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ORIGINAL ARTICLE

Consolidation of remote fear memories involves corticotropin releasing hormone (CRH) receptor type 1-mediated enhancement of AMPA receptor GluR1 signaling in the dentate gyrus

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Running title: CRH and consolidation of remote fear

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ABSTRACT

Persistent dreadful memories and hyperarousal constitute prominent psychopathological features of posttraumatic stress disorder (PTSD). Here we used a contextual fear conditioning paradigm to demonstrate that conditional genetic deletion of corticotropin-releasing hormone (CRH) receptor 1 within the limbic forebrain in mice significantly reduced remote, but not recent, associative and non-associative fear memories. Per os treatment with the selective CRHR1 antagonist DMP696 (3mg/kg) attenuated the consolidation of remote fear memories, without affecting their expression and retention. This could be achieved, if DMP696 was administered for one week starting as late as 24h after the foot shock. Furthermore, by combining electrophysiological recordings and Western blot analyses we demonstrate a delayed-onset and long-lasting increase in AMPA receptor GluR1-mediated signaling in the dentate gyrus of the dorsal hippocampus 1 month after foot shock. These changes were absent from CRHR1 deficient mice and after DMP696 treatment. Inactivation of hippocampal GluR1-containing AMPARs by antisense technology or local treatment with philanthotoxin confirmed the behavioral relevance of AMPA-type glutamatergic neurotransmission in maintaining the high levels of remote fear in shocked mice with intact CRHR1 signaling. We conclude that limbic CRHR1 receptors enhance the consolidation of remote fear memories in the first week after the foot shock by increasing the expression of Ca^{2+} -permeable GluR1-containing AMPA receptors in the dentate gyrus. These findings suggest both receptors as rational targets for the prevention and therapy, respectively, of psychopathology associated with exaggerated fear memories, such as PTSD.

INTRODUCTION

PTSD is a severe and disabling psychiatric disease that is triggered by the experience of a trauma. The life-time prevalence of experiencing a trauma-like incident is up to 75% for an individual (Yehuda and Ledoux, 2007), and up to 8% people of the general population develop the disease (Kessler *et al*, 1995). Pharmacotherapeutic strategies with PTSD-approved drugs, such as selective-serotonin reuptake inhibitors, achieve remission rates in only 66% of the patients (Albucher and Liberzon, 2002). This asks for the development of better therapies, which requires a deeper understanding of the neurobiological mechanisms involved in the development of PTSD. However, only little is known about the neurobiology of the processes underlying the consolidation of remote fear memories (Cui *et al*, 2004; Frankland *et al*, 2004; Pastalkova *et al*, 2006). Moreover, some of the identified targets such as BDNF (Bekinschtein *et al.*, 2007) are not readily accessible for pharmacological interventions in human patients. This explains the interest in the contribution of neuropeptides to the development and maintenance of PTSD (Ressler *et al.*, 2011). Among them, CRH appears to be particularly promising. CRH was identified as the major component of the body's hormonal stress response (Vale *et al*, 1981). Along with this activation of the hypothalamus-pituitary-adrenocortical (HPA) system, CRH controls anxiety responses in extrahypothalamic brain areas (Müller *et al*, 2003; Walker *et al*, 2003) via two G-protein coupled receptors, CRHR1 and CRHR2 (Bale and Vale, 2004). CRH signaling within the amygdala or the hippocampus has also been shown to affect acquisition and consolidation of recent fear memories (Kolber *et al*, 2008; Pitts *et al*, 2009; Radulovic *et al*, 1999; Roozendaal *et al*, 2002; Tovote *et al*, 2005), including the development of non-associative fear responses (Adamec *et al.*, 2010). Noteworthy, clinical data suggest an involvement of CRH in long-lasting psychopathological anxiety observed in PTSD (Bremner *et al*, 1997). However, the specific contribution of CRH to the development, expression and/or retention of remote fear memories has not been explored in animal models of this disorder.

Therefore, the present study assessed the role of CRH in consolidation of trauma-related fear in a mouse model of PTSD. This model involves exposure to a brief, but intense, electric foot shock and the subsequent assessment of associative (i.e. contextual) and non-associative (i.e sensitized response to an a priori neutral tone) fear (Siegmund and Wotjak, 2007). This dichotomy refers to the different

psychopathological components of PTSD: Associative learning accounts for intrusive memories and avoidance behaviour, whereas non-associative (or stress-related) learning results in hyperarousal (Siegmund and Wotjak, 2006).

The first part of the study assessed whether CRH affects remote fear memories (i.e. contextual and sensitized fear), and identified the CRH receptor subtype involved. This was achieved by testing different mutant mice lacking expression of CRHR1/CRHR2 either throughout the whole body or selectively within the brain/limbic forebrain 1 month after the foot shock. The second part dissected the involvement of CRHR1 signaling in consolidation vs. retention/expression of remote fear by pharmacological means. The third part dealt with potential effectors of CRHR1 signaling. In this context we focused on AMPA receptor (AMPA) trafficking, which has been repeatedly shown to underlie the consolidation of different types of aversive memories (Clem and Huganir, 2010; Hu *et al*, 2007; Mitsushima *et al*, 2011; Rumpel *et al*, 2005; Whitlock *et al*, 2006). These studies, however, did not resolve whether remote fear memories in general, and the fear-promoting effects of limbic CRHR1 signaling in particular, involve long-term changes in AMPAR neurotransmission. We focused specifically on changes at level of the dentate gyrus as the entrance point of the hippocampus formation. Several lines of evidence suggest a crucial role for the dentate gyrus in the formation of episodic memories by the process of pattern separation (Treves *et al*, 2008), which may involve neurogenesis in the subgranular zone (Sahay *et al*, 2011). The remote fear responses observed in our animal model largely resemble fear generalization, most probably on the basis of impaired context discrimination, i.e. pattern separation (Golub *et al.*, 2009; Pamplona *et al.*, 2011). Interestingly, excessive fear generalization is considered a main feature of PTSD (Peri *et al*, 2000).

Our experiments revealed a scenario, according to which exposure to an intense, aversive foot shock leads to a transient activation of CRHR1 signaling within the limbic forebrain within the first week after the traumatic incident that enhances the consolidation and retention of remote fear memories via increased expression of Ca²⁺-permeable GluR1-containing AMPA receptors at level of the dorsal hippocampus.

MATERIAL and METHODS

Mice

Experiments were performed with male mice at an age of 8 to 17 weeks. Upon arrival in the animal facility of the Max Planck Institute of Psychiatry, all mice were kept under standardized, single housing conditions (i.e., Makrolon type II cages with wood shavings, inverse 12:12h light-dark schedule with lights off at 09:00h, at $22 \pm 2^\circ\text{C}$ room temperature and $55 \pm 5\%$ humidity). Water and food was provided *ad libitum*. Animals were allowed to habituate to the housing conditions for 2 weeks before starting the experiment.

C57BL/6N mice. C57BL/6NCrl inbred mice were purchased from Charles River Germany at an age of 6-7 weeks.

Mouse mutants. All mutant mice (KO) and their respective wild-type littermate controls (WT) were generated in our local breeding facilities (MPIP, MPIBC) and genotyped as previously described [Total-CRHR1 (i.e. R1-KO/WT; Timpl *et al*, 1998); CRHR1^{loxP/loxP;Nes-Cre} (i.e. R1^{Nes}-KO/WT; Schmidt *et al*, 2006); CRHR1^{loxP/loxP;CaMKII-Cre} (i.e. R1^{CaMKII}-KO/WT; Müller *et al*, 2003); Total-CRHR2 (i.e. R2-KO; Coste *et al*, 2000)]. Mice were kept at different genetic backgrounds (R1-KO/WT: 129S2/SvPas x CD1; R1^{Nes}-KO/WT and R1^{CaMKII}-KO/WT: 129S2/SvPas x C57BL/6J; R2-KO/WT: C57BL/6J Mice). R1-KO/WT and R2-KO/WT were derived from heterozygous breeding pairs. In case of the R1^{CaMKII} line, mutants were maintained by breeding pairs with the genotypes Cre(-);CRHR1^{loxP/loxP} (i.e. WT) for the father and Cre(+);CRHR1^{loxP/loxP} (i.e. KO) for the mother in order to avoid germline recombination, which is frequently encountered in males of this line. In case of the R1^{Nes} line, the arrangement of the breeding pairs was vice versa. R1-KO and R2-KO lack expression of CRHR1 and CRHR2, respectively, throughout the entire body (i.e. including the anterior pituitary). As a consequence, stress-induced release of adrenocorticotrope hormone (ACTH) and corticosterone is reduced in R1-KO mice, whereas basal steroid release is not affected. In R1^{Nes}-KO, in contrast, deletion of CRHR1 expression is restricted to the brain, i.e. to all neurons/glia cells, but not the anterior pituitary, thus sparing the key regulatory brain centre of the hypothalamic-pituitary-adrenocortical (HPA) axis. R1^{CaMKII}-KO lack expression of CRHR1 in telencephalic and limbic brain structures, but not in the hypothalamus (and the

pituitary). Thus, basal or stress-induced activation of HPA axis remains unaffected in these mice (Müller *et al*, 2003).

All experimental procedures were approved by the Committee on Animal Health and Care of the local government (i.e., Regierung von Oberbayern) and performed in accordance with the guidelines for the care and use of laboratory animals set by the European Community Council (86/609/CEE).

Foot shock delivery and testing procedures

Experimental set-ups and procedures have been previously described in detail (Kamprath and Wotjak, 2004; Siegmund and Wotjak, 2007). Briefly, experiments were performed in two different contexts: Foot shock application and testing for conditioned contextual fear was done in a mouse conditioning chamber that has a cubic shape with two metal and two Plexiglas walls and a metal grid floor. Sensitized fear was assessed in a neutral context consisting of a Plexiglas cylinder with wood shavings as bedding. Both contexts were cleaned with detergents of different odours (conditioning chamber: 70% ethanol; neutral context: 1% acetic acid).

For application of the foot shock (day 0), animals were placed into the conditioning chamber, and after 198 sec a scrambled electric foot shock (2 sec duration, 1.5 mA current intensity) was delivered via the metal grid. Animals remained in the conditioning chamber for an additional 60 sec before they were returned to their home cages. Assessing sensitized (i.e. non-associative) fear, mice were placed in the neutral test context, and after 180 sec a 3 min neutral tone (80 dB, 9 kHz) was presented to the animals. Contextual (i.e. associative) fear was tested by re-exposing the animals to the conditioning chamber for 3 min. In general, testing for recent or remote fear memories was performed either within 1 to 3 (recent fear) or within 28 to 30 days (remote fear) after delivery of the foot shock. The differences in time points are related to different experimental interventions (e.g., intrahippocampal injections) before testing (for details see figures and figure legends).

As a measure of fear, we assessed freezing behavior defined as the absence of all movements except for respiration and the animal's head remaining in a horizontal position. Freezing behavior was recorded and analyzed offline by an observer blind to genotype or treatment.

Oral drug administration

We administered the specific CRHR1 antagonist DMP696 (kindly provided by Bristol Myers Squibb) orally due to its good pharmacokinetic characteristics (Li *et al*, 2003). The drug was dissolved in saccharose-flavored NaCl 0.9% containing 10% DMSO, 10% PEG 400 and Tween 80 (1 drop/ml). The drug suspension was delivered on an oat flake and then fed to the animals at a final dose of 3mg/kg. Controls received an oat flake soaked with saccharose-flavored vehicle only. The dose of 3mg/kg was chosen on the basis of pilot experiments where doses of 3mg/kg, 10mg/kg and 30mg/kg had been compared. The drug solutions were always freshly pipetted onto the flake and shortly dried prior to delivery. *Ad libitum* fed mice received some saccharose-flavored oat flakes 2 days before starting an experiment to habituate them to the novel food. Over the course of treatment, mice showed no signs of aversion against the drug-soaked flakes, but readily ate them within a few seconds after delivery.

Intrahippocampal treatment

Surgery. Mice were anesthetized with isoflurane (Forene®, Abbott, Germany) and placed in a stereotaxic apparatus (TSE systems, Germany) with adapted components to allow mouse inhalation anaesthesia. After exposure of the skull, a hole was drilled and guide cannulae (23G) was implanted bilaterally in the dorsal hippocampus. Coordinates based on the stereotaxic mouse brain atlas (Franklin and Paxinos, 2001) were -1.8 mm posterior to bregma, +/-1.3 mm lateral from midline and 1.0 mm below the surface of the skull. Cannulae were fixed to the skull with dental cement and two jeweller screws inserted into the skull. Mice received analgesic treatment before surgery (0.5 mg/kg meloxicam s.c., Metacam®, Boehringer Ingelheim, Germany) and 3 days afterwards via the drinking water at the same dose. Animals were allowed to recover from surgery for 2 weeks before starting the experiment.

Group assignment. Mice equipped with the guide cannulae received the electric foot shock as described. To ensure equal levels of sensitisation between the different groups before treatment with the AMPA receptor antagonists, we measured sensitised fear in response to a neutral tone essentially as described, except for the duration of the tone (1 min instead of 3 min) either on day 2 or on day 28 after

conditioning. Animals were assigned to treatment and control groups on the basis of their freezing responses to the tone in a way that all the groups showed the same level of sensitised fear before treatment (data not shown).

Intracerebral injections. Mice were slightly anaesthetised with isoflurane, and drugs were infused into the hippocampus (i.h.) in a volume of 0.5 µl (receptor antagonists) or 1 µl (antisense oligonucleotides) per injection site over the course of 1 - 2 min. Injections were performed by means of an injection cannula, which was connected to a microliter syringe via a calibrated tubing containing an air bubble for monitoring treatment progress. The injection cannula protruded from the guide cannula by 1 mm thus reaching the *stratum lacunosum moleculare* of the dorsal hippocampus. After completion of the injection, it was left in place for another minute before removal. Mice were excluded from the experiment, if fluid/blood was flowing out of the guide cannula.

Drugs. AMPA receptor signaling was attenuated/blocked either by the unspecific AMPA/kainate receptor antagonist NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-quinoline-7-sulfonamide disodium salt; 50µM; Tocris Bioscience, Germany) or by philanthotoxin 433, a specific blocker of AMPA receptors containing the Ca^{2+} – permeable GluR1, but not the Ca^{2+} – impermeable GluR2 subunit (100µM; Sigma-Aldrich, Germany). In both cases, Ringer's solution served as vehicle control (Fresenius-Kabi, Germany), and injections were performed 30 min before measuring contextual fear 29 days after foot shock. A third batch of mice was infused with GluR1 antisense (ASO; 5µM; 1 µl infusion volume per side) or missense (MSO) oligonucleotides (dissolved in Ringer's solution) 3 times (12h intervals) before testing for contextual fear memory on day 30 after foot shock administration (without prior tone testing on day 28). Oligonucleotides (Metabion International, Germany) were HPLC-purified 19-mer sequences with phosphorothioate bonds at both 5' and 3' ends: GluR1 ASO, 5'-TAA GCA TCA CGT AAG GAT C-3', GluR1 MSO, 5'-AGC GTA TCA CAG TAT AGA C-3'. GluR1 ASO was screened in public libraries and proven to be specific for mouse *Glur1* mRNA (Slipczuk *et al*, 2009), whereas control MSO sequences did not generate any full matches to identified gene sequences.

Histology. After each experiment, animals were killed with an overdose of isoflurane. Brains were removed, frozen in pre-chilled 2-methylbutane on dry ice and stored at – 80°C until sectioning in a cryostat microtome. Correct placement of the injection sites

within the dorsal hippocampus was assessed in the cryostat unaware of the treatment. This direct assessment enabled us to punch out the dentate gyrus of ASO/MSO treated mice for subsequent Western blot analysis of GluR1 content in addition to histological verification of the injection sites. Only data of mice with correct bilateral hippocampal injections were included into further analyses.

Electrophysiology

Brain slice preparation. Acute hippocampal slices were prepared from 12- 14 weeks old male C57BL/6N mice and 8-15 weeks old male R1^{CaMKII} KO/WT 28 days after conditioning or control exposure to the conditioning chamber without shock delivery. Coronal slices (350µm thick) were prepared using a vibratome (HM650V, Thermo Scientific) and maintained in artificial cerebrospinal fluid (aCSF) containing [in mM] 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 glucose, 25 NaHCO₃, 1 MgCl₂, 2 CaCl₂ at pH 7.4 saturated with 5% CO₂/95% O₂. Slices were allowed to recover in a storage chamber initially at 34°C for 45min and then at room temperature (23-25°C) for another 30min-1h before being transferred to the recording chamber.

AMPA-mEPSC recording. Whole-cell patch-clamp recordings were carried out from granule cells of the dentate gyrus (DG) in hippocampal brain slices by means of a SEC-10LX amplifier (npi Electronics, Tamm, Germany). An infrared videomicroscope equipped with the gradient contrast system was used to visualize somata of DG neurons. Whole-cell pipette internal solutions contained [in mM] 130 CH₃O₃SCs, 5 CsCl, 0.5 EGTA, 2 Mg-ATP, 10 HEPES, 5 glucose at pH 7.4-adjusted with CsOH. Patch-clamp electrodes (open-tip resistance of 4-6MΩ) were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) on a DMZ-Universal puller. Neurons were voltage-clamped at -60mV, and AMPAR-mEPSCs were measured while slices were continuously superfused with oxygenated aCSF containing 1µM tetrodotoxin (TTX), 50µM D(-)-2-Amino-5-phosphonopentanoic acid (AP5) and 10µM (-)-bicuculline methiodide (BIM). All recordings were performed at room temperature.

AMPA-mEPSCs were recorded in individual neurons during a 5min-period 5min after breaking into the cell. Amplitudes and frequencies of AMPAR-mEPSCs were analyzed offline using the Mini Analysis software (Synaptosoft, GA) with a detection threshold set at 4pA.

Chemicals for aCSF preparation were purchased from Merck (Germany), TTX and AP5 from Ascent Scientific (UK) and BIM from Tocris Biosciences (UK).

Western blot analysis of AMPA receptor GluR1 subunits

DG specimens. The amounts of total and synaptosomal AMPAR subunit GluR1 or GluR2 were determined in tissue of hippocampal DG in non-shocked vs. shocked mice. Mice were sacrificed under isoflurane anaesthesia, and brains were removed, frozen in pre-chilled methylbutane on dry ice and stored at -80°C . Frozen brains were sectioned at -20°C in a cryostat microtome and DG tissue of the dorsal hippocampus was dissected bilaterally using cylindrical punches (Fine Science Tools, Heidelberg, Germany; Dahlhoff *et al*, 2010) with internal diameters of 0.6mm. Specimens had a punch-length of approximately 1.0mm. The dissection site was verified by histological analysis.

Total GluR1 and GluR2. The punched hippocampal tissue of two brains was pooled and suspended in 60 μl lysis buffer (50mM TrisHCl, 2%SDS, pH 7.5), heated at 95°C for 10min and further centrifuged at 12.000rpm for 8min at 4°C . The supernatant was transferred to a novel vial and stored at -80°C until western blot analysis (see below).

Synaptosomal GluR1. Synaptosomal membrane preparation was performed with pooled punches of two brains. Tissue was homogenized on ice in 250 μl lysis buffer (0.5M EDTA, 1M Tris-HCl, pH 7.4, 10mM PMSF, 0.08% Calpain I, 0.08% Calpain II and a cocktail of protease inhibitors [Complete Protease Inhibitor Cocktail, Roche Applied Science, Germany]) and further centrifuged at 3000rpm for 4min. The supernatant was transferred to a novel vial and centrifuged at 70.000xg for 35min at 4°C . The membrane pellets were re-suspended in lysis buffer and stored at -80°C .

Immunoblotting. Samples were loaded onto SDS-PAGE gels (10%) after normalization of protein concentrations for each sample (measurement of BSA equivalents by a NanoDrop spectrophotometer; Thermo Fisher Scientific, Germany). The resulting gels were transferred to PVDF membranes (Immobilion-P, Millipore, Germany). The membranes were then blocked with 3% BSA and 1% Tween-20 in PBS over night at 4°C , incubated for 90min with anti-GluR1 (1:100; Anti-GluR1 pAB, Calbiochem, Germany) or anti-GluR2 (1:1000; Anit-GluR2 (extracellular), Alomone Labs, Israel) or the p44/42-MAPK antibody (1:1000; Cell Signaling Technology, MA). The antibodies were dissolved in a buffer containing 1% Tween-20, 5% BSA and 0.05%NaN₃ in PBS. Membranes were further washed three times for 10min with 1%

milk powder and 1% Tween-20 in PBS, and incubated for 1h with peroxidase-conjugated anti-rabbit immunoglobulin (1:50,000 in blocking buffer; Dianova, Germany). After washing with 1% milk powder and 1% Tween-20 in PBS (2*5min) and 1% Tween-20 in PBS (1*10min) the membranes were immersed in chemiluminescence substrate (ECL; Western Lightning Chemiluminescence, PerkinElmer, Germany) for 1min and then scanned in a Curix 60 – scanner (Agfa, Germany). The scanned digital images were quantified using Adobe Photoshop 7.0 software by an investigator unaware of the source of specimens. We analyzed the relative amount of GluR1 (or GluR2) by determining the ratio of the signals detected by GluR1 (GluR2) and the p44/42-MAPK antibodies. In any of the Western blot experiments, we did never observe differences in p44/42-MAPK-levels between the experimental groups (data not shown).

Data analysis and statistics

Data are presented as mean \pm s.e.m.. Behavioral data were averaged to the 3min-testing period and presented as a percentage of the analysis interval. In order to improve the comparability of the data obtained in the different mutant lines (which were kept on different genetic backgrounds) and the experiments with pharmacological treatment, freezing data were expressed as a percentage of the means of the respective controls. Non-normalized data and sample sizes are reported in the text or figures. Data were analyzed by two-group comparisons using unpaired student's t-tests and one-sample t-tests to the basis 100% (if data were expressed relative to the mean control levels) as indicated in the results section and figure legends. Analyses were performed using GraphPad Prism 5.0 (GraphPad, CA), Statistica 5.0 (StatSoft, OK) and SPSS 16.0 (SPSS, IL). Statistical significance was accepted if $p \leq 0.05$.

RESULTS

CRHR1 in the limbic forebrain enhances remote fear memories

We exposed mice with constitutive genetic deletions of either CRHR1 (R1 KO) or CRHR2 (R2 KO) to an electric foot shock and tested them for freezing to a neutral tone in a neutral context as a measure of sensitized fear and to the shock context as a measure of contextual fear 28 days later. R1 KO showed significantly reduced sensitized fear ($t_{22} = 2.4$; $p = 0.023$), but no significant differences in contextual fear ($t_{22} = 1.4$; $p = 0.134$; *WT control* freezing tone: $50.2 \pm 7.7\%$, context: $44.7 \pm 8.5\%$). Changes in fear responses were observed in R2 KO only with respect to an increase in contextual fear (Figure 1a; tone: $t_{23} = 0.6$; $p = 0.549$; context: $t_{23} = 1.7$; $p = 0.045$; *WT control* freezing tone: $64.3 \pm 3.0\%$, context: $73.4 \pm 6.2\%$).

R1 KO mice lack expression of the receptor also at level of the pituitary with the consequence of an impaired HPA axis activity (Timpl *et al*, 1998). To rule out that this deficiency accounts for the reduction in remote sensitized fear, we tested conditional CRHR1 mutants with undisturbed HPA axis activity due to restriction of CRHR1 deletion to the brain ($R1^{Nes}$) or specifically to the forebrain ($R1^{CaMKII}$). Both $R1^{Nes}$ and $R1^{CaMKII}$ KO mice showed significantly reduced sensitized ($R1^{Nes}$: $t_{20} = 2.5$; $p = 0.022$; $R1^{CaMKII}$: $t_{25} = 2.6$; $p = 0.015$) and contextual fear ($R1^{Nes}$: $t_{19} = 2.2$; $p = 0.019$; $R1^{CaMKII}$: $t_{23} = 1.7$; $p = 0.050$) 4 weeks after conditioning (Figure 1a; $R1^{Nes}$ *WT control* freezing tone: $41.1 \pm 8.5\%$, context: $41.8 \pm 5.6\%$; $R1^{CaMKII}$ *WT control* freezing tone: $29.7 \pm 5.2\%$, context: $40.4 \pm 7.5\%$).

Sensitized and contextual fear were not significantly reduced in $R1^{CaMKII}$ KO (tone: $t_{29} = 1.7$; $p = 0.092$; context: $t_{28} = 1.2$; $p = 0.240$), if the mice were tested 1 and 2 days after conditioning (Figure 1b; $R1^{CaMKII}$ *WT control* freezing tone: $55.9 \pm 3.4\%$, context: $63.7 \pm 6.0\%$).

Taken together, our data obtained in $R1^{CaMKII}$ mice indicate that remote fear memories are potentiated by CRHR1 signaling within the limbic forebrain.

The CRHR1 receptor antagonist DMP696 attenuates the development of remote fear memories

Studies in mutant mice preclude a more detailed dissection of CRH effects in terms of early vs. late consolidation, retention or expression of remote fear memories. Therefore, we treated C57BL/6N mice with the specific CRHR1 antagonist DMP696

(3mg/kg; p.o., twice daily) at different time points after the foot shock: Acute administration of DMP696 1h before testing did not affect the expression of sensitized fear 28 days after trauma (treatment group I, Figure 2; $t_{23} = 0.0$; $p = 0.987$; *control* freezing tone: $31.9 \pm 7.2\%$). In contrast, administration of DMP696 for one week starting either immediately (group II) or 24h after foot shock delivery (group III) resulted in a significant reduction of remote, trauma-related fear as assessed 21 days later (Figure 2; II, tone: $t_{25} = 2.4$; $p = 0.022$; III, tone: $t_{25} = 2.3$; $p = 0.033$; II, context: $t_{25} = 2.3$; $p = 0.031$; III, context: $t_{25} = 2.2$; $p = 0.038$; *group II* and *III control* freezing tone: $45.5 \pm 5.7\%$, context: $58.2 \pm 5.3\%$). Once long-term consolidation of remote fear memories was established, memory retention was unaffected by CRHR1, since treatment with DMP696 over the course of 2 weeks, starting 28 days after foot shock followed by two additional weeks of drug wash-out, had no influence on sensitized and contextual fear (Figure 2; IV, tone: $t_{30} = 0.1$; $p = 0.902$; context: $t_{30} = 0.1$; $p = 0.904$; *control* freezing tone: $63.7 \pm 6.2\%$, context: $82.1 \pm 2.1\%$).

AMPA receptor trafficking is associated with the development of remote fear memories

In vitro patch-clamp recordings of AMPAR-mediated miniature excitatory postsynaptic currents (AMPA-mEPSCs) revealed significantly increased amplitudes, but not frequencies, of AMPAR-mEPSCs in DG granule cells of C57BL/6N mice 28 days after the foot shock (Figure 3a; amplitude: $t_{47} = 2.2$; $p = 0.032$; frequency: $t_{47} = 1.1$; $p = 0.295$; resting membrane potentials [RMP]: $53.3 \pm 1.8\text{mV}$ in shocked and $56.1 \pm 1.6\text{mV}$ in non-shocked mice).

This increase in AMPAR-mEPSC amplitudes may result from an increased surface expression of AMPARs, especially GluR1-containing AMPARs (O'Brien *et al*, 1998). Therefore, we quantified GluR1 protein levels in the synaptosomal membrane fraction of dissected DG tissue of shocked vs. non-shocked wild-type (i.e. C57BL/6N) mice by Western blot. Exposure to the foot shock led to a significant increase in surface expression of GluR1 in the DG as assessed 28 days later (Figure 3b). A more detailed temporal analysis of GluR1 expression revealed wave-like changes in synaptosomal GluR1-containing AMPARs with peak levels 1h and 28 days and a trough 3 days after the foot shock (Figure 3b). In contrast, total DG GluR1 or GluR2 levels did not differ significantly between shock or non-shock groups 28 days after

delivery of the foot shock, as measured in a separate experiment [GluR1 (% normalized to controls): $116.3 \pm 7.7\%$, $p=0.07$ (one-sample t-test); GluR2 (% normalized to controls), $89.5 \pm 5.7\%$, $p=0.11$ (one-sample t-test); $n = 8-9/\text{group}$].

GluR1-containing AMPARs in the hippocampus contribute to retrieval and/or expression of remote fear memories

The involvement of the hippocampus in storage and/or retrieval of remote contextual and episodic-like memories is still a matter of debate (Kim and Fanselow, 1992; Kitamura *et al*, 2009; Pastalkova *et al*, 2006; Wiltgen *et al*, 2010; Winocur *et al*, 2010). Therefore, we confirmed the physiological relevance of hippocampal GluR1 receptor for remote contextual fear memories by various means: First, an acute pharmacological blockade of hippocampal AMPA and kainate receptors by NBQX significantly reduced contextual fear 29 days after conditioning (group I, Figure 3c; $t_{19} = 2.5$; $p = 0.021$; *control* freezing: $53.0 \pm 7.2\%$). Second, reduced remote contextual fear was observed after inhibition of GluR1 synthesis by locally infused antisense oligonucleotides (ASO) (group II) as compared to MSO treatment (Figure 3c, left; $t_{11} = 2.3$; $p = 0.043$; *control* freezing: $52.0 \pm 12.6\%$). Western Blot analysis confirmed that ASO treatment led to significantly reduced total GluR1 protein levels in the DG of the dorsal hippocampus ($t_{11} = 4.7$; $p < 0.001$; Figure 3c, right). Third, intrahippocampal administration of philanthotoxin 433 (PhTx), a specific blocker of Ca^{2+} -permeable GluR1 containing AMPARs, also reduced context freezing in the long-term aftermath of the trauma (group III, Figure 3c, left; $t_{23} = 4.7$; $p < 0.001$; *control* freezing: $69.3 \pm 4.6\%$). Noteworthy, as revealed in new groups of mice, the same treatment had no effects on contextual fear 3 days after the foot shock (Figure 3d; $t_{19} = 1.1$; $p = 0.284$; *control* freezing: $64.6 \pm 4.3\%$).

Together, these findings demonstrate that Ca^{2+} -permeable GluR1 containing AMPARs of the hippocampus play a prominent role in the retention and/or expression of remote fear memories only.

Limbic CRHR1 affects remote fear memories via changing the expression of GluR1-containing AMPARs

Finally, we looked for potential interactions between CRHR1 signaling and the expression of GluR1-containing AMPARs. In accordance to our observations in

C57BL/6N mice (cf. Fig. 3a), $R1^{CaMKII}$ WT showed a significant increase in the amplitudes of AMPAR-mEPSCs in DG granule cells 28 days after the foot shock, compared to non-shocked WT controls (Fig. 4a; $t_{41} = 2.2$; $p = 0.036$). $R1^{CaMKII}$ KO, in contrast, failed to show similar changes. Consequently, the increase in the amplitudes observed in shocked WT mice was significantly higher than that of shocked KO (Figure 4a; $t_{44} = 2.6$; $p = 0.011$; DG neuron RMPs: $RMP_{WT, no\ shock} = 54.6 \pm 2.0mV$, $RMP_{KO, no\ shock} = 56.3 \pm 1.9mV$; shock: $RMP_{WT, shock} = 54.8 \pm 1.7mV$, $RMP_{KO, shock} = 53.1 \pm 1.5mV$).

Shocked $R1^{CaMKII}$ KO mice also expressed significantly less surface GluR1 protein in the DG than shocked $R1^{CaMKII}$ WT (as measured by Western blots in synaptosomal membrane preparations of dissected DG tissue in the remote fear situation; Figure 4b; $t_8 = 2.5$; $p = 0.036$). No genotype differences were observed under non-shock conditions (data not shown). Similarly, wild-type mice treated with DMP696 for one week starting 24h after the foot shock (cf. group III, Figure 2) also showed reduced synaptosomal GluR1 levels compared to shocked vehicle-treated controls (Figure 4c; $t_8 = 3.7$; $p = 0.007$). In addition, there was a significant positive correlation between the contextual fear responses shown at day 28 and the levels of GluR1 in vehicle and DMP696 treated mice (Figure 4d; Pearson's $r = 0.71$; $p = 0.03$).

Together, these findings indicate that pharmacological blockade of CRHR1 within the first week after conditioning interferes with the consolidation of remote fear memories by preventing the long-lasting increase in GluR1 mediated neurotransmission at the level of the DG.

DISCUSSION

The present study demonstrates by genetic and pharmacological means that CRH enhances the consolidation, but does not affect expression or retention, of remote fear memories (i.e. freezing to context) and fear sensitisation (i.e. freezing to neutral tone), and that this effect depends on CRHR1 signaling in the limbic forebrain within the first week after perception of an aversive foot shock. It has already been reported that CRH facilitates the acquisition and consolidation of fear memories, as assessed within a few days after conditioning (Kolber *et al*, 2008; Pitts *et al*, 2009; Radulovic *et al*, 1999; Roozendaal *et al*, 2002). Our data substantially extend those observations by demonstrating that CRHR1 signaling plays a pivotal role in the consolidation of remote fear memories. In fact, conditional mutants lacking CRHR1 expression in the limbic forebrain (R1^{CaMKII} KO) showed significantly less sensitised and contextual fear 1 month, but not a few days, after delivery of the foot shock. In line with this reasoning, transient pharmacological blockade of CRHR1 attenuated remote fear memories even if initiated 24h after foot shock, i.e. after the initial consolidation period.

In a number of subsequent experiments we identified and characterized GluR1-containing AMPA receptors within the dorsal hippocampus as putative effectors of CRHR1-mediated consolidation of remote fear memories. Learning-induced trafficking and translation of this specific glutamate receptor subtype in the hippocampus or amygdala have consistently been shown to be required for the consolidation of context (Matsuo *et al*, 2008; Mitsushima *et al*, 2011) or auditory fear memory (Hu *et al*, 2007; Rumpel *et al*, 2005; Thoeringer *et al*, 2010) and inhibitory avoidance learning (Cammarota *et al*, 2004; Whitlock *et al*, 2006). These studies investigated GluR1-driven consolidation mechanisms at early time points after conditioning leaving the question about the role of hippocampal AMPA receptor regulation in the development and stabilization of long-lasting memories unanswered. In the present study, we observed a so far unrecognized delayed-onset and long-lasting increase in AMPAR GluR1-mediated neurotransmission in the dentate gyrus, which became evident by increased amplitudes of AMPAR mEPSCs and increased GluR1 surface expression in synaptosomal fractions. GluR1 expression peaked 1h and 28 days after the foot shock, but showed a trough after 3 days. Consequently, GluR1-containing AMPARs might be involved not only in acquisition and initial consolidation of fear, but also, and in particular, in the retention/expression of remote

fear memories. This conclusion was substantiated by the finding that both pharmacological blockade and antisense oligonucleotide-mediated down-regulation of GluR1-containing AMPARs attenuated remote contextual fear. A similar result has recently been published for the retrieval of detailed contextual memories in mice (Wiltgen *et al*, 2010). Noteworthy, treatment with philanthotoxin 433 attenuated contextual fear 29 days, but not 3 days, after conditioning, thus substantiating that Ca^{2+} -permeable GluR1 containing AMPARs play also a prominent role in the retention and/or expression of remote fear memories. As these effects were abolished in $\text{R1}^{\text{CaMKII}}$ KO and CRHR1 antagonist-treated mice, we conclude that CRH enhances the consolidation of remote fear memories by stabilizing the surface expression of GluR1-containing AMPA receptors.

It is still a matter of debate whether episodic and contextual memories are permanently stored in the hippocampus. Our study proves a major role of the dorsal hippocampus in the expression and/or retention of remote fear memories 1 month after conditioning. In this way it contradicts the hypothesis of a temporally limited involvement of the hippocampus in memory storage and/or recall (Kim and Fanselow, 1992). It is conceivable that the differences in the involvement of the hippocampus in recall/expression of remote fear memories relate, at least in part, to the intensity of the conditioning procedure employed in the present study (1.5 mA): Higher emotional load (stress) is associated with decreased neurogenesis at level of the dentate gyrus (Dranovsky and Hen, 2006) that, in turn, may account for the persistent involvement of the hippocampus in processing of remote fear memories (Kitamura *et al*, 2009). An enhanced postsynaptic AMPAR neurotransmission within the dentate gyrus may contribute to this process and, thus, accompany recurrent activation of NMDA receptors (Shimizu *et al*, 2004) as mediator of long-term retention of remote fear memories.

In the clinical situation, exaggerated remote fear memories and hyperarousal (i.e. fear sensitization) are core symptoms of PTSD. Given the fact that PTSD patients show elevated cerebrospinal fluid levels of CRH (Baker *et al*, 1999; Bremner *et al*, 1997), our findings provide the neurobiological rationale for a pharmacological blockade of CRHR1 in early aftermath of a trauma as a novel treatment strategy for attenuating the development of the disease. Despite potential side effects (Holsboer and Ising, 2008), treatment with CRHR1 antagonist might be a more promising approach than, for instance, the beta blocker propranolol (so far the only drug

extensively tested in the pharmaco-prophylaxis of PTSD), which has failed to meet initial hopes (Baker *et al*, 2009). Importantly, CRHR1 antagonist treatment might be initiated even 24 h after the traumatic incident and discontinued after a few days, thus demonstrating a sufficiently broad temporal window for pharmacological intervention, while minimizing the risk of potential side effects.

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CONFLICT OF INTEREST

The authors declare full disclosure of any financial interest.

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FIGURE LEGENDS

Figure 1: Limbic CRHR1 signaling promotes long-term consolidation of sensitized and contextual fear.

(a) Conventional (R1 KO) and, in particular, conditional CRHR1 knockouts (Cre-induced brain- [Nestin promoter, R1^{Nes} KO] and forebrain- [CaMKII promoter, R1^{CaMKII} KO] specific CRHR1 deletions), but not conventional CRHR2 knockouts (R2 KO), show reduced freezing to a 3-min tone in a novel context (○) and to the shock context (■) 28 and 29 days after exposure to an un-signaled electric foot shock (1.5mA, 2sec; ▩). Sample sizes were as follows: R1 (control/KO) = 8/16, R1^{Nes} = 10/12, R1^{CaMKII} = 14/13 and R2 = 13/12. For the sake of clarity, freezing levels were expressed as a percentage of the means of the respective wild-type controls. **(b)** Genotype differences of R1^{CaMKII} mice in freezing to tone and context were not significantly reduced, if the animals were tested 1 to 2 days after foot shock. n (control/KO) = 16/14. * $p < 0.05$ (one-sample t-test).

Figure 2: Effects of preventive treatment with the CRHR1 inhibitor DMP696 on development, expression and retention of remote fear memories.

Treatment with the CRHR1 antagonist DMP696 (3mg/kg, p.o.) at different time points after foot shock affects consolidation, but not expression and retention of sensitised and contextual fear. Group I, acute drug administration before neutral tone exposure at day 28 after the foot shock [n (vehicle/DMP696) = 12/13]; group II, immediate post-foot shock drug treatment lasting for 7 days (twice daily; n=14/13); group III, delayed post-foot shock (+24h) drug treatment lasting for 7 days (twice daily; n=14/13) and testing on day 28/29; group IV, therapeutic treatment starting 28 days after the foot shock and lasting for 2 weeks (twice daily; n=16/16) followed by a wash-out period of 2 weeks before testing on day 49/50. Freezing data of DMP696-treated mice were expressed as a percentage of the averaged freezing responses of the respective vehicle-treated controls (dotted line = 100%). * $p < 0.05$ (one-sample t-test).

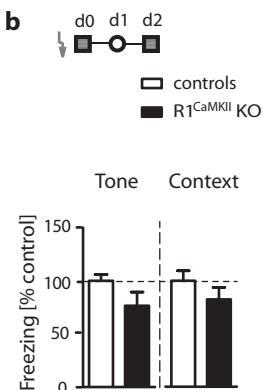
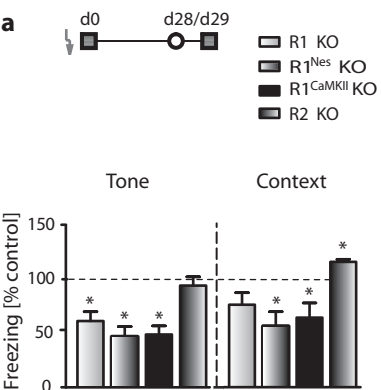
Figure 3: Hippocampal GluR1-containing AMPARs control the retention and/or expression of remote fear.

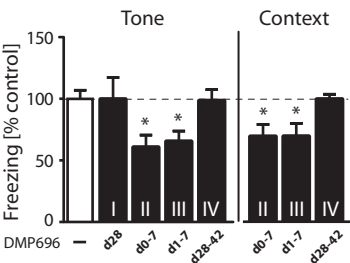
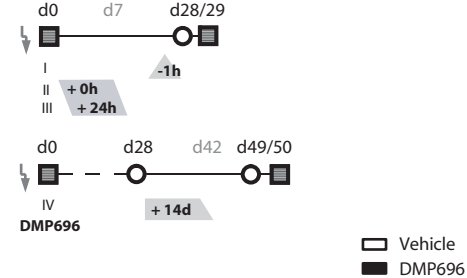
(a) Representative traces of *in vitro* patch-clamp AMPAR-mEPSC recordings from DG granule cells of C57BL/6N mice 28 days after exposure to a foot shock or control exposure (upper panel). Shocked mice showed significantly increased amplitudes, but not frequencies, of AMPAR-mEPSCs (N [no

shock/shock] = 7/12 mice; n [no shock/shock] = 20/29 neurons). **(b)** Western blot analyses of synaptosomal GluR1 protein in the DG of C57BL/6N mice 1h, 24h, 3 days, 7 days or 28 days after exposure to a foot shock or control exposure (controls for early group [1h – 7d] and separate controls for 28d-group). Shocked mice showed significantly higher levels of membrane-standing GluR1 at time points 1h and 28d and significantly lower levels 3 days after foot shock (shock: n = 6 [1h, 24h, 3d, 7d] and 10 [28d]/group; no shock: n = 6/early group, n = 10/28d-group). **(c) Left:** Attenuated AMPAR signaling in the dorsal hippocampus of C57BL/6N mice significantly reduced contextual fear 1 month after conditioning. Group I, acute infusion of NBQX (50 μ M; n = 11), an AMPA and kainate receptor antagonist; group II, infusion of GluR1 antisense oligonucleotides (ASO 5 μ M; n = 7; 3 infusions spaced 12 h apart); group III, acute treatment with philanthotoxin 433 (PhTx; 100 μ M; n = 13), a specific antagonist of Ca²⁺-permeable GluR1 containing AMPA receptors. Controls received vehicle (n = 10-12) or GluR1 missense oligonucleotides (MSO 5 μ M; n = 6). *Right:* Western blot analysis revealed that GluR1 ASO significantly reduced total GluR1 in the dentate gyrus of group II mice compared to MSO treated controls. **(d)** Acute infusions of PhTx (100 μ M; n = 10) into the hippocampus before contextual fear testing on day 3 after conditioning did not affect the fear response compared to vehicle-treated controls (n = 11). GluR1/MAPK ratios (B) and freezing data (C) were expressed as a percentage of the means of the respective controls (dotted line = 100%). [#]p<0.05 (unpaired t-test), *p<0.05 (one-sample t-test compared to control levels of 100%).

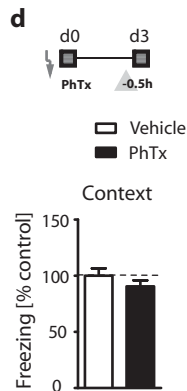
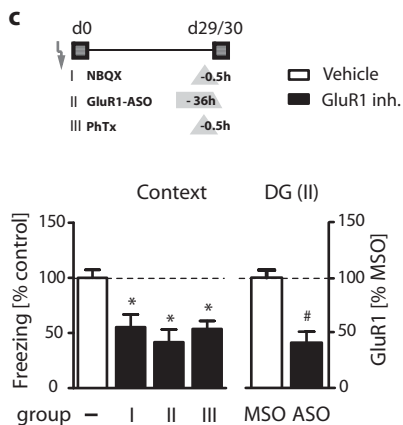
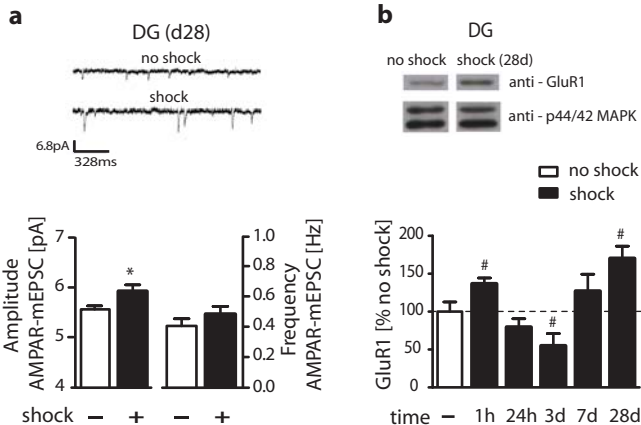
Figure 4: Hippocampal AMPAR neurotransmission is controlled by limbic CRHR1. **(a)** Amplitudes of AMPAR-mEPSCs were significantly increased in DG granule cells of wild-type littermate controls, but not in R1^{CaMKII} KO, 28 days after conditioning as compared to shocked KO and non-shocked WT. Sample sizes were as follows: n_{no shock} [control/R1^{CaMKII} KO] = 20/20, n_{shock} = 23/23 neurons measured in N_{no shock} [control/R1^{CaMKII} KO] = 6/7 and N_{shock} = 7/8 mice. **(b)** R1^{CaMKII} KO displayed significantly less synaptosomal GluR1 protein levels in the DG than littermate WT controls 28 days after foot shock (n = 5/group). **(c)** DMP696 treatment starting 24h after trauma and lasting for 7 days (cf. Fig. 2, group III) led to significantly reduced surface expression of GluR1 in the DG 36 days after foot shock, compared to vehicle-treated controls (n = 5/vehicle, n = 4/DMP696). **(d)** Significant positive

correlation of GluR1 and freezing levels in DMP696 and vehicle treated mice (cf. group III; Figure 2). AMPAR-mEPSC data were expressed relative to the non-shock levels of the same genotype; GluR1/MAPK ratios were expressed as a percentage of the mean WT levels. # $p < 0.05$ (unpaired t-test), * $p < 0.05$ (one-sample t-test).

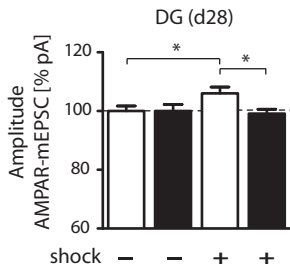
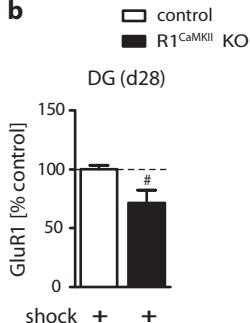
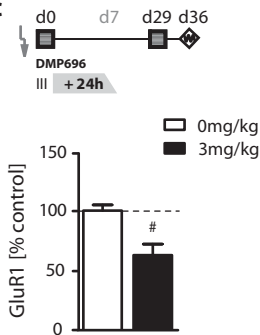




Thoeringer et al., **Figure 2**



Thoeringer et al., **Figure 3**

a**b****c****d**