Development of a Mouse Test for Repetitive, Restricted Behaviors: Relevance to Autism

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Abstract

Repetitive behavior, a core symptom of autism, encompasses stereotyped responses, restricted interests, and resistance to change. These studies investigated whether different components of the repetitive behavior domain could be modeled in the exploratory hole-board task in mice. Four inbred mouse strains, C57BL/6J, BALB/cByJ, BTBR T$^+$*tf*/J, and FVB/NJ, and mice with reduced expression of *Grin1*, leading to NMDA receptor hypofunction (*NR1$^{neo/neo}$* mice), were tested for exploration and preference for olfactory stimuli in an activity chamber with a 16-hole floor-board. Reduced exploration and high preference for holes located in the corners of the chamber were observed in BALB/cByJ and BTBR T+*tf*/J mice. All inbred strains had initial high preference for a familiar olfactory stimulus (clean cage bedding). BTBR T$^+$*tf*/J was the only strain that did not demonstrate a shift in hole preference towards an appetitive olfactory stimulus (cereal or a chocolate chip), following home cage exposure to the food. The *NR1$^{neo/neo}$* mice showed lower hole selectivity and aberrant olfactory stimulus preference, in comparison to wildtype controls. The results indicate that *NR1$^{neo/neo}$* mice have repetitive nose poke responses that are less modified by environmental contingencies than responses in wildtype mice. 25-30% of NMDA-receptor hypomorphic mice also show self-injurious responses. Findings from the olfactory studies suggest that resistance to change and restricted interests might be modeled in mice by a failure to alter patterns of hole preference following familiarization with an appetitive stimulus, and by high preference persistently

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demonstrated for one particular olfactory stimulus. Further work is required to determine the characteristics of optimal mouse social stimuli in the olfactory hole-board test.

Keywords

autism; exploration; olfaction; restricted interests; social preference; stereotypy

Introduction

The diagnostic criteria for autism include impaired social interaction, communication deficits, and repetitive, restricted interests and behaviors [2]. Recent work examining autistic-like traits in twin pairs has provided evidence that these three core symptoms of autism, while all highly heritable, are genetically heterogeneous [68] (see also [69]). The findings suggest that the genes that mediate the non-social component of autism are different from the genes underlying abnormalities in social interaction or communication. The non-social component, the domain of repetitive behavior, encompasses a broad range of clinical indices, including motor stereotypy, self-injury, obsessions and compulsions, an insistence on sameness, and other signs of inflexible, ritualistic responses. Studies using a factor analysis approach to examine repetitive behavior in autism have identified different dimensions within the domain, including the factors "repetitive sensory motor actions and resistance to change" [15] and "repetitive sensory and motor behaviours and insistence on sameness" [74].

In a recent review, Lewis et al. [35] noted that, overall, the many different forms of repetitive behavior may fall within two clusters, one composed of overt, "lower-order" motoric stereotypy and self-injury, and the other containing more complex, "higher-order" signs of cognitive rigidity, such as obsessions, repeated ritualistic acts, and an insistence on sameness in the environment (see also [80]). Repetitive behaviors from both clusters tend to co-occur in autistic populations with mental retardation, as well as in high-functioning autistic children or those with Asperger's syndrome [7,11,47,73]. The complexity of the repetitive behavior domain poses particular challenges for the development of animal models relevant to the autism phenotype [35].

Our research group has proposed a set of mouse behavioral tasks for modeling the core symptoms of autism [48,49,51]. For the lower-order repetitive behavior domain, periodic home cage observations and automated measures for activity are used to detect motor stereotypy, self-injurious responses, and other overt signs of aberrant repetitive movements. It is notable that, in mice, cage-related stereotypies can include remarkably high rates of repeated jumping, backward flipping, or cage-top "twirling" [63-66,79]. One concern with the use of measures of stereotyped motor actions to model autistic-like behavior in mice is that this may not provide a valid measure of the more cognitively-oriented or higher-order repetitive behaviors seen clinically in autism.

Pierce and Courchesne [60] used an environmental exploration task to assess another dimension of repetitive behavior, restricted interests, in normal and autistic children. In this study, subjects were instructed to play in a large room with many different containers, such as tins, boxes, and bags, holding stuffed animals, balls, "magic wands," and many other items. The researchers found that the autistic children showed significantly less exploration than the normal subjects. In particular, the autistic children spent much less time opening the various containers or examining the novel contents. These findings suggest that restricted interests characteristic of children with autism may interfere with their ability to adaptively explore novel environments. Based on this premise, we reasoned that exploration tasks may be a reasonable way to model higher-order repetitive behaviors like restricted interests in mice.

Thus, pairing exploration tasks with established procedures for measuring stereotyped motor behavior would, together, allow researchers to model both lower-order (stereotyped motor) and higher-order (restricted interest) features of the repetitive behavior phenotype of autism.

In the present studies, we have used an automated 16-hole nose poke task in mice to model the repetitive behavior and restricted interests observed in autism spectrum disorders. Previous work has shown that nose pokes or head dipping in a hole-board test provide measures of directed exploration [14,24,36,37,54]. In this procedure, mice are placed in a standard open field, with a hole-board on the floor, and measures are taken of the number of nose pokes into each hole. Similar to the novel items that were used by Pierce and Courchesne [60], different types of novel olfactory stimuli may be placed in the holes, with screen covers to prevent the mice from touching the stimuli.

Studies of locomotor patterns during exploration in an open field have shown that mice tend to remain in the corner regions or near the walls of the chamber, with less activity in the center region [53,58,67]. However, patterns of locomotion during exploration can show significant variations across inbred mouse strains [58,67]. Therefore, we predicted that mice tested in the nose poke exploration task would also demonstrate strain–dependent patterns of hole preference, with some strains showing the highest rates of nose pokes for holes in the corner regions, and the lowest rates of nose pokes in the center regions. Further, these patterns could be modified by the placement of novel olfactory stimuli in the less-preferred center holes. A lack of hole preference might indicate a resistance to modify nose poke responses in regards to environmental factors, such as hole location or olfactory stimuli. Overall, we conceptualized that a deficit in selective hole preference or persistent responses to one particular olfactory stimulus would reflect the resistance to change and restricted interests observed in the autism spectrum disorders.

In order to validate the test as relevant to mouse models of the autism phenotype, inbred mouse strains were chosen that had been characterized with varying levels of social approach or reversal learning in spatial tests [48,49,51]. These behavioral domains reflect the impaired social interaction and resistance to change learned patterns of behavior observed in autism [2]. C57BL/6J and FVB/NJ were selected as strains with behavioral profiles that include moderate to high levels of social approach, exploration, and reversal learning. C57BL/6J was of particular interest, since this strain provides the genetic background for many mouse models of neuropsychiatric disorders. Performance in this strain could indicate the patterns of exploration and olfactory preference that might be typical of wildtype groups for mutant line comparisons. Both young adult and older adult C57BL/6J mice were tested, providing information on the use of the assay in mice of differing ages. BALB/cByJ was chosen because the BALB substrains have been characterized as high in anxiety-like behavior and neophobia (dependent upon the behavioral measure; [16,26,27,34,49,78]), and with good reversal learning in spatial tasks [49]. Responses of the BALB/cByJ strain would provide information on the effects of anxiety on exploration and olfactory preference in the nose poke task. The fourth inbred mouse strain, BTBR T$^+$tf/J, has a behavioral profile that reflects some components of autism, including low social preference and a selective deficit in reversal learning, without high levels of anxiety-like behavior [49] (see also [8]). Given the initial findings of an autism-like phenotype, we predicted that BTBR T$^+$tf/J mice would also show low levels of general exploration and demonstrate preference for only one or two olfactory stimuli, similar to the low levels of exploration and restricted interests observed in autistic children [60].

The present studies also included *Grin1$^{neo/neo}$* (*NR1$^{neo/neo}$*) mice, which have reduced levels of the NR1-NMDA receptor subunit. This mutant line is characterized by an aberrant behavioral phenotype, including marked deficiencies in social behavior [21,46] and a tendency for self-injurious responses (present studies), suggesting that the *NR1$^{neo/neo}$* mice might

demonstrate repetitive behavior and restricted interests in the nose poke assay. In addition to the autism-like behavioral profile, the deficiency in glutamate function found in the *NR1neo/neo* mice may be relevant to the syndrome in humans. Carlsson [12] has proposed a hypoglutamatergic hypothesis for autism, based on the neuropathology observed in autistic patients. In particular, the reduced size of the hippocampus and amygdala observed in autism [5,71] might be associated with deficiencies in the glutamatergic neuronal projections that originate in these regions. Abnormal concentrations of glutamine/glutamate in the amygdala and hippocampus have been reported in adults diagnosed with autism spectrum disorders, in comparison to healthy subjects [55]. Changes in glutamatergic neurotransmission might also be relevant to the abnormal synaptic function found in many genetic mouse models for autism and related syndromes [4,29,30,42,84].

Materials and Methods

Subjects

Inbred mouse strains—Male mice from four inbred strains, C57BL/6J (B6), BALB/cByJ (BALB), BTBR T^+tf/J (BTBR), and FVB/NJ (FVB), were purchased from The Jackson Laboratory, Bar Harbor, ME (JAX). Mice were 3 to 4 weeks of age upon arrival at the University of North Carolina animal facility in Chapel Hill, NC. Mice were 6-9 weeks of age at the start of hole board testing, except for one group of B6 (n=8) and the BALB mice (n=10), which were 7 months of age at the start of testing. Animals were housed separately by strain, with 3 to 4 mice per plastic tub cage, and provided with Purina 5058 chow and water ad libitum. Behavioral testing was conducted during the light period of a 12-h light/dark cycle.

NR1-NMDA receptor subunit (NR1) hypomorphic mice—$NR1^{+/+}$ and $Grin1^{neo/neo}$ ($NR1^{neo/neo}$) mice were generated from heterozygous breeder pairs, as previously described [21,46]. Briefly, subjects were F1 hybrid mice, generated by the intercross of 129/Ola-$NR1^{neo/+}$ female mice with C57BL/6-$NR1^{neo/+}$ males. The 129-$NR1^{neo/+}$ are coisogenic for the $NR1^{neo}$ mutation. The C57BL/6-$NR1^{neo/+}$ mice were derived from twelve generations of backcrossing to B6 (The Jackson Laboratories, Bar Harbor, Maine). The $NR1$ hypomorphic mice carried an insertion of a neomycin resistance gene into intron 20 of the $NR1$ locus, leading to an underexpression, but not elimination, of the $NR1$ gene. Mice were 3 to 5 months in age at the time of testing. Animals were housed separately by gender, with 2 to 4 mice per plastic tub cage, and provided with Purina 5058 chow and water ad libitum.

For all mice in the study, the housing room was maintained at 23°C on a 12-h light/dark cycle (lights off at 7 PM). All procedures were conducted in strict compliance with the policies on animal welfare of the National Institutes of Health and the University of North Carolina (stated in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, 1996 edition), and approved by the University of North Carolina Animal Care and Use Committee.

Behavioral test procedures

Before the hole-board assays, all mice in the present study were tested using one or more of the following behavioral procedures: the elevated plus maze test (a 5-min procedure), activity in an open field (a 1-hour or 3-hour test), motor coordination on an accelerating rotarod (5 trials), social approach in a three-chambered test box (a 30-min test), an olfactory test to find a buried food (a 15-min test), or an acoustic startle test (a 35-min test). For most groups, this testing served two purposes: it allowed the mice to acclimate to handling, to general laboratory stimuli, and to exploration of novel environments. The testing also provided additional data for an on-going effort by our research group to characterize multiple inbred mouse strains across several domains of behavior. Detailed methods for these procedures are given in Moy

et al. [49,50]. Data from these procedures are not included in the present study, which is focused on performance in the hole-board test. The results section notes the previous testing experience of each experimental group.

Hole-board exploration—Nose poke responses were assessed by 1-hour trials (1 per day) in an open field chamber (40 cm × 40 cm × 30 cm), also utilized for activity tests. A floor-board with 16 equidistant holes was placed on the bottom of the chamber. Underneath the floor-board, each hole was crossed by a set of photobeams, allowing measurement of nose poke counts for each hole (Pokemon system, Accuscan Instruments, Columbus, Ohio). Wire-mesh screens were placed 15 mm underneath each hole. The screens were used to prevent mice from touching objects placed in the holes during the subsequent tests with olfactory stimuli. Each set of mice was given one or two trials with no stimuli in the holes, in order to determine patterns of hole preference during general exploration. The total distance traveled and time spent in the center region of the test chamber during each test were determined from breaks in a separate grid of photobeams above the floor-board (VersaMax, Accuscan Instruments). Hole-boards were removed after each test, washed with hot soapy water, and dried before the next mouse was tested.

In the present project, one group of female *NR1* mice (10 wildtype and 10 homozygous hypomorphs) was tested for general exploration in the hole-board assay. A larger set of *NR1* mice (described below in study three) was tested for both exploratory behavior, and for nose poke responses to olfactory stimuli.

Olfactory stimuli in the hole-board test—Four different studies were conducted, in order to determine if patterns of hole preference in the hole-board task could be altered by placing olfactory stimuli in the four center holes, and whether mice would show preference for one or more of the scented objects. All olfactory stimuli were placed in scintillation vial caps (unlined white polypropylene caps, 24 mm diameter, for 20 mL vials, Fisher Scientific, Pittsburgh, PA) before being placed into the holes, in order to facilitate cleaning of the hole-board apparatus. New sets of olfactory stimuli were used for each mouse. Stimuli, described in detail below, were selected for familiarity (such as the clean bedding used in the home cages of the mice), novelty (lemon scent), or possible appetitive or social valence (cereal, chocolate chips, mouse urine, soiled mouse cage bedding). For each test, one center hole was left empty as a control for baseline nose poke levels. The location of the different types of olfactory stimuli and the empty center hole (the control center hole) was randomly changed across tests, except in the case of repeated tests with chocolate chips or female mouse urine. Wire-mesh screens prevented the mice from touching the objects. Depending on thickness, stimuli were located from 8 to 14 mm below the surface of the screens, which were 15 mm below the surface of the hole-board.

Items used for olfactory stimuli

Familiar or novel stimuli—The following stimuli were used: familiar clean cage bedding, the type used in the home cages of the subjects (approximately 1.5 g per sample, bed-o-cobs, The Andersons, Maumee, Ohio), novel cage bedding that was not familiar to the mice (Paperchip, Shepherd Specialty Papers, Plainwell, MI), and a novel lemon scent (100% dilution of Pure Lemon Extract with water; 0.05 ml placed on a gauze square; McCormick & Co, Inc., Hunt Valley, MD).

Appetitive stimuli—Food items used as olfactory stimuli included three different types of cereal (Cinnamon Toast Crunch, General Mills, Minneapolis, MN; Honey-Nut Cheerio, Kellogg Co., Battle Creek, MI; or Peanut Butter Cap'n Crunch, The Quaker Oats Co., Chicago, IL); and chocolate chips (Nestle, Vevey, Switzerland).

Social olfactory stimuli—The olfactory stimuli included male or female mouse urine (0.05 ml; CD1 or B6 strain; Bioreclamation, Hicksville, NY). The mouse urine was purchased as frozen 1-ml samples (Bioreclamation, Hicksville, NY), and stored at −20 C° until the day of the test. The sample was thawed and aliquoted into scintillation vial caps (one aliquot per subject), and placed into the hole, with a screen above. Social stimuli also included soiled home cage bedding of either male or female PL/J mice. For these social stimuli, the home cages had not been changed in at least 24 hours. The bedding in the home cages was the same as used in the home cages of the test mice (familiar bedding). PL/J mice were chosen as an inbred strain different from the test mice (B6 and BALB). On the day of testing, the bedding material was mixed in the cage, in order to distribute odors of urine, feces, etc. across samples. Approximately 1.5 g per sample was used for each separate olfactory test.

Familiarization tests—The effects of novelty and familiarity were investigated by first presenting the mice with three olfactory stimuli in the center holes (familiar cage bedding, a novel olfactory stimulus such as unfamiliar cage bedding, and a novel cereal or chocolate chip) during a 1-hour hole-board test. Following this first test, mice were familiarized with the novel cereal or chocolate chip by having 4-5 of the treats added to the home cages each day for the next two days. Following familiarization, mice were tested again with three olfactory stimuli, including the familiar cage bedding and the now-familiar appetitive stimulus (cereal or chocolate chip) during a second 1-hour hole-board test. In the fourth study, this same procedure was used to examine familiarization to a social stimulus. In this case, mice were given a test before and after exposure to female mouse urine. In the first hole-board test, the following stimuli were placed in 3 center holes: familiar cage bedding, a novel cereal, and novel female mouse urine (0.05 ml; CD1 strain; Bioreclamation, Hicksville, NY). Familiarization to the urine was then conducted over two days, with 0.05 ml placed on a white cotton square (Nestlet; Ancare Corp., Bellmore, N.Y) and added to the home cages of the test mice. The second test (post- familiarization) was given three days following the first test, using the same stimuli.

Description of each study with olfactory stimuli

Study one—The first set of male mice tested for hole preference included three groups: young B6 mice (2 months in age, n = 16), and older groups of B6 (n=8) and BALB (n=10) mice, which were 7 months in age at the start of hole-board testing. Mice were first given two general exploration tests, one week apart, in the hole-board/activity chamber without any stimuli present. Data were lost for one exploration test from 8 of the younger B6 mice due to equipment malfunction. Testing with olfactory stimuli began at least one week following the general exploration tests. Mice were first given one olfactory test to determine initial levels of olfactory preference for familiar or novel stimuli. Following the first olfactory test, mice were given two additional tests to investigate preference before and after familiarization to a novel cereal.

Study two—The second set of mice tested for olfactory preference included three inbred mouse strains: B6 and BTBR (n = 10 for each strain), and a separate group of FVB mice (n=17). Mice were first given two tests, on two consecutive days, for general exploration in the hole-board test with no olfactory stimuli added. At least one week later, mice were given two olfactory tests to investigate preference before and after familiarization to a novel chocolate chip.

Study three—The same procedure as described in study two was used to investigate general exploration and familiarization in mice hypomorphic for the NMDA NR1 receptor subunit. Subjects were 16 $NR1^{+/+}$ mice (8 males and 8 females) and 13 $NR1^{neo/neo}$ mice (7 males and 6 females).

Study four—A series of additional hole-board tests were conducted with the mice described in study one, in order to assess preference for various types of mouse social stimuli. Mice were tested in separate hole-board assays with soiled cage bedding (male or female), before and after familiarization with female mouse urine, or in a two-choice test between familiar cage bedding, and familiar cage bedding with female mouse urine added to the sample (0.05 ml; B6 strain, Bioreclamation, Hicksville, NY).

Self-injurious behavior in NR1 +/+ and neo/neo mice—In order to determine the rates of self-injurious responses, records were kept (by an observer blind to mouse genotype) across several experiments for repeated scratching or persistent grooming leading to torn ears or skin lesions. Repeated scratching or grooming was defined as responses directed to the same body area for longer than 3-4 seconds. Any evidence of torn or lacerated ears, or skin lesions around the ears, face, or neck, was recorded. Overall, records for 290 mice (87 male $NR1^{+/+}$, 67 female $NR1^{+/+}$, 71 male $NR1^{neo/neo}$, and 64 female $NR1^{neo/neo}$) were examined.

Data presentation

One challenge with the hole-board task is that each test provides nose poke counts for 16 different holes. We needed to find an optimal way to present up to 16 measures per test in a graphic form. In addition, as shown in Results, inbred mouse strains can have very different levels of nose poke responses (see also [32,54]), yet it would be beneficial to present the patterns of hole preference in a way that was comparable across strains or experimental groups. One way to control for the differences in numbers of nose pokes was to present the data as percent of total nose pokes per hole for each experimental group. Therefore, for presentation of the overall patterns of hole preference (but not for data analysis), the number of nose pokes per group was summed across an hour, and the percent of the total nose pokes, per hole, was depicted.

Figure 1 shows the hole-board numbering system. It is evident from the schematic that holes 1, 4, 13, and 16 can be categorized as corner holes, while holes 6, 7, 10, and 11 are center holes. The remaining holes can be categorized as wall holes. If nose poke responses are evenly distributed across the 16 holes, then the percent of total nose pokes per hole will average 6.25%. However, we have predicted that some holes will receive a higher percentage of the total responses, and some holes will receive a lower percentage. Based on our initial results, we have set a criterion of 12.5%, or twice the expected average percent ($2 \times 6.25\%$), as an indication of high preference, and a criterion of 18.75%, or three times the expected average percent ($3 \times 6.25\%$), as extreme preference. Low preference for a particular hole would be indicated by a percent of half the expected frequency (3.125%).

Data analysis

Data (nose poke counts) were analyzed using repeated measures ANOVAs. Rather than compare counts for 16 different holes, numbers of nose pokes were summed, for each mouse, for the 4 corner holes, the 4 center holes, and the 4 wall holes closest to the back corner (holes 2, 3, 5, and 9), and the 4 wall holes closest to the front corner (holes 8, 12, 14, and 15). For statistical analysis, each mouse thus had four nose poke measures per test (one sum for each category: corner, center, back-wall, and front-wall). Activity data were compared using one-way ANOVAs. Within-strain or within-genotype repeated measures ANOVAs were used to determine significant hole preference in each experimental group. Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value was determined. For all comparisons, significance was set at $p < 0.05$.

Results

Exploration in the hole-board test without olfactory stimuli

B6 and BALB—The first mice tested for general patterns of exploration in the hole-board assay were young B6 (8-9 weeks in age; previous tests: elevated plus maze and activity test), and older groups of B6 and BALB, which were 7 months in age (previous tests, completed by 3 months in age: rotarod, activity, social approach test, olfactory test). For this study, mice were given two one-hour tests, with one week between each test. The distributions of percent total nose pokes for the first and second tests are shown in Figure 2. In the first test, both the older B6 mice and the BALB mice had high preference for one or more corner holes, and low preference for the center holes. A high preference for one corner hole was still evident in the BALB group, but not the B6 group, during the second test.

High preference for corner hole 1 was observed in both the older B6 and BALB groups. This hole was located at the back of the activity chamber, and was also closest to the back of the laboratory room. Therefore, this corner of the chamber was, at most times, farthest from sources of noise in the testing environment. Although the chambers were enclosed in sound-attenuating cubicles, it was possible that the mice were still aware of outside activity in the laboratory.

Nose poke counts—Analysis of the nose poke counts for each hole category (Figure 3) reflects the findings from the percent measures: on the first day of testing, the older groups of B6 and BALB preferred the corner holes significantly more than the wall holes or the center holes [within-group repeated measures ANOVAs, B6 $F_{(3,21)}=14.21$, $p<0.0001$; and BALB $F_{(3,27)}=33.97$, $p<0.0001$], while no significant selectivity was evident in the younger B6 mice. By the second test, only the BALB strain had a significant preference for the corner holes [within-group repeated measures ANOVAs, B6 $F_{(3,21)}=14.21$, $p<0.0001$; and BALB $F_{(3,27)}=33.97$, $p<0.0001$]. Figure 2 also indicates that the younger B6 mice had higher average numbers of nose pokes, while the BALB group had lower counts. Across-groups repeated measures analysis confirmed highly significant effects of group and hole category on number of responses for the first test [main effect of group, $F_{(2,23)}=31.06$, $p<0.0001$, and hole category, $F_{(3,69)}=19.09$, $p<0.0001$], and an interaction between group and hole category that approached significance [$F_{(6,69)}=2.20$, $p=0.053$]. This group x hole category interaction reached significance during the second test [$F_{(6,75)}=2.54$, $p=0.027$], as did the main effect of group [$F_{(2,25)}=98.49$, $p<0.0001$].

B6, BTBR, and FVB—The second set of mice included B6 and BTBR (6-7 weeks in age, previous tests: elevated plus maze, social approach test, activity test), and a separate group of FVB (8-9 weeks in age, previous tests: rotarod, social approach test, activity test), each given two one-hour hole-board tests on consecutive days. Figure 4 depicts the percent total nose poke distributions for the three mouse strains. The B6 mice tended to prefer the corner holes during the first test, although none of the percents reached the criterion for high preference. The same pattern was evident in the BTBR mice for the first test, with a stronger overall preference for the corner holes. During the second test, the BTBR group demonstrated a high preference for one corner hole (hole number 1, located at the back of the activity chamber). The FVB group did not evidence a clear preference for any of the holes for either test.

Nose poke counts—The number of responses for each hole category (Figure 5) indicates that selective hole preference was observed in the B6 and BTBR mice, but not the FVB mice, during the first hole board test [within-group repeated measures ANOVAs, B6 $F_{(3,27)}=5.43$, $p=0.0047$; and BTBR $F_{(3,27)}=14.77$, $p<0.0001$]. Only the BTBR mice had a significant preference for the corner holes in the second test [$F_{(3,27)}=5.12$, $p=0.0062$]. Across-group repeated measures ANOVAs for the B6 and BTBR groups confirmed strain differences in

numbers of nose pokes and a significant effect of hole category for the first test [main effect of strain, $F(1,18)=16.49$, $p=0.0007$, and hole category, $F(3,54)=15.81$, $p<0.0001$] and the second test [main effect of strain, $F(1,18)=8.14$, $p=0.0106$, and hole category, $F(3,54)=4.98$, $p=0.004$].

Hole selectivity in mice with reduced NMDA receptor function—One reason for testing mice partially deficient in the NR1-NMDA receptor subunit ($NR1^{neo/neo}$) for repetitive behavior in the hole-board assay is that these mice show home cage stereotyped responses, including self-scratching and grooming to the point of skin lesions (sometimes necessitating euthanasia). Records taken across several experiments indicated that no wildtype mice showed stereotyped scratching or signs of self-injury. However, 29.6% (21/71) of the male $NR1^{neo/neo}$ mice, and 25% (16/64) of the female $NR1^{neo/neo}$ mice, were noted to exhibit repeated scratching, torn ears, or skin lesions. These data suggested that the $NR1^{neo/neo}$ mice might provide an animal model for the self-injurious form of repetitive behavior commonly associated with other forms of repetitive behavior in autism, and therefore, were valuable candidates for assessment in the hole-board test.

Ten $NR1^{+/+}$ and ten $NR1^{neo/neo}$ female mice (previous tests: two 3-hour activity tests) were given a one-hour hole-board test. The wildtype mice demonstrated greater selectivity for the corner holes, with high preference for holes 1 and 13 (Figure 6A). In contrast, no clear hole preference was evident in the $NR1$-hypomorphic mice (Figure 6B). Analysis of the nose poke counts (Figure 7) confirmed that the $NR1^{+/+}$ group had a higher level of exploration for the corner holes, in contrast to holes in other locations [within-genotype repeated measures ANOVA, $F(3,27)=24.83$, $p<0.0001$]. No pattern of hole preference was seen in the $NR1^{neo/neo}$ mice. These group differences were reflected in the results from the across-groups repeated measures ANOVA, which indicated a significant genotype x hole category interaction [$F(3,54)=3.95$, $p=0.0128$], and a significant effect of hole category [$F(3,54)=4.55$, $p=0.0065$].

Activity during the hole-board tests—Levels of activity during the hole board tests were examined in order to determine if mice showing low nose poke counts, such as the BALB group, also had low general exploration in the activity chamber. Figure 8 depicts total distance traveled during the second hole board test from the first and second sets of mice. As previously noted, only the BALB and BTBR mice had significant corner hole preference during the second test. The activity levels of the $NR1^{+/+}$ and $NR1^{neo/neo}$ are included on the figure, since these groups showed overt differences in patterns of hole preference. The results for the first set of mice show that the higher rates of nose poke observed in the younger B6 mice were accompanied by higher levels of distance traveled, in comparison to the older B6 and BALB groups [post hoc comparisons following main effect of group, $F(2,25)=9.98$, $p=0.0007$]. The relatively low numbers of nose pokes evident in the BALB mice were not associated with general hypoactivity, in comparison to the older B6 mice. The BTBR group traveled less distance than the B6 group from the second set of mice [main effect of strain, $F(1,18)=11.23$, $p=0.0036$]. In line with previous reports [20,46], the $NR1^{neo/neo}$ showed higher levels of activity than the wildtype mice [main effect of strain, $F(1,18)=25.96$, $p<0.0001$]

Hole-board tests with olfactory stimuli

Study 1

First olfactory test: This test addressed two questions: would placement of olfactory stimuli in the center holes lead to a change in the pattern of hole-board exploration, and would mice demonstrate selective preference for the different olfactory stimuli? The same set of mice as shown in Figure 2 were tested in this study. In the first olfactory test, the following stimuli were placed in the center holes: familiar cage bedding, lemon scent placed on a gauze square, and unfamiliar cereal (one Cinnamon Toast Crunch). One center hole remained empty. The

results, depicted in Figure 9, clearly indicate that hole preference can be shifted from the corner holes to the center holes containing olfactory stimuli. Surprisingly, both groups of B6 mice demonstrated extreme preference for the hole containing the familiar cage bedding. The BALB mice also had very high preference for the bedding stimuli, but showed almost the same preference for the novel cinnamon cereal. Overall, the study suggested that the hole-board test could be used to examine different olfactory preference across inbred mouse strains.

Familiarization test: The extreme preference shown for the hole containing clean bedding was not expected, and, in fact, this stimulus had only been included as a possible neutral control. In the next test, the familiar bedding stimulus was retested, with two new novel stimuli: paper chip cage bedding, which was not familiar to the mice, and another type of cereal (a Cheerio). The results from the second olfactory test confirmed a high preference for clean, familiar home cage bedding in all three groups (Figure 10A, first sets of bars, percent distribution for center holes only). None of the other olfactory stimuli met the criterion for high olfactory preference.

After this test, mice were familiarized to the novel cereal across two days, by having 4-5 Cheerios added to the home cages. Mice were then re-tested with the familiar cage bedding, the now-familiar cereal, and a new novel stimulus (0.05 ml male mouse urine, CD1 strain) in a one-hour session. Following familiarization, all three groups evidenced high-to-extreme preference for the cereal olfactory stimulus (Figure 10B, first sets of bars). Only one group, the older B6 mice, maintained the same high levels of exploration of the hole containing the clean cage bedding. High preference was not observed for the social olfactory stimulus (the novel mouse urine) in any group.

Analysis of the nose poke counts (Figure 11A and B) supported the finding that most of the responses were directed to the familiar cage bedding before familiarization to the cereal, while more nose pokes were made to the familiar cereal, in the young B6 and BALB groups, after familiarization with the cereal [post-hoc comparisons following significant within-group repeated measures ANOVAs for effect of olfactory stimuli, before and after familiarization: young B6; before, $F_{(3,45)}=16.5$, $p<0.0001$, and after, $F_{(3,45)}=9.23$, $p<0.0001$; older B6, before, $F_{(3,21)}=8.31$, $p=0.0008$; and after, $F_{(3,21)}=13.87$, $p<0.0001$; and BALB, before, $F_{(3,27)}=5.99$, $p=0.0029$; and after, $F_{(3,27)}=5.94$, $p=0.003$].

Study 2

Familiarization to a chocolate chip: This study was conducted in order to confirm the finding of high preference for familiar cage bedding and the effect of familiarization in a separate set of B6 mice, and to see whether the same pattern of preference for olfactory stimuli would be observed in BTBR and FVB mice. Mice were given a one-hour test with the following stimuli: familiar cage bedding, novel cage bedding, and a novel chocolate chip. As presented in Figure 10A (last set of bars), all three strains showed extreme preference for the familiar bedding for the first olfactory stimuli test. Following familiarization with the chocolate chip in the home cage, the mice were given a second test with the same olfactory stimuli. Both the B6 and FVB mice demonstrated a dramatic increase in percent total nose pokes directed towards the hole containing the chocolate chip (Figure 10B). However, the BTBR group exhibited almost no change at all in olfactory stimuli preference from the first test to the second test. This was the only group that continued to demonstrate extreme preference for the familiar cage bedding following familiarization to the appetitive stimulus.

As shown in Figure 11A, before familiarization, all three strains had higher numbers of nose pokes into the hole containing familiar bedding, in comparison to the other olfactory stimuli [post-hoc comparisons following significant within-group repeated measures ANOVAs for effect of olfactory stimuli, before familiarization: B6, $F_{(3,27)}=6.82$, $p=0.0014$; BTBR, $F_{(3,27)}=7.24$, $p=0.001$; and FVB, $F_{(3,48)}=35.08$, $p<0.0001$]. After exposure to the chocolate chip in

the home cages, the B6 and FVB groups demonstrated a clear shift in preference to the chip olfactory stimuli, while the BTBR mice persisted in making more nose poke responses toward the familiar cage bedding [post-hoc comparisons following significant within-group repeated measures ANOVAs for effect of olfactory stimuli, after familiarization: B6, $F(3,27)=25.18$, $p<0.0001$; BTBR, $F(3,27)=8.34$, $p=0.0004$; and FVB, $F(3,48)=41.92$, $p<0.0001$].

Study 3

Familiarization test in mice with reduced NMDA receptor function: The same familiarization procedure as used in Study 2 was conducted with 16 $NR1^{+/+}$ mice and 13 $NR1^{neo/neo}$ mice (previous test: habituation in the acoustic startle paradigm). Mice were first given two one-hour sessions with no olfactory stimuli present. As found with the first group of $NR1$ mice, there were significant main effects of genotype on nose poke counts for the first test [$F(1,27)=15.53$, $p=0.0005$] and the second test [$F(1,27)=27.56$, $p<0.0001$]. A lack of hole selectivity was especially evident in the mutant mice by the second general exploration test: a within-genotype repeated measures ANOVA failed to reveal a significant effect for hole category in the $NR1^{neo/neo}$ group [$F(3,36)=0.22$, $p=0.8832$], while this effect was highly significant in the $NR1^{+/+}$ group [$F(3,45)=19.34$, $p<0.0001$] (data not shown).

Figure 12 depicts the results from the olfactory stimuli tests, before and after familiarization, in the wildtype and NMDA receptor-deficient mice. Before familiarization, the wildtype mice evidenced an extreme preference for the familiar cage bedding, with almost 40% of the total responses across the 16 holes directed toward this olfactory stimulus. The $NR1^{neo/neo}$ mice showed high preference for the familiar bedding, but, alone of all the groups, also had extreme preference for the novel cage bedding. Examination of the data suggests that this high value was partly due to one subject, which made 130 nose poke responses into the center hole containing the novel bedding. These responses were distributed across every 5-minute interval of the 1-hour test. Following the two-day familiarization to the chocolate chip, both groups of mice had a higher preference for this olfactory stimulus, with the $NR1^{+/+}$ group having extreme preference, and the $NR1^{neo/neo}$ group having high preference for the familiar appetitive stimuli.

Analyses of the nose poke counts confirm significant differences in hole selectivity for the first olfactory stimuli test [genotype x hole category interaction, $F(3,81)=4.19$, $p=0.0083$; trend for significant genotype effect, $F(1,27)=3.84$, $p=0.0604$], and a significant genotype main effect during the second test [$F(1,27)=8.05$, $p=0.0085$]. Within-genotype repeated measures ANOVAs confirmed that only the wildtype group demonstrated significant hole selectivity [first test, $F(3,45)=18.9$, $p<0.0001$; and second test, $F(3,45)=10.17$, $p<0.0001$].

Time spent in center of chamber: One general question about the addition of olfactory stimuli to the hole-board was whether overall locomotor patterns were altered during the test. As shown in Figure 13, most of the experimental groups in studies 1, 2, and 3 showed significant increases in time spent in the center region of the activity box during the post-familiarization olfactory test, in comparison to the second exploration test. This pattern was not observed in one of the B6 groups, which already demonstrated relatively high levels of center time, or in the $NR1^{neo/neo}$ mice. Interestingly, the BTBR strain was the only group that showed lower center time during the olfactory test.

Study four

Social stimuli in the hole-board test: A goal of the present series of experiments was to determine relative preference for social olfactory stimuli in the hole-board assay. The mice in the first familiarization study did not demonstrate high preference for male mouse urine. These same groups of mice were given additional olfactory tests, each with a different social stimulus: soiled bedding from a home cage containing male mice, soiled bedding from a home cage

containing female mice, and female mouse urine before and after familiarization. In each test, mice were also presented with a center hole containing familiar (clean) cage bedding, and either a novel cereal or novel chocolate chip. Since preference for the social stimuli was the primary measure of interest, only the data for the social olfactory stimuli, in comparison to the familiar cage bedding, are given in Table 1. The results showed that soiled cage bedding which contained odors from either male or female mice was not any more preferred than non-soiled cage bedding. High preference was not observed for female mouse urine, from the CD1 outbred strain, even after samples of the urine had been placed on nestlets and added to the home cages on two days. In the final olfactory test, female urine (0.05 ml; B6 inbred strain) was placed on familiar, clean cage bedding, and mice were given a two-choice test: familiar cage bedding in one center hole, and familiar cage bedding plus the female mouse urine in another hole. In this case, both groups of B6 mice demonstrated extreme preference for the "compound" social olfactory stimulus, while the BALB mice showed high preference. Within-group repeated measures ANOVAs, conducted for the nose poke counts, indicated that only the B6 groups had significantly higher numbers of nose pokes directed to the female urine + cage bedding stimulus, in comparison to the cage bedding alone [young B6, $F(1,15)=16.01$, $p=0.0012$; older B6, $F(1,7)=11.48$, $p=0.0116$] (data not shown).

Discussion

The purpose of the present studies was to determine if the hole-board test could be used to measure repetitive behaviors and restricted interests in mice, as part of an on-going initiative to develop mouse behavioral tasks relevant to the autism phenotype [48,49,51]. The strategy for this study was to characterize two strains (B6 and FVB) that, based on previous work in our laboratory [49], would not be expected to show any autism-like profiles of behavior. The patterns of exploration and olfactory preference in these strains were compared to behavior in BALB and BTBR, two strains with behavioral phenotypes including low social preference. In order to provide a first validation of the nose poke test as an assay for mutant mouse lines, we evaluated the *NR1neo/neo* mice. Since lower-order motoric symptoms tend to co-occur with higher-order signs of cognitive rigidity in autism or Asperger's syndrome [7,11,47,73], we reasoned that a mouse line with overt self-injurious behavior might also demonstrate repetitive, restricted responses in the nose poke task, in comparison to wildtype mice.

The results suggest that behavior in this assay can be used to model components of autism. Deficits in hole selectivity shown by the mice with reduced NR1 NMDA receptor function could reflect persistent, repeated behaviors that are less constrained by environmental conditions, such as hole location or olfactory stimuli, than responses in the wildtype controls. Possible indications for restricted interests include the failure to demonstrate a shift in hole preference following familiarization in the BTBR strain, and the aberrant interest in novel cage bedding seen in the *NR1neo/neo* group. Low numbers of nose poke responses in the BTBR mice could model the reduced exploration observed in autistic children [60]. Although the BALB mice also had low nose poke counts, the reduced exploration may have been due to more anxiety-like behavior in this strain. Overall, the findings suggest that the hole-board test can provide measures that reflect different dimensions of repetitive behavior in mice. However, interpretation of results may require complementary information from evaluations of activity, anxiety, sensory ability, and learning and memory.

Previous work has shown that inbred mouse strains are characterized by different levels of head dipping in hole-board tasks [32,54]. Pharmacological assays have indicated that numbers of head dips can be increased by treatment with diazepam or other anxiolytic drugs, and decreased by anxiogenic compounds [75], although these changes may be related to general drug effects on activity levels [33]. In the present study, BALB mice had lower numbers of nose poke responses and a higher preference for the corner holes than the B6 mice. Other

investigators have reported anxiety-like behavior and neophobia (dependent on the behavioral measure; [16,27,78]), and low social approach [9,10,72] in BALB/c inbred strains. We have also found a high-anxiety phenotype in the BALB/cByJ strain, including low open arm time on the elevated plus maze, and markedly low levels of sociability and entries in the three-chamber social approach task [49], suggesting that the low nose poke counts and significant preference for the corner holes in the hole-board assay might reflect a high-anxiety strain trait (e.g. [6]). The low levels of time that the BALB mice spent in the center of the activity chamber provide further confirmation of anxiety-like behavior. On the other hand, the BALB strain, like B6 and FVB, demonstrated a high or extreme preference for an appetitive olfactory stimulus following familiarization. These findings suggest that a shift in olfactory preference is not prevented by tendencies for anxiety-like behavior or neophobia.

BTBR mice also had reduced measures of exploration, such as nose poke counts and time in the center of the activity chamber, in comparison to B6 mice matched in age and training history. In addition, BTBR was the only inbred strain that failed to demonstrate high or extreme preference for an appetitive olfactory stimulus following familiarization. Instead, this strain had persistent preference for the familiar bedding stimulus in the second olfactory test. There is some evidence that this lack of interest in the familiarized appetitive stimuli is not due to a general deficit in food-related detection or motivation in this strain. We have previously shown that, in a buried food test for olfactory ability, BTBR and B6 had comparable performance: 83% of the BTBR mice, and 85% of the B6 mice, were able to uncover a hidden piece of cereal [49]. These results indicate that the majority of BTBR mice have intact olfactory ability, and can be motivated by appetitive olfactory stimuli. This strain also has enhanced acquisition in an appetitive T-maze task in comparison to B6, indicating that the failure to demonstrate a shift in olfactory preference following familiarization might not be related to a general memory deficit. We have also found that the BTBR strain is not hypoactive in the open field test, and is comparable to B6 in the percent time spent in the open arms of the elevated plus maze [49].

In contrast to these findings, BTBR mice have been characterized with a lack of significant sociability in the 3-chamber social approach task, without concomitant low numbers of chamber entries [49]. This strain was also found to have low social interaction in a cage setting [8]. BTBR mice do not demonstrate significant quadrant selectivity after reversal learning in the Morris water maze task, which may indicate a resistance to change in this strain [49]. It is possible that the behavioral phenotype of low social approach [8,49] and failure to modify behavioral patterns in the hole-board familiarization assay might be related to the overt neuroanatomical defects in these mice, including absent corpus callosum and reductions in the hippocampal commissure [82]. Some studies have reported deficiencies in corpus callosum volume or white matter concentration in autistic patients [1,13,41,61,81,83].

The FVB group did not evidence significant hole selectivity during the first hole-board exploration tests. These mice had also received several previous behavioral tests, including the 3-chamber social approach and activity procedures. The exposure to handling and novel environments may have led to decreased anxiety in the FVB group and, subsequently, to increased investigation of the center and wall holes. However, the two other inbred strains tested for hole-board exploration in study two had similar previous testing experience, and also had significant preference for the corner holes for the first exploration assay (B6), or for both exploration assays (BTBR). The genotype of the FVB strain includes the gene for retinal degeneration [77], and it is possible that visual impairment in the FVB mice led to deficits in hole selectivity based on spatial location of the holes. The lack of hole preference did not lead to overt differences in general locomotor patterns, since the time that the FVB mice spent in the center region was similar to that observed in the B6 group. In addition, the FVB and B6 inbred strains had very comparable levels of hole selectivity during the olfactory familiarization

procedure. Overall, the lack of hole preference in the first tests with the FVB mice may have been due to a high-exploration trait in this inbred strain, as evidenced by high social approach behavior in other studies [8,48,49,51], rather than previous testing experience or sensory deficits.

The present study showed altered hole-board behavior in the NR1-NMDA receptor subunit hypomorphic mice, including less hole selectivity than the wildtype mice. Home cage observations indicated that 25%-30% of the mice self-scratch or groom to the point of torn ears and skin lesions, sometimes necessitating euthanasia. Previous work has reported several behavioral abnormalities in the $NR1^{neo/neo}$ mice, including significant alterations in social behavior [21,46]. For example, using the 3-chamber social approach test, we have found marked deficits in sociability in the mutant mice, while in the standard resident-intruder test, both male and female $NR1^{neo/neo}$ mice show an impoverished repertoire of behaviors associated with social dominance [21]. $NR1^{neo/neo}$ mice consistently exhibit exaggerated startle responses and deficient prepulse inhibition in acoustic startle tests [19-21,25,50]. It is notable that, in two human studies, deficits in sensorimotor gating were reported for adults with autism [59] or Asperger's syndrome [43]. Overall, the $NR1^{neo/neo}$ mice have a behavioral phenotype that includes self-injurious repetitive responses and deficits in social behavior and sensorimotor gating, without overt motoric or sensory impairment, thus reflecting some components of autism.

Both groups of $NR1^{neo/neo}$ mice in the present study showed reduced hole selectivity, which could be interpreted as repeated responses emitted without regard of environmental conditions, such as hole location, that had significant effects on hole selectivity in the wildtype mice. The pattern of reduced hole selectivity was also seen during the olfactory tests, when the only percent hole preference meeting the "extreme" criterion in the mutant mice was for the hole containing the novel cage bedding – a pattern not observed in any other group of mice in the present studies. This high level of interest was especially evident in one $NR1^{neo/neo}$ male, suggesting that the hole-board task can be used to identify individual mice characterized by unusual restricted interests (extreme interest in one olfactory stimulus, especially a stimulus non-preferred by control mice), as well as repetitive behavior. In the past, exploration on the hole-board task has been described as the random dispersion of head dipping across holes, while repeated responses into one hole were a sign of stereotypy [39,40]. The results from the inbred strains and the $NR1^{+/+}$ and $NR1^{neo/neo}$ mice suggest that the environmental context, including location, type of olfactory stimuli, and previous experience, should be considered in the interpretation of repeated responses to one or more holes. In particular, the non-random patterns of hole selection can provide further information on normal or typical exploration in wildtype mice, versus altered patterns in the corresponding mutant lines.

Some of the findings from the present studies were unexpected. For example, the familiar cage bedding was initially added to the set of olfactory stimuli as a type of neutral, non-novel control. However, high or even extreme preference for this material was observed in all groups of mice during one or more tests (and for different center-hole locations). The familiarization tests provided more support for the premise that the mice preferred to investigate olfactory stimuli that had been presented in the home cage. This non-preference for novelty was in contrast to other paradigms using a preference for novelty to assess object recognition and episodic memory in mice and rats [17,22,32,70]. Neophobia for novel food, objects, or locations has been well-documented in rodents (see [23,31]), and can be altered by pharmacological challenge [6,26,76], rearing conditions [28], or genetic change [3,18,38,45,57,62]. Non-preference for novelty has been shown to be strain-dependent in mice [26,34]. In one study using a free exploration procedure, BALB/c mice preferred a familiar over a novel compartment, while B6 mice had a preference for the novel location [26]. Avoidance in the BALB/c mice could be reversed by adding familiar stimuli to the novel compartment. In the

present studies, the addition of novel olfactory stimuli to the center holes did not induce active aversion (except perhaps in the case of the lemon scent). However, the low baseline rates of nose poke responses made into the center holes might make the detection of avoidance of novelty, versus preference, difficult.

It is possible that the non-preference for novelty was the underlying basis for a lack of interest in almost all of the social stimuli selected for our tests. The mice did not prefer to investigate olfactory stimuli from small drops of mouse urine, either from males or females, and did not appear to distinguish between soiled cage bedding and clean cage bedding. The final test in the present project found a higher preference for female mouse urine when the sample was added to the familiar bedding stimuli. This suggests that the amount of urine used (0.05 ml) was adequate to provide detectable olfactory cues, and that the stimulus was not located too far below the surface of the hole-board to allow detection. Previous work has shown that mice detect urine sample sizes of 0.01 ml [56], and can discriminate mouse urine from plain water, even after four-fold [52] or 100-fold [85] dilution. In addition, wildtype mice on a B6 and 129 background have been found to have a significant preference for the olfactory investigation of either male-soiled or female-soiled cage bedding, in comparison to clean bedding [85]. One issue may be that the aliquots of urine were taken from previously frozen samples, and may not have contained the same volatile elements as fresh urine samples. Further work is needed to determine how, in the hole-board assay, the strain of mouse for the source of the urine or soiled cage bedding, the "compound" nature of the urine + bedding stimuli, or other factors affect preference for social stimuli.

Mice in the present study were not experimentally naïve at the start of the hole-board assays. Other researchers have reported that previous testing experience can alter behavior in some, but not all, assays in mice [44]. We controlled for the possible confounding effects of testing history by conducting statistical comparisons only between groups with the same testing experience. In particular, the sets of $NR1^{+/+}$ and $^{neo/neo}$ mice were matched for testing history. It is important to note that there can be beneficial consequences of repeated testing in mice. Evaluations across several assays can acclimate mice to handling, transport, and the laboratory setting, and thereby lessen anxiety or stress induced by general environmental factors. In many phenotyping studies, groups of wildtype and mutant mice are given a battery of tests, in order to assess behavior across multiple domains of function. Information on motor, sensory, and learning abilities, as well as levels of anxiety-like behavior and social preference, can be valuable for the interpretation of performance in the hole-board test or other assays. One strategy to optimize the use of a testing battery is to conduct the least aversive procedures early in the testing sequence, and the more aversive procedures (such as the Morris water maze test) at the end of the study [44].

Overall, this initial work using the hole-board test has provided possible methods for examining aberrant repetitive behaviors, resistance to change, and restricted interests, as well as reduced exploration, in mice. The method also allows complementary measures of activity levels and time in the center of the chamber (an index of anxiety-like behavior). Determination of optimal social stimuli for olfactory preference tests is an important goal for future studies on developing mouse behavioral tasks to model core symptoms of autism.

Acknowledgements

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Hole-board Numbering System

Back wall of chamber

1	2	3	4
5	6	7	8
9	10	11	12
13	14	15	16

Front wall of chamber

Figure 1.
Schematic of hole-board numbering system in the activity chamber, indicating four corner holes (1, 4, 13, and 16), eight wall holes (2, 3, 5, 8, 9, 12, 14, and 15), and four center holes (6, 7, 10, and 11).
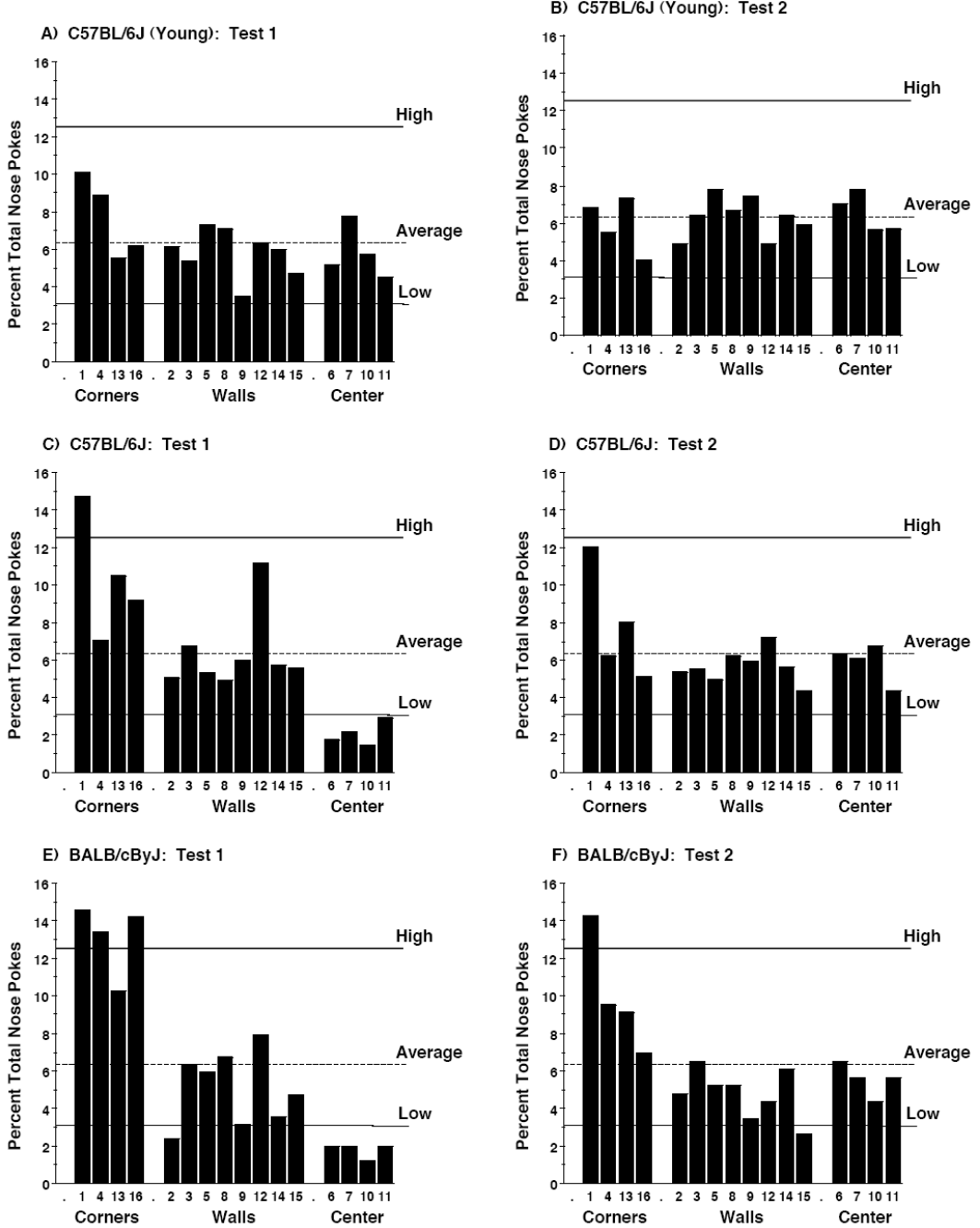
Figure 2.
Hole preference during a one-hour exploration test in C57BL/6J (B6; young, 8-9 weeks in age), B6 and BALB/cByJ (BALB; 7 months in age) male mice. Criterion levels for preference were 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low). Mean total number of nose pokes for data in each panel were: B6 (young) A, 212.5 (SEM=27.0), B, 178.3 (SEM=11.8), B6 C, 94.3 (SEM=14.9), D, 66.8 (SEM=7.6), and BALB E, 25.4 (SEM=7.2), and F, 23.1 (SEM=4.8).
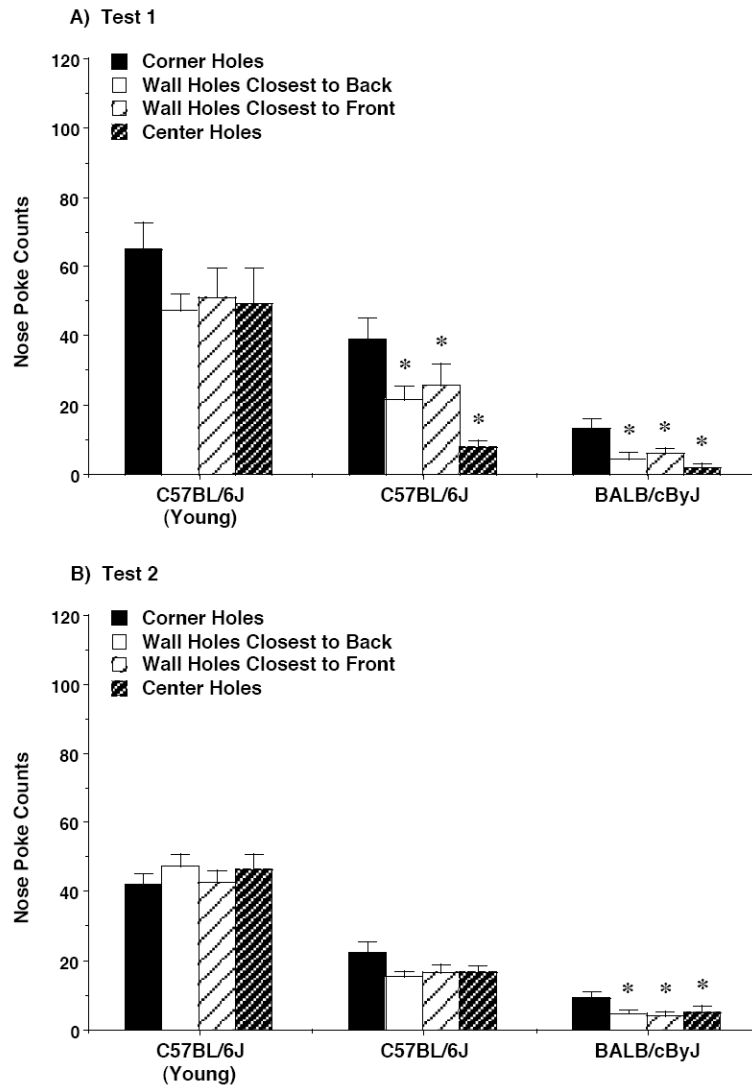
Figure 3.
Number of nose pokes during a one-hour exploration test in B6 (young, 8-9 weeks in age), B6 and BALB (7 months in age) male mice. Data shown are means (+SEM). * p< 0.05, within-group comparison to nose poke counts for center holes.
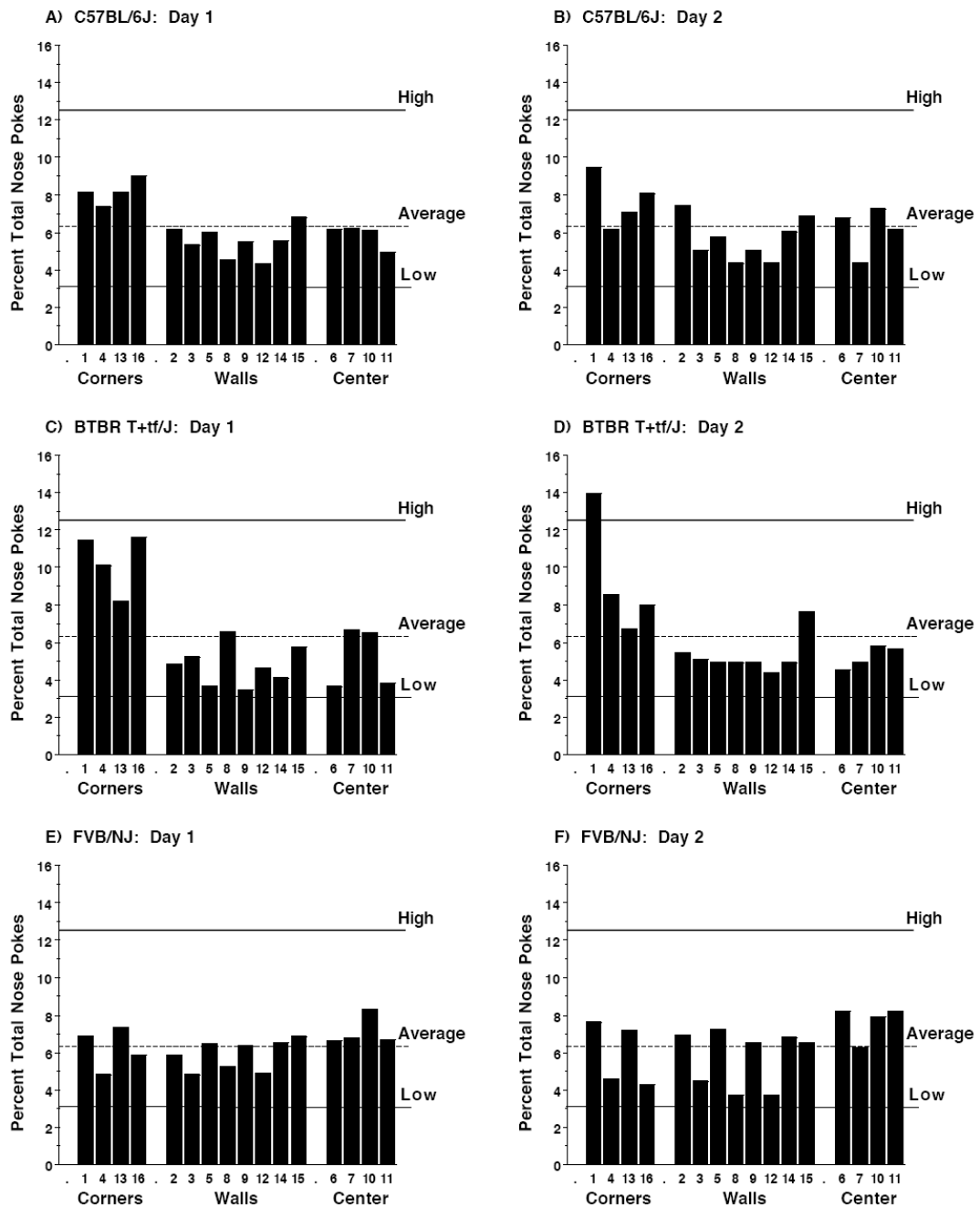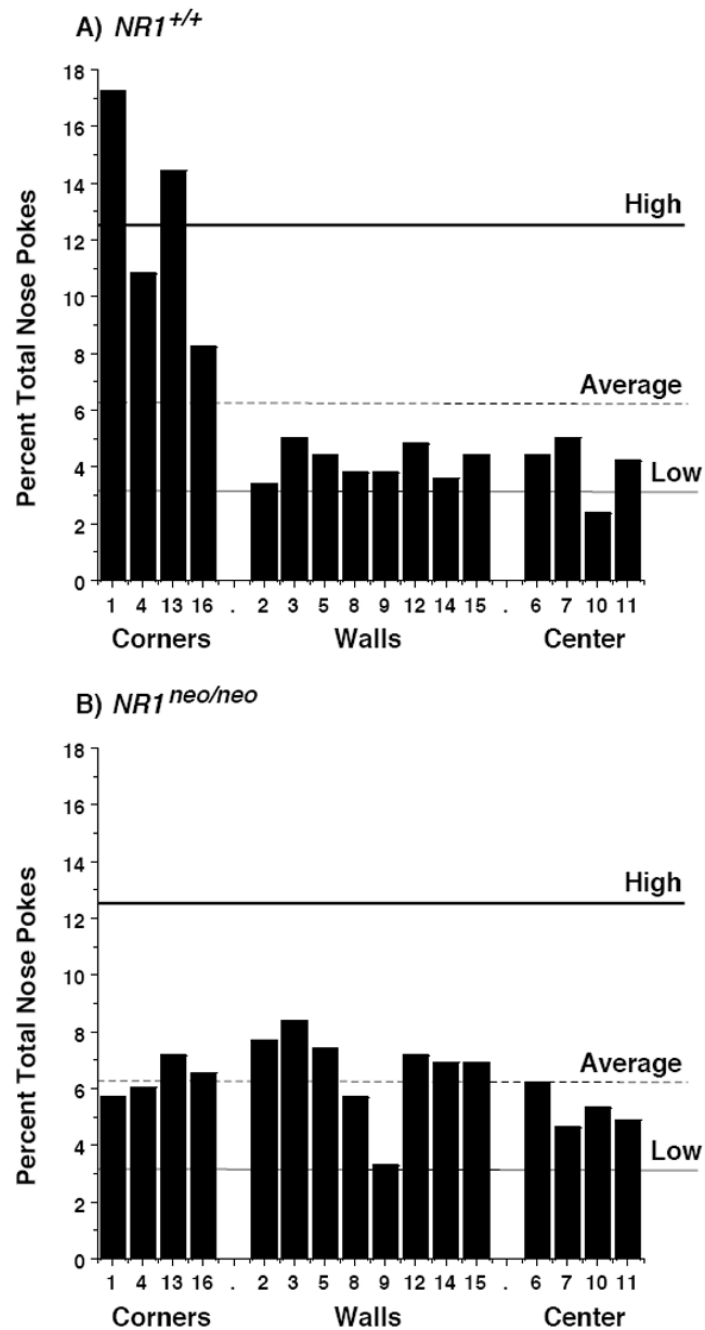
Figure 4.
Hole preference during a one-hour exploration test in B6, BTBR T+tf/J (BTBR), and FVB/NJ (FVB) male mice. Criterion levels for preference were 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low). Mean total number of nose pokes for data in each panel were: B6 A, 222.4 (SEM=25.0), B, 107.8 (SEM=16.1), BTBR C, 107.8 (SEM=13.2), D, 55.2 (SEM=8.9), and FVB E, 158.5 (SEM=8.7), and F, 121.9 (SEM=7.9).
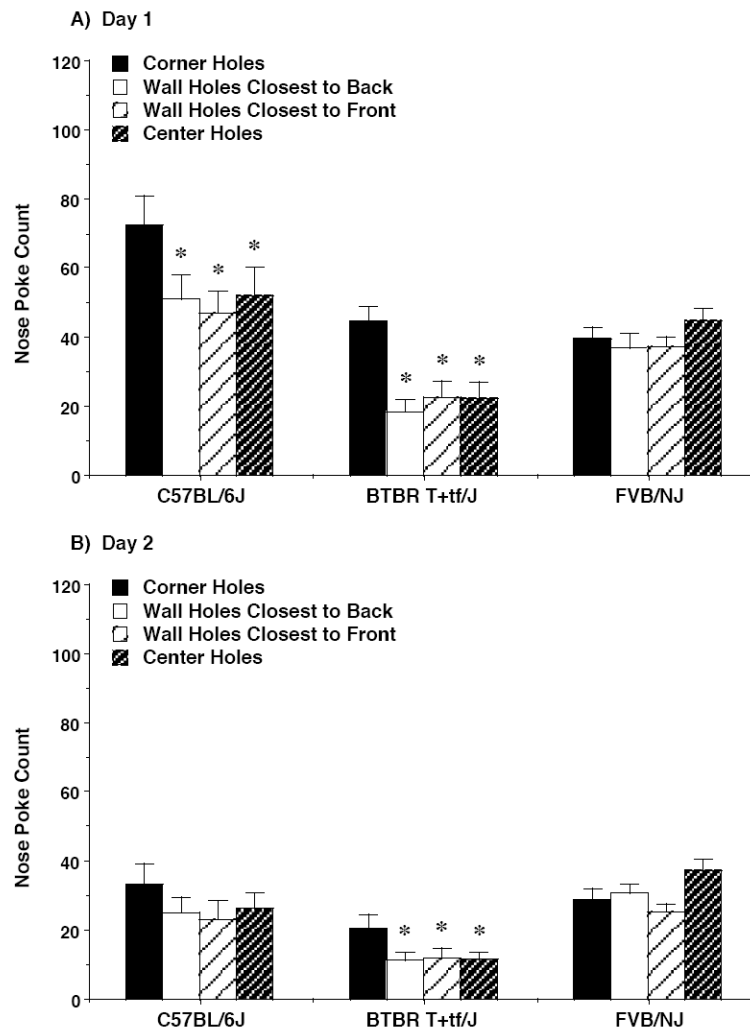
Figure 5.
Number of nose pokes during a one-hour exploration test in B6, BTBR, and FVB male mice. Data shown are means (+SEM). * $p < 0.05$, within-strain comparison to nose poke counts for center holes.

Figure 6.
Hole preference during a 1-hour exploration test in NR1 NMDA receptor-hypomorphic mice. Criterion levels for preference were 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low). Mean total nose pokes per subject: $NR1^{+/+}$, 49.9 (SEM=9.8), $NR1^{neo/neo}$, 82.3 (SEM=22.8).
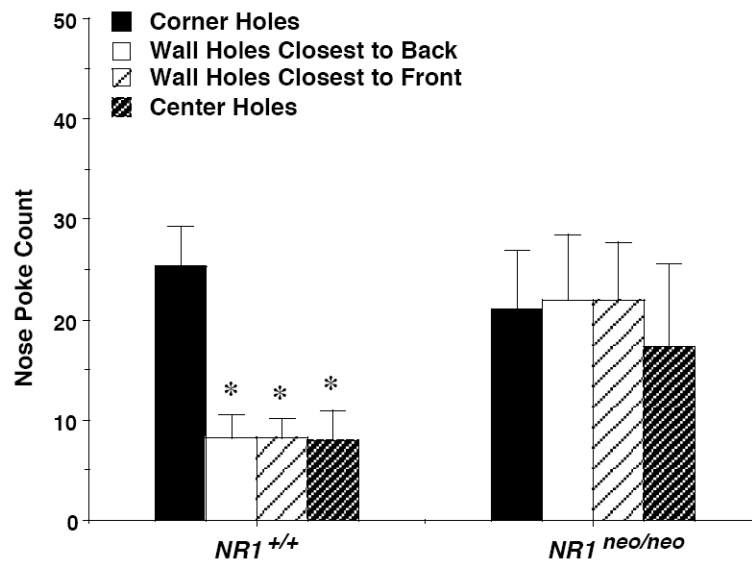
Figure 7.
Numbers of nose pokes during hole-board exploration in NR1 NMDA receptor-hypomorphic mice. Data shown are means (+SEM). * $p < 0.05$, within-genotype comparison to nose poke counts for center holes.
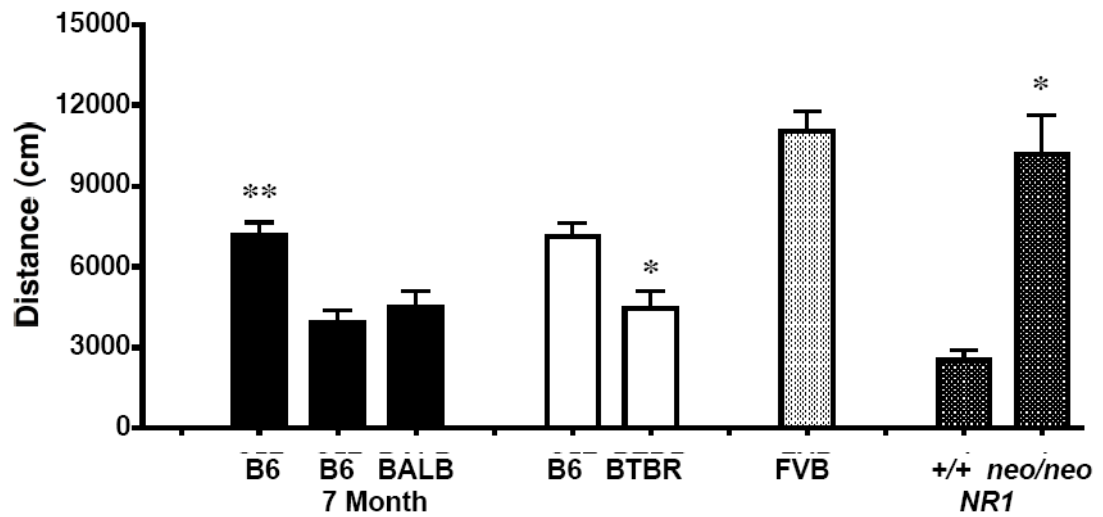
Figure 8.
Total distance traveled during hole-board exploration. Data shown are means (+SEM) for a 1-hour test. Inbred strains were 6-9 weeks in age for exploration test, except for one group of B6 and one group of BALB, which were 7 months of age. *NR1* mice were 3-5 months in age. *p<0.05, different from adjacent group; **p<0.05; different from both adjacent groups (black-filled bars).
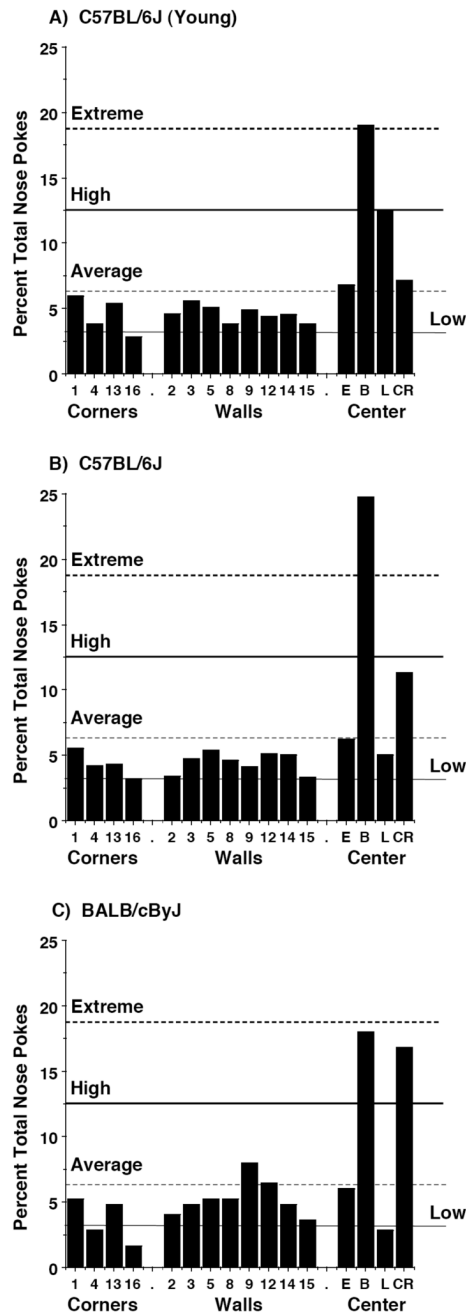
Figure 9.
Hole preference in a 1-hour olfactory stimuli test. Center holes were empty (E), or contained familiar, clean cage bedding (B), a gauze square with 0.05 ml dilute lemon extract (L), or a novel cinnamon cereal (CR). Criterion levels for preference were 18.75% (Extreme), 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low). Mean total number of nose pokes per subject: B6 (young), 106.1 (SEM=10.8), B6, 95.1 (SEM=13.9), BALB, 25 (SEM=4.2).
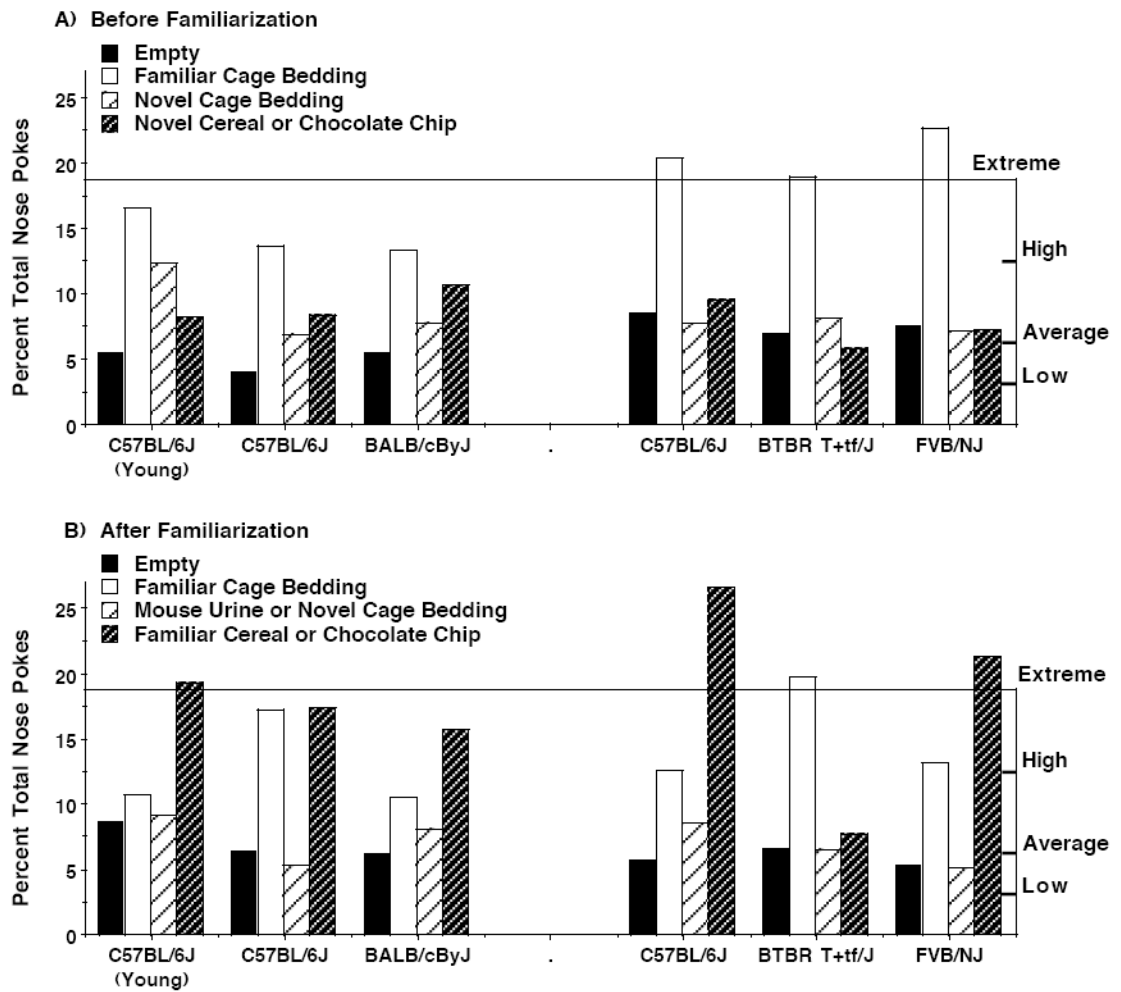
Figure 10.
Percent of total nose pokes toward the four center holes (A) before and (B) after familiarization with cereal (first three groups) or chocolate chips (last three groups). Criterion levels for preference were 18.75% (Extreme), 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low).
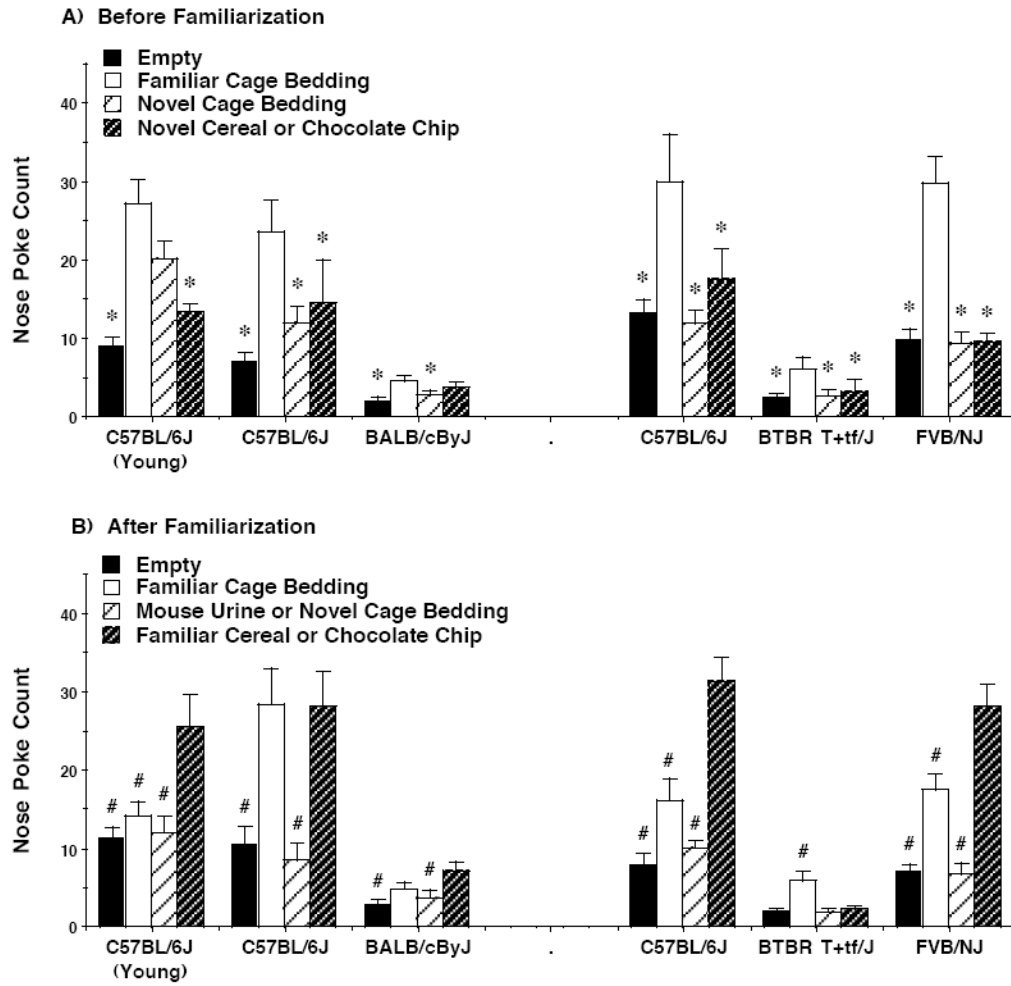
Figure 11.
Number of nose pokes into center holes (A) before and (B) after familiarization with cereal (first three groups) or chocolate chips (last three groups). Data shown are means (+SEM). * p< 0.05, within-group comparison to nose poke counts for hole containing familiar cage bedding. # p<0.05, within-group comparison to nose poke counts for hole containing familiar cereal or familiar chocolate chip.
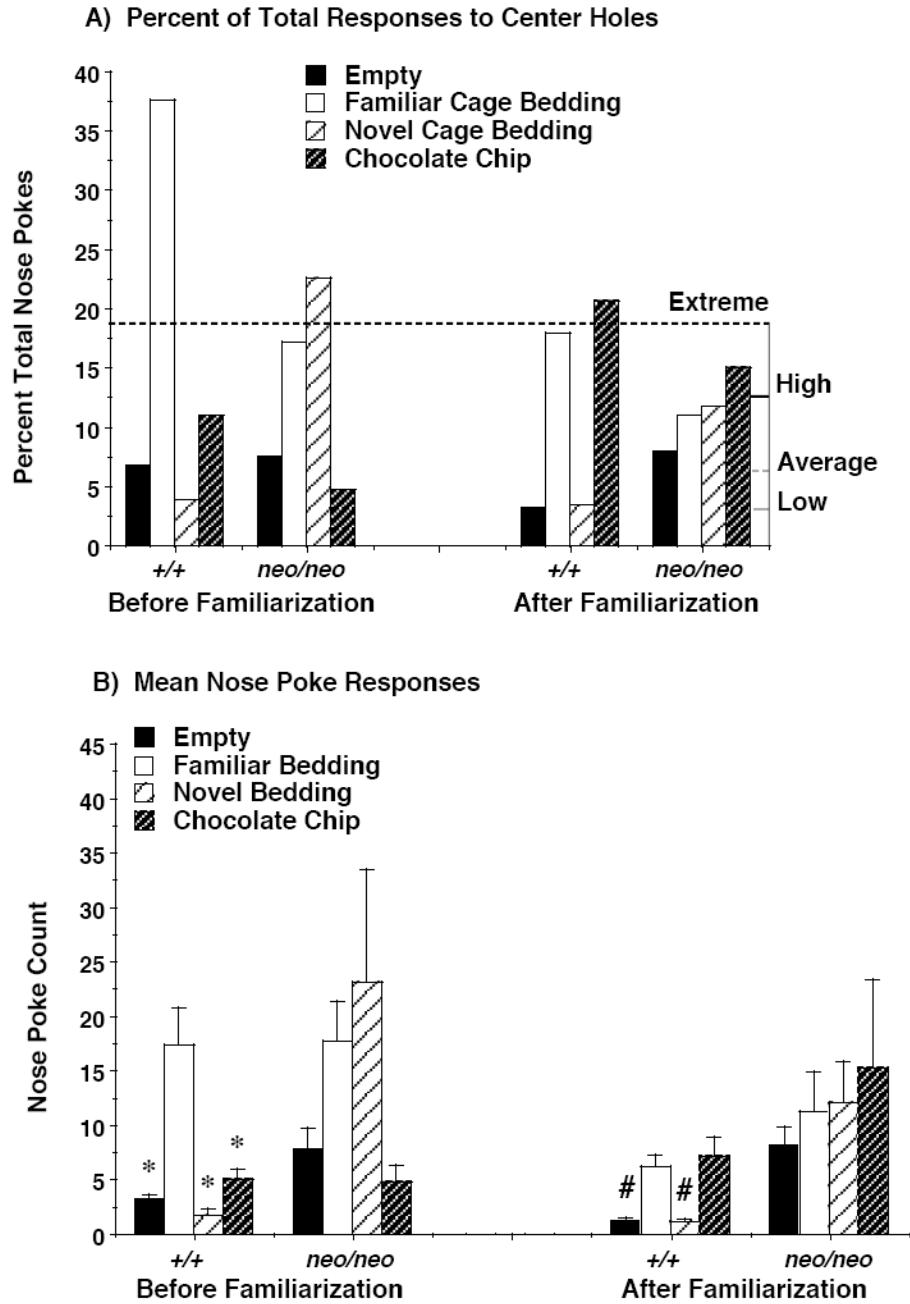
Figure 12.
Familiarization with a chocolate chip in NR1 NMDA receptor-deficient mice. Criterion levels for preference were 18.75% (Extreme), 12.5% (High), 6.25% (Average; equal distribution across holes), and 3.125% (Low). Data shown in B are means (+SEM). * p< 0.05, within-genotype comparison to nose poke counts for hole containing familiar cage bedding. # p<0.05, within-genotype comparison to nose poke counts for hole containing familiar chocolate chip.
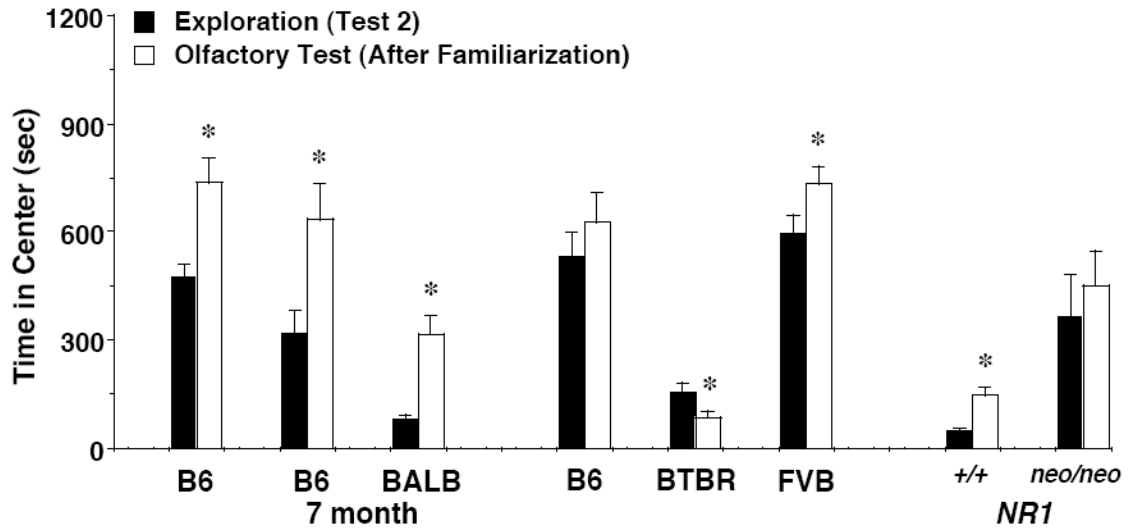
Figure 13.
Time in the center of the activity chamber during a hole-board test for general exploration, and during a hole-board test with olfactory stimuli, following familiarization to the appetitive stimulus. Data shown are means (+ SEM) for one-hour tests. Inbred strains were 6-9 weeks in age for exploration test, except for one group of B6 and one group of BALB, which were 7 months of age. *NR1* mice were 3-4 months in age. * p<0.05, within-group comparison.

Table 1

Percent total nose poke responses to social stimuli (SS), versus familiar cage bedding (FB), in the hole-board test.

Social stimuli	B6 (Young)		B6		BALB	
	FB	SS	FB	SS	FB	SS
Unfamiliar male cage bedding	15%[H]	16%[H]	21%[E]	19%[E]	15%[H]	15%[H]
Unfamiliar female cage bedding	14%[H]	15%[H]	21%[E]	18%[H]	19%[E]	13%[H]
CD1 female urine						
Before familiarization	16%[H]	7%	18%[H]	6%	13%[H]	9%
After familiarization	16%[H]	9%	17%[H]	7%	10%	6%
C57 female urine placed on familiar cage bedding (2-choice test)	11%	19%[E]	15%[H]	25%[E]	11%	13%[H]

Social stimuli included soiled cage bedding taken from home cages of PL/J male or female mice, CD1 female urine (0.05 ml) before and after familiarization (a 2-day exposure in the home cages of the test mice), or B6 female urine (0.05 ml) added to familiar, clean cage bedding. E: criterion level for extreme preference (18.75%). H: criterion level for high preference (12.5%). B6: C57BL/6J; BALB: BALB/cByJ.