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Deletion of forebrain glycine transporter 1 enhances conditioned freezing to a reliable, but not an ambiguous, cue for threat in a conditioned freezing paradigm

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Abstract

Enhanced expression of Pavlovian aversive conditioning but not appetitive conditioning may indicate a bias in the processing of threatening or fearful events. Mice with disruption of glycine transporter 1 in forebrain neurons exhibit such a bias, but they are at the same time highly sensitive to manipulations that hinder the development of the conditioned response (CR) – suggesting that the mutation may modify higher cognitive processes that extract predictive information between environmental cues. Here, we further investigated the development of fear conditioning in forebrain neuronal GlyT1 knockout mice when the predictiveness of a tone stimulus for foot shock was rendered ambiguous by interspersing [tone → no shock] trials in-between [tone → shock] trials during acquisition. The CR to the ambiguous tone CS (conditioned stimulus) was compared with that generated by an unambiguous CS that was always followed by the shock US (unconditioned stimulus) during acquisition. We showed that rendering the CS ambiguous as described significantly attenuated the CR in the mutants, but it was not sufficient to modify the CR in the control mice. It is concluded that disruption of GlyT1 in forebrain neurons does not increase the risk of forming spurious and potentially maladaptive fear associations.

Keywords

Fear; Glutamate; Glycine reuptake; Learning; NMDA receptor; Pavlovian conditioning

1. INTRODUCTION

Glycine transporter 1 (GlyT1) controls the extra-cellular levels of glycine in the brain through the active re-uptake of glycine into neurons and astrocytes [1,2]. In the

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microenvironment of synapses containing *N*-methyl-D-aspartate (NMDA) receptors, GlyT1 keeps the allosteric glycine-binding site (glycine-B site) located on the NR1 subunits of the NMDA receptor below saturation [3,4]. Since glycine-B site occupancy is necessary for the activation of NMDA receptor channel in response to the binding of L-glutamate to the NR2 subunits of the receptor [5,6], blockade of glycine re-uptake via GlyT1 can effectively boost the glutamate signals via NMDA receptors at excitatory synapses [4,7–9]. Given that the activation of NMDA receptors is linked to long-term changes in synaptic efficacy that underlie at least some forms of learning and memory [10–12], GlyT1 inhibition has been investigated as a potential remedy for cognitive deficiency, for instance in schizophrenia [13]. This strategy may avoid some of the excitotoxic effects associated with direct NMDA receptor agonists [14].

In mice, it has been shown that the selective disruption of GlyT1 in forebrain neurons is sufficient to potentiate the activity of the NMDA receptor and enhance Pavlovian learning [15], whereby the animals acquire a new response (conditioned response, CR) to an initially neutral stimulus following the pairing of that stimulus (as a conditioned stimulus, CS) with a significant event (an unconditioned stimulus, US). However, the enhancement of Pavlovian associative learning appears to be specific to aversive conditioning in which the US is an aversive stimulus (e.g., an electric foot shock), but not when the US is an appetitive/rewarding stimulus (e.g., a highly palatable food) [15, 16]. The impact of forebrain GlyT1 deletion on Pavlovian conditioning does not only depend on the valence of the US, but also seems to be critically determined by the reliability or accuracy of the CS as a predictor of the US. Selective disruption of forebrain neuronal GlyT1 also increases the sensitivity to the non-reinforced pre-exposure to the CS prior to conditioning [i.e., latent inhibition; 15] and the separation of CS and US in time during conditioning [i.e., temporal discontinuity; 17]. These findings clearly indicate that the modification of associative learning by GlyT1 deletion is far from simple. Instead, GlyT1 disruption may influence higher cognitive processes that govern the extraction of predictive information from incidental cues in the environment.

Here, we further explore this possibility by evaluating the conditioned freezing response to a tone CS that was rendered ambiguous by interspersing [tone → no foot-shock] trials in-between [tone → foot-shock] trials during conditioning. The procedure was titrated to generate minimal impact in control mice so as to maximize our ability to test the prediction that mice with forebrain neuronal GlyT1 disruption might be more sensitive to a reduction in the *prospective* conditional probability of receiving a foot-shock following a tone, $P(\text{shock}|\text{tone})$, to 0.5, while the *retrospective* conditional probability $P(\text{tone}|\text{shock})$ was maintained at 1. This was to be compared with the standard conditioning procedure [15,17] whereby both conditional probabilities equal 1 because acquisition solely consisted of [tone → foot-shock] trials. We also separately examined contextual conditioning with the same shock US in the absence of any discrete CS, because the situation may also be interpreted as ambiguous, since, between shock deliveries, the context might also be perceived as [context → no foot-shock]. This further allowed us to distinguish between conditioning to foreground versus background contextual cues, in the absence and presence of discrete CS's, respectively, given that this distinction is neurobiologically meaningful [18].

2. METHODS

2.1 Subjects

A homozygous Glyt1tm1.2fl/fl colony was established and maintained on a pure C57BL/6 background as described before [19]. Forebrain neuron specific deletion of GlyT1 was achieved by CaMKII α Cre-mediated recombination [see 15]. Appropriate heterozygous Cre mice were mated with Glyt1tm1.2fl/fl mice to generate the desired mutant (GlyT1^{fl/fl};CaMKII α ^{+/-}) and control (GlyT1^{fl/fl}) littermates. Animals of both sexes were employed in the present study. The mice were weaned at 21 days old, and littermates of the same sex were kept in groups of four to six in Makrolon Type-III cages (Techniplast, Milan, Italy). The subjects were housed in a temperature- and humidity-controlled (at 22°C and 55% R.H.) animal vivarium under a reversed light-dark cycle with lights off from 0700–1900hrs. Testing was always conducted in the dark phase of the diurnal cycle. The animals were maintained under *ad libitum* water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) throughout the study. All experimental procedures described had previously been approved by the Zurich Veterinary Office; they also conformed to the ethical standards stipulated by the Swiss Act and Ordinance on Animal Protection and were in accordance to the European Council Directive 86/609/EEC.

2.2 Apparatus

The apparatus consisted of two sets of four conditioning chambers. The two sets were distinct from each other, and were installed in separate testing rooms, providing two distinct contexts as fully described before [15]. The first set of chambers (context ‘A’) comprised four Coulbourn Instruments (Allentown, PA, USA) operant chambers (Model E10-10), each equipped with a grid floor made of stainless steel rods (4 mm in diameter) spaced at an interval of 10 mm centre to centre, and through which scrambled electric shocks (the US, set at 0.3 mA) could be delivered (model E13–14; Coulbourn Instruments). A transparent Plexiglas enclosure confined the animals to a rectangular region (17.5 × 13 cm). The inside of the chambers was illuminated by a house light (2.8 W) positioned on the panel wall, 21 cm above the grid floor. The second set of chambers (context ‘B’) comprised four cylindrical (19 cm in diameter) enclosures made of clear Plexiglas resting on a metal mesh floor. Illumination inside the chamber was provided by an infrared light source instead of visible light. The CS was an 86-dBA tone provided by a sonalert (model SC628; Mallory, Indianapolis, IN, USA). Each of the eight chambers contained a miniature digital camera mounted 30 cm directly above the centre of the area of interest. The algorithm of the freezing response detection procedure has been validated and fully described before [20].

2.3 Experiment 1

Mutant and control mice were randomly allocated into one of two subgroups (*ambiguous* vs. *standard* training procedure) with the following group sizes: Control/standard, n=17; Control/ambiguous, n=18; Mutant/ standard, n=17; and Mutant/ambiguous, n=18. In the ‘standard’ training procedure, the shock US always followed immediately the cessation of the CS, and three such CS-US pairings were administered. In the ‘ambiguous’ procedure, three additional CS-only presentations were intermixed with the three CS-US trials. On the day of conditioning (Day 1), three discrete trials of CS-US pairings were administered at 3,

6.3 and 10 min into the session that lasted for a total of 13 min 33 s. In each such trial, the CS and US were serially arranged with the termination of the 30-s CS coinciding with the onset of the 1-s US. Animals in the ambiguous procedure received in addition three CS-alone trials at 4.3, 9 and 11.3 min into the session. On the next day (Day 2, context test), the animals were returned to the training context *A* for a period of 8 min in the absence of any discrete stimuli to assess conditioning to the background contextual cues. On Day 3 (CS test), conditioned freezing to the tone-CS was evaluated in the neutral context *B* when the CS was presented for 8 min following an initial acclimatization period of 2 min without CS.

2.4 Experiments 2

The procedures have been fully described before [21]. In this experiment (controls $n=12$, mutants $n=12$), three foot-shock US (0.3 mA for 1s) were delivered on the conditioning day. Each shock delivery was preceded and followed by a 3-min inter-shock interval (ISI). Following conditioning in context *A* on day 1, conditioned freezing was assessed by re-exposing the animals to context *A* or *B* on alternate days in the order of *A-B-A-B* across the next four days. Comparison between the two contexts allowed us to gauge whether the observed freezing response was specific to the shocked context *A*. Each test of context freezing lasted for 4 min.

2.5 Statistical analysis

All data were analysed by parametric analysis of variance (ANOVA) with the between-subject factors Genotype (mutant vs. littermate control) and Training procedure (standard vs. ambiguous) in Experiment 1. Preliminary analyses also included the between-subject factor Sex, which was dropped from the final analysis to increase statistical power given that there was no evidence for any significant effect involving sex. Appropriate within-subject factors (time bins, trials, etc.) were included according to the nature of the considered dependent variables. To assist interpretation of the statistical outcomes, significant effects were further investigated by pair-wise comparisons based on the associated error terms taken from the overall ANOVA. All statistical analyses were carried out using SPSS for Windows (version 19, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows 7 operating system. A two-tailed criterion of $p < 0.05$ was taken as the yardstick for statistical significance.

3. RESULTS

3.1 Experiment

1 3.1.1 CS-freezing (Day 1)—Freezing levels during CS presentations in the ambiguous and standard training procedure were separately analysed because the latter involved only three discrete CS-US trials whereas the former procedure involved additional three CS-only trials. Separate genotype $\times$ CS-presentations ANOVAs indicated that freezing generally increased across successive CS presentations regardless of training procedure and genotype [standard procedure: $F_{(2,64)}=32.59$, $p<0.01$; ambiguous procedure: $F_{(5,170)}=15.17$, $p<0.01$] (Figure 1A & 1B).

As expected, the progressive increase of freezing recorded during the successive presentations of the CS was substantially weaker in mice under the ambiguous training procedure. This impression was confirmed by an additional ANOVA comparing directly the two training procedures by focusing on the three reinforced CS-presentations that were common to both training procedures. This $2 \times 2 \times 3$ (genotype $\times$ training procedure $\times$ CS-presentations) ANOVA yielded a significant effect of training procedure [$F_{(1,66)}=11.98$, $p<0.01$], CS-presentations [$F_{(2,132)}=48.09$, $p<0.01$] and their interaction [$F_{(2,132)}=12.49$, $p<0.01$]. Post hoc pair-wise comparison indicated that the contrast between the ambiguous and the standard training procedure achieved statistical significance in the third reinforced CS-presentation [$t_{(132)}=6.56$, $p<0.01$].

3.1.2 Context background conditioning (Day 2)—Expression of conditioned fear developed towards the training context was evaluated 24 h after conditioning by re-exposing the animals to the context. Contextual freezing was generally weak and comparable among the four groups of mice (Figure 2). Irrespective of genotype and training procedure, freezing levels gradually increased across time and peaked after 6 min before decreasing in the last 2 min of the test. This pattern was consistent with a $2 \times 2 \times 4$ (genotype $\times$ training procedure $\times$ 2-min bins) ANOVA of percent time freezing across successive 2-min bins during the 8-min observation period which revealed only a significant main effect of bins [$F_{(3,198)}=5.10$, $p<0.01$].

3.1.3 Pre-CS freezing (Day 3)—Expression of conditioned freezing to the tone CS was assessed on the next day in a neutral context, consisting of an initial 2-min pre-CS period followed by a period of 8-min CS presentation. Freezing was low and highly comparable across the four groups in the pre-CS period (Figure 3A). A 2-way (genotype $\times$ training procedure) ANOVA of percent freezing recorded during the 2-min Pre-CS period did not yield any significant effect.

3.1.4 CS freezing (Day 3)—Onset of the CS led to a clear rise in freezing. The increase was substantially weaker in mice trained in the ambiguous procedure than those trained in the standard procedure (Figures 3A). However, this effect was only visible in the mutants but not the control mice (Figures 3B). The mutants froze more strongly than controls in the standard procedure, whilst they exhibited marginally weaker CS-freezing than controls in the ambiguous procedure (Figure 3B). These effects of the mutation contributed to the emergence of a significant genotype by training procedure interaction [$F_{(1,66)}=5.07$, $p<0.05$] in a $2 \times 2 \times 4$ (genotype $\times$ training procedure $\times$ 2-min bins) ANOVA of percent time freezing across successive 2-min bins during the 8-min CS period. Post-hoc pairwise comparisons of the average freezing levels across the 8-min test indicated a significant effect of training procedure in the mutants ($p=0.01$) but not the controls, and that this contrast was associated with a significant difference between genotypes in the standard procedure ($p=0.04$). The ANOVA also yielded a significant effect of bins [$F_{(3,198)}=92.17$, $p<0.01$], as well as the 3-way interaction [$F_{(3,198)}=3.32$, $p<0.05$]. Post hoc pair-wise comparisons based on the error term associated with the 3 way ANOVA, were then performed to assess genotypic difference within either the standard or the ambiguous procedure at each successive bin. These indicated an increase in conditioned freezing in the mutants relative to

the controls in the standard procedure over the first two bins [$p < 0.05$]. Similarly, post hoc pair-wise comparisons of the training procedure for each genotype at each successive bin revealed an increase in conditioned freezing in the standard training procedure compared to the ambiguous procedure in the mutants over the first two bins [$p < 0.05$].

3.2 Experiment 2

On the day of conditioning, freezing increased at each inter-shock interval (Figure 4A) without any apparent difference between genotypes. A 2×4 (genotype $\times$ ISI's) ANOVA of freezing across the four successive ISI's revealed only a significant effect of ISI's [$F_{(3,66)} = 43.63$, $p < 0.01$]. Across the next four test days, freezing was strong in the training context 'A' but remained low in the neutral context 'B' (Figure 4B), demonstrating the context-specificity of the conditioned freezing response. A $2 \times 2 \times 2$ (genotype $\times$ contexts $\times$ days) ANOVA supported this conclusion with a significant effect of contexts [$F_{(1,22)} = 47.47$, $p < 0.001$]. Freezing on the second exposure to the training context was visibly lower than the first, reflecting extinction of this conditioned response. A 2×2 (genotype $\times$ days) ANOVA restricted to context 'A' yielded a significant effect of days [$F_{(1,22)} = 7.25$, $p < 0.05$].

4. DISCUSSION

The present study extended our thesis that the modulation of aversive Pavlovian conditioning, and in particular conditioned freezing, by deletion of forebrain neuronal GlyT1 critically depends on the informativeness of the CS as a predictor of the US. The results again show that the GlyT1 deletion did not indiscriminately strengthen the expression of the conditioned freezing response. A phenotype of increased conditioned freezing was only detected when acquisition was performed in the absence of ambiguous information that may reduce the predictive power/reliability of the CS for the eminent occurrence of the US. The introduction of CS-alone presentations during conditioning had reduced the *prospective* conditional probability ($P(\text{US}|\text{CS})$, i.e. the likelihood of being shocked given that a CS has occurred) to 0.5. This manipulation weakened the observed level of CS freezing in both mutants and controls, to a similar extent, by the end of conditioning in comparison to their counterparts that had experienced [CS $\rightarrow$ US] trials only (Figure 1). However, the behavioural impact of this manipulation in the retention test of CS freezing 48 h after acquisition was only detectable in the mutant mice (Figure 3B). Control littermates trained under the two different conditioning protocols did not differ significantly in the retention test of CS freezing.

It is tempting to compare this novel phenotype observed in our mutant mice here with their enhanced sensitivity to the CS pre-exposure effect – commonly known as latent inhibition (LI) [22]. Presentations of the to-be-conditioned CS alone prior to CS-US conditioning may retard the subsequent expression of the CR to the CS. Mice lacking forebrain neuronal GlyT1 readily exhibited LI when the number of CS pre-exposures was insufficient to generate robust LI in control littermates [15]. Thus, experience suggesting that a potential CS may not be a reliable predictor of the future occurrence of the US seems to be more strongly registered in the mutants as indicated in the CS test (Figure 3). A similar outcome was observed when the temporal contiguity of the CS and US was manipulated.

Interspersing the CS and US with a temporal gap (known as “trace interval”) also had a stronger negative impact on the magnitude of the CR in the mutants compared to controls [17]. These phenotypes (on LI and trace conditioning) as well as the one demonstrated here are all associated with an increase of CS-freezing in the standard $[CS \rightarrow US] \times 3$ procedure – this control procedure was employed in all these experiments. In all cases, the strengthening of CS-freezing induced by the GlyT1 deletion was specific to the standard $[CS \rightarrow S] \times 3$ protocol, with the expression of CS-freezing significantly weakened by the experimental protocol: CS-pre-exposure [15], trace conditioning [17] or the introduction of CS-only trials during conditioning (the present experiment). Sensitivity to this range of known manipulations on conditioning may indicate that the deletion of GlyT1 modifies the extraction of information (predictive of future significant events) from potential CSs in the environments.

The apparent lack of an effect on context foreground conditioning here (Figure 4) therefore may not be surprising. Unlike a discrete phasic CS, the static nature of contextual cues could not precisely predict the occurrence of the US in time. In this regard, the contextual cues are ambiguous predictors, resembling the CS in the ambiguous $[CS \rightarrow US; CS \rightarrow \text{no-US}] \times 3$ procedure. Hence, the contextual conditioning procedure by itself was not sufficient to elicit a clear genotype effect on the expression of conditioned freezing. Similarly, the observed difference in the ambiguous procedure between mutants and controls was not statistically significant in the tone-test here. The genotype effect on tone-freezing only became apparent statistically in the standard conditioning procedures. However, interpretation must take into account the behaviour of the mutants in both training procedures.

What mechanism could potentially strengthen the subsequent CS-freezing in the standard procedure but not when $[CS \rightarrow \text{no-US}]$ trials were introduced during conditioning? According to learning theories, exposure to each of the $[CS \rightarrow \text{no-US}]$ trials should revise the CS-US associative strength down from any gain resulting from the preceding $[CS \rightarrow US]$ trial. According to the computation model proposed by Rescorla & Wagner [23], adjustment of associative strength in either direction is modulated by the learning rate parameter, α . The change of associative strength after successive learning trials follows a simple error correction rule, such that the change in associative strength (ΔV_n) after the n^{th} trial may be expressed as: $\Delta V_n = \alpha (\lambda - V_{n-1})$, where λ refers to the maximum strength of the CS-US association achievable for a given magnitude of the US, and V stands for the current CS-US associative strength. The resulting (cumulative) associative strength after the n^{th} trial (V_n) is therefore $V_n = V_{n-1} + \Delta V_n$.

Application of the Rescorla-Wagner model to the two conditioning protocols here (standard vs. ambiguous) shows that increasing values of α produces in the ambiguous procedure an $\alpha \rightarrow V$ relationship that follows an inverted U-shape, which is substantially different from the monotonic increase of V as a function of increasing α generated by the standard $[CS \rightarrow US] \times 3$ procedure (see Figure 5). This qualitative difference may be relevant to our empirical findings. Our observation that the two conditioning procedures did not lead to substantial difference in the strength of tone-freezing expressed on the test day in the controls leads us to suggest that α_{control} could be approximated at 0.2. Increment of the value of α from this point will lead to a rapid increase in the final associative strength, V , in the standard

conditioning protocol and a less rapid decrease of V in the ambiguous [CS→US; CS→no-US] × 3 protocol. Graphically, a value of α_{mutant} near 0.6 would fit our empirical results in the mutants. This analysis also suggests that further increase of α_{mutant} might eventually lead to a bidirectional modulation of the final value of V between the two conditioning protocols (see Figure 3B).

It is worth noting that the inverted U-shaped function predicted by the Rescorla-Wagner model essentially stems from the fact that the last trial of the ambiguous conditioning protocol was a [CS→no-US] trial. This analysis would further suggest that the new phenotype identified here would be absent if the sequence of the ambiguous procedure employed had instead ended with a [CS→US] trial. If so, the sensitivity to information ambiguity would appear to be local rather than global. This certainly warrants consideration.

While this theorization may appeal to parsimony as the change of a single learning parameter in a simple mathematical model of associative learning could match the empirical data, it is not without difficulties. If the learning parameter α remained higher in the mutants than the controls, faster extinction would also be expected regardless of the initial training condition. Yet, there was no evidence that the extinction of CS-freezing was more rapid in the mutants (Figure 3A). This is consistent with our impression obtained in our previous studies on conditioned freezing with this mutant mouse line [24].

Finally, GlyT1 inhibition has been emphasized as a new pharmacological strategy to boost NMDA receptor function (see Introduction). It is in this context that GlyT1 inhibition has been suggested as a new pharmacotherapy for conditions in which deficient NMDA receptor function is implicated, including schizophrenia [13]. It may be conceivable that the associative phenotypes here and elsewhere stem from modifications of NMDA receptor function in our mutant mice. We have previously reported that hippocampal NMDA receptor current is selectively potentiated in our mutant mice [15]. It would be interesting to test if the new phenotype reported here may be silent by some appropriate downregulation of NMDA receptors in the mutant mice. A positive outcome would strengthen the possibility of a causal link. In this respect, it is important to note that our mutant mice with GlyT1 deletion restricted largely to forebrain neurons may only mimic some but not all of the effects associated with systemic administration of GlyT1 inhibitors [25]. While it is encouraging that the GlyT1 inhibitors SSR504732 and SSR103800, can enhance the expression of LI as observed in mice with forebrain neuronal GlyT1 deletion [15,26,27], other reported pro-cognitive effects of certain GlyT1 inhibiting drugs, such as working memory enhancement [28,29], have only been clearly seen when the genetic disruption of GlyT1 is extended to all cell types (namely, not only neurons but also glial cells where GlyT1 is normally expressed) in the telencephalon [30]. Although specific effects of pharmacological GlyT1 inhibition, such as object memory enhancement, appear more robustly seen in knockout mice models [13, 25], some reported drug effects (such as enhanced extinction learning) are absent in existing knockout mice models [see 25]. Hence, the possibility that cell-type specificity (as well as regional specificity) might critically alter the therapeutic scope of GlyT1 inhibition may point to an effect of GlyT1 inhibition beyond NMDA receptors. One clear candidate is inhibitory glycine receptor function, which can be potentiated by GlyT1 inhibition [13]. This deserves to be taken into account in the continual

development of pharmacotherapy based on GlyT1 inhibition. Indeed, such consideration may potentially explain some of the inconsistent effects seen across different classes (e.g., competitive vs noncompetitive; sarcosine-based vs non-sarcosine-based backbone) of GlyT1 inhibitors as discussed elsewhere [13].

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Highlights

- Forebrain GlyT1 may modulate Pavlovian fear conditioning
- The sensitivity to “informativeness” of potential CSs seems to be enhanced
- This study extends the finding to ambiguous CS due to partial reinforcement
- Forebrain GlyT1 disruption does not indiscriminately enhance conditioned fear
- GlyT1 disruption may not foster the acquisition of spurious maladaptive fear

Figure 1. Acquisition of the conditioned response on the conditioning day

(A). Percent time freezing at successive CS presentations for the standard training procedure, in which animals received three trials of CS presentations that were always followed by a US (indicated as "CS+"). (B) The percent time freezing obtained from animals trained in the ambiguous procedure, in which the CS was presented on six occasions. Three of these were followed by a US (i.e., trials 1, 3 and 5; and indicated by "CS+") and occurred at the same time that corresponded to the three CS+ trials in the standard procedure. The remaining presentations of the CS were not followed by a US (indicated as "CS-"). Error bars refer to $\pm$ standard error (SE).

Figure 2. Test of context freezing

The animals were returned to the conditioning context in the absence of the CS one day following conditioning. Context freezing did not significantly differ between the four groups throughout the 8 min test. Error bars refer to $\pm$ SE.

Figure 3. Test of CS freezing

The levels of freezing observed during the CS test (48 h following conditioning) are expressed across successive 2-min bins (**A**). The first bin refers to the initial baseline period prior to CS onset (labelled as “PreCS”) when no significant difference between groups was detected. Onset of the CS induced a rapid increase in freezing which subsided over the course of the 8-min test period. The average levels of freezing over the 8-min test period are shown in (**B**). While the mutants exhibited differential levels of CS freezing between the standard and ambiguous training procedures (indicated by *, $p < 0.05$), no such difference was detected in the controls. In addition, the mutants exhibited significantly stronger freezing than controls in the standard procedure (#, $p < 0.05$). The significant pairwise comparisons illustrated in (**A**) and (**B**) were obtained based on the error variance associated with the significant 3-way interaction and the genotype $\times$ training procedure interaction, respectively, in the overall ANOVA. Error bars refer to $\pm$ SE.

Figure 4. Acquisition and Test of context foreground conditioning in Experiment 2

Percent time freezing showed a monotonic increase across successive ISI-periods (each lasting 3 min) on day 1 (A). Context freezing was assessed by returning the animals to the shocked context (Context 'A') on days 2 and 4, in comparison to the freezing response to a neutral context (Context 'B') on days 3 and 5. The higher levels of freezing in Context 'A' in comparison to Context 'B' indicates the context specificity of the conditioned freezing response. No genotype difference was evident throughout the entire experiment. Error bars refer to $\pm$ SE.

Figure 5. Impact of α on associative strength under the ambiguous vs the standard training procedures

We applied the Rescorla-Wagner model to calculate the final associative strength according to the ambiguous and standing training procedure as a function of α (see text for the exact formulae and parameters). λ is set to 1, and the initial value of CS-US associative strength is set to $V_0 = 0$ in these simulations. Increasing α leads to a monotonic increase in the final associative strength in the standard training procedure, but increasing α is associated with an inverted U-shaped curve in the ambiguous procedure. The outcome of the Experiment 1 may be accommodated by an increase in the value of α in the mutants (α_{mutant}). Controls are

assigned a value of $\alpha_{\text{control}} = 0.2$, which is the maximum value here that did not yield a difference in final CS-US associative strength between the two training procedures. An increase of α from this level to 0.6 ($\alpha_{\text{mutant}} = 0.6$) is associated with a rapid increase of the final associative strength in the mutant/standard condition and a weaker fall of the final associative strength in the mutant/ambiguous condition. The resulting pattern of divergence closely approximates the empirical data reported in Experiment 1.