Molecular Autism

RESEARCH Open Access

Enhanced fear limits behavioral flexibility in *Shank2*-deficient mice

Miru Yun[1,2], Eunjoon Kim[1,2*] and Min Whan Jung[1,2*]

Abstract

Background: A core symptom of autism spectrum disorder (ASD) is repetitive and restrictive patterns of behavior. Cognitive inflexibility has been proposed as a potential basis for these symptoms of ASD. More generally, behavioral inflexibility has been proposed to underlie repetitive and restrictive behavior in ASD. Here, we investigated whether and how behavioral flexibility is compromised in a widely used animal model of ASD.

Methods: We compared the behavioral performance of *Shank2*-knockout mice and wild-type littermates in reversal learning employing a probabilistic classical trace conditioning paradigm. A conditioned stimulus (odor) was paired with an unconditioned appetitive (water, 6 μl) or aversive (air puff) stimulus in a probabilistic manner. We also compared air puff-induced eye closure responses of *Shank2*-knockout and wild-type mice.

Results: Male, but not female, *Shank2*-knockout mice showed impaired reversal learning when the expected outcomes consisted of a water reward and a strong air puff. Moreover, male, but not female, *Shank2*-knockout mice showed stronger anticipatory eye closure responses to the air puff compared to wild-type littermates, raising the possibility that the impairment might reflect enhanced fear. In support of this contention, male *Shank2*-knockout mice showed intact reversal learning when the strong air puff was replaced with a mild air puff and when the expected outcomes consisted of only rewards.

Limitations: We examined behavioral flexibility in one behavioral task (reversal learning in a probabilistic classical trace conditioning paradigm) using one ASD mouse model (*Shank2*-knockout mice). Thus, future work is needed to clarify the extent to which our findings (that enhanced fear limits behavioral flexibility in ASD) can explain the behavioral inflexibility associated with ASD. Also, we examined only the relationship between fear and behavioral flexibility, leaving open the question of whether abnormalities in processes other than fear contribute to behavioral inflexibility in ASD. Finally, the neurobiological mechanisms linking *Shank2*-knockout and enhanced fear remain to be elucidated.

Conclusions: Our results indicate that enhanced fear suppresses reversal learning in the presence of an intact capability to learn cue-outcome contingency changes in *Shank2*-knockout mice. Our findings suggest that behavioral flexibility might be seriously limited by abnormal emotional responses in ASD.

Keywords: Shank2, Reversal learning, Fear, Classical conditioning

Background

Autism spectrum disorders (ASD) are developmental disorders that are associated with a diverse array of symptoms, including impaired social interaction and communication as well as repetitive and restrictive patterns of behavior [1]. Many genes implicated in ASD are expressed broadly in the cerebral cortex, and their

*Correspondence: kime@kaist.ac.kr; mwjung@kaist.ac.kr

[1] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea
Full list of author information is available at the end of the article

mutations may lead to aberrant circuit development in widespread cortical areas [2–4]. Given that the cerebral cortex (especially frontal cortical areas such as the orbitofrontal, anterior cingulate, and dorsolateral prefrontal cortices) plays a key role in the adaptive control of behavior [5–10], mutations in genes that play important roles in cortical development may lead to compromised behavioral flexibility in adults. People with ASD often show impairments in tasks requiring cognitive flexibility, such as the Wisconsin card sorting test [11, 12], and impaired cognitive flexibility has been proposed to underlie repetitive and restrictive patterns of behavior in this disorder [13]. People with ASD also show impairments in reversal learning, and their performance in reversal learning correlates with clinical ratings of restricted and repetitive behavior [14] and everyday symptoms of behavioral inflexibility [15]. Impaired behavioral flexibility has also been reported in mouse models of ASD [16–30]. Thus, a growing body of studies indicates that impaired behavioral flexibility is present and correlated with repetitive and restrictive behaviors in ASD, raising the possibility that repetitive and restrictive patterns of behavior associated with ASD might be manifestations of impaired flexibility.

Shank2 (SH3 and multiple ankyrin repeat domains 2), which is a multi-domain scaffolding protein enriched in the postsynaptic density of excitatory synapses [31, 32], is strongly implicated in ASD [33]. Genetic variations of the *SHANK2* gene have been identified in ASD [34–58]. Moreover, mutations/deletions in the *Shank2* gene lead to a diverse array of behavioral changes in mice; some of these mutant mice show impaired social interaction and repetitive behavior, which are core symptoms of ASD [59–71]. Mice with *Shank2* gene alterations have been used widely to investigate neurobiological mechanisms of ASD, and substantial amounts of behavioral and neural data have been accumulated. However, the relationship between *Shank2* mutations and behavioral flexibility is largely unknown.

In the present study, we investigated whether and how behavioral flexibility is compromised in *Shank2*-KO mice lacking exons 6 and 7 [60] by subjecting them to reversal learning under a probabilistic classical conditioning paradigm. In this task, two different odor cues were paired with a reward (water) or a punishment (air puff) probabilistically over a 1-s delay (trace classical conditioning), and anticipatory licking responses during the delay period were measured as an index for learning. Numerous studies have indicated that the orbitofrontal cortex plays a crucial role in reversal learning [72, 73]. We previously showed that an intact medial prefrontal cortex is also involved in probabilistic reversal learning in mice [74]. Shank2 is expressed broadly in the brain including the orbitofrontal and medial prefrontal cortices [75]. We have also shown various physiological abnormalities in the medial prefrontal cortex of *Shank2*-KO mice [67, 68]. These findings suggest a potential link between *Shank2*-KO and a reversal learning deficit.

We examined reversal learning of both male and female *Shank2*-KO mice because people with ASD are four times more frequent in males than females [76] and male–female differences in various behavioral, synaptic, molecular, and neuroanatomical phenotypes have been detected in mouse models of ASD [77–82]. We also examined reversal learning in juvenile *Shank2*-KO mice because ASD is characterized by early manifestations of symptoms and mouse models of ASD, including *Shank2*-KO mice, frequently show differential phenotypes at different postnatal stages [60, 67, 83]. We found that male *Shank2*-KO mice, both adult and juvenile, but not female adult *Shank2*-KO mice, show impaired reversal learning only when a strong air puff was used as an unconditioned stimulus (US). We also found that male, but not female, *Shank2*-KO mice display abnormally heightened fear responses to the strong air puff. The results suggest that abnormal emotional responses may limit behavioral flexibility in ASD under certain circumstances.

Methods

Subjects

We used *Shank2*-KO mice that harbor deletions in exons 6 and 7 of *Shank2* and thus mimic the ASD-related microdeletion of exons 6 and 7 in human *SHANK2* [60]. We used 45 adult (12–24 weeks old) male *Shank2*-KO mice, 45 adult male wild-type (WT) littermates, 17 adult female *Shank2*-KO mice, 12 adult female WT littermates, 22 juvenile (P30–45) male *Shank2*-KO mice, and 20 juvenile male WT littermates in this study. Different groups of mice were tested in three different versions of reversal learning (Tasks 1–3; see below). Some of the mice were tested for air puff-induced eye closure responses before being tested in reversal learning (Additional file 1: Table S1). The mice had a C57BL/6 N background and were characterized by PCR genotyping as previously reported [60, 67]. For classical conditioning, the mice were water-deprived. They were allowed to drink water only during the task (total amount per session, Tasks 1 and 2, ~ 0.9 ml; Task 3, ~ 1.2 ml) unless their body weights fell below 80% of their initial body weights. Additional water (1–4 ml) was provided 1 h after the task to those mice whose body weights fell below 80% of their initial body weights when assessed immediately after task completion. Mice used to measure eye closure responses to air puff were fed ad libitum. The estrous cycle of female mice was not assessed. All mice were housed individually and all experiments were performed during the dark phase of a 12 h light/dark cycle.

Surgery

General anesthesia was induced in mice with isoflurane (3.0% in 100% [v/v] oxygen) inhalation for 5 min. The anesthetized mice were head-fixed on a stereotaxic system, their head skin was removed to expose the skull, and the concentration of isoflurane was lowered to 1.5–2.0%. A customized aluminum head plate was briefly sterilized with 70% ethanol and placed on the skull near the lambdoid suture [74, 84]. The head plate was fixed by sterilized screws (M1 × 3 mm) and covered with dental cement. The mice were allowed to recover > 1 week before behavioral training began.

Probabilistic trace classical conditioning

All mice were trained in a probabilistic trace classical conditioning task under head fixation (Fig. 1A) as previously described [74]. The animal's head was fixed to a custom-built metal holder. A water port (a blunt 17-gauge needle) was placed slightly below the animal's nose, an air puff port (a blunt 18-gauge needle) was placed 3–5 mm away from the animal's left eye, and an odor port (silicon tube; diameter, 8 mm) was placed slightly above the animal's nose. Four different odors (citrus, isoamyl acetate, L-carvone, and 1-butanol) were dissolved in mineral oil (1:1000, v:v) and delivered to the animal using an air circulation system. The animal's licking behavior was detected by an infrared light sensor placed adjacent to the water port.

The behavioral phases consisted of habituation, acquisition, and reversal. For each animal, two odors were selected randomly from the four possible odors and used in the acquisition and reversal phases. For habituation, a small amount of water (6 µl) was provided from the water port initially every 5 s without an odor cue (~ 50 trials, day 1). Then the same amount of water was provided 1 s after the delivery of an odor cue (1 s) that was different from those used in the acquisition and reversal phases, and a 2.5–4.5 s inter-trial interval (uniform random distribution) was imposed. The habituation phase lasted 1–3 sessions (~ 400 trials per session).

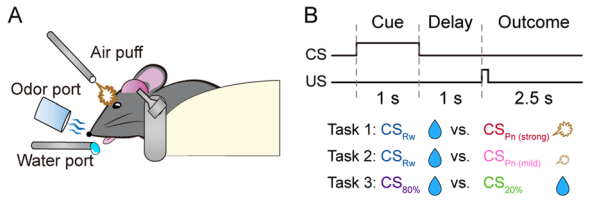

Fig. 1 Behavioral task. **A** The experimental setting. Head-fixed mice performed a probabilistic classical conditioning task. **B** Task schematic. A conditioned stimulus (CS; odor) was delivered for 1 s, there was a 1-s delay, and then an appetitive (water, 6 µl) or aversive (air puff) US was delivered in a probabilistic manner. Each CS was paired with 75% water (CS_{Rw}) or 75% strong air puff ($CS_{Pn(strong)}$) in Task 1, with 75% water or 75% mild air puff ($CS_{Pn(mild)}$) in Task 2, and with 80% water ($CS_{80\%}$) or 20% water ($CS_{20\%}$) in Task 3

In the acquisition phase, an odor cue randomly chosen from two different odors was delivered for 1 s, there was a delay of 1 s, and an associated outcome was delivered with a given probability (Fig. 1B). An inter-trial interval (2.5–4.5 s, uniform random distribution) was then imposed before the next trial began. The mice were trained for three daily sessions of 400 trials in the acquisition phase. We used three different sets of cue-outcome contingencies. A reward-predicting conditioned stimulus (CS_{Rw}) and a punishment-predicting conditioned stimulus (CS_{Pn}) were paired with water (6 µl) and air puff, respectively, as follows:

Task 1 (strong air puff): CS_{Rw} (75% delivery of water) and CS_{Pn} (75% delivery of strong air puff; 100 ms, 3 psi).
Task 2 (mild air puff): CS_{Rw} (75% delivery of water) and CS_{Pn} (75% delivery of mild air puff; 5 ms, 3 psi).
Task 3 (no air puff): $CS_{80\%}$ (80% delivery of water) and $CS_{20\%}$ (20% delivery of water).

In the reversal phase, the mice were first trained with the same cue-outcome contingency as in the acquisition phase until they reached the criterion for reversal onset, which was when the smoothed anticipatory lick rate (moving average of 20 trials) was significantly different between two cues (CS_{Rw} versus CS_{Pn} or $CS_{80\%}$ versus $CS_{20\%}$) in ≥ 75 of the prior 100 analysis windows. Then the cue-outcome contingency of the acquisition phase was reversed. For Tasks 1 and 2, the reward-predicting cue before reversal was paired with punishment after reversal ($CS_{Rw \rightarrow Pn}$) and the punishment-predicting cue before reversal was paired with a reward after reversal ($CS_{Pn \rightarrow Rw}$). For Task 3, the cue predicting 80% reward delivery before reversal was paired with 20% reward delivery after reversal ($CS_{80\% \rightarrow 20\%}$) and the cue predicting 20% reward delivery before reversal was paired with 80% reward delivery after reversal ($CS_{20\% \rightarrow 80\%}$). Each mouse was trained until it met the reversal criterion, which took 1–5 daily sessions (400 trials per session). The reversal criterion was five consecutive trials after cue-outcome contingency reversal during which the smoothed anticipatory lick rate (moving average of 25 trials) was significantly higher (*t* test, $p < 0.05$) following $CS_{Pn \rightarrow Rw}$ than $CS_{Rw \rightarrow Pn}$ (or $CS_{20\% \rightarrow 80\%}$ than $CS_{80\% \rightarrow 20\%}$).

Performance in reversal learning was assessed in two ways. The first measure was the number of trials needed to reach the reversal criterion. Note that this measure was obtained over a variable number of sessions (1 to 5) across animals. The second measure was the relative

cue-dependent lick frequency in the first reversal session. Given that the number of trials after cue-outcome contingency reversal in the first session varied between 50 and 289 across all animals and all tasks (Task 1, 62–270; Task 2, 50–283; Task 3, 64–289), we equalized the number of first-session trials to the smallest number for a given task (Task 1, 62; Task 2, 50; Task 3, 64) and then estimated the lick difference index (LDI) using the last 10 equalized trials. The LDI was calculated as follows:

$$LDI = (A - B)/(A + B),$$

where A is the anticipatory lick rate during the delay period in initially more rewarding trials ($CS_{Rw \rightarrow Pn}$ in Tasks 1 and 2 and $CS_{80\% \rightarrow 20\%}$ in Task 3) and B is the anticipatory lick rate during the delay period in initially less rewarding trials ($CS_{Pn \rightarrow Rw}$ in Tasks 1 and 2 and $CS_{20\% \rightarrow 80\%}$ in Task 3).

Eye closure response

The mice were habituated to the head-fixed experimental setting for 1 h per day for 3 days. Adult male mice received different durations (5, 10, 50, 100 ms) and intensities (3, 7, 15, 30 psi) of air puff (total, 16 combinations) five times each (Fig. 2A). Adult female mice were tested with three combinations of air puff duration and intensity (100 ms at 3 psi; 5 ms at 3 psi; and 100 ms at 30 psi). Consecutive air puffs were separated by 9–11-s intervals (uniform random distribution). The animal's eye closure response was quantified by measuring the area of the left pupil with an infrared camera (IDIS Co., Ltd., South Korea) at 30 Hz, as previously described [74, 85, 86]. Briefly, raw video images were converted to grayscale images, Gaussian-filtered ($\sigma = 2$ pixels), and thresholded to generate binary masked images. The pupil area was measured from the binary masked images and then normalized between 0 (fully open eye) and 1 (fully closed eye; Fig. 2B).

Pharmacological rescue

D-cycloserine (DCS; Sigma, C6880) was dissolved in filtered saline (6 mg/ml). DCS (20 mg/kg) or the same volume of saline was injected to the animal intraperitoneally at 30 min before measurement of eye closure responses. All mice were tested twice, once following DCS injection and once following saline injection. The sequence of drug injection was counterbalanced across mice. We tested three combinations of air puff duration and intensity (100 ms at 3 psi; 5 ms at 3 psi; and 100 ms at 30 psi), which enabled us to complete the test within the half-life of DCS (23 min).

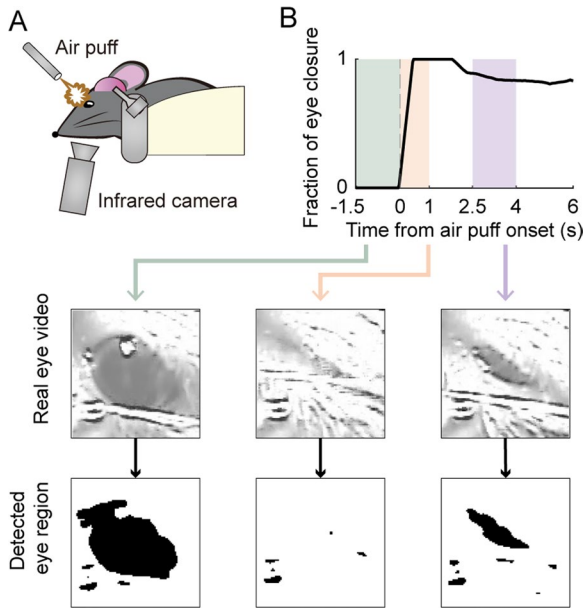

Fig. 2 Measurement of eye closure. The eye closure response to air puff was estimated by measuring pupil diameter with an infrared camera. **A** Schematic for the experimental setting. **B** A sample eye closure response to an air puff. Video images and detected eye regions before and after air puff delivery are shown at the bottom

Statistical analysis

All statistical tests were performed with MATLAB (version R2017a) and SPSS (version 25.0). Group comparisons were performed with Student's t test and multiple-way ANOVA (independent and mixed) with Bonferroni post hoc tests. All statistical tests were two-tailed. Statistical significance was accepted at $p < 0.05$. All data are expressed as mean ± SEM.

Results

Impaired reversal learning in male Shank2-KO mice

We first tested adult (12–24-week-old) male mice (10 WT and 10 *Shank2*-KO) on Task 1, in which one odor cue was paired with a small amount of water (6 μl) and another with an air puff (100 ms, 3 psi), each with 75% probability (Fig. 3A). The mice were trained in the task for three daily sessions of 400 trials (acquisition phase). We used the anticipatory lick rate during the delay period (1 s) as an index for discrimination between two odor cues throughout the study. The anticipatory lick rate diverged rapidly (in < 100 trials) according to CS during the first session (Fig. 3B). Overall, the anticipatory lick rate decreased gradually within each session, possibly reflecting a gradual decrease in thirst. Nevertheless, both genotypes showed higher anticipatory lick rates in CS_{Rw} trials than in CS_{Pn} trials throughout the three training sessions (Fig. 3B). We divided each session

into four stages (100 trials each) and subjected the LDI (see Section "Methods") to a two-way mixed ANOVA (Fig. 3C). We found a significant main effect of training ($F_{(11,198)}=8.014$; $p=1.7\times10^{-11}$) along with a significant training × genotype interaction effect ($F_{(11,198)}=3.318$; $p=3.3\times10^{-4}$; main effect of genotype, $F_{(1,198)}=1.193$, $p=0.289$). Post hoc Bonferroni tests revealed that the LDI was significantly higher in KO mice than in WT mice in the fourth stage of the first session (stages 1–4; $p=0.211$, 0.083, 0.073 and 0.004, respectively; second session, p values > 0.2; third session, p values > 0.2). These results indicate that although the initial learning rate was faster in *Shank2*-KO mice than in WT mice, both genotypes were over-trained to discriminate between CS_{Rw} and CS_{Pn}.

The mice were then trained until they reached the reversal criterion over 1–5 sessions (400 trials per daily session). The dynamic of lick rate changes during reversal varied widely across individual mice. The number of trials required to reach the reversal criterion differed significantly between WT and *Shank2*-KO mice (240.3 ± 35.7 and 570.7 ± 146.6 trials, respectively; *t* test, $t_{(18)}=2.190$, $p=0.042$; Fig. 3D, E). The mice sometimes consumed water in the subsequent trial rather than during the inter-trial interval following a rewarded trial. To rule out the influence of such invalid anticipatory licks, we deleted the trials during which the mice consumed water delivered in the previous trial (215 out of 8115 trials; 2.65%) and determined the number of trials needed to reach the reversal criterion. This analysis also found that there was a significant difference between WT and *Shank2*-KO mice in the number of trials needed to reach the reversal criterion (240.3 ± 35.7 and 571.2 ± 146.7 trials, respectively; *t* test, $t_{(18)}=2.190$, $p=0.042$).

To test whether reversal learning was influenced by the level of initial training, we examined the relationship between the number of trials needed to reach the reversal criterion and the number of licks in CS_{Rw} trials, the number of licks in CS_{Pn} trials, the difference in the number of licks between CS_{Rw} and CS_{Pn} trials, and the LDI during the last acquisition session. We found that there was no significant relationship between these measures and the number of trials needed to reach the reversal criterion (Fig. 3H). This indicates that the difference in reversal learning between WT and *Shank2*-KO mice cannot be accounted for by different levels of initial learning.

We also found that the LDI during the first reversal session (calculated using trials #53–62) differed significantly between WT and *Shank2*-KO mice (-0.010 ± 0.184 and 0.449 ± 0.101, respectively; *t* test, $t_{(18)}=2.189$, $p=0.042$; Fig. 3F, G). No significant relationship was found between the LDI and the number of licks in CS_{Rw} trials, the number of licks in CS_{Pn} trials, the difference in the number of licks between CS_{Rw} and CS_{Pn} trials, or the LDI during the last acquisition session (Fig. 3I). Together, these results indicate that reversal learning is slower in *Shank2*-KO mice than in WT mice.

Impaired reversal learning in juvenile male Shank2-KO mice

Given that ASD is a neurodevelopmental disorder and people with ASD may show signs of behavioral inflexibility in childhood [87–92], we tested whether juvenile *Shank2*-KO mice also show deficits in reversal learning. Toward this end, juvenile (P30–45) male mice (10 WT and 10 *Shank2*-KO) were tested in Task 1 as described for adult male mice (Fig. 4A). The mice were trained for three daily sessions during the initial acquisition phase; unfortunately, however, the third-session data were lost due to a procedural error. We therefore assessed initial learning based on the first two sessions. The anticipatory lick rate diverged rapidly according to CS in the first session, and this difference was maintained in the second session (Fig. 4B). Two-way mixed ANOVA of LDI revealed that there was a significant main effect of training ($F_{(7,126)}=8.400$; $p=2.1\times10^{-8}$), but no significant main effect of genotype ($F_{(1,126)}=0.601$; $p=0.448$) or training × genotype interaction effect ($F_{(7,126)}=1.628$;

(See figure on next page.)

Fig. 3 Reversal learning is impaired in adult male *Shank2*-KO mice. **A** Left, two odor cues, CS_{Rw} and CS_{Pn}, were paired with 75% reward (water, 6 µl; blue) and 75% strong air puff (100 ms, 3 psi; red), respectively (Task 1). Right, mean licking responses (lick density functions, $\sigma=100$ ms) of WT (left; $n=10$) and *Shank2*-KO (right; $n=10$) mice during the last acquisition session. Trials were grouped according to CS and outcome. **B** Mean delay period anticipatory lick rates in response to CS_{Rw} (blue) or CS_{Pn} (red) during initial acquisition (three sessions). **C** The LDI of WT (black) and *Shank2*-KO (red) mice in each stage (100 trials) during initial acquisition. **D** Sample reversal learning sessions. Blue and red lines indicate anticipatory licking responses to $CS_{Rw\rightarrow Pn}$ and $CS_{Pn\rightarrow Rw}$ cues, respectively, in a moving average of 25 trials. Gray and black asterisks at the top indicate significantly higher anticipatory licking response to the $CS_{Rw\rightarrow Pn}$ (gray) or $CS_{Pn\rightarrow Rw}$ (black) cue compared to the other cue, in a moving window of 25 trials ($p<0.05$, *t* test). Open triangles indicate the first trial since meeting the cue-outcome contingency reversal criterion. **E** The number of trials required to exceed the reversal criterion. **F** The LDI during the first reversal session (moving average of 25 trials). **G** The LDI during the last 10 trials in **F**. **H, I** Relationships between reversal learning performance (ordinate; **H**, the number of trials needed to reach the reversal criterion; **I**, LDI during the first reversal session [last 10 trials in F]) and the mean anticipatory lick rate in CS_{Rw} trials, that in CS_{Pn} trials, their difference, and the LDI during the last acquisition session (abscissa). Gray dashed lines represent least-squares linear fit. Circles indicate individual animal data **E**, **F**, **H** and **I**. Squares and bar graphs **B**, **C**, **E** and **H** present the mean across 10 mice. Shading and error bars indicate the SEM across 10 mice **A**–**C**, **E**, **G** and **H**. *$p<0.05$, *t* test

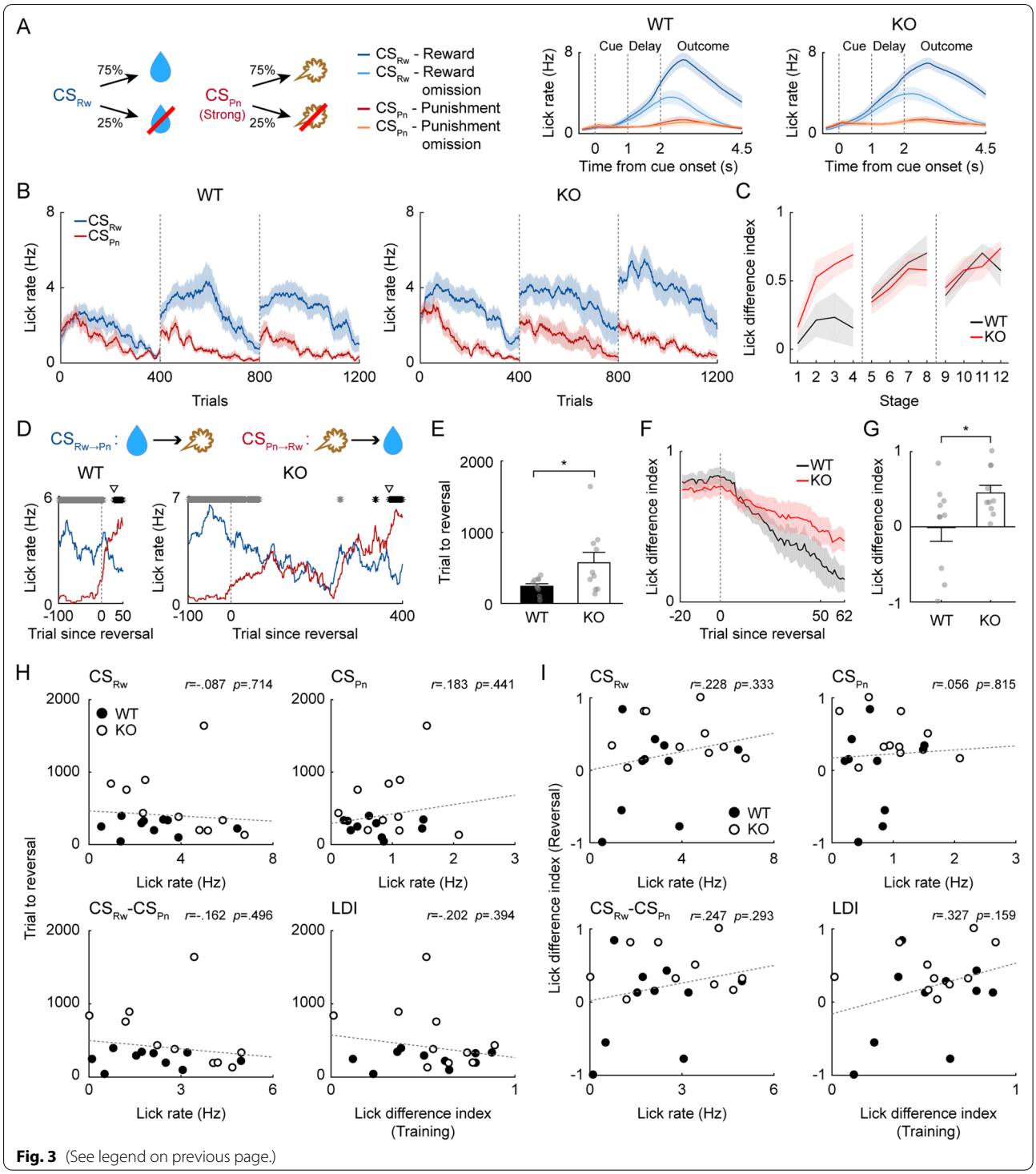

Fig. 3 (See legend on previous page.)

$p = 0.133$; Fig. 4C). Although we could not examine the animals' behavior during the third training session, the mice showed significantly different anticipatory licking responses between CS_{Rw} and CS_{Pn} trials at the outset (first 100 trials) of the first reversal session before reversal onset, and their rates did not differ significantly from those of the corresponding adult male mice (Additional file 1: Fig. S1). Moreover, the number of trials needed to reach the reversal onset criterion did not differ significantly from that of the corresponding adult male mice

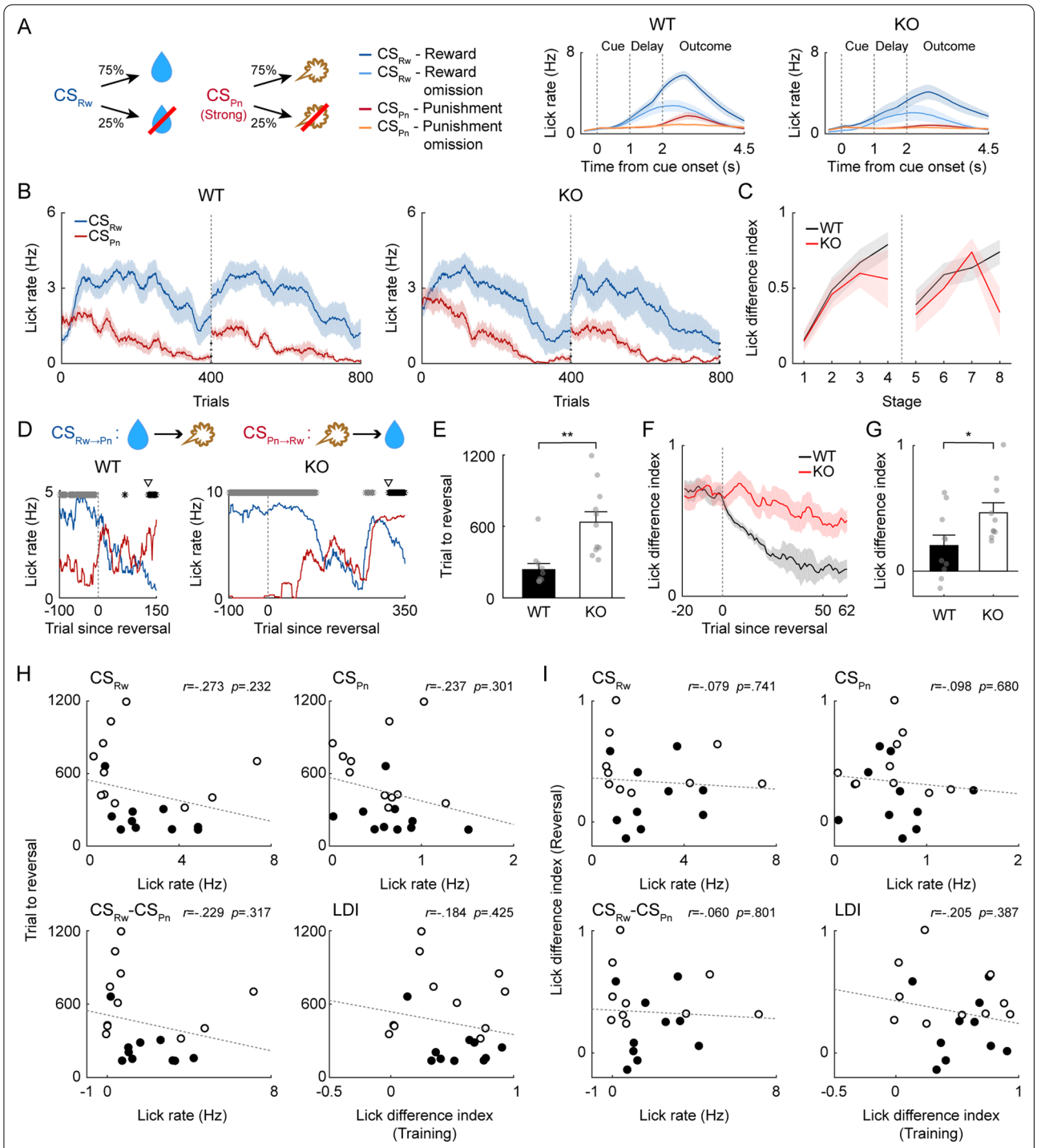

Fig. 4 Reversal learning is impaired in juvenile male *Shank2*-KO mice. Results of reversal training with a strong air puff (Task 1) in juvenile male WT ($n = 10$) and *Shank2*-KO ($n = 11$) mice. The results are presented as described for Fig. 3

(Additional file 1: Fig. S1). These results indicate that the juvenile WT and *Shank2*-KO mice were well trained to discriminate between CS_{Rw} and CS_{Pn}.

Upon CS-US contingency reversal (2–4 daily sessions of 400 trials until the reversal criterion was reached), the number of trials needed to reach the reversal criterion differed significantly between the juvenile male WT

and *Shank2*-KO mice (236.5 ± 50.6 and 634.9 ± 88.2 trials, respectively; *t* test, $t_{(19)} = 2.189$, $p = 0.001$; Fig. 4D, E; similar results were obtained after deletion of trials in which previously delivered water was consumed; 306 out of 8945 trials; 3.42%; *t* test, $t_{(19)} = 3.428$, $p = 0.003$). The LDI during the first reversal session (calculated using trials #53–62) also differed significantly between the two genotypes (0.203 ± 0.084 and 0.465 ± 0.079, respectively; *t* test, $t_{(18)} = 2.282$, $p = 0.035$; Fig. 4F, G). No significant relationship was found between these reversal learning measures (the number of trials needed to reach the reversal criterion and the LDI) and the number of licks in CS_{Rw} trials, the number of licks in CS_{Pn} trials, the difference in the number of licks between CS_{Rw} and CS_{Pn} trials, or the LDI during the last acquisition session (Fig. 4H, I). These results indicate that, as seen for adult males, juvenile male *Shank2*-KO mice were impaired in the reversal learning task studied herein.

Intact reversal learning in female Shank2-KO mice

Because the prevalence of ASD is strongly male-biased [76], we examined whether adult (12–24 weeks old) female *Shank2*-KO mice also show reversal learning deficits in Task 1 (Fig. 5A). Adult female WT ($n = 12$) and *Shank2*-KO (n = 10) mice showed preferential anticipatory licking in response to CS_{Rw} compared to CS_{Pn} throughout the initial training sessions (Fig. 5B). Two-way mixed ANOVA of LDI revealed that there was a significant main effect of training ($F_{(11,220)} = 6.812$; $p = 7.7 \times 10^{-10}$), but no significant main effect of genotype ($F_{(1,220)} = 0.392$; $p = 0.538$) or training × genotype interaction effect ($F_{(11,220)} = 1.611$; $p = 0.097$; Fig. 5C).

Upon CS-US contingency reversal (2–4 daily sessions of 400 trials until reaching the reversal criterion), we found that there was no significant difference in the number of trials needed to reach the reversal criterion between female WT and *Shank2*-KO mice (387.9 ± 45.0 and 386.9 ± 77.2 trials, respectively; *t* test, $t_{(20)} = 0.012$, $p = 0.991$; Fig. 5D, E; similar results were obtained after deletion of trials in which previously delivered water was consumed; 347 out of 8598 trials; 4.04%; *t* test, $t_{(20)} = 0.408$, $p = 0.687$). We also failed to find a significant difference in the LDI (calculated using trials #53–62 of the first reversal session; 0.396 ± 0.100 and 0.502 ± 0.127, respectively; *t* test, $t_{(19)} = 0.661$, $p = 0.516$; Fig. 5F, G). These reversal learning measures showed no significant relationship with the number of licks in CS_{Rw} trials, the number of licks in CS_{Pn} trials, the difference

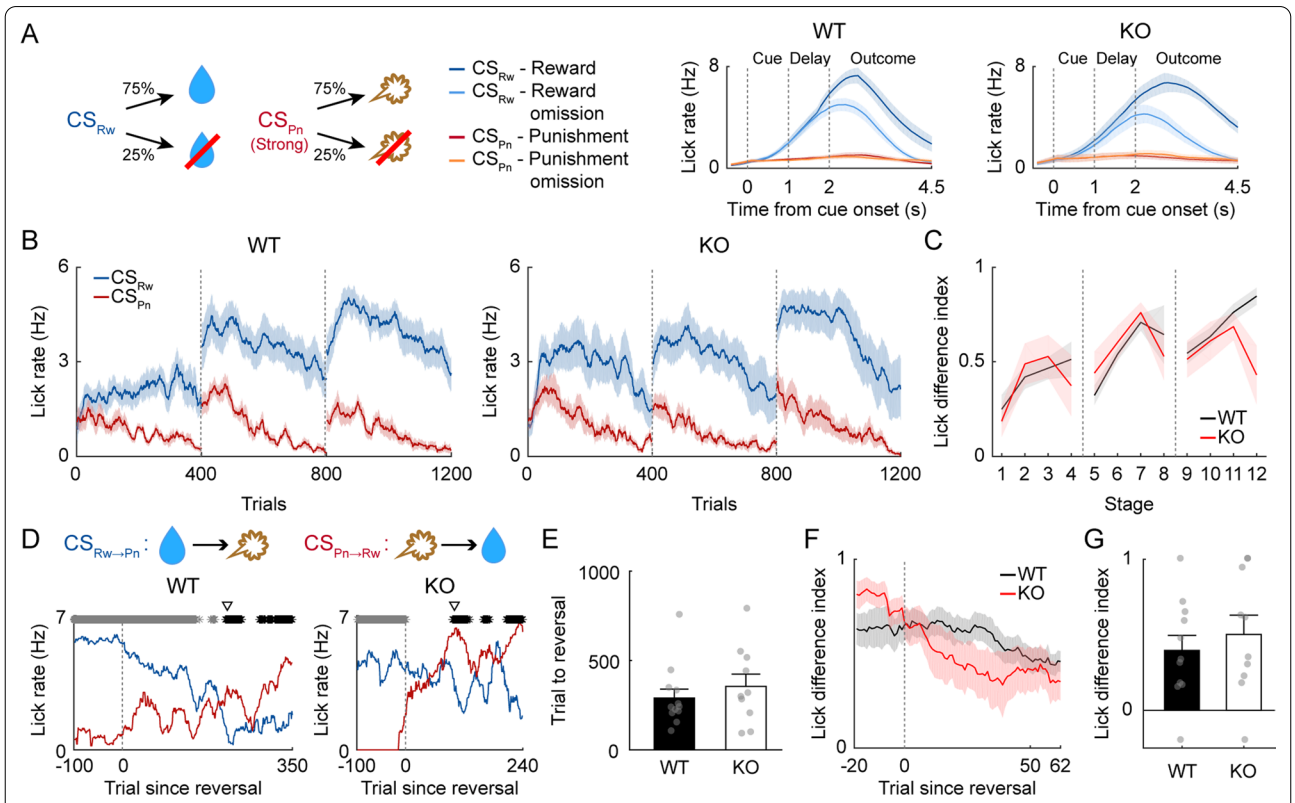

Fig. 5 Intact reversal learning in female *Shank2*-KO mice. Results of reversal training with a strong air puff (Task 1) in adult female WT ($n = 12$) and *Shank2*-KO ($n = 10$) mice. The results are presented as described for Fig. 3A–G

in the number of licks between CS_{Rw} and CS_{Pn} trials, or the LDI during the last acquisition session (Additional file 1: Fig. S2). These results indicate that the reversal learning of female *Shank2*-KO mice is intact.

Enhanced eye closure responses in male Shank2-KO mice

Since ASD is associated with atypical sensory responses and heightened anxiety [93–95], we examined whether WT and *Shank2*-KO mice show differential eye closure responses to an air puff. Specifically, we delivered the air puff used in Task 1 (100 ms, 3 psi) without any preceding sensory cue (inter-trial interval, 9–11 s, uniform random distribution) and measured the fraction of eye closure before, during, and after air puff delivery (Fig. 2). Two-way mixed ANOVA revealed that there were significant main effects for genotype ($F_{(1,40)}=10.958$, $p=0.004$), time ($F_{(2,40)}=24.723$, $p=1.0\times10^{-7}$), and their interaction ($F_{(2,40)}=3.729$, $p=0.033$) on eye closure responses (Fig. 6A). Post hoc Bonferroni tests indicated that the eye closure response was significantly stronger in *Shank2*-KO than WT mice before (1.5-s time window before air puff onset; $p=0.001$) and after (between 2.5 and 4 s since air puff onset; $p=0.005$), but not during (1-s time window since air puff onset; $p=0.073$) air puff delivery. These results indicate that anticipatory eye closure responses differ between adult male WT and *Shank2*-KO mice.

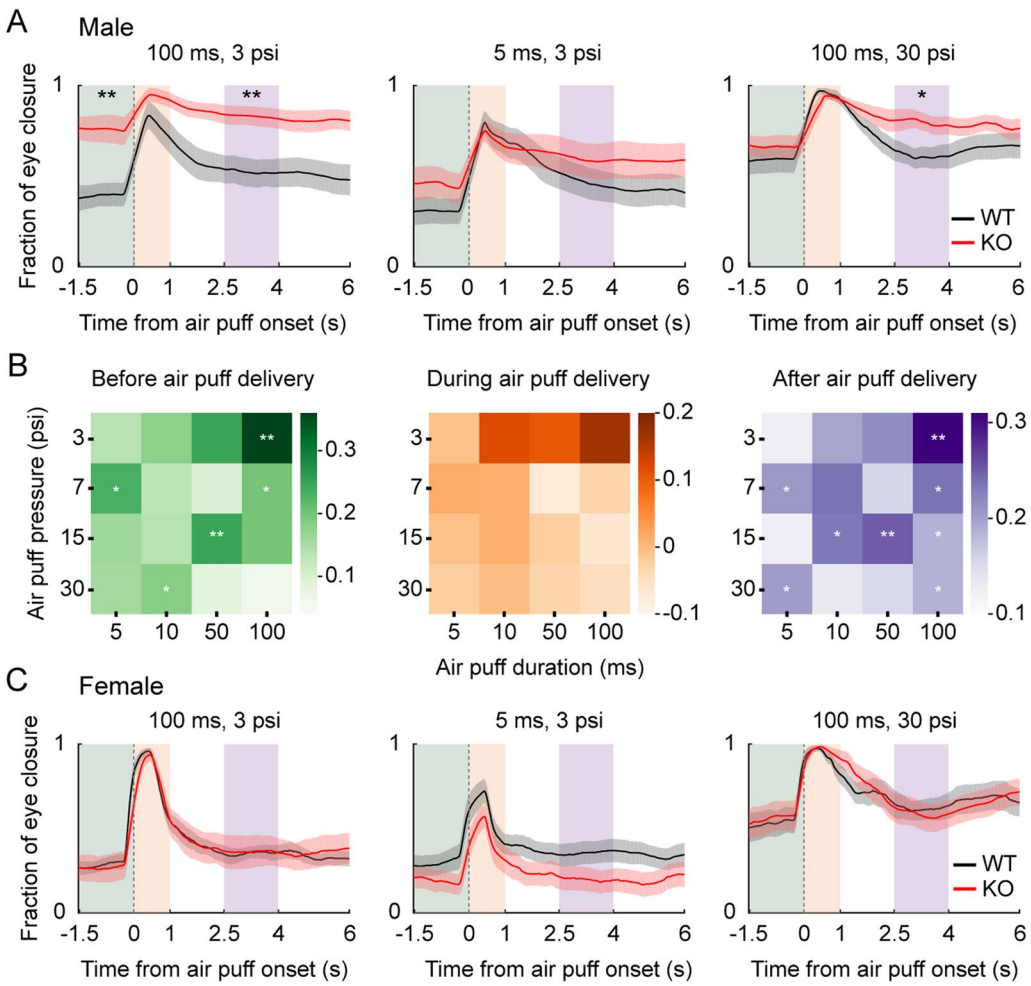

Fig. 6 Eye closure response to air puff differs between male, but not female, WT and *Shank2*-KO mice. **A** Eye closure responses (average of 5 trials) of adult male WT (black; $n=12$) and *Shank2*-KO (red; $n=10$) mice (shading, SEM across mice) to strong (100 ms, 3 psi; left), mild (5 ms, 3 psi; middle), and very strong (100 ms, 30 psi; right) air puffs. Shaded rectangles indicate time periods before (green, 1.5 s before air puff onset), during (orange, 1 s after air puff onset), and after air puff delivery (purple, between 2.5 and 4 s after air puff onset). **B** The difference in eye closure response between *Shank2*-KO and WT mice to 16 different combinations of air puff duration (abscissa) and pressure (ordinate) before (left), during (middle), and after (right) air puff delivery. **C** Eye closure responses of adult of female WT (black; $n=11$) and *Shank2*-KO (red; $n=10$) mice. The results are presented as described for panels A–C. $^{*}p<0.05$, $^{**}p<0.01$, using two-way mixed ANOVA followed by post hoc Bonferroni test

The above results raised the possibility that abnormal emotional responses to air puff may negatively affect reversal learning in male *Shank2*-KO mice. One way to explore this possibility would be to test male *Shank2*-KO mice using a mild air puff that does not induce an abnormal eye closure response. For this, we examined the relationship between air puff strength and eye closure response by systematically varying the duration and intensity of air puff (15 combinations other than the original one [100 ms, 3 psi]). Eye closure responses during air puff delivery (1-s time period since air puff onset) did not differ significantly between the two genotypes for any combination of air puff duration and intensity (*t* test, *p* values > 0.05; Fig. 6B). However, anticipatory eye closure responses before (1.5-s time window before air puff onset) and after (between 2.5 and 4 s since air puff onset) air puff delivery differed significantly between the two genotypes for some combinations of air puff duration and intensity (Fig. 6B). As expected, mild air puffs induced similarly low levels of anticipatory eye closure responses (Fig. 6A) in male WT and *Shank2*-KO mice. Based on these results, we chose to use the mildest air puff (5 ms, 3 psi; Fig. 6A and 6B) to further examine the reversal learning of male *Shank2*-KO mice.

Because female KO mice showed intact reversal learning in Task 1, we examined whether they would also show normal levels of eye close responses. In females, unlike males, *Shank2*-KO and WT mice showed similar levels of eye closure before, during, and after the delivery of the air puff used in Task 1 (100 ms, 3 psi; two-way mixed ANOVA, main effect of genotype, $F_{(1,38)} = 0.057$, $p = 0.814$; main effect of time, $F_{(2,38)} = 109.621$, $p = 1.7 \times 10^{-16}$; genotype × time interaction effect, $F_{(2,38)} = 0.222$, $p = 0.802$; Fig. 6C). We also failed to find a significant difference in eye closure response between female WT and *Shank2*-KO mice to mild (5 ms, 3 psi; main effect of genotype, $F_{(1,38)} = 2.371$, $p = 0.140$; main effect of time, $F_{(2,38)} = 62.549$, $p = 9.5 \times 10^{-13}$; genotype × time interaction effect, $F_{(2,38)} = 0.279$, $p = 0.758$; Fig. 6C) and very strong (100 ms, 30 psi; main effect of genotype, $F_{(1,38)} = 0.002$, $p = 0.964$; main effect of time, $F_{(2,38)} = 47.561$, $p = 4.5 \times 10^{-11}$; genotype × time interaction effect, $F_{(2,38)} = 0.274$, $p = 0.762$; Fig. 6C) air puffs. These results indicate that female *Shank2*-KO mice lack the heightened fear response observed in male *Shank2*-KO mice.

Intact reversal learning of male Shank2-KO mice in the presence of mild air puff

Using the mildest air puff (5 ms, 3 psi), to which adult male WT and *Shank2*-KO mice showed similar anticipatory eye closure responses, we tested another group of adult male mice (10 WT and 10 *Shank2*-KO) for reversal learning (Task 2; Fig. 7A). Both male WT and *Shank2*-KO mice quickly developed and maintained preferential anticipatory licking responses to CS_{Rw} versus CS_{Pn} during the initial training sessions. Two-way mixed ANOVA of LDI revealed that there was a significant main effect of training ($F_{(11,187)} = 14.010$; $p = 1.9 \times 10^{-19}$) and genotype ($F_{(1,187)} = 14.561$; $p = 0.001$), but no significant interaction effect between the two ($F_{(11,187)} = 1.092$; $p = 0.370$; Fig. 7B, C). Thus, both genotypes learned the CS-US contingencies well, but the initial learning was stronger in WT mice than in *Shank2*-KO mice.

The mice were then trained until they reached the reversal criterion over 2–4 daily sessions (400 trials each). The number of trials needed to reach the reversal criterion did not vary significantly between the male WT and *Shank2*-KO mice (373.6 ± 117.5 and 379.0 ± 79.9 trials, respectively; *t* test, $t_{(18)} = 0.038$, $p = 0.970$; Fig. 7D, E; similar results were obtained after deletion of trials in which previously delivered water was consumed; 114 out of 7697 trials; 1.48%; *t* test, $t_{(18)} = 0.084$, $p = 0.934$). The LDI (calculated using trials #41–50 of the first reversal session) also did not differ significantly between the two genotypes (0.328 ± 0.059 and 0.386 ± 0.153, respectively; *t* test, $t_{(18)} = 0.348$, $p = 0.732$; Fig. 7F, G). These measures of reversal learning had no significant relationship with the number of licks in CS_{Rw} trials, the number of licks in CS_{Pn} trials, the difference in the number of licks between CS_{Rw} and CS_{Pn} trials, or the LDI during the last session of the initial training (Additional file 1: Fig. S3). These results indicate that male *Shank2*-KO mice exhibit intact reversal learning in Task 2. Similar results were obtained with juvenile (P30–45) male WT and *Shank2*-KO mice tested on Task 2 (Additional file 1: Fig. S4).

Intact reversal learning of Shank2-KO mice in the absence of aversive outcome

To further confirm that the behavioral flexibility of male *Shank2*-KO mice is intact in the absence of a strong aversive outcome, we tested another group of adult male WT ($n = 10$) and *Shank2*-KO ($n = 10$) mice in reversal learning using only appetitive outcomes. In Task 3, two different odor cues were paired with the same amount of water (6 µl), but with two different probabilities (80 and 20%; $CS_{80\%}$ and $CS_{20\%}$, respectively; Fig. 8A). In both genotypes, anticipatory licking responses were higher to $CS_{80\%}$ than $CS_{20\%}$ throughout the acquisition session (Fig. 8B). Two-way mixed ANOVA of LDI revealed that there was a significant main effect of training ($F_{(11,198)} = 5.117$; $p = 4.8 \times 10^{-7}$) but no significant main effect of genotype ($F_{(1,198)} = 0.447$; $p = 0.513$) or training × genotype interaction effect ($F_{(11,198)} = 1.329$; $p = 0.211$; Fig. 8C).

During the reversal training (2–4 daily sessions of 400 trials until the reversal criterion), the number of trials

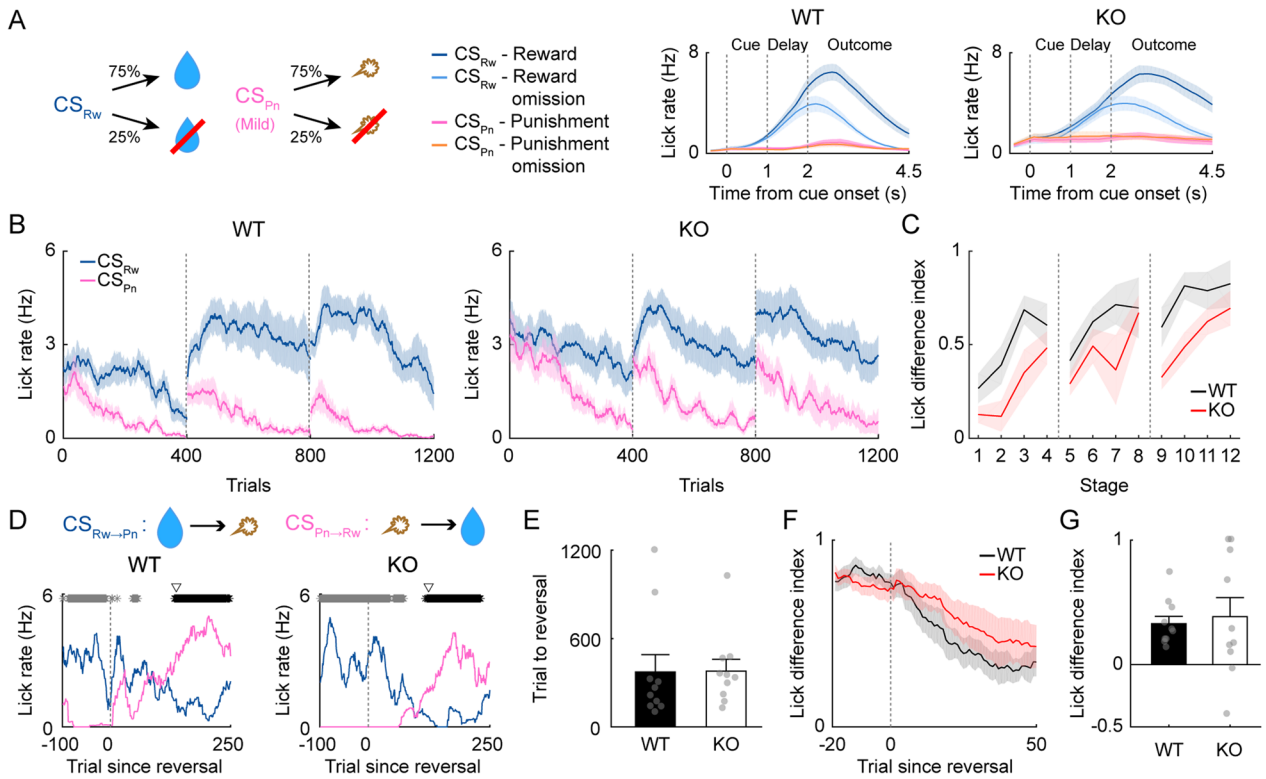

Fig. 7 Adult *Shank2*-KO mice exhibit intact reversal learning with the use of a mild air puff. Results of reversal training with a mild air puff (Task 2) in adult male WT ($n=10$) and *Shank2*-KO ($n=10$) mice. The results are presented as described for Fig. 3A–G, except that pink lines indicate anticipatory lick rates in response to the CS_{Pn} predicting a mild air puff (5 ms, 3 psi)

needed to reach the reversal threshold did not differ significantly between the male WT and *Shank2*-KO mice (594.4 ± 97.1 and 614.2 ± 177.4 trials, respectively; *t* test, $t_{(18)}=0.098$, $p=0.923$; Fig. 8E; similar results were obtained after deletion of trials in which previously delivered water was consumed; 210 out of 10,686 trials; 1.97%; *t* test, $t_{(18)}=0.950$, $p=0.355$). Also, the LDI (calculated using trials #53–64 of the first reversal session) did not differ significantly between the male WT and *Shank2*-KO mice (0.207 ± 0.061 and 0.108 ± 0.042, respectively; *t* test, $t_{(16)}=0.076$, $p=0.940$; Fig. 8F, G), and these reversal learning measures had no significant relationship with the number of licks in $CS_{80\%}$ trials, the number of licks in $CS_{20\%}$ trials, the difference in the number of licks between $CS_{80\%}$ and $CS_{20\%}$ trials, or the LDI during the last acquisition session (Additional file 1: Figure S5). These results verify that the reversal learning of *Shank2*-KO mice is intact in the absence of a strong aversive outcome.

Effect of DCS on fear response

Previous studies [60, 67] showed that DCS, a partial agonist of NMDA receptor, rescues the social interaction deficits of *Shank2*-KO mice. We therefore tested whether DCS would also rescue the behavioral deficits of *Shank2*-KO mice found in the current study. As repeated administration of DCS causes tachyphylaxis [96–99] and the drug's half-life in mice is only 23 min [100, 101], it would have been difficult to test the effects of DCS on the above-described reversal learning, which takes a relatively long time (1–5 days of training). Given that our results suggested that enhanced fear is the source of reversal learning deficit in male *Shank2*-KO mice, and eye close responses can be tested within a short period of time, we examined whether DCS could rescue the abnormal eye closure responses (i.e., fear responses) of adult male *Shank2*-KO mice. We tested the effects of DCS on eye closure responses to the strong (100 ms, 3 psi), mild (5 ms, 3 psi), and very strong (100 ms, 30 psi) air puffs. For the strong air puff (100 ms, 3 psi), three-way mixed ANOVA revealed that there were significant main effects of genotype ($F_{(1,38)}=4.714$, $p=0.043$) and time ($F_{(2,38)}=45.503$, $p=8.2\times10^{-11}$), but no significant interaction between them ($F_{(2,38)}=0.719$, $p=0.494$). The main effect of drug ($F_{(1,38)}=0.122$, $p=0.731$) and the other interaction effects were not statistically significant (genotype × time, $F_{(2,38)}=0.719$, $p=0.494$; drug × genotype, $F_{(1,38)}=0.130$, $p=0.723$; drug × time, $F_{(2,38)}=0.331$,

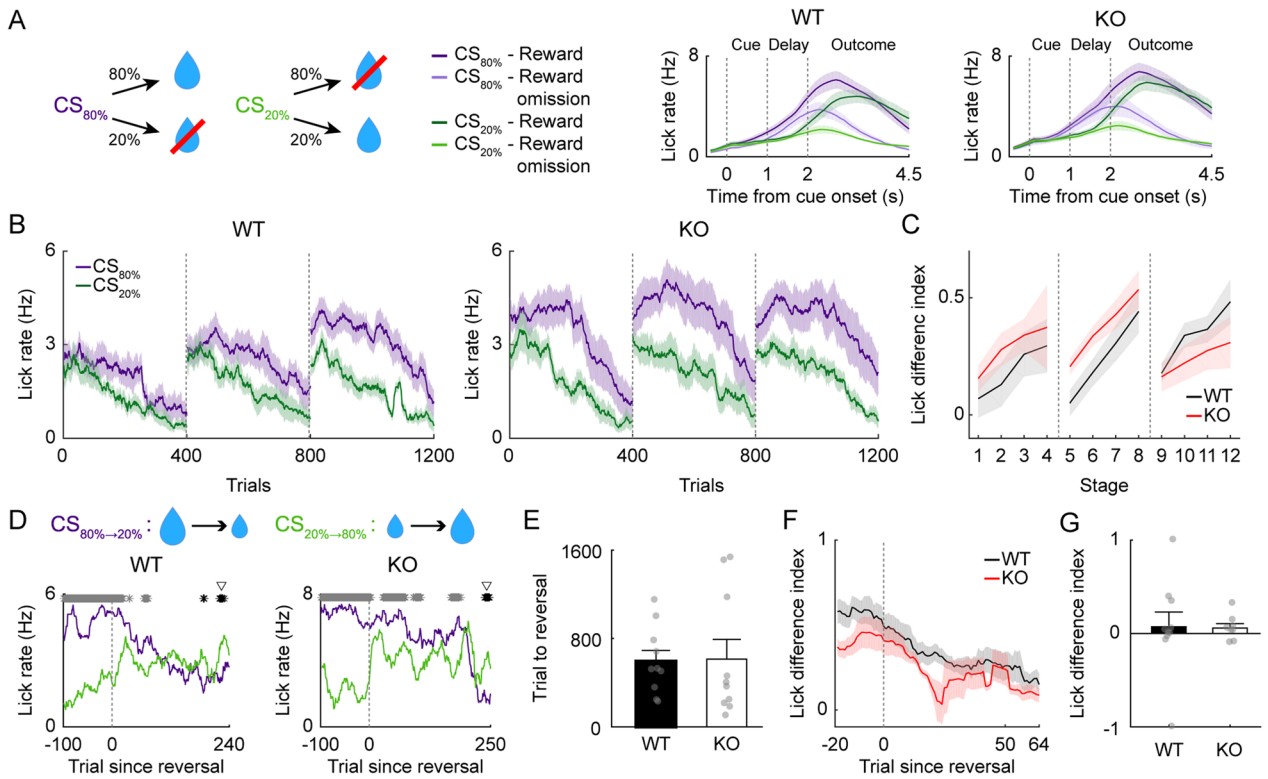

Fig. 8 *Shank2*-KO mice exhibit intact learning in reward probability reversal. Results of reversal training using only appetitive outcomes (Task 3) in adult male WT ($n=10$) and *Shank2*-KO ($n=10$) mice. The results are presented as described for Fig. 3A–G, except that the purple and green lines denote anticipatory lick rates in response to $CS_{80\%}$ and $CS_{20\%}$, respectively

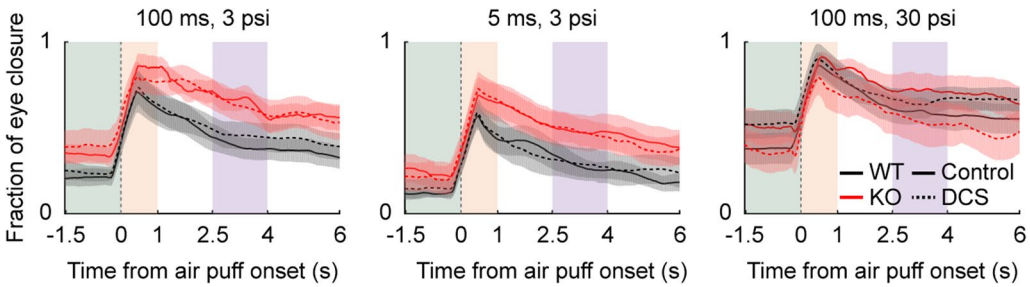

Fig. 9 DCS does not rescue the abnormal eye closure response of male *Shank2*-KO mice. Eye closure responses (average of 5 trials) of adult male WT (black; $n=11$) and *Shank2*-KO (red; $n=10$) mice (shading, SEM across mice) treated with DCS (dashed line) or saline (solid line) injection and exposed to the strong, mild, and very strong air puffs

$p=0.720$; drug × genotype × time, $F_{(2,38)}=0.489$, $p=0.617$; Fig. 9A). Similarly, the main effect of drug and the effects of interactions involving the drug were not statistically significant for the mild (main effect of drug, $F_{(1,38)}=0.020$, $p=0.888$; drug × genotype, $F_{(1,38)}=0.012$, $p=0.914$; drug × time, $F_{(2,38)}=0.050$, $p=0.951$; drug × genotype × time, $F_{(2,38)}=0.189$, $p=0.829$; Fig. 9B) and very strong (main effect of drug, $F_{(1,38)}=0.186$, $p=0.671$; drug × genotype, $F_{(1,38)}=2.888$, $p=0.106$; drug × time, $F_{(2,38)}=0.362$, $p=0.700$; drug × genotype × time, $F_{(2,38)}=0.559$, $p=0.577$; Fig. 9C) air puffs. These results indicate that DCS does not rescue the enhanced fear response of male *Shank2*-KO mice to the air puff.

Discussion

We herein examined the behavioral flexibility of *Shank2*-KO mice by testing their capability for reversal learning in a probabilistic trace classical conditioning paradigm using the anticipatory lick rate as an index for learning. Compared to WT mice, male *Shank2*-KO mice showed significantly slower reversal learning when a water reward and a strong air puff were used as the appetitive and aversive outcomes, respectively (Task 1). Moreover, male *Shank2*-KO mice showed stronger eye closure responses than WT mice to the anticipated air puff. On the one hand, eye closure responses did not vary significantly between male *Shank2*-KO and WT mice immediately after an air puff was delivered. This suggests that the reversal learning deficit of *Shank2*-KO mice observed in Task 1 is unlikely to reflect abnormal sensory processing, which is often observed in people with ASD [102, 103] and animal models of ASD [103, 104], or abnormal reactivity to an aversive stimulus. On the other hand, male *Shank2*-KO mice showed significantly stronger eye closure responses than WT mice before and after air puff delivery, indicating that repeated exposure to strong air puffs altered the basal level of eye closure in these mice. Both nonspecific emotional (fear) and specific motor (eyelid movement) responses develop during eyeblink conditioning. Conditioned fear, such as an increase in blood pressure/heart rate and pupillary dilation, emerges only after a few CS-US pairings, whereas conditioned eyeblink (with the peak responses precisely timed to the expected delivery of the US) typically emerge after many CS-US pairings [105–107]. Our results are consistent with the idea that male *Shank2*-KO mice show enhanced fear to an anticipated aversive stimulus, rather than an enhanced reflexive motor response to the delivery of an aversive stimulus. This elevated fear may suppress the behavioral expression of learned cue-outcome contingencies during reversal training in Task 1.

Consistent with this possibility, when we replaced the strong air puff with a mild air puff that induced similar anticipatory eye closure responses between male *Shank2*-KO and WT mice, male *Shank2*-KO mice showed intact reversal learning (Task 2). Male *Shank2*-KO mice also showed intact reversal learning between two cues predicting rewards with different probabilities (Task 3). Together, these results are consistent with the notion that male *Shank2*-KO mice show enhanced fear but intact learning of cue-outcome contingency changes in our behavioral settings. This is further supported by the finding that female *Shank2*-KO mice were intact in reversal learning in the presence of a strong air puff and also showed WT-level anticipatory eye closure responses to the strong air puff. A previous study found that reversal learning was delayed in people with ASD in a fear conditioning paradigm in which an air puff was used as an US [15]. Our results raise the possibility that people with ASD might show intact reversal learning if the US is replaced with a mild air puff, which remains to be tested. Our present results suggest that the neural machinery needed to keep track of changes in cue-outcome contingency and to control behavior according to such evaluations are likely to be intact in these brain structures of *Shank2*-KO mice, but their behavioral expressions are suppressed by enhanced fear in male *Shank2*-KO mice.

Previous studies on reversal learning in ASD model mice yielded mixed results. Both intact and impaired reversal learning have been reported for a diverse array of ASD mouse models in diverse tasks. Regarding classical conditioning using aversive outcomes, *Tsc1* mutant mice were reported to be impaired in the reversal of eyeblink conditioning [24]. Given that *Tsc1* and *Tsc2* form a complex for signal transduction and *Tcs2* deletion increases anxiety [108], the impairment of Tsc1 mutant mice in the reversal of eyeblink conditioning is in line with our finding that an altered emotional response may limit behavioral flexibility in a mouse model of ASD. Some previous studies found that reversal learning is intact in ASD model mice exposed to tasks involving no aversive outcome. For example, mice with mutations in postsynaptic density protein-95 were intact in reversal learning on a touch screen task in which one target was associated with a reward and another with no reward [109]. These results are consistent with our finding that *Shank2*-KO mice were intact in reversal learning when only appetitive outcomes were used. However, numerous other studies found that reversal learning was impaired in various ASD model mice in the absence of an aversive outcome [16–20, 22–29]. It is difficult to compare findings across studies because the studied genotypes and experimental procedures have varied widely. For example, the utilized experimental procedures have varied in their choice availability (classical vs. instrumental tasks), outcome valence (appetitive vs. aversive), reinforcement type (positive vs. negative), outcome certainty (deterministic vs. probabilistic delivery), freedom of movement (head-fixed vs. freely moving), stimulus modality (olfactory, visual, etc.), and choice modality (spatial vs. nonspatial). These factors may directly or indirectly influence performance in reversal learning. For example, anxiety is elevated under uncertainty [110, 111] and anxiety disorder patients prefer to play a passive rather than an active role in decision-making [112]. Thus, it is possible that uncertain outcomes under a free-choice condition may elevate anxiety and thereby impair reversal

learning in certain ASD model mice. Consistent with this, BTBR T + Itpr3tf/J and C58 mice showed intact reversal learning when a reward was delivered in an all-or-none manner, but exhibited impaired reversal learning when the reward was delivered probabilistically [17, 23]. Our results raise the possibility that altered emotional responses may limit behavioral flexibility despite the presence of intact learning capability in ASD.

Shank2-KO mice lacking exons 6 and 7, such as those used in the present study, show reduced NMDA receptor (NMDAR) function in several brain regions, including the medial prefrontal cortex, hippocampus, and amygdala, at juvenile and adult stages [60, 67, 68, 71]. This NMDAR hypofunction has been causally associated with paradoxically increased NMDAR function at early postnatal stages [67], which highlights the long-lasting impacts of early postnatal pathophysiology [83]. Normalizing the NMDAR hypofunction in *Shank2*-KO mice by direct DCS-dependent NMDAR activation or by indirect NMDAR activation (through the zinc chelator, clioquinol, or early postnatal memantine treatment) rescues mainly social deficits but not other behavioral deficits, including hyperactivity, repetitive behaviors, and anxiety-related behaviors [60, 67, 71, 113]. Whether fear responses are altered by and associated with NMDAR dysfunction has not been tested in our *Shank2*-KO mice or other *Shank2*-KO mouse lines [65]. Nevertheless, our present finding that DCS does not alter the eye closure fear responses of *Shank2*-KO mice does not seem to disagree with the previous report that NMDAR activation has the main effect on social rescue. Currently, Shank2-related circuit dysfunctions remain largely unexplored [68], partly because Shank2 is widely expressed in various brain regions, including the cerebellum [32, 61, 62], and in various cell types, including excitatory and inhibitory neurons [68]. Clearly, further studies are needed to elucidate the critical Shank2-related circuit dysfunction that leads to enhanced fear.

The male–female difference in the reversal learning and anticipatory eye closure responses in *Shank2*-KO mice is intriguing in light of previous reports that male and female *Shank2*-KO mice display similar NMDAR hyperfunction and behavioral deficits in social, repetitive behavioral, locomotor, and anxiety-like domains as well as similar pharmacological rescue profiles of these deficits [60, 67]. However, male–female differences in mouse models of ASD have been differentially detected in various behavioral, synaptic, molecular, and neuroanatomical phenotypes [77–82]. Also, male–female differences in eyeblink conditioning have been reported in rats, mice, and humans [114–116]. Therefore, it is possible that reversal learning and fear responses represent two behavioral tests through which male–female differences in *Shank2*-KO mice may be detected. Although the related mechanisms remain to be determined, previous transcriptomic studies on human and mouse samples have identified sexually dimorphic expression patterns among astrocyte- and microglia-related genes, suggesting that there are sex differential interplays between neuronal and glial cells [76, 77, 117].

Limitations

Our study has limitations in many respects. First, our conclusions are drawn from the results obtained from only one mouse model of ASD (*Shank2*-KO mice) tested in one type of behavioral task (reversal learning in a probabilistic trace classical conditioning paradigm). Therefore, future work is needed to clarify the extent to which altered emotional responses contribute the behavioral inflexibility associated with ASD. Second, it remains possible that abnormalities in processes other than emotional processing contribute to the behavioral inflexibility seen in ASD. We examined only the influence of enhanced fear on reversal learning; meanwhile, behavioral flexibility can be caused by abnormalities in many different underlying processes, such as inhibitory control, value-based decision-making under free-choice conditions, and the ability to capture complex stimulus–response–outcome contingencies (task rules) under cognitively demanding situations. In this regard, our results do not argue directly against the cognitive inflexibility hypothesis for ASD [13]. Third, we did not investigate the neurobiological mechanisms linking *Shank2*-KO and enhanced fear in this study. The brain structures involved in the fear-induced suppression of reversal learning in male *Shank2*-KO mice are unclear, as are the physiological and molecular processes leading to enhanced fear in male *Shank2*-KO mice. All of these details remain to be elucidated.

Conclusions

Our results demonstrate that one consequence of *Shank2*-KO in male mice is an abnormally heightened fear that may limit behavioral flexibility under certain circumstances. Our findings suggest that behavioral flexibility may be seriously limited by abnormal emotional responses in ASD. Further studies are needed to determine the extent to which people with ASD and animal models of ASD show impairments in their ability to flexibly adjust behavior due to altered emotional responses under diverse behavioral settings. Going forward, the neurobiological mechanisms linking *Shank2*-KO and enhanced fear remain to be determined.

Abbreviations
ASD: Autism spectrum disorder; CS: Conditioned stimulus; DCS: D-cycloserine; KO: Knockout; LDI: Lick difference index; PCR: Polymerase chain reaction; Shank2: SH3 and multiple ankyrin repeat domains 2; US: Unconditioned stimulus; WT: Wild-type.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13229-022-00518-1.

Additional file 1: Table S1, Table S2, Fig. S1, Fig. S2, Fig. S3, Fig. S4 and Fig. S5. Statistical test results for eyelid closure responses to diverse combinations of air puff duration and pressure.

Acknowledgements
We thank Jeansok Kim and Junsik Choi for helpful discussions and Chan Mee Bae for animal care.

Author contributions
M.Y. and M.W.J. conceived the study. M.Y. collected and analyzed the data. M.W.J. and E.K. supervised the study. M.Y. and M.W.J. wrote the manuscript with input from E.K. All authors read and approved the final manuscript.

Funding
This work was supported by the Research Center Program of the Institute for Basic Science (IBS-R002-D1 to E.K. and IBS-R002-A1 to M.W.J.).

Availability of data and materials
Raw data and code to reproduce this work are archived at Mendeley Data (https://data.mendeley.com/datasets/bg8s4zpdk6/1).

Declarations

Ethics approval and consent to participate
All animal care and experimental procedures were performed in accordance with protocols approved by the directives of the Animal Care and Use Committee of Korea Advanced Institute of Science and Technology (approval number KA2018-08).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea. [2]Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon 34141, Korea.

Received: 6 June 2022 Accepted: 15 September 2022
Published online: 03 October 2022

References

1. American Psychiatric Association: Diagnostic and statistical manual of mental disorders (5th. ed). 2013.
2. Mundy P. Annotation: the neural basis of social impairments in autism: the role of the dorsal medial-frontal cortex and anterior cingulate system. J Child Psychol Psychiatry. 2003;44:793–809.
3. Hashem S, Nisar S, Bhat AA, Yadav SK, Azeem MW, Bagga P, Fakhro K, Reddy R, Frenneaux MP, Haris M. Genetics of structural and functional brain changes in autism spectrum disorder. Transl Psychiatry. 2020;10:229.
4. Yan Z, Rein B. Mechanisms of synaptic transmission dysregulation in the prefrontal cortex: pathophysiological implications. Mol Psychiatry. 2021;27(1):1–21.
5. Schoenbaum G, Roesch MR, Stalnaker TA. Orbitofrontal cortex, decision-making and drug addiction. Trends Neurosci. 2006;29:116–24.
6. Rushworth MF, Behrens TE, Rudebeck PH, Walton ME. Contrasting roles for cingulate and orbitofrontal cortex in decisions and social behaviour. Trends Cogn Sci. 2007;11:168–76.
7. Shenhav A, Botvinick MM, Cohen JD. The expected value of control: an integrative theory of anterior cingulate cortex function. Neuron. 2013;79:217–40.
8. Funahashi S. Neuronal mechanisms of executive control by the prefrontal cortex. Neurosci Res. 2001;39:147–65.
9. Miller EK, Cohen JD. An integrative theory of prefrontal cortex function. Annu Rev Neurosci. 2001;24:167–202.
10. Barbas H, Zikopoulos B. The prefrontal cortex and flexible behavior. Neuroscientist. 2007;13:532–45.
11. Hill EL. Executive dysfunction in autism. Trends Cogn Sci. 2004;8:26–32.
12. Leung RC, Zakzanis KK. Brief report: cognitive flexibility in autism spectrum disorders: a quantitative review. J Autism Dev Disord. 2014;44:2628–45.
13. Geurts HM, Corbett B, Solomon M. The paradox of cognitive flexibility in autism. Trends Cogn Sci. 2009;13:74–82.
14. D'Cruz AM, Ragozzino ME, Mosconi MW, Shrestha S, Cook EH, Sweeney JA. Reduced behavioral flexibility in autism spectrum disorders. Neuropsychology. 2013;27:152–60.
15. South M, Newton T, Chamberlain PD. Delayed reversal learning and association with repetitive behavior in autism spectrum disorders. Autism Res. 2012;5:398–406.
16. Bader PL, Faizi M, Kim LH, Owen SF, Tadross MR, Alfa RW, Bett GC, Tsien RW, Rasmusson RL, Shamloo M. Mouse model of timothy syndrome recapitulates triad of autistic traits. Proc Natl Acad Sci U S A. 2011;108:15432–7.
17. Amodeo DA, Jones JH, Sweeney JA, Ragozzino ME. Differences in BTBR T+ tf/J and C57BL/6J mice on probabilistic reversal learning and stereotyped behaviors. Behav Brain Res. 2012;227:64–72.
18. Tsai PT, Hull C, Chu Y, Greene-Colozzi E, Sadowski AR, Leech JM, Steinberg J, Crawley JN, Regehr WG, Sahin M. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. Nature. 2012;488:647–51.
19. Dickson PE, Corkill B, McKimm E, Miller MM, Calton MA, Goldowitz D, Blaha CD, Mittleman G. Effects of stimulus salience on touchscreen serial reversal learning in a mouse model of fragile X syndrome. Behav Brain Res. 2013;252:126–35.
20. Puścian A, Łęski S, Górkiewicz T, Meyza K, Lipp HP, Knapska E. A novel automated behavioral test battery assessing cognitive rigidity in two genetic mouse models of autism. Front Behav Neurosci. 2014;8:140.
21. Dong T, He J, Wang S, Wang L, Cheng Y, Zhong Y. Inability to activate Rac1-dependent forgetting contributes to behavioral inflexibility in mutants of multiple autism-risk genes. Proc Natl Acad Sci. 2016;113:7644–9.
22. Stoodley CJ, D'Mello AM, Ellegood J, Jakkamsetti V, Liu P, Nebel MB, Gibson JM, Kelly E, Meng F, Cano CA, et al. Altered cerebellar connectivity in autism and cerebellar-mediated rescue of autism-related behaviors in mice. Nat Neurosci. 2017;20:1744–51.
23. Whitehouse CM, Curry-Pochy LS, Shafer R, Rudy J, Lewis MH. Reversal learning in C58 mice: modeling higher order repetitive behavior. Behav Brain Res. 2017;332:372–8.
24. Badura A, Verpeut JL, Metzger JW, Pereira TD, Pisano TJ, Deverett B, Bakshinskaya DE, Wang SS. Normal cognitive and social development require posterior cerebellar activity. Elife. 2018;7:36401.
25. Nolan SO, Lugo JN: Reversal learning paradigm reveals deficits in cognitive flexibility in the Fmr1 knockout male mouse. F1000Research 2018; 7: 711–711.
26. Hughes RB, Whittingham-Dowd J, Clapcote SJ, Broughton SJ, Dawson N. Altered medial prefrontal cortex and dorsal raphé activity predict genotype and correlate with abnormal learning behavior in a mouse model of autism-associated 2p16.3 deletion. Autism Res. 2022;15:614–27.

27. Arnall S, Cheam LY, Smart C, Rengel A, Fitzgerald M, Thivierge JP, Rodger J. Abnormal strategies during visual discrimination reversal learning in ephrin-A2(-/-) mice. Behav Brain Res. 2010;209:109–13.
28. Sala M, Braida D, Lentini D, Busnelli M, Bulgheroni E, Capurro V, Finardi A, Donzelli A, Pattini L, Rubino T, et al. Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. Biol Psychiatry. 2011;69:875–82.
29. Huang HS, Burns AJ, Nonneman RJ, Baker LK, Riddick NV, Nikolova VD, Riday TT, Yashiro K, Philpot BD, Moy SS. Behavioral deficits in an Angelman syndrome model: effects of genetic background and age. Behav Brain Res. 2013;243:79–90.
30. Gandhi T, Lee CC. Neural mechanisms underlying repetitive behaviors in rodent models of autism spectrum disorders. Front Cell Neurosci. 2021. https://doi.org/10.3389/fncel.2020.592710.
31. Sheng M, Kim E. The Shank family of scaffold proteins. J Cell Sci. 2000;113(Pt 11):1851–6.
32. Sheng M, Kim E. The postsynaptic organization of synapses. Cold Spring Harb Perspect Biol. 2011;3: a005678.
33. Abrahams BS, Arking DE, Campbell DB, Mefford HC, Morrow EM, Weiss LA, Menashe I, Wadkins T, Banerjee-Basu S, Packer A. SFARI gene 2.0: a community-driven knowledgebase for the autism spectrum disorders (ASDs). Mol Autism. 2013;4:36.
34. Berkel S, Marshall CR, Weiss B, Howe J, Roeth R, Moog U, Endris V, Roberts W, Szatmari P, Pinto D, et al. Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. Nat Genet. 2010;42:489–91.
35. Pinto D, Pagnamenta AT, Klei L, Anney R, Merico D, Regan R, Conroy J, Magalhaes TR, Correia C, Abrahams BS, et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature. 2010;466:368–72.
36. Wischmeijer A, Magini P, Giorda R, Gnoli M, Ciccone R, Cecconi I, Franzoni E, Mazzanti L, Romeo G, Zuffardi O. Olfactory receptor-related duplicons mediate a microdeletion at 11q13. 2q13. 4 associated with a syndromic phenotype. Molecular syndromology. 2010;1:176–84.
37. Berkel S, Tang W, Trevino M, Vogt M, Obenhaus HA, Gass P, Scherer SW, Sprengel R, Schratt G, Rappold GA. Inherited and de novo SHANK2 variants associated with autism spectrum disorder impair neuronal morphogenesis and physiology. Hum Mol Genet. 2012;21:344–57.
38. Leblond CS, Heinrich J, Delorme R, Proepper C, Betancur C, Huguet G, Konyukh M, Chaste P, Ey E, Rastam M, et al. Genetic and functional analyses of SHANK2 mutations suggest a multiple hit model of autism spectrum disorders. PLoS Genet. 2012;8: e1002521.
39. Prasad A, Merico D, Thiruvahindrapuram B, Wei J, Lionel AC, Sato D, Rickaby J, Lu C, Szatmari P, Roberts W, et al: A discovery resource of rare copy number variations in individuals with autism spectrum disorder. G3 (Bethesda) 2012, 2(12):1665–1685.
40. Rauch A, Wieczorek D, Graf E, Wieland T, Endele S, Schwarzmayr T, Albrecht B, Bartholdi D, Beygo J, Di Donato N, et al. Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. Lancet. 2012;380:1674–82.
41. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, Ercan-Sencicek AG, DiLullo NM, Parikshak NN, Stein JL, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature. 2012;485:237–41.
42. Chilian B, Abdollahpour H, Bierhals T, Haltrich I, Fekete G, Nagel I, Rosenberger G, Kutsche K. Dysfunction of SHANK2 and CHRNA7 in a patient with intellectual disability and language impairment supports genetic epistasis of the two loci. Clin Genet. 2013;84:560–5.
43. Liu YY, Du YS, Liu WW, Yang CH, Liu Y, Wang HY, Gong XH. Lack of association between NLGN3, NLGN4, SHANK2 and SHANK3 gene variants and autism spectrum disorder in a Chinese population. PLoS ONE. 2013;8: e56639.
44. Schluth-Bolard C, Labalme A, Cordier M-P, Till M, Nadeau G, Tevissen H, Lesca G, Boutry-Kryza N, Rossignol S, Rocas D. Breakpoint mapping by next generation sequencing reveals causative gene disruption in patients carrying apparently balanced chromosome rearrangements with intellectual deficiency and/or congenital malformations. J Med Genet. 2013;50:144–50.
45. Guilmatre A, Huguet G, Delorme R, Bourgeron T. The emerging role of SHANK genes in neuropsychiatric disorders. Dev Neurobiol. 2014;74:113–22.
46. Leblond CS, Nava C, Polge A, Gauthier J, Huguet G, Lumbroso S, Giuliano F, Stordeur C, Depienne C, Mouzat K, et al. Meta-analysis of SHANK mutations in autism spectrum disorders: a gradient of severity in cognitive impairments. PLoS Genet. 2014;10: e1004580.
47. Costas J. The role of SHANK2 rare variants in schizophrenia susceptibility. Mol Psychiatry. 2015;20:1486.
48. Peykov S, Berkel S, Degenhardt F, Rietschel M, Nöthen M, Rappold G. Rare SHANK2 variants in schizophrenia. Mol Psychiatry. 2015;20:1487–8.
49. Peykov S, Berkel S, Schoen M, Weiss K, Degenhardt F, Strohmaier J, Weiss B, Proepper C, Schratt G, Nothen MM, et al. Identification and functional characterization of rare SHANK2 variants in schizophrenia. Mol Psychiatry. 2015;20:1489–98.
50. Homann OR, Misura K, Lamas E, Sandrock RW, Nelson P, McDonough SI, DeLisi LE. Whole-genome sequencing in multiplex families with psychoses reveals mutations in the SHANK2 and SMARCA1 genes segregating with illness. Mol Psychiatry. 2016;21:1690–5.
51. Yuen RKC, Merico D, Bookman M, Howe JL, Thiruvahindrapuram B, Patel RV, Whitney J, Deflaux N, Bingham J, Wang ZZ, et al. Whole genome sequencing resource identifies 18 new candidate genes for autism spectrum disorder. Nat Neurosci. 2017;20:602.
52. Bai Y, Qiu S, Li Y, Li Y, Zhong WJ, Shi MJ, Zhu XJ, Jiang HY, Yu YQ, Cheng Y, Liu YW. Genetic association between SHANK2 polymorphisms and susceptibility to autism spectrum disorder. IUBMB Life. 2018;70:763–76.
53. Lu ZA, Mu W, Osborne LM, Cordner ZA: Eighteen-year-old man with autism, obsessive compulsive disorder and a SHANK2 variant presents with severe anorexia that responds to high-dose fluoxetine. Case Reports 2018, 2018**:**bcr-2018–225119.
54. Mossa A, Giona F, Pagano J, Sala C, Verpelli C. SHANK genes in autism: defining therapeutic targets. Prog Neuropsychopharmacol Biol Psychiatry. 2018;84:416–23.
55. Aspromonte MC, Bellini M, Gasparini A, Carraro M, Bettella E, Polli R, Cesca F, Bigoni S, Boni S, Carlet O, et al. Characterization of intellectual disability and autism comorbidity through gene panel sequencing. Hum Mutat. 2019;40:1346–63.
56. Zhou WZ, Zhang J, Li Z, Lin X, Li J, Wang S, Yang C, Wu Q, Ye AY, Wang M, et al. Targeted resequencing of 358 candidate genes for autism spectrum disorder in a Chinese cohort reveals diagnostic potential and genotype-phenotype correlations. Hum Mutat. 2019;40:801–15.
57. Satterstrom FK, Kosmicki JA, Wang JB, Breen MS, De Rubeis S, An JY, Peng MS, Collins R, Grove J, Klei L, et al. Large-scale exome sequencing study implicates both developmental and functional changes in the neurobiology of autism. Cell. 2020;180:568.
58. Wang T, Hoekzema K, Vecchio D, Wu H, Sulovari A, Coe BP, Gillentine MA, Wilfert AB, Perez-Jurado LA, Kvarnung M. Large-scale targeted sequencing identifies risk genes for neurodevelopmental disorders. Nat Commun. 2020;11:1–13.
59. Schmeisser MJ, Ey E, Wegener S, Bockmann J, Stempel AV, Kuebler A, Janssen AL, Udvardi PT, Shiban E, Spilker C, et al. Autistic-like behaviours and hyperactivity in mice lacking ProSAP1/Shank2. Nature. 2012;486:256–60.
60. Won H, Lee HR, Gee HY, Mah W, Kim JI, Lee J, Ha S, Chung C, Jung ES, Cho YS, et al. Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. Nature. 2012;486:261–5.
61. Ha S, Lee D, Cho YS, Chung C, Yoo Y-E, Kim J, Lee J, Kim W, Kim H, Bae YC. Cerebellar Shank2 regulates excitatory synapse density, motor coordination, and specific repetitive and anxiety-like behaviors. J Neurosci. 2016;36:12129–43.
62. Peter S, Ten Brinke MM, Stedehouder J, Reinelt CM, Wu B, Zhou H, Zhou K, Boele HJ, Kushner SA, Lee MG, et al. Dysfunctional cerebellar Purkinje cells contribute to autism-like behaviour in Shank2-deficient mice. Nat Commun. 2016;7:12627.
63. Lim CS, Kim H, Yu NK, Kang SJ, Kim T, Ko HG, Lee J, Yang JE, Ryu HH, Park T, et al. Enhancing inhibitory synaptic function reverses spatial memory deficits in Shank2 mutant mice. Neuropharmacology. 2017;112:104–12.
64. Pappas AL, Bey AL, Wang XM, Rossi M, Kim YH, Yan HD, Porkka F, Duffney LJ, Phillips SM, Cao XY, et al. Deficiency of Shank2 causes

mania-like behavior that responds to mood stabilizers. Jci Insight. 2017. https://doi.org/10.1172/jci.insight.92052.
65. Eltokhi A, Rappold G, Sprengel R. Distinct phenotypes of Shank2 mouse models reflect neuropsychiatric spectrum disorders of human patients With SHANK2 variants. Front Mol Neurosci. 2018;11:240.
66. Kim R, Kim J, Chung C, Ha S, Lee S, Lee E, Yoo YE, Kim W, Shin W, Kim E. Cell-type-specific Shank2 deletion in mice leads to differential synaptic and behavioral phenotypes. J Neurosci. 2018;38:4076–92.
67. Chung C, Ha S, Kang H, Lee J, Urn SM, Yan H, Yoo YE, Yoo T, Jung H, Lee D, et al. Early correction of N-Methyl-D-aspartate receptor function improves autistic-like social behaviors in adult Shank2(-/-) mice. Biol Psychiat. 2019;85:534–43.
68. Lee E, Lee S, Shin JJ, Choi W, Chung C, Lee S, Kim J, Ha S, Kim R, Yoo T, et al. Excitatory synapses and gap junctions cooperate to improve Pv neuronal burst firing and cortical social cognition in Shank2-mutant mice. Nat Commun. 2021;12:5116.
69. Eltokhi A, Gonzalez-Lozano MA, Oettl L-L, Rozov A, Pitzer C, Röth R, Berkel S, Hüser M, Harten A, Kelsch W, et al. Imbalanced post- and extrasynaptic SHANK2A functions during development affect social behavior in SHANK2-mediated neuropsychiatric disorders. Mol Psychiatry. 2021;26:6482–504.
70. Grabrucker S, Pagano J, Schweizer J, Urrutia-Ruiz C, Schön M, Thome K, Ehret G, Grabrucker AM, Zhang R, Hengerer B, et al. Activation of the medial preoptic area (MPOA) ameliorates loss of maternal behavior in a Shank2 mouse model for autism. EMBO J. 2021;40: e104267.
71. Lee EJ, Lee H, Huang TN, Chung C, Shin W, Kim K, Koh JY, Hsueh YP, Kim E. Trans-synaptic zinc mobilization improves social interaction in two mouse models of autism through NMDAR activation. Nat Commun. 2015;6:7168.
72. Ragozzino ME. The contribution of the medial prefrontal cortex, orbitofrontal cortex, and dorsomedial striatum to behavioral flexibility. Ann N Y Acad Sci. 2007;1121:355–75.
73. Amodeo L, McMurray M, Roitman J. Orbitofrontal cortex reflects changes in response–outcome contingencies during probabilistic reversal learning. Neuroscience. 2017;345:27–37.
74. Jeong H, Kim D, Song M, Paik SB, Jung MW. Distinct roles of parvalbumin- and somatostatin-expressing neurons in flexible representation of task variables in the prefrontal cortex. Prog Neurobiol. 2020;187: 101773.
75. Boeckers TM, Kreutz MR, Winter C, Zuschratter W, Smalla KH, Sanmarti-Vila L, Wex H, Langnaese K, Bockmann J, Garner CC, Gundelfinger ED. Proline-rich synapse-associated protein-1/cortactin binding protein 1 (ProSAP1/CortBP1) is a PDZ-domain protein highly enriched in the postsynaptic density. J Neurosci. 1999;19:6506–18.
76. Werling DM, Geschwind DH. Sex differences in autism spectrum disorders. Curr Opin Neurol. 2013;26:146–53.
77. Jung H, Park H, Choi Y, Kang H, Lee E, Kweon H, Roh JD, Ellegood J, Choi W, Kang J, et al. Sexually dimorphic behavior, neuronal activity, and gene expression in Chd8-mutant mice. Nat Neurosci. 2018;21:1218–28.
78. Wong H, Hooper AWM, Niibori Y, Lee SJ, Hategan LA, Zhang L, Karumuthil-Melethil S, Till SM, Kind PC, Danos O, et al. Sexually dimorphic patterns in electroencephalography power spectrum and autism-related behaviors in a rat model of fragile X syndrome. Neurobiol Dis. 2020;146: 105118.
79. Matas E, Maisterrena A, Thabault M, Balado E, Francheteau M, Balbous A, Galvan L, Jaber M. Major motor and gait deficits with sexual dimorphism in a Shank3 mutant mouse model. Molecular Autism. 2021;12:2.
80. Bruce MR, Jones KL, Vernon AC, Silverman JL, Crawley JN, Ellegood J, Lerch JP, Van de Water J. Sexually dimorphic neuroanatomical differences relate to ASD-relevant behavioral outcomes in a maternal autoantibody mouse model. Mol Psychiatry. 2021;26:7530–7.
81. Ferri SL, Abel T, Brodkin ES. Sex differences in autism spectrum disorder: a review. Curr Psychiatry Rep. 2018;20:9.
82. Jeon SJ, Gonzales EL, Mabunga DFN, Valencia ST, Kim DG, Kim Y, Adil KJL, Shin D, Park D, Shin CY. Sex-specific behavioral features of rodent models of autism spectrum disorder. Experiment Neurobiol. 2018;27:321–43.
83. Chung C, Shin W, Kim E. Early and late corrections in mouse models of autism spectrum disorder. Biol Psychiatry. 2021. https://doi.org/10.1016/j.biopsych.2021.07.021.
84. Guo ZV, Hires SA, Li N, O'Connor DH, Komiyama T, Ophir E, Huber D, Bonardi C, Morandell K, Gutnisky D, et al. Procedures for behavioral experiments in head-fixed mice. PLoS ONE. 2014;9: e88678.
85. Heiney SA, Wohl MP, Chettih SN, Ruffolo LI, Medina JF. Cerebellar-dependent expression of motor learning during eyeblink conditioning in head-fixed mice. J Neurosci. 2014;34:14845–53.
86. Mount RA, Sridhar S, Hansen KR, Mohammed AI, Abdulkerim M, Kessel R, Nazer B, Gritton HJ, Han X. Distinct neuronal populations contribute to trace conditioning and extinction learning in the hippocampal CA1. Elife. 2021. https://doi.org/10.7554/eLife.56491.
87. McEvoy RE, Rogers SJ, Pennington BF. Executive function and social communication deficits in young autistic children. J Child Psychol Psychiatry. 1993;34:563–78.
88. Griffith EM, Pennington BF, Wehner EA, Rogers SJ. Executive functions in young children with autism. Child Dev. 1999;70:817–32.
89. Coldren JT, Halloran C. Spatial reversal as a measure of executive functioning in children with autism. J Genet Psychol. 2003;164:29–41.
90. Dichter GS, Radonovich KJ, Turner-Brown LM, Lam KS, Holtzclaw TN, Bodfish JW. Performance of children with autism spectrum disorders on the dimension-change card sort task. J Autism Dev Disord. 2010;40:448–56.
91. Yerys BE, Antezana L, Weinblatt R, Jankowski KF, Strang J, Vaidya CJ, Schultz RT, Gaillard WD, Kenworthy L. Neural correlates of set-shifting in children with autism. Autism Res. 2015;8:386–97.
92. Crawley D, Zhang L, Jones EJH, Ahmad J, Oakley B, San José Cáceres A, Charman T, Buitelaar JK, Murphy DGM, Chatham C, et al: Modeling flexible behavior in childhood to adulthood shows age-dependent learning mechanisms and less optimal learning in autism in each age group. PLOS Biology 2020; https://doi.org/10.1371/journal.pbio.3000908.
93. Vasa RA, Carroll LM, Nozzolillo AA, Mahajan R, Mazurek MO, Bennett AE, Wink LK, Bernal MP. A systematic review of treatments for anxiety in youth with autism spectrum disorders. J Autism Dev Disord. 2014;44:3215–29.
94. Kazdoba TM, Leach PT, Yang M, Silverman JL, Solomon M, Crawley JN. Translational mouse models of autism: advancing toward pharmacological therapeutics. Trans Neuropsychopharmacol. 2015. https://doi.org/10.1007/7854_2015_5003.
95. Chen Q, Deister CA, Gao X, Guo B, Lynn-Jones T, Chen N, Wells MF, Liu R, Goard MJ, Dimidschstein J, et al. Dysfunction of cortical GABAergic neurons leads to sensory hyper-reactivity in a Shank3 mouse model of ASD. Nat Neurosci. 2020;23:520–32.
96. Quartermain D, Mower J, Rafferty MF, Herting RL, Lanthorn TH. Acute but not chronic activation of the NMDA-coupled glycine receptor with D-cycloserine facilitates learning and retention. Eur J Pharmacol. 1994;257:7–12.
97. Nong Y, Huang Y-Q, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW. Glycine binding primes NMDA receptor internalization. Nature. 2003;422:302–7.
98. Parnas AS, Weber M, Richardson R. Effects of multiple exposures to D-cycloserine on extinction of conditioned fear in rats. Neurobiol Learn Mem. 2005;83:224–31.
99. Goff DC, Cather C, Gottlieb JD, Evins AE, Walsh J, Raeke L, Otto MW, Schoenfeld D, Green MF. Once-weekly d-cycloserine effects on negative symptoms and cognition in schizophrenia: An exploratory study. Schizophr Res. 2008;106:320–7.
100. Conzelman GM Jr, Jones RK. On the physiologic disposition of cycloserine in experimental animals. Am Rev Tuberc. 1956;74:802–6.
101. LeVine SM, Tsau S. Substrate reduction therapy for krabbe disease: exploring the repurposing of the antibiotic D-Cycloserine. Front Pediatr. 2022 Jan 18;9:807973. https://doi.org/10.3389/fped.2021.80797.
102. Marco EJ, Hinkley LB, Hill SS, Nagarajan SS. Sensory processing in autism: a review of neurophysiologic findings. Pediatr Res. 2011;69:48–54.
103. Balasco L, Provenzano G, Bozzi Y. Sensory abnormalities in autism spectrum disorders: a focus on the tactile domain, from genetic mouse models to the clinic. Front Psychiatry. 2019;10:1016.
104. Orefice LL, Zimmerman AL, Chirila AM, Sleboda SJ, Head JP, Ginty DD. Peripheral mechanosensory neuron dysfunction underlies tactile and behavioral deficits in mouse models of ASDs. Cell. 2016;166:299–313.

105. Konorski J. Integrative activity of the brain. Chicago: University of Chicago Press; 1967.
106. Rescorla RA, Solomon RL. Two-process learning theory: relationships between pavlovian conditioning and instrumental learning. Psychol Rev. 1967;74:151–82.
107. Lee T, Kim JJ. Differential effects of cerebellar, amygdalar, and hippocampal lesions on classical eyeblink conditioning in rats. J Neurosci. 2004;24:3242–50.
108. Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, Kwiatkowski DJ, Ramesh V, Silva AJ. Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nat Med. 2008;14:843–8.
109. Horner AE, Norris RH, McLaren-Jones R, Alexander L, Komiyama NH, Grant SGN, Nithianantharajah J, Kopanitsa MV. Learning and reaction times in mouse touchscreen tests are differentially impacted by mutations in genes encoding postsynaptic interacting proteins SYNGAP1, NLGN3, DLGAP1, DLGAP2 and SHANK2. Genes Brain Behav. 2021;20: e12723.
110. Hirsh JB, Mar RA, Peterson JB. Psychological entropy: a framework for understanding uncertainty-related anxiety. Psychol Rev. 2012;119:304–20.
111. Grupe DW, Nitschke JB. Uncertainty and anticipation in anxiety: an integrated neurobiological and psychological perspective. Nat Rev Neurosci. 2013;14:488–501.
112. Anderson WG, Arnold RM, Angus DC, Bryce CL. Passive decision-making preference is associated with anxiety and depression in relatives of patients in the intensive care unit. J Crit Care. 2009;24:249–54.
113. Yoo YE, Lee S, Kim W, Kim H, Chung C, Ha S, Park J, Chung Y, Kang H, Kim E. Early chronic memantine treatment-induced transcriptomic changes in wild-type and Shank2-mutant mice. Front Mol Neurosci. 2021;14: 712576.
114. Thanellou A, Schachinger KM, Green JT. Shortened conditioned eyeblink response latency in male but not female Wistar-Kyoto hyperactive rats. Behav Neurosci. 2009;123:650–64.
115. Löwgren K, Bååth R, Rasmussen A, Boele HJ, Koekkoek SKE, De Zeeuw CI, Hesslow G. Performance in eyeblink conditioning is age and sex dependent. PLoS ONE. 2017;12: e0177849.
116. Rapp AP, Weiss C, Oh MM, Disterhoft JF: Intact female mice acquire trace eyeblink conditioning faster than male and ovariectomized female mice. eNeuro 2021; 8.
117. Werling DM, Parikshak NN, Geschwind DH. Gene expression in human brain implicates sexually dimorphic pathways in autism spectrum disorders. Nat Commun. 2016;7:10717.

Publisher's Note