Mesopontine median raphe regulates hippocampal ripple oscillation and memory consolidation

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Sharp wave–associated field oscillations (~200 Hz) of the hippocampus, referred to as ripples, are believed to be important for consolidation of explicit memory. Little is known about how ripples are regulated by other brain regions. We found that the median raphe region (MnR) is important for regulating hippocampal ripple activity and memory consolidation. We performed in vivo simultaneous recording in the MnR and hippocampus of mice and found that, when a group of MnR neurons was active, ripples were absent. Consistently, optogenetic stimulation of MnR neurons suppressed ripple activity and inhibition of these neurons increased ripple activity. Notably, using a fear conditioning procedure, we found that photostimulation of MnR neurons interfered with memory consolidation. Our results demonstrate a critical role of the MnR in regulating ripples and memory consolidation.

Memory consolidation, a process that transforms newly acquired information into long-term memory, depends critically on the hippocampus. The hippocampus generates ripple activity, a sharp wave–associated fast field oscillation (100–300 Hz), which is predominantly detected in the CA1 region. Converging evidence suggests that ripple activity reflects the memory consolidation process. Previous studies have shown that hippocampus place cell firing during locomotor navigation is subsequently 'replayed' in the same sequence, but in a temporary compressed manner, and the replay coincides with ripples. Moreover, selective disruption of ripple activities after learning trials of a task results in poor performance of the task, an effect that is likely caused by disrupted memory consolidation. Ripple activity frequently occurs during inattentive states, including slow-wave sleep, awake immobility and feeding. However, little is known about how ripple activity is regulated.

A recent study reported that hippocampal ripple activity is accompanied by inhibition of subcortical regions. This observation raises the question of whether subcortical regions have a role in regulating ripple activity. The MnR, localized along the midline of the ventral mesopontine area, projects extensively to the forebrain and has been implicated in regulation of state. Notably, MnR neurons project to the entire hippocampal formation and participate in regulation of hippocampal theta activity and thereby possible regulation of learning and memory. We found that activation of MnR neurons suppressed hippocampal ripple activity and disrupted memory consolidation.

RESULTS

MnR neurons display hippocampal ripple-correlated activity

To investigate whether and how the MnR is involved in regulating hippocampal neural activity, we implanted a bundle of eight tetrodes in the MnR and another four tetrodes in the hippocampal CA1 region of C57BL/6j mice (Fig. 1a). The tetrodes were coupled with miniature micro-drives to achieve precise placement in the targeted CA1 pyramidal layer in the hippocampus (Supplementary Fig. 1a–c). In addition, this setup allowed us to record neural activity at several different depths in the MnR by driving the tetrodes down (~80-µm advancement per step), resulting in a higher yield in the total number of recorded MnR neurons (Online Methods). We simultaneously recorded local field potentials (LFPs) and single-neuron activity in the hippocampal CA1 and MnR in freely behaving mice (that is, without restrain or anesthesia) in their home cages, and analyzed neural data recorded when the mice were in a quiet immobile state that likely corresponded to slow-wave sleep (referred to as immobile/sleep; Online Methods and Supplementary Fig. 1d,e), unless otherwise noted. Hippocampal ripple activity was band-pass filtered at 150–250 Hz and ripple events were identified with peak amplitude exceeding 6 s.d. of baseline activity (Fig. 1b and Online Methods).

We recorded a total of 191 MnR neurons from 6 mice (see Supplementary Fig. 2a,b for electrodes placements) and first identified serotonergic and non-serotonergic neurons. About 15% (29/191; maintained firing rate = 1.34 ± 0.97 Hz, mean ± s.d.) were classified as putative serotonergic neurons according to established criteria (Supplementary Fig. 2c–f and Online Methods). Consistent with previous studies, these classified serotonin neurons displayed a slow, steady firing rate (that is, long inter-spike intervals), which was suppressed by administration of a serotonin 1A receptor agonist (Supplementary Fig. 2e). We then examined the timing of putative serotonin neuron firing relative to hippocampal ripple activity, and found that the majority (22 of 29) of neurons showed a small, but significant, decrease in activity coinciding with ripple events (peri-event histogram trough z score < −3.28 and P < 0.001, Wilcoxon signed-rank test; Fig. 1c,d).

In addition, we classified non-serotonergic neurons with respect to their activity and hippocampal ripples. The majority of non-serotonergic MnR neurons (59%, 96 of 162) displayed significant...
Figure 1 MnR neurons display hippocampal ripple correlated activity. (a) Schematic drawing of the simultaneous recording sites in the hippocampal CA1 and MnR (left) and representative coronal sections showing recording sites in the CA1 and MnR (middle and right, respectively). Dotted outline indicates boundary. Scale bars represent 0.5 mm. (b) Representative LFP and filtered ripple events recorded in the CA1 (left), and rasters and mean amplitude of 1,000 ripple events (right). Color bar represents voltage amplitude. (c) Representative activity of putative 5-HT (serotonin), type I and type II neurons plotted in relation to the ripple peak (time 0). The three neurons were recorded simultaneously. (d) Summary of all of the classified MnR neurons (recorded from six mice) identified as either putative 5-HT (15%, 29 of 191, left), type I (21%, 41 of 191, middle) or type II (29%, 55 of 191, right). Color bars represent z-scored neural firing frequency.

Ripple-coordinated activity changes (peri-event histogram z score absolute value > 3.28 and P < 0.001, Wilcoxon signed-rank test; Fig. 1c,d), and these neurons were further divided into two types. Type I neurons (43%, 41 of 96; 2.4 ± 1.7 Hz, mean ± s.d.) displayed a prolonged decrease in activity (80% of baseline or lower) that lasted 1 s or longer before ripple events (Fig. 1c,d and Supplementary Fig. 2g). Immediately after ripple events, about half of them transiently restored their firing rates to baseline or higher. On the other hand, type II neurons (57%, 55 of 96; 14.2 ± 12.3 Hz) displayed a diversity of robust transient activity (inhibitory, excitatory or both) that coincided with ripple events, but showed little change in firing frequency before ripple events (Fig. 1c,d and Supplementary Fig. 2g); thus, they appeared to be a heterogeneous collection of neurons. As noted above, ripple activity was present during feeding. The ripple-correlated type I neural activity during feeding differed from that of the immobile/sleep state (Supplementary Fig. 3b). During feeding, type I neurons were inhibited less before ripple event and excited more immediately after ripple event.

Rates and synchrony of type I neuron firing during ripple events

Our observation that type I neurons were silent just before the occurrence of ripple events (Fig. 1c,d) raises the question of whether suppression of type I neuron activity is a necessary condition for hippocampal ripples to occur. In other words, is activation of these neurons likely to suppress ripple events? To examine this possibility, we analyzed the raw data of spike trains of type I neurons with respect to ripple events. The absence of ripple activity was usually accompanied by increased firing rates of type I neurons (Fig. 2a). Because inter-spike intervals (ISIs) varied extensively among type I neurons (Supplementary Fig. 4a,b), we first examined ripple activity when type I neuron firing rates increased twofold above their mean firing rates (high instantaneous rate; Fig. 2b and Supplementary Fig. 4c). We found that ripple activity was largely suppressed when firing rates of type I neurons increased twofold or greater (Fig. 2c). The temporal relationship between type I neuron activity and ripple events was also verified by additional auto-correlation and cross-correlation analyses (Fig. 2d and Supplementary Fig. 4d,e). Notably, type I neurons fired immediately after a transient increase of ripple activity (Fig. 2c,d), raising the possibility that the post-ripple firing of type I neurons may be recruited for immediate suppression of subsequent ripple activity. In addition, we created a smoothed firing rate estimate for each type I neuron and a smoothed rate estimate for ripple events. Type I neuron firing rates were averaged every 500-ms epoch and then scatter-plotted with the corresponding ripple rates (Fig. 2e). We found that hippocampal ripple activity was strongly suppressed for a few seconds after fast firing of the type I neuron (Fig. 2f).

In addition, MnR type I neurons fired in synchrony during the immobile/sleep state (Fig. 3a), and a cross-correlation analysis confirmed strong synchronous firings among all of the simultaneously recorded type I neurons (cross-correlation histogram peak z score > 3.28 and P < 0.001, Wilcoxon signed-rank test; Fig. 3b,c). The averaged cross-correlation histogram revealed that most of the synchronized firings were within a time window of less than 200 ms (Fig. 3d). To determine how the co-firing in different size windows relates to ripple events, we divided the spikes of each type I neuron into five sets on the basis of the co-firing interval (that is, the interval of the closest co-fired spikes; Fig. 3e), with a smaller co-firing interval indicating a higher synchrony. We found that the more synchronized the firing between type I neurons, the larger the suppression of the ripple activity (Fig. 3e,f). These results suggest that MnR type I neurons strongly suppress hippocampal ripple activity by coordinating firing among themselves. In addition, we found that the MnR serotonergic neurons...
Figure 2: Ripple event frequency in relation to type I neuron firing-frequency. (a) Representative filtered ripple activity, identified ripple events and spikes of a type I MnR neuron. Asterisks indicate high instant activity of the type I neuron. (b) Instant firing rate of the type I neuron shown in a. For this neuron, firing rates of 80 Hz or greater are classified as high instant rate of firing. (c) Mean ripple-event frequency (solid line) and s.e.m. (dashed) referenced to the high instant rate firing of 32 type I neurons. Top inset, a finer time resolution display. (d) Mean cross-correlation histogram (solid line) and s.e.m. (dashed) between type I neurons (n = 32) and ripple events. Top inset, a finer time resolution display. (e) An example of the density scatter plot of ripple event frequency versus a type I neuron firing frequency. Type I neuron and ripple event frequencies were first smoothed with a Gaussian filter (filter width, 250 ms). Type I neuron firing frequency was averaged every 500-ms epoch (represented as a dot, and ripple event frequency was calculated between 0 and 1 s immediately after each 500-ms epoch of the type I neuron). (f, g) Mean ripple event frequency as a function of type I neuron firing frequency; shown are an example of a representative neuron (f) and the mean ± s.e.m. of 32 neurons (g). As with the 0–1 s block (e), ripple event frequency was calculated after each 500-ms epoch of the type I neuron for 1–2, 2–3 and 3–4 s. Data used in e and f are from the same set; the last ten highest frequencies of each type I neuron were combined with nearby windows in f and g. Type I neurons with mean firing frequency above 1 Hz were used for analyses.

Also synchronized with the type I neuron, although mostly with a brief ~200–300-ms delay (Supplementary Fig. 5). Hippocampal ripples tended to occur in clusters (Supplementary Fig. 6ab). We therefore examined type I neural activity may correlate with the ripple clusters, specifically the onsets and offsets of ripple clusters. Our analyses revealed that the greater the frequency of the type I neuron firing, the more synchronized the firing of MnR type I neurons emerged at the offset rather than onset of ripple clusters (Supplementary Fig. 6c–f), whereas low firing rates and unsynchronized firing showed little or no difference in relationship to the onset and offset of ripple clusters (Supplementary Fig. 6c–f).

Thus, increased firing rates and increased synchronicity of type I neurons predict the presence of streaks of ripple activity and may be needed to suppress them.

Up- and downregulation of ripple activity by MnR neurons

To determine whether there is a causal relationship between MnR neuron firing and ripple suppression, we employed an optogenetic approach. We injected a serotype-1 adeno-associated viral (AAV1) vector encoding channelrhodopsin-2 (ChR2) fused with enhanced yellow fluorescent protein (EYFP), driven by a general synapsin promoter, into the MnR. An optic fiber was then implanted in the
neurons. Both serotonergic (n = 5) and GABAergic (n = 6) neurons responded to a 200-ms continuous blue light (Fig. 5b,d). Although all of the GABAergic neurons (n = 6) responded to every light pulse of a four-pulse train (25 Hz; pulse width, 3 ms), only three of five serotonergic neurons were able to respond to every light pulse (Fig. 5b,d), and the other two serotonergic neurons only fired a single spike.

To examine the role of individual MnR populations in ripple regulation, we used multiple parameters, including 1, 2, 4 and 25 pulses of 3-ms blue light for MnR photostimulation in vivo (Fig. 5e). Consistent with our results (Fig. 1c,d), photostimulation of serotoninergic neurons resulted in moderate reduction of CA1 ripple activities compared with non-selective MnR photostimulation (Fig. 5f,g). Photostimulation of MnR GABAergic neurons resulted in a rebound suppression of ripple activity at the termination of photostimulation (Fig. 5h), suggesting that the MnR GABAergic neurons indirectly regulate ripple activity by inhibiting MnR non-GABAergic neurons. These results suggest that the MnR serotoninergic or GABAergic neurons alone have partial or minor roles in regulating hippocampal ripple activity.

To directly examine neurochemical identity of the three neural types (Fig. 1c,d), we employed an optogenetic tagging approach (Fig. 6). First, we injected the Cre-dependent ChR2 virus into the MnR of ePet-Cre or Vgat-ires-Cre mice. We then implanted an optrode (an optical fiber combined with four tetrodes) in the MnR and another four-tetrode bundle in the CA1 (Fig. 6a). The optrodes allowed us to determine, with subsequent photostimulation, whether recorded neurons had been serotoninergic or GABAergic. Our results indicate that most of the classified putative 5-HT neurons were indeed serotoninergic (Fig. 6b,c,f), confirming that our classification criteria for MnR 5-HT neurons were valid (Supplementary Fig. 2c–f). On the other hand, the majority of the type II neurons were GABAergic (Fig. 6d–f). However, the MnR type I neurons were mostly not serotoninergic or GABAergic (Fig. 6f). These results are consistent with our above results showing that the MnR serotoninergic and GABAergic neurons have partial or minor roles in regulating hippocampal ripple activity (Fig. 5f–h).
Impairment of fear memory by MnR photostimulation

The interruption of hippocampal ripple activity has been shown to disrupt memory consolidation, as indicated by poor performance of memory-guided behavior\(^{10,11,28}\). To determine whether MnR photostimulation that suppresses hippocampal ripple activity would disrupt memory consolidation, we employed a hippocampus-dependent contextual fear conditioning procedure\(^{29,30}\). Mice received MnR injections of either ChR2-EYFP or EYFP vector driven by the synapsin promoter and an optic-fiber implantation for subsequent photostimulation (Supplementary Fig. 8). We habituated the mice to the conditioning chamber for 10 min 3 weeks later; the next day, the mice received foot shock (0.8 mA, 2 s) in the chamber, followed by a 4-h session with photostimulation (2 pulses per 2 s only if mice were immobile; for ripple suppression efficiency, see Supplementary Fig. 9a) in their home cage; and on day 3, they were tested for freezing behavior in the chamber (Fig. 7a). ChR2-EYFP–expressing mice displayed significantly less conditioned freezing than EYFP-expressing control mice \((P = 0.0042, \text{unpaired} \ t\ \text{test}; \ Fig. 7f)\). This does not appear to be a result of possible neural damage caused by photostimulation (Supplementary Fig. 9c,d) or of possible sleep cycle dysregulation. Both slow-wave sleep (SWS) and rapid eye movement (REM) sleep were preserved (Fig. 7b,c and Supplementary Fig. 10), with a slight increase in REM sleep (Supplementary Fig. 9b) and a decrease in SWS delta (1–4 Hz) power \((P = 1.3 \times 10^{-6}, \text{unpaired} \ t\ \text{test}; \ Fig. 7c)\), which were probably the results of the reduction of ripple-associated sharp waves (~2–3 Hz). Consistently, during the 4-h photostimulation session, the total immobility time did not differ between the two groups \((P = 0.696, \text{unpaired} \ t\ \text{test}; \ Fig. 7d)\). These results suggest that MnR photostimulation immediately after fear conditioning impairs memory-consolidation processes.

**DISCUSSION**

We found that many MnR neurons were inactive when hippocampal ripple events occurred during the immobile/sleep state.
MnR serotonin neurons were inactive just before ripple events; however, this effect was generally small. Our observation that many of non-serotonergic neurons displayed prolonged suppression just before ripple events led us to the hypothesis that firing of those (type I) neurons suppresses ripple events. Consistent with this hypothesis, we found that, as the firing rate and synchronicity of MnR type I neurons increased, the suppression of ripple events grew stronger. Moreover, we observed that optogenetic excitation and inhibition of MnR neurons suppressed and enhanced ripple activity, respectively.

Selective excitation of serotonin neurons had small effects. Although selective excitation of GABA neurons had little effect, prolonged inhibition of GABA neurons was followed by clear rebound suppression of ripple activity, raising a question whether GABA neurons inhibit type I neurons. Finally, we found that optogenetic stimulation of MnR neurons after fear conditioning disrupted the acquisition of conditioned fear response, suggesting that there was interference in memory consolidation.

**Roles of MnR neurons in suppression of ripple activity**

It is important to note that MnR type I neurons fired immediately after ripple event (Fig. 1c,d). This observation implies that type I neurons receive information regarding hippocampal activity. In other words, the activity of type I neurons appears to be regulated in relation to ripple events. This implication raises two important questions. First, how is type I neuron activity coordinated in relation to ripple events? This question may not be easy to answer, as there is no direct projection from the hippocampal formation to the MnR. Second, what function does such interaction serve? The activity of type I neurons may be characterized as insertion of a ‘gap’ between ripple events and, consequently, it may be pacing memory consolidation.

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**Figure 6** Optical tagging of the MnR serotonergic and GABAergic neurons. (a) Schematic drawing showing a four-tetrode probe in the hippocampal CA1 and a MnR optrode consisting of an optic fiber and four tetrodes. ePet-Cre (n = 4) and Vgat-IRES-Cre (n = 3) mice received these implants and an injection of AAV-DIO-ChR2-EYFP into the MnR for the identification of serotonergic and GABAergic neurons, respectively. (b) Inter-spike interval histogram of a putative serotonergic neuron identified by optogenetic stimulation. Inset, cumulative spikes of the same neuron after an injection of the serotonin 1A receptor agonist 8-OH-DPAT (0.2 mg per kg of body weight, intraperitoneal). (d) Peri-ripple event histogram of a putative GABAergic neuron identified by optogenetic stimulation. (c,e) Peri-event rasters and histogram of the two neurons shown in b and d following four-pulse light trains (25 Hz; pulse width, 3 ms). (f) Percentages of putative 5-HT (n = 5 and 8 from ePet-Cre and Vgat-IRES-Cre mice, respectively), type I (n = 6 and 9, respectively) and type II (n = 15 and 8, respectively) neurons that responded to photostimulation. Neurons were considered to be responsive to photostimulation if peri-stimulation histogram bin with a z score of 3.28 (P < 0.001) or greater occurred within 5 ms of the onset of photostimulation. Response latencies varied between 2 and 5 ms.

**Figure 7** Impairment of fear memory consolidation by MnR photostimulation. (a) Contextual fear conditioning protocol. On day 2, immediately after fear conditioning, mice were returned to the home cage, where they received two-pulse trains of MnR photostimulation (25 Hz; pulse width, 3 ms) if they were immobile over the course of 4 h. (b) Representative hippocampal CA1 LFP during SWS and REM sleep with and without MnR photostimulation. Scale bars represent 2 s and 2 mW. (c) Mean power spectral density (PSD, black and dark blue lines) and s.d. (gray and light blue) with (light and dark blue lines) and without (gray and black lines) MnR photostimulation during SWS or REM sleep (n = 17 and 20 sessions, respectively). (d) Percentage of immobility time for individual mice during the 4-h stimulation session following fear conditioning (n = 11 per group). (e,f) Percentage of freezing time for individual mice before and after fear conditioning (n = 11 per group). **P = 0.0042, unpaired t test. Error bars represent s.e.m. in d and f. n.s., not significant, P = 0.696 in d and P = 0.364 in e.
The previous finding that stimulation of median raphe neurons produces a rapid excitation of hippocampal interneurons19,31 may have suggested a substantial contribution of serotonergic neurons for the suppression of ripple activity. However, our results suggest that MnR serotonergic neurons do not readily suppress hippocampal ripple activity. First, serotonergic neurons were inhibited just before ripple events to a lesser extent than type I neurons. In addition, selective excitation of serotonergic neurons suppressed ripple activity much less effectively than non-selective excitation of MnR neurons. Our electrophysiological criteria for identifying serotonin neurons were confirmed with pharmacological and optogenetic tests. Injections of the 5-HT1A receptor agonist 8-OH-DPAT inhibited serotonergic neurons identified by the electrophysiological criteria (Supplementary Fig. 2c,d). Moreover, none of type I or II neurons responded to optogenetic stimulation that selectively excited serotonin neurons (Fig. 6). However, we cannot conclude that serotonergic neurons have little involvement in ripple suppression, as our procedures were not completely irrefutable. Our optogenetic procedure involving ePet-Cre mice resulted in viral expression in 67% of MnR serotonergic neurons; thus, we cannot exclude the possibility that some unaffected serotonergic neurons would have effectively suppressed ripple activity. In addition, our electrophysiological recording procedure may have missed, for unknown reasons, those serotonergic neurons with prolonged inhibition before ripple events.

In any case, it is clear that non-serotonergic neurons are important for suppressing hippocampal ripple activity. Given that the majority of type I neurons are not serotonergic or GABAergic, they may be glutamatergic neurons. The majority of MnR neurons projecting to the hippocampus contain vesicular glutamate transporter type 3 (VGlut3)24,32–34. Indeed, previous studies have shown that the stimulation of MnR serotonergic neurons excites hippocampal interneurons with a short latency, its synapses contain glutamate receptors and its excitatory effect was blocked by glutamate receptor antagonists19,34. Given these previous findings and our current ones, it is reasonable to hypothesize that type I neurons are largely glutamatergic.

Roles of the MnR in memory consolidation

Using a fear conditioning procedure, we found that MnR neurons are important for memory consolidation via interrupting hippocampal ripple activity. Although we focused on MnR neural activity in relation to hippocampal ripple activity, the MnR may also regulate memory consolidation through its projections to thalamocortical regions. Interactions between the hippocampus, thalamus and cortical regions, including the medial prefrontal cortex, during slow-wave sleep are thought to be critical for memory consolidation35–38. Spindles are low-frequency oscillations that are generated by the reticular thalamic neurons37, and are detected in the medial prefrontal cortex whose spindles occur close in time with hippocampal ripples40. MnR neurons project directly to the medial prefrontal cortex18 and extensively to the midline thalamic regions, including the reuniens, mediodorsal, paracentral, parafascicular, central lateral and central medial nuclei18, which in turn, project to the medial prefrontal cortex40. These MnR projections may participate in regulation of memory consolidation, in addition to its projection to the hippocampus.

Roles of MnR in hippocampal functions and behavioral state

The MnR is involved in suppression of hippocampal theta oscillation and its mechanism differs from that in suppression of ripple activity. Stimulation of the MnR desynchronizes hippocampal LFPs41–46, whereas lesions of the MnR33,44 or pharmacological manipulations that inactivate MnR neurons47–49 elicit hippocampal theta oscillations.

Serotonergic neurons appear to be important for suppressing hippocampal theta activity. Stimulation of MnR serotonergic autoreceptors inactivates serotonergic neurons and elicits hippocampal theta oscillations49. Conversely, lesion-induced theta oscillations are readily abolished by injections of the 5-HT precursor L-5-hydroxytryptophan44. Similarly, the 5-HT synthesis inhibitor p-chlorophenylalanine disrupts MnR stimulation–induced desynchronized LFPs in the hippocampus41. In addition, suppression of hippocampal theta oscillations by MnR neurons appears to be mediated by the medial septum/diagonal band of Broca (MS/DB), which is involved in the generation of hippocampal theta rhythm41. MnR stimulation disrupts rhythmic discharge of MS/DB neurons. Conversely, inhibition of serotonergic neurons with intra-MnR injections of 8-OH-DPAT elicits rhythmic discharge in MS/DB neurons synchronized with hippocampal theta activity40. In summary, theta suppression involves serotonergic neurons projecting through the MS/DB, whereas ripple suppression largely involves non-serotonergic, most likely glutamatergic, neurons projecting through direct pathways.

Notably, we found that the firing of serotonergic neurons was correlated with that of type I neurons (Supplementary Fig. 5). Although the relevance of this observation has not yet been determined, it may indicate that the MnR regulates processes involving multiple forebrain regions, including the hippocampus and MS/DB, for coordinated global functions. It is also possible that serotonergic neurons work together with type I neurons to suppress ripple activity. In addition to cognitive functions involving the hippocampus and MS/DB, the MnR is involved in tonically suppressing motivated behaviors. The inhibitory manipulations of local MnR neurons that elicit hippocampal theta activity in anesthetized animals also induce feeding, drinking, reward-seeking and reward16. Although downstream neural mechanisms involved in cognitive and motivational processes may be markedly different, the MnR appears to regulate environmental stimuli and behavior.

In conclusion, we found that the MnR is critical for regulating hippocampal ripple oscillation and memory consolidation, and our results provide a fundamentally new perspective on the role of subcortical regions in hippocampal functions. Our findings stimulate a new line of research, and may lead to new insights for treatments of affective or anxiety disorders arising from dysregulated memory processes.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.V.W., H.-J.Y. and S.I. designed the experiments. D.V.W., H.-J.Y., C.I.B. and J.-H.T. performed the experiments. D.V.W. analyzed the data with help from H.-J.Y., C.I.B. and J.-H.T. D.V.W., H.-J.Y., A.B. and S.I. contributed to interpretation of the data. D.V.W. and S.I. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
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ONLINE METHODS

**Mice.** Male C57BL/6j mice (3–5 months old, Jackson Laboratories), ePet-Cre and Vgat-ires-Cre mice crossed with C57BL/6j (3–5 month old; NIDA animal facility) were used. After surgery, they were singly housed in a plastic cage (30 x 20 x 20 cm) containing woodchips and cotton material and kept on a 12-h light/dark cycle and had ad libitum access to food and water except during testing. All procedures were approved by the Animal Care and Use Committee of the Intramural Research Program, NIDA, and were in accordance with National Research Council’s Guide for the Care and Use of Laboratory Animals.

**Viral vectors.** The NIDA Optogenetics and Transgenic Technology Core produced adenovirus-associated virus serotype-1 (AAV1) encoding channelrhodopsin-2 (ChR2), halorhodopsin (eNpHR-3.0) and enhanced yellow fluorescent protein (EYFP): AAV1-hSyn-ChR2 (H134R)-EYFP, AAV1-hSyn-(eNpHR-3.0)-EYFP and AAV1-hSyn-EYFP (control vector). The final viral concentration was ~10^{12} genome copies (GC) per ml. The University of Pennsylvania Penn Vecr Core produced Cre-dependent AAV encoding ChR2 and EYFP, the pAAV-Ef1a-DIO- hChR2 (H134R)-EYFP-VPRE-hGH and pAAV-Ef1a-DIO-EYFP-VPRE-hGH. The final viral concentration was 9 x 10^{12} GC per ml.

**Sterotoxic surgery.** Mice were anesthetized with ketamine/xylazine mixture (~80/12 mg per kg, intraperitoneal). Some mice received a bundle of electrodes implanted in the hippocampal CA1, whereas others received two bundles of electrodes implanted in the CA1 and MnR, consisting of the median and para-median raphe nuclei. The coordinates for the MnR were AP ~4.5 mm posterior from Bregma, ML 0.4 mm lateral from midline, DV 3.9 mm ventral from the dorsal surface of the brain, tilted with a 6-degree angle on a coronal plane with the tip pointing toward the midline (to avoid sinus); the coordinates for CA1 were AP ~2.3 mm, ML 1.8 mm and DV 1.1 mm in the right hemisphere. The electrode bundles were slowly lowered toward the MnR and CA1, respectively, and secured on the skull with stainless screws and dental cement. After ~3 d recovery from surgery, electrodes were screened for neural activity.

The coordinates for MnR viral injections are AP ~4.5 mm, ML 0.0 mm, DV 4.2 mm. AAV viral vectors (0.25 or 0.5 μl) were microinjected into the MnR with a syringe pump (Micro 4, World Precision Instruments) over 5 or 10 min, with additional 10 min before removal of the injection needle (34 gauge, beveled). An optic fiber (200-μm diameter) or an optetrode (100-μm diameter optical fiber glued together with four tetrodes) was then chronically implanted 0.2 mm dorsal to the injection site, and was secured on the skull with stainless screws and dental cement. Experiments were started 3–4 weeks after surgery, to allow gene expression.

**TPH staining.** 3 weeks after receiving an AAV1-DIO-EYFP injection, 3 mice were intracardially perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde (wt/vol). Brains were coronally sectioned with a 30-μm thickness, and then TPH was visualized with a rabbit anti-TPH (1:500; Product No. T0678, Sigma) and a donkey anti-mouse secondary antibody (1:50; Code No. 715-025-151, Jackson ImmunoResearch Laboratories). Mounted sections were counterstained with DAPI (4,6-diamidino-2-phenylindole), a fluorescent nuclear stain. EYFP and TPH expression were examined with a confocal microscope (BX61WI, Olympus).

**Glial fibrillary acidic protein (GFAP) staining.** Six mice received an injection of AAV1-DIO-ChR2-EYFP into the MnR, and were randomly assigned to one of two groups. Three of the mice received a 4-h MnR photostimulation procedure (2-pulse train per 2 s when mice were immobile), while the other three received no photostimulation. 24 h later, they were intracardially perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains were coronally sectioned at 40 μm and immunostained with a rabbit anti-GFAP (1:500; Product No. Z0034, DAKO) and a donkey anti-rabbit secondary antibody (1:500; Code No. 711-605-152; Jackson ImmunoResearch Laboratories). Mounted sections were counterstained with DAPI. Three sections (from each mouse) with clear optic fiber tract were used for quantification.

**RNA in situ hybridization.** To colocalize EYFP and GABA neurons, RNAseq 2.0 assay (Advanced Cell Diagnostics) was performed for detection of GAD in Vgat-IRES-Cre mice that had received an injection of AAV1-DIO-EYFP into the MnR. Briefly, the fresh frozen brain was embedded in cryo-embedding medium (OCT) and then coronally sectioned at 14 μm. Mounted sections were then fixed in chill 10% neutral buffered formalin (NBF, vol/vol), dehydrated in graded ethanol (50%, 70% and 100%), digested with protease and followed by hybridizations with target-specific probes. Sections were counterstained with DAPI, and the images were examined with the confocal scope.

**Electrodes and optetrode.** A bundle of four or eight tetrodes was coupled with the moveable (screw-driven) microdrive assembly (~1 g weight; Supplementary Fig. 1a,b). The MnR probe consisted of eight tetrodes, and CA1 probe consisted of four tetrodes. Each tetrode consisted of four wires (90% platinum and 10% iridium; 18-μm diameter with an impedance of ~1–2 MΩ for each wire; California Fine Wire). The optetrode consisted of an optic fiber (100-μm diameter) and 4 tetrodes glued together, and it was coupled with the moveable microdrive (Fig. 6a,b). The distance between the tips of optical fiber and tetrodes were ~0.3–0.6 mm; the photostimulation (~10–50 mW mm⁻²) was delivered with a variable interval schedule of 10–15 s.

**In vivo electrophysiology.** Neural signals were pre-amplified, digitized and recorded using a Neuralyx Digital Lynx acquisition system; the animals’ behaviors were simultaneously recorded. Neural signals for LFP were digitized at 2 kHz and filtered at 1–500 Hz or 150–250 Hz (ripples), using ground as the reference. If no clear ripple activity was detected, the electrode array was lowered by ~80 μm daily until a clear ripple activity was detected (with the ripple amplitude reaching at least > 6 s.d.). Spikes recorded at the hippocampal CA1 were digitized at 32 kHz and filtered at 600–6,000 Hz, using ground as the reference. Spikes recorded at the MnR were digitized at 32 kHz and filtered at 250–8,000 Hz, using one recording electrode that lacked obvious spike signals as the reference. The MnR electrode bundle was lowered by ~80 μm after a recording session. Each mouse received 3–6 recording sessions (up to 6 h per session).

**In vitro slice electrophysiology.** The mice that had received an AAV1-DIO-ChR2-EYFP injection were anesthetized with isoflurane and intracardially perfused with a 30-ml ice-cold NMDG-based artificial cerebrospinal fluid (aCSF) solution containing (in mM): 92 NMDG, 20 HEPES, 2.5 KCl, 1.2 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 30 NaHCO₃, 25 glucose, 2 thiourea, 5 sodium ascorbate, 3 sodium pyruvate and 12 N-acetyl-l-cysteine (300–310 μM, pH 7.3–7.4)⁴. After perfusion, the brain was quickly removed and coronal brain slices containing the MnR were made in 250 μm with VT-1200 vibratome (Leica). The brain slices were then recovered for less than 15 min at 32 °C in NMDG-based aCSF. After recovery, the slices were transferred and stored in room temperature HEPES-based aCSF containing (in mM): 92 NaCl, 20 HEPES, 2.5 KCl, 1.2 NaH₂PO₄, 1 MgCl₂, 2.4 CaCl₂, 30 NaHCO₃, 11 glucose, 0.1 picrotoxin, and 2 kynurenic acid, and was saturated with 95% O₂ and 5% CO₂ at 32–34 °C. To obtain a whole cell recording, glass pipette (KC-30, King Precision Glass) with pipette resistance 2.8–30 MΩ was used and filled with K⁺-based internal solution containing (in mM): 140 KMeSO₄, 5 KCl, 0.05 EGTA, 2 MgCl₂, 2 Na₂ATP, 0.4 NaGTP, 10 HEPES and 0.05 Alexa Fluor 594 (Invitrogen), pH 7.3, 290 mOsm. Whole-cell configuration was made using a MultiClamp 700B amplifier (Molecular Devices) from identified EYFP-positive cell in the MnR slice. A 200-μs pulse of blue (473 nm; intensity 0.3–4 mW) was delivered via a fiber optic attached to 40× lens and positioned just above the slice to test the expression of ChR2. The expression of ChR2 in the cell was confirmed by a light-induced inward current under voltage clamp. Then a train of 2 or 4 light pulses (pulse width, 3 ms; 25 Hz) were flashed above the slice under current clamp to examine the effect of light-induced depolarization on neuron excitability. Series resistance (10–30 MΩ) was continually monitored online with ~20 pA, 300 ms current injection given after every current injection step; if the series resistance changed by >20%, data were not included in the analysis. During the recording, signal was sampled at 20 kHz and filtered at 10 kHz. Data were acquired in Clampex 10.3 (Molecular Devices), and was analyzed off-line in Clampfit 10.3 (Molecular Devices) and IGOR Pro 6.0 (WaveMetrics).
Fear conditioning and optical stimulation procedures. The fear conditioning experiment used 11 mice per group; this number is generally considered sufficient for behavioral study. Mice were randomly assigned into 2 groups, which received the ChR2-EYFP and EYFP-Control virus injections, respectively, and were kept on a 12-h light/dark cycle (lights on at 06:00 a.m.). Pairs of mice, consisting of an experimental (ChR2-EYFP) mouse and a control (EYFP only) mouse, had gone through the following procedure at the same time in two separate chambers. The assignments of the experimental and control mice between the two chambers were counterbalanced among pairs; thus although the experiment was not a blind one, it was conducted in a highly systematic manner. Each day’s session started at 1:30 p.m. On days 1 and 2, each mouse was handled for 10–15 min. On days 3–5, mice were habituated to the photostimulation environment (~60 dB white noise background; ~23 °C room temperature) and procedure without actual stimulation. Each homecage (30 × 20 × 20 cm) containing a mouse was placed under a video camera, and the mouse was connected with optic fiber cable for 4 h. On day 5 before the stimulation habituation session, mice were also habituated to the conditioning chamber (32 × 25 × 25 cm) encased in a sound-attenuated box, by leaving them there for 10 min. On day 6, mice received single-trial contextual fear conditioning: they were allowed to freely explore the conditioning chamber for 3 min, followed by one foot shock (0.8 mA; scrambled; 2 s). 30 s after the foot shock, mice were placed back to their homecages under a video camera and connected with the optical fiber. They received two-pulse trains of optical stimulation (25 Hz; pulse width, 3 ms; laser power, 16 mW) when the video-tracking system (EthoVision tracking system, Noldus) detected that they were immobile (rest/sleep) for 2 s, and this procedure lasted for 4 h. On day 7, each mouse was placed in the conditioning chamber for 5 min, and their freezing response was assessed with the video-tracking software VideoFreeze (Med-Associates) with the previously established criteria52 of animals’ motion index assessed with the video-tracking software VideoFreeze (Med-Associates). Low-frequency firing neurons (recorded for 1 h or longer) were plotted on a histogram with the bin of 10 ms. Some neurons displayed only tonic activity; thus, these neurons had a single inter-spike interval (ISI), as previously described53,54. ISIs of each neuron (recorded for 1 h or longer) were plotted on a histogram with the bin of 10 ms. Some neurons displayed only tonic activity; thus, these neurons had a single histogram peak. The latency value of the histogram peak was determined as its ISI (Supplementary Fig. 2d). On the other hand, others displayed both tonic and high-frequency activity, which sometimes have two clear histogram peaks.

We assigned the second peak as their ISI. Neurons with the ISI longer than 200 ms were defined as putative serotonergic neurons (Supplementary Fig. 2f).

Non-serotonergic type I and type II neurons. Firing rate changes were determined by sorting the data of non-serotonergic neural firing with respect to ripple events using a peri-event analysis (NeuroExplorer). A 3−bin Gaussian filter was then used to smooth the peri-ripple histogram (bin = 10 ms; averaged over 1,000 ripple events). Neurons that, with 3 or more consecutive bins (within ±1 s from ripple peak), differ from the baseline (a 2-s period that occurred between 2 and 4 s before ripple peak) by z-score of 3.28 or greater were defined as ripple-correlated neurons. These ripple-correlated neurons were classified into two types: type I neurons are defined as neurons that decreased firing rate by 20% or greater from the baseline, lasting 1 s or longer before ripple peak (Supplementary Fig. 2g), and the rest of the ripple-correlated neurons were classified as type II.

Rates and synchrony of type I neuron firing during ripple events. For analyses shown in Figure 2e–g, type I neuron and ripple event histograms (bin = 50 ms) were first smoothed with a Gaussian filter (filter width, 250 ms). Type I neuron firing-frequency was averaged every 500-ms epoch; ripple event frequency was averaged between 0–1, 1–2, 2–3 and 3–4 s after each 500-ms epoch of the type I neuron. Firing rates of type I neurons were classified into three levels with respect to change from the baseline firing-rates for analyses shown in Supplementary Figure 6c.d. Type I neuron’s rates were high, medium and low, if firing rates exceeded twofold of mean firing-frequency, fall between half and twofold of the mean, or fall below half-fold of the mean, respectively. Moreover, firing-synchrony of type I neurons were classified into three levels for Supplementary Figure 6e.f. Type I neuron’s synchrony were high, medium, and low, if two type I neurons fired within a time window of 25 ms, 25.1–200 ms or 200.1 s or longer, respectively. The medium-synchrony time window (25.1–200 ms) were further divided into 25.1–50 ms, 50.1–100 ms and 100.1–200 ms windows for analyses in Figure 3e.f. Synchrony between type I neurons was determined significant if three or more consecutive bins from the cross-correlation histogram (bin = 5 ms) exceed the z-score of 3.28 (P < 0.001, Wilcoxon signed rank test).

Histology for implantation verification. At the completion of the electrophysiology recordings, the final electrode position was marked by passing a 20-s, 10-μA current using a linear constant current stimulus isolator (Neurolog System) through two selected tetrodes. Mice were deeply anesthetized and intracardially perfused with ice-cold phosphate-buffered saline followed by 4% paraformaldehyde. Brains were then removed and post-fixed in PFA for at least 24 h. Brains were rapidly frozen and sliced on a cryostat (50-μm coronal sections). Sections from the dual-site recording mice were stained with cresyl violet for microscopic examination of electrode placements, while other sections were mounted with the Mowiol mounting medium mixed with DAPI (Vector Laboratories) for fluorescent microscopic examination of viral vector expression and optical fiber placements.

Statistical analyses. Sample sizes were based on the authors’ experience, previous similar studies and preliminary experiments performed in our lab. To determine firing-rate change, the value that deviates from the baseline mean by z-score of 3.28 or more was considered to be significant. Since distributions of baseline data were not necessarily normal, Wilcoxon signed-rank tests were performed on z-score-indicated changes, to assure that firing-rate changes are significant at P < 0.001. To determine the latency of stimulation-evoked neural activity change, the firing-rate value that deviates from the baseline mean by z-score of 1.96 or more was considered significant (Supplementary Fig. 7b). Wilcoxon signed-rank tests were used for comparisons of firing rates between two conditions (Supplementary Fig. 6c–f), and significance level was set at P = 0.001. Shapiro–Wilk test confirmed normality assumption for r-tests (Fig. 7e, Supplementary Fig. 9b), significance level was set at P = 0.05. When Mauchly’s test detected violation of the sphericity assumption for repeated measures of ANOVAs (Fig. 4e, Supplementary Fig. 9d), the degrees of freedom for respective tests were adjusted with the Greenhouse–Geisser method. Tukey’s honestly significant difference post hoc test was performed when appropriate. All statistical tests were two-sided.

A Supplementary Methods Checklist is available.
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