Dentate spikes and learning: Disrupting hippocampal function during memory consolidation can improve pattern separation

Running head: Dentate spikes and learning

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Abstract

Hippocampal dentate spikes (DSs) are short-duration, large-amplitude fluctuations in hilar local-field potentials and take place while resting and sleeping. During DSs, dentate gyrus (DG) granule cells increase firing while CA1 pyramidal cells decrease firing. Recent findings suggest DSs play a significant role in memory consolidation after training on a hippocampus-dependent, non-spatial associative learning task. Here, we aimed to find out if DSs are important in other types of hippocampus-dependent learning tasks as well. To this end, we trained adult male Sprague-Dawley rats in a spatial reference memory task, a fixed interval task and in a pattern separation task. During a rest period immediately after each training session, we either let neural activity to take place as usual, timed electrical stimulation of the ventral hippocampal commissure (vHC) to immediately follow DSs, or applied the vHC stimulation during a random neural state. We found no effect of vHC stimulation on performance in the spatial reference memory task or in the fixed interval task. Surprisingly, vHC stimulation, especially contingent on DSs, improved performance in the pattern separation task. In conclusion, the behavioral relevance of hippocampal processing and DSs seems to depend on the task at hand. It could be that in an intact brain, offline memory consolidation by default involves associating neural representations of temporally separate but related events. In some cases this might be beneficial for adaptive behavior in the future (associative learning), while in other cases it might not (pattern separation).

New & Noteworthy

The behavioral relevance of dentate spikes seems to depend on the learning task at hand. We suggest that dentate spikes are related to associating neural representations of temporally separate but related events within the dentate gyrus. In some cases this might be beneficial for adaptive behavior in the future (associative learning), while in other cases it might not (pattern separation).

Keywords

hippocampus, dentate spike, learning, memory consolidation

Introduction

Hippocampal electrophysiological activity reflected in the local-field potential (LFP) is characterized by alternating epochs of large-amplitude, irregular activity (LIA) and regular slow activity also called theta (3-12 Hz). Two types of hippocampal events are evident during LIA: Sharp-wave ripples (SPW-Rs) and dentate spikes (DSs). SPW-Rs are ~100-ms bursts of high-frequency (110-200 Hz), high-amplitude oscillations riding on a sharp slow wave, and visible in the CA1 (Buzsaki, 1986; 2015). DSs are short-duration (up to 40 ms), large amplitude (up to 4 mV) spikes visible in LFPs recorded from the hilus of the dentate gyrus (DG) (Bragin et al. 1995; Headley et al. 2017; Penttonen et al. 1997). While SPW-Rs take place during a variety of behavioral states including slow-wave sleep, immobile awake state, grooming, eating and drinking (Buzsaki, 1986), DSs are almost exclusively limited to slow-wave sleep and awake immobility (Bragin et al., 1995). A recent study by Headley and colleagues (2017) in rats found that both SPW-Rs and DSs take place preferentially during neocortical UP-states in slow-wave sleep. Further, DSs occur especially when cortical activity is highly synchronized, indicated by increased power at the delta-band (0.5-4 Hz) (see also Bragin et al., 1995). Headley and colleagues (2017) also report that the probability of a DS is tripled immediately (~50 ms) following hippocampal SPW-Rs. In addition, DSs seem to suppress SPW-Rs, as coincidence of both results in smaller-amplitude SPW-Rs. This is in line with the original report by Bragin et al., (1995) stating that SPW-Rs virtually never took place within 200 ms of a preceding DS. On the other hand, DSs that take place right before an SPW-R are smaller in amplitude (Headley et al., 2017) and thus possibly not very effective in suppressing the SPW-R. Interestingly, eliminating entorhinal input to the hippocampus reduces DSs but increases the occurrence of SPW-Rs (Bragin et al. 1995) suggesting that normally entorhinal input to the hippocampus evokes DSs and suppresses

SPW-Rs. In conclusion, the majority of both SPW-Rs and DSs take place during slow-wave sleep UP-states and their occurrence is bi-directionally related.

Upon closer inspection, during SPW-Rs, synchronous neuronal activation spreads from the pyramidal cells of the CA3 to the pyramidal cells of the CA1 and from there on to principal cells in the subiculum and the neocortex [for a review, see (Buzsaki 2015)]. On the contrary, during DSs, entorhinal cortical activation arrives to the DG via the perforant path and evokes firing of both DG granule cells and interneurons. Coincidently, pyramidal cells in the CA3 and CA1 decrease firing rate for a period of up to 100 ms (Penttonen et al. 1997). That is, whereas SPW-Rs result in increased firing of CA1 pyramidal cells, the effects of DSs are opposite. Considering the relation of SPW-Rs and DSs to neocortical activity, both are associated with an increase in the firing rate of neocortical neurons and an increase in neocortical gamma-band (35-100 Hz) oscillatory power and coherence that peaks roughly 40 ms after the hippocampal event (Headley et al., 2017). However, interregional neocortical coherence at the gamma-band is increased more during DSs compared to SPW-Rs. This increase in gamma-band synchronization across the neocortex is time-locked only to the DSs but not to the SPW-Rs (Headley et al., 2017). To summarize, both the intra-hippocampal and neocortical activity patterns that accompany SPW-Rs and DSs are different.

Little doubt remains about the importance of SPW-Rs in memory consolidation. Namely, it has been repeatedly demonstrated (using different learning paradigms and different SPW-R manipulation methods) that interrupting the normal course of SPW-Rs in both sleep and awake rest impairs learning (Girardeau et al. 2009; for a recent review, please see Girardeau et al. 2017). On the other hand, boosting hippocampo-neocortical communication related to SPW-Rs enhances learning (Maingret et al., 2016; see also Tang & Jadhav, *in press*). On the contrary, the meaning of DSs for behavior is still largely unclear. A recent study from our group suggests DSs might be important for memory consolidation

(Nokia et al. 2017): Disrupting DS-related silencing of hippocampal CA1 pyramidal cell firing consistently after the training session impaired the learning of trace eyeblink conditioning, a hippocampus-dependent pavlovian conditioning task (Kim et al. 1995). Here, to further probe the importance of DSs for neural plasticity and behavior, we trained adult male Sprague-Dawley rats in hippocampus-dependent learning tasks probing spatial reference memory, pattern separation and temporal interval learning. After each training session, during a period of supposed memory consolidation in rest or sleep, we either disrupted the neural processing that normally follows DSs, disrupted neural processing at random or let the neural activity take its normal course. For the disruption, we used electrical stimulation of the ventral hippocampal commissure (Nokia et al. 2017). We expected disruption of DS-related neural processing to impair learning in all three tasks.

Materials and Methods

Ethical Approval

All experimental procedures were approved by the Animal Experiment Board of Finland and implemented in accordance with directive 2010/63/EU of the European Parliament and of the Council on the care and use of animals for research purposes. The study complies with the ARRIVE guidelines (Kilkenny et al. 2010).

Subjects

The subjects were 36 healthy adult male Sprague-Dawley rats (Envigo, Netherlands) weighing ~300 g at surgery. Animals were single-housed on the premises of the animal research unit at the University of Jyväskylä. Food (R36, Lantmännen, Sweden) and tap-water were freely available, and room temperature and humidity were controlled at 21 ± 2°C and 50 ± 10%, respectively. All rats had aspen chips (Tapvei, Kaavi, Finland) and paper towels at the bottom of the cage as bedding and nesting material. Rats were maintained on a 12 h–12 h light–dark cycle, with lights on at 08.00 h. All procedures were conducted during the light portion of the cycle, and the training was done in random order to prevent the possible effects of circadian rhythm on the outcome. A subset of the animals was trained in the 8-arm radial maze task only, and the rest were trained first in the context-object discrimination (COD) task and then in the fixed interval (FI) task. At least a week of rest was given in between the tasks. The timeline for the experiments is presented in Figure 1.

Surgery and recording

Two bundles of 4 recording electrodes were implanted unilaterally to record hippocampal local-field potentials (LFPs) from the hilus (3.6-4.5 mm posterior, 1.5-2.2 mm lateral and 3.6-4.0 mm below bregma) and stimulation electrodes were placed bilaterally on the ventral hippocampal commissure (vHC, 1.3 mm posterior, 1.0 mm lateral and 4.0 mm

below bregma) to stimulate the hippocampus (see Figure 2A and 2B) (Paxinos and Watson 1998). Recording electrodes were made of Formvar insulated nichrome wire (bare diameter 50 µm, #762000, A-M Systems Inc., Carlsboro, WA, USA) glued together using cyanoacrylate with a tip separation of 200 to 250 µm. Bipolar stimulation electrodes were made of Formwar coated stainless steel dual-wire with a diameter of 100 µm and a tip separation of ~500 µm. For a detailed description of the surgery, please see (Nokia et al. 2017).

To acquire neural measures, a wireless headstage [bandwidth 1-5 kHz, W2100-HS8, MultiChannel Systems (MCS), Reutlingen, Germany] was attached to the electrode connector anchored with dental acrylic to the rat's head. The filtered and digitized data was conveyed to a wireless W2100-System (MCS) for storage. A flexible, insulated cable was used to connect the animal to a stimulator (model STG4008, MCS). Movement was detected by an accelerometer (EVAL-ADXL335Z, Analog Devices Inc., Norwood, MA, USA) attached to the cable going from the animal to the stimulator. In the W2100-System, the LFP signals were further low-pass filtered (1^{st} order IIR, cutoff frequency 800 Hz) and all signals were recorded with Experimenter software (MCS) using a 2-kHz sampling rate. Labview (National Instruments Corporation, Austin, TX, USA) was used for online signal analysis and triggering of events.

Behavioral training

Eight-arm radial maze task

Hippocampal SPW-R involvement in spatial learning was demonstrated by Girardeau and colleagues almost a decade ago (Girardeau et al. 2009). They used a reference memory protocol in the 8-arm radial maze task. Specifically, rats were trained to search for reward in three stable locations over the course of several days. Disrupting SPW-Rs by using electrical

stimulation impaired the learning of this task (Girardeau et al. 2009). Here we used a similar protocol to study the involvement of DSs in spatial reference memory. First, the rats received ~10-20 pieces of 45mg sugar pellets (Sandown Scientific, Hampton, Middlesex, UK) to their home cage every day for at least a week, to habituate them to eating them. Then, one day prior to habituation to the maze, the animals were put on a restricted diet of 15 g of feed per day. The rats remained on the restricted diet Sun-Fri throughout the maze task, which was conducted on weekdays for three weeks. Each animal was weighed daily.

Habituation to the maze consisted of an initial 10-minute exploration trial during which all arms were baited with three pellets scattered throughout the arm. Next, each animal was allowed three 3-minute trials of exploration with three pellets in each arm. The intertrial interval was 12 min. The next day, training was started. Four trials per session per animal were conducted, the inter-trial interval again being 12 minutes. During each trial, all arms were open and the same three arms were baited, each with one 45-mg sugar pellet. The animal was placed in the center facing a random direction and let to search for the sugar pellets until it ate all of them or up to three minutes. After each trial, the maze was turned at least 45 degrees and cleaned with 70% ethanol. All sessions were recorded with a standard web cam facing the maze from above. The behavior of the animal was scored on-line, during the session but the videos were stored as back-up. Altogether 15 sessions were conducted adding up to 60 trials.

Context-Object Discrimination (COD) task

Dentate gyrus granule cells are capable of creating sparse (Senzai and Buzsaki 2017), non-overlapping representations of highly similar yet distinct events. This ability to make distinctions between places or events alike is often referred to as pattern separation and is thought to be governed mostly by the DG (Knierim and Neunuebel 2016). COD is a task that relies on efficient pattern separation and the formation of non-overlapping representations of

each context-objects entity. Performance in this task is dependent on an intact hippocampus in rats (Mumby et al. 2002). Thus, we studied the involvement of DSs in pattern separation using a COD protocol modified from Czerniawski and colleagues (Czerniawski et al. 2015). The basic set-up is illustrated in Figure 1.

For COD we used two different arenas in three different rooms and six different pairs of identical objects. One type of arena was a square, 74 cm x 74 cm, with 30-cm-high, plexiglass walls and the other type was round (~74 cm diameter, 30-cm high walls) with walls of matt white metal sheet. To modify the arenas to provide six different variations, the walls of both arenas were decorated with prominent visual cues. The floor was also modified, the material being either plexiglass, black rubber, brown plywood or grey plastic carpet. The distal visual cues and lighting in the rooms were also varied. Note, that no two identical contexts were used for the same animal. The objects used were halogen lamps, metallic saltshakers, and objects made of glass (transparent bowl, dark brown bottle with white, plastic cap, green cube) or stone (gray, round candle holders). The pairs of objects used in each context was pseudorandomized. Objects were placed side-by-side, 20 cm from the wall. After each exposure, the arena and the objects were cleaned with 70% ethanol solution.

As in Czerniawski et al. (2015), on two consecutive days, the rat was placed in contexts A and B and allowed to freely explore the objects and the arena for 5 minutes. Between visits 1 and 2 (Day 1) and 3 and 4 (Day 2), each rat was returned to its home cage for 20 minutes. The daily sessions were conducted with an interval of ~24 hrs. The order of visits to A and B was pseudorandomized. The test session took place on Day 3 and consisted of 5 min in either context A or B (pseudorandomized). This time, one of the objects in the arena was consistent with training (in context) but the other one was one that had previously been presented in the other context (out of context). All sessions were recorded with a standard web cam. The behavior of the animal was scored off-line (see Data analysis).

Fixed interval (FI) task

To evaluate the animal's ability to learn a temporal interval, a fixed interval (FI) task was used. Learning to time responses accurately in a fixed or peak interval task is dependent on the dorsal hippocampus: Animals with lesions tend to respond too early [see for example (Yin and Meck 2014)]. Here we used a protocol in which the rat was trained to poke its nose for a reward every 113 seconds. We wanted to be sure the length of the interval is not divisible and thus picked a prime number. A very long interval (compared to that used in Yin and Meck 2014) was selected because we have plans to record DG single-unit activity during the FI task, and the DG granule cells are known to fire at a very low rate (~0.1 Hz) in awake state (see Senzai & Buzsaki, 2017). Thus, long recordings are needed to catch cells.

FI training took place inside a cylinder (diameter 20 cm, height 40 cm) made of clear acrylic and placed inside a sound insulated cubicle (ENV-018V, Med Associates Inc., Fairfax, VT, USA) dimly lit. A nose poke port (diameter ~2 cm) was on one side of the cylinder, approximately 5 cm from the bottom. On the opposite side, there was another hole for the delivery of the reward pellet onto a black concave acrylic block tray (1.5 cm x 2 cm). Reward was delivered using a standard pellet dispenser (ENV-203-45, Med Associates Inc., Fairfax, VT, USA) connected to the pellet tray with silicone tubing. The experiment was controlled by an Arduino® microcontroller and nosepokes were detected using a reflective optical sensor with transistor output (TCRT5000L, Vishay Intertechnology, Inc., Malvern, PA, USA). More precisely, the infrared LED and the phototransistor were positioned on opposite sides of the opening in the cylinder wall, outside the cylinder, meaning that the animal had to purposely extend its neck to break the light beam for a reward. Only the first nosepoke that started after the target interval had elapsed was rewarded and one 45-mg pellet was delivered at a time.

During the FI task, animals were maintained on a restricted diet of 15 g of food pellets per day starting from the day prior to the first FI session. The rats were not habituated to the task to keep them completely naïve prior to training. Our pilot studies did not show marked effects of pre-training using a shorter, 17-s interval. In line with this, the rats were performing the maximum number of trials from the 3rd session onwards. Each session lasted 1 h allowing for ~30 trials. Altogether 10 sessions were conducted, one per day. Nosepokes and pellet deliveries were recorded as TTL pulses using the Analog inputs of the W2100 system. Performance was analyzed offline using Matlab (see Data analysis).

Hippocampal stimulation via vHC

After each training session the animals were let to rest in plastic cages placed inside sound insulated cubicles (ENV-018V, Med Associates Inc.). This procedure was carried out for 2 h following each training session in the 8-arm maze task and the COD-task, and for 1 h following the FI task. Note that after the FI task, the animals remained in the same training cylinder but bright lights were switched on to signal the end of the FI-session.

In the experimental condition (EXP), the hippocampus was stimulated via bilateral stimulation electrodes, immediately following the peak of the DS (see also Nokia et al., 2017). In the yoked control (YC) condition, stimulations were delivered during immobility and at a similar rate as for the EXP animal, but irrespective of neural state. To deliver stimulation based on the DSs, signal from a recording electrode showing clear DSs and a large (> 1 mV) positive response to the vHC stimulation was conveyed to Labview. DSs were detected based on a fast rise in signal amplitude and a simple peak amplitude threshold. It was also required that the animal was still, i.e. that the accelerometer signal showed minimal activity. When the conditions for a DS were met, the Labview produced a TTL pulse. This triggered the delivery of a bipolar electrical pulse (STG4008, MCS) to the vHC of the EXP animal (~5-20 ms variable delay). Stimulation duration and amplitude (0.2 to 0.24 ms, max.

160 µA) were adjusted for each animal so that the amplitude of the hippocampal response in the electrode showing spontaneous DSs was 1 to 1.5 mV in amplitude.

In the normal control (NC) condition, no hippocampal stimulation was given. Note that stimulation was omitted also in the EXP and YC groups during recordings performed after the 8^{th} (middle) and 15^{th} (last) 8-arm maze training session and after the 10^{th} (last) FI task training session. Data from these recordings were used to study possible link between DS occurrence rate and learning.

Histology

Rats were euthanized by exposure to a rising concentration of CO_2, and death was verified by rapid decapitation. The locations of the electrode tips in the brain were marked by passing a DC anodal current (200 mA, 5 s) through them. The brain was then removed and stored in 4% paraformaldehyde solution (pH 7.4) for at least 48 h at +4 °C. After that, the brain was kept in phosphate buffered saline solution in +4 °C until sectioning. The brain was coronally sectioned with a vibratome (Leica VT1000) into 40-µm slices. The slices were attached to slides, dried, and stained with Prussian blue and Cresyl violet. The electrode tip locations were determined with the help of a conventional light microscope and a brain atlas (Paxinos and Watson 1998).

Data analysis

Behavior

Eight-arm radial maze task

During maze training, behavior was scored on-line and verified later from video, if needed. The animal was considered to have entered an arm when the whole body (excluding tail) was inside an arm. Entries to empty arms were considered errors, as were entries to a baited arm and not eating the sugar pellet, and re-entries to a baited, already emptied arm.

Correct response (maximum 3) was scored when the animal entered a baited arm and ate the pellet. A performance index was calculated as in Girardeau et al. (2009) based on the number of errors and correct responses as follows: Performance index = $2 / \pi * \arcsin(\sqrt{(\text{correct} / (\text{correct} + \text{errors}))})$. Perfect score is 1 (one) and indicates no errors and one (and only one) visit to each baited arm while eating all the available pellets.

COD task

For the context-object -discrimination task, behavior was evaluated off-line from the recorded videos by an experimenter blind to animal identity. Exploration was defined as pointing the nose towards the object within < 2 cm distance or otherwise physically interacting with the object (i.e. leaning on it, climbing over it etc.). The time spent exploring each object during the first minute of the test session was scored in seconds. Then the percentage of time spent exploring the out-of-context object was calculated relative to total time spent exploring either object.

FI task

To evaluate timing performance in the FI task, data was analyzed off-line using MATLAB (MathWorks Inc., Natick, MA, USA). First, the probability of a nose poke was determined for the first half of the fixed interval (false response) and for the period exceeding 90% of the fixed interval (correct). Then the ratio between the correct response probability and the summed probability of both false and correct responses was calculated (0 = only false responses, 1 = only correct responses). A value of 0.7 was set as a criterion for learning.

Dentate spikes

Dentate spikes were detected off-line using a custom-made script in MATLAB. Data was analyzed from recordings during which no vHC stimulation was applied. Only periods of immobility were included in analysis, when accelerometer signal was available. The mean and standard deviation (SD) of the hilar LFP signal during immobility were derived. A

threshold was set at mean + x*SD. The factor x varied between 3 and 4, depending on electrode location, i.e. how large were the DSs in relation to background activity. Then, positive deflections in LFP amplitude that exceeded the threshold were detected using a 20 ms window split in half: Both the maximum LFP amplitude during the latter half (peak) and the difference between the maximum LFP amplitudes during the former and latter halves (rise) had to exceed the threshold to qualify the event as a DS. Accuracy of DS detection was verified by qualitative analysis of detected events by a trained human eye.

Statistics

IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY, USA) was used for statistics. Sigmaplot (Systat Software Inc., San Jose, CA, USA) was used for data visualization. Analysis of variance for repeated measures (ANOVA-RM) was used to analyze changes across training and differences between conditions. Whenever a significant interaction emerged, separate repeated measures ANOVAs were conducted for each group. Paired samples t-test was used for comparing two related measures. Greenhouse–Geisser corrected p-values are reported when the sphericity assumption was violated according to Mauchly's test. Bonferroni-corrected p-values were used for post-hoc comparisons when appropriate.

Results

Recording electrodes were placed in the hilus and stimulation electrodes in the vHC

In all animals assigned to the YC or the EXP conditions, stimulation electrodes were verified to be located in the vHC and recording electrodes in the hilus (Figure 2, panels A and B). In animals only used as normal controls, the electrodes were either misplaced in different hippocampal cell layers or the headstage was damaged in some way. Representative examples of dentate spike and vHC stimulation responses in the hilus are illustrated in Figure 2, panel C.

Stimulation of the vHC had no effect on spatial reference memory

Fourteen animals successfully completed the 8-arm radial maze -task and were included in the analyses. There were six animals in the normal control group (NC), four animals in the yoked control group (YC) and four animals in the experimental group (EXP). No significant weight loss was detected due to the restrained diet (data not shown). For a graphical presentation of the results, please see Figure 3 panels A (behavior) and D (DSs and vHC stimulations).

To study the rate of occurrence of DSs at baseline and during spatial learning we analyzed data from 7 of the 8 animals with hilar recording electrodes (EXP and YC). One animal in the YC group was excluded as the dentate spikes were lower in amplitude than in the others (i.e. recording electrode was not optimally placed). The number of DSs per immobile minute was 4.7 ± 1.1 (mean ± standard error of mean) at baseline, 3.6 ± 1.0 in the middle of training (8^{th} session) and 3.2 ± 0.4 in the end of training (15^{th} session). On average 4.5 ± 0.3 vHC stimulations per minute were delivered to the YC and the EXP animals during the 2-hr recordings conducted after training sessions 1-7 and 9-14. ANOVA-RM showed no change in the rate of stimulation between the first and the 14^{th} session ($n = 8$, $F [1, 7] = 0.52$,

p = 0.496). That is, DS-contingent vHC stimulation rates were comparable or even slightly higher than that expected based on the rate of DSs detected off-line.

To examine behavioral performance in the 8-arm radial maze, the performance index from three consecutive sessions was averaged into five blocks. ANOVA-RM revealed a significant main effect of block ($F [4, 44] = 51.82$, $p < 0.001$), no interaction of block and group ($F [8, 44] = 0.80$, $p = 0.607$) and no main effect of group ($F [2, 11] = 0.14$, $p = 0.875$). Statistical analysis was also performed on the number of errors made and the time (s) taken to retrieve all three pellets. The results were the same, i.e. robust learning and no difference between groups (data not shown). That is, animals in all groups learned the 8-arm maze task equally well. In the end of training, the fastest animals were able to retrieve all three reward pellets in less than 10 seconds.

Stimulation of the vHC contingent on dentate spikes had a positive effect on pattern separation

Nine animals were trained in COD using a within-subjects design and were included in the analysis. Each animal was trained and tested three times, once without stimulation (normal control, NC), once with DS -contingent stimulation (experimental, EXP) and once with random stimulation (yoked control, YC). The order in which the animals were assigned to NC, YC and EXP was randomized, and there was at least one week between repetitions. For a graphical presentation of the results, please see Figure 3 panels B (behavior) and E (DSs and vHC stimulations).

First, we analyzed the occurrence of spontaneous DSs and vHC stimulations. In the NC condition, the number of DSs per minute was 2.2 ± 0.3 after the first training session and 2.8 ± 0.3 after the second training session. Note that during these recordings no movement data was acquired so detection was based on LFP quality only. This means that the rate per minute is an underestimation compared to all other conditions where time periods with movement

have been excluded. On average 7.2 ± 0.8 and 8.4 ± 1.1 vHC stimulations per minute were delivered to the animals in the YC and the EXP conditions while immobile during the 2-hr recordings conducted after the two training sessions, respectively. ANOVA-RM revealed no effect of training day on either the rate of DSs or the rate of vHC stimulations. That is, DS-contingent vHC stimulation rates were again higher than that expected based on the rate of DSs detected off-line (even if taking into account the underestimation due to inclusion of awake movement periods).

Analysis of behavioral performance in the COD task indicated that even though animals seemed to perform best during the first exposure to the COD task, this was not a statistically significant effect (ANOVA-RM, three rounds of COD: $F [2, 16] = 2.99$, $p = 0.079$). However, ANOVA-RM indicated a clear effect of experimental condition (NC – YC – EXP: $F [2, 16] = 3.80$, $p = 0.045$). Paired comparisons (t-test) further revealed that performance on the COD task was better after DS -contingent vHC stimulation compared to no stimulation (NC vs. EXP: $t (8) = 2.66$, $p = 0.029$; EXP vs. YC: $t (8) = 0.76$, $p = 0.467$; NC vs. YC: $t (8) = 1.84$, $p = 0.104$) (see Figure 3, panel B). In fact, rats only performed above chance level (50 %) after the experimental manipulation (one sample t-test, EXP: $t (8) = 3.03$, $p = 0.016$; NC: $t (8) = 0.31$, $p = 0.765$; YC: $t (8) = 1.85$, $p = 0.101$). To summarize, contrary to expectation, DS-contingent vHC stimulation during the memory consolidation period improved pattern separation performance on the context-object –discrimination task.

Stimulation of the vHC had no effect on temporal interval learning

Eleven animals completed the FI task. Five of these animals were assigned to the EXP group, four to the NC group and two to the YC group. For a graphical presentation of the results, please see Figure 3 panels C (behavior) and F (vHC stimulations and DSs). All animals lost some weight due to the restricted diet (start vs. end of training, ANOVA-RM: interaction of group and time: $F [2, 8] = 0.84$, $p = 0.47$; main effect of time: $F [1, 8] = 17.98$,

p = 0.003; main effect of group: F [2, 8] = 0.13, p = 0.880). Animals weighed 461 ± 7 g (mean ± standard error of mean) in the beginning and 448 ± 6 g in the end of FI training. Thus, the weight loss was on average 2.85 ± 0.57 %.

During the 1-hr recordings conducted after the first nine training sessions, 6.1 ± 0.6 vHC stimulations per minute were delivered to the YC and the EXP animals. ANOVA-RM showed no change in the rate of stimulation across training (first vs. 9^{th} session, n = 7, F [1, 6] = 0.32, p = 0.593). To study the rate of occurrence of DSs, we analyzed data from five animals from the last (10^{th}) recording during which no vHC stimulation was performed. The number of off-line detected DSs was 3.0 ± 0.4 per immobile minute. In sum, DS-contingent vHC stimulation rates were again higher than that expected based on the rate of DSs detected off-line. To study learning, FI performance index values from two consecutive sessions were averaged to produce five blocks (see Figure 3 panel C). ANOVA-RM indicated equal learning in all groups (interaction of block and group: F [8, 32] = 0.59, p = 0.780; main effect of block: F [4, 32] = 48.95, p < 0.001; main effect of group: F [2, 8] = 0.07, p = 0.932). That is, hippocampal stimulation via the vHC during a period supposed to support memory consolidation had no effect on learning of a simple temporal interval task.

Discussion

Our aim was to study whether DSs are relevant for memory formation in three different hippocampus-dependent learning tasks. To this end, we trained animals in a spatial reference memory task, a temporal fixed interval task and in a pattern separation task. Immediately after training, we interfered with the normal course of DS-related neural processing (Bragin et al. 1995; Headley et al. 2017; Penttonen et al. 1997) by stimulating the hippocampus using a weak electrical pulse to the vHC. This interference had an effect only on performance in the pattern separation task. Contrary to our assumptions, our manipulation improved performance compared to a non-stimulated normal control condition. Thus, while DS-related neocortico-hippocampal processing that takes place during rest and sleep after the initial experience seems crucial for associative learning (Nokia et al. 2017), disrupting it actually might sometimes be beneficial. This may happen in situations that require the preservation of separate representations of similar events.

It is somewhat surprising that hippocampal stimulation either contingent on DSs or irrespective of neural state had no effect on learning the location of the reward pellets in the eight-arm radial maze, a task measuring spatial reference memory. A previous study shows that performance in this task is dependent on the normal occurrence of hippocampal SPW-Rs (Girardeau et al. 2009). As in our current experiment, Girardeau et al. (2009) also used electrical stimulation of the hippocampus via the vHC as the method to interfere with SPW-Rs. Thus, the difference in results between our current study and that of Girardeau and colleagues (2009) should not be due to methodological differences. The vHC stimulation method and rate, as well as the rate of DS occurrence, in our present experiments are also comparable to those in our earlier study (Nokia et al. 2017), in which we *did* find a clear effect of disrupting DS-related processing on learning. If anything, in our current studies, the on-line criteria for stimulation of the vHC contingent on DSs seemed to be somewhat looser

than the off-line criteria for detecting DSs. That is, stimulations were delivered upon detection of all notable DSs in the experimental condition. Based on these observations and our current results, it seems that for simple place learning or spatial reference memory, SPW-Rs but not DSs are needed. Further studies could probe the effects of interfering with DSs during a certain phase of the training. However, it is unlikely that effects would be seen then, either.

We also found no effect of vHC stimulation on learning the fixed interval task. Earlier studies show that accurate performance in tasks requiring evaluation of temporal duration is dependent on an intact hippocampus: Animals with hippocampal lesions tend to seek for reward earlier or more often than normal animals (Meck et al. 2013; Yin and Meck 2014; Bannerman et al. 1999). However, in previous studies, the intervals used have been much shorter (from 2 to 45 s) compared to what we used here (113 s). Although most definitely hippocampus-dependent, it might be that our paradigm was not sensitive enough to show differences between stimulated animals and normal controls. In further studies, it might be a good idea to use a shorter interval for FI training. Nevertheless, our finding is in line with the fact that direct stimulation of the CA1 pyramidal cell layer has no impact on learning or performance of an operant conditioning task (Jurado-Parras, Gruart and Delgado-García 2012). To our knowledge, apart from trace eyeblink conditioning (Nokia et al. 2012), the role of SPW-Rs in temporal interval learning or timing ability has not been studied. Thus, further research probing the role of different hippocampal oscillations in fixed interval learning using variable interval lengths could be feasible. Regardless, our results clearly suggest that disrupting the normal course of DS-related neocortico-hippocampal neural processing does not dramatically impair the ability to acquire an appetitive operant conditioning task and to learn the duration of a temporal interval.

Last, we did observe a clear effect of vHC stimulation on performance on a context-object –discrimination task that measures a phenomenon termed pattern separation. Our assumption was that after the experience, offline memory consolidation would involve mostly the strengthening of an association between objects and the context in which they were encountered. Much to our surprise, animals in which hippocampal stimulation was targeted to DSs showed better recognition for the out-of-context object in the testing situation, that is, improved pattern separation. Thus, contrary to our assumption, it seems that normal offline memory consolidation involves either formation of "false" associations between entities that are similar but not related or the degradation of accurate associations. As vHC stimulation improved pattern separation especially when targeted at DSs, we suggest that some of this processing takes place during or right after DSs. This proposed process might be especially important during tasks that require either the formation of associations between temporally separate events (Nokia et al. 2017) or retaining separate representations of similar events such as during pattern separation – a function often assigned to the DG (Knierim and Neunuebel 2016).

Some aspects of our results merit further discussion. First, of all the hippocampus-dependent learning tasks that we have probed, the effects of DS-contingent vHC stimulation were limited to trace eyeblink conditioning (Nokia et al. 2017) and COD (study at hand). It is not very clear what makes both of these tasks so special from the viewpoint of the hippocampus let alone the DG. However, as discussed above, performance on both tasks relies on accurate formation of associations. Related, saturation of the CA3-CA1 synapse by inducing long-term potentiation impairs learning of trace eyeblink conditioning (Gruart, Muñoz and Delgado-García 2006). In addition, selective inactivation of DG granule cells has a remarkably deleterious effect on acquisition and performance of the learned response during trace conditioning but only if the inactivation overlaps with conditioning (Madronal et

al. 2016). Madronal and colleagues (2016) propose that the entorhinal input via the perforant path to the DG potentiates the connection between the CA3 and the CA1, while the direct input from EC to CA1 depotentiates this connection. According to them, the role of this depotentiation from the EC via the PP to the CA1 would be to weaken old associations to clear space for new learning. This is at least partially in line with an idea that DSs, together with SPW-Rs, might be crucial for learning due to their possible role in maintaining the excitation/inhibition balance in the hippocampo-neocortical circuit. This notion is related to the more general idea of homeostasis as the necessary basis for adaptive synaptic plasticity (Tononi and Cirelli 2016).

Second, for the pattern separation task, there was also a modest but not statistically significant positive effect of random stimulation of the hippocampus. This is not surprising taken that non-specific electrical interference with hippocampal function during the memory consolidation period has profound effects on long-term memory (Kesner and Wilburn 1974). These findings underline the obvious fact that hippocampal processing crucial for memory consolidation also takes place outside DSs. Third, for the pattern separation task, we used a within-subjects design as opposed to a between-subjects design. This might have made it possible to detect differences between conditions more reliably than in the FI and maze tasks in which the effects might have been partially masked by inter-subject variability.

In further studies, it might be interesting to more closely investigate hippocampal neural activation during a pattern separation task and the following rest period. Specifically, it would be interesting to see if different DG granule cells (and possibly mossy cells) fire in the different contexts, and whether these two neural assemblies are then co-activated during DSs that are observed at rest following the behavioral exposure. It is possible that DSs reflect the reactivation of a large population of recently activated DG cells belonging to separate cell assemblies, and that this is the mechanism by which associations between neural

515 representations are modified off-line to produce associations between events that in fact did
516 not take place in the exact same moment.
517 In conclusion, in some cases DSs seem beneficial for accurate memory formation
518 [associative learning, (Nokia et al. 2017)] while in other cases (pattern separation) the
519 performance is actually enhanced if the DS-related processing is disrupted.

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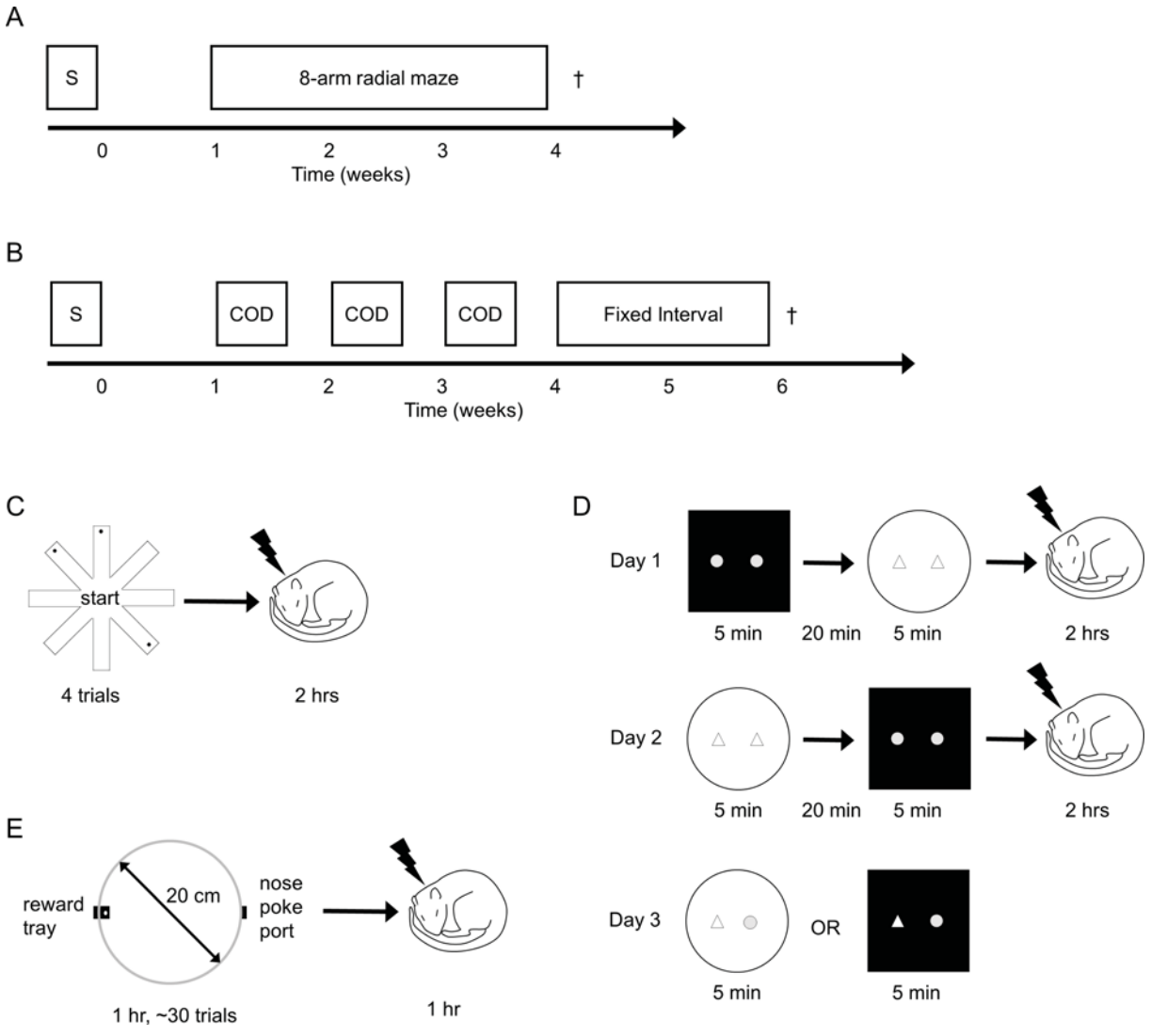
Figure Captions

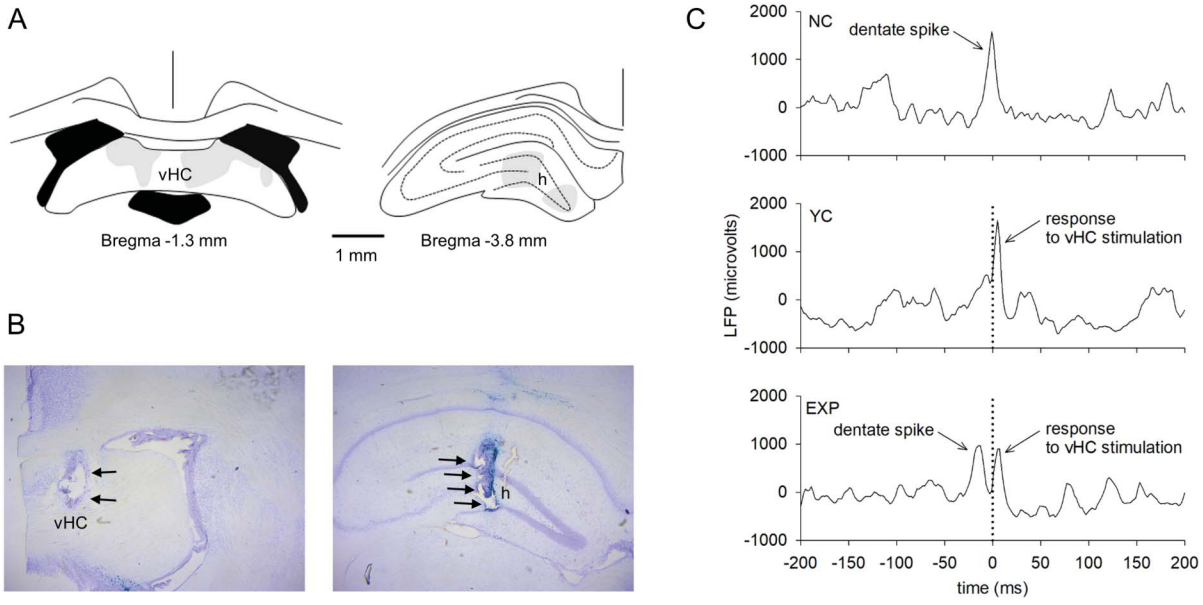
Figure 1. Experimental protocol. A) One group of animals was used for examining the role of DSs in spatial reference memory using the 8-arm radial maze task. Surgery (S) to implant electrodes was conducted first, followed by a week of recovery and then training in the maze. B) Another group of animals was used for studying the role of DSs in pattern separation (context-object discrimination, COD) and in temporal interval learning (fixed interval task). C) In the 8-arm radial maze task, the same three reward locations were always used. Animals were trained for 15 days, 4 trials per day. vHC stimulation was applied during a 2-hr rest period after each session (excluding sessions 8 and 15). More specifically, during this supposed memory consolidation period, local-field potentials from the hilus were monitored and recorded and vHC stimulation was either withheld (normal control condition), administered at random (yoked control condition) or administered when DSs were detected (experimental condition). D) During COD training, animals were exposed to two contexts, each containing a pair of identical objects, for 5 min each, in random order. A 20-minute break was held in between exposures, for which the animals were placed in home cage. After the latter exposure, vHC stimulation was conducted for 2 hours, as for the maze task. Training was conducted on two consecutive days. On the third day, animals were placed in either of the two contexts but now containing two different objects, one from each context. The animal was assumed to indicate memory of the context-object entities by preferentially exploring the out-of-context –object. Note that COD training was conducted thrice so that each animal experienced the normal control condition, experimental condition and a yoked control condition. E) For the fixed interval task, animals were placed in the chamber for training for 1 hour, during which they received a reward when they poked the nose port when at least 113 s had elapsed since the last reward. Again, vHC stimulation was carried out after the training session, but this time for only 1 hour.

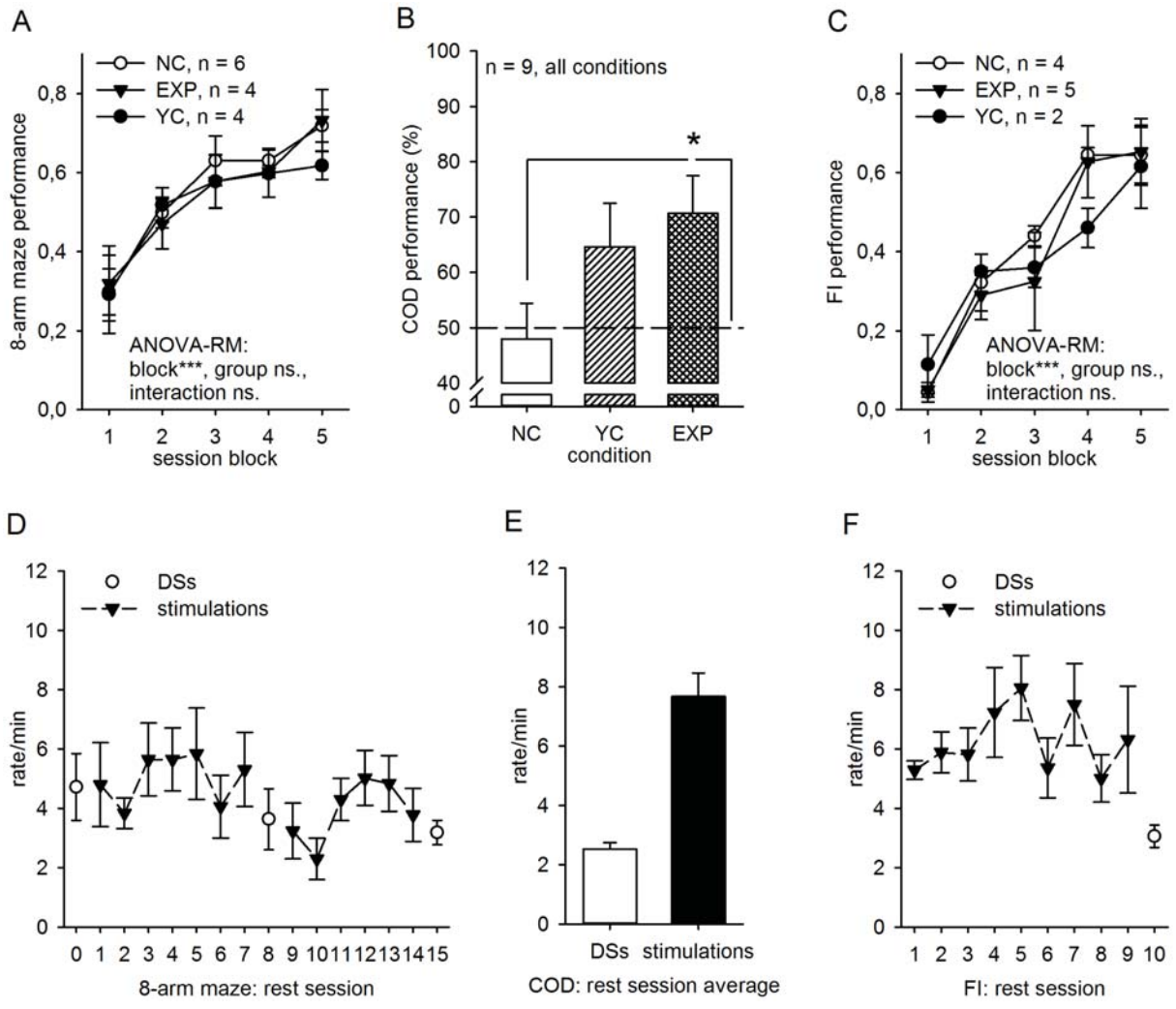
Figure 2. Recording electrodes were placed in the hilus (h) to detect dentate spikes and stimulation electrodes in the ventral hippocampal commissure (vHC) to stimulate the hippocampus. Electrode placement was verified from histological samples. The coordinates for implantation were based on the atlas of Paxinos and Watson (1998). The stimulation electrodes were aimed at 1.3 mm posterior to, 1.0 mm lateral to and 4.0 mm below bregma whereas the recording electrodes were aimed at 3.6 to 4.5 mm posterior to, 1.5 to 2.2. mm lateral to and 3.6 to 4.0 mm below bregma. A) Light grey shading indicates the range of locations for stimulation electrodes in the vHC (left panel) and recording electrodes in the hippocampus, aimed at the hilus (h) (right panel). B) Representative examples of stimulation electrode placement in the right vHC (left) and of a recording electrode in the hippocampus (right). Arrows point to electrode tips. Hilus is indicated by the letter "h". C) Local-field potentials from the hilus of one rat illustrating a representative dentate spike recorded after training in the context-object discrimination task in the normal control (NC) condition (top), a response to vHC stimulation recorded in the yoked control (YC) condition (middle) and a dentate spikes followed by a response to vHC stimulation recorded in the experimental (EXP) condition (bottom).

Figure 3. Stimulation of the hippocampus, especially contingent on dentate spikes improved pattern separation performance in a context-object discrimination task (COD). A) Stimulation of the hippocampus via the vHC either contingent on dentate spikes (experimental group, EXP) or randomly, during immobility (yoked control, YC) after each conditioning session had no effect on learning a hippocampus-dependent spatial reference memory task in the 8-arm radial maze. Animals learned the fixed locations of the rewards equally well as those in the normal control (NC) group. Note that for statistical analysis

(repeated measures ANOVA, ANOVA-RM), performance indexes from three consecutive sessions (days) were averaged to produce five blocks. Vertical lines indicate standard error of mean. Asterisks refer to statistical significance ($p < 0.001$). B) Pattern separation in the COD task was improved by hippocampal stimulation during the memory consolidation period, especially when the stimulation was triggered by dentate spikes. Note that for this experiment, the same nine animals were trained three times, once in each condition. The animals indicated memory for the objects in context by preferring to explore the out-of-context object only when the hippocampal stimulation had been delivered contingent on dentate spikes (EXP). This is indicated by the statistically significant difference (one sample t-test, $p < 0.05$) from chance (dashed horizontal line at 50 %). There was also a statistically significant difference between performance in the non-stimulated control condition (NC = normal control) and that in the EXP condition (paired sample t-test, $p < 0.05$). C) Stimulation of the hippocampus via the vHC during the memory consolidation period had no effect on learning a fixed interval (FI) task. Animals received a reward when they poked their nose to a hole in the wall of the chamber when at least 113 s had elapsed since the last reward delivery. D-F) Rate per minute for DSs and vHC stimulations in each task.


A
S
8-arm radial maze
†
0
1
2
3
4
Time (weeks)
B
S
COD
COD
COD
Fixed Interval
†
0
1
2
3
4
5
6
Time (weeks)
C
start
4 trials
2 hrs
D
Day 1
5 min
20 min
5 min
2 hrs
Day 2
5 min
20 min
5 min
2 hrs
Day 3
OR
5 min
5 min
E
reward tray
20 cm
nose poke port
1 hr, ~30 trials
1 hr


A
vHC
h
Bregma -1.3 mm
1 mm
Bregma -3.8 mm
B
vHC
h
C
NC
dentate spike
YC
response
to vHC stimulation
EXP
dentate spike
response
to vHC stimulation
LFP (microvolts)
2000
1000
0
-1000
-200
-150
-100
-50
0
50
100
150
200
time (ms)


A
NC, n = 6
EXP, n = 4
YC, n = 4
8-arm maze performance
ANOVA-RM:
block***, group ns.,
interaction ns.
session block
B
n = 9, all conditions
COD performance (%)
*
NC
YC
EXP
condition
C
NC, n = 4
EXP, n = 5
YC, n = 2
FI performance
ANOVA-RM:
block***, group ns.,
interaction ns.
session block
D
DSs
stimulations
rate/min
8-arm maze: rest session
E
rate/min
DSs
stimulations
COD: rest session average
F
DSs
stimulations
rate/min
FI: rest session