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Pharmacological interference with metabotropic glutamate receptor subtype 7 but not subtype 5 differentially affects within- and between-session extinction of Pavlovian conditioned fear☆

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ABSTRACT

Fear extinction is defined as the attenuation of a conditioned-fear memory by re-exposing animals to the conditioned stimulus without the aversive stimulus. This process is known to be effectively enhanced via administration of D-cycloserine (DCS), a partial NMDA-receptor agonist. However, other glutamatergic mechanisms, such as interference with metabotropic glutamate receptor (mGluR) subtypes 5 and 7 in the extinction of aversive memories are insufficiently understood. Using the allosteric mGluR5 receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP), the mGluR7 allosteric agonist *N,N′*-dibenzyhydryl-ethane-1,2-diamine dihydrochloride (AMN082), and DCS for comparison, we aimed to study how pharmacological blockade of mGluR5 and activation of mGluR7 influenced within- and between-session conditioned-fear extinction training and extinction retention in rats. We show that when injected before extinction training, mGluR7 activation with AMN082 enhanced freezing and thereby attenuated within-session fear extinction, whereas both DCS and the mGluR5 receptor antagonist MPEP had no effect on this process. However, these differential drug effects were not long lasting, as no difference in extinction retention were observed 24 h later. Therefore, we assessed whether the compounds affect 24 h consolidation of extinction training following incomplete extinction training (between-session extinction). Similar to DCS, AMN082- but not MPEP-treated rats showed facilitated extinction retention, as exhibited by decreased freezing. Finally, using fluoxetine, we provide evidence that the effect of AMN082 on between-session extinction retention is most likely not via increasing 5-HT transmission. These findings demonstrate that mGluR7 activation differentially modulates conditioned-fear extinction, in dependence on the protocol employed, and suggests drugs with AMN082-like mechanisms as potential add-on drugs following exposure-based psychotherapy for fear-related human disorders.

1. Introduction

Several psychiatric illnesses involve learned components that contribute to the development of the symptom complexes. For instance, in specific phobias, social anxiety disorder and post-traumatic stress disorder (PTSD), conditioned stimuli (learned associations) may elicit fear, anxiety and intrusive memories. Also, in addiction disorders, drug-associated cues can trigger withdrawal responses, craving and relapse effects (Hofmann et al., 2006b; Myers et al., 2010; Rothbaum and Davis, 2003). Such conditioned stimuli can also be formed experimentally by repeatedly pairing initially neutral cues (e.g. odours, tones, visual signals) with an unconditioned stimulus (e.g. physical punishment or onset of drug effect). Consequently, the neutral cue acquires the ability to elicit classical conditioned responses, such as freezing. The most efficient way to reverse conditioned responses is through the process of extinction, which usually involves a protocol of repeated or prolonged exposure to the conditioned stimulus in the absence of the adverse event it once predicted, a procedure termed extinction training (McCallum et al., 2010; Myers et al., 2010; Myers and Davis, 2002).

Like acquisition of Pavlovian conditioning, extinction is also a form of associative learning. It can be sub-divided into mechanistically distinct memory formation processes, most notably encoding,

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consolidation and retention of extinction memory. Encoding occurs during the extinction training session(s), and experimental assessment is often attempted by measuring the amplitude of within-session extinction changes of the behavioural response that was initially triggered by the aversive stimulus, which is a short-term reduction (usually within less than 1 h). Thus, within-session extinction is measured during repeated or prolonged exposure to the conditioned stimulus, but in the absence of the aversive stimulus. Consolidation and retention of extinction, on the other hand, are mostly addressed by measuring between-session extinction, where extinction memory is generally assessed by an extinction retention test usually performed at least 24 h after completion of extinction training (McCallum et al., 2010; Myers et al., 2010).

The brain circuitry underlying fear conditioning and extinction is highly conserved across species (Myers et al., 2010). Therefore, modern research makes extensive use of rodent animal models and aims to uncover the exact neural circuitry as well as molecular and neurophysiological mechanisms underlying the various behavioural characteristics of extinction. Mechanistically, much has been learned about the contribution of various neurotransmitter systems to extinction. In particular, the main excitatory neurotransmitter of the mammalian brain, L-glutamate, and its pre- and postsynaptic receptors have received a lot of attention during the past years (Fendt et al., 2008; Myers et al., 2010; Zushida et al., 2007). The actions of L-glutamate are mediated by ionotropic and metabotropic receptor subtypes (iGluR and mGluR protein families, respectively). *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors constitute the main iGluR subtypes, and their role in extinction has already been studied in depth (Akirav, 2007; Myers et al., 2010; Walker et al., 2002b; Zushida et al., 2007). In fact, one of the best examined pharmacological mechanisms to exert control on fear extinction is partial allosteric activation of ionotropic NMDA receptors at the glycine modulatory site with the clinically approved antibiotic D-cycloserine (DCS). In rodents, systemic administration of DCS before or after extinction training facilitates fear extinction, an effect which could be localized within the basolateral amygdala (Ledgerwood et al., 2003, 2005; Myers et al., 2010; Richardson et al., 2004; Walker et al., 2002b). Interestingly, extinction of conditioned drug craving and withdrawal is facilitated by DCS as well (Botreau et al., 2006; Groblewski et al., 2009; Nic Dhonnchadha et al., 2010; Paolone et al., 2009; Thanos et al., 2009; Torregrossa et al., 2010). Also, at the clinical level, DCS broadly enhances extinction-based psychotherapy, e.g. for fear of heights, social anxiety or panic disorders, and nicotine as well as cocaine addiction (Guastella et al., 2008; Hofmann et al., 2006a; Otto et al., 2010; Price et al., 2009; Ressler et al., 2004; Santa Ana et al., 2009).

In contrast to iGluRs, studies on the contribution of mGluRs to fear extinction have only appeared very recently, and the knowledge at this time is still limited. In the mammalian central nervous system, mGluRs exist as eight receptor subtypes with multiple pharmacological sites and modes of action (Conn and Niswender, 2006; Flor et al., 2002; Niswender and Conn, 2010). In the present study, we focus on two mGluR-directed mechanisms that represent promising potential for neurological and possibly psychiatric disorders (Bird and Lawrence, 2009; Krystal et al., 2010; O'Connor et al., 2010; Yang, 2005), which also emerge in the field of conditioned fear and extinction research: first, negative allosteric modulation of mGluR5 (e.g. with the prototypical drug MPEP), a mechanism that is under clinical investigation in several nervous system disorders and has also demonstrated effects against acquisition and retention of conditioned fear responses (Fontanez-Nuin et al., 2010; Gasparini et al., 2008; Kim et al., 2007; Riedel et al., 2000; Schulz et al., 2001; Xu et al., 2009); second, allosteric activation of the mGluR7 subtype, e.g. with the agent AMN082 that shows preclinical antiparkinsonian-, anxiolytic-, and antidepressant-like activity (Greco et al., 2010; O'Connor et al., 2010; Palucha et al., 2007; Stachowicz et al., 2008). Interestingly, AMN082 blocks acquisition of conditioned fear but also facilitates extinction of conditioned aversion and fear in two amygdala-dependent paradigms [i.e. conditioned taste aversion and fear-potentiated startle (Fendt et al., 2008; Siegl et al., 2008)]. Furthermore, a recent study, also targeting a closely related metabotropic glutamate receptor, revealed that mGluR8-deficient mice had a significant decrease in the freezing response to the conditioning context (Fendt et al., 2010). Thus, the available evidence suggests that mGluRs may represent promising candidates for pharmacologically improving the outcome of exposure-based therapy.

Therefore, the primary aim of our present study was to assess whether pharmacological interference with mGluR5 and mGluR7 differentially affects the stages of extinction memory, as assessed by conditioned freezing. Further, we compare the effects of the mGluR-directed mechanisms to those of DCS. Overall, our studies aim to inform future clinical anxiety and drug addiction trials about MPEP- and AMN082-like agents as possible pharmacological add-on aids to exposure-based psychotherapy in man.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Charles River, Sulzfeld, Germany) weighing 220–250 g were housed in groups of four and kept under standard laboratory conditions (12:12 light/dark cycle, lights on at 6 am, 22 °C, 60% humidity and given free access to water and standard rat chow). All behavioral procedures took place during the light phase (8 am–2 pm). Experimental procedures were approved by the local government of the Oberpfalz (Bavaria, Germany) and followed the European Communities Council directive (86/609/EEC).

2.2. Drugs

All drugs were administered intraperitoneally (i.p.) at a volume of 1 ml/kg. DCS (from Sigma-Aldrich, Germany) was freshly dissolved in saline. AMN082 and MPEP (synthesized by Novartis Pharma AG; Basel, Switzerland) were freshly dissolved in 0.5% methylcellulose (AMIMED, Allschwil, Switzerland). The dose of DCS (15 mg/kg) was selected because it has previously been reported to facilitate fear extinction in rats (Bertotto et al., 2006; Langton and Richardson, 2008; Ledgerwood et al., 2003; Walker et al., 2002b). The dose of AMN082 (10 mg/kg) was chosen based on previously published studies, which all showed a very narrow useful dose-range for AMN082 (Bahi et al., in press; Fendt et al., 2008; Mitsukawa et al., 2005; Stachowicz et al., 2008). Higher doses of AMN082 in rats (20–60 mg/kg) induced motor side-effects such as mild ataxia or body tremor and/or showed reduced efficacy in several behavioural tests (compared to 10 mg/kg; see e.g. Bahi et al., in press). Lower doses than 10 mg/kg of AMN082 in rats are usually not efficacious in behavioural models (Bahi et al., in press; Fendt et al., 2008). In addition, previous studies have shown that 10 mg/kg of MPEP was also a safe and effective dose in fear conditioning experiments and did not affect spontaneous locomotor activity (Backstrom et al., 2004; Fontanez-Nuin et al., 2010; Herzig and Schmidt, 2004; Schulz et al., 2001; Varty et al., 2005). Fluoxetine (Sigma- Aldrich, Germany) was freshly dissolved in saline. The selected dose of fluoxetine (10 mg/kg) has previously been reported to alter fear conditioning (Burghardt et al., 2007; Santos et al., 2006).

2.3. Apparatus

The cued-fear experiments were performed in two different contexts, A and B, which differed in visual, tactile and olfactory cues. Fear conditioning occurred in context A, which consisted of a transparent perspex box (45 × 45 × 40 cm) with a transparent lid. The floor was made up of 25 × 0.6 cm stainless steel bars set 1.2 cm apart. Context A was cleaned with a neutral smelling detergent before each trial. Extinction training and retention occurred in context B, which consisted of a black perspex box (45 × 45 × 40 cm) with a smooth floor. Context B was cleaned with a lemon-scented detergent before each trial. The boxes were enclosed in a wooden chamber to reduce external noise and visual stimulation. A low level of background noise was produced by ventilation fans within the chamber. Illumination (300 lx for context A and 20 lx for context B) was provided by four white LEDs. Auditory stimuli were delivered through a speaker attached approximately 30 cm above the floor of the box. A video camera was placed at the top of the chamber and allowed the recording of the animal's behavior. As a measure of fear, freezing/inactivity, defined

as the absence of all movement except those required for respiration (Fanselow, 1980), was measured.

Inactivity was measured with the TSE fear conditioning system (TSE System GmbH, Bad Homburg, Germany). The conditioning chamber contained two horizontal detection fields. The first detection field was located 1.7 cm above the floor and allowed the detection of movement. The second detection field was located 7.7 cm above the floor and enabled the detection of jumping and rearing behavior. Each detection field contained 32 infra-red light-beams set 1.3 cm apart. Inactivity was measured by the infra-red beans and defined as no light-beam interruption for at least 3 s. In preliminary studies we compared freezing to hand scoring and inactivity as measured by connecting the camera to a computer with Noldus Ethovision XT 7.1(Wageningen; Netherlands) (Noldus et al., 2001; Pham et al., 2009). No difference between freezing (hand-scoring), TSE-determined inactivity or Noldus Ethovision XT 7.1-determined inactivity was detected (data not shown). Therefore, all experiments were scored using the TSE-based inactivity system (Fendt et al., 2008; Siegl et al., 2008).

2.4. Procedure

2.4.1. Fear conditioning (day 1)

The same fear conditioning procedure was used for all experiments. Rats were placed in the conditioning chamber (context A) and, after a 5 min adaptation period were exposed to 5 CS-US pairings with a 2 min inter-trial interval. The conditioned stimulus (CS) was a 80 dB, 4.5 kHz, 30 s white noise, which co-terminated with a mild electric foot-shock (unconditioned stimulus, US, 0.7 mA, 2 s). After the CS-US pairings, rats were left in the conditioning chamber for 5 min before they were returned to their home-cage.

2.4.2. Extinction training (day 2)

To determine whether mGluR compounds differentially affected within-session extinction or extinction consolidation/retention (between-session extinction) two different protocols were employed (see Table 1).

Between-session procedure: Approximately 24 h after fear conditioning, rats were placed in context B and allowed a 5 min adaptation period. Rats were then given 6 CS presentations (white noise, 80 dB, 4.5 kHz, 30 s, 5 s inter-stimulus interval) – so that no detectable decrease in freezing behavior occurred within the session. Immediately after, rats were injected i.p. with either DCS or saline (experiment 1); with MPEP, AMN082 or 0.5% methylcellulose (experiment 4) or with fluoxetine or saline (experiment 5). Experiments 1, 4 and 5 were designed to investigate the effects of DCS, MPEP, AMN082, and fluoxetine on the consolidation of extinction memory. The freezing duration was shown individually during these 6 CS, to demonstrate the lack of decrease in freezing between trials.

Within-session procedure: In experiment 2, rats were injected i.p. with DCS or saline 40 min before extinction training. In experiment 3, rats were injected i.p. with MPEP, AMN082 or 0.5% methylcellulose 40 min before extinction training. Extinction training occurred approximately 24 h after fear conditioning. Rats were placed in context B and, after a 5 min adaptation period received 30 CS presentations (white noise, 80 dB, 4.5 kHz, 30 s, 5 s inter-stimulus interval). These 30 CS were then collapsed into 10 blocks with the mean inactivity time of 3 CS presentations represented in each block. Rats were returned to their home-cage 5 min after the last CS presentation. Experiments 2 and 3 were designed to investigate the effects of DCS, MPEP and AMN082 on within-session extinction.

2.4.3. Extinction retention (day 3)

The same extinction retention procedure was used for all experiments. Approximately 24 h after extinction training, rats were placed in context B and allowed a 5 min adaptation period. All rats received one CS presentation (white noise, 80 dB, 4.5 kHz, 30 s). Afterwards, rats were returned to the animal facility.

2.5. Statistical analysis

Fear conditioning and extinction training data were analyzed using repeated measures ANOVA, followed by a Bonferroni *post-hoc* analysis whenever ANOVA was significant. Freezing duration in extinction retention was analyzed using either an unpaired *t*-test (DCS and fluoxetine experiments) or a one-way ANOVA followed by a Bonferroni *post-hoc* analysis (AMN082 and MPEP experiments). The criterion for significance for all comparisons was $p < 0.05$.

3. Results

3.1. Effect of DCS on between-session extinction (experiment 1)

In this experiment, we examined whether DCS has an effect on between-session extinction of conditioned fear. Therefore, vehicle ($n = 18$) or DCS ($n = 12$) was administered i.p. immediately after extinction training to rats that were not fully extinguished.

Conditioning. Fear conditioning was successful, as the level of freezing increased across trials ($F_{(4,112)} = 12.477$; $p < 0.001$; Fig. 1A). Rats were assigned into two treatment groups, which did not differ in conditioning ($F_{(1,28)} = 0.003$; n.s.).

Extinction training. Analysis of CS-freezing levels revealed successful fear-expression, as shown by increased freezing during CS presentation across trials 24 h after conditioning ($F_{(5,140)} = 8.603$; $p < 0.001$). There was no difference between the vehicle- and DCS-treated rats ($F_{(1,28)} = 0.719$; n.s.; Fig. 1B).

Extinction retention. There was a significant difference in CS-elicited freezing, with DCS-treated rats exhibiting lower levels of freezing than vehicle-treated rats ($T_{(28)} = 2.179$; $p < 0.05$; Fig. 1C).

3.2. Effect of DCS on within-session extinction (experiment 2)

In this experiment, we examined whether DCS has an effect on within-session extinction of conditioned fear. Therefore, 40 min before extinction training, rats were injected i.p. with either vehicle ($n = 6$) or DCS ($n = 12$).

Conditioning. Fear conditioning was successful, as the level of freezing increased across trials ($F_{(4,64)} = 7.229$; $p < 0.001$; Fig. 2A). Rats were assigned into two treatment groups, which did not differ in conditioning ($F_{(1,16)} = 0.006$; n.s.).

Extinction training. Extinction was successful in both groups, as the high levels of freezing during the first trials decreased substantially by the last trial ($F_{(9,144)} = 4.119$; $p < 0.001$; Fig. 2B). There was no difference between the vehicle- and DCS-treated rats ($F_{(1,16)} = 0.537$; *n.s.*).

Extinction retention. There was a significant difference in CS-elicited freezing, with DCS-treated rats exhibiting lower levels of freezing than vehicle-treated rats ($T_{(16)} = 2.631$; $p < 0.05$; Fig. 2C).

3.3. Effect of AMN082 and MPEP on within-session extinction (experiment 3)

In this experiment, we examined whether pharmacological modulation of mGluR5 and mGluR7 influenced within-session cued-fear extinction. Therefore, 40 min before extinction training, rats were injected i.p. with either vehicle ($n = 32$), MPEP ($n = 12$), or AMN082 ($n = 27$).

Conditioning. Fear conditioning was successful, as the level of freezing increased across trials ($F_{(4,272)} = 30.425$; $p < 0.001$; Fig. 3A). Rats were assigned into three treatment groups, which did not differ in conditioning ($F_{(2,68)} = 1.395$; n.s.).

Extinction training. Extinction was successful, as the high levels of freezing during the first trials decreased substantially by the last trial ($F_{(9,612)} = 10.430$; $p < 0.001$; Fig. 3B). There was a significant

Table 1
Time-lines of the experimental procedure.

	Day 1	Day2	Day 3
Experiment 1	Conditioning	Extinction (6 CS) – DCS immediately after	Retention
Experiment 2	Conditioning	DCS 40 min before – Extinction (30 CS)	Retention
Experiment 3	Conditioning	MPEP/AMN082 40 min before – Extinction (30 CS)	Retention
Experiment 4	Conditioning	Extinction (6 CS) – MPEP/AMN082 immediately after	Retention
Experiment 5	Conditioning	Extinction (6 CS) – fluoxetine immediately after	Retention

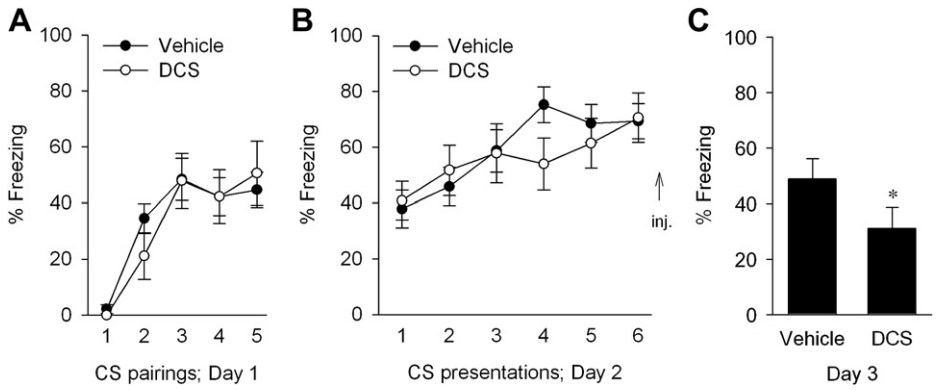

Fig. 1. DCS enhanced retention of between-session extinction of learned fear. (A) All rats were subjected to cued-fear conditioning on day 1. (B) On day 2, expression of learned fear was evaluated and, immediately after, rats were injected i.p. with either vehicle ($n = 18$) or DCS (15 mg/kg; $n = 12$). (C) Extinction retention was measured on day 3. Data represent mean time of CS-elicited freezing ±SEM. $^*p < 0.05$ compared with vehicle treatment.

effect of treatment ($F_{(2,68)} = 5.292$; $p < 0.01$), with AMN082-treated rats exhibiting higher levels of freezing during trial 8 and 9 than vehicle-treated rats ($p < 0.05$; Fig. 3B), while MPEP did not affect extinction training. As AMN082 appeared to enhance the original fear memory, we performed a separate analysis of freezing during the first CS block, which indeed confirmed that the AMN082 group displayed enhanced freezing compared with the vehicle group (Mann Whitney U test; $p < 0.05$).

Extinction retention. There was no difference in CS-elicited freezing between vehicle-, MPEP-, and AMN082-treated rats ($F_{(2,68)} = 0.743$; n.s.; Fig. 3C).

3.4. Effect of AMN082 and MPEP on between-session extinction (experiment 4)

In this experiment, we examined whether pharmacological modulation of mGluR5 and mGluR7 influenced between-session cued-fear extinction. Therefore, vehicle ($n = 20$), MPEP ($n = 11$), or AMN082 ($n = 14$) was administered i.p. immediately after extinction training to rats that were not fully extinguished.

Conditioning. Fear conditioning was successful, as the level of freezing increased across trials ($F_{(4,168)} = 20.769$; $p < 0.001$; Fig. 4A). Rats were assigned into three treatment groups, which did not differ in conditioning ($F_{(2,42)} = 0.228$; n.s.).

Extinction training. Analysis of CS-freezing levels revealed successful fear-expression, as shown by increased freezing during CS presentation across trials 24 h after conditioning ($F_{(5,210)} = 15.997$; $p < 0.001$). There was no difference between the vehicle-, AMN082-, and MPEP-treated rats ($F_{(2,42)} = 0.160$; n.s.; Fig. 4B).

Extinction retention. There was a significant difference in CS-elicited freezing ($F_{(2,42)} = 4.168$; $p < 0.05$; Fig. 4C), with AMN082-treated rats exhibiting lower levels of freezing than vehicle-treated rats ($p < 0.05$), while MPEP did not affect extinction retention.

3.5. Effect of fluoxetine on between-session extinction (experiment 5)

In this experiment, we examined whether fluoxetine has an effect on between-session extinction of conditioned fear as it was previously described that AMN082 has, in addition to its mGluR7 activating properties, fluoxetine-like pharmacology at serotonin (and noradrenaline) reuptake transporters (Sukoff Rizzo et al., 2011) and because the effect of SSRIs on between-session extinction of conditioned fear is not well documented. Therefore, vehicle ($n = 8$) or fluoxetine ($n = 11$) were administered i.p. immediately after extinction training to rats that were not fully extinguished.

Conditioning. Fear conditioning was successful, as the level of freezing increased across trials ($F_{(4,68)} = 7.863$; $p < 0.001$; Fig. 5A). Rats were assigned into two treatment groups, which did not differ in conditioning ($F_{(1,17)} = 0.064$; n.s.).

Extinction training. Analysis of CS-freezing levels revealed successful fear-expression, as shown by increased freezing during

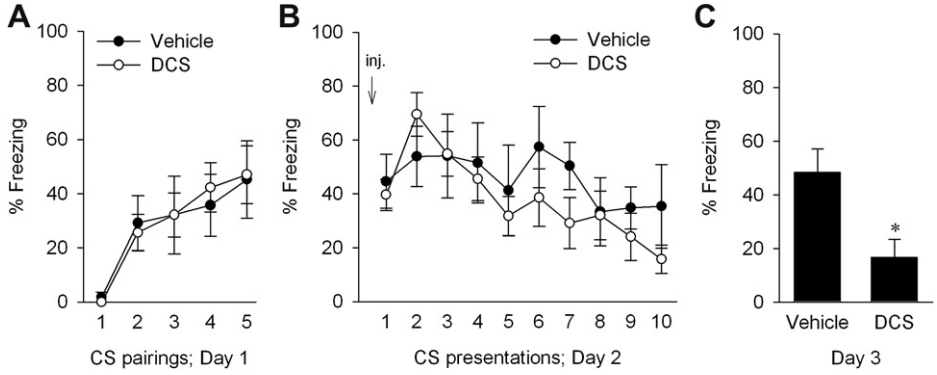

Fig. 2. DCS enhanced retention of within-session extinction of learned fear, without affecting extinction training. (A) All rats were subjected to cued-fear conditioning on day 1. (B) On day 2, rats were injected i.p. with either vehicle ($n = 6$) or DCS (15 mg/kg; $n = 12$) and 40 min later extinction training was evaluated. (C) Extinction retention was measured on day 3. Data represent mean time of CS-elicited freezing ±SEM. $^*p < 0.05$ compared with vehicle treatment.

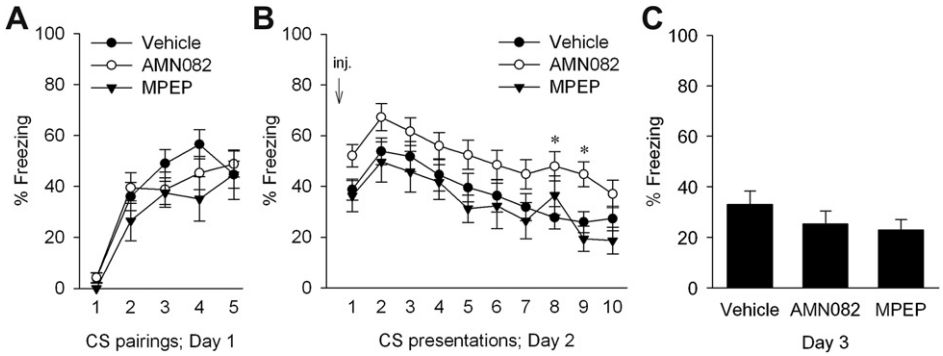

Fig. 3. AMN082, but not MPEP, enhanced freezing and attenuated within-session extinction training of learned fear, without affecting extinction retention. (A) All rats were subjected to cued-fear conditioning on day 1. (B) On day 2, rats were injected i.p. with either vehicle ($n = 32$), MPEP (10 mg/kg; $n = 12$), or AMN082 (10 mg/kg; $n = 27$) and 40 min later extinction training was evaluated. (C) Extinction retention was measured on day 3. Data represent mean time of CS-elicited freezing ±SEM. $^*p < 0.05$ compared with vehicle treatment.

CS presentation across trials 24 h after conditioning ($F_{(5,85)} = 11.855$; $p < 0.001$). There was no difference between the vehicle- and fluoxetine-treated rats ($F_{(1,17)} = 0.895$; n.s.; Fig. 5B).

Extinction retention. There was no significant difference in CS-elicited freezing between vehicle- and fluoxetine-treated rats ($T_{(17)} = 0.263$; n.s.; Fig. 5C).

4. Discussion

The present findings demonstrate that pharmacological modulation of mGluR7, but not mGluR5, alters the extinction of conditioned fear memories. In more detail, blockade of the mGluR5 system via systemic administration of the antagonist MPEP affected neither within-session- nor between-session extinction training or extinction retention. In contrast, administration of the mGluR7 activator AMN082 enhanced freezing and thereby attenuated within-session extinction training but improved between-session extinction retention, to a greater degree than DCS. As a major metabolite of AMN082 also blocks serontonin- and to a lesser extent catecholamine-transporters (Sukoff Rizzo et al., 2011), we assessed the effect of fluoxetine administration on between-session extinction retention revealing that it has no effect on this process. Together, these findings support an mGluR7-mediated facilitatory mechanism of action of AMN082 on extinction retention. These results have important clinical connotations as they suggest that mGluR7-, but not mGluR5-, directed drugs could be used to facilitate extinction retention of learned fear during long-term exposure therapy. Moreover, they reveal that careful choice of when to modulate the mGluR7 system is required (see below).

Progress towards a better understanding of neural systems involved in learned fear extinction and extinction retention could lead to novel therapeutic strategies for the treatment of anxiety disorders. The most widely acknowledged system involved in these processes is the NMDA receptor, specifically its positive modulation by DCS (Myers et al., 2010). In the present study, we demonstrate that systemic DCS administration facilitates between-session extinction retention (Figs. 1 and 2), which concurs with the present literature, in both rodents and humans (Myers et al., 2010). Moreover, it has been recently shown that intra-hippocampal administration of spermine, which is also an NMDA-receptor modulator, facilitates extinction retention in an inhibitory avoidance paradigm (Gomes et al., 2010). However, fewer studies exist assessing the ability of DCS to affect within-session extinction training with those performed suggesting that DCS has no effect on this process (Lee et al., 2006; Walker et al., 2002a), which our present findings are in agreement with (Fig. 2).

While a great deal is known regarding NMDA-receptors in extinction processes, only a very limited number of studies addressed the potential role of mGluR5 and mGluR7 in the extinction of learned fear memories (Callaerts-Vegh et al., 2006; Fendt et al., 2008; Goddyn et al., 2008; Xu et al., 2009). In the present study, we demonstrate that administration of the selective mGluR5 antagonist MPEP prior to extinction training did not affect within-session extinction or extinction retention (Fig. 3). In contrast, systemic administration of the mGluR7 agonist, AMN082, at the same time point, enhanced freezing and thereby attenuated within-session extinction training but not extinction retention (Fig. 3). Similarly, MPEP administration, after incomplete extinction

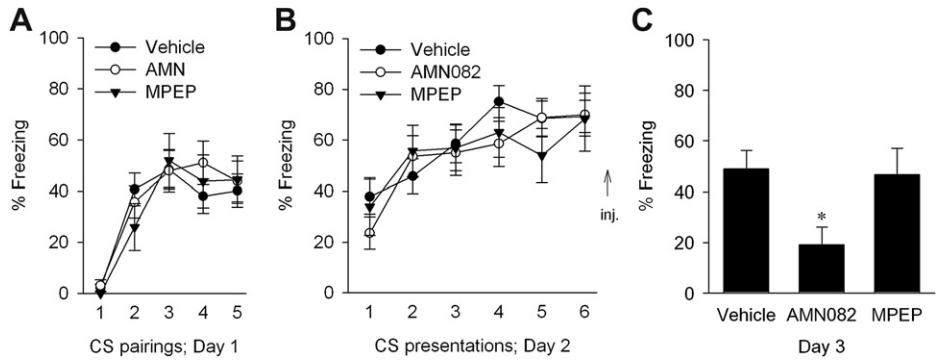

Fig. 4. AMN082, but not MPEP, enhanced retention of between-session extinction of learned fear. (A) All rats were subjected to cued-fear conditioning on day 1. (B) On day 2, expression of learned fear was evaluated and, immediately after, rats were injected i.p. with either vehicle ($n = 20$), MPEP (10 mg/kg; $n = 11$), or AMN082 (10 mg/kg; $n = 14$). (C) Extinction retention was measured on day 3. Data represent mean time of CS-elicited freezing ±SEM. $^*p < 0.05$ compared with vehicle treatment.

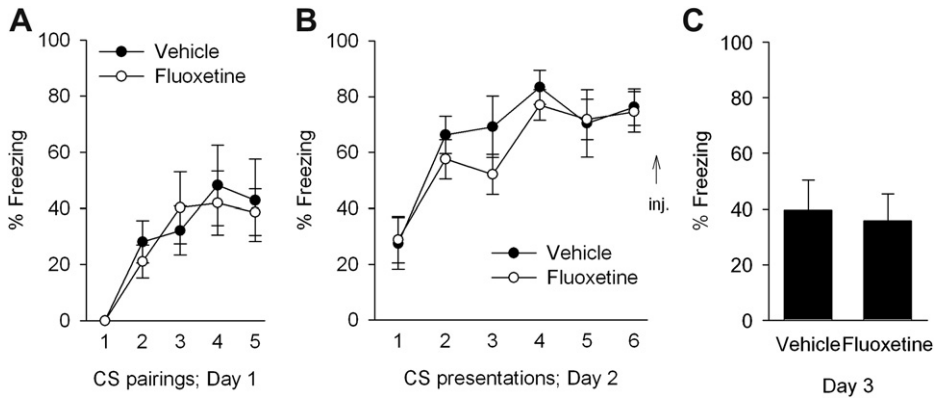

Fig. 5. Fluoxetine did not affect retention of between-session extinction of learned fear. (A) All rats were subjected to cued-fear conditioning on day 1. (B) On day 2, expression of learned fear was evaluated and, immediately after, rats were injected i.p. with either vehicle ($n = 8$) or fluoxetine (10 mg/kg; $n = 11$). (C) Extinction retention was measured on day 3. Data represent mean time of CS-elicited freezing ±SEM.

training, did not affect, whereas AMN082 facilitated, between-session extinction retention (Fig. 4). Taken together, the findings from the different extinction protocols suggest a differential role of mGluR7 pharmacological modulation with the studied modes of action in extinction learning.

One study employing mGluR5 knockout mice demonstrated deficits in conditioned fear behaviour (Xu et al., 2009). In more detail, mGluR5 knockout mice displayed impaired cued-fear acquisition (i.e. impaired learning) but were able to retain the fear memory, albeit at a lower level compared to wild-type controls over several extinction sessions (Xu et al., 2009). A more recent study, assessing the effect of systemic or infralimbic cortex administration of MPEP on extinction revealed that neither manipulation affected within-session extinction training but impaired extinction retention (Fontanez-Nuin et al., 2010). The fact that MPEP-treated rats of our study were shown to have almost complete extinction for the majority of the extinction training implies that the animals had a more significant number of CS presentations to consolidate this memory. This may explain the discrepancy with the study by Fontanez-Nuin et al. (2010) as in the present study MPEP-treated rats were exposed to 30 CS presentations compared to only 20 in the aforementioned study.

The present findings add to our previous results showing that mGluR7 activation facilitates between-session extinction in fear-potentiated startle tests and accelerates learned taste avoidance extinction (Fendt et al., 2008). Additionally, mGluR7 knockout mice have also been shown to have delayed extinction (Callaerts-Vegh et al., 2006). Here, we extend and support these findings to a different, cued-fear conditioning, paradigm. A novel aspect reported here is that activation of the mGluR7 system prior to extinction training enhances freezing and thereby attenuates within-session extinction. Although this was not observed in the former conditioned taste aversion trial (Fendt et al., 2008), it is possible that the high aversion on the first day after conditioning represented a ceiling level, which could not be further increased.

The recent discovery that AMN082 shows, in addition to its mGluR7-activating property, physiologically relevant binding activity to serotonin- and catecholamine-transporters, via a metabolite (Sukoff Rizzo et al., 2011), provides the interesting possibility that facilitation of monoaminergic transmission may contribute, at least in part, to some of our observed effects of AMN082 on extinction of conditioned fear. Interestingly, blockers of serotonin/catecholamine-transporters have also been shown to impair within-session extinction (Burghardt et al., 2007; Santos et al., 2006) but less is known about their effect on between-session extinction. Here, we were able to demonstrate that administration of fluoxetine (which blocks serotonin and to a lesser extent catecholamine-transporters) after an incomplete extinction training session does not affect between-session extinction retention (Fig. 5). Therefore, while the effect of AMN082 on within-session extinction training, i.e. attenuation, is similar to those of acute SSRI treatment, its effect on between-session extinction retention differs. In further support of the hypothesis, while AMN082 enhanced freezing and thereby attenuated within-session extinction training, mediated, at least in part, by its metabolite, extinction retention was not impaired. This is suggestive of a facilitatory effect of mGluR7-activation on extinction retention in both protocols used in the present studies.

In terms of neuronal mechanism behind this facilitatory effect on extinction retention, it has been reported that mGluR7 is located presynaptically (Kinoshita et al., 1998; Ohishi et al., 1995; Shigemoto et al., 1997) and its activation reduces both L-glutamatergic and GABAergic synaptic transmission. These effects are presumably mediated through the inhibition of calcium channels and via down-regulation of cAMP levels; however more recently, mGluR7 activation was also shown to facilitate L-glutamatergic transmission (Martin et al., 2011, 2010; Millan et al., 2002). In the context of our study, it is currently unknown which of the two transmitters is modulated by mGluR7; but the fact that AMN082 also affects the physiology of the amygdala during a blockade of GABAergic activity by picrotoxin (Fendt et al., 2008) lead to the suggestion that the observed AMN082 effects on fear extinction retention are mainly mediated by a modulation of L-glutamate transmission. In addition, it is generally accepted that the ventromedial prefrontal cortex plays a contributing role in between-session extinction retention of conditioned fear (Fontanez-Nuin et al., 2010) and the abundance of mGluR7 protein within the prefrontal cortex and amygdala is relatively high (Kinoshita et al., 1998). Specifically, it is therefore possible that mGluR7 activation in the amygdala inhibits L-glutamate release while activation in the prefrontal cortex may facilitate release. This would result in the amygdala being more inhibited whilst the prefrontal cortex becomes more activated with AMN082, possibly leading to the observed facilitating effect of AMN082 on between-session extinction retention. Future studies involving site-specific injections into the prefrontal cortex will aid in the further elaboration of whether there is a regional basis of the effects of AMN082 on the different forms of extinction.

Taken together, the present findings suggest that pharmacological interference with mGluR7, but not mGluR5, could play an important role in fear extinction. In detail, our results suggest mainly that mGluR7 activation improves consolidation of

extinction training. The finding that activation of mGluR7 facilitates between-session extinction retention is of potential therapeutic importance for the future use of such drugs in human anxiety disorders including PTSD. In particular, we would like to suggest that mGluR7 allosteric activators could be used as add-on drugs following exposure-based psychotherapy sessions and may thus find application for longer-term treatment of incompletely extinguished/persistent fear and aversion memories.

Disclosure

The authors declare no conflict of interest, direct or indirect, in submitting this work.

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