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Increased cholecystokinin labeling in the hippocampus of a mouse model of epilepsy maps to spines and glutamatergic terminals

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Abstract

The neuropeptide cholecystokinin (CCK) is abundant in the central nervous system and expressed in a subset of inhibitory interneurons, particularly in their axon terminals. The expression profile of CCK undergoes numerous changes in several models of temporal lobe epilepsy. Previous studies in the pilocarpine model of epilepsy have shown that CCK immunohistochemical labeling is substantially reduced in several regions of the hippocampal formation, consistent with decreased CCK expression as well as selective neuronal degeneration. However, in a mouse pilocarpine model of recurrent seizures, increases in CCK-labeling also occur and are especially striking in the hippocampal dendritic layers of strata oriens and radiatum. Characterizing these changes and determining the cellular basis of the increased labeling were the major goals of the current study. One possibility was that the enhanced CCK labeling could be associated with an increase in GABAergic terminals within these regions. However, in contrast to the marked increase in CCK-labeled structures, labeling of GABAergic axon terminals was decreased in the dendritic layers. Likewise, cannabinoid receptor 1-labeled axon terminals, many of which are CCK-containing GABAergic terminals, were also decreased. These findings suggested that the enhanced CCK labeling was not due to an increase in GABAergic axon terminals. The subcellular localization of CCK immunoreactivity was then examined using electron microscopy, and the identities of the structures that formed synaptic contacts were determined. In pilocarpine-treated mice, CCK was observed in dendritic spines and these were proportionally increased relative to controls, whereas the proportion of CCK-labeled terminals forming symmetric synapses was decreased. In addition, CCK-positive axon terminals forming asymmetric synapses were readily observed in these mice. Double labeling with vesicular glutamate transporter 1 and CCK revealed co-localization in numerous terminals forming asymmetric synapses, confirming the glutamatergic identity of these terminals. These data raise the possibility that expression of CCK is increased in hippocampal pyramidal cells in mice with recurrent, spontaneous seizures.

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Keywords

Cannabinoid receptor 1 (CB1); CCK; interneuron; pilocarpine; inhibition; electron microscopy

The neuropeptide cholecystokinin (CCK) is extensively expressed throughout the brain (Vanderhaeghen et al., 1975) at robust concentrations (Schneider et al., 1979; Crawley, 1985), especially in the cerebral cortex, striatum, hypothalamus and limbic regions (Innis et al., 1979; Beinfeld et al., 1981). A number of diverse changes to the hippocampal CCK expression profile have been documented in various models of epilepsy. In several instances, CCK immunoreactivity is reduced, either indicating decreased expression, as in the mossy fiber pathway (Gall, 1988a; Fetissov et al., 2003; Arabadzisz et al., 2005), or, potentially, degeneration, as in mossy cell and basket cell terminals (Schwarzer et al., 1995; Sayin et al., 2003; Wyeth et al., 2010). In other cases, CCK is increased (Iadarola et al., 1986; Meyer et al., 1986; Gruber et al., 1993; Schwarzer et al., 1995). In epileptic spiking tissue resected from human patients, CCK was reduced relative to nonepileptic tissue, but CCK mRNA was increased – potentially reflecting increased CCK release at the epileptic focus (Iadarola and Sherwin, 1991).

Echoing other seizure-induced reductions in CCK in the mossy fibers (Gall, 1988b), the normally strong CCK expression in the mossy fiber pathway and in the inner molecular layer of the dentate gyrus is decreased in the mouse pilocarpine model of temporal lobe epilepsy (Wyeth et al., 2010). CCK-containing basket cell terminals in the pyramidal cell layer are also reduced, with evidence that they degenerate shortly following status epilepticus (Wyeth et al., 2010). In contrast to these changes, CCK-labeling in the dendritic layers of s. radiatum and s. oriens is chronically enhanced in pilocarpine-treated mice. This study seeks to elucidate the subcellular localization of the increased CCK immunoreactivity.

CCK is expressed in a subgroup of interneurons, where it colocalizes with GABA in axon boutons (Greenwood et al., 1981; Hendry et al., 1983b; Somogyi et al., 1984; Kosaka, et al., 1985; Nunzi et al., 1985; Freund et al., 1986). Therefore, one possibility is that CCK is increased in inhibitory axon boutons in the pilocarpine model. However, pyramidal cells also contain mRNA for CCK (Ingram et al., 1989; Burgunder and Young, III, 1990; Schiffmann and Vanderhaeghen, 1991; Tricoire et al., 2011), leaving open the possibility that increased protein expression could occur within these neurons.

The goals of this study were to determine whether increased diffuse labeling in s. radiatum of CA1 could be resolved with confocal microscopy, to compare the pattern of CCK-expressing terminals with that of additional markers of these terminals (including labeling of GABAergic boutons and cannabinoid receptor 1), and finally to localize CCK immunoreactivity with electron microscopy, in order to better understand the nature of the observed increase in the pilocarpine model.

Experimental Procedures

Animals and pilocarpine treatment

C57BL/6 mice (6 to 8 weeks of age) were injected with methyl scopolamine nitrate (1mg/kg s.c.), followed 30 min later by the muscarinic receptor agonist pilocarpine (340 mg/kg, i.p.) to induce status epilepticus (saline was substituted for age-matched controls). Behavioral seizures were alleviated after 2 hours with diazepam (5 mg/kg, s.c.). Recurrent spontaneous seizures were subsequently documented by video recording in all pilocarpine-treated mice used in this study. 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 and the Veterans Administration Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their discomfort.

Tissue preparation for light microscopic immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3, at 4 to 8 weeks post status epilepticus. After 1 hour at 4°C, brains were removed and post-fixed for another hour. Following thorough rinsing, brains were cryoprotected in 30% sucrose overnight at 4°C, embedded in OCT Compound (Sakura Finetek, Torrance, CA), frozen on dry ice, and sectioned coronally at 30 µm with a cryostat.

Antisera

A mouse monoclonal antibody to CCK (No. 9303) was generously provided by the Center for Ulcer Research and Education (CURE, Los Angeles, CA). This antibody has been characterized previously (Kovacs et al., 1989; Ohning et al., 1994; Ohning et al., 1996), used extensively in rodent brain (Pawelzik et al., 2002; Klausberger et al., 2005; Fuentealba et al., 2008; Karson et al., 2009), and the labeling corresponds with the hippocampal pattern of other antibodies to CCK (Kosaka et al., 1985; Gall, 1988a; Fetissov et al., 2003). To verify the specificity of the CCK antibody, absorption tests were performed with 1 µg/ml sulfated-CCK8 and gastrin 17. Such absorption eliminated specific labeling in the hippocampus in sections from both control and pilocarpine-treated mice. When the antibody was tested in tissue from CCK knockout mice, no labeling of CCK was found (Gabor Nyiri, personal communication).

The CCK antibody was used at 1:8000 for light microscopy, or 1:2000 for electron microscopy, as determined by dilution series experiments for each and consistent with the different tissue fixation required for light and electron microscopy (Karson et al., 2009). Cannabinoid receptor 1 (CB1) selectively labels CCK-expressing presynaptic terminals (Katona et al., 1999) and was utilized as an additional marker for these axon boutons. Rabbit anti-CB1 (L14) was generously provided by Ken Mackie and used at 1:8000 (Nyiri et al., 2005). Antibody to glutamate decarboxylase 65 (GAD65) was obtained from Developmental Studies Hybridoma Bank (GAD6, mouse) and used at 1:2000 to label GABAergic axon boutons. Vesicular glutamate transporter 1 (VGLUT1) antiserum was procured from Millipore (Billerica, MA; AB5905, guinea pig) and used at 1:3000 for electron microscopy to label excitatory terminals (Bellocchio et al., 1998; Takamori et al., 2000; Kaneko et al., 2002; Freneau, Jr. et al., 2004; Burette et al., 2009).

Immunoperoxidase labeling for light microscopy

Free-floating cryosections (30 µm) were rinsed in 1% hydrogen peroxide for 30 min. Antigen retrieval methods were used for CCK and CB1 labeling: tissue was immersed in 0.05 M citrate buffer (1% NaCl, pH 8.6) at 90°C for 70 min. Sections were rinsed in 0.1 M Tris-buffered saline (TBS; 1% NaCl, pH 7.3), pre-incubated in blocking solution (1:10 normal serum and 1:5 avidin in TBS with 0.3% triton and sodium azide) for 1 hour, then incubated overnight in primary antibody (diluted in 1:50 normal serum and 1:5 biotin in TBS with sodium azide) at room temperature. On the next day, sections were incubated in secondary antiserum (1:200 in TBS with 30% normal serum), and then in Vectastain Elite ABC in TBS (1:100; Vector Laboratories, Inc, Burlingame, CA) for 1 hour each. Following rinses in 0.075 M phosphate buffered saline (PBS), sections were processed with diaminobenzidine tetrahydrochloride (Stable DAB; Invitrogen, Carlsbad, CA) for 10 min and treated with 0.001% osmium tetroxide for 30 seconds. Sections were subsequently mounted

onto gelatin-coated slides, dehydrated and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Immunofluorescence labeling and quantification

Immunofluorescence was conducted as above with a few modifications. Sections were pre-incubated in blocking solution for 3 hours, transferred directly to CCK (or GAD6) primary antiserum for 3 nights, and then incubated in secondary solution containing Alexa Fluor 555 goat anti-mouse IgG (Invitrogen) at 1:500 with 1:30 normal serum in TBS for four hours. Sections were mounted and coverslipped with ProLong Gold antifade reagent (Invitrogen) for examination with a Zeiss LSM 510 META confocal microscope (Zeiss; Thornwood, NY).

Five pairs of age-matched mice were analyzed for semi-quantification of CCK-positive puncta. Images in CA1 s. radiatum were taken bilaterally from 2 sections per mouse (approximately 300 μ m apart) using a 100x objective with 4x digital zoom. ImageJ's "Find Maxima..." (command based on a plugin contributed by Michael Schmid) was used to quantify CCK-positive puncta. An appropriate standard noise tolerance level was set to differentiate particles from surroundings (maxima were ignored if they did not stand out from surroundings by more than a value of 1500), and the contiguous area around a supra-threshold local maximum was used for semi-quantitative analysis of CCK-labeled puncta. Nonparametric statistical analysis was employed to determine significance at a level of $p < 0.05$.

Tissue preparation for electron microscopy immunohistochemistry

At 4 to 8 weeks post status epilepticus, anesthetized mice were transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min. After 1 hour at 4°C, brains were removed and post-fixed overnight at 4°C and thoroughly rinsed in 0.12 M phosphate buffer.

Pre-embedding peroxidase immunohistochemistry for electron microscopy and quantification

Coronal vibratome sections (60 μ m) were cut from post-fixed brains, thoroughly rinsed in phosphate buffer (pH 7.3), and pre-treated with 1% sodium borohydride for 30 min. Following 1 hour in blocking solution with 1:10 normal horse serum in TBS, sections were incubated in CCK primary antibody (1:2000, in TBS with 1:50 normal horse serum) at room temperature overnight. The following day, tissue was rinsed and transferred to 1:200 horse anti-mouse IgG secondary antiserum in TBS with 1:30 normal horse serum for 2 hours, and then 1:100 ABC in TBS for 2 hours. Rinsed sections were developed in Stable DAB for 20 min. After thorough rinsing, tissue was post-fixed in 2% paraformaldehyde with 2.5% glutaraldehyde for 1 hour, treated with 1% osmium tetroxide in PBS, dehydrated in ethanol and treated with propylene oxide. Sections were polymerized in Durcupan resin for 24 hours and then embedded in Durcupan capsules for further polymerization at 56°C for another 24 hours. Ultrathin sections were picked up onto mesh grids, stained with 0.3% lead citrate, and studied with a JEOL transmission electron microscope (Tokyo, Japan).

For semi-quantitative analysis, the tissue from 4 pairs of mice was comprehensively analyzed. CCK-labeled profiles with intact synapses (at least 100 per mouse) were photographed for classification as presynaptic axon terminals forming symmetric or asymmetric synapses, or as postsynaptic spines or dendrites. Due to the strict criteria and variations of reagent penetration, it was not possible to extract precise measurements of the number of CCK-labeled structures per unit area. However, the analysis allowed for comparison of the fractions of profile types observed in control and pilocarpine-treated mice. Nonparametric statistical analysis was used to determine significance ($p < 0.05$).

Double-labeling for electron microscopy

The protocol began as described for peroxidase immunohistochemistry. Following overnight incubation in VGLUT1 and CCK antisera, the tissue was rinsed repeatedly before a brief incubation in 1:50 NGS and transferred to Nanogold goat anti-guinea pig secondary antibody (Nanoprobes, Yaphank, NY) at 1:100, with 1:50 NGS for 4.5 hours at room temperature for labeling VGLUT1. After thorough rinses in TBS and two 3 min rinses in distilled water, the sections were developed in GoldEnhance solution (Nanoprobes, prepared according to company protocol) for 12.5 min. Two more 3 min rinses in distilled water and 15 min rinses in TBS were followed by 1 hour in 1:50 NGS and incubation in mouse anti-CCK overnight. Immunoperoxidase labeling for CCK continued as described above.

Single-immunogold labeling for electron microscopy

Pre-embedding immunogold labeling for CCK was used to confirm the identity of the immunoperoxidase-labeled profiles and allow clear visualization of the structures without occlusion by peroxidase reaction product. The basic immunogold labeling methods were identical to those described for VGLUT1 labeling, in the previous section. Briefly, sections were incubated in CCK mouse antibody (1:2000) overnight, and, after thorough rinsing, were incubated in Nanogold goat anti-mouse IgG for 4.5 hours at room temperature. Sections were developed in GoldEnhance solution as described above.

Results

CCK was increased in dendritic layers of pilocarpine-treated mice

In pilocarpine-treated mice, CCK expression in the hippocampal dendritic layers of s. radiatum and s. oriens (and cerebral cortex) was markedly increased relative to controls (Fig. 1A,C). This diffuse labeling was visualized with confocal microscopy as discrete punctate structures, and greater numbers of puncta were evident in pilocarpine-treated mice (Fig. 1B,D). Automated semi-quantification of CCK-positive puncta estimated an increase of approximately 58%, from 58 ± 9 to 91 ± 8 puncta/ μm^2 ($p=0.032$, Mann-Whitney test, $n=5$ pairs) (Fig. 1E). This increase in number rather than mere intensity suggested that protein levels were not just elevated in the same location, but up-regulated in additional compartments, the identity of which was further investigated.

The increased CCK expression in dendritic regions of the hippocampus had a distinct laminar pattern. Strong homogeneous increases in CCK labeling were evident throughout s. radiatum and oriens in CA3 through CA1, but little change was evident in s. lacunosum-moleculare of these same regions (Fig. 1A,C). In contrast to the increased labeling in the hippocampus, CCK immunoreactivity was strikingly reduced in the inner molecular layer of the dentate gyrus and the mossy fiber pathway of pilocarpine-treated mice compared to controls (Fig. 1A,C).

GABAergic boutons were not correspondingly increased in CA1 s. radiatum

In s. radiatum, the CCK neuropeptide is a marker for a subclass of Schaffer collateral-associated interneurons whose axon boutons innervate pyramidal cell dendrites (Klausberger et al., 2005; Klausberger, 2009). An antibody to glutamate decarboxylase 65 (GAD65) labeled a network of GABAergic boutons across s. radiatum of CA1 (Fig. 2A) which could be more clearly visualized with immunofluorescence at higher magnification (Fig. 2B). In juxtaposition to the increase of CCK-immunoreactivity, a parallel increase of GAD-labeled boutons was not observed in s. radiatum of chronic pilocarpine-treated mice (Fig. 2C,D). However, because GAD65 labels additional inhibitory boutons beyond the subset containing CCK which is of interest here, a more restrictive marker was also used.

CB1-labeled boutons were not increased in CA1 s. radiatum

CCK-containing interneurons also express CB1 on their presynaptic terminals (Katona et al., 1999). Thus, CB1 was used as a secondary marker for CCK-positive axon boutons. An extensive network of CB1-expressing boutons was evident in s. radiatum of control mice (Fig. 3A). This terminal labeling was considerably diminished in s. radiatum of the pilocarpine-treated mice (Fig. 3B), in contrast with the increased labeling of CCK (Fig. 1C,D). Together, the absence of increases in either GAD- or CB1-containing boutons in mice with spontaneous seizures prompted exploration of alternate locations to account for the increase in CCK.

CCK was not confined exclusively to inhibitory axon terminals in control and pilocarpine-treated mice

Because CCK-labeled structures were small, numerous, and tightly packed in the neuropil, electron microscopy was used to provide the necessary resolution to determine the subcellular localization of CCK labeling with pre-embedding peroxidase immunohistochemistry.

As expected in control mice, the majority of labeled profiles were axon terminals that formed symmetric synapses (inhibitory), usually with postsynaptic dendrites (Fig. 4A–D). However, CCK-labeled terminals that formed asymmetric synaptic contacts (excitatory) and CCK-labeled spines were occasionally observed (Fig. 4E,F).

CCK-positive terminals making symmetric synapses were also found in s. radiatum of CA1 in pilocarpine-treated mice (Fig. 5A,B). Unexpectedly, thick postsynaptic densities were noticed opposite some active zones of CCK-labeled terminals in s. radiatum of CA1 (Fig. 5C,D). Such CCK-containing terminals forming asymmetric contacts were more common in pilocarpine-treated mice than in control mice.

CCK was expressed in glutamatergic terminals

In order to verify the glutamatergic identity of these terminals, double-labeling for CCK and VGLUT1 was undertaken with peroxidase and gold-enhanced immunogold methods, respectively. Terminals single-labeled for VGLUT1 forming asymmetric synapses (Fig. 6B,D), and terminals single-labeled for CCK forming symmetric synapses (Fig. 6A,C) were present in both control and pilocarpine treated mice. Additionally, terminals that contained both immunogold labeling for VGLUT1 and peroxidase labeling for CCK, and also formed asymmetric synaptic contacts, were plentiful in the pilocarpine condition (Fig. 6E,F). Double-labeled terminals with asymmetric synapses were also evident in control mice, though much more rarely.

CCK was increased in postsynaptic spines in pilocarpine-treated mice

Surprisingly, in addition to axon terminals, a large proportion of CCK-labeled profiles in pilocarpine-treated mice were postsynaptic spines (Fig. 7A–F). The CCK-labeled spines often emerged from long, unlabeled dendrites, suggestive of pyramidal cell dendrites (Fig. 7A–C). The labeled spines were frequently contacted by axon terminals that established synapses with thick postsynaptic densities (Fig. 7E). Although CCK-positive spines were occasionally detected in control mice, (Fig. 4F), they were considerably more common in the pilocarpine-treated mice.

To corroborate the immunoperoxidase labeling of spines, tissue labeled for CCK with immunogold was also examined. Terminals synapsing onto clearly labeled spines were evident in CA1 s. radiatum of pilocarpine-treated mice (Fig. 7F). In immunogold-labeled spines, the thick postsynaptic densities were particularly clear (Fig. 7F).

Relative distribution of CCK-immunoreactive structures

In order to estimate the change in the expression pattern of CCK in s. radiatum of CA1 in pilocarpine-treated mice, an extensive ultrastructural study was made of CCK-labeled profiles in the single-labeling peroxidase condition. Immunoreactive profiles forming an intact synapse were identified as presynaptic terminals or postsynaptic spines (or rarely, dendrites) and the type of associated contact was determined to be either symmetric or asymmetric. Immunopositive profiles were more prevalent in tissue from pilocarpine-treated mice. However, this is not conveyed by the current analysis which describes changes in the relative ratios of labeled profile types.

In s. radiatum of CA1, the fraction of CCK-immunoreactive terminals forming symmetric synapses decreased on average from $58 \pm 7\%$ to $35 \pm 8\%$ ($X^2 = 11.0$, $p < 0.001$, $n = 4$ pairs) (Fig. 8). CCK-labeled terminals forming asymmetric synapses accounted for approximately 20% in both conditions ($19 \pm 2\%$ and $20 \pm 5\%$ respectively; $X^2 = 0.0778$, $p = 0.8$), although they were observed to be more plentiful in pilocarpine-treated mice. CCK-positive spines constituted $19 \pm 4\%$ of labeled profiles in controls, but were significantly increased in mice with chronic seizures – doubling their proportion to $38 \pm 8\%$ on average ($X^2 = 22.6$, $p < 0.001$, $n = 4$ pairs). Labeled dendrites were infrequently found in both control ($4 \pm 1\%$) and pilocarpine-treated mice ($7 \pm 3\%$; $X^2 = 2.88$, $p = 0.09$, $n = 4$ pairs).

Discussion

A major finding of this study is that CCK was increased in the hippocampal dendritic layers, particularly s. radiatum of CA1, in a mouse model of temporal lobe epilepsy, contrasting with decreases in other layers. This enhanced CCK immunoreactivity was particularly surprising in light of the absence of parallel increases in labeling of either GABAergic boutons in the same region or the corresponding population of CB1-positive boutons. Closer inspection with electron microscopy unexpectedly revealed substantially increased labeling of CCK in postsynaptic dendritic spines in the pilocarpine model. Further investigation demonstrated CCK-positive terminals forming VGLUT1-positive asymmetric synapses that are also likely to contribute to the overall increase in CCK immunoreactivity. The layer-specific increases and decreases in CCK do not seem to be a result of altered trafficking within GABAergic neurons. Rather than pertaining to aspiny hippocampal interneurons, these findings point to increased CCK expression in spiny neurons as well as glutamatergic terminals that innervate s. radiatum and oriens. Hippocampal pyramidal cells appear to be well-suited candidates, although extra-hippocampal projection neurons cannot be ruled out as a source of the glutamatergic terminals. Overall, the bidirectional changes in cellular localization of CCK immunoreactivity in CA1 s. radiatum of mice with spontaneous seizures suggest up-regulation in new subcellular compartments of excitatory neurons coupled with reduced contribution from inhibitory interneurons.

Lack of enhanced CCK labeling of inhibitory terminals

Because CCK demarcates a subgroup of interneurons, increased CCK immunoreactivity could reasonably be expected to occur in GABAergic terminals. However, the apparent reduction in labeling of GABAergic boutons in the dendritic layers of the hippocampus in the pilocarpine-treated mouse is consistent with previous reports of decreased GAD-immunoreactivity in a similar rat model of epilepsy (Cossart et al., 2001; Dinocourt et al., 2003). In addition, the reduced labeling of CB1-positive boutons, together with the reduction in the proportion of CCK-positive terminals forming symmetric synapses, argues against an increase in CCK-labeled interneuron terminals.

Increased CCK labeling in dendritic spines

Considering the apparent decrease in inhibitory axon terminals, the increase in CCK labeling must be accounted for otherwise. Ultrastructural analysis revealed that on average the proportion of CCK-positive postsynaptic dendritic spines was approximately doubled in the pilocarpine model. This was particularly surprising in light of the well-recognized CCK localization to presynaptic terminals of interneurons in CA1 (Kosaka et al., 1985).

As a neuromodulator, CCK is conventionally situated in axon boutons, but CCK-like immunoreactivity has previously been detected in some dendrites and dendritic spines of the cat inferior colliculus (Paloff et al., 1996). Although the CCK-labeled spines could be from interneurons, this appears unlikely in the current study. In CA1, CCK is associated with a specific subset of inhibitory interneurons. Like many interneurons, the Schaffer collateral-associated CCK-containing interneurons that target s. oriens and s. radiatum, are largely aspiny (Harris et al., 1985). Furthermore, it seems unlikely that CCK-expressing interneurons would be sufficiently numerous to account for the robust, uniform increase in neuropil labeling.

Although the CCK protein is associated with local inhibitory cells in the hippocampus (Kosaka et al., 1985; Nunzi et al., 1985; Leranth and Frotscher, 1986; Sloviter and Nilaver, 1987; Cope et al., 2002; Pawelzik et al., 2002), *in situ* hybridization studies have also demonstrated CCK mRNA in pyramidal cells (Ingram et al., 1989; Burgunder and Young, 1990; Schiffmann and Vanderhaeghen, 1991; Tricoire et al., 2011). The absence of complementary CCK protein from these and cortical pyramidal cells, given their expression of CCK mRNA, has long puzzled researchers.

The findings in this study, however, would be consistent with CCK protein expression in pyramidal cells. Localization in numerous pyramidal cells would fit with the blanket, diffuse increase of CCK levels in pilocarpine-treated mice that is evident with light microscopy. In addition, pyramidal cells are embellished with spines. Although CCK was also observed in some spines of control mice with electron microscopy, the increase of labeled spines in pilocarpine-treated mice was striking.

The possible role for CCK in this postsynaptic location is unclear. One possibility is that the antibody recognized CCK bound to internalized receptors. Some labeled spines were postsynaptic to CCK-immunoreactive terminals, consistent with this interpretation, but the vast majority was contacted by unlabeled terminals. Another possibility is that CCK is released from spines as a retrograde transmitter, perhaps in a different manner than via the large dense core vesicles of presynaptic boutons. Alternatively, CCK may serve some yet to be determined postsynaptic function as an effector molecule in the cytoplasm, or in regulating membrane-bound proteins, potentially related to the ability of CCK to bind iron and calcium ions (Baldwin et al., 2008). Increased CCK immunoreactivity in s. radiatum but not in s. lacunosum-moleculare could suggest some presynaptic influence on expression.

Increased CCK labeling of glutamatergic terminals

It is likely that glutamatergic CCK-containing terminals forming asymmetric synapses also contribute to the overall increase in CCK immunoreactivity in s. radiatum of CA1 in pilocarpine-treated mice. Whereas many ultrastructural studies of normal animals have demonstrated that CCK-labeled terminals form symmetric synapses in cat and monkey cortex, and in cat and rat hippocampus (Hendry et al., 1983a; Somogyi et al., 1984; Kosaka et al., 1985; Nunzi et al., 1985; Freund et al., 1986), a few studies have also noted suggestive postsynaptic thickenings in rat and monkey cortex (Hendry et al., 1983b), and demonstrated CCK-labeled terminals forming asymmetric contacts that were attributed to bipolar cells in rat cortex (Peters et al., 1983). In the rat hippocampus, one study reported finding a limited

number of CCK-positive asymmetric synapses, along with a preponderance of symmetric synapses formed by labeled terminals, occasionally onto dendritic spines (Hendry and Jones, 1985). Another study found that in the rat hippocampus, CCK-immunoreactive terminals forming asymmetric synapses were absent or rare, whereas in the monkey hippocampus, immunoreactive terminals formed asymmetric contacts with dendritic spines (Leranth et al., 1988).

A conditional knockout of CB1 in GABAergic neurons retains light, diffuse CB1-labeling in hippocampal dendritic layers that is attributed to glutamatergic terminals (Monory et al., 2006). In the current study, the profuse neuropil CCK immunoreactivity in s. radiatum is similarly suggestive of localization to more abundant excitatory boutons rather than to distinctive punctate labeling associated with the relatively sparse interneuron population (Figs. 2,3). The CCK-containing glutamatergic terminals may therefore originate from pyramidal cells of CA3 and/or CA1. This would be consistent with the presence of CCK mRNA expression in principal cells. However, CCK is broadly expressed throughout the brain, and CCK expression is also increased in the cerebral cortex of pilocarpine-treated mice. Thus contribution from extrahippocampal projections cannot be excluded.

Interference with a prohormone convertase involved in processing the CCK neuropeptide has been shown to have opposing effects on CCK mRNA versus the peptide in certain brain regions (Beinfeld et al., 2005), and the functionality of some such regulatory protein could contribute to the imbalance between message and protein seen in certain neurons, like pyramidal cells. Pituitary corticotrophs (Rehfeld, 1986; Rehfeld, 1987) contain substantial amounts of the precursor proCCK, but the octopeptide was undetectable, potentially due to rapid degradation (Migaud et al., 1995), or post-translational attenuation of the final carboxyamidation (Rehfeld, 1986; Rehfeld, 1989).

Ultrastructural analysis did not identify an increase in the fraction of CCK-containing terminals forming asymmetric synapses in pilocarpine-treated mice. However, after factoring in the overall increase in CCK expression evident with light microscopy, it follows that CCK may be up-regulated in excitatory axon boutons in mice with spontaneous seizures. The conditions for, and function of, CCK release from glutamatergic axon boutons and the reason for its increase following pilocarpine injection remain to be established.

CCK function in epilepsy

In normal conditions, the sulfated octopeptide is postulated to be released under strong, repetitive stimulation (Verhage et al., 1991; Karson et al., 2008; Deng et al., 2010), activating predominantly G protein-coupled CCK₂ receptors in the brain (Gaudreau et al., 1983; Hill et al., 1987; Carlberg et al., 1992; Harro et al., 1993; Wang et al., 2011; Lee et al., 2011). In general, the functional role of CCK is not entirely understood, although that is not for lack of investigation (for review see Lee and Soltesz, 2011). Even reliably effecting endogenous release has proven difficult. There is evidence that activation of CCK₂ receptors in the hippocampus produces a depolarizing response by decreasing potassium conductance, whether in interneurons (Miller et al., 1997) or pyramidal cells (Shinohara and Kawasaki, 1997). Accordingly, CCK reportedly enhances neurotransmitter release of GABA (Deng and Lei, 2006; Foldy et al., 2007; Karson et al., 2008), as well as glutamate in the hippocampus, and in this latter case increases pyramidal cell excitability (Dodd and Kelly, 1981; Jaffe et al., 1987; Boden and Hill, 1988; Bohme et al., 1988; Hughes et al., 1990; Gronier and Debonnel, 1995; Migaud et al., 1995; Breukel et al., 1997; Deng et al., 2010; Wang et al., 2011). The overall effect on the network would consequently depend upon the cell types and precise synapses involved.

There are several accounts that CCK has anti-epileptogenic, anti-convulsant properties (Zetler, 1981; Kadar et al., 1983; Kadar et al., 1984; Schwarzer et al., 1996) and protects against excitotoxicity by preventing glutamate-induced increases in internal calcium (Shinohara et al., 1992; Tamura et al., 1992; Tirassa et al., 2002; Tirassa et al., 2005; Ferraro and Sardo, 2009). On the other hand, another study suggests that CCK may be pro-convulsant, fitting with an excitatory action for CCK (Arabadzisz et al., 2005).

CCK plasticity in epilepsy and its implications

Following acute seizures induced by systemic administration of kainate, amygdala-kindling, PTZ-kindling or unilateral electrolytic lesion of the dentate gyrus in rats or mice, hippocampal CCK mRNA and protein levels have been found to be adjusted up and down in the subsequent hours and days (Iadarola et al., 1986; Meyer et al., 1986; Gall, 1988a; Marksteiner et al., 1989; Olenik et al., 1989; Gruber et al., 1993; Schwarzer et al., 1995; Sayin et al., 2003; Arabadzisz et al., 2005). Gruber et al. (1993) demonstrated increased CCK mRNA in the CA1 pyramidal cell layer by *in situ* hybridization in the first couple of days following an acute kainate-induced seizure in rat. The increased mRNA usually returned to control values in a few days, while the protein remained elevated in select layers for 2 months (in CA1 s. pyramidale, lacunosum-moleculare, the alveus, and inner molecular layer of the dentate gyrus). Although the cellular localization of the CCK labeling was not determined, it was hypothesized that CCK-expressing neurons may be damaged early, and that the later protein increase reflected an augmentation in afferent fibers that would only be released upon sustained firing, possibly protecting against seizures (Gruber et al., 1993).

In the current study, adjustments up and down in protein immunoreactivity were also observed across various regions in this model. Increased CCK expression in excitatory terminals in CA1 s. radiatum, and a possible role for CCK in facilitating glutamate release, could indicate a CCK contribution to increased network excitability.

Conclusion

CCK expression in the hippocampus undergoes considerable and varied changes during the development and progression of spontaneous seizures in the mouse pilocarpine model of epilepsy. In CA1 s. radiatum, CCK labeling was observed in glutamatergic axon terminals and proportionally increased in dendritic spines. This then does not merely suggest altered protein trafficking of CCK in interneurons, but greater functional remodeling of the network involving multiple cell types.

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Abbreviations

CB1	cannabinoid receptor 1
CCK	cholecystokinin

GAD65	glutamate decarboxylase 65
NGS	normal goat serum
Pilo	pilocarpine
PBS	phosphate-buffered saline
TBS	Tris-buffered saline
VGLUT1	vesicular glutamate transporter 1

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Research Highlights

- CCK-immunoreactivity is increased in CA1 s. radiatum of pilocarpine-treated mice
- The proportion of CCK-labeled axon terminals forming symmetric synapses is reduced
- The proportion of CCK-containing postsynaptic dendritic spines is increased
- CCK is also identified in axon terminals forming asymmetric synapses

Fig. 1.

CCK is increased in s. radiatum of CA1 in pilocarpine-treated mice. (A) In a control mouse, CCK is heavily expressed in the hilus (h), inner molecular layer of the dentate gyrus (arrows) and mossy fiber pathway (arrowheads), with lighter labeling in the neuropil of s. oriens (O) and s. radiatum (R). (B) Higher magnification reveals CCK-immunofluorescent puncta in s. radiatum of CA1 in a control mouse. (C) One month post-pilocarpine treatment, CCK immunoreactivity is reduced in the inner molecular layer of the dentate gyrus, hilus, and mossy fiber pathway. By contrast, diffuse labeling of CCK in s. radiatum and s. oriens of CA1 and CA3 is elevated. (D) In the pilocarpine model, many more CCK-positive puncta are evident in CA1 s. radiatum. (E) The density of punctate CCK-immunoreactive structures in CA1 s. radiatum increases approximately 58% in pilocarpine-treated mice relative to controls ($p = 0.032$, Mann-Whitney test, $n = 5$). Scale bars (shown in C and D) = 200 μm (A, C); 2 μm (B, D).

Fig. 2. GABAergic axon boutons are not increased in s. radiatum of CA1 in pilocarpine-treated mice. (A) In a control mouse, GAD65-positive puncta are abundant in s. radiatum (R) of CA1. Labeled boutons are also apparent in the pyramidal cell layer (P). (B) Confocal imaging captures GAD65-immunoreactive axon boutons in s. radiatum of CA1 in a control mouse. (C) GAD65-labeled punctate structures in s. radiatum of CA1 appear decreased in a mouse with recurrent seizures. (D) In a pilocarpine-treated mouse, fewer GAD65-expressing axon boutons are evident. Scale bars (shown in C and D) = 20 μ m (A, C); 5 μ m (B, D).

Fig. 3. Cannabinoid receptor 1 (CB1)-expressing presynaptic boutons are decreased in CA1 s. radiatum of pilocarpine-treated mice. (A) CB1 labels a dense plexus of interneuron axon boutons that are also known to contain CCK in CA1 s. radiatum (R) of a control mouse. Basket cell boutons in the pyramidal cell layer (P) are especially dense. (B) In a mouse with spontaneous seizures, the network of CB1-positive axon boutons is reduced. Scale bar (shown in B) = 20 μ m (A, B).

Fig. 4.

In control mice, the majority of CCK-labeled profiles are axon terminals that form symmetric synapses. (A–D) Many CCK-immunoreactive vesicle-filled axon terminals (T) make symmetric contacts (arrowheads), primarily onto dendrites (D). (E) As observed infrequently, a CCK-containing terminal forms an asymmetric synapse (open arrowhead), with a probable dendritic spine (S). (F) A rare CCK-labeled spine (S) extends from an unlabeled dendrite and forms an asymmetric contact (open arrowhead) with an unlabeled axon terminal. Scale bar (shown in F) = 0.2 μ m (A, B, C, F); scale bar (shown in E) = 0.2 μ m (D, E).

Fig. 5.

In pilocarpine-treated mice, CCK-containing profiles are increased and include axon terminals that form asymmetric as well as symmetric synapses. (A) A CCK-labeled axon terminal (T) makes a typical symmetric synapse onto a dendrite (arrowhead). (B) Multiple symmetric contacts (arrowheads) are made by a CCK-containing terminal onto a spine that contains a spine apparatus. (C,D) CCK-immunoreactive terminals form distinct asymmetric synapses (open arrowheads) with probable dendritic spines, a configuration more common in pilocarpine-treated mice. Scale bar (shown in D) = 0.2 μm (A–D).

Fig. 6.

Pre-embedding electron microscopy demonstrates colocalization of CCK and VGLUT1 in axon terminals that form asymmetric synapses in pilocarpine-treated mice. (A) In s. radiatum of CA1 of a control mouse, a CCK-positive terminal (T) forms a symmetric contact (arrowhead) onto a dendrite and is single-labeled with peroxidase reaction product. (B) Immunogold particles label VGLUT1 in a terminal forming asymmetric synapses (open arrowheads) in the same mouse. (C) In a pilocarpine-treated mouse, a single-labeled CCK-positive terminal making a symmetric synapse (arrowhead) onto a proximal dendrite can still be found in CA1 radiatum. (D) Likewise, in a pilocarpine-treated mouse, a terminal forming an asymmetric synapse (open arrowhead) contains VGLUT1 but does not express CCK. (E,F) In a pilocarpine-treated mouse, many peroxidase-labeled CCK-positive terminals contain immunogold labeling for VGLUT1 amongst the synaptic vesicles. These double-labeled terminals form asymmetric synapses, evident by the thick postsynaptic density (open arrowheads). Scale bar (shown in F) = 0.2 μ m (A–F).

Fig. 7.

In pilocarpine-treated mice, CCK-containing profiles include dendritic spines. (A–D) CCK-labeled spines (S) extend from unlabeled dendrites (D) and form synapses (open arrowheads) with unlabeled vesicle-filled terminals (T), a pattern commonly observed in mice with recurrent seizures. (E) A CCK immunoperoxidase-labeled spine forms a synaptic contact with an unlabeled axon terminal, and a distinct postsynaptic density (arrowhead) is evident within the spine. (F) A CCK-containing spine is labeled with immunogold particles that are located within and near the surface of the spine. A thickened postsynaptic density (arrowhead) is clearly visible within the labeled spine and is in contact with an unlabeled axon terminal. Scale bars (shown in A – D, F) = 0.2 μ m (A–D); 0.1 μ m (E,F).

Fig. 8.

Semi-quantification of the relative apportionment of CCK-labeled profiles as pre- or postsynaptic structures in control and pilocarpine-treated mice. The contribution of CCK-labeled terminals forming symmetric synapses is decreased in pilocarpine-treated mice (-40% , $p < 0.001$), while the average proportion of labeled dendritic spines is increased two-fold ($p < 0.001$). The ratio of terminals forming asymmetric synapses is unchanged near 20% , but is consistent with an overall increase given the previously established change in density of CCK-positive puncta by confocal microscopy. CCK-labeled dendritic profiles were quite rare in both groups of mice. (Chi-squared test, $n = 4$ pairs)