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# Activity-dependent plasticity of mouse hippocampal assemblies *in vitro*

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Memory formation is associated with the generation of transiently stable neuronal assemblies. In hippocampal networks, such groups of functionally coupled neurons express highly ordered spatiotemporal activity patterns which are coordinated by local network oscillations. One of these patterns, sharp wave-ripple complexes (SPW-R), repetitively activates previously established groups of memory-encoding neurons, thereby supporting memory consolidation. This function implies that repetition of specific SPW-R induces plastic changes which render the underlying neuronal assemblies more stable. We modeled this repetitive activation in an *in vitro* model of SPW-R in mouse hippocampal slices. Weak electrical stimulation upstream of the CA3-CA1 networks reliably induced SPW-R of stereotypic waveform, thus representing re-activation of similar neuronal activity patterns. Frequent repetition of these patterns (100 times) reduced the variance of both, evoked and spontaneous SPW-R waveforms, indicating stabilization of pre-existing assemblies. These effects were most pronounced in the CA1 subfield and depended on the timing of stimulation relative to spontaneous SPW-R. Additionally, plasticity of SPW-R was blocked by application of a NMDA receptor antagonist, suggesting a role for associative synaptic plasticity in this process. Thus, repetitive activation of specific patterns of SPW-R causes stabilization of memory-related networks.

**Keywords:** plasticity, sharp wave-ripple, SPW-R, oscillation, hippocampus, assembly, neuronal assemblies, neuronal plasticity

## Introduction

The mammalian hippocampus supports the formation and consolidation of spatial, episodic and declarative memories. At the network level, neuronal activity is organized in coherent spatio-temporal patterns which correlate with distinct processes in memory formation (Sirota and Buzsáki, 2005). Recording of multiple single units in freely moving rodents has revealed that hippocampal network oscillations go along with precisely timed action potentials of specific neurons which fire in a place-specific manner. Sequential activation of such place cells during spatial exploration forms a representation of the animal's trajectory (O'Keefe and Recce, 1993). In subsequent phases of slow-wave sleep, the same sequences are repetitively activated, probably mediating memory consolidation (Wilson and McNaughton, 1994; Lee and Wilson, 2002). In this state, sequential activation of place cells occurs on top of sharp wave-ripple complexes (SPW-R; Ylinen et al., 1995; Csicsvari et al., 1999). It has been suggested that this re-activation of neuronal activity sequences mediates memory consolidation by

transferring transiently stable activity into the neocortex for long-term storage (Buzsáki, 1989). In line with this concept, memory consolidation is associated with irregular oscillatory activity containing SPW-R (Girardeau et al., 2009; Mölle and Born, 2011; Sadowski et al., 2011).

Work during the past decades has revealed much insight into the cellular mechanisms underlying memory formation, including Hebbian plasticity of synapses in major hippocampal projection pathways (Bliss and Lomo, 1973; Köhr et al., 2003; Buschler et al., 2012; Goh and Manahan-Vaughan, 2013). Oscillation-associated assembly formation constitutes a potential link between synaptic plasticity and network-level information processing. Indeed, theta-gamma states support synaptic plasticity (Palmer et al., 2004; Lisman et al., 2005) and may thus allow for transient assembly formation. In accordance, *in vitro* data show that high frequency stimulation can induce spontaneous SPW-R oscillations in rat hippocampal slices (Behrens et al., 2005). Additionally, this induction is NMDA-R activation dependent and indicates an essential role of synaptic strengthening in hippocampal assembly formation. Interestingly, SPW-Rs also favor associative strengthening of synapses (King et al., 1999), and their selective disruption impairs spatial memory formation (Girardeau et al., 2009). Together, these processes may account for the rapid formation and subsequent consolidation of space-encoding assemblies in hippocampal networks (Frank et al., 2003; Carr et al., 2012). It is, thus, likely that repeated activation of assemblies during SPW-R strengthens connectivity between participating neurons, resulting in transiently stable, highly specific activity patterns (Buzsáki and Draguhn, 2004). While we know much about plasticity of synapses, the activity-dependent formation of neuronal assemblies within the different hippocampal subfields remains, however, poorly understood.

In previous work we showed that different neuronal assemblies are reflected in different waveforms of spontaneously occurring SPW-R (Reichinnek et al., 2010). Therefore, extracellular recordings of SPW-R (Maier et al., 2003) and subsequent waveform sorting can be taken as a proxy of monitoring distinct neuronal assemblies. Here, we tested the effect of repetitive activation of such neuronal groups on the diversity and stability of spontaneous network activity patterns.

## Materials and Methods

All animal procedures were performed in accordance with the guidelines of the European Community Council and approved by the state government of Baden-Württemberg.

### Experimental Outline

#### Slice Preparation

Experiments were performed on slices of four to 8 weeks old male mice (C57Bl6). Animals were decapitated after being deeply anesthetized with CO<sub>2</sub>. Subsequently, the brain was separated and transferred into cooled (0–4°C) artificial cerebral spinal fluid (ACSF: NaCl 124 mM, KCl 3.0 mM, MgSO<sub>4</sub> 1.8 mM, CaCl<sub>2</sub> 1.6 mM, Glucose 10 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, NaHCO<sub>3</sub> 26 mM), saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (corresponding to

pH 7.4 at 37°C). Before slicing, frontal lobe and cerebellum were removed, and the remaining tissue block was glued to the holding chamber of a Leica Vibratome (VT1000S). Horizontal slices of 450 μm thickness were cut and subsequently transferred to a Haas-type interface recording chamber where they recovered for at least 2 h at 32–34°C at the border between ACSF (flow: 1.5–2.0 ml/min) and humidified gas (95% O<sub>2</sub> and 5% CO<sub>2</sub>).

### Recording and Stimulation

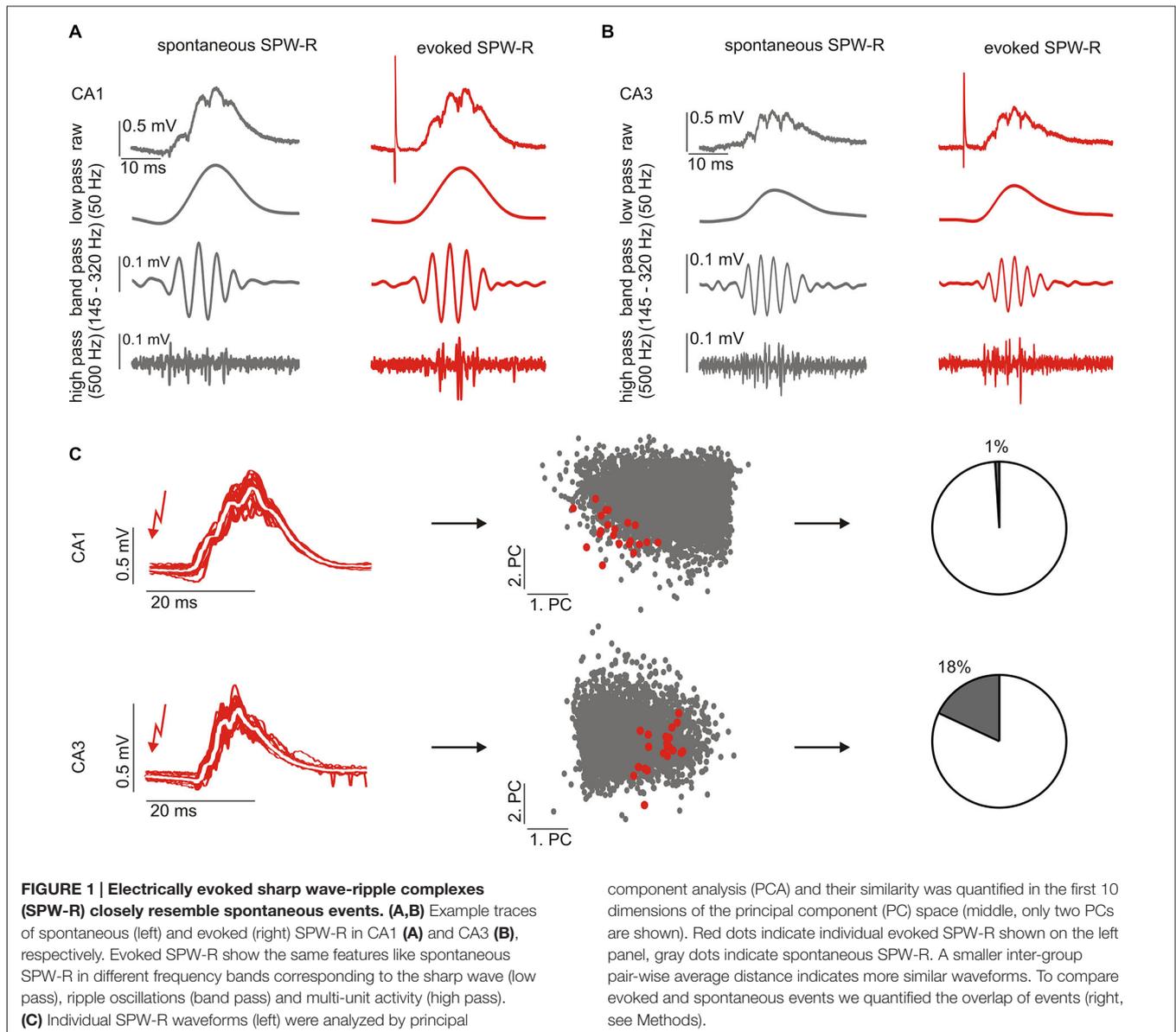
Extracellular field potentials were recorded in stratum pyramidale of CA3a, CA3b, and CA1 by use of three tetrodes, each consisting out of four twisted wires (Tungsten California Fine Wire, 12.5 μm) connected to an EXT-T2 amplifier (npi electronics, Tamm, Germany). Signals were amplified ( $\times 200$ ), low pass filtered at 8 kHz and digitized at 20 kHz for offline analysis (CED Power 1401 mkII expanded by a CED 2805SA-8 Analog BCN box, recording program Spike 2.0, CED, Cambridge, UK).

Two stimulation electrodes (“Micro Probes” Platinum/Iridium, 100 kΩ at 1 kHz, 75 μm tip separation) were placed into the granule cell layer of the supra-pyramidal blade of area dentata. Electrode positions and stimulation strengths were optimized such that unipolar stimulation pulses evoked field events in CA3 and CA1 which resembled waveforms of spontaneously occurring SPW-R (Figures 1A,B). In order to avoid direct interference with ongoing network activity, pulses were triggered at a delay of 150 ms after a preceding spontaneous SPW-R waveform. Alternating stimulation of both electrodes (at intervals of 60 s) caused two different waveform sets of evoked SPW-R at recording sites in CA3a, CA3b and CA1. After 20 stimulations performed at each site, we applied a series of 100 repetitive stimulations at 10 s intervals to one of the two stimulation electrodes, followed again by 20 alternating stimulations at both sites. The first group of control slices underwent the baseline stimulation protocol (alternating stimulation at both sites at intervals of 60 s) but not the repetitive stimulation at 0.1 Hz (1000 s pause at the respective time). Since there was no repetitively stimulated and not repetitively stimulated pattern in this condition we subsequently recorded two evoked waveform data sets in each slice. In a second control condition the 100 repetitive stimulations were triggered without delay on a spontaneously occurring SPW-R while baseline stimulations were still performed with a delay of 150 ms. In a third control the original experiment was repeated in the presence of the NMDA-R antagonist APV (30 μM).

### Data Analysis

#### Analysis of Field Potentials

Raw data were analyzed with custom-made routines written in Matlab. To extract SPW-R events, data were low-pass filtered (50 Hz) and local maxima with amplitudes above 0.15 mV were detected within 30 ms time windows. This threshold corresponds to  $\sim 4$  standard deviations of event-free baseline noise (Both et al., 2008) and allows for reliable detection of SPW-R. Identified SPW-Rs were analyzed within a time interval starting 33 ms before and ending 67 ms after the peak of the sharp



wave. Waveforms of evoked SPW-R-like events were processed similarly to spontaneous SPW-R after removal of the stimulus artifact from raw data traces. Extracellular multi-unit activity was detected by high-pass filtering at 500 Hz and setting a negative threshold at 4.5 SDs from background noise.

The stability of the extracellular signal was controlled by monitoring amplitude and frequency of spontaneous SPW-R during baseline recording. These parameters were regarded stable when rejecting the null hypothesis of finding a change at 5% significance level according to the Wald-Wolfowitz test. In some slices stimulation failed to evoke a downstream response pattern at one site during the course of the experiment. We suspected this to be a local effect since stimulation at the other site and spontaneous SPW-Rs were not affected. Subsequently we excluded experiments with less than ten evoked waveforms to ensure sufficient numbers of evoked events.

### Waveform Analysis

For analysis of SPW-R waveforms, signals were down-sampled to 5 kHz, resulting in 500 sample points within a 100 ms window. This data set was further reduced by principal component analysis (PCA; Nicolelis et al., 1995; Lopes-dos-Santos et al., 2013). In accordance with our previous work (Reichinnek et al., 2010) we used the first 10 principal components to describe events within a 10 dimensional feature space. The first PC mainly reflected waveform amplitude and was divided by two in order to reduce the relative contribution of this parameter.

Similarity or difference of waveforms was analyzed by calculating the pairwise Euclidian distance between waveforms within the 10-dimensional principal component space. Subsequently, we calculated the pair-wise Euclidian distance between all spontaneous as well as evoked events before and after repetitive stimulation. This resulted in distance distributions

for evoked and spontaneous events, respectively. To compare changes in the variety of waveforms, we divided the median distance after the repetitive stimulation by the median distance before the repetitive stimulation.

In a different approach, we measured the overlap between the repetitive evoked waveform distribution and the evoked SPW-Rs from the site without repetitive stimulation. To quantify this overlap we calculated the distances between the group of evoked but not repetitively stimulated events and the distribution of the group of evoked and repetitively stimulated events. In this case, we used the Mahalanobis distance which is a multi-dimensional way of measuring how many standard deviations a specific event is distant from the center of a distribution. Events from the group of evoked but not repetitively stimulated events were considered similar to the repetitive stimulated SPW-R waveform distribution if the Mahalanobis distance was smaller than the first percentile of the Mahalanobis distances within the distribution of the group of repetitively stimulated events itself. Subsequently, the overlap between both groups of evoked events was defined as the percentage of events from the group of evoked events that were not repetitively stimulated but were within the distribution of evoked events that underwent repetitive stimulation. In analogy the overlap between spontaneous SPW-R and the repetitive stimulated waveform pattern was calculated.

## Statistics

Quantitative results are given as median, the 25<sup>th</sup> and the 75<sup>th</sup> percentiles. Whiskers in the box plots correspond to the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles. For statistical analysis we tested whether data were normally distributed by Kolmogorov-Smirnoff-Test (significance level  $> 0.05$ ). Paired normally distributed data were analyzed by paired *t*-test, whereas non-paired data were tested by two sided *t*-test. Non-normally distributed data were compared by Wilcoxon signed-rank test and Wilcoxon rank sum test, respectively. In all cases,  $p < 0.05$  was regarded as significant.

## Results

### Weak Stimulation of the Dentate Gyrus Elicits Physiological SPW-R Waveforms

Compound potentials similar to spontaneous SPW-R can be evoked by weak electrical stimulation in CA3 (Reichinnek et al., 2010). In order to evoke such events in both hippocampal subfields, CA3 and CA1, we stimulated the upstream network of the DG which is powerfully connected to CA3 pyramidal cells and inhibitory neurons (Acsády et al., 1998). To avoid interference with the ongoing network activity stimuli were applied at 150 ms after the occurrence of a spontaneous SPW-R event. Under these conditions, weak electrical stimulation within the supra-pyramidal blade of the granule cell layer (100  $\mu$ s, 0.5–5 V) reliably evoked field potential transients in the pyramidal cell layers of CA3 ( $n = 12$  slices) and CA1 ( $n = 10$  slices), respectively. These transient potentials resembled spontaneous sharp wave-ripple events and had characteristic, rather stereotypic waveforms (**Figures 1A,B**). Although we

observed a lower number of unit-events on evoked waveforms overall quantitative analysis further confirmed the similarity between evoked field events and spontaneous SPW-R (**Table 1**). Moreover, it should be noted that spontaneously occurring SPW-R show heterogeneous levels of multi-unit activity not reflected by the median value across slices.

For further comparison of spontaneous and evoked waveform patterns we described the events by a reduced set of parameters following principal component analysis (PCA, see Methods). The principal components describing evoked events in both hippocampal subfields (CA1 and CA3) were reliably located within the parameter space covered by spontaneous events (**Figure 1C**), which underlines the similarity of both patterns. In summary, we conclude that stimulation-evoked network events in CA3 and CA1 are very similar to spontaneous ongoing spontaneous SPW-R activity and are therefore referred to as “evoked SPW-R”.

### Evoked SPW-R Waveforms are Stabilized by Repetitive Stimulation

Memory consolidation has been suggested to depend on the repetitive re-play of hippocampal activity patterns during SPW-R (Wilson and McNaughton, 1994; Lee and Wilson, 2002; Girardeau et al., 2009). We therefore asked for the effect of repetitive activation of similar sets of neurons through electrical stimulation. Twenty highly stereotypic SPW-R waveforms were evoked every 60 s alternating at two stimulation sites within the DG. Subsequently, one site was selected and 100 repetitive pulses were performed at 10 s intervals followed by 20 more stimulations every 60 s at both sites (**Figure 2A**). Stability of evoked waveform sets prior and after the 100 repetitive stimulation pulses was analyzed within the 10-dimensional PCA parameter space by calculating the pair-wise Euclidian distance between individual SPW-R.

In CA1, median pair-wise distances of evoked SPW-R were consistently reduced following the 100 repetitive stimulations. This was quantified by the ratio of median distances after/before stimulation which was 0.81 ( $p = 0.029$ ,  $n = 8$ ; **Figure 2D**). In contrast, slices that did not undergo the 100 repetitive stimulations (1000 s stimulation pause respectively) showed no reduction in waveform variability (ratio of median distances late/early recording period 1.05,  $p = 0.25$ ,  $n = 11$  data sets from 7 slices). The difference between both conditions was significant ( $p = 0.0023$ ) indicating that repetitive activation mediates stabilization of evoked SPW-R waveform patterns in CA1. Within CA3, SPW-Rs were recorded at two different sites (CA3a and CA3b). Under control conditions (1000 s stimulation pause), waveforms in CA3a showed a trend towards increased diversity over time (median distances late/early recording period 1.08,  $p = 0.051$ ,  $n = 11$  data sets from 6 slices), which reached significance in CA3b (median 1.14,  $p = 0.016$ ,  $n = 17$  data sets from 9 slices). The increase in variance in both CA3 regions was prevented by 100 repetitive stimulations within the DG, which is similar to the results obtained in CA1. In stimulated slices, median distance after/before repetitive stimulations was 0.95 ( $p = 0.72$ ,  $n = 8$ ) in CA3a and in CA3b, respective value was 0.97 ( $p = 0.55$ ,  $n = 12$ ). When compared to un-stimulated slices,

**TABLE 1 | Spontaneous (top) and evoked (bottom) waveforms are compared regarding amplitude (left), ripple occurrence (middle), and unit occurrence (right) in CA1 and CA3.**

	SPW-R amplitude (mV)		Number of ripple cycles (per SPW-R)		Multi-unit activity (spikes per SPW)	
	CA1	CA3	CA1	CA3	CA1	CA3
Spontaneous events	median 0.23	median 0.21	median 6.0	median 5.5	median 3.0	median 4.4
	25 <sup>th</sup> 0.17	25 <sup>th</sup> 0.17	25 <sup>th</sup> 5.7	25 <sup>th</sup> 3.6	25 <sup>th</sup> 1.9	25 <sup>th</sup> 1.2
	75 <sup>th</sup> 0.28	75 <sup>th</sup> 0.25	75 <sup>th</sup> 6.2	75 <sup>th</sup> 6.5	75 <sup>th</sup> 4.3	75 <sup>th</sup> 10
Evoked events	median 0.26	median 0.29	median 5.8	median 4.7	median 1.0	median 0.8
	25 <sup>th</sup> 0.19	25 <sup>th</sup> 0.19	25 <sup>th</sup> 5.6	25 <sup>th</sup> 4.0	25 <sup>th</sup> 0.95	25 <sup>th</sup> 0.63
	75 <sup>th</sup> 0.61	75 <sup>th</sup> 0.37	75 <sup>th</sup> 6.9	75 <sup>th</sup> 5.4	75 <sup>th</sup> 1.0	75 <sup>th</sup> 1.0
p-value	0.14	0.0051	0.38	0.10	0.096	0.10
	(n = 10)	(n = 12)	(n = 10)	(n = 12)	(n = 10)	(n = 12)

Median, 25<sup>th</sup> percentile, and 75<sup>th</sup> percentile represent the distribution formed by the medians observed in each experiment and p-values (bottom line) are calculated between spontaneous and evoked events.

the reduction of variability was significant for CA3a ( $p = 0.045$ ) while the ratio of waveform distances in CA3b did not reach significance ( $p = 0.10$ ).

As stated above, baseline stimulation was alternating between two different sites within the supra-pyramidal blade of the DG. Repetitive stimulation at 0.1 Hz (100 pulses) was, however, restricted to one of the two locations. Interestingly, SPW-R waveforms in CA1 elicited from the other site (which was not repetitively stimulated) did also show reduced variance following the 100 repetitive stimulations at the other site. This was reflected by the ratio of the median distances after/before stimulation of 0.66 (25<sup>th</sup> 0.64, 75<sup>th</sup> 0.75,  $p = 0.0039$ ,  $n = 7$ ,  $p = 0.00037$  in comparison to the “un-stimulated” control condition). In CA3, similar to the site which was repetitively stimulated, evoked events at the site which was not repetitively stimulated did not show any change in variability (CA3a ratio after/before median 1.01, 25<sup>th</sup> 0.92, 75<sup>th</sup> 1.31,  $p = 0.67$ ,  $n = 8$ ,  $p = 0.71$  in comparison to “un-stimulated” control condition; CA3b, ratio after/before median 1.01, 25<sup>th</sup> 0.84, 75<sup>th</sup> 1.09,  $p = 0.76$ ,  $n = 8$ ,  $p = 0.051$  in comparison to the “un-stimulated” control condition).

### Spontaneous Events Mimic Evoked Events and Reduce Their Variability

Surprisingly, reduction of waveform variability after 100 repetitive stimulations was also observed for spontaneous SPW-R in CA1 and CA3. Median Euclidian distances of spontaneously occurring events in CA1 were significantly smaller in slices that had undergone 100 repetitive stimulation pulses as compared to “un-stimulated” slices ( $p = 0.0044$ ). Accordingly, the ratio of distances after/before stimulation was 0.80 ( $p = 0.0035$ ,  $n = 10$ ) in the repetitively stimulated slices. In “un-stimulated” control condition this value was 0.97 ( $p = 0.64$ ,  $n = 7$ ). In CA3, variability of spontaneous SPW-R increased under control conditions, similar to the evoked events (CA3a: median 1.10,  $p = 0.075$ ,  $n = 6$ ; CA3b: median 1.12,  $p = 0.0054$ ,  $n = 9$ ). Again, this progressive increase in variability was reduced by 100 intermittent repetitive stimulations (CA3a: median 0.93,  $p = 0.24$ ,  $n = 10$ ; CA3b: median 0.99,  $p = 0.82$ ,  $n = 12$ ). The differences in variability ratios between the repetitively stimulated condition and the “un-stimulated” control were significant in CA3a ( $p = 0.015$ ) and CA3b ( $p = 0.0069$ ), respectively.

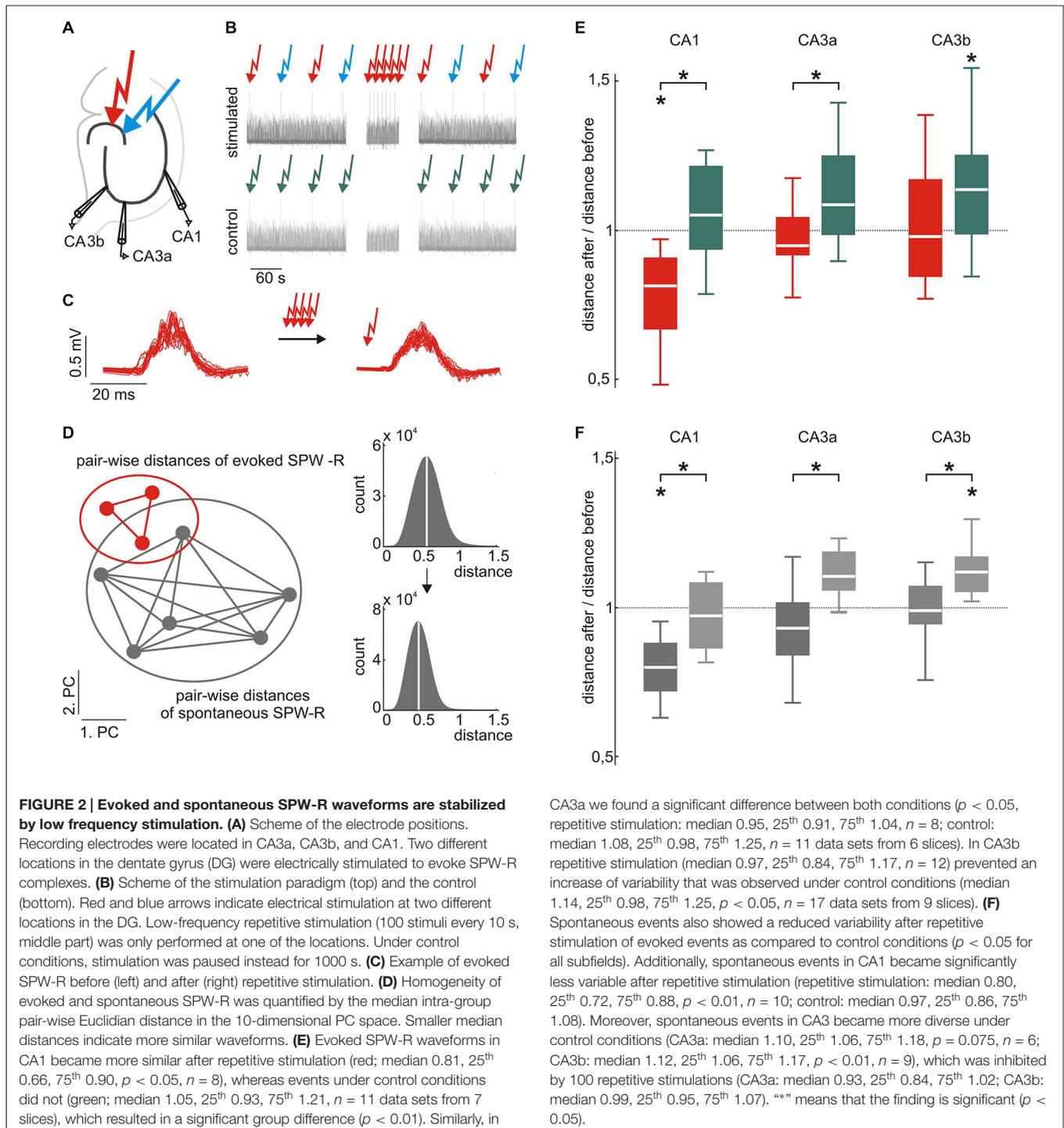
Overall, these results indicate that repetitive activation of limited neuronal subsets in the hippocampal formation reduces variance of subsequent network patterns, indicating activity-dependent stabilization of hippocampal assemblies.

### Stimulation-Evoked Waveform Sets Increase Similarity

Since evoked waveform sets from both stimulation sites became more homogeneous despite the fact that only one location was repetitively stimulated 100 times (see **Figure 2**), we analyzed the distinctiveness of both patterns. To do this, we calculated the Mahalanobis distance between both groups of evoked events within the principal component space (see Methods). In CA1 we found an initial overlap of 60%, which significantly increased to 94% ( $p = 0.000095$ ,  $n = 5$ ) by the 100 repetitive stimulations (see **Figure 3**). In contrast, in CA3 the overlap between both evoked patterns did not change significantly. In CA3a the initial overlap was 51% before, and 59% after the 100 stimulations ( $p = 0.71$ ,  $n = 6$ ). In CA3b we observed an overlap of 81% prior to and 80% after the repetitive stimulation ( $p = 0.80$ ,  $n = 8$ , **Figure 3C**).

In slices that had not undergone 100 repetitive stimulations, the overlap between events was calculated in both directions (data not shown). In contrast to the stimulated condition, overlap remained stable in CA1 ( $p = 0.25$ ,  $n = 10$  data sets within 5 slices) and percentages of overlap were lower than in the above described repetitive stimulation paradigm (initial overlap: median 17%, 25<sup>th</sup> 5%, 75<sup>th</sup> 48%; after stimulation pause: median 5%, 25<sup>th</sup> 5%, 75<sup>th</sup> 32%). Likewise, CA3 overlap did not change significantly (CA3a:  $p = 0.19$ ,  $n = 10$  data sets within 5 slices; CA3b:  $p = 0.38$ ,  $n = 16$  data sets within 8 slices; CA3a initial overlap 38%, 25<sup>th</sup> 31%, 75<sup>th</sup> 88%; past stimulation pause 28%, 25<sup>th</sup> 13%, 75<sup>th</sup> 60%; CA3b initial overlap 33%, 25<sup>th</sup> 6%, 75<sup>th</sup> 78%; past stimulation pause 22%, 25<sup>th</sup> 2%, 75<sup>th</sup> 50%).

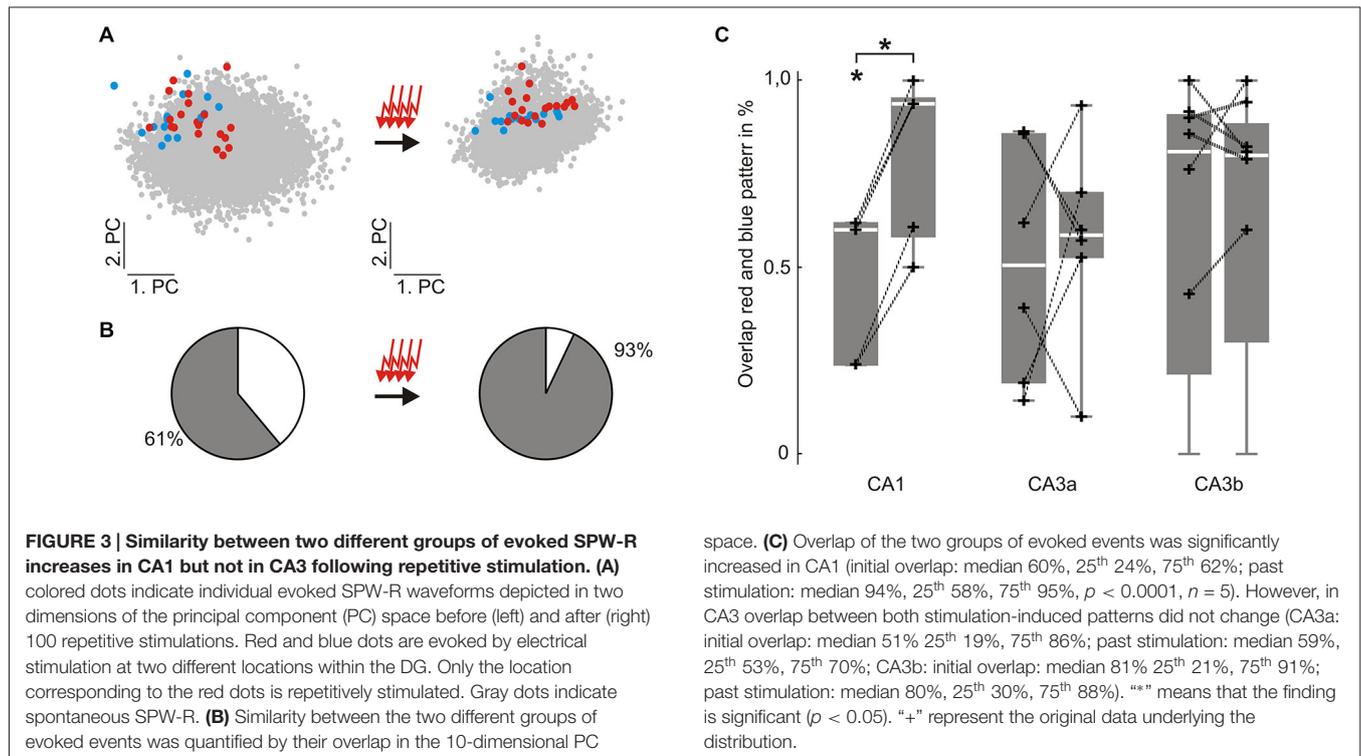
In summary, both evoked SPW-R waveform sets in CA1 became more similar to each other when one of the stimulation locations was repetitively stimulated 100 times. No significant change in similarity could be observed in CA3 for the repetitive stimulation paradigm. Moreover, similarity of SPW-R waveforms remained the same in the whole hippocampus, when no repetitive stimulation was performed.



### SPW-R Stabilization is Affected by the Timing of Stimulation

Electrical stimulation during an ongoing SPW-R did not elicit a typical SPW-R waveform. This situation can be used to distinguish between the effects of the electrical stimulus itself and the effects of pattern repetition. We therefore tested the effects of 100 repetitive stimulations ( $\sim 0.1$  Hz) timed on a spontaneously occurring SPW (Figure 4A) and compared it

to the above described stimulation paradigm (SPW-R evoked by stimulating 150 ms after a spontaneous event). In CA1, stimulating during spontaneous SPW-R did not induce any change in evoked or spontaneous SPW-R waveforms (evoked: median 0.96,  $p = 0.75$ ,  $n = 7$ ; spontaneous: median 1.01,  $p = 0.99$ ,  $n = 8$ ). Subsequently, when compared to the above described original repetitive stimulation paradigm (100 times, 150 ms after spontaneous SPW-R at 0.1 Hz) we found significant difference



for evoked ( $p = 0.024$ ) and spontaneous ( $p = 0.00035$ ) waveform stability (Figures 4B,C).

In CA3, 100 repetitive stimulations during an ongoing spontaneous SPW-R did not induce changes in SPW-R waveforms of spontaneous or evoked events (Figures 4B,C). Thus, findings were similar to the ones from the above-described 100 repetitive stimulation 150 ms after a spontaneous SPW-R (CA3a  $p = 0.66$  for evoked and  $p = 0.12$  for spontaneous events; CA3b  $p = 0.90$  for evoked events and  $p = 0.19$  for spontaneous events). In CA3a, the after/before distance ratios of evoked (median 1.01,  $p = 0.80$ ,  $n = 6$ ) and spontaneous SPW-R (median 1.02,  $p = 0.30$ ,  $n = 8$ ) were stable. Neither in CA3b SPW-R variability alterations were observed for evoked (median 1.03,  $p = 0.79$ ,  $n = 7$ ) nor spontaneous SPW-R (median 1.04,  $p = 0.11$ ,  $n = 8$ ).

### Modulation of SPW-R Waveform Stability Depends on NMDA Receptor Activation

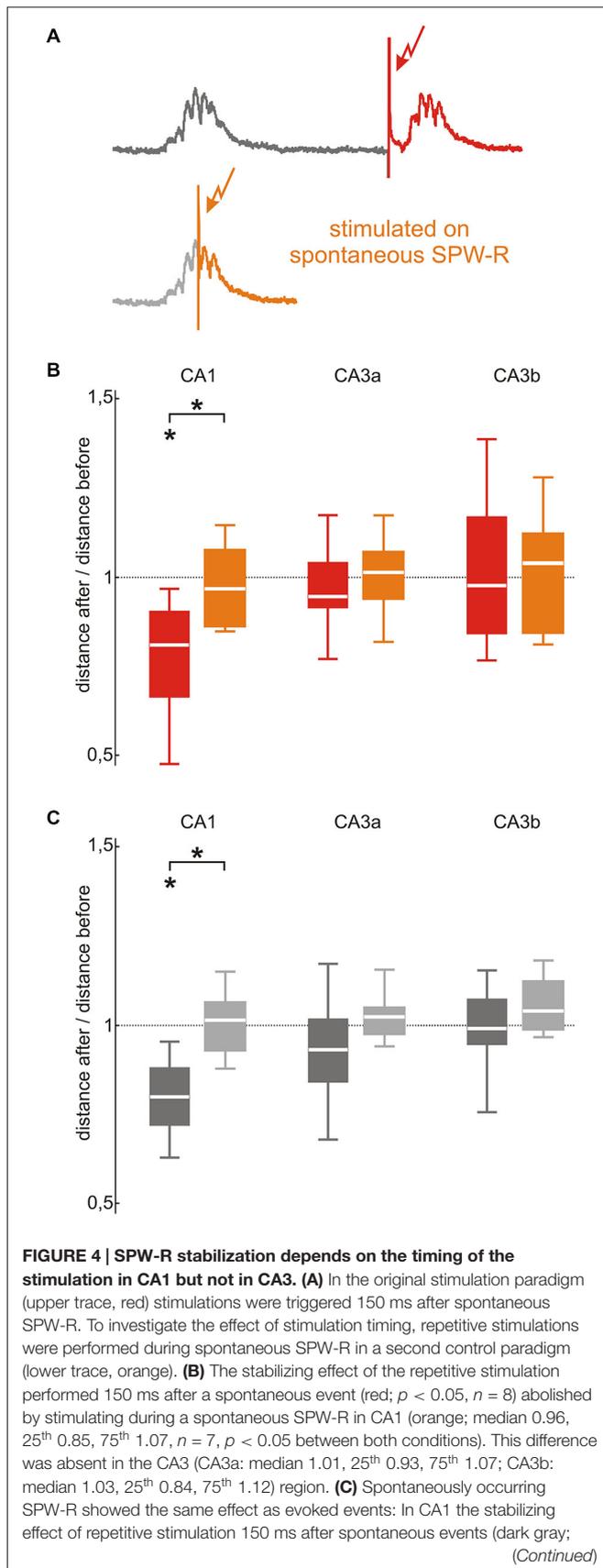
Finally, we asked whether NMDA receptor-dependent synaptic plasticity plays a role for stimulation-induced changes of hippocampal assemblies. We therefore repeated the original repetitive stimulation paradigm under bath application of the NMDA receptor antagonist APV (30  $\mu$ M) over the entire course of the experiment (Figure 5). Under these conditions, median pair-wise distances of evoked events were not affected by 100 repetitive stimulations (CA1 median 0.96,  $p = 0.41$ ,  $n = 6$ ; CA3a median 0.95,  $p = 0.28$ ,  $n = 7$ ; CA3b median 1.03,  $p = 0.54$ ,  $n = 7$ ). Likewise, spontaneously occurring SPW-R remained stable (CA1 median 0.84,  $p = 0.051$ ,  $n = 8$ ; CA3a median 1.0,  $p = 0.56$ ,  $n = 7$ ; CA3b median 1.08,  $p = 0.16$ ,  $n = 8$ ). Compared

to the original stimulation paradigm in absence of APV evoked SPW-R stability was significantly lower in CA1 ( $p = 0.047$ ) but not in CA3 (CA3a  $p = 0.89$ ; CA3b  $p = 0.68$ ). Interestingly, we found no significant difference in spontaneous SPW-R variability between slices recorded with to slices recorded without APV bath application (CA1  $p = 0.33$ ; CA3a  $p = 0.44$ ; CA3b  $p = 0.14$ ).

## Discussion

The formation, stabilization and retrieval of precisely timed activity of specific neuronal assemblies may represent a network-level correlate of memory. Conserved temporal relationships between discharges of selected neurons have been observed in various brain regions (Wilson and McNaughton, 1994; Pizzagalli et al., 2003; Pitzalis et al., 2013). In the present study, we made use of an *in vitro* model of hippocampal sharp wave-ripple (SPW-R) complexes. We evoked field events in mouse hippocampal slices by a sparse and distributed activation of CA3 neurons through electrical stimulation of presynaptic neurons in the upstream DG. On evoked downstream response pattern we observed lower multi-unit activity than on average spontaneous SPW-R which possibly originate from not completely resolved peri-somatic inhibition. Since different levels of multi-unit activity are also found in spontaneously occurring SPW-R and stimulation induced waveform were highly similar to spontaneous SPW-R patterns, we hoped to activate physiological neuronal assemblies in the hippocampal subfields of CA1 and CA3.

We observed that specific and repetitive activation of evoked SPW-R complexes induced a stabilization of the stimulated

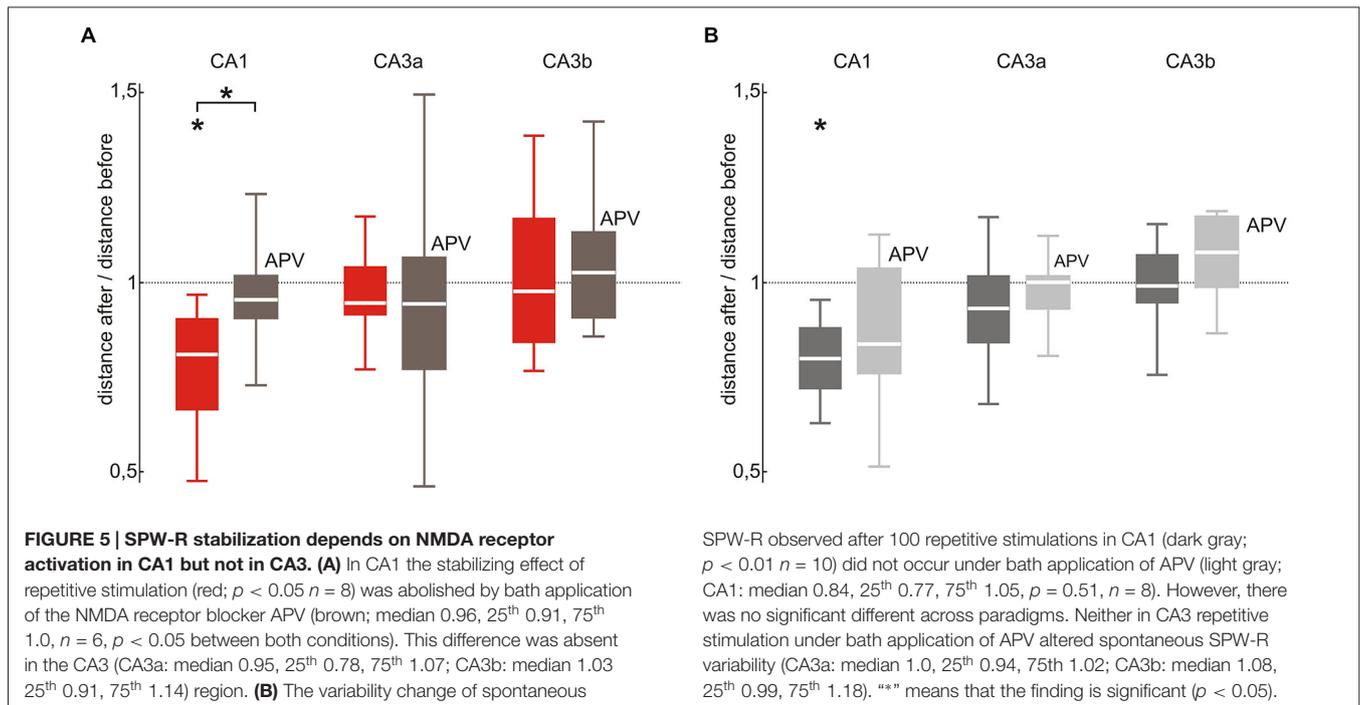
**FIGURE 4 | Continued**

$p < 0.01$ ,  $n = 10$ ) was abolished by stimulating during spontaneous SPW-R (light gray; median 1.01, 25<sup>th</sup> 0.93, 75<sup>th</sup> 1.07,  $n = 8$ ,  $p < 0.001$  between both conditions) but not in CA3 (CA3a: median 1.02, 25<sup>th</sup> 0.97, 75<sup>th</sup> 1.05; CA3b: median 1.04, 25<sup>th</sup> 0.99, 75<sup>th</sup> 1.12). “\*\*” means that the finding is significant ( $p < 0.05$ ).

patterns in a region specific manner. In CA1, repetitive stimulation (0.1 Hz, 100 times) of defined patterns led to an increased stability of the evoked assembly activity. Surprisingly, we also observed a stabilization of the evoked pattern that was not repetitively stimulated. This may be explained by the large overlap between parameters of both patterns which did even increase following stimulation. Interestingly, spontaneously occurring SPW-R also appeared to be stabilized by the repetitive activation of a specific assembly, as reflected by a reduced variability of spontaneously occurring waveforms. In CA3, repetitive stimulation prevented the increased distance between evoked and spontaneous SPW-R which was observed under control conditions without repetitive stimulation.

Local field potentials are the excitatory and inhibitory postsynaptic potentials as well as action potentials of individual cells (Schomburg et al., 2012). Features of well-defined field potential waveforms can be used to investigate the underlying assembly composition *in vitro* (Reichinnek et al., 2010) and *in vivo* (Agarwal et al., 2014). Specifically, we have shown previously that the stability and plasticity of neuronal assemblies can reliably be investigated by analyzing the distinct waveforms of SPW-R complexes (Reichinnek et al., 2010; Zylla et al., 2013). While neuronal assemblies cannot be precisely defined with this method, the assembly composition of the slice as a whole can be appropriately analyzed and compared (Reichinnek et al., 2010). We strived to activate a sparse and distributed population of neurons in the CA3 region by stimulation within the supra-pyramidal blade of the dentate gyrus (DG). At this location we expect to stimulate both, DG granule cells and fibers of the perforant path which both innervate CA3 pyramidal cells. As horizontal slices only maintain about 10% of the fibers between the DG and the hippocampus proper, this method of stimulation will generate a sparse and distributed activation of postsynaptic neurons in the CA3 region (Bischofberger et al., 2006; Geiger J, personal communication).

The stabilizing effect of repetitive activation on SPW-R complexes in CA1 was dependent on NMDA receptors (suggesting a contribution of synaptic plasticity, e.g., at the Schaffer collateral synapse) and on the timing of stimulation with respect to spontaneous SPW-R. Plasticity was induced when stimuli were set at  $\sim 150$  ms after a spontaneous SPW-R while stimulation during an ongoing SPW-R event was without any effect. In this case, the evoked events were clearly different from spontaneous SPW-R and showed high variability. These data suggest that stimulation during spontaneous SPW-R leads to aberrant, incomplete or overlapping activation of assemblies, thereby preventing stabilization of pre-existing neuronal connections. Interestingly, neither NMDA receptor blockade nor incomplete and unspecific assembly activation



altered the stabilizing effect of repetitive stimulation in the CA3 region. This finding is compatible with a role of mossy fiber synapses for changes in assemblies (Nicoll and Schmitz, 2005; Evstratova and Tóth, 2014). Thus, our results indicate different mechanisms of activity-dependent assembly plasticity within the CA3 and the CA1 sub-networks.

Classical protocols for long term potentiation (LTP) at the Schaffer collateral synapse use stimulation frequencies at around 100 Hz (Larson et al., 1986; Barnes et al., 1994; Durand et al., 1996; Abraham and Huggett, 1997; Colgin et al., 2004). The same paradigm is also able to induce spontaneous SPW-R oscillations in “silent” hippocampal slices (Behrens et al., 2005). Additionally, it has been shown that stimulation frequencies below 10 Hz induce hardly any potentiation and that a 1 Hz stimulation induces long term depression (LTD) rather than LTP (Dunwiddie and Lynch, 1978). Interestingly, memory consolidation by SPW-R complexes *in vivo* is characterized by a low frequency occurrence of SPW at 2–4 Hz (Steriade et al., 1993; Ylinen et al., 1995; Csicsvari et al., 1999; Wolansky et al., 2006; Schall et al., 2008; Mölle and Born, 2011; Sadowski et al., 2011) while each of the complexes generates several cycles of high frequency oscillations (Ylinen et al., 1995). One consequence of superimposed ripple oscillations is the highly synchronized and near coincident spiking of neurons (Wilson and McNaughton, 1994) that might provide a privileged condition for plasticity processes during SPW-R (Csicsvari et al., 1999; King et al., 1999; Girardeau et al., 2009; Sadowski et al., 2011).

Why do CA3 and CA1 behave differently? The CA3 region has been suggested to form an auto-associative network for transient storage of memories (Kesner, 2013; Rolls, 2013).

Moreover, it supports pattern completion upon partial cue activation (Nakazawa et al., 2002; Kesner and Rolls, 2015). Additionally, CA3 serves as the initiator of SPW-R oscillations during sleep and might play an important role in guaranteeing the replay of stored network patterns (Buzsáki, 1989; Wilson and McNaughton, 1994). The underlying pronounced plasticity might explain the tendency of CA3 to form new assemblies following repetitive consolidation of specific sub-sets of neurons. CA1, on the other hand, forms a critical interface between the direct input of environmental information by the entorhinal cortex and the pre-processed information from the CA3 region. This region has been proposed to act as a comparator between previously stored input patterns from CA3 and the actual input coming directly from the entorhinal cortex (Vinogradova, 2001; Kumaran and Maguire, 2007). In addition, CA1 serves as a major source of outputs from hippocampal to neocortical areas and thus plays a crucial role in the transfer process of memory from the hippocampus to the neocortex (Chrobak and Buzsáki, 1994, 1996). These properties might require a higher stability of information which is reflected by both, a stable re-activation of neuronal assemblies under control condition and the tendency to stabilize pre-existing assemblies by repetitive activation.

In summary, low-frequency repetitive activation of specific fast oscillating neuronal activity patterns causes a stabilization and consolidation of the underlying neuronal assemblies. This process of network-level plasticity expresses characteristic regional differences, in line with the proposed differential functions of CA3 (pattern completion and auto-associative memory) and CA1 (novelty detection and readout of existing assemblies).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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