2014-3

Endocannabinoid Biosynthetic Enzyme mRNA: Patterns of Expression in Hippocampus and Ventral Tegmental Area and Effects on Synaptic Plasticity

Collin Brutch Merrill
Brigham Young University - Provo

Endocannabinoid Biosynthetic Enzyme mRNA: Patterns of Expression in Hippocampus and Ventral Tegmental Area and Effects on Synaptic Plasticity

Collin B. Merrill

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Jeffrey G. Edwards, Chair
Scott C. Steffensen
Sterling N. Sudweeks
Dixon J. Woodbury
David D. Busath

Department of Physiology and Developmental Biology

Brigham Young University

March 2014

ABSTRACT

Endocannabinoid Biosynthetic Enzyme mRNA: Patterns of Expression
in Hippocampus and Ventral Tegmental Area
and Effects on Synaptic Plasticity

Collin B. Merrill
Department of Physiology and Developmental Biology, BYU
Doctor of Philosophy

Endocannabinoids (eCBs) are lipophilic signals that are produced by postsynaptic neurons in an activity-dependent manner, and signal in a retrograde fashion to modulate neurotransmitter release. As such, eCBs are highly involved in synaptic plasticity, a process that strengthens or weakens synapses. eCB-mediated synaptic plasticity is involved in many brain processes including learning, short-term memory, and adaptive reward, which are processed in the hippocampus and ventral tegmental area (VTA), respectively. However, the expression of eCB biosynthetic enzyme mRNA within hippocampal and VTA neurons, as well as the relationship between these mRNA species and the occurrence of synaptic plasticity, remains unclear. The goal of these studies was to demonstrate the expression pattern of eCB biosynthetic enzyme mRNA within hippocampal and VTA neurons, and to describe the relationship between synaptic plasticity and mRNA expression. Using whole-cell electrophysiology and real-time quantitative PCR, I tested hippocampal and VTA neurons for the presence of eCB biosynthetic enzyme mRNA and described the relationship between these enzymes and synaptic plasticity. The data presented herein demonstrate the importance of eCB signaling within the hippocampus and VTA and the expression patterns of eCB biosynthetic machinery within several neuron types. These data provide evidence that eCB signaling plays a critical role in learning, short-term memory, and adaptive reward.

Keywords: DAGLα, NAPE-PLD, 12LO, mGluR, long-term depression, dopamine, GABA

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Jeffrey Edwards, for his advice and guidance through graduate school and this project. I would also like to thank the members of my committee, Dr. Sterling Sudweeks, Dr. Scott Steffensen, Dr. Dixon Woodbury, and Dr. David Busath for their guidance.

I would like to thank the various members of the Edwards lab for their friendship and support during my graduate work. In particular, special thanks go to Michael McNeil, Ryan Williamson, Brian Poole, Blake Nelson, and Zach Hopkins for their assistance in performing RT-qPCR experiments. I would also like to thank Lindsey Friend and Scott Newton for their assistance in performing immunohistochemical experiments.

Finally, I would like to thank my wife, Adrianne, and my children, Garrett, Gabrielle, and Bentley, for their love and support through all this work. Without them, I would not have made it.

TABLE OF CONTENTS

TITLE PAGE i

ABSTRACT ii

ACKNOWLEDGMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER 1: Introduction 1

The Hippocampus 1

Hippocampal Cell Types 2

The Ventral Tegmental Area 2

Ventral Tegmental Area Cell Types 3

Synaptic Plasticity 3

Endocannabinoids 4

Endocannabinoid-mediated Synaptic Plasticity 4

Specific Aims 5

CHAPTER 2: Identification of mRNA for Endocannabinoid Biosynthetic Enzymes Within Hippocampal Pyramidal Cells and CA1 Stratum Radiatum Interneuron Subtypes Using Quantitative Real-time Polymerase Chain Reaction 6

Abstract 6

Introduction 7

Methods 10

Preparation of Slices 10

Electrophysiological Recordings and Extraction 10

Reverse Transcription Reaction 11

Primer Design, Verification, and Optimization 12

Preamplification (Multiplex) Reaction 12

Quantitative PCR Reaction 13

Data Analysis 13

Results 14

Discussion 18

Interneuron Subtypes 19

Endocannabinoid Biosynthetic Enzyme mRNA Expression Within Interneurons 20

Type I mGluR Expression 22

TRPV1 Expression in Hippocampal Neurons and Components Involved in mGluR/TRPV1-mediated Interneuron Plasticity 23

CA1/CA3 Pyramidal Cell Expression of Endocannabinoid Biosynthetic Enzymes 24

Conclusion 24

Acknowledgements 25

Table 2.1. Primer Sequences for qPCR. 26

Table 2.2. eCB mRNA Expression in Interneuron Subtypes. 27

Table 2.3. eCB mRNA Coexpression in Interneuron Subtypes. 27

Figure 2.1. Endocannabinoid Biosynthetic Pathways and Receptor Targets. 28

Figure 2.2. Optimization and Verification of RT-qPCR Primers and Probes. 29

Figure 2.3. Identification of CA1 Stratum Radiatum Interneuron Subtypes by Their Expression of Endocannabinoid Biosynthetic Enzyme mRNA and Spiking Pattern. 30

Figure 2.4. Endocannabinoid Biosynthetic Enzyme mRNA Expression in CA1 Stratum Radiatum Interneuron Subtypes. 31

Figure 2.5. Expression of Endocannabinoid Biosynthetic Enzyme mRNA in Hippocampal Pyramidal Cells. 32

Figure 2.6. TRPV1 mRNA Expression in a CA3 Pyramidal Cell. 33

CHAPTER 3: Ventral Tegmental Area Dopamine and GABA Neurons: Physiological Properties and Expression of mRNA for Endocannabinoid Biosynthetic Enzymes and Type I Metabotropic Glutamate Receptors 34

Abstract 34

Introduction 36

Methods 39

Slice Preparation 39

Electrophysiological Recordings and Extraction 39

Reverse Transcription Reaction 40

Primer Design, Verification, and Optimization 41

Preamplification (Multiplex) Reaction 41

Quantitative PCR Reaction 42

Data Analysis 42

Immunohistochemistry 43

Results 44

Discussion 49

VTA Neuron Identification 49

Calcium Binding Protein mRNA Within VTA Neurons 51

Endocannabinoid Biosynthetic Enzyme mRNA Within VTA Neurons 52

Type I mGluR mRNA Within VTA Neurons 54

Conclusion 54

Acknowledgements 55

Table 3.1. Mouse Primer and Probe Sequences. 56

Table 3.2. Calcium-binding Protein and Neuropeptide Expression in Rat Ventral Tegmental Area Neurons. 57

Table 3.3. eCB Biosynthetic Enzyme and Type I mGluR mRNA Expression in Rat and Mouse VTA Neurons. 57

Figure 3.1. Gene Expression and Electrophysiological Profiles of Ventral Tegmental Area Dopaminergic and GABAergic Neurons. 58

Figure 3.2. Relative mRNA Expression of mGluR1/5 and DAGLα in Ventral Tegmental Area Neurons. 59

Figure 3.3. Immunolabeling of Endocannabinoid Biosynthetic Enzymes and Type I mGluRs Within Mouse Ventral Tegmental Area. 60

CHAPTER 4: Subtype-specific Synaptic Plasticity Within Hippocampal CA1 Stratum Radiatum Interneurons is Influenced by eCB Biosynthetic Enzyme mRNA Expression 61

Abstract 61

Introduction 62

Methods 64

Slice Preparation 64

Electrophysiological Recordings and Extraction 64

Reverse Transcription Reaction 66

Primer Design, Verification, and Optimization 66

Preamplification (Multiplex) Reaction 67

Quantitative PCR Reaction 67

Results 68

Discussion 70

Acknowledgements 74

Table 4.1. Relationship Between Subtype, Synaptic Plasticity, and eCB Biosynthetic Enzyme and Type I mGluR Expression in Stratum Radiatum Interneurons. 75

Figure 4.1. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-positive Basket Cell. 76

Figure 4.2. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-CB Positive Basket Cell. 77

CHAPTER 5: Conclusion 78

REFERENCES 79

CURRICULUM VITAE 91

LIST OF TABLES

Table 2.1. Primer Sequences for qPCR 26

Table 2.2. eCB mRNA Expression in Interneuron Subtypes..... 27

Table 2.3. eCB mRNA Coexpression in Interneuron Subtypes..... 27

Table 3.1. Mouse Primer and Probe Sequences..... 56

Table 3.2. Calcium-binding Protein and Neuropeptide Expression in Rat Ventral Tegmental Area Neurons 57

Table 3.3. eCB Biosynthetic Enzyme and Type I mGluR mRNA Expression in Rat and Mouse VTA Neurons. 57

Table 4.1. Relationship Between Subtype, Synaptic Plasticity, and eCB Biosynthetic Enzyme and Type I mGluR Expression in Stratum Radiatum Interneurons. 75

LIST OF FIGURES

Figure 2.1. Endocannabinoid Biosynthetic Pathways and Receptor Targets. 28

Figure 2.2. Optimization and Verification of RT-qPCR Primers and Probes. 29

Figure 2.3. Identification of CA1 Stratum Radiatum Interneuron Subtypes by Their Expression of Endocannabinoid Biosynthetic Enzyme mRNA and Spiking Pattern. 30

Figure 2.4. Endocannabinoid Biosynthetic Enzyme mRNA Expression in CA1 Stratum Radiatum Interneuron Subtypes. 31

Figure 2.5. Expression of Endocannabinoid Biosynthetic Enzyme mRNA in Hippocampal Pyramidal Cells. 32

Figure 2.6. TRPV1 mRNA Expression in a CA3 Pyramidal Cell. 33

Figure 3.1. Gene Expression and Electrophysiological Profiles of Ventral Tegmental Area Dopaminergic and GABAergic Neurons. 58

Figure 3.2. Relative mRNA Expression of mGluR1/5 and DAGLα in Ventral Tegmental Area Neurons. 59

Figure 3.3. Immunolabeling of Endocannabinoid Biosynthetic Enzymes and Type I mGluRs Within Mouse Ventral Tegmental Area 60

Figure 4.1. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-positive Basket Cell. 76

Figure 4.2. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-CB Positive Basket Cell 77

CHAPTER 1: Introduction

Endocannabinoid (eCB) signaling is a critical modulator of neurotransmission throughout the brain, and is a critical component of the circuitry underlying learning, short-term memory, and adaptive reward processing. Learning and short-term memory take place in the hippocampus, while adaptive reward processing occurs in the ventral tegmental area (VTA). Within both these areas, modulation of neurotransmission occurs through synaptic plasticity, which can be mediated by endocannabinoids. While the effects of eCBs on neurotransmission are well documented, the cellular localization of receptors and enzymes involved in eCB biosynthesis in the hippocampus and VTA is not well understood. Further, the effects of having differential expression of these receptors and enzymes among various cell types remain unclear.

The Hippocampus

The hippocampus is located in the medial temporal lobe of the brain in humans, and is the site of short-term memory and learning processes. The region is divided into four major parts: the dentate gyrus, hippocampus proper, and entorhinal cortex. The hippocampus proper is further divided into subfields, consisting of the CA3 area, CA1 area, and subiculum. In addition, the hippocampus proper contains several layers, which include the stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare. Information flow through the area follows a consistent pattern from the dentate gyrus to CA3 to CA1 to subiculum, and finally to the entorhinal cortex. In addition to this main circuit, there are additional connections between CA3 stratum pyramidale and CA1 stratum radiatum via Schaeffer collaterals and several reciprocal connections between CA1 stratum oriens to CA3 stratum pyramidale.

Hippocampal Cell Types

The hippocampal circuit involves two main cell types: pyramidal cells and interneurons. Pyramidal cells are located in the stratum pyramidale throughout all subfields of the hippocampus proper, and they send projections throughout all subfields. The synapses between CA3 cells and CA1 cells are the major site of learning and memory processing. A distinguishing feature of all pyramidal cells is their homogeneity—CA3 and CA1 neurons are remarkably similar in morphology, gene expression, and electrophysiological characteristics. Interneurons are located throughout the hippocampus, within all subfields and layers, and function to modulate pyramidal cell activity. Unlike pyramidal cells, interneurons do not project to other subfields, and also display incredible heterogeneity. Using criteria such as morphology, gene expression, and electrophysiological characteristics, interneurons can be divided into more than twenty different subtypes, each with its own function. Of the subtypes contained in CA1 stratum radiatum, the three critical groups are basket cells, which can be either cholecystokinin (CCK) or CCK-calbindin (CB) positive, bistratified cells, which are CB-positive, and interneuron-selective cells, which are distinguished by calretinin (CR) expression. These interneuron subtypes can all be innervated by CA3 pyramidal cells, and in turn, innervate CA1 pyramidal cells, providing an activity-based source of modulation of pyramidal cell populations.

The Ventral Tegmental Area

The mesocorticolimbic pathway is the main site of reward processing in the brain, and involves the projection of the VTA to the nucleus accumbens and prefrontal cortex, reciprocal connections between these areas, and projections to other brain areas such as the hippocampus, amygdala, and hypothalamus. The projection of the VTA to the nucleus accumbens is the major site of dopamine release, which is a critical factor in adaptive reward and motivational

processing. Altered dopamine (DA) release from the VTA underlies perturbed reward processing which is a hallmark of addiction.

Ventral Tegmental Area Cell Types

Within the VTA, two cell types underlie adaptive reward processing. DAergic neurons comprise the majority of cells within the area. These neurons project to the nucleus accumbens and release DA in response to rewarding stimuli. DA cells can be identified via a combination of electrophysiological characteristics and gene expression. These neurons generally fire at low frequencies and express tyrosine hydroxylase (TH) and dopamine transporters (DAT), enzymes involved in DA synthesis and packaging.

GABAergic neurons are the other main cell type found within the VTA. These neurons can project to other brain areas or can remain within the VTA, and modulate DA cell activity. GABA neurons can also be identified by electrophysiological characteristics and gene expression, firing at higher frequencies and expressing glutamate decarboxylase 65 or 67 (GAD65/67), enzymes involved in GABA synthesis.

Synaptic Plasticity

Synaptic plasticity describes activity-dependent synaptic changes that underlie modulation of neurotransmission, and occurs at many synapses within the brain. The two types of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP occurs when a synaptic connection is strengthened, either by a presynaptic increase in neurotransmitter release or by a postsynaptic increase in receptors. Either mechanism allows activation of the postsynaptic cell with a smaller stimulus. LTD occurs when a synapse becomes weaker, either by a presynaptic decrease in neurotransmitter release or a postsynaptic decrease in receptors. In this case, the postsynaptic cell requires a larger stimulus for activation.

Endocannabinoids

eCBs are a group of lipophilic molecules, similar to the active compound in marijuana, THC, and are derived from plasma membrane phospholipids. In contrast to typical neurotransmission, eCBs generally signal in a retrograde fashion from the postsynaptic neuron to the presynaptic neuron. The most prevalent endocannabinoids are 2-arachidonylglycerol (2-AG), anandamide, and 12-HPETE, which are synthesized by diacylglycerol lipase α (DAGLα), N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), and 12-lipoxygenase. 2-AG activates cannabinoid receptor 1 (CB1), 12-HPETE activates transient receptor vanilloid 1 (TRPV1), and anandamide activates both CB1 and TRPV1.

Endocannabinoid-mediated Synaptic Plasticity

Because retrograde eCB signaling has the potential to modulate neurotransmission, they also have the potential to mediate synaptic plasticity. For example, hippocampal CA1 stratum radiatum interneurons exhibit LTD that is mediated by TRPV1. In this type of plasticity, high glutamate levels activate perisynaptic metabotropic glutamate receptors (mGluRs), which causes 12LO activity and produces 12-HPETE, which then activates presynaptic TRPV1 receptors and decreases neurotransmitter release. Additional synaptic plasticity occurs at multiple synapses within the hippocampus via 2-AG and anandamide signaling as well. However, most evidence relies largely on electrophysiology and immunohistochemistry to demonstrate the effects of eCBs and related synaptic plasticity at hippocampal synapses. In addition, experiments describing eCB-mediated synaptic plasticity demonstrate that interneurons can also undergo short-term depression or no depression in response to high frequency stimulation.

Within the VTA, one source of DA modulation is mediated by eCB signaling at synapses onto DA neurons. This type of plasticity is largely mediated by 2-AG activation of presynaptic

CB1 receptors and causes decreased DA release. Anandamide was also demonstrated to play a role in synaptic plasticity of DA neurons through the use of CB1 receptor antagonists and blockade of anandamide degradation. eCB-mediated synaptic plasticity can occur at various synapses within the VTA, both onto DA and GABA neurons, with differential effects on DA release. However, these effects were described using electrophysiological techniques, with very little direct evidence for the cellular localization of eCB biosynthetic enzymes and type I mGluRs within VTA neurons.

Specific Aims

Though the effects of eCB-mediated synaptic plasticity are well documented within the hippocampus and VTA using electrophysiological techniques, there is very little evidence for the expression pattern of eCB biosynthetic enzymes and type I mGluRs within CA1 stratum radiatum interneurons or VTA dopamine or GABA neurons using molecular techniques. Therefore, my first goal was to describe the expression pattern of eCB biosynthetic enzyme and type I mGluR mRNA within CA1 stratum radiatum interneurons using real-time quantitative PCR (RT-qPCR). My second goal was to perform RT-qPCR experiments within the VTA to describe the localization of eCB biosynthetic enzyme and type I mGluR mRNA within DA and GABA cells, and to examine the protein expression of these targets using immunohistochemistry. Finally, the relationship between interneuron subtype and synaptic plasticity remains unclear. Therefore, my third goal was to examine the correlation between interneuron subtypes, eCB biosynthetic enzyme and type I mGluR mRNA expression, and synaptic plasticity.

CHAPTER 2: Identification of mRNA for Endocannabinoid Biosynthetic Enzymes Within Hippocampal Pyramidal Cells and CA1 Stratum Radiatum Interneuron Subtypes Using Quantitative Real-time Polymerase Chain Reaction

C.B. Merrill, M. McNeil, R.C. Williamson, B.R. Poole, B. Nelson, S. Sudweeks, and J.G. Edwards

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 α, 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 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, 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-arachidonylglycerol, synthesized by diacylglycerol lipase α (DAGLα) (Tanimura et al., 2010), activates 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 2.1). 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 (Fuentealba 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.

Methods

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 μm thick coronal slices, and stored for at least one hour submerged on a net in artificial cerebrospinal fluid containing (in mM) 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 11 glucose, saturated with 95% O_2/5% CO_2 (pH 7.4). Slices were then transferred to a submerged recording chamber and bathed in oxygenated artificial cerebrospinal fluid.

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 40x 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. 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.

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 μL. This mixture was then cycled in a C1000 Thermocycler (BioRad) under the following conditions: 25.0 ºC for 8 minutes, 42.0 ºC for 60 minutes, and 70 ºC 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.

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 2.2). 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/μL from whole brain homogenate.

Preamplification (Multiplex) Reaction

Once the reverse transcription reaction was complete, each cell was divided into two portions of approximately 5 μL each. The primers for each target were divided into two groups, and then a mixture including iQ Supermix (BioRad), ddH_2O, and one group of 10-fold diluted primers (see Table 2.1) 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 ºC

hot start for 3 minutes, followed by 15 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC for 25 seconds.

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 Figure 2.1), and iQ Supermix (BioRad). Each cell was run on a CFX96 qPCR machine (BioRad) according to the following protocol: 95 ºC hot start for three minutes, followed by 50 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC 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 2.3b). For TRPV1, we sequenced the amplification product to verify amplicon identity. Because CB1 lacks introns, 5 cells were tested to determine if the amplified PCR product came from expressed mRNA, rather than genomic DNA. These cells were extracted in the same manner as all other cells, except that the reaction mixture lacked reverse transcriptase. These cells were then multiplexed and evaluated by qPCR. Ct values from these cells were compared to the Ct values from all other tested interneurons using a T-test and were found to be significantly different from the other interneurons ($P < 0.07$), indicating that detected CB1 was indeed from expressed mRNA, rather than genomic DNA.

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 ΔCt value for each target. Any target with a ΔCt value greater than 20 was excluded from analysis as non-specific. To quantify mRNA expression levels, the ΔΔCt 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. 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 2.3) 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 2.3A 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 DAGLα, the enzymes responsible for synthesis of 12-HPETE, anandamide, and 2-arachidonylglycerol, respectively (see Figure 2.1). 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). DAGLα was examined in 20 of these 29 cells and was present in 10 cells (Table 2.2).

We next examined the distribution of these components within different interneuron subtypes (Figures 2.3-2.4 and Table 2.2). 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 DAGLα 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 2.3-2.4 and Table 2.3). Indeed, we identified that mGluR5 was coexpressed with NAPE-PLD, DAGLα, or both in all interneuron subtypes (see Figure 2.4a and Table 2.3) 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 2.3,2.4b and Table 2.3). We also noted mGluR1 and mGluR5 expressed together with endocannabinoid biosynthetic enzymes in CCK-CB cells (Figure 2.4a). 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 VGlut1and 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 DAGLα. We observed 12-LO and NAPE-PLD expression in CA3 pyramidal cells (Fig. 2.5a & 2.5b), but not in CA1 cells (Fig. 2.5c), while DAGLα expression was detected in both CA3 and CA1 pyramidal cells (Fig. 2.5a-2.5c). 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 2.6). 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 2.6b). 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. DAGLα 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 ± 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.

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 trilaminar or Schaeffer-collateral associated cells, which are present in stratum radiatum (Ferraguti et al., 2004, Boscia et al., 2008, Szilagyi 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).

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 DAGLα 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.

DAGLα, 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 DAGLα 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 DAGLα.

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 produced 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 DAGLα 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 DAGLα 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.

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-principle cells in the radiatum (Kerner et al., 1997, van Hooft et al., 2000, Ferraguti et al., 2004), where mGluR1 expression was identified in aspiny 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.

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, Toth 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.

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 DAGLα are expressed in CA3 pyramidal cells, while 12-LO and DAGLα 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.

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.

Acknowledgements

We acknowledge Andrew Martin for technical assistance. This work was supported by National Institute of Health Grant R15NS078645. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders and Stroke or the National Institutes of Health. This work was also supported by institutional Brigham Young University mentoring grants.

Table 2.1. Primer Sequences for qPCR.

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

Table 2.2. eCB mRNA Expression in Interneuron Subtypes.

Subtype	12-LO	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 2.3. eCB mRNA Coexpression in Interneuron Subtypes.

Subtype	mGluR5/12-LO	mGluR5/NAPE-PLD	mGluR5/DAGLα
CCK		X	X
CCK-CB		X	X
CB			X
CR			
	mGluR1/12-LO	**mGluR1/NAPE-PLD**	**mGluR1/DAGLα**
CCK			
CCK-CB	X	X	X
CB		X	
CR			

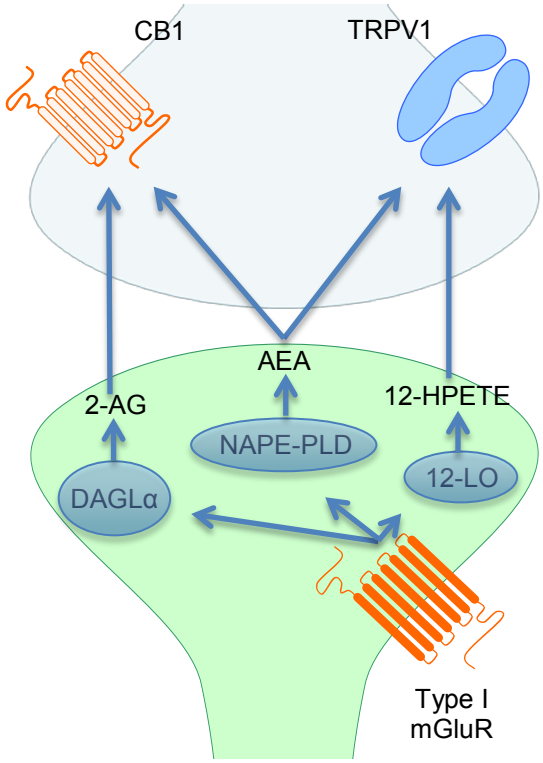

Figure 2.1. Endocannabinoid Biosynthetic Pathways and Receptor Targets. Postsynaptic type I metabotropic glutamate receptor activation commonly produces metabolites used in endocannabinoid and eicosanoid synthesis. Endocannabinoid biosynthetic enzymes such as diacylglycerol lipase α (DAGLα), N-acyl-phosphatidylethanolamine-specific 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).

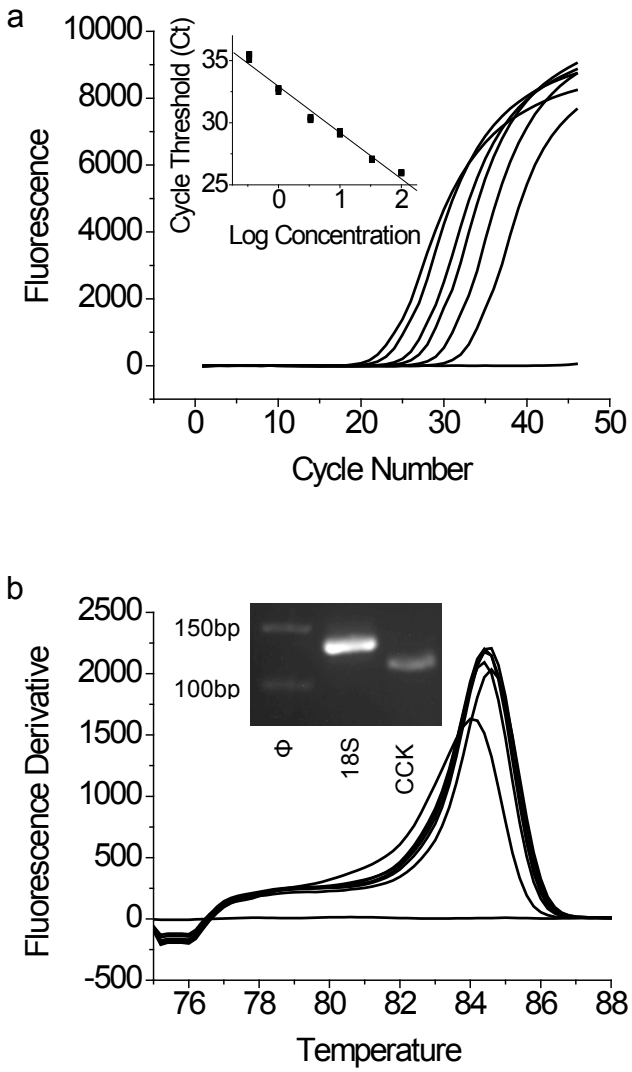

Figure 2.2. Optimization and Verification of RT-qPCR Primers and Probes. a) A dose-response set of fluorescent curves of the primer/probe set for CCK ranging from 100 ng to 0.3 ng cDNA. Inset: The linear fit of the dose response from (a) in log scale for CCK. Ct values for all triplicates from each concentration 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.

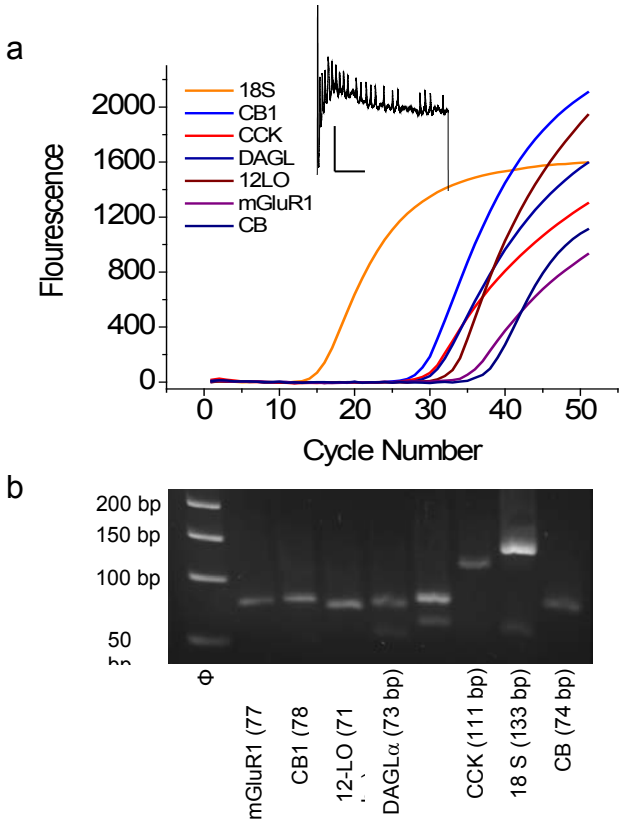

Figure 2.3. Identification of CA1 Stratum Radiatum Interneuron Subtypes by Their Expression of Endocannabinoid Biosynthetic Enzyme mRNA and Spiking Pattern. a) A representative CCK-CB cell expressing DAGLα (royal), 12-LO (wine), and mGluR1 (purple). Data are displayed as fluorescence from a FAM-TAMRA hydrolysis probe from a RT-qPCR reaction. Inset: A representative irregular spiking CA1 stratum radiatum interneuron. Membrane potential before stimulation 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, DAGLα, CCK, 18S, and CB, with their respective amplicon sizes.

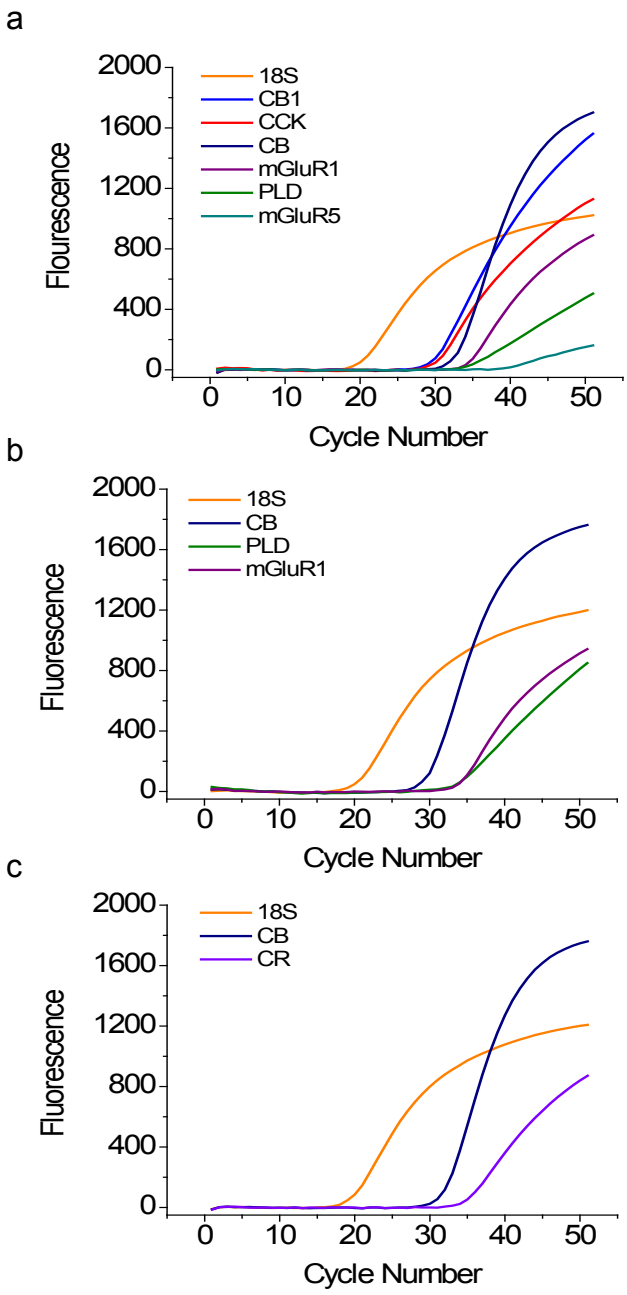

Figure 2.4. Endocannabinoid Biosynthetic Enzyme mRNA Expression in CA1 Stratum Radiatum Interneuron Subtypes. a) A representative CCK-CB cell demonstrates the presence of mGluR1 (purple), mGluR5 (dark cyan) and NAPE-PLD (olive). Note that the scaling of this figure did not allow for an accurate depiction 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.

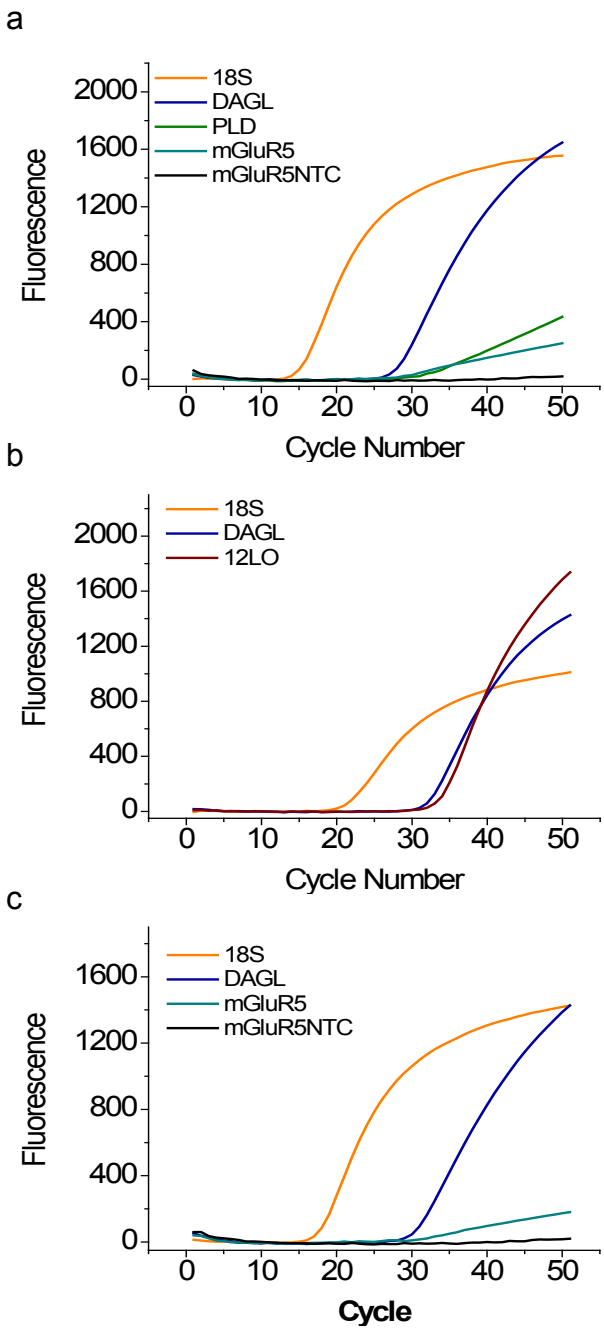

Figure 2.5. Expression of Endocannabinoid Biosynthetic Enzyme mRNA in Hippocampal Pyramidal Cells. a) A representative CA3 pyramidal cell demonstrates the presence of DAGLα (royal), NAPE-PLD (olive), and mGluR5 (dark cyan). Note the mGluR5 no-template control (mGluR5 NTC). b) A second CA3 pyramidal cell demonstrates the presence of DAGLα (royal) and12-LO (wine). c) A representative CA1 pyramidal cell demonstrates the presence of DAGLα (royal) and mGluR5 (dark cyan). Data are displayed as fluorescence from a RT-qPCR reaction.

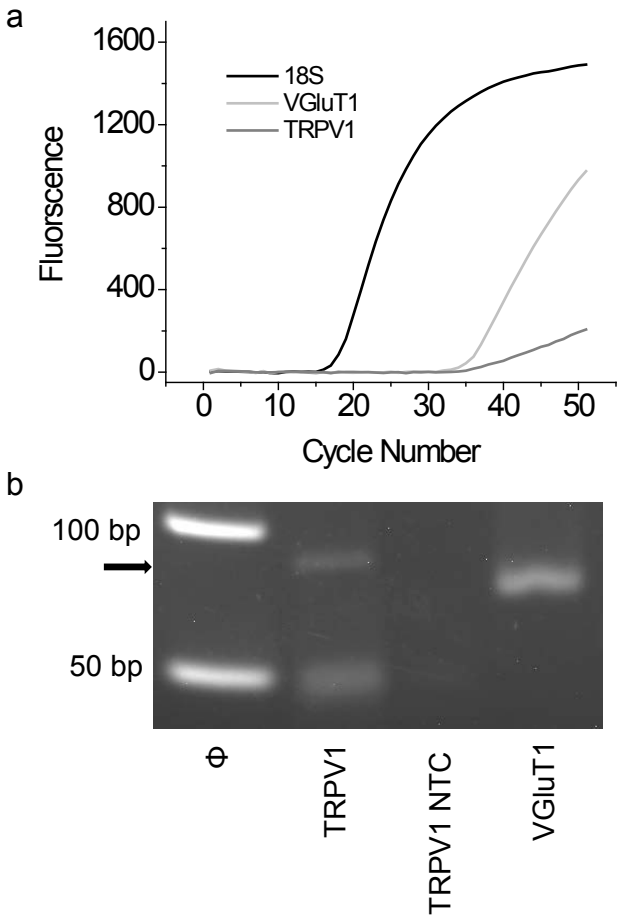

Figure 2.6. TRPV1 mRNA Expression in a CA3 Pyramidal Cell. a) A representative CA3 pyramidal cell expressing VGluT1 (light gray) and TRPV1 (dark gray). b) A 4% agarose gel of the cell in a), showing, from left to right, 50 bp ladder (50 bp and 100 bp shown), TRPV1 (86 bp, arrow), TRPV1 no-template control, and VGluT1 (74 bp).

CHAPTER 3: Ventral Tegmental Area Dopamine and GABA Neurons: Physiological Properties and Expression of mRNA for Endocannabinoid Biosynthetic Enzymes and Type I Metabotropic Glutamate Receptors

Collin B. Merrill, Lindsey N. Friend, Scott T. Newton, Zachary H. Hopkins,
Jeffrey G. Edwards

Abstract

The ventral tegmental area (VTA) is highly involved in adaptive reward and motivation processing in the brain and is composed of both dopaminergic and GABAergic neurons. In order to more completely understand not only reward, but also addictive mechanisms in the brain, an understanding of elements regulating the activity and synaptic plasticity of both of these cells is critical. However, some elements such as endocannabinoid production in the VTA are poorly understood. In this study DA and GABAergic cells were identified with a multipronged approach using electrophysiology, cell-specific markers, and a transgenic mouse model where GABA cells express green fluorescent protein. We examined physiological properties and generally observed slower firing frequencies and larger I_h potentials in DA neurons. Using single-cell RT-qPCR and immunohistochemistry, we probed for the presence of mRNA and protein for enzymes that form several endocannabinoids and eicosanoids such as diacylglycerol lipase α, N-acyl-phosphatidylethanolamine-specific phospholipase D, and 12-lipoxygenase. We also examined expression profiles of type I metabotropic glutamate receptors, which often activate pathways necessary for endocannabinoid production. Our results demonstrate endocannabinoid biosynthetic enzyme and type I mGluR mRNA and protein expression in both DAergic and GABAergic neurons of the VTA. Collectively, these data provide the first molecular evidence for the expression patterns of endocannabinoid biosynthetic enzyme and type I mGluR mRNA in VTA neurons and suggest that both GABAergic and DAergic cells have the potential to produce

various types of endocannabinoids. Therefore, the endocannabinoids implicated in adaptive motivational reward or addiction via modulating neuronal activity or mediating plasticity in the VTA could potentially be produced in both major cell types of the VTA.

Keywords: Parvalbumin, calretinin, calbindin, DAG lipase α, anandamide, NAPE-PLD, RT-PCR, firing frequency

Introduction

The mesocorticolimbic circuit is the reward-processing system in the brain. This reward system allows adaptation to an environment by attaching salience to novel rewarding stimuli and mediates feelings of pleasure. This system is of particular interest to examine as alterations in its activity by drugs of abuse can lead to addiction, a disorder in which natural reward systems are usurped by substances or behaviors.

The ventral tegmental area (VTA) is a key region in the mesocorticolimbic system implicated in adaptive reward and motivational processing, and is composed of two predominant cell types: Dopamine (DA) and GABA neurons. Reward stimuli are processed by increased VTA DA neuron activation causing DA release to their downstream target, primarily in the nucleus accumbens (NAc) (For review, see (Fields et al., 2007). DA release in the NAc is therefore an important aspect of reward induction and the resulting reward response (McBride et al., 1999, Ferrari et al., 2002, Nicola et al., 2005, Brown et al., 2012). Of similar importance, VTA GABAergic cells, which innervate and inhibit DA cells, can also modulate DA cell activity and DA release (Mathon et al., 2005b, van Zessen et al., 2012). For example, increased or decreased GABA signaling within the VTA, respectively, decreases and increases DA release in the NAc. Therefore, decreased GABA activity can disinhibit DA neurons and thus increase DA levels (Parker et al., 2011, Tan et al., 2012). In addition, VTA GABA projections into the NAc are involved in DAergic signaling, reward, and associative learning (Mathon et al., 2005a, Brown et al., 2012, van Zessen et al., 2012). This illustrates that GABA neurons as well as DA neurons are involved in reward processing and thus both cell types are likely critical for appropriate reward processing.

While DA release is critical for behavioral responses to reward, long-term synaptic changes, known as synaptic plasticity, are thought to be the cellular correlate mediating the addiction component of reward. As synaptic plasticity can modulate adaptive or learned reward responses, understanding the cellular expression of elements involved in known forms of synaptic plasticity in GABA and DA cells becomes critical. A necessary element in many forms of plasticity are N-methyl-D-aspartate (NMDA) receptors, which mediate NMDA receptor-dependent long-term potentiation (LTP) and long-term depression (LTD) (Luscher and Malenka, 2011, Madsen et al., 2012). However other signaling molecules such as endocannabinoids (eCBs) and eCB receptors mediate several forms of VTA plasticity including eCB-dependent LTD (Perra et al., 2005, Matyas et al., 2007, Matyas et al., 2008, Pan et al., 2008, Kortleven et al., 2011, Oleson et al., 2012). This eCB-dependent plasticity is most often mediated by retrograde activation of presynaptic eCB/vanilloid receptors such as cannabinoid receptor 1 (CB1) or transient receptor potential vanilloid 1 (TRPV1), following postsynaptic eCB production mediated by type I metabotropic glutamate receptors (mGluRs) (Maejima et al., 2001, Varma et al., 2001). However, the location of eCB synthesis in the VTA is poorly understood.

Electrophysiological data suggest eCBs involved in synaptic plasticity are produced within DAergic cells in the VTA (Perra et al., 2005, Pan et al., 2008, Kortleven et al., 2011), but there is no molecular evidence yet examining this. In addition, evidence of any kind is lacking regarding eCB production within GABA neurons. Production of eCBs by inhibitory neurons was considered in the past by many to be much less likely. However, recent data in the hippocampus illustrates GABAergic neurons do indeed express eCB biosynthetic enzyme mRNA (Merrill et al., 2012) and the eCBs produced by the GABA cells can directly modulate

synaptic plasticity of the interneuron (Gibson et al., 2008, Peterfi et al., 2012). Our goal was to determine which VTA neurons express eCB biosynthetic enzyme and type I mGluR mRNA, thus suggesting their potential ability to produce eCBs.

Using electrophysiology, gene expression, and immunocytochemistry, we characterized and examined VTA GABA and DA cells for the enzymes that produce endocannabinoids/eicosanoids, namely diacylglycerol lipase α (DAGLα), 12-lipoxygenase (12-LO), and N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), which respectively synthesize 2-arachidonylglycerol (2-AG), 12-(S)-hydroperoxyeicosa-5Z, 8Z, 10E, 14Z-tetraenoic acid (12-HPETE) and anandamide (AEA) (Di Marzo et al., 1994, Hwang et al., 2000, Ueda et al., 2005, Tanimura et al., 2010). 2-AG activates CB1(Sugiura et al., 1995), 12-HPETE activates TRPV1 (Huang et al., 2002), and AEA interact with both CB1 and TRPV1 (Berrendero et al., 1999, Smart et al., 2000). We also probed for the presence of mGluR1/5. Our data demonstrate that eCB biosynthetic enzymes and type I mGluRs are coexpressed in both VTA DA and GABA cells, suggesting eCB production can occur within either cell type. This suggests eCB-mediated mechanism such as synaptic plasticity within the mesocorticolimbic system could be induced by either cell type, which is important to consider as both LTP and LTD are often altered or occluded by drugs abuse in the VTA (Kauer, 2003, Saal et al., 2003, Borgland et al., 2004, Niehaus et al., 2010, Dacher and Nugent, 2011), which may underlie altered reward processing leading to addiction. Finally, as electrophysiological characteristics to distinguish VTA DA and GABA cells has been debated in the past, we positively identify GABA cells using a genetic GFP label of these cells and describe their firing frequencies and Ih potentials as compared to DA neurons.

Methods

Slice Preparation

All experiments were performed in accordance with Institutional Animal Care and Use Committee protocols and followed 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. Male Sprague-Dawley rats (16-28 days old) were used for RT-qPCR experiments and male GAD67-GFP (16-28 day) mice (Tamamaki et al., 2003) were used for RT-qPCR and immunohistochemistry experiments. All animals were anesthetized using isoflurane and decapitated using a rodent guillotine. The brain was rapidly removed, sectioned into 300 μm (mouse) or 400 μm (rat) thick horizontal slices, and stored for at least one hour submerged on a net in artificial cerebrospinal fluid containing (in mM) 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 11 glucose, saturated with 95% O_2/5% CO_2 (pH 7.4). Slices were then transferred to a submerged recording chamber and bathed in oxygenated artificial cerebrospinal fluid.

Electrophysiological Recordings and Extraction

Slices were continuously perfused with filtered artificial cerebrospinal fluid at a flow rate of 2-3 mL/min. Ventral tegmental area neurons were visually selected using infrared or fluorescence optics, CCD camera and monitor, with an Olympus BX51WI microscope with a 40x 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, 2 mM ATP-Na, 0.3 mM GTP-Na, and 0.6 nM EGTA-K (pH 7.28, 275-285 mOsm). Spiking patterns were acquired in whole cell current clamp configuration by injecting current in 50 pA steps from -200 pA to 600 pA for 1 sec. Electrophysiological data

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 10.3 Clampfit software (Molecular Devices).

After recordings, each cell was extracted from the slice with gentle suction. Once free of the slice, the entire cell was carefully aspirated into the pipette tip and transferred immediately into a chilled reverse transcription mixture and processed within two hours. To ensure cellular DNA or contaminating mRNA brought up along with the cell soma that was not from within the cell itself did not cause false-positive results, cytosol-only control samples were extracted by gentle suction under visual observation. The pipette was then carefully withdrawn and removed from the slice. The presence of cytosol was verified by observation of GFP in the pipette tip. An artificial cerebrospinal fluid control sample was extracted for every slice, where the electrode was first placed in the slice, removed and then artificial cerebrospinal fluid was aspirated just above the slice 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 positive results. All samples were processed in the same manner.

Reverse Transcription Reaction

The reverse transcription reaction was performed with iScript cDNA Synthesis Kit (BioRad), following the prescribed protocol, with a final reaction mixture of 12 µL. This mixture was then cycled in a C1000 Thermocycler (BioRad) under the following conditions: 25.0 ºC for 8 minutes, 42.0 ºC for 60 minutes, and 70 ºC for 15 minutes.

For primer optimization (see below for more details) a cDNA library was created by reverse transcription of mRNA from homogenized brain tissue. Homogenates were obtained

from rats and mice. 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.

Primer Design, Verification, and Optimization

All primers for selected cDNA of eCB biosynthetic enzymes, type I mGluRs, 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 (for rat primer/probe sequences, see (Merrill et al., 2012); for mouse primer/probe sequences, see Table 3.1). All primer sets were designed to cross an intron-exon boundary and amplify from exon to exon in order to avoid nuclear DNA amplification. For control purposes each primer was tested using a serial dilution series of cDNA from rat or mouse 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). 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/μL from whole brain homogenate.

Preamplification (Multiplex) Reaction

For rat and mouse neuron experiments, each cell was divided into three equal portions of approximately 4 μL each. Each primer was assigned into one of three groups, and then a mixture including iQ Supermix (BioRad), ddH_2O, and one group of 10-fold diluted primers was added to

each aliquot. All cells were tested using the same species-specific primer groups. All aliquots were then placed in a C1000 Thermocycler (BioRad) and processed as follows: 95 ºC hot start for 3 minutes, followed by 15 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC for 25 seconds.

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 and species, and iQ Supermix (BioRad). Each cell was run on a CFX96 qPCR machine (BioRad) according to the following protocol: 95 ºC hot start for three minutes, followed by 50 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC 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. Proper amplification of each cellular target was also examined using 4% agarose gel electrophoresis to verify amplicon size.

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 within 5 cycles of the cell Ct value, it was excluded from the cell analysis, on a target-by-target basis. However, some artificial cerebrospinal fluid samples displayed expression of most 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 ΔCt value for each target. Any target with a ΔCt value greater than 20 was excluded from analysis as non-

specific. To quantify mRNA expression levels, the ΔΔCt method (Livak and Schmittgen, 2001) was used. Expression data was obtained using CFX Manager software (BioRad). All expression levels were tested for significance ($p < 0.05$) using an unpaired two-way Student's T-test.

Immunohistochemistry

Mouse GAD67-GFP brains were either transcardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) or rapidly dissected and fixed overnight in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were cryoprotected in 30% sucrose solution, frozen in OCT, sliced into 30 μm sections and collected into 1M PBS for a free-floating staining procedure. Slices were permeablized with a 20 minute wash with 0.2% Triton-X (Fisher Bioreagents), washed with 1% bovine serum albumin in 1 M PBS for 2 hours, and treated with primary antibody for rabbit anti-NAPE-PLD (1:500, kindly provided by Dr. Ken Mackie), rabbit anti-DAGLα (1:1000, kindly provided by Dr. Ken Mackie), or rabbit anti-mGluR5 (1:500, Abcam) in 10% normal goat serum and 1% bovine serum albumin in PBS overnight at 10°C. Slices were then washed twice with 1 M PBS, followed by a wash of 0.2% Triton-X (Fisher Bioreagents) in 1 M PBS for 20 minutes, a wash of 1% bovine serum albumin and 5% normal goat serum in 1 M PBS for 2 hours, and a final wash of anti-sheep (1:1000, DAPI, Invitrogen) secondary antibody in 10% normal goat serum, 1% bovine serum albumin in PBS for 2 hours at room temperature. Slices were washed three times with tris-buffered saline and mounted onto Superfrost Plus microscope slides (VWR). After drying overnight, slides were coverslipped with Fluoromount G (Southern Biotech) and imaged on an Olympus FluoView FV1000 laser scanning confocal microscope. Image capture was performed by sequential excitation of each fluorophore to prevent any cross-excitation.

Following imaging, each 20X-magnified image was divided into nine equal sections for semi-quantitative analysis. Positively labeled GAD67-GFP, single immunolabeled, and double immunolabeled cells were visually identified, counted in each section, and totaled. Positive double-labeled cells are expressed as a percentage of total GAD67-GFP-labeled cells. Two individuals independently performed cell counting. Semi-quantitative percentages were compared to mRNA expression percentages from RT-qPCR experiments to compare mRNA expression and protein expression detected using immunocytochemistry.

Results

One primary goal was to identify the eCB biosynthetic enzyme and type I mGluR mRNA expression pattern in distinct VTA neurons and thus their capacity to produce eCBs. Because little is known about the eCB system within VTA GABAergic neurons and their role in adaptive reward processes, and because of the difficulty in positively identifying VTA GABA neurons in the past, we particularly focused on VTA GABA neurons. We extracted single cells from the VTA of rats and mice and analyzed gene expression using RT-qPCR. Cells were classified based on cellular markers. We confirmed DAergic cell identity as tyrosine hydroxylase (TH) or DA transporter (DAT) positive, and GABAergic cells as GAD65 or GAD67 positive. Of 75 neurons extracted from rat brain slices, 16 were identified as DAergic, 12 were identified as GABAergic, 20 were unable to be classified based on the cell marker genes we examined, and 27 were failures. To examine mouse VTA neurons we employed a genetic mouse line where GAD67-expressing cells display targeted knock-in of GFP. We extracted 30 cells including 12 GABAergic-GFP neurons and again used TH or DAT expression to identify DA cells. Using GAD67-GFP allows us to positively confirm a cell as GABAergic rather than rely solely on cellular markers. This further enabled us to compare mouse VTA GABA cells to rat VTA

GABA cells to verify mRNA expression patterns detected in rat GABA cells. Twenty GFP-positive cytosol-only samples were also extracted as a control to verify positive expression of mRNA rather than genomic DNA.

To further assist in identification and classification of rat VTA DAergic and GABAergic neurons identified using RT-qPCR, we obtained electrophysiological profiles of tested neurons to compare their spiking frequency, firing pattern, and sag potentials generated by I_h currents in response to hyperpolarizing current injection. In rat VTA, DAergic neurons fired usually in a regular pattern with a few adapting, and spiking frequencies from 17.7 to 29.6 Hz (25.0 Hz average) in response to one-second current injection. Upon returning to resting membrane potential DA neurons generally fired a regular train or a burst of approximately five action potentials (Figure 3.1e). Rat GABAergic neurons fired in a regular, irregular or adapting pattern, with firing frequencies from 12.2 to 54.2 Hz (31.9 Hz average), and generated either one to two, or no action potentials upon return to a resting state (see Figure 3.1e). In addition, DA neurons in general produced large sag potentials during hyperpolarization while sag potentials in GABA neurons were generally smaller, though there was some overlap.

To provide a positive control comparator for electrophysiological profiles of rat GABAergic cells, which are morphologically very difficult to distinguish from DA cells in brain slices, we examined physiological profiles in genetically-identified mouse GABAergic neurons. Electrophysiological parameters from mouse GAD67-GFP GABAergic cells were similar to those of neurons classified via RT-qPCR as GABAergic from rat VTA. Within GAD67-GFP positive cells, the average action potential frequency was 32.9 Hz and each cell generally fired either one to two, or no action potentials upon return to a resting state. In addition, these cells generally displayed very small or absent sag potentials during hyperpolarizing current injection

(Figure 3.1e). Collectively, these data provide support of rat GABA cell classification using electrophysiological parameters and RT-qPCR.

GABAergic neurons from many brain regions can often be categorized into subtypes based on coexpression of calcium binding proteins and neuropeptides, and in many cases, principal neurons and GABA neurons differ in calcium binding protein and neuropeptide expression as well. We therefore tested rat VTA neurons for the presence of parvalbumin, calbindin, calretinin, and CCK to attempt to identify VTA GABA neuron subtypes and to assist in establishing VTA neuron identity. We detected parvalbumin, calbindin, calretinin, and CCK expression (see Table 3.2) with a large degree of colocalization within identified rat GABAergic neurons. Only one cell displayed no calcium-binding protein or neuropeptide expression. In addition, almost all CCK-positive GABA neurons coexpressed both parvalbumin and calbindin (n=5, 83.3%). In DAergic cells, expression of parvalbumin, calbindin, calretinin expression was sparse, but CCK expression was frequently observed (see Table 3.2), though four cells did not display calcium binding protein or neuropeptide mRNA expression. These data demonstrate that multiple calcium binding proteins and CCK co-expression is prevalent in VTA GABA cells and calcium binding proteins expression occur more frequently in GABAergic neurons than DAergic neurons.

One main emphasis of this study was to examine the co-expression of eCB biosynthetic enzyme and type I mGluR mRNA within VTA neurons to determine their ability to produce eCBs. We therefore probed for eCB biosynthetic enzyme mRNA coding for DAGLα, NAPE-PLD, and 12LO, and for mGluR1/5 mRNA. Within identified rat DA and GABAergic cells, we detected expression to some degree of all of these (Figure 3.1a-b, Table 3.3). Indeed, the

expression of these between DA and GABA neurons is strikingly similar. Notably, NAPE-PLD expression occurred more frequently in DAergic neurons (Table 3.3).

To confirm these results, particularly in GABA cells, we examined expression of these targets in neurons genetically identified as GABAergic from GAD67-GFP mice. In 12 GABA neurons, we observed expression of all eCB enzymes and both type I mGluRs (Figure 3.1d, Table 3.3). These expression patterns were reasonably similar to our classified rat VTA GABAergic cells. Mouse non-GFP cells classified as DA neurons by TH or DAT also expressed all these targets, though at slightly lower percentages (Figure 3.1c). Cytosol-only control samples devoid of DNA also displayed expression of all three endocannabinoid biosynthetic enzymes and both type I mGluRs, verifying our rat and mouse whole-cell data.

Colocalization in both rat DA and GABA cells of eCB biosynthetic enzyme mRNA and type I mGluR mRNA was also noted (Figure 3.1, Table 3.3), with especially high co-expression of mGluR5 and DAGLα in GABA neurons (Figure 3.1a-b,Table 3.3). Within GAD67-GFP-positive mouse whole-cell samples, mGluR1/5 co-localized with these enzymes as well (Figure 3.1d, Table 3.3), consistent with expression patterns from rat VTA GABAergic neurons. Taken together, these data demonstrate that VTA DA and GABAergic neurons express the cellular machinery necessary for eCB synthesis.

Quantification of expression levels of eCB biosynthetic enzyme and type I mGluR mRNA within VTA neurons was also examined. Figure 3.2 summarizes some of the largest differences in expression levels between DA and GABA neurons. Within rat brain slices (3.2a), DAGLα mRNA levels were 254% higher in GABA neurons (n=7) than DA neurons (n=7), though this difference was not significant (p=0.34). Expression of mGluR1 mRNA levels in GABA neurons (n=4) were 32% of DA neuron expression (n=3), and mGluR5 expression in

GABA neurons (n=7) were 142% higher than DA neuron expression (n=5), though these differences were not significantly different (p=0.26 and p=0.60), respectively. In mouse VTA neurons (Figure 3.2b), mGluR1 expression in mouse GABA neurons (n=5) was 7% of DA neuron expression (n=3, p=0.05) and GABA neurons mGluR5 expression (n=5) was 6% of DA neuron expression (n=7, p=0.02).

To confirm that mRNA correlates to actual expression we probed for the presence of protein, particularly in GABA neurons. Immunohistochemical experiments were performed for DAGLα, NAPE-PLD, and mGluR5 in mouse horizontal VTA slices, with a focus on GAD67-positive neurons. Importantly, GAD67 never co-localized with DAT or TH in RT-qPCR experiments, and has not been reported to occur, suggesting that the GAD67-GFP positive neurons are likely all GABAergic. We observed positive immunolabeling of DAGLα, mGluR1, and mGluR5 within many GFP labeled cells. Semi-quantitative subjective analysis was performed to assess estimates of positive immunolabeling in VTA GABA neurons using low magnification images. NAPE-PLD (Figure 3.3a-f) co-localized with GAD67 in 56% of GFP-positive GABA neurons (n = 75) and DAGLα (Figure 3.3g-i) co-localized with GAD67 in 66% of GFP-positive GABA neurons (n = 68), with positive labeling occurring in the cytosol for both targets. mGluR5 (Figure 3.3j-l) was observed in 51% of GAD67-GFP-positive neurons (n = 70), with positive labeling appearing cytosolically within GABA cell bodies. Collectively, percentages of expression from immunohistochemistry were very similar to expression noted in qPCR experiments, albeit at higher protein expression levels compared to mRNA expression levels, which would be expected.

Discussion

Modulation of DA transmission by eCBs, especially via synaptic plasticity, plays a role in long-term disruptions to DA transmission associated with addiction (Melis et al., 2004b, Riegel and Lupica, 2004, Perra et al., 2005, Matyas et al., 2008, Pan et al., 2008, Kortleven et al., 2011, Oleson et al., 2012), but until now, the neuronal source of eCBs within the VTA was poorly understood. Additionally, GABAergic neurons have recently been demonstrated as critical to VTA function and DA transmission (Matyas et al., 2008, Nugent and Kauer, 2008, Niehaus et al., 2010, Dacher and Nugent, 2011, Michaeli and Yaka, 2011, van Zessen et al., 2012), and evidence for their role in eCB modulation of DA signaling is very sparse. Our data demonstrate that both VTA DAergic and GABAergic neurons co-express mRNA for enzymes and receptors involved in eCB signaling, suggesting that both these cell types can potentially play a role in eCB modulation of DA signaling via eCB-mediated activities such as synaptic plasticity.

VTA Neuron Identification

In vitro, there is no one reliable method other than positive genetic identification to determine neuron identity, so combining several methods such as physiology and gene expression is needed to characterize VTA neurons. Physiologically, DA neurons generally fire at lower frequencies and display large sag potentials in current-clamp mode during hyperpolarizing current injection (Koyama et al., 2005), while GABA neurons tend to fire at higher frequencies and display very small or absent sag potentials (Steffensen et al., 1998, Korotkova et al., 2003, Koyama et al., 2005, Margolis et al., 2006), though this is not always the case (Margolis et al., 2006). Our data demonstrating higher firing rates and very small or absent sag potentials in GABA neurons corroborates previous data, but in some cases, we observed

some GABA neurons with slower firing rates and large sag potentials--characteristics associated with DA neurons. We also observed some DA neurons with faster firing frequencies and small sag potentials, demonstrating some overlap in physiological properties. While these physiological properties in general hold true for VTA cells, they cannot be used exclusively in order to characterize VTA cells types.

Concerning gene expression, standard cellular markers such as DAT, TH and GAD65/67 sometimes overlap between cell types. Traditional DA cell markers such as TH were observed to co-localize with GAD65 and GABA (Gonzalez-Hernandez et al., 2001, Korotkova et al., 2003, Olson and Nestler, 2007), especially in GABA-releasing DA neurons that project to lateral habenula (Stamatakis et al., 2013). Our data demonstrated GAD65 co-expression with TH in exactly half of tested cells, but importantly, GAD67/TH co-expression was not detected. Previous studies that examined GAD65/67 expression did not observe co-localization of TH with GAD67 (Chieng et al., 2011), which corroborates our data and demonstrate that GAD67 is a good marker to identify VTA GABA neurons. This also provides evidence that the GAD67-GFP mouse model we employed is an appropriate model to specifically study VTA GABA cells. In addition, TH/DAT mRNA expression never co-localized with GAD67 mRNA expression in mouse GAD67-GFP positive neurons, which provides further verification of neuron identity by expression of these cell type markers. Collectively, the similarity in our data between rat GABA cells identified by PCR and genetically identified GABA cells in mouse support our classification methodology as being accurate.

Regarding RT-qPCR, it is important to note that when interpreting results, lack of detection does not necessarily indicate absence of the tested target. Therefore, data and analyses discussed herein may underestimate actual mRNA expression levels, which is common for RT-

qPCR experiments. For example, we classified 20 cells as unidentified due to lack of TH/DAT and GAD65/67 expression. These cells were positive for many eCB biosynthetic enzyme and type I mGluR mRNA expression, and were likely DA, GABA, or glutamatergic cells, where cell identity markers were false negatives. Potential false negatives also explain why mRNA expression levels were slightly lower compared to protein levels in GABA cells. In addition, performing single cell RT-qPCR on an entire cell maximizes the amount of mRNA recovered and avoids apparent false negatives. However, with combined RT-qPCR and immunohistochemical data, we are confident that the positive expression of detected mRNA products we report within VTA neurons is accurate.

Calcium Binding Protein mRNA Within VTA Neurons

In many brain areas such as the hippocampus, the presence of the neuropeptides and calcium binding proteins is used as a classification scheme for GABA interneuron subtypes (Ascoli et al., 2008). In VTA GABA neurons, previous studies have observed parvalbumin, calbindin, calretinin and CCK co-localization with GAD65/67 (Rajakumar et al., 1994, Tamamaki et al., 2003, Korotkova et al., 2004, Olson and Nestler, 2007), as well as CCK expression within VTA GABA neurons (Olson and Nestler, 2007). Our data was very similar in that there was a high degree of calcium-binding protein and CCK expression in GABA cells. Interestingly, three cells expressed calretinin with no colocalization of PV, CB, or CCK, which in the hippocampus and cortex is indicative of a GABA cell that innervates and inhibits other GABA cells (Wierenga et al., 2010). Collectively, our data demonstrate the co-expression of multiple calcium-binding proteins and neuropeptides within VTA GABA cells was common, unlike other brain regions where high degree of co-expression is less common. However, expression of these targets did not differentiate GABA cells into similar subtypes as has been

demonstrated in the hippocampus and cortex (Freund and Buzsáki, 1996, Ascoli et al., 2008). This suggests that either distinct GABAergic subtypes are not as common in the VTA or that these subtypes, if present, must be classified using other criteria such as axon innervation pattern or projection target.

Regarding DA cells, colocalization of TH with CCK, calbindin or calretinin is well-established (Hokfelt et al., 1980, Seroogy et al., 1989, Rogers, 1992, Isaacs and Jacobowitz, 1994, Koyama et al., 2005), as well as some colocalization of TH with both calbindin and calretinin (Nemoto et al., 1999). Importantly, parvalbumin expression has never been directly tested in VTA DA neurons. This study is the first to describe positive parvalbumin mRNA expression in some DA neurons.

While our data is supportive of prior data regarding calcium binding protein and neuropeptide mRNA expression patterns in GABAergic and DAergic neurons, we note a high degree of co-expression in GABA neurons compared to DA neurons. These data also suggest that calcium binding protein and neuropeptide expression alone are not sufficient to distinguish VTA principal and non-principal neurons.

Endocannabinoid Biosynthetic Enzyme mRNA Within VTA Neurons

DAGLα is expressed within VTA DA neurons and is involved in synaptic plasticity, which was observed electrophysiologically at excitatory synapses onto DA cells (Melis et al., 2004a, Melis et al., 2004b, Matyas et al., 2008, Kortleven et al., 2011). However, regarding VTA GABA neurons, molecular evidence for the expression of DAGLα was demonstrated only via in situ hybridization and electron microscopy techniques (Matyas et al., 2008). Our data represent the first description of DAGLα mRNA within VTA GABA neurons using RT-qPCR and immunohistochemistry. Identification of similar levels of DAGLα expression in both DA

and GABA cells suggests that 2-AG can be produced at multiple locations within the VTA and may allow modulation of DA transmission via 2-AG mediated plasticity at multiple synapses within the circuit.

NAPE-PLD produces anandamide, which activates multiple presynaptic eCB receptors such as CB1 or TRPV1. The effects of anandamide within the VTA have only been indirectly tested using CB1 or fatty acid amide hydrolase antagonists, and suggest anandamide is not involved in CB1-mediated synaptic plasticity at VTA inputs (Melis et al., 2004a, Melis et al., 2004b, Perra et al., 2005, Pillolla et al., 2007). However, some effects of nicotine and cocaine on medium spiny neurons of the NAc are blocked by NAPE-PLD antagonists (Luchicchi et al., 2010). The data presented here is the first data demonstrating expression of NAPE-PLD mRNA within DA cells by RT-qPCR, and the first description of NAPE-PLD mRNA or protein within VTA GABA neurons. Interestingly, NAPE-PLD expression and subsequent anandamide production is thought to perform an autoregulatory function in other brain areas (Bacci et al., 2004, Marinelli et al., 2009). This suggests that VTA neurons may have intrinsic autoregulatory processes, by which NAPE-PLD could be another mechanism of DA modulation within the VTA circuit.

Finally, 12LO activity is thought to play a role in the acute phase of addiction via alterations to glutamate activation of DA cells (Manzoni and Williams, 1999, Walters et al., 2003). Further, 12-HPETE produced by 12LO is involved in TRPV1-mediated synaptic plasticity (Gibson et al., 2008) and TRPV1 was observed to excite DA neurons (Marinelli et al., 2005). However, we detected 12LO at very low levels within tested DA and GABA neurons. Taken together, the mechanism of 12LO function within the VTA remains unclear and requires further investigation.

Type I mGluR mRNA Within VTA Neurons

Type I mGluRs are involved in eCB biosynthesis in many brain areas (Maejima et al., 2001, Varma et al., 2001). mGluR1 is expressed within the VTA (Testa et al., 1994) and is involved in several types of synaptic plasticity, particularly mGluR-LTD (Bellone and Luscher, 2005, 2006, Mameli et al., 2007). 2-AG is synthesized via an mGluR5-induced pathway (Jung et al., 2005). In addition, mGluR5 activation increases DA levels in NAc and prefrontal cortex (Romano et al., 1995, Chau et al., 2011, Tronci and Balfour, 2011) and is a key factor in cocaine sensitization (Chiamulera et al., 2001, Bird et al., 2010, Ghasemzadeh et al., 2011, Timmer and Steketee, 2012). Our data represent the first description of type I mGluR mRNA expression in VTA DA and GABA neurons by RT-qPCR and the first description of type I mGluR mRNA within VTA GABA neurons using immunohistochemistry. Higher levels of mGluR1 expression within rat and mouse DA neurons suggests that these cells may be more likely to induce mGluR-LTD compared to GABA cells. Curiously, expression levels of mGluR5 in DA and GABA neurons were the opposite in rats versus mice. While we don't have a rationale to explain this, higher mGluR5 expression in distinct neuron populations may suggest 2-AG synthesis may be more likely within that cell type. Collectively, these data provide additional evidence for the role of type I mGluRs in modulation of DA transmission in the VTA.

Conclusion

In summary, our data provide the first description of eCB biosynthetic enzyme localization and expression pattern within VTA DA and GABA neurons and demonstrate remarkable similarities in expression patterns between the two cell types. Similar type I mGluR mRNA expression in DA and GABA neurons provide further evidence that both cell types possess the cellular machinery necessary for eCB production. This expression pattern further

supports the potential for eCB biosynthesis at multiple locations within the VTA circuit, any of which could modulate DA signaling during adaptive reward and motivational processing. The localization of these targets provides a better understanding of the role of eCB modulation of VTA DA signaling, whose perturbation may be a key factor in addiction.

Acknowledgements

The authors would like to thank Dr. Ken Mackie for the generous gift of anti-NAPE-PLD and anti-DAGLα antibodies used in this study. We also thank Scott Steffensen for his careful review of this manuscript. National Institute of Health Grant R15NS078645 supported this work. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders and Stroke or the National Institutes of Health. This work was also supported by institutional Brigham Young University mentoring grants.

Table 3.1. Mouse Primer and Probe Sequences.

Target	Direction	Primer Sequence	Probe Sequence
18S	Forward	GTGCATGGCCGTTCTTAGTTG	TGGAGCGATTTGTCTGGTTAATTCCGATAAC
133 bp	Reverse	GCCACTTGTCCCTCTAAGAAGTTG	
mGluR1	Forward	ATATCGTCAAGCGGTACAACTG	TGCAGTCCACACAGAAGGGAATTACGG
102 bp	Reverse	GGCAGCCAACTCTTTGAAAG	
mGluR5	Forward	CTGCACACCTTGTAAGGAGAATG	TACACCTGCAAGGCGTGCCAACTG
103 bp	Reverse	CAAATCACAACCTGTCAAGTCG	
DAGLα	Forward	AGAAGAAGTTGGAGCAGGAGATG	ACCTGGGCCGTGGAACCAAACACTA
100 bp	Reverse	AAGGAGTGGCCTACCACAATC	
NAPE-PLD	Forward	CTGGACTGCATCCTCAAACG	AGCTAGCCCTCGGGATCAACAGCG
114 bp	Reverse	CAACGTCCGCTTGCTGTAC	
12LO	Forward	GCCAAGAGAAGCAGCAAGATG	AAGACTCGCTCTCAGATGCCCTACAAAGTG
100 bp	Reverse	CATCCTCAGTCCCAGAAAAGTG	
GAD67	Forward	ATCATGGCTGCTCGTTACAAGTAC	CATGGCGGCTGTGCCCAAACT
100 bp	Reverse	AATAGTGACTGTGTTCTGAGGTGAAG	
TH	Forward	GGACAAGCTCAGGAACTA	TCTCGTATCCAGCGCCCATTCTC
67 bp	Reverse	GGTGTACGGGTCAAACTTC	
DAT	Forward	AACCTGTACTGGCGGCTATG	CCCCTGCTTCCTTCTGTATGTGGTCG
87 bp	Reverse	GGGTCTGAAGGTCACAATGC	

Table 3.2. Calcium-binding Protein and Neuropeptide Expression in Rat Ventral Tegmental Area Neurons.

Calcium-binding protein	DA neuron (n=16)	GABA neurons (n=12)
Parvalbumin (PV)	3	7
Calbindin (CB)	2	8
Calretinin (CR)	1	7
CCK	7	6
Calcium-binding protein and neuropeptide mRNA coexpression in rat ventral tegmental area neurons		
PV, CB, CR, CCK	0	3
PV, CB, CCK	0	2
PV, CB	1	0
CB, CCK	1	1
CR only	1	3

Table 3.3. eCB Biosynthetic Enzyme and Type I mGluR mRNA Expression in Rat and Mouse VTA Neurons.

Cell type	DAGLα	NAPE-PLD	12LO	mGluR1	mGluR5
Rat DA (n=16)	7	6	2	3	5
Rat GABA (n=12)	7	2	2	4	7
Mouse GABA (n=12)	5	1	4	5	5
eCB biosynthetic enzyme and type I mGluR mRNA coexpression in VTA Neurons					
	mGluR1/DAGLα	**mGluR1/NAPE-PLD**	**mGluR1/12LO**		
Rat DA	X	X			
Rat GABA	X	X	X		
Mouse GABA	X	X	X		
	mGluR5/DAGLα	mGluR5/NAPE-PLD	mGluR5/12LO		
Rat DA	X	X	X		
Rat GABA	X	X	X		
Mouse GABA	X	X	X		

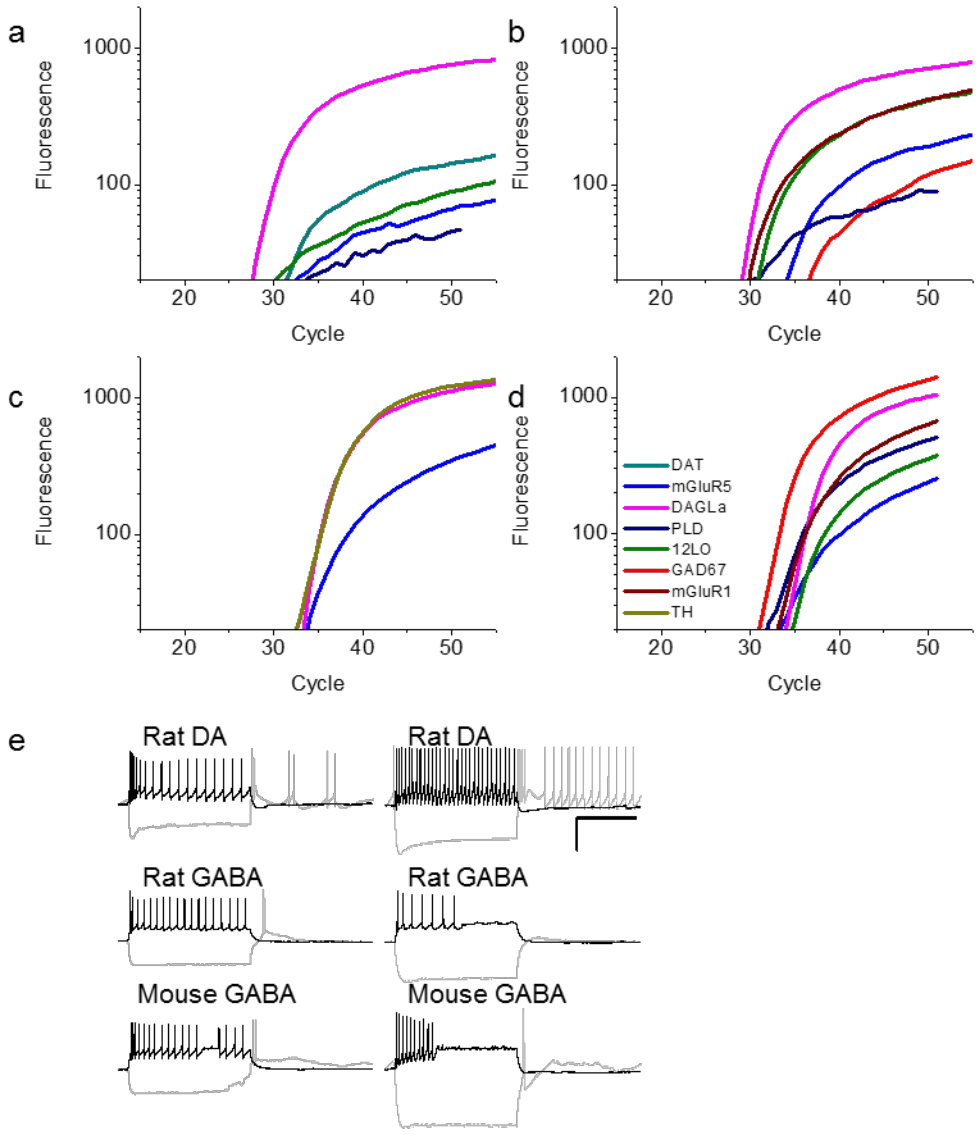

Figure 3.1. Gene Expression and Electrophysiological Profiles of Ventral Tegmental Area Dopaminergic and GABAergic Neurons. a) A representative rat DAergic cell expressing DAGLα, 12LO, DAT, mGluR5, and NAPE-PLD. b) A representative rat GABAergic neuron expressing DAGLα, mGluR1, 12LO, NAPE-PLD, mGluR5, and GAD67. c). A representative mouse DAergic neuron expressing TH, DAGLα, and mGluR5. d) A representative mouse GABAergic neuron expressing GAD67, NAPE-PLD, mGluR1, mGluR5, DAGLα, and 12LO. Fluorescence was generated by FAM-TAMRA probes and graphed relative to the number of PCR cycles performed. e) Representative electrophysiological profiles of VTA dopaminergic and GABAergic neurons.

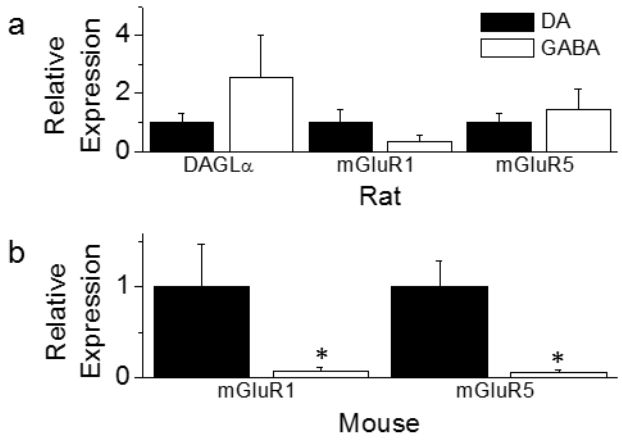

Figure 3.2. Relative mRNA Expression of mGluR1/5 and DAGLα in Ventral Tegmental Area Neurons. a) Relative mRNA levels of DAGLα, mGluR1, and mGluR5 in rat ventral tegmental area neurons. b) Relative mRNA levels of mGluR1 and mGluR5 in mouse ventral tegmental area neurons. Asterisks indicate a significant difference between DA and GABA neurons ($p<0.05$). Error bars represent SEM. Expression data was normalized to DA neurons in all quantification analyses.

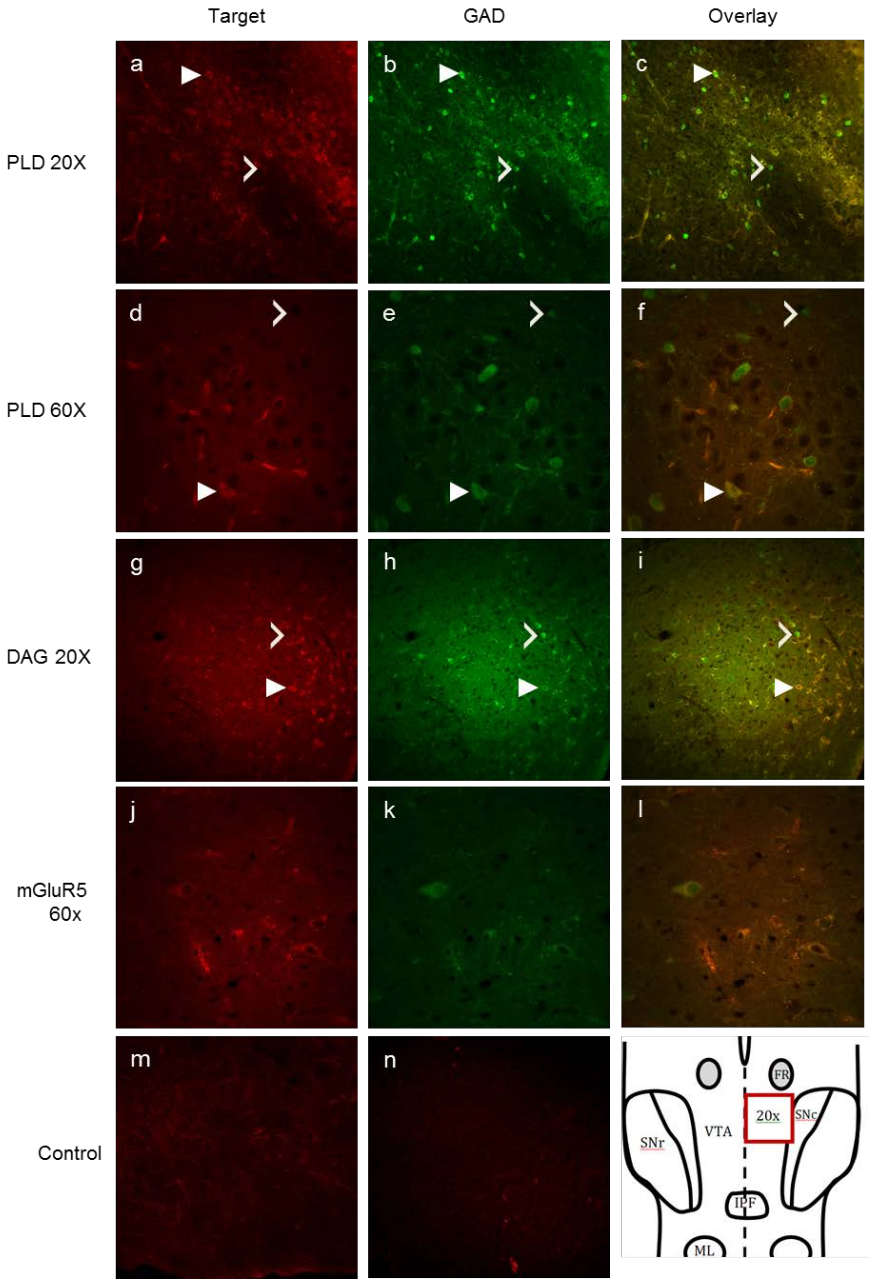

Figure 3.3. Immunolabeling of Endocannabinoid Biosynthetic Enzymes and Type I mGluRs Within Mouse Ventral Tegmental Area. a-c) NAPE-PLD immunoreactivity within GAD67-GFP positive neurons at 20X magnification. d-f) NAPE-PLD immunoreactivity within GABAergic neurons at 60X magnification. g-i) DAGLα immunoreactivity within GABAergic neurons at 20X magnification. j-l) mGluR5 immunoreactivity within GABAergic neurons at 60X magnification. m) Control image showing secondary antibody staining only. n) Control image with preabsorption using a blocking peptide for DAGLα. o) A schematic diagram of VTA showing area imaged in immunohistochemical experiments. Open arrows represent GFP-positive GABA neurons that are negative for the protein of interest. Arrowheads identify positive immunolabeling of each target in GFP-positive GABA neurons.

CHAPTER 4: Subtype-specific Synaptic Plasticity Within Hippocampal CA1 Stratum Radiatum Interneurons is Influenced by eCB Biosynthetic Enzyme mRNA Expression

C.B. Merrill, L.N. Friend, Z.H. Hopkins, J.G. Edwards

Abstract

Hippocampal CA1 stratum radiatum interneurons are a heterogeneous population of inhibitory cells that modulate pyramidal cell activity. The occurrence of synaptic plasticity is well documented within hippocampal interneurons, but synaptic plasticity within specific subtypes is not well understood. In addition, we recently observed that endocannabinoid (eCB) biosynthetic enzyme mRNA is expressed in a subtype-specific manner within hippocampal CA1 stratum radiatum interneurons. These data suggest that synaptic plasticity in stratum radiatum interneurons may also be subtype-specific, and may be related to eCB biosynthetic enzyme mRNA expression. Our goal was to elucidate the relationship between interneuron subtype and synaptic plasticity and the expression of eCB biosynthetic enzyme mRNA expression. We therefore tested individual CA1 stratum radiatum interneurons for the occurrence of synaptic plasticity and extracted tested interneurons and probed for the presence of calcium binding proteins and the neuropeptide CCK via RT-qPCR. We also examined eCB biosynthetic enzyme mRNA for diacylglycerol lipase α, N-acyl-phosphatidylethanolamine-specific phospholipase D, and 12-lipoxygenase. We observed differences in synaptic plasticity among CCK and CCK-CB basket cells and CB-positive cells, which also correlate to differences in eCB biosynthetic enzyme mRNA expression. These data suggest that synaptic plasticity is not equal among all hippocampal interneurons, and may be linked to the eCBs that are produced within a given interneuron. These results demonstrate the importance of interneuron subtypes and eCB signaling during modulation of hippocampal activity.

Introduction

Learning and short-term memory processing occurs in the hippocampus, part of the medial temporal lobe of the brain. These processes are thought to be mediated by synaptic plasticity, which are defined as alterations to neurotransmission. Increased synaptic activity strengthens the synaptic connection, termed long-term potentiation (LTP) (Bliss and Lomo, 1973), while decreased activity weakens the synapse, termed long-term depression (LTD) (Dudek and Bear, 1992). Both LTP and LTD occur at hippocampal synapses involving excitatory pyramidal cells and inhibitory interneurons.

LTP and LTD can be modulated by endocannabinoids (eCBs) (Feinmark et al., 2003, Abush and Akirav, 2010, Oudin et al., 2011, Alger, 2012), lipophilic molecules derived from plasma membrane phospholipids. In contrast to traditional neurotransmission, eCB signaling often occurs in a retrograde manner to modulate neurotransmission. Three prevalent eCBs are 2-arachidonylglycerol (2-AG), anandamide, and 12-(S)-hydroperoxyeicosa-5Z, 8Z, 10E, 14Z-tetraenoic acid (12-HPETE), which are synthesized by diacylglycerol lipase α (DAGLα) (Tanimura et al., 2010), N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Ueda et al., 2005), and 12-lipoxygenase (12LO), respectively (Hwang et al., 2000). 2-AG activates cannabinoid receptor 1 (CB1) (Ludanyi et al., 2011), 12-HPETE activates transient receptor potential vanilloid 1 (TRPV1) (Hwang et al., 2000), and anandamide activates both receptors (Smart et al., 2000, De Petrocellis and Di Marzo, 2005).

eCB-mediated plasticity is common within the hippocampus, occurring at several synapses involved in the hippocampal circuit (Heifets and Castillo, 2009, Abush and Akirav, 2010, Peterfi et al., 2012). One example is eCB-mediated interneuron LTD that occurs at the synapse of CA3 pyramidal cells and CA1 stratum radiatum interneurons. In this type of LTD,

high frequency stimulation causes activation of postsynaptic type I metabotropic glutamate receptors (mGluRs), which leads to 12-HPETE synthesis by 12LO. 12-HPETE then activates presynaptic TRPV1 and decreases neurotransmitter release (Gibson et al., 2008).

Interneurons of the stratum radiatum display remarkable heterogeneity. Electrophysiological, gene expression, and morphological experiments demonstrate many interneuron subtypes, each with their own function in regulation of hippocampal pyramidal cell activity. Within CA1 stratum radiatum, at least four important subtypes can be distinguished based on expression of the calcium binding proteins calbindin (CB), calretinin (CR), and the neuropeptide cholecystokinin (CCK). These subtypes are CCK-CB basket cells, CCK-positive basket cells, CB-positive bistratified cells, and CR-positive interneuron selective cells (Freund and Buzsáki, 1996, Gulyas et al., 1996, Parra et al., 1998, Ascoli et al., 2008, Wierenga et al., 2010). Importantly, each interneuron subtype displays distinct innervation of downstream targets, with basket cells innervating pyramidal cell somata (Bartos and Elgueta, 2012) and CB-positive cells innervating pyramidal cell dendrites (Freund and Buzsáki, 1996). We recently observed that eCB biosynthetic enzymes and type I mGluR mRNA is differentially expressed among these four interneuron subtypes (Merrill et al., 2012), suggesting that the potential for eCB-mediated synaptic plasticity may differ among these subtypes as well.

Our goal was to demonstrate the relationship between hippocampal CA1 stratum radiatum interneuron subtypes and synaptic plasticity, and the relationship between synaptic plasticity and eCB biosynthetic enzyme and type I mGluR mRNA expression. While the occurrence of eCB-mediated synaptic plasticity within the hippocampus is well documented, there is little evidence for the involvement of specific interneuron subtypes in different forms of synaptic plasticity. Our data suggest that synaptic plasticity within stratum radiatum

interneurons varies among cells, and appears to be more dependent on eCB biosynthetic enzyme and type I mGluR mRNA expression. In particular, cells displaying short-term depression (STD) express eCB biosynthetic enzymes, while cells displaying LTD coexpress these targets and type I mGluRs. These data provide further evidence for varying degrees of synaptic plasticity within stratum radiatum interneurons, which may be important in activity-dependent modulation of pyramidal cell activity by individual interneuron subtypes.

Methods

Slice Preparation

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. Male Sprague-Dawley rats (16-28 days old) were used for RT-qPCR experiments. All animals were anesthetized using isoflurane and decapitated using a rodent guillotine. The brain was rapidly removed, sectioned into 400 μm thick horizontal slices, and stored for at least one hour submerged on a net in artificial cerebrospinal fluid containing (in mM) 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 11 glucose, saturated with 95% O_2/5% CO_2 (pH 7.4). Slices were then transferred to a submerged recording chamber and bathed in oxygenated artificial cerebrospinal fluid.

Electrophysiological Recordings and Extraction

Slices were continuously perfused with filtered artificial cerebrospinal fluid containing 4 μM picrotoxin to block GABA transmission at a flow rate of 2-3 mL/min. CA1 stratum radiatum interneurons were visually selected using infrared optics, CCD camera and monitor, with an Olympus BX51WI microscope with a 40x 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, 2 nM ATP-Na, 0.03 nM GTP-Na, and 0.6 nM EGTA-K (pH 7.28, 275-285 mOsm). Spiking patterns were acquired in whole cell current clamp configuration by injecting current in 50 pA steps from -200 pA to 600 pA for 1 sec. Following the spiking protocol, EPSCs were stimulated at 0.1 Hz (100 μsec) using a bipolar stainless steel stimulating electrode placed in stratum radiatum at least 200 μm from the recorded cell. CA1 interneurons were voltage clamped at −65 mV, and EPSCs were evoked by paired 100 μsec pulses with an interval of separated by 50 msec (stimulation intensity ~100-300 μA). Following a 10-minute baseline recording, two 1 second trains at 100 Hz with a 20 second inter-train interval, at 150% current intensity were delivered to induce synaptic plasticity. During all experiments, cell input resistance and series resistance were monitored; if these values changed by more than 10% during the experiment, that cell was excluded from further electrophysiological and RT-qPCR analysis. 15 cells were excluded due to excessive changes to series resistance. Electrophysiological data 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 10.3 Clampfit software (Molecular Devices). Following plasticity experiments, each cell was extracted from the slice with gentle suction. Once free of the slice, the entire cell was carefully aspirated into the pipette tip and transferred immediately into a chilled reverse transcription mixture and processed within two hours. 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 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 positive results.

Reverse Transcription Reaction

The reverse transcription reaction was performed with iScript cDNA Synthesis Kit (BioRad), following the prescribed protocol, with a final reaction mixture of 12 μL. This mixture was then cycled in a C1000 Thermocycler (BioRad) under the following conditions: 25.0 ºC for 8 minutes, 42.0 ºC for 60 minutes, and 70 ºC for 15 minutes.

For primer optimization (see below for more details) a cDNA library was created by reverse transcription of mRNA from homogenized rat whole 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.

Primer Design, Verification, and Optimization

All primers for selected cDNA of eCB biosynthetic enzymes, type I mGluRs, 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 (for rat primer/probe sequences, see Merrill et al., 2012). All primer sets were designed to cross an intron boundary and amplify from exon to exon in order to avoid nuclear DNA amplification. 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). 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/μL from whole brain homogenate.

Preamplification (Multiplex) Reaction

For RT-qPCR experiments, each cell was divided into three equal portions of approximately 4 μL each. Each primer was assigned into one of three groups, and then a mixture including iQ Supermix (BioRad), ddH_2O, and one group of 10-fold diluted primers was added to each aliquot. All aliquots were then placed in a C1000 Thermocycler (BioRad) and processed as follows: 95 ºC hot start for 3 minutes, followed by 15 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC for 25 seconds.

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, and iQ Supermix (BioRad). Each cell was run on a CFX96 qPCR machine (BioRad) according to the following protocol: 95 ºC hot start for three minutes, followed by 50 cycles of 95 ºC for 15 seconds, 57 ºC for 20 seconds, and 72 ºC 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.

Results

Our goal was to elucidate the relationship between hippocampal CA1 stratum radiatum interneuron subtypes and synaptic plasticity. We evaluated synaptic plasticity by measuring evoked EPSC amplitude, followed by extraction of tested interneurons for RT-qPCR analysis. High-frequency stimulation was used to induce synaptic plasticity in stratum radiatum interneurons using whole-cell voltage clamp. LTD was identified by decreased EPSC amplitude following high-frequency stimulation that did not return to baseline values. Short-term depression (STD) was identified by a decrease in EPSC amplitude that returned to baseline values 3-5 minutes after high frequency stimulus. Short-term potentiation was identified by increased EPSC amplitude that returned to baseline values 3-5 minutes after high frequency stimulus. No synaptic plasticity was identified as having no change in EPSC amplitude following high-frequency stimulation. Tested cells displayed a range of plasticity, with cells displaying STD and LTD, and in some cases, the absence of any evoked plasticity. Each cell was then tested for interneuron subtype by examining interneuron cell type markers parvalbumin, CB, CR, and the neuropeptide CCK. 10 cells were assigned to an interneuron subtype based on calcium-binding protein and neuropeptide mRNA expression, 2 cells were classified as unknown due to lack of detection of subtype markers, and 3 were classified as failures, due to lack of detection of gene expression. The 10 remaining cells were classified as CCK-positive (n=5), CCK-CB positive (n=4), or CB positive (n=1) and tested for eCB biosynthetic enzyme and type I mGluR mRNA.

To further assist in and support subtype identification, we examined physiological profiles of identified cells. CCK-positive cells displayed an average firing frequency of 47.2 Hz, CCK-CB positive cells fired with an average frequency of 37.2 Hz, and the single CB-positive

cell fired at 27.2 Hz. Further, CCK-positive cells fired in regular or irregular patterns, while CCK-CB cells fired in a burst, regular, or strongly adapting pattern. The CB-positive cell fired in a regular pattern. While spiking profiles varied within subtypes and displayed a degree of overlap between subtypes, general spiking patterns support our subtype classification.

We next examined the synaptic plasticity observed within each interneuron subtype. In tested cells, 5 cells displayed STD, 8 displayed LTD, one displayed short-term potentiation, and one did not display any plasticity (See Table 4.1). CCK-positive neurons generally underwent STD (Figure 4.1b,c), with one cell displaying LTD and one displaying no plasticity. CCK-CB interneurons tended to undergo LTD (Figure 4.2b,c) or STD, with one cell displaying short-term potentiation. The CB-positive neuron underwent LTD (See Table 4.1).

Next, because synaptic plasticity can be mediated by eCBs, we examined the relationship between synaptic plasticity and eCB biosynthetic enzyme mRNA expression. Following EPSC analysis, we tested cells displaying synaptic plasticity for the expression of eCB biosynthetic enzyme mRNA for DAGLα, NAPE-PLD, and 12LO (See Table 4.1). Within cells displaying STD, we detected either DAGLα, NAPE-PLD, or 12LO. Cells that underwent LTD also displayed either DAGLα, NAPE-PLD, or 12LO, and in most cases, coexpressed mGluR5. The cell that displayed short-term potentiation expressed only NAPE-PLD and the cell that displayed no plasticity expressed only 12LO.

Finally, we examined the relationship between eCB biosynthetic enzymes and synaptic plasticity by interneuron subtype. Neurons that displayed STD tended to be CCK-positive and expressed either DAGLα, NAPE-PLD, or 12LO, but not mGluR1/5 (Figure 4.1). Cells that underwent LTD were usually CCK-CB positive and coexpressed mRNA for at least one eCB biosynthetic enzyme and mGluR5 (Figure 4.2), though there was some overlap between

interneuron subtype, synaptic plasticity, and eCB biosynthetic enzyme mRNA expression. These results suggest that synaptic plasticity in stratum radiatum interneurons may be dependent on the coexpression of eCB biosynthetic enzyme and type I mGluR mRNA within individual cells, rather than interneuron subtype.

Discussion

Synaptic plasticity is an important modulator of neurotransmission in the brain, and is involved in processes such as learning and memory in the hippocampus. eCBs underlie many types of synaptic plasticity, with TRPV1-mediated long-term depression of hippocampal CA1 stratum radiatum interneurons (Gibson et al., 2008) being particularly relevant to this study. Recently, we described the cellular localization of eCB biosynthetic enzyme and type I mGluR mRNA within radiatum interneurons (Merrill et al., 2012), demonstrating that the expression of these enzymes is subtype specific. However, the correlation between interneuron subtype and the potential for synaptic plasticity remains unclear. Our goal was to describe the relationship between stratum radiatum interneuron subtype and the occurrence of synaptic plasticity and eCB biosynthetic enzyme mRNA expression.

The use of RT-qPCR to evaluate gene expression provides many advantages, such as the ability to quantify mRNA expression and to determine mRNA expression levels between samples. When evaluating RT-qPCR data, it is important to note that lack of detection does not necessarily equate to lack of expression. Therefore, the expression patterns that we describe herein may be lower than actual expression. For example, in this study, mGluR1 mRNA was not detected in tested cells. However, mGluR1 expression occurs within CA1 stratum radiatum interneurons, particularly within CCK-positive neurons (Romano et al., 1995, Lujan et al., 1996, Huber et al., 2001, Ferraguti et al., 2004, Merrill et al., 2012). The lack of mGluR1 expression in

this study is likely due to lack of detection, rather than absence in hippocampal interneurons. In spite of this, we are confident in the description of gene expression and conclusions we present in this study.

Hippocampal interneurons represent a very heterogeneous population of inhibitory cells, whose function is to modulate pyramidal cell activity and therefore information flow through the hippocampal circuit. Many different subtypes within CA1 stratum radiatum have been identified, including basket, bistratified, and interneuron-selective (interneurons that innervate other interneurons). Stratum radiatum basket cells can be further subdivided into CCK-positive and CCK-CB positive populations, while bistratified cells can be identified as CB-positive and interneuron-selective cells as being CR-positive (Kosaka et al., 1985, Freund and Buzsáki, 1996, Gulyas et al., 1996, Jinno and Kosaka, 2002, 2006, Ascoli et al., 2008, Wierenga et al., 2010). In this study, CCK and CCK-CB basket cells, as well as CB-positive bistratified cells were tested, though the cells classified as failures or of unknown subtype could be other interneuron subtypes, such as CR-positive interneuron-selective or Schaeffer collateral-associated cells. Action potential pattern was traditionally used to identify interneuron subtypes, but due to overlap in electrophysiological profiles both between and among subtypes, it can be difficult to employ (Ascoli et al., 2008, Wierenga et al., 2010). However, our action potential data demonstrating basket cells as fast spiking, with a regular or irregular pattern and CB-positive bistratified cells as slower, regular spiking is in accordance with previous observations (Buhl et al., 1996, Merrill et al., 2012).

In addition to heterogeneity in morphology, physiology, and gene expression, stratum radiatum interneurons also display key differences in innervation targets. In particular, basket cells tend to innervate target somata (Bartos and Elgueta, 2012), while CB-positive basket cells

innervate pyramidal cell dendrites (Freund and Buzsáki, 1996). Because of this distinct innervation pattern, these subtypes have different effects on pyramidal cell activity, with basket cells having a greater effect in modulating their postsynaptic targets (Freund and Katona, 2007, Tukker et al., 2007). Importantly, the synaptic plasticity expressed by these cells has not been well elucidated. A recent study examined plasticity within parvalbumin and cannabinoid receptor 1 expressing basket cells and demonstrated that the plasticity within these basket cell populations is subtype specific, with parvalbumin-positive basket cells displaying long-term plasticity and cannabinoid receptor 1 positive cells displaying short-term or no plasticity (Nissen et al., 2010). However, these cells were not evaluated for CCK or CB expression, though cannabinoid receptor 1 positive cells were classified as either basket or non-basket cells. CCK-positive cells also express cannabinoid receptor 1 (Katona et al., 1999), so our data demonstrating STD in CCK-positive basket cells corroborates previous data. However, our data demonstrating LTD in CCK-CB positive and CB-positive cells suggests that subtype-specific synaptic plasticity is also present in other cell types. Collectively, these data demonstrate that distinct interneuron subtypes in stratum radiatum display distinct synaptic plasticity, which may differentially modulate pyramidal cell activity during learning and memory processing.

The eCBs 2-AG, anandamide, and 12-HPETE play a key role in hippocampal function, especially during processes involving synaptic plasticity (Feinmark et al., 2003, Katona et al., 2006, Cristino et al., 2008, Egertová et al., 2008, Chavez et al., 2010, Ludanyi et al., 2011, Peterfi et al., 2012). However, in studies of eCB-mediated LTD, all interneurons tested did not respond equally to high-frequency stimulation (McMahon and Kauer, 1997, Gibson et al., 2008, Nissen et al., 2010). Our data demonstrate that interneuron subtypes can undergo eCB-mediated

synaptic plasticity that is somewhat subtype-specific, but overlap in evoked plasticity between subtypes suggests that the type of plasticity may be dependent on factors other than subtype.

In many cases, eCB synthesis requires type I mGluR activation (Maejima et al., 2001, Varma et al., 2001, Feinmark et al., 2003), and type I mGluRs are expressed in a cell-type specific fashion (Romano et al., 1995, Kerner et al., 1997, Shigemoto et al., 1997, van Hooft et al., 2000, Merrill et al., 2012). These data suggest that synaptic plasticity mediated by activation of type I mGluRs, followed by subsequent eCB production, may also be interneuron subtype-specific. Indeed, our data demonstrates that many cells exhibiting LTD express both eCB biosynthetic enzyme and type I mGluR mRNA, whereas cells exhibiting STD express only eCB biosynthetic enzyme mRNA. Type I mGluRs were suggested to initiate eCB production via phospholipase activity (Piomelli, 2003, Jung et al., 2005), suggesting pathways that activate these cellular cascades may also activate eCB production. In addition, postsynaptically produced eCBs can diffuse laterally, activating adjacent synapses in a heterosynaptic fashion (Chevaleyre and Castillo, 2003). This may explain the difference between the plasticity observed in our experiments and the differential type I mGluR expression. Long-lasting synaptic changes may be type I mGluR-dependent, whereas short-term plasticity may be induced via heterosynaptic eCB transmission.

In summary, our data elucidate the relationship between hippocampal stratum radiatum interneuron subtype and synaptic plasticity, demonstrating that the induction of synaptic plasticity within interneurons is somewhat subtype-specific. Further, our data provide evidence linking long-term synaptic plasticity to coexpression of eCB biosynthetic enzymes and type I mGluRs, whereas short-term synaptic changes may be mGluR-independent. Collectively, these

data demonstrate the potential of distinct interneuron populations to differentially modulate learning and memory processing within the hippocampus.

Acknowledgements

This work was supported by National Institute of Health Grant R15NS078645. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders and Stroke or the National Institutes of Health. This work was also supported by institutional Brigham Young University mentoring grants.

Table 4.1. Relationship Between Subtype, Synaptic Plasticity, and eCB Biosynthetic Enzyme and Type I mGluR Expression in Stratum Radiatum Interneurons.

Cell type	Plasticity	DAGLα	NAPE-PLD	12LO	mGluR5
CCK	STD	**X**	**X**	**X**	
	LTD	**X**			**X**
	None			**X**	
CCK-CB	STD			**X**	
	LTD			**X**	**X**
	STP	**X**		**X**	
CB	LTD		**X**	**X**	**X**

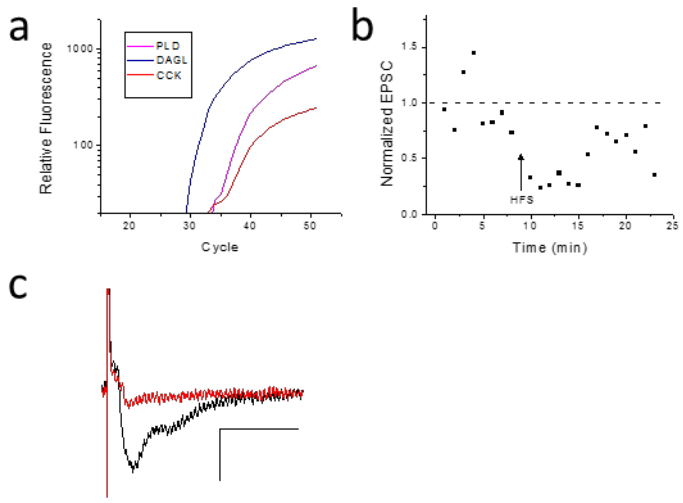

Figure 4.1. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-positive Basket Cell. A CCK-positive basket cell demonstrating a) fluorescence data from a RT-qPCR reaction, showing expression of DAGLα (navy), NAPE-PLD (magenta), and CCK (red); b) short-term depression following high-frequency stimulation; and c) evoked EPSCs before (black) and after (red) high frequency stimulation, showing EPSC depression.

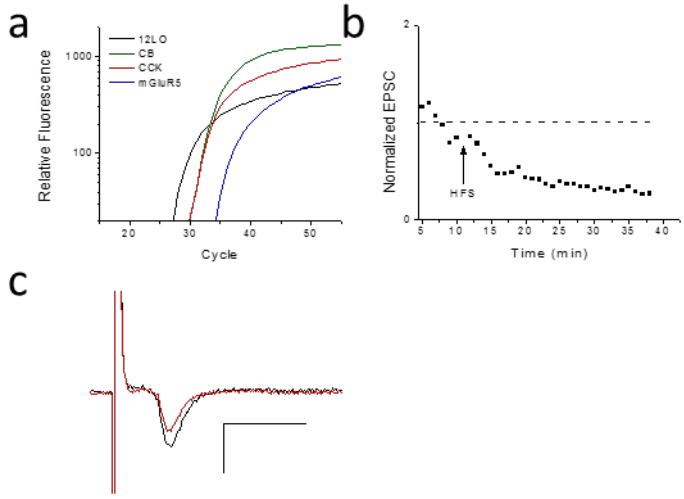

Figure 4.2. Characterization of a Hippocampal CA1 Stratum Radiatum CCK-CB Positive Basket Cell. A CCK-CB positive basket cell demonstrating a) fluorescence data from a RT-qPCR reaction, showing expression of 12LO (black), CCK (red), CB (olive), and mGluR5 (blue); b) long-term depression following high-frequency stimulation; and c) evoked EPSCs before (black) and after (red) high frequency stimulation, showing long-term EPSC depression.

CHAPTER 5: Conclusion

eCB signaling is an important modulator of neurotransmission in the brain. The projects described herein demonstrate the localization of eCB biosynthetic enzyme mRNA within hippocampal interneurons and VTA DAergic and GABAergic neurons, as well as the relationship between stratum radiatum interneuron subtype, synaptic plasticity, and eCB biosynthetic enzyme mRNA expression.

Collectively, these data provide evidence for the importance of eCB biosynthetic enzyme and type I mGluR mRNA expression within inhibitory cells of the hippocampus and VTA. Because eCB signaling has the potential to modulate neurotransmission, understanding the localization of these enzymes within hippocampal interneurons provides insight into learning and memory, processes that are disrupted during pathological states such as dementia and Alzheimer's disease. The expression pattern of eCB biosynthetic enzyme and type I mGluR mRNA within ventral tegmental area neurons provides evidence for eCB modulation of pleasure and adaptive reward processing, which may be an underlying factor in the development of addiction. Finally, understanding the normal function of these eCB signaling systems and their role during pathological states such as Alzheimer's disease, dementia, or addiction, could potentially provide additional therapeutic targets to treat these debilitating diseases.

REFERENCES

Abush H, Akirav I (2010) Cannabinoids modulate hippocampal memory and plasticity. Hippocampus 20:1126-1138.

Alger BE (2012) Endocannabinoids at the synapse a decade after the Dies Mirabilis (29 March 2001): what we still do not know. J Physiol.

Alger BE, Pitler TA (1995) Retrograde signaling at GABAA-receptor synapses in the mammalian CNS. Trends in Neurosciences 18:333-340.

Ascoli GA, Alonso-Nanclares L, Anderson SA, Barrionuevo G, Benavides-Piccione R, Burkhalter A, Buzsaki G, Cauli B, Defelipe J, Fairen A, Feldmeyer D, Fishell G, Fregnac Y, Freund TF, Gardner D, Gardner EP, Goldberg JH, Helmstaedter M, Hestrin S, Karube F, Kisvarday ZF, Lambolez B, Lewis DA, Marin O, Markram H, Munoz A, Packer A, Petersen CC, Rockland KS, Rossier J, Rudy B, Somogyi P, Staiger JF, Tamas G, Thomson AM, Toledo-Rodriguez M, Wang Y, West DC, Yuste R (2008) Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. Nature Reviews Neuroscience 9:557-568.

Bacci A, Huguenard JR, Prince DA (2004) Long-lasting self-inhibition of neocortical interneurons mediated by endocannabinoids. Nature 431:312-316.

Bartos M, Elgueta C (2012) Functional characteristics of parvalbumin- and cholecystokinin-expressing basket cells. J Physiol 590:669-681.

Beierlein M, Regehr WG (2006) Local interneurons regulate synaptic strength by retrograde release of endocannabinoids. J Neurosci 26:9935-9943.

Bellone C, Luscher C (2005) mGluRs induce a long-term depression in the ventral tegmental area that involves a switch of the subunit composition of AMPA receptors. Eur J Neurosci 21:1280-1288.

Bellone C, Luscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. Nat Neurosci 9:636-641.

Bennion D, Jensen T, Walther C, Hamblin J, Wallmann A, Couch J, Blickenstaff J, Castle M, Dean L, Beckstead S, Merrill C, Muir C, St Pierre T, Williams B, Daniel S, Edwards JG (2011) Transient receptor potential vanilloid 1 agonists modulate hippocampal CA1 LTP via the GABAergic system. Neuropharmacology 61:730-738.

Berrendero F, Sepe N, Ramos JA, Marzo VD, Fernández-Ruiz JJ (1999) Analysis of cannabinoid receptor binding and mRNA expression and endogenous cannabinoid contents in the developing rat brain during late gestation and early postnatal period. Synapse 33:181-191.

Bird MK, Reid CA, Chen F, Tan HO, Petrou S, Lawrence AJ (2010) Cocaine-mediated synaptic potentiation is absent in VTA neurons from mGlu5-deficient mice. The international

journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 13:133-141.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. The Journal of Physiology 232:331-356.

Borgland SL, Malenka RC, Bonci A (2004) Acute and chronic cocaine-induced potentiation of synaptic strength in the ventral tegmental area: electrophysiological and behavioral correlates in individual rats. J Neurosci 24:7482-7490.

Boscia F, Ferraguti F, Moroni F, Annunziato L, Pellegrini-Giampietro DE (2008) mGlu1α receptors are co-expressed with CB1 receptors in a subset of interneurons in the CA1 region of organotypic hippocampal slice cultures and adult rat brain. Neuropharmacology 55:428-439.

Brown MT, Tan KR, O'Connor EC, Nikonenko I, Muller D, Luscher C (2012) Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning. Nature 492:452-456.

Buhl EH, Szilagyi T, Halasy K, Somogyi P (1996) Physiological properties of anatomically identified basket and bistratified cells in the CA1 area of the rat hippocampus in vitro. Hippocampus 6:294-305.

Caterina M, Schumacher M, Tominaga M, Rosen T, Levine J, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816 - 824.

Cavanaugh DJ, Chesler AT, Jackson AC, Sigal YM, Yamanaka H, Grant R, O'Donnell D, Nicoll RA, Shah NM, Julius D, Basbaum AI (2011) Trpv1 reporter mice reveal highly restricted brain distribution and functional expression in arteriolar smooth muscle cells. The Journal of neuroscience 31:5067-5077.

Chau P, Soderpalm B, Ericson M (2011) The mGluR5 antagonist MPEP elevates accumbal dopamine and glycine levels; interaction with strychnine-sensitive glycine receptors. Addict Biol 16:591-599.

Chavez AE, Chiu CQ, Castillo PE (2010) TRPV1 activation by endogenous anandamide triggers postsynaptic long-term depression in dentate gyrus. Nature Neuroscience 13:1511-1518.

Chevaleyre V, Castillo PE (2003) Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. Neuron 38:461-472.

Chiamulera C, Epping-Jordan MP, Zocchi A, Marcon C, Cottiny C, Tacconi S, Corsi M, Orzi F, Conquet F (2001) Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice. Nat Neurosci 4:873-874.

Chieng B, Azriel Y, Mohammadi S, Christie MJ (2011) Distinct cellular properties of identified dopaminergic and GABAergic neurons in the mouse ventral tegmental area. J Physiol 589:3775-3787.

Chuang SC, Zhao W, Young SR, Conquet F, Bianchi R, Wong RK (2002) Activation of group I mGluRs elicits different responses in murine CA1 and CA3 pyramidal cells. J Physiol 541:113-121.

Cristino L, de Petrocellis L, Pryce G, Baker D, Guglielmotti V, Di Marzo V (2006) Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. Neuroscience 139:1405-1415.

Cristino L, Starowicz K, De Petrocellis L, Morishita J, Ueda N, Guglielmotti V, Di Marzo V (2008) Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain. Neuroscience 151:955-968.

Dacher M, Nugent FS (2011) Morphine-induced modulation of LTD at GABAergic synapses in the ventral tegmental area. Neuropharmacology 61:1166-1171.

De Petrocellis L, Di Marzo V (2005) Lipids as regulators of the activity of transient receptor potential type V1 (TRPV1) channels. Life Sciences 77:1651-1666.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz J-C, Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686-691.

Dudek SM, Bear MF (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proceedings of the National Academy of Sciences of the United States of America 89:4363-4367.

Edwards DA, Kim J, Alger BE (2006) Multiple mechanisms of endocannabinoid response initiation in hippocampus. J Neurophysiol 95:67-75.

Egertová M, Simon GM, Cravatt BF, Elphick MR (2008) Localization of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression in mouse brain: A new perspective on *N*-acylethanolamines as neural signaling molecules. The Journal of Comparative Neurology 506:604-615.

Feinmark SJ, Begum R, Tsvetkov E, Goussakov I, Funk CD, Siegelbaum SA, Bolshakov VY (2003) 12-Lipoxygenase Metabolites of Arachidonic Acid Mediate Metabotropic Glutamate Receptor-Dependent Long-Term Depression at Hippocampal CA3-CA1 Synapses. J Neurosci 23:11427-11435.

Ferraguti F, Cobden P, Pollard M, Cope D, Shigemoto R, Watanabe M, Somogyi P (2004) Immunolocalization of metabotropic glutamate receptor 1alpha (mGluR1alpha) in distinct classes of interneuron in the CA1 region of the rat hippocampus. Hippocampus 14:193-215.

Ferrari R, Le Novere N, Picciotto MR, Changeux JP, Zoli M (2002) Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. Eur J Neurosci 15:1810-1818.

Fields HL, Hjelmstad GO, Margolis EB, Nicola SM (2007) Ventral Tegmental Area Neurons in Learned Appetitive Behavior and Positive Reinforcement. Annual Review of Neuroscience 30:289-316.

Freund TF, Buzsáki G (1996) Interneurons of the hippocampus. Hippocampus 6:347-470.

Freund TF, Katona I (2007) Perisomatic inhibition. Neuron 56:33-42.

Fuentealba P, Begum R, Capogna M, Jinno S, Márton LF, Csicsvari J, Thomson A, Somogyi P, Klausberger T (2008) Ivy Cells: A Population of Nitric-Oxide-Producing, Slow-Spiking GABAergic Neurons and Their Involvement in Hippocampal Network Activity. Neuron 57:917-929.

Galarreta M, Erdelyi F, Szabo G, Hestrin S (2004) Electrical coupling among irregular-spiking GABAergic interneurons expressing cannabinoid receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:9770-9778.

Gee CE, Benquet P, Gerber U (2003) Group I metabotropic glutamate receptors activate a calcium-sensitive transient receptor potential-like conductance in rat hippocampus. The Journal of Physiology 546:655-664.

Ghasemzadeh MB, Vasudevan P, Giles C, Purgianto A, Seubert C, Mantsch JR (2011) Glutamatergic plasticity in medial prefrontal cortex and ventral tegmental area following extended-access cocaine self-administration. Brain Res 1413:60-71.

Gibson HE, Edwards JG, Page RS, Van Hook MJ, Kauer JA (2008) TRPV1 Channels Mediate Long-Term Depression at Synapses on Hippocampal Interneurons. Neuron 57:746-759.

Gonzalez-Hernandez T, Barroso-Chinea P, Acevedo A, Salido E, Rodriguez M (2001) Colocalization of tyrosine hydroxylase and GAD65 mRNA in mesostriatal neurons. Eur J Neurosci 13:57-67.

Gulyas AI, Hajos N, Freund TF (1996) Interneurons Containing Calretinin Are Specialized to Control Other Interneurons in the Rat Hippocampus. J Neurosci 16:3397-3411.

Heifets BD, Castillo PE (2009) Endocannabinoid signaling and long-term synaptic plasticity. Annu Rev Physiol 71:283-306.

Hill EL, Gallopin T, Ferezou I, Cauli B, Rossier J, Schweitzer P, Lambolez B (2007) Functional CB1 Receptors Are Broadly Expressed in Neocortical GABAergic and Glutamatergic Neurons. J Neurophysiol 97:2580-2589.

Hoffman AF, Riegel AC, Lupica CR (2003) Functional localization of cannabinoid receptors and endogenous cannabinoid production in distinct neuron populations of the hippocampus. European Journal of Neuroscience 18:524-534.

Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, Markey K (1980) Evidence for coexistence of dopamine and CCK in meso-limbic neurones. Nature 285:476-478.

Holderith N, Nemeth B, Papp OI, Veres JM, Nagy GA, Hajos N (2011) Cannabinoids attenuate hippocampal gamma oscillations by suppressing excitatory synaptic input onto CA3 pyramidal neurons and fast spiking basket cells. J Physiol 589:4921-4934.

Huang S, Bisogno T, Trevisani M, Al-Hayani A, De Petrocellis L, Fezza F, Tognetto M, Petros T, Krey J, Chu C, Miller J, Davies S, Geppetti P, Walker J, Di Marzo V (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. Proc Natl Acad Sci U S A 99:8400 - 8405.

Huber KM, Roder JC, Bear MF (2001) Chemical Induction of mGluR5- and Protein Synthesis-Dependent Long-Term Depression in Hippocampal Area CA1. J Neurophysiol 86:321-325.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D, Oh U (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc Natl Acad Sci U S A 97:6155-6160.

Isaacs KR, Jacobowitz DM (1994) Mapping of the colocalization of calretinin and tyrosine hydroxylase in the rat substantia nigra and ventral tegmental area. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 99:34-42.

Jinno S, Kosaka T (2002) Patterns of expression of calcium binding proteins and neuronal nitric oxide synthase in different populations of hippocampal GABAergic neurons in mice. The Journal of Comparative Neurology 449:1-25.

Jinno S, Kosaka T (2006) Cellular architecture of the mouse hippocampus: a quantitative aspect of chemically defined GABAergic neurons with stereology. Neuroscience research 56:229-245.

Jung KM, Mangieri R, Stapleton C, Kim J, Fegley D, Wallace M, Mackie K, Piomelli D (2005) Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. Mol Pharmacol 68:1196-1202.

Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF (1999) Presynaptically Located CB1 Cannabinoid Receptors Regulate GABA Release from Axon Terminals of Specific Hippocampal Interneurons. J Neurosci 19:4544-4558.

Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. The Journal of neuroscience 26:5628-5637.

Kauer JA (2003) Addictive drugs and stress trigger a common change at VTA synapses. Neuron 37:549-550.

Kawaguchi Y, Wilson CJ, Augood SJ, Emson PC (1995) Striatal interneurones: chemical, physiological and morphological characterization. Trends in Neurosciences 18:527-535.

Kerner JA, Standaert DG, Penney JB, Jr., Young AB, Landwehrmeyer GB (1997) Expression of group one metabotropic glutamate receptor subunit mRNAs in neurochemically identified neurons in the rat neostriatum, neocortex, and hippocampus. Molecular brain research 48:259-269.

Klausberger T (2009) GABAergic interneurons targeting dendrites of pyramidal cells in the CA1 area of the hippocampus. European Journal of Neuroscience 30:947-957.

Kofalvi A, Oliveira C, Cunha R (2006) Lack of evidence for functional TRPV(1) vanilloid receptors in rat hippocampal nerve terminals. Neurosci Lett.

Korotkova TM, Ponomarenko AA, Brown RE, Haas HL (2004) Functional diversity of ventral midbrain dopamine and GABAergic neurons. Molecular neurobiology 29:243-259.

Korotkova TM, Sergeeva OA, Eriksson KS, Haas HL, Brown RE (2003) Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. J Neurosci 23:7-11.

Kortleven C, Fasano C, Thibault D, Lacaille JC, Trudeau LE (2011) The endocannabinoid 2-arachidonoylglycerol inhibits long-term potentiation of glutamatergic synapses onto ventral tegmental area dopamine neurons in mice. Eur J Neurosci 33:1751-1760.

Kosaka T, Kosaka K, Tateishi K, Hamaoka Y, Yanaihara N, Wu JY, Hama K (1985) GABAergic neurons containing CCK-8-like and/or VIP-like immunoreactivities in the rat hippocampus and dentate gyrus. The Journal of Comparative Neurology 239:420-430.

Koyama S, Kanemitsu Y, Weight FF (2005) Spontaneous activity and properties of two types of principal neurons from the ventral tegmental area of rat. J Neurophysiol 93:3282-3293.

Le Duigou C, Holden T, Kullmann DM (2011) Short- and long-term depression at glutamatergic synapses on hippocampal interneurons by group I mGluR activation. Neuropharmacology 60:748-756.

Liu J, Wang L, Harvey-White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan AC, Zhou Z, Huang BX, Kim H-Y, Kunos G (2006) A biosynthetic pathway for anandamide. Proceedings of the National Academy of Sciences 103:13345-13350.

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods 25:402-408.

Luchicchi A, Lecca S, Carta S, Pillolla G, Muntoni AL, Yasar S, Goldberg SR, Pistis M (2010) Effects of fatty acid amide hydrolase inhibition on neuronal responses to nicotine,

cocaine and morphine in the nucleus accumbens shell and ventral tegmental area: involvement of PPAR-alpha nuclear receptors. Addict Biol 15:277-288.

Ludanyi A, Hu SS, Yamazaki M, Tanimura A, Piomelli D, Watanabe M, Kano M, Sakimura K, Magloczky Z, Mackie K, Freund TF, Katona I (2011) Complementary synaptic distribution of enzymes responsible for synthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in the human hippocampus. Neuroscience 174:50-63.

Lujan R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 8:1488-1500.

Luscher C, Malenka RC (2011) Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. Neuron 69:650-663.

Madsen HB, Brown RM, Lawrence AJ (2012) Neuroplasticity in addiction: cellular and transcriptional perspectives. Frontiers in molecular neuroscience 5:99.

Maejima T, Hashimoto K, Yoshida T, Aiba A, Kano M (2001) Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron 31:463-475.

Mameli M, Balland B, Lujan R, Luscher C (2007) Rapid synthesis and synaptic insertion of GluR2 for mGluR-LTD in the ventral tegmental area. Science 317:530-533.

Manzoni OJ, Williams JT (1999) Presynaptic regulation of glutamate release in the ventral tegmental area during morphine withdrawal. J Neurosci 19:6629-6636.

Margolis EB, Lock H, Hjelmstad GO, Fields HL (2006) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? The Journal of Physiology 577:907-924.

Marinelli S, Pacioni S, Cannich A, Marsicano G, Bacci A (2009) Self-modulation of neocortical pyramidal neurons by endocannabinoids. Nat Neurosci 12:1488-1490.

Marinelli S, Pascucci T, Bernardi G, Puglisi-Allegra S, Mercuri N (2005) Activation of TRPV1 in the VTA excites dopaminergic neurons and increases chemical- and noxious-induced dopamine release in the nucleus accumbens. Neuropsychopharmacology 30:864 - 870.

Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. European Journal of Neuroscience 11:4213-4225.

Mathon DS, Lesscher HM, Gerrits MA, Kamal A, Pintar JE, Schuller AG, Spruijt BM, Burbach JP, Smidt MP, van Ree JM, Ramakers GM (2005a) Increased gabaergic input to ventral tegmental area dopaminergic neurons associated with decreased cocaine reinforcement in mu-opioid receptor knockout mice. Neuroscience 130:359-367.

Mathon DS, Ramakers GM, Pintar JE, Marinelli M (2005b) Decreased firing frequency of midbrain dopamine neurons in mice lacking mu opioid receptors. Eur J Neurosci 21:2883-2886.

Matyas F, Urban GM, Watanabe M, Mackie K, Zimmer A, Freund TF, Katona I (2008) Identification of the sites of 2-arachidonoylglycerol synthesis and action imply retrograde endocannabinoid signaling at both GABAergic and glutamatergic synapses in the ventral tegmental area. Neuropharmacology 54:95-107.

Matyas F, Watanabe M, Mackie K, Katona I, Freund TF (2007) Molecular architecture of the cannabinoid signaling system in the core of the nucleus accumbens. Ideggyogy Sz 60:187-191.

McBride WJ, Murphy JM, Ikemoto S (1999) Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behavioural brain research 101:129-152.

McMahon LL, Kauer JA (1997) Hippocampal Interneurons Express a Novel Form of Synaptic Plasticity. Neuron 18:295-305.

Melis M, Perra S, Muntoni AL, Pillolla G, Lutz B, Marsicano G, Di Marzo V, Gessa GL, Pistis M (2004a) Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. J Neurosci 24:10707-10715.

Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL (2004b) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. J Neurosci 24:53-62.

Merrill CB, McNeil M, Williamson RC, Poole BR, Nelson B, Sudweeks S, Edwards JG (2012) Identification of mRNA for endocannabinoid biosynthetic enzymes within hippocampal pyramidal cells and CA1 stratum radiatum interneuron subtypes using quantitative real-time polymerase chain reaction. Neuroscience 218:89-99.

Michaeli A, Yaka R (2011) Dopamine-related drugs act presynaptically to potentiate GABA(A) receptor currents in VTA dopamine neurons. Neuropharmacology 61:234-244.

Morishita J, Okamoto Y, Tsuboi K, Ueno M, Sakamoto H, Maekawa N, Ueda N (2005) Regional distribution and age-dependent expression of *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D in rat brain. Journal of neurochemistry 94:753-762.

Nemoto C, Hida T, Arai R (1999) Calretinin and calbindin-D28k in dopaminergic neurons of the rat midbrain: a triple-labeling immunohistochemical study. Brain Res 846:129-136.

Nicola SM, Taha SA, Kim SW, Fields HL (2005) Nucleus accumbens dopamine release is necessary and sufficient to promote the behavioral response to reward-predictive cues. Neuroscience 135:1025-1033.

Niehaus JL, Murali M, Kauer JA (2010) Drugs of abuse and stress impair LTP at inhibitory synapses in the ventral tegmental area. Eur J Neurosci 32:108-117.

Nishiyama M, Watanabe T, Ueda N, Tsukamoto H, Watanabe K (1993) Arachidonate 12-lipoxygenase is localized in neurons, glial cells, and endothelial cells of the canine brain. Journal of Histochemistry & Cytochemistry 41:111-117.

Nissen W, Szabo A, Somogyi J, Somogyi P, Lamsa KP (2010) Cell Type-Specific Long-Term Plasticity at Glutamatergic Synapses onto Hippocampal Interneurons Expressing either Parvalbumin or CB1 Cannabinoid Receptor. J Neurosci 30:1337-1347.

Nugent FS, Kauer JA (2008) LTP of GABAergic synapses in the ventral tegmental area and beyond. J Physiol 586:1487-1493.

Oleson EB, Beckert MV, Morra JT, Lansink CS, Cachope R, Abdullah RA, Loriaux AL, Schetters D, Pattij T, Roitman MF, Lichtman AH, Cheer JF (2012) Endocannabinoids shape accumbal encoding of cue-motivated behavior via CB1 receptor activation in the ventral tegmentum. Neuron 73:360-373.

Olson VG, Nestler EJ (2007) Topographical organization of GABAergic neurons within the ventral tegmental area of the rat. Synapse 61:87-95.

Oudin MJ, Hobbs C, Doherty P (2011) DAGL-dependent endocannabinoid signalling: roles in axonal pathfinding, synaptic plasticity and adult neurogenesis. European Journal of Neuroscience 34:1634-1646.

Pan B, Hillard CJ, Liu QS (2008) Endocannabinoid signaling mediates cocaine-induced inhibitory synaptic plasticity in midbrain dopamine neurons. J Neurosci 28:1385-1397.

Parker JG, Wanat MJ, Soden ME, Ahmad K, Zweifel LS, Bamford NS, Palmiter RD (2011) Attenuating GABA(A) receptor signaling in dopamine neurons selectively enhances reward learning and alters risk preference in mice. J Neurosci 31:17103-17112.

Parra P, Gulyás AI, Miles R (1998) How Many Subtypes of Inhibitory Cells in the Hippocampus? Neuron 20:983-993.

Pawelzik H, Bannister AP, Deuchars J, Ilia M, Thomson AM (1999) Modulation of bistratified cell IPSPs and basket cell IPSPs by pentobarbitone sodium, diazepam and Zn2+: dual recordings in slices of adult rat hippocampus. European Journal of Neuroscience 11:3552-3564.

Perra S, Pillolla G, Melis M, Muntoni AL, Gessa GL, Pistis M (2005) Involvement of the endogenous cannabinoid system in the effects of alcohol in the mesolimbic reward circuit: electrophysiological evidence in vivo. Psychopharmacology 183:368-377.

Peterfi Z, Urban GM, Papp OI, Nemeth B, Monyer H, Szabo G, Erdelyi F, Mackie K, Freund TF, Hajos N, Katona I (2012) Endocannabinoid-mediated long-term depression of

afferent excitatory synapses in hippocampal pyramidal cells and GABAergic interneurons. J Neurosci 32:14448-14463.

Pillolla G, Melis M, Perra S, Muntoni AL, Gessa GL, Pistis M (2007) Medial forebrain bundle stimulation evokes endocannabinoid-mediated modulation of ventral tegmental area dopamine neuron firing in vivo. Psychopharmacology 191:843-853.

Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nature reviews Neuroscience 4:873-884.

Placzek EA, Okamoto Y, Ueda N, Barker EL (2008) Mechanisms for recycling and biosynthesis of endogenous cannabinoids anandamide and 2-arachidonylglycerol. J Neurochem 107:987-1000.

Rajakumar N, Elisevich K, Flumerfelt BA (1994) Parvalbumin-containing GABAergic neurons in the basal ganglia output system of the rat. The Journal of comparative neurology 350:324-336.

Riegel AC, Lupica CR (2004) Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. J Neurosci 24:11070-11078.

Rogers JH (1992) Immunohistochemical markers in rat brain: colocalization of calretinin and calbindin-D28k with tyrosine hydroxylase. Brain Res 587:203-210.

Romano C, Sesma MA, McDonald CT, O'Malley K, Van den Pol AN, Olney JW (1995) Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. The Journal of Comparative Neurology 355:455-469.

Saal D, Dong Y, Bonci A, Malenka RC (2003) Drugs of Abuse and Stress Trigger a Common Synaptic Adaptation in Dopamine Neurons. Neuron 37:577-582.

Sanchez J, Krause J, Cortright D (2001) The distribution and regulation of vanilloid receptor VR1 and VR1 5' splice variant RNA expression in rat. Neuroscience 107:373 - 381.

Seroogy K, Schalling M, Brene S, Dagerlind A, Chai SY, Hokfelt T, Persson H, Brownstein M, Huan R, Dixon J, et al. (1989) Cholecystokinin and tyrosine hydroxylase messenger RNAs in neurons of rat mesencephalon: peptide/monoamine coexistence studies using in situ hybridization combined with immunocytochemistry. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 74:149-162.

Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17:7503-7522.

Simon GM, Cravatt BF (2010) Characterization of mice lacking candidate N-acyl ethanolamine biosynthetic enzymes provides evidence for multiple pathways that contribute to endocannabinoid production in vivo. Mol Biosyst 6:1411-1418.

Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, Davis JB (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). British Journal of Pharmacology 129:227-230.

Stamatakis AM, Jennings JH, Ung RL, Blair GA, Weinberg RJ, Neve RL, Boyce F, Mattis J, Ramakrishnan C, Deisseroth K, Stuber GD (2013) A unique population of ventral tegmental area neurons inhibits the lateral habenula to promote reward. Neuron 80:1039-1053.

Steffensen SC, Svingos AL, Pickel VM, Henriksen SJ (1998) Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. J Neurosci 18:8003-8015.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochemical and biophysical research communications 215:89-97.

Szilagyi T, Orban-Kis K, Horvath E, Metz J, Pap Z, Pavai Z (2011) Morphological identification of neuron types in the rat hippocampus. Rom J Morphol Embryol 52:15-20.

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J-I, Obata K, Kaneko T (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. The Journal of Comparative Neurology 467:60-79.

Tan KR, Yvon C, Turiault M, Mirzabekov JJ, Doehner J, Labouebe G, Deisseroth K, Tye KM, Luscher C (2012) GABA neurons of the VTA drive conditioned place aversion. Neuron 73:1173-1183.

Tanimura A, Yamazaki M, Hashimotodani Y, Uchigashima M, Kawata S, Abe M, Kita Y, Hashimoto K, Shimizu T, Watanabe M, Sakimura K, Kano M (2010) The Endocannabinoid 2-Arachidonoylglycerol Produced by Diacylglycerol Lipase α Mediates Retrograde Suppression of Synaptic Transmission. Neuron 65:320-327.

Testa CM, Standaert DG, Young AB, Penney JB, Jr. (1994) Metabotropic glutamate receptor mRNA expression in the basal ganglia of the rat. J Neurosci 14:3005-3018.

Timmer KM, Steketee JD (2012) Examination of a role for metabotropic glutamate receptor 5 in the medial prefrontal cortex in cocaine sensitization in rats. Psychopharmacology 221:91-100.

Toth A, Boczan J, Kedei N, Lizanecz E, Bagi Z, Papp Z, Edes I, Csiba L, Blumberg P (2005) Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. Brain Res Mol Brain Res 135:162 - 168.

Tronci V, Balfour DJ (2011) The effects of the mGluR5 receptor antagonist 6-methyl-2-(phenylethynyl)-pyridine (MPEP) on the stimulation of dopamine release evoked by nicotine in the rat brain. Behavioural brain research 219:354-357.

Tukker JJ, Fuentealba P, Hartwich K, Somogyi P, Klausberger T (2007) Cell Type-Specific Tuning of Hippocampal Interneuron Firing during Gamma Oscillations In Vivo. J Neurosci 27:8184-8189.

Ueda N, Okamoto Y, Morishita J (2005) N-acylphosphatidylethanolamine-hydrolyzing phospholipase D: A novel enzyme of the β-lactamase fold family releasing anandamide and other N-acylethanolamines. Life Sciences 77:1750-1758.

van Hooft JA, Giuffrida R, Blatow M, Monyer H (2000) Differential Expression of Group I Metabotropic Glutamate Receptors in Functionally Distinct Hippocampal Interneurons. J Neurosci 20:3544-3551.

van Zessen R, Phillips JL, Budygin EA, Stuber GD (2012) Activation of VTA GABA neurons disrupts reward consumption. Neuron 73:1184-1194.

Varma N, Carlson GC, Ledent C, Alger BE (2001) Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. J Neurosci 21:RC188.

Walters CL, Wang BC, Godfrey M, Sun D, Funk CD, Blendy JA (2003) Augmented responses to morphine and cocaine in mice with a 12-lipoxygenase gene disruption. Psychopharmacology 170:124-131.

Wierenga CJ, Mullner FE, Rinke I, Keck T, Stein V, Bonhoeffer T (2010) Molecular and electrophysiological characterization of GFP-expressing CA1 interneurons in GAD65-GFP mice. PLoS One 5:e15915.

Wittner L, Henze DA, Zaborszky L, Buzsaki G (2006) Hippocampal CA3 pyramidal cells selectively innervate aspiny interneurons. Eur J Neurosci 24:1286-1298.

CURRICULUM VITAE

Collin Merrill
1077 West 1290 South
Orem, UT 84058
208-716-1205
collmerr@gmail.com

Degrees

2006 Brigham Young University-Idaho
BS-Biology
2014 Brigham Young University
PhD Candidate—Physiology and Developmental Biology
Graduating April 2014

Honors and Awards

2012 Graduate Fellowship Award: $15,000 for academic year
2008-2014 Teaching Assistantships and Research Assistantships from Department of Physiology and Developmental Biology
2008-2014 Physiology and Developmental Biology Scholarships

Teaching Experience

2010-2013 Brigham Young University, Teaching Aid

PDBio 363: Advanced Physiology Lab. This course is taught for pre-professional students, covering topics including osmotic equilibrium, resting membrane potentials and electrophysiology, neuron function, skeletal muscle function, electromyography, endocrinology, electrocardiography and cardiac regulation, hematology, spirometry, urinalysis, and glucose metabolism. I taught 2-3 sections per year, with approximately 20 students per section.

2008-2009 Brigham Young University, Teaching Aid

PDBio 305: Physiology for Non-Majors. This course is taught for non-physiology majors to fulfill either a general science requirement or a prerequisite for health science-related majors, covering topics including scientific calculations, pH, osmolarity, neuron function, muscle function, heart function, blood pressure and ECGs, hematology, respiration, and glucose metabolism. Each semester, I taught 3-4 sections, with approximately 25-30 students each.

2008-2009 Provo College, Teaching Aid and Tutor.

Biology 2420: Human Physiology. This course is taught for pre-nursing students to fulfill a prerequisite for admission to the nursing program. Subjects covered in lab included histology, electrocardiography, spirometry, and urinalysis. Each semester, I taught 2 lab sections, with approximately 20 students per section and tutored approximately 30 students per week in various subjects including physiology, microbiology, inorganic chemistry, introductory algebra, and

college algebra.

Abstracts and Presentations

Society for Neuroscience 2009 Annual Meeting:

- Hippocampal interneuron expression of endocannabinoid signaling components.

Society for Neuroscience 2010 Annual Meeting Society for Neurosci
Chapter Annual Snowbird Symposium:

- Expression of Endocannabinoid Signaling Components in Hippocampal CA1 Interneurons

2010 Utah State University Intermountain Graduate Research Symposium:

- Expression of Endocannabinoid Signaling Components in Hippocampal Interneurons

Society for Neuroscience 2010 Annual Meeting:

- The role of connexin-36 gap junctions between GABA neurons in the ventral tegmental area in alcohol intoxication and reward.
- Acute and chronic effects of nicotine on GABA neurons in the ventral tegmental area.
- Identification of endocannabinoid components within hippocampal CA1 interneuron subtypes.

2011 Utah State University Intermountain Graduate Research Symposium:

- Identification of Endocannabinoid Components in Hippocampal CA1 Interneuron Subtypes

2011 Society for Neuroscience Annual Meeting:

- Expression of endocannabinoid biosynthesizing enzymes in hippocampal CA1 neurons.
- Acute and chronic effects of nicotine on GABA neurons in the ventral tegmental area.
- Transient receptor potential vanilloid 1 agonists modulate hippocampal CA1 LTP via the GABAergic system.

2012 Society for Neuroscience Annual Meeting:

- Identification of endocannabinoid biosynthetic enzyme mRNA in hippocampal pyramidal cells and CA1 stratum radiatum interneurons
- Endocannabinoid biosynthesizing enzyme expression in hippocampal stratum oriens neurons
- Expression of endocannabinoid biosynthetic enzyme mRNA within ventral tegmental area neurons

Publications

Nicotine Enhances the Excitability of Gaba Neurons in the Ventral Tegmental Area via Activation of Alpha 7 Nicotinic Receptors on Glutamate Terminals. Taylor DH, Burman PN, Hansen MD, Wilcox RS, Larsen BR, et al. Biochem & Pharmacol 2013 doi:10.4172/2167-0501.S1-007

Identification of mRNA for endocannabinoid biosynthetic enzymes within hippocampal pyramidal cells and CA1 interneuron subtypes. Merrill CB, McNeil M, Williamson RC, Poole BR, Nelson B, Edwards JG. Neuroscience 218, 30 August 2012: 89–99.

Transient receptor potential vanilloid 1 agonists modulate hippocampal CA1 LTP via the GABAergic system. Bennion D, Jensen T, Walther C, Hamblin J, Wallmann A, Couch J, Blickenstaff J, Castle M, Dean L, Beckstead S, Merrill C, Muir C, St Pierre T, Williams B, Daniel S, Edwards JG. Neuropharmacology. 2011 Sep;61(4):730-8.

The role of connexin-36 gap junctions in alcohol intoxication and consumption. Steffensen SC, Bradley KD, Hansen DM, Wilcox JD, Wilcox RS, Allison DW, Merrill CB, Edwards JG. Synapse. 2011 Aug;65(8):695-707. doi: 10.1002/syn.20885.

Service and Outreach

Brain Awareness Week: A Society for Neuroscience sponsored event held annually in March from 2010-2013. This is a national program whose goal is to educate the public about brain function, health, and safety. My outreach activities associated with this event involve 1-1.5 hour classroom presentation at local secondary schools, usually in a science or health-related class, presenting topics such as neuron morphology and function, brain anatomy, perception, and safety.

BYU Undergraduate Fair 2010: I represented the Physiology and Developmental Biology department at an event hosted by BYU, whose goal was to recruit undergraduate students to the PDBio department. My activities involved describing the research projects in Dr. Jeff Edwards' lab, in order to provide mentored research experience for undergraduate students.

BYU Grad Expo 2013: This event showcased the research performed at BYU by graduate students across the university. I represented the Physiology and Developmental Biology department at the expo, and spoke with attendees about the learning and memory and addiction research performed in Dr. Jeff Edwards' lab.