ORIGINAL RESEARCH REPORT

Long-term changes in the CA3 associative network of fear-conditioned mice

Gürsel Çalışkan1,2*, Anne Albrecht1,3,4*, Jan O. Hollnagel2, Anton Rösler2, Gal Richter-Levin3,4, Uwe Heinemann2, and Oliver Stork1,5

1Department of Genetics & Molecular Neurobiology, Institute of Biology, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany, 2Institute for Neurophysiology, Charité-Universitätsmedizin Berlin, Berlin, Germany, 3Sagol Department of Neurobiology, University of Haifa, Haifa, Israel, 4The Institute for the Study of Affective Neuroscience, University of Haifa, Haifa, Israel, and 5Center for Behavioral Brain Sciences, Magdeburg, Germany

Abstract

The CA3 associative network plays a critical role in the generation of network activity patterns related to emotional state and fear memory. We investigated long-term changes in the corticosterone (CORT)-sensitive function of this network following fear conditioning and fear memory reactivation. In acute slice preparations from mice trained in either condition, the ratio of orthodromic population spike (PS) to antidromic PS was reduced compared to unconditioned animals, indicating a decrease in efficacy of neuronal coupling within the associative CA3 network. However, spontaneous sharp wave–ripples (SW-R), which are thought to arise from this network, remained unaltered. Following CORT application, we observed an increase in orthodromic PS and a normalization to control levels of their ratio to antidromic PS, while SW-R increased in slices of fear conditioned and fear reactivated mice, but not in slices of unconditioned controls. Together with our previous observations of altered hippocampal gamma activity under these learning paradigms, these data suggest that fear conditioning and fear reactivation lastingly alters the CORT-sensitive configuration of different network activity patterns generated by the CA3 associational network. Observed changes in the mRNA expression of receptors for glutamate, GABA and cannabinoids in the stratum pyramidale of area CA3 may provide a molecular mechanism for these adaptive changes.

Keywords

CA3 associative network, corticosterone, fear memory, glutamatergic receptors, sharp wave–ripples, ventral hippocampus

Introduction

The ventral hippocampal CA3 region, in close interaction with the hypothalamic–pituitary–adrenal (HPA) stress axis (Jacobson & Sapolsky, 1991; Maggio & Segal, 2007), plays a key role in anxiety modulation and formation of fear memory (Bannerman et al., 2004; Trivedi & Coover, 2004). Chronic changes in circulating levels of corticosterone (CORT) or deficits in the HPA stress axis response are characteristic for affective and anxiety disorders (Ströhle & Holsboer, 2003). We have previously shown that a single fear reactivation session is sufficient to induce long-term changes in circulating CORT levels as well as in gamma frequency oscillations in area CA3 of the ventral hippocampus (Albrecht et al., 2013). Using slice preparations with and without CORT supplementation, it is possible to address the functional network changes in this region that may mediate the behavioral consequences of conditioned fear stress.

The ventral hippocampal CA3 region is characterized by an associative network built of recurrent axon collaterals that target other pyramidal cells and interneurons in which sequential information can be stored (Le Duigou et al., 2014). In addition to gamma oscillations, this collateral associative network generates sharp wave–ripples (SW-R), positive field potential (FP) transients, which occur spontaneously in ventral hippocampal slices of mice (Maier et al., 2002), and are superimposed by ripples with a frequency of ~200 Hz (Kranig et al., 2013; Maier et al., 2003, 2009). Gamma oscillatory activity and SW-R are believed to participate in memory formation, but while gamma oscillations occur during more active behaviors, e.g. exploration of a context (Hájos & Paulsen, 2009; Montgomery & Buzsáki, 2007), SW-R occur during quiescent behavior. SW-R are relevant for memory consolidation by replay during sleep as well as for decision-making in a new situation in relation to previous memories in awake animals (Girardeau et al., 2014; Jadhav et al., 2012).

Therefore, we determined the effects of fear conditioning and fear reactivation on the strength of collateral associative network activity in area CA3 and its responsiveness to CORT. To address molecular factors potentially involved in the

*These authors contributed equally to this work.

Correspondence: Gürsel Çalışkan, Institute of Biology, Otto-von-Guericke-University, Leipziger Str. 44, Haus 91, 39120, Magdeburg, Germany. E-mail: caliskan.gursel@gmail.com
altered network function, we examined the mRNA expression of several receptors (NMDA-R, AMPA-R, GABA<sub>A</sub>-R, cannabinoid receptor 1 [CB1-R]) that have previously been related to generation and maintenance of SW-R in the ventral hippocampal area CA3. Our data show a differential long-term modification of the CA3 collateral associative network and its sensitivity to CORT following fear learning and fear reactivation.

Materials and methods
Seven-week-old male C57B/6BomTac mice were obtained from M&B Taconic (Berlin, Germany) and acclimatized to our animal facility for one week (12 h light/dark cycle with lights on at 19:00 h and 30 min dawn phases; food and water were provided ad libitum). After another week of single housing, animals were randomly assigned to the different experimental groups. All experiments were conducted in accordance with the European and German regulations for animal experiments and were approved by the Landesverwaltungsamt Sachsonia-Anhalt (AZ 2-618).

Fear conditioning and fear reactivation
Data were obtained from fear conditioned, fear reactivated and control animals described previously (Albrecht et al., 2013). Different sets of animals underwent auditory-cued fear conditioning and its reactivation and were either used for examining oscillatory activity of the ventral hippocampus in slice preparations or gene expression changes in ventral CA3 sublayers via laser capture microdissection and real-time PCR. In brief, all animals were adapted in four sessions (twice a day) to the fear conditioning chamber, a 16 cm × 32 cm × 20 cm acrylic glass arena with a grid floor, loudspeaker and ventilation fan in a sound isolation cubicule (background noise 70 dB SPL, light intensity <10 lux; TSE, Bad Homburg, Germany). Each adaptation session consisted of 2-min exposure to the conditioning context, followed by six exposures to a neutral tone (CS−: 2.5 kHz for 10 s, 80 dB; 20 s inter stimulus intervals (ISI)). One day later, after 2 min of context exposure, mice were trained to associate a tone (CS+: 10 kHz for 10 s, 80 dB) immediately followed by a footshock (US: 0.4 mA for 1 s) in three pairings, separated by 20 s ISI. Reactivation of fear memory took place 24 h later by re-exposure to the training context (2 min) and to four CS− (10 s each, 20 s ISI) and four CS + (10 s each, 20 s ISI) delivered in the training context. While the fear reactivation group received the full protocol (group R), the reactivation session 24 h posttraining was omitted in the non-reactivation group (NR). An additional control group (CTL) received only tones (3 × 10 kHz, 10 s, 80 dB) but no foot shocks during the training session. During sessions, the animal’s behavior was assessed online via a photo beam detection system. All mice were left undisturbed except for animal care and then either slice electrophysiology or gene expression was studied in ex vivo preparations four weeks after fear conditioning.

Electrophysiological recordings of SW-R activity and population spikes in the ventral CA3
Animals of groups reactivation (R, N = 7), NR (N = 7) and control (CTL, N = 6) underwent the respective fear conditioning and reactivation protocols as described above and were decapitated 30 d later under deep isoflurane anesthesia. Horizontal ventral hippocampal slices (400 μm) were cut at an angle of about 12° in the fronto-occipital direction. The preparation of slices was done in ice-cold, carbogenated (5% CO<sub>2</sub>/95% O<sub>2</sub>) artificial cerebrospinal fluid (aCSF) containing (in mM) 129 NaCl, 21 NaHCO<sub>3</sub>, 3 KCl, 1.6 CaCl<sub>2</sub>, 1.8 MgCl, 1.25 Na<sub>2</sub>HPO<sub>4</sub> and 10 glucose. Slices were transferred to an interface chamber perfused with aCSF at 34 ± 0.1°C (flow rate: 1.8 ± 0.2 ml/min, pH 7.4, osmolarity ~300 mosmol/kg). Slices were incubated at least for an hour before starting recordings.

Extracellular field recordings were obtained from stratum pyramidale (SP) of area CA3. FP responses were evoked by constant voltage stimulation of stratum radiatum (SR) in area CA1 using bipolar platinum wire electrodes with exposed tips of 50–80 μm and tip separations of 100–200 μm (electrode resistance in aCSF: ~10 KOhm). Drugs were applied via continuous bath perfusion: CORT (1 μM, Sigma-Aldrich, Steinheim, Germany) was diluted freshly prior to the experiment using a stock solution with dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany). Microelectrodes were filled with aCSF (resistance: 5–10 MΩ). Signals were pre-amplified using a custom-made amplifier and low-pass filtered at 3 kHz. Signals were sampled at a frequency of 5 kHz and stored on a computer hard disc for off-line analysis.

Statistical data were reported as mean ± standard error of the mean. Before statistical comparison of different groups, normality test (Shapiro–Wilk test) and equal variance test were performed. Group differences were determined by one-way ANOVA. Post hoc Fisher’s LSD or Dunn’s method was used for pair-wise comparison. To statistically compare the CORT effect, Student’s t test or Mann–Whitney U test was used (SigmaPlot for Windows Version 11.0, 2008, Systat Software, Erkrath, Germany).

Analysis of population spikes in the ventral CA3
Orthodromic population spike (PS) generation is due to activation of recurrent excitatory interactions between CA3 pyramidal cells (Behrens et al., 2005; Fano et al., 2012). Schaffer collateral (SC) stimulation induces an antidromic PS in the CA3 network, which leads to generation of a secondary orthodromic PS (Behrens et al., 2005; Fano et al., 2012). To obtain a stimulus response curve of CA3 PS, constant voltage stimuli ranging from 2 V to 10 V were delivered to SR in area CA1. SR stimulation results in activation of SCs, which induces first an antidromically evoked PS in area CA3, strength of which indicates the number of activated fibers. This is followed by a field EPSP superimposed by a secondary PS (orthodromic) due to activation of recurrent axon collaterals between CA3 pyramidal cells and between CA3 pyramidal cells and interneurons (Figure 1A). The strength of PS was measured by calculating the area (mV.ms) of the PS. To measure the strength of collateral associative network activation in area CA3, the orthodromic PS was normalized to the antidromic PS for all stimulation intensities. Stimulus intervals of 20 ms, 30 ms, 40 ms, 50 ms and 100 ms were used to calculate paired-pulse ratios by dividing the...
second orthodromic PS area to the first orthodromic PS area. Paired-pulse ratios were measured using the responses obtained from stimulations with 6–8 V intensities. Data analysis was performed off-line using Spike2, version 6, software (Cambridge Electronic Design, Cambridge, UK).

Analysis of SW-R in the ventral CA3

After one hour of incubation, SW-R appeared in SP of area CA3 of ventral horizontal hippocampal slices (Supplementary Figure 1). For analysis of SW-R, 2-min data files were extracted to be further analyzed using a MATLAB-based code (MathWorks, Natick, MA). Sharp waves (SWs) were detected by low-pass filtering the data at 45 Hz (Butterworth, 8th order). The threshold for event detection was set to 2.5 times the standard deviation (SD) of the low-pass-filtered signal. Minimum interval between two subsequent SW was set to 80 ms. Data stretches of 125 ms centered to the maximum of SW event were stored for further analysis. To analyze the area under the curve of SW, the points crossing the mean of the data were used as the start and the end point of SW. The SW area was measured using trapezoidal numerical integration of low pass-filtered data.

To isolate the ripples, the raw data was band-pass filtered at 120–300 Hz (Butterworth, 8th order). Data stretches of 15 ms before and 10 ms after the maximum of SW event (25 ms) were stored for further analysis. Threshold for ripple detection was set to three times SD of the band-pass filtered signal. To analyze the ripple amplitude, triple-point-minimax-determination was used. If the difference between falling and rising component of a ripple was higher than 75%, ripples were discarded from analysis. Frequencies were calculated only from subsequent ripples. Furthermore, to decompose a time-series into time-frequency space, a wavelet analysis using the Morlet wavelet transform was performed (Erchova et al., 2004; Farge, 1992).
mRNA expression in sublayers of ventral hippocampal CA3 region

Mice from groups CTL, NR and R (N = 6 each) were killed by cervical dislocation 30 d after fear conditioning, at a day time with low internal CORT plasma levels (2.00–3.00 pm at an inverse light–dark cycle; Albrecht et al., 2013). Brains were quickly removed, embedded in Tissue Tek freezing compound and snap-frozen in methylbutane cooled by liquid nitrogen. For laser capture microdissection, 8–10 horizontal cryosections per animal (20 μm thick) at the level of the ventral hippocampus were mounted on 0.05% poly-l-lysine-coated RNase-free PEN membrane slides (Carl Zeiss, Jena, Germany), fixated in −20°C cold 70% ethanol and briefly stained with 1% cresyl violet acetate (Sigma-Aldrich) under nuclease-minimized conditions. Using a laser capture microdissection system (Carl Zeiss), the SR, SP and stratum oriens (SO) of the ventral CA3 were microdissected and collected in an adhesive cap capture device (Carl Zeiss). Sample lysis, removal of genomic DNA and isolation of total RNA was done with the RNeasy Micro Plus kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. First-strand synthesis of cDNA was performed with the Sensiscript Reverse Transcription kit (Qiagen) for low amounts of RNA, in the presence of 2.5 mM dNTPs, Oligo (dT)18/random decamer first-strand primer mix (50 μM each; Life Technologies, Darmstadt, Germany) and RNase Inhibitor (SuperaseIN; 20 U/μl; Life Technologies) for 60 min at 37°C. Determination of relative gene expression levels of different glutamate receptor subunits was done in a 1:5 dilution of cDNA via quantitative multiplex PCR (ABI Prism Step One real-time PCR; Life Technologies). TaqMan® reagents and predesigned assays were used with different fluorescent dyes labeling glutamatergic and GABAergic targets (TaqMan gene expression assays, Life Technologies; assay IDs: GlnA1 (Gria1): Mm00433753_m1; GlnN1 (Gri1): Mm00433790_m1; GluN2A (Grin2a): Mm00433802_m1; GluN2B (Grin2b): Mm00433820_m1; GABA_Aα2 (Gabra2): Mm00433435_m1; GABA_Aα3 (Gabra3): Mm01294271_m1; CB1 (Cnr1): Mm01212717_s1) or the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; endogenous control, Life Technologies). All samples were run in triplicates in 50 cycles of 15 s at 95°C and 1 min at 60°C, preceded by a 2-min decontamination step at 95°C with uracil-N-glycosidase and 1 min at 95°C for 10 min.

For data analysis, the cycle thresholds (CTs) in each triplicate assay and each target gene was determined and relative quantification (RQ) was conducted with the ddCT method (Livak & Schmittgen, 2001) by normalizing each sample to the overall content of cDNA using GAPDH as an internal control (ddCT; dCT = dCT (target gene) − dCT (GAPDH)). All ddCT values were normalized to the mean of control group for each target gene and area with ddCT = dCT (sample) − mean dCT (control group). Transformation to RQ values was done according to RQ = 2−ddCT × 100 (%) with RQ (control) = 100%. Statistical analysis was performed based on RQ% values by one-way ANOVA for the factor training group followed by LSD tests for post hoc comparison in each layer and for each target gene.

Results

Changes in the associative network of CA3 after fear conditioning and/or its reactivation

We analyzed the relationship of antidromically induced PS to orthodromically induced PS, which depends on secondary activation during synaptic interactions within area CA3 (Figure 1A1–2). The antidromic PS (n = 5–7 slices for each group) was not altered in different groups for stimulus intensities of 2–10 V (Figure 1B1; stimulation intensity: 6 V; one-way ANOVA: F(2, 17) = 1.43, p = 0.267; CTL: 5.1 ± 0.9 mVms, NR: 3.4 ± 0.9 mVms, R: 3.4 ± 0.4 mVms). In addition, CORT (1 μM) had no significant effect on antidromic PS (Student’s t-test or Mann–Whitney U test: p > 0.05; Figure 1C1–E1) for all stimulation intensities in all groups. The orthodromic PS (n = 5–7 slices for each group) was also not significantly altered in both NR and R (Figure 1B2; stimulation intensity: 8 V; one-way ANOVA on ranks: H(2) = 5.62, p = 0.06; CTL: 0.5 ± 0.2 mVms, NR: 0.1 ± 0.04 mVms, R: 0.3 ± 0.1 mVms). Interestingly, CORT increased the orthodromic PS area for higher stimulation intensities in both group NR (Figure 1D2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.002; 0.1 ± 0.04 mVms versus 1.0 ± 0.3 mVms) and group R (Figure 1E2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.022; 0.3 ± 0.1 mVms versus 1.0 ± 0.4 mVms), while in control no significant change was observed (Figure 1C2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.589; 0.5 ± 0.2 mVms versus 0.9 ± 0.4 mVms). Finally, when the orthodromic PS was normalized to the antidromic PS, we obtained a measure of synaptic interactions between CA3 pyramidal cells. Both group NR (0.038 ± 0.009, p = 0.009) and group R (0.059 ± 0.015, p = 0.027) had decreased orthodromic versus antidromic PS ratio (one-way ANOVA on Ranks: H(2) = 9.363, p = 0.009; Figure 1F) compared to the CTL group (0.102 ± 0.019). The decrease in this ratio could be rescued by CORT in both groups NR (Mann–Whitney U test: p ≤ 0.001; 0.038 ± 0.009 versus 0.097 ± 0.014) and R (Mann–Whitney U test: p = 0.007; 0.059 ± 0.015 versus 0.162 ± 0.040). This was not the case in CTL group (Mann–Whitney U test: p = 0.359; 0.102 ± 0.019 versus 0.128 ± 0.023).

Finally, we analyzed the paired-pulse ratio of orthodromic PS (n = 5–7 slices for each group) for intervals ranging from 20 ms to 100 ms (Figure 2). Below 30 ms, second orthodromic PS values tended to be lower than those for the first orthodromic PS values without any significant change between the groups (stimulation interval: 20 ms; one-way ANOVA on ranks: H(2) = 5–7 slices for each group) was also not significantly altered in both NR and R (Figure 1B2; stimulation intensity: 8 V; one-way ANOVA on ranks: H(2) = 5.62, p = 0.06; CTL: 0.5 ± 0.2 mVms, NR: 0.1 ± 0.04 mVms, R: 0.3 ± 0.1 mVms). Interestingly, CORT increased the orthodromic PS area for higher stimulation intensities in both group NR (Figure 1D2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.002; 0.1 ± 0.04 mVms versus 1.0 ± 0.3 mVms) and group R (Figure 1E2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.022; 0.3 ± 0.1 mVms versus 1.0 ± 0.4 mVms), while in control no significant change was observed (Figure 1C2; stimulation intensity: 8 V; Mann–Whitney U test: p = 0.589; 0.5 ± 0.2 mVms versus 0.9 ± 0.4 mVms). Finally, when the orthodromic PS was normalized to the antidromic PS, we obtained a measure of synaptic interactions between CA3 pyramidal cells. Both group NR (0.038 ± 0.009, p = 0.009) and group R (0.059 ± 0.015, p = 0.027) had decreased orthodromic versus antidromic PS ratio (one-way ANOVA on Ranks: H(2) = 9.363, p = 0.009; Figure 1F) compared to the CTL group (0.102 ± 0.019). The decrease in this ratio could be rescued by CORT in both groups NR (Mann–Whitney U test: p ≤ 0.001; 0.038 ± 0.009 versus 0.097 ± 0.014) and R (Mann–Whitney U test: p = 0.007; 0.059 ± 0.015 versus 0.162 ± 0.040). This was not the case in CTL group (Mann–Whitney U test: p = 0.359; 0.102 ± 0.019 versus 0.128 ± 0.023).

Finally, we analyzed the paired-pulse ratio of orthodromic PS (n = 5–7 slices for each group) for intervals ranging from 20 ms to 100 ms (Figure 2). Below 30 ms, second orthodromic PS values tended to be lower than those for the first orthodromic PS values without any significant change between the groups (stimulation interval: 20 ms; one-way ANOVA on ranks: H(2) = 1.830, p = 0.401) indicating that fast inhibition was unaffected. Similarly, CORT did not change the paired-pulse facilitation in area CA3 of ventral hippocampal slices in all groups for all intervals (Student’s t-test or Mann–Whitney U test: p > 0.05).

Combined, these data suggest that fear conditioning and its reactivation decrease the strength of collateral coupling within the associative network in CA3 of ventral hippocampal slices and that this can be normalized by CORT.

SW-R activity in fear conditioned mice

We hypothesized that changes we observed in CA3 associative network strength would be reflected in changes in a
CA3-dependent network activity. Thus, we recorded SW-R recorded from SP of area CA3 (Figure 3A–E), which did not differ with respect to incidence (one-way ANOVA: \( F(2, 17) = 0.317, p = 0.732, n = 6–7 \) per group; Figure 3A–B) in the “no reactivation group” \( (NR, 1.08 \pm 0.11 \text{ Hz}) \) and the “reactivation group” \( (R, 0.96 \pm 0.16 \text{ Hz}) \) compared to control \( (0.97 \pm 0.08 \text{ Hz}) \). However, pre-application of CORT \( (1 \mu M, \text{Figure 3A–B}, n = 5–7 \text{ per group or treatment}) \) for 30 min increased incidence of SW-R in both group NR \( (t\text{-test: } t(10) = -2.897, p = 0.016, 1.08 \pm 0.11 \text{ Hz versus } 1.49 \pm 0.06 \text{ Hz}) \) and group R \( (t\text{-test: } t(10) = -2.154, p = 0.05, 0.96 \pm 0.16 \text{ Hz versus } 1.42 \pm 0.09 \text{ Hz}) \), while in control group, the CORT effect was not significant \( (t\text{-test: } t(9) = 0.913, p = 0.385, 0.97 \pm 0.08 \text{ Hz versus } 1.19 \pm 0.26 \text{ Hz}) \). Likewise, the SW area was not different between groups (one-way ANOVA: \( F(2, 17) = 0.055, p = 0.947; \) Figure 3C). However, when CORT was applied, we noted an increase in SW area in the NR group only \( (t\text{-test: } t(9) = 2.365, p = 0.04; 0.84 \pm 0.18 \text{ mV ms versus } 2.21 \pm 0.65 \text{ mV ms; Figure 3C}) \). Ripple amplitudes were not significantly altered and remained unaffected by CORT (one-way ANOVA: \( F(2, 17) = 0.312, p = 0.736; \) Figure 3D). Similarly, ripple frequency was not altered in groups NR and R compared to control (one-way ANOVA: \( F(2, 17) = 0.073, p = 0.93; \) Figure 3E). However, CORT significantly decreased ripple frequency in group NR \( (t\text{-test: } t(9) = 4.279, p = 0.002; 205 \pm 2 \text{ Hz versus } 195 \pm 1 \text{ Hz}) \). Finally, in order to eliminate any possible effects of DMSO (0.1%) on SW-R, control experiments were performed. DMSO (0.1%) did not alter SW-R activity (data not shown). These results suggest that SW-R occurrence is increased after CORT application in mice exposed to fear conditioning.

mRNA expression of glutamatergic, GABAergic and cannabinoid receptor subunits

Assessment of mRNA levels with quantitative real-time PCR revealed a long-lasting reduction of various receptor subunits in the SP of the ventral CA3 region after fear conditioning, but not after its reactivation (Figure 4; for expression in SO and SR, see Figure S2–3). Specifically, the GluA1 subunit of the AMPA receptor was reduced in NR \( (F(2,15) = 4.239, p = 0.035; \text{LSD post hoc: } p = 0.011, \text{NR versus CTL}) \). While the expression of the GluN2A subunit of the NMDA receptor showed no training effect \( (F(2,15) = 0.652, p = 0.535) \), significant long-term effects of fear conditioning were observed on the expression of the GluN1 \( (F(2,15) = 7.370, p = 0.006) \) and GluN2B subunit \( (F(2,15) = 6.563, p = 0.009) \). Post hoc analysis revealed a reduction of both subunits after fear conditioning alone \( (\text{GluN1: } p = 0.005, \text{NR versus CTL}; \text{GluN2B: } p = 0.003, \text{NR versus CTL}) \) but not after reactivation of the conditioned fear \( (\text{NR1: } p = 0.004, \text{NR versus R}; \text{NR2B: } p = 0.034, \text{NR versus R}) \). However, the expression of selected targets related to GABAergic signaling was affected in a different way. After both, fear conditioning and its reactivation, expression levels of the GABA\(_A\) receptor \( \alpha 2 \) subunit were reduced \( (F(2,15) = 6.892, p = 0.008; \text{NR versus CTL}; \text{R versus CTL}) \). A similar expression pattern was observed for the CB1-R \( (F(2,15) = 7.124, p = 0.007; \text{NR versus CTL}; \text{R versus CTL}) \), while the expression of the \( \alpha 3 \) subunit was not changed \( (F(2,14) = 0.729, p = 0.585) \).

Together, while fear conditioning induces a long-lasting reduced expression of both, glutamatergic and GABAergic modulators of SW-R in the SP of the ventral CA3, fear memory reactivation only restores the expression of glutamatergic receptor subunits, indicating differential experience-induced mechanism of SW-R modulation.

Discussion

In this study, we report long-term changes in the function of the mouse CA3 collateral associative network following fear conditioning and fear memory reactivation. In acute slice preparations, network patterns with similar characteristics observed in vivo can be obtained. They thus are well suited to study the mechanisms underlying different behaviorally relevant network activity patterns and their change following behavioral stimulation (Albrecht et al., 2013; Fisahn et al., 1998; Maier et al., 2009; Lu et al., 2011). In addition to analyzing slices from differently trained animals, we simulated states of high and low CORT levels during
recording. Without supplementation of exogenous CORT, we observed reduced orthodromic/antidromic PS ratio indicating reduced excitatory synaptic interaction within the CA3 network (Figure 1) as well as decreased gamma oscillations (Albrecht et al., 2013), but normal spontaneous SW-R activity. In contrast, under high CORT levels, PS ratios and gamma activity were both comparable to those in slices from control animals, whereas SW-R were increased in fear-conditioned and fear-reactivated groups. Thus, fear learning appears to lastingly alter the CORT-sensitive configuration of different network activity patterns generated by the CA3 associational network. This is in line with the different functions attributed to gamma oscillations and SW-R in the behaving animal. For example, we recently showed that in naïve animals, the effects of the stress-related neuromodulators including CORT, corticotropin-releasing factor and tetrahydrodeoxycorticosterone on spontaneous SW-R are rather mild compared to their effects on gamma oscillations (Calıskan et al., 2015).

In vivo, gamma oscillations occur during exploratory behavior while the hippocampus receives a strong cholinergic input (Hironaka et al., 2001; Lee et al., 1994; Montgomery & Buzsáki, 2007). Similarly, gamma oscillations can be induced in hippocampal slice preparations by challenging the network via cholinergic or kainate receptor activation (Fano et al., 2012; Fisahn et al., 1998; Wójtowicz et al., 2009). This type of perturbation depolarizes the cells in area CA3, where the gamma network activity emerges due to extensive axon collaterals interacting with pyramidal cells and inhibitory interneurons (Hájos & Paulsen, 2009). Contrary to gamma oscillations, in vivo, SW-R mostly occur during quiescent behavior such as grooming and slow-wave sleep when the level of ACh in the hippocampus is low. In vitro, SW-R are observed in hippocampal slice preparations of mice as spontaneous events, which often originate from area CA3 (Maier et al., 2003).

In fact, different local circuit mechanisms are thought to underlie the generation of SW-R and gamma oscillations in the CA3, whereas gamma oscillations require cycle-by-cycle reciprocal interactions (PING model) between pyramidal cell and inhibitory interneurons (parvalbumin containing basket cells, etc.); SW-R are more dependent on reciprocal inhibitory interactions (FINO model) (Schlingloff et al., 2014). In our experiments, as paired-pulse behavior of orthodromic PS was affected neither by fear conditioning nor by its reactivation, the effects observed appear to be rather of postsynaptic than of presynaptic nature (Zucker & Regehr, 2002).

We found that CORT pre-application augmented SW-R activity in slices from mice after fear conditioning and its reactivation, but not in control slices. A previous study described the concentration-dependent effects of CORT on the SW-R activity in naïve slices obtained from male C57Bl6 mice (Weiss et al., 2008). Weiss et al. recorded the SW-R activity in area CA1, whereas in our study, we recorded SW-R in area CA3, where they are generated (Kranig et al., 2013; Maier et al., 2002). However, similar to our study, after 1 μM CORT, relatively mild effects on the occurrence of SW-R were reported (Weiss et al., 2008). Detailed comparison of training groups reveals a somewhat attenuated impact of CORT after fear memory reactivation, as compared to simple fear conditioning, whereas in NR slices, CORT increased the incidence and the size of SW-R as well as the ripple frequency; the additional reactivation of fear memory increased only SW-R incidence but had no effect on SW-R size or ripple frequency. A differential response to prior training experience has also been observed concerning gamma oscillations and their rescue by CORT supplementation (Albrecht et al., 2013). Thus, fear conditioning and fear
reactivation appear to lastingly alter the CORT sensitivity of
network activity patterns generated in the CA3 associative
network. The increase in SW-R occurrence in the fear-
exposed mice might be due to augmenting effects of CORT
on excitatory neurotransmission within the 30-min CORT pre-
exposure interval. This suggests an involvement of fast non-
genomic mechanisms rather than CORT-induced changes in
gene expression that would just onset in this phase (Joe¨ls
et al., 2012). On the other hand, cannabinoid signaling in the
ventral CA3 is not altered. (E) GABA receptor α2 subunit (GABRA2) expression is
reduced in both groups NR and R. (F) Cannabinoid receptor 1 (CB1-R) expression is
also reduced in both groups NR and R. Values are mean RQ
% ± SEM (normalized to average expression in CTL group). *Indicates
significant difference between groups with $p<0.05$, **$p<0.01$ ($n=6$
per group, one-way ANOVA).

Figure 4. Long-lasting changes in mRNA expression of glutamatergic,
GABA and cannabinoid receptors subunits in the stratum pyramidale
(SP) and differential recovery by fear memory reactivation. (A) The
expression of the AMPA receptor subunit GluA1 is reduced in group NR
(no reactivation). (B) The expression of NMDA receptor subunits GluN1
and (D) GluN2B are reduced in group NR, while (C) GluN2A expression
is not altered. (E) GABA$_A$ receptor α2 subunit (GABRA2) expression is
reduced in both groups NR and R. (F) Cannabinoid receptor 1 (CB1-R)
expression is also reduced in both groups NR and R. Values are mean RQ
% ± SEM (normalized to average expression in CTL group). *Indicates
significant difference between groups with $p<0.05$, **$p<0.01$ ($n=6$
per group, one-way ANOVA).

We propose that the differential change in expression of glutamate, GABA$_A$, and cannabinoid receptors may not only
underlie CORT-sensitive changes in the CA3 associative
network but could also be involved in modulation CORT-
responsive behavior in these animals. SW-R reflect a
temporally condensed replay of learning-activated neuronal
ensemble activity during sleep. Recent studies furthermore
suggest a role for SW-R in decision-making based on
previously acquired memory (Jadhav et al., 2012). Their
increase under CORT supplementation indicates a sensitiza-
tion of the CA3 network in animals with previous fear
reactivation experience that may alter the behavioral
response to challenging situations with increased levels of
circulating CORT. In fact, we have previously shown an
increased generalization to the background context of mice
that had undergone memory reactivation, and thus presented
with increased levels of endogenous CORT (Figure 5; Albrecht et al., 2013).

This interpretation is supported by reported functions of
the receptors changed in expression: on the one hand, GABA$_A$
receptors are closely related to anxiety-like behavior and may
exert anxiolytic actions in conjunction with CORT (Engin
et al., 2012; Raud et al., 2009; Wislowska-Stanek et al.,
2012). On the other hand, cannabinoid signaling in the
in the mRNA expression of receptors known to control the
activity of the CA3 collateral associative network.

Strikingly, mRNA expression changes were exclusively
observed in the SP, but not SO or radiatum of the ventral CA3
region. A recent study by Hájos et al. (2013) investigated the
contribution of anatomically identified CA3 neurons to SW-R and
found that the majority of cells firing during SW-R are
interneurons, specifically basket cells and axo-axonic cells
located within the SP. Moreover, specific firing properties
were observed for different types of basket cells that express
either parvalbumin or CB1. Our data thus raise the exciting
possibility that fear conditioning alters CA3 network activity
by lastingly and differentially modulating the activity and
molecular composition of particular types of interneurons.
Future studies using cell-type specific analysis methods are
required to investigate this hypothesis further.

It can be assumed that altered mRNA levels four weeks
after the inducing experience reflect changes in their steady-
state expression. Therefore, the observed differential expres-
sion changes may be directly relevant for adaptive and
maladaptive cellular function in the ventral CA3 region.
Indeed, the generation and maintenance of hippocampal
SW-R are critically dependent on both, glutamatergic and
GABAergic receptor activation (Ellender et al., 2010; Hájos
et al., 2013; Papanicolaou et al., 2007), while activation of
CB1-R disrupts CA3 network activity by suppression of
excitatory transmission (Holderith et al., 2011; Maier et al.,
2012; Sun et al., 2012). Thus under low CORT conditions,
reduced NMDA and AMPA receptor expression could prevent
over-excitation of area CA3 and thus maintain SW-R within
a normal range in fear conditioned animals. A single fear
reactivation session, however, is sufficient to disrupt the
balanced regulation of excitatory and inhibitory factors,
normalizing NDMA and AMPA receptor expression to
control levels while GABA$_A$ receptor alpha2 subunit and
CB1R remain low.

We propose that the differential change in expression of
 glutamate, GABA$_A$, and cannabinoid receptors may not only
underlie CORT-sensitive changes in the CA3 associative
network but could also be involved in modulation CORT-
responsive behavior in these animals. SW-R reflect a
temporally condensed replay of learning-activated neuronal
ensemble activity during sleep. Recent studies furthermore
suggest a role for SW-R in decision-making based on
previously acquired memory (Jadhav et al., 2012). Their
increase under CORT supplementation indicates a sensitiza-
tion of the CA3 network in animals with previous fear
reactivation experience that may alter the behavioral
response to challenging situations with increased levels of
circulating CORT. In fact, we have previously shown an
increased generalization to the background context of mice
that had undergone memory reactivation, and thus presented
with increased levels of endogenous CORT (Figure 5; Albrecht et al., 2013).

This interpretation is supported by reported functions of
the receptors changed in expression: on the one hand, GABA$_A$
receptors are closely related to anxiety-like behavior and may
exert anxiolytic actions in conjunction with CORT (Engin
et al., 2012; Raud et al., 2009; Wislowska-Stanek et al.,
2012). On the other hand, cannabinoid signaling in the
The hippocampus modulates anxiety and memory dependent on previous stress experience (Akirav, 2011). Cannabinoids are further believed to modulate adaptation to stress, ameliorating the impact of severe and chronic stress pre-exposure on plasticity, cognitive tasks, fear memory extinction and expression of GR (Ganon-Elazar & Akirav, 2013; Segev et al., 2014). Furthermore, NMDA receptors in the hippocampus are critical for the formation of context fear memories (McHugh & Tonegawa, 2009; Stiedl et al., 2000; Zhang et al., 2001). A shift toward glutamatergic receptor expression thus also is in line with the generalization to background context in fear reactivated mice (see also Figure 5).

We observed that fear reactivation attenuates the sensitization of CA3 associative network to CORT and recovers normal glutamatergic receptor expression. After reactivation, a fear memory becomes labile and can be updated with non-fearful information (Alberini, 2011). Reorganization of CA3 network activities following fear learning and reactivation may provide a powerful mechanism to support such adaptive

Figure 5. Summary of results from this and our previous (Albrecht et al., 2013) study. Thirty days after auditory cued fear conditioning alone (NR), anxiety-like behavior in the elevated plus maze was decreased. At the same time, mRNA expression levels of glucocorticoid (GR) and mineralocorticoid receptors (MR) as well as of AMPA and NMDA receptor subunits were decreased in ventral CA3 sublayers in only NR while the GABA<sub>A</sub>-R α2 subunit (GABRA2) and the cannabinoid receptor 1 (CB1) were decreased in both NR and R suggesting a specific recovery of excitatory subunit expression by fear memory reactivation. Moreover, fear conditioning reduced lastingly network activity in the ventral CA3 region, reflected by gamma oscillations and associative excitatory interactions as indicated by the ratio of orthodromic to antidromic population spike (PS). These changes were reversible by acute application of CORT in vitro. In addition, CORT augmented SW-R incidence and magnitude. Reactivation of fear memory (R) induced a differential pattern of changes. While anxiety-like behavior was reduced as well, the remote fear memory toward the background context was enhanced and CORT plasma levels were lastingly increased. The mRNA expression levels of MR, GR and glutamatergic receptors were comparable to control levels while gamma oscillations and associative excitatory interactions within area CA3 were reduced as well. However, the impact of CORT on network activity appears reduced after reactivation.
changes; it will be interesting to see how these behave during other modifications of fear memory such as extinction learning.

Acknowledgements
We are grateful to C. Obst, F. Webers and S. Stork for excellent technical assistance and to A. Deter and T. Porcuzek for animal care.

Declaration of interest
The authors declare no conflict of interest. The German Declaration of interest for animal care.

We are grateful to C. Obst, F. Webers and S. Stork for excellent technical assistance and to A. Deter and T. Porcuzek for animal care.

References

Ganon-Elazar E, Akirav I. (2013). Cannabinoids and traumatic stress on emotional learning and LTP in a rat model of depression. Neurosci Biobehav Rev 37:394:186–97.

Hajós N, Karlóci MR, Németh B, Ulbert I, Monyer H, Szabó G, Erdélyi F, et al. (2013). Input-output features of anatomically identified CA3 neurons during hippocampal sharp wave/ripple oscillation in vitro. J Neurosci 33:11677–91.

Hironaka N, Tanaka K, Izaki Y, Hori K, Nomura M. (2001). Memory-related acetylcholine efflux from the rat prefrontal cortex and hippocampus: a microdialysis study. Brain Res 901:143–50.

Holderith N, Németh B, Papp Ol, Veres Jm, Nagy GA, Hajós N. (2011). Cannabinoids attenuate hippocampal γ oscillations by suppressing excitatory synaptic input onto CA3 pyramidal neurons and fast spiking basket cells. J Physiol 589:4921–34.

Jacobson L, Sapolsky R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118–34.

Jadhav SP, Kemere C, German PW, Frank LM. (2012). Awake hippocampal sharp-wave ripples support spatial memory. Science 336:1454–8.

Joëls M, Sarabdjitsingh RA, Karst H. (2012). Unraveling the time domains of corticosterone hormone influences on brain activity: rapid, slow, and chronic modes. Pharmacol Rev 64:901–38.

Kranig SA, Duhme N, Waldeck C, Drugguh A, Reichinnek S, Both M. (2013). Different functions of hyperpolarization-activated cation channels for hippocampal sharp waves and ripples in vitro. Neuroscience 228:325–33.

Le Duigou C, Simonnet J, Telecčuk MT, Fricker D, Miles R. (2014). Recurrent synapses and circuits in the CA3 region of the hippocampus: an associative network. Front Cell Neurosci 7:262.

Lee MG, Chrobak JJ, Sik A, Wiley RG, Buzsáki G. (1994). Hippocampal theta activity following selective lesion of the septal cholinergic system. Neuroscience 62:1033–47.

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–8.

Lu CB, Jefferys JG, Toescu EC, Veugdenhilt M. (2011). In vitro hippocampal gamma oscillation power as an index of in vivo CA3 gamma oscillation strength and spatial reference memory. Neurobiol Learn Mem 95:221–30.

Maggio N, Segal M. (2007). Striking variations in corticosterone modulation of long-term potentiation along the septotemporal axis of the hippocampus. J Neurosci 27:5757–65.

Maier N, Guldénagel M, Söhl G, Siegmund H, Willecke K, Drugguh A. (2002). Reduction of high-frequency network oscillations (ripples) and pathological network discharges in hippocampal slices from connexin 36-deficient mice. J Physiol 541:521–8.

Maier N, Nimmrich V, Drugguh A. (2003). Cellular and network mechanisms underlying spontaneous sharp-wave–ripple complexes in mouse hippocampal slices. J Physiol 550:873–87.

Maier N, Norris G, Johenning FW, Schmitz D. (2009). An approach for reliably investigating hippocampal sharp wave-ripples in vitro. PLoS One 4(9):e6925.

Maier N, Norris G, Schuchmann S, Korotkova T, Ponomarenko A, Böhm C, Wozny C, Schmitz D. (2012). Cannabinoids disrupt hippocampal sharp wave-ripples via inhibition of glutamate release. Hippocampus 22:1350–62.

McHugh TJ, Tonegawa S. (2009). CA3 NMDA receptors are required for the rapid formation of a salient contextual representation. Hippocampus 19:1153–8.

Mongtomery SM, Buzsáki G. (2007). Gamma oscillations dynamically couple CA3 and CA1 region during memory task performance. Proc Natl Acad Sci USA 104:14495–5000.

Papatheodoropoulos C. (2007). NMDA-receptor-dependent high-frequency network oscillations (100-300 Hz) in rat hippocampal slices. Neurosci Lett 414:197–202.

Raud S, Sütt S, Luuk H, Plaas M, Innos J, Köks S, Vasar E. (2009). Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wsf1-deficient mice. Neurosci Lett 460:138–42.

Schrölolf D, Káli S, Freund TF, Hajós N, Gulyás AI. (2014). Mechanisms of sharp wave initiation and ripple generation. J Neurosci 34:11385–98.

Segev A, Rubin AS, Abush H, Richter-Levin G, Akirav I. (2014). Cannabinoid receptor activation prevents the effects of chronic mild stress on emotional learning and LTP in a rat model of depression. Neuropsychopharmacology 39:919–33.

Changes; it will be interesting to see how these behave during other modifications of fear memory such as extinction learning.

Acknowledgements
We are grateful to C. Obst, F. Webers and S. Stork for excellent technical assistance and to A. Deter and T. Porcuzek for animal care.

Declaration of interest
The authors declare no conflict of interest. The German Israeli Project Cooperation (DIP RI 1922/1-1 HE 1128/16-1) to G. R. L., U. H. and O. S., the Collaborative Research Program SFB779 TPB5 to OS, the excellence cluster NeuroCure to U. H. (257 EXC). The DFG graduate college GRK 1123 “Cellular mechanisms of learning and memory consolidation” provided a stipend to G. C.
Stiedl O, Birkenfeld K, Palve M, Spiess J. (2000). Impairment of conditioned contextual fear of C57BL/6J mice by intracerebral injections of the NMDA receptor antagonist APV. Behav Brain Res 116:157–68.

Ströhle A, Holsboer F. (2003). Stress responsive neurohormones in depression and anxiety. Pharmacopsychiatry 36(Suppl 3):S207–14.

Sun Y, Norimoto H, Pu XP, Matsuki N, Ikegaya Y. (2012). Cannabinoid receptor activation disrupts the internal structure of hippocampal sharp wave-ripple complexes. J Pharmacol Sci 118:288–94.

Trivedi MA, Coover GD. (2004). Lesions of the ventral hippocampus, but not the dorsal hippocampus, impair conditioned fear expression and inhibitory avoidance on the elevated T-maze. Neurobiol Learn Mem 81:172–84.

Weiss EK, Krupka N, Bähner F, Both M, Draguhn A. (2008). Fast effects of glucocorticoids on memory-related network oscillations in the mouse hippocampus. J Neuroendocrinol 20:549–57.

Wisłowska-Stanek A, Lehner M, Skórzewska A, Maciejak P, Szyndler J, Turzyńska D, Sobolewska A, Płaźnik A. (2012). Corticosterone attenuates conditioned fear responses and potentiates the expression of GABA-A receptor alpha-2 subunits in the brain structures of rats selected for high anxiety. Behav Brain Res 235:30–5.

Wójtowicz AM, van den Boom L, Chakrabarty A, Maggio N, Haq RU, Behrens CJ, Heinemann U. (2009). Monoamines block kainate- and carbachol-induced gamma-oscillations but augment stimulus-induced gamma-oscillations in rat hippocampus in vitro. Hippocampus 19: 273–88.

Zhang WN, Bast T, Feldon J. (2001). The ventral hippocampus and fear conditioning in rats: different anterograde amnesias of fear after infusion of N-methyl-D-aspartate or its noncompetitive antagonist MK-801 into the ventral hippocampus. Behav Brain Res 126:159–74.

Zucker RS, Regehr WG. (2002). Short-term synaptic plasticity. Annu Rev Physiol 64:355–405.