Selective Reduction of Cholecystokinin-Positive Basket Cell Innervation in a Model of Temporal Lobe Epilepsy

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Perisomatic inhibition from basket cells plays an important role in regulating pyramidal cell output. Two major subclasses of CA1 basket cells can be identified based on their expression of either cholecystokinin (CCK) or parvalbumin. This study examined their fates in the mouse pilocarpine model of temporal lobe epilepsy. Overall, immunohistochemical labeling of GABAergic boutons in the pyramidal cell layer of CA1 was preserved in the mouse model. However, CCK-labeled boutons in this layer were chronically reduced, whereas parvalbumin-containing boutons were conserved. Immunohistochemistry for cannabinoid receptor 1 (CB₁), another marker for CCK-containing basket cells, also labeled fewer boutons in pilocarpine-treated mice. Hours after status epilepticus, electron microscopy revealed dark degenerating terminals in the pyramidal cell layer with lingering CCK and CB₁ immunoreactivity. In mice with recurrent seizures, carbachol-induced enhancement of spontaneous IPSCs (sIPSCs) originating from CCK-containing basket cells was accordingly reduced in CA1 pyramidal cells. By suppressing sIPSCs from CCK-expressing basket cells, a CB₁ agonist reverted the stimulatory effects of carbachol in naive mice to levels comparable with those observed in cells from epileptic mice. The agatoxin-sensitive component of CA1 pyramidal cell sIPSCs from parvalbumin-containing interneurons was increased in pilocarpine-treated mice, and miniature IPSCs were reduced, paralleling the decrease in CCK-labeled terminals. Altogether, the findings are consistent with selective reduction in perisomatic CA1 pyramidal cell innervation from CCK-expressing basket cells in mice with spontaneous seizures and a greater reliance on persisting parvalbumin innervation. This differential alteration in inhibition may contribute to the vulnerability of the network to seizure activity.

Introduction

Basket cells provide inhibition to the cell bodies and proximal dendrites of CA1 pyramidal cells. This confers them with strong influence over principal cell output and strategically positions them to regulate spike timing, oscillation rate, and synchronization of their targets (Cobb et al., 1995; Ylinen et al., 1995; Miles et al., 1996; Toth et al., 1997; Penttonen et al., 1998; Pouille and Scanziani, 2001; Cobb and Davies, 2005; Mann et al., 2005; Mann and Paulsen, 2007; Glickfeld et al., 2008). These attributes also make basket cells of interest in investigations of the mechanisms underlying temporal lobe epilepsy.

Two major subclasses of basket cells have been described in CA1 that are similar in their morphology but distinctive in their functional characteristics (Klausberger et al., 2003; Somogyi and

Klausberger, 2005; Tukker et al., 2007). These subtypes can be differentiated by their protein expression profiles, because it is well established that discrete populations of hippocampal basket cells express either the calcium binding protein parvalbumin (PV) or the neuropeptide cholecystokinin (CCK) (Nunzi et al., 1985; Ribak et al., 1990; Pawelzik et al., 2002; Mátyás et al., 2004; Klausberger et al., 2005; Freund and Katona, 2007). Other distinguishing proteins that can be used to discriminate between these subclasses of basket cells are the cannabinoid receptor type I (CB₁) and M₁/M₃ acetylcholine receptors, which are expressed by CCK- but not PV-containing basket cells, as well as voltage-gated calcium channels, with PV-containing basket cells expressing P/Q-type and CCK-containing basket cells expressing N-type calcium channels (Katona et al., 1999; Freund and Katona, 2007).

Interneurons are differently affected in temporal lobe epilepsy: some prove to be vulnerable and others more resilient (Houser and Esclapez, 1996; Morin et al., 1998). Previous anatomical and electrophysiological studies have demonstrated that, although GABAergic inputs to CA1 pyramidal cell dendrites are reduced in animals with spontaneous seizures, inhibition to pyramidal cell bodies is not lost and may be increased (Morin et al., 1998, 1999; Hirsch et al., 1999; Cossart et al., 2001; Wittner et al., 2005).

The present study used a multipronged approach to investigate the independent fates of basket cell subtypes in the pilocarpine model of temporal lobe epilepsy to ascertain whether consideration as a broad group could mask alterations in the

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Table 1. Antibody details

Antiserum	Source	Number	Host	IHC	IMF	EM
NeuN	Millipore Corporation	MAB377	Mouse	1:5000		
GAD67	Millipore Corporation	MAB5406	Mouse	1:3000		
PV	Provided by Dr. Marco Celio, University of Fribourg, Fribourg, Switzerland		Rabbit	1:8000	1:10,000	
CCK	CURE, University of California, Los Angeles, Los Angeles, CA	9303	Mouse	1:10,000	1:2000	1:5000
CB ₁	Provided by Dr. Ken Mackie, Indiana University, Indianapolis, IN	L14	Rabbit	1:8000		
CB ₁	Frontier Science	Af380-1	Rabbit			1:500–1:1000

IHC, Immunohistochemistry; IMF, immunofluorescence; EM, electron microscopy.

subgroups. Immunohistochemistry was used to examine PV- and CCK-containing basket cell terminals with light and electron microscopy, and postsynaptic GABAergic currents were recorded from CA1 pyramidal cells under various pharmacological conditions to determine whether the sources of perisomatic inhibition were changed.

Materials and Methods

Mouse pilocarpine model. Pilocarpine was administered to 7- to 8-week-old male C57BL/6 mice to induce status epilepticus, as described previously by Peng et al. (2004). In brief, mice were pretreated with a low dose of the cholinergic antagonist methylscopolamine nitrate (1 mg/kg, s.c.; Sigma-Aldrich) to reduce peripheral cholinergic effects. After 30 min, mice were injected with either pilocarpine hydrochloride (320 mg/kg, i.p.; Sigma-Aldrich) to induce status epilepticus or saline for control mice that otherwise received the same series of injections. Two hours after the onset of status epilepticus, behavioral seizures were alleviated with diazepam (5 mg/kg, s.c.; Abbott Laboratories). Pilocarpine-treated mice were monitored for spontaneous seizures after a period of recovery. All mice exhibited spontaneous seizures by the fourth week after status epilepticus. Nine pairs of control and pilocarpine-treated mice (9 h, 1 and 2 d, 1 week, 1 and 2 months after status epilepticus) were used for light microscopy, 12 pairs were used for electron microscopy (9 h and 1, 3, 5, and 7 d after status epilepticus), and six pairs were used for electrophysiology (2 months after status epilepticus). An additional nine age-matched naive mice were used in the electrophysiological studies. All animal use protocols conformed to National Institutes of Health guidelines and were approved by the University of California, Los Angeles, Chancellor's Animal Research Committee.

Antisera. A monoclonal antibody to neuron-specific nuclear protein (NeuN) (Mullen et al., 1992) was used to assess possible loss of CA1 pyramidal cells after status epilepticus. A monoclonal antibody against glutamate decarboxylase 67 (GAD67) was used to label GABAergic interneuron boutons. During preliminary studies, we evaluated vesicular GABA transporter VGAT, GABA transporter GAT-1, GAD65, and GAD67 as markers for perisomatic GABAergic terminals in CA1. Although GAD65 is generally a preferred marker for GABAergic terminals (Esclapez et al., 1994), GAD67 has been identified as a particularly sensitive marker of terminals in the CA1 pyramidal cell layer (Fukuda et al., 1998). We confirmed these findings before selecting GAD67 for use in these studies.

PV and CCK antisera were used to label basket cell terminals of different subclasses. Two antisera were used against the presynaptic CB₁, which is an additional marker for CCK-expressing basket cell terminals. The CB₁ antiserum L14 was raised in rabbit against the last 15 aa of the C terminus of the rat CB₁ receptor (Nyiri et al., 2005). This antibody to the CB₁ receptor may be specific to GABAergic boutons because it is possibly occluded from binding in glutamatergic cells by the cannabinoid receptor-interacting protein CRIP1a (Katona et al., 2006; Niehaus et al., 2007). The CB₁ antiserum from Frontier Science, used for ultrastructural studies, was raised against 31 aa of the mouse C terminus in rabbit. The characteristics and specificity of these antisera have been described previously (Fukudome et al., 2004; Yoshida et al., 2006; Uchigashima et al., 2007). Antibody details are given in Table 1.

Tissue preparation for light microscopy. Mice were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. Matched controls were perfused along with pilocarpine-treated mice at 9 h, 1 and 2 d, 1 week, and 1 or 2 months after status epilepticus. After cooling *in situ* at 4°C for 1 h, brains were postfixed with the same solution for 1 h at room temperature (RT). After thorough rinsing, the brains were cryoprotected in a 30% sucrose solution before embedding in OCT compound (Sakura Finetech) and freezing on dry ice. Coronal slices (30 μm) were sectioned with a cryostat. Every 10th section was stained with cresyl violet for histological analyses, whereas the remaining sections were stored in cryoprotectant at −20°C until processing.

Immunohistochemistry for light microscopy. For consistency, sections from control and experimental mice were processed in parallel at RT. To reduce the effects of endogenous peroxidase activity, sections were pretreated for 30 min with 1% H₂O₂. Those sections processed for CCK and CB₁ (L14) immunohistochemistry were first incubated for 70 min in a citrate buffer (0.05 M sodium citrate in 1% NaCl, pH 8.6) at 90°C for antigen retrieval. After a 15 min rinse in 0.1 M Tris-buffered saline (TBS), pH 7.3, all sections were incubated in 10% normal horse serum or normal goat serum (NGS) in TBS with 200 μl/ml avidin for 1–3 h to reduce nonspecific binding. Blocking solution also included 0.3% Triton X-100 to maximize labeling in all cases except for NeuN. Sections were then incubated overnight with the primary antiserum diluted in TBS containing 2% normal serum and biotin (200 μl/ml). The next day sections were rinsed and incubated for 1 h in biotinylated secondary antiserum (1:1000; Vector Laboratories): horse anti-mouse IgG or goat anti-rabbit IgG, as appropriate to the primary antiserum. Sections were rinsed in TBS before incubation in avidin–biotin peroxidase complex (1:200; Vectastain Elite ABC; Vector Laboratories) for 1 h, rinsed, and processed for 10 min in stable diaminobenzidine tetrahydrochloride (Invitrogen) for visualization of peroxidase labeling. After rinsing in PBS, sections were treated with 0.003% osmium tetroxide in PBS for 30 s to enhance and preserve immunolabeling. Sections were given a final rinse in PBS and were mounted on gelatin-coated slides, dehydrated, and coverslipped.

Double-immunofluorescence labeling. To confirm the distinct protein expression profiles of basket cell subgroups, double-immunofluorescence labeling for PV and CB₁ was performed. After rinsing in TBS and a 30 min pretreatment in 1% H₂O₂, free-floating sections were incubated for 3 h in blocking solution containing 10% NGS in 0.1 M TBS with 0.3% Triton X-100. Sections were again rinsed before incubating for three nights at RT in a mixture of primary antisera that included 2% NGS and 0.1% sodium azide in TBS. After another TBS rinse, sections were incubated for 4 h at RT in a 3% NGS/TBS mixture of fluorescent dye-labeled secondary antisera (1:500; both from Invitrogen): goat anti-rabbit IgG labeled with Alexa Fluor 488 was used to visualize PV labeling, and goat anti-mouse IgG conjugated to Alexa Fluor 555 was used for visualizing CB₁. After a final rinse in PBS, sections were mounted on slides and coverslipped with the antifade medium Prolong Gold (Invitrogen).

Analysis of light microscopy. Digital images of immunoperoxidase-labeled sections were obtained with a Zeiss Axio Imager.Z1 AX10 microscope equipped with an AxioCam digital camera system and AxioVision 4.6 software. Qualitative analyses of terminal labeling were conducted for

Figure 1. Pyramidal cells in CA1 were not noticeably reduced in pilocarpine (Pilo)-treated mice selected for this study. **A–D**, NeuN immunolabeling demonstrates preservation of the pyramidal cell layer (P) in CA1 in a pilocarpine-treated mouse (**B, D**) compared with a control mouse (**A, C**). However, cell loss is evident in the hilus (h) of this pilocarpine-treated mouse (**B**). **E, F**, GABAergic interneurons and boutons were labeled for GAD67 and are highly concentrated in the pyramidal cell layer in a control mouse, especially toward stratum oriens (O) (**E**). In a pilocarpine-treated mouse, a dense band of labeling remains in the pyramidal cell layer, and basket cell boutons innervating pyramidal cell bodies in CA1 are preserved (**F**). In contrast, in stratum radiatum (R), labeling of GABAergic boutons appears to be reduced in the pilocarpine-treated mouse (**F**). Scale bars: **A, B**, 200 μ m; **C–F**, 10 μ m.

all mice. In addition, semiquantitative analyses of CCK-, PV-, and CB₁-labeled terminals in the CA1 pyramidal cell layer were performed for control and pilocarpine-treated mice at 1 and 2 months after status epilepticus at similar levels of the dorsal hippocampus. Counting frames (3500 μ m²) along the CA1 pyramidal cell layer were outlined bilaterally in each section, and the number of terminals was determined in four to six sections from five pairs of control and pilocarpine-treated mice that were processed together and matched for date of birth, injections, and perfusion. Grayscale images for quantification were obtained using a 100 $\times$ objective and analyzed with the AxioVision Automated Measurement System. Threshold and watershed parameters were established to best approximate manual determinations of labeled puncta. No brightness, contrast, sigma filtering, shading correction, edge enhancement, artifact deletion, or hole filling alterations were performed. A segmentation setting for threshold was calibrated to include intensities $\sim$ 2 SDs below the mean to best detect all labeled terminals. Watershed with 0 tolerance was applied for auto object separation to partition any clusters. The program was set to exclude objects with a radius <0.25 μ m, and automated terminal counts were conducted. Data were analyzed for significance by two-tailed *t* test.

Double-labeled sections were scanned, and digital images were obtained with a Zeiss LSM 510 META confocal microscope. Confocal images were analyzed with Zeiss LSM 5 Image Examiner software.

Tissue preparation for electron microscopy. Electron microscopy was used to check for morphological evidence of dark degenerating terminals at 1, 3, 5, and 7 d after pilocarpine-induced status epilepticus. In preparation for structural analysis, mice were perfused as described previously but with a fixative solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3. After cooling *in situ* at 4°C for 2 h, brains were postfixed overnight. The tissue was rinsed, and a

VT1000S Leica Microsystems vibratome was used to cut 250- μ m-thick coronal sections for electron microscopy, with 50 μ m sections interspersed for staining with cresyl violet to verify minimal CA1 pyramidal cell loss.

Immunoelectron microscopy was used to determine the neurochemical identity (CCK/CB₁) of degenerating terminals. Mice were perfused as described above, 9 h after status epilepticus, with a fixative solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3.

Processing for electron microscopy. Specimens were cut from the CA1 pyramidal cell layer, rinsed in 0.12 M phosphate buffer, and incubated in 1% osmium tetroxide. The tissue was rinsed, processed through ethanol and propylene oxide, and then incubated for 1 h at 56°C in Durcupan before being transferred to fresh Durcupan for overnight incubation at RT. The next day, sections were embedded in capsules with Durcupan and polymerized at 56°C for 48 h. Ultrathin sections were cut with an ultramicrotome (Reichert-Jung), and gold-reflecting ($\sim$ 20 nm) sections were picked up on nickel mesh grids that were freshly coated with a Coat-Quick G pen (Electron Microscopy Sciences). Sections were treated for 20 min with 2% uranyl acetate. After rinsing, sections were treated with 0.3% lead citrate for 3 min.

Preembedding immunogold labeling. Hippocampal tissue was sectioned coronally at 60 μ m with a Leica Microsystems vibratome. Select sections were processed for Nissl staining to verify that the CA1 pyramidal cell layer was intact. After rinsing, sections for immunogold labeling were sequentially pretreated with 1% sodium borohydride and 0.5% H₂O₂ for 30 min each. Sections were rinsed and incubated for 2 h in 10% normal serum and then overnight at RT in primary antiserum (CCK or CB₁) diluted in 0.1 M TBS containing 1% NaCl, 0.1% Na₂S₂O₃, and 2% normal serum. The next day, sections were rinsed in TBS, processed in 2% NGS for 10 min, and incubated in nanogold-labeled goat anti-mouse or goat anti-rabbit secondary antiserum (1.4 nm colloidal gold particles) diluted 1:100 in TBS with 0.01% NGS for 4.5 h at RT. Sections were thoroughly rinsed in TBS and then double-distilled H₂O. Gold enhancement of the nanogold particles was conducted using a kit from Nanoprobes (catalog #2002, #2004, and #2113) according to company specifications: equal parts enhancer and activator solutions were mixed 5–10 min before the addition of initiator solution, followed by the buffer solution. Sections were processed for 12.5 min, rinsed well, and stored overnight at 4°C. The next day, sections were postfixed for 1 h in 2% paraformaldehyde with 2.5% glutaraldehyde, rinsed in phosphate buffer, and processed for electron microscopy as above.

Electrophysiology. Carbachol, an acetylcholine receptor agonist, has been used to selectively stimulate CCK- but not PV-containing basket cells (Karson et al., 2008). This provided a convenient method for testing whether the inputs from CCK-containing basket cells to their CA1 pyramidal cell targets are altered in mice with spontaneous seizures. In a complimentary experiment, ω -agatoxin IVa was used to selectively inhibit P/Q-type calcium channels on PV-containing interneuron boutons (but not N-type calcium channels on CCK- expressing interneuron boutons) to test whether inhibitory currents from PV-expressing basket cells are changed in pilocarpine-treated mice. Subsequently, cadmium was used to block all calcium-dependent transmitter release to assess whether miniature IPSCs (mIPSCs) are reduced in pilocarpine-treated mice.

Spontaneous IPSCs (sIPSCs) were recorded from CA1 pyramidal cells by whole-cell patch clamp in slices prepared from six pilocarpine-treated C57BL/6 mice, six age-matched controls, and nine age-matched naive

mice. Mice were anesthetized deeply with halothane and guillotined, and their brains sectioned coronally at 350 μm on a Leica Microsystems vibratome. Oxygenated cutting solution contained the following: 135 mM *N*-methyl-D-glucamine, 20 mM choline bicarbonate, 10 mM D-glucose, 1.5 mM MgCl_2 , 1.2 mM KH_2PO_4 , 1 mM KCl, and 0.5 mM CaCl_2 , 310 mOsm, pH adjusted to ~ 7.4 with HCl. Slices were stored at RT, immersed in oxygenated artificial CSF (ACSF): 126 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , and 10 mM D-glucose, pH ~ 7.4 , ~ 310 mOsm.

For recordings, sections were perfused (~ 7 ml/min) with oxygenated ACSF at $\sim 33^\circ\text{C}$, and CA1 pyramidal cells were identified using infrared video microscopy (Versascope; E. Marton Electronics). Cells were voltage clamped at 10 mV (~ 5 M Ω pipette resistance) to record sIPSCs with internal solution containing the following: 140 mM Cs-methylsulfonate, 10 mM HEPES, 5 mM NaCl, 2 mM MgATP, 0.2 mM EGTA, 0.2 mM NaGTP, and 5 mM QX-314 [2-(triethylamino)-*N*-(2,6-dimethylphenyl) acetamine], pH adjusted to ~ 7.3 with CsOH, 280–290 mOsm.

In the first set of experiments, carbamylcholine chloride (carbachol, 10 μM ; C-4382; Sigma-Aldrich) and CB₁ agonist WIN55,212-2 mesylate [R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate] (5 μM ; catalog #1038 dissolved in Tocrisolve, catalog #1684; Tocris Bioscience) were bath applied to stimulate or inhibit CCK/CB₁-containing basket cells, respectively. Preliminary experiments were conducted in naive mice in the presence of kynurenic acid to suppress glutamatergic transmission. However, after establishing that its omission resulted in no significant difference in the effect of carbachol (frequency peaked at 11.9 ± 2.2 Hz in 3 mM kynurenic acid and regular ACSF at 10.1 ± 2.8 Hz; $t > 0.05$), kynurenic acid was left out of the perfusing solution.

In the second set of experiments, 500 nM ω -agatoxin IVa (H-1544; Bachem) was used to silence sIPSCs from interneurons expressing P/Q-type calcium channels. The ACSF was recirculated and included 5% bovine serum albumin (SP-5050; Vector Laboratories) to prevent the drug from sticking to the tubing. After 10 min (usually the effect of ω -agatoxin IVa stabilized after ~ 3 min), 50 μM cadmium chloride was added to the recirculating solution to block calcium-dependent neurotransmitter release, isolating mIPSCs.

The signal was low-pass filtered at 2 kHz and digitized at 4096 Hz, and sIPSCs were detected and analyzed using EVAN (custom-designed LabView-based software from Thotec) and IGOR Pro software (WaveMetrics).

Analysis of electrophysiology. Digitized recordings were plotted for every 20,480 points (5 s) in IGOR, and the baseline was normalized to the mean current. As described previously (Klaassen et al., 2006), an alpha function was fit to the mean phasic current using the following equation: $i_{\text{mean}}(t) = A \times [(t - t_0)/\tau] \times \exp[1 - (t - t_0)/\tau] + y$, where A is peak amplitude, t_0 is latency, τ is decay, and y is the control baseline. Results are expressed as mean $\pm$ SEM values. Data were examined by two-tailed t tests, one-way ANOVA with Tukey's multiple comparison test, or two-way ANOVA with Bonferroni's *post hoc* tests using Prism (GraphPad Software), and differences were considered significant at $p < 0.05$.

Results

CA1 basket cell targets and extensive GABAergic innervation remained in pilocarpine-treated mice

In the mouse pilocarpine model, there is generally little loss of CA1 pyramidal cells, but there can be patchy, and occasionally

Figure 2. Subpopulations of basket cell terminals in CA1 were disparately affected in pilocarpine (Pilo)-treated mice. **A**, Immunohistochemistry for PV labels a subset of basket cell terminals that innervate CA1 pyramidal cells (P) and proximal dendrites in a control mouse. Terminal labeling in the dendritic layers of strata radiatum (R) and oriens (O) is less dense. **B**, Boutons immunoreactive for PV are not reduced in a pilocarpine-treated mouse compared with a control. **C**, In a control mouse, CCK labels punctate structures—presumptive basket cell boutons—throughout the pyramidal cell layer of CA1. **D**, Punctate labeling of CCK-containing basket cell boutons is decreased in the pyramidal cell layer in a pilocarpine-treated mouse, whereas diffuse labeling in strata radiatum and oriens is often increased. Scale bar: **A–D**, 10 μm .

severe, loss. To ensure that the postsynaptic targets of basket cells remained, cresyl violet or NeuN labeling was used to screen for mice with an intact pyramidal cell layer for inclusion in the study. Among mice with spontaneous seizures, only those whose pyramidal cell layer was preserved were included (Fig. 1*A–D*).

GAD67 immunohistochemistry was used to compare the overall pattern of GABAergic perisomatic terminal fields in CA1 between controls and mice with spontaneous seizures. In control mice, the pyramidal cell layer was strongly labeled by a dense plexus of GABAergic terminals (Fig. 1*E*). The boutons encircled the pyramidal cell bodies and were especially dense toward stratum oriens (Fig. 1*E*). In pilocarpine-treated mice, GAD67 labeling of the CA1 pyramidal cell layer was not appreciably reduced compared with controls (Fig. 1*F*). In contrast, GAD67-labeled boutons appeared to be reduced in CA1 dendritic layers of pilocarpine-treated mice compared with controls (Fig. 1*E,F*), consistent with previous reports (Houser and Esclapez, 1996; Cossart et al., 2001).

Perisomatic innervation from subclasses of basket cells diverged

Subgroups of basket cell boutons in CA1 were then examined to determine whether this would unveil changes in pilocarpine-treated mice that were masked by generic consideration of basket cell boutons. Two distinct populations of basket cells were distinguished by their expression of either PV or CCK. PV-immunoreactive boutons were numerous and surrounded the pyramidal cell bodies (Fig. 2*A*). The plexus of PV-labeled basket cell boutons innervating CA1 pyramidal cell bodies was preserved in pilocarpine-treated mice (Fig. 2*B*). Semiquantitative analysis identified 13.1 ± 0.4 PV-immunoreactive boutons per 100 μm^2 in controls and 13.6 ± 0.4 boutons in mice treated with pilocarpine ($p > 0.05$, two-tailed t test).

Figure 3. The reduction of CCK-labeled basket cell boutons occurred soon after status epilepticus and persisted long term. **A, B.** In a control mouse, CCK labeling is present in all layers of CA1, including strata radiatum (R) and oriens (O), and with distinct terminal-like structures evident in the pyramidal cell layer (P). The hilus (h), inner molecular layer of the dentate gyrus (arrowheads), and mossy fiber path (arrows) are densely labeled. **C, D.** A decrease in CCK-immunoreactive boutons in the CA1 pyramidal cell layer compared with controls was visible as early as 9 h after injection of pilocarpine (Pilo). **E, F.** At 1 week after status epilepticus, the reduction in CCK labeling is still evident in the pyramidal cell layer and slightly more pronounced. Labeling of CCK is also reduced in the hilus (h) and mossy fiber pathway (arrows). **G, H.** CCK labeling in the pyramidal cell layer remains reduced at 2 months after status epilepticus, whereas labeling in strata oriens and radiatum is stronger than at earlier time points. Scale bars: **A, C, E, G,** 200 μm ; **B, D, F, H,** 10 μm .

In control mice, CCK-containing boutons were sparser than PV-containing boutons, and puncta were scattered evenly throughout the pyramidal cell layer (Fig. 2C). However, in mice at 2 months after status epilepticus, CCK-immunoreactive boutons were substantially reduced relative to controls (Fig. 2D). Semiquantitative analysis revealed an 88% decrease of CCK-labeled boutons in mice with spontaneous seizures (from 10.9 ± 0.9 to 1.7 ± 0.6 per 100 μm^2 ; $p < 0.001$, two-tailed t test). The reduction in CCK-immunoreactive boutons was evident throughout the rostrocaudal extent of the hippocampus.

Timeline of reduction in bouton labeling

A qualitative time course study was conducted to determine the timing of the decrease in CCK expression (Fig. 3). At 9 h after status epilepticus, there was a perceptible reduction in the number of

labeled boutons in CA1 compared with controls (Fig. 3A–D). Within 1 week, very few CCK-immunoreactive boutons remained in the pyramidal cell layer (Fig. 3E, F). The number of labeled boutons remained low 2 months after pilocarpine (Fig. 3G, H), indicating that this reduction persists long term and is likely permanent. By this time, diffuse CCK immunoreactivity was commonly increased in the dendritic layers of strata oriens and radiatum, in contrast to the reduced punctate labeling in the cell body layer. A comparable decrease in CCK-labeled boutons was also evident in the CA3 pyramidal cell layer. These changes in the hippocampus were more marked than those in the granule cell and molecular layers of the dentate gyrus in which only a minor decrease in CCK boutons was observed. However, over time a reduction in CCK labeling was evident in the mossy fiber pathway and hilus (Fig. 3, compare A, E). A similar, species-specific reduction in CCK immunoreactivity in the dentate gyrus and mossy fibers was reported previously (Gall et al., 1986).

Although the decrease in CCK labeling could be attributed to a decrease in immunoreactivity in some regions, such as the mossy fiber path and inner molecular layer, a loss of CCK-expressing interneurons could have occurred in other regions, including CA1. With current methods, it was not possible to consistently label the cell bodies of CCK-containing neurons in the mouse hippocampus, and thus we were unable to obtain reliable counts of CCK basket cells in control and pilocarpine-treated mice. Other immunohistochemical and electrophysiological studies were therefore used to determine whether the reduction of CCK-labeled boutons in CA1 reflected loss of immunoreactivity or degeneration.

Decrease in CCK-labeled boutons was mirrored by a decrease in CB₁

CB₁ was used as another marker of CCK-containing boutons to distinguish between the possibilities that the reduction in CCK-labeled boutons reflects loss of immunoreactivity versus degeneration. The axon terminals of hippocampal basket cells that contain CCK also express presynaptic CB₁, whereas boutons of PV-containing basket cells primarily do not. The CB₁ antiserum heavily labeled the pyramidal cell layer, demonstrating the dense innervation of pyramidal cell bodies by CB₁-expressing basket cells (Fig. 4A). In mice with spontaneous seizures, the number of labeled boutons was greatly diminished (Fig. 4B). The reduction was especially prominent in the pyramidal cell layer but also occurred in the dendritic layers of strata oriens, radiatum, and lacunosum. Semiquantitative analysis indicated a 57% reduction in CB₁-labeled boutons in the CA1 pyramidal cell layer of pilocarpine-treated mice (from 10.6 ± 0.2 per 100 μm^2 in controls to 4.5 ± 0.2 per 100 μm^2 ; $p < 0.0001$, two-tailed t test).

Figure 4. Immunolabeling of CB₁-expressing basket cell boutons was decreased in pilocarpine (Pilo)-treated mice. **A**, Labeling for CB₁ revealed an especially dense terminal field in the CA1 pyramidal cell layer (P) of a control mouse. **B**, In a pilocarpine-treated mouse, CB₁-immunoreactive boutons are decreased in the CA1 pyramidal cell layer (arrows). CB₁ labeling was also reduced in strata oriens (O) and radiatum (R) of CA1. Scale bar: **A**, **B**, 200 μ m.

CB₁ labeling was reduced soon after status epilepticus and persisted long term

To clarify the timing of the decline in bouton immunoreactivity for CB₁, mice were studied at intermediate intervals (including 9 h, 1 and 2 d, 1 week, and 1 and 2 months) after status epilepticus. In controls, labeling for CB₁ showed a thick network of basket cell boutons that outlined the contours of unlabeled pyramidal cell bodies (Fig. 5A). A decrease in labeled boutons in CA1 was evident 48 h after status epilepticus (Fig. 5B). Loss of labeling for CB₁-containing boutons was progressive over a period of days after status epilepticus, but consistent levels of labeling at 1 and 2 months indicated that it stabilized and was permanent (Fig. 5C). During the chronic period, the paucity of CB₁-immunoreactive boutons remaining in the pyramidal cell layer in mice with spontaneous seizures was in stark comparison with the plentiful labeling in control mice.

Subtypes of basket cell boutons were heterogeneously affected in pilocarpine-treated mice

Finally, double labeling was used to confirm the decrease in CB₁-labeled boutons and preservation of PV-labeled terminals in the same tissue sections. Double labeling for CB₁ and PV revealed nonoverlapping populations of boutons, confirming that they belong to two unique subclasses of basket cells (Fig. 6). The profusion of PV-expressing boutons in controls (Fig. 6A) was maintained in mice with spontaneous seizures (Fig. 6B), concurrent with a substantial and long-lasting reduction in the number of boutons labeled for CB₁ (Fig. 6C,D). This analysis confirmed the divergent fates of these subgroups in the same tissue (Fig. 6E,F).

Basket cell terminals degenerate after status epilepticus

For direct evidence of basket cell terminal degeneration after status epilepticus, electron microscopy was used to identify dark

Figure 5. Reduced labeling of CB₁-containing boutons was evident shortly after status epilepticus. **A**, CB₁-immunoreactive boutons are abundant in the pyramidal cell layer (P) in a control mouse and encircle the cell bodies. **B**, Within 48 h after status epilepticus, labeling of CB₁-containing boutons targeting pyramidal cell bodies is diminished. **C**, The number of CB₁-labeled boutons remains low up to 2 months after pilocarpine (Pilo) administration. Labeled boutons are also decreased in adjacent dendritic layers, strata oriens (O) and radiatum (R). Scale bar: **A–C**, 10 μ m.

degeneration. Because dark degeneration can occur rapidly, tissue was examined at 1, 3, 5, and 7 d after pilocarpine injection and in matched controls. Healthy basket cell terminals contained well defined mitochondria and numerous synaptic vesicles, and they formed synapses onto pyramidal cell bodies in CA1 (Fig. 7A). Although healthy terminals remained abundant in the pyramidal cell layer after status epilepticus, degenerating terminals were found at all time points. Degeneration was recognized as electron-dense profiles of irregular shape whose organelles were no longer distinct (Fig. 7B). Numerous dark profiles were nevertheless identifiable as terminals by their remaining synaptic contacts.

Figure 6. Subclasses of CA1 basket cell boutons were distinct and differentially affected in pilocarpine (Pilo)-treated mice. **A, B**, Numerous PV-containing boutons surround the somata of pyramidal cells in a control mouse and remain abundant in a pilocarpine-treated mouse. **C, D**, In the same tissue sections, CB₁-labeled boutons are reduced from a mouse with recurrent seizures compared with a control. **E, F**, Double-labeled sections show that the PV- and CB₁-expressing boutons are from non-overlapping subpopulations and demonstrate the differential decrease in CB₁-labeled boutons. Scale bar: **A–F**, 5 μ m.

The swift degeneration into unidentifiable profiles and subsequent phagocytosis, compounded by the protracted period of asynchronous degeneration across the population of terminals, precluded an overall quantitative analysis of degeneration at the ultrastructural level. There is the possibility of limited degeneration under normal conditions, but the qualitative data showed that it was nowhere near as widespread as after status epilepticus, in keeping with other examples of cell loss in epilepsy and its models. Examination of matched control tissue yielded one possible degenerating terminal, in contrast to the numerous degenerating terminals identified in pilocarpine-treated mice over the expanse of 1 week.

To determine whether these degenerating terminals were from CCK/CB₁-expressing basket cells, gold-enhanced immunogold preembedding methods were used. Even shorter intervals (9 h) after status epilepticus were used to detect residual protein in degenerating terminals. With these methods, healthy CCK-expressing terminals in the CA1 pyramidal cell layer were labeled with nanogold particles (Fig. 7C). Immunogold particles also labeled CCK in dark degenerating profiles that could be identified as basket cell terminals by their persisting synapses onto pyramidal cell bodies (Fig. 7D).

The same methods were undertaken with CB₁ antiserum. In this case, immunogold particles labeled cannabinoid receptors on the plasma membrane, lining the perimeter of healthy terminals in control mice (Fig. 7E). Preembedding immunogold also identified CB₁ in dark degenerating CA1 basket cell terminals 9 h after status epilepticus, and the characteristic receptor pattern around the surface of the terminal was observed (Fig. 7F). This established that some CCK- and CB₁-expressing basket cell terminals that synapse onto CA1 pyramidal cells were degenerating at short intervals after status epilepticus.

Reduced pyramidal cell sIPSCs generated by CCK-containing basket cells

Carbachol, an acetylcholine receptor agonist, has been used previously to stimulate CCK- but not PV-containing basket cells (Karson et al., 2008). The selective targeting allowed for testing whether the inputs from CCK-containing basket cells to their CA1 pyramidal cell targets were altered in pilocarpine-treated mice. Whereas PV-containing basket cells express M₂ receptors on their terminals, CCK-containing cells express M₁/M₃ receptors on their cell bodies (Hájos et al., 1998; Freund and Katona, 2007). A low concentration of carbachol (10 μ M) was used to selectively activate M₁/M₃ receptors on CCK-expressing cells in slices, and sIPSCs were monitored in pyramidal cells by whole-cell patch-clamp recording. If pilocarpine-treated mice had fewer CCK-containing terminals, a diminished response to carbachol compared with controls would be expected. However, if those terminals had merely lost CCK immunoreactivity, they could still respond to carbachol.

Perfusion of carbachol increased inhibitory currents recorded from individual pyramidal cells in slices from control mice, increasing both frequency and amplitude of sIPSCs (Figs. 8A, 9). The mean phasic current was calculated in 5 s bins using a macro run on IGOR software after any linear trends in the mean baseline current were subtracted (Glykys and Mody, 2007). Because carbachol produced a transient peak in mean phasic current, an alpha function was fit to smooth the noise in the response (Fig. 8B). Carbachol had a much less pronounced effect in CA1 pyramidal cells of pilocarpine-treated mice on sIPSCs and the mean phasic current (Fig. 8C,D). Carbachol did not significantly change the amplitude and frequency of sIPSCs in pilocarpine-treated mice (Figs. 8C, 9), nor did it significantly enhance the mean phasic current (Figs. 8D, 10).

Bath perfusion of carbachol significantly increased sIPSC frequency in control pyramidal cells (from a baseline of 3.1 ± 1.4 to 10.1 ± 2.8 Hz; $p < 0.01$, two-way ANOVA; $n = 12$) (Fig. 9A) but had negligible effect in cells from pilocarpine-treated mice (increasing frequency from 4.6 ± 1.1 to 6.1 ± 1.1 Hz; $p > 0.05$, two-way ANOVA; $n = 15$). The $369 \pm 68\%$ average enhancement in sIPSC frequency seen in control slices is significantly greater than the $114 \pm 50\%$ change in slices from pilocarpine-treated mice ($p < 0.01$, one-way ANOVA, Tukey's test) (Fig. 9B). The mean percentage enhancement was calculated from the increase in each cell normalized to its baseline.

In control slices, carbachol significantly increased the amplitude of recorded sIPSCs (from 42.3 ± 4.3 to 75.3 ± 6.3 pA; $p < 0.001$, two-way ANOVA; $n = 12$) (Fig. 9C). Carbachol did not significantly increase sIPSC amplitude in slices from pilocarpine-treated mice (33.8 ± 3.3 to 42.1 ± 9.1 pA; $p > 0.05$, two-way ANOVA; $n = 15$). This corresponds to a $28 \pm 27\%$ increase in amplitude over baseline in CA1 pyramidal cells from pilocarpine-treated mice compared with an $84.8 \pm 16\%$ increase in controls ($p > 0.05$, one-way ANOVA, Tukey's test) (Fig. 9D).

Carbachol increased the peak of the alpha function fit to the mean phasic current by $685 \pm 133\%$ to 13.9 ± 2.9 pA in control slices from a baseline of 3.3 ± 1.7 pA ($p < 0.001$, two-way ANOVA; $n = 12$ cells), in contrast to a more subtle $152 \pm 57\%$ increase in slices from pilocarpine-treated mice to a peak of 3.4 ± 1.0 pA from a baseline of 1.4 ± 0.2 pA ($p > 0.05$, two-way ANOVA; $n = 15$ cells) (Fig. 10*A,B*). Once again, carbachol had a significantly greater effect in control slices than slices from pilocarpine-treated mice ($p < 0.001$, two-way ANOVA). The reduced ability of carbachol to stimulate sIPSCs from CCK-expressing cells in pilocarpine-treated mice is most consistent with the idea that the terminals are no longer in place to be stimulated.

Specific activation of CB₁-containing cells by carbachol

Pyramidal cells also express M₁/M₃ receptors, and carbachol can activate other muscarinic receptors at higher concentrations. To confirm that our experiments measured a response to carbachol specifically from CCK-expressing interneurons, the effect of carbachol on slices from naive mice was also tested in the presence of the CB₁ agonist WIN55,212-2, which leads to reduced GABA release from CCK-containing interneurons (Hájos et al., 2000). Bath application of WIN55,212-2 stifled the response to carbachol recorded in CA1 pyramidal cells from naive mice (Fig. 8*E,F*).

The presence of WIN55,212-2 tempered the carbachol-induced increase in sIPSC frequency (from 4.6 ± 0.6 to 9.8 ± 1.2 Hz; $p < 0.001$, two-way ANOVA; $n = 20$ cells) (Fig. 9*A*), corresponding to a $155 \pm 39\%$ increase over baseline that was significantly less than in controls ($p < 0.01$, one-way ANOVA, Tukey's test) (Fig. 9*B*) but was not different from cells from pilocarpine-treated mice. The use of averaged relative increases in sIPSC frequency produced by carbachol (Fig. 9*B*) corrects for variation in absolute frequencies between slices and animals and clearly illustrates the reduced effect of carbachol in WIN55,212-2.

Carbachol had a marginal effect on the amplitude of sIPSCs in the presence of WIN55,212-2, inducing an increase from 40.5 ± 5.6 to 47.2 ± 3.6 pA ($p > 0.05$, two-way ANOVA; $n = 14$) (Fig. 9*C*). This $30 \pm 9\%$ increase over baseline (Fig. 9*D*) was less than the increase of sIPSC amplitude in controls ($p < 0.01$, one-way ANOVA) and more similar to that in cells from pilocarpine-treated mice ($p > 0.05$, one-way ANOVA).

Relative to controls, the peak of the alpha function fit to the carbachol-induced enhancement in mean phasic current was also

Figure 7. CCK- and CB₁-expressing CA1 basket cell terminals degenerated within hours of status epilepticus in pilocarpine (Pilo)-treated mice. *A*, A week after status epilepticus, mitochondria and synaptic vesicles are clearly visible in a healthy terminal that synapses (arrows) with CA1 pyramidal cell bodies. *B*, An electron-dense degenerating terminal, with an irregular shape and indistinct organelles, is found among healthy terminals in the CA1 pyramidal cell layer a few days after status epilepticus. It remains in synaptic contact (arrow) with a cell body. *C*, Gold-enhanced immunogold particles label a normal CCK-containing terminal in contact with a CA1 pyramidal cell body in a control mouse. *D*, At 9 h after status epilepticus, immunogold labeling of CCK is detected in a degenerating terminal. *E*, Immunogold labeling of CB₁ forms a ring near the plasma membrane of a normal CA1 basket cell terminal. *F*, CB₁ immunogold labeling is evident in a degenerating basket cell terminal in the pyramidal cell layer at 9 h after pilocarpine injection. Scale bars: *A–F*, 0.25 μ m.

dampened in CA1 pyramidal cells bathed in WIN55,212-2 (baseline, 2.0 ± 0.3 pA; carbachol, 5.8 ± 0.9 pA; $p > 0.05$, two-way ANOVA; $n = 14$ cells) (Fig. 10*A,B*). This suggests that the effect of carbachol was essentially elicited from CCK-containing cells, although some feedback inhibition from pyramidal cell activation of parvalbumin basket cells cannot be ruled out.

Figure 8. Carbachol (CCh) enhanced sIPSCs recorded in a CA1 pyramidal cell from a control mouse but was less effective in pilocarpine (Pilo)-treated mice or when WIN55,212-2 (WIN) selectively suppressed inhibition from CCK-expressing cells. **A**, In a control slice, CCh ($10 \mu\text{M}$) increased sIPSC activity in a CA1 pyramidal cell voltage clamped at $+10 \text{ mV}$. **B**, CCh also enhanced the mean phasic current, which could be fit with an alpha function (gray line). **C**, The response to CCh in a CA1 pyramidal cell from a pilocarpine-treated mouse is less robust than in controls. **D**, The mean phasic current and the fitted alpha function are essentially unchanged by the addition of CCh in a pilocarpine-treated mouse. **E**, Inclusion of $5 \mu\text{M}$ WIN55,212-2 in the perfusing ACSF to activate CB_1 receptors and inhibit GABAergic transmission from CB_1 -expressing CCK basket cells reduces the response to CCh in a slice from a naive mouse. **F**, In the presence of WIN55,212-2, the application of CCh does less to enhance the mean phasic current than in a control cell. Calibration: **A**, **C**, **E**, 100 pA, 200 ms (top traces), 25 s (bottom traces); **B**, **D**, **F**, 2 pA, 25 s.

Increased CA1 pyramidal cell sIPSC sensitivity to ω -agatoxin IVa

CA1 basket cells also differ in the voltage-gated calcium channels they express in their terminals. Whereas GABA release from PV-containing basket cells is mediated by P/Q-type calcium channels that can be blocked by ω -agatoxin IVa, CCK-containing basket cells express N-type calcium channels on their boutons (Poncer et al., 1997; Wilson et al., 2001; Hefft and Jonas, 2005). Selective blockade of sIPSCs from agatoxin-sensitive interneurons provides a means to investigate whether inhibitory input to CA1 pyramidal cells from putative PV-containing basket cells is altered in pilocarpine-treated mice.

Exposure to 500 nM ω -agatoxin IVa for 10 min reduced the inhibitory input to voltage-clamped CA1 pyramidal cells in control mice (Fig. 11A) and even more substantially in pyramidal cells of pilocarpine-treated mice (Fig. 11B). Perfusion of agatoxin reduced sIPSC frequency by half in control slices (from 18.7 ± 2.0 to $9.0 \pm 0.7 \text{ Hz}$; $p < 0.01$, two-way ANOVA; $n = 11$) and by three-quarters in cells from the pilocarpine model (from 23.7 ± 4.8 to $5.5 \pm 1.2 \text{ Hz}$; $p < 0.001$, two-way ANOVA; $n = 8$), leaving sIPSC frequency in agatoxin significantly lower in CA1 pyramidal cells of pilocarpine-treated mice ($p < 0.05$, two-tailed t test) (Fig. 11C).

The agatoxin-sensitive component was calculated from the basal frequency by subtraction (Fig. 11D). In control slices, the agatoxin-sensitive sIPSC component was $9.6 \pm 1.9 \text{ Hz}$, or $46 \pm 6\%$ of the basal frequency. In cells from pilocarpine-injected mice, agatoxin-sensitive sIPSCs comprised $18.1 \pm 3.9 \text{ Hz}$, or $76 \pm 3\%$ (Fig. 11C,D). The greater fraction (Fig. 11D) of agatoxin-sensitive inhibitory activity in CA1 pyramidal cells of pilocarpine-treated mice relative to controls is consistent with a more influential role of PV-expressing basket cells in inhibition after epilepsy, potentially as a result of a reduced role of CCK-expressing basket cells.

There was no significant difference in the agatoxin-insensitive fraction of the sIPSCs (control, $1.4 \pm 0.5 \text{ Hz}$, or $8 \pm 2\%$ of total activity; pilocarpine-treated, $2.2 \pm 1 \text{ Hz}$, or $7 \pm 3\%$ of total

activity; $p > 0.05$, two-way ANOVA) (Fig. 11D). This was calculated using the mIPSC data detailed below. The lack of change is most likely attributable to low basal activity levels of CCK-containing cells in the absence of stimulation, e.g., by carbachol (Pitler and Alger, 1994; Karson et al., 2008).

Miniature IPSCs are reduced

After the addition of ω -agatoxin IVa, cadmium chloride was added to block all calcium entry-dependent transmitter release, leaving only mIPSCs from perisomatic inputs (Andrásfalvy and Mody, 2006; Glykys and Mody, 2007). A change in the number of mIPSCs in the pilocarpine model would potentially indicate a change in the number of synaptic inputs.

Perfusion of $50 \mu\text{M}$ CdCl_2 further tamped down inhibitory currents in CA1 pyramidal cells of both control (Fig. 11A) and pilocarpine-treated (Fig. 11B) mice. The mIPSC frequency after 10 min in CdCl_2 was significantly lower in cells from pilocarpine-treated mice than in controls (3.3 ± 0.5 vs $7.6 \pm 0.7 \text{ Hz}$, respectively; $p < 0.0001$, two-tailed t test). Miniature IPSCs contributed $46 \pm 6\%$ of total IPSC activity in control cells but less than half that ($17 \pm 3\%$) in pyramidal cells of pilocarpine-treated mice ($p < 0.001$, two-way ANOVA). The difference in average mIPSC frequency between CA1 pyramidal cells from control and pilocarpine-treated mice is consistent with a reduced inhibition from CCK-containing basket cell boutons.

Discussion

This study found that basket cell subtypes in CA1 are differentially affected after status epilepticus. A selective decrease in CCK- and CB_1 -, but not PV-, immunolabeled boutons was identified in mice with recurrent spontaneous seizures. Immunogold labeling of CCK and CB_1 in electron-dense terminals suggested that the reduction in immunoreactivity was associated with degeneration of axon terminals at short intervals after status epilepticus. Furthermore, application of carbachol to stimulate CCK-containing interneurons was less effective at enhancing inhibitory currents in CA1 pyramidal cells in mice with chronic

seizures than in control animals. Pyramidal cells from mice with persistent seizures also had lower mIPSC frequencies than control cells and were more sensitive to agatoxin blockade of P/Q-type calcium channels on PV-expressing basket cells. These findings support the interpretation that the particular inhibition of CA1 pyramidal cells by CCK-expressing basket cells is compromised in the mouse pilocarpine model of temporal lobe epilepsy, leaving greater reliance on perisomatic inhibition from PV-expressing basket cells. This parallels the finding that, in postischemic rats, CCK-expressing basket cells in CA3 are preferentially reduced, whereas PV-expressing basket cell innervation is maintained, suggesting to the authors “selective impairment of long-lasting somatic inhibition with a good preservation of high-frequency oscillations” (Epsztein et al., 2006).

Evidence supports terminal loss over reduced CCK expression

In the current studies, several lines of investigation favor the interpretation of degenerating CCK-labeled boutons over reduced protein expression. In a manner strikingly parallel to CCK, CB₁-labeled CA1 basket cell boutons were decreased by a substantial degree on a comparably brief timescale after status epilepticus and with apparently similar permanence.

Other studies have also reported reduced CB₁ immunoreactivity in animals and patients with chronic seizures (Falen-ski et al., 2007; Ludanyi et al., 2008), which was ascribed to a reduction in protein expression. However, those studies were in different species (rat and human), and one focused on a different region, i.e., the dentate gyrus (Ludanyi et al., 2008). In the current study, some CCK- and CB₁-expressing basket cell terminals in CA1 exhibited dark degeneration within hours of status epilepticus, supporting the decrease in immunoreactivity reflecting an actual loss of axon terminals.

The muted carbachol-induced increase in IPSC frequency, amplitude, and mean phasic current by selective activation of M₁/M₃ receptors on CCK basket cells from the pilocarpine model is consistent with a reduction in the number of CCK-containing interneurons available to respond to carbachol in mice with spontaneous seizures.

Suppression of sIPSCs with cadmium revealed fewer mIPSCs in CA1 pyramidal cells of pilocarpine-injected mice than controls, further supporting reduced inhibitory innervation. However, Hirsch et al. (1999) found no difference in number or size of boutons making perisomatic symmetric synapses in CA1 in kainate- or pilocarpine-treated rats. Rather, they ascribed decreased mIPSC frequency to reduced GABA quantal release evi-

Figure 9. Summary data of carbachol (CCh) effects on sIPSC frequency and amplitude in cells from control, pilocarpine (Pilo)-treated, or naive mice in the presence of WIN55,212-2 (WIN). **A**, CCh (10 μ M) significantly increased sIPSC frequency in cells from control slices ($p < 0.01$, two-way ANOVA; $n = 12$) but not in cells from pilocarpine-treated mice ($p > 0.05$, two-way ANOVA; $n = 15$). In the presence of WIN55,212-2 to suppress neurotransmission from CCK/CB₁-expressing cells in slices from naive mice, CCh significantly increased sIPSC frequency ($p < 0.05$, two-way ANOVA; $n = 14$) but by a lesser extent than in controls. **B**, The percentage increase in frequency was calculated for each cell using the formula $(\text{CCh max} - \text{baseline}) \times 100/\text{baseline}$, and the individual ratios were averaged. CCh increased sIPSC frequency by 369% ($n = 12$) in CA1 pyramidal cells from control mice, but the effect of carbachol on sIPSC frequency was significantly weaker in cells from pilocarpine-treated mice (114%; $n = 15$; $p < 0.01$, one-way ANOVA) and from cells in the presence of 5 μ M WIN55,212-2 (155%; $n = 14$; $p < 0.01$, one-way ANOVA). **C**, Similarly, CCh significantly increased sIPSC amplitude in CA1 pyramidal cells from control slices ($p < 0.001$, two-way ANOVA; $n = 12$) but not in cells from pilocarpine-treated mice ($p > 0.05$, two-way ANOVA; $n = 12$), or in cells from naive mice treated with WIN55,212-2 ($p > 0.05$, two-way ANOVA; $n = 14$). **D**, On average, the CCh-induced increase in sIPSC amplitudes in control cells ($85 \pm 16\%$) was significantly greater than in cells from naive mice perfused with WIN55,212-2 ($30 \pm 9\%$; $p < 0.01$, one-way ANOVA), and this was similar to the $28 \pm 27\%$ increase in cells from pilocarpine-treated mice ($p > 0.05$, one-way ANOVA). More detailed values are given in Results.

denced by a 52% reduction in the density of synaptic vesicles. They reflected that a particular subpopulation of perisomatic synapses may have a decreased probability of GABA quantal release, whereas the current study has presented evidence for some selective terminal loss. It remains possible that, in the pilocarpine model, some terminals may have reduced Ca²⁺ entry-independent release.

The greater proportion of P/Q-type calcium channel-dependent (agatoxin-sensitive) sIPSCs, most likely originating from PV-containing basket cells, in pilocarpine-treated mice cor-

Figure 10. Carbachol (CCh)-induced increase of mean phasic inhibitory current was reduced in CA1 pyramidal cells of pilocarpine (Pilo)-treated mice. **A**, The averaged peaks of the alpha functions fit to the mean phasic currents recorded in CA1 pyramidal cells in each condition were significantly smaller in slices from pilocarpine-treated mice compared with controls ($p < 0.001$, two-way ANOVA; $n = 12$). The maximum response to carbachol was also significantly suppressed in the presence of WIN55,212-2 (WIN) relative to controls ($p < 0.001$, two-way ANOVA), whereas it was similar to the effect in cells from pilocarpine-treated mice ($p > 0.05$, two-way ANOVA). **B**, The average baseline, peak, and time constant of the fitted alpha functions were calculated and used to generate a single trace for each condition (solid lines). Broken lines show the accompanying $\pm$ SEM. In controls, the peak of the alpha function increased $685 \pm 132\%$ over the baseline current ($p < 0.001$, two-way ANOVA; $n = 12$), whereas in slices from pilocarpine-treated mice, the peak increased $152 \pm 57\%$ over the baseline current ($p > 0.05$, two-way ANOVA; $n = 15$). When carbachol was applied to naive slices in the presence of WIN55,212-2, the peak of the alpha function increased $228 \pm 55\%$ over the baseline current ($p > 0.05$, two-way ANOVA; $n = 14$). More detailed values are given in Results.

robored a greater inhibitory contribution from the enduring PV-containing subtype. Altogether, the current findings strongly suggest that CCK-expressing basket cells are selectively damaged after status epilepticus, whereas PV-containing interneurons remain to provide perisomatic inhibition to CA1 pyramidal cells in mice with spontaneous seizures.

Overall perisomatic inhibition

Considering the loss of CCK- and CB₁-immunoreactive boutons, one might expect a concordant decrease in GABAergic innervation, but a decrease in GAD67-labeled boutons was not observed in the CA1 pyramidal cell layer of pilocarpine-treated mice. This is consistent with previous immunohistochemical studies that did not find a loss of GABAergic terminals or sIPSC frequency in the region (Williams et al., 1993; Esclapez et al., 1997; Hirsch et al., 1999; Morin et al., 1999; Cossart et al., 2001; Wittner et al., 2005). It is possible that a reduction is concealed by some shrinkage of the hippocampus or that increased immunoreactivity for GAD67 disguises a decrease in GABAergic boutons in pilocarpine-treated mice. Alternatively, it raises the possibility that the axons of surviving interneurons subsequently sprout to fill in vacancies left by lost innervation from CCK-containing basket cells. Even without sprouting, it could be that PV-expressing basket cell activity adjusts to functionally compensate for reduced inhibition from CCK-expressing cells. In the current study, the baseline mean phasic current and the frequency of sIPSCs recorded in CA1 pyramidal cells of pilocarpine-treated mice were maintained at comparable levels relative to controls, despite a decrease in the carbachol-amplified peak attributed to CCK-containing basket cells. This correlates with a corresponding functional increase in sIPSCs mediated by PV-expressing basket cells. If such an increase resulted from new synapses, they

would not necessarily have normal functionality, perhaps evidenced by the reduction in mIPSC frequency.

Implications of fewer CCK-expressing basket cell terminals and reduced CCK

A possible decrease in inhibition from missing CCK-expressing basket cell terminals could compromise a brake on the system at a time when it is most critical: as a hyperexcitable network builds toward seizure activity. Whether or not there is compensation from PV-expressing basket cells, the network would be altered by reduced inhibition from CCK-expressing interneurons because, despite their overlapping location and targets, there are numerous differences between the two subpopulations of basket cells as summarized in several review articles (Pawelzik et al., 2002; Klausberger et al., 2005; Freund and Katona, 2007). Because the PV-containing and CCK-containing subclasses are not fully interchangeable, subtype-specific loss would be disruptive to the circuit.

Several studies have recently demonstrated communication between the PV- and CCK-containing basket cell subgroups, with CCK providing a potentiating effect on inhibition of pyramidal cells by PV-containing cells (Földy et al., 2007; Karson

et al., 2008). Whether or not the CCK-containing basket cells remain in mice with spontaneous seizures, the reduced availability of the neuropeptide could decrease the ability for CCK-expressing basket cells to enhance inhibition from PV-expressing basket cells. Here, too, a deficit in CCK could be particularly detrimental on those occasions when it is most needed; as activity is escalating toward a seizure, CCK would not be available to recruit extra inhibition from PV-containing basket cells.

Increased weight to PV basket cells

Somewhat paradoxically, the reduced GABAergic innervation of PV-expressing basket cells by their CCK counterparts could relieve PV-containing basket cells from some inhibitory control. This, compounded with decreased innervation of pyramidal cells by CCK-containing basket cells, would give increased weight to PV-containing basket cell inputs to pyramidal cells, with a likely impact on network function. This shift was demonstrated by the increased fraction of agatoxin-sensitive sIPSCs recorded in CA1 pyramidal cells of pilocarpine-treated mice.

Whereas the CCK-expressing cells have been suggested to be relevant to mood disorders, PV-expressing cells have received greater emphasis than their CCK associates with regard to epilepsy conditions (Cossart et al., 2005; Maglóczy and Freund, 2005; Monory et al., 2006; Freund and Katona, 2007; Ludanyi et al., 2008). The oscillatory rhythm generation capacity of fast-spiking PV-containing cells confers the ability to induce broad synchrony among pyramidal cells across an extensive region (Penttonen et al., 1998; Tamás et al., 2000; Maglóczy and Freund, 2005; Bartos et al., 2007; Mann and Paulsen, 2007; Tukker et al., 2007; Klausberger and Somogyi, 2008; Cardin et al., 2009). The current study raises the possibility that the loss of CCK-containing basket cells could be a contributing factor to the

Figure 11. ω -Agatoxin IVa suppressed sIPSCs to a greater extent in CA1 pyramidal cells from pilocarpine (Pilo)-treated mice than in cells from control mice. **A**, Agatoxin (500 nM) reduced sIPSC activity by blocking sIPSCs generated by interneurons expressing P/Q calcium channels, and 10 min later addition of 50 μ M CdCl₂ further blocked all calcium entry-dependent neurotransmission, leaving only mIPSC activity. Top traces are clipped at 800 pA for clarity. Arrows indicate where time is expanded for bottom traces. **B**, Perfusion of agatoxin reduced sIPSCs to a greater extent in cells from pilocarpine-treated mice, and CdCl₂ isolated mIPSC activity. **C**, Although the overall baseline sIPSC frequency was similar in cells from control and pilocarpine-treated mice ($p > 0.05$, two-tailed t test), application of agatoxin more profoundly suppressed sIPSC frequency in cells from pilocarpine-treated mice than in controls ($*p < 0.05$, two-tailed t test). Addition of CdCl₂ blocked remaining sIPSC activity, revealing mIPSC frequency in cells from pilocarpine-treated mice to be half that in controls ($*p < 0.001$, two-tailed t test). **D**, Whereas agatoxin (Aga)-sensitive sIPSCs made up 46 $\pm$ 6% of activity in controls, this contribution was increased to 76 $\pm$ 3% in pilocarpine-treated mice ($*p < 0.001$, two-way ANOVA). Agatoxin-insensitive sIPSCs comprised <10% of activity in both groups ($p > 0.05$, two-way ANOVA). mIPSCs constituted 46 $\pm$ 6% of activity in controls but only 17 $\pm$ 3% in pilocarpine-treated animals ($*p < 0.001$, two-way ANOVA). More detailed values are given in Results.

proposed role in epilepsy of their PV-containing counterparts. A shift in the balance of perisomatic inhibition primarily to PV-containing cells could have the effect of amplifying synchrony as a result of excessive influence. In addition, the skewed network of basket cell innervation could contribute to secondary pathologies that accompany temporal lobe epilepsy in some patients, including memory deficits and affective disorders (Klausberger et al., 2005).

CB₁ in the chronic model of temporal lobe epilepsy

Endocannabinoids are retrograde signaling molecules that activate presynaptic G-protein-coupled CB₁ with the downstream effect of suppressing exocytosis: usually limiting the release of GABA as these receptors are primarily expressed on interneuron boutons (Katona et al., 1999; Tsou et al., 1999; Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001; Alger, 2004). There has been a longstanding interest in the role of the cannabinoid system in epilepsy that recently is burgeoning. Conflicting reports on the role of CB₁ receptors reflect the complexity contributed by differences in cell types, brain region, species, and epilepsy etiologies (Wallace et al., 2002, 2003;

Mechoulam and Lichtman, 2003; Lutz, 2004; Monory et al., 2006; Deshpande et al., 2007a,b; Armstrong et al., 2009).

Because CB₁ receptor activation has a silencing effect on the neurons that express them, reduced CB₁ protein expression in basket cells could impair a constraint on inhibition. In contrast, lost innervation from basket cells would remove a source of inhibition. The findings of the current study are consistent with a hypothesis that CB₁-containing interneurons are reduced in number and thus unavailable to respond in chronically epileptic animals, translating to decreased inhibition. However, during an acute seizure in an otherwise healthy animal, the interneurons would be intact and able to respond, for example, to a CB₁ antagonist, which in such a case could potentially boost inhibition to antiepileptogenic effect.

Conclusion

The current findings suggest a revision of the view that perisomatic inhibition to hippocampal pyramidal cells is unchanged in the pilocarpine model of epilepsy. The selective decrease in CCK-

and CB₁-immunoreactive boutons in the CA1 pyramidal cell layer, backed with ultrastructural evidence of their degeneration, points to a preferential loss of GABAergic innervation from CCK-containing basket cells. This interpretation is corroborated by reduced mIPSC frequency in CA1 pyramidal cells, decreased inhibition of pyramidal cells by CCK-containing neurons, and increased contribution from PV-containing interneurons as determined by electrophysiology. These results demonstrate another instance of the heterogeneous fates of diverse interneurons in epilepsy and may contribute to the evolution of dysfunctional circuitry in this model of acquired temporal lobe epilepsy.

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