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Characterization of a Novel Subtype of Hippocampal Interneurons That Express Corticotropin-Releasing Hormone

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Abstract

A subset of corticotropin-releasing hormone (CRH) neurons was previously identified in the hippocampus with unknown function. Here we demonstrate that hippocampal CRH neurons represent a novel subtype of interneurons in the hippocampus, exhibiting unique morphology, electrophysiological properties, molecular markers, and connectivity. This subset of hippocampal CRH neurons in the mouse reside in the CA1 pyramidal cell layer and tract tracing studies using AAV-Flex-ChR2-tdTomato reveal dense back-projections of these neurons onto principal neurons in the CA3 region of the hippocampus. These hippocampal CRH neurons express both GABA and GAD67 and using *in vitro* optogenetic techniques, we demonstrate that these neurons make functional connections and release GABA onto CA3 principal neurons. The location, morphology, and importantly the functional connectivity of these neurons demonstrate that hippocampal CRH neurons represent a unique subtype of hippocampal interneurons. The connectivity of these neurons has significant implications for hippocampal function.

Keywords

GABA; interneuron; hippocampus; CRH; inhibition

INTRODUCTION

Corticotropin-releasing hormone (CRH) neurons have been identified in the hippocampus of both mice and rats (Swanson et al., 1983; Merchenthaler, 1984; Yan et al., 1998; Chen et al., 2001) [for review see (Chen et al., 2012; Maras and Baram, 2012)]. This unique subset of neurons in the hippocampus was identified by immunolabeling for CRH, but much remains to be discovered about the function of these neurons and their impact on the hippocampal network. Outside the hippocampus, in the paraventricular nucleus (PVN) of the hypothalamus, CRH neurons play a critical role in mounting the physiological response to stress [for review see (Ulrich-Lai and Herman, 2009)]. However, it remains unclear whether these neurons serve as stress mediators in extrahypothalamic regions.

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The hippocampus is particularly vulnerable to the effects of stress and prolonged glucocorticoid exposure, resulting in changes in hippocampal network excitability, due to altered synaptic plasticity, dendritic atrophy, and a loss of neurogenesis (Joels and Krugers, 2007; Joels and Baram, 2009; McEwen and Gianaros, 2011). The majority of these studies have focused on the impact of peripherally derived stress hormones, but the adverse effects of stress on the hippocampus may also involve local CRH signaling (Chen et al., 2010) [for review see (Chen et al., 2012)]. Interestingly, stress has been demonstrated to increase the expression of CRH in the hippocampus (Givalois et al., 2000) and CRH immunoreactivity in the neuropil in CA3 (Chen et al., 2004) [for review see (Chen et al., 2012)], suggesting a potential role for local stress-induced CRH signaling in the hippocampus. Insight into the mechanisms underlying the adverse effects of stress on the hippocampus will have significant implications for understanding the pathophysiology of stress-related disorders. However, to date, the role of local CRH signaling has been largely unexplored.

Although the effects of CRH on the hippocampus are thought to be largely excitatory (Hollrigel et al., 1998) [for review see (Baram and Hatalski, 1998)], hippocampal CRH neurons also express GAD67 (Chen et al., 2001, 2004), demonstrating that they may be GABAergic. To date, the existence of these neurons has only been demonstrated using immunohistochemistry, making it impossible to assess their function. The objective of the current study is to characterize a subset of hippocampal CRH neurons whose cell bodies reside in the CA1 pyramidal cell layer, which has been hindered by the inability to identify or specifically activate this subset of neurons in the hippocampus. We have overcome these technical limitations by developing mice which express GFP or tdTomato specifically in CRH neurons (Crh-GFP or Crh-Ai9 mice, respectively) (Sarkar et al., 2011) to easily identify this subset of neurons within the hippocampus. In addition, we generated mice which express Channelrhodopsin in CRH neurons (*ChR/Crh* mice), enabling us to specifically activate this subset of neurons. Using these tools, we demonstrate that hippocampal CRH neurons are back-projecting interneurons in the hippocampus which release GABA onto principal neurons in CA3. The location, morphology, electrophysiological properties, and connectivity of these interneurons suggest that hippocampal CRH neurons represent a novel subtype of hippocampal interneurons.

MATERIALS AND METHODS

Animals

Adult (8–12 week old) mice of either sex were housed at the Tufts University School of Medicine, Division of Laboratory Animal Medicine, in clear plastic cages (five mice/cage) in a temperature- and humidity-controlled environment with a 12 h light/dark cycle (lights on at 0700 h) and *ad libitum* access to food and water. Animals were handled according to protocols approved by the Tufts University Institutional Animal Care and Use Committee.

Mice which express GFP specifically in CRH neurons were characterized in a previous publication (Crh-GFP mice) (Sarkar et al., 2011). Mice which express tdTomato specifically in CRH neurons (Crh-Ai9 mice) were generated by crossing floxed Ai9 mice (Jackson Lab, Stock #007909) with the Crh-Cre mice [obtained from the Mutant Mouse Regional Resource Center (MMRRC) and described in (Sarkar et al., 2011)]. Floxed

Channelrhodopsin mice were obtained from Jackson Lab (Stock # 017455) and crossed with Crh-Cre mice to generate mice in which Channelrhodopsin and tdTomato are expressed specifically in CRH neurons (*ChR/Crh* mice).

Single-Cell PCR

The CRH-Cre mice were generated by insertion of the coding sequence for Cre recombinase into the mouse genomic BAC after the ATG transcription initiation codon of the corticotropin releasing hormone gene (Gong et al., 2007). This BAC transgene inserts at random into the genome and therefore is unlikely to disrupt the endogenous gene. Therefore, single-cell PCR was used to detect the full length endogenous CRH transcript.

Corticotropin-releasing hormone expression in hippocampal CRH neurons was confirmed using single-cell PCR performed on the cellular contents harvested from either Crh-GFP or adjacent GFP-negative CA1 pyramidal neurons as previously described (Luther et al., 2002; Sarkar et al., 2011). The primers and experimental details for the single-cell PCR for CRH were performed as described in a previous manuscript (Sarkar et al., 2011). The cytoplasm of GFP-positive or GFP-negative neurons in the CA1 pyramidal cell layer was aspirated into the patch pipette without aspiration of the nucleus. The cytoplasmic contents were transferred to a 0.5 ml PCR tube containing 2.9 μ l of DEPC-treated water, 1.4 μ l of BSA, 1.4 μ l of oligo-dt (0.5 μ g μ l⁻¹), and 1.4 μ l of RNasin (40 U μ l⁻¹), heated to 70°C for 10 min and then placed on ice for 1 min. RT-PCR was performed by adding 8 μ l of DEPC-treated water, 2 μ l of 10 $\times$ first strand buffer, 2 μ l of MgCl₂ (25 mM), 2 μ l of DTT (0.1 M), 1 μ l of dNTPs (10 mM), and 0.7 μ l of SuperScript III reverse transcriptase (200 U μ l⁻¹) and incubated at 42°C for 50 min. The reaction mixture was incubated at 70°C for 15 min and RNase H (0.5 μ l of 2 U μ l⁻¹) was added and incubated at 37°C for 20 min. PCR was performed by adding 5 μ l of primer mix (2 μ M each), 15 μ l of RNase-free water, and 5 μ l of cDNA template and cycling through 50 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 70 s. Total brain, control cDNA (Clontech) was used as a positive control. Reactions without specific primers were used as negative controls. The primers used were as follows:

CRH	F	GGGGAAAGCAAAGAAAAGG
	R	GACAGAGCCACCAGCAGCAT
VGlut2	F	CCCGCAAAGCATCCAACCA
	R	TGAGAGTAGCCAACAACCAGAAGCA
GAD65	F	TCTTTTCTCCTGGTGGTGCC
	R	CCCCAAGCAGCATCCACAT
Parvalbumin	F	CGGATGAGGTGAAGAAGGTGT
	R	TCCCCATCCTTGTCTCCAGC
CCK	F	ATACATCCAGCAGGTCCGCAA
	R	CAGACATTAGAGGCGAGGGGT
Somatostatin	F	GCATCGTCCTGGCTTTGGG
	R	GGGCTCCAGGCATCATTCT

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Lee and Maguire, 2013). Immunostaining was performed on Crh-GFP, Crh-Ai9, or on Crh-Cre mice injected with AAV-Flex-ChR2-tdTomato. Mice were anesthetized with isoflurane, euthanized by decapitation, the brain rapidly removed and fixed by immersion fixation overnight at 4°C. For the CRH immunostaining, mice were transcardially perfused with 4% paraformaldehyde prior to immersion fixation to prevent detection of circulating CRH. The brains were cryoprotected in a series of 10–30% sucrose in phosphate buffered saline, rapidly frozen in isopentane on dry ice, and stored at –80°C until 40 µm sections were prepared using a Leica cryostat. Sections were incubated with a monoclonal antibody against GAD67 (1:1,000, mouse, Millipore), GABA (1:5,000, rabbit, Sigma), parvalbumin (1:1,000, mouse, Sigma), somatostatin (1:1,000, rat, Millipore), calbindin (1:5,000, mouse, Cell Signaling or Synaptic Systems), calretinin (1:5,000, mouse, SWANT), or CRH (1:10,000, rabbit, provided by Dr. Paul Sawchenko on behalf of Dr. Wylie Vale) followed by either anti-rabbit, anti-rat, or anti-mouse Alexa 488 or 647 (1:200, Invitrogen). Images were collected on a Nikon A1R confocal microscope and colocalization was determined using the Just Another Colocalization Plugin (Bolte and Cordelieres, 2006) for Image J as previously described (Sarkar et al., 2011). For the GAD67 immunostaining, colchicine (50 µg in a 2 µl volume) was injected unilaterally into the lateral ventricle using the following stereotaxic coordinates (0.5mm posterior, 1-mm lateral, 2-mm depth) 24 h prior to perfusion with 4% paraformaldehyde. This treatment was required to visualize the immunoreactivity in the cell body of hippocampal CRH neurons rather than the terminals.

Tract Tracing

Tract tracing was performed using a protocol adapted from (Atasoy et al., 2008). Adult Crh-Cre mice were stereotaxically injected with AAV-Flex-ChR2-tdTomato (Addgene plasmid 18917 produced by Dr. Scott Sternson; virus obtained from the Penn Vector Core). The stereotaxic coordinates used for targeting the CA1 region of the hippocampus were as follows: 2.0-mm posterior; 1.5-mm lateral; 1.0-mm depth. A 5 µl model 75 Hamilton syringe was used to inject 1.5 µl of virus (500 nl per minute over 3 min) into each hemisphere. Following injection, the syringe was left in the injection site for 5 min before withdrawal. Animals were sacrificed and their brains harvested 2 weeks after virus injection to allow maximal expression. The labeled neurons and projections were visualized using a Nikon A1R confocal microscope.

Electrophysiology

Slices were prepared from adult mice 10–12 weeks of age and electrophysiological recordings were performed as previously described (Sarkar et al., 2011; Lee and Maguire, 2013). Both coronal and transverse 350-µm slices were used. Current clamp recordings were performed on visually identified GFP-or tdTomato-labeled CRH neurons from Crh-GFP or Crh-Ai9 mice, respectively, or in tdTomato-expressing neurons from *ChR/Crh* mice residing in the CA1 pyramidal cell layer using electrodes with DC resistance of 5–8 MΩ and an intracellular solution containing 130 mM potassium gluconate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.25; 280–290

mOsm). For biocytin filling, microelectrodes contained 2% neurobiotin (Vector Laboratories) and 100 ms, 1nA current pulses were injected at 5 Hz for a minimum of 20 min. The electrophysiological profile of CRH neurons from Crh-GFP or Crh-Ai9 mice and CA1 pyramidal neurons (GFP or Ai9-negative) was examined in response to a series of 1s current injections from -100pA to +100pA in 20pA steps. The average input-output data for each experimental group was fit with a Boltzmann function: $f(I) = \text{MAX} / (1 + \exp(-(I_{50} - I)/k))$, where I is current injected, MAX is the maximum response, k is a slope factor, and I_{50} is the current injection amplitude that elicits 50% of MAX. To calculate the action potential threshold, a linear regression was fit to the first four non-identical data points. The properties of the action potential were assessed using phase plane plots where the first derivative of the voltage is plotted against the voltage. The input resistance was calculated using the Nernst equation by measuring the change in voltage in response to the -100 pA current injection. The spontaneous firing rate of hippocampal CRH neurons from *ChR/Crh* mice was measured over a 5-min period under laser off conditions and then measured over a 5 min period in the presence of optogenetic stimulation with a fiber optic (200 μ m, Thor Labs) placed above the CA1 region of the hippocampus, delivering 100 ms, 1 Hz pulses with a 473-nm laser.

Voltage clamp recordings were carried out in CA3 neurons from *ChR/Crh* mice using an intracellular recording solution containing: 140 mM cesium methanesulfonate, 10 mM HEPES, 5 mM NaCl, 0.2 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.25; 280–290 mOsm). Electrodes with DC resistance of 5–8 M Ω were used for recording spontaneous EPSCs at $V_H = -60$ mV and IPSCs at $V_H = 0$ mV. A fiber optic (200 μ m, Thor Labs) was placed into either the CA1 or CA3 region of the hippocampus of *ChR/Crh* mice and *Cre*^{-/-} littermates to activate the CRH-positive neurons impinging on principal neurons. The latency and peak amplitude of events was measured in a 1 min window throughout the 1Hz optogenetic stimulation (60 events). The latency to the evoked IPSC was measured from the beginning of the optogenetic stimulation. Events which occurred within 10 ms of the laser stimulation were considered to be evoked responses. The evoked responses were identified based on amplitude and kinetics of the response which differed from the spontaneously recorded events. SR95531 (>200 μ M) and kynurenic acid (3 mM) were added where indicated to confirm that the evoked responses were mediated by GABA. Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance was >20 M Ω or increased by >20% during the course of the experiment.

Statistical Analysis

Either paired or unpaired Student's t tests were used to determine statistical significance between experimental groups. Data is presented as mean $\pm$ SEM. All statistical tests were performed using Prism 6.0 software (GraphPad).

RESULTS

Identification of Hippocampal CRH Neurons

We previously characterized a mouse model which expresses GFP specifically in CRH neurons (Crh-GFP mice) (Sarkar et al., 2011). We utilized this mouse model to further characterize a subset of hippocampal CRH neurons whose cell bodies reside in the CA1 pyramidal cell layer (Fig. 1a), as previously described (Yan et al., 1998; Chen et al., 2001). In addition to the GFP-positive cell body labeling in the CA1 region of the hippocampus (Fig. 1a1), there are dense GFP-labeled CRH terminals observed in the CA3 region of the hippocampus (Fig. 1a2). We also generated Crh-Ai9 reporter mice, which express tdTomato specifically in CRH neurons (data not shown), exhibiting a similar pattern of expression to Crh-GFP mice. We utilized both of these reporter mice to further characterize this subset of hippocampal CRH neurons.

To demonstrate that the GFP- and tdTomato-expressing neurons in the hippocampus of Crh-GFP and Crh-Ai9 mice, respectively, do in fact express CRH, we performed immunolabeling for CRH as well as single-cell PCR on hippocampal CRH neurons. Immunoreactivity for CRH was detected (b2, green) in $40.1\% \pm 5.8\%$ of tdTomato-positive neurons (b1, red) (Fig. 1b) ($n=232$ hippocampal CRH neurons, four mice). These data demonstrate that Ai9-positive CRH neurons exhibit colocalization with CRH (b4, yellow). We also observe a population of CRH-immunopositive cells in the CA1 region of the hippocampus which do not express the Ai9 reporter ($37.6\% \pm 6.5\%$), possibly indicating that our reporter mice are identifying a subpopulation of these hippocampal CRH neurons. Consistent with the notion that our reporter is only labeling a subset of hippocampal CRH neurons, rather than exhibiting ectopic expression of the reporter, we observe colocalization with parvalbumin (PV) in $18.3\% \pm 3.6\%$ of neurons reactive for endogenous CRH in the hippocampal CA1 subregion; however, our reporter does not exhibit colabeling with this population of PV-positive CRH neurons. To further assess CRH expression in hippocampal Ai9-expressing neurons, we performed single-cell PCR on the contents of a subset of labeled hippocampal CRH neurons with their cell bodies in CA1 and adjacent unlabeled CA1 pyramidal neurons. Single-cell PCR confirms that the GFP- and tdTomato-positive hippocampal CRH neurons express transcript for CRH, whereas nearby CA1 pyramidal cells do not. Our data demonstrate that $100\% \pm 0.0\%$ of GFP-labeled hippocampal CRH neurons express CRH mRNA (Fig. 1c1). In contrast, $0.0\% \pm 0.0\%$ of neighboring CA1 pyramidal neurons express transcript for CRH (Fig. 1c1) ($n=5-12$ cells per experimental group). In addition, these neurons express transcripts for glutamate decarboxylase (GAD65, 71.4%), somatostatin (SOM, 28.6%), cholecystokinin (CCK, 100%), but not parvalbumin (PV, 14.3%) or the vesicular glutamate transporter 2 (VGlut2, 0%) (Fig. 1c2). The finding that neurons expressing the reporter also express transcript for CRH and exhibit colocalization with CRH immunolabeling validate this model for further characterization of this subset of hippocampal CRH neurons.

Molecular Markers Expressed in Hippocampal CRH Neurons

Consistent with previous reports suggesting that hippocampal CRH neurons are GABAergic (Yan et al., 1998), $93.1\% \pm 2.5\%$ of Crh-Ai9 neurons in the hippocampus colabeled with

GABA (Fig. 2a; Table 1) ($n=290$ cells, 4 mice) and $56.4\pm1.6\%$ with GAD67 (Fig. 2b, Table 1) ($n=202$ cells, three mice). Interneurons in the hippocampus are classified, in part, based on the expression of molecular markers. None of the hippocampal CRH neurons labeled in this model express PV ($0.0\pm0.0\%$) (Fig. 2c, Table 1) ($n=167$ cells, four mice). However, $39.5\pm4.0\%$ express SOM, $23.2\pm8.4\%$ express calretinin (CR), and $26.5\pm7.6\%$ express calbindin (CB) (Figs. 2d–f, Table 1; $n=167$ – 290 cells, four mice per experimental group).

Electrophysiological Profile of Hippocampal CRH Neurons

Interneurons are further classified based on their electrophysiological profile, including firing properties. We assessed the electrophysiological profile of hippocampal CRH interneurons by measuring the input-output curves and firing properties of GFP- or Ai9-expressing CRH neurons in the CA1 pyramidal cell layer in response to a series of current injections 1s in duration from -100 pA to $+100$ pA, in 20 -pA steps (Fig. 3). Representative responses to -100 pA and $+100$ pA current injections are shown in Figure 3b. Hippocampal CRH neurons and CA1 pyramidal cells were filled with biocytin and the morphology of the neurons is shown in Figures 3j–o. Phase plane plots were generated to assess the properties of the action potentials in hippocampal CRH neurons and CA1 pyramidal neurons (Fig. 3c, Table 2). There were no obvious differences in the properties of the action potentials, including action potential threshold, action potential peak, AP rise time, or the peak of the after hyperpolarization between these cell types. Values for all these parameters are provided in Table 2 and are similar to previously reported values for CA1 pyramidal neurons (Reid et al., 2014). However, hippocampal CRH neurons were significantly less excitable compared to CA1 pyramidal neurons, exhibiting a shift in the input-output curve (CA1: $W_{50}=54.5$; CRH: $W_{50}=91.6$) (Figs. 3e–f) and a decrease in the maximum number of action potentials elicited in response to a $+100$ mV current injection (CA1: 31.0 ± 6.3 ; CRH: 16.8 ± 4.7) (Fig. 3g). Hippocampal CRH neurons also exhibit a depolarized resting membrane potential (RMP) (-57.7 ± 3.2 mV) compared to CA1 pyramidal neurons (-68 ± 1.1 mV) (Fig. 3d). The latency to evoke an action potential was delayed in hippocampal CRH neurons (25.8 ± 9.0 ms) compared to CA1 pyramidal neurons (12.7 ± 2.3 ms) (Fig. 3h). The decrease in the excitability of hippocampal CRH neurons may be due, in part, to a decrease in the input resistance (R_i) of these neurons (32.1 ± 3.9 MOhm) compared to CA1 pyramidal neurons (53.7 ± 5.0 MOhm) (Fig. 3i) ($n=14$ – 15 cells, 7–8 mice; $P<0.05$ using an unpaired Student's t test). Interestingly, biocytin labeling of hippocampal CRH neurons which reside in stratum pyramidale resulted in biocytin expression in neighboring tdTomato-positive CRH neurons, consistent with gap junction coupling of these neurons (Fig. 3m). In contrast, biocytin filling of CA1 pyramidal neurons resulted in biocytin expression restricted to the filled neuron (Fig. 3j). These data are the first steps in the classification of interneurons in the hippocampus expressing CRH and distinguishing these neurons from surrounding, morphologically similar CA1 pyramidal neurons.

Connectivity of Hippocampal CRH Neurons

To determine whether the dense network of CRH terminals observed in the CA3 region of the hippocampus (Fig. 1a2) originated from the cell bodies of hippocampal CRH neurons residing in the CA1 pyramidal cell layer (Fig. 1a1), we performed tract tracing studies using

AAV-Flex-ChR2-tdTomato, which has previously been utilized for circuit mapping (Atasoy et al., 2008). AAV-Flex-ChR2-tdTomato was injected into the CA1 region of the hippocampus in *Crh*-Cre mice (Fig. 4a), resulting in expression specifically in hippocampal CRH neurons whose cell bodies reside in the CA1 pyramidal cell layer (Figs. 4b,c) ($n=10$ mice). We confirmed the restricted expression of AAV-Flex-ChR2-tdTomato to hippocampal CRH neurons in the CA1 pyramidal cell region and exclusion in other brain regions with populations of CRH neurons, such as the bed nucleus of the stria terminalis (BnST), amygdala, and PVN (Figs. 4h–j) ($n=10$ mice). Using this tract tracing method, we were able to identify AAV-Flex-ChR2-tdTomato-expressing terminals in the CA3 region of the hippocampus (Fig. 4d) emanating from labeled neurons in the CA1 region of the hippocampus (Fig. 4c) in $100.0\pm0.0\%$ of mice ($n=10$ mice). Colocalization of AAV-Flex-ChR2-tdTomato with GAD67 confirmed that these terminals are GABAergic (Figs. 4d–g) ($n=6$ mice). These data demonstrate that hippocampal CRH neurons are GABAergic and back-project from CA1 to the CA3 subregion of the hippocampus.

Functional Connectivity of Hippocampal CRH Neurons

To determine whether hippocampal CRH neurons make functional inhibitory connections onto CA3 pyramidal neurons, we optogenetically activated hippocampal CRH neurons in *ChR/Crh* mice and recorded the evoked IPSCs (eIPSCs) in CA3 pyramidal neurons. Channelrhodopsin and tdTomato expression in hippocampal CRH neurons from *ChR/Crh* mice (Fig. 5a) resembles the pattern observed in the *Crh*-GFP and *Crh*-Ai9 reporter mice (Fig. 1a), with cell bodies localized to the CA1 pyramidal cell layer and terminals in the CA3 region (Fig. 5a). Optogenetic activation of hippocampal CRH neurons in stratum pyramidale (Fig. 5b) with continuous 100ms, 1Hz laser stimulation was sufficient to increase the spontaneous firing rate of these neurons from 0.2 ± 0.3 Hz in the absence of laser stimulation to 6.0 ± 1.3 Hz in the presence of optogenetic stimulation (Figs. 5c,d) ($n=7$ cells, two mice; $P<0.05$ using a paired Student's *t* test). Optogenetic activation of hippocampal CRH neurons in *ChR/Crh* mice using either 1, 10, or 100 ms laser pulses was sufficient to evoke action potentials in these neurons (Fig. 5e). The number of action potentials elicited by optogenetic stimulation increased from 1.1 ± 0.1 at 1 ms to 2.2 ± 0.2 at 10 ms and 7.5 ± 0.1 at 100 ms (Figs. 5e,f). These data demonstrate the optogenetic activation of hippocampal CRH neurons *in vitro*.

Optogenetic activation of hippocampal CRH neurons in *ChR/Crh* mice evoked IPSCs in CA3 principal neurons (Figs. 6b–h), which varied with laser intensity and fiber optic placement (data not shown). Evoked IPSCs in response to 100 ms, 1 Hz 473-nm laser pulses were observed in $74.1\pm8.6\%$ of CA3 principal neurons (Figs. 6b,c,g) ($n=27$ cells, six mice). Similarly, evoked IPSCs were observed in 100% of CA3 pyramidal neurons in response to 10 ms, 1 Hz 473-nm laser stimulation (Figs. 6d,g). The latency to onset of evoked IPSCs was 6.4 ± 1.0 ms in response to 10ms laser stimulation and 4.2 ± 1.0 ms in response to 100ms laser stimulation (Fig. 6h). The short latency to the evoked IPSC following the onset of optogenetic stimulation (marker preceding event) can be appreciated in the average evoked IPSC trace in response to 100 ms optogenetic stimulation (Fig. 6c). The peak amplitude of the evoked IPSC was larger in response to 100 ms optogenetic stimulation (100.6 ± 17.6 pA) compared to the shorter, 10ms stimulation protocol (64.4 ± 11.1

pA) (Fig. 6e). Similarly, the time to decay of the evoked IPSC is prolonged in response to 100 ms optogenetic stimulation (379.5 ± 95.6 ms) compared to the shorter, 10 ms stimulation protocol (40.4 ± 17.6 ms) (Fig. 6f) ($n=12-13$ cells, 4–5 mice per experimental group; $P<0.05$ using an unpaired Student's t test).

The optogenetically evoked IPSCs in CA3 principal neurons were blocked by bath application of Gabazine (>200 μ M SR95531) (Figs. 6j,k); however, the optogenetically-evoked IPSCs in CA3 pyramidal neurons were not blocked with a kynurenic acid, a non-selective antagonist of NMDA and AMPA/kainate receptors (Fig. 6q). Evoked IPSCs were not observed in response to 100 ms, 1 Hz 473-nm laser pulses in CA1 pyramidal neurons from *ChR/Crh* mice (Fig. 6l) or in either CA1 or CA3 neurons from *Cre*^{-/-} littermates (Figs. 6m,n). In addition, evoked excitatory postsynaptic currents (EPSCs) were never observed in CA1 or CA3 pyramidal neurons in either *ChR/Crh* mice or *Cre*^{-/-} littermates upon optical stimulation (Figs. 6o,p). These data demonstrate that hippocampal CRH neurons make functional inhibitory connections onto CA3 principal neurons.

DISCUSSION

Interneuron classification is based on electrophysiological properties, molecular expression profiles, morphology, and connectivity [for review see (Maccaferri and Lacaille, 2003)]. The morphology and connectivity of hippocampal CRH neurons are distinct from other interneurons whose cell bodies reside in the CA1 pyramidal cell layer [for review see (Somogyi and Klausberger, 2005; Klausberger and Somogyi, 2008)]. To-date, a very small number (2) of back-projecting interneuron subtypes have been described in the hippocampus (Sik et al., 1994, 1995) [for review see (Somogyi and Klausberger, 2005)]. However, the location, morphology, electrophysiological properties, and connectivity of hippocampal CRH neurons differ from those described previously (Sik et al., 1994, 1995) [for review see (Somogyi and Klausberger, 2005)]. Thus, it appears that hippocampal CRH neurons represent a novel subtype of interneurons in the hippocampus, consistent with the GABAergic phenotype of the majority of CRH neuron populations (Kubota et al., 2011).

This subset of interneurons likely evaded detection previously due to the similar morphology to and distribution amongst CA1 pyramidal neurons. The identification of CRH neurons in the hippocampus was first described by Wylie Vale's laboratory using immunohistochemical techniques (Swanson et al., 1983). In the present study, identification of these neurons relied on reporter expression in *Crh*-*Cre* mice. However, it is not clear whether all of these neurons produce and/or secrete CRH. For example, in this model only $40.7\% \pm 5.8\%$ of neurons express immunoreactivity for the CRH peptide. However, 100% of them express transcript for CRH. It is possible that these hippocampal CRH neurons no longer express the peptide in the adult or that the CRH is secreted making immunodetection difficult. In fact, previous studies have demonstrated an increase in CRH immunolabeling in the neuropil surrounding CA3 following stress (Chen et al., 2004) [for review see (Chen et al., 2012)], which is suggestive of CRH release. Whether or not the entirety of this population of neurons expresses and secretes CRH in the adult remains to be determined but does not detract from the fact that this method enabled us to identify a homogeneous population of novel interneurons in the hippocampus, evident by the fact that GABA is expressed in 93.1%

$\pm 2.5\%$ of these neurons, the reporter colocalizes with GAD67 (Fig. 2, Table 1), and we demonstrate functional GABA release onto CA3 pyramidal neurons.

Here we demonstrate using Cre-dependent viral tracing techniques that hippocampal CRH neurons back-project onto CA3 principal neurons (Fig. 4). We ensured that only hippocampal CRH neurons whose cell bodies reside in stratum pyramidale in CA1 expressed the Cre-dependent viral tracer demonstrating that these neurons sent projections to the CA3 region of the hippocampus. Previous studies suggested that hippocampal CRH neurons also formed synapses on CA1 pyramidal neurons (Yan et al., 1998). However, we did not observe any functional connections of hippocampal CRH neurons with CA1 pyramidal neurons (Fig. 6), though this lack of response may be due to differences in the density of terminals in CA1 compared to CA3. It is also important to note that the current study focused only on a subset of hippocampal CRH neurons whose cell bodies reside in the stratum pyramidale of CA1. There are several other discrepancies between the characterization of this subset of hippocampal CRH neurons and previous description of these neurons, including the lack of synaptic connections onto CA1 pyramidal neurons as mentioned above (Yan et al., 1998) and previous reports of colocalization of CRH with PV in these neurons (Yan et al., 1998). However, lack of colocalization of CRH with PV has been demonstrated in other CRH-Cre lines (Taniguchi et al., 2011). One potential explanation for these discrepancies is the fact that this reporter strain is not labeling all CRH-positive neurons in the hippocampus. It is possible that we are not capturing the population of hippocampal CRH neurons which express PV or those that exhibit connections onto CA1 pyramidal neurons. Consistent with the notion that our reporter is labeling a subset of CRH neurons in the hippocampus, excluding the parvalbumin population, we observe co-localization of PV in a small percentage of neurons expressing endogenous CRH (18.3%). However, the expression of molecular markers in hippocampal CRH neurons described in this study is consistent with previous reports in an independent CRH-ires-Cre line (Taniguchi et al., 2011). In this way, this model may uniquely characterize CA3 back-projecting hippocampal CRH neurons. The unique connectivity of the interneuron subtype described here makes them ideally suited to modulate hippocampal network excitability.

The GABAergic and CRH phenotype of hippocampal CRH neurons suggests that they may play dual roles in the modulation of CA3 principal neurons. There is a dichotomy between the excitatory actions of CRH presumably released from these neurons (Chen et al., 2004) [for review see (Baram and Hatalski, 1998; Chen et al., 2012)] and the inhibitory GABAergic phenotype of these hippocampal CRH neurons (Fig. 6). Here we directly demonstrate functional GABAergic signaling of hippocampal CRH neurons onto CA3 pyramidal neurons (Fig. 6). It is interesting to note that the terminals of hippocampal CRH neurons surround the cell bodies of pyramidal neurons in CA3 (Figs. 1 and 4) (Chen et al., 2004, 2012), exerting robust GABAergic control over these neurons. In contrast, the corticotropin-releasing factor type 1 (CRFR₁) receptors are localized to pyramidal neuron dendrites, $>100\mu\text{m}$ away from the potential release sites (Chen et al., 2012). Thus, the physiological significance of hippocampal CRH neuron-mediated GABAergic transmission versus CRH signaling remains to be determined and will undoubtedly be the focus of future studies.

Corticotropin-releasing hormone has been demonstrated to play a role in spine remodeling and dendritic atrophy associated with chronic stress in the hippocampus (Chen et al., 2008; Andres et al., 2013). The hippocampus is particularly vulnerable to the effects of stress (Joels and Krugers, 2007; Joels and Baram, 2009; McEwen and Gianaros, 2011); however, it is largely assumed that the adverse effects of glucocorticoids on the hippocampus are from peripheral sources. The existence of CRH neurons in the hippocampus make them ideally suited to mediate the effects of stress on the hippocampus. However, under what circumstances CRH is released in the hippocampus remains to be determined. Our data suggest that the spontaneous firing rate of hippocampal CRH neurons is low which would suggest minimal CRH or GABAergic signaling under basal conditions. Given the inhibitory back-projections of these neurons, it is tempting to speculate that these neurons might be recruited and serve as a fail-safe inhibitory circuit in the event of excessive excitability in the hippocampus. Activation of hippocampal CRH neurons would induce direct inhibition of CA3 pyramidal neurons. Further, excessive activation of hippocampal CRH neurons would result in CRH release and accumulation which could potentially impact the CRFR¹ receptors located at an increased distance from the release site (Chen et al., 2012) and at which point could remodel dendritic spines to impact neuronal excitability through an independent mechanism. Future studies are required to evaluate the specific roles for GABA and CRH signaling mediated by hippocampal CRH neurons under both physiological and pathophysiological conditions.

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References

- Andres AL, Regev L, Phi L, Seese RR, Chen Y, Gall CM, Baram TZ. NMDA receptor activation and calpain contribute to disruption of dendritic spines by the stress neuropeptide CRH. *J Neurosci*. 2013; 33:16945–16960. [PubMed: 24155300]
- Atasoy D, Aponte Y, Su HH, Sternson SM. A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J Neurosci*. 2008; 28:7025–7030. [PubMed: 18614669]
- Baram TZ, Hatalski CG. Neuropeptide-mediated excitability: A key triggering mechanism for seizure generation in the developing brain. *Trends Neurosci*. 1998; 21:471–476. [PubMed: 9829688]
- Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. 2006; 224:213–232. [PubMed: 17210054]
- Chen Y, Bender RA, Frotscher M, Baram TZ. Novel and transient populations of corticotropin-releasing hormone-expressing neurons in developing hippocampus suggest unique functional roles: A quantitative spatiotemporal analysis. *J Neurosci*. 2001; 21:7171–7181. [PubMed: 11549728]
- Chen Y, Brunson KL, Adelmann G, Bender RA, Frotscher M, Baram TZ. Hippocampal corticotropin releasing hormone: Pre- and postsynaptic location and release by stress. *Neuroscience*. 2004; 126:533–540. [PubMed: 15183503]

- Chen Y, Dube CM, Rice CJ, Baram TZ. Rapid loss of dendritic spines after stress involves derangement of spine dynamics by corticotropin-releasing hormone. *J Neurosci*. 2008; 28:2903–2911. [PubMed: 18337421]
- Chen Y, Rex CS, Rice CJ, Dube CM, Gall CM, Lynch G, Baram TZ. Correlated memory defects and hippocampal dendritic spine loss after acute stress involve corticotropin-releasing hormone signaling. *Proc Natl Acad Sci USA*. 2010; 107:13123–13128. [PubMed: 20615973]
- Chen Y, Andres AL, Frotscher M, Baram TZ. Tuning synaptic transmission in the hippocampus by stress: The CRH system. *Front Cell Neurosci*. 2012; 6:13. [PubMed: 22514519]
- Givalois L, Arancibia S, Tapia-Arancibia L. Concomitant changes in CRH mRNA levels in rat hippocampus and hypothalamus following immobilization stress. *Mol Brain Res*. 2000; 75:166–171. [PubMed: 10648901]
- Gong S, Doughty M, Harbaugh CR, Cummins A, Hatten ME, Heintz N, Gerfen CR. Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. *J Neurosci*. 2007; 27:9817–9823. [PubMed: 17855595]
- Hollrigel GS, Chen K, Baram TZ, Soltesz I. The proconvulsant actions of corticotropin-releasing hormone in the hippocampus of infant rats. *Neuroscience*. 1998; 84:71–79. [PubMed: 9522363]
- Joels M, Baram TZ. The neuro-symphony of stress. *Nat Rev Neurosci*. 2009; 10:459–466. [PubMed: 19339973]
- Joels M, Krugers HJ. LTP after stress: Up or down? *Neural Plast*. 2007; 2007:93202. [PubMed: 17502912]
- Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: The unity of hippocampal circuit operations. *Science*. 2008; 321:53–57. [PubMed: 18599766]
- Kubota Y, Shigematsu N, Karube F, Sekigawa A, Kato S, Yamaguchi N, Hirai Y, Morishima M, Kawaguchi Y. Selective coexpression of multiple chemical markers defines discrete populations of neocortical GABAergic neurons. *Cereb Cortex*. 2011; 21:1803–1817. [PubMed: 21220766]
- Lee V, Maguire J. Impact of inhibitory constraint of interneurons on neuronal excitability. *J Neurophysiol*. 2013; 110:2520–2530. [PubMed: 24027099]
- Luther JA, Daftary SS, Boudaba C, Gould GC, Halmos KC, Tasker JG. Neurosecretory and non-neurosecretory parvocellular neurones of the hypothalamic paraventricular nucleus express distinct electrophysiological properties. *J Neuroendocrinol*. 2002; 14:929–932. [PubMed: 12472873]
- Maccaferri G, Lacaille JC. Interneuron diversity series: Hippocampal interneuron classifications GÇô making things as simple as possible, not simpler. *Trends Neurosci*. 2003; 26:564–571. [PubMed: 14522150]
- Maras PM, Baram TZ. Sculpting the hippocampus from within: Stress, spines, and CRH. *Trends Neurosci*. 2012; 35:315–324. [PubMed: 22386641]
- McEwen BS, Gianaros PJ. Stress- and allostasis-induced brain plasticity. *Annu Rev Med*. 2011; 62:431–445. DOI: 10.1146/annurevmed-052209-100430.431-445 [PubMed: 20707675]
- Merchenthaler I. Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. *Peptides*. 1984; 5(Suppl 1):53–69.
- Reid CA, Leaw B, Richards KL, Richardson R, Wimmer V, Yu C, Hill-Yardin EL, Lerche H, Scheffer IE, Berkovic SF, Petrou S. Reduced dendritic arborization and hyperexcitability of pyramidal neurons in a Scn1b-based model of Dravet syndrome. *Brain*. 2014; 137:1701–1715. [PubMed: 24747835]
- Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J. Neurosteroidogenesis is required for the physiological response to stress: Role of neurosteroid-sensitive GABAA receptors. *J Neurosci*. 2011; 31:18198–18210. [PubMed: 22171026]
- Sik A, Ylinen A, Penttonen M, Buzsaki G. Inhibitory CA1-CA3-hilar region feedback in the hippocampus. *Science*. 1994; 265:1722–1724. [PubMed: 8085161]
- Sik A, Penttonen M, Ylinen A, Buzsaki G. Hippocampal CA1 interneurons: An in vivo intracellular labeling study. *J Neurosci*. 1995; 15:6651–6665. [PubMed: 7472426]
- Somogyi P, Klausberger T. Defined types of cortical interneurone structure space and spike timing in the hippocampus. *J Physiol*. 2005; 562:9–26. [PubMed: 15539390]

- Swanson LW, Sawchenko PE, Rivier J, Vale WW. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology*. 1983; 36:165–186. [PubMed: 6601247]
- Taniguchi H, He M, Wu P, Kim S, Paik R, Sugino K, Kvitsani D, Fu Y, Lu J, Lin Y, Miyoshi G, Shima Y, Fishell G, Nelson SB, Huang ZJ. A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron*. 2011; 71:995–1013. [PubMed: 21943598]
- Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci*. 2009; 10:397–409. [PubMed: 19469025]
- Yan XX, Toth Z, Schultz L, Ribak CE, Baram TZ. Corticotropin-releasing hormone (CRH)-containing neurons in the immature rat hippocampal formation: Light and electron microscopic features and colocalization with glutamate decarboxylase and parvalbumin. *Hippocampus*. 1998; 8:231–243. [PubMed: 9662138]

FIGURE 1.

Characterization of hippocampal CRH neurons. **a**, A representative image of the hippocampus from Crh-GFP mice. **a1**, Higher magnification of the CA1 pyramidal cell layer highlights the morphology of hippocampal CRH neurons. **a2**, Higher magnification of the CA3 subregion of the hippocampus reveals dense GFP-expressing terminals surrounding the principal neurons. **b**, Representative image of a tdTomato-expressing hippocampal CRH neurons (red; **b1**) that is also immunoreactive for CRH (green; **b2**) ($n=232$ neurons, four mice). **c1**, Single-cell PCR detects transcript for CRH in seven individual Crh-GFP neurons in the hippocampus and in the positive controls (total brain cDNA) but not the negative control (no template) (top panel). The contents of five Crh-GFP neurons express transcript for CRH, whereas, adjacent non-GFP CA1 pyramidal neurons did not (bottom panel) ($n=5-12$ cells per experimental group). **c2**, Single cell PCR in seven individual hippocampal CRH

neurons detects transcripts for GAD65, CCK, SOM, and PV, but not VGlut2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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FIGURE 2.

Molecular markers expressed by hippocampal CRH neurons. a, Representative image of GABA immunoreactivity (green) in Ai9-positive CRH neurons (red) in the CA1 region of the hippocampus. b, Representative GAD67 immunoreactivity (green) and colocalization in Ai9-positive hippocampal CRH neurons (red). c, Representative immunolabeling for parvalbumin (green) which does not overlap with Ai9 expression in CRH neurons (red). d, Representative images demonstrating the co-localization (right panel) of somatostatin (green) in Ai9-positive CRH neurons (red) in the CA1 region of the hippocampus. Representative images for calretinin (e; green) and calbindin (f; green) in Ai9-expressing CRH neurons (red) in the hippocampus. Colocalization of molecular markers and Ai9 expression is shown in the right panels. DAPI is shown in blue. $n=167-290$ cells, three to four mice per experimental group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Electrophysiological properties of hippocampal CRH neurons. a, A diagram depicting the electrophysiological recording of hippocampal CRH neurons in Crh-GFP mice. b, Representative traces of CA1 pyramidal neurons (left panel) and hippocampal CRH neurons (right panel) in response to -100 pA and $+100$ pA current injections. c, Representative phase plane plots highlighting the properties of the first action potential fired in response to a $+100$ pA current injection in CA1 pyramidal neurons (black trace) and hippocampal CRH neurons (gray trace). d, The average resting membrane potential (RMP) is depolarized in hippocampal CRH neurons compared to CA1 pyramidal neurons. e, The average input-output curves from CA1 pyramidal neurons (black line) and hippocampal CRH neurons (gray line). f, The average input-output curves from CA1 pyramidal neurons (black line) and hippocampal CRH neurons (gray line) were fit with a Boltzman function. g, The average

maximum number of action potentials fired in response to a +100 pA current injection is decreased in hippocampal CRH neurons (gray bars) compared to CA1 pyramidal neurons (black bar). h, The average latency to the first action potential is longer in hippocampal CRH neurons (gray bar) compared to CA1 pyramidal neurons (black bar). i, The average input resistance is decreased in hippocampal CRH neurons (gray bar) compared to CA1 pyramidal neurons (black bar). $n=11-13$ cells, six to seven mice per experimental group. * denotes statistical significance of $P < 0.05$ determined using a Student's t test. j, A representative image of a CA1 pyramidal neuron filled with biocytin at $\times 20$ magnification. k, The morphology of a representative CA1 pyramidal neuron is reconstructed. l, Higher magnification ($\times 80$) of a apical dendrite of a CA1 pyramidal neuron. m, A representative, biocytin filled hippocampal CRH neuron whose cell body resides in stratum pyramidale. n, Reconstruction of the representative hippocampal CRH neuron allows better appreciation of the morphology. o, Higher magnification ($\times 80$) of a apical dendrite of a hippocampal CRH neuron whose cell body resides in stratum pyramidale. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 4.

Unique connectivity of hippocampal CRH neurons. a, A schematic of the tract tracing method based on Atasoy et al., 2008. AAV-Flex-ChR2-tdTomato was stereotactically injected into the CA1 region of the hippocampus in Crh-Cre mice. After 2 weeks, the expression of tdTomato and projections of hippocampal CRH neurons were visualized using confocal microscopy. b, Representative image of AAV-Flex-ChR2-tdTomato targeting to hippocampal CRH neurons. c, CRH neurons expressing AAV-Flex-ChR2-tdTomato were observed in the CA1 pyramidal cell layer and projections were observed surrounding principal neurons in the CA3 subregion (d). Immunostaining for GAD67 (e) demonstrated colocalization of

GAD67 in AAV-Flex-ChR2-tdTomatoexpressing terminals in CA3 (f–g). g, Higher magnification highlights the colocalization of GAD67 in AAV-Flex-ChR2-tdTomatoexpressing terminals in CA3. AAV-Flex-ChR2-tdTomato targeted to and solely expressed in hippocampal CRH neurons with cell bodies residing in the CA1 pyramidal cell layer (b, arrows) and not in other populations of CRH neurons, such as in the PVN (h), BnST (i), or amygdala (j). AAV-Flex-ChR2-tdTomato=red; DAPI=blue ($n=6–10$ mice). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Optogenetic activation of hippocampal CRH neurons. a, tdTomato expression in *ChR/Crh* mice. a1, Higher magnification of the CA1 pyramidal cell layer highlights the expression of tdTomato in hippocampal CRH neurons, which strongly resembles the expression in the CRH-GFP mice. a2, Higher magnification of the CA3 subregion of the hippocampus reveals dense tdTomato-expressing terminals surrounding the principal neurons, similar to that observed in the *Crh*-GFP mice. b, A diagram depicting the electrophysiological recording and optogenetic activation of hippocampal CRH neurons in *ChR/Crh* mice. c, Continuous optogenetic activation increases the spontaneous firing rate of tdTomato-positive CRH neurons in *ChR/Crh* mice. d, The average spontaneous firing rate of tdTomato-positive CRH neurons in *ChR/Crh* mice in response to continuous optogenetic activation. e, Optogenetic activation at 1 μ s (e1), 10 μ s (e2), or 100 μ s (e3) at 1 Hz elicits action potentials in tdTomato-positive CRH neurons in *ChR/Crh* mice. f, The average number of spikes fired in response to optogenetic activation at 1, 10, or 100 μ s delivered at 1 Hz. $n=7$ cells, three mice per experimental group. * denotes statistical significance of $P < 0.05$ determined using a paired

Student's t test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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FIGURE 6.

Hippocampal CRH neurons make inhibitory connections onto principal neurons in CA3. a, A diagram depicting the optogenetic activation of hippocampal CRH neurons with simultaneous electrophysiological recording of principal neurons in the CA3 or CA1 region of the hippocampus in *ChR/Crh* mice. b, A representative traces of evoked IPSCs recorded in a CA3 pyramidal neuron in response to 100ms, 1Hz 473nm laser pulses. c, The average evoked IPSCs recorded in a representative CA3 pyramidal neuron in response to 100 ms, 1 Hz 473-nm laser pulses. The start of the optogenetic activation is shown with a marker preceding the average evoked IPSC making it easy to appreciate the short latency to evoke

the IPSC in the postsynaptic cell. d, IPSCs can also be evoked in CA3 pyramidal neurons with shorter, 10ms, 1Hz 473-nm laser pulses. e, The average peak amplitude of evoked IPSCs recorded in CA3 pyramidal neurons in response to either 10 ms (black bar) or 100 ms (gray bar) 1 Hz 473-nm laser pulses. f, The average 10–90% time to decay is shown for IPSCs evoked with either 10 ms (black bar) or 100 ms (gray bar) 1 Hz 473-nm laser pulses. g, The average number of CA3 pyramidal cells which exhibited evoked IPSCs in response to either 10 ms (black bar) or 100 ms (gray bar) 1 Hz 473-nm laser pulses. h, The average latency to evoke an IPSC in CA3 pyramidal neurons from the beginning of optogenetic activation using either 10 ms (black bar) or 100 ms (gray bar) 1 Hz 473-nm laser pulses. $n=12$ –13 cells, four to five mice per experimental group. * denotes statistical significance of $P < 0.05$ determined using a Student's t test. i, A representative recording of evoked IPSCs in CA3 pyramidal neurons in response to 100 ms, 1 Hz 473 nm optogenetic stimulation. j, Representative traces of evoked IPSCs in CA3 pyramidal neurons in the presence of nACSF which are blocked in the presence of $>200 \mu\text{M}$ SR95531 (k) ($n=26$ cells, 6 mice per experimental group; $P < 0.0001$ compared to CA3 neurons from *ChR/Crh* mice). l, A representative trace demonstrating the lack of evoked IPSCs in CA1 pyramidal neurons from *ChR/Crh* mice in the presence of 100 ms, 1 Hz 473-nm laser pulses. Representative traces demonstrating the inability to evoke IPSCs in CA1 (m) or CA3 (n) pyramidal neurons from *Cre^{-/-}* littermates in the presence of 100 ms, 1 Hz 473-nm laser pulses. o–p, Representative traces demonstrating the inability to evoke EPSCs in CA3 pyramidal neurons from *ChR/Crh* mice (o) or *Cre^{-/-}* littermates (p) in the presence of 100 ms, 1 Hz 473-nm laser pulses ($n=12$ cells, three to four mice per experimental group). q, Optogenetically evoked IPSCs in CA3 pyramidal neurons were not blocked by the glutamate receptor antagonist, kynurenic acid ($n=6$ cells, two mice). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

Colocalization of Interneuron Markers with Hippocampal CRH Neurons

Marker	% of CRH cells positive	Manders' coefficient	<i>n</i>
GABA	93.1 (270/290 cells)	0.739	4
GAD67	56.4 (114/202 cells)	0.688	3
Parvalbumin	0 (0/167 cells)	N/A	4
Somatostatin	39.5 (85/215 cells)	0.351	4
Calretinin	23.2 (51/220 cells)	0.314	4
Calbindin	26.5 (52/196 cells)	0.304	4
Somatostatin and calretinin	0 (0/94 cells)	N/A	4
Somatostatin and calbindin	0 (0/150 cells)	N/A	4
Calretinin and calbindin	1.29 (2/155 cells)	N/A	4

The percentage of hippocampal CRH neurons which express GABA, GAD67, parvalbumin, somatostatin, calretinin, and calbindin are provided with the Mander's Coefficient characterizing the extent of the colocalization of these markers with the reporter.

TABLE 2

Properties of Action Potentials in Hippocampal CRH Neurons and CA1 Pyramidal Neurons

Parameter	Hippocampal CRH	CA1 pyramidal
AP threshold (mV)	-44.0±1.1	-42.7±1.2
AP peak (mV)	63.9±3.8	58.0±3.5
AP rise (ms)	0.39±0.04	0.32±0.04
AP minimum (mV)	-62.4±1.8	-65.1±3.6
AHP peak (mV)	18.4±1.9	22.3±3.3

The mean values for AP threshold, AP peak, AP rise, AP minimum, and AHP peak are provided±SEM. No statistically significant differences in the properties of the action potentials were observed between hippocampal CRH neurons and CA1 pyramidal neurons.

None of the action potential parameters reached statistical significance of $P<0.05$ using a Student's t test.