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Research report

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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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.

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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 (GlyT1) 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.

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 microenvironment of synapses containing *N*-methyl-D-aspartate (NMDA) receptors, GlyT1s keep 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

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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 aversive (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 discontiguity [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*(shock|tone), to 0.5, while the *retrospective* conditional probability *P*(tone|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 CSs, respectively, given that this distinction is neurobiologically as well as psychologically 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 CaMKIIαCre-mediated recombination (see [15]). Appropriate heterozygous Cre mice were mated with Glyt1tm1.2fl/fl mice to generate the desired mutant ($GlyT1^{fl/fl}$:$CaMKII\alpha^{+/-}$) 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 0700–1900 h. 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 center to center, 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 cm × 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-dB_A 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 center of the area of interest. The algorithm of the freezing response detection based on image analysis of successive digital frames 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 the CS.

2.4. Experiment 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 1 s) 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 4 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.