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

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## Abstract

**Existing assays of social interaction are suboptimal, and none measure 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 co-occupancy 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, social and environmental conditions. In outbred mice and rats, familiarity determined willingness to co-occupy the tube, with siblings and/or cagemates of both sexes exhibiting higher co-occupancy behavior than strangers. Subsequent testing using multiple genotypes revealed that inbred strain siblings do not co-occupy 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* knockin) displayed significantly decreased co-occupancy.**

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 to strangers versus familiars (social novelty preference). The assay has been used to establish various inbred (e.g., BTBR $T^{+}$ *tf*/J) and mutant mouse strains (e.g., *Fmr1* null mutants) 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 either the home cage or a neutral environment (3). Such comprehensive recording is extremely labor-intensive and prone to errors (4) and almost impossible to automate using present technology, although some progress is being made using machine learning algorithms (5).

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 (4), the incarceration of one of the social partners (6), and confounds related to location of the stimulus animals (7). We would suggest as well 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 (8). At the very least, the measurement, over an extended period of time, of voluntary social proximity—propinquity—among rodents placed in a neutral, novel environment might add usefully to our social neuroscience armamentarium, especially as propinquity itself is far more easily automated than fine-grained individual measures of reciprocal social interactions. To these ends we developed the tube co-occupancy 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: 1) they are genetically homozygous at every locus, 2) they have been artificially selected for both tameness and reproductive success under laboratory conditions, and 3) post-weaning 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 (9-11). 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 over the 3-hour testing period (Fig. 1*A*, leftmost bar). Same-sex dyadic groups (siblings, non-sibling cagemates, separated siblings [i.e., siblings housed separately since weaning], and strangers) spent different proportions of their time sharing the tube (co-occupancy), 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 co-occupancy 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 compared to 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 early (0–10 min) social interactions occurring outside the tube (Fig. S1*C*). A visual inspection of the time course of tube co-occupancy (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 co-occupancy were displayed by stranger dyads by the end of testing, although still lower than other groups. Sex differences in tube co-occupancy were only observed in one group, with female separated siblings spending significantly more time together than male separated siblings (condition x 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 co-occupancy (Fig. S1*D*). Strangers also displayed sibling-like levels of co-occupancy after repeated daily exposures to each other in the arena (Fig. S1*E*) or co-housing prior to testing for as little as one day (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 co-occupancy is highly preferred among related and/or familiar mice even if each mouse had the option of hiding in their own tube. Sibling/cagemate versus stranger differences in co-occupancy were also seen in outbred Sprague Dawley rats tested for one hour 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-week old mice displaying much lower tube co-occupancy, 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/sec) measure of its displacement such that occupancy by zero, one or two mice can be inferred and summed over the

testing period or any portion thereof. See Fig. 2*A* for photograph of the automated version of the TCOT. The accuracy of the automated scoring compared to manual scoring (via sampling) was found to be very high ($r$=0.96, $p$<0.001) (Fig. 2*B*,*C*). Subsequently presented data were obtained largely using the automated TCOT.

**Stress-Dependence of TCOT Behavior.** We have previously shown that in both mice (12) and humans (13), 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 compared to siblings (time x condition: $F_{3,91}$ = 6.5, $p$=0.001); significantly so at 30–60 min (Fig. 3*A*). In hypothalamic tissue obtained at the 120 min time point, strangers displayed higher mRNA expression of the corticotrophin releasing hormone gene (*Crh*; $t_4$ = 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. 3*B*). To investigate the dependence of performance in the TCOT on stress, we pretreated mice with compounds known to reduce or enhance stress. The corticosterone synthesis inhibitor, metyrapone (50 mg/kg, i.p.), while having no effect in siblings, significantly increased co-occupancy in strangers (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 x social condition interaction: $F_{1,33}$ = 3.4, $p$=0.08) (Fig. 3*C*). Conversely, the anxiogenic $\alpha_2$-adrenergic receptor blocker, yohimbine (2.5 mg/kg, i.p.), while having no effect in strangers, significantly decreased co-occupancy in siblings (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 x social condition interaction: $F_{1,31}$ = 12.9, $p$=0.001) (Fig. 3*D*).

**Genotype-Dependence of TCOT Behavior.** A variety of inbred mouse strains were tested on the TCOT (Fig. 4*A*), including three strains showing high levels of sociability on the three-chamber test (AKR/J, C3H/HeJ, and C57BL/6J) and three strains showing low sociability (129S1/SvImJ, BALB/cJ, and BTBR $T^+$ *tf*/J). All strains displayed statistically equivalent levels of tube occupancy in the isolated (alone) condition ($F_{4,46}$=1.2, $p$=0.32). 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. 3*E*). Only AKR mice displayed significantly higher co-occupancy in sibling versus stranger dyads ($p$=0.01; all other $p$'s>0.33). Notably, the lowest levels of co-occupancy in sibling dyads were displayed by the BTBR $T^+$ *tf*/J strain. Although 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 "new" stranger – time spent with "old" stranger; $r$=0.05) on the three-chamber test (14). This pattern was also 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 is not unexpected, since 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 new outbred strain/supplier combinations: ICR mice from Taconic, and Swiss Webster mice from both Charles River and Taconic. All outbred populations displayed higher co-occupancy in siblings compared to strangers (Fig. 4*B*). 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 $p$'s$>0.29$).

This finding raises the possibility of major differences in social preferences between outbred and inbred mice, 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, we tested cross-fostered adult offspring of wild mice (*M. musculus domesticus*) trapped in a semi-rural area of Montreal. As shown in Fig. 4*C*, they too displayed greater co-occupancy in sibling compared to 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. 4*A*–*C*).

**Reduced Co-Occupancy 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*$^{\text{Ser209}\rightarrow\text{Ala}}$ knockin mice, expressing a nonphosphorylatable form of the eukaryotic translation initiation factor 4E (eIF4E) (15). *Fmr1*$^{-/y}$ and *Eif4e* knockin mice demonstrated dramatically reduced tube co-occupancy compared to wildtype 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. 4*D*,*E*). We also tested mutant mice with haploinsufficiency of SH3 and multiple ankyrin repeat domains 3 (*Shank3*$^{+/-}$), which have shown contradictory phenotypes on existing social tests; they were found to be indistinguishable from wildtypes on the TCOT (Fig. S2).

## Discussion

Herein we describe and characterize a new, automatable test of social behavior in mice and rats, in which willingness to co-occupy a "safe" tube in a stressful novel environment is measured. Two animals sharing a tube is a qualitatively different situation than two animals sharing a nesting area within a home cage, because tube co-occupancy involves a motivational conflict between the desire to hide from potential predators and the desire to avoid a stranger conspecific. Thus, we find that outbred rodents are less likely to co-occupy the tube with strangers compared to siblings or non-sibling cagemates; co-occupancy with familiars is preferred even when both mice could hide separately. This preference for familiars can be found in human studies of interpersonal distance and is impaired in children with autism spectrum disorders (16).

The reluctance to co-occupy with strangers appears to be due to 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, such that social tolerance as assessed by propinquity can only occur if stress levels are low. As a practical matter this might represent an interpretational challenge for experiments. However, this challenge is equally relevant to the three-chamber test, and almost certainly more so, since the experiment is performed in the first 10–15 minutes, when stress levels are highest. We note that stress is not a confound of the main conclusions of our study regarding genotype differences, as the highly social C57BL/6 strain did not exhibit lower stress levels in the TCOT than either CD-1 or BTBR mice. Aggression is also not responsible for our findings, since even between male strangers agonistic behaviors were

exceedingly rare, probably because the 3-hour duration of the TCOT is nowhere near 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 such that the familiar vs. stranger preference—typical of outbred and rederived wild mice—is absent in them. This might be due to the reduced olfactory differentiability of inbred mice (17), although we observed that C57BL/6 mice displayed high co-occupancy (82.3 ± 9.6%) even when the other member of the dyad was a stranger of a different strain (C3H/He) (data not shown). It is also clear that inbred mice can differentiate between familiars and strangers, as social phenomena like emotional contagion have been shown to be more pronounced between C57BL/6 cagemates compared to strangers (18). Dramatic phenotypic (especially stress-related) and gene expression differences between mutants placed on C57BL/6 ("domesticated") versus rederived wild mice have been recently reported (11).

Given the apparently poor representativeness of inbred laboratory mouse strains in terms of their social behavior, and the fact that mutations can now easily be placed on outbred genetic backgrounds using CRISPR-Cas9 (19), we suggest that social neuroscientists should reconsider the ubiquitous use of inbred mice. The current data also identify a number of novel social phenomena (e.g., preference for separated siblings in females but not males, stress-dependence of social behavior, decreasing tolerance for co-occupancy with age) deserving of further study.

## Materials and Methods

**Animals.** In most experiments, naïve 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 (Bourcherville, QC) or Taconic Biosciences, Inc. (Albany, NY). 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 (Bar Harbor, ME). 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 Sonenberg laboratory (*Eif4e*$^{Ser209\rightarrow Ala}$ knockin, *Eif4e*$^{+/+}$) (20). Mice procured from other facilities were P21-P28 when they were transferred to the in-house vivaria at McGill University or University of Edinburgh.

Prior to testing, mice were group-housed (3–5 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 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 relevant local animal care and use committees according to appropriate national regulations.

Mice were tested behaviorally as young adults (6–10 weeks of age) except in one experiment in which mice were tested at 12 or 18 weeks 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 naïve, 5-7 week old male Sprague Dawley rats purchased from Charles River Ltd., UK, which were habituated to the facility for a minimum of 2 weeks before testing. Rats were kept on a 12/12-hour light-dark cycle (lights on at 07:00 h), at 22 °C, with *ad libitum* access to food (RM1; Special Diets Services, DBM Scotland) and group housed in cages of four.

**Tube Co-Occupancy Test (TCOT).** 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 x 26 x 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 (≈3000 lux). Each open field box contained a single opaque polyvinyl chloride (PVC) cylinder (7.5 x 3 cm diameter; or in one experiment, a larger 10 x 3 cm diameter tube) placed against one long wall. In the "two-tube" variant of the TCOT, two 7.5 x 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 the TCOT arena (except in one experiment featuring 30- or 90 min-habituation), in one of the following social conditions, all same-sex: 1) *Siblings* – born of the same parents and raised together (same-sex siblings only) in a single home cage from birth until testing; 2) *Cagemates* – born of different parents but living in the same home cage from weaning at P21 until testing; 3) *Separated Siblings* – born of the same parents but living in different home cages from weaning at P21 until testing; and, 4) *Strangers* – born of different parents with no contact prior to testing.

For stranger habituation experiments, stranger mice from two different cages were put together as a dyad into a clean cage and co-housed for 1, 4, 7 or 14 days prior to testing.

All animals were age-matched and tested only once in the TCOT, except in repeat exposure experiments, where mice were tested multiple (2–4) times. After placing animals in the TCOT arena, male or female experimenters turned on the automated recording system and/or video cameras and then quickly left the room.

For the rat study, the arena measured 60 x 60 x60 cm, with floors and walls made of white acrylic. A black plastic sewage pipe measuring 14.5 cm long x 11 cm in diameter was fixed to the floor with Velcro. Two bright lights (≈3000 lux) were shone down onto the arena. Between experiments the arena floor, walls and the tube itself were cleaned using 70% alcohol. Rats were handled for 20 min per cage for 3 days prior to testing. The duration of TCOT testing was 1 h; the experimenter exited the room promptly after rat placement in the arena. The social conditions were identical to the mouse TCOT, except that no separated sibling group was tested.

**TCOT Scoring.** Scoring occurred either by video time-sampling (one 10-s sample every minute), generating percentages of samples featuring tube co-occupancy, single occupancy, or vacancy, or 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 of the tube.

The automated system consists of PVC cylinders hanging magnetically from 780 g-capacity load cells. 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/sec) display of the current weight of the cylinder, which is exported for analysis.

Tube vacancy, single occupancy and co-occupancy (measured in seconds) can easily be inferred from the weight data. Fully automated data collection generally precluded the need for blinding of investigators.

**Social Behaviors Outside 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.** In order to test whether acute stress impacts behaviour in the TCOT, a separate cohort of CD-1 mice received one of three compounds: 1) metyrapone (50 mg/kg, i.p.), a corticosterone synthesis inhibitor, 2) yohimbine (2.5 mg/kg, i.p.), a known anxiogenic compound, or 3) saline (10 ml/kg, i.p.) as a control. All drugs were injected in the home cage 30 min prior to testing. Mice were randomized to these conditions within-cage, and investigators were blind to drug condition.

**Corticosterone Levels.** A separate cohort of CD-1 mice were tested in the TCOT and euthanized, 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 (15,000 rpm, 4 °C) for 15 min and blood plasma was collected. Samples were normalized and diluted 1:800 and 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 relative concentration of corticosterone for each sample as compared to the included kit standards.

**qPCR.** Following TCOT testing, mice were euthanized 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 1½ needle was used to obtain tissue from the paraventricular nucleus of the hypothalamus. Relative expression levels of the corticotropin-releasing hormone (*Crh*) and glucocorticoid receptor (*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 to the housekeeping gene *Gapdh* (cat#4308313), and were made following the ΔΔCt standard curve method.

**Three-Chamber Test.** We followed the experimental protocol described in Yang et al. (21). "Test" mice were habituated to the center of the three-chambered apparatus for 10 min. The doors to the side chambers were then raised and mice were allowed to explore the entire space for an additional 10 min (baseline). A single naïve mouse was then placed in an inverted pencil cup in one or the other side chamber (in a counterbalanced fashion). These "stimulus" mice were previously trained to reside in the pencil cup, to avoid agitation and excessive movement in the cup. Test mice were then videotaped for 10 min in the presence of the stimulus mice. Total number of entries and total time spent in each side chamber (one containing the stimulus mouse; 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), one-way or two-way ANOVA followed by Tukey 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 >2 standard deviations from the group mean).

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# FIGURE LEGENDS

**Figure 1**. **Reduced Tube Co-occupancy 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 ± SEM percentage of samples featuring tube co-occupancy (Co-occ.), single occupancy (Single) and no occupancy (Vacant). (*B*) Time course of tube co-occupancy behavior. Symbols represent mean ± SEM percentage of samples featuring tube co-occupancy (using sampling of digital videotape) per 20-min period. (*C*) Tube occupancy in the second hour of testing. Bars as in graph *A*, but for the 60–120 min period. (*D*) Tube occupancy by subject sex. Bars as in graph *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 ± SEM percentage of samples featuring co-occupancy 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 version of the test. Bars as in graph *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 to sibling/cagemate groups, or indicated comparison.

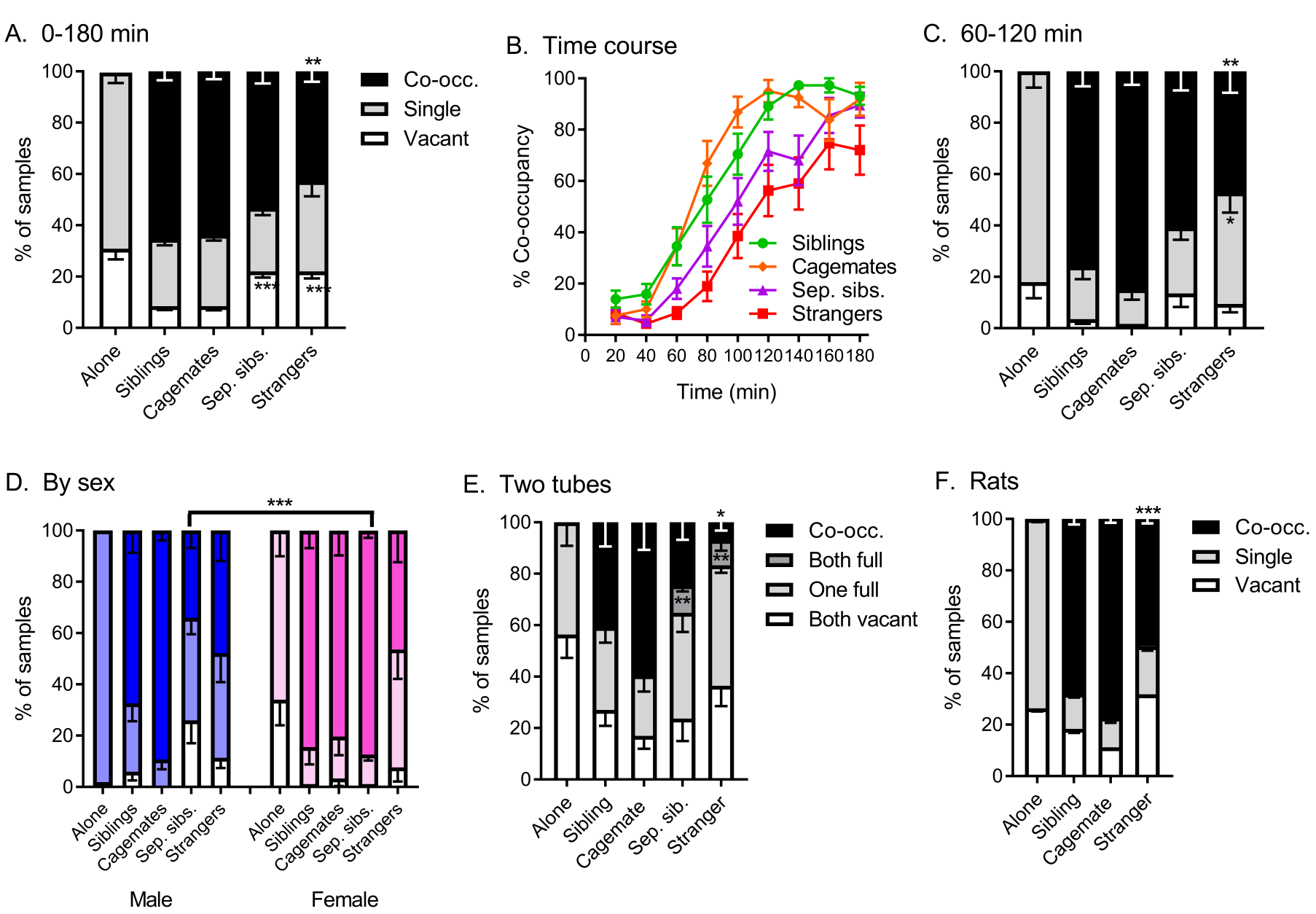
**Figure 2. Automation of the TCOT**. (*A*) A photograph of the automated version of the TCOT. Dimensions are provided in metric and Imperial. (*B*) The automated version of the TCOT provides accurate quantification of tube status compared to manual scoring using sampling (repeated measures: $F_{1,18} = 1.2$, $p=0.29$). Bars represent mean ± SEM percentage of samples

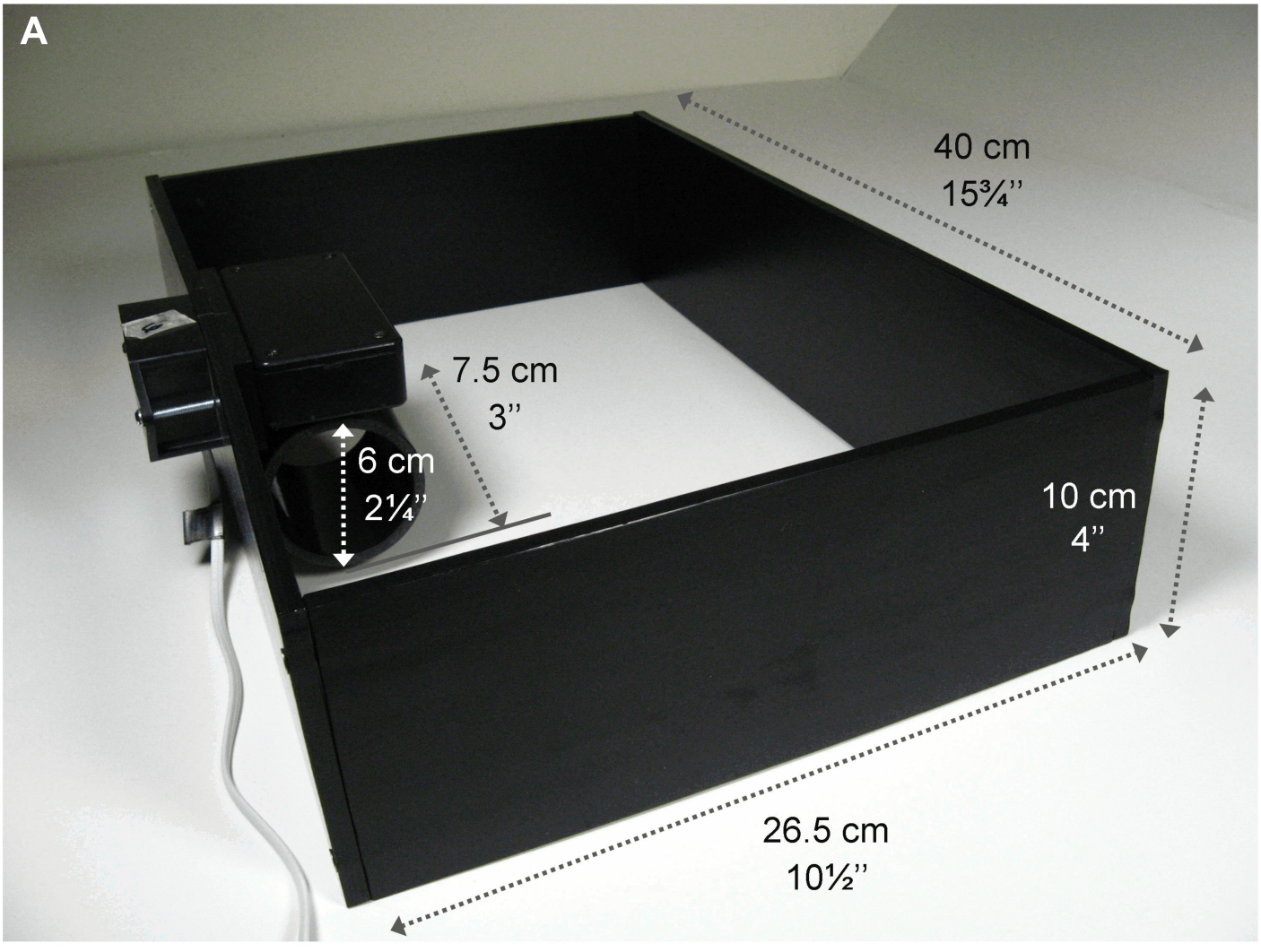
(manual) or percentage of time (automated) featuring co-occupancy (co-occ.), single occupancy and a vacant tube from 60-120 min in sibling mice ($n$=17 dyads/measurement technique). (*C*) Correlation between manual and automated TCOT scoring. Symbols ($n$=19) represent percentage co-occupancy as in graph *B*.

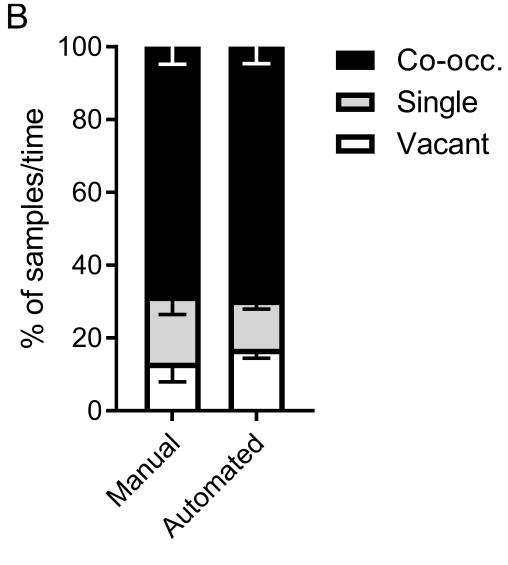
**Figure 3. Dependence of TCOT Behaviors on Social Stress**. (*A*) Compared to mice tested alone in the TCOT, strangers in dyads have elevated corticosterone levels compared to siblings in dyads. Symbols represent mean ± SEM plasma corticosterone concentration (ng/μl) for mice sacrificed at 30, 60, 120, or 180 min after the start of TCOT testing ($n$=11–14 dyads/group/time point). ***$p$<0.001, •$p$<0.1 compared to sibling group at same time point. Note that corticosterone levels are high in all groups, likely due to the stress of the novel environment. (*B*) Altered expression of stress-relevant genes *Crh* (corticotrophin-releasing hormone) and *Nr3c1* (glucocorticoid receptor) in the periventricular nucleus (PVN) of the hypothalamus in stranger dyads compared to sibling dyads. Bars represent mean ±SEM mRNA levels in arbitrary units compared to the housekeeping gene, *Gapdh* ($n$=3–4 biological replicates/group). *$p$<0.05, **$p$<0.01 compared to corresponding Sib. (*C*) Increased co-occupancy behavior in stranger dyads pretreated with the corticosterone synthesis inhibitor, metyrapone. **$p$<0.01, ***$p$<0.001 compared to corresponding vehicle group. Bars represent mean ± SEM percentage co-occupancy in sibling or stranger dyads pretreated with vehicle (Veh.) or 50 mg/kg metyrapone (MET); $n$=8–11 dyads/social condition/drug. (*D*) Decreased co-occupancy behavior in sibling dyads pretreated with the anxiogenic $\alpha_2$-adrenergic antagonist, yohimbine. *$p$<0.05, ***$p$<0.001 compared to corresponding vehicle group. Bars represent mean ± SEM percentage

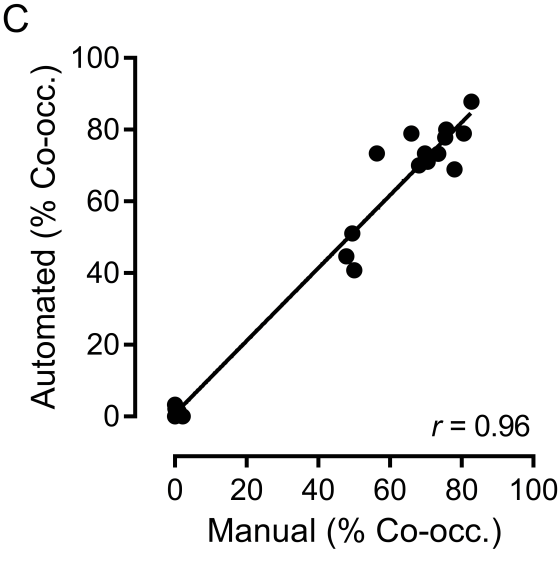
co-occupancy in sibling or stranger dyads pretreated with vehicle (Veh.) or 2.5 mg/kg yohimbine (YOH); *n*=8–10 dyads/social condition/drug.

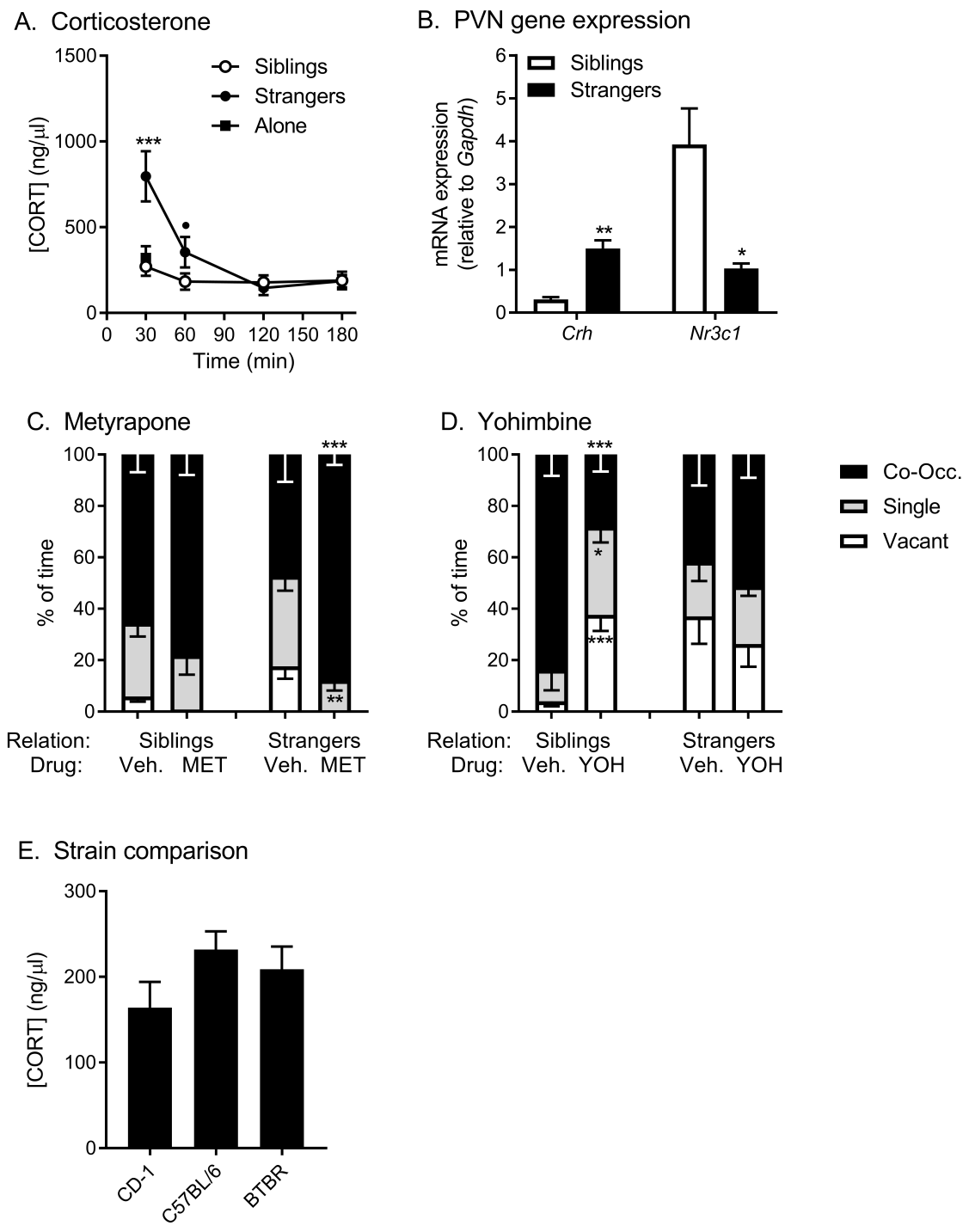
**Figure 4**. **Genotype-dependence of Tube Co-occupancy in Sibling Versus Stranger Dyads**. (*A*) Tube occupancy behavior in six inbred mouse strains, and their average (Inbred). Bars represent mean ± SEM percentage of time (60–120 min; using automated measurement) with tube co-occupancy (Co-occ.), single occupancy (Single) and no occupancy (Vacant); *n*=12 dyads/social condition/genotype except for 129S1 strangers (*n*=8). (*B*) Tube occupancy behavior in six outbred mouse stocks, and their average (Outbred). Bars as in graph A; *n*=12–26 dyads/social condition/genotype. (*C*) Tube occupancy behavior in rederived wild mice. Bars as in graph *A*; *n*=12–13 dyads/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-E) Tube occupancy behavior in *Fmr1*$^{-/y}$ mice (-/y) (*D*) and *Eif4e* knockin (KI) mice (*E*) compared to controls (+/+). Bars as in graph *A*; *n*=7–12 dyads/social condition/genotype. **p*<0.05, ***p*<0.01, ****p*<0.001 compared to sibling group, or indicated comparison. $^{+}p$<0.05 compared to BALB/c, C3H/He and C57BL/6.

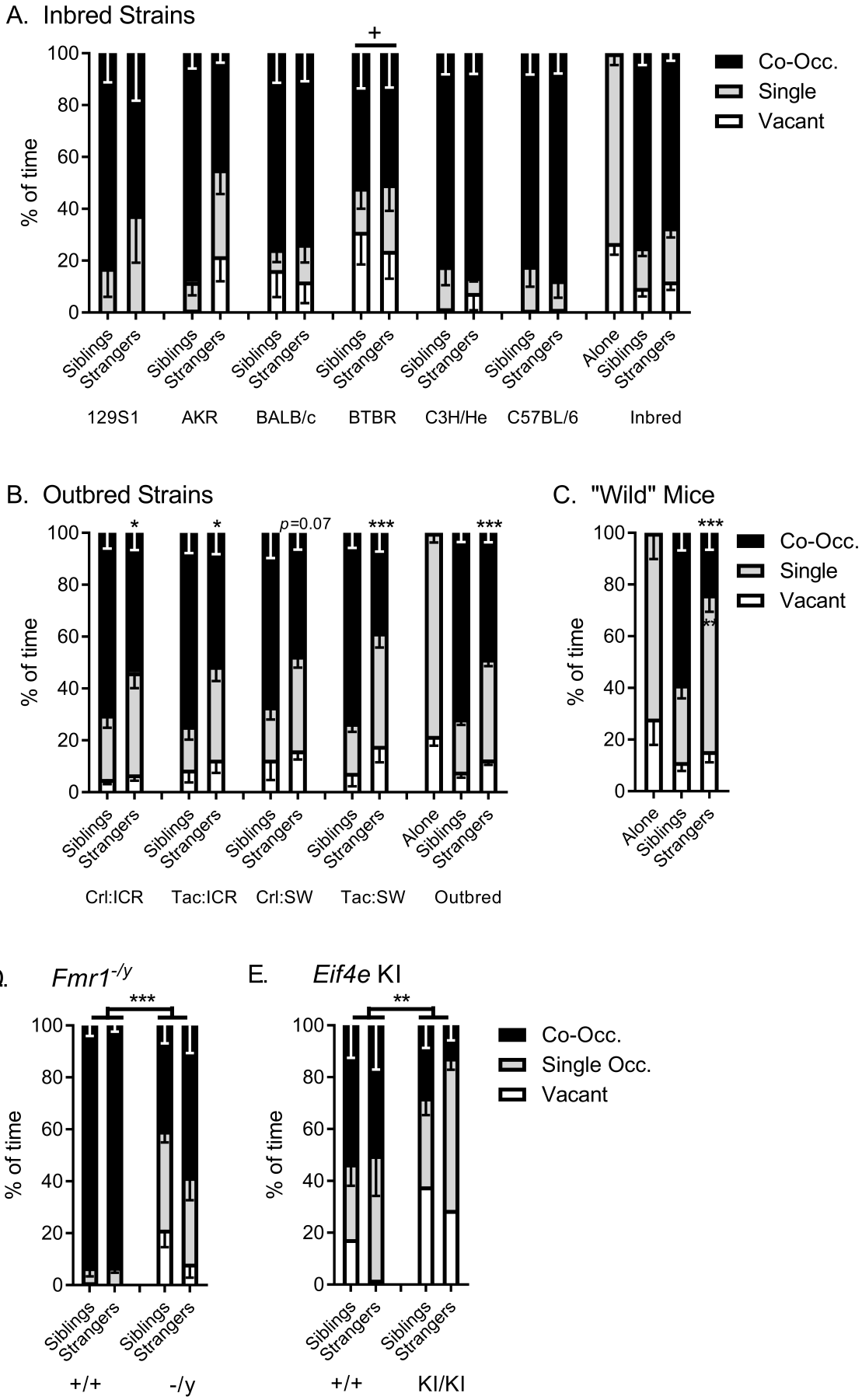

A. 0-180 min
% of samples
Co-occ.
Single
Vacant
Alone
Siblings
Cagemates
Sep. sibs.
Strangers
B. Time course
% Co-occupancy
Time (min)
Siblings
Cagemates
Sep. sibs.
Strangers
C. 60-120 min
D. By sex
Male
Female
E. Two tubes
Both full
One full
Both vacant
Sibling
Cagemate
Sep. sib.
Stranger
F. Rats


A
40 cm
15¾”
7.5 cm
3”
6 cm
2¼”
10 cm
4”
26.5 cm
10½”


B
% of samples/time
100
80
60
40
20
0
Co-occ.
Single
Vacant
Manual
Automated


C
Automated (% Co-occ.)
100
80
60
40
20
0
r = 0.96
0
20
40
60
80
100
Manual (% Co-occ.)


A. Corticosterone
[CORT] (ng/µl)
1500
1000
500
0
Siblings
Strangers
Alone
***
0 30 60 90 120 150 180
Time (min)
B. PVN gene expression
mRNA expression
(relative to Gapdh)
6
5
4
3
2
1
0
Siblings
Strangers
**
*
Crh
Nr3c1
C. Metyrapone
% of time
100
80
60
40
20
0
***
**
Relation: Siblings Strangers
Drug: Veh. MET Veh. MET
D. Yohimbine
% of time
100
80
60
40
20
0
***
*
***
Relation: Siblings Strangers
Drug: Veh. YOH Veh. YOH
Co-Occ.
Single
Vacant
E. Strain comparison
[CORT] (ng/µl)
300
200
100
0
CD-1
C57BL/6
BTBR


A. Inbred Strains
+
100
80
60
40
20
0
% of time
Co-Occ.
Single
Vacant
Siblings
Strangers
Alone
129S1
AKR
BALB/c
BTBR
C3H/He
C57BL/6
Inbred
B. Outbred Strains
*
*
p=0.07
***
***
Crl:ICR
Tac:ICR
Crl:SW
Tac:SW
Outbred
C. "Wild" Mice
***
**
D. Fmr1-/y
***
+/+
-/y
E. Eif4e KI
**
KI/KI
Single Occ.

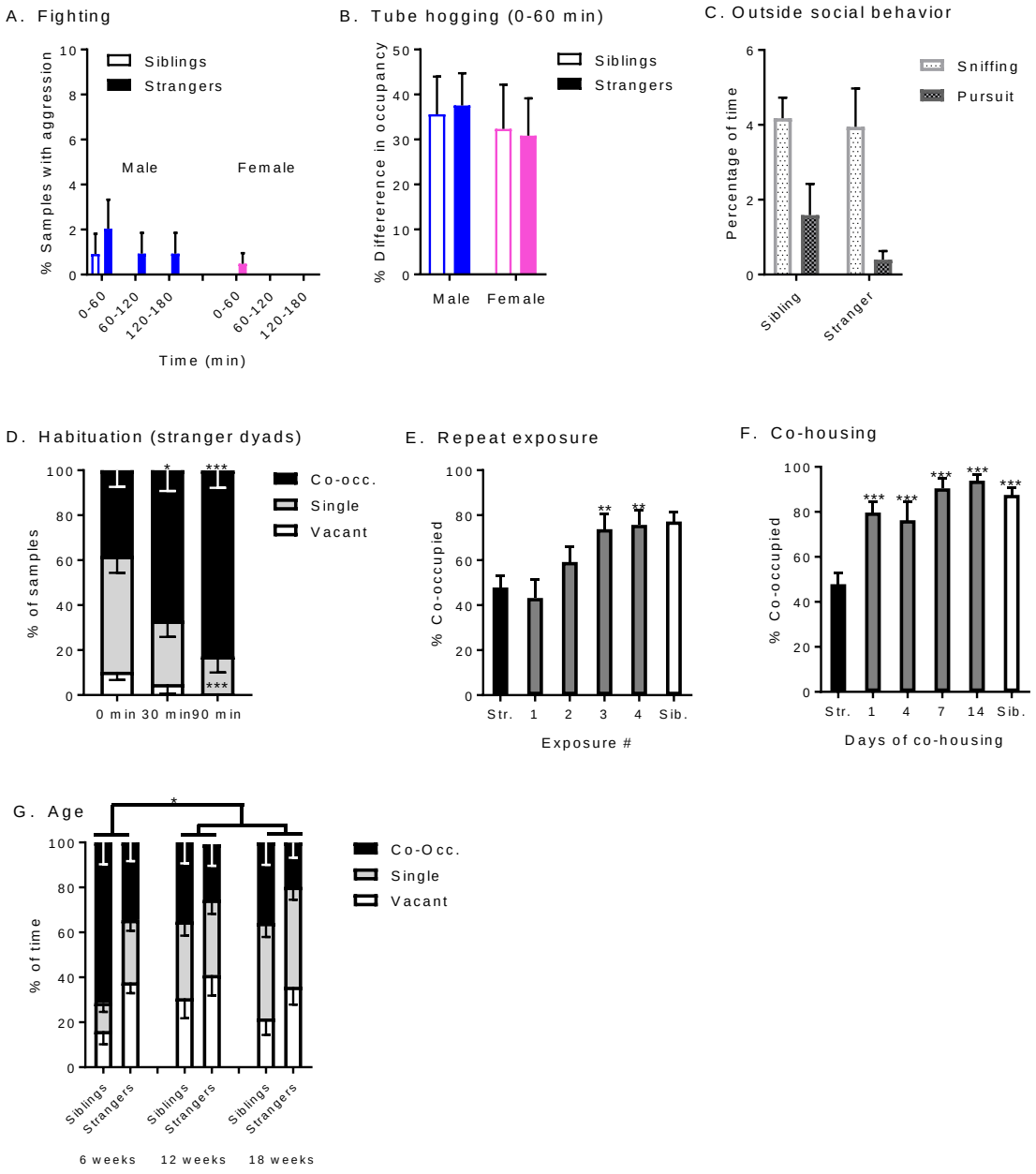



**Figure S1. TCOT behaviors and parametric considerations affecting co-occupancy in the TCOT.** Related to Figure 1. (*A*) Extremely limited fighting behavior in the TCOT. Bars represent mean ± SEM percentage of 10-s/min samples (60 samples per hour) featuring aggressive behavior (biting or scuffling) in a subset of sibling or stranger dyads; $n$=11 dyads/social condition/sex. No group at any time point displayed aggressive behavior significantly greater than zero (one-sample $t$-tests: $t_{10}$ = 1.0–1.5; 0.08<$p$<0.17). (*B*) Differential single occupancy behavior by each member of the dyad (i.e., "hogging" the tube for oneself) does not vary by sex or familiarity (main effect of sex: $F_{1,44}$ = 0.4, $p$=0.56; main effect of social condition:

$F_{1,44}$ = 0.0, $p$=0.98; sex x social condition: $F_{1,44}$ = 0.0, $p$=0.84). Bars represent mean ± SEM percentage difference in single occupancy of one mouse in the dyad compared to the other over the first hour in the TCOT (featuring the highest levels of single occupancy); $n$=11–13 dyads/social condition/sex. As can be seen, in most cases, one mouse in the dyad dominated the tube (spending ≈30% more time alone in the tube compared to the other mouse), but this did not depend on their sex or familiarity status. (*C*) Analysis of early (0–10 min) social interaction behaviors occurring outside of the tube in the TCOT arena. Bars represent mean ± SEM percentage of time engaging in anogenital sniffing and pursuit behaviors of mice in sibling versus stranger dyads; $n$=10–12 dyads/social condition. No group differences were observed in either behavior ($t_{20}$=0.2, $p$=0.84, $t_{20}$=1.3, $p$=0.22, respectively). (*D*) Habituation of stranger mice to the TCOT before co-exposure greatly increases co-occupancy behavior ($F_{2,45}$ = 7.6, $p$=0.001). Bars represent mean ± SEM percentage of samples featuring co-occupancy (co-occ.), single occupancy and a vacant tube from 60-120 min after co-exposure in stranger mice separately habituated to the TCOT for 0 min, 30 min or 90 min ($n$=16 dyads/habituation time). *$p$<0.05, ***$p$<0.001 compared to 0 min group by Dunnett's case-comparison posthoc test. (*E*) Repeat exposure of stranger dyads (Str.) to the TCOT (and each other) increases co-occupancy behavior (repeated measures: $F_{3,63}$ = 7.5, $p$<0.001) to the level displayed by sibling dyads (Sib.). Bars represent mean ± SEM percentage of samples featuring co-occupancy of mice exposed to each other 1, 2, 3 or 4 times; $n$=24 dyads. **$p$<0.01 compared to first exposure by posthoc testing with Sidak correction for multiple comparisons. (*F*) One or more days of co-housing yields sibling-like levels of co-occupancy in previously stranger mice ($F_{5,62}$ = 14.3, $p$<0.001). Bars represent mean ± SEM percentage of samples featuring co-occupancy of stranger dyads (Str.), sibling dyads (Sib.), or stranger dyads having been co-housed for 1, 4, 7 or 14 days before testing; $n$=6–12 dyads/co-housing time. ***$p$<0.001 compared to Str. group. (*G*) High levels of co-occupancy only in young adult mice (main effect of age: $F_{2,65}$ = 4.6, $p$=0.01; main effect of social condition: $F_{1,65}$ = 7.5, $p$=0.008; age x social condition: $F_{2,65}$ = 1.4, $p$=0.25). Bars represent mean ± SEM percentage of samples featuring co-occupancy (co-occ.), single occupancy and a vacant tube from 60-120 min in mice of 6, 12 or 18 weeks of age; $n$=10–13 dyads/age/social condition. *$p$<0.05 compared to 12- and 18-week-old mice. Note that although co-occupancy behavior decreased, older mice still preferred to co-occupy with siblings compared to strangers.

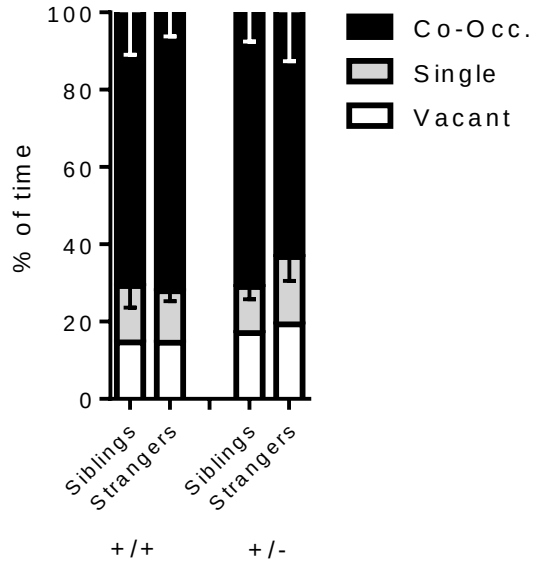



**Figure S2**. **No altered tube occupancy behavior in *Shank3*$^{+/-}$ mice.** ANOVA revealed: a main effect of genotype: $F_{1,19}$ = 0.2, $p$=0.68; main effect of social condition: $F_{1,19}$ = 0.1, $p$=0.76; genotype x social condition interaction: $F_{1,19}$ = 0.2, $p$=0.66). Bars represent mean ± SEM percentage co-occupancy in *Shank3* wildtype (+/+) and heterozygous mutant (+/-) mice; $n$=4–7 dyads/social condition/genotype. There is considerable controversy in the literature about whether these mutants do or do not display social deficits (see ref. [1]).

1. Jiang, Y-H & Ehlers, MD (2013) Modeling autism by SHANK gene mutations in mice. *Neuron* 78:8-27.