Network state-dependent inhibition of identified hippocampal CA3 axo-axonic cells in vivo

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Hippocampal sharp waves are population discharges initiated by an unknown mechanism in pyramidal cell networks of CA3. Axo-axonic cells (AACs) regulate action potential generation through GABAergic synapses on the axon initial segment. We found that CA3 AACs in anesthetized rats and AACs in freely moving rats stopped firing during sharp waves, when pyramidal cells fire most. AACs fired strongly and rhythmically around the peak of theta oscillations, when pyramidal cells fire at low probability. Distinguishing AACs from other parvalbumin-expressing interneurons by their lack of detectable SATB1 transcription factor immunoreactivity, we discovered a somatic GABAergic input originating from the medial septum that preferentially targets AACs. We recorded septo-hippocampal GABAergic cells that were activated during hippocampal sharp waves and projected to CA3. We hypothesize that inhibition of AACs, and the resulting subcellular redistribution of inhibition from the axon initial segment to other pyramidal cell domains, is a necessary condition for the emergence of sharp waves promoting memory consolidation.

RESULTS

Identification of hippocampal AACs

To assess the contributions of AACs to the hippocampal network, we recorded the activity of single interneurons in both urethane-anesthetized and freely moving rats, followed by juxtacellular labeling (Fig. 1 and Supplementary Fig. 1). We identified nine AACs (from nine rats) on the basis of the presence of radial rows of boutons (cartridges) around the sPyr-sOri border (Fig. 1a,c). Dendrites of seven AACs crossed stratum lucidum (sLuc), demonstrating the cells’ location in CA3. The dendrites were radially distributed across all strata, with a broad tuft aligned with the entorhinal cortical input in stratum lacunosum-moleculare (sLacMol) (n = 8 AACs, Fig. 1a), representing 34.7 ± 7.6% (mean ± s.d.) of the total dendritic length (n = 3 AACs, Fig. 1a,b). A smaller proportion was found in sRad (11.8 ± 8.3%) and only minor proportions in sRad (1.9 ± 0.4%) and sPyr (4.9 ± 2.4%). The highest proportion of dendrites was in sOri and the alveus (46.7 ± 6.7%). Thus, CA3 AAC dendrites are aligned with pyramidal cell dendrites. One CA3 AAC had dendrites and axon in CA2 (Fig. 2a). From two AACs recorded in freely moving rats, one was located in CA2 and had axon innervating both CA2 and CA3 but not CA1; the other was located in CA1 and innervated CA1 pyramidal cells.

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Next we tested the molecular profiles, postsynaptic targets and firing patterns of AACs (Table 1). All AACS were immunoreactive for parvalbumin (PV) (n = 9 of 9 cells examined), and their somatodendritic membrane expressed small puncta and large patches of ErbB4 (ref. 16) (n = 5 of 5 cells) (Fig. 1d,e). In contrast to other identified PV+ cells, such as CA1 bistratified cells and oriens lacunosum-moleculare cells12,13, AACS lacked detectable immunoreactivity for neuropeptide Y (NPY) and somatostatin (SOM) (n = 0 of 2 for both molecules) and were also immunonegative for neuronal calcium-binding protein 1 (NECAB1)17 (n = 0 of 4 tested cells).

Testing for postsynaptic targets using ankyrin G, a marker for the AIS18, showed that AAC boutons targeted the AISs of pyramidal cells14 (n = 3 cells, Fig. 2b). These AISs were covered by vesicular GABA transporter (VGAT)-immunoreactive boutons, the labeled AAC terminals being a subset of these (Fig. 2b). Electron microscopy confirmed that these sites were GABAergic synapses14 with membrane specialization (100% targeted AISs15; n = 7, 13, 11, 14, 10 and 9 synapses for cells B10a, BK24g, TV34n, B45a, B44a and B53b, respectively; Fig. 2c and Supplementary Figs. 1a and 2). Thus, CA3 AACS release GABA selectively on the AIS.

Firing patterns of AACS during sharp waves

To explore the firing patterns of CA3 AACS, we identified SWRs (90–200 Hz oscillations) and theta oscillations (3–6 Hz) in local field potentials (LFPs) recorded in both CA3 and CA1 of anesthetized rats (Figs. 1f and 2a). Transitions between different states occurred spontaneously or were promoted by administration of anesthetics (SWRs and non-theta states) or a tail pinch (theta). Coincident with the occurrence of SWRs, firing of AACS often ceased completely, despite firing heavily before and after (Fig. 1f). The number of action potentials fired by individual CA3 AACS was less during SWRs than expected from their firing in a ±10 s window around the SWRs (SWRs versus ‘peri-SWRs’ significant at α = 0.05 with Mann-Whitney U-test in 3 of 3 and 5 of 5 cells for CA3 and CA1 SWRs, respectively; Fig. 2c and Table 1). Mean (± s.d.) firing rates dropped from 24.6 ± 4.0 Hz and 22.8 ± 3.1 Hz peri-SWR to 4.4 ± 3.7 Hz and 4.8 ± 3.5 Hz during SWRs (P = 0.039 and 6 × 10−4; t1 = 4.9 and t2 = 9.7; n = 3 cells and 5 cells, respectively) for SWRs detected in CA3 and CA1, respectively; paired Student’s t-tests (Fig. 2e). We detected significant variation in the mean firing rate of AACS as a function of normalized SWR time (P = 4 × 10−8 and 6 × 10−11; F31,64 = 4.88 and F31,128 = 5.74; n = 3 and 5 cells for CA3 and CA1 SWRs, respectively; one-way ANOVA), with a significant reduction in the rate associated with SWR occurrence (Tukey’s post hoc multiple comparisons test; Fig. 2d). Consistent with the generation of SWRs in CA3 (ref. 5), we found that 30 ± 10%
Figure 2 GABAergic input to CA3 pyramidal cell AISs is withdrawn during sharp waves in vivo. (a) Locations of CA1 electrodes (black asterisk, rats B44, B10, B45, J67, B62 and B53; brown asterisk, rat J54), tetrode tips (color-coded open circles) and somata of AACs (color-coded filled circles). TV34n and LK24g are from drug-free, freely moving rats. (b,c) Examples of AACs (b, J67d; c, B10a and LK24g) innervating pyramidal cell AISs. (b) Neurobiotin-filled axonal boutons (green, arrows), immunoreactive for VGAT (yellow, right) are apposed to AISs, identified by membrane undercoating (asterisk) and linked microtubules (arrowhead). An unlabeled bouton also makes a synapse (open arrow). SV, synaptic vesicles. (d) Variation of firing rate of AACs before, during (gray box) and after SWRs detected in CA3 (top) or CA1 (middle; lines, individual cells; gray bars, mean ± s.d.; asterisks significant at α = 0.05 by Tukey’s post hoc comparisons). Bottom: firing rates for identified AACs TV34n and LK24g recorded in drug-free rats; SWRs detected from the electrode recording the cell. (e) Overall firing rates during and outside SWRs (peri-SWR) detected in CA3 or CA1. Individual cells are color-coded as in a (mean ± s.d. in black; asterisks, significant at α = 0.05 with paired t-test; P = 0.039 and 6 × 10^{-4} for SWRs detected in CA3 and CA1, respectively). (f) Firing of LK24g during sleep. Top to bottom traces: Electroencephalogram (EEG) in frontal cortex; band-pass filtered (130–230 Hz) LFP at the recording site of the cell (CA2), SWRs in red; action potentials; LFP. Scale bars: a, 1 mm; b, 5 μm; c, 0.5 μm. Panel b, maximum intensity projection of 9 optical sections (2.8-μm-thick z-stack). DG, dentate gyrus.

(mean ± s.d.) of detected SWRs occurred coincidentally in CA3 and downstream CA1 (n = 3 experiments from 3 rats). Some SWRs (27 ± 4.5%) detected by the CA3 electrode did not recruit the CA1 area at the CA1 electrode site; others detected only in CA1 (43 ± 14%) could have originated from sites other than that of the CA3 electrode. Suppression of CA3 AAC firing was similar irrespective of where the SWRs were detected (reduction to 19 ± 14% versus 21 ± 13% during SWRs in CA3 and CA1, n = 3 and 5 cells, respectively; P = 0.9; t_k = 0.13; two-sample Student’s t-test; Fig. 2e). In both areas, small increases in the power of 90–200 Hz oscillations, below the threshold of SWR detection and possibly reflecting distant, localized SWRs, were often associated with AAC silencing (Fig. 1f), suggesting that the suppression of AACs occurs on most SWRs.

To test whether the observed suppression of firing during SWRs could be detected in animals without anesthesia, we examined firing patterns of identified AACs recorded in drug-free, freely moving rats. During SWS and quiet wakefulness, we observed a reduction in AAC firing during local SWRs (Fig. 2f and Supplementary Fig. 1b; 130–230 Hz oscillations). Identified AACs in both CA2 and CA1 significantly decreased their firing rate during SWRs compared to peri-SWR periods (CA2, cell LK24g: 0.72 Hz versus 8.8 Hz, P = 6 × 10^{-7}, n = 112 SWRs; CA1, cell TV34n: 2.4 Hz versus 6.9 Hz, P = 0.026, n = 61 SWRs; Mann-Whitney U-test; Fig. 2d and Table 1). The overall firing rate was very low during resting states, when SWRs occurred (Supplementary Fig. 3 and Table 1). Occasionally, AACs fired in bursts (Fig. 2f) uncorrelated to SWRs. We conclude that AACs are inhibited during SWRs under drug-free conditions also.

Theta- and gamma-modulated firing of AACs

During theta oscillations under anesthesia, CA3 AACs fired rhythmically at 28.8 ± 4.2 Hz (mean ± s.d.; n = 5 cells from 5 rats), with six to eight action potentials per cycle (Fig. 1g and Table 1). Firing of AACs was lowest around theta troughs detected either locally in CA3 or in CA1 (Figs. 2a and 3a), suggesting a reduction in GABAergic input to CA3 AISs at this phase (Fig. 2bc). Correspondingly, the preferred theta phase of firing was close to the peak, at 146° ± 28.1° for CA3 theta oscillations (circular mean ± circular s.d.; n = 6 cells; Fig. 3a and Table 1) and at 183° ± 21.7° for CA1 theta (n = 5 cells; only CA1 electrode in pSyr included; all AACs significant at α = 0.05, Rayleigh test; Fig. 3a and Table 1). Consistent with the strong theta modulation of AACs, the mean vector lengths were similarly high (0.44 ± 0.07 in CA3 versus 0.45 ± 0.06 in CA1, mean ± s.d.; P = 0.48, t_k = 0.75, n = 6,
Table 1 Properties of identified AACs

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<tr>
<th>Region</th>
<th>CA3</th>
<th>CA3</th>
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<td>B62a</td>
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<td>NA</td>
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<td>NA</td>
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<td>10^{-10}</td>
<td>5 \times 10^{-19}</td>
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<td>23 (4)</td>
<td>NA</td>
<td>80 (28)</td>
<td>50 (3)</td>
<td>NA</td>
<td>112 (5)</td>
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CA1 SWRs

| Rate during SWRs (Hz) | NA | 1.5 | 10.6 | 4.1 | 4.5 | 3.0 | 2.6 | NA | 7.4 |
| Rate peri-SWR (Hz) | NA | 18.5 | 23.1 | 23.5 | 22.1 | 27 | 8.3 | NA | 6.9 |
| U-test P value | NA | 4 \times 10^{-32} | 5 \times 10^{-5} | 3 \times 10^{-6} | 6 \times 10^{-21} | 10^{-13} | 0.250 | NA | 0.026 |
| Total n SWRs (active n) | NA | 106 (11) | 28 (12) | 20 (6) | 147 (35) | 49 (7) | 7 (1) | NA | 61 (7) |
| Theta firing rate (Hz) | NA | 19.4 | NR | NR | 19.7 | 27.9 | 7.63 | |
| Non-theta firing rate (Hz) | NA | 25.9 | 29.0 | 32.1 | 23.5 | 33.4 | 3.2 | 20.4 | 17.5 |

In vivo firing patterns

CA2/3 SWRs

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<th>U-test P value</th>
<th>Total n SWRs (active n)</th>
<th>Theta firing rate (Hz)</th>
<th>Non-theta firing rate (Hz)</th>
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<tr>
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<td>194.9</td>
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<td>20,706</td>
<td>3,880</td>
<td>1,691</td>
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CA1 AACs fired at a similar rate to CA1 PV + basket cells (PVBCs) during theta oscillations in drug-free rats (non-theta firing rate (Hz) NA 19.4f NR NR 19.7f 27.9 7.63)

Pairing AAC firing with strong GABAergic synaptic input (VGAT puncta) confirmed that AAC dendrites receive GABAergic synaptic input paired t-test). In freely moving rats, theta oscillations were observed during head movements and postural shifts; the animals remained in the same location throughout the recording period. Firing of AACs during movement occurred at a high rate and was rhythmic but intermittent (Fig. 3b, Supplementary Figs. 1c and 3a,b, and Table 1). Firing rates and consequent GABA release on CA2 and CA3 pyramidal cells (LK24g) and on CA1 pyramidal cells (TV34n) were lowest close to theta troughs, with preferred firing phases of 191° and 225°, respectively (phase distributions significantly different from uniform at $\alpha = 0.05$, Rayleigh test; Fig. 3b and Table 1). Notably, AACs fired at a similar rate to CA1 PV + basket cells (PVBCs) during theta oscillations in drug-free rats (Supplementary Fig. 3a,c) but were more strongly theta-modulated (AAC vector lengths: 0.48 in CA2, LK24g: 0.42 in CA1, TV34n; mean of mean vector lengths ± s.d. for 5 PVBCs: 0.22 ± 0.06). Differences in firing of AACs and PVBCs were evident across behavioral states (Supplementary Fig. 3b,d).

Identified CA3 AACs are not coupled to kainate-induced gamma oscillations in vitro, whereas CA1 AACs are coupled locally in vivo. We have examined the relationship between AAC firing and 15–100 Hz LFP oscillations under anesthesia. We observed coupling (at $\alpha = 0.01$, Rayleigh test; $n = 33,003, 30,870, 9,633, 14,673, 26,474$ and 34,760 spikes for cells B10a, B45a, B3b, B62a, J54a and J67d, respectively) to CA1 gamma oscillations (mean vector length (reflecting phase coupling strength) $r = 0.06 ± 0.04$ at the frequency of strongest coupling, 39 ± 12 Hz; mean ± s.d.; $n = 6$ cells; Fig. 3f) and relative to CA3 gamma oscillations (Fig. 3d–f) from either the local electrode ($r = 0.16 ± 0.09$ at the strongest coupling frequency, 71 ± 24 Hz; $n = 6$; $P = 0.066$, $t_5 = 2.35$; for $r$ compared to CA1, paired $t$-test; Fig. 3f) or a nearby tetrode in CA3 sPyr ($n = 2$; Fig. 3d–f). Variability in firing phase may have resulted from the variable position of the electrode tips (compare Fig. S2a and 3f). Nevertheless, CA3 AACs show significant phase-coupling to gamma oscillations, comparable in strength and phase to those of CA3 pyramidal cells and PVBCs.

Identification of inputs to AAC dendrites

Next we investigated synaptic inputs to AAC dendrites that could shape their firing patterns. Immunoreactivity for the presynaptic active-zone protein bassoon25 showed that 5.7 ± 0.7% (mean ± s.d.) of putative synapses colocalized with VGAT puncta ($n = 215$ bassoon puncta, 3 cells from 3 rats; Fig. 4a), suggesting a relatively small proportion of GABAergic synapses on dendrites. Electron microscopy confirmed that AAC dendrites receive GABAergic synaptic input.
Figure 3  AACs in CA3 fire rhythmically around the peak of theta oscillations and are coupled to gamma oscillations in vivo. (a) Firing rate of AACs (color-coded) as a function of theta phase (sinusoid shown above) recorded locally in CA3 (top) and at a reference site in CA1 (bottom; black trace is the mean). (b) Firing rate versus theta phase (local electrode) for identified AACs recorded from drug-free rats in CA2 (LK24g) and CA1 (TV34n). (c) Firing patterns of LK24g during head movement of the rat. Top to bottom traces: electroencephalogram (EEG); action potentials of the cell; LFP in CA3 pyramidal layer; LFP in CA3 tetrode; Unprocessed spikes (orange). (d) Modulation of spike timing (cell B45a) by the phase of gamma oscillations from a CA3 tetrode. Left, mean vector length (\(|\mathbf{v}|\)) as a function of gamma frequency (15–100 Hz, B5 logarithmically equidistant bins). Right, spike count (color-coded) binned as a function of gamma phase (20 bins per cycle) and frequency (15–100 Hz, B5 logarithmically equidistant bins). White line, mean phase spectrum. (e) Representative LFP and spikes for the same cell as in d. Unprocessed CA3 tetrode LFP (gray), filtered tetrode LFP (gamma, 30–80 Hz, black), spikes (orange). (f) Summary of gamma coupling for all cells recorded in anesthetized rats (color code as in a; B53b, purple). Position of circle represents the frequency at which the coupling is maximal and the mean phase observed at this frequency. Size of circle represents coupling strength (vector length, \(r\)) at the same frequency. Filled, open and dashed circles represent AAC coupling to gamma oscillations recorded by CA1 electrode, local CA3 electrode and a tetrode wire in CA3, respectively (for positions, consult Fig. 2a).

\((n = 22)\) VGAT\(^+\) terminals with synaptic junctions on dendrites of AAC B10a (Fig. 4b). These inputs could originate from the medial septum or from hippocampal interneurons. Two molecular markers differentiating the source of GABAergic terminals are parvalbumin and vasoactive intestinal polypeptide\(^{26}\), but the AAC-dendrite-innervating VGAT\(^+\) puncta were immunonegative for both molecules (data not shown).

Large mossy fiber terminals of dentate granule cells target pyramidal cell spines in sLUC, whereas thin filopodial extensions target interneuron dendrites\(^{27}\). Surprisingly, we have also found synapses from large mossy fiber terminals on identified AAC dendrites, which also targeted the thorny excrescences of pyramidal cells (Fig. 4c and Supplementary Fig. 4). Smaller boutons in sLUC, representing the filopodial extensions, also made synapses on AAC dendrites. From a total of 52 (cell B10a) and 82 (cell B45a) synaptic junctions sampled on 2 dendrites of each cell in sLUC, 6 and 3 synapses were made by large mossy fiber terminals, respectively. The metabotropic glutamate receptor type 7b (mGluR7b) is localized to the presynaptic active zone of mossy fiber terminals predominantly in synapses to interneurons\(^{28}\). However, mGluR7b-immunoreactive puncta were not found around AAC dendrites, in contrast to adjacent highly decorated unlabeled (neurobiotin-lacking) interneuron dendrites (Fig. 4d). These data show that AAC dendrites receive similar mGluR7b-negative glutamateric granule cell input to that of pyramidal cells\(^{29}\).

Immunonegativity for SATB1 identifies PV\(^+\) AACs

Specific transcription factors mark neuronal subpopulations. We investigated the immunoreactivity for special AT-rich sequence binding protein 1 (SATB1; Fig. 5), which labels unidentified hippocampal interneurons\(^{30}\). We found SATB1 immunoreactivity in the nuclei of some PV\(^+\) (Fig. 5a), NPY\(^+\), SOM\(^+\), calbindin\(^+\), preprocholcytokinin\(^+\) and ErbB4\(^+\) interneurons, but not in neurons immunoreactive for neuronal nitric oxide synthase (data not shown). In both CA3 and CA1, some PV\(^+\) neurons, among other, SATB1\(^+\)PV\(^+\) double-positive neurons, lacked detectable immunoreactivity for SATB1 (were SATB1\(^-\)) (Fig. 5b,c).

Of PV\(^+\) interneuron types with somata in or near sPyr—AACs, basket cells and bistratified cells\(^{31}\)—only the bistratified cells express SOM and NPY\(^{13}\). We detected SATB1 immunoreactivity in all PV\(^+\) neurons that were either SOM\(^+\), NPY\(^+\) or double-positive in sPyr of both CA3 (n = 236 cells), CA1 (n = 46 of 46 cells). Of all PV\(^+\) somata located in CA3 sPyr (n = 236 cells), 10.7 \(\pm\) 4.4% (mean \(\pm\) s.e.m. in 4 sections from 3 rats) were immunoreactive for both SATB1 and either SOM or NPY (Fig. 5e). All PV\(^+\) neurons were positive for the GABA\(_{A}\) receptor \(\alpha1\) subunit\(^{32}\) (Fig. 5b–d). Similar proportions of PV\(^+\) somata were observed in CA1 (13.7 \(\pm\) 4.2% SATB1\(^+\)SOM\(^+\), SATB1\(^+\)NPY\(^+\) or SATB1\(^+\)SOM\(^+\)NPY\(^+\); 59.7 \(\pm\) 3.3% SATB1\(^+\) only; 26.5 \(\pm\) 3.8% SATB1\(^-\); n = 362 cells, 4 sections, 3 rats; Fig. 5e). The proportion of PV\(^+\)SATB1\(^+\) neurons is consistent with previous counts of putative PVBCs\(^{31}\). We found similar proportions of PV\(^+\) cells in mouse, suggesting that certain mouse PV\(^+\) interneurons may be homologous to those characterized in the rat\(^{32}\) (mouse CA3: 13.5 \(\pm\) 3.6% SATB1\(^+\)SOM\(^+\); 59.8 \(\pm\) 3.4% SATB1\(^+\) only; 26.7 \(\pm\) 3.8% SATB1\(^-\)).
Innervation of AACs by the medial septum

Medial septal GABAergic neurons innervate diverse types of hippocampal interneurons. We labeled the axons of medial septal neurons with *Phaseolus vulgaris* leucoagglutinin (PHA-L; Fig. 6a). In the medial septum, we observed clusters of labeled neurons, and axonal targets were consistent with known septal projections, including the hippocampus. In a control, PHA-L injected in the lateral septum produced very sparse labeling in the hippocampus.

Septo-hippocampal axons in CA3 and CA1 were of either type I (thick, putatively GABAergic) or type II (thin, putatively cholinergic). Individual type I axons had boutons apposed to the somata and proximal dendrites of some PV+ neurons but they bypassed others, suggesting preferential targeting of distinct interneuron types (n = 41 PV+ neurons innervated in CA3 and CA1, 36 axons; Fig. 6b). Some axons strongly innervated SATB1−PV+ neurons (putative AACs; n = 18 targeted cells in CA3, 17 axons) and bypassed adjacent SATB1+PV+ neurons (Fig. 6c). We also followed single septal axons that targeted other CA3 PV+ interneurons but not putative AACs, and in one case observed two SATB1+PV+ neurons each with seven PHA-L boutons apposed to their somata in a 'basket-like' arrangement (Supplementary Fig. 5a.b). Similarly to those in CA3, SATB1+PV+ neurons in CA1 were targeted by axons (n = 6 cells, 6 axons) that did not innervate nearby SATB1+PV+ neurons (Supplementary Fig. 5d.e). We also immunolabeled CA1 pyramidal neurons using the marker SATB2 (ref. 34) but did not observe any septal axons in sPyr innervate these neurons in a basket-like manner around the somata (Supplementary Fig. 5d). These data suggest a medial septal input preferentially targeting AACs in both CA3 and CA1.

One population of GABAergic septo-hippocampal axons are immunoreactive for parvalbumin in rat. We tested the axons targeting the SATB1−PV+ neurons for parvalbumin and VGAT using confocal microscopy. Two out of ten axons targeting SATB1−PV+ somata were immunoreactive for parvalbumin, in seven axons parvalbumin was undetectable and in one axon we could not test immunoreactivity. Of the septal boutons apposed to SATB1−PV+ somata, 25.0% were PV+ and in 57.7% parvalbumin was undetectable (17.3% were undetermined; n = 104 boutons; 10.4 ± 6.0 per soma observed in fluorescence; mean ± s.d.; Fig. 6c). Notably, five of ten axons targeting SATB1+PV+ neurons showed immunoreactivity for parvalbumin (undetectable in two, undetermined in three; Supplementary Fig. 5c). Most (72.9%) of septal boutons on SATB1−PV+ somata were PV+ (undetectable in 10.4% and undetermined in 16.6%; n = 48 boutons; 4.8 ± 3.2 per soma; Supplementary Fig. 5c). Therefore, compared to other PV+ interneurons using immunofluorescence, putative AACs (SATB1−PV+...
neurons) appeared to be mainly innervated by septal afferents with undetectable levels of parvalbumin. We observed both strongly and weakly parvalbumin-immunoreactive somata in the medial septum (data not shown).

To test the neurotransmitter in soma-innervating septal terminals, we analyzed VGAT-immunoreactivity around PV+ neurons. We observed, for example, a SATB1− soma but not an adjacent SATB1+ soma innervated by PHA-L+ boutons, which contained VGAT puncta (Fig. 6d). GABAergic synapses are rich in the postsynaptic scaffolding protein gephyrin that regulates clustering of GABA_A receptors. Gephyrin-immunoreactive puncta on SATB1−PV+ neurons were aligned with PHA-L+ boutons (Fig. 6e). Finally, we asked whether all apposed septal boutons established synapses with the SATB1−PV+ neurons. We identified PHA-L-labeled boutons in apparent contact with putative AAC somata in the light microscope (Supplementary Fig. 6). Boutons were electron microscopically sampled from three SATB1−PV+ cells (n = 15 of 39 boutons for a cell in CA3, Supplementary Fig. 6a–c; n = 13 of 29 boutons for another cell in CA3; n = 11 of 11 boutons for a cell in CA1), and all were apposed to the plasma membrane of the target cell. Synaptic junctions were found between 11 of 15, 12 of 13 and 10 of 11 boutons and the somata or dendrites (Supplementary Fig. 6f–j), some with multiple active zones. In the remaining boutons, the presence of synapses could not be tested owing to an oblique cutting angle. In no case did the boutons innervate any other cells; they made synapses only with the putative AAC. We conclude that all medial septal boutons made synapses with the target AACs, confirming the prediction derived from the gephyrin-immunoreactive puncta (Fig. 6e). Therefore, a subpopulation of GABAergic septo-hippocampal axons targets SATB1−PV+ but not SATB1+PV+ somata.

**Septo-hippocampal neurons activated during sharp waves**

Some medial septal cells increase their firing during hippocampal SWRs but whether they project to the hippocampus is unknown. We asked whether any GABAergic septo-hippocampal neurons were activated during sharp waves. We extracellularly recorded and juxtaglomerularly labeled single medial septal neurons in anesthetized rats (Fig. 7). From 31 theta-modulated cells (at α = 0.05 with Rayleigh test) that were recorded during >20 SWR events, 12 (38.7%; recorded from 11 rats) showed a variable and significant increase in firing during SWRs (at α = 0.05 with Mann Whitney U-tests), on average from 12.0 ± 9.9 Hz peri-SWR to 28.5 ± 15.6 Hz (mean ± s.d.; Fig. 7a and Supplementary Table 1). In some cases, the SWR-activated cells fired rhythmically during non-theta periods (not analyzed here), and we also observed activation during events that remained below the threshold for SWR detection with our criteria at the CA1 recording site (Fig. 7c). Five of the 12 cells could be labeled and identified, with 5 of 5 and 4 of 4 immunopositive for parvalbumin and hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4), respectively (Fig. 7a, Supplementary Fig. 5g and Supplementary Table 1). We note that parvalbumin immunoreactivity in the septum does not necessarily imply parvalbumin immunoreactivity in axonal boutons in distant target areas. Notably, 4 of 4 cells were also immunoreactive for SATB1. Of four cells, which were well labeled, two had axons in the fimbria with collaterals entering CA3 (ref. 35) (Fig. 7b and Supplementary Table 1), but their targets could not be identified. The other two cells had axons in the dorsal fornix and may have projected to the cortex; one of these cells had additional minor branches that entered medial CA1 and subiculum. Septal neurons (3 of 3 tested; Supplementary Table 1) were VGAT+ in their local medial septal terminals (Fig. 7d) and were thus GABAergic.
All 12 cells that showed increased firing during SWRs were strongly phase-locked to different phases of theta oscillations (vector lengths of 0.46–0.76; Supplementary Fig. 5h,i and Supplementary Table 1). Interestingly, the two CA3-projecting cells fired in a counter-oscillatory phase manner to the firing of hippocampal AACs (Fig. 3a), preferentially at the trough of CA1 theta oscillations (vector length of 0.46; Supplementary Fig. 5g). We have demonstrated that the firing of CA3 AACs is suppressed in levels of parvalbumin immunoreactivity in PHA-L-labeled septohippocampal terminals targeting SATB1−PV+ somata and the differing firing patterns of septal cells, inhibition of AAs may be mediated by more than one medial septal cell type.

DISCUSSION

We have demonstrated that the firing of CA3 AAs is suppressed during sharp waves in vivo, resulting in a temporally and spatially restricted reduction of GABA release on the AISs of CA3 pyramidal cells. This is consistent with a key role of AAs in coordinating cell...
assembly reactivation by allowing excitatory interactions in the CA3 recurrent system selectively during sharp waves. We propose that the inhibition of AACs causes a subcellular redistribution of inhibition from the AIS to the soma and dendrites of pyramidal cells, creating conditions permissive for sharp wave initiation.

We show that the somata of AACs are innervated by a subset of GABAergic medial septal cells that do not target nearby SATB1+PV+ hippocampal neurons and that AACs also receive some dendritic inhibition. Although we recorded SWR-activated septal cells that were immunopositive for parvalbumin, levels of this marker in PHA-L-labeled septal terminals targeting AACs varied. Subsets of septal GABAergic cells that project to CA3 fired strongly around the trough of theta oscillations and could innervate AACs. Silencing AACs leads to rhythmic disinhibition of pyramidal cell AISs every 90–200 ms, which could contribute to the theta dipole across hippocampal strata, resulting in the population of pyramidal cells firing with highest probability at theta trough, the minimal firing phase of this cell type, along with strongly activated bistratified cells targeting PVBCs. As SATB1 is expressed by some septo-hippocampal neurons in rats and is also found in the mouse medial septum, recording from similar neurons in both wild-type and SATB1 mutant mice might provide clues as to how they influence sharp wave generation. To our knowledge, SATB1 represents the first molecular marker that differentiates AACs and PVBCs in the hippocampus, and the septal input represents the first evidence of an identified source of GABAergic input to a single type of hippocampal interneuron. The firing increase of PVBCs and decrease of AACs at the onset of SWRs causes a subcellular redistribution of inhibition in pyramidal cells. According to this concept, AACs, acting as gatekeepers, select and control the firing of CA3 pyramidal cell assemblies during the replay of memory sequences.

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
T.J.V., B.L., I.K., M.G.C., J.J.T., T.K. and P.S. collected and analyzed data and wrote the paper. To expand on the equal-contributions footnote, each of the first four authors made important—though different—contributions, and hence they should be considered equal first authors.

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Spectra for all spikes but those during SWRs were included. For spike phase spectra (15–100 Hz; extracted with wavelet transformation), as previously described23, Rayleigh test23, taking the mean phase and mean vector length as descriptive statistics for phase preference and coupling strength, respectively. Cell 5154a was excluded from mean CA1 theta phase calculations because the CA1 electrode was in sRad instead of sPyr. Cell B53b was excluded from mean firing rate calculations because of unusually deep anesthesia.

To identify effects of SWRs on firing rates, the distribution of the numbers of action potentials during SWRs were compared to the distribution in identical time windows randomly placed 100 times in the ±10-s vicinity of the SWR (with other SWRs excluded; peri-SWR data)23. Distributions were compared by a Mann–Whitney U-test.

Tissue processing. After transcardial perfusion (4% paraformaldehyde, 15% v/v saturated picric acid and 0.05% glutaraldehyde in 0.1 M PB), brains were stored in 0.1 M PB with 0.05% sodium azide (PB-Az) at 4 °C. Coronal sections (70 µm; Leica VT 1000S vibratome) were washed three times in 0.1 M PB and stored in PB-Az at 4 °C. To find neurobiotin-labeled neurons, sections were either permeabilized in Tris-buffered saline (TBS) with 0.1% Triton X-100 (Tx) or subjected to one or two rounds of freeze-thaw over liquid nitrogen after cryoprotecting in 20% sucrose. After three or four washes in 0.1 M PB, sections were incubated in 1:1,000 streptavidin-conjugated Alexa Fluor 488 (Invitrogen) in TBS or TBS-Tx for 4 h at room temperature (RT) or overnight at 4 °C. After three washes, sections were mounted on slides in VectaShield (Vector Laboratories). For analysis, electron microscopy and reconstruction, sections were processed with the horseradish peroxidase (HRP) reaction to reveal neurobiotin with a diaminobenzidine (DAB) reaction product. Sections were incubated in 1:100 avidin–biotinylated peroxidase complex (ABC; Vectastain ABC Elite kit, Vector Laboratories) in TBS-Tx or TBS for 2–3 d at 4 °C. If sections were previously incubated in streptavidin, sections were additionally incubated with the biotinylated peroxidase complex for 4 h at RT or overnight at 4 °C before incubating in ABC. After three washes in 0.1 M PB, sections were incubated in a 0.5 mg/ml DAB (Sigma-Aldrich) solution containing glucose, ammonium chloride and 0–4% nickel ammonium sulfate. After 15 min, 0.2% glucose oxidase was added to generate H2O2 for the oxidation of DAB by peroxidase, forming a blue-black precipitate in the presence of nickel. After 40–60 min, depending on the darkness of the reaction product, sections were washed four times in 0.1 M PB. Next, sections were incubated in 0.2–1% osmium tetroxide in 0.1 M PB for 1 h and washed three times in 0.1 M PB. Sections were dehydrated in successive rounds of ethanol (50%, 70%, 90%, 95%, 100%), followed by propylene oxide (VWR International). Between the 70% and 90% ethanol washes, sections were incubated for 40 min in 2% uranyl acetate in 70% ethanol to enhance contrast for electron microscopy (see below). Next, sections were submerged in epoxy resin (Durcupan ACM Fluka, Sigma–Aldrich) and left overnight. Finally, sections were mounted on slides under a coverslip and the resin was polymerized at 60 °C for ~24 h.

lot 72156, 715-175-151 lot 88814, 706-175-148 lot 72155, respectively; donkey anti-rabbit, anti-goat or anti-mouse DyLight 649 (1:250, Jackson; 711-495-152 lot 98016, 705-495-147 lot 97534, 715-495-151 lot 94507, respectively), donkey anti-sheep or anti–guinea pig Alexa Fluor 647 (1:250, Jackson; 713-605-147 lot 102770, 706-605-148 lot 100905, respectively); donkey anti–guinea pig HRP (1:100, Jackson); and swine anti–rabbit HRP (1:100, Dako, Glostrup, Denmark; P0217). After 4 h at room temperature or 4 °C overnight, sections for immunofluorescence were washed three times for 10 min each and mounted on glass slides in VectaShield. For two-color DAB-based HRP enzyme reactions (rabbit anti-VGAT and neurobiotin), freeze-thawed sections were incubated in primary antibody as above, processed with ABC and then processed with DAB (without Ni2+) and glucose oxidase to react the neurobiotin-labeled cell. Next sections were washed four times in 0.1 M PB and incubated overnight in anti-rabbit HRP secondary antibody solution. Sections were processed for DAB but using H2O2 as the substrate directly for 2–7 min. Processing continued as above. In experiments that used the biotinylated anti–PH-A L primary antibody, streptavidin Alexa Fluor 488 was used for fluorescence visualization followed by conversion with DAB as above (4% nickel ammonium sulfamate).

Confocal imaging. An LSM710 confocal microscope (Axio Imager.Z1, © 2013 Nature America, Inc. All rights reserved.) was used to acquire multi-channel fluorescence images. DIC M27 Plan-Apochromat 63×/1.4 n.a. and alpha Plan-Apochromat 100×/1.46 n.a. immersion objectives were used with the following channels (laser and excitation wavelength, fluorophores, beam splitters, emission spectral filter): 405-30 solid state 405 nm with attenuation filter NF04, DyLight405, MBS-405, 409–499 nm; argon 488 nm, Alexa Fluor 488, MBS-488, 493–542 nm; HeNe 543 nm, Cy3, MBS-458/543, 552–639 nm; HeNe 633 nm, Cy5/DyLight 649/Alexa Fluor 647, MBS 488/543/633, 637–757 nm. Channels were acquired sequentially with an eight-bit dynamic range, and the pinhole size was set at 1 Airy unit for the shortest wavelength used; the pinhole sizes of the other channels were adjusted to values <1 Airy unit, giving all channels the same optical slice thickness. With the 63× lens and the 405-30 laser, the optical slice thickness was typically 0.6 µm. Using the argon laser as the shortest excitation wavelength, 1 Airy unit gave an optical slice thickness of 0.7 µm. Pixel number was calculated with Nyquist sampling, optimized for the channel having the shortest wavelength. At 63× with the 405-30 laser, sampling resolution was typically 12.686 pixel µm−1, 0.08 × 0.08 × 0.43 µm voxels. At 100×, sampling was 13.173 pixel µm−1. We used the 63× lens for the majority of 3D images due to higher transmission, including for montages (zoomed out by 0.8). Assignment of antibody fluorophores was based on minimizing spectral overlap between channels. Synaptic markers were assigned the highest resolution channel. In some cases where markers showed no spatial overlap, two markers were sometimes labeled with the same fluorophore, increasing the number of markers used within one section: for example, gephyrin DyLight405 (post-synaptic puncta); SATB2 and PHA-L AF488 (nuclei and axons); SATB1 and NPY Cy3 (nuclei and Golgi apparatus); and parvalbumin DyLight649 (cytoplasm). Images were analyzed in ImageJ. Deconvolution (ZEN 2008) was used before quantifying bassoon puncta.

Quantification of immunolabeled neurons. To quantify SATB1 immunoreactivity in PY+ neurons, cells in dorsal CA1 and CA3 of 70-µm-thick sections (from both adult rats and C57/B6 mice) were counted using widefield epi-fluorescence on a Leitz DMRB microscope (Leica) equipped with PL Fluotar objectives. We counted only cells that had whole somata within sPyr, with the condition that the nucleolus must be visible in the fluorescence channel corresponding to parvalbumin. Cells were marked SATB1-immunonegative only when the mean pixel intensity of the nuclear region was the same as a background region of interest in the SATB1 channel. To confirm immunopositivity or immunonegativity, counted cells were sometimes scanned with the confocal microscope.

To quantify parvalbumin immunoreactivity in PHA-L-labeled septal axon terminals in the hippocampus, confocal microscopic z-stacks were analyzed. As a positive control, reference PHA-L-labeled axons immunopositive for parvalbumin were located at different depths in the z-stack of each brain section. Axons deep in the stack were excluded because of the lower signal-to-noise ratio. “Immunopositive” was defined as positive pixels in the axon or in axon boutons, similar to those in the positive control, compared to the background. “Undetectable” defines the limit of our method; we cannot rule out low parvalbumin immunoreactivity. “Undetermined” corresponds to PHA-L-labeled axons or axon boutons that were obscured by the parvalbumin immunoreactivity of larger structures.

Electron microscopy. Target areas (for example, axon-rich regions, or dendrites in sLUC) were cut from the resin-embedded 70-µm-thick sections and reembedded for ultramicrotome sectioning. Serial electron microscopic sections were cut and mounted on single-slot, piezoform-coated copper grids. A Philips CM100 transmission electron microscope fitted with a Gatan UltraScan 1000 CCD camera was used to acquire images. All boutons cut by the section plane were followed in serial sections and searched for synaptic junctions. AISs were identified as previously described11. All synapses found innervated AISs; none of the boutons could be shown to make a synapse with a dendrite or a soma. As the AISs are relatively small in diameter and may have tortuous courses, the membrane of boutons and the apposed AIS membrane is cut at an oblique angle in many cases, which makes it impossible to reveal the synaptic cleft even with tilting the sections in the electron microscope. Such cases were not counted. Initial identification of axon cartridges23 at the light microscopic level (and their association with AISs using ankG immunogold) correlated with the electron microscopic results. Mossy fibers were scanned in the vicinity of AAC dendrites in sLuc. Only clearly identified synapses were quantified. Nonlinear contrast adjustment was applied to all images.

Digital reconstruction and quantification. The full dendritic trees of three AACs were reconstructed in 3D from the series of nominally 70-µm-thick coronal sections. Dendrites were traced using NeuroLucida (v9, MBF Bioscience) on a Nikon Eclipse 80i microscope equipped with a Lucivid and a 100× 1.4 n.a. oil immersion Plan Apo VC objective lens. Laminar boundaries were drawn for all sections in NeuroLucida and crossing points for all dendrites were checked individually and recorded. The outline of each section was also traced as a general reference for rotational alignment and confirmation of shrinkage correction. To aid the delineation of laminar and areal boundaries, immunoreactivity to mGlurR2/3 (for sRad–sLacMol and sLuc–sRad)28 and calbindin (for sLac–sRad and CA3–CA2)53 was sometimes tested on the sections with immunofluorescence and in immunoperoxidase reactions. Because of tissue shrinkage, each section was expanded to its original unprocessed dimensions by applying shrinkage correction ratios, whenever possible using individual section values. In order to compensate for z-axis shrinkage, the section thickness was measured by visualizing the top and bottom surfaces of the tissue using DAPI staining, a Colibri 405-nm LED and the Axio Imager. Z1 microscope with the 100× 1.46 n.a. oil immersion objective lens. The mean thickness was calculated for each section from at least five z measurements taken along CA1, CA3 and dentate gyrus. Postprocessing (epoxy resin–embedded) section thickness was measured in NeuroLucida. In some cases, the correction was done using the mean preprocessing thickness calculated from nondendritic sections. The same methods were applied for the lateral dimensions from images taken before and after processing.

Dendrites of B45a, B10a, J54a spanned 11, 11 and 9 consecutive sections, respectively. For B45a, freeze-thawed and Triton-treated dendritic sections had mean z dimension shrinkage correction ratios of 1.31 ± 0.07 (s.d.) and 1.40 ± 0.1, respectively. For x-y dimensions, the mean ratio for the freeze-thawed dendritic sections was 1.06 ± 0.02; dimensions of Triton-treated dendritic sections were scaled manually in NeuroLucida to match the other corrected sections. For B10a, the mean shrinkage correction ratios were 1.07 ± 0.17 (freeze-thawed dendritic sections) and 1.27 ± 0.15 (Triton-treated dendritic sections) for the z dimension. For x-y dimensions, mean ratios were 1.05 ± 0.02 (freeze-thawed) and 1.08 ± 0.01 (Triton-treated); four dendritic sections were scaled manually. For J54a, the mean shrinkage correction ratios were 1.13 ± 0.11 (freeze-thawed) and 1.20 ± 0.14 (Triton-treated) for the z dimension. For x-y dimensions, the mean shrinkage correction ratios were 1.04 ± 0.02 (freeze-thawed) and 1.12 ± 0.03 (Triton-treated).

The total dendritic length per stratum in the shrinkage-corrected and spliced reconstructions was calculated using NeuroLucida Explorer. For figures, reconstructions were rotated to obtain a semi-coronal projection, providing the maximum overlap between corresponding laminar boundaries from consecutive sections. A representative part of the B45a axon, initially drawn on

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paper with a drawing tube, was photographed and superimposed onto the reconstructed dendrites.


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Erratum: Network state-dependent inhibition of identified hippocampal CA3 axo-axonic cells in vivo

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