the central zone of the OF continues to increase over test days. These response patterns suggest that DA transmission may be potentiated in heterozygotes. Although augmentation in center zone activities across days is reminiscent of the sensitization effects of cocaine in normal animals (Carey and Gui, 1997), there are several reasons to suspect that they are related to exploration. In object tests, HZ mice interact with objects more frequently than the WT controls. Additionally, when placed into a free-exploration paradigm, most heterozygotes repeatedly exit their home cage and explore the novel environment, whereas few WT mice engage in this behavior. Finally, although the numbers of transitions between open and closed areas of the zero maze is not different between vehicle-treated WT and HZ animals, diazepam increases the number of transitions to a greater degree in HZ mice. Since benzodiazepines may enhance dopaminergic tone (Soderpalm *et al*, 1991), the zero maze data suggest that DA function in heterozygotes may be more sensitive to GABAergic control than in WT mice.

The results from tests of center zone activity, object interaction, and free exploration in the OF may suggest that disruption of a single *Dat1* allele leads to reduced anxiety and emergence of a novelty-seeking phenotype (see Misslin and Ropartz, 1981; Treit and Fundytus, 1988; Griebel *et al*, 1993; Dellu *et al*, 1996). It should be emphasized that anxiety and novelty-seeking constructs are often tightly connected, where high novelty seeking often accompanies low anxiety and *vice versa* (Gray, 1982). Interestingly, separate brain systems appear to subserve anxiety and novelty seeking (Gray, 1982). Since dysfunction of the DA system has been proposed to contribute to novelty-seeking behaviors in both humans (Cloninger, 1987; Benjamin *et al*, 1996) and animals (Dellu *et al*, 1996; Bardo *et al*, 1996), the reduced anxiety in HZ mice may contribute to a

novelty-seeking phenotype. The novelty-seeking personality trait is a well-recognized dimension of human personality (Cloninger, 1987; Zuckerman, 1994). Human sensation seekers desire variety and change in their environment and they frequently seek novel situations and engage in risk-taking behaviors. In the present experiments, the response to novelty by HZ mice is independent of their activity in a number of different experimental settings. Interestingly, a similar relationship has also been described for human patients (Zuckerman, 1994). Together, these data suggest that there are many similarities between behaviors of *Dat1* HZ mice and humans diagnosed with low anxiety and a novelty-seeking personality trait.

In comparison to behaviors of heterozygotes, the phenotype of the homozygous mutants is quite different. When initially placed into the OF, activity of KO mice is similar to that of HZ and WT animals. After approximately 10 min, however, horizontal and vertical activities are preferentially increased for KO mice. These findings are somewhat different from those reported by Gainetdinov *et al* (1999) who found activity to be increased immediately for KO animals. Several variables that may have contributed to these differences include 1- *vs* 5-min sampling times, illumination of the OF, acclimation to the test room, and sensitivity of the KO mice to external stimuli. With respect to the latter variable, we found that repeated exposure to the OF or handling of mice for vehicle/diazepam injection is sufficient to promote increased activity upon exposure to the OF. Regardless, in Gainetdinov *et al*'s (1999) and our own experiments, the enhancement in activities of KO mice may be due to several different mechanisms that include increased exploration, enhanced emotional responses, or stereotypic activation of behavior. Exploration was evaluated in the free exploration and object paradigms. When KO mice are given free access to the OF, they rarely exit their home cage to explore the novel environment. In the object test, the number of object contacts is comparable between WT and KO animals, but the duration of contacts is reduced for KO mice. When hyperactivity of KO mice is restricted by the size of the OF or reduced by haloperidol administration, object exploration is increased. Collectively, these data show that KO mice do not display a novelty-seeking phenotype and their hyperactivity is not due to enhanced exploration.

Another reason for the hyperactivity and thigmotaxis of KO animals may be attributed to increased emotional responses. When the homozygous mutants are initially placed into the OF, their activity is not distinguished from HZ or WT animals; thereafter, it is preferentially increased. This delay in hyperactivity may be due to anxiety-like responses because the latency to enter the center zone of the OF is prolonged compared to HZ and WT animals and diazepam counteracts the initial 'hypoactivity' of KO mice so that hyperlocomotion and thigmotaxis are immediately evident. Parenthetically, the locomotor-activating effects of anxiolytics are well known and they are usually described as increased exploration due to reduced anxiety (see Gentsch *et al*, 1989; Gray, 1982). A similar attenuation of anxiety-like responses is also seen with diazepam in the zero maze at 0–5 min when the percent of time in the open areas and the numbers of transitions between open to closed areas increase. These data suggest that the KO mice display an initial anxiety phenotype when first exposed to a novel environment. Over time or with repeated exposure, this emotional response may become dissipated and replaced by hyperactivity and stereotyped responses. Hence, the hyperactivity and thigmotaxis of the homozygous mutants does not appear to be related to their emotional responses.

A third mechanism that may subserve the hyperactivity of KO mice is stereotypic activation of behavior. Here, stereotypy refers to an excessive repetition of one type of motor response (Ridley, 1994). In the OF stereotypy of KO mice is not restricted to repetition of locomotor pathways as in thigmotaxis (cf. Ralph *et al*, 2001), but it is also evident as repetitive rearing, jumping, and continuous climbing responses. It is unlikely that these stereotyped behaviors represent escape responses for several reasons. First, if these behaviors are escape responses, one may anticipate that they will be displayed by most KO animals. Instead, the responses of KO mice are not uniform but are highly individualized as has been noted in other settings that include social behavior (Rodriguiz *et al*, 2004). Second, anxiety-like responses disappear at the time stereotypical behaviors emerge. Finally, escape responses are typically goal-directed (Sheffield and Temmer, 1950) and the stereotyped or perseverative behaviors of the KO mice do not appear to be associated with any particular goal. Together, our results suggest that the hyperactivity and stereotypical responses of KO animals are not indicative of escape behaviors.

While KO mice do not appear to engage in escape behaviors, there is some evidence that their anxiety-like behavior is transient. For instance, the low activity upon initial exposure to the OF is replaced by increased activity within the first session. Locomotion of these mutants is also augmented over the 7 days of testing and is confined almost exclusively to the perimeter of the OF. Similarly, activity of KO mice in the zero maze at 0–5 min is low, but it is significantly augmented at 30–35 min when they display an increased number of transitions between the open and closed arms of the maze. Importantly, diazepam potentiates locomotion of the homozygous mutants in both the OF and zero maze. In KO mice, the dissipation of anxiety-like responses and the increased stereotyped activity in both the zero maze and OF is striking, and they suggest that the stereotypic activation of behavior may serve an arousal-reducing function in these animals.

Although disruption of both *Dat1* alleles in mice leads to a number of different biochemical and neurochemical changes, one of the more salient features involves their hyperdopaminergia (Jones *et al*, 1998). Since no alteration in DA release was detected at the time of OF exposure, we sampled DA release ventral striatum given the importance of this area for psychostimulant-induced hyperlocomotion (Pijnenburg *et al*, 1976). Although our microdialysis experiment demonstrates that DA release is negligible in KO mice at the time when the stereotyped activation of behavior is clearly evident, it should be emphasized that our haloperidol studies clearly show that their behavioral stereotypies are dependent upon DA tone. In the microdialysis study, potential alterations in DA levels in KO mice may have been obscured both by high basal levels of the catecholamine and by compromised evoked DA release. Since serotonergic agents suppress the hyperactivity of KO mice (Gainetdinov *et al*, 1999), we hypothesize that their

abnormal behavior is due to a decrease in serotonergic tone. This reduction in tone may be achieved through an increase in 5-HT metabolism in ventral striatum as is shown by the increase in tissue and extracellular levels of 5-HIAA in KO mice. The mechanism of behavioral activation in KO mice is quite different from that of psychostimulant-induced stereotypies. Psychostimulants at high doses induce hyperlocomotion and thigmotaxis in the OF (Simon *et al*, 1994) by eliciting a massive DA release in the basal ganglia of normal rodents (Kuczenski and Segal, 1997). Interestingly, tissues contents of DA and 5-HT are reduced under basal conditions in frontal cortex, dorsal striatum, and ventral striatum of KO mice. When tissue levels of monoamines are measured in KO mice at a time of stereotypic activation, 5-HT metabolism is increased in all three brain regions. By contrast, in WT animals, OF exposure alters DA levels in frontal cortex and 5-HIAA is increased only in frontal cortex and caudate putamen which may reflect stress of the test conditions (Berridge and Dunn, 1989; Ge *et al*, 1997). Hence, in KO mice interactions between dopaminergic and serotonergic neurotransmission are perturbed. Together, these findings suggest that stimulation of 5-HT systems in the ventral striatum may contribute to the activation of stereotyped behavior in the KO mouse. In mutants the decreased tissue stores in ventral striatum of 5-HT at the expense of metabolism may provide them with a constitutive predisposition to develop stereotypical behaviors.

In KO mice, an imbalance between the DA and 5-HT systems in the basal ganglia may exert several consequences on behavior. First, inadequate opposing 5-HT neurotransmission may allow DA-activated motor responses to override regulation by the frontal cortex (see Norman and Shallice, 1986). A disruption of this process can lead to detachment of behaviors from their consequences such that once the KO mice become stereotypically activated their behavior becomes inflexible regardless of experimental context as in the OF, zero maze, radial arm maze (Gainetdinov *et al*, 1999), and social interactions (Rodriguiz *et al*, 2004). A second consequence of DA and 5-HT imbalance is that stereotypical activation of behavior leads to a restriction in the behavioral repertoire (see Lyon and Robbins, 1975). Hyperlocomotion becomes a dominant response in KO mice and any other responses that may normally compete with it in novel settings, such as exploratory or emotional responses, disappear, or become truncated. Hence, object exploration is significantly diminished when the KO mice become stereotypically activated in the OF. Once the hyperlocomotion is suppressed by physical constraints, this behavior can switch to another category (eg rearing). The stereotyped behavior can also become eliminated by haloperidol through reduction of DA tone and, presumably by a rebalancing of DA and 5-HT transmission, such that object exploration is increased.

In the present studies, we have shown that the *Dat1* heterozygotes display an anxiety-reducing and novelty-seeking phenotype, while stereotypic activation of behavior is a major characteristic of the repertoire of the homozygous mutants. Novelty seeking is a personality trait associated with increased sensitivity to novelty, as well as reward. This trait represents a basic feature of risk-taking behavior (Zuckerman, 1994), ADHD (Downey *et al*, 1997), and drug abuse (Cloninger, 1987; Bardo *et al*, 1996). The present findings support the idea that disturbance in the DA system may represent a biological basis of a psychopathology associated with sensation-seeking in humans. In this context, the HZ animals may serve as a useful model for study of reward-related behaviors and impulse control. By contrast, the highly stereotyped and anxiety-like behaviors of KO mice may be useful for elucidation of basic DA mechanisms that may contribute to certain psychiatric conditions such as schizophrenia, autism, obsessive-compulsive disorder, and mental retardation (American Psychiatric Association, 1994; Ridley, 1994). Although the etiologies of these conditions are complex and cannot be merely ascribed to changes in DAT function, the stereotypical-perseverative phenotype of KO mice may represent a fundamental model for the study of the psychological components of these disorders. Collectively, the HZ and KO mice may provide new insights into how alterations in DA function can influence neurotransmitter interactions and, thereby, contribute to a spectrum of disorders that are associated with DA dysfunction.

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