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Identification of mRNA for endocannabinoid biosynthetic enzymes within hippocampal pyramidal cells and CA1 stratum radiatum interneuron subtypes using quantitative real-time PCR

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Abstract

The hippocampus is required for short-term memory and contains both excitatory pyramidal cells and inhibitory interneurons. These cells exhibit various forms of synaptic plasticity, the mechanism underlying learning and memory. More recently, endocannabinoids were identified to be involved in synaptic plasticity. Our goal was to describe the distribution of endocannabinoid biosynthetic enzymes within CA1 stratum radiatum interneurons and CA3/CA1 pyramidal cells. We extracted mRNA from single interneurons and pyramidal cells and used real-time quantitative PCR to detect the presence of 12-lipoxygenase, N-acyl-phosphatidylethanolamine-specific phospholipase D, diacylglycerol lipase, and type I metabotropic glutamate receptors, known to be involved in endocannabinoid production and plasticity. We observed that the expression of endocannabinoid biosynthetic enzyme mRNA does occur within interneurons and that it is coexpressed with type I metabotropic glutamate receptors, suggesting interneurons have the potential to produce endocannabinoids. We also identified that CA3 and CA1 pyramidal cells express endocannabinoid biosynthetic enzyme mRNA. Our data provide the first molecular biological evidence for putative endocannabinoid production in interneurons, suggesting their potential ability to regulate endocannabinoid-mediated processes, such as synaptic plasticity.

Keywords

mGluR; LTD; CCK; calbindin; calretinin; eicosanoid

Introduction

The hippocampus is the brain region involved in learning and declarative memory. The process of learning and memory formation is thought to occur through synaptic plasticity. Long-term potentiation is the strengthening of a synapse (Bliss and Lomo, 1973), while

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long-term depression is the weakening of a synapse (Dudek and Bear, 1992). Within the hippocampus there are fairly homogeneous excitatory pyramidal cells and heterogeneous interneurons, which can both exhibit various types of plasticity.

Recently, some types of synaptic plasticity have been reported to either be modulated by or require endocannabinoids (Feinmark et al., 2003, Abush and Akirav, 2010; for review see Oudin et al., 2011, Alger, 2012). Endocannabinoids are a group of lipid soluble molecules, often arachidonic acid metabolites, that can function in retrograde neurotransmission (Alger and Pitler, 1995). 2-arachidonylethanolamide, synthesized by diacylglycerol lipase (DAGL) (Tanimura et al., 2010), binds cannabinoid receptor 1 (CB1) (Hill et al., 2007, Ludanyi et al., 2011). 12-(S)-Hydroperoxyeicosa-5Z, 8Z, 10E, 14Z-tetraenoic acid (12-HPETE), which is synthesized by 12-lipoxygenase (12-LO) (Hwang et al., 2000), can activate transient receptor potential vanilloid 1 (TRPV1) receptors. Anandamide is produced by N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Di Marzo et al., 1994, Ueda et al., 2005) and can bind TRPV1 (Smart et al., 2000, De Petrocellis and Di Marzo, 2005) or CB1 (Figure 1). Importantly, while most studies have examined the role of endocannabinoids in pyramidal cell synaptic plasticity, few have investigated their role in hippocampal interneuron plasticity.

However, a recent paper suggested CA1 stratum radiatum interneurons do indeed produce endocannabinoids (Gibson et al., 2008). In this example, endocannabinoids mediated a novel interneuron long-term depression at the CA3 pyramidal cell-CA1 stratum radiatum interneuron synapse. This was identified to be elicited by retrograde endocannabinoid signaling. It was proposed that postsynaptic type I metabotropic glutamate receptor (mGluR) activation induced formation of arachidonic acid, which was then converted to the endocannabinoid 12-HPETE by 12-LO. 12-HPETE retrogradely activated TRPV1 receptors, decreasing neurotransmitter release onto the interneuron. The data suggested that the interneuron itself produced 12-HPETE. However, whether interneurons or various interneuron subtypes have the capability to synthesize endocannabinoids or express endocannabinoid biosynthetic enzymes is unclear and remains controversial as no molecular data has been presented to provide evidence for the presence of endocannabinoid synthesizing enzymes in hippocampal interneurons.

As there are many interneuron subtypes, various classification schemes have been developed to distinguish between them. These schemes are based on gene expression, physiology or anatomy (Ascoli et al., 2008). Classified subtypes include axo-axonic, basket, bistratified, and interneuron-selective subtypes, based on the innervation patterns of their axons. Using the expression of calcium binding proteins such as parvalbumin, calbindin (CB) and calretinin, as well as neuropeptides such as cholecystokinin (CCK), neuropeptide Y, and somatostatin one can generally categorize interneurons into these anatomical subtypes. Parvalbumin-positive cells are generally axo-axonic cells or basket cells found in stratum pyramidale. Another population of basket cells found in the stratum radiatum expresses CCK and can coexpress CB. Many bistratified cells express CB (Freund and Buzsáki, 1996), as well as other subtype markers (Fuentesalba et al., 2008, Klausberger, 2009). Interneuron-selective cells are identified by the expression of calretinin and these cells may express CB (Gulyas et al., 1996, Ferraguti et al., 2004). Because of the remarkable heterogeneity of interneurons, it is plausible that different subtypes could produce different varieties of endocannabinoids, and therefore express different endocannabinoid synthesizing enzymes.

Pyramidal cells are the other major cell type involved in CA3-CA1 hippocampal circuitry. Pyramidal cells are mostly homogeneous in gene expression, morphology, and electrophysiological properties. In pyramidal cells, endocannabinoid involvement in mediating plasticity has been noted physiologically (Edwards et al., 2006, Heifets and

Castillo, 2009, Abush and Akirav, 2010) and endocannabinoid biosynthetic enzymes have been identified using immunocytochemistry (Cristino et al., 2008). However, none of these studies have utilized real-time quantitative PCR (RT-qPCR) to describe the distribution of endocannabinoid biosynthetic enzyme mRNA expression in pyramidal cells.

Our first goal was to use RT-qPCR to determine if CA1 stratum radiatum interneurons possess the cellular machinery to synthesize endocannabinoids and to correlate this if possible with interneuron subtype. Our second goal was to examine CA3 and CA1 pyramidal cells for the presence of endocannabinoid biosynthetic enzyme mRNA. To date, there are no studies published using this technique to examine endocannabinoid biosynthetic enzyme mRNA in hippocampal neurons. Our data clearly suggest that CA1 stratum radiatum interneurons indeed express the enzymes necessary for endocannabinoid synthesis, which appear to be fairly widespread in different interneuron subtypes, with the exception of calretinin interneuron-selective cells. Also, our data demonstrate the expression of endocannabinoid biosynthetic enzymes within hippocampal pyramidal cells. Collectively, our data suggest that interneurons have the putative capacity to produce endocannabinoids and thus could directly be involved in endocannabinoid signaling, including modulating synaptic plasticity, and even possibly regulating their own plasticity independent of pyramidal cell endocannabinoid production. This is the first molecular study to suggest the potential involvement of interneurons in endocannabinoid signaling.

Experimental Procedures

2.1 Preparation of Slices

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and followed the NIH guidelines for the care and use of laboratory animals. These guidelines include minimizing animal suffering and the number of animals used to perform the required experiments. Sprague-Dawley rats (16–28 days old) were used in all experiments. Animals were anesthetized using isoflurane and decapitated using a rodent guillotine. The brain was rapidly removed, sectioned into 400 μ m thick coronal slices, and stored for at least one hour submerged on a net in artificial cerebrospinal fluid containing (in mM) 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1.0 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄, and 11 glucose, saturated with 95% O₂/5% CO₂ (pH 7.4). Slices were then transferred to a submerged recording chamber and bathed in oxygenated artificial cerebrospinal fluid.

2.2 Electrophysiological Recordings and Extraction

Slices were continuously perfused with filtered artificial cerebrospinal fluid at a flow rate of 2–3 mL/min. Hippocampal CA1 stratum radiatum interneurons were visually selected using infrared optics, CCD camera and monitor, with an Olympus BX51WI microscope with a 40 $\times$ water immersion objective. Upon selection, each cell was patched with a borosilicate glass pipette filled with filtered internal solution composed of (in mM) 117 potassium gluconate, 2.8 NaCl, 20 HEPES, 5 MgCl₂, and 0.6 EGTA-K (pH 7.28, 275–285 mOsm). Spiking patterns were acquired in whole cell current clamp configuration by injecting 1000 pA positive current for 500 msec. Most cells recorded in current clamp mode were approximately –60 to –70 mV, while those cells depolarized by more than –40 mV were excluded from our spiking analysis; though these cells as analyzed by PCR were very consistent with others. Electrophysiological traces were recorded with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Signals were filtered at 4 kHz and digitized with an Axon 1440A digitizer (Molecular Devices) connected to a Dell personal computer with pClamp 10.2 Clampfit software (Molecular Devices). Each cell was then extracted from the slice with gentle suction. Once free of the slice, the entire cell was carefully aspirated into the pipette tip to avoid aspiration of artificial cerebrospinal fluid and

transferred immediately into a chilled reverse transcription mixture and processed within two hours. The entire cell was harvested in order to attain sufficient mRNA to examine the large number of desired targets we investigated. An artificial cerebrospinal fluid control sample was extracted for every slice, where the electrode was first placed in the slice and then artificial cerebrospinal fluid was aspirated just above the slice. This was done to ensure any contaminating mRNA if seen in these artificial cerebrospinal fluid controls from the slice could be eliminated from single cell analysis to avoid false positives.

2.3 Reverse Transcription Reaction

The reverse transcription reaction was accomplished using iScript cDNA Synthesis Kit (BioRad), following the prescribed protocol, with a final reaction mixture of 10 μ L. This mixture was then cycled in a C1000 Thermocycler (BioRad) under the following conditions: 25.0 °C for 8 minutes, 42.0 °C for 60 minutes, and 70 °C for 15 minutes.

For primer optimization (see below for more details) a cDNA library was created by reverse transcription of total RNA from homogenized brain tissue. Homogenization and mRNA extraction were performed using TriZol reagents (Invitrogen), according to its published protocol, followed by mRNA conversion to cDNA using iScript cDNA synthesis kit (BioRad), according to its published protocol.

2.4 Primer Design, Verification, and Optimization

Primers for selected cDNA of endocannabinoid signaling components, calcium-binding proteins, and other targets were designed using Vector NTI software (Invitrogen) and PrimerExpress software (Applied Biosystems Inc.), using identical parameters (T_m , GC content, minimum primer length) for each primer set. All primer sets were designed to cross an intron boundary and amplify from exon to exon in order to avoid nuclear DNA amplification, with the exception of CB1 because it is intron-less. For control purposes each primer was tested using a serial dilution series of cDNA from rat whole brain and SsoFast EvaGreen Supermix (BioRad), followed by melt curve analysis to verify amplification of one product. The resulting amplification mixture was tested by 4% agarose gel electrophoresis to confirm that the size of the amplified cDNA fragment matched the designed amplicon size. Once primers were verified, each primer set was optimized to 90–95% amplification efficiency using probes specific to the amplified fragment and iQ Supermix (BioRad) (Figure 2). The primers were also grouped and tested to ensure that no primer cross binding occurred during the multiplex reaction by performing the multiplex reaction using mixed primers with cDNA template of 10 ng/ μ L from whole brain homogenate.

2.5 Preamplification (Multiplex) Reaction

Once the reverse transcription reaction was complete, each cell was divided into two portions of approximately 5 μ L each. The primers for each target were divided into two groups, and then a mixture including iQ Supermix (BioRad), ddH₂O, and one group of 10-fold diluted primers (see Table 1) was added to each aliquot. The same two groups were used for both interneurons and pyramidal cells, except interneurons were not examined for VGlut1. Next, both aliquots were then placed in a C1000 Thermocycler (BioRad) and processed as follows: 95 °C hot start for 3 minutes, followed by 15 cycles of 95 °C for 15 seconds, 57 °C for 20 seconds, and 72 °C for 25 seconds.

2.6 Quantitative PCR Reaction

For qPCR, cDNA from the pre-amplified multiplex reaction was used for probe-based gene detection. Each target was run individually in triplicate, with undiluted primers, the

appropriate FAM-TAMRA probe (Applied BioSystems, Inc.) specific to each target (see Table 1), and iQ Supermix (BioRad). Each cell was run on a CFX96 qPCR machine (BioRad) according to the following protocol: 95 °C hot start for three minutes, followed by 50 cycles of 95 °C for 15 seconds, 57 °C for 20 seconds, and 72 °C for 25 seconds. Amplification was measured by increased relative fluorescence during each cycle and a cycle threshold (Ct) value was assigned to each target using BioRad CFX Manager software. Each target from a cell was also examined using 4% agarose gel electrophoresis to verify amplicon size (see Figure 3B). For TRPV1, we sequenced the amplification product to verify amplicon identity. In addition, because CB1 lacks introns, 5 cells were tested as controls to determine whether the amplified CB1 PCR product came from genomic DNA rather than reverse transcribed mRNA (cDNA). These cells were extracted in the same manner as all other cells, except that half the reaction mixture lacked reverse transcriptase and was processed for CB1, while the other half was run with reverse transcriptase for 18S in order to determine Ct values. These cells were then multiplexed and evaluated by qPCR. Fluorescence curves for CB1 were extremely weak and shifted back compared to CB1 positive cells. Ct values from these 5 cells were significantly different from the other interneurons ($P < 0.05$; T-test), indicating that detected CB1 was indeed from expressed mRNA, rather than genomic DNA.

2.7 Data Analysis

Ct value data from the qPCR reaction from each cell was compared to Ct data from artificial cerebrospinal fluid samples extracted from each slice. If any target noted in artificial cerebrospinal fluid samples was either within 5 cycles of the cell Ct value or not significantly different from the Ct value of the cell, it was excluded from the cell analysis. Some artificial cerebrospinal fluid samples displayed expression of several targets; in this case, the cells corresponding to the artificial cerebrospinal fluid sample were classified as failures and not fully analyzed. Ct values for 18S were subtracted from the Ct value for each target in a cell wise manner to obtain a Δ Ct value for each target. Any target with a Δ Ct value greater than 20 was excluded from analysis as non-specific. To quantify mRNA expression levels, the $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) was used. Expression data was obtained using CFX Manager software (BioRad) or by double-derivative analysis using GraphPad Prism 4 (GraphPad Software, Inc.). All expression levels were tested for significance ($p < 0.05$) using an unpaired two-way Student's T-test.

Results

We extracted 56 putative interneurons from CA1 stratum radiatum and analyzed these cells by RT-qPCR. Interneuron identity was confirmed by selecting cells located in stratum radiatum that were not near the pyramidal cell layer, as well as their expression of GAD65, GAD67, or CCK. The majority of cells expressed GAD65 and/or GAD67, while others were positive for CCK, which was not noted in pyramidal cells. Of the 56 putative interneurons, 30 cells were classifiable based on their expression of our selected interneuron markers, 12 cells were unidentifiable and 14 were failures. Interneuron markers included the calcium binding proteins parvalbumin, CB, calretinin, and the neuropeptide CCK. While there are many interneuron subtypes, we selected markers that allowed us to distinguish subtypes more common in stratum radiatum. After analysis, we categorized interneurons into the following types: parvalbumin positive (1 cell), CCK positive (7 cells), CB positive (5 cells), CCK-CB positive (11 cells; Figure 3) and calretinin positive (6 cells). We also examined interneuron spiking as a way to distinguish between interneuron subtypes. Spiking profiles were measured in most, but not all interneurons, including 5 CCK, 6 CCK-CB, 5 CB and 4 calretinin cells. We noted that regular spiking interneurons firing at 60–80 Hz were identified among the CCK and CCK-CB positive cells, with the majority of CCK cells (3)

firing in irregular patterns (Figure 3A inset) and the majority of CCK-CB cells (4) firing about 5–10 spikes before strongly adapting. CB and calretinin cells were not regular spiking and varied from irregular to adaptive to only spiking a few times, and in general tended towards lower spiking numbers and frequencies particularly in the calretinin cells. While there was not homogeneity of spiking among all the cells of each subtype, our data illustrate general types of spike patterns within each group, supporting our characterization of subtypes based on calcium binding and neuropeptide expression profiles.

Our main goal was to examine the expression of endocannabinoid biosynthetic enzyme mRNA and type I mGluRs known to be involved in interneuron synaptic plasticity within interneuron subtypes. We tested for the presence of mGluR1, mGluR5, as well as 12-LO, NAPE-PLD and DAGL, the enzymes responsible for synthesis of 12-HPETE, anandamide, and 2-arachidonylglycerol, respectively (see Figure 1). In examining the four major interneuron subtypes represented by 29 cells (excluding the single parvalbumin positive cell due to small sample size), we identified expression of mGluR1 (in 6 cells), mGluR5 (in 7 cells), 12-LO (in 5 cells) and NAPE-PLD (in 7 cells). DAGL was examined in 20 of these 29 cells and was present in 10 cells (Table 2).

We next examined the distribution of these components within different interneuron subtypes (Figures 3–4 and Table 2). While most of these components were fairly widely expressed, some differential expression was noted. For example, 12-LO expression was not observed in CCK or CB cells and neither DAGL nor mGluR5 were observed in calretinin cells. In addition, as type I mGluRs are usually needed for endocannabinoid production, if interneurons have any capacity to produce endocannabinoids we would expect to identify coexpression of mGluR1 and/or mGluR5 along with endocannabinoid biosynthetic enzymes (Figures 3–4 and Table 3). Indeed, we identified that mGluR5 was coexpressed with NAPE-PLD, DAGL, or both in all interneuron subtypes (see Figure 4a and Table 3) except calretinin cells. mGluR1 was coexpressed with one or more of all the endocannabinoid biosynthetic enzymes we examined in CCK-CB and CB interneuron subtypes (see Figure 3,4b and Table 3). We also noted mGluR1 and mGluR5 expressed together with endocannabinoid biosynthetic enzymes in CCK-CB cells (Figure 4a). Taken together, these results indicate that interneurons indeed express the receptors together with the enzymes necessary to produce endocannabinoids and do so in a subtype specific fashion.

We then examined pyramidal cells to investigate endocannabinoid biosynthetic enzyme expression. This is the first study to examine these enzymes in pyramidal cells using RT-qPCR. Pyramidal cells were identified based on their expression of VGlut1 and classified as CA3 or CA1 by pyramidal cell layer subfield. 17 of 18 cells were positive for VGlut1 expression and used for analysis, including 10 CA3 and 7 CA1 pyramidal cells. All were negative for GAD65/67, and as noted by others we never detected expression of CCK in these cells (Freund and Buzsáki, 1996). We tested pyramidal cells for the presence of type I mGluRs, 12-LO, NAPE-PLD, and DAGL. We observed 12-LO and NAPE-PLD expression in CA3 pyramidal cells (Fig. 5a & 5b), but not in CA1 cells (Fig. 5c), while DAGL expression was detected in both CA3 and CA1 pyramidal cells (Fig. 5a–5c). This suggests CA3 specific expression of 12-LO and NAPE-PLD. We also examined type I mGluR expression and identified mGluR5 mRNA in CA3 and CA1 pyramidal cells, while mGluR1 mRNA was only detected in CA3 cells.

Next, we probed for the presence of TRPV1 mRNA, as the model proposed by Gibson et al. (2008) suggests presynaptic CA3 TRPV1 was a key factor in long-term depression of CA1 stratum radiatum interneurons. In support of this model, TRPV1 mRNA was identified in 3 of 6 CA3 cells examined (Figure 6). While TRPV1 was more weakly expressed as measured using probe based RT-qPCR, gel electrophoresis did display a band for TRPV1 of the

appropriate size that was absent in no-template controls, suggesting TRPV1 mRNA was indeed expressed in at least some CA3 pyramidal cells (Figure 6b). In addition, amplified cDNA created using our primers and isolated by gel electrophoresis was sequenced as TRPV1. We did not test CA1 pyramidal cells for TRPV1.

Finally, we examined quantitative differences in mRNA expression between cells. The mGluR5 expression level in interneurons was very similar to pyramidal cells, which are known to express significant levels of mGluR5. The relative expression levels were $1.0 \pm 0.5\%$ in interneurons ($n=9$) compared to $1.0 \pm 0.4\%$ in pyramidal cells ($n=5$, $p > 0.9$, normalized to interneurons). Regarding endocannabinoid biosynthetic enzymes, 12-LO was more highly expressed in interneurons than pyramidal cells, but a small sample size prevented good statistical comparison. DAGL α expression was not significantly ($p > 0.5$) different between pyramidal cells and interneurons, where expression levels were $1.0 \pm 0.7\%$ in interneurons ($n=14$) compared to $1.9 \pm 1.0\%$ in pyramidal cells ($n=10$, normalized to interneurons). There were too few NAPE-PLD positive pyramidal cells for a good statistical comparison, though expression levels tended to be lower in pyramidal cells than in interneurons. Regarding interneuron subtype markers, while the expression of most target mRNA was very similar, noted differences existed in CCK expression between CCK-CB and CCK-only expressing cells. CCK levels in CCK-CB cells were significantly ($p < 0.05$) greater, where expression levels were $1.0 \pm 0.5\%$ in CCK-only cells ($n=11$) compared to $21.1 \pm 8.7\%$ in CCK-CB cells ($n=8$, normalized to CCK-only cells). While expression levels of some targets differed between interneurons and pyramidal cells, expression levels of most markers of cell identity were extremely consistent between subtypes, supporting our qPCR methodology. As an important note, because expression levels of the reference gene 18S were not significantly different ($p > 0.05$) between interneuron subtypes or interneurons and pyramidal cells, we assume fairly equal harvesting of mRNA among these cells.

Discussion

Until now, it was unclear whether hippocampal interneurons possessed the cellular components to produce endocannabinoids, or how endocannabinoid biosynthetic enzymes were distributed within the hippocampus. Our data represent the first time that the distribution of endocannabinoid biosynthetic enzymes within hippocampal interneurons has been studied using RT-qPCR and also correlated to specific interneuron subtypes. In this study, we have examined the expression of genes involved in endocannabinoid signaling in hippocampal stratum radiatum interneurons. Our data provide evidence that hippocampal interneurons of at least 3 subtypes possess the machinery to synthesize endocannabinoids. CA3 and CA1 pyramidal cells also express mRNA coding for endocannabinoid biosynthetic enzymes, though they display differences with regard to 12-LO and NAPE-PLD expression.

During the discussion of the results, it should be noted that when using RT-qPCR to evaluate gene expression, failure to identify a particular target is not proof that mRNA for that target is not present in the cell. As such, data and analyses that we present may tend toward lower expression levels and ratios than are actually present in these cells, which is common for RT-qPCR. For example, mGluR5 expression levels are likely higher than we report. This is because while mGluR5 is likely expressed by most CA1 pyramidal cells, we identified it in half of them, suggesting mGluR5 expression levels we report in interneurons are also likely lower than actual expression levels. However, we are confident that the conclusions we present for positive identification of endocannabinoid biosynthetic enzyme mRNA expression and type I mGluRs are correct and reflect an accurate accounting of their expression profiles. Lastly, while mRNA expression suggests the presence of endocannabinoid biosynthetic enzymes and normally indicates expression of protein encoded by the mRNA, it does not necessarily indicate proof of protein expression.

4.1 Interneuron Subtypes

In undertaking this study, we first verified that the interneuron subtypes we categorized matched those of previously published studies. Our results were consistent with ratios of stratum radiatum interneurons and molecular profiles outlined previously (Freund and Buzsáki, 1996, Jinno and Kosaka, 2002, 2006, Klausberger, 2009). In addition, we noted all CCK containing cells also expressed CB1, as would be expected (Katona et al., 1999, Marsicano and Lutz, 1999). These prior reports mainly use immunocytochemistry or western blot methodologies. These current experiments using qPCR still reveal a similar pattern of calcium binding protein and neuropeptide expression, supporting our qPCR methodology as a valid technique for identification of interneuron subtypes. Indeed, using selected targets to classify only some of the many interneuron subtypes in the hippocampus, we identified four major subpopulations within stratum radiatum using this technique. Other subtypes, such as trilinear or Schaeffer-collateral associated cells, which are present in stratum radiatum (Ferraguti et al., 2004, Boscia et al., 2008, Szilagy et al., 2011), could possibly be among the cells we did not attempt to categorize. Many of the 14 unidentified cells also expressed these endocannabinoid-producing enzymes and type I mGluRs. While spiking is often used to help discriminate interneuron subtypes, it is difficult to employ as a clear identifier of interneuron subtypes due to variability in spiking among subtypes (Ascoli et al., 2008, Wierenga et al., 2010). However, our data suggests that general spiking patterns support our characterization of subtype groups based on calcium binding proteins and CCK as compared to others (Kawaguchi et al., 1995, Buhl et al., 1996, Pawelzik et al., 1999, Galarreta et al., 2004).

4.2 Endocannabinoid biosynthetic enzyme mRNA expression within interneurons

The major find of this study is the description of mRNA for endocannabinoid biosynthetic enzymes NAPE-PLD, 12-LO, and DAGL in hippocampal interneurons. NAPE-PLD produces anandamide in the brain (Morishita et al., 2005, Ueda et al., 2005, Placzek et al., 2008) and is reported to be present and active in the hippocampus (Morishita et al., 2005). Anandamide activates endocannabinoid receptors such as CB1 and TRPV1 (Caterina et al., 1997), and may also be produced by other enzymes (Liu et al., 2006, Simon and Cravatt, 2010). While some stratum radiatum interneurons in mice were identified to express NAPE-PLD using immunocytochemistry (Cristino et al., 2008), the expression pattern of NAPE-PLD in specific interneuron subtypes was not described. We determined that NAPE-PLD is most highly expressed in CCK-CB and CB expressing cells.

DAGL, responsible for the synthesis of 2-arachidonylglycerol (Tanimura et al., 2010, Ludanyi et al., 2011), was previously identified using in situ hybridization in CA3 and CA1 pyramidal cells, and was either absent or expressed at undetectable levels for this technique in interneurons and glia (Katona et al., 2006). Our qPCR data confirm the expression of DAGL in both CA3 and CA1 pyramidal cells and also suggest its presence in CA1 stratum radiatum interneurons. Expression was highest in CCK-CB and CB cells and somewhat lower in CCK-only cells. This is the first report of hippocampal interneurons expressing DAGL.

12-LO synthesizes 12-HPETE (Hwang et al., 2000), and has been identified in some stratum radiatum interneurons in mice (Cristino et al., 2008). The interneuron subtypes expressing 12-LO, however, were not identified. Our data indicate that 12-LO is mainly expressed in the radiatum by CCK-CB interneurons.

While others have identified protein expression to some degree of these endocannabinoid biosynthetic enzymes in the hippocampus using immunocytochemistry, which provides support for our RT-qPCR data, we now identify which cells types they are expressed in and

their co-expression with type I mGluRs demonstrating their capacity to produce endocannabinoids. In short, endocannabinoid biosynthetic enzymes are indeed likely expressed by interneurons and this expression is at least partly subtype specific, where NAPE-PLD and DAGL expression is fairly broadly distributed, unlike 12-LO, and calretinin cells had very little expression of mRNA for these enzymes. While the ability of hippocampal interneurons to produce endocannabinoids has been debated (Hoffman et al., 2003), our data suggest it as a strong possibility, as has been described for cerebellar interneurons (Beierlein and Regehr, 2006). Therefore, signaling (retrograde and otherwise) and plasticity that is mediated by endocannabinoids produced within hippocampal interneurons, appears to be a possibility. Furthermore, a recent study has shown that activation of CB1 receptors on interneurons decreases gamma oscillations in the hippocampus (Holderith et al., 2011). This suggests that the coexpression of CB1 and DAGL or NAPE-PLD by CA1 stratum radiatum interneurons could lead to autoregulation of oscillatory behavior or self-inhibition as described in neocortical interneurons (Bacci et al., 2004), and further highlights the importance of our findings.

4.3 Type I mGluR expression

As type I mGluR activation often results in production of endocannabinoid precursors such as arachidonic acid, they are usually necessary for endocannabinoid production within cells that modulate plasticity (Huber et al., 2001, Edwards et al., 2006). Therefore, it was critical to identify type I mGluR coexpression with endocannabinoid biosynthetic enzymes to provide evidence for interneurons' role in endocannabinoid production. Regarding type I mGluRs, it was previously shown that mGluR1 is expressed by non-principal cells in the radiatum (Kerner et al., 1997, van Hooft et al., 2000, Ferraguti et al., 2004), where mGluR1 expression was identified in spiny interneurons (Wittner et al., 2006). Our data using RT-qPCR demonstrate mGluR1 expression in radiatum interneurons, particularly in CB and CCK-CB cells, which also coexpressed CB1. This supports prior immunocytochemical evidence that CB1 is coexpressed with mGluR1, particularly in CCK-CB interneurons in both rats and mice (Boscia et al., 2008). Boscia et al. also identified mGluR1 in other interneuron populations, including CCK positive and CCK negative cells. Our data suggest that some of these previously unidentified mGluR1 expressing interneuron subtypes may include CB cells as well as CCK-CB cells. While mGluR1 expression in calretinin containing cells has been described previously (Ferraguti et al., 2004), we did not observe this coexpression, possibly because of our sample size for calretinin cells.

Expression of mGluR5, which is widely present in hippocampal pyramidal cells (Kerner et al., 1997, van Hooft et al., 2000, Huber et al., 2001), was previously noted immunocytochemically in some CA1 stratum radiatum cells that appeared to be GABAergic, but were not classified as such (Romano et al., 1995). We are the first to positively identify mGluR5 expression in stratum radiatum interneurons, which appear to be mainly in CCK expressing cells. This is also the first report to specifically examine coexpression of type I mGluR and endocannabinoid biosynthetic enzyme mRNA in CA1 stratum radiatum interneurons, where our data support the potential capacity of interneurons to produce endocannabinoids. Finally, the identification or suggestion that type I mGluRs are at least present in some interneurons using immunocytochemistry or physiological, as listed above, also supports our identification of these receptors using RT-qPCR in radiatum interneurons.

4.4 TRPV1 expression in hippocampal neurons and components involved in mGluR/TRPV1-mediated interneuron plasticity

TRPV1 has been shown to be expressed in the hippocampus (Sanchez et al., 2001, Tóth et al., 2005, Cristino et al., 2006, Cristino et al., 2008, Bennion et al., 2011) with some

important exceptions (Kofalvi et al., 2006, Cavanaugh et al., 2011), and has been demonstrated to be involved in hippocampal synaptic plasticity (Gibson et al., 2008, Chavez et al., 2010, Bennion et al., 2011). We recently used RT-qPCR to identify TRPV1 mRNA present in whole hippocampal homogenates (Bennion et al., 2011). Indeed, TRP conductance mediated by type I mGluRs was identified in the hippocampus previously (Gee et al., 2003), and endocannabinoid signaling via TRPV1 is known to be initiated by the activity of type I mGluRs (Gibson et al., 2008, Bennion et al., 2011). Presently, we identified weak TRPV1 mRNA expression in CA3 pyramidal cells using RT-qPCR, supporting prior reports that it is present in the hippocampus. The low levels of TRPV1 noted might suggest that high TRPV1 expression is not necessary to influence synaptic plasticity or that TRPV1 mRNA is transported toward its axonal expression site, which would reduce the amount of TRPV1 mRNA within the soma, where mRNA was harvested for our study. Because we noted TRPV1 expression in CA3 pyramidal cells as well as type I mGluRs and 12-LO in CA1 stratum radiatum interneurons, it supports physiological data of the proposed mechanism for presynaptic TRPV1-mediated long-term depression in stratum radiatum interneurons (Gibson et al., 2008). Additionally, 12-LO-expressing cells, such as CCK-CB cells, or those expressing NAPE-PLD that produce anandamide could potentially induce TRPV1-mediated interneuron long-term depression by themselves.

4.5 CA1/CA3 pyramidal cell expression of endocannabinoid biosynthetic enzymes

In addition to interneurons, endocannabinoid biosynthetic enzymes are also expressed in pyramidal cells. Prior studies using immunocytochemistry indicate that 12-LO, NAPE-PLD, and DAGL are expressed in CA3 pyramidal cells, while 12-LO and DAGL are expressed in CA1 pyramidal cells (Nishiyama et al., 1993, Cristino et al., 2008, Egertová et al., 2008, Tanimura et al., 2010). 12-LO was also shown to be physiologically active in CA3 Schaeffer collaterals (Feinmark et al., 2003). Overall, our data corroborate the published evidence for endocannabinoid biosynthetic enzyme expression in pyramidal cells, except that we did not detect 12-LO expression in CA1 pyramidal cells. Additionally, mGluR5 was noted in both CA3 and CA1 pyramidal cells, while mGluR1 was noted to be expressed solely in CA3 cells using immunocytochemistry or physiology (Lujan et al., 1996, Shigemoto et al., 1997, Chuang et al., 2002, Le Duigou et al., 2011). Our qPCR data confirm this finding. Collectively, our data support previously published data and represent the first time that the expression of these endocannabinoid biosynthetic enzymes in pyramidal cells has been described using RT-qPCR. This also provides support for the reliability of our RT-qPCR methodology, as it closely models prior physiological and immunocytochemical data, supporting RT-qPCR as a viable method to study neuronal gene expression.

4.6 Conclusion

In summary, our qPCR data indicate that CCK, CCK-CB, and CB expressing CA1 stratum radiatum interneurons have the potential to produce some endocannabinoids due to their coexpression of type I mGluRs and endocannabinoid biosynthetic enzymes. This suggests that these subtypes could be involved in some forms of endocannabinoid-mediated signaling, including synaptic plasticity and regulating oscillatory behavior. Our data also indicate that calretinin cells display very little mRNA for endocannabinoid biosynthetic enzymes or the receptors involved in their activation. Collectively, our data clearly demonstrate the capacity for hippocampal stratum radiatum interneurons to produce endocannabinoids, providing evidence that interneuron involvement in endocannabinoid signaling in the hippocampus may be greater than previously thought.

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Highlights

- Hippocampal CA1 interneurons express mRNA for endocannabinoid biosynthetic enzymes
- The expression of mRNA for these enzymes occurs in a subtype-specific manner
- Coexpression with type I mGluRs suggests interneurons can produce endocannabinoids
- Pyramidal cells also express mRNA for these enzymes that differ between CA1 and CA3

Figure 1.

Endocannabinoid biosynthetic pathways and receptor targets. Postsynaptic type I metabotropic glutamate receptor activation commonly produces metabolites used in endocannabinoid and eicosanoid synthesis. Endocannabinoid biosynthetic enzymes such as diacylglycerol lipase (DAGL), N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and 12-lipoxygenase (12-LO) produce the endocannabinoids 2-arachidonylglycerol (2-AG), anandamide (AEA), and 12-(S)-Hydroperoxyeicosa-5Z, 8Z, 10E, 14Z-tetraenoic acid (12-HPETE), respectively. Endocannabinoids are lipophilic substances that can act retrogradely on presynaptic terminals to modulate neurotransmitter

release via 2-AG or AEA activating cannabinoid receptor 1 (CB1), or 12-HPETE or AEA activating transient receptor potential vanilloid 1 (TRPV1).

Figure 2.

Optimization and verification of RT-qPCR primers and probes. a) A dose-response set of fluorescent curves of the primer/probe set for CCK ranging from 100 ng to 0.3 ng cDNA. Inset: The linear fit of the dose response from (a) in log scale for CCK. Ct values for all triplicates from each concentration are included. b) Melt curve peaks of CCK from the same cDNA dose response in (a). Inset: Electrophoresis gel of CCK, showing, from left to right, 50 base pair (bp) ladder, 18S amplification product (133 bp), and CCK amplification product (111 bp). Curves produced for dose response and melt curves in (a) and (b) were triplicate averages.

Figure 3.

Identification of CA1 stratum radiatum interneuron subtypes by their expression of endocannabinoid biosynthetic enzyme mRNA and spiking pattern. a) A representative CCK-CB cell expressing DAGL (royal), 12-LO (wine), and mGluR1 (purple). Data are displayed as fluorescence from a FAM-TAMRA hydrolysis probe from a RT-qPCR reaction. Inset: A representative irregular spiking CA1 stratum radiatum interneuron. The membrane potential of this cell (not visible here) was -70 mV. Scale bar: 150 ms, 15 mV. b) A 4% agarose gel of the cell presented in (a) showing, from left to right, 50 bp ladder, mGluR1, CB1, 12-LO, DAGL, CCK, 18S, and CB, with their respective amplicon sizes.

Figure 4.

Endocannabinoid biosynthetic enzyme mRNA expression in CA1 stratum radiatum interneuron subtypes. a) A representative CCK-CB cell demonstrates the presence of mGluR1 (purple), mGluR5 (dark cyan) and NAPE-PLD (olive). Note that the scaling of this figure did not allow for an accurate depiction of mGluR5, which under different scaling demonstrates a sigmoid curve, and the correct amplicon size is noted using gel electrophoresis. b) A representative CB-expressing cell demonstrates the presence of NAPE-PLD (olive) and mGluR1 (purple). c) A representative calretinin-expressing cell lacking expression of endocannabinoid components or type I mGluRs. Data are displayed as fluorescence from a FAM-TAMRA hydrolysis probe in the RT-qPCR reaction.

Figure 5.

Expression of endocannabinoid biosynthetic enzyme mRNA in hippocampal pyramidal cells. a) A representative CA3 pyramidal cell demonstrates the presence of DAGL (royal), NAPE-PLD (olive), and mGluR5 (dark cyan). Note the mGluR5 no-template control (mGluR5 NTC). b) A second CA3 pyramidal cell demonstrates the presence of DAGL (royal) and 12-LO (wine). c) A representative CA1 pyramidal cell demonstrates the presence of DAGL (royal) and mGluR5 (dark cyan). Data are displayed as fluorescence from a RT-qPCR reaction.

Figure 6.

TRPV1 mRNA expression in a CA3 pyramidal cell. a) A representative CA3 pyramidal cell expressing VGluT1 (light gray) and TRPV1 (dark gray). b) A 4% agarose gel of the cell in a), showing, from left to right, 50 bp ladder (50 bp and 100 bp shown), TRPV1 (86 bp, arrow), TRPV1 no-template control, and VGluT1 (74 bp).

Table 1

Primer and probe sequences used for RT-qPCR, and whether primers are intron spanning.

Target	Direction	Primer Sequence	Probe Sequence	Intron Spanning
mGluR1 77 bp	Forward Reverse	AGGAGGTGTGGTTCGATGAG ATTAGCTTCTGTGTACTGCAGATTC	ATGCTCCCGGAAGGTATGACATTA	Y
TRPV1 86 bp	Forward Reverse	TCCTGACGGCAAGGATGAC TGATGATACCCACATTGGTGTTC	TGGTGTTTCAGGGTGGACGAG	Y
GAD67 58 bp	Forward Reverse	CATCCTGGTCAAGGAAAAGG TGCTTGTCTGGCTGGAAGAG	TATACTCCAAGGATGCAACCAGATGTGTGC	Y
CB1 78 bp	Forward Reverse	CTTTATGGACATGGAGTGTCTTAT CCCAGTGTGAGGGACAGTACA	TCTGAATCCCAGCCAGCAGCTG	N
NAPE-PLD 75 bp	Forward Reverse	GCCAAGGTGGTTTATGAAATACC TTGGCTTGAACGTCAATGTG	CATGCTGACCCAGAGGATGCTGTAAGG	Y
12-LO 71 bp	Forward Reverse	TGGTGTCTGGGAGATCACTGA GGACTGGAAGGAGACAGGGAAT	TGTGCCATGCCAGGACAGAG	Y
PV 71 bp	Forward Reverse	CAAGAAGGCGATAGGAGCCTTT GGCCACCATCTGGAAGAA	CTGCTGCAGACTCCTTCGACCACAAAAA	Y
DAGLα 73 bp	Forward Reverse	CAGATGCCTATTGAGAAATTGC ATGTCTGGAGGGCACTATGTC	CCTCTTGTCTGAATTTTCCGTGACC	Y
GAD65 155 bp	Forward Reverse	AGTGCCACAGCTGGAACCA ACACCGTTCAGCTTCCACTTGT	TCTCTTGGCTGTAGCTGACATCTGCAAAAAATA	Y
mGluR5 79 bp	Forward Reverse	TTTCTGGAGATATGATCCTGTTTG CCCATTTCTTGAAATTCATTAT	TGAAAATGGAGACTCTCCAGGAGGGTATG	Y
CCK 111 bp	Forward Reverse	TGTAGTCCCGGTCACTTATCC TGTCTAGCCCGATACATCCA	AAAGCTCCCTCTGGCCGCAT	Y
VGlut1 74 bp	Forward Reverse	TGCGCAGTCGTCATATAATGTC AGCTTCCATCCCGAAACC	ACGACCAATGTGCGAAAGCTGATG	Y
18S 133 bp	Forward Reverse	GTGCATGGCCGTTCTTAGTTG GCCACTTGTCCCTCTAAGAAGTTG	TGGAGCGATTTGTCTGGTTAATTCCGATAAC	Y
CR 78 bp	Forward Reverse	CCGTCCCTATGATGAACCTAAGC TTGCCGTCTCCATTTAAGTCAA	CCAGGAGTACACCCAAACCATACTACGCATG	Y
CB 74 bp	Forward Reverse	GCTTCTGCAGGCACGAAAAG GCCCATATTGATCCACAAAGGT	CATCTCAGGTGATAGCTCCAATCCAGCCT	Y

Table 2

The expression of endocannabinoid biosynthetic enzyme and type I metabotropic glutamate receptor mRNA in interneuron subtypes.

Subtype	12-L O	NAPE-PLD	mGluR1	mGluR5	DAGLα
CCK (n=7)	0	1	1	3	2 of 6
CCK-CB (n=11)	4	3	3	3	5 of 8
CB (n=5)	0	2	1	1	3 of 4
CR (n=6)	1	1	1	0	0 of 2
All (n=29)	5	7	6	7	10 of 20

Table 3

Coexpression of endocannabinoid biosynthetic enzyme and type I metabotropic glutamate receptor mRNA in interneuron subtypes.

Subtype	mGluR5/12-LO	mGluR5/NAPE-PLD	mGluR5/DAGL α
CCK		x	x
CCK-CB		x	x
CB			x
CR			
	mGluR1/12-LO	mGluR1/NAPE-PLD	mGluR1/DAGL α
CCK			
CCK-CB	x	x	x
CB		x	
CR			