



Differential roles of mGlu₇ and mGlu₈ in amygdala-dependent behavior and physiology



Markus Fendt^{a,*}, Stefan Imobersteg^a, Daniel Peterlik^b, Frédérique Chaperon^a, Catherine Mattes^{a,1}, Christina Wittmann^a, Hans-Rudolf Olpe^a, Johannes Mosbacher^{a,2}, Ivo Vranesic^{a,1}, Herman van der Putten^a, Kevin H. McAllister^{a,1}, Peter J. Flor^b, Christine E. Gee^{a,**}

^a Novartis Institutes for BioMedical Research, Neuroscience DA, Forum 1, Novartis Campus, CH-4056 Basel, Switzerland

^b University of Regensburg, Faculty of Biology and Preclinical Medicine, Universitätsstrasse 31, D-93053 Regensburg, Germany

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ABSTRACT

Glutamate transmission and synaptic plasticity in the amygdala are essential for the learning and expression of conditioned fear. Glutamate activates both ionotropic glutamate receptors and eight subtypes of metabotropic glutamate receptors (mGlu_{1–8}). In the present study, we investigated the roles of mGlu₇ and mGlu₈ in amygdala-dependent behavior and synaptic plasticity. We show that ablation of mGlu₇ but not mGlu₈ attenuates long-term potentiation (LTP) at thalamo-lateral amygdala (LA) synapses where a strong association between LTP and learning has been demonstrated. mGlu₇-deficient mice express a general deficit in conditioned fear whereas mGlu₈-deficient mice show a dramatic reduction in contextual fear. The mGlu₇ agonist AMN082 reduced thalamo-LA LTP and intra-amygdala administration blocked conditioned fear learning. In contrast, the mGlu₈ agonist DCPG decreased synaptic transmission but not LTP at thalamo-LA synapses. Intra-amygdala DCPG selectively reduced the expression of contextual fear but did not affect the acquisition and expression of cued fear. Taken together, these data revealed very different roles for mGlu₇ and mGlu₈ in amygdala synaptic transmission, fear learning and its expression. These receptors seem promising targets for treating anxiety disorders with different underlying pathologies with exaggerated fear learning (mGlu₇) or contextual fear (mGlu₈).

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1. Introduction

The amygdala plays a critical role in all phases of Pavlovian fear conditioning, i.e. in acquisition, consolidation, expression and extinction of conditioned fear (Davis, 1992; Fendt and Fanselow, 1999; LeDoux, 1995). In the lateral nucleus of the amygdala (LA), sensory information converges from the neutral to-be-conditioned stimulus (CS) and the noxious fear-inducing unconditioned

stimulus (Romanski and LeDoux, 1992). It is here that both acquisition and consolidation of the learned association occur (Fanselow et al., 1994; Miserendino et al., 1990). The potentiation of sensory-evoked activity in the LA, long-term potentiation (LTP), and the acquisition of conditioned fear have common underlying mechanisms (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). The LA consists mainly of glutamatergic neurons and a much smaller number of interspersed GABAergic neurons (e.g., Ehrlich et al., 2009; Pare et al., 2004). Increasing the GABAergic tone in the amygdala of experimental animals, e.g. by local administration of agonists or positive modulators of the GABA_A receptor, reduces or blocks the learning and retrieval of conditioned fear (Harris and Westbrook, 1995; Helmstetter and Bellgowan, 1994). Furthermore, the amygdala is crucial for the expression of conditioned fear (Kim et al., 1993) and is involved in its extinction (Falls et al., 1992). In humans, hyperexcitability of the amygdala is believed to be a common neuropathological hallmark of anxiety disorders (Etkin and Wager, 2007). Positive modulators of the GABA_A receptor, such as benzodiazepines, decrease amygdala activity (Paulus et al., 2005) and are clinically efficacious in patients with anxiety

* Corresponding author. Current address: Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Leipziger Straße 44, D-39120 Magdeburg, Germany. Tel.: +49 391 6721982.

** Corresponding author. Current address: Center for Molecular Neurobiology, University Medical Center Hamburg-Eppendorf, Falkenried 94, D-20251 Hamburg, Germany.

E-mail addresses: markus.fendt@med.ovgu.de (M. Fendt), christine.gee@zmnh.uni-hamburg.de (C.E. Gee).

¹ Current address: Novartis Pharma, Forum 1, Novartis Campus, CH-4056 Basel, Switzerland.

² Current address: F. Hoffmann-La Roche AG, Pharma Research & Early Development, DTA Neuroscience, Grenzacherstrasse 124, CH-4070 Basel, Switzerland.

disorders (Nemeroff, 2003). However, due to the side effects and/or the modest efficacy of the currently clinically established anxiolytic drugs, there is still a strong medical need for mechanistically novel pharmacological treatments.

Modulating glutamatergic transmission in the amygdala can also decrease conditioned fear and anxiety disorders. Local administration of AMPA/kainate or NMDA receptor antagonists reduces learning and/or expression of conditioned fear (Kim et al., 1993; Miserendino et al., 1990). In an open-label clinical study, the partial NMDA receptor antagonist memantine showed some promise as an add-on therapy for anxiety disorders (Schwartz et al., 2012). However, targeting these receptors systemically for therapeutic applications is extremely challenging as they are widely distributed and involved in many other key brain functions such as learning and memory and the control of autonomic functions.

Glutamatergic transmission can also be modulated by targeting the G-protein coupled metabotropic glutamate receptors (mGlu). Unlike mGlu₁ and mGlu₅ which are mainly postsynaptic, other subtypes including mGlu₂, mGlu₃, mGlu₇ and mGlu₈ are preferentially located close to presynaptic neurotransmitter release sites (Ferraguti et al., 2005; Neki et al., 1996; Petralia et al., 1996; Shigemoto et al., 1997). Recently, mGlu_{2/3} agonists, which decrease neurotransmitter release, have shown efficacy in the treatment of generalized anxiety disorders (Dunayevich et al., 2008). Here, we focus on mGlu₇ and mGlu₈ that are exclusively presynaptically located and that are often co-expressed in brain areas involved in the control of emotions, including the amygdala (Corti et al., 1998).

The goal of the present study was to further elaborate and compare the roles of mGlu₇ and mGlu₈ in amygdala-dependent fear learning and synaptic plasticity. To this end, we used the allosteric mGlu₇ agonist AMN082 (Mitsukawa et al., 2005), the orthosteric mGlu₈ agonist DCPG (Thomas et al., 2001) and mGlu₇- and mGlu₈-deficient mice (Duvoisin et al., 1995; Sansig et al., 2001). First, we compared the effects of AMN082, DCPG, and mGlu₇- and mGlu₈-ablation on synaptic transmission and thalamo-LA LTP in amygdala slices. Second, we compared the behavior of mGlu₇- and mGlu₈-deficient mice in Pavlovian fear conditioning and tested the effects of intra-amygdala administered AMN082 and DCPG on acquisition, retention and extinction of conditioned fear memory.

2. Material and methods

2.1. Animals

Male C57BL/6J (Janvier, Le Genest Saint Isle, France), mGlu₇-deficient mice (Cryan et al., 2003; Sansig et al., 2001), and mGlu₈-deficient mice (Duvoisin et al., 2005) were used for this study. The animals were housed in groups of 2–4 in a humidity (55%) and temperature (22 °C) controlled room under a 12 h/12 h day–night cycle with lights on at 07:00 am. Water and food were available ad libitum. Experiments were in accordance with the Swiss law and international guidelines for the care and use of animals and approved by the local ethics committee (Kantonales Veterinäramt Basel-Stadt, Switzerland).

2.2. Electrophysiology

For the preparation of acute coronal brain slices containing the amygdala, mice were anaesthetized with isoflurane and sacrificed by decapitation. The skull covering the cortex was removed and the brain was quickly excised and placed in ice-cold artificial cerebrospinal fluid (ACSF) equilibrated with 95% O₂/5% CO₂ containing (in mM): NaCl (124), KCl (2.5), KH₂PO₄ (1.2), CaCl₂ (2.5), MgSO₄ (1.3), NaHCO₃ (26), glucose (10) and saccharose (4) (pH 7.4, osmolarity adjusted to 320 ± 2 mOsm by reducing the amount of H₂O). The brain was trimmed and affixed, caudal side down, to the stage of a vibrating microtome with cyanoacrylate glue. 350–400 μm thick coronal slices were cut and those containing the amygdala complex were maintained at room temperature in the same solution but fully diluted to give osmolarity 306 ± 2 mOsm.

For field recordings, slices were transferred to an interface-type recording chamber and superfused with ACSF containing 5 μM picrotoxin (to partially block inhibition) at 27 °C. Stimulation and recording electrodes were positioned to activate thalamic inputs and to record field excitatory post-synaptic potentials (fEPSPs)

from the lateral amygdala (LA; see Chaperon et al., 2012; Humeau et al., 2003). Where indicated the stimulating electrode was positioned to activate cortical inputs. The stimulation electrodes were made from twisted nichrome wires (50 μm diameter) and stimuli were delivered with a constant current stimulus isolation unit to evoke fEPSPs in the LA. Glass recording electrodes had resistances of 3–5 MΩ when filled with 4 M NaCl. Responses were recorded with an Axoprobe 1 A amplifier and pClamp 9.0 software. Input/output curves were obtained and the stimulation intensity was adjusted to evoke a fEPSP that was 25–40% of the maximum. Test stimuli were delivered every 20 s. After recording a stable baseline, substances were perfused for 20 min where indicated and a second input–output curve was obtained. If necessary, the stimulus intensity was re-adjusted.

After recording test responses for a further 10 min baseline period, long-term potentiation was induced with five 1 s trains of 100 Hz stimuli delivered every 20 s. Test responses were then recorded for an additional 40 min. AMN082 was dissolved in DMSO and diluted at least 1000× into the artificial cerebrospinal fluid so that the final concentration of DMSO did not exceed 0.1%. DCPG was dissolved in ACSF. Control experiments were performed with an equal amount of DMSO or ACSF added to the perfusate.

To record excitatory post-synaptic currents, the same ACSF was used and patch electrodes (~5 MΩ) were filled with (in mM): K-gluconate (120), HEPES (10), Mg-ATP (4), Phosphocreatine (10), KCl (20), Na-GTP (0.3), MgCl (1), pH 7.25. Whole-cell patch-clamp recordings were obtained under visual control from LA pyramidal neurons. Series resistance was less than 20 MΩ. Membrane potential was clamped at –65 mV.

2.3. Behavior

2.3.1. Apparatus

To measure conditioned freezing, a computerized fear-conditioning system (TSE, Bad Homburg, Germany) was used. The system consisted of four identical transparent Plexiglas boxes (46 cm × 46 cm × 32 cm) placed inside an animal detection infrared sensor frame. Each box was located in a sound-attenuating chamber provided with loudspeakers for the acoustic stimuli (background noise of 60 dB SPL and the tone stimuli for fear conditioning), with light sources (continuous illumination of ca. 10 lux), and with a ventilation fan. The floor of the boxes consisted of removable stainless steel grids (bars: 4 mm diameter, distance from rod center to rod center: 9 mm) that were connected to a shock unit and able to deliver foot shocks of defined duration and intensity. Delivery of all stimuli was controlled by a personal computer. Four additional boxes of the same size as those described above but made of black Perspex (including the floor) served to create a different context for the cued fear test.

Movements of the animals were detected by the infrared sensors. The time spent freezing (immobility) was automatically recorded (no infrared beam crosses for more than 1 s) during all phases of the experiments. Automatically measured freezing in this system is highly correlated with human observer scoring of freezing (Andres et al., 2007; Misane et al., 2005).

2.3.2. Experimental procedure (transgenic mice)

On day 1, fear conditioning was performed in the transparent Perspex boxes. The animals were placed individually into the boxes. Sixty seconds later, the first of six pairings of a tone stimulus (8 kHz, 80 dB, 30 s) and a scrambled foot shock (0.6 mA, during the last two second of the tone stimulus) were presented. The inter trial intervals were 60 s. Thirty seconds after the last pairing, the mice were returned to the home cage. Then, the boxes were cleaned with 70% ethanol and dried.

On the next day, the animals were put into the conditioning context (transparent boxes) for 5 min to evaluate conditioned context fear. Then, they were put back into the homecage. Two hours later, a test on cued fear was performed. For this test, the second context (black boxes) was used and the tone stimulus, which was used during the conditioning, was presented 10 times with an interstimulus interval of 60 s. On the following two or three days, the test on cued fear was repeated. The transgenic mice were not treated during the experiment.

2.3.3. Experimental procedure (intra-amygdala injections)

Mice were anesthetized with ketamine/xylazine (110 mg/kg, 10:1, i.p.) and placed into a stereotaxic frame. The skull was exposed and stainless steel guide cannulae (diameter: 0.35 mm; length: 6 mm) were bilaterally implanted aiming at the amygdala. The following coordinates were used (Paxinos and Franklin, 2001): 1.5 mm caudal from Bregma, ±3.5 mm lateral from Bregma, –3.7 mm ventral from dura. The guide cannulae were fixed to the skull with dental cement and 2–3 anchoring screws (2 × 2.75 mm; Laubscher, Täuffelen, Switzerland). To prevent post-surgery pain, the analgesic buprenorphine (0.01 mg/kg, i.p.) was given twice a day on the first two days following surgery. Following full recovery (5–6 days), behavioral tests identical to the first two test days described above (conditioning, test on cued and contextual fear) began.

In three different experiments, 1 μM AMN082, 3.3 μM DCPG, or the respective vehicle was bilaterally injected into the amygdalae either before fear conditioning, before testing contextual fear, or before testing cued fear. The other phases of each experiment were without treatment. AMN082 or DCPG were injected into the amygdala at concentrations previously demonstrated to be efficacious in rats

(Schmid and Fendt, 2006; Siegl et al., 2008). Injectors with a diameter of 0.15 mm (connected to Hamilton syringes by tubes) were introduced into the guide cannulae and a total volume of 0.3 μ l solution/side were injected with a speed of 0.1 μ l/min, controlled by a microinfusion pump. The injector was removed after an additional 60 s, and the mice were returned to their home cage for 10 min. Then, the animals were placed in the experimental setup.

2.3.4. Histology

Immediately after the final behavioral test, all mice with intra-amygdala cannulae were euthanized and the brains were removed for histological analysis to verify the injection sites were within the amygdala. The brains were immersion-fixed with 4% formaldehyde and stored in fixative until further processing. Prior to sectioning, the brains were transferred to phosphate buffered 30% sucrose for at least 12 h. Frontal sections (100 μ m) were cut on a freezing microtome and stained with cresyl violet. The injection sites were localized and the extent of tissue lesion due to cannulation was assessed under a light microscope. The injection sites were drawn onto plates taken from a mouse brain atlas (Paxinos and Franklin, 2001).

2.4. Statistical analyses

Data were analyzed with custom written analysis routines in VBA and Excel, or with SYSTAT (SPSS Inc, version 11). Electrophysiological data were normalized to the baseline fEPSP slope. All data were normally distributed (Kolmogorov–Smirnov test) and are expressed as mean \pm SEM. For statistical analysis, either Student *t*-tests, paired *t*-tests or analyses of variance (ANOVA) were used. The ANOVAs had treatment or genotype between-subject factor and trial number as within-subject factor. In case of significance ($p < 0.05$), post-hoc Dunnett's or Tukey tests were carried out.

3. Results

3.1. Electrophysiology

3.1.1. Basal synaptic transmission in thalamo-LA synapses

Field potentials in the LA were evoked by stimulation of thalamic inputs (see Humeau et al., 2003). Application of the mGlu₈ agonist, DCPG (5 μ M) reduced thalamo-LA field potentials in wild-type mice (C57BL/6 background, Fig. 1A; paired *t*-test, $p = 0.006$). This depression was specific to activation of mGlu₈ receptors as DCPG had no effect on thalamo-LA field potentials in mice lacking mGlu₈ (Fig. 1A; paired *t*-test, $p = 0.95$). Similarly to what has been reported previously, neither application of the mGlu₇ agonist AMN082 (1 μ M in 0.01% DMSO) nor vehicle (0.01% DMSO) had any effect on thalamo-LA field potentials recorded from wildtype C57BL/6 mouse brain (Fig. 1B, DMSO paired *t*-test, $p = 0.83$; AMN082 paired *t*-test, $p = 0.10$; see also Schmid and Fendt, 2006). Also the very first responses during wash-in of AMN082 were unaffected. Thus, allosteric activation mGlu₇ had no effect on basal transmission at thalamo-LA connections whereas these were depressed by activation of mGlu₈.

Others reported that EPSPs in the basal amygdala evoked at 2 Hz but not at 0.05 Hz were sensitive to application of 10 μ M AMN082 (Ugolini et al., 2008). We tested whether this may also be the case in the LA by evoking EPSCs at 2 Hz for 2 min in ACSF after the slice had been superfused for 15–20 min with ACSF containing either vehicle (0.1% DMSO) or 10 μ M AMN082 (in 0.1% DMSO). After 1.5 min at 2 Hz, EPSC magnitudes reached steady-state levels and we analyzed the last 50 responses during each 2 min train (Fig. 1C; $n = 8$, mean ACSF $82.2\% \pm 2.3$, mean DMSO $63.5\% \pm 4.8$, mean AMN082 $68.1\% \pm 6.2$, ANOVA $F_{2,21} = 4.2$, $p = 0.03$). EPSCs in the presence of 10 μ M AMN082 were not significantly different from EPSCs evoked in the presence of vehicle only (Fig. 1C; paired *t*-test, $p = 0.27$, DMSO vs AMN082). However, we did observe a DMSO-specific reduction in EPSCs (paired *t*-test, $p = 0.02$ ACSF vs AMN082).

3.1.2. Effects of AMN082 and DCPG on thalamo-LA LTP

LTP, evoked by tetanic stimulation of the thalamo-LA pathway, was strongly reduced by 1 μ M AMN082 compared with LTP evoked in the presence of vehicle (Fig. 2A; *t*-test, $p = 0.05$, AMN082 vs

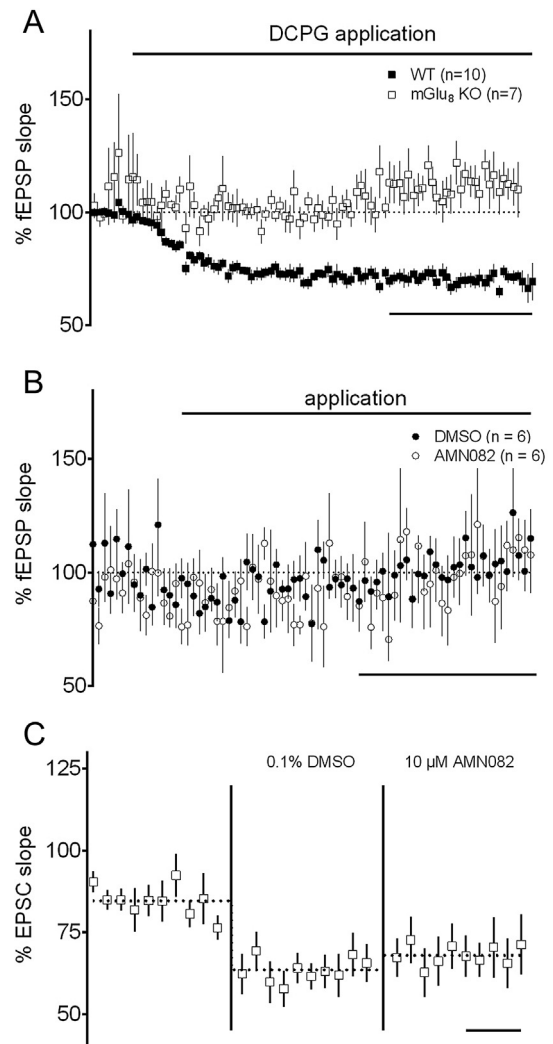


Fig. 1. Effects of mGlu₇ and mGlu₈ agonists on synaptic transmission in the lateral amygdala. A) The mGlu₈ agonist DCPG (5 μ M) was applied as indicated by the horizontal bar in WT and mGlu₈-deficient mice. fEPSPs were evoked by stimulating thalamic inputs and recorded in the LA. Scale bar: 10 min. DCPG decreases fEPSP slope in wildtype but not in mGlu₈-deficient mice. B) Vehicle (0.01% DMSO) or 1 μ M AMN082 were applied in wildtype mice as indicated by the horizontal bar. fEPSPs were evoked by stimulating thalamic inputs and recorded in the LA. Scale bar: 10 min. AMN082 does not affect fEPSPs. C) The vehicle DMSO 0.1% but not AMN082 decreases cortico-LA EPSC slopes ($n = 8$). Cortico-LA EPSCs were evoked in slices from wildtype mice at 2 Hz for 2 min and normalized to test pulses evoked at 0.05 Hz in ACSF. Shown are the pulses from the last 25 s of each of three trains. For illustrative purposes only, 5 pulses were binned prior to averaging. The first train was evoked in ACSF followed sequentially by trains collected after 15–20 min superfusion with ACSF containing vehicle (DMSO 0.1%) followed by 10 μ M AMN082. Scale bar: 10 s.

0.01% DMSO). This effect of AMN082 was mGlu₇-dependent and did not occur in mice lacking mGlu₇ (Fig. 2B; *t*-test, $p = 0.3$ AMN082 vs 0.01% DMSO). DCPG had no effect on LTP evoked by tetanic stimulation of the thalamo-LA pathway (Fig. 2C; *t*-test, $p = 0.96$). Therefore, pharmacological activation of mGlu₇ but not mGlu₈ reduced thalamo-LA LTP.

3.1.3. Thalamo-LA LTP in mGlu₇- and mGlu₈-deficient mice

Interestingly, mice lacking mGlu₇ had reduced thalamo-LA LTP (Fig. 2D; *t*-test, $p = 0.016$, WT vs KO,) which is contrary to what one might have expected considering that the mGlu₇ agonist AMN082 also reduced LTP (Fig. 2A). In contrast, ablation of mGlu₈ had no effect on thalamo-LA LTP (Fig. 2E; *t*-test, $p = 0.90$, WT vs KO) which is in line with the lack of effect of its agonist, DCPG (Fig. 2C).

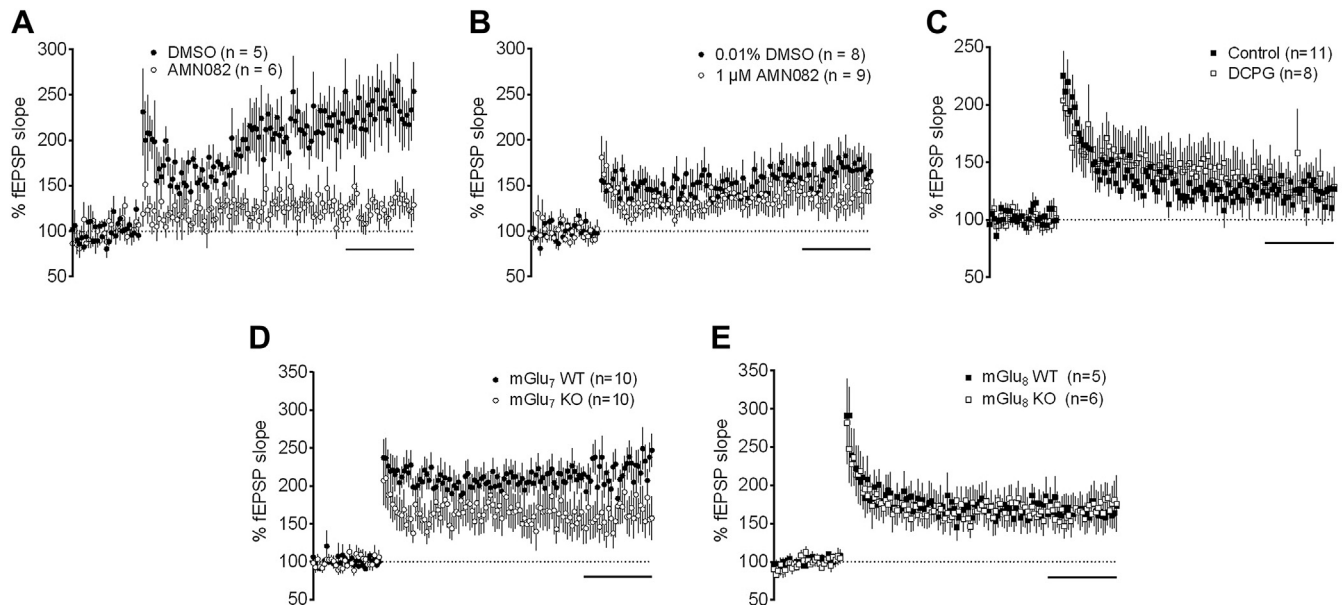


Fig. 2. Contrasting roles of mGlu₇ and mGlu₈ on thalamo-LA plasticity. Field EPSPs were recorded in the lateral amygdala and evoked by stimulation of thalamic inputs. After 10 min stable baseline, LTP was induced by 5×1 s long trains of 100 Hz stimuli evoked at the test intensity. Trains were delivered at 20 s intervals. Responses were recorded for a further 40 min. A) AMN082 blocks LTP in wildtype mice, B) AMN082 has no effects on LTP in mGlu₇-deficient mice, C) DCPG (5 μ M) does not affect LTP in wildtype mice, D) mGlu₇-deficiency decreases LTP, E) mGlu₈-deficiency does not affect LTP. Scale bars: 10 min.

3.2. Behavior

3.2.1. Conditioned fear in mGlu₇-deficient mice

During fear conditioning on day 1, mGlu₇-deficient mice expressed less overall freezing than their wildtype littermates. These mice also showed less increase in freezing with successive pairings of tone and footshock than did the wildtype mice (Fig. 3A; ANOVA, factor genotype: $F_{(1,25)} = 5.03$, $p = 0.03$; factor pairing number: $F_{(5,125)} = 6.83$, $p < 0.001$; interaction genotype \times pairing number: $F_{(5,125)} = 4.41$, $p = 0.003$). Thus mGlu₇-deficient mice showed reduced associative learning of the CS–US.

Prior to the first retention test for cued fear on day 2, mGlu₇-deficient and wildtype littermates showed no differences in freezing (Fig. 3A; t -test, $p = 0.69$). When the CS (tone) was presented, mGlu₇-deficient mice again expressed less freezing than their wildtype littermates confirming that the lack of mGlu₇ had also resulted in a reduced conditioned fear response 24 h after learning (genotype: $F_{(1,25)} = 6.56$, $p = 0.017$). Both the factor presentation number and the interaction genotype \times presentation number failed to reach statistical significance indicating that there was no significant within session extinction on day 2 (F 's < 1.44 , p 's > 0.18). Notably, also the freezing response to the context in which the animals were conditioned, was significantly reduced in mGlu₇-deficient mice on day 2 (t -test, $p < 0.01$) as reported previously (Masugi et al., 1999).

Repeated testing for cued fear on days 2–5 revealed that freezing behavior in wildtype mice decreased on subsequent days whereas mGlu₇-deficient mice showed a marked attenuation of cued fear extinction (Fig. 3A; genotype \times day interaction: $F_{(3,75)} = 16.79$, $p < 0.001$; factor day: $F_{(3,75)} = 31.19$, $p < 0.001$; factor genotype: $F_{(1,25)} = 0.05$, $p = 0.83$). Therefore, a lack of mGlu₇ is associated with reduced expression of contextual fear, a reduced ability of associative cued fear learning and reduced extinction of cued fear.

3.2.2. Conditioned fear in mGlu₈-deficient mice

Both wildtype and mGlu₈-deficient mice showed increased freezing during successive pairings of tone and foot shock indicating fear conditioning was equally successful in both groups of

mice (Fig. 3B; factor pairing number: $F_{(5,95)} = 20.02$, $p < 0.001$). In contrast to mGlu₇-deficient mice, freezing behavior during the fear conditioning process was not significantly affected by the lack of mGlu₈ (Fig. 3B; factor genotype: $F_{(1,19)} = 2.13$, $p = 0.16$; interaction genotype \times pairing number: $F_{(5,95)} = 1.84$, $p = 0.11$). Therefore, genetic ablation of mGlu₈ unlike mGlu₇ did not affect fear learning in our CS–US paradigm.

In the retention test for cued fear on day 2, freezing behavior in mGlu₈-deficient mice was reduced prior to presentation of the first CS (t -test, $p = 0.01$). However, freezing to the CS (tone) was of similar magnitude in mGlu₈-deficient and wildtype littermates ($F_{(1,19)} = 1.71$, $p = 0.21$). There was also neither an effect of trial number ($F_{(9,171)} = 1.00$, $p = 0.43$) nor an interaction between trial number and genotype ($F_{(9,171)} = 1.28$, $p = 0.25$). Thus, unlike mice lacking mGlu₇, mGlu₈-deficient mice showed no CS–US associative learning defect and their ability to respond and remember the CS–US association one day later was similar to wildtype demonstrating that ablation of mGlu₈ had no effect on CS–US fear memory consolidation. However, mGlu₈-deficient mice showed an impaired freezing response when tested for the retention of contextual fear memory, like mGlu₇-deficient mice (t -test, $p < 0.01$).

There was no difference in extinction between mGlu₈-deficient mice and wildtype littermates (factor genotype: $F_{(1,19)} = 1.55$, $p = 0.23$; interaction genotype \times test day: $F_{(2,38)} = 0.29$, $p = 0.73$). Analysis of mean freezing values of the cued fear tests on days 2–4 revealed only a slight trend for between-session extinction of conditioned fear (factor test day: $F_{(2,38)} = 2.94$, $p = 0.06$).

3.2.3. Conditioned fear after intra-amygdala injection of AMN082 and DCPG

Wildtype mice received bilateral implants of guide cannulae and were subjected to cue and context fear conditioning as above. Of the 117 mice that received the implants, 22 animals were excluded from analysis because post hoc histological assessment of the injection sites revealed either a misplaced injection or a tissue lesion in the amygdala. Fig. 4 depicts the positions of all injection sites in the remaining 95 mice. Most injection sites were located either in the lateral, the basolateral or the central nucleus of the amygdala.

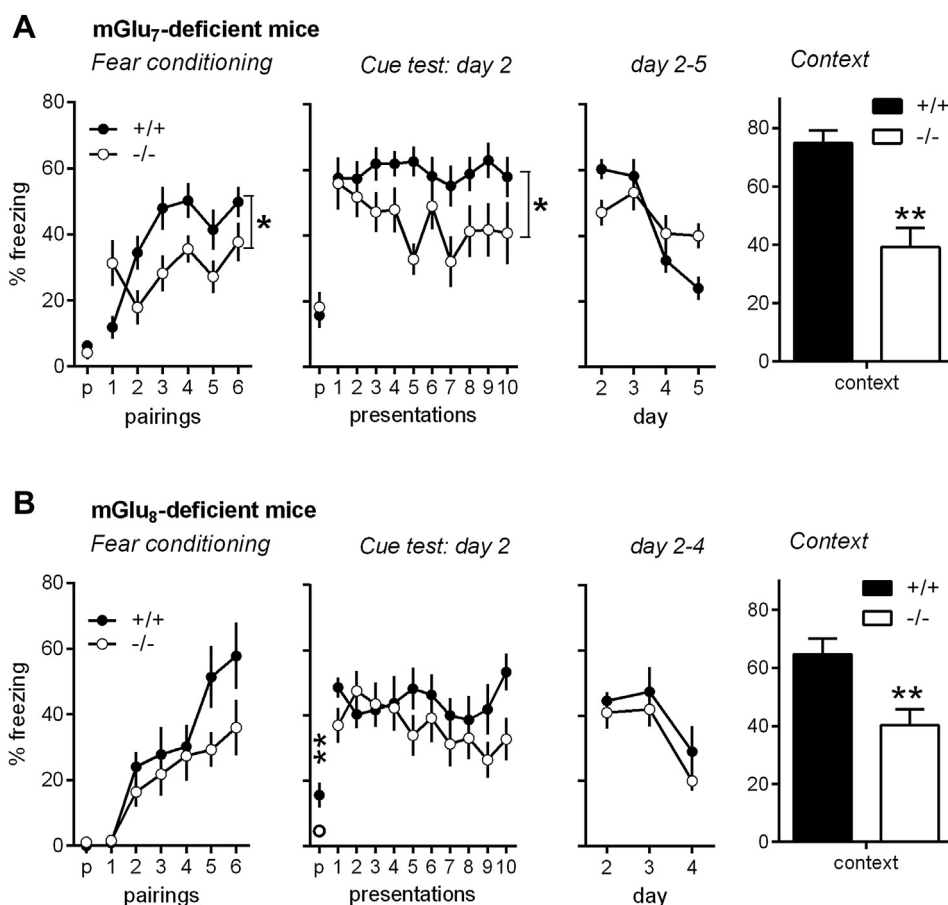


Fig. 3. Contrasting roles of mGlu₇ and mGlu₈ deficiency on acquisition (fear conditioning), expression (cue test, context), and extinction (day 2–5) of conditioned fear measured by freezing. A) mGlu₇-deficient mice ($n_s = 13–14$) express less freezing during fear conditioning and during the tests on cued and contextual fear. B) mGlu₈-deficient mice ($n_s = 9–12$) have a deficit in contextual fear. p = pre-CS freezing, ** $p < 0.01$, * $p < 0.05$, Student t -test (pre-CS, context) or ANOVA (fear conditioning, cue test, day 2–5).

As the injection radius is on the order of 0.5–1 mm (Allen et al., 2008; Martin, 1991), the entire mouse amygdala would be affected by all injections included in the analysis. Vehicle, 1 μ M AMN082, or 3.3 μ M DCPG were injected at a volume of 0.3 μ l either before conditioning or shortly before testing for the contextual or cued fear response. Each animal received drug only once.

Fig. 5A shows the freezing behavior of mice that received AMN082 immediately before fear conditioning. The freezing behavior during fear conditioning was not affected by AMN082 (ANOVA, factor treatment: $F_{(1,23)} = 0.02$, $p = 0.90$; factor pairing number: $F_{(5,115)} = 7.76$, $p < 0.001$; interaction: $F_{(5,115)} = 1.54$, $p = 0.20$). However, freezing responses to both the context as well as to the tone cue were significantly attenuated when fear memory retention was tested on day 2 (factor treatment, context: $F_{(1,23)} = 6.81$, $p = 0.02$; cue: $F_{(1,23)} = 4.60$, $p = 0.04$). The results in both tests are reminiscent of an extinction of freezing behavior (factor minute or cue presentation number: $F_s > 2.42$, $p_s < 0.05$) and it occurred to a similar extent (interaction treatment \times minute/presentation number: $F_s < 1.63$, $p_s > 0.17$).

AMN082 administered just before testing the retention of contextual fear memory significantly increased the contextual fear response (Fig. 5B; factor treatment: $F_{(1,16)} = 6.68$, $p = 0.02$) but it did not affect subsequent extinction of contextual fear (factor minute: $F_{(4,64)} = 7.98$, $p < 0.001$; interaction: $F_{(4,64)} = 0.47$, $p = 0.76$). AMN082 did also not significantly alter freezing during the other phases of the experiment ($F_s < 0.45$, $p_s > 0.51$).

AMN082 injected into the amygdala prior to the memory retention test for cued fear (Fig. 5C), had no effect on freezing

behavior in response to the CS (factor treatment: $F_{(1,17)} = 1.91$, $p = 0.18$). However, as compared to vehicle AMN082 strongly attenuated within-session extinction of cued fear and this phenotype is similar to what we observed in mGlu₇-deficient mice. For reference, there was a significant decrease in freezing with increasing CS presentations in the vehicle-injected mice ($F_{(4,68)} = 4.59$, $p = 0.002$; factor presentation number: $F_{(4,68)} = 9.02$, $p < 0.001$). Again, AMN082 did not alter freezing behavior during the other phases of the experiment ($F_s < 0.22$, $p_s > 0.64$).

Injection of DCPG into the amygdala before fear conditioning significantly enhanced freezing behavior prior to presenting the first CS–US pairing (Fig. 6A; t -test, $p = 0.04$). There was also a trend toward increased freezing during the conditioning session that followed (factor treatment: $F_{(1,25)} = 3.89$, $p = 0.06$; factor trial number: $F_{(5,125)} = 6.18$, $p < 0.001$; interaction: $F_{(5,125)} = 0.41$, $p = 0.85$) but this difference was short-lived and freezing levels were similar by the end of the conditioning session (t -test, $p = 0.46$). Pre-CS freezing and freezing during the retention tests for contextual and cued fear were also not affected by DCPG administered before conditioning ($F_s < 1.43$, $p_s > 0.24$).

DCPG injected right before testing contextual fear memory responses significantly increased freezing behavior (Fig. 6B; factor treatment: $F_{(1,17)} = 9.81$, $p = 0.006$) but had no effect on freezing behavior during the other phases of the experiment ($F_s < 0.09$, $p_s > 0.76$).

DCPG injections shortly before testing the retention of cued fear memory did not affect freezing to the cue (Fig. 6C; $F_{(1,17)} = 0.90$, $p = 0.36$) and also had no effect on extinction behavior during the

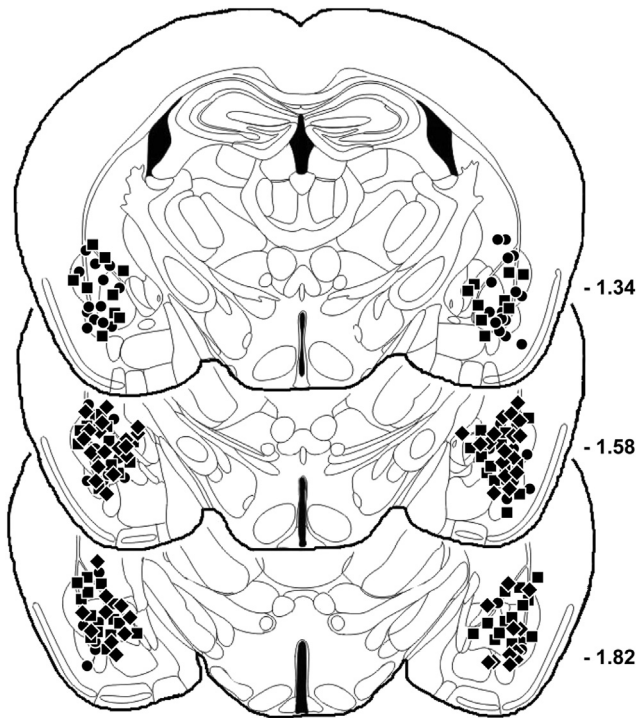


Fig. 4. Frontal sections of the amygdala depicting the injection sites of AMN082 and DCPG. Squares, injections before fear conditioning; diamonds, injections before context test; circles, injections before cue test.

subsequent phase ($F_s < 0.32$, $p_s > 0.58$). In summary, DCPG had pronounced effects on the expression of learned contextual fear only when the compound was injected just prior to testing for context memory retention.

4. Discussion

The present study revealed remarkably different roles for mGlu₇ and mGlu₈ in amygdala-dependent conditioned fear and thalamo-LA synaptic transmission and LTP (Table 1). In vitro, the mGlu₇ agonist AMN082 had no effect on thalamo-LA field potentials or cortico-LA EPSCs but strongly reduced thalamo-LA LTP as did genetic ablation of mGlu₇. In sharp contrast, the mGlu₈ agonist DCPG strongly decreased field potentials in amygdala slices but, like genetic ablation of mGlu₈, had no effect on thalamo-LA LTP. Therefore, these two closely related receptors seem to play very different and distinct roles in modulating sensory signal input function in the amygdala. Basic synaptic transmission is under the influence of mGlu₈ whereas mGlu₇ plays an important role in synaptic plasticity.

Different roles for mGlu₇ and mGlu₈ were also apparent in Pavlovian fear conditioning. mGlu₇-deficient mice expressed reduced freezing during fear conditioning and when tested for retention of conditioned cued fear memory. In addition, mice lacking mGlu₇ showed a deficit in extinction of conditioned cued fear. Although these mice initially expressed less fear to cue during and after conditioning, they exhibited more freezing than their wildtype littermates after 4 days of repeated testing. In contrast, mGlu₈-deficient mice showed no changes in conditioning, retention or extinction of cued fear. Interestingly, fear to the conditioned context was attenuated by the absence of either receptor.

Focal pharmacological manipulation of mGlu₈ and mGlu₇ inside the amygdala confirmed the different and distinct roles of these two receptors in modulating conditioned fear. As the mouse

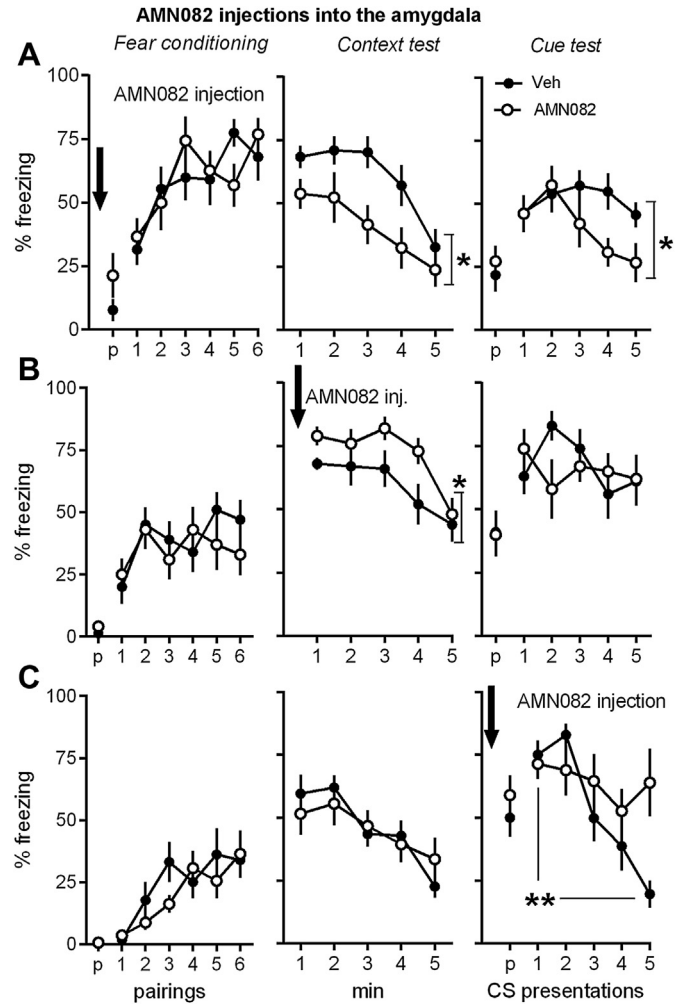


Fig. 5. Effects of intra-amygdala injection of AMN082 on conditioned fear ($n_s = 8-13$). The different horizontal panels represent the different experiments with different injection time points (vertical arrows). A) AMN082 injections before fear conditioning reduce the expression of fear in the context and cue test on the following day. B) AMN082 injections before the context test increased contextual fear. C) Injections before the cue test attenuate the within-session extinction of conditioned fear which is observed after vehicle (Veh) injections in this test. p = pre-CS freezing, ** $p < 0.01$, * $p < 0.05$, Student t -test (pre-CS) or ANOVA (fear conditioning, context test, cue test).

amygdala has a volume of about 1 mm³, it is not reasonable to separate data based on subnuclei as all injections would be expected to deliver drug to the entire amygdala. The mGlu₇ agonist did not affect the retention of fear memory to the conditioned cue and slightly enhanced contextual fear. In contrast, injection of the mGlu₈ agonist had only effects on freezing that occurred prior to the start of the conditioning trial and when injected immediately before the context memory retention test, this agonist increased freezing. Altogether, these findings demonstrate that amygdala-dependent fear behaviors were more dramatically and broadly affected by modulating mGlu₇ as compared to mGlu₈.

Recently, Dobi et al. (2013) have described clear differences between the presynaptic localizations of mGlu₇ and mGlu₈. Both excitatory and inhibitory synapses onto the large mGlu_{1α} expressing GABAergic cells located at the border to the intercalated cell clusters are heavily targeted by afferents containing mGlu₇ and/or mGlu₈. Also, mGlu₇ was prominent in afferents arising from the thalamus and basal amygdala. In contrast, mGlu₈ was less abundant and was not found on those afferents. Thus, the striking differences in behavioral effects evoked by intra-amygdala administered mGlu₇

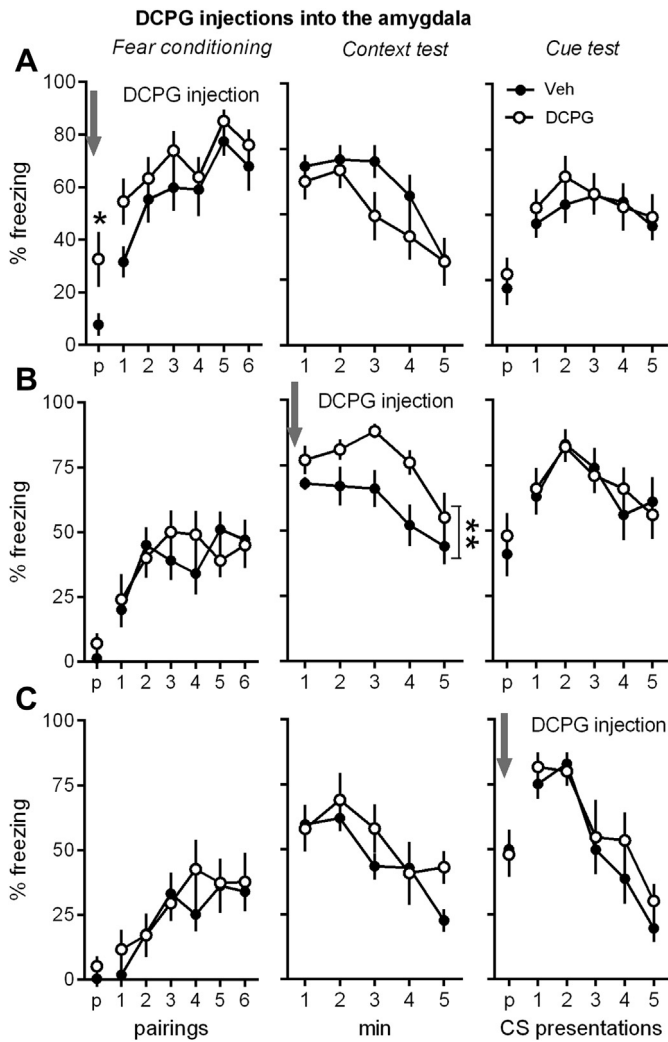


Fig. 6. Effects of intra-amygdala injection of DCPG on conditioned fear ($n_s = 9-14$). The different horizontal panels represent the different experiments with different injection time points (vertical arrow). A) DCPG injections before fear conditioning did not affect contextual and cued fear on the following test day. B) DCPG injections before the context test increased contextual fear. C) Injections before the cue test had no effect. p = pre-CS freezing, $**p < 0.01$, $*p < 0.05$, Student t -test (pre-CS) or ANOVA (fear conditioning, context test, cue test).

and mGlu₈ agonists and genetic ablation of these receptors are mirrored by their distinct localizations in the fear circuitry.

Our study confirmed the deficits in fear learning and expression reported previously in mGlu₇-deficient mice (Masugi et al., 1999) and additionally demonstrated that these mice exhibit impaired cued fear and impairment in its extinction. It is somewhat puzzling that the mGlu₇ agonist AMN082 and genetic ablation of mGlu₇ had similar effects on conditioned fear (see also: Dobi et al., 2013; Fendt et al., 2008; Toth et al., 2012; O'Connor et al., 2013). Concerns were raised recently about the interpretation of in vivo data generated following systemic administration of AMN082 because of a liver-derived metabolite that has activity on multiple monoaminergic transporters (Rizzo et al., 2011). It is however highly unlikely that this metabolite forms in brain slices ex vivo or when AMN082 is injected directly into the amygdala. Furthermore, it has been shown that AMN082 induces internalization of the mGlu₇ receptor (Pelkey et al., 2007). Therefore, AMN082 may work functionally as an antagonist and block mGlu₇ signaling, which would explain how this agonist and ablation of its receptor can result in similar

Table 1
Comparison of mGlu₇ and mGlu₈ effects.

	mGlu ₇ agonist	mGlu ₇ knockout	mGlu ₈ agonist	mGlu ₈ knockout
Synaptic transmission	No effect	–	Reduced	–
LTP	Reduced	Reduced	No effect	No effect
Context learning	Reduced	Reduced	No effect	Reduced
Context retention	Enhanced	–	Enhanced	–
Cue learning	Reduced	Reduced	No effect	No effect
Cue retention	No effect	–	No effect	–
Cue extinction (within-session)	Enhanced	Enhanced	No effect	No effect
Cue extinction (between-session)	–	Reduced	–	No effect

Summary of results using application of the mGlu₇ agonist AMN082, the mGlu₈ agonist DCPG and the respective knockout mice. In the experiments using the knockout mice it cannot be determined whether effects seen in the fear conditioning experiments were on learning or retention. Therefore, there is a single entry for each measure.

phenotypes. Alternatively, developmental compensation of mGlu₇-deficiency may occur and account for this apparent discrepancy. In contrast to mGlu₇, mGlu₈ played no role in the acquisition and extinction of cued fear in our experimental paradigms. In contrast, we confirmed earlier findings showing a decrease of contextual fear in mGlu₈-deficient mice (Fendt et al., 2010) whereas DCPG was shown to enhance contextual fear responses after intra-amygdala administration in this and another study (Dobi et al., 2013). Altogether, these findings seem consistent with the notion that antagonizing mGlu₈ may find therapeutic application in specific forms of context-dependent anxiety.

With regard to roles of mGlu₈ in unconditioned fear, DCPG has been reported to decrease unconditioned fear after systemic administration (Duvoisin et al., 2010). We found that immediately after its intra-amygdala application, DCPG increased freezing in a novel environment, which perhaps reflects an activity of DCPG that relates to unconditioned fear. However, this effect is opposite to the reduction of unconditioned fear described by Duvoisin et al. (2010) and perhaps this difference is due to differential effects of DCPG on brain circuits following systemic versus focal application. Irrespective, it seems not likely that DCPG differentially affects conditioned and unconditioned fear and similar treatments in different species having different outcomes (for example see: Gengler et al., 2007; Schmid and Fendt, 2006; Wilsch et al., 1998) simply underlines the need for results from proof-of-concept studies in human to understand the therapeutic potential.

Our study also revealed very different roles of mGlu₇ and mGlu₈ in the extinction of conditioned fear. The decrease of conditioned fear within a test session is referred to as within-session extinction or acquisition of extinction. The other form of extinction, the decrease of conditioned fear from test-session to test-session, is called between-session extinction or retention of extinction. Neither within-session nor between-session extinction was affected by manipulating mGlu₈ in agreement with a previous report (Dobi et al., 2013). In contrast, we observed decreased between-session extinction in mGlu₇-deficient mice, as found previously in other behavioral paradigms (Callaerts-Vegh et al., 2006; Goddyn et al., 2008). Interestingly, within-session extinction was apparent during the first test session in mGlu₇-deficient mice but not in their wildtype littermates with the note of caution that the paradigm we used does not normally induce very robust within-session extinction. Activation of mGlu₇ by intra-amygdala administration of AMN082 blocked within-session extinction (present study) and facilitated between-session extinction (Fendt et al., 2008; Toth et al., 2012) contrary to what we saw in mGlu₇-deficient mice and unlike the very similar effects AMN082 and

genetic ablation of mGlu₇ had on the acquisition and retention of conditioned fear memory. It seems likely that mGlu₇ located on different synapses are responsible for those different effects and also, AMN082-induced internalization of mGlu₇ receptors might differ between synapses. Irrespective, these findings demonstrate an important role of mGlu₇ in both within- and between-session extinction.

Hence, mGlu₇ is important for both forms of plasticity usually observed in fear learning experiments, namely acquisition and extinction of conditioned fear. It should be noted that extinction of conditioned fear is not simply forgetting (unlearning, erasure) of previously learned fear but is a new, active, and cue-specific learning process (e.g., discussed in Myers and Davis, 2007). The finding that mGlu₇-deficiency reduced both learning and between-session extinction of conditioned fear suggests that chronic blockade of mGlu₇ might reduce exaggerated fear learning and be of benefit to patients with certain types of anxiety disorders. On the other hand, chronic blockade may make the fear circuit rigid and inflexible, which may prevent the extinction of already learned (pathological) fear and exacerbate phobias or post-traumatic stress disorders. In contrast, temporarily agonizing mGlu₇ as exemplified here using AMN082 seems to evoke favorable effects: fear learning is decreased and between-session extinction of fear is facilitated (Fendt et al., 2008; Toth et al., 2012) with the note of caution that the latter effect was seen after systemic application and could be mediated by off-target effects of an AMN082 metabolite (Rizzo et al., 2011). Future studies will be needed to unravel the mechanisms underlying the extinction-facilitating effects ascribed to mGlu₇ and the appropriate drugs needed before this may become a fruitful approach to treat anxiety disorders where fear extinction is impaired (Ressler et al., 2004).

In contrast to mGlu₇, mGlu₈ played no significant role in modulating cue but had some role in context conditioned fear and might have a role in processes related to generalized or innate anxiety-like behaviors. For example, the reduction of freezing in a new context on the day after fear conditioning might point to less generalization of conditioned fear in mGlu₈-deficient mice. However, both anxiogenic-like (Duvoisin et al., 2005; Linden et al., 2003, 2002; Robbins et al., 2007) and anxiolytic-like phenotypes (Fendt et al., 2010; Gerlai et al., 2002; present study) have been reported for mGlu₈-deficient mice and further studies are needed to clarify this receptor's exact role.

Taken together, both receptors seem interesting targets for developing novel therapies to treat distinct anxiety disorders linked to either pathological acquisition of fear and its extinction (mGlu₇) or disorders associated with exaggerated contextual fear such as generalized anxiety disorders (mGlu₈).

Conflict of interest

All authors except DP were or still are employees of Novartis Institutes for BioMedical Research who funded this project. JM is employee of F. Hoffmann-La Roche AG.

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