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Social Approach in Genetically-Engineered Mouse Lines Relevant to Autism

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Abstract

Profound impairment in social interaction is a core symptom of autism, a severe neurodevelopmental disorder. Deficits can include a lack of interest in social contact and low levels of approach and proximity to other children. In this study, a three-chambered choice task was used to evaluate sociability and social novelty preference in five lines of mice with mutations in genes implicated in autism spectrum disorders. *Fmr1^{tm1Cgr/Y}* (*Fmr1^{-y}*) mice represent a model for fragile X, a mental retardation syndrome that is partially co-morbid with autism. We tested *Fmr1^{-y}* mice on two genetic backgrounds, C57BL/6J and FVB/N-129/OlaHsd (FVB/129). Targeted disruption of *Fmr1* resulted in low sociability on one measure, but only when the mutation was expressed on FVB/129. Autism has been associated with altered serotonin levels and polymorphisms in *SLC6A4* (*SERT*), the serotonin-transporter gene. Male mice with targeted disruption of *Slc6a4* displayed significantly less sociability than wildtype controls. Mice with conditional overexpression of *Igf-1* (*Insulin-like growth factor-1*) offered a model for brain overgrowth associated with autism. *Igf-1* transgenic mice engaged in levels of social approach similar to wildtype controls. Targeted disruption in other genes of interest, *En2* (*Engrailed 2*) and *Dhcr7*, was carried on genetic backgrounds that demonstrated low levels of exploration in the choice task, precluding meaningful interpretations of social behavior scores. Overall, results show that loss of *Fmr1* or *Slc6a4* gene function can lead to deficits in sociability. Findings from the fragile X-model suggest that the FVB/129 background confers enhanced susceptibility to consequences of *Fmr1* mutation on social approach.

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Keywords

autism spectrum disorders; endophenotype; Engrailed; *Fmr1*; fragile X; Sert; *Slc6a4*; sociability

Introduction

Autism is a severe neurodevelopmental disorder characterized by abnormal social interaction and communication, restricted and unusual interests, and aberrant repetitive behavior (American Psychiatric Association 2000). Twin studies have demonstrated a strong genetic component for disease etiology (Bailey *et al.* 1995, Folstein & Rosen-Sheidley 2001, Steffenburg *et al.* 1989). However, genetic analyses to determine specific heritable factors underlying susceptibility for autism have suggested that the majority of cases involve the interaction between multiple genes, as well as possible environmental factors (Abrahams & Geschwind 2008, Freitag 2007, Polleux & Lauder 2004). One approach for the study of polygenic clinical disorders is to determine specific endophenotypes, or measurable, simplified indexes of complex disease phenotypes, that may be associated with a single gene or a limited number of genes (Braff *et al.* 2008, Gottesman & Gould 2003). Identification of endophenotypes may provide functional markers for disease diagnosis and classification, and for the genetic dissection of disease symptomatology. Recent investigations using endophenotyping approaches in neuropsychiatric disorders have included assessments for neuropsychological function or cognition as heritable, quantifiable markers for broader domains of impairment (Boonstra *et al.* 2008, da Rocha *et al.* 2008, Gur *et al.* 2007, Horan *et al.* 2008).

While mouse models cannot fully recapitulate diverse behavioral elements of complex neuropsychiatric disorders such as autism, engineered mutations in mouse lines provide a way to investigate the association between candidate endophenotypes and specific genes or signaling pathways implicated in the human disease (Hranilovic & Bucan 2001). The following studies utilized genetic mouse models to investigate social approach deficits as a possible endophenotype for the broad domain of abnormal social function in autism. Mutant lines were selected for alterations in genes linked to heritable, biochemical, or neuropathological aspects of autism. Our hypothesis was that one or more of these mouse lines would demonstrate deficient social approach, and thus provide a link between a single gene (*Fmr1*, *Slc6a4*, *Igf-1*, *En2*, or *Dhcr7*) and a quantifiable social endophenotype for more global social impairment.

In humans, fragile X syndrome is associated with mental retardation, physical abnormalities, and autistic symptoms (Hagerman *et al.* 1986). The disease is caused by disrupted function of the *FMR1* (*Fragile X Mental Retardation 1*) gene, which has been modeled in the *Fmr1*^{-y} mouse (Bakker *et al.* 1994). Numerous studies have provided evidence for significant parallels between alterations observed in children with fragile X and abnormal behavior in *Fmr1*^{-y} mice, including deficits in attention and learning (Bakker *et al.* 1994, Kooy *et al.* 1996, Moon *et al.* 2006, Paradee *et al.* 1999), changes in reactions to sensory stimuli (Chen & Toth 2001, Frankland *et al.* 2004), and abnormal social responses (McNaughton *et al.* 2008, Mineur *et al.* 2006, Spencer *et al.* 2005). One interesting feature of this mouse model is that the phenotype of *Fmr1* loss-of-function may be dependent upon the genetic background. For example, researchers have proposed that the C57BL/6J background confers resistance to effects of *Fmr1* deficiency on spatial learning, while FVB/N-129/OlaHsd (FVB/129) leads to greater susceptibility (Dobkin *et al.* 2000, see also Paradee *et al.* 1999). The direction of neuroanatomical changes in *Fmr1*-null mice, such as labeling of mossy fiber terminals, can also be dependent on background strain (Ivanco & Greenough 2002, Mineur *et al.* 2002), suggesting that modifier genes play an important role

in *Fmr1*-related phenotypes. The present report evaluates *Fmr1*^{-/-} mice on both C57BL/6J and FVB/129 backgrounds for alterations in social approach.

Several lines of evidence support the involvement of dysregulated serotonergic signaling in autism, such as repeated findings of platelet hyperserotonemia in the disorder (Anderson *et al.* 1990, Hranilovic *et al.* 2007, Mulder *et al.* 2004, Piven *et al.* 1991, Whitaker-Azmitia 2005). Studies in autism populations have identified possible candidate genes in serotonergic pathways, including the serotonin transporter (*SLC6A4* or *SERT*) (Brune *et al.* 2006, Devlin *et al.* 2005, Sutcliffe *et al.* 2005, Tordjman *et al.* 2001). One study examining gene expression profiles in monozygotic twins discordant for symptoms of autism found that expression of *SLC6A4* was significantly reduced in the twin with greater symptom severity (Hu *et al.* 2006). Mice with disruptions of *Slc6a4* function have an abnormal behavioral phenotype, including low levels of exploration and reduced social interactions (Holmes *et al.* 2002, 2003, Kalueff *et al.* 2006, 2007a,, Kalueff *et al.* b; see Murphy & Lesch 2008 for review), suggesting that these mice may also demonstrate deficient social approach in a choice task.

Both cross-sectional and longitudinal studies have shown that a subset of autistic children demonstrate age-dependent brain overgrowth (Courchesne *et al.* 2001, 2003, Hazlett *et al.* 2005). Brain overgrowth at an early age can be modeled by the conditional overexpression of *Igf-1* (*Insulin-like growth factor-1*) in brain, which induces significant increases in brain volume during the embryonic and early postnatal period (Popken *et al.* 2004). In comparison to wildtype controls, *nestin-Igf-1* transgenic mice exhibit an almost 30% greater brain size, without concomitant changes in overall body weight (Popken *et al.* 2004). Interestingly, Mills *et al.* (2007) reported both greater head circumference and higher levels of plasma IGF-1 in children with autism and autism spectrum disorder (ASD), compared to normal controls. The correlation between head size and IGF-1 levels was highly significant in the autism/ASD group, but not in the control group. The present studies investigated whether conditional overexpression of *Igf-1* was associated with autism-like social behavior in mice.

Mice deficient for *Engrailed-2* (*En2*), a gene crucial for normal development of the cerebellum, were also assessed. Several studies in human populations have reported that variants of *En2* may confer risk for ASDs (Benayed *et al.* 2005, Brune *et al.* 2008, Gharani *et al.* 2004, Wang *et al.* 2008), although not all findings have been positive (Zhong *et al.* 2003). Researchers have observed parallels between neuroanatomical changes in brain of *En2*^{-/-} mice and alterations in cerebellar structure reported in autistic children (Kuemerle *et al.* 2007, Murcia *et al.* 2005). Lastly, we examined a genetic mouse model for Smith-Lemli-Opitz syndrome (SLOS), a disease with a markedly high co-occurrence with autism (Bukelis *et al.* 2007, Sikora *et al.* 2006, Tierney *et al.* 2001). SLOS arises from mutations in *DHCR7* (*7-dehydrocholesterol reductase 7*), the last enzyme in the cholesterol biosynthesis pathway, with subsequent disruption of cholesterol synthesis. During the prenatal period, *Dhcr7*-null mice have overt increases in serotonin immunoreactivity (Waage-Baudet *et al.* 2003). Unfortunately, the loss of *Dhcr7* function is lethal in mice (Fitzky *et al.* 2001). Our study evaluated heterozygous animals (*Dhcr7*^{tm1Gst/+} or *Dhcr7*^{+/-}), which have a mild reduction in the *Dhcr7* enzyme.

An important issue for interpreting results from social approach tests is that low preference for the social partner may be associated with changes in activity and/or anxiety-like behavior (Kalueff *et al.* 2007a, Moy *et al.* 2007, Spencer *et al.* 2005). In the present studies, information from one or more control measures, including motor coordination, activity levels in an open field, and anxiety-like behavior in an elevated plus maze, was considered in the interpretation of results from the social approach assays.

Materials and Methods

Animals

Fmr1^{+y} and ^{-y} mice for testing and for breeding pairs were obtained from Dr. William T. Greenough (Beckman Institute, University of Illinois, Urbana-Champaign, IL) and shipped to the University of North Carolina (UNC; Chapel Hill, North Carolina). The *Fmr1*-null allele was placed on two strain backgrounds: C57BL/6J (B6.129P2-*Fmr1*^{tm1Cgr} (Grossman *et al.* 2006, McKinney *et al.* 2005, Miyashiro *et al.* 2003; originally described in Bakker *et al.* 1994)); and FVB.129P-*Fmr1*^{tm1Cgr} (FVB/129; a sighted strain, as described in Errijgers *et al.* 2007, Irwin *et al.* 2002). The C57BL/6J mice were derived from original C57BL/6J × FVB/N × 129/OlaHsd mice, backcrossed for 6 generations to C57BL/6J mice (McKinney *et al.* 2005, Miyashiro *et al.* 2003). The FVB/129 mice were originally derived from 129/OlaHsd embryonic stem (ES) cells and backcrossed to FVB/N for multiple generations (Dobkin *et al.* 2000, Ivanco & Greenough 2002). Subjects for Cohort 1 of the present studies were sent from the Greenough laboratory, and started testing at 13–14 weeks of age; subjects for Cohort 2 were obtained by wildtype male × female heterozygote within-strain crosses at UNC, and started testing at 6–8 weeks of age. Only males were used in the behavioral assays. Number of litters, number of subjects (offspring of litters), and other characteristics are given in Table 1.

Slc6a4 mice (^{+/+}, ^{+/-}, and ^{-/-}) were male and female littermate offspring bred at UNC from pairs provided by Dr. Dennis L. Murphy (Laboratory of Clinical Science, NIMH, Bethesda, MD). Mice had been backcrossed onto a C57BL/6 background for 12–15 generations (see Holmes *et al.* 2003) from an original mixed background (129/P1ReJ (ES cells), C57BL/6J, and CD-1; Bengel *et al.* 1998, Salichon *et al.* 2001). Subjects were taken from 11 litters, and were 6–8 weeks in age at the beginning of testing. Subject numbers for the *Slc6a4* mice, as well as the three other mutant mouse lines described below, are given in Table 2.

Igf-1 mice (^{+/+} and ^{Tg}) were male and female littermate offspring bred at UNC. The nestin/*Igf-1* transgenic (^{Tg}) mice were created on a C57BL/6 background using standard microinjection methods by the Mutant Mouse Resource Center at UNC (Popken *et al.* 2004). Lines were initiated by breeding the C57BL/6 transgenic heterozygous mice to C57BL/6 wildtype mice (Charles River Laboratories, Wilmington, MA). The *Igf-1*^{Tg} mice used in the present study were heterozygous for the transgene (*Igf-1* mice homozygous for the transgene die *in utero* (Popken *et al.* 2004)). Subjects were taken from 7 litters, and were 2–4 months in age at the beginning of testing.

En2^{+/+} and *En2*^{tm1Alj/tm1Alj} (*En2*^{-/-}) mice were male littermate offspring bred at UNC from pairs provided by Dr. Karl Herrup (Rutgers, The State University of New Jersey, Nelson Biological Laboratories, Piscataway, NJ). *En2*^{-/-} mice carried a null allele derived from D3 129/Sv ES cells (Joyner *et al.* 1989, 1991, Millen *et al.* 1994), with the mutation transferred from a 129S2/SvPas background to a 129/S1 background (Gerlai *et al.* 1996, Kuemerle *et al.* 2007). Subjects were taken from 5 litters, and were 5–6 weeks in age at the beginning of testing.

Dhcr7 mice (^{+/+} and ^{+/-}) were male littermate offspring on a 129/SvEv background (Waage-Baudet *et al.* 2003), obtained from pairs bred at UNC. The mutation was produced by targeted disruption of the coding sequence of the last *Dhcr7* exon, the proposed active site of the human gene (Fitzky *et al.* 2001). Subjects were taken from 6 litters, and were 5–7 weeks in age at the beginning of testing.

Mice from each study were separated by strain and sex, and housed in ventilated plastic tub cages, with free access to water and Purina 5058 chow. The housing room had a 12-hr light/

dark cycle (lights off at 7:00 p.m.). For groups bred at UNC, genotyping was conducted from tail tissue by PCR. Testing methods were designed to minimize pain and discomfort in the mice. All procedures were conducted in strict compliance with the policies on animal welfare of the National Institutes of Health and UNC (stated in the “Guide for the Care and Use of Laboratory Animals,” Institute of Laboratory Animal Resources, National Research Council, 1996 edition). All procedures were approved by the UNC Institutional Animal Care and Use Committee.

Behavioral Testing

Order of testing for each group was: *Fmr1* mice, Cohort 1: 1) elevated plus maze, 2) test for sociability. *Fmr1* mice, Cohort 2: 1) elevated plus maze, 2) activity in an open field, 3) rotarod, 4) social approach test. *Slc6a4*, *Igf-1*, *En2*, and *Dhcr7* mice: 1) neurobehavioral screen and home cage observation, 2) activity in an open field, 3) rotarod, 4) social approach test, 5) buried food test for olfactory ability, 6) elevated plus maze. Only 1 procedure was conducted per day. Detailed descriptions of these tests have been previously published (Moy *et al.* 2007).

Control measures

Home cage behaviors—Observations of mice in their home cages were taken at 3 different time points: 8:00 AM, 12:00 noon, and 7:00 PM. Records were taken by a human experimenter for 20 min at each time point, for a total of 60 min of home cage observation. Two hours before the noon observation, 1 white cotton nestlet square (Ancare Corp., Bellmore, NY) was added to each cage, in order to assess nest-building behavior. The evening observation was conducted 10 min before and 10 min after the lights had gone off, using red light illumination. Records were taken for nestlet shredding (amount shredded), nest building and structure (flattened nest, short walls, spherical nest), sleeping in huddles (percent of mice in huddle), activity, fighting, and any aberrant behaviors, such as tremor or seizures, or possible stereotyped responses, such as repeated “jack-hammer” jumping or cage-lid flipping. When possible, records included individual subject identification (based on a simple ear punch system) for mice that remained outside of a huddle, or that showed unusual responses. Scoring included percent of cages observed with a nest, percent of mice observed huddling, and percent of mice showing aberrant behavior.

General health and neurological reflexes—Mice were evaluated for general health, including body weight, appearance of fur and whiskers, body posture, and normality of gait. Reflexive reactions to a gentle touch from a cotton swab to the whiskers on each side of the face, the approach of the cotton swab to the eyes, and the sound from a metal clicker (Preyer reflex) were assessed. Animals were observed for the visual placing reflex (forepaw extension when lowered toward a visible surface), and for ability to grasp a metal grid with forepaws and hindpaws.

Elevated plus-maze test for anxiety-like behavior—Mice were given one 5-min trial on the plus-maze, which had 2 closed arms, with walls 40 cm in height, and 2 open arms. The maze was elevated 50 cm from the floor, and the arms were 21 cm long. Animals were placed on the center section (9.5 cm × 9.5 cm), and allowed to freely explore the maze. Arm entries were defined as all four paws entering an arm. Entries and time in each arm were recorded during the trial by a human observer via computer coding. Percent open arm time was calculated as $100 \times (\text{time spent on the open arms} / (\text{time in the open arms} + \text{time in the closed arms}))$. Percent open arm entries was calculated using the same formula.

Open field—Exploratory activity in a novel environment was assessed in a photocell-equipped automated open field (40 cm × 40 cm × 30 cm; Versamax system, Accuscan

Instruments). Parameters included ambulation (total distance traveled), rearing movements, and time spent in the center region of the chamber. Activity chambers were contained inside sound-attenuating boxes, equipped with houselights and fans.

Rotarod performance—Mice were assessed for balance and motor coordination on an accelerating rotarod (Ugo-Basile, Stoelting Co., Wood Dale, IL). Revolutions per minute (rpm) were set at an initial value of 3, with a progressive increase to a maximum of 30 rpm across the 5-min test session. Each animal was given a test session consisting of 2 trials, with 45 seconds between each trial. Latency to fall, or to rotate off the top of the turning barrel, was measured by the rotarod timer.

Olfactory test following food deprivation—Several days before the olfactory test, an unfamiliar food (Froot Loops, Kellogg Co., Battle Creek, MI) was placed overnight in the home cages of the subject mice, in order to avoid food neophobia on the day of testing. 16–20 hours before the test, all food was removed from the home cage. On the day of the test, each mouse was placed in a large, clean tub cage (46 cm L × 23.5 cm W × 20 cm H), containing 3 cm deep paper chip bedding (Canbrands Product, Moncton NB, Canada), and allowed to explore for 5 min. The animal was removed from the cage, and 1 Froot Loop was buried in the cage bedding, approximately 1 cm below the surface of the litter. The subject mouse was then returned to the cage for a 15 min test. Measures were taken of latency to find the buried food.

Sociability and preference for social novelty

Fmr1 mice were tested in a non-automated 3-chambered box, with measures taken by a human observer blind to mouse genotype (Duncan *et al.* 2004, Moy *et al.* 2004). All other mutant lines were tested in an automated 3-chambered box (Moy *et al.* 2007, Nadler *et al.* 2004). Dividing walls had retractable doorways allowing access into each chamber. The automated box had photocells embedded in each doorway to allow quantification of entries and duration in each chamber of the social test box. The chambers of the apparatus were cleaned with water and dried with paper towels between each trial. At the end of each test day, the apparatus was sprayed with 70% ethanol and wiped clean with paper towels.

The choice test had three 10-min phases: A) *Habituation*. The test mouse was first placed in the middle chamber and allowed to explore, with the doorways into the 2 side chambers open. Each of the 2 sides contained an empty wire cage (11 cm H, 10.5 bottom diameter, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, Ohio). B) *Sociability*. After the habituation period, the test mouse was enclosed in the center compartment of the social test box, and an unfamiliar mouse (stranger 1; an adult C57BL/6J male) was enclosed in one of the wire cages and placed in a side chamber. The location for stranger 1 alternated between the left and right sides of the social test box across subjects. Following placement of stranger 1, the doors were re-opened, and the subject was allowed to explore the entire social test box. Measures were taken of the amount of time spent in each chamber and the number of entries into each chamber by the automated testing system. In addition, a human observer scored time spent sniffing each wire cage, using a computer keypad and software (Johns *et al.* 1998). C) *Preference for social novelty*. At the end of the sociability test, each mouse was further tested for preference to spend time with a new stranger. A new unfamiliar mouse was placed in the wire cage that had been empty during the previous session. The test mouse then had a choice between the first, already-investigated mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The same measures were taken as with the sociability test.

Cohort 1 of *Fmr1* mice was tested for sociability, but not for social novelty preference. For this single set of mice, the test for sociability involved a choice between a side containing the unfamiliar stranger, and an empty side (without the empty wire cage present for the other groups). The measure for sniffing was not taken for the first cohort.

Statistical analysis

Data from each mutant mouse line were first analyzed using 1-way or 2-way ANOVAs (analysis of variance) or repeated measures ANOVAs, with the factor targeted mutation (genotype) and, for the *Fmr1* lines, background strain (C57BL/6J or FVB/129). The repeated measures included week of testing (for body weight), time during the open field test, rotarod trial, and chamber side in the social approach test. These analyses determined main effects of genotype, background strain, and the repeated measure, and interactions between the different factors. In the *Fmr1* lines, each overall ANOVA was followed by separate analyses within each background strain, in order to further examine effects of the *Fmr1* genotype. The *Slc6a4* and *Igf-1* mouse lines included both males and females; separate analyses were conducted for each sex. Significant effects of altered genotype found in the ANOVAs were further explored using post-hoc Fisher's PLSD (protected least-significant difference) tests to determine differences between group means. For all comparisons, significance was set at $p < 0.05$.

Separate analyses were used to determine levels of social preference within each experimental group. Sociability and social novelty preference were evaluated using within-genotype repeated measures ANOVAs, with the factor of chamber side (e.g., stranger 1 side or the opposite side). For all comparisons, significance was set at $p < 0.05$.

Results

Control measures in *Fmr1* mice

***Fmr1* genotype effects on body weight, elevated plus maze and rotarod performance**—Overall, no differences in the control measures were found between wildtype and *Fmr1*^{-/-} mice on the C57BL/6J background. Significant effects of *Fmr1* genotype on body weight and one measure from the plus maze were observed in the FVB/129 lines, but only in the first cohort (Table 1). In this case, the mutant mice on the FVB/129 background weighed more than the control mice at the beginning of testing [post-hoc tests following a repeated measures ANOVA, main effect of *Fmr1* genotype, $F(1,49)=15.16$, $p=0.0003$; *Fmr1* genotype \times strain interaction, $F(1,49)=7.28$, $p=0.0096$]. This same mutant group made significantly fewer entries than wildtype controls on the elevated plus maze [post-hoc analyses following two-way ANOVA, main effect of *Fmr1* genotype, $F(1,49)=10.45$, $p=0.0022$; *Fmr1* genotype \times strain interaction, $F(1,49)=6.16$, $p=0.0165$]. There were no effects of *Fmr1* loss on the percent time and entries on the open arms of the maze, indicating that anxiety-like behavior was similar in the mutant and control mice. Similarly, *Fmr1* deficiency had no effects on latency to fall from the rotarod.

Strain effects on elevated plus maze and rotarod performance—Two-way ANOVAs indicated significant effects of background strain in the first cohort for two measures on the elevated plus maze, percent time [$F(1,49)=8.71$, $p=0.0048$] and percent entries [$F(1,49)=10.19$, $p=0.0025$], reflecting generally higher percentages in the FVB/129 groups, in comparison to the C57BL/6J groups. In the second cohort, strain had a significant main effect on rotarod performance, with the C57BL/6J groups having longer latencies [repeated measures ANOVA across trials, $F(1,90)=28.14$, $p<0.0001$].

Open field exploration—The second cohort group was further assessed for activity levels in a novel open field (Figure 1). The *Fmr1*^{-/-} mice on the FVB/129 background had higher levels of distance traveled during most intervals of the 1-hour test [post-hoc tests following a repeated measures ANOVA, main effect of *Fmr1* genotype, $F(1,90)=7.1$, $p=0.0091$; main effect of strain, $F(1,90)=47.46$, $p<0.0001$]. Deficiency of *Fmr1* did not have significant effects on rearing movements or time spent in the center region of the open field (data not shown).

Social approach in *Fmr1* mice

Social preference in the sociability assay—Both the mice obtained from the University of Illinois and the group of mice bred at the University of North Carolina demonstrated a similar pattern in the choice task (Figure 2a,b). A significant preference for spending time in the side of the test box containing the stranger mouse, versus the opposite side, was observed in *Fmr1*^{+/-} and *Fmr1*^{-/-} mice on the C57BL/6J background; however, on the FVB/129 background, only the *Fmr1*^{+/-} mice had significant sociability [post-hoc tests following within-group repeated measures ANOVA, main effects of side (the repeated measure) for Cohort 1, $F(1,49)=86.23$, $p<0.0001$; Cohort 2, $F(1,90)=47.06$, $p<0.0001$]. In both cohort groups, the *Fmr1*^{-/-} mice on the FVB/129 background failed to demonstrate a significant preference for proximity to an unfamiliar mouse.

***Fmr1* genotype and background strain effects in the sociability assay**—Repeated measures ANOVAs indicated significant group differences in amount of time spent in the two side chambers in the first cohort [main effect of strain, $F(1,49)=9.34$, $p=0.0036$, and a three-way interaction between *Fmr1* genotype, strain, and side that approached significance; $F(1,49)=3.85$, $p=0.0554$], and in the second cohort [*Fmr1* genotype × side interaction, $F(1,90)=4.32$, $p=0.0406$]. Further analyses indicated significant group differences for time spent in the side with the stranger mouse [Cohort 1, main effect of strain, $F(1,49)=19.01$, $p<0.0001$, and *Fmr1* genotype × strain interaction, $F(1,49)=5.65$, $p=0.0214$; Cohort 2, main effect of *Fmr1* genotype, $F(1,90)=5.63$, $p=0.0198$]. Post-hoc tests showed that, in Cohort 2, the *Fmr1*^{-/-} mice on the C57BL/6J background spent less time in proximity to stranger 1 than the wildtype mice.

Entries in the sociability assay—There were no significant effects of *Fmr1* genotype on numbers of entries during the test for sociability (Figure 2c). Therefore, the differences in social preference were not due to a lack of exploration in the *Fmr1*^{-/-} mice on the FVB/129 background. In both cohort groups, there were significant effects of strain on numbers of entries [repeated measures ANOVA; Cohort 1, main effect of strain, $F(1,49)=8.34$, $p=0.0057$, and strain × side interaction, $F(1,49)=9.07$, $p=0.0041$; Cohort 2, strain × side interaction, $F(1,90)=5.66$, $p=0.0195$].

Sniffing during the sociability assay—For the second cohort of mice, measures were taken of sniffing at each cage during the test (Figure 3a). All of the experimental groups demonstrated a preference for sniffing the cage containing the unfamiliar stranger, versus sniffing the empty cage. Although an overall repeated measures ANOVA indicated a significant *Fmr1* genotype × side interaction for the sniff measure [$F(1,88)=6.21$, $p=0.0146$], separate analyses for each side did not reveal any other significant effects of *Fmr1* genotype.

Preference for social novelty—Cohort 2 was further tested for social approach toward a second novel stranger, in comparison to the first stranger mouse (Figure 3b). In this assay, a second unfamiliar mouse (stranger 2) was placed in the cage that had been empty during the sociability assay. No effects of *Fmr1* genotype were evident for time spent with stranger 2, versus time spent with the first stranger, although there was a main effect of strain [repeated

measures ANOVA; $F(1,90)=13.76$, $p=0.0004$]. Overall, the FVB/129 lines tended to spend less time than the C57BL/6J lines in the two side chambers.

Control measures in *Slc6a4*, *Igf1*, *En2*, and *Dhcr7* mouse lines: As shown in Table 2, there were no differences between wildtype and mutant mice within each study for body weight, anxiety-like behavior on the elevated plus maze, motor coordination on an accelerating rotarod, or performance in the buried food test for olfactory ability. The home cage observations and neurobehavioral screen did not reveal any overt changes in huddling behavior, motor ability, or simple reflexive responses in any of the mouse lines (data not shown).

Social approach in *Slc6a4* mice

Time spent in each side during the sociability and social novelty assays—

Neither the male *Slc6a4*^{-/-} mice, nor the female *Slc6a4*^{+/-} or ^{-/-} mice, demonstrated significant preference for spending time with the stranger mouse in the test for sociability (Figure 4a) [post-hoc tests following repeated measures ANOVAs, main effect of side for males, $F(1,27)=8.95$, $p=0.0059$; females, $F(1,30)=14.98$, $p=0.0005$]. Repeated measures ANOVAs conducted on the data from the male experimental groups indicated a significant *Slc6a4* genotype × side interaction [$F(2,27)=5.12$, $p=0.013$]. Significant main effects of *Slc6a4* genotype were observed for time spent in the side with the stranger [$F(2,27)=5.62$, $p=0.0091$] and time spent in the side with the empty cage [$F(2,27)=3.81$, $p=0.0349$]. Fisher's PLSD tests revealed that the *Slc6a4*^{-/-} males spent less time in the stranger side, and more time in the empty cage side, than either the *Slc6a4*^{+/+} or ^{+/-} males. There were no significant effects of *Slc6a4* genotype in the female mice during the test for sociability, or in either the male or female mice during the test for social novelty preference (Figure 4b).

Sniffing and entries during the social approach test—All of the experimental groups demonstrated a significant preference for sniffing at the cage containing the stranger mouse, versus the empty cage (Figure 5a) [post-hoc tests following repeated measures ANOVAs, main effect of side for males, $F(1,26)=72.26$, $p<0.0001$; females, $F(1,30)=98.62$, $p<0.0001$]. In the male groups, there was a non-significant trend for reduced sniffing at the stranger mouse cage by the *Slc6a4*-null mice [repeated measures ANOVA, main effect of *Slc6a4* genotype, $F(2,26)=2.86$, $p=0.0751$]. Overall, there were no significant effects of *Slc6a4* genotype on the measure of sniffing during the sociability assay, or during the subsequent test for social novelty preference (Figure 5b), in either the male or female mice. No group differences were observed for number of entries into the side chambers during the social approach tests (see Table 3 for entries during the sociability test), suggesting that the lack of social preference in the *Slc6a4*^{-/-} mice could not be attributed to general hypoactivity and a failure to explore.

Social approach in *Igf-1* mice: During the test for sociability, both the male and female *Igf-1* lines had a significant preference for spending time in the side with the stranger mouse (Figure 6a) [post-hoc tests following repeated measures ANOVA, main effect of side for males, $F(1,11)=23.87$, $p=0.0005$; and females, $F(1,20)=24.65$, $p<0.0001$]. In contrast, none of the groups demonstrated a significant side preference when a new unfamiliar mouse (stranger 2) was introduced during the social novelty test (Figure 6b). Overexpression of *Igf-1* had no significant effects in the male experimental groups for any of the measures taken in the social approach tests. In the female groups, the *Igf-1* transgenic mice spent significantly more time in the side containing the empty cage than the wildtype mice during the test for sociability [post-hoc tests following repeated measures ANOVA, main effect of *Igf-1* genotype, $F(1,20)=4.9$, $p=0.0386$]. No other significant effects of *Igf-1* genotype were found in the female mice.

Low exploration in *En2*^{-/-} and *Dhcr7*^{+/-} mice: None of the *En2* or *Dhcr7* experimental groups, either wildtype or mutant, showed social preference in the choice tests (data not shown). Examination of the data suggests that low numbers of entries (Table 3), especially in the *En2*^{+/+} and ^{-/-} mice, confounded findings from the choice task. Overall, 74% of the mice from the *En2* groups, and 25% of the mice from the *Dhcr7* groups, had zero entries for one or two of the side chambers. In contrast, no mice from the *Slc6a4* or *Igf-1* experimental groups had a zero entry score. Findings from a 5-min activity test in a novel open field confirm the intrinsic low levels of exploration in the *En2* and *Dhcr7* mice (Table 3). In particular, these mouse lines had markedly deficient rearing movements and time spent in the center region of the open field.

Discussion

In addition to profound deficits in social interaction, the core symptoms of autism include aberrant repetitive behavior and restricted interests (American Psychiatric Association, 2000). The impairments in social function may involve a different set of genes than symptoms related to the repetitive behavior domain (Ronald *et al.* 2006; see also Ronald *et al.* 2005). Complex neuropsychiatric disorders with this type of genetic heterogeneity and phenotypic diversity present difficulties for large-scale genome linkage and candidate gene association studies. Recently, investigators have focused on endophenotyping approaches for genetic analysis of clinical syndromes such as autism or schizophrenia, measuring social or neurocognitive traits (Duvall *et al.* 2007, Gur *et al.* 2007, Horan *et al.* 2008). The present studies used genetically-engineered mouse lines to evaluate a quantifiable social trait as a heritable marker for impaired social function relevant to autism.

One challenge for the development of mouse models for autism is that the fundamental mechanisms underlying symptomatology are unknown. However, several of the candidate genes implicated in autism play a role in synaptic function, suggesting that disruption of synaptic mechanisms may be a common factor across ASDs (Abrahams & Geschwind 2008). In the present studies, the *Fmr1*- and *Slc6a4*- null mouse lines provided models of dysregulated synaptic function associated with specific candidate genes for autism. *Fmr1* silencing can lead to abnormal synaptic plasticity, which has been linked to prolonged glutamatergic signaling (Hou *et al.* 2006, Huber *et al.* 2002, Nakamoto *et al.* 2007, Nosyreva & Huber 2006). Synaptic disruption in *Fmr1*-null mice includes aberrant dendritic morphology, characterized by longer, thinner spines and a higher spine density, comparable to abnormalities observed in fragile X syndrome (Comery *et al.* 1997, Irwin *et al.* 2002, McKinney *et al.* 2005). Similarly, activation of serotonergic pathways is regulated by the serotonin transporter. Loss of *Slc6a4* results in prolonged signaling, which may have a profound impact on normal brain development and function (Murphy & Lesch 2008). Regionally-specific alterations in dendritic morphology and increased spine density have been reported in *Slc6a4*-null mice (Wellman *et al.* 2007).

Our results show that deficits in social approach are found with the targeted disruption of either *Fmr1* or *Slc6a4*. The mice null for *Fmr1* on an FVB/129 background failed to demonstrate significant preference for spending time in the social-partner side in the choice task, in contrast to *Fmr1*^{-/y} mice on a C57BL/6J background. The lack of preference could not be attributed to low exploration, low activity, or higher levels of anxiety-like behavior in the mutant mice. In line with these findings, male *Slc6a4*^{-/-} mice (on a C57BL/6J background) spent significantly less time than wildtype controls in the proximity of the unfamiliar social partner. As with the fragile X model, lack of social preference was not associated with low total number of entries during the test, nor with changes in anxiety-like behavior. A similar link between genetic changes leading to altered synaptic function and deficient social approach has been reported for *Gabrb3* (GABA_A receptor subunit β3) -null

mice (DeLorey *et al.* 2008), *Mecp2* (methyl-CpG-binding protein-2) -mutant mice (Moretti *et al.* 2005), and *Nlgn3* (neuroligin-3) R451C knockin mice (Tabuchi *et al.* 2007). It is notable that *GABRB3*, *MECP2*, and *NLGN3*, as well as *FMR1* and *SLC6A4*, are found on chromosomal loci associated with susceptibility for ASDs (Abrahams & Geschwind 2008).

In addition to specific behavioral characteristics, age-dependent brain overgrowth has been observed in autism (Aylward *et al.* 2002, Courchesne *et al.* 2003, Hazlett *et al.* 2005) and fragile X syndrome (Chiu *et al.* 2007). *Igf-1* transgenic mice were used to model this neuroanatomical abnormality. A previous study with *Igf-1* null mice provided evidence that this gene is important for dendritic growth and synaptogenesis (Cheng *et al.* 2003). Interestingly, loss of *Igf-1* led to significant decreases in dendritic spine length and density, which are opposite to the alterations observed with *Fmr1* deficiency (Comery *et al.* 1997, Irwin *et al.* 2002, McKinney *et al.* 2005). These findings suggest that *Igf-1* overexpression might induce abnormal growth of dendritic spines, and therefore, have detrimental effects on synapse function similar to targeted disruption of *Fmr1*. However, in contrast to the fragile X-model mice, the *Igf-1* transgenic mice did not show deficits in social approach or in any other behavioral measure. The unchanged phenotype of the *Igf-1* mutants demonstrates that even overt alterations in normal brain development do not necessarily lead to social endophenotypes.

En2-null mice served as a model of altered cerebellar morphology observed in autism (Kuemerle *et al.* 2007, Murcia *et al.* 2005). Cheh *et al.* (2006) found that *En2*^{-/-} mice have higher levels of serotonin than wildtype controls in cerebellum, but not frontal cortex, hippocampus, or striatum. Thus, the *En2*^{-/-} mice could provide information on the behavioral effects of a regionally-specific enhancement of serotonin signaling. The *Dhcr7*^{+/-} mice were investigated as another interesting mutant with dysregulation of serotonin signaling. These mice reflect the disrupted cholesterol biosynthesis observed in Smith-Lemli-Opitz syndrome (Fitzky *et al.* 2001). There is evidence that reductions in cholesterol lead to decreased activity of the serotonin transporter (Magnani *et al.* 2004, Nomura *et al.* 2008, Scanlon *et al.* 2001), which could underlie increases in hindbrain serotonin observed in *Dhcr7*-null mice during prenatal development (Waage-Baudet *et al.* 2003). Unfortunately, the behavioral phenotypes of the *En2* and *Dhcr7* lines, both wildtype and mutant, included markedly low exploration. Other researchers have reported general hypoactivity in mutant mouse lines on a 129S2/SvPas (Gerlai *et al.* 1996) or a 129S6/SvEvTac (Holmes *et al.* 2003) background. Inbred strain distributions of anxiety-like behavior (Bouwknicht & Paylor 2002, Brooks *et al.* 2005, Rodgers *et al.* 2002; see also Cook *et al.* 2002) confirm low exploration in specific 129 substrains. In the present studies, the lack of exploration in the social approach task precluded the detection of social endophenotypes in the *En2* and *Dhcr7* mutant mice.

Our findings with the *Fmr1*-null mice illustrate the importance of background strain in determining the effects of genetic alteration. Recently, *Fmr1*-null mice on a C57BL/6J × FVB/NJ hybrid background were reported to have normal social preference in a three-chambered choice task (McNaughton *et al.* 2008). However, depending upon the behavioral assay, *Fmr1*^{-y} mice on a C57BL/6J background can exhibit altered social responses. *Fmr1*-null C57BL/6J mice have been found to have deficits in social interaction with repeated presentations of an ovariectomized female during a habituation procedure (Mineur *et al.* 2006). Spencer *et al.* (2005) evaluated *Fmr1*-null C57BL/6J mice across several domains of social behavior. In a repeated partition test, the mutant mice had decreased social interest for the unfamiliar stranger mouse at the beginning of the twenty-minute procedure, and increased social interest by the end of the testing period. We observed a similar lack of significant preference in the *Fmr1*^{-y} FVB/129 mice during the first ten-minute assay (the sociability test), but not the following ten-minute assay (the social novelty test). Spencer *et*

al. (2005) have suggested that decreased social interest at the beginning of a test may reflect increased social anxiety in *Fmr1* mutants. However, changes in anxiety-like behavior may be dependent upon the particular assay. In line with previous reports (Mineur *et al.* 2002, Nielsen *et al.* 2002), our study on elevated plus maze performance did not indicate a general increase in anxiety-like behavior in *Fmr1*^{-y} mice on either background strain.

Other researchers have found changes in social behavior in *Slc6a4*^{-/-} mice on a C57BL/6J background. Holmes *et al.* (2002) noted decreased aggression in male *Slc6a4*-null mice during a resident-intruder test, without any changes in investigatory social interest. The mutant mice were also hypoactive in the home cages and in an open field. Kalueff *et al.* (2007a) found that female *Slc6a4*^{-/-} mice had less initiation of sniffing directed toward the social partner in a free interaction test. The *Slc6a4*^{-/-} mice also had decreased exploration in an open field test, as well as reduced approaches in a novel object test. Therefore, results from these social interaction tests may have reflected hypoactivity and higher levels of neophobia in the *Slc6a4*-null mice, rather than an intrinsic deficit in social interest. The issue of hypoactivity and low exploration is also problematic for the evaluation of depression-like behavior in *Slc6a4*-null mice (Kalueff *et al.* 2006). In the present study, reduced social approach was observed in *Slc6a4*^{-/-} mice without decreases in approach toward a non-social novel object (the empty wire cage) or fewer entries during the test. The low percent time (ranging from 5% to 9%) spent in the open arms of the elevated plus maze by the *Slc6a4* line may have prevented the detection of increases in anxiety-like behavior in the mutant mice.

The dependence of social preference on background strain in the *Fmr1*^{-/-} mice suggests that modifier genes can attenuate or exacerbate the consequences of *Fmr1* loss. One important conclusion from the findings in the *Slc6a4* line is that the C57BL/6J background does not necessarily confer protection from the effects of genetic alteration on social approach in the three-chambered choice task. In the *Slc6a4*-null mice, modifier genes present in the C57BL/6J background did not prevent the changes induced by disrupted transporter function, which may indicate a stronger association of the serotonin signaling pathway, rather than *Fmr1*-mediated events, with fundamental alterations in social motivation. However, many other factors could have affected social behavior in the mutant lines, including altered learning ability, deficits in sustained attention, subtle olfactory dysfunction, or other traits not assessed in these experiments.

The social approach test used in the present studies included an assay for social novelty preference to provide a secondary measure of social approach, based on discrimination between two partners (stranger 1 and the more-novel stranger 2). Previous work has shown that high sociability does not predict subsequent preference for social novelty in inbred mouse strains, suggesting that the two assays are measuring different components of social behavior (Moy *et al.* 2007, 2008). A similar dissociation between sociability and social novelty preference was evident in the mutant mouse lines of the present studies. In particular, neither the *Slc6a4* nor the *Igf-1* wildtype groups had significant preference for the stranger 2 mouse, even though both groups had significant sociability, and were on a background characterized by positive social novelty preference (C57BL/6J; Moy *et al.* 2007, 2008). The lack of social novelty preference suggests that, across multiple generations, the *Slc6a4* and *Igf-1* mouse lines have diverged from the original C57BL/6J background.

In the *Slc6a4* groups, only the heterozygous mice showed a significant preference for social novelty. Previous work has shown that *Slc6a4*^{+/-} mice retain about 50% of normal serotonin transporter binding (Bengel *et al.* 1998, Montanez *et al.* 2003). Behavior in *Slc6a4*^{+/-} mice is usually not different from wildtype mice, or else parallels, to a lesser extent, changes observed in *Slc6a4*^{-/-} mice, supporting a gene dose-dependent function for some behavioral

domains (Holmes *et al.* 2002, 2003, Kalueff *et al.* 2007a). However, one study found that serotonin levels in the frontal cortex were significantly increased in *Slc6a4*^{+/-} mice, but decreased in null mutant mice, in comparison to controls (Bengel *et al.* 1998). It is possible that a reduction, versus a loss, of transporter function could lead to qualitatively different alterations in specific brain regions, and to different profiles of social behavior.

Our findings, together with published reports in other mutant mouse lines (DeLorey *et al.* 2008, Moretti *et al.* 2005, Tabuchi *et al.* 2007), provide evidence that synaptic dysfunction through various mechanisms can lead to similar deficits in social approach. The results are in line with human genetic analyses that have identified disruption of synaptic function as a possible cellular mechanism underlying symptoms in ASDs (Abrahams & Geschwind 2008). In addition, recent work has shown that genetic alterations thought to restore normal synaptic function can reverse, either fully or partially, abnormalities in dendritic morphology, plasticity, and behavior in *Fmr1*-null double transgenic mice (Dolen *et al.* 2007, Hayashi *et al.* 2007). Overall, these studies support the utility of mouse models to link specific genes and signaling pathways to heritable social endophenotypes, and to examine possible underlying mechanisms relevant to autism.

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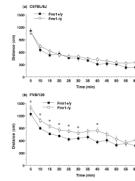


Figure 1. Open field locomotion in *Fmr1* mouse lines from Cohort 2

Significant increases in distance traveled were seen in the *Fmr1*-null mice on an FVB/129, but not a C57BL/6J, background. Activity was assessed by a 1-hour trial in an open field chamber. Data shown are mean \pm SEM. * $p < 0.05$.

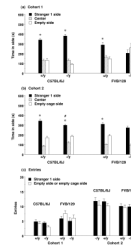


Figure 2. Time spent in each side during the test for sociability in (a) Cohort 1 and (b) Cohort 2 of the *Fmr1* mouse lines, and (c) numbers of entries during the test

Fmr1-null mice on the FVB/129 background, from both cohorts, did not have a significant preference for proximity to stranger 1. The loss of *Fmr1* did not have significant effects on number of entries in either background strain. Side choice for Cohort 1 included an empty side (without any wire cage); for Cohort 2, an empty cage side. Data shown are mean + SEM. * $p < 0.05$, within-group comparison, stranger 1 side different from empty (Cohort 1) or empty cage (Cohort 2) side. # $p < 0.05$, comparison with same measure in $+/y$ mice with C57BL/6J background.

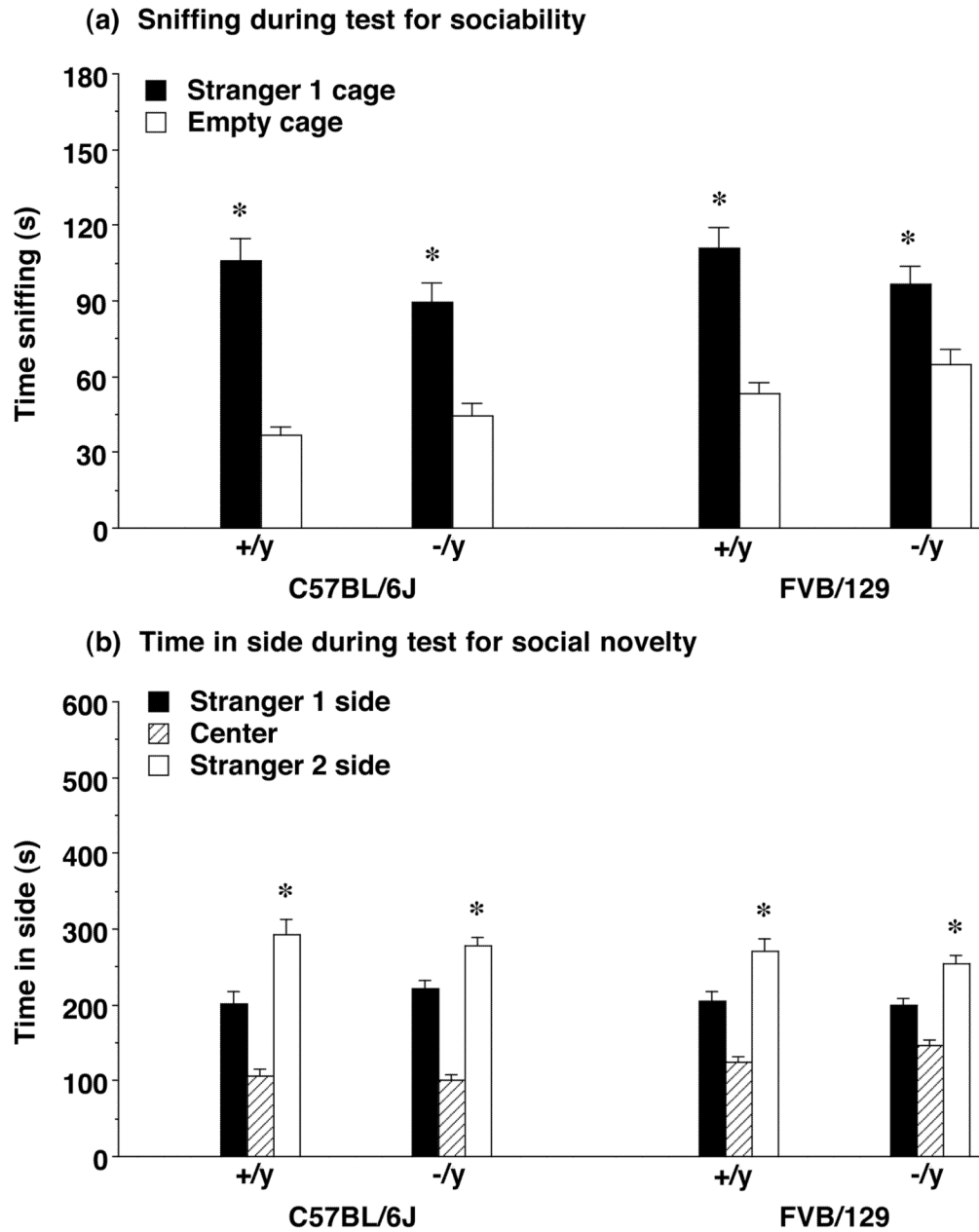


Figure 3. (a) Time spent sniffing each cage during the test for sociability and (b) time spent in each side during the test for social novelty preference in *Fmr1* mouse lines from Cohort 2 All groups had a significant preference for the wire cage containing an unfamiliar mouse, stranger 1, in comparison to an empty cage (a), and a significant preference for proximity to the more-novel stranger 2 (b). Data shown are mean + SEM. * $p < 0.05$, within-group comparison, stranger 1 side different from opposite side.

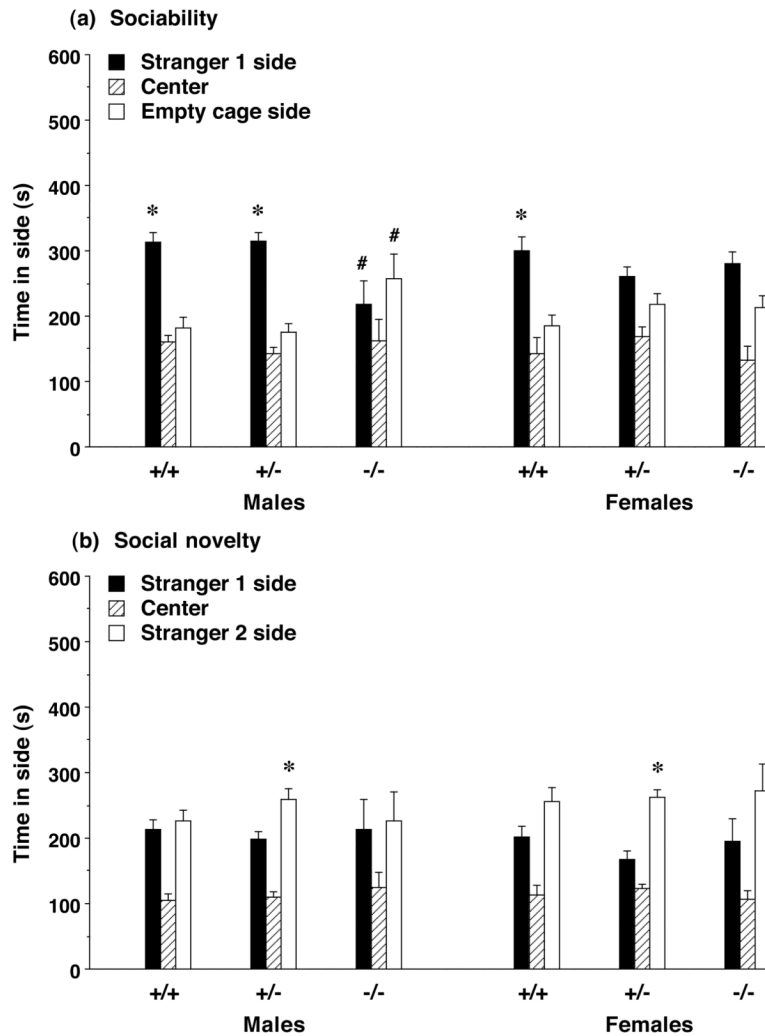


Figure 4. Time spent in each side by *Slc6a4* mice during the tests for (a) sociability and (b) preference for social novelty

Neither male nor female *Slc6a4*-null mice, or female heterozygous mice, had a significant preference for proximity to stranger 1. Data shown are mean + SEM for each group. * $p < 0.05$, within-group comparison, stranger 1 side different from empty cage side (a) or stranger 2 side (b). # $p < 0.05$, comparison with same measure in both $+/+$ and $+/-$ male mice.

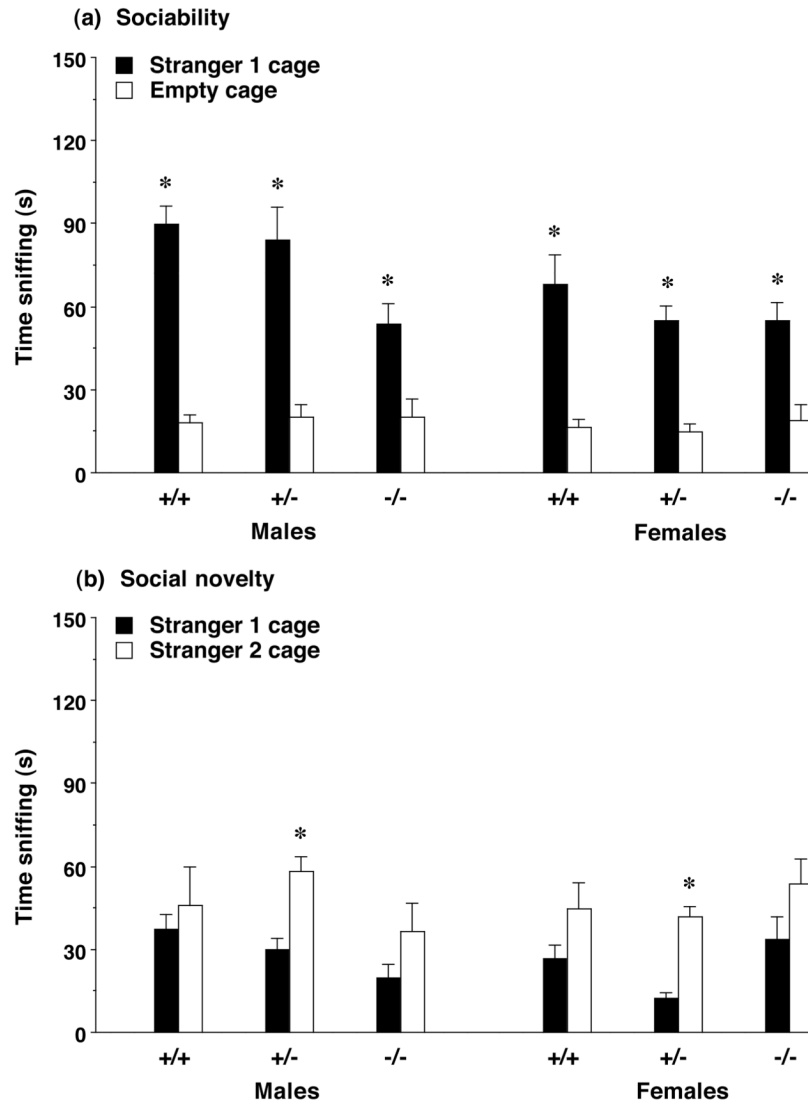


Figure 5. Time spent sniffing each cage by *Slc6a4* mice during the tests for (a) sociability and (b) preference for social novelty

During the test for sociability, all groups had a significant preference for the cage containing stranger 1. Data shown are mean + SEM for each group. * $p < 0.05$, within-group comparison, stranger 1 side different from empty cage side (a) or stranger 2 side (b).

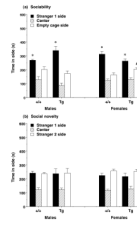


Figure 6. Time spent in each side by *Igf-1* mice during the tests for (a) sociability and (b) preference for social novelty

All groups had a significant preference for proximity to stranger 1 in the test for sociability. Data shown are mean + SEM for each group. * $p < 0.05$, within-group comparison, stranger 1 side different from empty cage side. # $p < 0.05$, comparison with same measure in $+/+$ female mice.

Table 1

Control measures in *Fmr1* mouse lines. Data shown are means \pm SEM for body weight, percent time in and percent entries into the open arms of an elevated plus maze, total arm entries on the plus maze, and latency to fall from an accelerating rotarod

	# Litters	N	Body weight (g)	% Open arm		Total entries	Latency on rotarod (s)
				time	entries		
Cohort 1 ^a							
C57BL/6J	8						
<i>Fmr1</i> ^{+/-}	10	27 \pm 1	20 \pm 5	23 \pm 2	23 \pm 1		Not tested
<i>Fmr1</i> ^{-/-}	16	29 \pm 1	27 \pm 2	31 \pm 3	25 \pm 2		Not tested
FVB/129							
<i>Fmr1</i> ^{+/-}	14	28 \pm 1	32 \pm 4	37 \pm 4	40 \pm 4		Not tested
<i>Fmr1</i> ^{-/-}	13	32 \pm 1*	39 \pm 6	39 \pm 4	28 \pm 3*		Not tested
Cohort 2 ^b							
C57BL/6J	9						
<i>Fmr1</i> ^{+/-}	13	21 \pm 1 ^c	23 \pm 4	24 \pm 3	25 \pm 2		222 \pm 15
<i>Fmr1</i> ^{-/-}	24	24 \pm 1	18 \pm 2	22 \pm 2	26 \pm 1		227 \pm 15
FVB/129							
<i>Fmr1</i> ^{+/-}	31	24 \pm 1 ^d	26 \pm 3	28 \pm 2	28 \pm 2		147 \pm 14
<i>Fmr1</i> ^{-/-}	26	26 \pm 1	26 \pm 4	25 \pm 3	27 \pm 2		139 \pm 14

^aSource: Dr. William Greenough, University of Illinois.

^bSource: Bred at the University of North Carolina.

^cBody weight means from 10 ^{+/-} and 14 ^{-/-} mice.

^dBody weight means from 24 ^{+/-} and 17 ^{-/-} mice.

* p<0.05, comparison to ^{+/-} group.

Table 2

Control measures in *Slc6a4*, *Igf-1*, *En2*, and *Dhcr7* mouse lines. Data shown are means \pm SEM for body weight, percent time in and percent entries into the open arms of an elevated plus maze, total arm entries on the maze, latency to fall from a rotarod, latency to find buried food and percent of group finding the food in a test for olfactory ability.

	N	Body weight (g)	% Open arm		Total entries	Rotarod latency (s)	Olfactory test	
			time	entries			latency (s)	% group
<i>Slc6a4</i>								
Males								
<i>Slc6a4</i> ^{+/+}	8	25 \pm 1	8 \pm 2	18 \pm 4	26 \pm 4	198 \pm 31	115 \pm 41	100%
<i>Slc6a4</i> ^{+/-}	13	23 \pm 1	6 \pm 1	15 \pm 2	26 \pm 2	162 \pm 22	272 \pm 101	77%
<i>Slc6a4</i> ^{-/-}	9	23 \pm 2	6 \pm 1	14 \pm 2	21 \pm 4	134 \pm 23	100 \pm 30	100%
Females								
<i>Slc6a4</i> ^{+/+}	8	18 \pm 1	9 \pm 3	14 \pm 3	30 \pm 3	146 \pm 18	57 \pm 11	100%
<i>Slc6a4</i> ^{+/-}	13	18 \pm 1	7 \pm 3	14 \pm 3	25 \pm 2	139 \pm 20	162 \pm 68	92%
<i>Slc6a4</i> ^{-/-}	12	20 \pm 1	5 \pm 1	10 \pm 3	23 \pm 3	147 \pm 9	266 \pm 78	92%
<i>Igf-1</i>								
Males								
<i>Igf-1</i> ^{+/+}	7	23 \pm 1	4 \pm 1	9 \pm 2	20 \pm 4	171 \pm 36	184 \pm 121	86%
<i>Igf-1</i> ^{f8}	6	24 \pm 1	1 \pm 1	4 \pm 2	19 \pm 2	145 \pm 10	117 \pm 85	100%
Females								
<i>Igf-1</i> ^{+/+}	10	20 \pm 1	3 \pm 1	7 \pm 2	26 \pm 3	180 \pm 33	199 \pm 89	90%
<i>Igf-1</i> ^{f8}	13	20 \pm 0.4	2 \pm 1	5 \pm 1	23 \pm 2	155 \pm 16	139 \pm 72	92%
<i>En2</i> ^{+/+}	11	21 \pm 1	4 \pm 1	6 \pm 2	17 \pm 2	123 \pm 16	690 \pm 106	36%
<i>En2</i> ^{-/-}	8	20 \pm 1	4 \pm 3	6 \pm 4	12 \pm 1	129 \pm 12	581 \pm 137	50%
<i>Dhcr7</i> ^{+/+}	8	17 \pm 1	7 \pm 1	13 \pm 2	24 \pm 1	175 \pm 12	327 \pm 94	100%
<i>Dhcr7</i> ^{+/-}	12	18 \pm 1	7 \pm 2	12 \pm 3	23 \pm 2	195 \pm 12	356 \pm 99	83%

Table 3

Entries in the test for sociability and activity measures in a novel open field. Data shown are means \pm SEM for entries into the side containing a stranger mouse or an empty cage, and for total distance traveled, rearing movements, and time spent in the center region during a 5-min activity test

	Stranger	Empty cage	Distance (cm)	Rears	Center time (s)
<i>Slc6a4</i>					
Males					
<i>Slc6a4</i> ^{+/+}	12 \pm 1	12 \pm 1	1408 \pm 134	50 \pm 4	26 \pm 3
<i>Slc6a4</i> ^{+/-}	11 \pm 1	9 \pm 1	1197 \pm 135	39 \pm 4	26 \pm 4
<i>Slc6a4</i> ^{-/-}	10 \pm 2	10 \pm 2	1376 \pm 110	38 \pm 4	29 \pm 5
Females					
<i>Slc6a4</i> ^{+/+}	13 \pm 3	13 \pm 3	1433 \pm 109	41 \pm 4	24 \pm 4
<i>Slc6a4</i> ^{+/-}	12 \pm 1	12 \pm 1	1646 \pm 148	40 \pm 3	25 \pm 5
<i>Slc6a4</i> ^{-/-}	10 \pm 1	10 \pm 2	1614 \pm 103	38 \pm 4	27 \pm 4
<i>Igf-1</i>					
Males					
<i>Igf-1</i> ^{+/+}	11 \pm 2	9 \pm 2	1173 \pm 161	28 \pm 4	28 \pm 9
<i>Igf-1</i> ^{l/l8}	10 \pm 1	9 \pm 2	1215 \pm 78	31 \pm 5	24 \pm 7
Females					
<i>Igf-1</i> ^{+/+}	15 \pm 1	14 \pm 1	1409 \pm 165	30 \pm 3	35 \pm 5
<i>Igf-1</i> ^{l/l8}	14 \pm 1	14 \pm 1	1505 \pm 196	30 \pm 3	36 \pm 6
<i>En2</i> ^{+/+}	2 \pm 0.6	2 \pm 1.0	783 \pm 154	0.3 \pm 0.1	3 \pm 2
<i>En2</i> ^{-/-}	1 \pm 0.5	1 \pm 0.4	733 \pm 154	0.0 \pm 0.0	2 \pm 1
<i>Dhcr7</i> ^{+/+}	4 \pm 1	5 \pm 1	510 \pm 128	1.9 \pm 1.0	4 \pm 2
<i>Dhcr7</i> ^{+/-}	5 \pm 1	5 \pm 1	740 \pm 87	2.8 \pm 1.4	4 \pm 2