NEUROPHYSIOLOGY

GABAergic interneurons excite neonatal hippocampus in vivo

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GABAergic interneurons are proposed to be critical for early activity and synapse formation by directly exciting, rather than inhibiting, neurons in developing hippocampus and neocortex. However, the role of GABAergic neurons in the generation of neonatal network activity has not been tested in vivo, and recent studies have challenged the excitatory nature of early GABA. By locally manipulating interneuron activity in unanesthetized neonatal mice, we show that GABAergic neurons are excitatory in CA1 hippocampus at postnatal day 3 (P3) and are responsible for most of the spontaneous firing of pyramidal cells at that age. Hippocampal interneurons become inhibitory by P7, whereas visual cortex interneurons are already inhibitory by P3 and remain so throughout development. These regional and age-specific differences are the result of a change in chloride reversal potential, because direct activation of light-gated anion channels in glutamatergic neurons drives CA1 firing at P3, but silences it at P7 in CA1, and at all ages in visual cortex. This study in the intact brain reveals that GABAergic interneuron excitation is essential for network activity in neonatal hippocampus and confirms that visual cortical interneurons are inhibitory throughout early postnatal development.

INTRODUCTION

GABA (γ-aminobutyric acid)–releasing (GABAergic) interneurons are critical for normal development of cortical circuits (1–3). This function is, in part, activity dependent (4, 5). Despite their importance, how interneurons regulate activity within developing cortical circuits is poorly understood. This is largely due to the technical challenges of manipulating interneuron activity in vivo at very early ages while recording network activity under naturalistic conditions.

One persistent hypothesis is that GABA, a largely inhibitory neurotransmitter in adults, depolarizes, and in some cases directly excites, young neurons as a result of their low expression of the neuronal K-Cl cotransporter KCC2 (6). According to this theory, early excitatory action of GABAergic neurons, primarily mediated by medial ganglion eminence–derived somatostatin-expressing neurons, drives synchronized activity in vitro (7, 8) and supports glutamatergic and GABAergic synapse formation in cortical circuits (9, 10). The switch of GABA’s function from excitatory to inhibitory has further been hypothesized to drive the maturation of cortical activity patterns in vivo in human neonates and rodent neonates (2, 11, 12). A more complete understanding of early GABA’s role in network function will be essential for the design of treatments for seizures in infants, for whom standard augmentation of GABA type A (GABAₐ) receptor signaling is often ineffective (13, 14).

So far, in vivo studies have failed to find evidence that GABAergic interneurons excite cortical networks. Inhibition of GABAₐ receptors in sensory cortex increases firing rates and causes bursting (15, 16). However, such blockade has similar effects in hippocampal slices, where GABA is clearly excitatory (17, 18). Two recent studies showed that GABA and GABAergic neurons are inhibitory in vivo, even when they are excitatory in vitro at the same ages (19, 20). In addition to the question of GABAergic polarity, the changing role of local interneurons themselves in the generation of early synchronized activity in vivo is unknown.

To directly test the role of local GABAergic neurons in early activity, we used a variety of chemogenetic and optogenetic approaches to acutely and bidirectionally manipulate the activity of GABAergic interneurons while recording network activity locally using multielectrode array recordings in unanesthetized neonatal mice. We show that local GABAergic interneurons exert a net excitatory effect in the CA1 region of hippocampus, but not in visual cortex, during the first postnatal week. The excitatory action of interneurons is regionally and age correlated with the excitatory effect of anion conductance in pyramidal neurons, suggesting that changes in the cellular CI⁻ reversal potential mediate GABAergic polarity in the network.

RESULTS

To locally manipulate activity of putative interneurons expressing glutamic acid decarboxylase 2 (GAD2), we used a Cre-dependent adeno-associated virus (AAV) to express either the inhibitory κ-opioid receptor Designer Receptors Exclusively Activated by Designer Drugs (KOR-DREADD or KORD) (21) or the excitatory DREADD (hM3Dq) (22) in the hippocampus of GAD2-Cre mice (23). Promoters and AAV subtype were optimized so that after viral injection at P0, KORD and hM3Dq could be detected by P3. In CA1 of hippocampus, expression was limited to GABAergic neurons (Fig. 1, B and C), and within the injection site, the large majority (~85%) of GABAergic neurons expressed the DREADD (Fig. 1C). Whole-cell current-clamp recordings in slices from these animals were then used to verify the effects of the DREADDs. These confirmed a hyperpolarization caused by the KORD agonist, salvinorin B (SalB) and a depolarization by the hM3Dq agonist, clozapine N-oxide (CNO) in CA1 GABAergic neurons by P3 that was similar to that obtained at P11 (Fig. 1D). Thus, this approach allowed suppression (KORD-SalB) or enhancement (hM3Dq-CNO) of the large majority of GABAergic neurons in neonatal hippocampus between P3 and P11.

To study the effects on hippocampal activity in vivo, a 32-channel linear array was inserted into CA1 of dorsal hippocampus (Fig. 1E). Only animals with viral expression surrounding the recording
Fig. 1. GABAergic interneurons are excitatory in 3-day-old hippocampus in vivo. (A) Experimental design. (B) Colabeling of AAV-dF-KORD-IRES-mCitrine expressed in GAD2-Cre mouse with anti-GABA. Scale bars, 100 and 20 μm. (C) Percentage of mCitrine(KORD)–expressing neurons coexpressing GABA and percentage of GABA-expressing neurons coexpressing mCitrine(KORD) (mean ± 95% CI, n = 3, 3). (D) Change in membrane potential in hippocampal slices at P3 and P11. SalB hyperpolarized KORD–expressing neurons and CNO depolarized hM3Dq–expressing neurons at both ages (n = 6, 6, 5, and 7; ANOVA, P < 0.001). (E) Representative localization of electrode and viral expression in P3 animal. (F) Representative recording for P3 reduction of GABAergic neuron excitability. MUA of spontaneous activity in CA1 hippocampus, along with associated stratum radiatum LFP and thoracic movement detection and electromyography. Activity is dominated by early sharp waves (eSPW) whose spike density is reduced following subcutaneous SalB (KORD agonist) injection. (G) Quantification of KORD-induced suppression of GABAergic neuron excitability and control conditions. [Pyramidal cell layer firing rate (n = 10, 6, and 8; ANOVA, P < 0.001), eSPW LFP amplitude (n = 10, 6, and 8; P = 0.002), and normalized (to mean of 1- to 100-Hz baseline) spectral power for stratum radiatum LFP, n = 10]. (H) Quantification of hM3Dq-induced increase in GABAergic excitability (n = 7, 6, and 6; P = 0.005; P = 0.33; n = 7). All values and statistics are listed in table S1.
firing rates and LFP power (Fig. 2, E and F). These results demonstrate a powerful net excitatory drive to hippocampus at this age. The amplitude and occurrence of eSPWs were also decreased at P3 (Fig. 1G and fig. S2C), resulting in a reduction of the power of the local field potential (LFP) in a broad frequency range (Fig. 1G). By contrast, in different animals, enhancing GABAergic neuron excitability by injecting CNO into animals expressing the excitationary DREADD increased pyramidal layer MUA but did not alter eSPW amplitude or LFP power (Fig. 1H and fig. S2, A and B). In a test for off-target effects of the injections, neither saline injection into animals expressing KORD/hM3Dq nor SalB or CNO injections into green fluorescent protein (GFP)–only mice altered activity.

These results suggest that local GABAergic interneurons excite the hippocampal network at P3 and are responsible for at least half the excitation in presumptive pyramidal cells in CA1. Although our MUA rates may include some spikes from interneurons, they are likely to be a small minority, as pyramidal cells account for 80 to 90% of neurons in the pyramidal cell layer, which, due to their large size, are much more likely to be picked up by the electrodes at these ages. In confirmation of this, when only pyramidal cell firing was directly driven by stGtACR2 (see below, Fig. 4), light stimulation caused immediate MUA activity at P3, showing the pyramidal cell dominance at this age.

The net excitatory action of GABAergic neurons on CA1 pyramidal cell layer firing was no longer observed by P7 (Fig. 2). At this age, suppressing GABAergic neuron excitability actually increased pyramidal layer firing rates, indicative of a net loss of inhibition. Reducing interneuron excitability also reduced the power of 6- to 14-Hz frequencies in the LFP but did not substantially change the occurrence, duration, or amplitude of eSPWs (Fig. 2, B and C, and fig. S3A), suggesting that the transmission or initiation of network events has largely become independent of interneurons by this age. Enhancing interneuron excitability decreased pyramidal layer firing and reduced LFP power across a broad range of frequencies (Fig. 2D), without significantly affecting the eSPW statistics (fig. S3C). By P11, modulating GABAergic neuronal activity had similar effects on firing rates and LFP power (Fig. 2, E and F). These results demonstrate a reversal of hippocampal GABAergic interneuron function, from excitatory to inhibitory, between P3 and P7.

The early excitatory action of GABAergic interneurons we observed in CA1 was not consistent with previous observations in vivo (20). To determine whether such an excitatory effect was consistent across cortical structures, we investigated the role of GABAergic interneurons in visual cortex (Fig. 3). As in CA1, local viral injections resulted in expression of the DREADDs that was specific to GABAergic neurons as well as expressed in nearly 90% of GABAergic neurons (Fig. 3C and fig. S1, B and C).

However, in contrast to hippocampus, reducing the excitability of GABAergic interneurons in visual cortex at P3 increased MUA firing rates recorded from the presumptive input layer [which can be identified as the peak of the negative LFP (27) within the injected region (Fig. 3, E and F)]. Increasing their excitability had the opposite effects (Fig. 3H). Neither manipulation significantly changed LFP spectral power, nor the occurrence, duration, or amplitude of spontaneous spindle bursts (fig. S4), the cortical activity driven by spontaneous retinal activity at this age (28). As in CA1, control assays showed that neither saline injection in DREADD-expressing animals nor active drug in GFP-expressing animals had an effect. In cortex, modulation of GABA neuron excitability at P7 and P11 had similar effects (Fig. 3, G and I). Thus, GABAergic interneurons have a net inhibitory role in visual cortex at ages at which they are excitatory in hippocampus.

To verify that the regional heterogeneity in the DREADD response was a direct result of our activity modulation of interneurons, we used a mechanistically independent method to reduce GABAergic neuron firing (fig. S5). Expressing a Cre-dependent JAWS, a light-driven inward chloride pump (29), reduced interneuron activity without relying on the G protein second messenger system. Like the DREADDs, 3 days of viral expression in GABAergic interneurons was sufficient to generate a significant hyperpolarization of the membrane potential, with similar magnitude of effect at P11. Optogenetic suppression of GABAergic neuron activity in vivo using JAWS showed similar effects to DREADD-based suppression, namely, decreasing pyramidal cell layer firing at P3 but increasing it at P7.

Such regional heterogeneity of interneuron function suggests that a systemic modulation of GABAergic activity, as might be used in epilepsy treatment, would produce complex and unpredictable outcomes. To examine the effects of global, rather than local, manipulation of GABAergic neurons, we expressed KORD and hM3Dq across a broader region including hippocampus, entorhinal cortex, and somatosensory cortex, the structures that provide the major drive to CA1 at these ages (25, 26). Activity measured from the ipsilateral CA1 at P3 showed that suppressing GABAergic neurons caused increased CA1 firing and their enhancement reduced CA1 firing (fig. S6). Thus, at P3, inhibition by interneurons in cortex (and possibly entorhinal cortex) can overwhelm local excitation by interneurons in hippocampus, causing a net inhibitory effect in CA1.

The regional heterogeneity of GABAergic neuron function might be a direct result of differences in the excitation caused by the GABA_A receptor–mediated anion conductance or by differential network effects such as desynchronization of local firing by depolarization (30, 31). To distinguish between these possibilities, we directly assayed the effect of activating an anion conductance on pyramidal neurons in hippocampus and cortex using stGtACR2, a light-gated anion channel with a soma-targeting motif (32) virally expressed in EMX1-Cre mice (Fig. 4, A and C) (33). In vivo photostimulation of stGtACR2 in glutamatergic neurons of the visual cortex (VC) reduced firing at both P3 and P7 (Fig. 4E), indicating an inhibitory role for anions in cortex at these ages, as observed in adults (32). In CA1 at P3, as predicted by the manipulation of GABAergic neurons, photostimulation of stGtACR2 in glutamatergic neurons induced immediate firing in the pyramidal cell layer (Fig. 4D). The induced firing lasted for about 150 ms (157.6 ± 29.5 ms) before becoming suppressed. Such suppression due to continuous activation of anion channels is expected even if they are initially excitatory, because the Cl⁻ conductance induces shunting inhibition during persistent activation (14). By P7, stGtACR2 activation became strongly inhibitory in CA1 (Fig. 4D). Thus, the heterogeneity of interneuron roles
Fig. 2. Hippocampal GABAergic neurons are inhibitory by P7. (A) Experimental design. (B) Representative recording for GABAergic neuron suppression in P7 hippocampus.  
(C and D) Quantification of suppression (C) and enhancement (D) of GABAergic neuron excitability at P7 [(C): CA1 firing rate: KORD-SalB: 1.14 ± 0.62 (n = 5), KORD-saline: 0.04 ± 0.35 (n = 4), GFP-SalB: −0.04 ± 0.43 (n = 4), P = 0.001; LFP spectra: P < 0.05 at 6.9 to 14.7 Hz (n = 5); (D): CA1 firing rate: hM3Dq-CNO: −2.37 ± 2.02 (n = 5), hM3Dq-saline: 0.17 ± 0.21 (n = 4), GFP-CNO: 0.13 ± 0.43 (n = 5), P = 0.003; LFP spectra: P < 0.05 at 7.7 to 93.3 Hz (n = 5)].  
(E and F) Similar quantification at P11 [(E): CA1 firing rate: KORD-SalB: 1.11 ± 0.57 (n = 6), KORD-saline: 0.12 ± 0.22 (n = 8), GFP-SalB: −0.03 ± 0.28 (n = 4), P < 0.001; LFP spectra: not significant (n.s.) (n = 6); (F): CA1 firing rate, hM3Dq-CNO: −2.09 ± 1.29 (n = 7), hM3Dq-saline: 0.18 ± 0.22 (n = 5), GFP-CNO: −0.07 ± 0.37 (n = 5), P = 0.001; LFP spectra, P < 0.05 at 2.3 to 93.3 Hz (n = 7)].
Fig. 3. GABAergic neurons in visual cortex have a net inhibitory action as early as P3. (A) Experimental design. (B and C) Colocalization of KORD expression with GABA [93.7 ± 6.7% (n = 3)]. Scale bars, 50 and 10 µm. (D) Change in membrane potential of GAD2+ neurons by activation of KORD and hM3Dq at P3 and P11 in visual cortical slices [P3 KORD: −6.62 ± 1.88 (n = 5), P11 KORD: −8.03 ± 2.14 (n = 7), P3 hM3Dq: 7.18 ± 2.23 (n = 6), P11 hM3Dq: 7.23 ± 2.06 (n = 8), P < 0.001]. (E) Representative recording of visual cortex at P3 and the effect of GABAergic neuron suppression. LFP spectrogram is from the presumptive input layer. (F) Quantification of change in superficial layer firing rate [KORD-SalB: 1.25 ± 0.5 (n = 6), KORD-saline: 0.22 ± 0.4 (n = 4), GFP-SalB: −0.03 ± 0.35 (n = 5), P = 0.001] and LFP spectral power [n.s. (n = 6)] following suppression of GABAergic neuron excitability by KORD activation. (G) Firing rate change at P3, P7, and P11 to KORD activation [P3: 1.25 ± 0.5 (n = 6), P7: 1.11 ± 0.71 (n = 6), P11: 1.17 ± 0.43 (n = 5), P = 0.89]. (H and I) As (F) and (G) but for GABAergic neuron enhancement by hM3Dq activation [(H): VC firing rate: hM3Dq-CNO: −1.51 ± 1.18 (n = 6), hM3Dq-saline: 0.11 ± 0.46 (n = 4), GFP-CNO: 0.02 ± 0.32 (n = 5), P = 0.007; LFP spectra: n.s. (n = 6)]; (l): P3: −1.51 ± 1.18 (n = 6), P7: −1.43 ± 1.02 (n = 5), P11: −1.94 ± 1.35 (n = 6), P = 0.71].
between regions and ages likely reflects the differential intracellular concentration of anions, particularly chloride.

**DISCUSSION**

For this study, we have overcome a major technical barrier to the acute manipulation of activity in unanesthetized neonatal mice. Using viral-mediated expression restricted to specific neural populations via the Cre-lox expression system, we reliably and bidirectionally manipulated activity with channelrhodopsins or DREADDs as early as P3. This allowed us to investigate the role of interneurons in generating endogenous spontaneous activity fundamental to circuit formation (34). We provide direct evidence that GABA can be excitatory in vivo and that this excitatory action is mediated by the activity of local interneurons that increase spiking in CA1 of hippocampus. Under the same experimental conditions, we further show that the net excitatory effect of local interneurons is more developmentally and regionally restricted within cortical circuits than predicted by ex vivo studies (6, 35). Heterogeneity of Cl⁻ concentration has been reported between cortical and subcortical regions.
of the same sensory system (35, 36) as well as within regions depending on neuronal birth date (37), but how such heterogeneity of Cl− regulation plays out in terms of excitation and network regulation in vivo has not been clear. In sensory neocortex, pharmacological experiments have suggested an inhibitory role for GABA_A receptors in vivo, even when they are excitatory in vitro (19). Along with Valeeva et al. (20), our results confirm that GABA is inhibitory in sensory cortex in vivo. We further show that activity-dependent release from local interneurons is an important source for GABA and that these neurons critically contribute to the reduction of excitability in cortex, likely restricting the spread of activity to aid map formation (4, 15). In CA1, our results diverge from those of Valeeva et al. (20), who observed a decrease in spontaneous glutamatergic currents in response to optogenetic activation of GABAergic neurons. The reason for this divergence is unclear. Our studies differ in terms of population, stimulation method, and readout, suggesting that complicated network effects beyond simple GABA reversal potential contribute to the generation of excitation and inhibition in the developing networks.

Our results suggest that the regional and developmental heterogeneity of interneuron function is the result of changes in Cl− concentration in pyramidal cells (9), because activation of a light-activated anion channel drove firing in CA1 pyramidal cells when interneurons were excitatory, but suppressed activity in visual cortex and in CA1 when interneurons were inhibitory. During development, this shift is driven by the increased expression of KCC2 in both visual cortex and hippocampus (6, 36), but [Cl−]i can also be modulated on a rapid time scale by hormones (38). Until we understand why GABA responses differ between ex vivo and in vivo preparations at P3 (19), it will be difficult to mechanistically determine why sensory cortex is dominated by shunting inhibition in response to GABA_A activation, while CA1 cells are excited.

Modulation of GABAergic interneuron excitability had an inconsistent effect on the occurrence and amplitude of spontaneous events, measured at the level of the LFP. This is likely a result of the complex generative mechanisms of this activity. In both visual cortex and CA1, the primary generators of the LFP are glutamatergic synapses from the primary input (relay thalamus and CA3/entorhinal cortex, respectively) (26, 28). Therefore, the number of events and their LFP amplitude would not be expected to change markedly as a result of local interneuron activity, only the firing response of local neurons receiving this input. P3 CA1 eSPW occurrence and amplitude were reduced following reduction of interneuron excitability, suggesting that either the probability of sharp wave initiation or spread is increased by excitatory GABA in vivo.

Our results suggest important differences between the roles of interneurons in circuit formation in sensory cortex and hippocampus. Reducing excitability in CA1 reduced the number of network events in CA1 but did not affect their number in visual cortex. This is consistent with ex vivo studies showing that when the glutamatergic input to both regions is severed, hippocampal spontaneous activity is dependent on GABA release while cortical activity depends on glutamatergic transmission (39). This may indicate a fundamental difference in the role for inhibitory circuits in each region and the computations each perform. In sensory cortex, early oscillations are generated in a complicated interaction between input to relay thalamus and the thalamocortical loop (28), and cortical inhibition largely serves to restrict activation, likely to aid accurate map formation (4, 15). By contrast, sensory information is not organized topographically, nor are sensory responses in hippocampal neurons exclusive to sensory processing (40). Interneurons here may act to extend, rather than restrict, the spread of incoming activity. A final reason for the observed difference between regions is that hippocampal circuit development may be delayed relative to sensory cortex, obeying an outside-in gradient for maturation. By showing that the textbook model of excitatory GABA does exist in vivo and that local interneurons are critical to the amplification of early hippocampal activity, though in a much more heterogeneous group of regions and ages than previously expected, our results here support the hypothesis that regulation of the chloride reversal potential by hormones (38), epilepsy (41), and autism risk (42, 43) may have important effects on developing networks, although our results also show that it is essential to test these effects in vivo.

**MATERIALS AND METHODS**

**Experimental design**

We used chemogenetic and optogenetic approaches combined with the Cre-lox system to specifically manipulate activity of GABAergic neurons using GAD2-Cre mice, including suppression by KORD with its ligand SalB, enhancement by hM3Dq with its ligand CNO, and suppression by JAWS with green-yellow light-emitting diode (LED), as well as activating anion channels in glutamatergic neurons using EMX1-Cre mice and stGtACR2 with blue LED. The effects of chemogenetic and optogenetic manipulation on endogenous network activity were monitored using multielectrode array recordings in unanesthetized neonatal mice.

**In vivo recording**

All experiments were conducted with approval from The George Washington University School of Medicine and Health Sciences Institutional Animal Care and Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH)].

GAD2-IRES-Cre (Gad2tm2(cre)Zjh, stock no.: 010802) (23) and EMX1-IRES-Cre (B6.129S2-Emx1tm1(cre)Krj/J, stock no.: 005628) (33) mice were acquired from The Jackson Laboratory. C57BL/6 mice were acquired from The Jackson Laboratory and Hilltop Lab Animals Inc. All GAD2-Cre and EMX1-Cre mice used were heterozygous, obtained by crossing homozygous GAD2-Cre or EMX1-Cre males and C57BL/6 females. Animals were housed one litter per cage on a 12-hour light/12-hour dark cycle. Both males and females were used.

In vivo recording methods are described previously (44–46). Topical lidocaine (2.5%) and systemic carprofen (5 mg/kg) were used for preoperative analgesia. To place the head plate, under isoflurane anesthesia (3% induction, 0.5 to 1.5% maintenance, verified by toe pinch), the scalp was resected, the skull was cleaned, and a stainless plate with a hole was placed so that the region over occipital cortex and hippocampus was accessible. The plate was fixed to the skull with Vetbond and dental cement. Pups were monitored for signs of stress after recovery from anesthesia. For recording, the animal was head-fixed, and the body was supported within a padded enclosure. Body temperature was monitored with a thermocouple placed under the abdomen and maintained at 34° to 36°C by heating pad placed under the support chamber. Body movement was detected using a piezo-based detector placed under the enclosure. Electromyogram was recorded from the ventral neck by a single
by identifying a triggering threshold of root mean square (RMS; than 20% changes between the start and end of recording), eight or with electrical or movement artifacts) or spike activity (more amplitudes larger than the maximum amplitude of visual response animals were excluded for unstable baseline LFPs (periods with LFP Neural signals were imported into MATLAB (MathWorks). Spike Analysis μV (P3) or −50 μV (P7) was extracted by threshold crossing of −40 V to a contact site located in the underlying white matter. Hippocampus 9 kHz and digitized at 32 kHz. Cortical recordings were referenced a with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) (Life Technologies) before insertion for histological verification of electrode location. For VC recording, the monocular primary visual cortex was targeted with the following coordinates: −0.2 to +0.2 mm anterior, 1.5 to 2.1 mm lateral from the lambda at P3; −0.2 to +0.2 mm and 1.7 to 2.3 mm at P7; and −0.2 to +0.2 mm and 2.1 to 2.9 mm at P11. For hippocampal recording, the dorsal CA1 was targeted with the following coordinates: 0.5 to 0.8 mm anterior and 1.3 to 1.7 mm lateral from the lambda at P3; 0.7 to 1.1 mm and 1.6 to 2.0 mm at P7; and 0.9 to 1.5 mm and 1.7 to 2.3 mm at P11. SalB [1 mg/kg, 0.1 mg/ml in saline with 1% dimethyl sulfoxide (DMSO)] was subcutaneously injected to activate KORD, and CNO (10 mg/kg; 1 mg/ml in saline with 1% DMSO) was subcutaneously injected to activate hM3Dq (21). Saline with 1% DMSO was subcutaneously injected as control. For activating JAWS, a 400-μm-diameter optic fiber (Thorlabs, M128L01) coupled with green-yellow LED (Thorlabs, MINTF4, peak at 554 nm, 80% intensity at 520 to 586 nm, 21.2 mW at the fiber tip) was placed above the skull over the dorsal hippocampus or visual cortex. One-second LED stimulation was given at every 15 to 20 s. For activating stGtACR2, photostimulation using 470-nm LED (Thorlabs, M470F3, peak at 467 nm, 80% intensity at 464 to 471 nm, 12.5 mW at the fiber tip) were similarly performed. One-second stimuli were chosen to allow a full temporal characterization of the network and cellular responses to stGtACR2 as described in the original description (32). Electrical signals were digitized using the Neuralynx Digital Lynx S hardware with Cheetah v5.6 software. Depth electroencephalogram (dEEG) signals were bandpass-filtered between 0.1 Hz and 9 kHz and digitized at 32 kHz. Cortical recordings were referenced to a contact site located in the underlying white matter. Hippocampus recordings were referenced to a contact just dorsal to hippocampus. MUA was extracted by threshold crossing of −40 μV (P3) or −50 μV (P7 and P11) following 300-Hz to 9-kHz bandpass filtering.

Analysis
Neural signals were imported into MATLAB (MathWorks). Spike times and dEEG were down-sampled to 1 kHz. Before analysis, six animals were excluded for unstable baseline LFPs (periods with LFP amplitudes larger than the maximum amplitude of visual response or with electrical or movement artifacts) or spike activity (more than 20% changes between the start and end of recording), eight animals were excluded for viral injection spread outside the recording region (hippocampus or visual cortex), and five animals were excluded because post-recording histology showed that the electrode was not located in CA1 of the hippocampus.

For hippocampus, the pyramidal cell layer was identified in each recording as the channel with the highest MUA spike rate, and the strata radiatum was identified as the channel with the largest negative LFP deflection. Average distance of the strata radiatum from the pyramidal cell layer was ~300 μm at all ages. eSPWs were detected by identifying a triggering threshold of root mean square (RMS; 9-ms window) of the strata radiatum LFP at 7 SDs above the mean. The beginning and end of the eSPW is defined as the points when the RMS remained above 2 SDs and contained the initiation threshold.

For cortex, at P3 and P7, presumptive layer 4 was identified in each recording as the layer with the largest negative LFP deflection during spindle bursts (27). In P11 animals, cortical layer 4 was identified in each recording as the channel with the earliest negative LFP deflection and the fastest spike response in the mean visual evoked response as previously described. (27). Spindle bursts were identified by at least one negative trough of the layer 4 LFP that was greater than 5 SDs from the mean; additional cycles with negative LFP greater than 2 SDs within 1 s of each other were used to define the length of the burst.

For all analyses, after a recovery period of at least 30 min following electrode insertion, baseline activity was calculated from the entirety of a continuous 20-min period, while the presentation condition (KORD-SalB, KORD-saline, GFP-KORD, hM3Dq-CNO, hM3Dq-saline, GFP-CNO) was calculated from a 20-min period beginning 10 min after injection. For spectral analysis, LFP spectra from presumptive layer 4 (visual cortex) or strata radiatum (hippocampus) were obtained by averaging 2-s multitaper windows [time-bandwidth 3 with five tapers (Chronux package) (47)]. To reduce the effect of the 1/f relationship, mean multitaper spectra were multiplied by frequency. The frequency axis was resampled on a log scale to equalize the representation of high and low frequencies and reduce the multiple-comparisons problem. For normalization, frequency power at each band was divided by the mean 1- to 100-Hz power of baseline. For optogenetic stimulation, spike rates for baseline (900-ms duration before LED stimuli) and for during photostimulation [900-ms duration starting 20 ms after the onset of LED stimuli to exclude potential photo-artifacts and antidromic spikes by stGtACR2 (32)] were analyzed.

Virus injection
AAV8-hSyn-dF-HA-KORD-ires-mCitrine (65417-AAV8, tier >7×10^{12} GC/ml) (21), AAV8-hSyn-DIO-hM3D(Gq)-mCherry (44362-AAV8, tier >1×10^{13} GC/ml), AAV8-hSyn-DIO-hM3D(Gq)-ires-mCitrine (50454-AAV8, tier >1×10^{13} GC/ml) (22, 48), and AAV1-hSyn1-SIO-stGtACR2-FusionRed (105677-AAV1, tier >1×10^{13} GC/ml) (32) were obtained from Addgene, and AAV1-CAG-FLEX-GFP(AAV1-AllenInstitute854, tier >2.9×10^{13} GC/ml) and AAV1-CAG-FLEX-tdTomato (AAV1-AllenInstitute864, tier 8.5×10^{12} GC/ml) were obtained from the University of North Carolina Vector Core (courtesy of A. Chuong and E. Boyd). Mouse pups on the day of birth (P0) were cold-anesthetized, and 30 to 100 nl of viral solution were injected locally into the hippocampus (0.4 to 0.8 mm anterior and 1.2 to 1.8 mm lateral from the lambda, 1.0 to 1.5 mm in depth) or visual cortex (−0.2 to 0.0 mm anterior and 1.5 to 2.0 mm lateral from the lambda, 0.4 to 0.7 mm in depth) using Nanoject II (Drummond) (45). Two-day post-injection yielded visible expression of fluorescent around the injected sites. Expression local to the recording electrode and limited to the region of interest was verified in all animals recorded. For chemogenetic manipulation, about the half of animals were injected with only AAV-FLEX-GFP for control, and the rest were injected with AAV-df-KORD and/or AAV-DIO-hM3Dq and/or AAV-FLEX-GFP. Similarly, for optogenetic manipulation, about half of the animals...
were injected only with AAV-FLEX-GFP or AAV-FLEX-tdTomato and the rest were injected with AAV-encoding opsins. Recordings and analyses were conducted in a blind manner.

**Slice recording**

Slice recordings were conducted as previously described with slight modifications (49). Mice were anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in ice-cold cutting solution containing 110 mM choline chloride, 2.5 mM KCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 12 mM glucose, 1 mM MgCl₂, and 2 mM CaCl₂. Coronal cortical and hippocampal slices (300 μm) were cut using a Leica Vibratome VT 1000S. The slices were kept in artificial cerebrospinal fluid (ACSF) containing 127 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 12 mM glucose, 1 mM MgCl₂, and 0.07 mM CaCl₂, brought to ~290 mOsm with ~28 mM sucrose and pH 7.3 with KOH. GABAergic cortical and hippocampal neurons with fluorescence were patched under a 40× objective, and whole-cell current-clamp recordings were conducted to measure the changes in resting membrane potential following bath application of 100 nM SalB or 100 nM CNO. At least 1 min of stable membrane potential was analyzed using Clampfit software (Molecular Devices) before and after treatment. For activating JAWS, a series of 1-s photostimulations with ~290 mOsm with light were delivered using a Leica DMLB microscope equipped with a ×40/1.3 NA oil immersion objective (Leica). Electrical signals were amplified with an Axopatch 200B amplifier, and pClamp (Molecular Devices) was used to acquire and analyze data.

**Immunohistochemistry**

Animals were perfused and postfixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were sectioned by vibratome at 150 μm in the coronal plane. For cryosectioning, brains were cryoprotected in 10% and 30% sucrose in PBS and then sectioned at 40 μm. The following antibodies were used: rabbit anti-GABA (Sigma, A2052, lot no. 103 M4793), chicken anti-GFP (Abcam, ab13970, lot no. GR319050-12), chicken anti–red fluorescent protein (Rockland, 600-901-379S, lot no. 42717), anti-chicken Alexa 488, anti-chicken Alexa 555, anti-rabbit Alexa 488, anti-rabbit Alexa 555, and DAPI (4’,6-diamidino-2-phenylindole) (Thermo Fisher Scientific). Confocal images were taken with Zeiss 710 using a 10× objective and analyzed with Fiji (ImageJ).

**Statistical analysis**

Mean ± 95% confidence interval (CI) are reported, and individual data were presented except for spectra. Hypothesis tests were conducted using nonparametric methods when n < 10. One-way analysis of variance (ANOVA) was used for all tests of manipulation and age dependence, and post hoc test (Tukey’s honestly significant difference) was used to examine differences between specific manipulation and age groups. Significant differences by post hoc test (P < 0.05) are reported as asterisks on the relevant figure. P values of <0.001 are rounded to nearest power of 10. Spectra were examined at each frequency for significant difference using nonparametric permutation tests corrected for multiple comparisons by the method of Cohen (50) with P < 0.05. All tests were performed in MATLAB. The number of animals, the statistical test, results, and P values are reported in table S1.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/24/eaba1430/DC1

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**

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