Social propinquity in rodents as measured by tube cooccupancy differs between inbred and outbred genotypes

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Existing assays of social interaction are suboptimal, and none measures propinquity, the tendency of rodents to maintain close physical proximity. These assays are ubiquitously performed using inbred mouse strains and mutations placed on inbred genetic backgrounds. We developed the automatable tube cooccupancy test (TCOT) based on propinquity, the tendency of freely mobile rodents to maintain close physical proximity, and assessed TCOT behavior on a variety of genotypes and social and environmental conditions. In outbred mice and rats, familiarity determined willingness to cooccupy the tube, with siblings and/or cagemates of both sexes exhibiting higher cooccupancy behavior than strangers. Subsequent testing using multiple genotypes revealed that inbred strain siblings do not cooccupy at higher rates than strangers, in marked contrast to both outbred and rederived wild mice. Mutant mouse strains with "autistic-like" phenotypes (*Fmr1*$^{-/y}$ and *Eif4e* Ser209Ala) displayed significantly decreased cooccupancy.

propinquity | autism | social interaction | rodent behavior | genetics

Autism spectrum disorders feature abnormal social interactions, which are most often assessed in rodents using the three-chamber sociability test (1). In this assay a subject mouse freely explores an arena containing two wire cups, under which are familiar or stranger conspecifics, inanimate objects, or nothing. Sociability is defined as greater time spent in proximity to conspecifics versus objects or empty cups (social approach) and as greater time spent in proximity to strangers versus familiars (social novelty preference). The assay has been used to establish various inbred (e.g., BTBR T+tf/J) and mutant (e.g., *Fmr1*-null) mouse strains as "autistic-like" mouse models (2). As an alternative to the three-chamber test, one may record the appearance of myriad social behaviors (e.g., anogenital and nose-to-nose sniffing, chasing, wrestling, allogrooming) in the home cage, a visible burrow, or a neutral environment (3, 4). Such comprehensive recording is extremely labor intensive and prone to errors (5) and is almost impossible to automate using present technology, although some progress is being made using machine-learning algorithms (6). Operant or classical conditioning techniques also have been used to quantify or infer sociability (7, 8), but these require laborious training sessions.

As a broad measure of sociability, the three-chamber test is potentially limited in a number of ways, including the extremely short (≤15-min) testing period (5), the incarceration of one of the social partners (9), and confounds related to location of the stimulus animals (10). We also suggest that a preference for strangers over familiars is not at all consistent with human social preferences; humans prefer to interact socially with familiars and maintain closer distances to friends than strangers (11). At the very least, the measurement over an extended period of voluntary social proximity—propinquity—among rodents placed in a neutral, novel environment might add usefully to our social neuroscience armamentarium, especially because propinquity itself is far more easily automated than fine-grained individual measures of reciprocal social interactions. To these ends we developed the tube cooccupancy test (TCOT).

Virtually all extant research in the preclinical autism field has been conducted on inbred mice or mutant mice bred onto inbred genetic backgrounds. Laboratory mouse strains are poor representatives of the species in at least three ways: (*i*) they are genetically homozygous at every locus; (*ii*) they have been artificially selected for both tameness and reproductive success under laboratory conditions; and (*iii*) postweaning same-sex housing deprives them of an ethologically relevant social environment. Robust differences in stress and social behaviors between inbred mice and wild mice have been reported (12–14). We thus evaluated TCOT behavior in outbred, inbred, and wild-derived mice.

Results

TCOT Behavior of Outbred Mice and Rats. Mice placed singly in the TCOT arena—a brightly lit rectangular box containing a single opaque PVC tube—showed a strong motivation to occupy the tube, spending ∼70% of their time inside the tube during the 3-h testing period (Fig. 1*A*, leftmost bar). Same-sex dyadic groups [siblings, nonsibling cagemates, separated siblings (i.e., siblings

Significance

We developed an assay of social behavior, the tube cooccupancy test. This assay is able to identify deficits in social behavior of known "autistic-like" mouse strains. Using the test, we revealed a major difference in social behavior between inbred and outbred strains, with only the latter behaving similar to wild mice.

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housed separately since weaning), and strangers] spent different proportions of their time sharing the tube (cooccupancy), occupying the tube one at a time (single occupancy), or with both mice outside the tube (vacant tube) (Fig. 1*A*). Stranger dyads displayed significantly less cooccupancy than (identically performing) sibling/cagemate dyads, $F(3, 68) = 5.5$, $P = 0.002$. Conversely, strangers and separated siblings spent significantly more time with a vacant tube than did sibling–cagemate dyads, $F(3, 65) = 12.0$, $P < 0.001$. Aggressive behavior in this 3-h assay was extremely rare, even among male strangers (Fig. S1*A*). There were no sex or sibling-versus-stranger differences in single-occupancy ratios (Fig. S1*B*), or in early (0–10 min) social interactions occurring outside the tube (Fig. S1*C*). A visual inspection of the time course of tube cooccupancy (Fig. 1*B*) revealed the second hour of testing (60–120 min) as most differentiating the groups, $F(3, 68) = 6.0$, $P = 0.001$ (Fig. 1*C*); all further analyses were performed on data from this time period. Fairly high levels (~70%) of cooccupancy were displayed by stranger dyads by the end of testing, although cooccupancy by these dyads was still lower than for other groups. Sex differences in tube cooccupancy were observed in one only group, with female separated siblings spending significantly more time together than male separated siblings: Condition × Sex, $F(3, 64) = 5.3$, $P = 0.002$ (Fig. 1*D*). Habituation of both stranger mice, separately, to the empty arena for 30 or 90 min before dyadic testing increased subsequent cooccupancy (Fig. S1*D*). Strangers also displayed sibling-like levels of cooccupancy after repeated daily exposures to each other in the arena (Fig. S1*E*) or after cohousing before testing for as little as 1 d (Fig. S1*F*). Highly similar results were obtained in a version of the TCOT featuring two tubes instead of one, $F(3, 35) = 6.4$, $P = 0.001$ (Fig. 1*E*), demonstrating that tube cooccupancy is highly preferred among related and/or familiar mice even if each mouse has the option of hiding in its own tube. Sibling–Cagemate versus stranger differences in cooccupancy were also seen in outbred Sprague–Dawley rats tested for 1 h in a larger TCOT arena, $F(2, 27) = 60.5$, $P < 0.001$ (Fig. 1*F*). One organismic factor greatly affecting propinquity was age, with 12- and 18-wk-old mice displaying much lower tube cooccupancy than younger mice, although the preference for familiars was preserved (Fig. S1*G*).

Automation of the TCOT. We fully automated data collection in the TCOT by attaching the PVC cylinders magnetically to load cells, which provide a real-time (1-s) measure of displacement so that occupancy by no mice, one mouse, or two mice can be inferred and summed over the testing period or any portion thereof. (See Fig. S2*A*

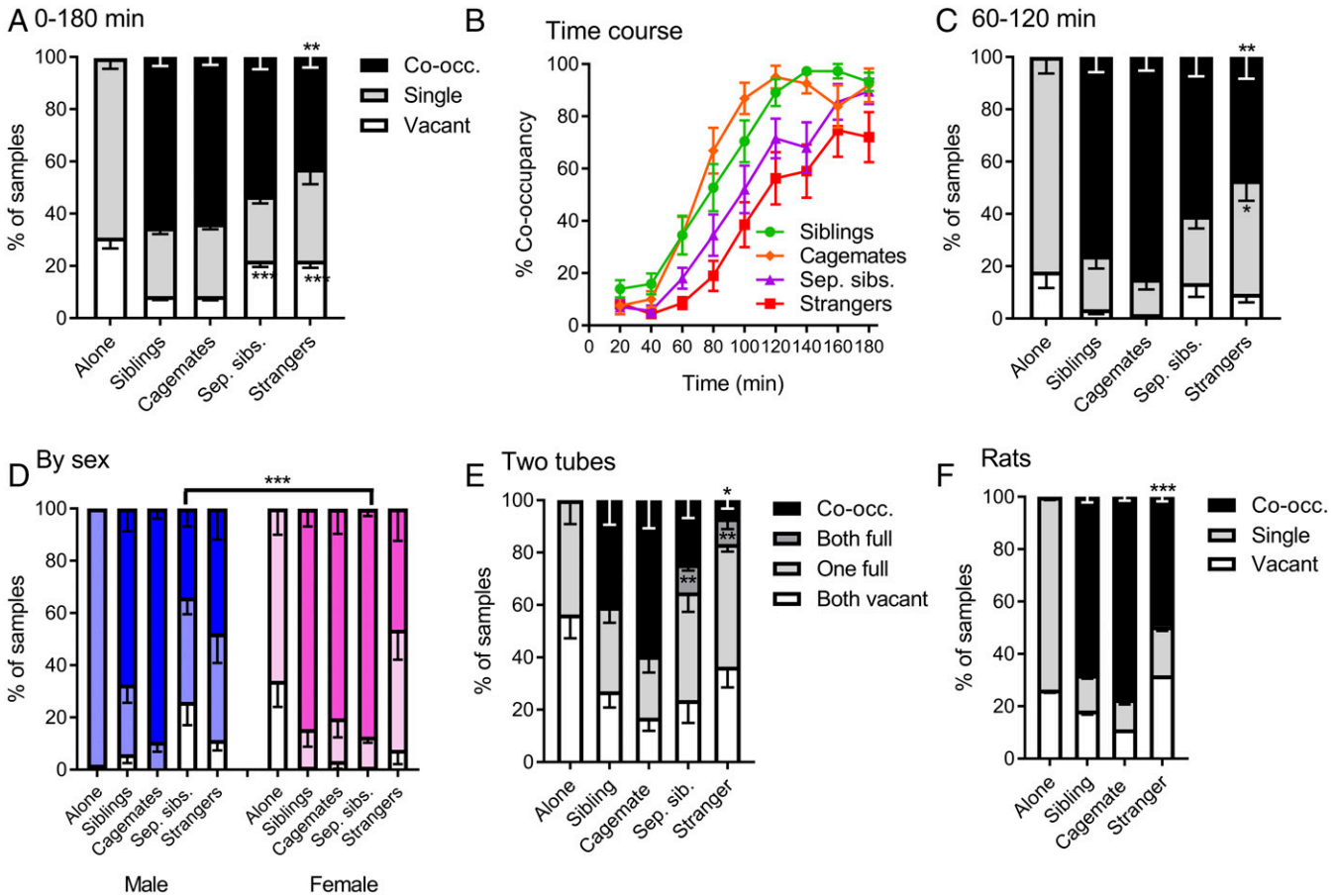

Fig. 1. Reduced tube cooccupancy in rodent stranger dyads. (*A*) Tube-occupancy behavior in various social situations (alone, two siblings, two cagemates, two separated siblings, or two strangers; $n = 18$ mice or dyads per social condition) over the full 180-min testing period. Bars represent mean percentage ± SEM of samples featuring tube cooccupancy (Co-occ.), single occupancy (Single), and no occupancy (Vacant). (*B*) Time course of tube cooccupancy behavior. Symbols represent mean percentage ± SEM of samples featuring tube cooccupancy (using sampling of digital videotape) per 20-min period. (*C*) Tube occupancy in the second hour of testing. Bars are as in *A*, but for the 60–120 min period. (*D*) Tube occupancy by subject sex. Bars are as in *C*; $n = 9$ mice or dyads per social condition per sex. (*E*) Tube-occupancy behavior in an arena with two tubes instead of one. Bars represent mean percentage ± SEM of samples featuring cooccupancy of either tube (Co-occ.), simultaneous single occupancy of both tubes (Both full), single occupancy of one tube (One full), or no occupancy (Both vacant); $n = 8$–12 mice or dyads per social condition. (*F*) Tube-occupancy behavior in various social situations in outbred, Sprague–Dawley rats in a larger arena. Bars are as in *A* over a 60-min testing period; $n = 10$ rats or dyads per social condition. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with sibling–cagemate groups or as indicated.

for a photograph of the automated version of the TCOT.) The accuracy of the automated scoring compared with manual scoring (via sampling) was found to be very high ($r = 0.96$, $P < 0.001$) (Fig. S2 *B* and *C*). Subsequently presented data were obtained largely using the automated TCOT.

Stress-Dependence of TCOT Behavior. We have previously shown that in both mice (15) and humans (16) the mere proximity of a stranger conspecific can produce measurable increases in adrenal stress hormones. We first confirmed that plasma corticosterone levels in the TCOT were increased in strangers as compared with siblings (Time × Condition), $F(3, 91) = 6.5$, $P = 0.001$; significantly so at 30–60 min (Fig. 2*A*). In hypothalamic tissue obtained at the 120-min time point, strangers displayed higher mRNA expression of the corticotrophin-releasing hormone gene (*Crh*), Student's $t(4) = 11.1$, $P < 0.01$, and correspondingly lower expression of the glucocorticoid receptor gene (*Nr3c1*), $t(5) = 2.9$, $P < 0.05$, in the periventricular nucleus of the hypothalamus (Fig. 2*B*). To investigate the dependence of performance in the TCOT on stress, we pretreated mice with compounds known to reduce or enhance stress. Although the corticosterone synthesis inhibitor metyrapone (50 mg/kg, i.p.) had no effect in sibling dyads, it significantly increased cooccupancy in stranger dyads: main effect of drug, $F(1, 33) = 12.1$, $P = 0.001$; main effect of social condition, $F(1, 33) = 0.3$, $P = 0.57$; Drug × Social Condition interaction, $F(1, 33) = 3.4$, $P = 0.08$ (Fig. 2*C*). Conversely, the anxiogenic α_2-adrenergic receptor blocker yohimbine (2.5 mg/kg, i.p.) had no effect in stranger dyads but significantly decreased cooccupancy in sibling dyads: main effect of drug, $F(1, 31) = 6.4$, $P = 0.02$; main effect of social condition, $F(1, 31) = 1.1$, $P = 0.30$; Drug × Social Condition interaction, $F(1, 31) = 12.9$, $P = 0.001$ (Fig. 2*D*).

Genotype Dependence of TCOT Behavior. A variety of inbred mouse strains were tested on the TCOT (Fig. 3*A*), including three strains (AKR/J, C3H/HeJ, and C57BL/6J) showing high levels of sociability on the three-chamber test and three strains (129S1/SvImJ, BALB/cJ, and BTBR T+tf/J) showing low sociability. All strains displayed statistically equivalent levels of tube occupancy in the isolated (alone) condition, $F(4, 46) = 1.2$, $P = 0.32$. An ANOVA revealed a significant main effect of genotype, $F(5, 127) = 2.7$, $P = 0.02$, but not social condition (sibling vs. stranger), $F(1, 127) = 1.8$, $P = 0.18$, or their interaction, $F(5, 127) = 1.2$, $P = 0.32$. Strain differences were unrelated to stress levels, $F(2, 41) = 1.8$, $P = 0.18$ (Fig. 2*E*). Only AKR mice displayed significantly higher cooccupancy in sibling than in stranger dyads ($P = 0.01$; all other Ps > 0.33). Notably, the lowest levels of cooccupancy in sibling dyads were displayed by the BTBR T+tf/J strain. Although the restricted numbers of strains precluded assessing statistical significance, we note the reasonable correlation between stranger dyad performance on the TCOT and sociability scores (time spent with stranger – time spent with empty cage; $r = 0.61$) but not social novelty preference (time spent with novel stranger – time spent with a previously encountered stranger; $r = 0.05$) on the three-chamber test (17). This pattern also was observed for mice of four strains (AKR/J, C3H/He, CD-1, and BTBR T+tf/J) tested on the three-chamber test in our laboratory (TCOT vs. sociability: $r = 0.68$; TCOT vs. social novelty: $r = -0.82$). This result is expected, because the much longer duration of the TCOT would preclude its ability to assess social novelty.

To investigate further the apparent difference in sibling versus stranger preference between outbred CD-1 (Crl:ICR) mice and inbred mice, we tested three other outbred strains from two different suppliers: ICR mice from Taconic and Swiss Webster mice from both Charles River and Taconic. All outbred populations displayed higher cooccupancy for siblings than for strangers (Fig. 3*B*). An ANOVA revealed a significant main effect of social condition, $F(1, 145) = 20.0$, $P < 0.001$, but not strain, $F(1, 145) = 1.3$, $P = 0.26$; supplier, $F(1, 145) = 0.04$, $P = 0.83$; or any interactions (all Ps > 0.29).

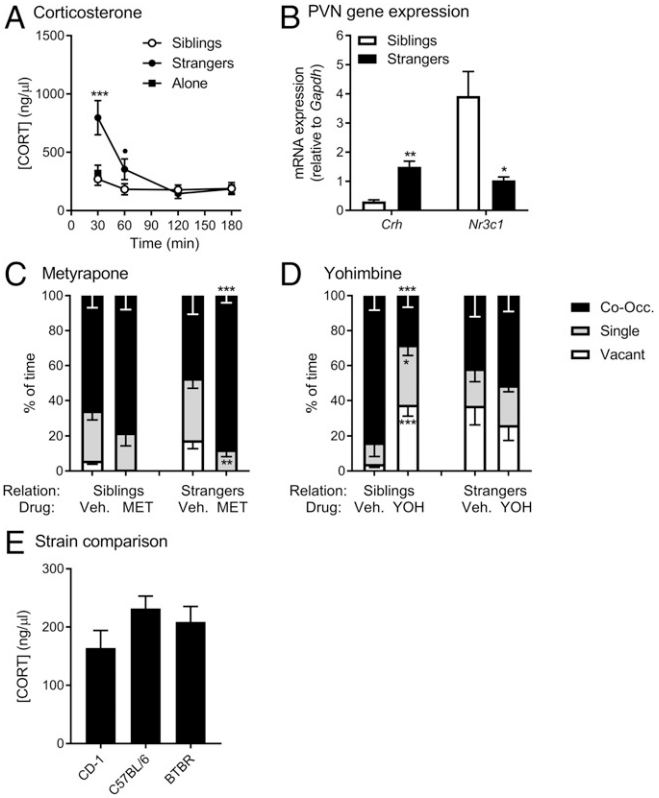

Fig. 2. Dependence of TCOT behaviors on social stress. (*A*) Compared with mice tested alone in the TCOT, strangers in dyads have higher corticosterone levels than siblings in dyads. Symbols represent mean corticosterone concentration (in nanograms per microliter) ± SEM in plasma for mice killed at 30, 60, 120, or 180 min after the start of TCOT testing (n = 11–14 dyads per group at each time point). ***$P < 0.001$, *$P < 0.1$, compared with the sibling group at same time point. Note that corticosterone levels are high in all groups, likely because of the stress of the novel environment. (*B*) Altered expression of stress-relevant genes *Crh* and *Nr3c1* in the periventricular nucleus (PVN) of the hypothalamus in stranger dyads compared with sibling dyads. Bars represent mean mRNA levels in arbitrary units ± SEM compared with the housekeeping gene *Gapdh* (n = 3 or 4 biological replicates per group). *$P < 0.05$, **$P < 0.01$, compared with corresponding sibling group. (*C*) Increased cooccupancy behavior in stranger dyads pretreated with the corticosterone synthesis inhibitor metyrapone. Bars represent mean percentage cooccupancy ± SEM in sibling or stranger dyads pretreated with vehicle (Veh.) or 50 mg/kg metyrapone (MET); n = 8–11 dyads per social condition per drug. **$P < 0.01$, ***$P < 0.001$, compared with corresponding vehicle group. (*D*) Decreased cooccupancy behavior in sibling dyads pretreated with the anxiogenic α_2-adrenergic antagonist yohimbine. Bars represent mean percentage cooccupancy ± SEM in sibling or stranger dyads pretreated with vehicle (Veh.) or 2.5 mg/kg yohimbine (YOH); n = 8–10 dyads per social condition for each drug. *$P < 0.05$, ***$P < 0.001$, compared with the corresponding vehicle-treated group. (*E*) Plasma corticosterone levels do not differ among three mouse strains with very different TCOT behavior. Bars represent mean ± SEM plasma corticosterone concentration ([CORT]) in nanograms per microliter.

This finding raises the possibility that outbred and inbred mice may have major differences in social preferences and begs the question as to which pattern (sibling > stranger vs. sibling = stranger) is "normal," that is, more representative of the species in general. To address this question, we tested cross-fostered adult offspring of wild mice (*Mus musculus domesticus*) trapped in a semirural area of metropolitan Montreal. As shown in Fig. 3*C*, these mice also displayed greater cooccupancy in sibling than in stranger dyads, $t(23) = 3.7$, $P = 0.001$. Overall, inbred, outbred, and wild mice displayed equivalent preference for tube occupancy, $F(2, 106) = 0.4$, $P = 0.64$, when tested alone (Fig. 3 *A*–*C*).

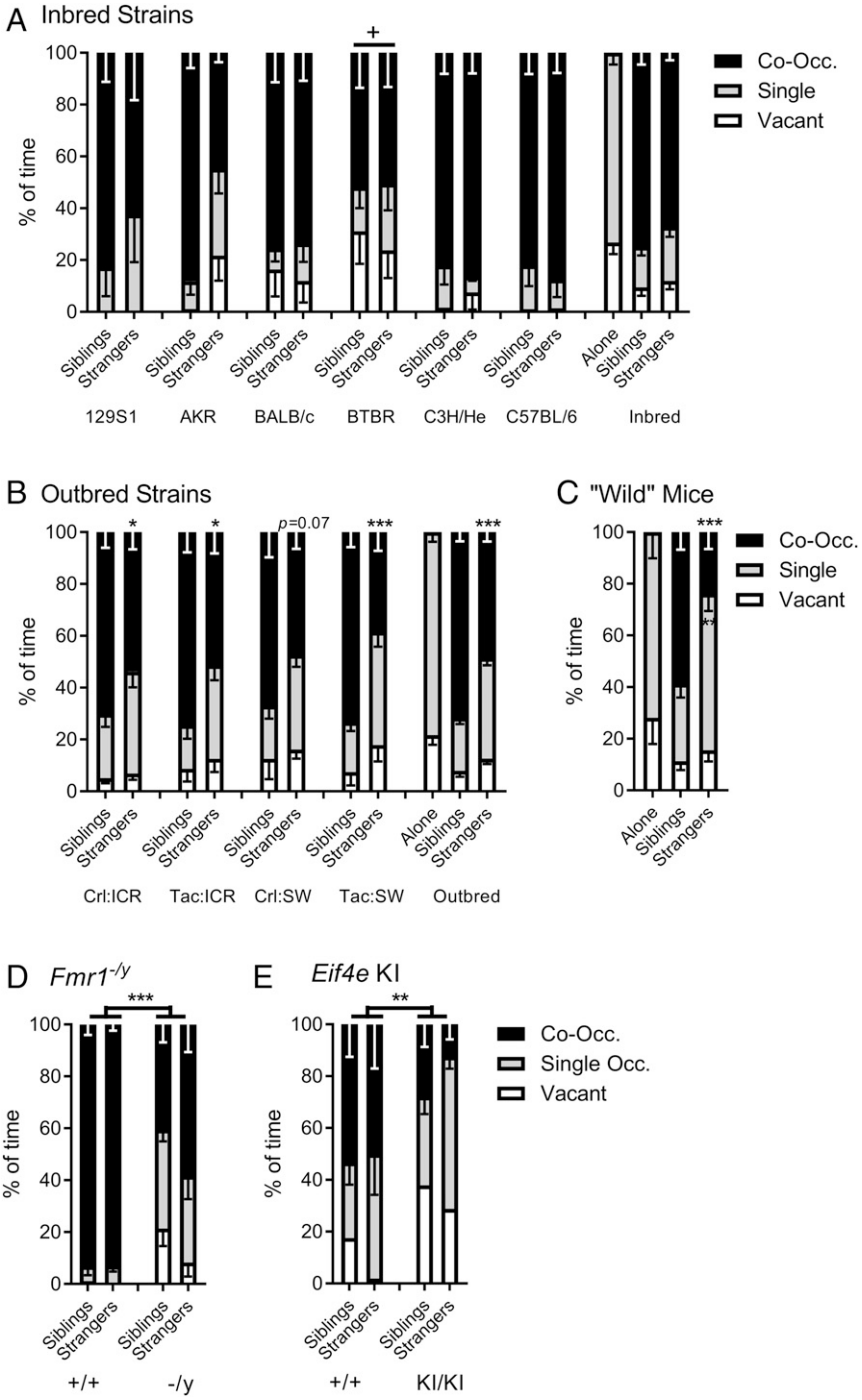

Fig. 3. Genotype-dependence of tube cooccupancy in sibling versus stranger dyads. (*A*) Tube-occupancy behavior in six inbred mouse strains and their average (Inbred). Bars represent the percentage of time (60–120 min; using automated measurement) of tube cooccupancy (Co-occ.), single occupancy (Single), or no occupancy (Vacant); $n = 12$ dyads per social condition for each genotype except for 129S1 strangers ($n = 8$). Data are presented as mean ± SEM. (*B*) Tube-occupancy behavior in six outbred mouse stocks and their average (Outbred). Bars are as in *A*; $n = 12$–26 dyads per social condition for each genotype. (*C*) Tube-occupancy behavior in rederived wild mice. Bars are as in *A*; $n = 12$–13 dyads per social condition. Data from mice tested alone in graphs *A*–*C* are presented averaged across strain; $n = 52$, $n = 50$, and $n = 7$, respectively. (*D* and *E*) Tube-occupancy behavior in *Fmr1*$^{-/y}$ (-/y) mice (*D*) and *Eif4e* Ser209Ala (knockin; KI) mice (*E*) compared with controls ($^{+/+}$). Bars are as in *A*; $n = 7$–12 dyads per social condition for each genotype. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with sibling group or as indicated. $^{+}P < 0.05$, compared with BALB/c, C3H/He, and C57BL/6 mice.

Reduced Cooccupancy Behavior of Mutant Strains. Finally, we wished to use the TCOT to examine social propinquity in autistic-like mutant mice. We examined two available genotypes featuring null mutations of autism-relevant genes: *Fmr1*$^{-/y}$ mutant mice lacking expression of fragile X mental retardation protein and *Eif4e*$^{\mathrm{Ser209\rightarrow Ala}}$-knockin mice expressing a non-phosphorylatable form of the eukaryotic translation initiation factor 4E (eIF4E) (18). *Fmr1*$^{-/y}$ and *Eif4e*-knockin mice demonstrated dramatically reduced tube cooccupancy than wild-type controls, regardless of social condition: main effects of genotype,

$F(1, 35) = 27.5$, $P < 0.001$, and $F(1, 27) = 6.9$, $P = 0.01$, respectively (Fig. 3 *D* and *E*). We also tested mutant mice with SH3 haploinsufficiency and multiple ankyrin repeat domains 3 (*Shank3*$^{+/-}$), which have shown contradictory phenotypes on existing social tests; they were found to be indistinguishable from wild types on the TCOT (Fig. S3).

Discussion

Here, we describe and characterize an automatable test of social behavior in mice and rats that measures willingness to cooccupy a "safe" tube in a stressful novel environment. Two animals sharing a tube is a qualitatively different situation from two animals sharing a nesting area within a home cage, because tube cooccupancy involves a motivational conflict between the desire to hide from potential predators and the desire to avoid a stranger conspecific. We find that outbred rodents are less likely to cooccupy the tube with strangers than with siblings or nonsibling cagemates. This preference for familiars can be found in human studies of interpersonal distance and is impaired in children with autism spectrum disorders (19). We observe that cooccupancy with familiars occurs at high levels even when it was possible for each of the two mice to hide separately, suggesting that behavior in the TCOT is driven by motivation for social propinquity and not just by the need to hide. The two-tube version of the TCOT might be preferable to the standard version for some experiments, although interpretation of data and automation are more complex.

The reluctance to co-occupy with strangers appears to result from stress associated with the stranger itself, over and above the stress associated with the novel environment. In fact, we find that the TCOT is strongly dependent on stress levels. Our data strongly suggest that sociality in rodents is itself highly dependent on anxiety/stress, so that social tolerance as assessed by propinquity can occur only if stress levels are low. As a practical matter, this stress-dependency might represent an interpretational challenge for experiments. However, this challenge is equally or even more relevant to the three-chamber test, because the experiments are performed in the first 10–15 min, when stress levels are highest. We note that stress is not a confound of the main conclusions of our study regarding genotype differences, because the highly social C57BL/6 strain did not exhibit lower stress levels in the TCOT than either CD-1 or BTBR mice. Aggression also is not responsible for our findings, because agonistic behaviors were exceedingly rare even between male strangers, probably because the 3-h duration of the TCOT is not nearly long enough for dominance-related fighting to begin.

The TCOT is able to identify mouse strains with impaired sociability, including BTBR T+tf/J mice and *Fmr1*$^{-/y}$ and *Eif4e* knockin mutants. However, our study suggests that all such conclusions may be confounded by the abnormal social behavior of inbred mice. That is, inbred mouse strains—especially the C57BL/6 mice that serve as the genetic background of most transgenic strains—appear to have been artificially selected for unusual social gregariousness so that they lack the preference for familiar vs. stranger typical of outbred and rederived wild mice. This gregariousness might be caused by the reduced olfactory differentiability of inbred mice (20), although we observed that C57BL/6 mice displayed high cooccupancy (82.3 ± 9.6%) even when the other member of the dyad was a stranger of a different strain (C3H/He). It is also clear that inbred mice can differentiate between familiars and strangers, because social phenomena such as emotional contagion have been shown to be more pronounced between C57BL/6 cagemates than between strangers (21). Dramatic phenotypic (especially stress-related) and gene expression differences between mutants placed on C57BL/6 ("domesticated") versus rederived wild mice have been reported recently (14).

Given the apparently poor representativeness of inbred laboratory mouse strains in terms of their social behavior, and because mutations can now be placed on outbred genetic backgrounds easily using CRISPR-Cas9 (22), we suggest that social neuroscientists should reconsider the ubiquitous use of inbred mice. The current data also identify a number of social phenomena (e.g., preference for separated siblings in females but not males, stress-dependence of social behavior, decreasing tolerance for cooccupancy with age) deserving of further study.

Materials and Methods

Animals. In most experiments, naive male and female CD-1 [Crl:CD1(ICR)] outbred mice were bred in-house at our animal facility at McGill University. Additional outbred mice of both sexes were purchased from Charles River Laboratories or Taconic Biosciences, Inc. Male and female inbred mice (129S1/Sv1mJ, AKR/J, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, and C57BL/6J) were purchased from The Jackson Laboratory. Wild mice were collected using live traps at two different agricultural facilities on McGill's MacDonald campus (45°24′N 73°56′W). Trapped wild mice were bred in quarantine, and their offspring were cross-fostered on P2–P3 to a CD-1 dam to minimize exposure to zoonotic agents. Offspring were screened for pathogens at P21 and brought to the laboratory after testing negative for pathogens. Finally, mutant strains and appropriate control strains were either purchased from The Jackson Laboratory (B6.129-*Shank3*$^{tm2Gfng-/+}$/J, B6.129-*Shank3*$^{tm2Gfng+/+}$/J, C57BL/6J-*Fmr* $^{-/y}$, C57BL/6J-*Fmr1*$^{+/+}$) or supplied by the N.S. laboratory (*Eif4e*$^{Ser209\rightarrow Ala}$ knockin, *Eif4e*$^{+/+}$) (23). Mice procured from other facilities were P21–P28 when they were transferred to the in-house vivaria at McGill University or the University of Edinburgh.

Before testing, mice were group-housed (three to five mice per standard shoebox cage) with same-sex companions (littermates for mice bred in-house). All mice were given tap water and Harlan Teklad 2020x (McGill) or Special Diets Services RM1 (Edinburgh) diet ad libitum and were maintained at 22 °C on a 12/12-h light–dark cycle (lights on at 07:00 h). All protocols and procedures were approved by the McGill University Downtown Animal Care Committee and the UK Home Office according to appropriate national regulations.

Mice were tested behaviorally as young adults (6–10 wk of age) except in one experiment in which mice were tested at 12 or 18 wk of age. All experiments included approximately equal numbers of male and female mice; mice were only used once. Experiments occurred near mid-photoperiod; all runs began no earlier than 09:00 h and no later than 15:00 h.

One experiment used naive, 5- to 7-wk-old male Sprague–Dawley rats purchased from Charles River Ltd., which were habituated to the facility for a minimum of 2 wk before testing. Rats were kept on a 12/12-h light–dark cycle (lights on at 07:00 h), at 22 °C, with ad libitum access to food (RM1; Special Diets Services, DBM Food Hygiene Supplies) and were group-housed in cages of four.

TCOT. The two members of the same-sex mouse dyads (or, as a control, a single mouse) were placed at the same time into an arena with opaque Plexiglas walls (39 cm wide × 26 cm long × 12 cm high). The arenas were situated on top of a glass shelf 105 cm above the ground to create a visual cliff and were brightly illuminated with a 250-W LED light (~3,000 lux). Each open-field box contained a single opaque PVC cylinder (7.5 cm long × 3 cm in diameter or, in one experiment, a larger tube 10 cm long and 3 cm in diameter) placed against one long wall. In the two-tube variant of the TCOT, two 7.5 × 3 cm cylinders were placed 4 cm apart from one another along the long wall of the arena. Mice were tested for 3 h, without prior habituation to the room or to the TCOT arena (except in one experiment featuring 30- or 90-min habituation), in one of the following social conditions (all same-sex): (*i*) siblings born of the same parents and raised together (same-sex siblings only) in the same home cage from birth until testing; (*ii*) cagemates born of different parents but living in the same home cage from weaning at P21 until testing; (*iii*) separated siblings born of the same parents but living in different home cages from weaning at P21 until testing; and (*iv*) strangers born of different parents with no contact before testing. Strangers were housed in different cages, but all mice in the study were housed in the same room. For stranger-habituation experiments, stranger mice from two different cages were put together as a dyad into a clean cage and were cohoused for 1, 4, 7, or 14 d before testing.

All animals were age-matched and were tested only once in the TCOT, except in repeat-exposure experiments, in which mice were tested multiple (two to four) times. After placing animals in the TCOT arena, male or female experimenters turned on the automated recording system and/or video cameras and then left the room quickly.

For the rat study, the arena measured 60 × 60 × 60 cm with floors and walls made of white acrylic. A black plastic sewage pipe measuring 14.5 cm long by 11 cm in diameter was fixed to the floor with Velcro. Two bright lights (~3,000 lux) shone down onto the arena. Between experiments the

arena floor and walls and the tube itself were cleaned using 70% alcohol. Rats were handled for 20 min per cage for 3 d before testing. The duration of TCOT testing was 1 h; the experimenter exited the room promptly after placing the rats in the arena. The social conditions were identical to those in the mouse TCOT, except that no separated-sibling group was tested.

TCOT Scoring. Scoring occurred either by video time-sampling (one 10-s sample/min), generating percentages of samples featuring tube cooccupancy, single occupancy, or vacancy, or by automated scoring, generating percentages of total time featuring these behaviors. For "manual" scoring, a digital video camera was placed directly over the arena. The resultant video file was also used to score behaviors (e.g., fighting) occurring outside the tube.

The automated system consists of PVC cylinders hanging magnetically from load cells with a 780-g capacity. Electrical signals from the load cells are amplified and conditioned for input into a digital processor. The processor outputs data into a computer programmed to store and present a real-time (1-s) display of the current weight of the cylinder, which is exported for analysis. Tube vacancy, single occupancy, and cooccupancy (measured in seconds) can be inferred easily from the weight data. Fully automated data collection generally precluded the need for blinding of investigators.

Social Behaviors Outside the Tube. A randomly chosen 5-min clip was selected during the first 10 min of the TCOT run. Blinded observers continuously coded this abbreviated clip for the following social interactions occurring outside of the tube (3): one mouse pursuing the other mouse in the open field or one mouse sniffing any part of the other mouse's body.

Stress Manipulations. To test whether acute stress impacts behavior in the TCOT, a separate cohort of CD-1 mice received one of three compounds: (*i*) metyrapone (50 mg/kg, i.p.), an inhibitor of corticosterone synthesis; (*ii*) yohimbine (2.5 mg/kg, i.p.), a known anxiogenic compound; or (*iii*) saline (10 mL/kg, i.p.) as a control. All drugs were injected when mice were in the home cage, 30 min before testing. Mice were randomized to these conditions while within the cage, and investigators were blind to drug condition.

Corticosterone Levels. A separate cohort of CD-1 mice was tested in the TCOT and killed by decapitation under isoflurane/oxygen anesthesia 30, 60, 120, or 180 min later. Trunk blood was collected for corticosterone enzyme immunoassay (EIA). Blood samples collected for EIA were spun down ($5.53 \times g$, 4 °C) for 15 min, and blood plasma was collected. Samples were normalized and diluted 1:800 and were run against a standard curve as part of a validated corticosterone EIA kit (Cayman Chemical). Linear regression analyses were performed on the results to determine the relative concentration of corticosterone for each sample as compared with the included kit standards.

Quantitative PCR. Following the TCOT, mice were killed by cervical dislocation under isoflurane/oxygen anesthesia. The whole brain was surgically removed from the skull, and the cerebellum and anterior frontal lobe were blocked off. Brain tissue was placed in a 1:10 PBS:water solution, after which 300-nm slices were obtained with a vibratome (Leica VT 1000S). A 21-gauge 1-1/2 in needle was used to obtain tissue from the paraventricular nucleus of the hypothalamus. Relative expression levels of *Crh* and *Nr3c1* genes were measured by quantitative RT-PCR using Applied Biosystems TaqMan probes (assay IDs: Mm04206019 m1 and Mm00433832 m1, respectively). Results are based on the relative quantification compared with the housekeeping gene *Gapdh* and were made following the ΔΔCt standard curve method.

Three-Chamber Test. We followed the experimental protocol described by Yang et al. (24). Test mice were habituated to the center of the three-chambered apparatus for 10 min. Then the doors to the side chambers were raised, and mice were allowed to explore the entire space for an additional 10 min (baseline). A single naive mouse then was placed in an inverted wire pencil cup in one side chamber (in a counterbalanced fashion). These "stimulus" mice were previously trained to reside in the pencil cup and to avoid agitation and excessive movement while in the cup. Test mice then were videotaped for 10 min in the presence of the stimulus mice. The total number of entries and the total time spent in each side chamber (one containing the stimulus mouse and the other empty) was coded by a blinded experimenter.

Statistics. Data were confirmed as being normally distributed (Shapiro–Wilk statistic) and featuring homogeneity of variance (Bartlett's test) among groups. Thus, data were analyzed by *t* test (two-sided) and by one-way or two-way ANOVA followed by Tukey's post hoc analyses where appropriate. For all analyses, an alpha level of 0.05 was considered significant.

Because of the novelty of the phenomenon, it was not possible to perform power analyses. Sample sizes were determined primarily by breeding success and our experience with other social phenomena in mice.

Behavioral runs were excluded in their entirety if the tube became detached from the load cell or the video camera was unable to record to the end of the behavioral run. In four cases, data were excluded after being identified as statistical outliers (Studentized residuals >3 SDs from the group mean).

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