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# Differential roles of $mGlu_7$ and $mGlu_8$ in amygdala-dependent behavior and physiology

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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 $mGlu_7$ and $mGlu_8$ in amygdala-dependent behavior and synaptic plasticity. We show that ablation of $mGlu_7$ but not $mGlu_8$ attenuates long-term potentiation (LTP) at thalamo-lateral amygdala (LA) synapses where a strong association between LTP and learning has been demonstrated. $mGlu_7$-deficient mice express a general deficit in conditioned fear whereas $mGlu_8$-deficient mice show a dramatic reduction in contextual fear. The $mGlu_7$ agonist AMN082 reduced thalamo-LA LTP and intra-amygdala administration blocked conditioned fear learning. In contrast, the $mGlu_8$ 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 $mGlu_7$ and $mGlu_8$ 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 ($mGlu_7$) or contextual fear ($mGlu_8$).



## 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

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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 $mGlu_1$ and $mGlu_5$ which are mainly postsynaptic, other subtypes including $mGlu_2$, $mGlu_3$, $mGlu_7$ and $mGlu_8$ 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 $mGlu_7$ and $mGlu_8$ 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 $mGlu_7$ and $mGlu_8$ in amygdala-dependent fear learning and synaptic plasticity. To this end, we used the allosteric $mGlu_7$ agonist AMN082 (Mitsukawa et al., 2005), the orthosteric $mGlu_8$ agonist DCPG (Thomas et al., 2001) and $mGlu_7$- and $mGlu_8$-deficient mice (Duvoisin et al., 1995; Sansig et al., 2001). First, we compared the effects of AMN082, DCPG, and $mGlu_7$- and $mGlu_8$-ablation on synaptic transmission and thalamo-LA LTP in amygdala slices. Second, we compared the behavior of $mGlu_7$- and $mGlu_8$-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), $mGlu_7$-deficient mice (Cryan et al., 2003; Sansig et al., 2001), and $mGlu_8$-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 sacrified 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% $O_2$/5% $CO_2$ containing (in mM): NaCl (124), KCl (2.5), $KH_2PO_4$ (1.2), $CaCl_2$ (2.5), $MgSO_4$ (1.3), $NaHCO_3$ (26), glucose (10) and saccharose (4) (pH 7.4, osmolarity adjusted to 320 ± 2 mOsm by reducing the amount of $H_2O$). 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 (Endres 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