HHS Public Access

Author manuscript

Behav Brain Res. Author manuscript; available in PMC 2016 February 01.

Published in final edited form as:

Behav Brain Res. 2015 February 1; 278: 115–128. doi:10.1016/j.bbr.2014.09.012.

Repetitive grooming and sensorimotor abnormalities in an ephrin-A knockout model for Autism Spectrum Disorders

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Abstract

EphA receptors and ephrin-A ligands play important roles in neural development and synaptic plasticity in brain regions where expression persists into adulthood. Recently, EPHA3 and EPHA7 gene mutations were linked with Autism Spectrum Disorders (ASDs) and developmental neurological delays, respectively. Furthermore, deletions of ephrin-A2 or ephrin-A3, which exhibit high binding affinity for EphA3 and EphA7 receptors, are associated with subtle deficits in learning and memory behavior and abnormalities in dendritic spine morphology in the cortex and hippocampus in mice. To better characterize a potential role for these ligands in ASDs, we performed a comprehensive behavioral characterization of anxiety-like, sensorimotor, learning, and social behaviors in ephrin-A2/-A3 double knockout (DKO) mice. The predominant phenotype in DKO mice was repetitive and self-injurious grooming behaviors such as have been associated with corticostriatal circuit abnormalities in other rodent models of neuropsychiatric disorders. Consistent with ASDs specifically, DKO mice exhibited decreased preference for social interaction in the social approach assay, decreased locomotor activity in the open field, increased prepulse inhibition of acoustic startle, and a shift towards self-directed activity (e.g., grooming) in novel environments, such as marble burying. Although there were no gross deficits in cognitive assays, subtle differences in performance on fear conditioning and in the Morris water maze resembled traits observed in other rodent models of ASD. We therefore conclude that ephrin-A2/-

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No Conflict of Interest

A3 DKO mice have utility as a novel ASD model with an emphasis on sensory abnormalities and restricted, repetitive behavioral symptoms.

Keywords

ephrin-A; Autism Spectrum Disorder; ASD mouse model; grooming; behavior

1. Introduction

Autism Spectrum Disorders (ASD) are heterogeneous neurodevelopmental disorders presenting a varying constellations of social communication impairments and restricted, repetitive patterns of behavior [1]. Despite being highly heritable (~90%), most ASD cases are not accounted for by single gene mutations. Rather, ASDs are associated with many diverse genetic mutations, which may partially account for the clinical heterogeneity of ASDs [2, 3]. A substantial proportion of identified ASD gene candidates encode synaptic proteins, and several of these are associated with common changes in synaptic physiology, neuronal morphology, cortical connectivity, and/or abnormal behaviors [4]. Recently, a family of developmental guidance molecules with these capabilities, the A-class Eph receptors and associated ephrins, joined the list with the identification of EPHA3 as an ASD candidate gene [2] and EPHA7 deletions being linked to developmental neurological delays during early childhood [5].

Eph/ephrins are cell-surface molecules with important functions during development, including regulating neuronal migration and sorting [6], topographical organization of neuronal connections [7-10], synaptogenesis [11], and synaptic plasticity [12-16]. Eph receptors and ephrin ligands have A-class and B-class sub-families, which exhibit a hierarchical within class binding specificity, with select members also possessing across-class binding [17]. A key feature of Eph/ephrin action is their exquisite sensitivity to gradients of complementary ligand and receptor concentrations in afferent and target sites [9]. Thus, even subtle changes in relative levels of Eph/ephrins can have marked effects on neuronal organization and function, as exemplified by retinotectal topographic mapping [18-21].

Deletions of A-class ephrins and EphA receptors also are associated with behavioral and anatomical phenotypes in animal models relevant to neuropsychiatric disorders. For example, deletion of ephrin-A3 results in impairments of context related hippocampal learning/memory [22]. In contrast, mice with ephrin-A2 deletions have intact hippocampal learning and memory, but impaired behavioral flexibility in a reversal learning/set shifting task [23]. This behavior bears a striking resemblance to core symptoms of Autism Spectrum Disorders (ASDs) seen both in humans and in animal models of ASD [24], and may likewise be relevant to Obsessive Compulsive Disorders (OCDs) and Tourette Syndrome (TS) [25]. Observations of mice in our colony with combined deletions of ephrin-A2 and ephrin-A3 provide a further link between ephrins-A2/-A3 and the above disorders since these mice develop abnormal increased repetitive facial grooming behaviors that result in lesions and abrasions around the eyes and snouts. Such “compulsive grooming” behaviors in

rodents are considered to model repetitive, rhythmic, patterned, and coordinated motor movement stereotypies in humans [26], a common symptom that may occur in ASDs, OCD, or TS.

Interestingly, similar “compulsive grooming” phenotypes have been observed in other mouse models with candidate ASD gene deletions, such as SAPAP2/Shank3 knockouts [27, 28]. EphA3, ephrin-A2, ephrin-A3 are all located at excitatory synapses, which provides a compelling link with other synaptic proteins encoded by candidate genes for ASD (including Shank3). Mutations in several of these genes are associated with changes in synaptic physiology, neuronal morphology, cortical connectivity, and/or abnormal behaviors [4, 29]

Despite the intriguing initial behavioral finding in animals and humans with deletions of A-class Eph/ephrins, surprisingly few studies have thoroughly investigated the behavioral phenotypes of mice with ephrin-A deletions. To bridge this gap, we evaluated sensorimotor, exploratory, anxiety, and learning/memory function in mice lacking both ephrins-A2 and -A3. To compare the pattern of performance of ephrin-A2/3 double knockout (DKO) mice across these behavioral domains (which are associated with the core features of ASDs, OCD, and TS), mice were tested on a broad array of assays (Open field, Marble Burying, Elevated Plus Maze, Sociability, Grooming, Prepulse Inhibition of Acoustic Startle, Morris Water Maze, and Fear Conditioning). Behavioral results across assays were then compared with control mice to assess whether DKOs may serve as a more specific animal model for one or more of these disorders.

2. Materials and Methods

2.1. Animals

Founder mice for the ephrin-$A2^{-/-}A3^{-/-}$ (ephrin-A2/3 double knockout; “DKO”) and ephrin-$A2^{+/+}A3^{+/+}$ (wildtype; “WT”) mice were generously provided by Dr. David Feldheim at UC, Santa Cruz. Founding breeders were on a mixed Swiss-Webster/C57BL-6/129 background and contained various combinations of gene deletions for ephrin-A2, ephrin-A3, and ephrin-A5. The original ephrin-A2 mutant mice were generated on a Swiss-Webster mouse strain background by Feldheim et al. [20]. Ephrin-A3 mutant mice were generated on a C57BL-6 strain background as described in Cutforth et al. [30]. The Ephrin-A5 mutant mice were generated in mice of a 129 strain background as described by Frisén et al. [21]. For a parallel set of physiological and anatomical experiments (manuscripts in preparation), the ephrin-A mouse colony was also crossed with mice that contained transgenes for either D1-tomato (BAC-Drd1a-tomato [31] courtesy of Dr. Calakos, Duke University), D2-EGFP (BAC-Drd2-EGFP [32] courtesy of Dr. Lovinger, National Institutes of Health), or EphA2-EGFP [MMRRC Tg(EphA2-EGFP)DE51Gsat/Mmmh; Stock #000298-MU]. These mice also were on either a C57BL-6 or a Swiss-Webster background. Since individuals with ASD comprise a heterogeneous genetic population, we decided to maintain our colony on a C57BL-6/Swiss-Webster hybrid background so the effects of the genetic background of a single strain would not bias our results. As performance on many common behavioral assays can vary between inbred strains [33-35], genetic drift between the DKOs and WTs was minimized by interbreeding these mice and then limiting the number of subsequent breeding cycles for each genotype to a

maximum of 3 generations before back-crossing with mice from an earlier generation. Further control for effects of genetic drift on behavior in the C57BL-6/Swiss-Webster hybrids was evaluated by statistical analysis of behavioral data between mice generated from early versus late breeding generations. These analyses indicated no significant difference between groups for either genotype.

Mice were housed on a 12-hour/12-hour light-dark cycle and all behavioral tests were performed during the light phase. Behavioral testing was conducted with a total of 73 mice, aged 4-6 months (WT n=42; DKO n=31).

This study strictly conformed to National Institutes of Health guidelines and the policies of the Georgetown University Animal Care and Use Committee. Care and housing was provided by the Georgetown University Division of Comparative Medicine, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

2.2. Testing order

At least 10 mice per genotype were tested on all observational behavioral assays (in the following order: Open Field, Marble Burying, Elevated Plus Maze, Sociability, and Grooming assays) followed by testing for prepulse inhibition of acoustic startle response (PPI/ASR). Additional mice underwent behavioral testing on more than one, but not all assays. For the Morris water maze (MWM) testing was performed on 10 mice per genotype (5 naive and 5 with prior behavioral testing).

Testing order was chosen so that mice always underwent low-stress tests before high-stress tests. All mice were males with the exception of PPI/ASR and Open-Field (OF) tests, which had larger sample sizes that included females. Females were included in these assays because power analyses of pilot data indicated that the variability in these assays required greater sample sizes. There was no significant effect of sex on any of the measures and post-hoc analysis confirmed that there were no sex-based differences in performance on either test. In both OF and PPI/ASR assays, females comprised approximately one-third of each group.

2.3. Behavioral tasks

2.3.1. Open-field (OF) Testing—Open-field experiments were performed in a sound controlled room with low light (white noise at 55 dB and 15 lux illumination). Spontaneous locomotor activity was assessed in a standard mouse TruScan photobeam arena (Coulbourn Instruments, Allentown PA) capable of detecting motion in the X-Y and Z- planes. Software recorded the position of the mouse at a rate of 1 Hz and was used to calculate horizontal, vertical (rearing) and thigmotactic behavior. Mice (WT n=29, DKO n=26) were habituated to the testing room for at least 30 min before testing on a 1 hour test. Each test lasted one hour, and data were analyzed in 10-min bins.

2.3.2. Elevated plus maze (EPM)—Mice (WT n=27, DKO n=26) were tested in the EPM using the methods previously described [36] in a standard white mouse elevated plus maze (26 inch arms, elevated 15 cm; San Diego instruments).

2.3.3. Marble-Burying (MB)—The procedure for marble burying was adapted from prior studies [37]. Mice (WT n=11, DKO n=16) were placed in a standard plastic mouse cage (29.85 cm × 18.42 cm) that contained 20 marbles evenly spaced in a 5 by 4 grid on top of 4.5 cm of bedding. After thirty minutes the mouse was removed and the number of buried marbles (>2/3rds covered) were counted. All mice were tested twice, on separate days, and non-parametric statistical analyses applied (Mann-Whitney test).

2.3.4. Grooming—Mice (WT n=16, DKO n=21) were placed in a standard mouse cage and grooming behavior was induced by misting the animal's face with water from a small spray bottle. This procedure mildly wet, but did not soak, the animal's face and head. Mice were then videotaped for 5 min and the total amount of time spent grooming was quantified by two separate observers blinded to the animal's genotype. All videos were rated by both observers, and there was no significant difference in means between raters. The average of the two values was used for statistical analysis.

2.3.5. Prepulse inhibition (PPI) of acoustic startle response (ASR)—Mice (WT n=37, DKO n=27) were tested for ASR and PPI of the ASR as previously described [36, 38] using the SR-LAB startle reflex system (San Diego Instruments, San Diego, CA). Background noise (70 dB) was present throughout the session. The startling stimulus was a broadband acoustic pulse with an intensity of 120 dB and a duration of 30 milliseconds (ms), and was either administered alone (“pulse-alone” trials) or paired with the prior presentation of a 30 ms duration prepulse (“prepulse” trials). The prepulse stimulus intensity was set to 3, 6, or 12 dB above background noise and was delivered with an interstimulus interval (ISI) of 130 ms (onset-to-onset). A testing session contained a total of 25 trials (10 pulse-alone and 5 prepulse trials at each prepulse intensity). Inter-trial intervals ranged from 5-25 sec. Startle magnitude was calculated as the average of the startle responses to the pulse-alone trials. PPI was calculated according to the formula: %PPI = (1 - (startle response for prepulse + pulse trials/startle response for pulse alone trials)) × 100.

2.3.6. Three-chambered social interaction test—Mice (WT n=12, DKO n=20) were tested in a three-chamber social behavior paradigm as described in prior studies [34]. This apparatus consisted of a 40 cm × 40 cm × 40 cm plexiglass box containing 3 chambers separated by two clear Plexiglas partitions to create two 40 cm × 15 cm side (social) chambers and a center “home” chamber (40 cm × 10 cm). Each partition had one drop-door that could be lifted to allow the animal access to either side chamber which contained a 6 inch high cylindrical cage made of mesh wire. Time spent in each chamber was recorded by using ANYMaze software (Stoelting Co.) over 3 bins of 5 min each.

Test mice were placed in the center (home) chamber and acclimated for 5 minutes (“acclimation period”). A novel mouse was then placed in one of the mesh cylinders (“social chamber”) while leaving the other mesh cylinder empty (“empty chamber”). The drop doors were raised and the animal was allowed 10 additional min to freely explore the apparatus (“test period”). Sociability was assessed using the formula: (time spent in the social chamber/(time spent in social chamber + time spent in the empty chamber)) × 100. Thus, a score of 50 indicates no significant social preference, a score higher than 50 indicates a social preference, and a score less than 50 indicates a social aversion.

2.3.7. Morris water maze (MWM)—Testing was carried out in a standard mouse water maze (4 foot in diameter; San Diego Instruments, San Diego, CA) in a 6 square foot room illuminated at 720 lux. A plexiglass platform (4 inches in diameter) was placed in one quadrant and submerged 1cm under opaque water. Visual markings were located at various positions on the maze walls and large extra-maze visual cues were attached to the walls. A camera placed above the pool recorded animal behavior in the maze and various parameters of interest (position, swim path, speed) were tracked using ANYMaze software (Stoelting Co).

Acquisition training: Mice (WT n=10, DKO n=10) were trained to locate the platform with 4 trials per day lasting 90 sec each, with a 25 min inter-trial interval. Each day the mouse was released from each of four starting points. Training and testing occurred over a 2 week period with a 2 day non-training rest between training days 5 and 6. The “escape latency” was recorded for each trial (latency for the mouse to find and remain on the platform for 3 consecutive seconds (training days 1-3) or 6 consecutive seconds (training days 4-9)). If a mouse failed to find or remain on the platform during the 90 second trial, a latency of 90 sec was assigned, and the mouse was placed on the platform for 10 sec before being returned to its home cage. Mice were considered to have reached criterion when they successfully found the platform at least 3 out of four trials for 2 consecutive days.

Probe Trial: On the tenth day, a retention probe trial was conducted in which the platform was removed and the mice were allowed to swim for 90 sec.

Visible Platform Trial: A visible platform probe trial was conducted to control for possible vision differences between genotypes due to the effects of the ephrin-A2/3 gene deletions on visual performance [20], as well as possibly absent motivation to “escape”. For this trial, the platform was replaced to its original location with a visible marker placed in the center. Mice were released in the same quadrant as during the probe trial and given 90 sec to reach and remain on the platform for 3 sec.

Reversal Trial: Two days after the probe trial, mice (WT n=5, DKO n=5) underwent reversal training with the hidden platform located in a different quadrant. Mice underwent 5 days of reversal training in which the trial was ended after 3 consecutive sec on the platform for reversal-training day 1 and after 6 sec for reversal training days 2-5. On the sixth consecutive day, another series of probe trials were conducted to assess reversal learning.

Data are expressed as weighted latency values per day, which normalized the average escape latency of all four trials per day by a factor that reflects the number of successful trials that day. For each day of training, a weighted latency value was calculated as [0.5] × [avg. trial latency] × [normalization factor reflecting the number of successful trials]. The normalization factor was calculated as equal to: [1 + (1 – ([number of trials with latencies < 90 sec] / 4))]. Thus, weighted latency values per day were used for statistics as a measure of learning that takes into account the number and latency of successful trials for each animal.

2.3.8. Fear conditioning (FC)—FC testing was carried out as previously described [39] with slight modifications. The apparatus consisted of a moderately lit plexiglas FC chamber

with foot-shock bars (Med-Associates Inc., St Albans, VT), and the procedure and video analysis of freeze time was driven by FreezeScan software (Clever Sys Inc., Reston, VA). Mice (TWT n=7, DKO n=7) were transported to the testing room in their home cages and allowed to acclimate for at least 30 min before testing. A single FC chamber was used for conditioning, context testing, and cue testing, but was disguised for cue testing by covering the bare chamber walls and shock bars with laminated and sporicidin-wiped colored panels, and by placing blocks and cardboard pieces on the Plexiglas ceiling. 24 hours prior to the conditioning session, mice were acclimated to the undisguised bare chamber sprayed with 70% ethanol ("conditioning environment") for 15 minutes, then returned to their home cage for 1 hour before being acclimated to the disguised environment ("unconditioned environment") for another 15 minutes. The next day, mice underwent conditioning procedures in the FC chamber configured as the conditioning environment. This procedure entailed a 180 sec baseline period ("Pre-Conditioning"), followed by three trials (separated by 120 sec inter-trial intervals) consisting of a 30 sec presentation of a 1000-Hz/70-dB tone as conditioned "cue" stimulus, delivered through speakers mounted on the inner chamber, which terminated with 2 sec delivery of a 1.5 mA foot shock as unconditioned stimulus. FreezeScan software was used to analyze the amount of time the animal spent "frozen" throughout the baseline period, three tone-shock presentations, and inter-trial intervals. Context-dependent fear memory was assessed 24 hours later by again placing mice in the conditioning environment and measuring time spent freezing during a 180 sec trial ("Context"). One hour after context-dependent fear memory testing, cue-dependent fear memory was assessed by placing the mouse in the unconditioned environment and measuring freezing activity throughout a 120 sec baseline period ("Pre-Cue") followed by three presentations of the 30-sec tone ("Cue") separated by 90 sec intervals.

2.4. Statistics

The following measures were analyzed using unpaired student's t-tests (assuming equal standard deviations): total distance travelled in the OF; rearing in the OF; thigmotaxis in the OF; distance travelled, time spent in the open arms, % entries to open arms, and number of entries to the open arms in the EPM; time spent grooming; ASR; and social index values. 1-way ANOVA with Bonferroni's multiple comparisons test for post-hoc analysis was used to analyze the % time spent in each quadrant in the MWM probe trial for each genotype. The following measures were analyzed using 2-way ANOVA: total distance moved over 10-min time bins in the OF; PPI at different prepulse intensities; time spent in each zone of the social assay; weighted latency values in the MWM; and % time spent in each quadrant during the MWM probe trial. All post-hoc analysis for 2-way ANOVAs used Bonferroni's multiple comparisons test. Main effects from 1-way and 2-way ANOVA are expressed in the text as an F-statistic and P-value within brackets, and for post-hoc comparisons p-values are represented in figures. In text and figures, data are presented as mean ± SEM.

3. Results

3.1. Locomotor Activity, Exploratory Behavior, and Anxiety

The open field (OF), elevated plus maze (EPM), and marble burying (MB) assays are complementary assays for different facets of anxiety in rodents [40]. In these assays, the

natural tendency for mice to explore novel areas and objects is pitted against their natural aversion to open spaces and bright lights [41, 42]. Locomotor activity in these assays also reflects overall activity levels and exploratory behavior. Thus, it is not necessarily straightforward to classify measures in the EPM, OF, and MB assays as reflecting either anxiety or exploratory behavior [40]. For example, lower values for the number of hindlimb rears in the OF, time spent in the open arms of the EPM, increased values for the proportion of time spent in the margins versus the center of the OF ("thigmotaxis"), and number of marbles buried in the MB assay are all taken as indicators of "state" anxiety (i.e., increased anxiety induced by an anxiogenic stimulus) [37, 40, 41, 43]. However, total horizontal distance travelled, rearing behavior, zone changes, and digging in the MB assay are also indicative of overall locomotor activity levels and exploratory behavior [40, 44]. Furthermore, anxiety measures in inbred strains are not always replicable across different assays. Although locomotor-related behaviors are more reliable measures across assays, differences in locomotor activity levels can confound measures of anxiety [40]. Therefore, exploratory behaviors were examined alongside anxiety measures, and inferences about anxiety as an innate "trait" of a mouse strain drawn only from a pattern of such measures over several assays.

3.1.1. Open Field (OF)—Figure 1 illustrates spontaneous locomotor activity as measured over 1 hour in the OF. DKO mice travelled significantly less distance compared with WTs (Fig. 1a) [t-test: p<0.001, t=4.244, df=53; *DKO, 4954 ± 671.5cm; WT, 8427 ± 488.3cm*]. On visual examination, all mice exhibited normal gait and ambulatory coordination.

To test whether differences in spontaneous locomotor activity reflect differences in overall activity level versus exploratory behavior in a novel environment, total horizontal distance travelled was analyzed in 10-min bins (Fig. 1b). This enabled an evaluation of whether differences in spontaneous locomotor activity between genotypes increased over time. DKOs were significantly hypolocomotive compared with WT mice at all time bins [Bonferroni: time= 0-10min, p<0.01; 10-20min, p<0.01; 20-30min, p<0.01; 30-40min, p<0.001; 40-50min, p<0.05; 50-60min, p<0.001]. However, the decline in exploratory activity was identical between genotypes based on non-linear regression analysis, indicating that DKOs had a comparable exploratory response [P>0.05, F(2,324)=0.02, R^2=0.396]. Although there were significant effects of time [2-way ANOVA: P<0.001, *F*(5,265)=83.75] and genotype [2-way ANOVA: P<0.001, *F*(1,53)=18.01] on total distance travelled, there was no significant time × genotype interaction [2-way ANOVA: P>0.05, *F*(5,265)=0.42]. Thus, the relative hypoactivity of DKOs as compared to WTs did not change over time. These data indicate that although DKO mice are hypoactive overall, their decreased rate of exploratory activity over time was comparable to that of WT mice.

DKOs reared significantly fewer times than WTs (Fig. 1c) [t-test: p<0.001, *t*=5.80, df=53; *DKO, 222.2 ± 25.08; WT, 395.3 ± 17.13*] and exhibited significantly increased thigmotaxis (i.e., the proportion of time in the margins of the OF) (Fig. 1d) [t-test: p=<0.01, *t*=3.22, df=53; *DKO, 0.93 ± 0.14; WT, 0.45 ± 0.07*]. Examined together, these data suggest that DKOs exhibited evidence of decreased overall exploratory behavior in the OF. However, decreased rearing and increased thigmotaxis may or may not be indicative of increased anxiety since both measures could be impacted by the overall decrease in locomotor activity.

3.1.2. Elevated Plus Maze (EPM)—During the 5 min testing period WTs and DKOs did not differ on measures of time spent in the open arms [t-test: p>0.05, *t*=0.06, df=51; *DKO, 64.95 ± 9.23sec; WT, 65.69 ± 8.23sec*], the number of entries into the open arms [t-test: p>0.05, *t*=0.00, df=51; *DKO, 7.00 ± 0.85; WT, 7.00 ± 0.79*], the percent of open arm entries [t-test: p>0.05, *t*=0.53, df=51; *DKO, 22.33 ± 1.91%; WT, 21.0 ± 1.6%*], nor distance travelled in the EPM [t-test: p>0.05, *t*=0.84, df=51; *DKO, 5.73 ± 0.54m; WT, 6.38 ± 0.57m*]. These results indicate that DKOs did not display signs of increased anxiety-like behaviors in this assay.

3.1.4. Marble Burying (MB)—Given the conflicting results for both locomotor activity levels and anxiety measures in the EPM and OF, mice were tested using the marble burying (MB) assay to probe both anxiety-related behavior and locomotor activity. Consistent with the significant decrease in locomotor activity and exploratory behavior of DKOs in the OF, they also buried significantly fewer marbles than WTs (Fig. 2a) [Mann-Whitney: p<0.01, *U*=35, df=27; *DKO, 11.16 ± 1.305; WT, 16.45 ± 1.012*].

However, in this assay DKOs again demonstrated a normal exploratory response to a novel environment. Genotype was not a significant factor affecting the time spent exploring the apparatus over 10-min bins [2-way ANOVA: P=0.107, *F*(1,25)=2.79], and time spent engaged in exploratory behavior (ambulation, rearing, or digging) was highest in the first 10 min of the MB assay , with no significant difference between DKOs and WTs (Fig. 2b) [Bonferroni: p>0.05, *t*=0.68, df=75; *DKO, 523.13 ± 19.37s; WT, 552.91 ± 14.12s*]. However, post-hoc comparisons indicated DKOs engaged in exploratory behavior significantly less in the second 10 min of testing [Bonferroni: p<0.05, *t*=2.51, df=75; *DKO, 390.38 ± 36.65s; WT, 500.73 ± 24.1s*]. Video analysis revealed that DKOs spent non-exploratory time mostly engaging in grooming behaviors and that time spent grooming increased in proportion to the decreased time spent exploring. DKOs were also observed to paw and sniff the marbles in a comparable fashion as WTs. These observations suggest that the decreased marble burying observed in DKOs most likely reflects an increased shift towards self-directed behavior in a novel environment, rather than an attenuated exploratory response due to general hypoactivity.

3.2. Grooming Behaviors

3.2.1. Observed phenotype—DKO mice manifest a compulsive facial grooming phenotype that becomes apparent around 3 months of age and increases in severity over time. This leads to the appearance of significant hair loss or skin lesions at the site of over-grooming between 4-6 months of age. Figure 3a illustrates a representative DKO mouse with typical self-induced hair loss and abrasions around the eyes and snout. Hair loss, abrasions, and skin lesion sites (including on the snout, around and beneath the eyes, and occasionally behind the ears) often differed between mice, but always corresponded with the particular grooming motion that the mouse was observed to perform excessively. While lesions and hair loss tend to be symmetrical on the snout, consistent with the stereotyped bilateral snout grooming motion [45], they often appeared asymmetrical for the ears and eyes. Where lesions were unilateral, these tended to be on the side where the ear tag was located. Interestingly, several mice eventually pull out their ear tag due to asymmetrical

compulsive grooming. After being re-tagged on the opposite side, two mice developed new grooming-related lesions on this side. While not quantified, no obvious difference between males and females in compulsive grooming phenotype was observed. However, this was difficult to assess as the prevalence of grooming increased with age.

3.2.2. Grooming Assay—To quantify time spent in a facial grooming behavior, differences in grooming time between DKO and WT mice were assessed using a stimulated grooming test. Figure 3b shows that during the 5 minutes after receiving a facial spritz of water, DKOs spent significantly more time grooming compared with WTs [t-test: p<0.001, *t*=3.74, df=35; *DKO, 205.3 ± 13.45sec; WT, 135.1 ± 12.28sec*].

3.3. Sensorimotor integration

3.3.1. Acoustic Startle Response (ASR) and Prepulse Inhibition (PPI)—ASR and PPI assay sensorimotor gating in humans and animals by measuring the attenuation of a reflexive startle response when a startling stimulus is delivered after a weak pre-stimulus or prepulse. Figure 4a illustrates the acoustic startle responses that were measured during PPI testing in trials where no prepulse was presented. DKOs had a significantly attenuated auditory startle compared to WTs as measured (in arbitrary units) on the trials without prepulses [t-test: p<0.001, *t*=4.38, df=62].

PPI was evaluated by delivering prepulses at intensities of 3 dB, 6 dB, and 12 dB above background noise 130 ms prior to administering the AS. As shown in Figure 4b, DKOs have significantly greater PPI of ASR (measured as % decrease) than WTs at all prepulse intensities. Main effects were identified for both prepulse intensity [2-way ANOVA: P<0.001, *F*(2,124)=102.57] and genotype [2-way ANOVA: P<0.001, *F*(1,62)=51.27]. This indicates that there was an increase in PPI with increased prepulse intensity and that PPI differed between genotypes. A significant genotype by prepulse intensity interaction [2-way ANOVA: P<0.001, *F*(2,124)=34.182] was also found. Thus, the increase in prepulse intensity did not affect PPI equivalently among genotypes.

3.3.2. ASR influence on PPI—To determine if differences in ASR could account for the differences seen in PPI, we performed an analysis of covariance (ANCOVA) with ASR as a covariate. The main effects and interactions seen in the above ANOVA were preserved and there was no main effect [ANCOVA: P>0.05, *F*(1,61)=2.90] or interaction between ASR and any other variable [ACNOVA: P>0.05, *F*(2,122)=0.39]. These results support the interpretation that decreased ASR cannot directly account for the increased PPI in DKOs.

3.4. Social Approach Behavior

In the three-chamber social behavior assay, DKOs exhibited a clear social aversion (Fig. 5a), with social index values significantly less than WTs [t-test: p<0.01, *t*=2.89, df=30; *DKO, 33.36 ± 6.749; WT, 59.52 ± 2.94*]. As in the OF, DKOs exhibited less spontaneous locomotor activity in this assay, even during the first 5 minutes of habituation to the novel environment of the apparatus while restricted to the center zone (not shown) [t-test: p<0.01, *t*=3.34, df=30; *DKO, 8.12 ± 1.17m; WT, 14.67 ± 1.631m*]. Therefore, the amount of time spent in each zone was analyzed using a 2-way ANOVA with multiple comparisons to

assess whether the difference in social index reflected a preference for non-social object interaction or decreased overall exploration of the apparatus (Fig. 5b). As expected, DKO mice spent significantly less time in the social zone than WT mice [Bonferroni: $p<0.001$, $t=3.86$, df=90; *DKO, 138.47 ± 29.44sec; WT, 287.48 ± 15.1sec*]. However, they did not spend more time in the empty chamber; rather, DKO mice spent significantly more time in the center zone than WT mice [Bonferroni: $p<0.01$, $t=3.49$, df=90; *DKO, 228.2 ± 26.63sec; WT, 93.77 ± 12.71sec*]. There was no significant difference between genotypes for the time spent in the empty zone [Bonferroni: $p>0.05$, $t=1.93$, df=90; *DKO, 142.93 ± 29.37sec; WT, 217.17 ± 13.99sec*]. Furthermore, while there was a significant effect of genotype [2-way ANOVA: $P<0.05$, $F(1,30)=4.71$] and a genotype by zone interaction [2-way ANOVA: $P<0.001$, $F(2,60)=11.06$], 2-way ANOVA revealed no significant main effect of zone. These data suggest that DKOs are less exploratory but nevertheless exhibit a strong social avoidance preference.

3.5. Learning and Memory

3.5.1. Morris Water Maze (MWM) assay—The MWM tests hippocampal-dependent spatial learning and memory [33]. To quantify learning over the 9 days of training, weighted latency to escape the maze was calculated for mice on each training day as an index of performance that encompasses both time values on individual trials and the number of successful trials per day. Analysis revealed a significant effect of training day [2-way ANOVA: $P<0.001$, $F(8,144)=21.58$] but no significant effect of genotype on weighted latency values or training day by genotype interaction. As illustrated in Figure 6a, DKOs had comparable learning curves for finding the platform as WTs. As learning is reflected on the probe trial by increased swimming time in the maze quadrant (NE) that contained the platform, percent time spent swimming in each quadrant (Fig. 6b) and the number of entries into the platform zone (Fig. 6c) was analyzed by genotype for the probe trial. No significant main effect of genotype or genotype by quadrant interaction was found, but there was a significant main effect of quadrant [2-way ANOVA: $P<0.001$, $F(3,54)=16.76$]. Both DKO and WT mice spent significantly more time swimming in the NE quadrant relative to the other quadrants [1-way ANOVA: DKO: $P<0.001$, $F(3,36)=7.58$; NE, 34.19 ± 4.27sec; NW, 17.43 ± 1.75sec; SE, 21.35 ± 2.29sec; SW, 17.04 ± 2.74sec; WT: $P<0.001$, $F(3,36)=16.08$; NE, 37.92 ± 3.01sec; NW, 10.64 ± 3.18sec; SE, 27.43 ± 4.04sec; SW, 14.01 ± 1.98sec]. Moreover, there was no significant difference in the number of entries into the platform zone. On the visible platform trial, 10/10 WT and 10/10 DKO mice quickly reached and mounted the platform (not shown).

3.5.2. Swim Patterns during MWM testing—Observation of swim patterns during training and the probe trial hint that WT and DKO mice might have employed different strategies during learning. Observations of swimming behaviors indicated that, in general, WT mice initially explored the maze using a combination of large circular swim patterns interspersed with some cross-maze swimming. In contrast, DKOs tended to exhibit small spiral swim patterns along the wall of the pool (edge-spirals) interrupted by bursts of random swimming that criss-crossed the maze. Figure 6d illustrates the swim patterns at four time-points: the first trial, the final day of training, the probe trial, and the visible platform control trial for a representative WT and DKO mouse. Interestingly, on the probe

trial DKO mice tend to revert back to the “criss-cross” or “edge-spiral” swim patterns that were evident during early training, whereas WT mice tend to swim in small circles around the original location of the hidden platform, and do not revert to early learning swim patterns.

3.5.3. MWM Reversal Learning—Mice were able to quickly learn the new location of the hidden quadrant during reversal training. There were no significant differences between WTs and DKOs in time to reach reversal learning criteria, nor was there a significant effect of genotype on daily weighted latency values [2-way ANOVA: P>0.05, $F(1,8)=0.63$] or significant differences between genotypes (n=5 each) in daily weighted latency values, according to Bonferroni post-tests [Bonferonni: Day 1: p>0.05, $t=0.51$, df=40, *DKO 40.99 ± 6.59, WT 48.08 ± 17.29*; Day 2: p>0.05, $t=1.31$, df=40, *DKO 18.99 ± 2.19, WT 37.08 ± 17.26*; Day 3: p>0.05, $t=0.11$, df=40, *DKO 11.53 ± 2.73, WT 10.05 ± 4.96*; Day 4: p>0.05, $t=1.13$, df=40, *DKO 8.28 ± 1.17, WT 23.83 ± 16.54*; Day 5: p>0.05, $t=0.18$, df=40, *DKO 6.79 ± 0.58, WT 9.30 ± 2.52*]. Observations of swim patterns on the reversal learning probe trial did not differ from those in the initial probe trial (data not shown). In the reversal learning probe trial, there was a only a significant effect of quadrant [2-way ANOVA: P<0.01, $F(3,24)=7.44$] and no significant difference between genotypes on the percent time spent in each quadrant [Bonferroni: all: NE, p>0.05, $t=0.14$, df=32; NW, p>0.05, $t=0.47$, df=32; SE, p>0.05, $t=0.93$, df=32; SW, p>0.05, $t=1.54$, df=32].

3.5.4. Fear Conditioning—As context-dependent and cue-dependent aspects of fear learning/memory are thought to differentially involve hippocampal and amygdala function respectively, a fear conditioning (FC) assay was used to probe whether function in these regions is affected in DKO mice. In this test freezing behavior was compared across three sessions (Fig. 7) for 4 conditions: first, in the conditioning environment prior to the first presentation of the tone (conditioned “cue” stimulus) and foot shock (“Pre-Conditioning” session); second, in the conditioned environment 24 hours post-conditioning (“Context” session); third, in the unconditioned environment prior to re-exposure to the tone (“Pre-Cue” session); and fourth, in the unconditioned environment during the 30 sec presentation of the tone (“Cued” session). Analysis of variance, testing for main effects of session (e.g., testing for contextual vs cue-related fear behavior) and genotype showed a significant main effect of session [2-way ANOVA: P<0.001, $F(3,39)=13.12$], but not genotype; and a significant genotype by session interaction [2-way ANOVA: P<0.05, $F(3,39)=3.36$]. Bonferroni post-tests only identified a significant difference in DKO mice freezing time during the “Pre-Cue” session relative to WT mice [Bonferroni: p<0.05, $t=2.81$, df=52; *DKO, 58.79 ± 11.76sec; WT, 18.66 ± 6.17sec*]. However, there was a significant increase in freezing time for all genotypes between “Pre-Conditioning” and the “Context” [Bonferroni: *DKO*, p<0.05, $t=2.90$, df=39; *pre-conditioning 33.77 ± 8.34sec, context 68.97 ± 13.50sec*; *WT*, p<0.05, $t=2.85$, df=39; *pre-conditioning 39.66 ± 9.03sec, context 71.95 ± 11.49sec*] and “Cued” test sessions [Bonferroni: *DKO*, p<0.001, $t=4.44$, df=39; pre-conditioning 33.77 ± 8.34sec, cue 87.57 ± 8.64sec; *WT*, p<0.05, $t=2.51$, df=39; *pre-conditioning 39.66 ± 9.03sec, cue 68.13 ± 10.29sec*].

4. Discussion

According to the Diagnostic and Statistical Manual of Mental Disorders V, ASD is diagnosed based on dysfunction in 2 core symptom domains: “social communication” and “restricted and repetitive patterns of behaviors, interests, or activities” (RRB), with sensory processing abnormalities included as a variable symptom in the RRB domain. The present behavioral characterization of ephrin-A2/3 DKO mice suggest an ASD-like phenotype with prominent RRB domain symptoms, featuring repetitive and self-injurious grooming behaviors accompanied by a decreased preference for social interaction, a shift towards self-directed activity (e.g., grooming) in novel environments, sensorimotor integration abnormalities, and intact learning and memory. In addition to excessive self-grooming, DKOs exhibited decreased exploratory activity in the open field and 3-chamber social apparatus, decreased marble burying, reduced social approach, and increased prepulse inhibition of acoustic startle reflex. The overarching pattern of decreased environmental exploration across MB, OF, and Social assays, combined with the excessive self-grooming phenotype in DKOs reflects a RRB phenotype that may or may not correlate with increased anxiety.

4.1 ASD-like Anxiety and RRB phenotypes

RRBs in ASD have a different relationship to anxiety compared with other disorders manifesting RRB. For example, RRBs are performed to alleviate anxiety in OCD, however in ASD this is not necessarily so [46, 47]. While DKOs manifest an obvious RRB phenotype, the role of anxiety in these behaviors is difficult to interpret since behavioral assays with measures thought to reflect anxiety in mice present inconsistent and conflicting results. For example, the increased grooming, increased thigmotaxis, and decreased rearing we observed in DKO mice is consistent with an increase in anxiety-like behavior. By contrast, the decreased marble burying in DKO mice could be interpreted as decreased anxiety-like behavior, while the lack of differences between DKO and WT mice in the EPM could be interpreted as “normal” levels of anxiety-like behavior. Although our data are inconclusive regarding whether DKOs manifested increased anxiety, by looking at the overall pattern of these results and considering alternate interpretations of the measurements, a more consistent interpretation of a DKO anxiety phenotype becomes possible.

At first, MB results seem to conflict with thigmotaxis and rearing results that suggest increased anxiety, but this depends on the meaning ascribed to mice burying marbles. The interpretations of marble burying behavior as reflecting anxiety-like behavior or altered environmental engagement and locomotor activity are based largely on evidence that selective serotonin reuptake inhibitors (SSRIs), benzodiazepines, and antipsychotics all decrease marble-burying behavior [37, 43]. Another interpretation of marble-burying behavior considers it a restricted, repetitive behavior akin to rituals in OCD or stereotypy in TS and ASD [37].

These interpretations are unlikely to apply since DKOs exhibited normal exploratory behavior in the first ten minutes, followed by hypolocomotion with increased grooming rather than hypoactivity. Although DKOs bury fewer marbles, they otherwise clearly exhibit higher-than normal RRB, as evidenced by grooming activity in the MB test and grooming-

related lesions. Therefore, we suggest that decreased marble burying may indicate that the novel stimuli in the MB task trigger grooming behavior in DKO mice. Engaging in RRB (i.e., grooming) in these mice appears to preclude spending time burying marbles.

Grooming behavior in rodents is often suggested to reflect restricted, repetitive behavior, and/or a measure of anxiety [40, 45, 48]. The repetitive and self-injurious grooming phenotype in DKOs could be interpreted as either. Increased grooming in rodents can occur in periods of very high or very low stress [45]. These observations have led to the hypothesis that differences in grooming phenotypes among mouse strains are due to a "complex interplay between anxiety, motor, and displacement activity" [45]. Thus, while repetitive behaviors such as abnormal self-grooming may be considered as coping or displacement behaviors, they may not necessarily be displacing anxiety, per se. For instance, motor stereotypies in children with ASD were found to relate specifically to abnormal sensory processing [49]. Sensorimotor gating deficits associated with abnormal fronto-striatal circuitry in adults with ASDs also could account for difficulty inhibiting RRB [50]. Thus, repetitive grooming in DKOs may be related to "sensory overload" and defective sensorimotor gating, which may be heightened in novel environments.

If self-grooming is a shift towards a self-directed stimulatory motor displacement activity in response to novel environments, and not necessarily due to anxiety, then this helps reconcile the conflicting results regarding anxiety measures in DKOs. If grooming were prompted by anxiety, it would be expected to override the normal exploratory activity observed in DKOs to the novel environment of the OF and MB apparatuses. Instead, like WTs, their exploratory activity is substantially higher during the first 10 minutes of both assays compared with subsequent time-points. Because the EPM assay is limited to 5 minutes, differences in grooming or ambulatory activity would not be expected. Thus, similar EPM performance between DKOs and WTs also supports a non-anxiogenic interpretation of the DKO shift to self-directed behavior. This is consistent with evidence in humans indicating that anxiety may be secondary and indirectly related to ASD-like sensory integration or communication problems [46, 47, 51, 52].

4.2 Sensorimotor abnormalities

The pattern of eye and snout lesions in DKO mice also supports the possibility that compulsive grooming in DKOs may be related to a sensory abnormality. For example, abrasive lesions around the eyes of DKO mice were nearly always more severe on the side where the ear tag was placed. This led us to hypothesize that excessive grooming might be related to a sensory stimulus associated with ear tag placement, and may reflect sensorimotor gating problems. Sensorimotor gating deficits are associated with increased rates of restricted and repetitive behaviors and difficulty inhibiting repetitive thoughts, speech, and actions in adults with ASD [50, 53]. Furthermore, in humans with ASD, motor stereotypies are correlated with the severity of sensory abnormalities [49]. Thus, the grooming pattern observed in DKOs may represent further evidence of a "sensory modulation behavior" in reaction to aversive sensory experiences, as is reported in (and by) individuals with ASD [52, 54].

Autistic traits and sensory abnormalities are highly correlated in the general population [51, 55]. In ASD, auditory, visual, and tactile sensory processing and sensorimotor gating abnormalities are highly prevalent [56, 57], but their manifestation is highly variable in individuals. For example, unresponsiveness and hypersensitivity to auditory stimuli are both common in ASD. Evidence for sensorimotor gating deficits is also mixed among PPI/ASR studies in human and animal models of ASD. While many human studies found an increased startle reflex and decreased PPI, our data support results from other studies that found decreased startle and increased PPI associated with ASD risk genes [58-61].

Insufficient sensory filtering is hypothesized to explain both hypo- and hyper-responsivity to auditory stimuli in ASD; however, increased PPI as observed in DKOs suggests that the ability to filter startling stimuli with advanced pre-pulse warning is enhanced, not deficient. Nevertheless, as reflected by a recent study of ASR & PPI in children with ASD [59], this increase does not necessarily mean there is no neurobehavioral sensory abnormality. Thus, unlike typically-developing controls, PPI in ASD children did not decrease with less intense pre-stimuli, suggesting that children with ASD process lower-intensity stimuli differently. Interestingly, we also found that DKOs exhibited increased PPI with similar stimulation parameters. Namely, PPI was elevated at all prepulse intensities and there was a greater percent increase in PPI with increasing prepulse intensities relative to WTs. Thus, prepulses may be more salient for DKOs than WTs. There are several possible explanations for this. Since Eph/ephrins are expressed in multiple brain regions processing auditory stimuli [62-64], it is likely that alterations in expression of ephrin-A2/3 could result in altered excitability in ascending auditory pathways in DKOs which may cause less filtering of stimuli not usually salient enough to pass the filter in controls (as speculated in [59]). There also could be heightened excitability of auditory forebrain networks resulting in stronger frequency-specific descending inhibition of the primary startle circuit both in the presence and absence of prepulses. Either of these could lead to enhanced PPI, but the latter could also account for the attenuated ASR.

4.3 Learning & Memory Behavior

In the FC paradigm, DKO mice did not show deficits in cue-related or context-related fear conditioning, but time spent freezing remained increased for all testing 24 hours after conditioning. Because time spent freezing is similarly high in the unconditioned environment before and during the cue presentation, it is difficult to assess whether cue-dependent fear conditioning occurred normally or whether learned fear in DKOs became generalized. However, increased freezing in the “pre-cue” and “cue” sessions does suggest intact amygdala activity [65]. Given that the amygdala is heavily involved in PPI and cue-related fear conditioning, it is possible that DKOs may have generalized fear conditioning by becoming sensitized to environmental stimuli not processed as relevant to WTs. In addition to amygdala involvement, it is possible that altered hippocampal activity may contribute to FC results, as increases in freezing in the “context” and “pre-cue” sessions suggest that context-dependent FC is normal or possibly enhanced.

Hippocampal-dependent spatial learning in the MWM appeared intact in DKOs, as reflected by performance during learning and on probe trials. This is consistent with results evaluating

spatial learning in ephrin-A3 single KO mice in the Barnes maze [23]. However, the observed crisscross and spiral swim patterns observed during the early training and probe trials suggest DKOs have subtle cognitive or motor differences in learning strategy. This is consistent with evidence from ephrin-A2 null mice which show intact hippocampal learning and memory in a visual discrimination task, but exhibit more inflexibly on a particular strategy in a set-shifting paradigm [23].

4.4 Plausibility as an ASD model

ASDs are heterogeneous neuropsychiatric syndromes that are often conceived as synaptic disorders characterized by imbalances both in local synaptic microcircuitry and regional brain interconnectivity. Moreover, phenotypic diversity among ASD populations corresponds with the diversity of identified ASD risk genes, which regulate functions such as: neuronal proliferation and migration, axonal pathfinding, maturation of postsynaptic structures, neurotransmitter receptors and transporters, and activity-dependent synaptic remodeling [29]. Unfortunately, despite recent advances in understanding ASD genetics and accumulating evidence linking synaptic and intercortical connectivity abnormalities to ASD phenotypes, the precise circuitry defects and possible molecular abnormalities involved in autistic behaviors are poorly understood [66, 67]. Thus, the use of the ephrin-A2/3 DKO model for studying ASD provides a link for studying the role of molecules that regulate both synaptic function and formation of axonal connections in the etiology of ASD.

Genome-wide association studies have identified several risk genes in subsets of ASD patients. Candidate ASD genes include transynaptic neuronal cell adhesion molecules (e.g. neurexin1, neuroligin3-4) [68-73], postsynaptic density-associated proteins (e.g. Shank1-3) [27, 28, 74-79], neurotransmitter trafficking (e.g. Met & EphA3 receptor tyrosine kinases) [2, 80], and several downstream signaling molecules involved in gene transcription and protein synthesis (e.g. Fmr1, TSC1/2, MeCP2) [60, 81-87]. A synaptic etiology for ASD has been proposed based on observations that multiple ASD risk genes encode proteins whose signaling converges at excitatory glutamatergic synaptic complexes [27, 29]. Of these, Shank3 forms a scaffold with PSD-95 and SAPAP that also interacts with the neurexin/neuroligin and EphA/ephrinA complexes. This suggests that ASD patients with impaired functions of these molecules may share a convergent synaptic mechanism that is responsible for similar ASD-like behaviors [29].

The present study supports the supposition that other proteins involved in regulating excitatory synaptic function, including A-ephrins, could contribute to ASDs. In addition to signaling through the same intracellular pathways as other “synaptic” ASD risk genes, ephrin-A2 and ephrin-A3 also can affect dendritic spine morphology and excitatory synaptic function via their expression by perisynaptic astrocytes [22, 88, 89]. Ephrin-A2 and ephrin-A3 are expressed by astrocytes in the cortex and hippocampus, respectively, where they have opposing effects on regulating astrocytic glutamate transporters and dendritic spine morphology. For example, ephrin-A2 null mice have decreased glutamate transport and increased NMDA receptor-mediated synaptic pruning in cortex [89]. In contrast, ephrin-A3 null mice have increased glutamate transport and exhibit hippocampal dendritic spine elongation [22]. This indicates that the effects on synaptic glutamate levels and dendritic

spine morphology in cortical versus hippocampal regions should be opposite in eprhin-A2/3 DKO mice. Thus, the present study of ephrin-A2/3 mutants not only contributes to the growing body of evidence implicating excitatory postsynaptic proteins in ASD, but represents a possible novel expansion of that mechanism to include astrocytes and regulators of astrocytic glutamate transport as possible targets for dysfunction in ASDs.

The ephrin-A2/3 DKO model for ASDs has an additional advantage because ephrin-A proteins are involved in regulating both long distance wiring and synaptic pruning of neural circuitry; both of which likely contribute to ASD. Thus, the resemblance of ephrin-A2/3 DKO behavioral phenotypes to other ASD models associated with synaptic abnormalities suggests that this mouse ASD model could be useful in future studies to link synaptic and circuit endophenotypes with specific subsets of ASD behaviors. A comparison of the behavioral characteristics of ephrinA2/3-DKO mice to ASD mouse models with mutations of Neurexin1α (Nrxn1), Neuroligins (Nlgn3-4), Shank1-3, Fragile × (Fmr1), and MeCP2 is provided in Table 1. The behavioral phenotype of DKOs most closely resembled that reported for Shank3, Nrxn1α, and Fmr1 KO mice, although there also was considerable overlap with MeCP2 and Shank2 mutant mice.

Overall, DKOs have face validity for ASDs with strong core domain 2 features, i.e. RRB and sensory processing abnormalities. As such, DKOs are a potentially valuable model for understanding the relationship between synaptic processes affected by various ASD risk genes and circuit-level changes in neural structures (e.g. frontocorticostriatal circuits) associated with both abnormal sensory integration and motor stereotypy. Support for this comes from the strongest resemblance of DKO phenotypes to Shank3-KO mice [27, 28]. Like Shank3-KOs, DKOs exhibit excessive, self-injurious grooming, a decreased preference for social interaction and anxiety-like behaviors, but normal spatial learning and memory. As Shank3 expression is specifically enhanced in striatum, which is strongly implicated in motor RRB (such as excessive self-grooming in mice), the similarity in the behavioral phenotype of DKOs to Shank3-KOs suggests similar corticostriatal circuits may be involved in both ASD models. In addition, Nrxn1 - KO mice, like DKOs, also exhibit a mixed anxiety-like phenotype, increases in grooming stereotypy, and hypolocomotion. Like neurexin, ephrins-A2 and -A3 are presynaptic partners that influence excitatory synaptogenesis through a postsynaptic receptor that crosstalks with the same MAPK signaling pathways activated by neuroligins through Shank [90].

Similar to the Fmr1-KOs and MeCP2-flox ASD models, DKOs also had reduced startle reflex and enhanced prepulse inhibition, suggesting possible overlapping abnormalities in ascending auditory and sensorimotor circuits among these models [60, 61]. Eph/ephrin signaling converges with the same downstream TSC2/mTOR signaling pathways that mediate the effects of Fmr1 on protein synthesis and MeCP2 on transcription and cell motility. Since alterations in the TSC2/mTOR signaling or EphA/ephrin-A signaling result in visual system abnormalities [91], interactions between the TSC2/mTOR and EphA/ephrin-A signaling pathways also could account for the disruptions that occur in auditory circuits when Eph/ephrin signaling is altered [62-64]. Thus, the DKO mouse model may allow further clarification of how disruptions in these sensory signaling pathways influence development of primary sensory circuits, which may be altered in ASD [92].

Despite being highly heritable, there is a relative paucity of single nucleotide polymorphisms and/or coding mutations associated with ASDs. It is unclear if this is because a wide variety of potential candidate genes each affects a very small subset of patients, or whether there is an alternative way in which ASD may be inherited that doesn't necessarily require SNPs or coding mutations. One alternate hypothesis is that spectrum disorders such as ASD result more from epigenetic factors affecting the developmental time-course and expression levels of molecules that regulate connectivity at the level of axonal circuitry, as well as synapses [93]. Thus, Eph/ephrins not only share with other ASD candidate gene products the ability to modulate excitatory synaptic activity and plasticity, but they have an additional property that would permit for graded presentations of anatomical and clinical phenotypes. Unlike other candidate gene products, the effects of Eph/ephrins are mediated according to their relative, not absolute, expression levels [18, 19, 94]. Given their complex spatial and temporal pattern of expression within the brain during development [6, 7, 9, 63, 95], EphA/ephrin-As are prime candidates for generating models of developmental spectrum disorders that involve abnormal brain connectivity [96, 97]. Therefore, the notion that alterations in the relative expression levels of receptors and cell-surface ligands may be sufficient to change developmental patterning and modify synaptic functions that lead to behavioral deficits is of great interest. Based on the overlapping behavioral characteristics of the DKO mice with other ASD mouse models, the DKO model should provide a unique model for understanding the mechanism by which these convergent synaptic signaling factors affect circuits relevant for sensorimotor and social behavior.

Acknowledgements

We would like to acknowledge J. Baiocco for assisting with preliminary studies. This work was supported by the Tourette Syndrome Association and the National Institutes of Health (NIH F31 MH098636 and a supplement to NIH R01 NS047700).

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Highlights

- Ephrin-A2/-A3 double knockout mice (DKOs) present an ASD behavioral phenotype.
- DKOs underwent assays of anxiety-like, sensorimotor, learning, and social behavior.
- DKOs exhibit self-injurious repetitive grooming and abnormal sensorimotor gating.
- DKOs mice show decreased preference for social interaction.
- DKOs mice exhibit a shift towards self-directed activity in novel environments.

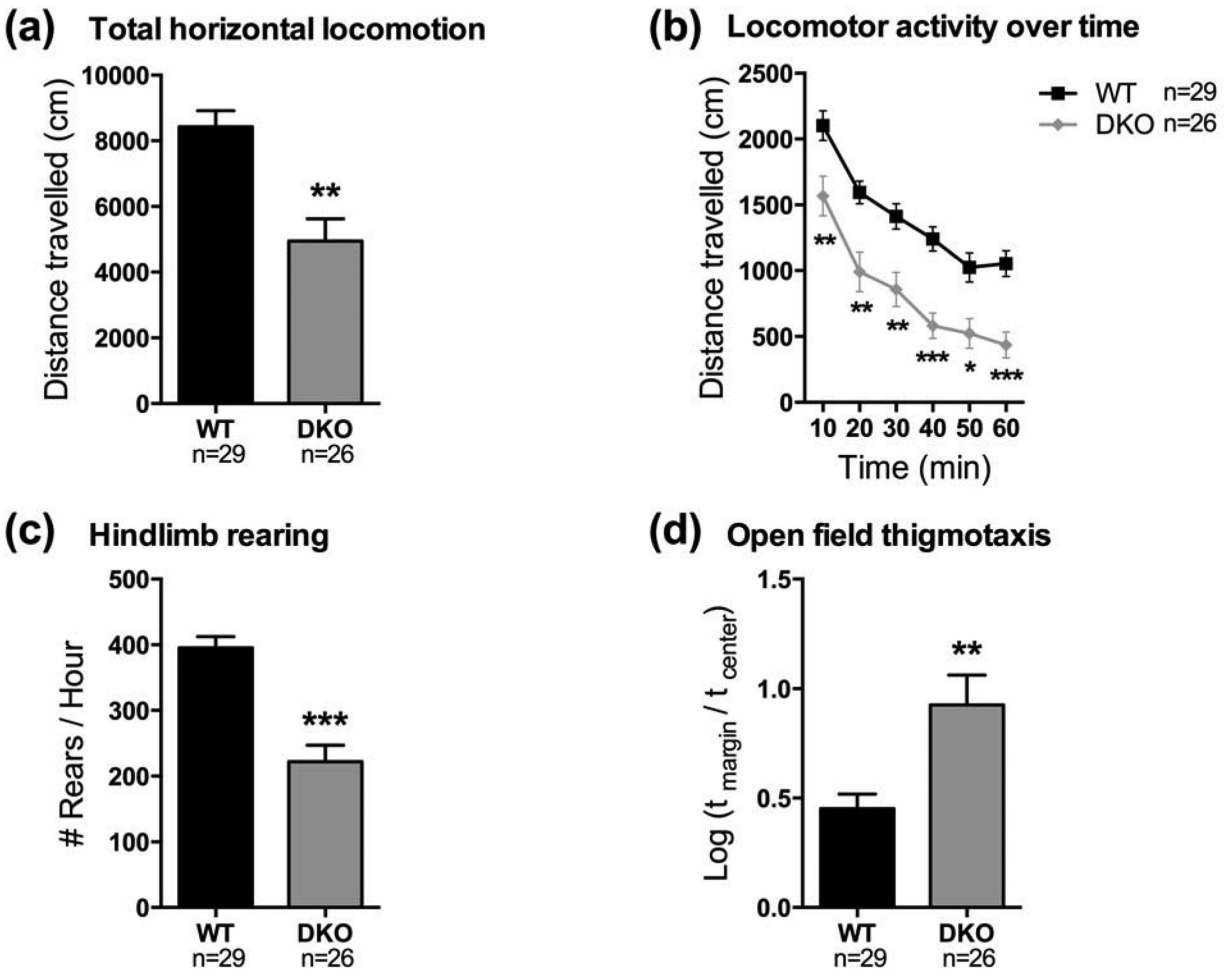

Figure 1. DKO mice are hypolocomotive and exhibit anxiety-like behavior in the Open Field

(a) DKOs travel significantly less horizontal distance over 1 hour. **(b)** DKOs exhibit a significantly reduced activity for each time bin, but their decrease in exploratory behavior over time is comparable to WT. **(c)** DKOs display decreased hindlimb rearing behavior. **(d)** DKOs spend more time in the open field margins relative to the center (thigmotaxis). Data are expressed as Log of the ratio (t_{margin}/t_{center}) to collapse data for graphical representation. (Data expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001).

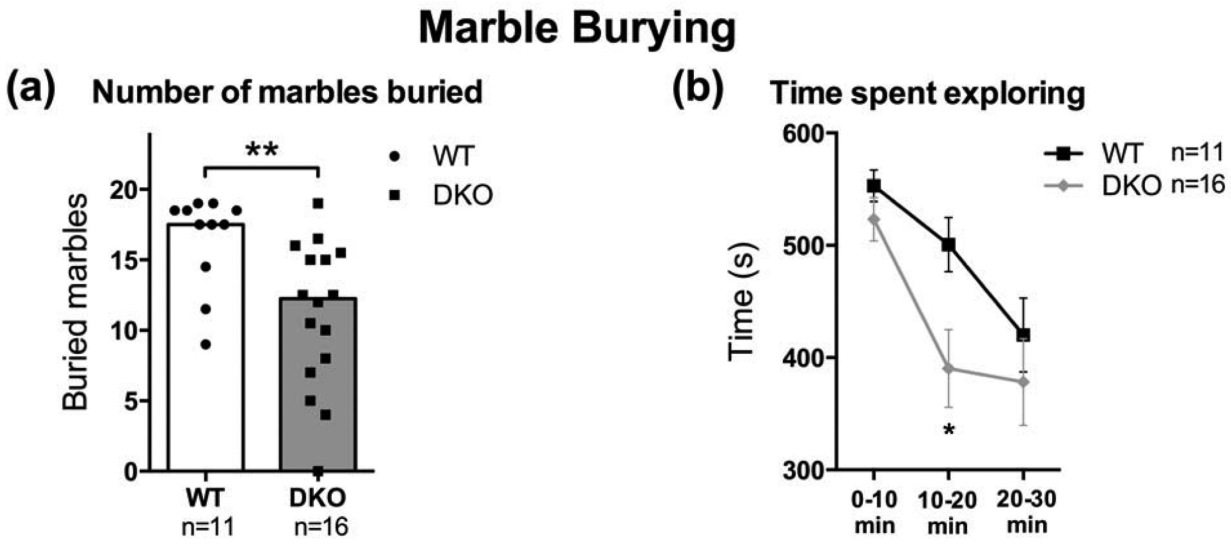

Figure 2. DKO mice have decreased marble burying behavior

(a) DKO mice bury significantly fewer marbles over 30 minutes compared to WT mice. (Data expressed as median (bars) and 2-trial means per mouse (points) **(b**) DKO mice spend less time in exploratory behaviors (ambulating, rearing, or digging) than WTs in the second 10 minutes. (*Data expressed as mean ± SEM; *p<0.05, **p<0.01)*

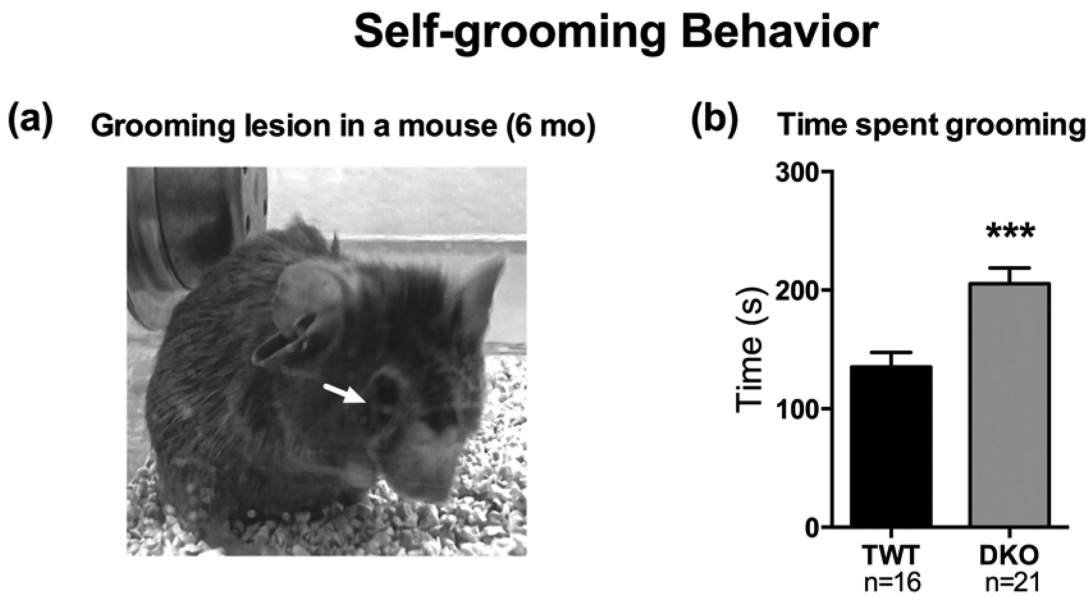

Figure 3. DKO mice display a self-injurious grooming phenotype

(a) Photograph of grooming lesion phenotype in a 6 month old DKO mouse. In addition to hair loss on the snout, there is often a characteristic lesion that forms around one or both eyes (arrow). **(b)** DKOs spend more time grooming than WTs. Grooming time was measured over a 5 minute period following stimulation by a mild spritz of water to the face. *(Data expressed as mean ± SEM; ***p<0.001)*

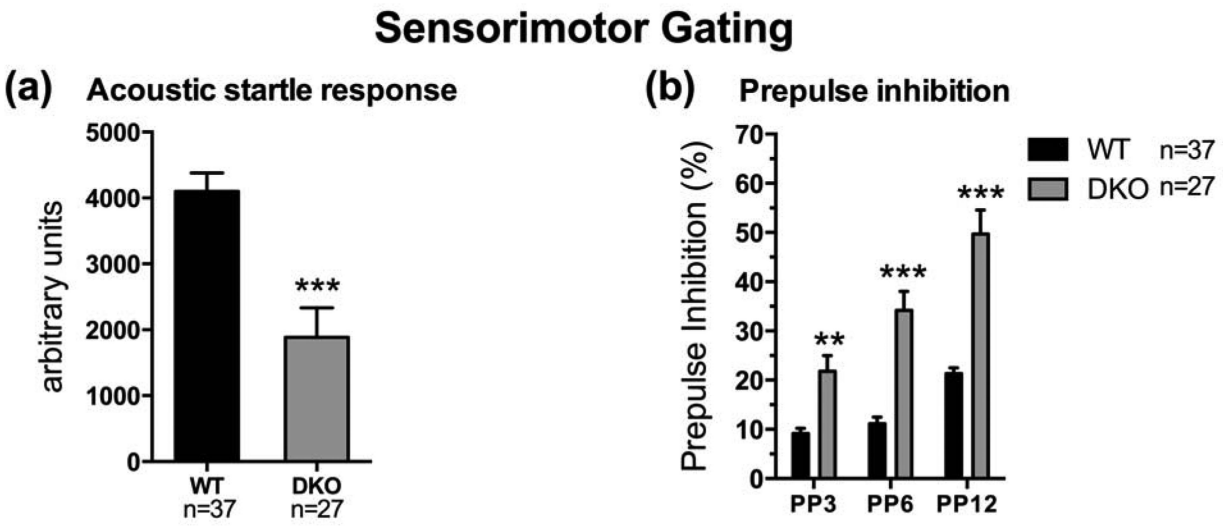

Figure 4. DKO mice exhibit abnormal auditory sensorimotor gating

(a) DKOs have decreased auditory startle response to a 120dB broadband noise pulse. **(b)** DKOs have significantly increased prepulse inhibition (PPI) of ASR compared with WTs when prepulses were delivered at intensities of 3dB (PP3), 6dB (PP6), and 12dB (PP12) above background noise (70dB). (Data expressed as mean ± SEM; **p<0.01, ***p<0.001)

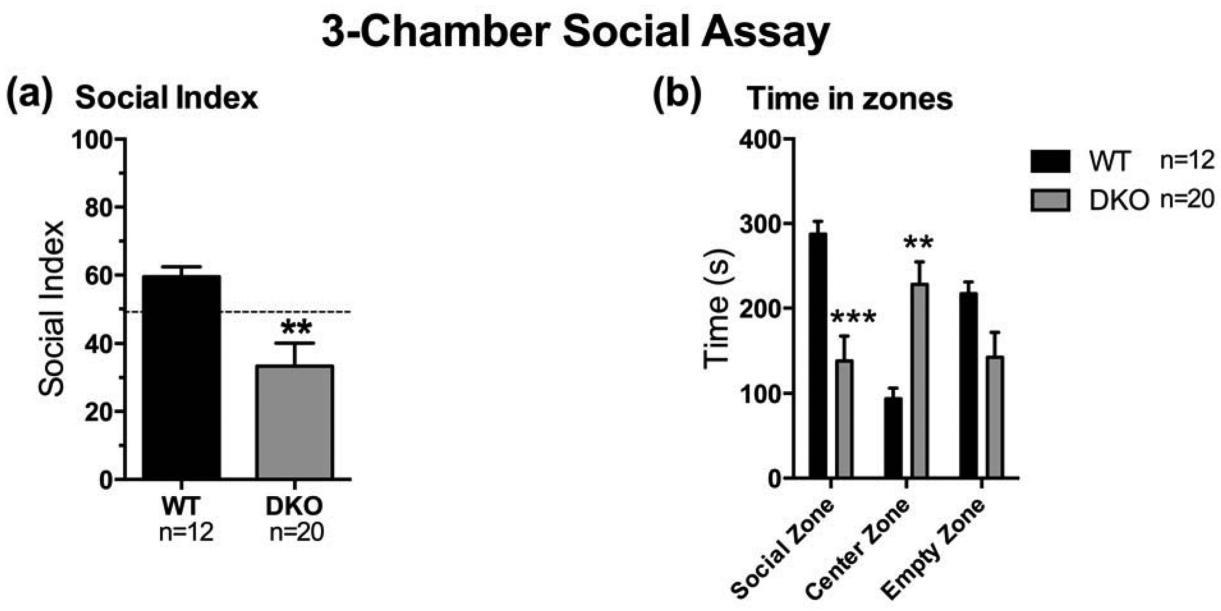

Figure 5. DKO mice exhibit decreased social approach behavior in a three-chamber social behavior assay

(a) Social index (calculated as: time spent near cage with novel mouse / [time spent near cage with novel mouse + time spent near empty cage] *100) was decreased in DKOs. Thus, a social index <50 indicates a social aversion, while >50 indicates a preference for social affiliation. **(b)** DKOs spent significantly less time in the social zone and significantly more time in the center (home) zone. (Data expressed as mean ± SEM; **p<0.01, ***p<0.001)

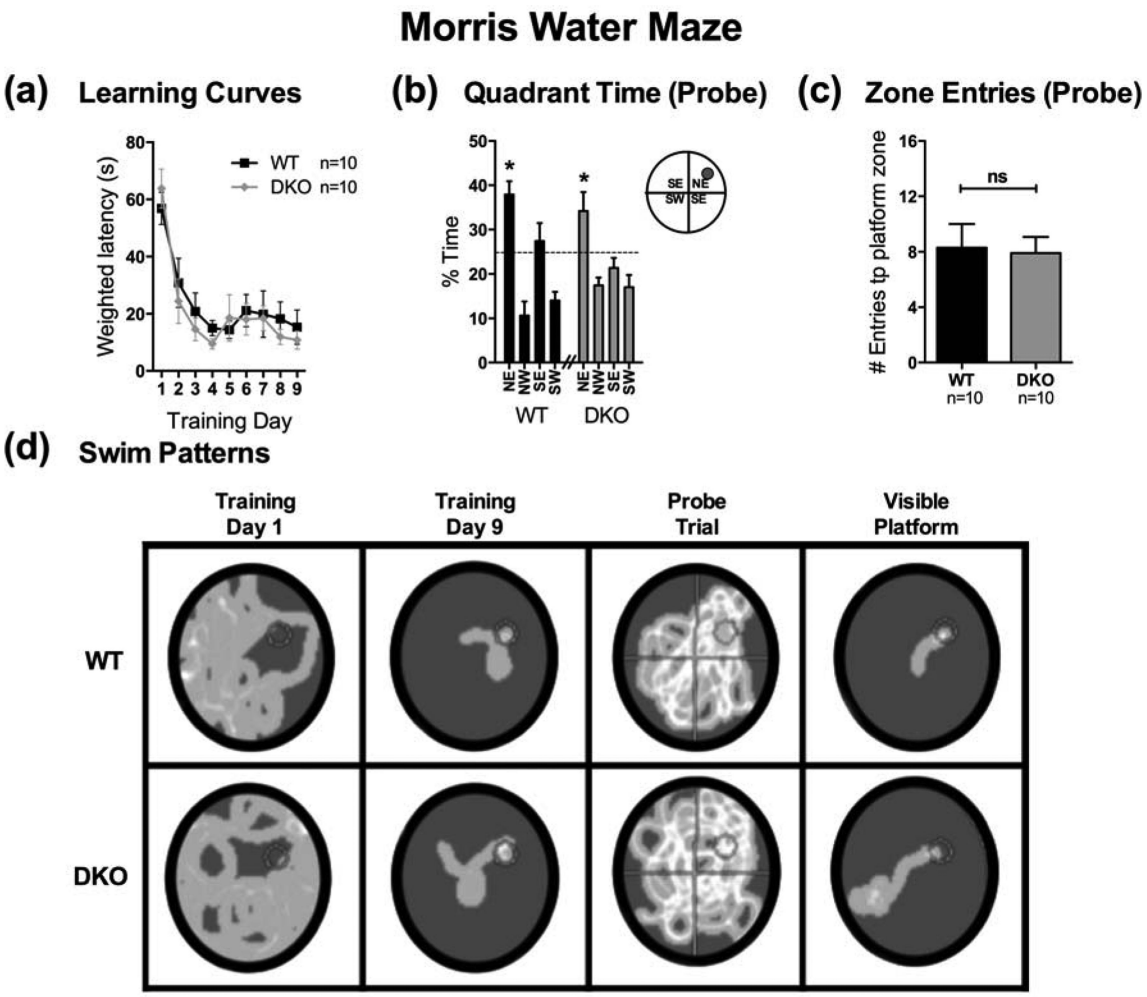

Figure 6. DKO mice perform normally in the MWM, but may utilize different learning strategies

(a) Learning curve data are expressed as weighted latency values which represent the average time for mice to find the platform, adjusted by the number of successful trials per training day. **(b).** During the probe trial both WT and DKO mice spent significantly more time in the quadrant that originally contained the hidden platform. **(c)** Number of entries into the platform zone during the probe trial did not differ between WTs and DKOs. **(d)** DKOs revert to early training swim patterns in the probe trial. Heat maps from sample trials illustrate the swim paths of representative mice. (*Data in a & c expressed as mean ± SEM; *p<0.001)*

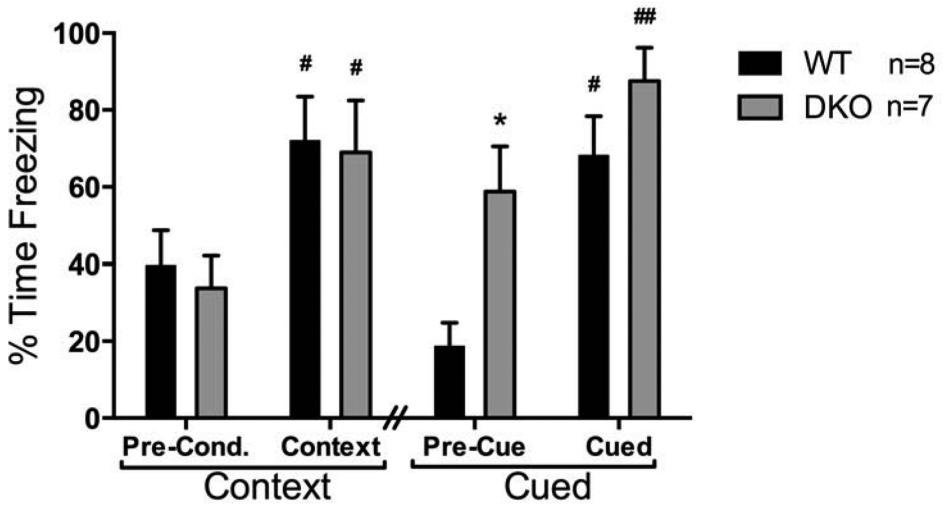

Figure 7. DKOs exhibited normal cue-dependent and context-dependent fear memory after fear conditioning

Both DKO and WT mice froze significantly more at 24 hours post-conditioning during contextual fear memory testing ("Context") and cued fear memory testing ("Cued") compared with their "Pre-Conditioning" baselines ($^{\#}p<0.05$, $^{\#\#}p<0.001$). In the unconditioned environment prior to cue testing ("Pre-Cue") at 24 hours post-conditioning, freezing in DKO and WTs was not significantly changed from their "Pre-conditioning" baseline. However, DKOs froze significantly more than did WTs during this session ($^{*}p<0.05$). (*Data expressed as mean ± SEM*)

Table 1

Comparison of ephrin-A2/3 DKO phenotype to other ASD models with synaptic constructs.

		Anxiety	Sensory Gating	Motor/RRB	Social Behavior	Learning & Memory	Refs
Cell Adhesion & Excitatory Synaptic Microcircuitry	*EfnA2/3*-DKO	≈ elevated plus maze (open arm *t*) ↑ anxiety-like behaviors (open field) ↑ freezing & grooming (novel environments)	↑ prepulse inhibition ↓ startle	↑ self-grooming • self-injurious grooming ↓ locomotion	↓ sociability (novel vs non-social)	≈ spatial learning •stereotypic swim pattern in MWM probe ≈ reversal learning •generalized anxiety on fear conditioning	
	Nrxn1α-KO	**↕ anxiety-like behaviors** ≈ thigmotaxis* **≈ elevated plus maze (open arm *t*)*** ↓ time in anxiogenic part of mazes†	↓ prepulse inhibition ≈ startle (120 dB) ↑ startle (80-100 dB)	**≈ locomotion in novel environment** **↓ locomotion activity in home cage** **↕ self-grooming (↑*; ≈†)** ↑ motor learning	↕ social behavior ≈ sociability (novel vs non-social)* ↑ sociability (intact social memory)† ↑ aggression (males)† ↓ nesting behavior	**≈ spatial learning** ≈ novel object recognition memory	[69]* [71]†
	Nlgn3-KO	≈ anxiety-like behaviors	≈ prepulse inhibition	≈ locomotion ≈ stereotypies ≈ motor learning	↕ social interactions ↓ USV rate	**≈ spatial learning** ↑ reversal learning ↓ fear conditioning	[72] [73]
	Nlgn4-KO	≈ anxiety-like behaviors	≈ prepulse inhibition ≈ startle	≈ locomotion ≈ stereotypies ≈ motor learning	↓ social interactions ↓ USV rate	**≈ spatial learning** **≈ reversal learning** **≈ fear conditioning**	[70] [68]
Excitatory Postsynaptic Scaffolding & Signaling	***Shank1-KO***	**↑ anxiety-like behaviors (partial)**	≈ prepulse inhibition ≈ startle	**↓ locomotion** **↑ self-grooming** **• repetitive behaviors** ↕ motor learning	↓ male to female social sniffing ↓ male to female USV rate	↑ spatial learning ↑ reversal learning ↓ fear conditioning	[74] [77] [76]
	Shank2-KO	**↑ anxiety-like behaviors**	N/A	↑ locomotion **↑ self-grooming** **↑ grooming with novel objects**	**↓ sociability (novel vs non-social)** ↕ USV (sex differences) ↓ nesting behavior	↓ spatial learning ≈ novel object recognition memory	[75] [78] [79]
	Shank3-KO	**↑ anxiety-like behaviors**	≈ prepulse inhibition ≈ startle	≈ locomotion **↑ self-grooming** **≈ self-injurious grooming** ↑ head pokes in hole-board test •stereotyped novel object manipulation	**↓ sociability (novel vs non-social)**	**≈ spatial learning** • delayed learning & reversal (MWM)	[27] [28]
Synaptic Gene Transcription Regulation	***Fmr1* -KO**	**↕ anxiety-like behaviors (↓†; ≈‡)**	**↑ prepulse inhibition** ↕ startle **(↑*;≈†)**	↕ locomotion(↑**; ≈†)	↕ social behavior **↕ sociability** (↓*; ≈†; ↑*)	**↕ spatial learning (↓*; ≈†; ↑‡)** **≈ reversal learning** **↕ fear conditioning (↑*; ≈†)** **↓ extinction of contextual fear**	[81]* [82, 83]† [86]†

		Anxiety	Sensory Gating	Motor/RRB	Social Behavior	Learning & Memory	Refs
						↕ novel object recognition memory (novel object recognition memory (	
	Mecp2-KO **Mecp2-cKO*** ***Mecp2-*** **flox †**	↓ time in anxiogenic part of mazes **↑ freezing & grooming in maze tasks** ↓ anxiety-like behavior*†	**↑ prepulse inhibition†** **↓ startle†**	**↕ locomotion & exploration (↓KO; ≈*)** ↓ motor skill in swim tasks ↓ coordination **↕ self-grooming** **↑ forepaw stereotypies***	↑ USV rate ↑ sociability ↓ nesting behavior	↓ cued fear conditioning ↓ spatial memory	[84]* [85] [60]† [87]

[Bolded text indicate overlap with the present results in DKO.]

Symbols indicate reported findings (↑increased; ↓decreased; ↕ mixed results; ≈ similar to controls; • observations).