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Chapter IV

Fear conditioning enhances spontaneous AMPA receptor mediated synaptic transmission in mouse hippocampal CA1 area

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Summary

AMPA receptor mediated synaptic modifications in the amygdala have been reported to sustain cued fear conditioning. However, the hippocampal formation is also critically involved in fear learning. Therefore, we examined if fear conditioning is also accompanied by changes in AMPA receptor mediated synaptic transmission in the hippocampus; we focused on spontaneous synaptic activity (mEPSCs). Young adult mice were trained using tone-footshock pairings; contextual / cued memories were tested 3-4 hrs and 1 day later. We found that mEPSC frequency was enhanced by 70% when recorded 3 hours, but not 24 hours, after fear conditioning training. Fear training induced a significant enhancement in the mEPSC amplitude at both 3 and 24 hours after training; synaptic GluA2 expression was enhanced 24 hours after training. The increased mEPSC frequency and amplitude was absent in animals that were only exposed to shock or novelty, implying an association between aversive learning and enhanced spontaneous synaptic transmission. We conclude that fear conditioning not only elicits clear changes in synaptic efficacy in the amygdala but also in the hippocampus, which may contribute to the encoding of contextual aspects of the fearful situation.

Introduction

Long-term potentiation (LTP) reflects an increase in synaptic efficacy and it is believed that such changes in synaptic weight underlie learning and memory processes (Neves et al., 2008). Indeed LTP-like changes have been reported in the hippocampal CA1 area after passive avoidance training and in the lateral amygdala after fear conditioning (Rogan et al., 1997b; Whitlock et al., 2006), and mechanisms maintaining LTP sustains spatial memory (Pastalkova et al., 2006).

Long term potentiation involves the activity-dependent recruitment of AMPA receptors to the postsynaptic membrane and a concurrent increase in AMPA-mediated transmission (Malinow and Malenka, 2002; Plant et al., 2006). Recent studies demonstrated that not only LTP is associated with a rapid delivery of AMPA receptors but that fear conditioning requires delivery of AMPA receptors to synapses of postsynaptic neurons in the lateral amygdala (Rumpel et al., 2005). Earlier it was shown that cued fear conditioning, a strong form of associative learning, is accompanied by enhanced glutamatergic transmission in subnuclei of the amygdala (Humeau et al., 2007; McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002).

However, behavioural studies suggest that not only the amygdala, but also the hippocampus is critically involved in aspects of fear conditioning (Maren, 2008; Sanders et al., 2003). Moreover, after cued fear conditioning rhythmical synchronized activity at theta frequencies increases between the lateral amygdala and the hippocampal CA1 area (Seidenbecher et al., 2003) suggesting that interactions between both regions might be critical to establish fearful memories. We therefore examined in detail whether fear learning alters hippocampal CA1 synaptic efficacy. To this end we recorded AMPA receptor mediated synaptic transmission in hippocampal CA1 neurons at different time points after fear conditioning, focusing on spontaneous excitatory synaptic currents (mEPSCs).

Materials and Methods

Animals

Male C57 black 6 mice (6-8 weeks old, Harlan, The Netherlands) were individually housed upon arrival in enriched cages for at least one week, with a light/dark cycle of 12 h (lights on at 8am; room temperature kept around 20°C). Food and water were given without restriction. The experiments were carried out with permission of the local Animal Committee of the University of Amsterdam.

Fear conditioning

Animals were trained in a fear conditioning chamber (30 x 24 x 26; W x L x H). The grid floor was made of 37 stainless steel rods, which were connected to a shock generator (Med-Farm LION-ELD) that was in-house developed. On day 1 (8:30-9:00 a.m.) one mouse at a time was trained in the foot shock chamber (cleaned with 1% acetic acid). The animal was allowed to freely explore the chamber for 3 minutes, which was then followed by 3 tone-foot shock pairs (with an interval of 1 min). Each tone (100 dB, 2.8 kHz) lasted 30 seconds, accompanied by a foot shock (0.8 mA) during the last 2 sec; 30 sec after the end of the last pairing, the mouse was taken back to its home cage. Freezing behavior during this training session was scored before the onset of the first tone and after the end of each tone-foot shock. Either 3 hours (FC/3h group) or 24 hours (FC/24h group) after training, the mouse was introduced into the same chamber and freezing behavior was scored for 3 minutes (contextual memory test). One hour later, the mouse was introduced into another chamber with different contextual background and cleaned with 70% ethanol. After free exploration for three minutes the animal was exposed to one (30 sec) tone without foot shock and freezing behavior was scored before and during the tone presence (tone cue memory test). One control group consisted of mice

introduced into the foot shock chamber for 7 min without receiving any tone or foot shock (novelty group). A second control group (shock group) received 3 foot shocks (2 sec intervals) immediately after being put into the chamber, after which they were immediately placed back in their home cage, so that association between the shocks and the environment in which these were received was unlikely to have occurred.

Freezing behavior was determined for each 2 sec throughout the experiment. Freezing was defined as no body movements except those related to respiration. The percentage of freezing time versus total experimental time was used for statistical analysis.

Electrophysiology

Mice were decapitated between 9 a.m. and 11:30 a.m. Dorsal hippocampal slices (350 μm) were made on a vibratome (LEICA VT 1000S; Germany) and stored in normal aCSF containing (in mM): 120 NaCl, 3.5 KCl, 1.3 MgSO₄, 1.25 NaH₂PO₄, 2.5 CaCl₂, 10.0 glucose and 25.0 NaHCO₃, pH7.4, and continuously gassed (mixture of 95% O₂-5% CO₂) at room temperature for >1h. One slice at a time was placed in a recording chamber mounted on an upright microscope (Nikon E600FN), continuously perfused with aCSF (32°C, 2-3ml/sec) and kept fully submerged. After the debris on the surface of the CA1 cell layer was removed with a cleaning pipette, whole cell patch clamp recordings were made using an AXOPATCH 200B amplifier (Axon Instruments, USA), with electrodes from borosilicate glass (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany). The pipette solution contained (in mM): 120 Cs methane sulfonate; 17.5 CsCl; 10 HEPES; 5 BAPTA; 2 Mg-ATP; 0.5 Na-GTP; 10 QX-314; pH7.4, adjusted with CsOH; pipette resistance was between 3-6 M Ω . Under visual control (40 \times objective and 10 \times ocular magnifications) the electrode was directed towards a CA1 neuron with positive pressure. Once sealed on the cell membrane (resistance above 1G Ω) the membrane patch under the electrode was ruptured by

gentle suction and the cell was kept at a holding potential of -70 mV. The liquid junction potential caused a shift of no more than 10 mV, which was not compensated during mEPSCs recording. Recordings with an uncompensated series resistance of <15 M Ω and <2.5 times of the pipette resistance with a shift of $<20\%$ during the recording, were accepted for analysis. Data acquisition was performed with Pclamp 8.2 and analyzed off-line with Clampfit 9.0.

Miniature (m)EPSCs were recorded at a holding potential of -70 mV as described before (Karst and Joels, 2005). Tetrodotoxin (0.25 μ M, Latoxan, Rosans, France) and bicuculline methobromide (20 μ M, Biomol) were added to the buffer to block action potential induced glutamate release and GABA_A receptor mediated mIPSCs, respectively. During some recordings the non-NMDA-receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μ M, Tocris) was perfused to confirm that the mEPSCs were indeed mediated by AMPA receptors. The events were identified as mEPSCs when the rise time was faster than the decay time. mEPSCs were recorded for 5 min in each cell. Of all cells, the frequency and peak amplitude of mEPSCs were determined.

Biochemistry

Hippocampi from naive or fear conditioned (3 or 24 hours after training), shock and novelty mice were isolated and stored at -80°C . For preparation of synaptoneurosomes, tissue samples were homogenized in ice-cold homogenization buffer (10 mM Hepes/1.0 mM EDTA/2.0 mM EGTA/0.5 mM DTT/0.1 mM PMSF/10 mg/l leupeptin/100 nM microcystin) with an eppendorf homogenizer, and homogenates were passed through two 100- μ m-pore nylon mesh filters. At this stage aliquots of whole hippocampus were taken and stored at -80°C for future analysis. The remaining tissue was passed through two further 5- μ m-pore filters. Filtered homogenates were centrifuged at 3600g for 10 min at 4°C . Resultant pellets were

resuspended in 100 μ L 1% SDS, boiled for 10 min and stored at -80°C .

Whole and synaptoneurosome hippocampal samples were quantified using the RC DC protein assay (Biorad). Equal protein samples were prepared at a concentration of 0.5 $\mu\text{g}/\text{ml}$ in 33 mM NaCl, 70 mM Tris-HCl, 1 mM EDTA, 2% (w/v) SDS, 0.01 % (w/v) bromophenol blue, 10% glycerol, pH 6.8. Proteins were resolved on 10% polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in TBS-0.1% Tween 20 (TBS-T). Membranes were then incubated with primary antibody (GluA2 1: 2000 (AbCAM), GluA1 1:10000 (Assay Design); GluA3 1:1000 (Invitrogen); Pan-actin 1:20000 (Sigma) for 2 h at room temperature. The membranes were washed 3 times in TBS-T for 10 min and then incubated over night at 4°C with the appropriate secondary horseradish peroxidase-linked antibodies diluted in blocking buffer. Following membrane washing with TBS-T buffer, the immunocomplexes were visualized using a chemiluminescence peroxidase substrate (SuperSignal West Dura Extended Duration Substrate) and immunoreactivity detected using the Biorad ChemiDoc XRS system. Densitometry analysis on the bands was calculated using Biorad Quantity One 4.2.3 software (Biorad Laboratories AG, Switzerland). Each band was normalized to the actin level determined in the corresponding sample. On each gel at least 2 naïve controls were used and protein changes were represented as a percentage of the normalized naïve value.

Data analysis

Data are expressed as mean \pm S.E.M. Behavioral and western blots data were analyzed using (repeated measures) ANOVA or two-tailed t-test. Electrophysiological data (Cumulative distribution of the peak amplitude and intervals of mEPSCs) were analyzed using Kolmogorov-Smirnov (KS) test. Statistical significance was set at $p < 0.05$.

Results

Fear Conditioning

Freezing behavior during training progressively and significantly increased after the end of each pair of tone and foot shock (Figure 1A, repeated measures ANOVA, $F(3,140)=52.6$; $P<0.001$). Three or 24 hours later, when contextual memory was tested for 3 minutes, both FC/3h and FC/24h groups displayed moderate amount of freezing behavior (Figure 1B: FC/3h, $29.4\% \pm 6.9$ of total time, $n=7$; FC/24h, $44.5\% \pm 5.0$, $n=6$) and significantly more when compared to the 3-minutes free exploration prior to the exposures of tone-shock pairs during training (two tailed paired t-test, $P<0.01$), when animals showed no freezing at all. Although the total amount of contextual freezing over 3 minutes was not significantly different between animals that were tested 3 versus 24 hrs after training (Figure 1B, $P>0.05$), contextual memory in FC/3h group was significantly less stable when compared to FC/24h group (Figure 1C, ANOVA: $F(1,11)=9.204$, $P<0.05$). Novelty or shock groups showed no freezing behavior (Figure 1B, novelty versus FC/3h: $F(1,8)=7.2$, $P<0.05$; shock versus FC/3h: $F(1,14)=18.9$, $P<0.01$), showing that associative learning is necessary for freezing to occur in the familiar context. During the tone-cue test, animals that were placed in a novel test cage 1 hr after contextual memory testing did not show significant amount of freezing behavior during the free exploration. In contrast, when the tone was applied, animals displayed significantly more freezing when compared to free exploration (Figure 1D, FC/3h: $F(1,12)=238.087$, $P<0.01$; FC/24h: $F(1,10)=48.62$, $P<0.01$)

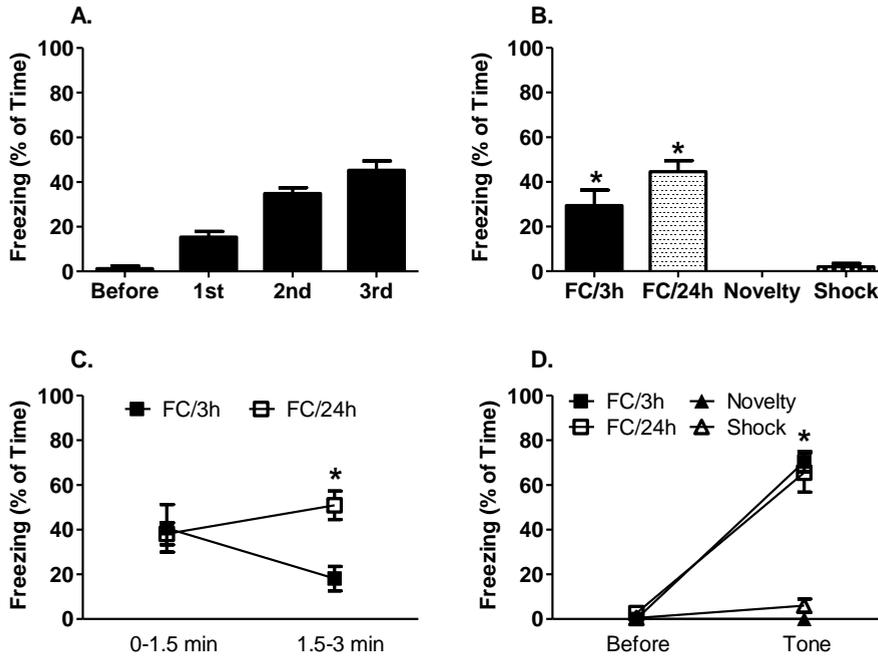


Figure 1. Fear Conditioning. A) Acquisition: Tone-shock pairings progressively and significantly increased freezing behavior. Before: 3min free exploration period; 1st, 2nd and 3rd: after the end of 1st, 2nd and 3rd pair of tone and foot shock respectively B) Context memory test: Three (FC/3h) and 24 hours after training animals displayed freezing, while no contextual freezing was observed in animals from novelty and shock groups. C) Context memory: Twenty four hours after training (FC/24h) animals show higher freezing during the last 1.5 minutes of the total of 23 minutes of testing. D) Tone cue memory test: Animals show significant higher freezing upon tone exposure when compared to the free exploration period and when compared to novelty and shock only animals. FC/3h: n=7; FC/24h: n=6; shock: n=9; novelty: n=3. *P<0.05.

mEPSCs recordings after Fear Conditioning

We next addressed the question if spontaneous AMPA receptor mediated synaptic events in the CA1 area – an area essential for contextual fear conditioning - were altered in parallel with associative learning. To this end, animals were first trained and then decapitated 3 hrs or 24 hrs later (i.e. in

the absence of any retention trials); we also included the naïve, novelty and shock control groups (6-8 samples per group). mEPSCs were recorded from hippocampal CA1 pyramidal cells kept at a holding potential of -70 mV, which favors the generation of AMPA- rather than NMDA-receptor mediated events. A typical example of a mEPSC recording is shown in Figure 2 (upper left trace). mEPSCs were completely blocked by application of the non-NMDA receptor antagonist CNQX ($10\ \mu\text{M}$, $n=3$; typical example in Figure 2, upper right traces), supporting that the mEPSCs indeed represented spontaneous AMPARs mediated synaptic events.

We observed frequency of mEPSCs in animals tested three hours after training was enhanced by 70% when compared to naïve animals. Thus, training significantly increased mEPSC frequency (inter-event intervals) in FC/3h group compared to naïve animals (Figure 2 A&B, $P<0.001$, KS test). Interestingly, the increase in frequency was transient, since animals tested 24 hrs after training had a mEPSC frequency that was back to the level of the naïve controls and significantly lower when compared to FC/3h group (Figure 2 A&B, $P<0.05$, KS test). The mEPSC frequency was significantly lower in the shock ($P<0.01$, KS test) and novelty group ($P<0.01$, KS test) compared to FC/3h group, indicating that the increase of mEPSC frequency in FC/3h group was related to associative learning and not to the stress of the environment or exposure to shocks.

The mEPSC peak amplitude was significantly increased both 3 hrs ($P<0.01$, KS test) and 24 hrs ($P<0.05$, KS test) after training when compared to naïve group (Figure 2 C&D). The distribution of peak amplitudes was skewed and significantly shifted to the right 3 hrs and 24 hrs after training (Figure 2D). It is unlikely that the skew we observed arises from differences in the electrotonic distance of synapses from the somatic recording site, since no correlation was found between the kinetics of mEPSCs (rise time or decay time) and their peak amplitudes (data not shown). Comparable mEPSC peak amplitude was found in novelty ($P>0.05$, KS test) and shock

($P > 0.05$, KS test) groups versus naïve control.

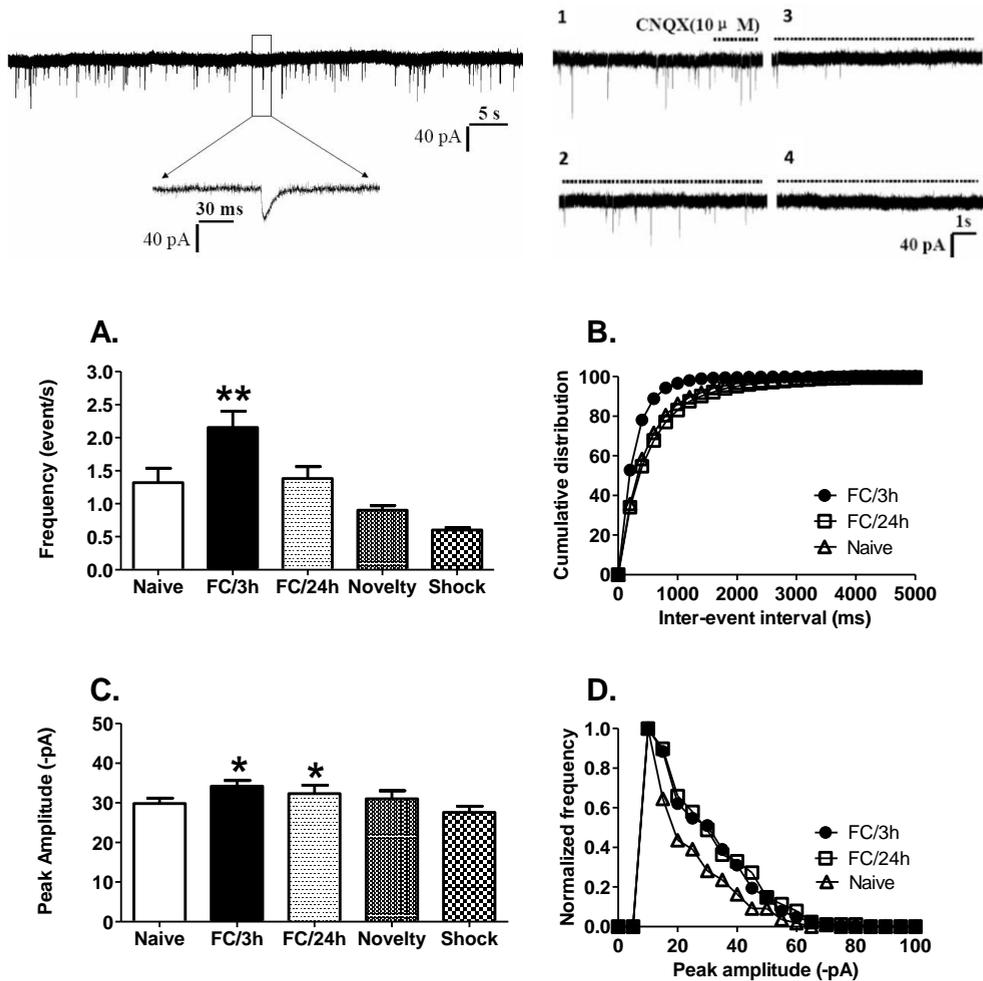
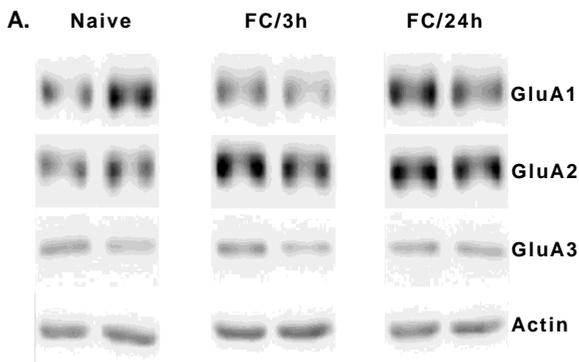


Figure 2. mEPSC recordings in hippocampal CA1 area. Upper left: Typical example of mEPSC recordings in hippocampal CA1 area. Upper right: mEPSCs gradually disappear within 5 minutes (traces 1-4) during the application of CNQX (10 μ M). A) mEPSC frequency three hours (FC/3h) and 24 hours (FC/24h) after fear conditioning training as well as in naïve, shock and novelty exposed animals. B) Cumulative distribution of mEPSC intervals in naïve animals and three (FC/3h) and 24 hours (FC/24h) after training. C) mEPSC amplitude three hours (FC/3h) and 24 hours (FC/24h) after training in fear conditioning task as well as in naïve, shock and novelty exposed animals. D) Cumulative distribution of mEPSC amplitude in naïve animals and three (FC/3h) and 24 hours (FC/24h) after training. ** $P < 0.01$ and

* $P < 0.05$ when compared to naïve controls by KS test. Naïve: $n=6$; FC/3h: $n=6$; FC/24h: $n=7$; shock: $n=8$; novelty: $n=6$.

Biochemistry

In view of the altered mEPSC peak amplitude, we examined whether postsynaptic AMPA receptor subunits (GluA1-3) were modulated by fear conditioning (Figure 3A-D). Three hours after training no change in synaptic GluA2 subunit was found ($113.9\% \pm 14$ of naïve animals, $P > 0.05$), while there was a trend for synaptic GluA1 to decrease ($78.4\% \pm 7.5$ of naïve animals, $P = 0.06$). In contrast, 24 hours after training synaptic GluA2 subunit was significantly enhanced ($122.3\% \pm 6.4$ of naïve animals, $P < 0.05$) while no change was detected in synaptic GluA1 expression ($97.2\% \pm 14.1$ of naïve animals, $P > 0.05$). Control groups (shock and novelty) did not show significant changes compared to naïve group, with the exception of an increase in synaptic GluA2 expression in novelty group. No changes were found in synaptic GluA3 subunits in all groups (data not shown).



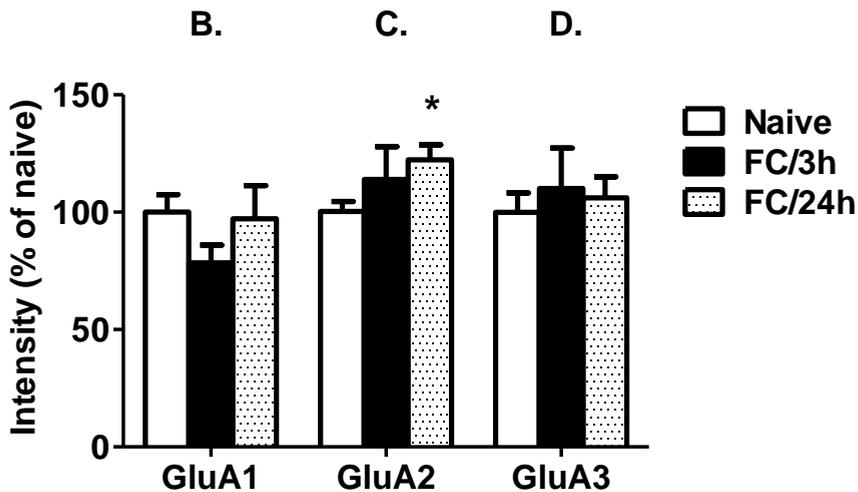


Figure 3. Synaptic AMPARs subunits expression after fear conditioning. A) Typical western blot examples (in duplo) of synaptic GluA1-3 and actin expressions in naïve, FC/3h and FC/24h groups. B-D) Quantification of synaptic GluA1-3 expression in different groups. Synaptic GluA2, but not GluA1 or GluA3, was enhanced 24 hrs after fear conditioning. No significant changes were found 3hrs after training. Naïve: n=10; FC/3h: n=8; FC/24h: n=8; *P<0.05 when compared to naïve.

Discussion

Activity dependent changes in functional postsynaptic AMPARs contribute to the two main forms of synaptic plasticity that are believed to underlie learning and memory in the hippocampus (Neves et al., 2008). Long term potentiation (LTP) involves the activity-dependent recruitment of AMPARs to the postsynaptic membrane and a concurrent increase in AMPA-mediated transmission whereas long term depression (LTD) is a decrease in synaptic AMPAR function (Malinow and Malenka, 2002). Importantly, modifications of synaptic strength have also been observed after learning. Fear conditioning, a strong form of associative learning, is accompanied by enhanced glutamatergic transmission in subnuclei of the amygdala (Humeau et al.,

2007; McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002). Moreover, trafficking of AMPA receptors to the postsynaptic membrane in the lateral nucleus of the amygdala is essential for forming fearful memories (Rumpel et al., 2005). Here we report that fear conditioning also enhances spontaneous AMPA receptor-mediated synaptic transmission in the hippocampal CA1 area, implying that changes in synaptic efficacy after fear conditioning are not confined to the amygdala. Although we did not assess learning and electrophysiology in the same animals - a single retention test may already influence the electrophysiological outcome -, there is nevertheless strong support for the view that the observed electrophysiological changes are associated with contextual fear learning. First, in the behavioral experiment all animals (without exception) displayed significantly enhanced freezing 3 hrs after training compared to the pre-shock period, i.e. learned the task; there is no reason to believe that the set of animals used for electrophysiology would respond differently. Secondly, the increased synaptic efficacy occurred only in animals which were trained to associate the context and footshock.

The most obvious effect of fear conditioning turned out to be a 70% increase in the mEPSCs frequency 3 hrs after training. The altered frequency suggests a presynaptic origin of the changes in efficacy but it remains to be investigated whether these changes are related to enhanced release probability, or to generation of new spines, as was reported to occur 24 hrs after fear conditioning (Matsuo et al., 2008). The fact that the current changes in frequency are transient suggests that they might be related to release rather than structural changes. This would be in line with earlier experiments in the amygdala reporting that cued fear training results in enhanced glutamatergic transmission (McKernan and Shinnick-Gallagher, 1997), involving enhanced presynaptic glutamate release probability as well as postsynaptic changes (Humeau et al., 2007; Tsvetkov et al., 2002; Zinebi et al., 2001).

In agreement with postsynaptic modifications of synaptic efficacy we observed that fear conditioning persistently enhanced mEPSC amplitude - although it should be emphasized that the effects were smaller when compared to the changes in frequency. In agreement with postsynaptic modifications after learning we observed that synaptic GluA2 levels were enhanced at 24 hours, but not 3 hours after training while synaptic GluA1 levels showed a tendency to decrease and synaptic GluA3 levels were unaffected. These results are strikingly similar with earlier findings that report a protein synthesis dependent increase in GluA2/3 levels during LTP *in vivo* (Williams et al., 2007) while one hour after LTP lateral diffusion of GluA2 containing AMPARs from extrasynaptic to synaptic sites is enhanced (Yao et al., 2008). Taken together, our current results may signify a role for postsynaptic GluA2 containing AMPARs in long-term establishment of fearful memories (Shimshek et al., 2006).

We conclude that fear conditioning not only enhances synaptic transmission in the lateral amygdala – a region critically involved in cued fear conditioning (LeDoux, 2000) – but also in the hippocampal CA1 area, presumably through both a pre- and post-synaptic mechanism. The findings strengthen the neurobiological basis for the idea that the hippocampus is critically involved in contextual aspects of fear learning (Maren, 2008). It is tempting to speculate that these synaptic modifications in both hippocampus and amygdala underlie synchronization of theta activities in the amygdala-hippocampal network to represent a neuronal correlate of conditioned fear (Seidenbecher et al., 2003).

Acknowledgements

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