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Long-lasting input-specific experience-dependent changes of hippocampus synaptic function measured in the anesthetized rat

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Abstract

How experience causes long-lasting changes in the brain is a central question in neuroscience. The common view is that synaptic function is altered by experience to change brain circuit functions that underlie conditioned behavior. We examined hippocampus synaptic circuit function *in vivo,* in three groups of animals, to assess the impact of experience on hippocampus function in rats. The "Conditioned" group acquired a shock-conditioned place response during a cognitively-challenging, hippocampus synaptic plasticity-dependent task. The "No-Shock" group had similar exposure to the environmental conditions but no conditioning. The "Home-Cage" group was experimentally naive. After the 1-week retention test, under anesthesia, we stimulated the perforant path inputs to CA1, which terminate in *stratum lacunosum moleculare*, and to the dentate gyrus, which terminate in the molecular layer. We find synaptic compartment specific changes that differ amongst the groups. The evoked field EPSP and pre-spike field response are enhanced only at the dentate gyrus input layer and only in Conditioned animals. The dentate gyrus responses, measured by the population spiking activity and post-spike responses, are enhanced in both the Conditioned and No-Shock groups compared to Home-Cage animals. These changes are pathway specific because no differences are observed in *stratum lacunosum moleculare* of CA1. These findings demonstrate long-term, experience-dependent, pathway-specific alterations to synaptic circuit function of the hippocampus.

Significance Statement

We investigated whether a hippocampus-dependent place avoidance memory causes long-lasting changes of hippocampus circuit function, as is commonly assumed. Immediately after testing 1-week memory retention in rats, under anesthesia, the entorhinal cortex projection to dentate gyrus is strengthened, as estimated by the synaptic response to stimulation as well as the amplitude of the population action potential response. These changes are pathway specific because no differences are observed in the *stratum lacunosum moleculare* of CA1 where the entorhinal cortex projection also terminates. These findings of experience-dependent, pathway-specific alterations to synaptic circuit function in hippocampus are consistent with theories that posit that memory formation causes persistent alterations of neural circuit function.

Introduction

How experience alters the brain to enable conditioned responses is an open question. The current view is that changes to synaptic circuit function underlie conditioned behavior. Changes to synaptic function have been difficult to identify and have focused on long term potentiation (LTP) immediately after conditioning a behavior (Whitlock et al., 2006), although changes in synaptic function have also been observed during (Fernández-Lamo et al., 2018) or one or more days after conditioning (Gruart et al., 2006; Park et al., 2015; Pavlowsky et al., 2017).

The two-frame active place avoidance task conditions a place response that depends on synaptic modifications in the dorsal hippocampus, specifically PKMzeta- and CaMKII-dependent LTP (Pastalkova et al., 2006; Tsokas et al., 2016; Hsieh et al., 2017; Rossetti et al., 2017). These studies have not, however, identified if the modifications to synaptic circuit function are universal or pathway-specific. In mice, following two-frame active place avoidance conditioning, robust changes were observed specifically in the CA3-CA1 synaptic response, but not in the entorhinal cortex (EC)-CA1 response, in *ex vivo* hippocampus slice recordings (Pavlowsky et al., 2017). In anesthetized mice, place avoidance training enhanced the excitability of granule cells in the dentate gyrus (DG) in response to EC stimulation (Park et al., 2015). Here we extend these findings to the rat. Immediately after retraining one week after task acquisition, under anesthesia, we find training-induced changes in hippocampus function that are specific to the DG but not the CA1 targets of the EC inputs.

Materials & Methods

All methods complied with Public Health and Service Policy on Humane Care and Use of Laboratory Animals and were approved by New York University Animal Welfare Committee, which follow NIH guidelines.

Animals

Twenty-two male Long-Evans (Taconic Farms, Germantown, NY) rats arrived at the New York University animal facilities and were given at least a week to acclimate. The rats had free access to food and water and were single-housed.

Two-frame active place avoidance

Sixteen rats were used for the behavioral experiments. Rats were handled ~5 min/day for five days before behavioral training. The training took place on an 81-cm diameter circular disk-shaped rotating arena with transparent walls (Bio-Signal Group, Acton, MA). The arena could be stationary or rotate at 1 rpm. The position of a rat on the arena was monitored at 30 frames per second from an overhead camera using video-tracking software (Tracker, Bio-Signal Group, Acton, MA). The software could track the rat's position in the room as well as on the rotating arena and could deliver a mild constant current foot shock (0.3 mA, 60-Hz 500-ms) whenever the rat was detected in a 60° sector that was designated the shock zone. Although the arena rotated, the shock zone was stationary and was defined by stationary landmarks that were fixed in the room.

The rats were given two 10-min pre-training trials to habituate to the stationary arena. During the two training days (Sessions 1 and 2), the rats were given eight 10-min trials per day on the rotating arena. Rats in the Conditioned group (n = 8) received shocks in the shock zone and rats in the No-Shock group (n = 8) experienced the same physical conditions, except they were never shocked. The time between trials was ~10 min. One week later, the rats were all

returned to the rotating arena for 10 minutes to test memory retention. The shock was on in the shock zone for rats in the Conditioned group. Immediately after the 1-week experience the rats were anesthetized and prepared for assessing *in vivo* hippocampus evoked responses. The Home-Cage (n = 6) group consisted of rats that were never exposed to the behavioral room and were not handled (Figure 1A).

Behavioral assessment

Behavioral end-point measures were computed from the position timeseries using TrackAnalysis (Bio-Signal Group, Acton, MA). We evaluated the distance walked on the arena, the number of entrances made into the shock zone, the time spent in each quadrant, and the time to enter the shock zone for the first time. The distance walked on the arena assesses locomotion. The shock zone entrances and the time in each quadrant estimate place avoidance and preference. The time to first enter the shock zone estimates avoidance memory across-sessions.

In vivo hippocampus evoked responses

Surgical protocol: Immediately after the 1-week retention trial, rats were anesthetized with 1.2 g/kg urethane (i.p.) and placed in a stereotaxic frame. A 16-site linear-array silicon electrode with 30 µm diameter recording contacts and 100 µm spacing (Neuronexus, Ann Arbor, MI; p/n: A 1 X 16 - 5mm - 100 - 703) was placed in the dorsal hippocampus to span the somatodendritic axis of dorsal CA1 and DG (Figure 1B). A six-wire stimulating electrode bundle was placed in the ipsilateral perforant path. The stimulating electrodes were made by twisting six nichrome wires (75 µm) together. The wires were cut at an angle to allow the six sites to span 1 mm. The following coordinates were used to target the perforant path fibers: relative to Bregma, anteroposterior (-7.6 mm), mediolateral (4.1 mm), dorsoventral (2.5-3.0 mm). The two-wire bipolar combination that evoked the largest response was selected for the stimulation

153 experiments. A constant current stimulus isolation unit (WPI, Sarasota, FL; model: A365RC)
154 was used to deliver individual 150 μs stimulus pulses across the electrode pair.
155 The response to stimulation was recorded using Axona USBdacq software (St. Albans,
156 U.K.) and the Axona hardware that was optimized for recording evoked potential responses. An
157 attenuating resistor (either 18 kΩ or 47 kΩ) was connected to the Axona input to maximize the
158 effective number of bits (16) used for digitization. The signals were low-pass filtered < 5 kHz
159 and digitized at 48 kHz.
160
161 Localization of the recording electrodes: Current Source Density (CSD) analysis was performed
162 for source localization, minimizing volume conducted signals in the analyzed data. The CSD is
163 computed from the second spatial derivative of the voltage along the recording depth:

$$CSD(z) = -\frac{V(z-\Delta z) + V(z+\Delta z) - 2 * V(z)}{\Delta z^2}$$

164 where $CSD(z)$ is the CSD at depth z, $V(z)$ is the Voltage at depth z and Δz = 100 μm is the
165 distance between adjacent recording sites. The CSD is calculated in units of mV/μm^2 and can
166 be multiplied by a conductivity constant σ to measure it in units of A/μm^3. By convention, CSD is
167 computed as the negative of the second spatial derivative with negative CSD indicating an
168 extracellular current sink and positive CSD an extracellular current source. Because it is
169 isotropic (Hrabetová, 2005), we assume that the conductivity is homogeneous within the
170 hippocampus as it was shown that variations in the conductivity had little effect on hippocampus
171 CSD estimates (Holsheimer, 1987). Similarly, the conductivity was assumed to be similar across
172 the groups.
173 The pattern of sinks and sources in each animal was used to localize the electrode
174 recording sites to each somatodendritic location for an individual rat (Figure 1C) as previously
175 described (Brankack et al., 1993; Wu and Leung, 2003). Briefly, the DG granule cell layer was
176 identified by the largest source in the lower channels that also included a sink, which was the

177 population spike (PS) activity. The molecular layer was identified as the largest sink 1-2
178 channels above the granule cell layer. The *stratum lacunosum moleculare* (*slm*) was identified
179 by two channels: the large sources above the molecular layer and an early latency sink in the 1-
180 2 channels just above the largest source. *Stratum radiatum* was identified as a late latency sink
181 and the pyramidal layer was identified as a late source above *stratum radiatum*. S*tratum oriens*
182 was identified as the sink above the pyramidal layer. Based on the sinks and sources from one
183 animal from the Conditioned group and two animals from the No-Shock group, the recording
184 and/or stimulation electrodes did not appear to be properly placed and these three animals were
185 excluded from the physiology analyses. Thus, for the physiology data, the final group numbers
186 were Conditioned n = 7, No-Shock n = 6, Home-Cage n = 6.
187
188 Analysis of the responses evoked by stimulation: All analyses were performed offline using
189 custom MATLAB software. We performed all estimates of the evoked responses on the CSD
190 traces to minimize the impact of volume conduction. Accordingly, we refer to these evoked
191 responses as source-localized. Input-output (I-O) curves were generated by measuring the
192 evoked responses to stimulus intensities ranging from 100 to 1000 μA in 100 μA steps. At each
193 stimulus intensity, four voltage responses were recorded and the CSD computed for each
194 response. All measurements were performed on each of the four responses and then averaged.
195 Spike times were estimated at the DG-granule cell layer for each rat.
196 To visualize the responses along the somatodendritic axis, we aligned all recordings to
197 the DG granule cell layer compartment and computed the group average CSD response to 800
198 μA stimulation for each rat. We chose to compute the average at 800 μA because that was the
199 highest stimulation intensity delivered to all animals. The CSDs obtained at 800 μA were then
200 averaged for each group to visualize the group-specific sinks and sources. The data were
201 plotted with interpolation across adjacent electrodes using the *shading interp* MATLAB function.

Unbiased Quantification: Area under curve – We computed the area-under-the-curve (AUC) of the source localized (sl-) evoked response as an unbiased estimate of the response since it includes both direct and polysynaptic responses as well as spiking. The source-localized area-under-the-curve (sl-AUC) was measured before and after population spiking at each compartment and separately for sources and sinks (Figure 1D). Analysis of the individual sources and sinks within the CSD profile allowed us to quantify specific components of the response, such as the post-spike activity, that is otherwise difficult to identify and measure as it is comprised of excitatory and inhibitory responses. We note that interpreting an (inward) current sink or (outward) current source depends on knowing the functional anatomy of the relevant sites because active sinks and sources are associated with passive return sources and sink currents, respectively.

Feature-directed Quantification - The evoked response contributions to the field excitatory postsynaptic potential (fEPSP) and to the population spike (PS) are well-characterized and commonly used to estimate the responses of DG to entorhinal stimulation. Feature directed analysis was performed on the evoked CSD traces to measure the source-localized population spike (sl-PS) and fEPSP (sl-fEPSP; Figure 1D). The sl-PS was measured in the granule cell layer of the DG and two measures of the sl-fEPSP were made: the positive slope in the granule cell layer and the negative slope in the molecular layer. The time window for estimating sl-fEPSP was 1.5 ms after the stimulus was delivered until 1 ms prior to population spiking activity. This time restriction was chosen to prevent the inclusion of (variable) dendritic spiking activity (Herreras, 1990).

Boltzmann function fits: We examined fEPSP and PS (E-S) coupling in the DG by fitting a Boltzmann function to the data using the following equation for the *sl-PS* as a function of the *sl-fEPSP*:

$$sl\text{-}PS = \frac{sl\text{-}PS_{max}}{1 + \exp\left(\frac{sl\text{-}fEPSP_{50} - sl\text{-}fEPSP}{S}\right)}$$

Where $sl\text{-}PS_{max}$ is the maximum *sl-PS*, $sl\text{-}fEPSP_{50}$ is the *sl-fEPSP* associated with the 50% $sl\text{-}PS_{max}$ response, and *S* is the slope (the slope being steeper as *S* decreases). The equation was fit for each animal and the $sl\text{-}PS_{max}$, $sl\text{-}fEPSP_{50}$ and slopes were compared amongst the groups.

Paired-pulse inhibition: Stimulation to assess paired-pulse inhibition was performed at 65% of the intensity required to elicit the maximal sl-PS response. Stimulus pairs were delivered at increasing inter-stimulus intervals between the first and second pulses (5, 10, 20, 40, 80, 160, 320, and 640 ms). We allowed 30 s between each pair of stimuli. At each inter-stimulus interval, four responses were recorded and CSD analysis was performed to attenuate effects of volume conduction. The responses were measured and the ratio of the second to first response was used to estimate the amount of inhibition on the second stimulation due to the initial stimulation. These ratios were averaged across the four recordings.

Verification of stimulation and recording sites: At the end of the recordings, the rats were transcardially perfused with 1X phosphate buffered saline (PBS) followed by 10% formalin. The brains were extracted and stored in formalin overnight and stored in 30% sucrose in 1X PBS until they were cut on a cryostat (40 μm) and thaw mounted onto gelatin-coated slides. The sections were dried overnight at room temperature and then Nissl stained. The slides were

scanned at 10X with an Olympus VS120 microscope and the images were subsequently examined for electrode tracks to verify the stimulation and recording locations (Figure 1E).

Statistical Analysis: Statistical analysis was performed in JMP 14 (SAS, Cary, NC). Behavioral data from all the animals were included in the analysis (Conditioned: n = 8; No-Shock n = 8). Multivariate analysis of variance (MANOVA) was conducted for each behavioral phase (Pretraining, Training, Retention) separately. For the Pretraining and Training phases, we conducted MANOVA analyses with the trials used as a repeated measure; Hotelling's trace correction was used when sphericity was violated. Additionally, when relevant, two-group comparisons were made using Student's t tests, with degrees of freedom adjusted for unequal variances. Electrophysiological data were assessed by Analysis of variance (ANOVA) to identify statistically significant group, stimulation intensity, and group x stimulation intensity differences between Conditioned, No-Shock, and Home-Cage rats. Stimulation intensity was treated as a continuous variable. Tukey's honest significant difference (HSD) post-hoc tests evaluated pair-wise differences. The effect size η^2 is given when a non-significant statistical trend is observed. Statistical significance was set at $p < 0.05$.

Results

Conditioned behavior

The arena is stationary during the Pretraining trials, allowing the assessment of locomotor behavior, similar to that done in an open field test. Because the rats were randomly assigned to the Conditioned and No-Shock groups, we did not expect to see group differences in locomotor activity on the stationary arena. Indeed, prior to training, the Conditioned and No-Shock control groups are identical in their exploratory activity (Figure 2B). The average time spent in each location for all rats in each group (Figure 2B, heat maps), with example traces of a rat from each group (Figure 2B, grey traces), indicates that during Pretraining, rats do not avoid

274 going into the region that will eventually be designated as the shock zone. However, to
275 investigate biases for locations on the arena, we analyzed the proportion of time spent in each
276 quadrant. There is a quadrant effect ($F_{3,56}$ = 3.12, p = 0.03) because rats show a preference to
277 spend time in at least one quadrant, but the group interactions are not significant indicating no
278 influence of group (group x quadrant: $F_{3,56}$ = 0.41, p = 0.74; group x quadrant x trial: $F_{3,56}$ =
279 0.16, p = 0.92), and neither is the trial x quadrant interaction significant, indicating steady state
280 preferences ($F_{3,56}$ = 0.82, p = 0.49). Although it appears from the heat maps (Figure 2B) that
281 the animals are spending more time in the quadrant opposite the eventual shock zone, post hoc
282 analysis on the quadrants indicates no preference for this specific quadrant. Furthermore, the
283 two groups are indistinguishable in their behaviors during this initial exploratory period (Figure
284 2C-E) in the total distance walked on the stationary arena (group: $F_{1,14}$ = 0.04, p = 0.85; trial:
285 $F_{1,14}$ = 10.52, p = 0.006; group x trial: $F_{1,14}$ = 0.78, p = 0.39), the number of times they enter the
286 eventual shock zone (group: $F_{1,14}$ = 0.23, p = 0.64; trial: $F_{1,14}$ = 2,27, p = 0.15; group x trial: $F_{1,14}$
287 = 0.95, p = 0.34) and the latency to first enter the eventual shock zone (group: $F_{1,14}$ = 8 x 10^{-4}, p
288 = 0.98; trial: $F_{1,14}$ = 4.77, p = 0.05; group x trial: $F_{1,14}$ = 0.55, p = 0.47).

289 Conditioning the rats to avoid the location of the shock during the training trials results in
290 multiple behavioral differences. Conditioned rats learn to avoid the location of shock on the
291 rotating arena in the two-frame active place avoidance task whereas rats exposed to the same
292 environment without shock (No-Shock rats) do not express a conditioned place response
293 (Figure B). During training, an effect of quadrant is observed ($F_{3,112}$ = 10.53, p = 10^{-6}) as well as
294 a group x quadrant interaction ($F_{3,112}$ = 26.34, p = 10^{-13}) and a group x trial x quadrant effect
295 ($F_{21,215.08}$ = 1.7, p = 0.032), but no other interaction (trial x quadrant effect : $F_{21,215.08}$ = 1.54, p =
296 0.067; day x quadrant effect : $F_{3,112}$ = 0.20, p = 0.90; group x day x quadrant effect : $F_{3,112}$ =
297 0.65, p = 0.58; trial x day x quadrant effect : $F_{21,215.08}$ = 1.37, p = 0.13; group x trial x day x
298 quadrant effect : $F_{21,215.08}$ = 1.32, p = 0.17). Post-hoc tests done separately for each group
299 during training show an effect of sector for both groups but with a preference for the opposite

sectors: Trained animals spend the least time in the shock quadrant while No-Shock animals spend the most time there. Both groups of rats appear to have an increase in distance walked early in Session 1 compared to what is observed in Pretraining, which may be due to the novelty of being on a rotating arena that was previously stationary. As seen in the example trajectory and the group-averaged time spent in each location on the arena in the last training trial of Session 2 (Figure 2B), the Conditioned rats learn to avoid the shock zone. The Conditioned rats walk 18% less than the No-Shock rats during training (Figure 2C), which is because of the No-Shock rats' increased locomotion, and to the restriction of the Conditioned rats walking in only three of the four quadrants (Distance walked; group: $F_{1,28} = 9.76$, $p = 4.12 \times 10^{-3}$; day: $F_{1,28} = 0.82$, $p = 0.37$; trial: $F_{7,22} = 1.57$, $p = 0.20$; group x day: $F_{1,28} = 0.67$, $p = 0.42$; group x trial: $F_{7,22} = 1.4$, $p = 0.26$; day x trial: $F_{7,22} = 1.26$, $p = 0.31$; group x day x trial: $F_{7,22} = 0.60$, $p = 0.75$). The Conditioned group quickly reduces the number of entries into the shock zone while the No-Shock rats continue to enter the shock zone location throughout the entire training period (Figure 2D, Shock zone entrances; group: $F_{1,28} = 171.12$, $p = 10^{-13}$; day: $F_{1,28} = 0.97$, $p = 0.33$; trial: $F_{7,22} = 1.50$, $p = 0.22$; group x day: $F_{1,28} = 2.04$, $p = 0.16$; group x trial: $F_{7,22} = 2.55$, $p = 0.044$; day x trial: $F_{7,22} = 0.7$, $p = 0.67$; group x day x trial: $F_{7,22} = 0.65$, $p = 0.71$; post hoc Student's t tests to evaluate group differences at each trial are all significant at $p < 0.05$). Conditioned rats also show avoidance memory by increasing their latency to enter the shock zone over the course of conditioning (Figure 2E, Time to first entry; group: $F_{1,28} = 29.57$, $p = 10^{-6}$; day: $F_{1,28} = 1.43$, $p = 0.24$; trial: $F_{7,22} = 0.84$, $p = 0.56$; group x day: $F_{1,28} = 1.91$, $p = 0.18$; group x trial: $F_{7,22} = 1.60$, $p = 0.19$; day x trial: $F_{7,22} = 0.36$, $p = 0.92$; group x day x trial: $F_{7,22} = 0.14$, $p = 0.99$).

The impact of the training experience on behavioral measures persists for at least one week following the end of conditioning. During the Retention trial, an effect of quadrant ($F_{3,56} = 4.49$, $p = 6.80 \times 10^{-3}$) and a group x quadrant interaction is still observed ($F_{3,56} = 9.36$, $p = 10^{-5}$). Again, post-hoc tests done separately on each group during the retention trial show that

Conditioned rats spend the least amount of time in the shock quadrant ($F_{3,28}$ = 9.42, p = $1.82x10^{-4}$ with Tukey's post-hoc indicating preference for the quadrant opposite to the shock quadrant), which is also seen in the reduced number of entrances into the shock zone compared to No-Shock rats ($t_{9.81}$ = 5.95, p = $1.52x10^{-4}$). During the retention trial, the No-Shock rats show no preference for any of the quadrants ($F_{3,28}$ = 0.97, p = 0.42) and there is no significant group difference in the distance walked ($t_{13.18}$ = 1.91, p = 0.078). In addition to displaying a preference for avoiding the shock quadrant, the Conditioned rats have an increased latency to enter the shock quadrant ($t_{7.09}$ = 2.42, p = 0.046), another indicator that the Conditioned rats have a 1-week old place avoidance memory.

Evoked potential results

We examined synaptic function in the dorsal hippocampus under anesthesia immediately following the retention trial. As is seen by the overlapping traces of the voltage and the CSD response waveforms (Figure 3A), performing CSD analysis reduces the influence of volume conduction on the waveforms and is therefore a better localized estimate of the evoked activity at the different somatodendritic compartments of CA1 and DG. We registered the data to the somatodendritic locations and then calculated group average CSD traces (Figure 3B), which appear different, although we did not further analyze the spatial profile of the CSD.

All estimates of evoked activity were performed on the CSD-corrected responses and are referred to as "source-localized" (sl-). To evaluate the stimulation responses, we chose to take two separate approaches, a feature-directed quantification and an unbiased evaluation of the evoked responses. The first approach is to quantify specific features of the evoked response that are traditionally used to estimate the synaptic and spiking components, the slope of the evoked source-localized synaptic fEPSP response (sl-fEPSP) and the amplitude of the source-localized population spike (sl-PS), respectively. The second approach evaluates the evoked activity localized to a single electrode at a specific location along the somatodendritic axis.

Without relying on assumptions about the waveform of the response, we measured the area-under-the-curve (AUC) of the CSD trace at this site. The activity following spiking is particularly difficult to quantify using traditional methods but can be captured in a straightforward manner by measuring the AUC of the response that occurs after the spike activity. Whether this activity corresponds to active transmembrane currents or passive return currents cannot be determined from the CSD alone and requires knowledge of the functional anatomy for a complete interpretation, and we therefore analyzed the CSD source and sink components separately. In contrast, the activity preceding the population spike standardly estimated by the slope of the fEPSP can also be estimated by measuring the AUC before the PS, which quantifies the entire response instead of the response at a specific time point; these two estimates of the synaptic response at the molecular layer are correlated in our data set ($r = 0.87$, $p = 10^{-57}$).

To get an initial sense of where training-induced changes in the evoked response might occur, we quantified the source-localized activity prior to and after the population spike activity by measuring the AUC of the source-localized signal (sl-AUC) in response to 800 μA stimulation, which is the maximum stimulation intensity that was recorded for all animals (Figure 3C). Because we wanted to separately quantify the activity prior to and after the sl-PS, we first determined if the sl-PS times differed amongst the groups. While the sl-PS times do differ across the three groups ($F_{2,89}= 3.72$, $p = 0.03$) as well as by stimulation Intensity ($F_{1,89}= 9.68$, $p = 2.5 \times 10^{-3}$), there is no interaction ($F_{2,89}= 0.01$, $p = 0.99$) and no post-hoc group differences. While the pre-spike activity at the molecular layer is not different between groups, ($F_{2,16}= 2.05$, $p = 0.16$, $\eta^2=0.20$; Figure 3C, left), the post-spike sink at the molecular layer is significantly different amongst the groups ($F_{2,16}= 3.65$, $p = 0.0496$). The difference between the Conditioned and Home-Cage groups was confirmed by post-hoc tests, but neither group is different from the No-Shock group (Figure 3C, right). Although the pre-spike molecular layer sink represents the perforant path input, it is uncertain to what extent this difference in the post-spike molecular

layer sink reflects a training-induced difference in either an active transmembrane current or a passive return current.

We evaluated the input-output curves of pre- and post-spiking responses (sl-AUC) at the molecular layer (Figure 3D), both of which show a significant effect of group due to greater statistical sensitivity of repeating the measurements for different stimulus intensities. The pre-spike response plateaus at approximately 300 μA stimulation for all groups and is greater for the Conditioned group (Group: $F_{2,180} = 17.38$, $p = 10^{-7}$; Stimulation Intensity: $F_{1,180} = 2.93$, $p = 0.09$; Interaction: $F_{2,180} = 0.39$, $p = 0.68$; with post hoc differences between the Conditioned group and the other groups; Figure 3D, top). After the spike, the response increases with stimulation intensity and is different between the groups such that Conditioned > No-Shock > Home-Cage (Group: $F_{2,180} = 23.92$, $p = 10^{-10}$; Stimulation Intensity: $F_{1,180} = 99.25$, $p = 10^{-19}$; Interaction: $F_{2,180} = 6.04$, $p = 0.003$; with post hoc differences between all groups; Figure 3D, bottom).

Traditionally, the early slope of the field excitatory post-synaptic potential (fEPSP) is used to estimate the synaptic response and the PS amplitude is used to estimate synchronous neuronal discharge. We estimated these values from the CSD-corrected responses. The Conditioned group sl-fEPSP at the molecular layer is larger than that of the No-Shock and Home-Cage rats (Group: $F_{2,180} = 13.65$, $p = 10^{-6}$; Stimulation Intensity: $F_{1,180} = 48.33$, $p = 6.41 \times 10^{-11}$; Interaction: $F_{2,180} = 2.30$, $p = 0.10$; Conditioned > No-Shock = Home-Cage; Fig. 3E).

Although the overall CSD responses (Figure 3C) do not appear different amongst the groups at the granule cell layer, there is a group effect on both the sl-fEPSP slope (Group: $F_{2,180} = 3.79$, $p = 0.02$; Stimulation Intensity: $F_{1,180} = 35.23$, $p = 10^{-8}$; Interaction: $F_{2,180} = 0.79$, $p = 0.46$; Conditioned > No-Shock = Home-Cage; Figure 3F) and the sl-PS amplitude (Group: $F_{2,180} = 5.92$, $p = 0.003$; Stimulation Intensity: $F_{1,180} = 160.76$, $p = 10^{-27}$; Interaction: $F_{2,180} = 1.64$, $p = 0.20$; Conditioned = No-Shock > Home-Cage; Figure 3G). None of these parameters measured in the physiology correlate with the behavior, in particular with the time to first enter the shock zone on the retention trial (data not shown). These differences are also not accompanied by

differences in E-S coupling (Figure 4, molecular layer sl-fEPSP to granule cell layer sl-PS (panels A,B): sl-PS_{max}: $F_{2,16}$ = 1.24, p = 0.32, η^2=0.13; sl-$fEPSP_{50}$: $F_{2,16}$ = 1.00, p = 0.39, η^2=0.11; slope: $F_{2,16}$ = 1.82, p = 0.19, η^2=0.19; granule cell layer sl-fEPSP to granule cell layer sl-PS (panels C,D): sl-PS_{max}: $F_{2,16}$ = 0.86, p = 0.44, η^2=0.10; sl-$fEPSP_{50}$: $F_{2,16}$ = 0.36, p = 0.70, η^2=0.04; slope: $F_{2,16}$ = 1.18, p = 0.33, η^2=0.13). Although the slopes of the E-S coupling appear different (Figure 4B,D, right panel), significance is not reached, likely due to the small effect sizes. Indeed, a power analysis with power set to 0.80, indicates 3-4 times larger sample sizes are needed to detect group differences in the E-S coupling. Finally, the differences in sl-fEPSP and sl-PS cannot not be easily attributed to changes in feedback inhibition, as there is no difference in paired-pulse inhibition measured as the ratio sl-PS2/sl-PS1 (Group: $F_{2,113}$ = 0.13, p = 0.88; ISI: $F_{1,113}$ = 2.73, p = 0.10; Interaction: $F_{2,113}$ = 0.49, p = 0.61; data not shown). Nonetheless, other estimates of altered inhibition merit assessing (Ruediger et al., 2011).

Discussion

Summary

Here we show that one week after two-frame active place avoidance conditioning, rats retain the conditioned place response and express an altered synaptic circuit response to perforant path stimulation of the entorhinal cortical input to DG. This indicates that learning the hippocampus-dependent place avoidance is associated with altered neocortical-hippocampal circuit function. The functional alteration of this neural circuit component is specific to the population synaptic component of the response localized to the molecular layer of DG where the stimulated pathway terminates, as observed in both the pre-Spike sl-AUC (Fig. 3D) and the sl-fEPSP (Fig. 3E). No change is detected in *lacunosum moleculare* of CA1 where perforant path fibers also terminate. The change in synaptic circuit function of the entorhinal-to-DG pathway is not observed in No-Shock control rats that experienced the identical physical conditions as the

Conditioned rats with one exception - they were never shocked. Because on average, the Conditioned rats only enter the shock zone approximately 5 times during each 10-min training session (Figure 2D), the physical experience of the environment only differs between the conditioned and No-shock groups during shock, which comprised ~2.5 s (5 x 500 ms) or less than 1% of each training session's duration. While the conditioning is sufficient to alter population synaptic function, it also results in increased neuronal discharge, evidenced as an increased population spike response at the granule cell layer, whereas the enhanced sl-fEPSP at the granule cell layer is harder to interpret (Fig. 3F) because the PP terminates at the molecular layer. Within the Conditioned DG, the evoked responses are greater (Figure 3E-G) and both the pre-spike population synaptic response and the post-spike population response are enhanced compared to the corresponding responses in Home-Cage DG (Figure 3D). In contrast, the conditioning does not alter E-S coupling, indicating that synaptic input responses change, likely without an effective change in the corresponding excitability, and the changes are input specific. Some components of the evoked responses are altered in both the Conditioned and No-Shock groups while other components are altered only in the Conditioned group. An enhanced synaptic component of the evoked response was only observed in the Conditioned rats, whereas compared to Home-Cage rats, the measures of neuronal discharge and post-spike activity were enhanced after either the conditioned or non-conditioned experience. Perhaps these changes reflect learning because even the No-Shock rats' experience leads to spatial learning as measured by hippocampal physiology (Muller and Kubie, 1987; Wilson and McNaughton, 1993; Radwan et al., 2016).

The observed increases in sl-PS amplitude in the Conditioned and No-shock groups compared to the Home-Cage group appears not to be due to increased intrinsic excitability as no significant effect of experience on E-S coupling is observed. However, E-S coupling reflects both intrinsic excitability and the balance of excitation/inhibition (Lu et al., 2000; Daoudal et al., 2002), which cannot be distinguished without further experiments. Alternatively, either altered

feedback or feedforward inhibition could account for the observations, but although the paired-pulse paradigm used in these studies did not detect a group difference, other estimates of feedback inhibition are warranted before concluding that feedback inhibition is unchanged by active place avoidance training. Nonetheless, because learning-induced changes in interneuron connectivity have been described in DG (Ruediger et al., 2011), and inhibition-sensitive increases of Schaffer collateral synapse effectiveness have been observed in Conditioned mice (Pavlowsky et al., 2017), it may be the case that learning causes more complex neural circuit changes that might be obscured in the present study by use of anesthesia, which can differentially impact excitatory and inhibitory neurotransmission. The impact of the conditioning and no-shock experiences on feedforward inhibition requires further examination, ideally in awake, freely-behaving subjects.

Relationship to prior work

Previous work in both awake rats (Whitlock et al., 2006) as well as *ex vivo* mouse hippocampus slices (Pavlowsky et al., 2017) observed changes in CA3-CA1 synaptic function after avoidance learning, whereas in the mouse slice experiments EC-CA1 synaptic responses were unchanged after place avoidance learning (Pavlowsky et al., 2017), similar to the Conditioned rats in the present study. *In vivo* recordings of anesthetized mouse DG responses to stimulation of EC input showed an increased fEPSP response in trained vs naïve animals while the amplitude of the PS and the E-S coupling was unchanged (Park et al., 2015). While our results are similar to Park et al. in that we find that the sl-fEPSP is increased with conditioning, our results contrast with these previous results because we also see an impact of conditioning on the sl-PS amplitude. This discrepancy could be due to the differences in analyzing our signals; by recording at multiple, evenly spaced sites, we were able to reduce the influence of volume conduction and better localize our measurements by computing the CSD and making our measurements on the CSD waveforms. Consistent with prior work, we conclude

that active place avoidance conditioning causes long-lasting changes to hippocampus circuit function in both mice and rats.

Other forms of conditioning have also been reported to change hippocampus circuit function. Forms of eyeblink conditioning cause LTP-like NMDAR- and PKMζ-dependent enhancement of the CA3-CA1 pathway, assessed in awake mice (Gruart et al., 2006; Gruart and Delgado-García, 2007; Madroñal et al., 2010). Non-aversive, non-associative learning, such as in an object recognition memory task was also associated with enhancement of CA3-CA1 input (Clarke et al., 2010). Changes in EC-DG synaptic responses were found over the course of operant conditioning, as were changes in EC-CA1. The changes were moderate in both cases and were behavior-dependent. Remarkably, the EC-DG changes were only measurable when animals approached the lever, and these were only observed during the learning phase of the paradigm and returned to baseline after the task was acquired. Furthermore, changes in EC-CA1 were only significantly altered when animals were going to retrieve food, eating the food, or grooming (Fernández-Lamo et al., 2018). Although it appears that some of these changes are transient (Fernández-Lamo et al., 2018), other changes measured *in vivo* can persist for days (Park et al., 2015) and cannot be easily explained by a behaviorally-induced, temporary rise in brain temperature (Moser et al., 1993a; 1993b; Moser, 1995). This is especially the case when we consider data from *ex* vivo slice experiments (Pavlowsky et al., 2017) and under constant conditions of anesthesia (Park et al., 2015), as in the present study. While the present findings unequivocally demonstrate that hippocampus-dependent learning is accompanied by persistent changes to hippocampus circuit function, changes in circuit function may not be unique to hippocampus and may be observable only in specific behavioral states (Fernández-Lamo et al., 2018). It is also not known whether the functional changes indicate LTP and other synaptic changes that, according to the synaptic plasticity and memory hypothesis, underlie long-term memory – definitive evidence remains elusive (Takeuchi et al., 2014; Ryan et al., 2015; Tonegawa et al., 2015).

Changes in synaptic function and synaptic plasticity

As described above, we, like others, have observed learning-related group differences in hippocampus circuit function that were detected *in vivo* by measurements of neural population activity such as the evoked responses of the present study. Because the groups only differ in their experience, we interpret those differences to indicate changes in circuit function consequent to different experiences that are our experimental manipulations. Such changes must be widespread if they are measurable in population activity and are thus at odds with expectations that memory-related functional changes are sparse (Whitlock et al., 2006; Takeuchi et al., 2014). Nonetheless they have been observed, but according to sparsity predictions, they are unlikely to simply reflect the long-term potentiation or depression of the specific synapses that store memory.

Here we observed that some components of the evoked responses are altered in both the Conditioned and No-Shock groups while other components are altered only in the Conditioned group, highlighting that behavioral history is important for interpreting estimates of neural circuit function, and that it can be difficult to definitively attribute measurable changes to specific changes in synaptic function (Moser et al., 1994; Andersen and Moser, 1995; Moser, 1995; Talbot et al., 2018). In particular, we observe altered sl-fEPSP and pre-spike activity in the Conditioned group and altered sl-PS and post-spike activity in both the Conditioned and No-Shock groups. These changes in both the Conditioned and No-Shock rats could be explained by various mechanisms that have been observed after exposing rodents to enriched environments. In the current study, both the Conditioned and No-Shock rats were handled as well as exposed to a novel environment, which may serve as enrichment compared to the Home-Cage rats. The mechanisms by which enrichment induced neuronal alterations include increased neurogenesis (Kempermann et al., 1997; 2002), increased dendritic length (Faherty et al., 2003), increased spine density (Moser et al., 1994), increased synaptic density (Rampon

et al., 2000), and increased synaptic protein expression (Frick and Fernandez, 2003; Nithianantharajah et al., 2004) to name a few. Given the impact of enrichment on these mechanisms, it is possible that pre- vs. post-spike alterations are a generalizable feature of conditioning vs. experience. While more experiments need to be performed to determine the generality as well as the specific nature of the present observations, the findings nonetheless demonstrate that experience-induced alterations to hippocampus circuit function are widespread but not global; they are instead pathway and compartment specific.

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Figure 1. Evoked response paradigm. **A)** Immediately after the retention trial, the Conditioned and No-Shock rats were anesthetized and underwent surgery for the evoked potential experiments. Home-Cage rats were never handled prior to the evoked response recordings. **B)** A 16-site linear electrode array was placed in the dorsal hippocampus, spanning the somatodendritic axis of CA1 and the dentate gyrus. A bundle of stimulating electrodes was placed in the perforant path to stimulate EC inputs. **C)** The recording probes were localized by performing CSD analysis, which identifies sinks and sources of the stereotypical response to perforant path stimulation at each layer. CA1: so = stratum oriens, pyr = pyramidal cell layer, strat. rad. = stratum radiatum, slm = stratum lacunosum moleculare, DG: mol = molecular layer, grc = granule cell layer. **D)** Quantification of the synaptic and evoked activity from the CSD was performed either by calculating the area under the curves for sinks and sources before or after the population spike activity and was performed for each layer of CA1 and DG. The other approach quantified the field EPSP of the molecular and granule cell layers and population spiking activity of the granule cell layer. **E)** Recording sites were verified histologically. Red lines indicate positions of the recording probes for the experiments performed. Anteroposterior coordinates relative to Bregma are indicated next to the coronal sections.

Figure 2. Memory-related behavior is different between Conditioned and No-Shock rats. **A)** Rats were placed on a rotating arena and conditioned to avoid a mild foot shock within a 60° sector that was stationary within the room. The No-Shock group was exposed to the same conditions, but without foot shock. **B)** The rats were given two pretraining trials on a stationary arena without shock. The next day the rats started two days of conditioning (Sessions 1 and 2). Each day a conditioning session consisted of 8 training trials. One week after the end of conditioning, the rats were given a retention trial to test memory. For the Conditioned group, the retention trial was conducted with the shock on. All trials were 10 min with at least 10 min inter-trial intervals. Example paths (in grey) of one No-Shock rat and one Conditioned rat are shown

over the course of training. The red dots represent where the animal received shocks and blue dots indicate where the animal would have received shock if the shock had been present. The heat maps show average time spent for all rats in each 0.3 cm x 0.3 cm region for each group. Red represents an average dwell of > 0.017 s. By the end of the training period the Conditioned rats avoid the 60° sector (bottom) while the No-Shock rats do not exhibit a place bias. **C-E)** Box plots of behavioral measures for the pre-training session, the training sessions (Trials 1-16) and the retention trial show there are differences between No-Shock and Conditioned rats. Asterisks in the box plots (C-E, left) indicate a main effect of group, while asterisks in the line graph (C-E, right) indicate group differences. No-Shock, n = 8; Conditioned, n = 8. Plots to the right of the boxplots represent mean ± SEM on a trial-by-trial basis. *p < 0.05. **p < 0.01.

Figure 3. Behavior-induced alterations to DG circuit function assessed by response to perforant path stimulation. **A)** The CSD (black traces) was computed from the recorded voltage traces (grey) in order to remove volume conducted signals. The sites determined to be located in *slm*, molecular layer and granule cell compartments (orange arrows) were used for further evaluation. The average ± S.E.M (shading) sl-CSD are shown. **B)** Average CSD heatmaps were generated for each group. Cooler colors are sinks (inward current) and warmer colors are sources (outward current). The sink in the molecular layer appears to increase with experience and cognitive demand. **C)** The area under the curve (AUC) of the pre-spike response (1.5 ms after the stimulation was delivered until the spike occurred) and the post-spike response (from the spiking activity until 20 ms after stimulation) were quantified at the maximum stimulation given to all animals (800μA) for each layer of the somatodendritic axis of the dorsal hippocampus. The pre-spike sink at the molecular layer is not changed by experience whereas the post-spike sink in the molecular layer is significantly altered by experience. CA1: pyr = pyramidal cell layer, strat. rad. = stratum radiatum, slm = stratum lacunosum moleculare, DG: mol = molecular layer, grc = granule cell layer. **D)** Top: The input-output curve that characterizes

692 the pre-spike sink, synaptic population response to the full range of stimulus intensities, is
693 significantly increased in Conditioned rats compared to both No-Shock and Home-Cage groups.
694 Bottom: The post-spike response to increasing stimulation intensity is significantly increased in
695 Conditioned rats compared to the No-Shock response, which is also significantly increased
696 compared to Home-Cage rats. **E)** The sl-fEPSP characterizing the population synaptic response
697 at the molecular layer is significantly increased by conditioning, confirming the findings based on
698 the sl-AUC. **F)** The sl-fEPSP at the granule cell layer is significantly different between
699 Conditioned and Home-Cage groups. **G)** The population spike response at the granule cell layer
700 is significantly increased by experience. Example CSD waveforms are plotted in **E-G** in black for
701 illustration with schematization of the measurement corresponding to the figure. The voltage
702 trace is underlaid in gray. Home-Cage, n = 6; No-Shock, n = 6; Conditioned, n = 7. Data are
703 mean ± SEM.
704
705 **Figure 4.** Coupling between the field synaptic potentials and population spiking is not different
706 between groups. **A)** The sl-fEPSP measured at the molecular layer is plotted against the sl-PS
707 measured at the granule cell layer for each stimulation response, with intensity varying from 100
708 to 1000 μA. The Boltzmann fit to each animal's E-S coupling is overlaid. **B)** Average parameters
709 of the Boltzmann fit per group show no difference due to experience in the sl-PS_{max} (left), the sl-
710 $fEPSP_{50}$ (middle), and the slope (right). **C)** The sl-fEPSP measured at the granule cell layer is
711 plotted against the sl-PS measured at the granule cell layer. **D)** Average parameters of the
712 Boltzmann fit per group also show no difference due to experience in the sl-PS_{max} (left), the sl-
713 $fEPSP_{50}$ (middle), and the slope (right). The inset in **C** schematizes the role of the parameters of
714 the Boltzmann fit. The example CSD waveforms in **B** and **D** are plotted for illustration.