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Examining the sex- and circadian dependency of a learning phenotype in mice with glycine transporter 1 deletion in two Pavlovian conditioning paradigms

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Abstract

Behavioural characterisation of transgenic mice has been instrumental in search of therapeutic targets for the modulation of cognitive function. However, little effort has been devoted to phenotypic characterisation across environmental conditions and genomic differences such as sex and strain, which is essential to translational research. The present study is an effort in this direction. It scrutinised the stability and robustness of the phenotype of enhanced Pavlovian conditioning reported in mice with forebrain neuronal deletion of glycine transporter 1 by evaluating the possible presence of sex and circadian dependency, and its consistency across aversive and appetitive conditioning paradigms. The Pavlovian phenotype was essentially unaffected by the time of testing between the two circadian phases, but it was modified by sex in both conditioning paradigms. We observed that the effect size of the phenotype was strongest in female mice tested during the dark phase in the aversive paradigm. Critically, the presence of the phenotype in female mutants was accompanied by an increase in resistance to extinction. Similarly, enhanced conditioned responding once again emerged solely in female mutants in the appetitive conditioning experiment, which was again associated with an increased resistance to extinction across days, but male mutants exhibited an opposite trend towards facilitation of extinction. The present study has thus added hitherto unknown qualifications and specifications of a previously reported memory enhancing phenotype in this mouse line by identifying the determinants of the magnitude and direction of the expressed phenotype. This in-depth comparative approach is of value to the interpretation of behavioural findings in general.

Keywords

associative learning; circadian rhythm; cognitive enhancement; glycine; NMDA receptor; Pavlovian conditioning; sex differences

1. Introduction

The search for genetic control and regulation of cognitive functions has extensively relied on the use of engineered mouse models in which selected genes and their products are modified. These are valuable and powerful translational tools that are widely available.

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Characterization of the behavioural and cognitive phenotypes associated with engineered genes deletions or mutations represents an important step in the proof of concept, and is an essential component in the preclinical evaluation of potential drug targets. It is of utmost importance therefore to gauge the robustness and generality of any specific behavioural phenotypes. Often, initial findings are based on a single test paradigm and interpreted without reference to possible sex dependency or environmental modulation. However, phenotypic expression can be labile and dependent on sex (Caldarone et al., 2010; Wiltgen et al., 2005; Yee et al., 2004) or circadian phase (Hossain et al., 2004; Marques & Waterhouse, 1994) even within the same mouse strain. Ignoring such factors can result in a simplistic and biased interpretation which may be counterproductive to translational research. Zucker & Beery (2010) have pointed out the limitations associated with the predominant use of males in animal studies, and several studies have emphasised the ethological inappropriateness of conducting behavioural tests on rodents during the light phase, which corresponds to their inactive phase (Beeler et al., 2006; Hossain et al., 2004; Marques & Waterhouse, 1994; Roedel et al., 2006).

Generalization across test paradigms is often overlooked. The emphasis on high-throughput approaches inadvertently favours the sole reliance on the most efficiently implemented tests, such as the preferential use of conditioned freezing for the assessment of Pavlovian associative learning. Indeed, the origin of Pavlovian associative learning was predominantly based on appetitive procedures, which obviously shares common associative learning mechanisms with aversive procedures (Pavlov, 1927). Yet, they also diverge significantly in terms of the motivational significance of the unconditioned stimulus (Konorski, 1976). In the past, the extension from aversive to appetitive Pavlovian paradigms has enabled us to clarify the nature of a sex-dependent learning phenotype in genetically modified mice (Yee et al., 2004). Such between-paradigm comparison may also be instructive in revealing underlying mechanisms (Austin & Duka, 2010) and brain regions involved (Knapska et al., 2006).

The present study provided the necessary scrutiny of a procognitive phenotype in Pavlovian learning identified in mice carrying a genetic deletion of glycine transporter 1 in forebrain neurons (GlyT1^{ΔFBNeuron}). This phenotype is presumably attributed to enhanced *N*-methyl-D-aspartate receptor (NMDAR) function due to elevated synaptic availability of glycine, an obligatory co-agonist of NMDAR activation. This has been taken as support of the general thesis that NMDAR-dependent neural plasticity is central to learning and memory (Morris, Anderson, Lynch, & Baudry, 1986; Morris, 1989), and the suggestion that its augmentation could be beneficial for cognitive deficiency in a number of psychiatric disorders (Black et al., 2009; Depoortere et al., 2005; Ingram et al., 1996; Martin, Grimwood, & Morris, 2000; Singer et al., 2009). However, although the initial report of an enhanced Pavlovian conditioning phenotype in GlyT1^{ΔFBNeuron} mice has been demonstrated across three conditioning paradigms, the paradigms were all aversive in nature and the reported data were exclusively derived from female subjects maintained in a reversed light-dark cycle to allow testing in the dark phase (Yee et al., 2006). Although there is evidence that this phenotype is present in both sexes and persisted into old age (Dubroqua et al., 2010), some data suggested that the phenotype might be somewhat weaker statistically in the male sex (Philipp Singer, personal communication).

The present study therefore explicitly examined the possibility of a sex-dependent phenotypic expression and further incorporated the time of testing (light vs. dark phases) as an additional factor. This design was repeated across two separate experiments for comparison between aversive (conditioned freezing) and appetitive (conditioned approach response) conditioning paradigms. Spontaneous locomotor activity, anxiety-related

behaviour, and shock sensitivity were assessed in order to control for potential confounding effects to assist interpretation of the conditioning results.

2. Methods

2.1 Subjects

All mice were derived from a pure C57BL/6J back ground as fully described before (see Yee et al., 2006). The experimental subjects were bred by pairing CamKII α Cre:Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice to yield litters of an expected 1:1 ratio of CamKII α Cre:Glyt1tm1.2fl/fl (carrying one copy of CamKII α -driven Cre-expression with homozygously floxed GlyT1 gene) and Glyt1tm1.2fl/fl (with only homozygously floxed GlyT1 gene and no Cre expression) genotypes. The former CamKII α Cre:Glyt1tm1.2fl/fl genotype is referred to as GlyT1 Δ FBNeuron and denoted as the “mutant”, with the latter Glyt1tm1.2fl/fl genotype serving as comparison “control” littermates. Breeding took place in a specific-pathogen free (SPF) breeding facility (Laboratory of Behavioural Neurobiology, ETH Zurich, Schwerzenbach, Switzerland), and litters were weaned and sexed on postnatal day 21. Genotypes were determined by standard PCR on tail biopsies obtained within 10 days after weaning as previously described (Yee et al., 2006).

All manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office as required by the Swiss Act and Ordinance on Animal Protection, which conforms with the ethical standards stipulated in the European Council Directive 86/609/EEC and the NIH publication no. 86-23 (revised 1985) on animal experimentation.

2.2 Housing under different circadian cycle

At the age of 11 weeks, a cohort of 87 mice (cohort A in Table 1) was subdivided into two balanced groups, with respect to genotype and sex, for separate housing between two identical climatized ($21 \pm 1^\circ\text{C}$, relative humidity at $55 \pm 5\%$) vivaria differing only in their circadian rhythm. One vivarium was maintained under a “normal” 12/12h light-dark cycle (lights off: 1900–0700h), while the other was under a “reversed” light-dark cycle (lights off: 0700–1900hrs). It thus allowed the direct behavioural comparison between light and dark phases in a between-subjects manner. All behavioural experiments were conducted between 0800 and 1800 h, thus falling within the light phase of animals kept in the “normal” cycle and the dark phase of those kept under the “reversed” cycle. Henceforth, we refer to this between-subject factor as “light-dark phase”. Mice of the same sex were housed in groups of 4 to 6 in Makrolon® Type-III cages (Techniplast, Milan, Italy) with constant provision of water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) unless otherwise specified. The mice were approximately 12–16 weeks old at the time of testing.

Four cages (one for each genotype/sex group) each housing four mice of the same sex and genotype, were arranged in each animal vivarium; and a miniature digital camera operating in the visible and infrared spectrum was mounted above the grid cage to allow monitoring of home cage activities. Video outputs of the four cameras in each animal vivarium were fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a PC for digital storage and cage-by-cage analysis. Five-minute 8-bit gray-scale video footages were sampled at 30 min past every o'clock over the 11-day acclimatization period. An image analysis algorithm calculated the number of pixels changed after appropriate thresholding (Open eVision 1.1, Euresys s.a., Belgium) between successive (360×288 pixels) binary images 1s apart. The number of pixels changed was expressed as percentage of total pixels per frame, averaged across each hourly 5-min sample. This number was then normalized and expressed as z-

scores with respect to each cage's average second-by-second percent pixels changed across the 11-day period). For each cage the 5-h running average time series was calculated and depicted in Figure 1.

Because our mice were bred under a reversed light-dark cycle, animals allocated to the vivarium with the same circadian rhythm adapted quickly and exhibited the expected diurnal variation in activity, namely, higher in the dark phase and lower in the light phase (Figure 1A,C). In contrast, animals switched to the normal light-dark cycle experienced a shift of circadian rhythm, and therefore their previous diurnal activity pattern persisted in the first 48h. Thereafter, they underwent a period of adjustment for about two days before the emergence of a new stable diurnal activity rhythm matching the light-dark rhythm (Figure 1B,C). As shown in the average activity profiles obtained from the two rooms (Figure 1C), their activity cycles were 12-h out-of-phase with each other for the last 7-8 days prior to commencement of experiments. Because all behavioural tests were conducted between 0800 to 1800h, mice kept in the reversed cycle would be tested in the dark phase, and those in the normal cycle in the light phase. The comparison between cycles effectively served as a contrast between light and dark phases while keeping the time and conditions of testing identical.

2.3 Experimental Plan

The cohort of 87 mice, prepared as described above, was first evaluated in an elevated plus maze test of anxiety and an open field test of locomotor activity before being split randomly into two subsets: one destined for the aversive conditioning experiment, and the other for the appetitive conditioning experiment (see Table 1). A separate cohort of naive mice (cohort B), which comprised only female mice kept in reversed light-dark cycle, was prepared especially for test of shock sensitivity.

2.4 Elevated plus maze

The elevated plus maze test of anxiety has been fully described before (Hagenbuch et al., 2006). Briefly, the maze was made of acrylic and consisted of four equally spaced arms radiating from a central square measuring 5×5 cm. Each arm was 30 cm long and 5 cm wide. A gray removable plastic floor in-lay was placed in the entire maze surface. One pair of opposing arms was enclosed with opaque walls 14 cm high. The remaining two arms were exposed with a 3-mm-high perimeter border along the outer edges. The maze was elevated 70 cm above floor level, and positioned in the middle of a testing room with diffuse dim lighting (25 lux in the centre of the maze). A digital camera was mounted above the maze and images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) tracking system. A test trial began with the mouse being placed in the central square with its head facing one of the open arms. It was allowed to explore freely and undisturbed for 5 min. The percent time in open arms = $[\text{time in open arms} / \text{time in all arms}] \times 100\%$ was used to index anxiety. In addition, the total distance travelled in the entire maze (i.e., arms and central platform) was recorded.

2.5 Open Field Test for spontaneous locomotor activity

This was conducted 48 h after the elevated plus maze experiment. The apparatus consisted of four identical square arenas (40×40 cm) surrounded by 25 cm high walls. They were made of wood with a white waterproof plastic surface. The four arenas were arranged in a 2-by-2 configuration, located in a testing room under diffused dim lighting (25 lux). A digital camera was mounted directly above the four arenas, transmitting images to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) software which tracked the animals with a temporal resolution of 5Hz. Four mice were tested simultaneously. They were placed in the centre of the appropriate arena and allowed to explore undisturbed for 60

min. Afterwards, they were returned to their home cage and the arenas were cleansed with water and dried before the next squad of four mice. Locomotor activity was indexed by distance travelled (in meter) across consecutive 10-min bins.

2.6 Pavlovian aversive conditioning

Five animals from each of the eight experimental groups were selected for this experiment (see Table 1), which commenced 48h after the open field test.

Two sets of chambers were used to provide two distinct contexts. The first set of chambers (context A) comprised four operant chambers (30 × 25 × 29 (high) cm, model E10-10, Coulbourn Instruments, Allentown, PA) individually installed in a ventilated, sound insulated chest. Each chamber was 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 shock could be delivered by a shock generator (Model E13-14). The animal was confined to a rectangular region (17.5 × 13 cm) in the centre by a clear Plexiglas enclosure. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the right 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, and located in ventilated, sound-insulated, wooden cabinets. They were illuminated by an infrared light source instead of visible light. Each chamber also contained a sonalert tone module (Model SC628, Mallory), which provided an 86 dB_A tone, and a miniature digital camera (sensitive to visible and infrared range) mounted 30 cm directly above the centre of the area of interest. The output of the camera was fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a computer running the Open eVision 1.1 (Euresys, Liège, Belgium) software under the control of a customized Microsoft Visual Basic (version 6) script. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998). In brief, successive digitized images (192 × 144, at 8-bit gray scale) obtained at a rate of 1 Hz were compared. The difference in number of pixels between adjacent frames was then computed. If this was less than 0.05% of the total number of pixels in a frame, the animal was considered to be freezing in that 1-s interval. The procedures comprised three distinct phases: (i) conditioning, (ii) test of conditioned context freezing, and (iii) test of conditioned tone freezing across days.

On Day 1, all animals were given three conditioning trials in context A. Each trial consisted of a 30s tone stimulus (conditioned stimulus, CS) followed immediately by a 1-s 0.25 mA foot shock (unconditioned stimulus, US). The first trial was administered 3 minutes after the animals were placed into the chambers. Successive trials were administered every 3 minutes. The conditioning session was concluded with a final 3-min interval.

On Day 2, the animals were returned to context A. They were placed in the test chamber for a period of 8 min.

On Days 3 to 10, conditioned freezing to the tone stimulus was assessed in context B. The tone stimulus was administered 2 min after the animals were placed into the test chamber. The tone remained on for a period of 8 minutes. The expression of freezing or immobility was expressed as percent time freezing.

The three phases were separately analyzed.

2.7 Appetitive Pavlovian conditioning

The animals from cohort A not included in the aversive conditioning experiment (see Table 1) were used in the appetitive conditioning experiment, which commenced 12 days after

conclusion of the open field experiment. Five days prior to conditioning, the animals were gradually introduced and acclimatized to a food-restricted diet until they were finally maintained on free feeding 2h per day throughout the experimental period. Their body weight was closely monitored and prevented from falling below 85% of their ad libitum weight.

The apparatus consisted of eight Habitest System operant chambers (29 × 25.5 × 28 high cm, Model E10-10, Coulbourn Instruments Allentown, PA), each located in a ventilated, sound-insulated chamber. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the right panel wall, 21 cm above the grid floor. A partition wall (parallel to the panel wall) was installed to reduce the floor area to 17 × 25.5 cm. A magazine tray was positioned in the middle of the panel wall and 2 cm above floor level. Nose-poke responses to the magazine were detected by an infrared photocell beam (Model H14-01M) placed at the entrance. Liquid reward (0.01 ml of a 20% dilution of a commercial condensed milk, Milch Lait®, Switzerland) was delivered in the magazine by an automated dipper (Model H14-05R) attached to it. Each chamber also contained a sonalert (Model SC628, Mallory), which provided an identical tone (86 dB_A) to that used in the Pavlovian aversive conditioning experiment. Each set of four chambers were connected via an interface to a PC running the Graphic State software (Version 1.013) which provided independent control of each chamber and collected all response data.

The animals were first familiarized with consuming the milk reward from the liquid dispenser in a 15-min session, during which the liquid dipper was programmed to be raised (for 5s at a time) at random intervals (mean=30s, range = 5 to 60s). All animals learned to access the magazine and to consume the liquid reward. Conditioning commenced the next day and continued for 14 consecutive days. On each daily conditioning session, the animals were placed in the same test chamber with house light on throughout. Ten discrete conditioning trials, each consisting of a 5-s tone stimulus (the conditioned stimulus, CS) followed immediately by the delivery of the liquid food reward available for 5s (the unconditioned stimulus, US), were presented at random intervals (mean=120s, range=60–180s).

Eight days of extinction immediately followed the last conditioning session. An extinction session was identical to that of the conditioning session except that no reward was available (although the dipper still underwent the motion of delivery).

The approach response was measured by magazine nose pokes. Conditioned approach response was indexed by comparing the frequency of nose pokes during CS presentation with the 5-s pre CS period to control for baseline difference using the ratio: *CS nose pokes / (CS nose pokes + Pre-CS nose pokes)*, calculated daily. A value of 0.5 refers to chance performance such that the nose poking frequency does not differ between the pre-CS and the CS periods. Data collected in the conditioning and extinction phases were separately analysed.

2.8 Shock sensitivity

To evaluate if differences in sensitivity to shock between mutant and controls might contribute to the conditioning phenotypes observed in the aversive Pavlovian conditioning experiment, a separate cohort of naive mice (mutant: n=8, control: n=7) was prepared. Only female mice kept in the reversed cycle (therefore tested in the dark phase) were used here since it was the combination exhibiting the strongest effect size of enhanced conditioned freezing (see Discussion). Four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA) were used to measure the direct whole body motor response to electric foot shock. Each startle chamber comprised a cylindrical enclosure made

of clear Plexiglas attached horizontally on a lightweight mobile platform, which in turn was resting on a solid base inside a sound-attenuated isolation cubicle. A foot shock grid was located along the length of the animal enclosure and was remotely controlled by a programmable shocker. Whole body motion was converted into analogue signals by a piezoelectric unit attached underneath the platform. These signals (in arbitrary units proportional to the force produced) were digitized and stored by a computer. Testing was performed under a constant background noise of 65 dB_A produced by a high-frequency loudspeaker mounted directly above the animal enclosure inside each test chamber. The mice were first acclimatized to the apparatus for 2 min after being placed inside the enclosure; 0.5-s foot shocks were then administered in either ascending-descending or descending-ascending sequence (counter-balanced across animals) of the following intensity: 0 (baseline), 0.045, 0.075, 0.105, 0.135, 0.165, 0.195, 0.225 and 0.255 mA. A total of 18 readings were obtained. A 0.5-s response window was defined starting from the shock onset. The force (in arbitrary units) produced by whole body movements was sampled at a rate of 1 kHz. The peak response and the latency to peak response were taken to index the reaction to the shock.

2.9 Statistical analysis

All data were analysed by parametric analysis of variance (ANOVA) using the between-subject factors genotype, light-dark phase and sex. Additional within-subject factors were included as determined by the experimental design, such as days, bins, blocks and shock intensity, with polynomial orthogonal contrasts. Statistical significant main effects and interaction terms were further examined by post-hoc pair-wise analysis (based on the pooled error variance) and supplementary restricted analyses to assist interpretation. All statistical analyses were carried out using SPSS for Windows (version 18, SPSS Inc. Chicago IL, USA) implemented on a PC running the Microsoft Windows 7 operating system.

3. Results

3.1 Elevated plus maze test of anxiety

All animals in cohort A were first evaluated in the elevated plus maze (see Table 1). This revealed that the presence or absence of a genotype effect depended on sex as much as on light-dark phase (Figure 1). First of all, a sex difference across light and dark phase was seen in control mice; male controls were more anxious than female controls. Against this background, a pronounced anxiolytic phenotype was observed in male mice tested in the light phase, which was in contrast to the opposite trend (i.e., anxiogenic effect) observed in the female tested also in the light phase. These impressions were supported by a $2 \times 2 \times 2$ (genotype $\times$ sex $\times$ light-dark phase) ANOVA of percentage time spent in the open arms, which yielded a significant 3-way interaction [$F_{(1,79)}=5.20$, $p<0.05$]. Post-hoc pair-wise comparisons revealed a significant genotype difference in male mice tested in the light phase [$p=0.03$], but the opposite trend between female mutant and controls housed in the same condition did not achieve statistical significance [$p=0.09$]. Nonetheless, the appearance of a sex-dependent phenotype in the opposite direction gave rise to a significant genotype $\times$ sex interaction [$F_{(1,39)}=6.35$, $p<0.05$] in a restricted ANOVA confined to animals tested in the light phase. No evidence for such an interaction was found in the ANOVA restricted to mice tested in the dark phase [$F<1$], which revealed only a significant sex effect [$F_{(1,40)}=4.85$, $p<0.05$]. The main effect of sex, however, did not achieve significance in the overall ANOVA.

Separate analysis of locomotor activity confirmed that the observed effect on anxiety-related behaviour described above was not confounded by any group differences in spontaneous locomotor activity. Parallel analysis of the total distance travelled on the entire maze surface

during the test did not reveal any significant effect. The animals on average ($\pm$ SEM) had covered a total distance of 9.01 ± 0.19 m within the 5-min test period.

3.2 Locomotor activity in the open field

Locomotor activity was further evaluated in the open field for an extended period of time to allow for the assessment of locomotor habituation effect. Habituation was evident by the monotonic reduction of activity over the course of the 60 min period which resulted in a highly significant bins effect [$F_{(5,395)}=336.08$, $p<0.001$] in a $2 \times 2 \times 2 \times 6$ (genotype $\times$ sex $\times$ light-dark phase $\times$ 10-min bins) ANOVA of distance travelled. Open field activity was independently affected by light-dark phase (Figure 3A) and sex (Figure 3B). These were confirmed by the presence of a significant sex effect [$F_{(1,79)}=14.54$, $p<0.001$], its interaction with bins [$F_{(5,395)}=5.01$, $p<0.001$], and the light-dark phase by bins interaction [$F_{(5,395)}=2.55$, $p<0.05$]. Post-hoc pair-wise comparisons at successive bins revealed that testing in the dark phase facilitated habituation transiently, leading to lower activity in bins 2-4 when compared to mice tested in the light phase. Thus, the phase by bins interaction was predominantly explained by its quadratic component [$F_{(1,79)}=5.83$, $p<0.02$]. On the other hand, female mice were consistently more active than male mice. But this effect was visually most pronounced towards the session's end, suggesting that habituation was stronger in male mice. Consistent with this impression, the sex by bins interaction was strongest in its linear component [$F_{(1,79)}=8.95$, $p<0.005$]. However, there was no evidence of any genotype effect [all F 's <1] (Figure 3C).

3.3 Pavlovian aversive conditioning

3.3.1 Conditioning session (day 1)

CS-freezing: The amount of freezing observed in the presence of the tone increased across the three tone-shock pairings (Figure 4). This was not affected by genotype or light-dark phase (Figures 4A,C). However, female mice exhibited a stronger freezing response by the last trial (Figure 4B), which was independent of the other factors. A $2 \times 2 \times 2 \times 3$ (genotype $\times$ sex $\times$ light-dark phase $\times$ conditioning trials) ANOVA revealed a significant trials effect [$F_{(2,64)}=57.3$, $p<0.001$], and its interaction with sex [$F_{(2,64)}=4.70$, $p<0.05$]. Post-hoc pair-wise comparisons at successive trials indicated that this interaction stemmed from a sex difference specific to the last CS presentation [$t_{(64)}=4.10$; $p<0.01$].

ITI-freezing: A similar pattern emerged in the analysis of between-trials (ITI) freezing (Figure 4D-F), such that female mice again showed stronger freezing, which emerged over successive ITI-periods (Figure 4E). In addition, an effect of genotype was observed with mutant mice displaying a stronger progressive increase in freezing across ITI's (Figure 4F). These impressions were confirmed by a $2 \times 2 \times 2 \times 4$ (genotype $\times$ sex $\times$ light-dark phase $\times$ ITI-periods) ANOVA, which yielded an effect of ITI-periods [$F_{(3,96)}=49.99$, $p<0.001$] and of sex [$F_{(1,32)}=5.94$, $p<0.05$], with the genotype effect just missing the criterion for significance [$F_{(1,32)}=4.09$, $p=0.05$]. The temporal dependency of the sex and genotype effects was evident by the presence of the highly significant sex by ITI-periods interaction [$F_{(3,96)}=8.47$, $p<0.001$] and genotype by ITI-periods interaction [$F_{(3,96)}=4.54$, $p=0.005$]. Post-hoc pair-wise comparisons at successive ITI's indicated that the sex and genotype effects were essentially restricted to the last two ITI periods. A significant sex difference was only detected in the last two ITI's [$t_{(96)}=3.56$ and 5.97 , respectively; both p 's <0.01]; and a significant genotype difference was likewise detectable only in the last two ITI's [$t_{(96)}=2.67$ and 4.68 , respectively; p 's <0.01].

3.3.2 Test of context freezing (day 2)—Conditioned fear to the context acquired on the first day was assessed by re-exposing the animals to the same context 24h later. The levels

of freezing were generally low during the 8-min test and were not affected by sex, light-dark phases or genotype (Figure 5A,B,C). A $2 \times 2 \times 2$ (genotype $\times$ sex $\times$ light-dark phase) ANOVA of percent time freezing did not yield any significant effects.

3.3.3 CS freezing (days 3 – 10)—Expression of conditioned freezing to the tone CS was assessed across the next 8 days in a neutral context. Each daily test began with a 2-min “pre-CS” period followed by the presentation of the CS continuously for 8 min. The weakening of the conditioned response (CR) over time (within and across days) further provided a measure of extinction learning, since the CS was now no longer followed by any US.

Pre-CS freezing: The levels of freezing observed in the pre-CS period remained low and stable over days (Figure 6). Animals tested in the light phase exhibited a significantly higher level of Pre-CS freezing ($2.02 \pm 0.31\%$) than those tested in the dark phase ($0.87 \pm 0.31\%$). A $2 \times 2 \times 2$ (genotype $\times$ sex $\times$ light-dark phase) ANOVA of percent time freezing during the 2-min pre-CS period yielded a significant light-dark phase effect [$F_{(1,32)}=7.56$, $p<0.01$], and a marginal sex effect [$F_{(1,32)}=3.79$, $p=0.06$] with female mice also showing higher levels of Pre-CS freezing ($1.98 \pm 0.31\%$) compared with male mice ($0.98 \pm 0.31\%$). The analysis revealed no other significant effects.

CS freezing: Presentation of the CS induced a conditioned freezing response due to its previous pairing with the shock US. Because the shock no longer followed the CS, the level of freezing fell across days, as well as across bins (within-day); and the latter effect was progressively less pronounced across days. A 5-way $2 \times 2 \times 2 \times 8 \times 4$ (genotype $\times$ sex $\times$ light-dark phase $\times$ days $\times$ 2-min bins) ANOVA of percent time freezing revealed highly significant effects of days [$F_{(7,224)}=51.18$, $p<0.001$], bins [$F_{(3,96)}=32.83$, $p<0.001$] as well as their interaction [$F_{(21,672)}=10.40$, $p<0.001$].

The overall level of conditioned freezing to the CS was significantly modified by genotype [$F_{(1,32)}=4.69$, $p<0.05$], sex [$F_{(1,32)}=10.33$, $p<0.005$] and light-dark phase [$F_{(1,32)}=7.60$, $p<0.01$]. Freezing was generally higher in the mutants, amongst female mice tested in their light phase (see Figure 6). The genotype and sex effects were visibly stronger in the early phase of the test in terms of days, as well as time bins, leading to the emergence of these factors' interactions with days [genotype $\times$ days: $F_{(7,224)}=2.07$, $p<0.05$; sex $\times$ days: $F_{(7,224)}=7.79$, $p<0.001$], with bins [genotype $\times$ bins: $F_{(3,96)}=3.56$, $p<0.05$; sex $\times$ bins: $F_{(3,96)}=7.47$, $p<0.005$], and with bins across days [genotype $\times$ days $\times$ bins: $F_{(21,672)}=1.68$, $p<0.05$; sex $\times$ days $\times$ bins: $F_{(21,672)}=2.99$, $p<0.001$].

The 4-way genotype $\times$ sex $\times$ days $\times$ 2-min bins ($2 \times 2 \times 8 \times 4$) interaction also attained significance [$F_{(21,672)}=1.69$, $p<0.05$], which was attributable to a sex-dependent genotype effect on the extinction of CS-freezing across bins that was most pronounced on the first CS test day. Additional analyses restricted to each test day confirmed the emergence of a significant genotype $\times$ sex $\times$ bins interaction only on the first CS test day [$F_{(3,96)}=3.59$, $p<0.05$], which was further accompanied by a significant effect of genotype [$F_{(1,32)}=5.07$, $p<0.05$] and of sex [$F_{(1,32)}=19.20$, $p<0.001$], but not of light-dark phase [$F_{(1,32)}=2.03$, $p=0.16$]. As shown in Figure 7, the enhanced freezing observed in the female mutants (compared to female controls) was the strongest in the last two bins (Figure 7A), whereas it was the clearest in the first bin in the males (Figure 7B).

Evidence for an interaction between sex and light-dark phase was supported by a significant light-dark phase $\times$ sex $\times$ days interaction [$F_{(7,224)}=2.10$, $p<0.05$], which was attributed to the sex effect across days being stronger in animals tested in the dark phase than those tested in the light phase (Figure 8). Consistent with this interpretation, a significant sex $\times$ days

interaction was only detected in a supplementary analysis restricted to the dark phase [$F_{(7,112)}=12.65$ $p<0.001$], but not when restricted to the light phase [$p=0.2$].

Spontaneous Recovery of CS freezing between consecutive CS sessions: The freezing response to the CS in the initial bin tended to be higher to that seen in the last bin of the previous CS test (see Figure 6A). This effect represents a partial recovery from the previous day's extinction learning, and is commonly referred to as 'spontaneous recovery'. As extinction was progressively consolidated over test days, the magnitude of spontaneous recovery also weakened. However, it appeared that the spontaneous recovery effect was somewhat stronger and more persistent in mutant mice. To specifically gauge spontaneous recovery between days, a difference score was computed contrasting the magnitude of freezing of the first CS bin of a given test day to the last CS bin of the preceding day. The difference scores were then subjected to a $2 \times 2 \times 2 \times 7$ (genotype $\times$ sex $\times$ light-dark phase $\times$ days) ANOVA, which yielded a main effect of days [$F_{(1,32)}=12.04$, $p<0.01$] (see Figure 9A) and of genotype [$F_{(1,32)}=4.66$, $p<0.05$] (see Figure 9B), thus confirming the initial visual impression derived from Figure 6A. No other effects attained or approached statistical significance.

3.4 Pavlovian appetitive conditioning

Acquisition—The animals were subjected to CS-US conditioning for 14 days. Conditioning was evident in all groups as indicated by the progressive increase in magazine approaches during the CS presentation relative to the pre-CS period (Figure 10). A $2 \times 2 \times 2 \times 7$ (genotype $\times$ sex $\times$ light-dark phase $\times$ 2-day blocks) revealed only a significant effect of blocks [$F_{(6,234)}=37.05$, $p<0.001$].

Extinction—Next, the animals were subjected to eight daily extinction sessions when the tone CS was no longer followed by the reward US. There was a progressive reduction in the conditioned approach response, constituting extinction learning (Figure 10). A $2 \times 2 \times 2 \times 4$ (genotype $\times$ sex $\times$ light-dark phase $\times$ 2-day blocks) ANOVA revealed a significant blocks effect [$F_{(3,117)}=31.42$, $p<0.001$]. A sex-dependent genotype effect was evident by the emergence of a genotype $\times$ sex $\times$ blocks interaction [$F_{(3,117)}=3.10$, $p<0.05$]. As shown in Figures 11A-B, female mutants showed a trend towards resistance to extinction compared with female controls, while a weak and opposite trend was observed in the male mutants. Given that the critical 3-way interaction was solely attributed to the linear component of blocks [$F_{(1,39)}=10.37$, $p<0.005$], accounting for 97% of the variance explained by the interaction, further post hoc comparisons of the linear rate of extinction were conducted, which confirmed the impression that the mutation resulted in opposite effects on the linear rate of extinction across days between sexes (see Figure 11C).

3.5 Shock sensitivity

A separate cohort of female mice maintained under reversed cycle was used for the assessment of shock sensitivity. This was chosen because this particular sex/light-dark cycle combination resulted in the largest effect size of genotype in the conditioned freezing experiment (see Discussion later). Analysis of peak response (Figure 12A) as well as time to reach the peak response (Figure 12B) recorded in the startle chambers did not yield any genotype effect across the entire range of electric shocks (0.045 – 0.255mA) examined. A separate 2×9 (Genotype $\times$ Shock intensity) ANOVA only revealed a main effect of shock intensity [peak response magnitude: $F_{(8,104)}=17.37$, $p<0.001$; time to peak response: $F_{(8,104)}=12.20$, $p<0.001$].

4. Discussion

In our attempt to scrutinize the reported enhanced Pavlovian learning phenotype following forebrain neuronal GlyT1 deletion, we uncovered that the phenotypic expression was distinguishable between the sexes as demonstrated in the aversive as well as in the appetitive paradigms of Pavlovian conditioning employed here. These findings were free from any confounding genotypic effect on pain sensitivity (Figure 12) and spontaneous activity measured in the open field (Figure 3) or in the elevated plus maze. In the elevated plus maze test, we further identified a hitherto unknown phenotype in anxiety-related behaviour through extending the test to the light phase. This finding agrees with the anxiolytic profile of the GlyT1 inhibitor SSR504734 which was obtained in male animals tested during the light phase (Depoortere et al., 2005). At the same time, the lack of such anxiety effects in the dark phase agrees with previous reports based solely on results obtained in this phase (Yee et al., 2006), and therefore anxiety did not confound the previously established phenotypes (Dubroqua et al., 2010; Singer et al., 2007, 2009; Yee et al., 2006). This new finding in the elevated plus maze may be interpreted as a state-dependent genotypic effect, revealed here by the explicit comparison between mice kept in opposite light-dark cycles but tested at the same time under identical test conditions. In contrast, the Pavlovian phenotypes were essentially unaffected by the light-dark cycle, and against which evidence for sex-dependency was obtained.

4.1 Crossing the Sex Boundary

Jazin and Cahill (2010) have summarized many sex differences in the phenotypic expression of a host of genetically manipulated mice. Here, the sex comparison allowed us to specify the form and the extent to which the Pavlovian phenotype associated with forebrain neuronal GlyT1 deletion might differ between sexes. In the aversive paradigm, the phenotypic dependency on sex was primarily one of magnitude: the phenotype of enhanced conditioned freezing appeared stronger in the female mutants (Figure 7). As reported before (Yee et al., 2006), this phenotype was specific to CS-freezing, without any significant impact on context-freezing. Indeed, neither sex nor light-dark cycle exerted significant effect (Figure 5). However, given that the CS was a good predictor of shock US here, context-freezing was predictably low in the range of 12%, hence interpretation of these null effects should be exercised with caution due to possible floor effects. Lack of statistical power was unlikely of a concern here because the effective sample size in each of these contrasts was large (with sample size around 20 per sex, genotype or light-dark cycle).

By contrast, sex critically determined the direction of the Pavlovian learning phenotype in the appetitive paradigm. The conditioned approach response was similarly enhanced in the mutant females, but was surprisingly somewhat weakened in male mutants (Figure 11C). Overall our results suggest that the enhanced Pavlovian learning phenotype appeared more consistent and robust in females than males.

Evidence for sex-dependent phenotypic expression was obtained regardless of whether sex exerted an effect by itself on performance. Across the different stages (acquisition, context-test and extinction) of aversive conditioning, female mice were consistently showing a stronger freezing response than male mice (Figure 6D; in keeping with previous mice studies e.g., Wiltgen et al., 2005; Yee et al., 2004; but also see Dalla & Shors, 2009). Our data therefore concurred with the faster extinction rate in the female rats in fear conditioning reported by Milad et al. (2009), but did not replicate their observation of weaker conditioning in female rats during acquisition. Notably, the latter effect was weak and indeed non-significant. We observed instead stronger conditioning in females although this emerged only on the 3rd CS presentation and this minor divergence might stem from species differences. By contrast, no such sex difference was apparent throughout the appetitive

conditioning experiment. The apparent sex effect in conditioned freezing experiment (i.e. generally stronger freezing in female mice) cannot be attributed to sex differences in spontaneous activity given that the female mice were more active than the male mice (Figure 3B). If anything, this sex difference in spontaneous activity would under- rather than over-estimate freezing performance.

Another possible confound that might be expected to influence expression of the conditioned freezing response, but not the conditioned approach responses was general anxiety. The anxiety phenotype demonstrated in the elevated plus maze test was clearly determined by the combined influence of sex and light-dark cycle. Forebrain neuronal GlyT1 deletion appeared to be anxiolytic in males but anxiogenic in females, thus effectively reversing the sex-difference seen in control mice (Figure 2); and this cross interaction was only evident in the light phase. To what extent could this pattern of results in anxiety-related behaviour explain the sex-dependent outcomes seen in the Pavlovian aversive conditioning experiment?

Based on the premise that the expression of conditioned freezing depends on the development of conditioned fear in response to the CS, anxiousness to threatening stimuli in general might promote aversive associative learning. Hence, an anxious phenotype in female mutants might be linked to the conditioned freezing phenotype in the same animals. However, this account was undermined by two considerations. First, the conditioned freezing phenotype did not significantly depend on light-dark cycle; and if anything, it was the strongest in female mice tested in the dark phase of the cycle (kept in reversed light-dark cycle). Second, this account should also predict an opposite conditioned freezing phenotype in the male mutants, which was obviously not the case even though the magnitude of the enhanced freezing phenotype was visually weaker in the male than the female mutants. As summarized in Table 2, the critical effect sizes associated with the relevant genotype effect in the aversive conditioning experiment was highest in the female/dark-phase combination, when the comparison between mutants and controls yielded no difference in elevated plus maze behaviour (Figure 2). Indeed, the combination of a null effect in anxiety and enhanced conditioned freezing replicated our previous report (Yee et al., 2006). Re-calculation of the effect size from the original data reported by Yee et al. (2006) yielded a value that is closer to that obtained here in animals of the same sex and tested in the same phase (see Table 2).

The evidence thus did not support an emotional account of the conditioned freezing phenotype, but favoured instead a cognitive interpretation. Importantly, the cognitive perspective can readily accommodate the enhanced conditioned approach phenotype seen in the female mutants in the appetitive Pavlovian paradigm, which shared a similar associative learning mechanism (CS-US association), but did not involve any fear-related emotion.

4.2 Lost and Found in Translation across Paradigms

The inclusion of the appetitive conditioning experiment was highly instructive and meaningful, because it provided an alternative test with similar cognitive demand but differed in several psychological as well as procedural aspects. Appetitive conditioning is based on a motivational system antagonistic to that in aversive conditioning (Konorski, 1976) and is therefore ideally suited to test the generality of the reported Pavlovian phenotype that, so far, has been demonstrated solely in associative learning paradigms using aversive unconditional stimulus (Yee et al., 2006).

While this phenotype of enhanced conditioning was translatable to the current appetitive paradigm in the female sex, the results obtained in the male sex were surprising, as they yielded somewhat opposite effects between paradigms. The latter does defy an interpretation simply based on enhanced associative strength. Indeed, such a simple account is already

insufficient to explain the original finding that forebrain neuronal GlyT1 deletion enhanced the latent inhibition effect (i.e., the sensitivity to a CS's history of non-reinforcement prior to conditioning; Yee et al., 2006). Thus, there are situations in which the mutant mice were more sensitive to the negative modulation of learning and/or expression of learned behaviour.

There was no evidence here to suggest that acquisition of the CS-US association was facilitated across the three tone-shock pairing trials in the aversive conditioning experiment (Figure 4C). However, freezing recorded during the inter-trial intervals was enhanced in the mutants, with a notable difference emerging in the last two intervals – i.e., after the second and third tone-shock pairing (Figure 4F). Unlike the development of conditioned freezing, the acquisition of the (appetitively motivated) conditioned approach response is a relatively protracted process allowing a closer examination of its development over many CS-US trials conducted across days. The appetitive conditioning experiment here consolidated the lack of an effect on conditioned responding throughout the entire acquisition phase (Figures 10 & 11).

The present study also showed that, irrespective of conditioning paradigm, the Pavlovian phenotype was confined to the extinction phase – when the animals were confronted with extended/repeated CS's that was no longer followed by any US. This result suggests that forebrain neuronal GlyT1 deletion robustly modified the expression rather than the acquisition of the critical CS-US association. In the appetitive paradigm, evidence for enhanced conditioned responding seen in the female mutants only emerged across daily extinction tests (Figure 11B,C). Although the direction of the weaker effect seen in male mutants was in the opposite direction here (Figure 11A,C), the phenotypic expression was also confined to the extinction phase. In the conditioned freezing experiment, the phenotype remained visible across the eight daily extinction tests (Figure 6A), and the sex-dependent phenotypic expression was a quantitative one as shown in the first extinction test (Figure 7). Hence, the unique phenotypic element that is translatable across paradigms refers to the increased resistance to extinction in female mutants. This element was detected across days in the appetitive conditioning experiment, but solely on the first test day in the aversive conditioning experiment. Although not seemingly a sex-dependent effect, evidence that extinction learning across extinction days was also modified by GlyT1 deletion was obtained when we examined spontaneous recovery of the conditioned freezing response between successive tests (Figure 9). Overall, spontaneous recovery, attributed to the dissipation of inhibitory mechanisms responsible for extinction (Rescorla, 2004), was stronger in the mutants – the daily re-emergence of the CS evoked a stronger initial response in the mutants.

Taken together, extinction learning is most robustly affected by GlyT1 deletion in the female sex. Although we cannot explain why this phenotype might be weaker or somewhat reversed in the male sex when switched to the appetitive paradigm, this conclusion may be considered as being consistent with the previous finding in the female mutants that latent inhibition (LI) was enhanced (Yee et al., 2006). The demonstration of LI requires two distinct phases with contrasting CS-US relationships: the pre-exposure phase in which CS is not followed by any significant event [*CS* → *nothing*] precedes the conditioning phase. LI refers to the observation that CS pre-exposed subjects show weaker conditioned response (CR) to the CS after CS-US pairing compared to non-pre-exposed subjects. Several theories suggest that the weaker CR does not stem from a failure to acquire the critical CS-US association but reflects the [*CS* → *nothing*] memory trace competing over the control of behaviour during testing (Bouton, 1993; Kraemer and Spear, 1991, 1993; Weiner, 1990). Thus, a stronger influence of the prior experience of non-reinforced CS exposures leads to stronger LI. Procedurally, extinction is exactly the reverse of LI, whereby [*CS* → *nothing*]

follows [$CS \rightarrow US$]. Thus, the phenotype of increased resistance to extinction could be similarly understood as a stronger influence of the prior experience of [$CS \rightarrow US$] over current control of responding. The mutant mice appeared to be biased towards what they have learned first about the CS, and this might be described as a stronger primacy effect or proactive interference in memory terms. This contradicts however the finding that forebrain neuronal GlyT1 disruption enhanced rather than impaired reversal learning (Singer et al., 2009), as it has been taken to suggest resistance rather than vulnerability to proactive interference.

In discrimination learning, animals not only learn the opposing reward valence associated with the discriminanda provided by the experimenter, but they also learn to dichotomize their responses (approach vs avoidance) efficiently by focusing attention on one or few distinguishable dimensions/features separating the discriminanda. Achieving the latter would facilitate reversal learning, because the animals need only to reverse the contingency related to the few attended dimensions (Sutherland and Mackintosh, 1971). This has been offered as an explanation of the over-training reversal effect, whereby over-training paradoxically enhances subsequent reversal learning (Reid, 1953). Hence, it is conceivable that the reversal phenotype reported by Singer et al. (2009) reflects primarily enhanced selective attention learning, whereas the extinction phenotype here reflects a stronger primacy effect in memory recall.

The hypothesized impact on memory expression and selective attention are not mutually exclusive, even though they might be expected to yield opposing effects on specific paradigms, e.g., in discrimination reversal. When the separate effects act in the same direction then the observed behavioural effect would be substantial. The LI effect which is expected to be potentiated by a susceptibility to the primacy effect (Postman and Phillips, 1965) as well as enhanced attentional learning (e.g. Sutherland and Mackintosh, 1971), was highly sensitive to forebrain neuronal GlyT1 deletion (Yee et al., 2006). However, the possible sex and paradigm dependency of the LI phenotype awaits further evaluation.

4.3 Separating the light from the dark

Sex is strictly a between-subject factor. The factor referring to the contrast between light and dark phases, on the other hand, ought to be interpreted as a potential within-subject factor referring to the different states (of wakefulness) phase-locked with the diurnal cycle. For practical reasons, it was evaluated here in a between-subject design by shifting the light-dark cycles between two animal keeping rooms by 12h, thus enabling us to conduct all tests at the same absolute time. This approach allowed a more effective approximation to the contrast between laboratories that systematically keep their animals either in one or the other cycle. This contrast did not yield any substantial impact on the measures of conditioning behaviour here. Notably, even when it was effective in eliciting a sex-dependent phenotype in the elevated plus maze in the mutant mice, it was without an effect in the controls (Figure 2). Contrary to some previous studies, we did not observe an increase in spontaneous locomotor activity in the open field in mice tested in the dark phase in comparison with those tested in the light phase. If anything, a transient effect in the opposite direction was recorded (Figure 3A). However, increased activity was evident in the animals tested in the dark phase during the CS test of the aversive conditioning experiment in the form of reduced freezing, and this was already evident in the pre-CS phase. When the subsequent CS-freezing score on each day was corrected for baseline pre-CS freezing, the main effect of light-dark phases on CS-freezing remained significant [$F_{(1,32)}=6.94$, $p<0.02$], without altering any other significant effects in the original analysis. Therefore, the contrast between light-dark phases did appear to exert an effect on conditioned freezing rather than affecting the general propensity to freeze as such. Finally, the contrast between light-dark phases did not reveal any impact on behaviour in the appetitive conditioning test. Thus, Pavlovian learning of specific CS-US

association in general seems to be relatively stable across the light-dark phases, with limited impact on the magnitude (effect size) of the conditioned freezing phenotype identified in our mutant mice. Our results do not readily support the general assertion that behavioural testing in the dark phase is generally more appropriate than in the light phase solely on the basis that the former represents the active phase in rodents (Marques & Waterhouse, 1994). Yet, caution should be exercised when interpreting ethologically based behavioural tests, such as the elevated plus maze test of anxiety, and other such tests that might be highly sensitive to wakefulness or circadian fluctuation of hormonal activity.

5. Conclusions

In our attempt to test the generality and the robustness of a phenotype on conditioned freezing behaviour previously reported in mice with forebrain neuronal GlyT1 deletion, the present study has revealed (i) an hitherto unknown anxiety-related phenotype, and (ii) determinants that may influence the magnitude and perhaps even the direction of a Pavlovian learning phenotype in this particular genetic mouse model. Our results suggest the interaction between genomic (sex difference) and environmental (light-dark cycle) factors critically determined the expression of the anxiety phenotype, but sex-dependency predominates over the Pavlovian phenotype. A satisfactory neurobiological account of the emergence of these phenotypes must therefore take into account such specifications revealed here.

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References

- Austin AJ, Duka T. Mechanisms of attention for appetitive and aversive outcomes in Pavlovian conditioning. *Behav Brain Res.* 2010; 213:19–26. [PubMed: 20412818]
- Beeler JA, Prendergast B, Zhuang X. Low amplitude entrainment of mice and the impact of circadian phase on behavior tests. *Physiol Behav.* 2006; 87:870–880. [PubMed: 16600314]
- Black MD, Varty GB, Arad M, Barak S, De Levie A, Boulay D, et al. Procognitive and antipsychotic efficacy of glycine transport 1 inhibitors (GlyT1) in acute and neurodevelopmental models of schizophrenia: latent inhibition studies in the rat. *Psychopharmacology (Berl).* 2009; 202:385–396. [PubMed: 18709358]
- Bouton ME. Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychol Bull.* 1993; 114(1):80–99. [PubMed: 8346330]
- Caldarone BJ, Duman CH, Picciotto MR. Fear conditioning and latent inhibition in mice lacking the high affinity subclass of nicotinic acetylcholine receptors in the brain. *Neuropharmacology.* 2000; 39:2779–2784. [PubMed: 11044747]
- Dalla C, Shors TJ. Sex differences in learning processes of classical and operant conditioning. *Physiol Behav.* 2009; 97:229–238. [PubMed: 19272397]
- Depoortere R, Dargazanli G, Estenne-Bouhtou G, Coste A, Lanneau C, Desvignes C, et al. Neurochemical, electrophysiological and pharmacological profiles of the selective inhibitor of the glycine transporter-1 SSR504734, a potential new type of antipsychotic. *Neuropsychopharmacology.* 2005; 30:1963–1985. [PubMed: 15956994]
- Dubroqua S, Singer P, Boison D, Feldon J, Möhler H, Yee BK. Impacts of forebrain neuronal glycine transporter 1 disruption in the senescent brain: Evidence for age-dependent phenotypes in Pavlovian learning. *Behav Neurosci.* 2010; 124:839–850. [PubMed: 21038935]

- Hossain SM, Wong BK, Simpson EM. The dark phase improves genetic discrimination for some high throughput mouse behavioral phenotyping. *Genes Brain Behav.* 2004; 3:167–177. [PubMed: 15140012]
- Ingram DK, Shimada A, Spangler EL, Ikari H, Hengemihle J, Kuo H, et al. Cognitive enhancement. New strategies for stimulating cholinergic, glutamatergic, and nitric oxide systems. *Ann N Y Acad Sci.* 1996; 786:348–361. [PubMed: 8687034]
- Jazin E, Cahill L. Sex differences in molecular neuroscience: from fruit flies to humans. *Nat Rev Neurosci.* 2010; 11:9–17. [PubMed: 20019686]
- Knapaska E, Walasek G, Nikolaev E, Neuhäusser-Wespy F, Lipp HP, Kaczmarek L, et al. Differential involvement of the central amygdala in appetitive versus aversive learning. *Learn Mem.* 2006; 13:192–200. [PubMed: 16547163]
- Konorski, J. Integrative activity of the brain An interdisciplinary approach. The University of Chicago Press; Chicago and London: 1967.
- Kraemer PJ, Spear NE. The effect of nonreinforced stimulus exposure on the strength of a conditioned taste aversion as a function of retention interval: Do latent inhibition and extinction involve a shared process. *Animal Learning and Behavior.* 1991; 20:1–7.
- Kraemer, PJ.; Spear, NE. Retrieval Processes and Conditioning. In: Zentall, TR., editor. *Animal Cognition. A tribute to Donald A. Riley.* New Jersey: Lawrence Erlbaum; 1993. p. 87-110.
- Marques MD, Waterhouse JM. Masking and the evolution of circadian rhythmicity. *Chronobiol Int.* 1994; 11:146–155. [PubMed: 8082223]
- Martin SJ, Grimwood PD, Morris RG. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci.* 2000; 23:649–711. [PubMed: 10845078]
- Milad MR, Igoe SA, Lebron-Milad K, Novales JE. Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience.* 2009; 164:887–895. Milad MR, Igoe SA, Lebron-Milad K, Novales JE. [PubMed: 19761818]
- Morris RG. Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. *J Neurosci.* 1989; 9:3040–3057. [PubMed: 2552039]
- Morris RG, Anderson E, Lynch GS, Baudry M. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature.* 1986; 319:774–776. [PubMed: 2869411]
- Pavlov, IP. *Conditioned reflexes.* London: Oxford University Press; 1927.
- Postman L, Phillips LW. Short-term temporal changes in free recall. *Quarterly journal of experimental psychology.* 1965; 17:132–138.
- Reid LS. The development of noncontinuity behavior through continuity learning. *J Exp Psychol.* 1953; 46:107–112. [PubMed: 13084854]
- Rescorla RA. Spontaneous recovery. *Learn Mem.* 2004; 11:501–509. [PubMed: 15466300]
- Richmond MA, Murphy CA, Pouzet B, Schmid P, Rawlins JNP, Feldon J. A computer controlled analysis of freezing behaviour. *J Neurosci Methods.* 1998; 86:91–99. [PubMed: 9894789]
- Roedel A, Storch C, Holsboer F, Ohl F. Effects of light or dark phase testing on behavioural and cognitive performance in DBA mice. *Lab Anim.* 2006; 40:371–381. [PubMed: 17018208]
- Singer P, Boison D, Mohler H, Feldon J, Yee BK. Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons. *Behav Neurosci.* 2007; 121:815–825. [PubMed: 17907814]
- Singer P, Boison D, Mohler H, Feldon J, Yee BK. Deletion of glycine transporter 1 (GlyT1) in forebrain neurons facilitates reversal learning: enhanced cognitive adaptability? *Behav Neurosci.* 2009; 123:1012–1027. [PubMed: 19824767]
- Sutherland, NS.; Mackintosh, NJ. *Mechanisms of animal discrimination learning.* New York: Academic Press; 1971.
- Weiner I. Neural substrates of latent inhibition: the switching model. *Psychol Bull.* 1990; 108:442–461. [PubMed: 1980149]

- Wiltgen BJ, Sanders MJ, Ferguson C, Homanics GE, Fanselow MS. Trace fear conditioning is enhanced in mice lacking the delta subunit of the GABAA receptor. *Learn Mem.* 2005; 12:327–333. [PubMed: 15897254]
- Yee BK, Balic E, Singer P, Schwerdel C, Grampp T, Gabernet L, et al. Disruption of glycine transporter 1 restricted to forebrain neurons is associated with a procognitive and antipsychotic phenotypic profile. *J Neurosci.* 2006; 26:3169–3181. [PubMed: 16554468]
- Yee BK, Hauser J, Dolgov VV, Keist R, Mohler H, Rudolph U, et al. GABA receptors containing the alpha5 subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. *Eur J Neurosci.* 2004; 20:1928–1936. [PubMed: 15380015]
- Zucker I, Beery AK. Males still dominate animal studies. *Nature.* 2010; 465:690. [PubMed: 20535186]

Figure 1. Acclimatization to separate housing conditions with opposite light-dark (12h/12h) cycle
Activity levels were determined by imaging analysis per cage. The hourly time series data obtained from each cage lasted for 11 days. The data from each cage were normalized and expressed as z-scores (with respect to the cage's overall average and standard deviation) and then smoothed by a moving average operator with a 5-h window (-2h to +2h). (A) shows the four cages kept under reversed cycle, and (B) the four cages in the normal cycle. The lighting conditions in each room are indicated by appropriate shading of the background (gray = dark phase, white = light phase). (C) illustrates the average profile obtained in the two vivaria. The time-shift between light and dark phases are indicated by the vertical gray lines. By the end of the acclimatization period, the diurnal activity patterns between the two housing conditions were perfectly out-of-phase with each other.

Figure 2. The elevated plus maze test

Anxiety-like behaviour was indexed by the percentage of time spent in the open arms. A significant anxiolytic effect was observed in male mutant mice compared with male controls tested during the light phase (* $p < 0.005$). No genotypic difference was apparent in animals tested in the dark phase, although a sex effect was revealed in a restricted ANOVA performed in these animals. ☼ = light phase, ☾ = dark phase, ♂ = male, ♀ = female. Error bars refer to $\pm$ SEM.

Figure 3. Locomotor activity in the open field test

Activity was indexed by distance travelled across successive 10 min-bins. The reduction of activity over time constituted locomotor habituation. The effects of light-dark phase (A) and sex (B) are shown with data collapsed across the other factors. * = $p < 0.05$ based on pair-wise comparisons between phases (in A) or sexes (in B). The genotype effect is separately plotted according to sex and light-dark phase (C). ☀ = light phase, ☾ = dark phase, ♂ = male, ♀ = female. Error bars refer to $\pm$ SEM

Figure 4.

Conditioning session. Freezing is quantified as percent time during tone presentation across three tone-shock trials (A-B), or during the inter-trials intervals (ITIs) period (D-F). * refers to significant differences ($p < 0.01$) indicated by post-hoc comparison at specific trial or ITIs following emergence of the corresponding interaction effect depicted. Error bars refer to $\pm$ SEM

Figure 5. Test of context freezing

Overall levels of conditioned freezing to the context across the entire 8-min test were not affected by sex (A), light-dark phase (B) or genotype (C). Data refer to mean $\pm$ SEM.

Figure 6. Test of CS freezing (across 8 CS-test days)

The levels of freezing (indexed as percent time freezing) is expressed as a function of 2-min bins across the 8 consecutive CS-test days, illustrating the comparison between mutant and control mice (A) or between male and female mice (B), collapsed across the different time of testing. The effect of bins, collapsed across days (B and E), and the effect of days, collapsed across bins (C and F) are also illustrated, again either comparing between genotypes (B and C) or between sexes (E and F). * refers to significant differences ($p < 0.05$) obtained by post hoc pair-wise comparisons based on the error variance associated with the illustrated interactions. 'Pre' refers to the 2-min bins preceding the CS presentation at the beginning of each CS-test session. Data refer to mean $\pm$ SEM.

Figure 7. Test of CS freezing (during first CS-test day)

The genotype effects of conditioned freezing to the CS on the first CS test conducted 48h after CS-US conditioning are illustrated separately in the male (A) and female (B) mice. * refers to significant differences ($p < 0.05$) obtained from post hoc comparisons between genotypes at the specific 2-min bins based on the error variance associated with the significant genotype $\times$ sex $\times$ 2min-bins interaction effect. = male, = female. 'Pre' refers to the 2-min bins preceding the CS presentation at the beginning of each CS-test session. Data refer to mean $\pm$ SEM.

Figure 8. Modification of CS freezing by sex and light-dark phase across the 8 CS-test days
 The effects of sex, collapsed across other factors, was illustrated separately for animals tested in the light phase (A) and in the dark phase (B). * refers to significant differences ($p < 0.05$) based on the error variance associated with the significant light-dark phase sex days interaction. ☀ = light phase, ☾ = dark phase. Data refer to mean $\pm$ SEM.

Figure 9. Spontaneous Recovery effect from the second to the last CS-test day

Comparison between the spontaneous recovery of the mutant and control mice from the second CS-test and onwards, collapsed across the other factors as a function of days (A), or averaged across CS-test days 2 to 8 (B). Spontaneous recovery between successive CS-tests was indexed by a difference score = (levels of freezing during the first 2-min bins of day_N levels of freezing during last 2-min bin of day_{N-1}). A positive value refers to the spontaneous recovery effect. Data refer to mean ± SEM.

Figure 10. The acquisition and extinction of Pavlovian appetitive conditioning

Conditioned approach response was indexed by comparing the frequency of nose pokes during each 5-s CS presentation with the 5-s pre-CS period using the ratio: CS nose pokes / (CS nose pokes + Pre-CS nose pokes). The genotype effect across 2-day blocks is separately illustrated for the factor sex and light-dark phase (A-D). The 7 blocks of *acquisition* are demarcated by a vertical line from the last 4 blocks of *extinction*. The dotted line represents the level of chance performance. ☀ = light phase, ☾ = dark phase, ♂ = male, ♀ = female. Data refer to mean $\pm$ SEM.

Figure 11. The acquisition and extinction of Pavlovian appetitive conditioning

The genotypic difference over the course of the appetitive experiment (acquisition and extinction) is illustrated separately for male (A) and female (B) mice, collapsed across the other factors. Emergence of a significant genotype $\times$ sex $\times$ 2-day block interaction over the four extinction days was explained predominately by the linear trend in blocks. The linear component was calculated and the (genotype by sex) group average is illustrated in (C), which presents the original three-way interaction. ♂ = male, ♀ = female. Data refer to mean $\pm$ SEM.

Figure 12. Reactivity to shock

Reactivity to electric foot shock measured by peak response of whole body movement (**A**) and time to reach the peak response (**B**) as a function of the shock intensity in a separate naive cohort of female mutant and control mice tested during the dark phase. Data refer to mean $\pm$ SEM.

Table 1
Number and distribution of the separate cohorts of animals used in the present study

Animals in cohort A were all tested initially in the elevated plus maze (EPM) test of anxiety and in the open field test of locomotor activity. The animals were then subdivided for Pavlovian conditioning using either an aversive (conditioned freezing) or appetitive (conditioned approach) paradigm. Cohort B was behaviourally naive at the time of testing.

Cohorts	Experiments	Mutant				Control			
		Light		Dark		Light		Dark	
		♂	♀	♂	♀	♂	♀	♂	♀
A	EPM & Open field	8	10	11	8	11	14	12	13
A	Aversive conditioning	5	5	5	5	5	5	5	5
A	Appetitive conditioning	3	5	6	3	6	9	7	8
B	Shock sensitivity					8		7	

Table 2
Effect size (η_p^2) of the genotype

derived from the restricted analysis for each combination of sex and light-dark phase in the aversive Pavlovian paradigms and from the non pre-exposed group of the previous aversive conditioning experiment (Yee et al., 2006). Effect size provides a measure of the magnitude of a treatment effect, this index is independent of sample size and allowed us to directly compare each of the sex and phase of testing combination used. The largest effect size was found in the female mice tested during the dark phase of the light-dark cycle. The effect size of the previously reported genotype effect on aversive conditioning, with female mice tested during the dark phase was also, larger than the other sex/phase combinations in the present study.

<i>Experiments</i>	♀		♂	
	☀	☾	☀	☾
Aversive conditioning	0.09	0.45	0.03	0.16
Aversive conditioning (Yee et al., 2006)	n/a	0.29	n/a	n/a