Minimal Aberrant Behavioral Phenotypes of Neuroligin-3 R451C Knockin Mice

Kathryn K. Chadman, Shiaoching Gong, Maria L. Scattoni, Sarah E. Boltuck, Shruti U. Gandhy, Nathaniel Heintz, and Jacqueline N. Crawley

Neuroligin-3 is a member of the class of cell adhesion proteins that mediate synapse development and have been implicated in autism. Mice with the human R451C mutation (NL3), identical to the point mutation found in two brothers with autism spectrum disorders, were generated and phenotyped in multiple behavioral assays with face validity to the diagnostic symptoms of autism. No differences between NL3 and their wildtype (WT) littermate controls were detected on measures of juvenile reciprocal social interaction, adult social approach, cognitive abilities, and resistance to change in a spatial habit, findings which were replicated in several cohorts of males and females. Physical and procedural abilities were similar across genotypes on measures of general health, sensory abilities, sensorimotor gating, motor functions, and anxiety-related traits. Minor developmental differences were detected between NL3 and WT, including slightly different rates of somatic growth, slower righting reflexes at postnatal days 2–6, faster homing reflexes in females, and less vocalizations on postnatal day 8 in males. Significant differences in NL3 adults included somewhat longer latencies to fall from the rotarod, less vertical activity in the open field, and less acoustic startle to high decibel tones. The humanized R451C mutation in mice did not result in apparent autism-like phenotypes, but produced detectable functional consequences that may be interpreted in terms of physical development and/or reduced sensitivity to stimuli.

Keywords: autism; mouse model; neuroligin; behavior phenotyping; social behavior

Introduction

Neuroligins and neurexins are cell adhesion proteins that have been implicated in autism [Kim et al., 2008]. Neurexins in axons bind to neuroligins in dendrites at synaptic junctions to trigger synaptic differentiation [Arac et al., 2007; Budreck & Scheiffele, 2007; Chubykin et al., 2007, 2005; Conroy, Nai, Ross, Naughton, & Berg, 2007; Craig & Kang, 2007; Varoqueaux et al., 2006]. Mutations in neuroligin-3 (NL3) are reported to shift the balance between glutamatergic and GABAergic synapses [Chih, Engelman, & Scheiffele, 2005; Chubykin et al., 2005; Levinson & El-Husseini, 2007; Sheng & Hoogenraad, 2007]. An arginine to cysteine mutation at amino acid position 451 in *NL3*, a gene located on the X-chromosome, was discovered in two Swedish brothers, one with severe autism and one with Asperger's syndrome [Jamain et al., 2003]. This point mutation causes defective neuroligin-3 protein trafficking, resulting in NL3 retention in the endoplasmic reticulum and decreased NL3 reaching the cell surface [Chubykin et al., 2005; Comoletti et al., 2004; De Jaco et al., 2006]. In contrast, a large number of association studies have not detected *NL3* mutations in autistic individuals, indicating that mutations in the neuroligin-3 gene are unlikely to account for a substantial number of cases of autism [Blasi et al., 2006; Gauthier et al., 2005; Talebizadeh et al., 2004, 2006; Vincent et al., 2004; Wermter, Kamp-Becker, Strauch, Schulte-Korne, & Remschmidt, 2008; Yan et al., 2005; Ylisaukko-oja et al., 2005]. However, multiple reports of mutations in several cell adhesion synaptic genes, including neurexins, neuroligins, *SHANK3*, and *CNTNAP2*, each in a small number of cases of autism, prompt the hypothesis that a shift in the interplay between synaptic genes during development contributes to autism spectrum disorders [Alarcon et al., 2008; Arking et al., 2008; Autism Genome Project Consortium, 2007; Durand et al., 2007; Garber, 2007; Jamain et al., 2008; Jeffries et al., 2005; Kim et al., 2008; Laumonnier et al., 2004; Lawson-Yuen, Saldivar, Sommer, & Picker, 2008; Moessner et al., 2007].

To begin to test the hypothesis that a mutation in NL3 contributes to the symptoms of autism, we generated

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knockin mice with the R451C NL3 mutation that was discovered in the two Swedish brothers [Jamain et al., 2003]. A comprehensive analysis of the behavioral phenotype of three independent cohorts of NL3 and WT males and females from two different types of matings was conducted, employing multiple tests for traits relevant to juvenile and adult social interaction, developmental milestones including vocalizations, sensory abilities, motor functions, anxiety-related behaviors, habit reversal, and control measures of general health. Since synapse formation is essential to cognitive abilities, we also evaluated NL3 and WT on two learning and memory tasks. Results indicate that NL3 R451C mice do not display autism-like behavioral phenotypes or cognitive deficits. No genotype differences were detected on physical and procedural abilities, although unusual scores on a small number of behavioral measures indicate functional outcomes of the mutation.

As we were completing a comprehensive behavioral characterization at NIMH of the line of R451C NL3 knockin mice generated at Rockefeller University, a report was published of an independently generated line of R451C NL3 knockin mice [Tabuchi et al., 2007]. Striking differences between our results and those described in Tabuchi et al. [2007] are addressed in the Discussion section below.

Methods

Mice

The humanized R451C mutation, in which the nucleotide sequence CGT in exon 6 for arginine 451 was modified to TGC for cysteine 451, was generated as described in Supplementary Methods. The targeting vector and confirmation of the conditional null allele by Southern blot analysis are shown in Figure 1, along with the initial breeding strategy.

Twenty mice (ten male and ten female) homozygous for the neuroligin-3 R451C mutation were shipped from Rockefeller University in New York, NY, to NIMH in Bethesda, MD, for behavioral testing. Homozygotes were mated with C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) to produce Cohort 1. Because the gene that codes for NL3 is located on the X-chromosome, Cohort 1 contained litters in which all females were heterozygous (Het), and males were either full mutant (NL3) or full wildtype (WT), depending on the mother. Heterozygote females from Cohort 1 were mated with either C57BL/6J or NL3 knockin males to generate Cohorts 2 and 3, consisting of WT or NL3 males and WT, heterozygous (Het), or homozygous (NL3) females. Identical behavioral tests were conducted with offspring from both breeding schemes. When tests were run with both types of cohorts, they were analyzed for the genotype of the dam. Tailsnips

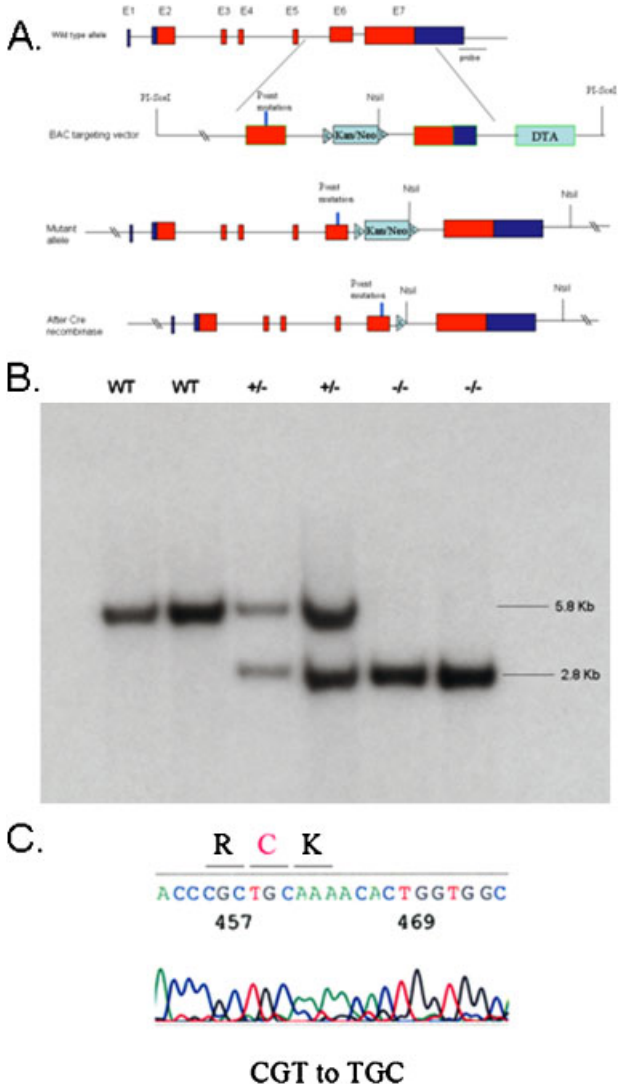

Figure 1. Gene targeting strategy and genotype analysis: (**A**) Diagram of the Nlgn3 locus with numbered exons, the BAC targeting vector with R451C mutation, the targeted Nlgn3 locus and the recombined locus after Cre-mediated excision of Kana/Neo marker cassette. PCR primers used for genotyping are noted by arrows. A floxed Kana/Neo cassette, a diphtheria toxin-negative selection cassette, and targeting arms are illustrated. The 3′ external probe used in the Southern blotting is indicated below the wildtype allele. Diagram is not to scale. (**B**) Southern blot analysis of NsiI-digested tail DNA isolated from control wildtype female, heterozygous female, and homozygous female mice. (**C**) DNA sequence of RT-PCR product. The homozygous R451C (CGT to TGC) missense mutation is indicated. RT-PCR, reverse transcriptase polymerase chain reaction.

were genotyped at Rockefeller University, as described in Supplementary Material. All mice were maintained in an NIH vivarium with a 12/12 light cycle (lights on at 7:00 a.m.) under temperature (20°C) and humidity (~48%) controlled conditions. Food and water were available ad libitum. Behavioral tests were conducted between

9:00 a.m. and 6:00 p.m. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the NIMH Animal Care and Use Committee.

Group sizes from each independent cohort used for behavioral testing were Cohort 1: 17 male WT, 12 male NL3; Cohort 2: 19 male WT, 15 male NL3, 5 female WT, 19 female Het, 4 female NL3; Cohort 3: 14 male WT, 16 male NL3, 7 female WT, 16 female Het, and 13 female NL3. Results from WT were consistent across cohorts for the same sex on the same test.

Behavioral Tests

Comprehensive behavioral phenotyping was conducted as described in Supplementary Materials. Pups were tested for developmental milestones, ultrasonic vocalizations during separation from the mother and siblings, and homing to familiar cage litter odors. Juveniles were tested for reciprocal social play and open field exploratory locomotion. Adults were tested for social approach in an automated three-chambered sociability apparatus, elevated plus maze and light↔dark anxiety-related behaviors, general health and home cage measures including nesting, neurological reflexes, open field locomotion, rotarod coordination and balance, forepaw reaching for vision, acoustic startle threshold for hearing, prepulse inhibition (PPI) of acoustic startle for sensorimotor gating, hot plate and tail flick for pain sensitivity, contextual and cued fear-conditioned learning and memory, and Morris water maze spatial learning and memory acquisition and reversal, as previously described [Bailey, Pavlova, Rohde, Hohmann, & Crawley, 2007; Crawley, 2007; Crawley & Goodwin, 1980; Crawley et al., 2007; McFarlane et al., 2008; Moy et al., 2007; Scattoni et al., 2008; Yang, Zhodzishsky, & Crawley, 2007) and described in detail in the Supplementary Methods.

Results

Juvenile Social Interaction

Figure 2A–D documents no significant differences between male genotypes for (A) follow ($F_{2,\ 28} = 0.157$, NS), (B) nose-to-nose sniff ($F_{2,\ 28} = 0.087$, NS), (C) push–crawl–touch ($F_{2,\ 28} = 2.031$, NS), or (D) social groom ($F_{2,\ 28} = 0.337$, NS). As shown in Supplementary Figure 1 (1S), there were also no significant differences between the genotypes for (A) nose-to-anogenital sniff ($F_{2,\ 28} = 1.01$, NS), (B) self-groom ($F_{2,\ 28} = 0.969$, NS), (C) jump ($F_{2,\ 28} = 3.127$, NS), and (D) exploration duration ($F_{2,\ 20} = 0.847$, NS).

Adult Social Approach

Figure 2, panels E–F, illustrates high levels of sociability for both NL3 and WT male mice. There was a significant effect of chamber time ($F_{1,\ 56} = 47.57$, $P < 0.0001$) but not of genotype ($F_{1,\ 56} = 0.04$, NS) or interaction of genotype and chamber time ($F_{1,\ 56} = 3.28$, NS). Both genotypes spent more time in the chamber with the stranger mouse than in the chamber with the novel object: WT ($F_{33,\ 1} = 18.778$, $P < 0.001$) and NL3 ($F_{23,\ 1} = 26.061$, $P < 0.0001$). There was a significant effect of sniff time ($F_{1,\ 56} = 90.87$, $P < 0.0001$) but not of genotype ($F_{1,\ 56} = 0.64$, NS) or interaction of genotype and sniff time ($F_{1,\ 56} = 3.34$, NS). Both genotypes spent more time sniffing the stranger mouse than the novel object: WT ($F_{1,\ 33} = 56.55$, $P < 0.0001$) and NL3 ($F_{1,\ 23} = 36.10$, $P < 0.0001$). There were no significant differences in exploration during the 10 min habituation period (Figure 2SA). There were no significant differences of entries ($F_{1,\ 56} = 0.24$, NS), genotype ($F_{1,56} = 0.90$, NS), or interaction of genotype and entries ($F_{1,56} = 0.39$, NS) for the number of entries into the chamber with the novel object vs. entries into the chamber with the stranger mouse for either genotype (Figure 2SB): WT ($F_{1,\ 23} = 0.451$, NS) and NL3 ($F_{1,\ 23} = 0.124$, NS), suggesting that there were no locomotor impairments or differential levels of general exploration that could have affected the social approach scores. Results for the preference for a social novelty phase, a control for social olfactory abilities, did not show any genotype differences and are presented in the Supplemental Material Figure 2S, panels C–E. Similar results obtained in the females are shown in Figure 3S, panels A–G.

Morris Water Maze Acquisition and Reversal

Acquisition. Figure 3, panels A–D (left side of graphs), illustrates performance on acquisition in the Morris water maze spatial learning and memory task, and reversal of the original position habit in the Morris water maze. During acquisition, male WT and NL3 mice similarly improved across day of training for latency to reach the hidden platform ($F_{4,\ 216} = 64.84$, $P < 0.0001$), with no main effect of genotype ($F_{1,\ 54} = 0.417$, NS) and no interaction between genotype and day ($F_{4,\ 216} = 1.094$, NS). Total distance traveled was significant for day of training ($F_{4,\ 216} = 31.064$, $P < 0.0001$) and for genotype ($F_{1,\ 54} = 4.378$, $P < 0.05$) but not for the interaction of genotype × day of training ($F_{4,\ 216} = 0.939$, NS). Swim speed showed a significant main effect of day of training (Figure 4S, panel A): ($F_{4,\ 216} = 7.731$, $P < 0.0001$) but not of genotype ($F_{1,\ 54} = 2.24$, NS) or interaction of genotype × day of training ($F_{4,\ 216} = 0.104$, NS). However, as shown in Figure 4S, panel B, a transient genotype difference was detected on thigmotaxis, a measure of distance from the perimeter wall during swimming. Thigmotaxis showed a significant main effect of day of training ($F_{4,\ 216} = 52.865$, $P < 0.0001$), and also of genotype ($F_{1,\ 54} = 4.291$, $P < 0.05$) and the interaction of genotype × day of training ($F_{4,\ 216} = 5.707$, $P < 0.001$). Newman–Keuls' post hoc analysis determined that

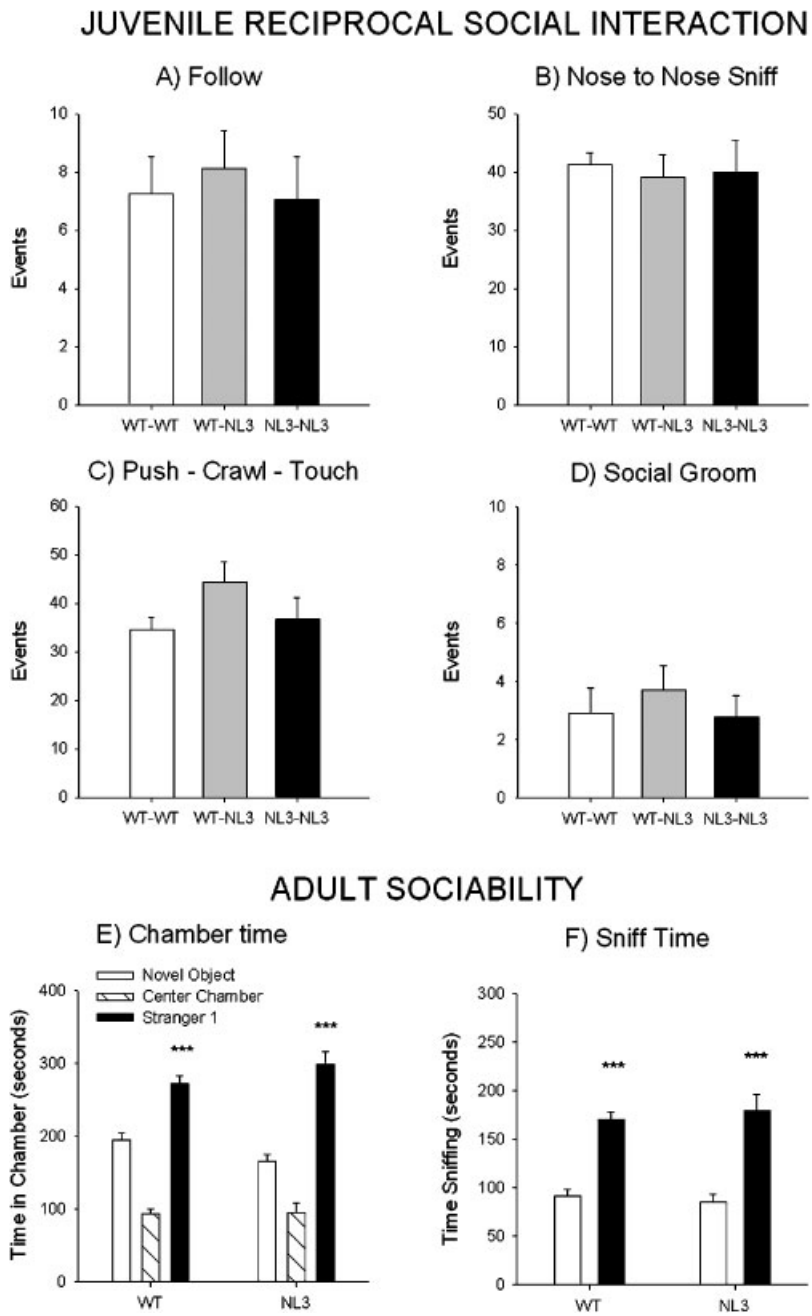

Figure 2. Juvenile social interaction and adult social approach in male neuroligin-3 (NL3) mutants and their wildtype (WT) littermate controls: No significant differences were detected between the genotype pairs on the number of bouts (events) for any of the reciprocal social interaction parameters: (**A**) follow; (**B**) nose-to-nose sniff; (**C**) push–crawl–touch; and (**D**) social groom. $N = 13$ pairs of WT–WT, $N = 9$ pairs of NL3–WT, and $N = 9$ pairs of NL3–NL3. Both genotypes displayed sociability as measured by social approach parameters in the automated three-chambered social approach test in adult males: (**E**) Both the WT and NL3 mice spent more time in the chamber with the stranger mouse as compared to time in the chamber with the novel object. (**F**) Both the WT and NL3 mice spent more time sniffing the stranger mouse as compared to the novel object. $N = 34$ WT, $N = 24$ NL3. In Figures 1–5, all data are from male mice and are shown as mean $\pm$ standard error of the mean, and $^{*} = P < .05$, $^{**} = P < .01$, $^{***} = P < .001$, for the comparison of NL3 and WT. Additional behavioral parameters for male mice, results for female mice, complete methods, tables and graphs, and procedures are located in Supplementary Material, as indicated in the text.

thigmotaxis was significantly different between the WT and NL3 mice on day 1 ($P < 0.01$). Probe trial results revealed that all genotypes spent significantly more time in the trained quadrant than in the other three quadrants ($F_{3,\ 165} = 28.84$, $P < 0.0001$, post hoc all comparison P-values < 0.05) and made significantly more platform

crossings over the former location of the hidden platform than over the comparable locations in the other three quadrants ($F_{3,165} = 32.10$, $P < 0.0001$, post hoc all comparison P-values < 0.05), demonstrating that both the WT and NL3 mice successfully learned the location of the hidden platform using distal spatial cues.

Reversal. Data from the reversal phase of the Morris water maze are shown in Figure 3, panels A–D (right side of graphs). During reversal training both WT and NL3 mice improved across day of training in latency to reach the hidden platform ($F_{4,\ 220} = 40.065$, $P < 0.0001$), but there was no main effect of genotype ($F_{1,55} = 1.05$, NS) and no genotype × day of training ($F_{4,220} = 0.956$, NS). Total distance traveled showed a significant main effect of day of training ($F_{4,220} = 36.863$, $P < 0.0001$) but not of genotype ($F_{1,\ 55} = 2.59$, NS) or genotype × day of training ($F_{4,220} = 0.51$, NS). Swim speed was significant for day of training ($F_{4,\ 220} = 7.68$, $P < 0.0001$) but not genotype ($F_{1,55} = 2.55$, NS) or genotype × day of training ($F_{4,220} = 0.48$, NS). Thigmotaxis (Figure 4SB) was significant for day of training ($F_{4,\ 220} = 3.00$, $P < 0.05$) but not for genotype ($F_{1,\ 55} = 2.86$, NS) nor genotype × day of training ($F_{4,\ 220} = 0.53$, NS). Probe trial testing revealed that all genotypes spent significantly more time (all comparison P-values < 0.05) in the reversal training quadrant as compared with the left and right adjacent quadrants. However, time spent in the opposite quadrant, where the hidden platform was originally located, was similar to time spent in the new quadrant where the hidden platform was located during the reversal training trials ($F_{3,165} = 14.25$, $P < 0.0001$, post hoc comparison for left and right quadrant P-values < 0.05, opposite quadrant NS). The WT group made significantly more platform crossings in the trained quadrant than in the other three quadrants during the reversal probe trial ($F_{3,\ 93} = 12.58$, $P < 0.0001$, post hoc all comparison P-values < 0.05). While the NL3 group showed a significant effect of platform crossings ($F_{3,72} = 3.48$, $P < 0.05$), the NL3 group crossed the reversal training platform significantly more than the right platform location but not any of the other platform locations.

Contextual and Cued Fear Conditioning

As shown in Figure 3E, no genotype differences were detected on contextual or cued fear conditioning in the standard delay learning and memory emotional task in male NL3 and WT mice. In the training phase, significantly more freezing was apparent during the 2 min after the cue–shock pairings than in the 2 min before the cue–shock pairings ($F_{1,59} = 92.39$, $P < 0.0001$). There was no main effect of genotype on freezing during training ($F_{1,\ 59} = 3.10$, NS), and no genotype × cue–shock pairing interaction ($F_{1,59} = 2.72$, NS). On the next day, when trained mice were placed back in the identical context, but with no footshock administered, there was no main effect of genotype on the levels of freezing ($F_{1,60} = 0.31$, NS). On the subsequent day, when trained mice were placed in a novel environmental context and given the auditory tone that had been paired with footshock during training, both genotypes displayed some freezing to the novel context and similarly high levels of freezing to the auditory cue ($F_{1,60} = 156.58$, $P < 0.0001$), with no significant differences between genotypes ($F_{1,60} = 0.10$, NS), and no interaction of cue × genotype ($F_{1,\ 60} = 0.41$, NS).

Pup Ultrasonic Vocalizations to Separation from the Mother and Siblings

Baseline measurements of ultrasonic vocalizations revealed a different profile of emission between male NL3 and WT at one time point during the first two postnatal weeks (Fig. 4B). On pnd 8, WT pups emitted significantly more calls (genotype: $F_{1,27} = 3.47$, $P = 0.07$; day: $F_{3,81} = 6.79$, $P < 0.001$; genotype × day interaction, $F_{3,81} = 2.85$, $P = 0.04$, pnd 8: $P < 0.001$), indicating a temporally shifted developmental profile of these distress calls. Duration, peak frequency, and amplitude of the calls did not differ between groups (duration, $F_{1,\ 27} = 1.61$, NS; peak frequency, $F_{1,\ 27} = 1.75$, NS; peak amplitude, $F_{1,27} = 0.80$, NS; Figure 5S, panels A–C). No differences were detected between the body temperatures of WT and NL3 mice ($F_{1,\ 27} = 0.01$, NS) as measured after each separation test. Female pups showed no significant genotype differences on any of the vocalization parameters (Figure 5S, panels D–G).

Homing Test (Postnatal 9)

In the homing test (Figure 6S), which measures the tendency of pups placed in a novel arena to move toward a familiar social odor, i.e. a location containing nesting material from the home cage, no significant effect of genotype was found in males on latency to reach the area containing the nest litter ($F_{1,\ 30} = 0.26$, NS) or time spent over the area containing the nest litter ($F_{1,\ 30} = 0.05$, NS). No significant effect of genotype was detected on general locomotor activity, as measured by the number of line crossings in the arena ($F_{1,\ 30} = 2.85$, NS; data not shown). Females (Figure 6S, panels C–D) displayed a significant genotype effect on latency to reach the area containing the nest litter (analysis of variance, ANOVA $F_{2,\ 33} = 3.81$, $P = 0.03$; Kruskal–Wallis $P = 0.02$). Post hoc comparisons revealed that female NL3 pups showed shorter latencies to reach the nest area in comparison with WT pups ($P < 0.01$). Female NL3 pups spent significantly more time in the nest area ($F_{2,\ 33} = 4.24$, $P = 0.02$, $P = 0.01$ for NL3 vs. WT). ANOVA did not detect a significant genotype effect on general locomotor activity, indicating that the three genotypes had similar activity levels in the homing arena ($F_{2,\ 33} = 2.60$, NS, data not shown).

Open Field Exploration

Figures 5 panels A and B and 8S, panels A and B, show open field exploratory locomotion in adult male WT and

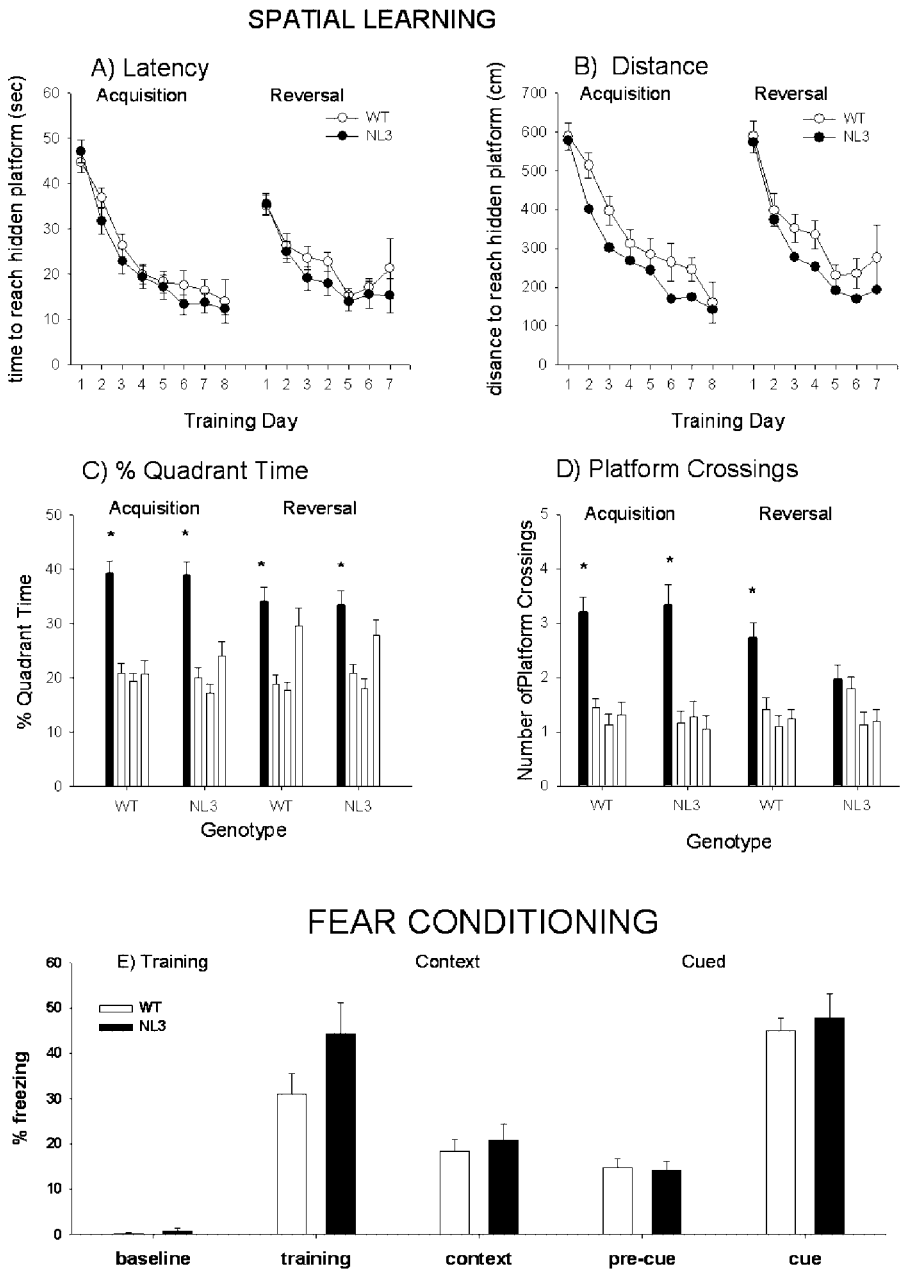

Figure 3. Morris water maze spatial learning and reversal, and contextual and cued fear conditioning measures of cognitive abilities in (wildtype) WT and NL3 knockin mice: (**A**) WT and NL3 displayed similar latencies to locate the hidden platform for both acquisition and reversal of the Morris spatial navigation task. (**B**) NL3 mice swam a shorter distance to find the hidden platform during acquisition but there was no genotype difference in the distance swum during reversal. (**C**) Both WT and NL3 spent more time in the previously trained quadrant (black bars) than the other three quadrants (white bars) during the probe trial following the initial acquisition phase. Both WT and NL3 spent more time in the previously trained quadrant than the left and right quadrants, but not the opposite quadrant, during the probe trial following the reversal phase. (**D**) Both WT and NL3 crossed the platform in the previously trained quadrant (black bars) significantly more than the platform locations in the other three quadrants (white bars) during the probe trial following the acquisition phase. WT crossed the platform in the previously trained quadrant (black bars) significantly more than the platform locations in the other three quadrants (white bars) during the probe trial following the acquisition phase, but NL3 crossed the platform in the trained quadrant more than the corresponding location in the quadrant to the right, but not the left and opposite locations, during the probe trial following the reversal phase. $N = 31$ WT, $N = 25$ NL3. (**E**) Contextual and cued fear conditioning. WT and NL3 did not differ in freezing, a species-specific fear response measured as the amount of time spent immobile, before training, and following training. WT and NL3 did not differ in freezing to the identical context 24 hr after training. WT and NL3 did not differ in freezing to a novel context 48 hr after training, either in the absence (pre-CS) or presence of the conditioned auditory cue. $N = 38$ WT, $N = 24$ NL3.

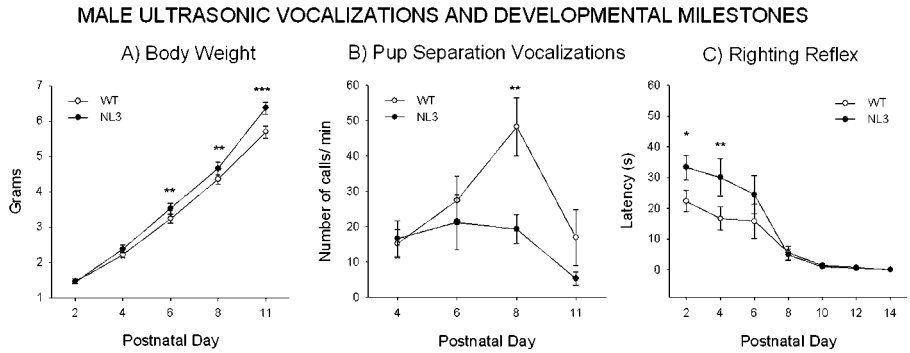

Figure 4. Ontogenetic profile at postnatal (pnd) ages 2–14 days of age: (**A**) Body weight (pnds 2–11); (**B**) ultrasonic vocalizations (pnds 4–11); and (**C**) righting reflex (pnds 2–14) in WT and NL3 male pups. NL3 pups had significantly higher body weights from pnd 6 through pnd 11. NL3 emitted fewer ultrasonic vocalizations at pnd 8 than WT. $N = 16$ WT, $N = 13$ NL3. Differences in the righting reflex were noted in NL3 pups displaying slower righting reflex latencies as compared with WT pups at pnds 2, 4, and 6, which normalized by pnd 8. $N = 16$ WT, $N = 16$ NL3.

NL3. Both groups were more active at the beginning of the test as compared with the end. Figure 5B illustrates a genotype difference in vertical activity ($F_{1,\ 58} = 8.40$, $P < 0.01$) and of a 5 min bin ($F_{5,290} = 5.43$, $P < 0.0001$) but no interaction of the genotype and 5 min bin ($F_{5,290} = 0.98$, NS), indicating that the NL3 made fewer upright rears. There was a significant effect of time on horizontal activity ($F_{5,295} = 56.76$, $P < 0.0001$; Figure 8S, panel A) and interaction of genotype by 5 min bin ($F_{5,295} = 5.43$, $P < 0.0001$) but no effect of genotype ($F_{1,59} = 0.099$, NS). Newman–Keuls' post hoc analysis did not detect any significant differences between the genotypes on any specific day ($P > 0.05$). There was a significant effect of 5 min time bins on total distance ($F_{5,295} = 75.87$, $P < 0.0001$) but no effect of genotype ($F_{1,59} = 0.001$, NS) or interaction of genotype by 5 min bin ($F_{5,295} = 2.02$, NS; Figure 5A). There was no effect of genotype on % center time ($F_{1,\ 95} = 0.175$, NS; Figure 8S, panel B) or interaction of genotype × 5 min bin ($F_{5,295} = 0.798$, NS), but there was a significant effect of 5 min bin ($F_{5,295} = 6.05$, $P < 0.0001$). The majority of the open field results were similar in the females, although genotype differences were detected in males but not in females on vertical activity (Figure 8S, panels C–F).

Accelerating Rotarod

Adult male NL3 mice displayed better performance on the rotarod than WT, as seen by longer latencies to fall (Figure 5C). There was a significant effect of genotype ($F_{1,32} = 8.31$, $P < 0.01$), day of training ($F_{2,64} = 52.70$, $P < 0.0001$), trial ($F_{2,64} = 23.11$, $P < 0.0001$), and interaction of day and trial ($F_{4,128} = 4.88$, $P < 0.01$) but no interaction of genotype × day of training ($F_{2,64} = 0.50$, NS), trial ($F_{2,\ 64} = 0.34$, NS), or day × trial ($F_{4,128} = 1.09$, NS). No genotype differences were found in the females (Figure 9S).

Acoustic Startle, Acoustic Startle Threshold, and PPI of Acoustic Startle

Adult male NL3 showed less startle response to sudden loud acoustic stimuli than WT (Figure 5D). There was a significant effect of genotype ($F_{1,\ 32} = 21.55$, $P < 0.0001$), trial type ($F_{5,160} = 66.58$, $P < 0.0001$), and interaction of genotype and trial type ($F_{5,\ 160} = 13.66$, $P < 0.0001$). Newman–Keuls' post hoc examination revealed significant differences between the genotypes on the 100, 110, and 120 dB trials ($P < 0.05$). Both genotypes showed minimal startle at 80 dB, suggesting similar acoustic thresholds. A separate cohort of NL3 mice showed a lower response to a 110 dB acoustic startle stimulus ($F_{1,47} = 6.79$, $P < 0.05$; Figure 5E) but normal PPI ($F_{1,47} = 2.58$, NS; Figure 5F). PPI increased with prepulse intensity up to 86 dB regardless of genotype ($F_{4,188} = 46.81$, $P < 0.0001$). There was no interaction between genotype and prepulse intensity ($F_{4,188} = 0.86$, NS). Similar results for females on acoustic startle and PPI are shown in Figure 10S, panels A–C.

General Health Measures

NL3 and WT were similar on almost all measures of general health, reflexes, and sensory function (Table I). General health included body weight, temperature, fur condition, body and limb tone. Empty cage observations included transfer freezing, wild running, stereotypies, exploration, and self-grooming. Motor reflexes included passivity, wire hang, grip strength, and trunk curl. The only genotype difference in males was detected in the wire hang where the WT hung on longer than the NL3 mice. The only genotype differences in females were on body weight (Table S1) and forepaw width in the footprint test (Table S2). General reactivity was assessed with petting escape, struggling and/or vocalizations, and

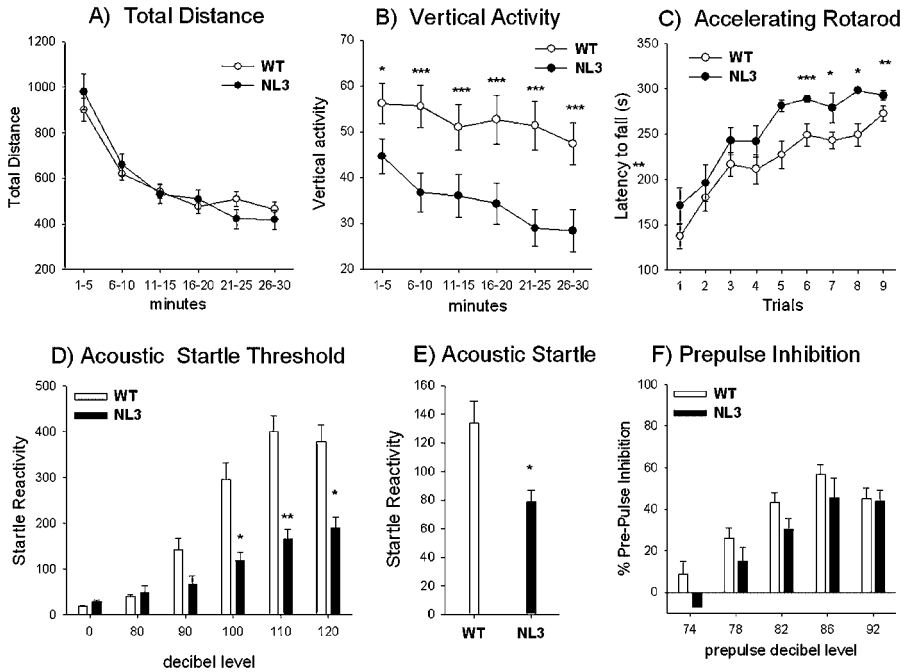

Figure 5. Motor performance, sensorimotor gating, and startle activity. (**A**) Open field. NL3 overall displayed significantly less vertical activity than WT but there were not genotype differences on. (**B**) total distance throughout the 30 min automated open field session. N = 36 WT, N = 25 NL3. Rotarod. (**C**) NL3 showed longer latencies to fall from the accelerating rotarod than WT across all trials. N = 19 WT, N = 15 NL3. (**D**) NL3 mice had significantly less startle response as compared to WT in the acoustic startle threshold test at the 100, 110 and 120 dB stimuli, but no differences at lower startle levels. N = 19 WT, N = 15 NL3. (**E**) Within the prepulse inhibition session, another cohort of NL3 mice similarly showed less startle to 110 dB than WT. (**F**) No genotype difference was detected in prepulse inhibition of acoustic startle. N = 30 WT, N = 19 NL3.

dowel biting. There were no significant differences between the genotypes. Observations of home cage behaviors in the vivarium indicated that NL3 mutants were normal on home cage activity, nest building, huddling, and grooming.

At young ages, body weight differed significantly between male WT and NL3 and between female genotypes (Fig. 4A, 7SA). ANOVA results showed that somatic growth rates differed between groups (genotype effect, $F_{1,27} = 3.67$, $P = 0.07$; days, $F_{4,108} = 1{,}098.27$, $P < 0.0001$; genotype × days interaction, $F_{4,108} = 5.16$, $P < 0.0008$). Post hoc comparisons revealed that NL3 pups had a significantly greater weight increase starting from pnd 6 through pnd 11 ($P < 0.001$ for NL3 vs. WT, Fig. 4A). In contrast with male data, NL3 female pups showed a significantly lower body weight on pnd 6, 8, and 11 (genotype effect, $F_{2,31} = 2.15$, NS; days, $F_{4,124} = 1{,}167.36$, $P < 0.0001$; genotype × age interaction, $F_{8,124} = 2.13$, $P = 0.03$; $P < 0.001$ for NL3 vs. WT; Figure 7SA). The same profile was seen for Het pups starting from pnd 8 to 11 ($P < 0.05$ for Het vs. WT).

No significant differences in body length ($F_{1,27} = 0.06$, NS), length of the tail ($F_{1,27} = 0.54$, NS), eyelid opening ($F_{1,27} = 3.13$, NS), incisors eruption ($F_{1,27} = 2.97$, NS), and fur development ($F_{1,27} = 0.82$, NS) between WT and NL3 were observed. Almost all reflex measurements were similar between the pup groups, including negative geotaxis ($F_{1,27} = 0.29$, NS), cliff aversion ($F_{1,27} = 0.72$, NS), forelimb stick grasp reflex ($F_{1,27} = 1.03$, NS), forelimb placing reflex ($F_{1,27} = 0.01$, NS), level and vertical screen test ($F_{1,27} = 1.32$, NS; $F_{1,27} = 0.26$, NS), and bar holding ($F_{1,27} = 0.18$, NS) (data not shown).

Transient delays in righting reflex were noted in NL3 compared with WT pups, leading to a different pattern of acquisition of the righting reflex (genotype × day interaction, $F_{6,180} = 2{,}18$, $P = 0.04$) (Fig. 4C). Newman–Keuls' post hoc analysis revealed that the righting reflex was significantly slower in the NL3 than in the WT on days 2 and 4 ($P < 0.05$ and 0.01, respectively), although both genotypes displayed the full righting reflex by day 8, indicating that the subsequent ontogenetic progression was similar in the two genotypes. Female NL3 showed similarly slower righting reflex latencies at earlier postnatal days, but no genotype differences in subsequent ontogenetic progression (Figure 7S, Panel B).

Anxiety-Related Traits

As described in the Supplementary Results and shown in Figure 11S, genotypes were generally similar on both the

Table I. General Health and Neurological Screening of Male R451C Neuroligin 3 Knockin (NL3) and Their Wildtype Littermate Controls (WT) at Age 5–12 Weeks

Genotypes	WT $N = 38$	NL3 $N = 25$	*P*-value
General health			
Body weight (g)	26.0 ± .67	24.5 ± .77	NS
Body temperature (°C)	38.1 ± .10	38.2 ± .11	NS
Fur Condition (3-point scale)	2	2	NS
Bald patches (%)	0	8	NS
Missing whiskers (%)	0	0	NS
Piloerection (%)	0	0	NS
Body tone (3-point scale)	2	2	NS
Limb tone (3-point scale)	2	2	NS
Empty cage behavior			
Transfer freezing (%)	8	8	NS
Wild running (%)	0	4	NS
Stereotypies (%)	0	0	
Exploration (3-point scale)	1.92	1.92	NS
Grooming (3-point scale)	2.18	2.4	NS
Motoric abilities			
Positional passivity (%)	42	28	NS
Wire hang (sec)	59.13 ± 0.65	52.99 ± 3.170	< 0.05
Grip strength (force)	113 ± 3.3	110 ± 3.8	NS
Curl (%)	97	100	NS
Reflexes			
Forepaw reach (%)	100	100	NS
Righting reflex (%)	100	100	NS
Corneal (%)	95	96	NS
Pinna (%)	100	100	NS
Vibrissae (%)	100	100	NS
Reactivity			
Petting escape (%)	45	32	NS
Struggle/vocalization (%)	53	36	NS
Dowel biting (3-point scale)	0.61	0.52	NS

Data are expressed as percentages, 3-point rating scales, or mean ± standard error of the mean. No genotype differences were detected.

elevated plus-maze and light ↔ dark transitions anxiety-related tests.

Tactile Sensory Abilities, Grooming, and Gait

Sensory abilities in adult males showed no significant effects of genotype (Table II). NL3 and WT were similar on latency to respond to thermal stimuli during testing in the hot plate ($F_{1,59} = 0.06$, NS) and tail flick ($F_{1,59} = 0.77$, NS) tests, on olfactory ability to locate buried food ($F_{1,71} = 2.23$, NS), and on time spent grooming in an empty cage ($F_{1,37} = 1.54$, NS). There was no significant effect of genotype on gait, as measured by forepaw width ($F_{1,32} = 3.58$, NS), hindpaw width ($F_{1,32} = 1.13$, NS), or stride length ($F_{1,32} = 1.30$, NS) in the footprint test.

Discussion

Comprehensive behavioral phenotyping of multiple cohorts of neuroligin-3 R451C knockin mice and their littermate controls revealed that the NL3 mutation did not significantly affect reciprocal social interactions in juveniles or social approach in adults. No perseveration was detected in any genotype on the ability to reverse a spatial position habit in the water maze. The great majority of measures of developmental milestones, pup separation vocalizations, general health, home cage behaviors, reflexes, sensory abilities including olfaction and nociception, and motor functions including locomotion, balance, and gait, were normal in NL3 male and female mice. Taken together, these corroborating results do not support the hypothesis that an R451C point mutation in NL3 produces behaviors in mice that are analogous to the first and third diagnostic symptoms of autism, abnormal reciprocal social interactions and repetitive behaviours with restricted interests. Furthermore, generally similar scores in NL3 and WT on the initial acquisition of the location of a hidden platform using distal environmental cues in the Morris water maze spatial task, along with similar scores on emotional learning and memory in contextual and cued fear conditioning, do not support the hypothesis that the R451C NL3 mutation directly affects learning and memory.

Table II. Measures of Sensory Abilities in Male Neuroligin 3 Knockin (NL3) and Wildtype (WT) Littermates

Genotypes	WT	NL3	*P*-Value
Pain sensitivity	$N = 38$	$N = 25$	
Hot plate (latency, sec)	8.90 ± 0.44	9.08 ± 0.62	NS
Tail flick (latency, sec)	2.02 ± 0.09	2.16 ± 0.13	NS
Olfactory sensitivity	$N = 17$	$N = 13$	
Buried food (latency to eat, sec)	35.44 ± 14.24	83.77 ± 30.31	NS
Self-grooming	$N = 24$	$N = 15$	
Time spent grooming (sec)	72.38 ± 6.1	89.64 ± 14.8	NS
Footprint test	$N = 19$	$N = 15$	
Forepaw width (cm)	1.80 ± 0.08	1.99 ± 0.05	NS
Hindpaw width (cm)	2.90 ± 0.08	2.78 ± 0.07	NS
Stride length (cm)	5.00 ± 0.15	4.76 ± 0.15	NS

Data are expressed as mean ± standard error of the mean. No genotype differences were detected.

Our findings of generally normal developmental milestones, physical attributes, simple procedural abilities, and complex behavioral traits in NL3 may be viewed in the broader context of the multiple neurexin and neuroligin proteins and the specific binding of their splice variants during synaptic formation [Budreck & Scheiffele, 2007; Chih et al., 2005; Chubykin et al., 2005, 2007; Conroy et al., 2007; Craig & Kang, 2007; Levinson & El-Husseini, 2007; Sheng & Hoogenraad, 2007; Varoqueaux et al., 2006]. Reduction in the cell surface expression of the neuroligin-3 protein may not be sufficient to affect a large number of synapses relevant to social and cognitive abilities, if other neuroligins

remain available. However, this does not appear to be the case for neuroligin-4, as an elegant study of behavioral phenotypes in neuroligin-4 knockout mice reported reduced social interactions and ultrasonic vocalizations [Jamain et al., 2008].

The present findings are at variance with some of the results and interpretations of behavioral phenotypes in another line of NL3 R451C mice [Tabuchi et al., 2007]. Both lines were normal on measures of general health, motor functions, and anxiety-related behaviors. However, social deficits and improved learning were reported in the other line [Tabuchi et al., 2007]. A careful examination of the social behavior results shown in the previous report [Fig. 5; Tabuchi et al., 2007] reveals that the NL3 R451C mutant did not differ from their WT controls on most measures of social behaviors. The first social task reported, shown in Tabuchi et al., Figure 5A and B, employed separate sequential test sessions. The subject mouse was first given 5 min to explore a contained novel object, and then given 5 min to explore a contained novel mouse. Unlike the many excellent and well-validated assays of social interaction used routinely by behavioral neuroscientists and behavioral neuroendocrinologists, this design of separate sequential opportunities is not a validated measure of interest in a social partner. Too many other factors could influence time spent with the novel mouse, such as generally increased exploration or heightened interest in the wire mesh container during the second 5 min.

Tabuchi et al., Figure 5C, employed the three-chambered social approach task which we originally developed and validated [McFarlane et al., 2008; Moy et al., 2007; Nadler et al., 2004; Yang et al., 2007]. The habituation and test sessions were performed using methods consistent with the ones in the present report. However, the statistical analyses were improperly conducted, using only pairwise *t*-tests. In fact, the data in Figure 5C of Tabuchi et al. [2007] appear to show that both genotypes spent more time with the novel mouse than with the novel object. It seems likely that both strains exhibited significant sociability, if properly compared with ANOVA statistics. This evidence for significant sociability in the NL3 group argues against a social deficit in the NL3 line tested in Tabuchi et al.

Further, in the conventional test for initial reciprocal social interactions between unfamiliar pairs of freely moving mice, there was no difference between WT and NL3 in the previous report [Fig. 5, panel D, Tabuchi et al., 2007], consistent with the lack of genotype differences between WT and NL3 on reciprocal social interactions during the juvenile test session in the present findings. The test of direct social interaction in Tabuchi et al. employed only a 2 min session, which is considerably shorter than social interaction tests used in the great majority of behavioral neuroscience assays. Further, the single parameter shown in Figure 5D, time spent in interaction, is a very gross composite measure. Most behavioral neuroscientists use a large number of specific parameters to characterize direct social interaction, as shown in our Figures 2 and 1S. The data shown in Tabuchi et al., Figure 5D, left bars, clearly demonstrate that both genotypes showed the same amount of social interaction. This lack of genotype difference in their best measure of social behaviors supports an interpretation that their R451C NL3 knockins display normal social behaviors.

The data shown in Tabuchi et al., Figure 5D, right bars, represent a social memory task. Again, the statistical analysis was incorrectly conducted, using a pairwise *t*-test. As a result, a misleading interpretation was given. Given the standard error bars of Figure 5D, it appears unlikely that there was a genotype difference in social memory if the proper ANOVA statistical analysis had been conducted.

Faster learning curves on both the initial acquisition and reversal learning in the Morris water maze were reported for one cohort of NL3 in Tabuchi et al. [2007] but not seen in the two large cohorts tested in the present studies, although the distance traveled during acquisition was somewhat shorter in NL3 during the initial acquisition in both the present study and in Tabuchi et al. [2007]. Different techniques for generating the knockin mutation, and different genetic backgrounds for the two NL3 lines, C57BL/6J in the present line from the Heintz laboratory vs. a mixed C57BL/6J and 129/ImJ in the line from the Sudhof laboratory, along with methodological differences between the two laboratories in conducting the cognitive assays, may have contributed to the divergent results on spatial learning.

In the present studies, NL3 differed from their WT littermates on several minor but potentially interesting developmental measures. NL3 mice displayed slower righting reflexes during the first few days after birth, emitted fewer ultrasonic vocalizations at postnatal day 8, varied from WT in body weights, engaged in less vertical activity in the open field, had longer latencies to fall from the rotarod, and spent less time near the walls in the Morris water maze. Females displayed more homing behaviors and a wider stance in the footprint test. Both males and females responded less strongly to acoustic startle stimuli. The lower startle response is not necessarily indicative of impaired hearing, as acoustic thresholds were similar across genotypes. In general, electrophysiological recordings during the startle response indicate no direct correlation between hearing and amplitude of startle response [Willott, Kulig, & Satterfield, 1984]. It remains possible that the few significant differences detected were simply the outcome of a large number of statistical comparisons. However, it seems likely that the several minor genotype differences, taken together,

represent small developmental abnormalities, and/or decreased arousal levels in response to sensory and motor stimuli, caused by the NL3 mutation.

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SUPPLEMENTARY MATERIAL
Chadman et al., Neuroligin-3 mice

Supplementary Methods

Generation of the R451C mutation in mice

A BAC targeting vector was constructed by using a genomic fragment comprising exons 6 to 7 of the neuroligin-3 gene from C57BL/6J. The nucleotide sequence, CGT in exon 6 for arginine451 was modified to TGC for cysteine451. A neomycin cassette flanked by loxP sites was inserted into the downstream of exon 6. A diphtheria toxin negative selection cassette was cloned into the 3' end of the vector. The linearized vector was electroporated into C2J ES cells (derived from a C57BL/6J line) and neomycin resistant clones were screened for homologous recombination by PCR and further confirmed by Southern blotting of EcoRI or Nsil-digested DNA using 3' external probes. The mutation was confirmed by direct sequencing of exon 6 after PCR amplification. Germline transmission was verified by Southern blotting using 3' external probes. In addition, the presence of the mutation was confirmed by direct sequence analysis after PCR amplification of tail DNA.

Chimeric founders were bred with wild type C2J mice. Offspring (F1) with the coat color characteristic for the ES background were tested for germ line transmission by PCR and further confirmed by Southern blot analysis. In order to remove the positive selection marker, 8 week old positive F1 mice were then bred with mild Cre deleter (EllaCre) transgenic mice (Lakso et al., 1996; Bunting et al., 1999; Xu et al., 2001; Plagge et al., 2004) which were in a C57BL/6 background. Offspring (F2) mice with partial and mosaic Cre-recombination patterns were then bred with C57BL/6 wildtype mice. The offspring (F3) mice containing the desired conditional null allele were identified by Southern blot analysis. Segregation of the targeted gene and Cre-recombinase alleles were also monitored by Southern blot analysis. Homozygous male mice and heterozygous female mice were subsequently bred to yield homozygous male and female mice.

Total RNA was isolated from wildtype or mutant brains using TRIzol reagent (Invitrogen). RT-PCR was performed using primers specific for exons 2 and 7 of neuroligin-3. The PCR product was electrophoresed on a 1% agarose gel, eluted, and the mutation was confirmed by direct sequencing.

Genotyping

Each tail snip was digested with TSEP (50 mM Tris,pH 8.0, 100 mM NaCl, 20 mM EDTA, 05% SDS, 100 ng/mL proteinase K) buffer at 55^0 C for 12 hours. The overnight sample was extracted with one volume of phenol/chloroform (1:1) solution and centrifuged at 4552 X g for 10 minutes (J6-MI Beckman-Coulter centrifuge, JS-4.2 rotor). The top layer was transferred to a new Eppendorf tube and 1.0 mL of 100% ethanol was added. The sample was mixed and centrifuged at 17,949 X g (Eppendorf microcentrifuge model 5417R) for 2 minutes. The precipitated DNA was washed with 70% ethanol and left to dry at room temperature for 2 minutes at 4^0 C. The pellet was dissolved in 50 µl 1X T.E buffer and 50ul of H2O, incubated at 55^0 C for 3 hours and stored at 4^0 C. For Southern blot analysis, the DNA was digested with a restriction enzyme at 37^0 C overnight, run on 0.7% agarose gel, transferred to nylon membrane and hybridized with the designed probe.

Comparison of cohorts and breeding strategies

Overall there were no major differences in behavioral results from pups obtained from breeding -/- or C57BL/6J dams versus heterozygous dams. The few minor differences included more activity (total distance, horizontal activity and vertical activity) in the mice from the non-heterozygous dams, for both WT and NL3. Similarly, both WT and NL3 mice from non-heterozygous dams swam faster in the Morris water maze and swam less distance. There was also more freezing during the cued part of the conditioned fear task in WT and NL3 mice from non-heterozygous dams. Thus, while there were a few differences in offspring phenotype across the breeding strategies, these differences were not genotype specific and did not appear to affect the general findings.

Sequence of behavioral testing

Behavioral phenotyping was conducted in the following sequences for the three male cohorts. Cohort 1: juvenile social interaction, adult social approach, Morris water maze, general health, open field, prepulse inhibition, hot plate, tail flick, contextual and cued fear conditioning. Cohort 2: pup ultrasonic vocalization test, juvenile social interaction, adult general health, open field, prepulse inhibition, hot plate, tail flick, social approach, Morris water maze, contextual and cued fear conditioning, grooming, accelerating rotorod, buried food test, acoustic startle threshold and footprint test. Cohort 3: pup developmental milestones, pup homing and open field test, juvenile social interaction, adult elevated plus-maze, light-dark exploration task, grip test, and buried food task. Female cohorts were tested in an order similar to the males except they were not run on the Morris water maze and contextual and cued fear conditioning. Cohort 3 females were used to complete acoustic startle threshold, accelerating rotarod and social approach.

All equipment was cleaned with 70% ethanol between subjects unless otherwise specified.

Statistical analysis

Most behavioral results were analyzed using a between subjects design Analysis of Variance (ANOVA) or Repeated Measures ANOVA where appropriate. Significant overall ANOVAs were further analyzed for individual group differences using Newman-Keuls posthoc tests. Males and females were analyzed independently because the neuroligin-3 gene is located on the X chromosome resulting in two different genotypes for males (WT & NL3) and three different genotypes for females (WT, Het, NL3).

Detailed methods for the behavioral tests

Behavioral methods are listed below in alphabetical rather than chronological order, for ease of access.

Acoustic startle (ASR) and prepulse inhibition (PPI)

ASR and PPI were measured using the SR-Lab System (San Diego Instruments, San Diego, CA) as described previously (Paylor and Crawley, 1997; Paylor et al., 1998; Holmes et al., 2001). Test sessions began by placing the mouse in the Plexiglas holding cylinder for a 5 min acclimation period. Over the next 10.5 min, mice were presented with each of seven trial types across six discrete blocks of trials for a total of 42 trials. The intertrial interval was 10-20 s. One trial type measured the response to no stimulus (baseline movement) and another measured the

response to the startle stimulus alone which was a 40 ms 110 dB sound burst. The other five trial types were acoustic prepulse plus acoustic startle stimulus trials. Prepulse tones were 20 ms at 74, 78, 82, 86, and 90 dB, presented 100 ms prior to the 110 dB startle stimulus. Startle amplitude was measured every 1 ms over a 65 ms period beginning at the onset of the startle stimulus. The maximum startle amplitude over this sampling period was taken as the dependent variable. A background noise level of 70 dB was maintained over the duration of the test session.

Acoustic startle threshold

To further address hearing ability and thresholds for startle, acoustic startle threshold was measured using the SR-Lab System (San Diego Instruments, San Diego, CA). Test sessions began by placing the mouse in the Plexiglas holding cylinder for a 5 min acclimation period. Over the next 8 min, mice were presented with each of six trial types across five discrete blocks of trials for a total of 30 trials. The intertrial interval was 10-20 s. One trial type measured the response to no stimulus (baseline movement). The other five trial types measured the response to a startle stimulus alone which was a 40 ms sound burst of 80, 90, 100, 110 and 120 dB. Startle amplitude was measured every 1 ms over a 65 ms period beginning at the onset of the startle stimulus. The maximum startle amplitude over this sampling period was taken as the dependent variable. A background noise level of 70 dB was maintained over the duration of the test session.

Analgesia

Responsiveness to painful stimuli was measured using the hot plate and tail flick tests. For the hot plate test, the mouse was placed on the hot plate surface at a constant temperature of 55°C (IITC Life Science, Inc., Woodland Hills, CA). Latency to first response was recorded. To prevent tissue damage, mice were removed from the test apparatus at a maximum cut off latency of 30 s. For the tail flick test, mice were gently restrained with the tail placed in the groove of the tail flick test apparatus (Columbus Instruments, Columbus, OH). An intense infrared light beam was focused on the tail and latency to move the tail away from the beam was recorded. To prevent tissue damage, a maximum latency cut off of 10 s was used.

Buried food olfactory test

Olfactory sensitivity was measured in the buried food test using methods previously described (Moy et al., 2007). Several days before the test, an unfamiliar food (Chocolate Chip Teddy Grahams, Kraft Foods, Northfield, IL) was placed overnight in the home cages of the subject mice to avoid food neophobia on the day of testing. Cages were checked for uneaten cookie the following day. Most of the mice began eating the cookie in a short amount of time, and cookie was consumed in all home cages. Mice were food deprived for 20 hours prior to testing. Testing was conducted in a clean empty mouse cage containing 3 cm deep clean home cage bedding. Mice were allowed to explore for 5 min and then removed from the cage. A cookie was then buried 1 cm deep in the cage bedding. The subject mouse was returned to the cage and the latency to dig for the buried cookie was measured with a 15 min cutoff.

Contextual and cued fear conditioning

Standard delay cued and contextual fear conditioning was conducted as previously described (Wrenn et al., 2004). Mice were trained and scored for freezing behavior to the same environmental context in a clear Plexiglas chamber (26 x 26 x 18 cm) with a metal rod floor for footshock delivery (Freeze Monitor, San Diego Instruments, San Diego, CA). A Dell Optiplex

computer connected to the shock generator delivered the unconditioned foot shock stimulus (0.5 mA, AC current, 2.5 s duration). The conditioned auditory stimulus (80 dB) was provided by a white noise generator (Research Services Branch, NIH/NIMH) using a toggle switch manual control consisting of 30 s presentations, which preceded and co-terminated with the foot shock. Timing of the auditory cue presentation and foot shock delivery were coordinated through San Diego Instruments software. The novel context chamber used for scoring the cued fear conditioning consisted of a white plastic triangular shaped chamber (36 x 36 x 51 cm) with 26 cm high walls and a solid floor. A novel odor (1:100 diluted McCormick vanilla extract) was spread with a cotton-tipped applicator on one wall of the triangle prior to the start of each test session. Freezing was manually scored as complete absence of movement except respiration at 10 s intervals during the scoring periods on each day of training and testing.

On the training day, mice were brought individually to the testing room, placed into the conditioning chamber, and presented with 3 pairings of auditory white noise (CS) and foot shock (US). CS-US parings were preceded and followed by 2 min exploration periods. Freezing was scored every 10 s during the initial 2 min period prior to the CS-US pairings and during the final 2 min after each of the 3 CS-US pairings by a highly trained observer. Twenty-four hours after training, mice were brought individually to the original test room and returned to the training chamber (same context), with the test room environment identical to the training day, for the contextual test. Mice were placed in the chamber and allowed to explore for 5 min in the absence of the auditory cue and foot shock. Freezing behavior was scored every 10 s over the 5 min test period. Forty-eight hours post-training, mice were brought to a different test room and placed in the triangular chamber (novel context) for auditory cue testing. The session consisted of a 3 min exploration period followed by 3 min of exploration with the identical 80 dB white noise. Freezing behavior was scored every 10 s for the 3 min exploration period and the 3 min white noise period.

Elevated plus-maze

Anxiety-like behavior was tested in the elevated plus-maze as previously described (Holmes et al., 2002; Bailey et al., 2007). The elevated (40 cm) plus-maze consists of two open arms (30 x 5 cm) and two closed arms (30 x 5 x 15 cm) extending from a central (5 x 5 cm) area. A raised lip (0.25 cm) around the open arms minimized falling off the edges of the open arms. Mice were placed in the central area facing an open arm and allowed to traverse the maze freely for 5 min. Arm entries (all 4 paws in the arm) and time spent in the open and closed arms were scored by a trained observer using ethological recording and analysis software (Hindsight, version 1.4).

Footprint analysis

Gait was assessed for ataxia using footprint analysis as previously described (Carter et al., 2003). The paws were painted with non-toxic tempera colors (forepaws green, hindpaws red; Crayola, Easton, PA). The mouse was placed on a piece of white paper, 8 x 11 cm, within a narrow runway (width 14 cm) 30 cm long going from light (13.7 cm) to dark (17.3 cm). The mouse quickly walked down the runway, leaving footprints on the paper. After the footprints had dried, the following parameters were measured with a centimeter ruler: forepaw width, hindpaw width, and stride length.

General health, neurological reflexes, sensory and motor abilities

The general health of the mice was evaluated at two time points: i) each pup was separated from the mother only for the duration of the test (3-5 min) and tested every other day from pnd 2 to 14 and ii) adults were tested once at age 5-13 weeks. Pups were tested according to a slightly modified Fox battery (Fox, 1965; Calamandrei et al., 1999). Adults were tested using measures described previously (Crawley and Paylor, 1997; Crawley, 1999; Bailey et al., 2007). Briefly, home cage observations involved scoring the activity of all mice in their home cages on their housing racks in the vivarium for approximately 15 min at three different time points (9:00 am, 3:00 pm, 8:00 pm). As the circadian schedule was lights on at 7:00 am, these time points of morning, afternoon, and early evening spanned the sleeping and early waking phases of the rodent home cage activity. The experimenter specifically noted incidence of excessive fighting, grooming, stereotypies, isolated mice, lack of huddling and quality of nest building. Empty cage behavior was scored in a separate session by placing the mouse into a clean, empty cage and noting incidents of transfer freezing, wild running, stereotypies, and grooming and exploration levels. General health assessment included assessing body weight, fur and whisker condition, limb and body tone. Limb strength was evaluated by placing mice on a wire cage lid that was then inverted over a standard mouse cage lined with a layer of bedding for a maximum of 60 s and latency to fall was recorded. Neurological reflex tests included forepaw reaching, righting reflex, trunk curl, whisker twitch, pinna twitch, eyeblink response and toe pinch. The reactivity level of the mice was assessed with tests measuring responsiveness to petting, intensity of dowel biting response and level of vocalization during handling.

Grip strength

Mice were brought to the testing room and allowed to acclimatize for 10 min. A Grip Strength Meter (Columbus Instruments. Columbus, OH) was used to measure forelimb grip strength as an indicator of neuromuscular function as previously described (Costa et al., 1999; Van Damme et al., 2003; Whittemore et al., 2003). The grip strength meter was positioned horizontally, and mice were held by the tail and raised toward the apparatus. Mice were allowed to grasp the smooth, metal, triangular pull bar (forelimbs only) and were then pulled backward in the horizontal plane. The force applied to the bar at the moment the grasp is released is recorded as the peak tension. The test was repeated 5 consecutive times within the same session, and the mean of all trials and the highest value from all trials were recorded for the grip strength for that animal. Mice were not trained prior to testing and each mouse was tested in only one session (5 trials equal one test session).

Homing test on postnatal day (pnd) 9

On pnd 9, the litter was separated from the dam and kept for 30 min in one holding cage. The cage was placed on a heating pad set at a temperature of 35°C to maintain normal body temperature of the pups in the nest. Individual pups were then transferred to a Plexiglas cage (36 cm × 22.5 cm, walls 10 cm high). Wood shavings from the home cage were evenly spread on one side (14 cm × 22.5 cm, nest area) while the rest of the cage was covered with clean bedding. The pup was placed in the middle of the Plexiglas cage and videorecorded for four minutes. The floor of the arena was virtually subdivided into squares of 7 cm × 7 cm each, to enhance scoring of locomotor activity from the video digital DVDs, using Noldus Observer 5.0 software. Homing performance was scored for latency to reach the area containing nest litter, time spent in the area containing nest litter and locomotor activity by square crossings.

Juvenile social interaction

The juvenile social interaction test was conducted in pairs of same-sex mice at postnatal day 21 ± 2 in the Noldus PhenoTyper arena (Noldus, Leesburg, VA, USA) as previously described (Terranova and Laviola, 2005; Yang et al., 2007; McFarlane et al., 2008). Fresh clean bedding was placed on the arena floor for each pair. On the testing day each subject mouse was isolated in a clean cage for at least 1 hour before its juvenile social interaction test session. Two non-littermates were then placed in the testing arena and their interactions recorded for 10 minutes. Because the genotypes were unknown at the time of testing, three pair types were obtained: WT-WT, NL3-WT, and NL3-NL3. The identities of each pair were determined after genotyping and the different pairings were subsequently arranged by genotypes into post facto groups for statistical analyses. Behaviors were scored from the video files using Noldus Observer 5.0 software. A highly trained observer unaware of the subjects' genotypes scored behavioral parameters including: nose-to-nose sniffing, nose-to-anogenital region sniffing, push-crawl-touch (pushing between the wall and the partner, crawling over or under the partner, and using the partner to balance on while standing on hindpaws), follow (closely following the partner at least halfway across the arena), self-grooming, and social grooming (grooming the partner). The investigator also scored non-social behavioral parameters including exploration and jumping. Each behavioral parameter was scored from the videotapes for frequency of occurrence except for exploration which was total duration.

Light ↔ dark exploration

The light ↔ dark exploration test was conducted as previously described (Crawley and Goodwin, 1980; Holmes et al., 2001; Bailey et al., 2007). The test apparatus consisted of a standard polypropylene rat cage (48 x 20 x 20 cm^3) divided into two unequal compartments (one 28 x 20 x 20 cm^3, the other 14 x 20 x 20 cm^3), by a black partition with a small opening at the base. The larger compartment was transparent and open from above. The smaller compartment was covered with a lid and the walls were painted black. Photocells located across the opening of the partition automatically detected transitions between the light and dark compartments and activated a timer recording time spend in the dark compartment. Mice were placed centrally in the open compartment facing away from the partition. Percentage of time spent in the light compartment and number of transitions between compartments were recorded for the 10 minute session.

Morris water maze acquisition and reversal

Spatial learning and reversal were assessed in the Morris water maze using established procedures and equipment (Holmes et al., 2001; Wrenn et al., 2004; Bailey et al., 2007). The training sequence was a) acquisition of hidden platform, b) probe trial, c) 3 days of no testing, d) reversal of hidden platform, e) probe trial. The apparatus was a circular pool (120 cm diameter) filled 45 cm deep with tap water rendered opaque with the addition of non-toxic (Crayola) white paint. Trials were videotaped and scored with WaterMaze video tracking software (Actimetrics, Inc., Wilmette, IL). Training consisted of 4 trials per session per day. The mouse was placed into the pool facing the pool edge in a new quadrant for each trial. The hidden platform remained in the same quadrant for all trials across all sessions of acquisition. The hidden platform was moved to the opposite quadrant for all trials across all sessions of reversal. Training and trials lasted 60

seconds (s). During the first of the 5 to 8 days of training, if the mouse did not locate the platform within 60 s, it was guided to the platform by the experimenter. Subjects were left on the platform for 15 s before being placed under a warming light for the 30-45 s intertrial intervals. Hidden platform training continued until the WT group met the criteria of 15 s or less latency to find the hidden platform. Cohorts reached criteria on different days during the initial acquisition training: Cohort 1A: Day 6, Cohort 1B: Day 7, Cohort 2A: Day 8, Cohort 2B Day 5.

Mice were tested on a 60 s probe trial 2-3 h after completing hidden platform testing on the day criteria was met. Parameters recorded during training were latency to reach the platform, total distance traveled, swim speed and thigmotaxis. Probe trial selective quadrant search was assessed by time spent in each quadrant and the number of crossings over the trained quadrant platform location compared to the analogous locations in the non-trained quadrants. Reversal training began 3 days after the probe trial. The hidden platform was moved to a new location in the pool in the opposite quadrant from acquisition training for each mouse. Acquisition and probe trials were conducted as before. During reversal, number of days to criteria were reached as follows: Cohort 1: Day 5, Cohort 2: Day 5, Cohort 3: Day 7; Cohort 4: Day 6.

Open field test at juvenile age pnd 18

General exploratory locomotion in a novel environment was tested by placing pups in a VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH, USA) for a 15-min test session. Each pup was separated from the mother only for the duration of the test. To compensate for the relatively small size of 18-day-old mice, the VersaMax vertical sensor was adjusted to the lowest setting of 7 cm, and the floor of the open field arena was elevated by 1.0 cm, so that the final height of the vertical sensor was 6.0 cm above the floor of the arena. The testing room was illuminated with a single 25-W red lamp and kept at a similar temperature as the colony room. The test was carried out between 10 AM and 1 PM.

Open field exploration (adults)

Exploratory locomotor activity was assayed in the same automated open field arena (Accuscan, Columbus, OH) as described previously (Bailey et al., 2007). Open field chambers consisted of clear Plexiglas sides and floor approximately 40 x 40 x 30.5 cm^3. Mice were placed in the center of the open field and allowed to explore the chamber for 30 min. Photocells at standard heights for recording horizontal activity were aligned 8 to a side, dividing the chamber into 64 equal squares. Vertical activity was assessed by an additional 8 photocells located above the horizontal photocells. Horizontal activity, total distance, vertical activity, and center time were automatically collected using the Versamax activity monitor and analyzer software system.

Self-grooming

The observational test was performed as previously described (Yang et al., 2007; McFarlane et al., 2008). The subject mouse was placed in a clean standard mouse cage for a 10 minute acclimation period. Following acclimation, subjects were observed for 10 min by a highly trained observer using a silenced stopwatch to score the cumulative time spent grooming.

Rotarod

Motor coordination,balance and motor learning were assessed using an accelerating rotarod (Ugo Basile, Schwenksville, PA). Mice were placed on a cylinder which slowly accelerated from 4 to 40 revolutions per minute over a 5 minute (300 s) test session. The task

requires the mice to walk forward in order to remain on top of the rotating cylinder rod. Mice were tested with 3 trials per day for 3 days. Latency to fall was recorded with a 300 second maximum latency.

Social approach test in adult mice ages 7 to 10 weeks

Social approach behaviors were tested in an automated 3 chamber apparatus as previously described (Moy et al., 2004; Nadler et al., 2004; Moy et al., 2007; Yang et al., 2007; McFarlane et al., 2008). Photocells embedded in the doorways automatically scored the time spent in each of the three chambers and number of entries into each chamber. A novel mouse (the "stranger") was placed in one side chamber and a novel object was placed in the other side chamber. Animals used as strangers were male 129Sv/ImJ mice obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Strangers were habituated to the testing chamber for 30 minute sessions across 3 consecutive days. Prior to addition of the stranger mouse, the subject mouse was acclimated to the apparatus for 10-min in the center chamber, and then for a second 10-min with access to all 3 empty chambers. The subject was then confined to the middle chamber while the novel object (an inverted wire cup, Galaxy Cup, Kitchen Plus, Streetsboro, OH) was placed into one of the side chambers and the stranger mouse inside an identical inverted wire cup was placed in the other side chamber. An upright plastic drinking cup weighed down with a lead weight was placed on top of each of the inverted wire cups to prevent the subject mouse from climbing on top. The location (left or right) of the novel object and stranger mouse alternated across subjects. The chamber doors were opened simultaneously and the subject had access to all 3 chambers for 10 minutes. The time spent in each chamber and the entries into each chamber were recorded. The time the subject mouse spent sniffing either the stranger mouse or the novel object was recorded by an observer with a stopwatch. The fourth 10-min session provided a measure of preference for social novelty, in which a second novel stranger was placed in the side chamber previously containing the novel object. The preference for social novelty test was included as a control to confirm olfactory abilities for detection and discrimination of social odors.

Ultrasonic vocalization

Baseline measurements of the ultrasonic vocalizations (USVs) were taken for every pup of each litter from pnd 4 to 11. Each pup was separated from the mother, placed into an empty plastic container (diameter, 5 cm; height 10 cm), located inside a sound-attenuating styrofoam box, and assessed for USVs during a three minute test. At the end of the recording session, each pup was weighed and its axillary temperature was measured by gentle insertion of the thermal probe in the skin pocket between upper foreleg and chest of the animal for about 30 seconds (Microprobe digital thermometer with mouse probe, Stoelting Co., Wood Dale, IL, USA). Pups of both sexes were tested for each litter.

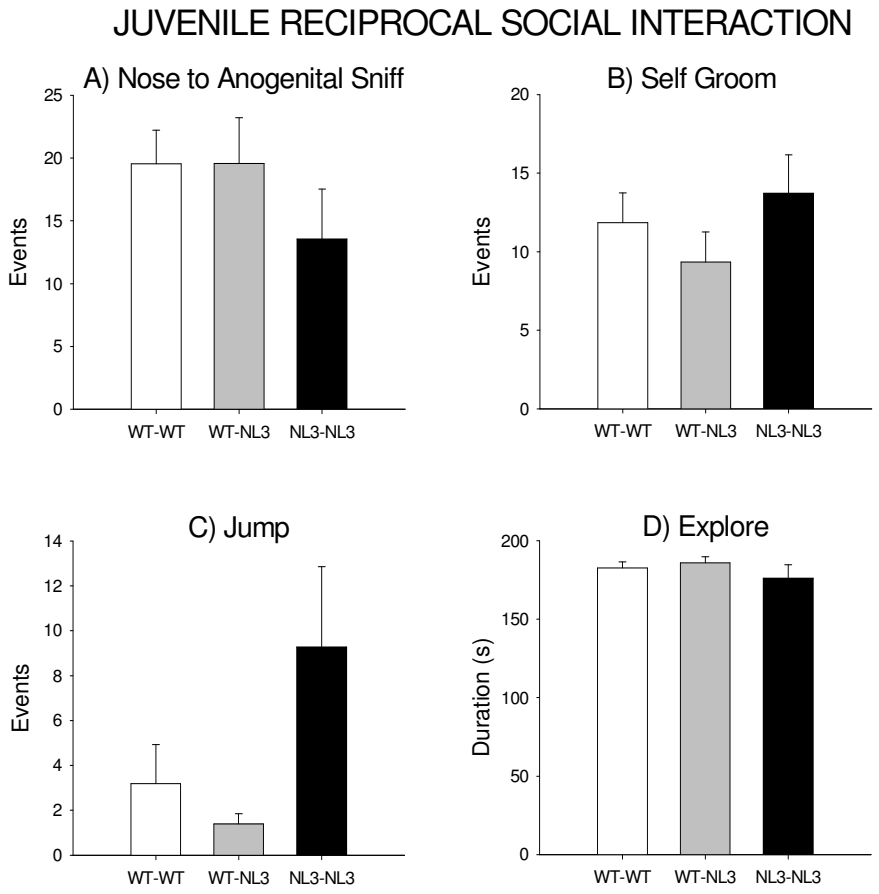
Ultrasonic calls were recorded in a sound-attenuating chamber by an Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10-180 kHz. The microphone was placed through a hole in the middle of the cover of the styrofoam sound-attenuating chamber, about 20 cm above the plastic container. The temperature of the room was maintained at 22 ± 1 °C. Vocalizations were recorded using Avisoft Recorder (Version 3.2). Settings included sampling rate at 250 kHz; format 16 bit. For acoustical analysis, recordings were transferred to

Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 1024 points and a time window overlap of 50% (100% Frame, Hamming window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency of 15 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Call detection was provided by an automatic threshold-based algorithm and a hold-time mechanism (hold time: 0.01 s). An experienced user checked the accuracy of call detection, and obtained a 100% concordance between automated and observational detection. The total number of calls and their duration were analyzed for each testing day. Additional qualitative and quantitative analyses included sound frequencies, measured in terms of peak frequencies (frequencies with the highest sound pressure), and peak amplitude at the peak frequency (maximum of the spectrum).

Supplementary Results

Juvenile social interaction

The results and statistics for the juvenile social interaction test shown in Figure 1S are described in the main manuscript.



Supplementary Figure 1S. Juvenile social interaction. No significant differences were detected between genotype pairs on additional social and non-social measures: A) Nose to Anogenital sniff, B) Self-groom, C) Jump and D) Explore. WT-WT N = 13, NL3-WT N=9, and NL3-NL3 N=9. Data are expressed as mean ± standard error of the mean in Figures 1S-11S.

Social approach in males, additional information

During the 10 min habituation phase (Figure 2S panel A), the subject mouse was able to explore all 3 chambers. There was a significant effect of chamber time ($F_{1,56}$ = 5.87, p<0.05) but not of genotype ($F_{1,56}$ = 0.09, NS) or interaction of genotype x chamber time ($F_{1,56}$ = 0.25, NS). The WT mice had a slight innate preference for the right chamber ($F_{1,33}$ = 4.267, p<0.05) while the NL3 mice did not show a side preference ($F_{1,23}$ = 2.247, NS). Although the WT mice showed a preference for the right side, there was no evidence of a side bias when the stranger mouse was present.

Preference for social novelty in males

As shown in Figure 3S, C) both genotypes spent more time in the chamber with stranger 2 than in the chamber with stranger 1, genotype ($F_{1,56}$= 2.03, NS) and chamber time ($F_{1,56}$= 43.91, p<0.0001), genotype x chamber time ($F_{1,56}$= 0.65, NS). As there was no effect of genotype but there was an effect of chamber time, individual ANOVAs were run for each genotype: WT ($F_{1,33}$= 39.95, p<0.001) and NL3 ($F_{1,23}$ = 11.69, p<0.01). D) Both genotypes spent more time sniffing the stranger 2 mouse than the stranger 1 mouse, genotype ($F_{1,56}$= 0.44, NS), sniff time ($F_{1,56}$= 105.98, p<0.0001) and genotype x sniff time ($F_{1,56}$= 0.31, NS). As there was no effect of genotype but there was an effect of chamber time, individual ANOVAs were run for each genotype: WT ($F_{1,33}$= 64.10, p<0.0001) and NL3 ($F_{1,23}$ = 43.57, p<0.0001). E) There was a significant difference in the number of entries into the chamber with stranger 2 versus entries into the chamber with stranger 1, entries ($F_{1,56}$= 4.56, p< 0.05) but it did not differ by genotype (genotype, $F_{1,56}$= 0.81, NS) or interact with genotype, genotype x entries ($F_{1,56}$= 0.73, NS). As there was no effect of genotype but there was an effect of chamber time, individual ANOVAs were run for each genotype: WT ($F_{1,33}$ = 11.13, p< 0.01) and NL3 ($F_{1,23}$ = 0.40, NS).

Social approach in females

As shown in Supplementary Figure 3S panels A-D, social approach behaviors did not differ between female NL3, Het and WT mice During the 10 min habituation phase (panel A), the subject mouse was able to explore all 3 empty chambers. There was no significant effect of genotype ($F_{2,37}$ = 1.90, NS), chamber time ($F_{1,37}$ = 0.02, NS) or interaction of genotype and chamber time ($F_{2,37}$ = 0.08, NS). There was no side bias during the habituation period that could have affected the social approach results: WT ($F_{1,9}$= 0.10, NS), Het ($F_{1,14}$= 0.03, NS) and NL3 mice ($F_{1,14}$ = 0.01, NS). Sociability seen in the males (Figure 2) was not as pronounced in the female genotypes during the 10 min choice session. There was no significant effect of genotype ($F_{2,37}$ = 1.60, NS) though there was an overall effect for chamber time ($F_{1,37}$ = 27.78, p< 0.0001) but there was no genotype x chamber time interaction ($F_{2,37}$ = 2.80, NS). The Het and NL3 groups spent significantly more time in the chamber with the stranger mouse than the novel object D) Het ($F_{1,14}$= 28.73, p<0.0001) and NL3 ($F_{1,14}$ = 9.70, p<0.01) but the WT ($F_{1,9}$= 1.85, NS) did not reach significance on sociability.

There was a significant effect for sniff time ($F_{1,37}$ = 54.21, p< 0.0001) but not for genotype ($F_{2,37}$ = 0.17, NS) or genotype x sniff time interaction ($F_{2,37}$ = 0.67, NS). All genotypes spent significantly more time sniffing the stranger mouse than the novel object: E) WT ($F_{1,9}$= 9.57, p<0.05), Het ($F_{1,14}$= 27.07, p<0.0001), and NL3 ($F_{1,14}$ = 22.69, p<0.001). There was a significant effect for entries ($F_{1,37}$ = 10.46, p< 0.01) but not for genotype ($F_{2,37}$ = 0.37, NS) or genotype x entries interaction ($F_{2,37}$ = 0.87, NS). The WT group had significantly more entries into the chamber with the stranger mouse F): WT ($F_{1,9}$ = 6.33, p<0.05) but the Het group did not

($F_{1,14}$= 1.52, NS) and NL3 ($F_{1,14}$= 2.67, NS) suggesting that there were no locomotor impairments that could have affected the experimental results. The most likely explanation of the lack of significant sociability in the WT and NL3 females is the small Ns available for testing.

Preference for social novelty in females

As shown in Figure 3S panel E, There was no significant effect of genotype ($F_{2,37}$ = 0.49, NS) though there was an overall effect for chamber time ($F_{1,37}$ = 23.89, p< 0.0001) but there was no genotype x chamber time interaction ($F_{2,37}$ = 0.67, NS). All genotypes spent more time in the chamber with stranger 2 than in the chamber with stranger 1,: WT ($F_{1,9}$= 46.72, p<0.001), Het ($F_{1,14}$= 6.30, p<0.05) and NL3 ($F_{1,14}$ = 5.36, p<0.05). F) There was a significant effect for sniff time ($F_{1,37}$ = 65.22, p< 0.0001) but not for genotype ($F_{2,37}$ = 0.98, NS) or genotype x sniff time interaction ($F_{2,37}$ = 0.28, NS). All genotypes spent more time sniffing the stranger 2 mouse than the stranger 1 mouse, WT ($F_{1,9}$= 35.05, p<0.001), Het ($F_{1,14}$= 14.48, p<0.001) and NL3 ($F_{1,14}$ = 14.48, p<0.01). G) There was a significant effect for entries ($F_{1,37}$ = 8.95, p< 0.01) but not for genotype ($F_{2,37}$ = 0.09, NS) or genotype x entries interaction ($F_{2,37}$ = 08, NS). None of the genotypes had a significant difference in the number of entries into the chamber with stranger 2 versus entries into the chamber with stranger 1: WT ($F_{1,9}$ = 2.81, NS), Het ($F_{1,14}$= 3.24, NS) and NL3 ($F_{1,14}$ = 4.13, NS).

Morris water maze acquisition and reversal

The results and statistics for the Morris water maze swim speed and thigmotaxis shown in Figure 4S are described in the main manuscript.

Pup Ultrasonic Vocalizations to Separation from the Mother and Siblings

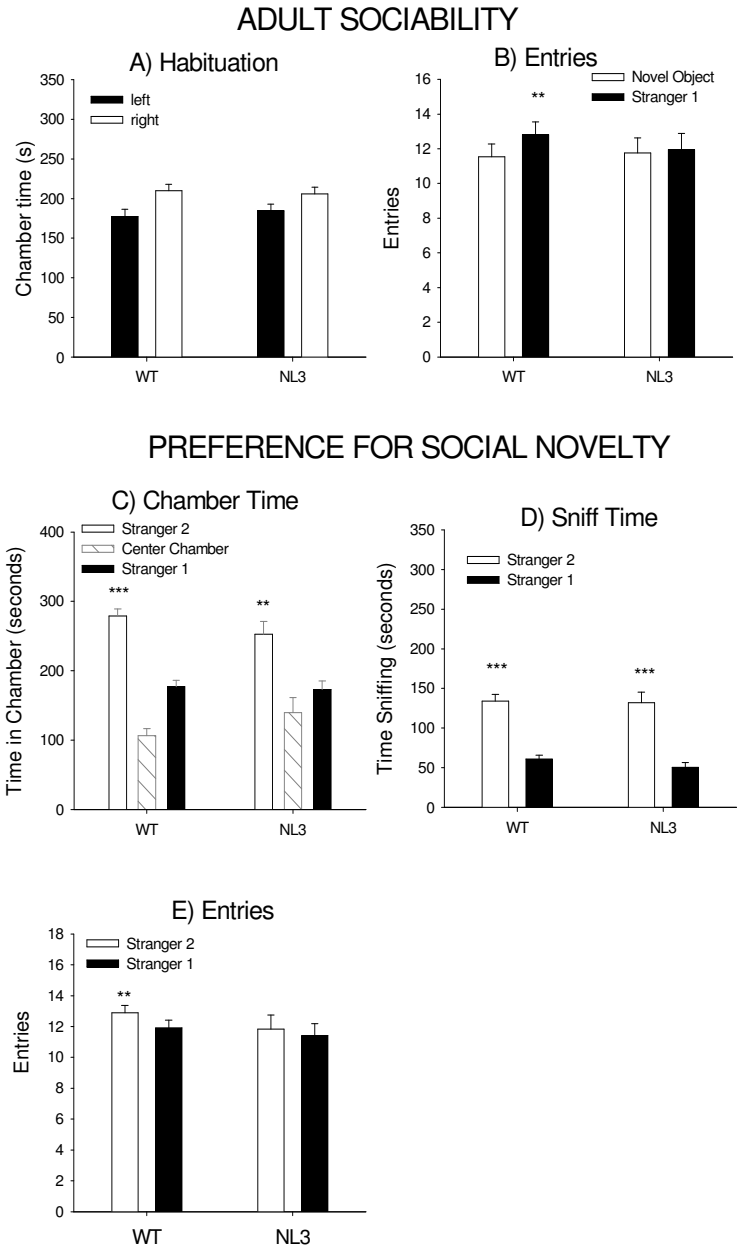
The results and statistics for the ultrasonic vocalizations for the male pups shown in Figure 5S panels A-C are described in the main manuscript. No significant differences were detected in number of calls ($F_{2,31}$= 0.31, NS), call duration ($F_{2,31}$= 1.20, NS), peak frequencies ($F_{2,31}$= 0.44, NS), or peak amplitudes ($F_{2,31}$= 2.15, NS) between WT, Het and NL3 female pups (Figure 5S panels D-G). No differences were detected between the body temperatures of the three groups ($F_{2,31}$= 0.97, NS) as measured after each separation test.

Homing test (postnatal day 9)

The results and statistics for the latency to reach the nest area and the time spent in the nest area by male and female pups as shown in Figure 6S are described in the main manuscript.

Developmental Milestones (pnd 2-14)

The results and statistics for the body weight and righting reflex as shown in Figure 7S A and B are described in the main manuscript.



Supplementary Figure 2S. Additional parameters for male adult sociability and male preference for social novelty. A) During the 10 min habituation to the three chambered apparatus, WT mice spent slightly more time in the right than left chamber but the NL3 mice showed no innate preference for either chamber. B) WT made significantly more entries into the chamber with the stranger mouse than into the chamber with the non-social novel object, although the number of entries made by the NL3 was within the range seen in previous studies with C57BL/6J mice from our laboratory (Yang et al., 2007; McFarlane et al., 2008). C) Both the WT and NL3 mice spent significantly more time in the chamber with the stranger 2 mouse than the chamber with the stranger 1 mouse. D) Both the WT and NL3 mice spent significantly more time sniffing the stranger 2 mouse than sniffing the stranger 1 mouse. E) The WT made significantly more entries into the chamber with the stranger 2 mouse but the NL3 did not. WT N = 34; NL3 N =24.

Open field test (pnd 18)

Locomotor activity of 18-day-old male pups in a non-social empty novel open field was similar between WT and NL3 ($F_{1,30}$=0.31, NS; data not shown). Both genotypes showed normal habituation to the open field arena across the 15 min session ($F_{2,60}$=51.12, p<0.0001). Vertical activity had a different profile in the pups than in the adult mice. In the pups, NL3 showed a higher activity level than WT ($F_{1,30}$=6.21, p=0.02). Adult NL3 showed lower vertical activity than the WT (Figure 5A). No significant genotype differences were detected for time spent in the center or horizontal activity in the open field arena ($F_{1,30}$=0.03, NS; $F_{1,30}$=0.72, NS).

On pnd 18, female WT and NL3 pups showed similar activity levels in an open field arena. ANOVA did not reveal any significant genotype differences in total distance traveled ($F_{2,33}$ = 1.33, NS), center time ($F_{2,33}$=0.31, NS), horizontal ($F_{2,33}$=0.85, NS) or vertical activity ($F_{2,33}$=1.30, NS).

Open Field Exploration (adults)

Males: The results and statistics for the horizontal activity and percent center time for male mice as shown in Figure 8S are described in the main manuscript.

Females: Figure 8S panels C-F, show open field exploratory locomotion in adult female WT, Het and NL3. All groups were more active at the beginning of the test as compared to the end. There was a significant effect of time on horizontal activity ($F_{5,135}$ = 13.40, p<0.0001; Figure 8S panel C) but no effect of genotype ($F_{2,27}$ = 0.76, NS) or interaction of genotype by 5 min bin ($F_{10,135}$ = 0.68, NS). There was a significant effect of 5 min time bins on total distance ($F_{10,135}$ = 9.27, p<0.0001) but no effect of genotype ($F_{2,27}$ = 1.47, NS) or interaction of genotype by 5 min bin ($F_{10,135}$ = 0.95, NS; Figure 8S panel D). There was no effect of genotype on % center time ($F_{2,27}$ = 0.795, NS; Figure 8S, panel E) or interaction of genotype x 5 min bin ($F_{10,135}$ = 1.10, NS), but there was a significant effect of 5 min bin ($F_{10,135}$ = 5.97, p<0.0001). There was a significant effect of time on vertical activity ($F_{5,135}$ = 4.42, p<0.001; Figure 8S panel F) but no effect of genotype ($F_{2,27}$ = 0.14, NS) or interaction of genotype by 5 min bin ($F_{10,135}$ = 0.71, NS).

Elevated plus-maze

Males: As shown in Figure 11S panels A-D, there was no difference between male NL3 and WT mice on anxiety-like behaviors on the elevated plus maze. A genotype difference was detected on B) number of open arm entries ($F_{1,28}$= 5.00, p<0.05), but not A) % time spent in the open arms ($F_{1,28}$= 2.236, NS), or C) number of closed arm entries ($F_{1,28}$= 2.549, NS) . D) There was a significant effect of genotype on number of total entries ($F_{1,28}$= 5.11, p< 0.05), indicating that higher total activity produced the higher number of open arm entries.

Females: As shown in Figure 11S panels G-J, there was no difference between female NL3, Het, and WT mice on anxiety-like behaviors on the elevated plus maze. No genotype differences were detected on G) % time spent in the open arms ($F_{2,31}$= 1.02, NS), H) number of open arm entries ($F_{2,31}$= 0.80, NS), I) number of closed arm entries ($F_{2,31}$= 0.97, NS) or J) total entries ($F_{2,31}$= 1.38, NS).
Het N = 16, NL3 N = 12.

Light ↔ Dark Exploration

Males: As shown in Figure 11S panels E-F, E) there was no significant effect of genotype on the % time spent in the light chamber ($F_{1,28}$ = 0.45, NS) or F) the number of transitions between the light and dark chambers ($F_{1,28}$ = 0.002, NS) in the 10 min light ↔ dark exploration test.

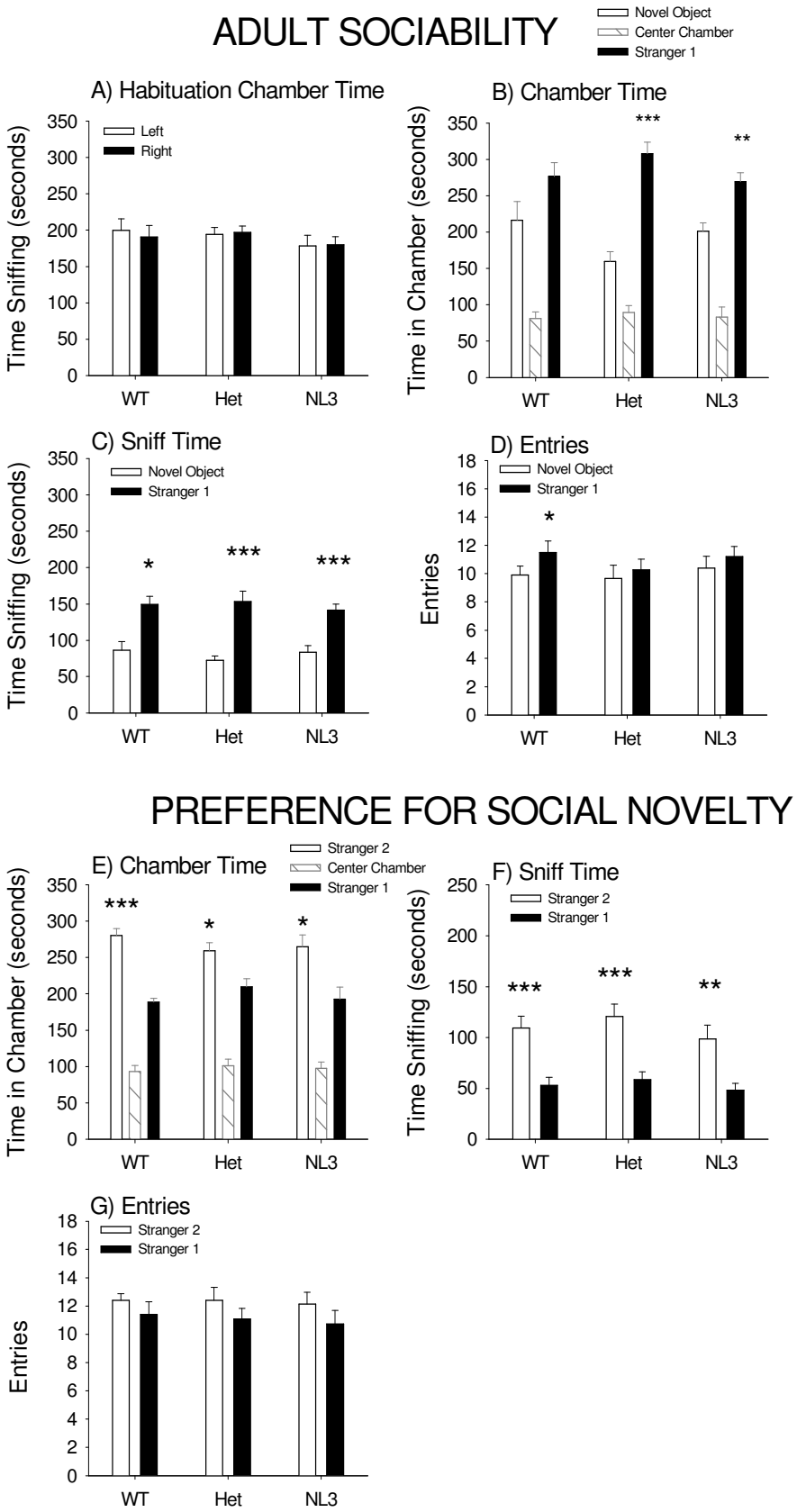
Females: As shown in Figure 11S panels K-L, K) there was no significant effect of genotype on the % time spent in the light chamber ($F_{2,26}$= 0.84, NS) or L) the number of transitions between the light and dark chambers ($F_{2,26}$= 1.57, NS) in the light ↔ dark exploration test.

General health measures in females

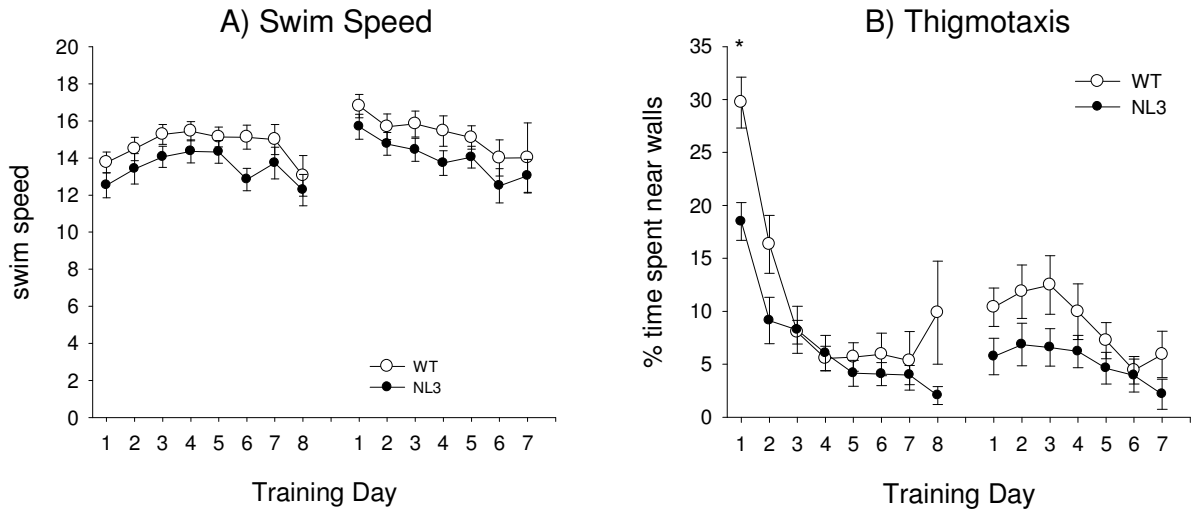
As shown in Table S1, female mice were examined on measures of general health, reflexes and sensory function. Genotypes did not differ significantly on any measure except body weight where the NL3 females weighed significantly less than WT and Het ($p<0.05$). General health parameters included temperature, fur condition, body and limb tone. During the empty cage observation, transfer freezing, wild running, stereotypies, exploration and grooming were scored and no significant differences were found between the genotypes. No genotype differences were found in motoric abilities using positional passivity, wire hang, grip strength and trunk curl. General reactivity was assessed with petting escape, struggling and/or vocalizations, and dowel biting. There were no significant differences between the genotypes.

Overall somatic growth did not differ between the three genotypes when tested on postnatal days 2 through 14. No significant differences in body or tail length ($F_{2,30}$= 0.05, NS; $F_{2,30}$= 0.35, NS), eyelid opening ($F_{2,30}$= 2.46, NS), incisors eruption ($F_{2,30}$= 2.04, NS) and fur development ($F_{2,30}$= 0.64, NS) were detected between WT, Het and NL3 (data not shown). Analysis of sensorimotor reflexes did not reveal any genotype effect (negative geotaxis ($F_{2,30}$= 0.26, NS), cliff aversion ($F_{2,30}$= 0.33, NS), forelimb stick grasp reflex ($F_{2,30}$= 0.73, NS), forelimb placing reflex ($F_{2,30}$= 0.21, NS), level and vertical screen test ($F_{2,30}$= 1.22, NS; $F_{2,30}$= 0.26, NS), bar holding ($F_{2,30}$= 0.99, NS) (data not shown).

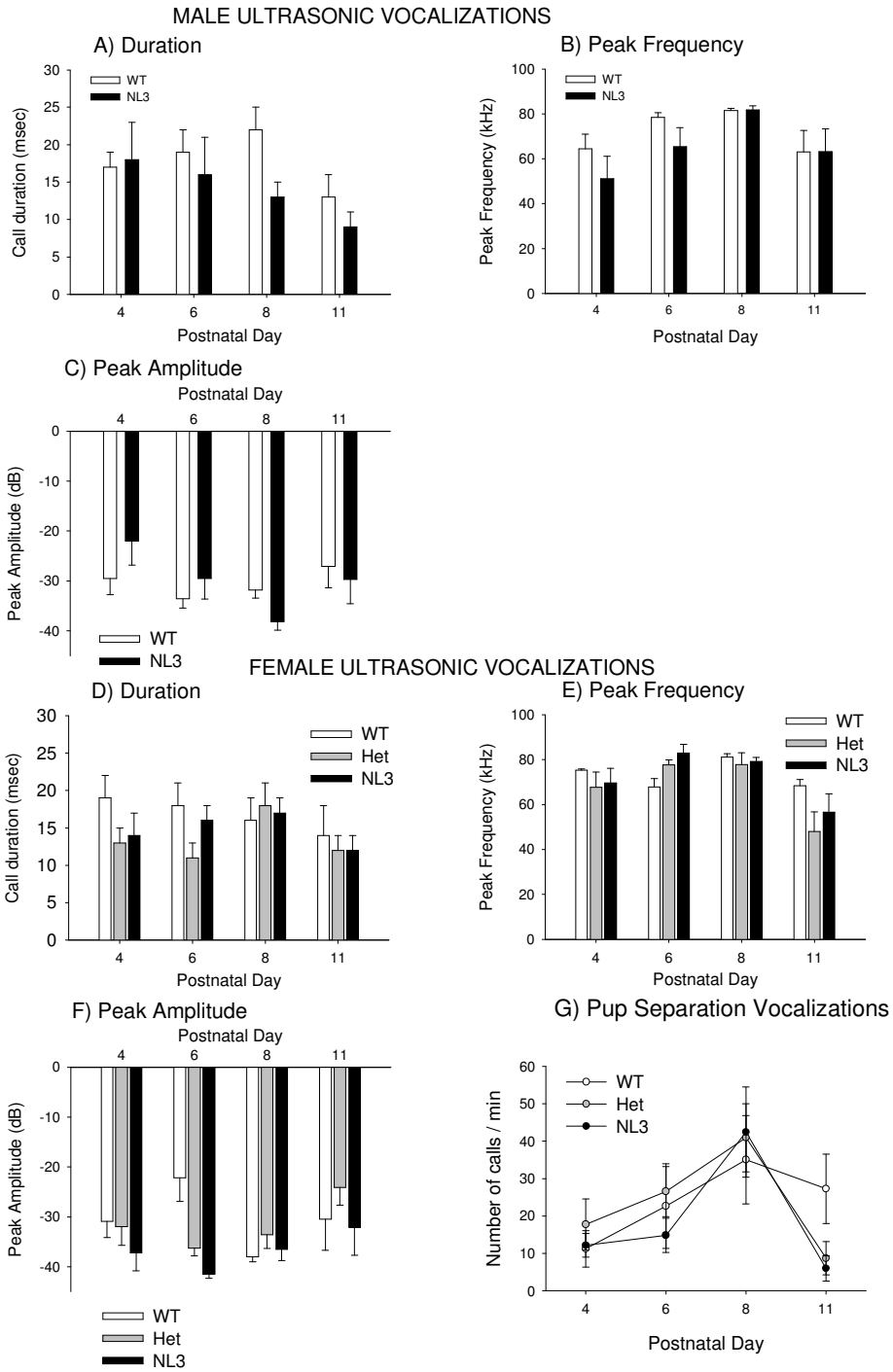
ANOVA revealed a significant difference in body weight of NL3 and Het pups in comparison to WT pups (genotype x age interaction, $F_{8,124}$=2.13, p=.03). In contrast with male data, NL3 female pups showed a significantly lower body weight on pnd 6, 8, and 11 ($p<.001$ for NL3 versus WT, Figure 7SA). The same profile was seen for Het pups starting from pnd 8 to 11 ($p<.05$ for Het versus WT). Transient differences in righting reflex latencies were noted in the females, consistent with findings in the males. NL3 and Het pups were slower to right themselves onto all four paws, as compared to WT pups, at the earliest days tested (genotype effect, $F_{2,33}$=6.66, $p<0.01$; genotype x age, $F_{12,198}$=3.36, $p<0.001$) (Figure 7S, Panel B). Newman-Keuls posthoc analysis revealed that righting reflex was significantly different between the WT, Het and NL3 mice on days 2 ($p<0.0001$ for Het and NL3 versus WT) and 4 ($p<0.005$ for Het and NL3 versus WT).


ADULT SOCIABILITY
Novel Object
Center Chamber
Stranger 1
A) Habituation Chamber Time
Left
Right
Time Sniffing (seconds)
WT
Het
NL3
B) Chamber Time
Time in Chamber (seconds)
***
**
C) Sniff Time
Novel Object
Stranger 1
*
***
***
D) Entries
Entries
PREFERENCE FOR SOCIAL NOVELTY
Stranger 2
Center Chamber
Stranger 1
E) Chamber Time
F) Sniff Time
G) Entries

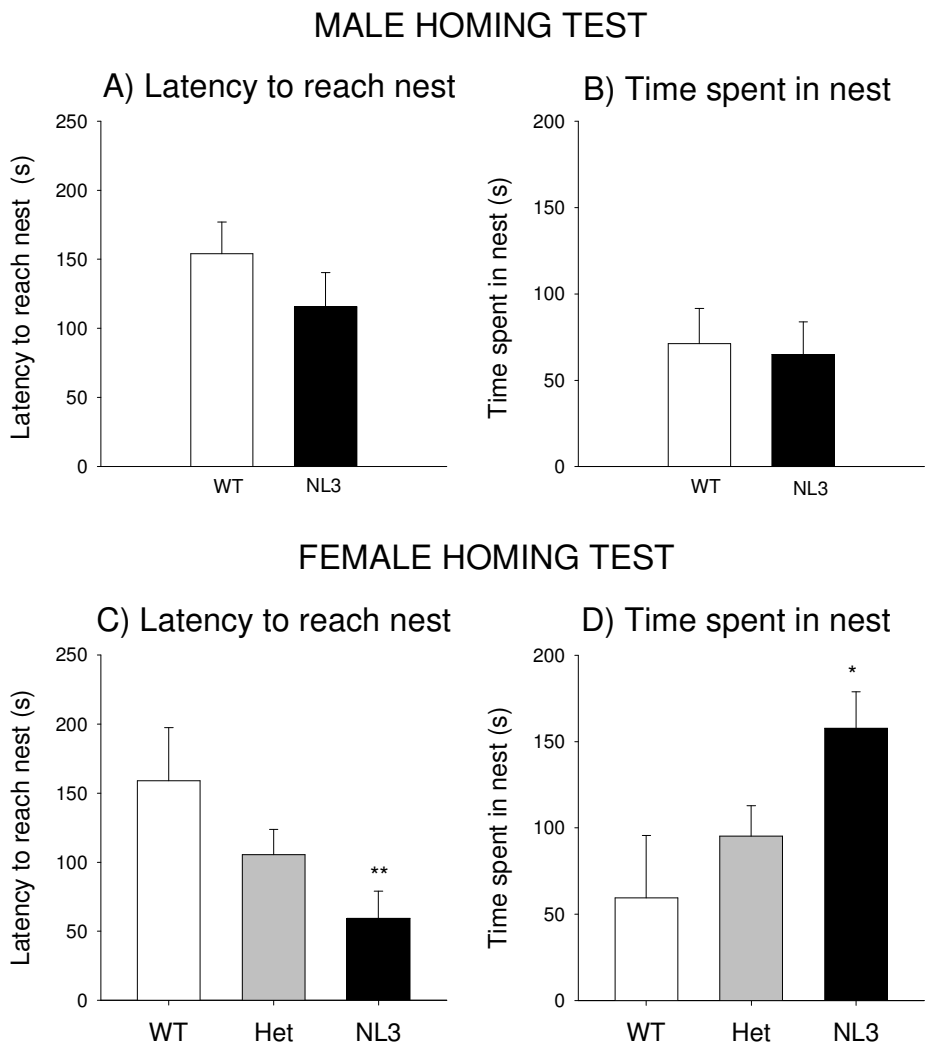
Supplemental Figure 3S. Female social approach and preference for social novelty. A) None of the female mice showed an innate side preference for either the right or left chamber during the habituation phase regardless of genotype. B) Het and NL3 spent significantly more time in the chamber with the stranger mouse than in the chamber with the novel object, but the WT group did not. C) All genotypes spent significantly more time sniffing the stranger mouse than the novel object. D) There was no difference between the number of entries into either chamber for any of the genotypes. E) All 3 genotypes spent significantly more time in the chamber with the stranger 2 than in the chamber with stranger 1. F) All genotypes spent significantly more time sniffing the stranger 2 than the stranger 1. G) There was no difference between the number of entries into either chamber for any of the genotypes. WT N = 10, Het N = 15, NL3 N = 15. Small Ns may be responsible for the lack of significant sociability in the WT females.



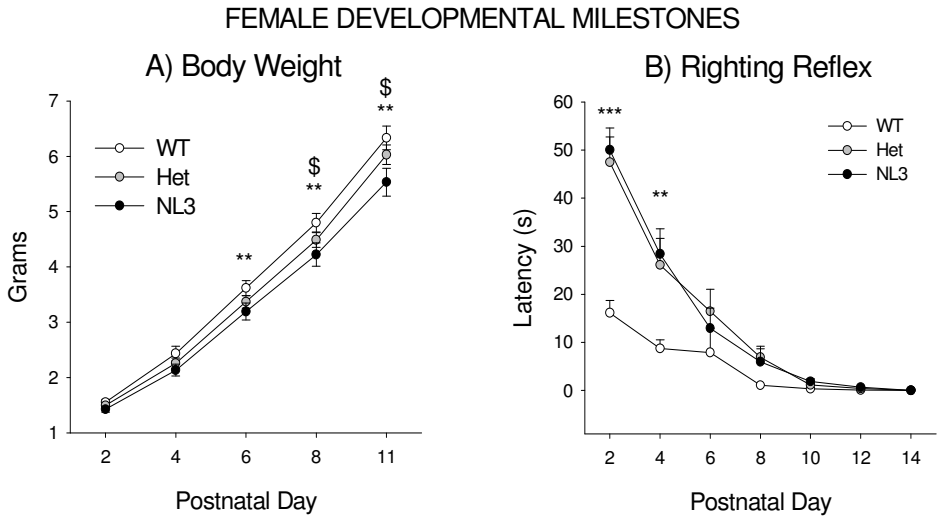
Supplementary Figure 4S. Additional Morris water maze parameters. A) There was no genotype difference detected in swim speed for either acquisition or reversal. B) The WT mice swam closer to the wall than the NL3 mice during acquisition but there were no differences during reversal. WT N = 31, NL3 N = 25.



Supplementary Figure 5S. Ultrasonic vocalizations to separation from the mother and siblings in male and female pups. Males: A) Duration, B) Peak frequency and C) Peak amplitude of vocalizations on PNDs 4, 6, 8 and 11 in response to social separation during a three minute session. No consistent genotype differences were detected across the four ages tested. Females: D) Duration, E) Peak frequency, F) Peak amplitude and G) Number of of USVs. Baseline measurements of vocalizations did not reveal a genotype difference in ontogenetic profile of emissions. Males: WT N = 16, NL3 N = 13. Females: WT N = 6, Het N = 16, NL3 N = 12.



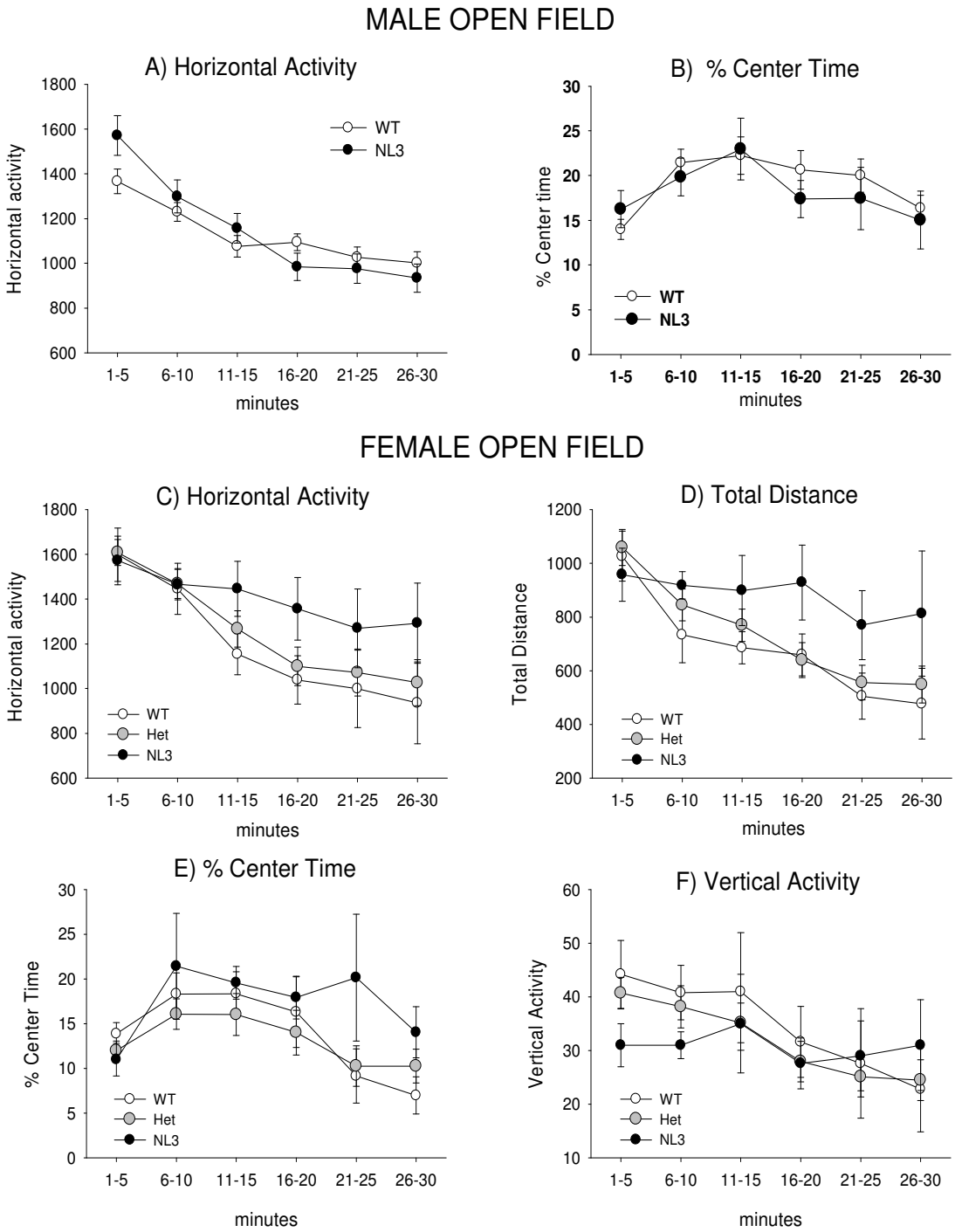
Supplementary Figure 6S. Homing test: A) Latency to reach nest material from the home cage. No significant effect of genotype was found on latency to reach the area containing the nest litter or time spent over the area containing the nest litter. B) Time spent in the nest material. C) Latency to reach nest material from the home cage. D) Time spent in the nest material. A significant genotype effect was found on latency to reach the area containing the nest litter and time spent in the nest area, with NL3 reaching the nesting litter faster and spending more time in the nesting litter than in the clean litter. Males: WT N = 16, NL3N = 16. Females: WT N = 7, Het N = 16, NL3 N = 13.



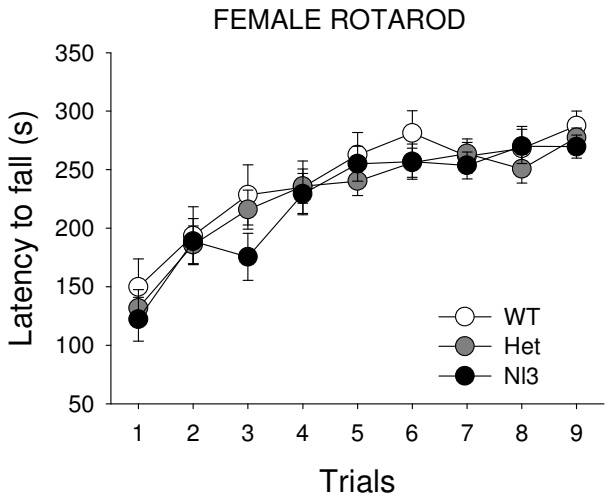
Supplementary Figure 7S. Developmental milestones of female pups. A) Body weights. NL3 pups had a significantly greater weight than WT from PND 6 through PND 11. B) Righting reflex. Transient differences in righting reflex were noted in NL3 and Het compared with WT pups, with female NL3 taking longer to right themselves than WT during the first postnatal day test. WT N = 6, Het N = 16, NL3 N = 12.

Sensory abilities

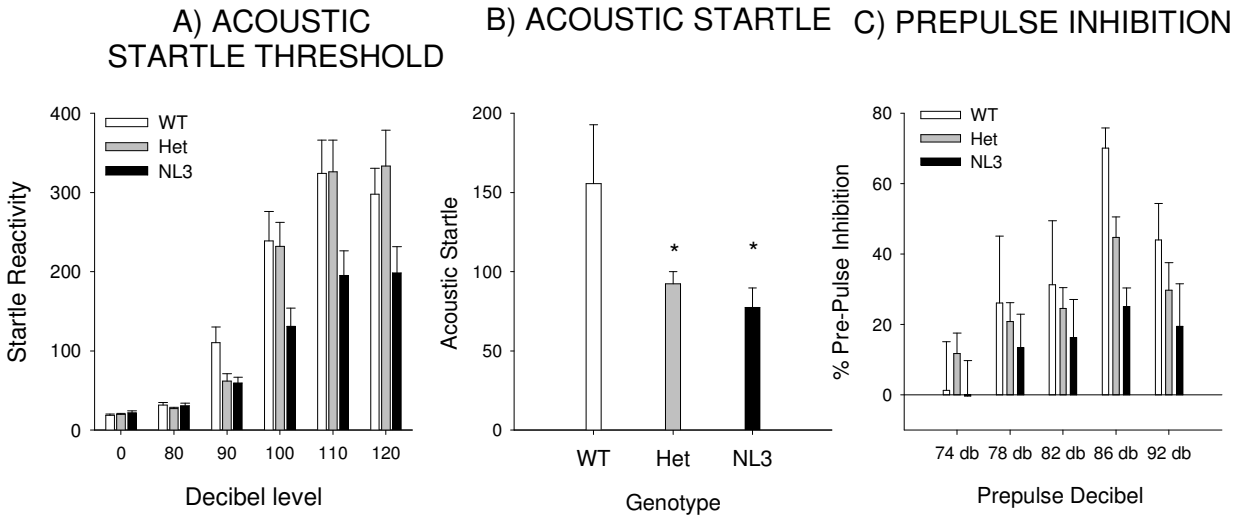
As shown in Table S2, in the females there was no significant effect of genotype on latency to respond to painful stimuli during testing in the hot plate ($F_{2,26}$=1.02, NS) and tail flick tests ($F_{2,26}$=1.82, NS). There was no significant effect of genotype on olfactory sensitivity during buried food testing ($F_{2,55}$=0.88, NS). There was no significant effect of genotype on time spent grooming in an empty cage ($F_{2,26}$=0.21, NS). The NL3 mice had significantly wider forepaw width ($p<0.05$) than the heterozygote and WT females ($F_{2,24}$=4.11, $p<0.05$) in the footprint test, but there were no significant differences in hindpaw width ($F_{2,24}$=1.83, NS) or in stride length ($F_{2,24}$=0.14, NS) between the genotypes.



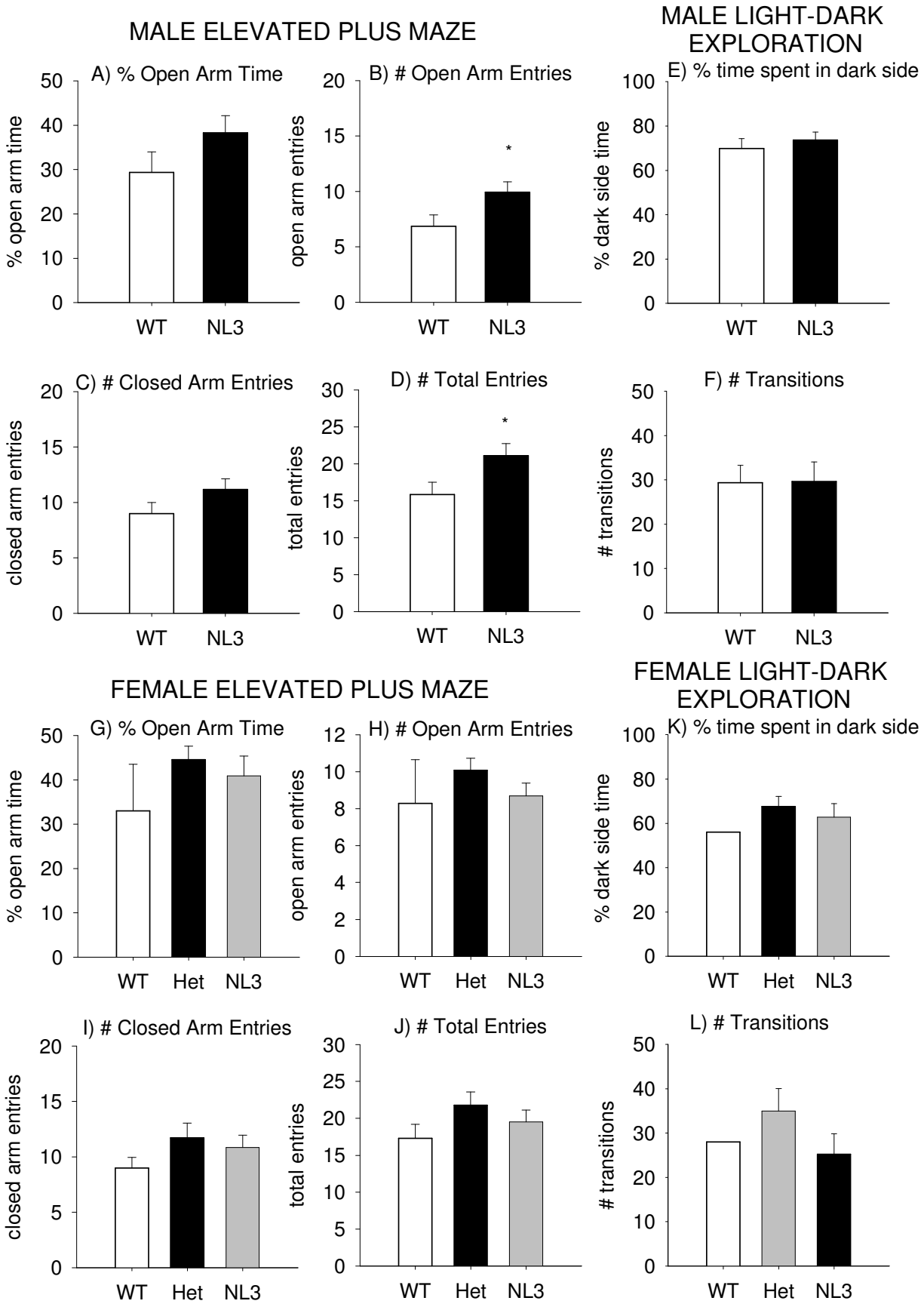
Supplementary Figure 8S. Open Field. Males: no genotype differences were observed between WT and NL3 in measures of A) horizontal activity and B) percentage of time spent in the center of the open field total distance traveled. Females: No genotype differences were detected during a 30 minute session in the open field on C) Horizontal activity, D) Total distance, E) Percentage of time spent in the center of the arena, and F) Vertical activity. Males: WT N = 36, NL3 N=25. Females: WT N=5; Het N = 20, NL3 N=5.



Supplementary Figure 9S. Female Rotarod. There were no genotype differences found on latency to fall from the accelerating rotarod across all trials. WT N = 5, Het N = 19, NL3 N = 5.



Supplementary Figure 10S. Acoustic startle threshold as measured by startle amplitudes to varying decibel levels, baseline acoustic startle response, and percentage of prepulse inhibition at increasing prepulse intensities in NL3, Het and WT female mice. A) NL3 differed from Het and WT, displaying significantly less startle response in the acoustic startle threshold test at the 100, 110 and 120 dB stimuli, but no differences at the lowest startle levels. WT N = 10, Het N = 29, NL3 N = 16. B) NL3 and Het mice showed less startle response than WT at 110 dB. C) No genotype differences were detected in PPI. WT N = 5, Het N = 19, NL3 N = 5.


MALE ELEVATED PLUS MAZE
A) % Open Arm Time
% open arm time
WT
NL3
B) # Open Arm Entries
open arm entries
*
MALE LIGHT-DARK EXPLORATION
E) % time spent in dark side
% dark side time
C) # Closed Arm Entries
closed arm entries
D) # Total Entries
total entries
F) # Transitions
transitions
FEMALE ELEVATED PLUS MAZE
G) % Open Arm Time
Het
H) # Open Arm Entries
FEMALE LIGHT-DARK EXPLORATION
K) % time spent in dark side
I) # Closed Arm Entries
J) # Total Entries
L) # Transitions

S

Supplementary Figure 11S. Anxiety-related behaviors. *males elevated plus maze:* There was a genotype difference detected in the elevated plus-maze on B) number of open arm entries but not A) % open arm time in the elevated plus maze, C) number of closed arm entries. D) The NL3 mice had significantly more total number of entries. WT N = 14, NL3 N = 16. *females elevated plus maze:* No genotype differences were detected on the elevated plus maze on G) % open arm time, H) number of open arm entries, I) number of closed arm entries. J) Total number of entries. WT N = 7, Het N = 14, NL3 N = 13. Light ↔ dark exploration task. *males light ↔ dark exploration.* No genotype differences were detected in E) % time spent in the light side, F) Number of transitions. WT N = 14, NL3 N = 15. *females light ↔ dark exploration.* No genotype differences were detected in K) % time spent in the light side, L) Number of transitions. WT N = 5, Het N = 12, NL3 N = 12.

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Supplemental Tables

Table S1. General health and neurological reflexes of female R451C neuroligin 3 knockin, heterozygote and wildtype mice at age 5-8 weeks.

Genotypes	WT N=5	Het N=19	NL3 N=6	P-Value
General Health				
Body weight (g)	20.8 +/- 1.2	17.6 +/- 2.0	16.5 +/- 2.1	< 0.01
Body temperature (°C)	38.3 +/- .26	38.5 +/- .21	38.6 +/- .24	NS
Fur condition (3 pt scale)	2	2	2	NS
Bald patches (%)	0	0	0	NS
Missing whiskers (%)	0	0	0	NS
Piloerection (%)	0	0	0	NS
Body tone (3 pt scale)	2	2	2	NS
Limb tone (3 pt scale)	2	2	2	NS
Empty Cage Behavior				
Transfer freezing (%)	0	5	33	NS
Wild running (%)	0	0	0	NS
Stereotypies (%)	0	0	0	NS
Exploration (3 pt scale)	2	2	2	NS
Grooming (3 pt scale)	2.4	1.9	1.17	NS
Motoric abilities				
Positional passivity (%)	40	11	17	NS
Wire hang (sec)	60	60	60	NS
Grip strength (force unit)	96+/- 5.6	110 +/- 4.2	111 +/- 4.1	NS
Trunk curl (%)	100	100	100	NS
Reflexes				
Forepaw reach (%)	100	100	100	NS
Righting reflex (%)	100	100	100	NS
Corneal (%)	100	100	100	NS
Pinna (%)	100	100	100	NS
Vibrissae (%)	100	100	100	NS
Reactivity				
Petting escape (%)	20	58	67	NS
Struggle/vocalization (%)	40	58	50	NS
Dowel biting (3 pt scale)	0.8	0.58	0.5	NS

Table S2. Measures of sensory abilities in female littermates neuroligin 3 knockin (NL3), heterozygote (Het) and wildtype (WT).

Genotypes	WT	Het	NL3	P-Value
Pain Sensitivity	N=5	N=19	N=5	
Hot plate (latency – sec)	8.9 +/- .5	9.8 +/- .4	8.8 +/- 1.1	NS
Tail flick (latency - sec)	2.04 +/- .2	2.11 +/- .1	2.47 +/- .3	NS
Olfactory Sensitivity	N= 10	N= 32	N= 16	
Buried food (latency to dig -sec)	42.2 +/- 11.4	86.8 +/- 27.2	46.8 +/- 9.6	NS
Self-Grooming	N= 5	N= 19	N= 5	
Time spent grooming (sec)	44.6 +/- 13.9	35.5 +/- 5.2	34.6 +/- 21.2	NS
Footprint test	N= 5	N= 17	N= 5	
Forepaw width (cm)	1.72 +/- .04	1.84 +/- .04	2.05 +/- .11	<.05
Hindpaw width (cm)	2.55 +/- .14	2.57 +/- .04	2.81 +/- .19	NS
Stride length (cm)	4.87 +/- .21	4.89 +/- .19	4.72 +/- .20	NS