Modulation of L-type Calcium Current by GABA-B Receptor Activation in the Neonatal Rat Hippocampus

Jennifer Grace Bray
Marquette University

MODULATION OF L-TYPE CALCIUM CURRENT BY $GABA_B$ RECEPTOR ACTIVATION IN THE NEONATAL RAT HIPPOCAMPUS

By

Jennifer G. Bray, B.S.

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ABSTRACT
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During the early postnatal period, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) facilitates current through voltage-dependent L-type calcium channels by activating metabotropic $GABA_B$ receptors in the rat hippocampus. In the present study, the effects of the $GABA_B$ receptor agonist baclofen on L-type currents were tested using whole-cell voltage clamp recording on neurons isolated from the superior region of hippocampi obtained from pups of various ages to determine the exact time course of L-type current facilitation. The facilitation of L-type current by $GABA_B$ receptors is more prominent during the second week of development.

One developmental process that L-type current may be involved in is changes in the expression of the K^+Cl^- co-transporter (KCC2) and $N^+K^+2Cl^-$ co-transporter (NKCC1), which are necessary in the maturation of the GABAergic system. To investigate whether calcium influx through L-type channels and $GABA_B$ receptor activation affects the expression of chloride transporters during the early neonatal period, hippocampal cultures isolated from day 0 pups were treated with a $GABA_B$ agonist or an L-type channel antagonist for one week. Steady state KCC2 and NKCC1 levels were determined by Western blot analysis. Blockade of L-type channels drastically reduced KCC2 expression but not NKCC1 expression, suggesting that the upregulation of KCC2 in the first postnatal week is dependent on calcium influx through L-type channels.

The involvement of protein kinase C (PKC) and A (PKA) in the signaling pathway of L-type current modulation by $GABA_B$ receptors was also investigated using electrophysiological experiments. The facilitatory response of baclofen was blocked in the presence of PKC inhibitors, but not PKA inhibitors. Direct activation of PKC using a phorbol ester mimicked the facilitation of L-type current seen with baclofen, whereas facilitation was not seen with direct activation of PKA with a cAMP analogue. Together, these experiments have demonstrated that the facilitation of L-type current by $GABA_B$ receptor activation is maximal during the second postnatal week in development and is mediated by PKC. In addition, calcium influx through L-type channels also contributes to the maturation of the GABAergic system.

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ABBREVIATIONS

VDCC, voltage-dependent calcium channels

CNS, central nervous system

GABA, γ-aminobutyric acid

LVA, low voltage-activated

HVA, high voltage-activated

PKC, protein kinase C

PKA, protein kinase A

CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II

DHP, dihydropyridines

GPCR, G-protein coupled receptors

KCC2, K^+Cl^- co-transporter

NKCC1, $Na^+K^+2Cl^-$ co-transporter

CCC, cation chloride cotransporter

PTX, pertussis toxin

GIRK Channels, G-protein-regulated inward-rectifier K^+ channels

DAG, diacylglycerol

PLC, phsopholipase C

PIP_2, phosphatidylinositol bisphosphate

IP_3, inositol 1,4,5-trisphosphate

cAMP, cyclic adenosine 3',5'-monophosphate

PDE, phosphodiesterase

ATP, adenosine-5'-triphosphate

8-Br-cAMP, 8-Bromoadenosine 3´, 5´- cyclic monophosphate

PMA, phorbol-12-myristate-13-acetate

DMSO, dimethyl sulfoxide

S.E.M**.,** standard error of the mean

PVDF, polyvinylidene difluoride

PBS, phosphate buffered saline

IOD, integrated optical density

BDNF, brain-derived neurotrophic factor

S6K1, p70 ribosomal protein S6 kinase 1

MSK1, mitogen- and stress-activated protein kinase

PSCs, postsynaptic currents

CREB, cAMP response element-binding

CHAPTER 1

INTRODUCTION

Understanding the function and regulation of voltage-dependent calcium channels (VDCC) is important because influx of calcium plays a fundamental role in the regulation of many cellular processes within the central nervous system (CNS). Calcium influx is crucial for the propagation of some action potentials, changes in synaptic plasticity, the release of neurotransmitters, and for the expression of certain genes (for review see West et al., 2001; Catterall and Few, 2008; Neher and Sakaba, 2008; Gover et al., 2009). Calcium signaling is not only important in processes of mature neurons, but it also plays a critical role in the differentiation, growth, and maturation of developing neurons. Due to the importance of calcium as an intracellular messenger, calcium channels are often regulated by neurotransmitters. One of the most common neurotransmitters that has been shown to modulate several types of calcium channels is γ-aminobutyric acid (GABA). Many inhibitory interneuron subtypes within the hippocampus utilize GABA to regulate the overall excitability of the hippocampal region.

Previous studies in the Mynlieff laboratory have shown modulation of calcium current by baclofen, a $GABA_B$ receptor agonist (Carter and Mynlieff, 2004). In acutely cultured hippocampal neurons isolated from 1 week old neonatal rat pups, N-type calcium current decreases in response to activation of $GABA_B$ receptors. In a subset of cells, L-type calcium current increases in response to activation of $GABA_B$ receptors. The facilitation of L-type current by $GABA_B$ receptors had not been previously observed in the mammalian nervous system. Thus, this phenomenon became an area of particular interest in the laboratory.

The focus of my studies has been to investigate the phenomenon of L-type calcium current facilitation by $GABA_B$ receptor activation in the rat hippocampus, a phenomenon that has only been demonstrated in hippocampal cultures isolated from neonates. The first goal of this project was to establish the exact time course of L-type calcium current facilitation by $GABA_B$ receptor activation during the first two weeks of development in the rat hippocampus. In addition, the functional significance of L-type calcium current modulation on the expression levels of chloride transporters during development was investigated as well as the second messenger systems involved in L-type calcium current modulation by $GABA_B$ receptors. A better understanding of the importance of $GABA_B$ receptors in the development of a mature nervous system can be achieved by increasing our knowledge of $GABA_B$ receptor modulation of calcium channels.

Voltage-Dependent Calcium Channels

Calcium channels have been classified into different subtypes according to voltage dependence, kinetics, pharmacology, and molecular biology. VDCCs can be characterized by channel activation into two different subgroups, low voltage-activated (LVA) and high voltage-activated (HVA) channels (Carbone and Lux, 1984; Bean, 1989a). LVA channels are activated at low voltages and produce a transient current (T-type channels; Nowycky et al., 1985; Bean and McDonough, 1998). HVA channels have been further divided into 5 different subgroups (L-type, P-type, Q-type, N-type, and R-type) based on pharmacology, kinetics, and molecular identification of the α_1 subunit (for review see Tsien et al., 1988; Ertel et al., 2000; Lipscombe, 2004).

Experiments in the laboratory have primarily focused on L-type calcium channels, which are found in neurons and different muscle types. Neuronal voltage-dependent L-type calcium channels are heteromeric complexes that contain an α_1 pore forming subunit, and several auxiliary subunits including an extracellular α_2 subunit, an intracellular β subunit, and a transmembrane δ subunit (Figure 1.1; for review see

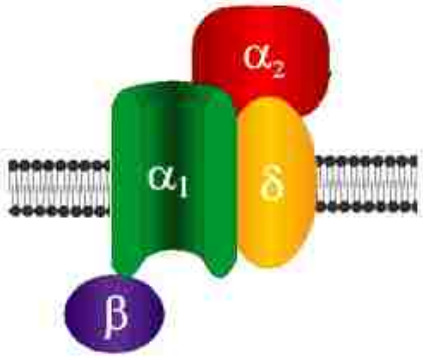

Figure 1.1: Structure of neuronal L-type calcium channels. The channel is made up of four subunits. The α_1 subunit forms the channel pore. The $\alpha_2\delta$ subunit associates with the α_1 subunit to modulate current amplitude. The intracellular β subunit regulates the activation, inactivation, current, and voltage dependence of the α_1 subunit (Singer et al., 1991).

Catterall, 1998, 2000). The $\alpha_2\delta$ and β auxiliary subunits modulate the trafficking and control the function of the α_1 subunit (Singer et al., 1991). The $\alpha_1$subunit is made up of 4 domains with each domain containing six transmembrane spanning segments (Figure 1.2; Catterall, 2000). Due to its role as the conduction pore, gating apparatus, and voltage

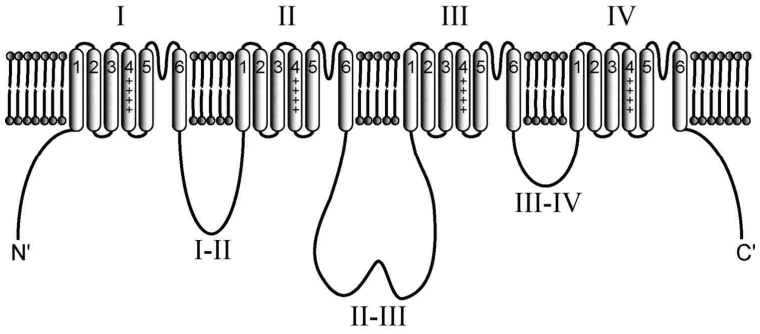

Figure 1.2: Structure of the α_1 subunit of VDCCs. The amino acid sequence for the α_1 subunit is approximately 2000 residues long and is organized in four repeated domains, each of which contain 6 transmembrane segments (S1 – S6). The S4 transmembrane segment contains several positively charged amino acids and is thought to be the voltage sensor. The N- and C- termini are cytoplasmic. The cytoplasmic regions are key sites for phosphorylation by kinases. Figure obtained from Tedford and Zamponi, 2006.

sensor, the α_1 subunit is a major site of regulation by drugs, toxins, and phosphorylation by protein kinase C (PKC), protein kinase A (PKA), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; for review see Catterall 1998, 2000).

Functional Identification	Molecular Identification	Pharmacological Antagonists
HVA L-type	Ca_v 1.1, 1.2,1.3, 1.4	Dihydropyridines
HVA P/Q-type	Ca_v 2.1	ω-Agatoxin IVA
HVA N-type	Ca_v 2.2	ω-Conotoxin GVIA
HVA R-type	Ca_v 2.3	Nickel, SNX-483
LVA T-type	Ca_v 3.1,3.2,3.3	Nickel, amiloride

Table 1: Summary of the identification and nomenclature of VDCCs. To distinguish between the various types of calcium channels different channels blockers can be used (Adapted from Ertel et al., 2000).

Currently, ten pore-forming α_1 subunits have been cloned and are used to further classify calcium channels into 3 different families Ca_v1, Ca_v2, and Ca_v3 (Table 1; Ertel et al., 2000; Lipscombe et al., 2004). The Ca_v1 family of α_1 subunits consists of several subtypes of L-type calcium channels, which produce long lasting calcium currents. These types of channels have relatively slow activation and inactivation kinetics, with some subtypes demonstrating calcium-dependent inactivation along with voltage-dependent inactivation (for review see Lipscombe et al., 2004). $Ca_v1.1$ channels are found in skeletal muscle where they are linked to ryanodine receptors in the sarcoplasmic reticulum. $Ca_v1.2$ channels are found in cardiac and smooth muscle where they initiate muscle contraction, in neurons where they are involved in gene regulation, and in endocrine cells where they aid in secretion (for review see McDonald et al., 1994; Catterall, 2000; Lipscombe et al., 2004). $Ca_v1.3$ channels have similar expression as Ca_v 1.2 channels and are expressed in neurons, cardiac muscle, and endocrine cells. $Ca_v1.4$ L-type calcium channels are located in retinal tissue and are linked to phototransduction (Baumann et al., 2004). $Ca_v1.2$ and $Ca_v1.3$ L-type calcium channels are both located on

the soma and dendrites of neurons (Westenbroek et al., 1990; Budde et al., 1998; McRory et al., 2004). These neuronal types of L-type calcium channels are modulated by protein phosphorylation and regulate a number of processes such as activity-dependent gene expression, mRNA stability, neuronal survival, synaptic efficacy, and the activity of neighboring ion channels (Murphy et al., 1991; Bading et al., 1993; Christie et al., 1995; Finkbeiner and Greenberg, 1998; Hardingham et al., 1998; Marrion and Tavalin, 1998; Charles et al., 1999; Schorge et al., 1999; Dolmetsch et al., 2001; Marshall et al., 2003; for review see Lipscombe, 2004; Pinato et al, 2009).

The Ca_v2 family consists of N-type, P/Q-type, and R-type calcium channels. These channels are most commonly found in neurons and regulate the release of neurotransmitters within synapses. T-type calcium channels make up the Ca_v3 family of α_1 subunits. T-type calcium channels, which can be found in the brain, heart, kidney, and liver play an important role in regulating overall cell excitability and hormone secretion (for review see Cueni et al., 2009; Iftinca and Zamponi, 2009). The HVA Ca_v1 and Ca_v2 calcium channels are primarily distinguished from one another in native neurons through pharmacological methods. L-type calcium channels are sensitive to dihydropyridines (DHP), whereas P/Q-type calcium channels are blocked by varying concentrations of a spider venom toxin ω-agatoxin IVA. N-type calcium channels are blocked by the cone-snail toxin ω-conotoxin GVIA and can not be blocked by DHP antagonists or activated by DHP agonists (Table 1; Fox, 1987; Cruz et al., 1987; Tsien et al., 1988; Bean, 1991; Mintz et al., 1992).

The use of heterologous expression systems to study calcium channel properties has given a wealth of information characterizing the different calcium channel subtypes.

When $Ca_v1.1$ L-type calcium channels were expressed in muscle cells from dysgenic mice, the currents were small and demonstrated slow activation kinetics (Tanabe et al., 1988; Adams et al., 1990). In comparison, when $Ca_v1.2$ L-type calcium channels were expressed, the activation kinetics were faster than seen with the $Ca_v1.1$ (Tanabe et al., 1990). Further heterologous expression studies have demonstrated different activation thresholds between $Ca_v1.2$ and $Ca_v1.3$ L-type calcium channels, with $Ca_v1.3$ channels exhibiting a lower activation threshold than $Ca_v1.2$ channels (Safa et al., 2001; Scholze et al., 2001; Xu and Lipscombe, 2001). $Ca_v1.3$ channels begin to activate at potentials around -55 mV, whereas $Ca_v1.2$ channels begin to activate closer to -30 mV. This suggests that $Ca_v1.3$ channels, but not $Ca_v1.2$ channels, may activate at subthreshold depolarizations. The differences in activation suggest that $Ca_v1.3$ and $Ca_v1.2$ may function in different signaling pathways within neurons. Calcium channels belonging to the Ca_v2 family can be distinguished from L-type calcium channels, because they require stronger depolarizations for activation (positive to -20 mV) and demonstrate moderate voltage-dependent inactivation (Tsien et al., 1988; Fujita et al., 1993; Delcour et al., 1993).

The Hippocampus

The hippocampus is a region of the brain that plays a prominent role in spatial learning and in the formation and retrieval of memories (for review see Bird and Burgess, 2008; Neves et al., 2008; Cutsuridis and Wennekers, 2009; Eichenbaum and Fortin, 2009). The hippocampus is also involved in the formation and spread of seizures (Falconer et al., 1964; Dam, 1980, 1982; Schwartzkroin, 1994). As a result of seizure

activity, several structural and functional changes occur in the neuronal connectivity of the hippocampus (for review see Lado et al., 2002; Naegele, 2009). This hippocampal damage can lead to recurrent seizures and ultimately may result in the development of epilepsy (Sutula et al., 1989; Overstreet-Wadiche et al., 2006).

As one of the most studied brain regions, the cell types of the hippocampus and their network connections have been characterized relatively well. The hippocampus is a highly organized structure that is divided into three major regions called the dentate gyrus, the CA3, and the CA1. The hippocampus acts as a unidirectional circuit that allows information to flow into, through, and then out to other neurons throughout the brain (Andersen et al., 1973). There are three major excitatory synaptic connections within the hippocampus (Figure 1.3). The main input into the hippocampus is through the perforant pathway in which axons from the entorhinal cortex project onto granule cells in the dentate gyrus. The mossy fiber pathway extends from granule cell axons that synapse onto pyramidal cells within the CA3 region. The Schaffer collateral pathway consists of axons from the CA3 pyramidal cells that project onto pyramidal cells within the CA1 region. The principle output from the hippocampus occurs through CA1 axons, which project to the subiculum and back to the entorhinal cortex (Amaral, 1993; Braak et al., 1996).

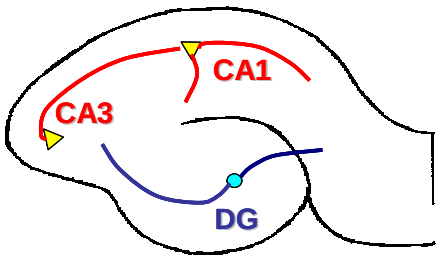

Figure 1.3: The Hippocampal Network. Input from the entorhinal cortex forms connections with the dentate gyrus (DG) through the perforant path. Granule cell axons in the dentate gyrus project to the CA3, which in turn projects to the CA1 through the Schaffer collateral pathway.

The overall excitability of the adult hippocampus is regulated by local inhibitory interneurons, which control the number of active pyramidal cells and their firing pattern by releasing the inhibitory neurotransmitter GABA. Pyramidal cells are modulated by a diverse population of GABAergic interneurons. There are over 21 distinguishable inhibitory interneurons that synapse either on specific regions of CA1 pyramidal cells or onto other interneurons (for review see Somogyi and Klausberger, 2005; Ascoli et al., 2008; Klausberger and Somogyi, 2008; Cutsuridis and Wennekers, 2009; Klausberger, 2009). These interneurons are classified based on their anatomical, morphological, and physiological characteristics along with antibody staining. Although the importance of interneurons in hippocampal functioning is clear, very little is known about the mechanisms involved in the maturation of inhibitory interneurons and synapses within the hippocampus. The experiments described here contribute to our knowledge of hippocampal development by investigating $GABA_B$ receptor facilitation of L-type calcium current during the maturation of the GABAergic circuitry within the neonatal rat hippocampus.

The hippocampus appears to be involved in severe illnesses such as schizophrenia, depression, bipolar disorder, and epilepsy. Seizures are more likely to occur in immature humans and animals, and in humans seizure susceptibility peaks during the first few months after birth (Sperber, 1999; for review see Lado et al., 2002; Holopainen, 2008; Rakhade and Jensen, 2009). This is most likely due to the fact that the inhibitory GABAergic system within the hippocampus is not yet fully developed and thus neurons are highly excitable due to the lack of inhibition. In addition, at least in rat hippocampus, the response to GABA is excitatory during the first few postnatal weeks.

Therefore, it is essential to understand the time course and mechanisms involved in the development of the hippocampus and the GABAergic system to aid in the treatment of early onset seizures.

GABA Receptors

The importance of calcium influx in neuronal function makes calcium channels a prime site for neurotransmitter modulation. One neurotransmitter within the mammalian CNS that can regulate calcium channels is GABA. GABA is the major inhibitory neurotransmitter in the mammalian CNS. It acts by binding to specific receptors, which produces a variety of cellular effects in both presynaptic and postsynaptic neurons. GABA receptors have been classified into 3 different subtypes, $GABA_A$, $GABA_B$, and $GABA_C$. The ionotropic $GABA_A$ and $GABA_C$ receptors have an ion channel incorporated into the receptor and mediate fast inhibitory synaptic transmission (Figure 1.4). When GABA binds to the receptor it causes a conformational change that allows chloride ions to flow through the channel causing the cell to hyperpolarize, which prevents it from reaching threshold. $GABA_B$ receptors are metabotropic and mediate their effects through G-proteins (Figure 1.4). Compared to the ligand-gated $GABA_A$ and $GABA_C$ channels, the effects of $GABA_B$ receptor activation are slower and often involve the regulation of calcium or potassium channels.

$GABA_B$ receptors are expressed on neurons both presynaptically, where they modulate neurotransmitter release and postsynaptically, where they modulate neuronal excitability. Functional $GABA_B$ receptors are heterodimeric complexes of two homologous subunits, $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann et al., 1997, 1998; Jones et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999; White et al., 2002).

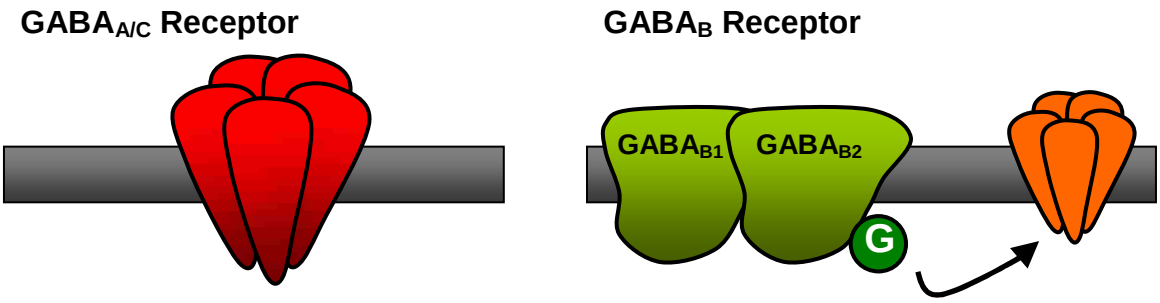

Figure 1.4: GABA Receptors. $GABA_A$ and $GABA_C$ receptors are ionotropic and form a channel that is permeable to chloride ions. $GABA_A$ receptors are pentameric structures that can be assembled from a number of different subunits (α, β, γ, δ, and ρ). The most common $GABA_A$ receptor found in the brain is composed of 2α:2β:1γ. $GABA_C$ receptors, which are constructed from ρ subunits, are insensitive to bicuculline and are found only in the retina. $GABA_B$ receptors are heterodimers, consisting of $GABA_{B1}$ and $GABA_{B2}$ subunits. $GABA_B$ receptors are metabotropic and are coupled to G-proteins and often regulate K^+ and Ca^{2+} channels.

There are two different isoforms of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$. These two isoforms are differentially localized within the hippocampus where $GABA_{B1a}$ is located presynaptically, while $GABA_{B1b}$ is primarily located postsynaptically (Vigot et al., 2006). In order for $GABA_B$ receptors to be functional, the $GABA_{B1}$ and the $GABA_{B2}$ subunits must form a dimer and be inserted into the membrane. It is thought that G-protein coupled receptors (GPCRs) form dimers to increase their surface area so that it is possible to interact with trimeric G-protein complexes (Bouvier, 2001; Hamm, 2001). To ensure that the complexes are assembled properly before being inserted into the membrane, $GABA_B$ receptors must first pass a dimerization-dependent trafficking checkpoint (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). Once functional heterodimeric $GABA_B$ receptors are inserted into the plasma membrane, agonists are able to bind to the ligand binding domain located on the large extracellular N-terminus of the $GABA_{B1}$ subunit (Galvez et al., 1999; 2000a; 2000b). Ligand binding causes a conformational change in the $GABA_{B2}$ subunit, which results in G-protein coupling and

activation. Subsequently, activated G-proteins are able to modulate downstream signaling pathways (Figure 1.5).

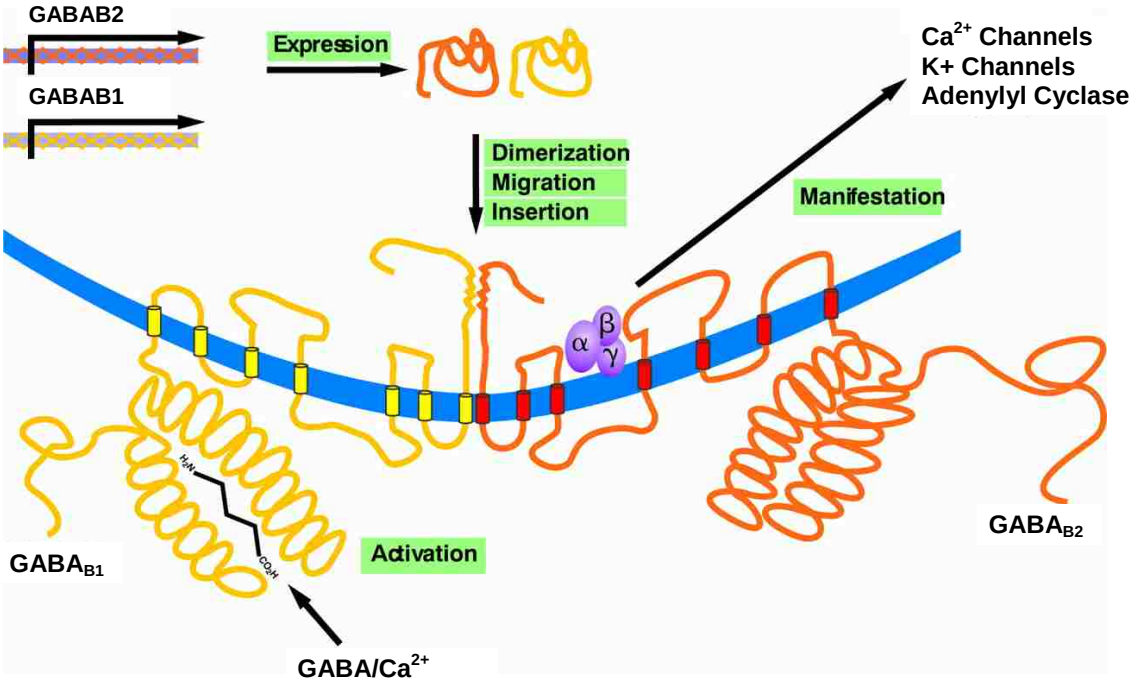

Figure 1.5: Structure of $GABA_B$ receptors. $GABA_{B1}$ and $GABA_{B2}$ heterodimers are trafficked and inserted into the plasma membrane. Activation of $GABA_{B1}$ by GABA binding to the N-terminus results in a conformational change in the $GABA_{B2}$ subunit, which allows recruitment and activation of G-proteins. Figure obtained from Enna, 2001.

$GABA_B$ receptors are commonly coupled to $G\alpha_i$ and $G\alpha_o$ proteins (Asano and Ogasawara, 1986; Morishita et al., 1990; Campbell et al., 1993; Menon-Johansson et al., 1993; Greif et al., 2000; Bettler et al., 2004). Modulation of ion channels and other downstream effectors can either occur via the α subunit or βγ subunits. Inhibition of N-type and P/Q-type calcium current by presynaptic $GABA_B$ receptor activation occurs through the Gβγ subunits that are associated with $G\alpha_i$ and $G\alpha_o$ proteins (Scholz and Miller, 1990; Menon-Johansson et al., 1993; Mintz and Bean, 1993; Pfrieger et al., 1994; Amico et al., 1995; Cardozo and Bean, 1995; Poncer et al., 1997; Kajikawa et al., 2001;

for review see Bettler et al., 2004). Inhibition of N-type and P/Q-type calcium channels in the presynaptic terminal following $GABA_B$ receptor activation will ultimately lead to a decrease in neurotransmitter release (Hirata et al., 1995; Doze et al., 1995; Takahashi et al., 1998). Postsynaptic $GABA_B$ receptor activation of inwardly rectifying K^+ current occurs through the $G\beta\gamma$ subunits that are associated with $G\alpha_i$ and $G\alpha_o$ proteins and results in the hyperpolarization of the postsynaptic cell (Gähwiler and Brown, 1985; Lüscher et al., 1997; Kaupmann et al., 1998; Yamada et al., 1998; for review see Bettler et al., 2004).

Using $GABA_B$ receptor knockout mice, it has been demonstrated that $GABA_B$ receptors are critically involved in neuronal development. $GABA_B$ receptor mutations or loss of functional $GABA_B$ receptors can lead to a number of abnormalities during development. In the absence of functional $GABA_{B1}$ subunits (site of GABA binding) neuronal excitability is not under control and mice exhibit epilepsy by postnatal week 2, hyperalgesia, hyperlocomotor activity, and have an impaired memory (Prosser et al., 2001; Schuler et al., 2001). The mean average life expectancy of $GABA_{B1}$ knockout mice is 21 days (Prosser et al., 2001). Mice that lack $GABA_{B2}$ subunits have similar symptoms as mice lacking $GABA_{B1}$ subunits, which is consistent with the heterodimeric structure of $GABA_B$ receptors (Gassmann et al., 2004; Mombereau et al., 2005). In comparison to the severe deficits seen in $GABA_{B1}$ or $GABA_{B2}$ knockout mice, adult animals treated with acute or chronic $GABA_B$ receptor antagonists do not develop such extreme behavioral changes (Bittiger et al., 1993; Mondadori et al., 1993; Vergnes et al., 1997). Although it is hard to get complete blockage of $GABA_B$ receptors *in vivo*, these experiments are suggestive that $GABA_B$ receptors may be critically involved in the

development of a mature nervous system. Therefore, it is essential to understand the role that $GABA_B$ receptors play in the development of neurons particularly within the hippocampus, where several of the behavioral deficits seen in the $GABA_{B1}$ and $GABA_{B2}$ knockout mice arise.

GABA Function During Development

It is well documented that GABA exerts an excitatory rather than inhibitory response early in neuronal development due to the high concentration of intracellular chloride ions (Mueller et al., 1984; Ben-Ari et al., 1989; Cherubini et al., 1991; Luhmann and Prince, 1991; Gaiarsa et al., 1995; Owens et al., 1999). During neuronal maturation $GABA_A$ receptor activation depolarizes the membrane. This GABA-induced depolarization shifts to a hyperpolarizing effect in the first few postnatal weeks due to a change in the reversal potential of chloride (Cherubini et al., 1990; Luhmann and Prince, 1991; Chen et al., 1996; Owens et al., 1999). The developmental switch of the GABAergic system has been demonstrated in both cultured hippocampal neurons as well as in hippocampal slices (Rivera et al., 1999; Ganguly et al., 2001). It is believed that the upregulation of the K^+Cl^- co-transporter, KCC2, and the downregulation of the, $N^+K^+2Cl^-$ co-transporter, NKCC1, may be responsible for shifting the chloride reversal potential in immature neurons (Plotkin et al., 1997; Lu et al., 1999; Rivera et al., 1999; Vu et al., 2000; Hübner et al., 2001a). NKCC1 expression predominates in immature neurons and mediates chloride influx, while KCC2 expression predominates in mature neurons and mediates chloride efflux (Figure 1.6; for review see Delpire, 2000; Payne et al., 2003).

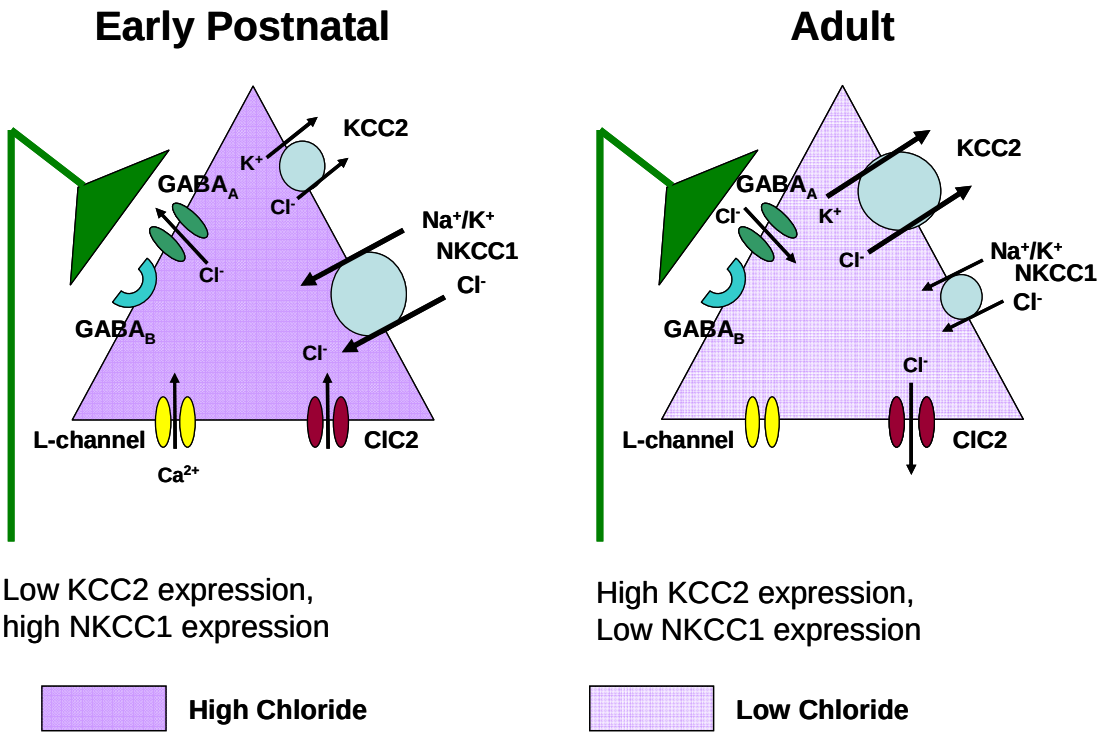

Figure 1.6: Maturation of the GABAergic system. In immature neurons the intracellular chloride concentration is kept relatively high due to the expression of NKCC1, which pumps chloride into the cell. $GABA_A$ receptor activation results in an efflux of chloride and excites the cell. In comparison, KCC2 expression predominates in mature neurons and mediates chloride efflux. $GABA_A$ receptor activation results in an influx of chloride and inhibits adult neurons. Figure adapted with permission from Fukuda et al., 1998. Abbreviations: KCC2, K^+ Cl^- co-transporter; NKCC1, Na^+ K^+ $2Cl^-$ co-transporter; ClC2, voltage-dependent chloride channel; L-channel, voltage-dependent L-type calcium channel.

There are nine types of cation chloride transporters (CCCs) found in mammals. There are two NKCCs (NKCC1 and NKCC2), four KCCs (KCC1-4), one Na^+Cl^- (NCC), and two additional CCCs (ClP1 and CCC9; for review see Delpire, 2000; Blaesse et al., 2009). NCC and NKCC2 are renal specific (for review see Knepper and Brooks, 2001; Castrop and Schnermann, 2008), whereas KCC2 is neuron specific (Payne et al., 1996; Lu et al. 1999). NKCC1 is expressed in neurons and also glial cells within the CNS (Kanaka et al., 2001; Hübner et al., 2001b). The three other types of KCCs (KCC1, KCC3, and KCC4) are expressed in various tissues including neurons, muscle, and the kidney (for review see Payne et al., 2003; Blaesse et al., 2009). CCC activity can be

regulated through phosphorylation by kinases. For example, NKCC1 activation occurs through phosphorylation of a regulatory domain in its N-terminus (Darman and Forbush, 2002; Flemmer et al., 2002). The two predominant CCCs within hippocampal neurons are KCC2 and NKCC1, however their expression levels are developmentally regulated during the first few postnatal weeks.

Due to the high intracellular chloride concentration in immature neurons, the activation of $GABA_A$ receptors causes an efflux of chloride, which depolarizes the cell. If sufficiently depolarized VDCCs will be activated leading to an increase of L-type calcium current. Using calcium imaging, Gangluy and others (2001) investigated the developmental timecourse of GABA-induced increases in intracellular calcium levels in hippocampal cultures isolated from E18 rats. In hippocampal neurons allowed to remain in culture for 4 to 9 days, increases in $[Ca^{2+}]_i$ were observed with GABA application. The increase in $[Ca^{2+}]_i$ was blocked with $GABA_A$ antagonists and L-type calcium channel blockers. These data demonstrate that $GABA_A$ receptor activation can lead to influx of calcium through L-type calcium channels during the early neonatal period. The facilitation of L-type calcium current could further increase calcium influx in response to GABA in a subset of cells.

The change in chloride transporter expression decreases the internal chloride concentration by increasing the extrusion of chloride, which results in a change of the reversal potential of $GABA_A$ mediated current (Ben-Ari et al., 1989; Leinekugel et al., 1995, 1997, 1998; Khazipov et al., 1997). It has been well documented that the upregulation of KCC2 transporter levels play a role in changing the chloride reversal potential (Payne, 1997; Lu et al., 1999; Rivera et al., 1999; Ganguly et al., 2001). Rivera

and others (1999) demonstrated that inhibition of KCC2 expression with antisense oligodeoxynucleotides delayed the shift in the reversal potential of $GABA_A$ responses from depolarizing-to-hyperpolarizing in cultured neonatal rat hippocampal slices. They demonstrated that in cultured hippocampal slices isolated from postnatal 0 to 4 day old rats, $GABA_A$ receptor activation evoked depolarizing responses, whereas in hippocampal slices isolated from postnatal rats 13 to 30 days old $GABA_A$ receptor activation evoked hyperpolarizing responses. When KCC2 expression levels were inhibited with antisense oligonucleotides in slices from postnatal 11 to 13 day old rats, the driving force of $GABA_A$ demonstrated a significant positive shift when compared to control slices (Rivera et al., 1999). These data demonstrate that KCC2 expression is involved in the fast hyperpolarizing $GABA_A$ receptor responses in mature hippocampal neurons.

In addition to the upregulation of KCC2, the downregualtion of NKCC1 expression may also play a role in changing the chloride reversal potential (Plotkin et al., 1997; Wang et al., 2002). However, there are several conflicting results when looking at NKCC1 mRNA levels within the developing rat hippocampus. Using *in situ* hybridization, Plotkin et al. (1997) reported that NKCC1 mRNA expression levels peak in the hippocampus of rats at postnatal day 7. In comparison, Wang et al. (2002) who also performed *in situ* hybridization reported higher NKCC1 mRNA levels in the hippocampus of postnatal day 15 rats through postnatal day 40 rats. Most of the studies on KCC2 and NKCC1 expression have measured mRNA levels. In theory, changes in mRNA levels should lead to changes in protein. However, mature protein levels are not only dependent on translation of mRNA, but also on the degradation of the protein. In the current study, protein levels of KCC2 and NKCC1 were measured rather than mRNA

levels since ultimately it is the protein that is the end product. Therefore, the developmental changes of NKCC1 and KCC2 protein levels within the developing rat hippocampus were further characterized.

It has been demonstrated that GABA may be responsible for the depolarizing-to-hyperpolarizing switch and that calcium influx is necessary for this $GABA_A$ mediated switch (Ganguly et al., 2001). Ganguly et al. (2001) showed that application of $GABA_A$ antagonists delayed the excitatory to inhibitory switch and also delayed the increase in KCC2 mRNA expression levels in cultured hippocampal neurons. In addition, chronic blockade of L-type calcium channels with the L-type channel antagonist nimodipine significantly delayed the developmental change in the $GABA_A$ reversal potential. These two observations taken together suggest that $GABA_A$ receptors directly depolarize the membrane to open L-type calcium channels and that influx of calcium is involved in the developmental maturation. Ganguly et al. (2001) did not directly look at the effects of L-type calcium current on KCC2 transporter protein levels. Calcium influx across L-type calcium channels may regulate the changes in chloride transporter expression. Therefore, the enhancement of L-type calcium current by $GABA_B$ receptor activation demonstrated by Carter and Mynlieff (2004) may play a role in increasing intracellular calcium levels that mediate the switch of $GABA_A$ responses from excitatory to inhibitory effects. The goal of the present study was to increase our knowledge of $GABA_B$ receptors and their downstream modulation to gain a better understanding of their functional role in the development of a mature CNS.

G-proteins

$GABA_B$ receptors are coupled to guanine nucleotide-binding proteins, also known as G-proteins. G-proteins linked to GPCRs are heterotrimeric proteins consisting of an α, β, and γ subunit. GPCRs belong to a family of seven transmembrane spanning receptors and are activated by a variety of extracellular neurotransmitters and hormones. When activated by ligands or agonists, GPCRs interact with G-proteins, which stimulate the exchange of GDP on the Gα subunit for cytoplasmic GTP. Following nucleotide exchange, the Gα subunit dissociates from the Gβγ subunits which remain anchored to the plasma membrane. Both the free Gα-GTP bound subunit as well as the Gβγ subunit complex are biologically active and can modulate downstream effector systems. The intrinsic GTPase activity of the α subunit converts GTP to GDP + Pi, which promotes the reassociation of the inactive Gα-GDP to the Gβγ complex (for review see McCudden et al., 2005; Wettschureck and Offermanns, 2005).

Gα subunits are classified into four groups based on their sequence similarity and activation of various cell signaling systems (Table 2). Each family consists of several different types of α-subunits, some of which show very specific expression patterns (for review see McCudden et al., 2005; Wettschureck and Offermanns, 2005). $G\alpha_s$ proteins activate adenylyl cyclase and are activated by cholera toxin-mediated ADP ribosylation, whereas the $G\alpha_{i/o}$ proteins inhibit adenylyl cyclase activity and are inhibited by pertussis toxin (PTX) mediated ADP ribosylation (Fields and Casey, 1997). $G\alpha_t$, also known as transducin, is found in cells of the retina and is coupled to the GPCR, rhodopsin. The G-protein that is primarily found in taste cells is called $G\alpha_{gust}$ or gustducin. $G\alpha_{q/11}$ proteins activate phospholipase C (PLC) and are not affected by either cholera toxin or PTX.

$G\alpha_{12/13}$ results in the activation of a small GTPase RhoA, which is overexpressed in various tumor cell types (Fritz et al., 1999; Abraham et al., 2001; Kamai et al., 2001; Fritz et al., 2002).

G-Protein Subfamily	G_{α}	Location	Pharmacology	Potential Effector Proteins
$G\alpha_s$	α_s α_{olf}	Ubiquitous Olfactory Epithelium	Cholera Toxin	Activates Adenylyl Cyclase
$G\alpha_{i/o}$	α_{i1}, α_{i2}, α_{i3} α_o α_z $\alpha_{t\text{-}r}$ $\alpha_{t\text{-}c}$ α_{gust}	Ubiquitous Neuronal Neuronal Retinal Rods Retinal Cones Taste Buds	Pertussis Toxin	Inhibits Adenylyl Cyclase, activates K^+ channels, inhibits Ca^{++} Channels, increases phosphodiesterase 6 activity (rods and cones)
$G\alpha_q$	α_q, α_{11} α_{14} $\alpha_{15/16}$	Ubiquitous Kidney, Lung Hematopoietic cells	Ym-254890	Activates Phosphlipase Cβ1-4
$G\alpha_{12/13}$	α_{12}, α_{13}	Ubiquitous		Activates Small G-proteins, RhoA, and Heat Shock Protein

Table 2: Pharmacology and signaling properties of the major G-protein subunit types. (Table adapted from Wettschureck and Offermanns, 2005; Tedford and Zamponi, 2006).

Following dissociation of Gα-GTP, the Gβγ subunit can activate a large number of effectors, including G-protein-regulated inward-rectifier K^+ channels (GIRK channels), PLCβ, and adenylyl cyclase (Camps et al., 1992; Smrcka and Sternweis, 1993; Park et al., 1993; Pian and Dobbs, 1995; Yamada et al., 1998). Due to the several different types of G-proteins, GPCRs, effectors, and regulatory proteins, there is a great deal of versatility among the signal transduction mechanisms throughout the different cell types of the body (for review see McCudden et al., 2005; Wettschureck and Offermanns, 2005).

Neuronal calcium channels are directly regulated by both Gα and Gβγ subunits as well as indirectly regulated by G-proteins through second messenger systems (for review see Schultz et al., 1990; Dolphin, 1998; Wettschureck and Offermanns, 2005). N-type and P/Q-type calcium channels are directly inhibited by Gβγ in a membrane delimited manner (for review see Tedford and Zamponi, 2006). Gβγ subunits remain anchored to the plasma membrane, whereas the Gα subunit can diffuse throughout the cytoplasm. (Herlitze et al., 1996; Ikeda et al., 1996). Binding of the Gβγ subunits to the intracellular loop between domains I and II of the α_1 subunit of VDCCs makes it more difficult for the channel to open (Bean, 1989b; Boland and Bean, 1993; Herlitze et al., 1997; Zamponi et al., 1997; for review see Tedford and Zamponi, 2006). In addition to direct binding, G-proteins can activate second messenger systems that activate protein kinases, which can in turn regulate calcium channel activity. Phosphorylation of the channel protein itself or a regulatory component can either stimulate or inhibit channel activity.

The signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptor activation in the mammalian nervous system has not been identified and was a main focus of my research project. $GABA_B$ receptor activation of PKC has been shown to facilitate L-type current in salamander retinal neurons (Shen and Slaughter, 1999). PKC activation within the hippocampus has also been shown to enhance L-type calcium current (Doerner et al., 1990; Doerner and Alger, 1992). However, this facilitation was not linked to $GABA_B$ receptor activation. Shen and Slaughter (1999) also demonstrated inhibition of the facilitation of L-type calcium current by $GABA_B$ receptor activation in the salamander retina in the presence of a PKA inhibitor, but the response was smaller than the blockade of facilitation seen with PKC inhibitors. The present

project directly investigated the role of PKC and PKA in $GABA_B$ receptor mediated facilitation of L-type calcium current within the neonatal rat hippocampus using multiple PKC and PKA inhibitors as well as activators for both PKC and PKA.

Protein Kinase C

PKC represents a family of serine/threonine protein kinases that are stimulated by diacylglycerol (DAG), calcium, and phospholipids. The structure of PKC includes a highly conserved catalytic domain along with a regulatory domain that keeps the kinase in an inactive conformation (Figure 1.7; for review see Steinberg, 2008). Activation of a $G\alpha_q$ family protein and certain $G\beta\gamma$ subunits results in the activation of PLC, which in turn cleaves the membrane lipid phosphatidylinositol bisphosphate (PIP_2) into DAG and inositol trisphosphate (IP_3). DAG stays within the membrane and activates PKC (Figure 1.8). Activated PKC can then phosphorylate target proteins, such as sodium, potassium, and calcium channels.

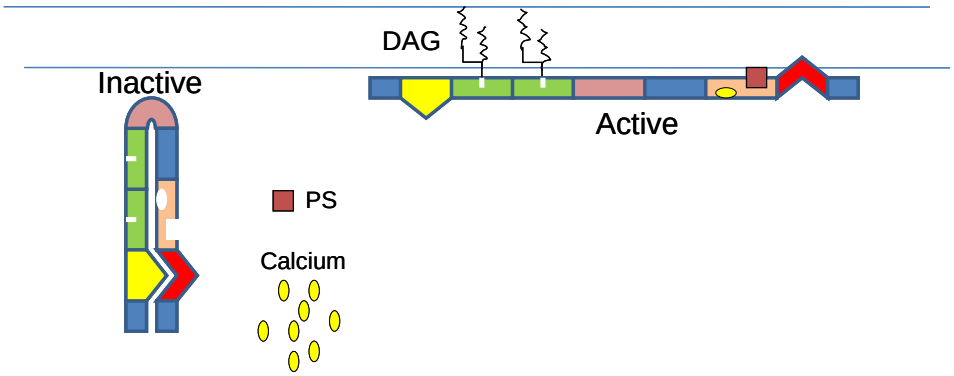

Figure 1.7: PKC is a monomeric protein. Certain isoforms require Ca^{2+}, DAG, and phosphatidylserine (PS) for activation and relief of autoinhibition.

There are at least 12 different isoforms of PKC, which are classified into three classes based on their activation requirements (Tanaka and Nishizuka, 1994; Nishizuka, 1995; Webb et al., 2000; Zarate and Manji, 2009). The conventional PKC isoforms are

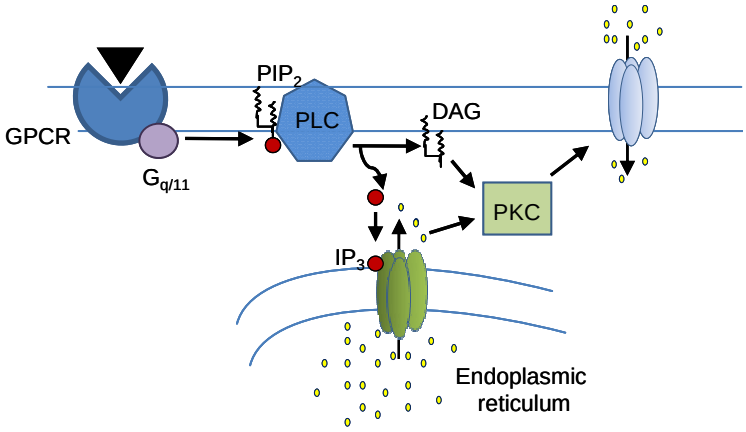

Figure 1.8: PKC pathway. PLC can be activated by the Gq family of Gα subunits as well as certain Gβγ subunits. The activated phospholipase cleaves PIP_2 into IP_3 (inositol trisphoshpate) and DAG. IP_3 initiates intracellular calcium release from the endoplasmic reticulum, which induces the translocation of PKC to the plasma membrane. PKC is activated by the combination of DAG, calcium, and phospholipids. Once activated, PKC is able to phosphorylate target proteins.

α, βI, βII, and γ. These isoforms require calcium and DAG for activation. There are five novel PKC isoforms, δ, ε, η, θ, and μ. The novel PKC isoforms do not require calcium for activation, but do require DAG for activation. Lastly, the atypical PKC isoforms, ζ, λ, and ι, require neither calcium nor DAG for activation (Tanaka and Nishizuka, 1994; Webb et al., 2000; Zarate and Manji, 2009). In addition to differences in their mode of activation, the different isoforms of PKC also vary in their structure, subcellular localization, and tissue specificity. Multiple PKC isoforms can exist within a single cell, but the subcellular localization may differ for each isoform. Thus, each isoform may be involved in regulating different cell functions throughout various regions of the cell (Roisin and Barbin, 1997).

Subfamilies of PKC	Isoforms	Activation Requirements
Conventional	α, β_I, β_{II}, γ	Ca^{2+}, DAG, Phospholipid
Novel	δ, ε, η, θ, μ	DAG and Phospholipid
Atypical	ζ, ι, λ	Phospholipid

Table 3: PKC isoforms are divided into three subfamilies based on their mode of activation.

All known PKC isoforms have been shown to be expressed in the mammalian CNS (Tanaka and Nishizuka, 1994). However, all of the PKC isoforms have a very specific tissue distribution at different developmental time points in the CNS (Oehrlein et al., 1998). PKC is present in high concentrations in hippocampal neurons and plays a large role in many developmental processes, such as neuronal differentiation (Webb et al., 2000). Various PKC isoforms are present in hippocampal neurons at different developmental stages. A study by Roisin and Barbin (1997) looked at the presence of several different PKC isoforms (α, β, ε, γ, and ζ) in hippocampal cultures isolated from embryonic rats as well as in hippocampal protein extracts isolated from postnatal rat pups of varying ages using Western blot analysis (Figure 1.9). When analyzing protein content at specific ages, PKC α (Δ) is expressed at low levels until postnatal day 4 where it begins to rise (Figure 1.9A). In comparison, PKC γ (▲) is barely detectable until postnatal day 14. PKC ε (●) is detectable in the hippocampal preparations isolated from embryonic rats with protein levels peaking during the first postnatal week. PKC β (○) levels are low in embryonic tissue, but begin to rise starting postnatal day 0. There were two immunoreactive bands for PKC ζ (75 kDa and 83 kDa), which demonstrate different expression patterns throughout development (Figure 1.9B). The 75 kDa band (■) is more highly expressed in the embryonic hippocampus and is not highly expressed past the second postnatal week, whereas the 83 kDa band (□) protein levels peak after the first postnatal week (Roisin and Barbin, 1997). This is one of the few studies to explore the presence of the different PKC isoforms within hippocampal neurons during development. Since this study is well over ten years old, not all of the PKC isoforms were investigated.

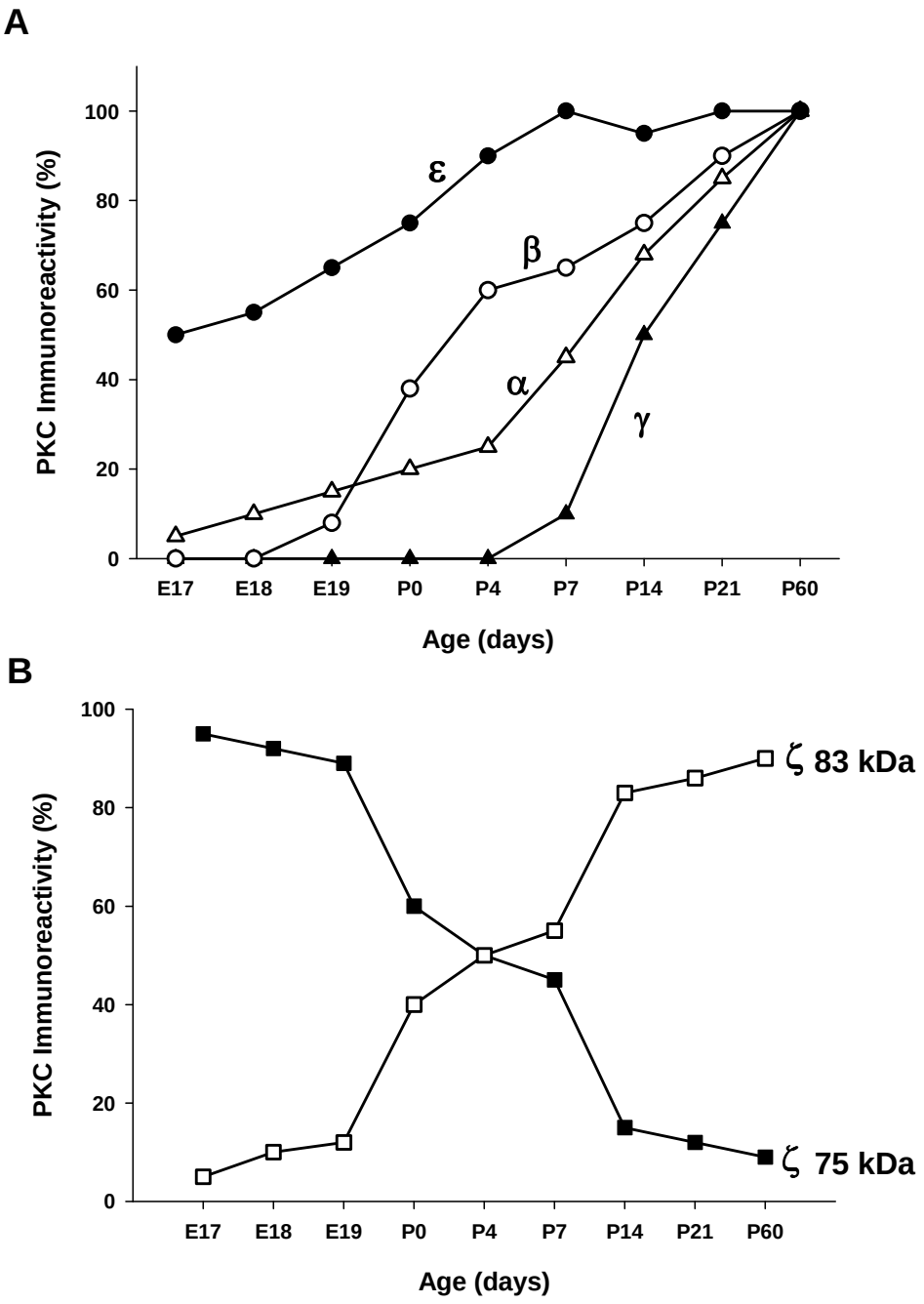

Figure 1.9: PKC isoforms vary throughout hippocampal development. Quantitative analysis by Western blot of PKC isoform expression in hippocampal protein extracts from rats of varying ages. (A) For PKC α, β, γ, and ε isoforms, data are expressed as the percentage of the value measured in the adult hippocampus. (B) For PKC ζ two bands were immunodetected. Each value is the mean of three determinations. Adapted from Neurochemistry International. Vol. 30, No. 3, Roisin MP, Barbin G, Differential expression of PKC isoforms in hippocampal neuronal cultures: Modifications after basic FGF treatment, 261-270, 1997, with permission from Elsevier.

Very few investigators have studied L-type calcium channel modulation by PKC and the studies that have been published demonstrate several conflicting results. There are many factors involved in determining whether phosphorylation of L-type calcium channels by PKC can either result in an increase or a decrease of calcium current. These include the type of cell, the type of calcium channel present, the type of accessory proteins associated with the calcium channels, as well as the specific isoform of PKC that is activated. PKC activation enhances L-type calcium current in cardiac myocytes, but CaMKII has also been shown to be involved in the signaling pathway (O-Uchi et al., 2008). When $Ca_v1.3$ channels were heterologously expressed in HEK cells, PKC activation with phorbol esters decreased whole-cell $Ca_v1.3$ current (Baroudi et al., 2006). Using single-channel recording, it was demonstrated that PKC activation reduced $Ca_v1.3$ current open probability, by decreasing open time and increased closed time (Chahine et al., 2007). Studies comparing the peak and sustained component of whole-cell currents in dorsal root ganglion cells from fetal mice in response to PKC activation, demonstrated a decrease in N-type calcium current with no effect on L-type calcium current (Gross and MacDonald, 1989). Each of these studies demonstrate a different response to PKC activation, either an increase, no change, or a decrease in L-type calcium current. These demonstrate the complexity of the signaling pathways involved in the regulation of calcium channels and the various factors that can affect the response to PKC activation.

PKC has also been shown to modulate the other HVA calcium channel types, N-type, P/Q-type and R-type calcium channels. When heterologously expressed in HEK cells, phosphorylation of both N-type and P/Q-type calcium channels by PKC increased the activity of the channels and also decreased the amount of inhibition of the channels

by Gβγ (Swartz, 1993; Zamponi et al., 1997; Herlitze et al., 2001). In Xenopus laevis oocytes, PKC activation enhanced both heterologously expressed N-type and R-type calcium currents, but did not affect either P/Q-type or L-type $Ca_v1.2$ calcium current (Stea et al., 1995). Stea et al., (1999) also determined that the enhancement of N-type and R-type calcium current was dependent on the coexpression of a β subunit. In comparison, other studies have shown that PKC activation can decrease N-type calcium current in the dorsal root ganglia of chick embryos through activation of α_2-adrenergic receptors by norepinephrine (Diversé-Pierlussi et al., 1993, 1995). In these cells N-type calcium current inhibition by activation of $GABA_B$ receptors with GABA is not dependent on PKC (Diversé-Pierlussi et al., 1993, 1995). PKC inhibitors had no effect on the GABA induced inhibition of N-type current in dorsal root ganglion cells. Using guinea pig hippocampus, Doerner et al. (1990) demonstrated N-type calcium current inhibition in response to direct activation of PKC in pyramidal cells, but they did not look for any effects in interneurons. Although expression of calcium channels in heterologous systems has provided a wealth of information about the characteristics of calcium channels and signaling mechanisms involved in their regulation, these mechanisms may be different to what is seen in native tissues.

Protein Kinase A

Many cellular functions involve the second messenger cyclic adenosine 3',5'-monophosphate (cAMP), whose concentration is modulated by adenylyl cyclase and phosphodiesterase (PDE) activity. Increased cAMP levels within neurons activates cAMP-dependent kinase or PKA, which phosphorylates target proteins. When in its

inactive form, PKA is a tetrameric structure consisting of two regulatory subunits along with two catalytic domains (Figure 1.10). There are four different regulatory subunits (RIα, RIβ, RIIα, RIIβ) and three catalytic subunits (Cα, Cβ, Cγ; for review see McKnight et al., 1988; Doskeland et al., 1993; Brandon et al., 1997). Each regulatory subunit contains two cAMP binding sites (Taylor et al., 1990). Following binding of cAMP to the regulatory subunit, a conformational change occurs that results in the dissociation of the catalytic subunits. Once released from the regulatory subunits, these monomeric catalytic subunits are now active and able to phosphorylate serine and threonine residues on downstream target proteins.

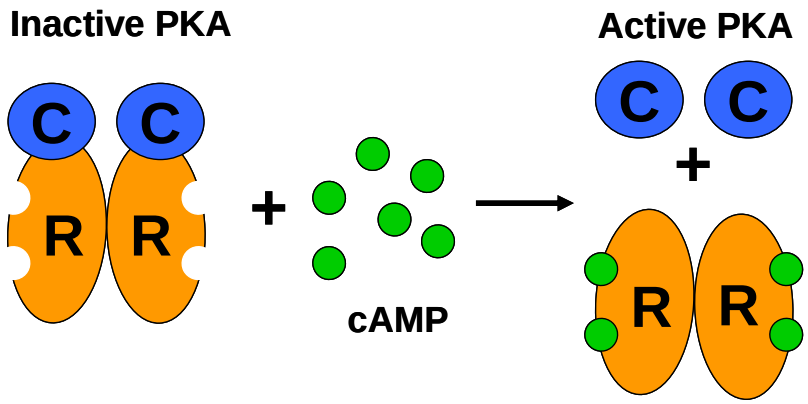

Figure 1.10: The structure of PKA consists of two regulatory subunits (R) and two catalytic subunits (C). In the absence of cAMP, the catalytic subunits associate with the regulatory subunits. As a result of cAMP binding, a conformational change in the regulatory subunit releases the catalytic subunits. The active catalytic subunits are free to phosphorylate proteins.

Adenylyl cyclase is an enzyme that synthesizes cAMP from adenosine-5'-triphosphate (ATP). Adenylyl cyclase activity can be either stimulated by $G\alpha_s$ resulting in an increase in cAMP levels or inhibited by $G\alpha_i$ resulting in decreased cAMP levels. cAMP levels are also controlled by PDE, an enzyme that breaks down cAMP. PKA regulates several functions within different cell types, including cellular metabolism,

changes in gene expression, cell and tissue development, neuronal excitability, ion channel conductivity, and changes in synaptic plasticity (for review see Brandon et al., 1997; Nguyen and Woo, 2003). Phosphorylation of L-type calcium channels by PKA often leads to the enhancement of intracellular Ca^{2+} concentration. For example in skeletal muscle, $Ca_v1.1$ L-type calcium current is enhanced through PKA phosphorylation of a single serine residue (Ser 1982) in the C-terminus of the α_1 subunit (De Jongh et al., 1996). This enhancement in intracellular Ca^{2+} levels ultimately increases the force of the contraction (Schmid et al., 1985; Arreola et al., 1987; Sculptoreanu et al., 1993a). Within the heart, β-adrenergic receptor activation leads to the enhancement of L-type calcium current through phosphorylation by PKA (for review see Kamp and Hell, 2000). Direct PKA activation by 8-Br-cAMP increases both the mean channel open time and probability of $Ca_v1.2$ L-type calcium channels in cultured rat cardiac myocytes (Reuter et al., 1982; Cachelin et al., 1983). Therefore, it is possible that PKA is involved in the signaling pathway of L-type calcium current by $GABA_B$ receptors in the neonatal rat hippocampus.

The overall goal of this research project was to continue exploring the novel phenomenon of L-type calcium current facilitation by $GABA_B$ receptor activation within the neonatal rat hippocampus. The first set of experiments investigated the developmental time course of L-type current facilitation by $GABA_B$ receptor activation in the first two developmental weeks to determine at which age facilitation was most prominent. Since many other investigators use either embryonic or adult hippocampal tissue for their studies rather than neonatal tissue, the facilitation of L-type calcium

current by $GABA_B$ receptor activation had not been previously demonstrated. The GABAergic system is not yet fully developed in embryonic hippocampal tissue and hippocampal cultures isolated from embryonic rats contain mostly excitatory pyramidal cells (Banker and Cowan, 1979; Scholz and Miller, 1991). Only 5-10% of neurons demonstrate GABA immunoreactivity in hippocampal cultures isolated from E17 rats, which is consistent with the excitatory synaptic potentials predominately observed in E17 embryonic cultures (Scholz and Miller, 1991). A greater percentage (12-36%) of neurons express GABA in cultures isolated from E18 rats (Scholz and Miller, 1991). Since there are very few GABAergic neurons in cultures isolated from early embryonic hippocampi, it could explain why facilitation of L-type calcium current has not been observed. Although the GABAergic system is fully developed in brain slices, it is problematic looking at receptor mediated effects on current in slices because the cells have many processes making it difficult to control the voltage. In acutely dissociated adult hippocampal damage occurs to surface proteins by either physical damage or trypsin mediated digestion and thus acute isolations are not an ideal preparation for these types of experiments. Hippocampal neurons isolated from adult tissue will not survive overnight in culture, whereas hippocampal neurons isolated from neonatal tissue will survive overnight in culture. Waiting overnight allows enough time for the neurons to recover from dissociation and for the reinsertion of channels or receptors. In addition, functional effects are likely to differ greatly in neonate versus adult due to major changes in the GABAergic system during the early neonatal period. Since facilitation has only been observed in neonatal cultures, it is possible that L-type calcium current facilitation could be involved in a developmentally regulated process.

Secondly, the role that L-type calcium current facilitation played in the regulation of chloride transporters during the maturation process of the GABAergic system was investigated. Due to the different subcellular distribution of N-type and L-type calcium channels on neurons *in vivo*, L-type channels are not generally linked to the regulation of neurotransmitter release. Rather activation of L-type calcium channels, which are localized on the cell body and dendritic region of neurons, can lead to a number of cellular effects ranging from gene activation to changes in postsynaptic activity (Westenbroek et al., 1990; Bading et al., 1993; Budde et al., 1998). It is possible that $GABA_B$ receptor mediated facilitation of L-type calcium current during the first few weeks of development may play a role in the regulation of chloride transporters that are necessary for the maturation of the GABAergic response in neurons.

Lastly, the signal transduction pathway of $GABA_B$ receptor modulation of L-type calcium channels in hippocampal neurons was explored using several kinase inhibitors as well as kinase activators. Since L-type calcium channels have several consensus sites for phosphorylation by kinases, the involvement of both PKC and PKA in the signaling pathway was investigated. Both PKC and PKA have been implicated in the signal transduction pathway of $GABA_B$ receptor facilitation of L-type calcium current in salamander retinal cells (Shen and Slaughter, 1999). Therefore, it is likely that PKC and PKA may be involved in the facilitation of L-type calcium current by $GABA_B$ receptor activation within the neonatal rat hippocampus.

CHAPTER 2

MATERIALS AND METHODS

Isolation of Hippocampal Neurons

All animal protocols were approved by the Marquette University Institutional Animal Care and Use Committee and followed the guidelines set forth by the U.S. Public Health Service. Neurons were isolated by a technique developed for the hippocampus of postnatal rats as published previously (Mynlieff, 1997). Sprague-Dawley rat pups of varying ages were anesthetized with CO_2 and sacrificed by decapitation. The head was immersed in 70% ethanol for 3 minutes for decontamination and rinsed in sterile rodent Ringer's solution with glucose (146 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 11 mM D-glucose, pH 7.4 with NaOH). The hippocampi were dissected from the brain in oxygenated, cold (~5°C), sterile rodent Ringer's solution with glucose using sterile technique. For electrophysiology experiments, dissections were restricted to the superior region of the hippocampus (excluding the dentate gyrus). For protein analysis of transporters, the entire hippocampus was used for cultures. The tissue was placed in PIPES-buffered saline (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM D-glucose, 20 mM piperazine-N,N'-bis[2-ethanesulfonic acid], pH 7.0 with NaOH) and sliced into ~1 mm^3 sections. The tissue was transferred into a small vial and incubated at room temperature in 1 ml of 0.5% Trypsin XI and 0.01% DNase I (Sigma-Aldrich, St. Louis, MO) in PIPES buffered saline at room temperature for 20-30 minutes with 100% oxygen blown over the tissue. This incubation was followed by another incubation for 40-60 minutes depending on the age of the pup at 35°C under continuous oxygen. The tissue was rinsed with 1 ml trypsin inhibitor solution (1 mg/ml trypsin

inhibitor, Type II-O: chicken egg white (Sigma-Aldrich, St. Louis, MO) and 1 mg/ml bovine serum albumin) in rodent Ringer's solution and rinsed again with 1 ml Neurobasal-A growth medium fortified with B27 supplement (Invitrogen, Carlsbad, CA), 0.5 mM glutamine, and 0.02 mg/ml gentamicin. The tissue was triturated with a fire-polished Pasteur pipette in fresh growth medium and plated onto the center of poly-L-lysine coated dishes for both electrophysiology and protein analysis. The culture dishes were coated with 1mg/ml of poly-L-lysine (MW 38,500 to 60,000, Sigma-Aldrich, St. Louis, MO) dissolved in 0.15 M boric acid (pH of 8.4 with NaOH). A detailed protocol for making poly-L-lysine dishes can be found in Mynlieff (1997). Cells were allowed to settle to the bottom of the dish for 5 minutes prior to the addition of growth medium to bring the volume up to 2 ml per dish. Neurons were incubated at 37°C in a 5% CO_2 water-jacketed incubator.

There are many advantages to using cultured cells for electrophysiological studies. Cultured cells have very few processes which allows for proper control over voltage and prevents space clamp issues when performing patch clamp recordings. In addition, it is possible to apply compounds directly to an individual cell without contaminating other cells within the culture by using a U-tube system (Mynlieff, 1997). Cultured cells provide much more stable recordings compared to acutely isolated hippocampal cells. Waiting 20 - 24 hours after dissociation allows time for the reinsertion of channels and receptors after the processes have been sheared off. For example, N-type and P/Q-type calcium channels, which are primarily found on processes, are reinserted into the cell body of the neuron (Figure 2.1; courtesy of Katie Halstead, a former undergraduate). Therefore, it is possible to study the modulation of channels

normally found in the synapse on the soma. Hippocampal cultured neurons isolated in the Mynlieff laboratory retain their electrophysiological properties and firing properties after dissociation (Mynlieff, 1997, 1999).

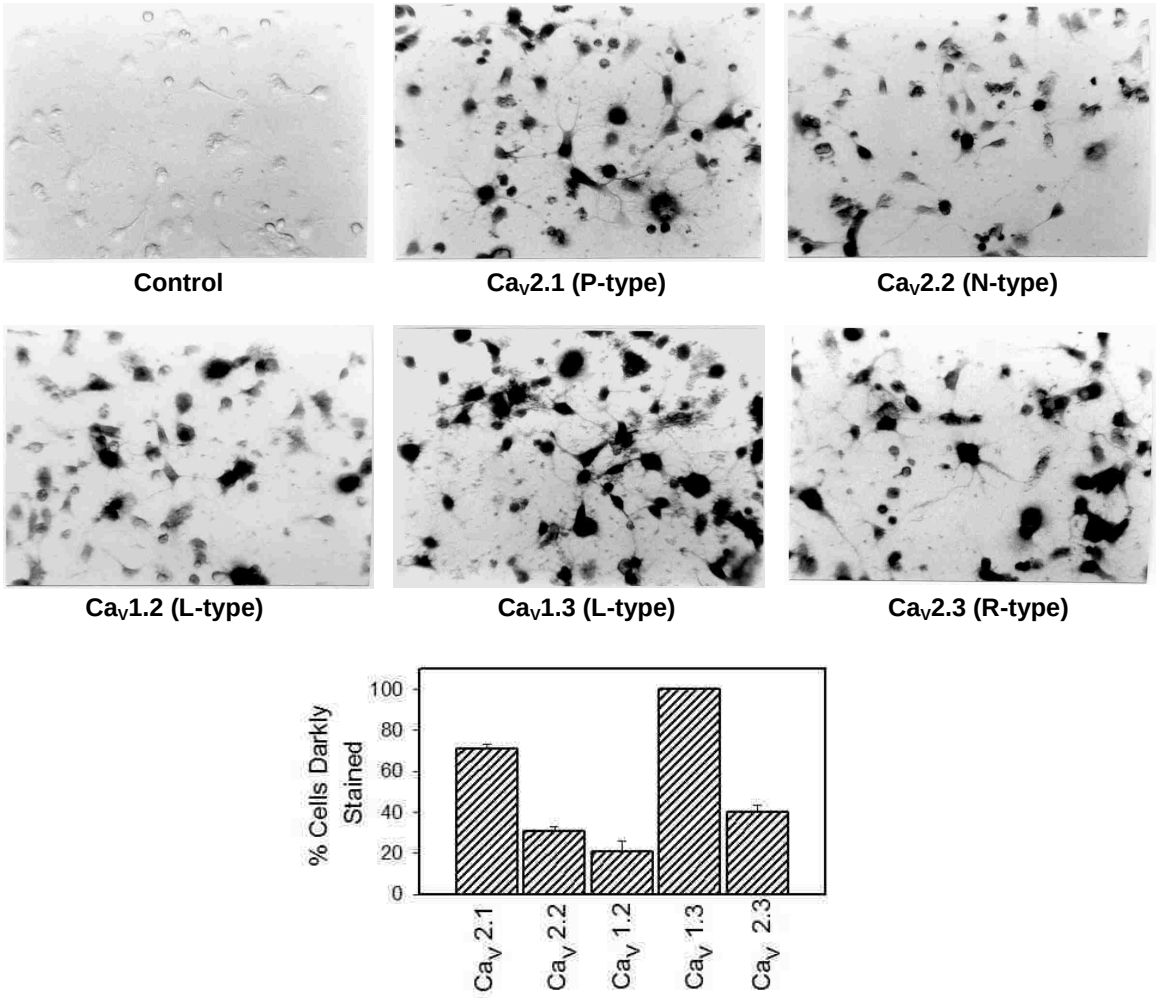

Figure 2.1: Reinsertion of HVA calcium channels. To determine the presence of different HVA calcium channel types, hippocampal cultures isolated from postnatal rats 6 to 9 days old were labeled with primary antibodies against the α_1 calcium channel subunits 24 hours following dissociation. All cultures treated with antibodies 24 hours after dissociation displayed staining for all of the α_1 subunits tested, suggesting that 24 hours is sufficient time for the reinsertion and stabilization of calcium channels in the membrane. Approximately only 20% of the cells stained darkly for the $Ca_V1.2$ (L-type) calcium channel, while almost 100% of the cells stained uniformly and intensely with the $Ca_V1.3$ (L-type) calcium channel. Data was obtained by Katie Halstead.

Hippocampal cultures isolated from embryonic rat pups are predominantly comprised of excitatory pyramidal cells, and thus are not ideal for the study of hippocampal interneurons (Banker and Cowan, 1979; Scholz and Miller, 1991). One of the benefits of using postnatal rat hippocampal cultures is that they are highly enriched in interneurons, making them an ideal system for studying the effects of $GABA_B$ receptor modulation of calcium channels during development. When cultures isolated from a 7 day old postnatal rat were labeled with anti-GABA antibodies, 41.8 ± 5.0% of the neurons contained GABA suggesting that almost 50% of the cells in the culture were GABAergic inhibitory interneurons (Mynlieff, 1997). One of the disadvantages to using cultured neurons is that the superior region of the hippocampus (CA1 and CA3) contains pyramidal cells along with several different types of inhibitory interneurons. After such a short time in culture it is not possible to identify the cells based on morphology (Mynlieff, 1997).

Electrophysiology

Whole cell patch clamp recording was used to measure calcium currents in voltage clamp mode. Data were collected using a Dagan 3900A patch clamp amplifier (Dagan Corporation, Minneapolis, MN) combined with a Digidata 1322 data acquisition system and a computer with pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). All electrophysiological experiments were performed at room temperature 20-24 hours following dissociation. Although voltage control is best immediately following dissociation, it was necessary to wait overnight to allow the cells time to recover from enzymatic dissociation and adhere to the bottom of the dish (Mynlieff, 1997). Recording

electrodes (3-7 MΩ) were made from borosilicate glass capillaries on a Flaming/Brown Micropipette Puller model P-87 (Sutter Instrument Co., Novato, CA). The internal solution used to fill the patch electrodes contained 140 mM Cs-aspartate, 5 mM $MgCl_2$, 10 mM Cs_2EGTA, 10 mM HEPES, 2 mM ATP-Na_2, and 0.1 mM GTP (pH of 7.4 with CsOH and osmolarity between 310-320 mOsm). The external solution used for calcium current measurements contained 10 mM $CaCl_2$, 145 mM TEACl, 10 mM HEPES and 1 μM tetrodotoxin (Sigma-Aldrich, St. Louis, MO) to block Na^+ currents (pH of 7.4 with CsOH and osmolarity between 300-310 mOsm). Whole cell currents were electronically filtered at 1 kHz and digitized at 2 kHz. Linear components of leak current were subtracted post-hoc by the passive resistance protocol in pClamp 9.0.

For the majority of the electrophysiology experiments, the sustained component of the calcium current was used versus the peak component of the calcium current (Figure 2.2). The peak current is a combination of several different types of calcium current, which can hamper the interpretation of these data. Since L-type calcium channels exhibit slow inactivation kinetics, the sustained current component at the end of the 300 msec depolarization pulse to +10 mV was measured to minimize the contribution of T-type, N-type, P/Q-type, and R-type calcium current while maximizing the contribution of L-type calcium current to the total measurement. The inactivation rate for L-type calcium current is very slow ($\tau > 500$ msec), whereas the inactivation rates for N-type, P/Q-type, and R-type calcium current is fast ($\tau \approx 50$-80 msec; Tsien et al., 1988). There may still be contributions of the other current types at the end of the 300 msec pulse, but the majority of the sustained current should be primarily comprised of L-type current due to its slower inactivation kinetics when compared to the other HVA calcium

current types. Previous studies in which nimodipine, an L-type calcium channel antagonist, blocked the ability of baclofen to facilitate sustained current have confirmed that the facilitatory effect of $GABA_B$ receptor activation on calcium currents was entirely mediated through L-type channels (Carter and Mynlieff, 2004).

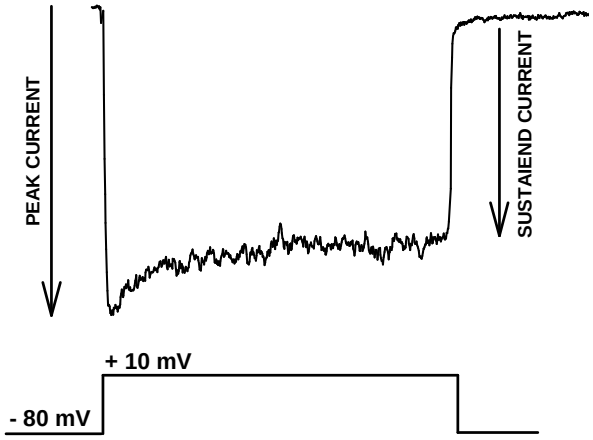

Figure 2.2: Peak versus sustained current. Calcium currents were elicited by a 300 msec depolarization to +10 mV from a holding potential of -80 mV. The peak current is measured at the beginning of the 300 msec pulse, while the sustained current is measured at the end of the 300 msec pulse.

Drugs and Enzymes Used in Electrophysiology Experiments

Multiple kinase inhibitors with different chemical structures as well as specific kinase activators were used for verification of results on whether PKC and/or PKA are involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptor activation. At certain concentrations kinase inhibitors can lose their specificity for a particular kinase, therefore the concentrations used for each compound were carefully chosen. Experimental concentrations were determined by examining the literature as well as comparisons with the IC_{50} / EC_{50} (Table 4). If a compound interacted with a single kinase a higher concentration could be used, whereas lower concentrations were necessary for compounds that were less specific for a particular kinase and may interact with other kinases at higher concentrations.

Compound	Action	Concentration Used	IC_{50} / EC_{50}
PKC fragment 19-36	inhibits PKC	2 μM	147 nM
GF-109203 X	inhibits PKC	500 nM	10 nM
Chelerythrine Chlroide	inhibits PKC	5 μM	0.66 μM
Phorbol Ester (PMA)	activates PKC	1 μM	1 nM
H-89 dihydrochloride hydrate	inhibits PKA	1 μM	48 nM
Rp-cAMPS	inhibits PKA	30 μM	11 μM
8-BR- cAMP	activates PKA	500 μM	166 μM

Table 4: Kinase activators and inhibitors for PKC and PKA. The above compounds were used to investigate the signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptor activation.

(RS)-baclofen (Tocris, Ellisville, MO) and 8-Bromoadenosine 3´, 5´- cyclic monophosphate (8-Br-cAMP, Sigma-Aldrich, St. Louis, MO) were directly dissolved in the external calcium solution. GF-109203X (AG Scientific, INC., San Diego, CA) and phorbol-12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA) were dissolved in dimethyl sulfoxide (DMSO) at 1000 - 5000 fold the final concentration. Rp-cAMPS triethylammonium salt hydrate, H-89 dihydrochloride hydrate, and chelerythrine chloride (Sigma-Aldrich, St. Louis, MO) were dissolved in water at 333 – 10,000 fold the final concentration. All concentrated stock solutions of the drugs were stored at -20°C until use up to two months. These compounds were diluted to their final concentration in the external calcium solution on the day of use. The drugs dissolved in the external calcium solution were applied to cells using a U-tube delivery system, constructed with PE-10 polyethylene tubing housed in a piece of glass tubing, which allowed for quick application and washout of compounds that were gravity fed onto the cell and removed by vacuum suction. A blue dye, Fast Green FCF (Sigma-Aldrich, St. Louis, MO), was added to the drug solution to visualize application of the drug onto the cell and to ensure complete washout of the drug off the cell. Protein kinase C fragment 19-36 (Sigma-Aldrich, St. Louis, MO) was dissolved in 1% acetic acid and included in the internal

solution with less than a 0.1 change on the pH of the internal solution. Pertussis toxin (PTX, Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water at 500 fold the final concentration with bovine serum albumin (4 mg/ml) added for stabilization. Cultures were exposed to 200 ng/ml PTX for a minimum of 16 hours starting two hours after dissociation. A longer exposure time is necessary with PTX treatment, to ensure complete inhibition of $G_{i/o}$ proteins. This concentration and time of pre-treatment is similar to the concentration (250 ng/ml) and time of PTX exposure (26 hrs) that Scholz and Miller (1991) used to demonstrate the effect of baclofen on calcium currents in hippocampal cell cultures. Higher concentrations as well as a longer PTX exposure time would be necessary for complete inhibition of $G_{i/o}$ proteins if hippocampal slices were being used for electrophysiological experiments.

Electrophysiology Data Analysis and Statistics

To analyze electrophysiological data, the magnitudes of the currents in response to a +10 mV depolarizing pulse were plotted as a scatter graph vs. time. The linear regression and 95% confidence intervals were determined for the control current data points and were used to determine the effect of 10 μM baclofen application on calcium current amplitude as described in Carter and Mynlieff (2004). Multiple control currents were measured before and after drug application. This allowed for compensation due to run-up or run-down of calcium currents (Figure 2.3). The percent change with baclofen application was determined by comparison to the regression line. The cells were grouped according to whether baclofen application caused a deviation in the magnitude of the calcium current when compared to the linear regression line determined by the control

data. The change in current magnitude was considered significant if it fell outside of the 95% confidence interval for the regression line. If the current with baclofen application fell above the 95% confidence interval the cells were grouped as demonstrating an increase in current and if it fell below the cells were grouped as demonstrating a decrease in current. In cells where the current with baclofen application fell within the 95% confidence interval, the cells were grouped together as demonstrating no change.

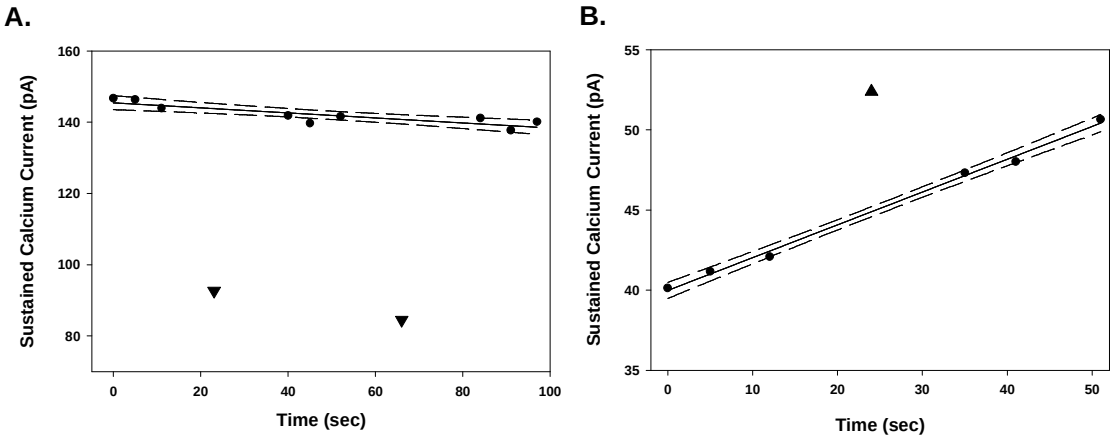

Figure 2.3: Analysis of calcium currents. Calcium currents were elicited by a 300 msec depolarization to +10 mV from a holding potential of -80 mV. These data represent sustained calcium currents recorded from cultured hippocampal neurons isolated from 7 day old rats. The linear regression (solid line) and 95% confidence interval (dashed line) were determined for the control currents (●). The percent change with 10 μM baclofen application (▲) was determined by comparison to the regression line. In all cells where the current with baclofen fell within the 95% confidence interval the effect was counted as "no change".

In experiments where kinase inhibitors or kinase activators were used, a contingency table was set up as a 2X3 table where row 1 and row 2 contained control cells and cells treated with a particular compound. The three columns contained the number of cells in each row demonstrating an increase, no change, or a decrease in sustained calcium current in the presence of baclofen. A chi-square was used to determine whether the columns in the contingency table vary from row to row. A Fisher exact test was used for pairwise comparisons. To determine whether the number of cells

that demonstrated an increase in calcium current with baclofen application in the absence and presence of a drug was significantly different, the number of cells that demonstrated no change or a decrease in calcium current with baclofen application were grouped together making the pair-wise comparison between the cells demonstrating an increase and "other" (cells demonstrating a decrease plus cells demonstrating no change). The opposite was done to determine whether the number of cells that demonstrated a decrease in calcium current with baclofen application in the absence and presence of a drug was significantly different. For this case the number of cells that demonstrated no change or an increase in calcium current with baclofen application were grouped together making the pair-wise comparison between the cells demonstrating a decrease and "other" (cells demonstrating an increase plus cells demonstrating no change). Averaged data are always given as mean ± standard error of the mean (S.E.M.).

Western Blot Analysis

For Western blot analysis, whole hippocampal tissue was obtained from rats ranging from 1 to 42 days old (D1 to D42) and from hippocampal cultures that were obtained from postnatal day 0 rats and kept in culture for 1 to 15 days (C0-1 to C0-15). A total volume of 2 ml of medium was maintained for cultures that were used for protein analysis of transporters. Twenty four hours after plating the cells, 1 ml of growth medium was removed and fresh medium was added to the dish. A volume control dish containing 2 ml of growth medium was placed in the incubator and the appropriate amount of fresh medium was added every other day to maintain the correct volume. For cultures treated with drugs, 2 μl of stock solutions of either 5 mM nimodipine (Sigma-

Aldrich, St. Louis, MO) dissolved in 5 M HCl or 10 mM (RS)-baclofen (Tocris, Ellisville, MO) dissolved in methanol, were added daily for a total of seven days to maintain concentrations of 5 μM or 10 μM, respectively. In addition, a few cultures were also treated with 2-hydroxysaclofen (Tocris, Ellisville, MO), a $GABA_B$ antagonist. The 2-OH-saclofen was dissolved in 1 M NaOH to give a 10 mM stock solution. To maintain a 10 μM concentration, 2 μl of the 2-OH-saclofen stock solution was added daily for a total of seven days. Chloride transporter levels were also tested in cultures treated with drug vehicles alone (methanol, HCl, and NaOH). No significant differences were found in transporter expression levels in comparison to the control cultures (Courtesy of Eddie Brotkowski).

Proteins were extracted by homogenization in an ice-cold sucrose buffer (250 mM sucrose, 10 mM Tris, 10 mM HEPES, 1 mM EDTA, pH 7.2) with fresh protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5 mg/ml pefabloc; Sigma-Aldrich, St. Louis, MO) followed by centrifugation at 3622 x g for 10 minutes at 4°C. The supernatant was centrifuged at 39,104 x g for 30 minutes at 4°C. The final pellet was resuspended in homogenizing buffer and stored at -80°C for less than 6 months. Protein concentration was measured with an enhanced BCA protein assay (Pierce, Rockford, IL).

NuPAGE lithium dodecyl sulfate (LDS) sample buffer and sample reducing agent (Invitrogen, Carlsbad, CA) were added to the membrane extracts and heated at 37°C for 30 minutes. The membrane extracts were run on a 3-8% Tris-acetate Novex Minigel and transferred to a polyvinylidene difluoride (PVDF) membrane (pore size 0.45 μm) for KCC2 experiments and a nitrocellulose membrane (pore size 0.45 μm) for NKCC1 experiments in NuPAGE transfer buffer (Invitrogen, Carlsbad, CA). The membranes

were washed with phosphate buffered saline (PBS, 134.4 mM NaCl, 4.36 mM KCl, 10.56 mM Na_2HPO_4, 1.66 mM NaH_2PO_4 and pH to 7.4 with HCl) and blocked for 1 hour in PBS containing 0.05% Tween, 5% nonfat dry milk, and 0.1% bovine serum albumin at room temperature. The membranes were decorated with primary antibodies in the PBS blocking solution against KCC2 (1:1000; Upstate Technologies, New York, NY) or NKCC1 (1:2000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) overnight at 4°C. Following a 1 hour wash in PBS, the membranes were incubated with either a goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibody (1:1000; Pierce, Rockford, IL) in the PBS blocking solution for 2 hours at room temperature. The SuperSignal West Dura Extended Duration chemiluminescent enhancement kit (Pierce, Rockford, IL) was used to visualize the protein bands with classic blue autoradiography film (Molecular Technologies, St. Louis, MO).

Quantification of Western Blots

To quantify protein on Western blots, the background intensity was first subtracted using ImageJ software (developed at U.S. National Institutes of Health and available at http://rsb.info.nih.gov/ij/). The integrated optical density (IOD) for each band was determined using Labworks 4.6 imaging and analysis software (UVP, Inc., Upland, CA). To control for gel loading, the IOD for each protein was divided by the IOD of a band in the same lane labeled with either anti-β-actin antibodies (1:1000; Cell Signaling Technologies, Danvers, MA) or anti-β-tubulin antibodies (1:5000; Sigma-Aldrich, St. Louis, MO). Multiple protein samples were analyzed per developmental

time point studied for both whole hippocampal protein extracts and cultured hippocampal protein extracts. Averaged data are always given as mean ± S.E.M.

CHAPTER 3

The developmental time course of L-type calcium current facilitation by $GABA_B$ receptor activation within the rat hippocampus

INTRODUCTION

The maturation and role of $GABA_A$ receptors early in neonatal development has been extensively studied. In comparison, there is considerably less known about the developmental changes of $GABA_B$ receptors. The current study was designed to enhance our knowledge of $GABA_B$ receptor function during development. Previous electrophysiological experiments in the lab performed on hippocampal neurons dissociated from the superior region of 5 to 7 day old rat pups have demonstrated both an inhibition and facilitation of calcium current in response to $GABA_B$ receptor activation (Carter and Mynlieff, 2004). Using specific calcium channel blockers Carter and Mynlieff (2004) confirmed that attenuation of calcium current was through N-type calcium channels while facilitation was through L-type calcium channels. $GABA_B$ receptor facilitation of HVA calcium current has been shown in a number of cell types, including salamander retinal neurons (Shen and Slaughter, 1999), adrenal chomaffin cell cultures (Parramón et al., 1995), and rat dorsal root ganglion (Fujikawa et al., 1997). In both salamander retinal neurons and adrenal chromaffin cell cultures, the facilitation of calcium current was blocked by DHPs, demonstrating that the effect was entirely through L-type calcium channels. The type of HVA calcium current involved in the facilitation by baclofen in dorsal root ganglion was not investigated (Fujikawa et al., 1997).

Calcium is one of the most important intracellular messengers within neurons. Calcium influx through VDCCs is not only important in processes of mature neurons, but it also plays a crucial role in many developmental processes. Calcium influx is involved in neuronal differentiation, growth, and maturation of developing neurons. Interestingly, during the early developmental period neuronal GABAergic synaptic transmission is

excitatory and switches to inhibitory within the first few postnatal weeks of development (Ben-Ari et al., 1989; Owens et al., 1999). The excitatory GABAergic potential can activate VDCCs, specifically L-type calcium channels, which results in an elevation of intracellular calcium concentrations (Yuste and Katz, 1991; Leinekugel et al., 1995; Khazipov et al., 1997). In turn the elevated intracellular calcium concentration can induce an increase in brain-derived neurotrophic factor (BDNF) levels, promote neuronal survival and differentiation, and may be necessary to form and strengthen synaptic connections (Berninger et al., 1995; Ikeda et al., 1997; Kirsch and Betz, 1998; Kneussel and Betz, 2000). Thus, it is possible that enhancement of L-type calcium current by $GABA_B$ receptor activation plays an important role during the first two postnatal weeks in hippocampal development.

There have only been a few studies to demonstrate facilitation of L-type calcium current by $GABA_B$ receptor activation (Parramón et al., 1995; Shen and Slaughter, 1999; Carter and Mynlieff, 2004). Since this phenomenon has only been demonstrated in hippocampal tissue isolated from neonatal rats 5 to 7 days old, it may only be present at a specific time point in development. The developmental time course of L-type calcium current facilitation by $GABA_B$ receptor activation was determined using whole-cell patch clamp experiments on hippocampal cultures dissociated from neonatal rats of varying ages. Neurons from the superior region of the hippocampus were isolated by a technique that has been well established in our laboratory (Mynlieff, 1997). Hippocampal cultures prepared in our laboratory contain excitatory pyramidal cells along with several different types of inhibitory interneurons. To decrease the heterogeneity of the cell cultures, only the superior region of the hippocampus containing the CA3 and CA1 regions was used.

These types of experiments are very difficult to perform using brain slices due to space clamp issues. Since cultured cells are spherical for the first 24 hours in culture and do not contain many processes, one has better control over the currents and the environment of the cell. The patch clamp technique allows for detailed analysis of ion currents and individual cell modulation. For the present study, electrophysiological experiments with the $GABA_B$ agonist baclofen were performed on cultured hippocampal neurons obtained from different aged rats to identify the timecourse of L-type calcium current facilitation by $GABA_B$ receptor activation. To determine if the varying baclofen responses observed at different developmental time points were due to changes in the number and type of calcium channels present, the density of HVA and LVA calcium current was measured in cultures obtained from different aged pups.

RESULTS

Calcium Currents in Cultured Hippocampal Neurons

Families of calcium currents were initially recorded in experiments to characterize the baclofen responses in hippocampal cultures isolated from neonatal rats. Families of calcium currents were elicited from a holding potential of -80 mV by 300 msec depolarizing test steps between -50 mV and +50 mV in 10 mV increments (Figure 3.1). Due to the heterogeneity of our cell cultures, each cell contains a different complement of calcium channels. Therefore, there can be a wide range of activation voltages seen in different cells. These values are also affected by the concentration and identity of the charge carrier used (Ba^{2+} and Ca^{2+}). Using 10 mM Ca^{2+} as the charge carrier gave currents that generally activated between -30 mV and -10 mV depending on the presence of T-type calcium channels (Figure 3.1). The current-voltage relationship shown in figure 3.1B shows that from a holding potential of -80 mV, the current turns on positively to the -10 mV test step and peaks at +20 mV. T-type calcium currents are activated at low voltages and inactivate very rapidly as can be seen in the family of currents represented in figure 3.1C. The current-voltage relationship shown in figure 3.1D shows that from a holding potential of -80 mV, this cell appears to have two activation voltages. The LVA current turns on around -40 mV with the HVA component appearing around -10 mV. The early activation voltage is due to the presence of T-type calcium current in this cell. In contrast to the cell represented in figure 3.1 C and D, the cell in figure 3.1 A and B, does not exhibit T-type current as demonstrated by the lack of a shoulder in the current-voltage relationship.

Calcium current demonstrates rapid run-up or run-down of currents. Thus, it was important to obtain control and baclofen data in the shortest time period possible. To investigate $GABA_B$ mediated effects on HVA currents, the cells were held at -80 mV and

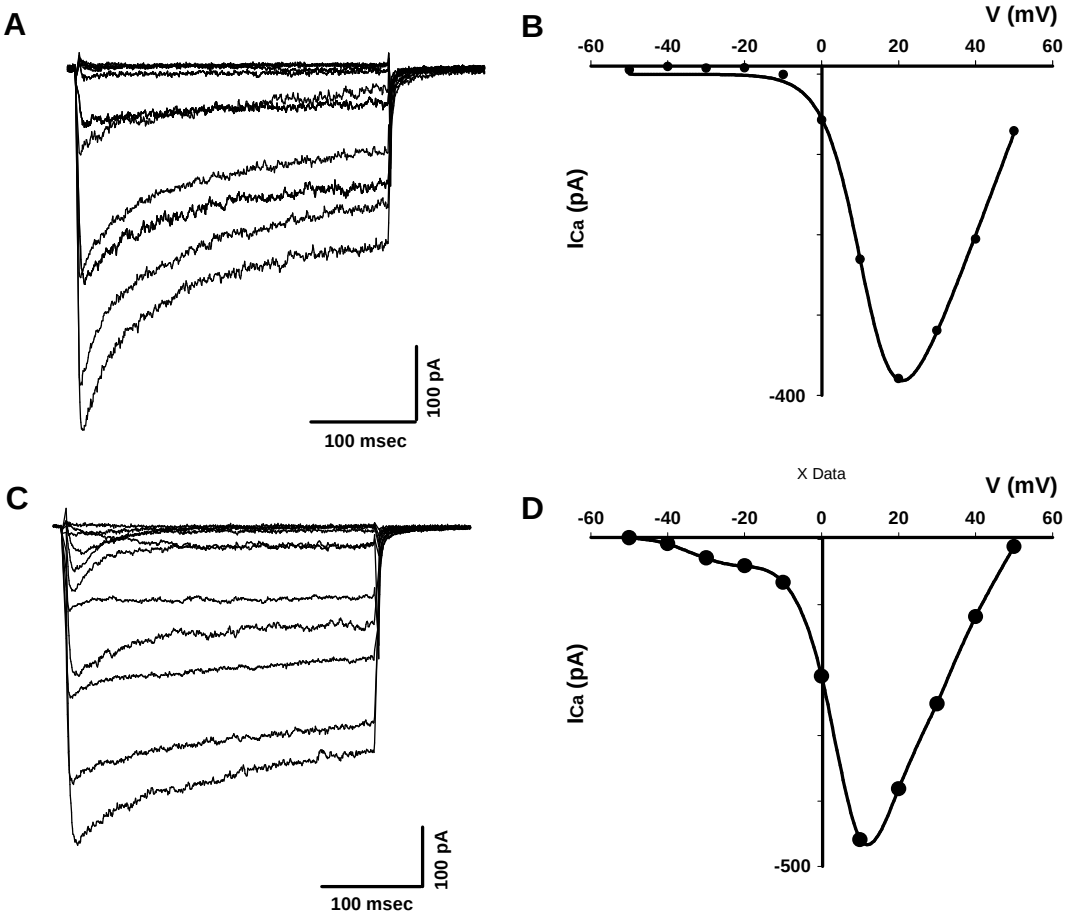

Figure 3.1: Calcium currents isolated in hippocampal neurons from 7 day old rats. (A) and (C) represent families of whole cell calcium currents elicited from a holding potential of -80 with 300 msec long depolarizing test steps from -50 mV to +50 mV in 10 mV increments. (B) and (D) represent the current-voltage relationship of the total calcium current from the same cells shown in (A) and (C).

depolarized with a 300 msec pulse to +10 mV. This allowed for rapid measurements in varying conditions (control, drug, and washout) minimizing run-up or run-down. Calcium currents in cultured hippocampal neurons isolated from postnatal rats of varying ages are modulated by activation of $GABA_B$ receptors with baclofen. Three different

responses are seen with baclofen application. Calcium currents either demonstrate a decrease, an increase, or no change in response to $GABA_B$ receptor activation (Figure 3.2).

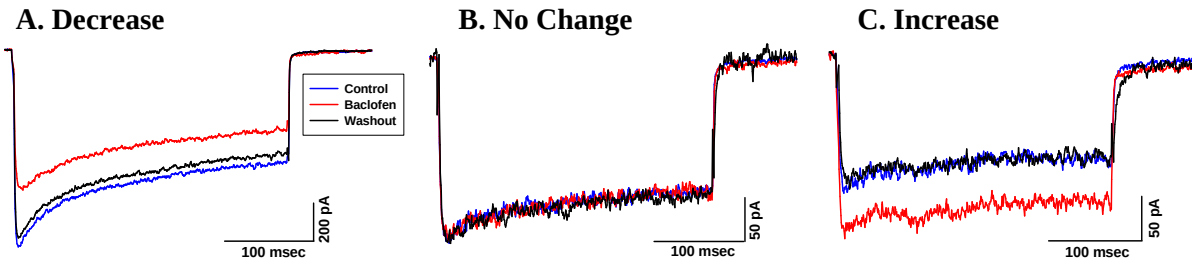

Figure 3.2: Total calcium currents were measured using whole-cell voltage clamp. The cells were held at -80 mV and stepped to +10 mV for 300 msec. The black trace represents the control current, the red trace represents when 10 μM baclofen was applied to the cell, and the blue trace represents the currents after baclofen was washed off of the cell. (A) This cell illustrates inhibition of total calcium current in the presence of 10 μM baclofen. (B) This cell did not show a response to 10 μM baclofen application. (C) This cell shows facilitation of total calcium current in response to 10 μM baclofen.

Time Course of Calcium Current Modulation by $GABA_B$ Receptors

To determine whether facilitation of L-type calcium current by $GABA_B$ receptor activation is developmentally regulated, calcium currents were recorded in neurons isolated from hippocampi of 0, 3, 7, and 14 day old rat pups using whole-cell voltage clamp recording in the absence and presence of the $GABA_B$ agonist baclofen (10 μM). Currents were elicited by a 300 msec depolarizing pulse from a holding potential of -80 mV as described in Chapter 2. A total of 22-32 cells from each developmental time point studied were analyzed to determine both the percent of total cells that exhibited each response to baclofen application and the average magnitude of the response. In addition, both the peak and sustained components of the currents were analyzed. Measuring the sustained component of the current at the end of a 300 msec pulse should minimize the

contributions from P/Q-type and N-type calcium currents, thereby increasing the contribution of L-type calcium current.

The percentage of cells demonstrating an increase, decrease, or no change in total calcium current depended on the age from which the neurons were isolated. At the early time points more neurons demonstrated a decrease in peak calcium current in response to application of 10μM baclofen, while more neurons demonstrated an increase in peak calcium current at the later time points studied (decrease, $P = 0.039$; increase $P < 0.001$; using a Chi-square, Figure 3.3A). In cultures isolated from 0 day old animals, 59.1% of the cells demonstrated an inhibition of peak calcium current in response to baclofen application. The percentage of cells demonstrating a decrease in peak calcium current was not significantly different in cultures isolated from 3 day old animals (30.5%). However, the percentage of cells demonstrating a decrease in peak calcium current was significantly lower in 7 day old animals (25%, $P = 0.022$, pairwise comparisons were determined with a Fisher's Exact test) and 14 day old animals (25%, $P = 0.035$, pairwise comparisons were determined with a Fisher's Exact test). In the cells that demonstrated a decrease in peak calcium current from hippocampal cultures isolated from 0 day old rats, the average decrease in total current was 20.30 ± 2.95 % (Figure 3.3B). The average percent decrease was not significantly different from the other time points studied ($P = 0.543$ using an ANOVA). In comparison to the cells demonstrating a decrease in peak current, more cells demonstrated an increase in peak current in response to baclofen application during the second postnatal week. The percent of cells that demonstrated facilitation of peak calcium current is different at the various ages studied ($P < 0.001$ using a Chi-square). Only 9.1% of cells isolated from 0 day old animals and no cells

isolated from 3 day old rat pups demonstrated an increase in peak calcium current. The percent of cells demonstrating an increase in peak calcium current was 31.3% in cultures isolated from 7 day old rat pups and 16.7% in cultures isolated from 14 day old rat pups.

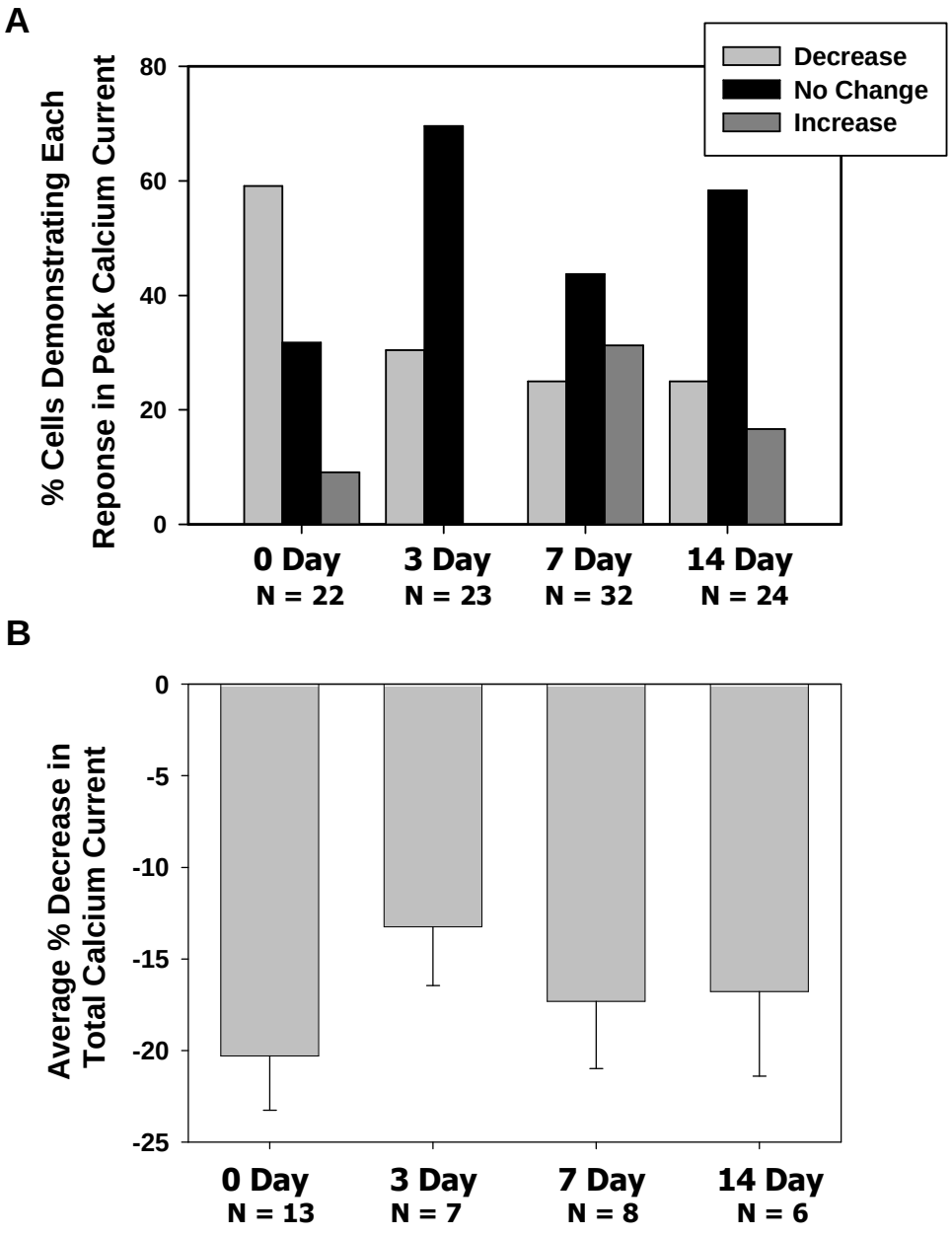

Figure 3.3: Modulation of peak calcium currents by $GABA_B$ receptor activation at different developmental time points. (A) Each bar represents the percentage of cells demonstrating a decrease, no change, or an increase in peak calcium current following application of 10 μM baclofen in hippocampal cultures isolated from postnatal rats ranging from 0 days to 14 days old. (B) Average percent decrease in peak current in cells demonstrating a decrease in response to baclofen (P = 0.543 using an ANOVA).

Peak current is a combination of L-type, N-type, P/Q-type, R-type, and T-type calcium current hampering interpretation of these data. Thus, the sustained current component at the end of a 300 msec depolarization pulse to +10 mV was measured minimizing the contribution of N-type, P/Q-type, and R-type calcium current while maximizing the contribution of L-type calcium current to the total measurement. Previous studies in the laboratory have already confirmed that the facilitatory effect of $GABA_B$ receptor activation on calcium currents was entirely mediated through L-type channels (Carter and Mynlieff, 2004). There was not a significant difference in the percent of cells demonstrating facilitation of sustained calcium current in response to baclofen application in hippocampal cultures isolated from 0, 3, 7, or 14 day old rat pups when time points were analyzed separately (Figure 3.4A; P = 0.054 using a Chi-Square). Since the number of cells isolated from 0 and 3 day old rats responding with facilitation were very low and similar and the numbers of cells isolated from 7 and 14 day old rats responding with facilitation were much higher, the data were re-analyzed and grouped into early postnatal (day 0-3) and late postnatal (day 7-14) time points. When the cells were grouped into early and late postnatal period, they were significantly different (P = 0.023 using a Chi-Square). The average percent increase in sustained calcium current in the cells that demonstrated an increase in calcium current with baclofen treatment were not significantly different at the developmental time points studied (Figure 3.4B; P = 0.136 using an ANOVA). The cells from 3 day old animals were not included in the statistical analysis, because there were only 2 cells that demonstrated an increase in response to baclofen. These experiments demonstrated that the percent of cells demonstrating facilitation of L-type calcium current by $GABA_B$ receptor activation does

vary throughout the first two weeks of development and is most prominent during the second postnatal week.

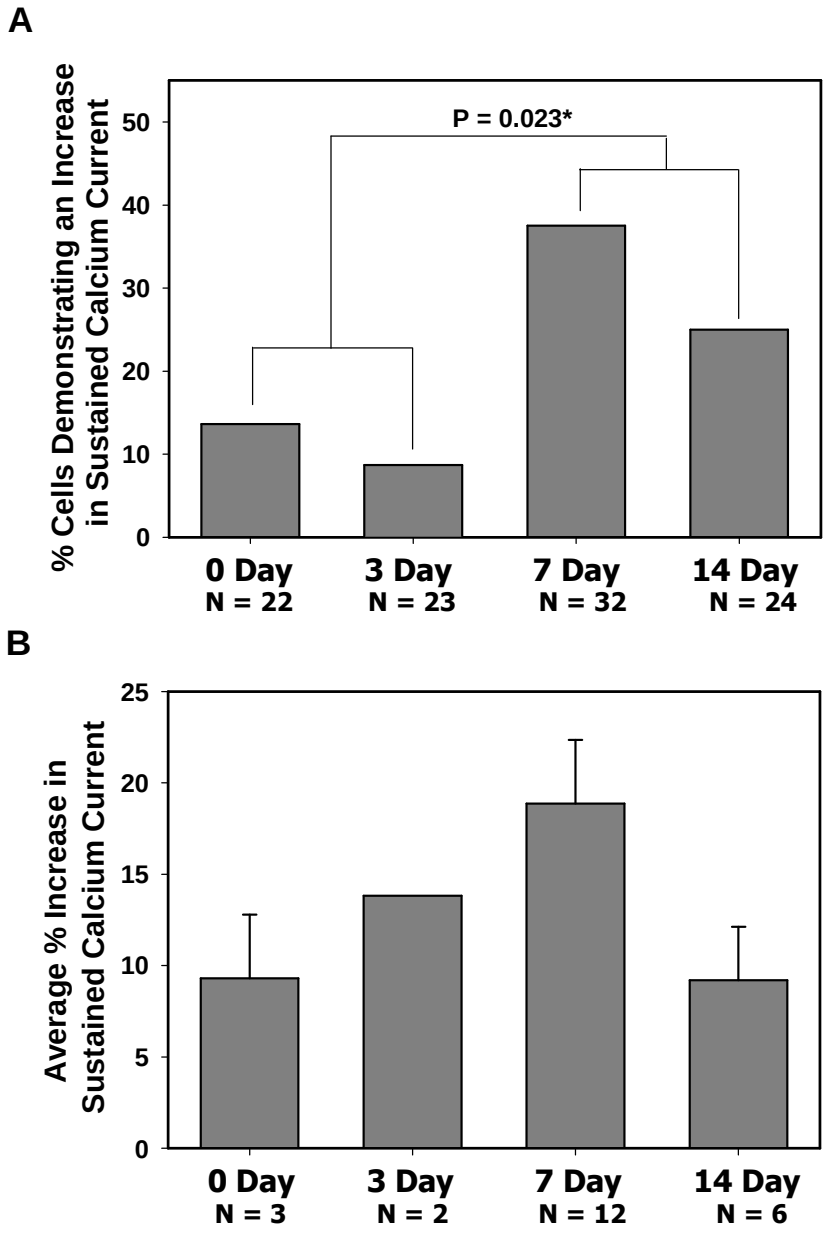

Figure 3.4: $GABA_B$ receptor facilitation of sustained calcium currents at different developmental time points. Currents were measured at the end of a 300 msec depolarizing pulse to +10 mV from a holding potential of – 80 mV. (A) Each bar represents the percent of cells demonstrating an increase in sustained calcium current following application of 10 μM baclofen in hippocampal cultures isolated from rats that were 0, 3, 7, and 14 days old ($p = 0.054$ using a Chi-Square when each age was analyzed separately; P = 0.023 using a Chi-Square when day 0 and day 3 were grouped into early postnatal period and day 7 and day 14 were grouped into late postnatal period). (B) Average percent increase of sustained current in cells demonstrating facilitation in response to baclofen (P = 0.136 using an ANOVA).

HVA and LVA Current Densities During the First Two Postnatal Weeks

One possible explanation for the varying baclofen responses seen at the different developmental ages studied could be due to changes in the number and type of channels present on the cell membrane. For example, if a particular HVA current type, such as L-type current is only modulated at earlier time points, it is possible that these channels are only expressed at that developmental time point. To determine whether there are changes in current density, both the mean LVA and HVA current densities were measured at the different ages studied. The LVA or T-type calcium current was elicited from a 300 msec pulse to -30 mV from a holding potential of -80 mV (Figure 3.5). LVA current density was determined by dividing the peak control current in these cells by the membrane capacitance. Since T-type calcium current varies throughout development, not all of the cells exhibited this type of current at the different time points studied (0 day (9/27), 3 day (9/29), 7 day (24/43) and 14 day (23/43)). T-type current density did not significantly differ when cultures were isolated from rats of varying ages (P = 0.107 using an ANOVA).

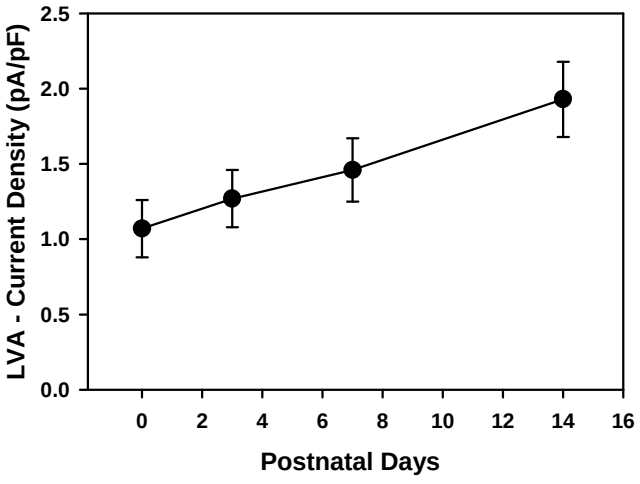

Figure 3.5: T-current density in hippocampal neurons isolated from rat pups of different ages. The low voltage activated T-type calcium current was elicited by a 300 msec pulse to -30 mV from a holding potential of -80 mV. Only a subset of cells exhibited T-current in cultures obtained from 0 day (9/27), 3 day (9/29), 7 day (24/43) and 14 day (23/43) old rats. The current density was determined by dividing the peak control current in these cells by the membrane capacitance. (P = 0.107 using an ANOVA).

In addition to the LVA current density, HVA current densities were also determined for all of the cells studied at the different time points. HVA currents were elicited with a 300 msec pulse to +10 mV from a holding potential of -80 mV (Figure 3.6). The peak and sustained components were separated and compared at each time point studied. The peak current was measured at the beginning of the 300 msec pulse and the sustained current was measured at the end of the 300 msec pulse. HVA current density was determined by dividing the current by the cell capacitance. Both the peak and sustained current densities did not significantly change during the first two postnatal weeks of development in the rat hippocampus (peak, P = 0.644; sustained, P = 0.508; using an ANOVA).

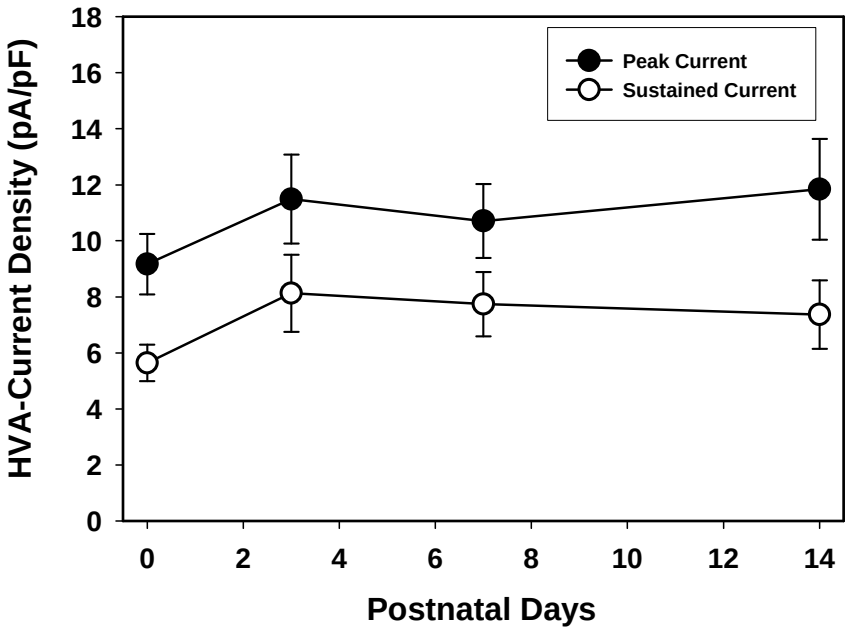

Figure 3.6: HVA current density in hippocampal neurons isolated from rat pups of different ages. HVA currents were elicited with a 300 msec pulse to +10 mV from a holding potential of -80 mV. The peak current was measured at the beginning of the 300 msec pulse and the sustained current was measured at the end of the 300 msec pulse. Current density was determined by dividing the current by the cell capacitance. Both the peak (solid circles) and sustained (open circles) current densities did not significantly change during the first two postnatal weeks of development in the rat hippocampus (peak, P = 0.644; sustained, P = 0.508; using an ANOVA). The N's ranged from 23-38 for each time point.

Due to the heterogeneity of our hippocampal cultures, it is possible that a cell type with a particular cell size or current density may demonstrate facilitation of L-type current by $GABA_B$ receptor activation. To determine whether cells demonstrating an increase in sustained calcium current in response to baclofen application were different in cell size or the number and type of calcium channels present when compared to cells demonstrating no change or a decrease in sustained calcium current in response to baclofen application, cell size and HVA current densities were compared for the different groups of cells. There were no significant differences in cell size ($P = 0.911$ using a Two Way ANOVA) as measured by cell capacitance or peak current density ($P = 0.690$ using a Two Way ANOVA) in the cells that exhibited an increase in sustained calcium current in response to 10 μM baclofen application when compared to cells that exhibited no effect or a decrease in sustained calcium current in response to 10 μM baclofen (Figure 3.7). Peak current density was determined by dividing the peak control current by the cell capacitance to normalize for cell size. There was no significant difference when determining the current density using the sustained component ($P = 0.633$ using a Two Way ANOVA) and the transient component ($P = 0.921$ using a Two Way ANOVA) of the current (Figure 3.8). The sustained control current was measured at the end of the 300 msec pulse and the transient component was calculated by subtracting the sustained current from the peak current for each cell and averaged for each group of cells. These experiments demonstrated that facilitation of L-type calcium current by $GABA_B$ receptor activation is most prominent in neurons isolated from 7 and 14 day old rats. However, the changes in response to baclofen can not be attributed to changes in HVA current density.

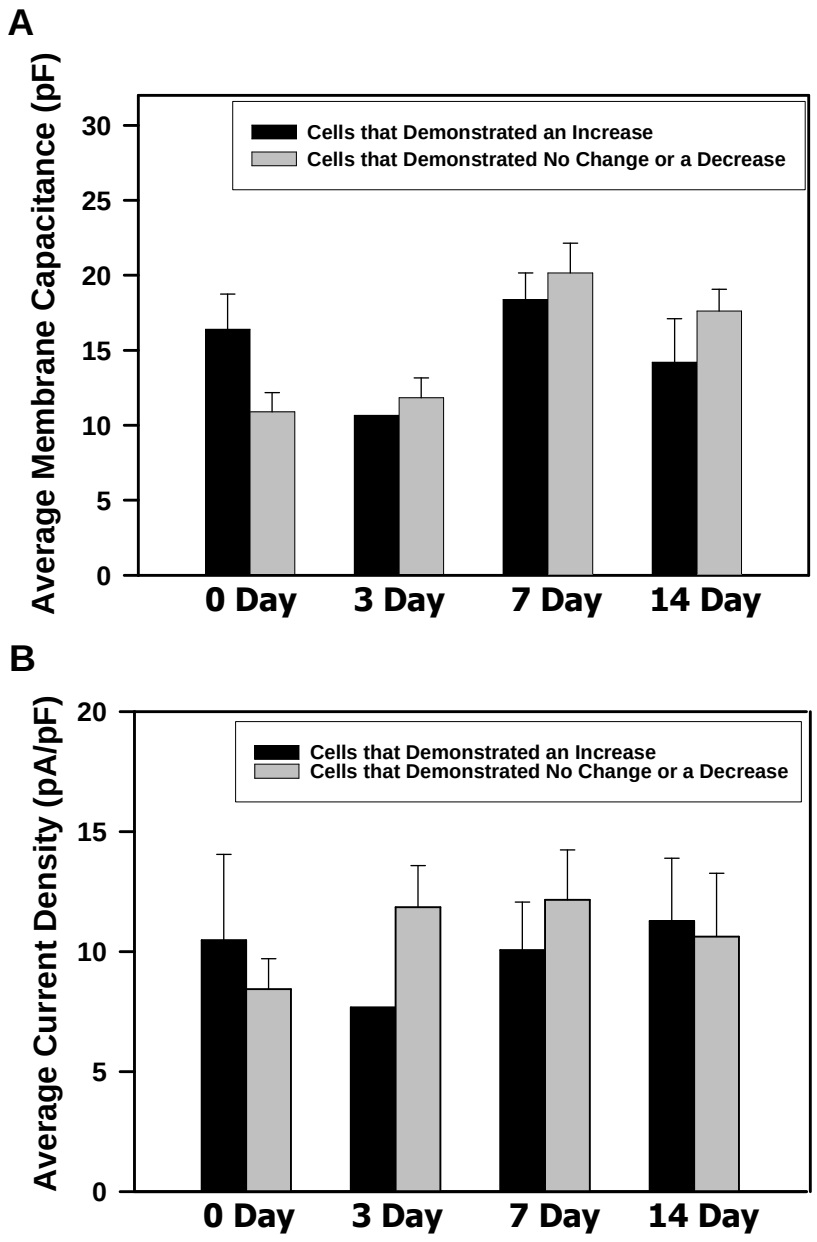

Figure 3.7: Membrane capacitance and HVA current density. (A) Membrane capacitance was measured as an indication of cell size. There was not a significant difference in capacitance between cells that demonstrated an increase in response to 10 μM baclofen (black bars; 0 day (3/22), 3 day (2/23), 7 day (10/32) and 14 day (6/24)) when compared to cells that demonstrated no change or a decrease in response to 10 μM baclofen (gray bars) at the different time points studied (P = 0.911 using a Two Way ANOVA). (B) Total current density was determined by dividing the peak control current by the cell capacitance to normalize for cell size. The average current densities were determined at each developmental time point for cells that exhibited an increase in sustained calcium current with 10 μM baclofen (black bars; 0 day (3/22), 3 day (2/23), 7 day (10/32) and 14 day (6/24)) and cells that exhibited no change or a decrease in sustained calcium current with 10 μM baclofen (gray bars). There was no difference in the average current densities based on the cell's response to baclofen at the different time points studied (P = 0.690 using a Two Way ANOVA).

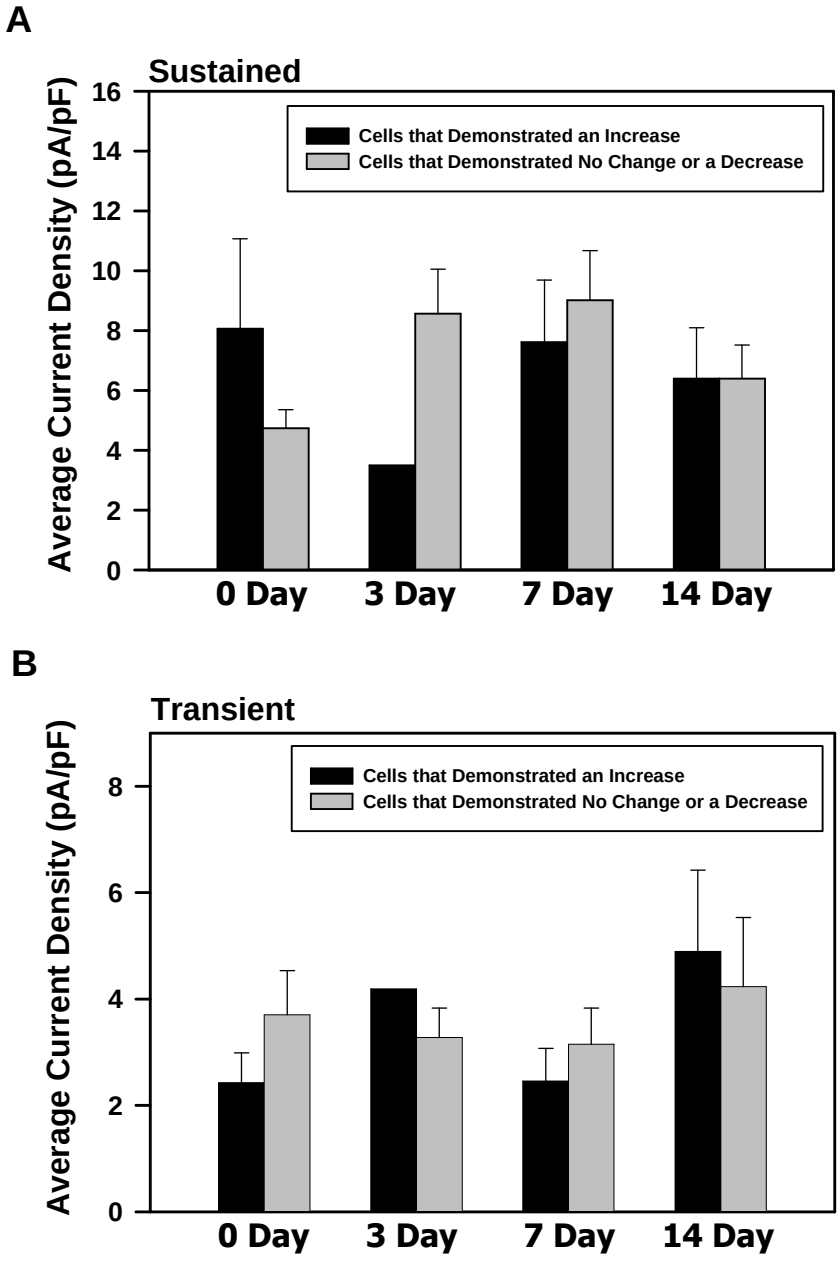

Figure 3.8: HVA current density. (A) Average current densities of the sustained component of current prior to the application of baclofen were determined for cells that exhibited an increase in sustained calcium current with 10 μM baclofen (black bars; 0 day (3/22), 3 day (2/23), 7 day (10/32) and 14 day (6/24)) and cells that exhibited no change or a decrease in sustained calcium current with 10 μM baclofen (gray bars). There were no differences in the average sustained current densities based on the cell's response to baclofen at the different time points studied (P = 0.690 using a Two Way ANOVA). (B) There were no significant changes in the transient current densities between cells that exhibited an increase in sustained calcium current with 10 μM baclofen (black bars; 0 day (3/22), 3 day (2/23), 7 day (10/32) and 14 day (6/24)) and cells that exhibited no change or a decrease in sustained calcium current with 10 μM baclofen (gray bars; P = 0.921 using a Two Way ANOVA).

DISCUSSION

$GABA_B$ receptors have been shown to attenuate N-type calcium current in a number of different cell types, including hippocampal neurons (Scholz and Miller, 1991; Dolphin and Scott, 1987; Mintz and Bean, 1993; Pfrieger et al., 1994; Amico et al., 1995; Cardozo and Bean, 1995; Lambert and Wilson, 1996; Shen and Slaughter, 1999). N-type calcium channels along with P/Q-type calcium channels are concentrated in synaptic terminals of CNS neurons and are involved in neurotransmitter release. N-type calcium channels contribute to neurotransmitter release earlier in development, whereas P/Q type calcium channels along with N-type calcium channels contribute to neurotransmitter release later in development (for review see Dolphin, 2003). This correlates to the present results, which demonstrated that the inhibition of peak calcium current by $GABA_B$ receptors predominates in hippocampal cultures isolated from 0 day old rat pups. Carter and Mynlieff (2004) have demonstrated that the decrease in peak current by $GABA_B$ receptor activation is through N-type calcium channels. *In vivo*, $GABA_B$ receptors involved in the inhibition of calcium current are likely to be localized on the presynaptic terminal providing a type of feedback inhibition of neurotransmitter release.

Much less is known about the facilitatory actions of $GABA_B$ receptor activation on calcium currents. To date, facilitation of L-type calcium current by $GABA_B$ receptor activation has only been observed in salamander retinal neurons (Shen and Slaughter, 1999) in adrenal chromaffin cell cultures (Parramón et al., 1995), and in hippocampal neurons (Carter and Mynlieff, 2004). Carter and Mynlieff (2004) were the first to demonstrate facilitation of L-type calcium current in the mammalian CNS. In their study, 30% of the cells demonstrated an enhancement of calcium current in response to baclofen application in hippocampal cultures isolated from 5 to 7 day old rat pups. This

enhancement of calcium current by $GABA_B$ receptors was blocked in the presence of the L-type channel blocker, nimodipine (Carter and Mynlieff, 2004). Since this phenomenon has only been observed in neonates and has not been previously observed in either embryonic or adult hippocampal tissue, it seemed likely that the facilitation of L-type calcium current by $GABA_B$ receptors is only present at a specific time in development.

The current study demonstrated that facilitation of L-type calcium current by $GABA_B$ receptor activation is a developmental phenomenon and is greatest in hippocampal cultured isolated from 7 and 14 day old rats. When analyzing the peak component of the current 31% of the cells demonstrated an increase in total calcium current and when analyzing the sustained current component 37.5% of the cells demonstrated an increase in calcium current in response to baclofen application in hippocampal cultures isolated from 7 day old rat pups. These percentages are consistent with the 30% of cells that Carter and Mynlieff (2004) found to be facilitated by $GABA_B$ receptor activation in hippocampal cultures isolated from 5 to 7 day old rats. Hippocampal cultures are heterogeneous and contain pyramidal cells along with several types of inhibitory interneurons. There are over 21 distinguishable inhibitory interneuron subtypes that have been classified in the hippocampus based on their morphological, neurochemical, and electrophysiological characteristics (for review see Klausberger and Somogyi, 2008; Cutsuridis and Wennekers, 2009; Klausberger, 2009). The variability in the responses to baclofen application is likely due to the heterogeneity of the cultures. Several factors such as the complement of calcium channels, the presence of $GABA_{B1a}$ versus $GABA_{B1b}$ receptors subtypes, the coupling with different G-proteins, the size, and the physical location of the cells can all vary in the different cell types present within the

hippocampus. Therefore, the cells demonstrating facilitation in response to baclofen application could represent a specific subset of cells present in the hippocampus.

Despite the heterogeneity of the cell cultures, experiments on HVA and LVA current density at various time points in this early postnatal period suggest that a global change in channel density or distribution of channel types in the cells cannot explain the differing effects of $GABA_B$ receptor activation at different time points. The sizes of the cells that demonstrated facilitation were not different when compared to cells that demonstrated no change or a decrease in calcium current in response to baclofen application. In addition, the HVA current density for cells that demonstrated facilitation was not different when compared to cells that demonstrated no change or a decrease in calcium current in response to baclofen application when the HVA current density was determined for the total, sustained, and transient component of the current. These studies suggest that cells demonstrating facilitation of calcium current by $GABA_B$ receptors do not differ significantly in the size or number of channels present from cells demonstrating no change or a decrease in calcium current by $GABA_B$ receptors.

Even though there are not changes in the total amount of sustained current throughout development, there may be variations in the contribution of the different forms of L-type calcium channels. The two types of L-type calcium channels that are ubiquitously expressed in the brain and are present in hippocampal neurons are $Ca_v1.2$ and $Ca_v1.3$ (Hell, 1993). It has been demonstrated that L-type $Ca_v1.2$ protein levels peak in hippocampal neurons isolated from 7 day old rats (Nuñez and McCarthy, 2007), whereas $Ca_v1.3$ protein levels begin low and increase throughout development (Ingraham and Mynlieff, unpublished data). Electrophysiological experiments in native neurons do

not distinguish between $Ca_v1.2$ and $Ca_v1.3$ current. A peak facilitation of L-type calcium current by baclofen at approximately one week may be a reflection of the peak expression of $Ca_v1.2$ protein levels in these neurons. This idea is also supported by the fact that immunocytochemical techniques have shown that less than 30% of hippocampal neurons taken from 7 day old pups demonstrate significant amounts of the $Ca_v1.2$ channels, which may reflect the subset of cells that demonstrate the facilitation of L-type calcium current by $GABA_B$ receptors.

Our lab has demonstrated that $GABA_B$ receptor activation within the neonatal rat hippocampus can modulate different types of VDCCs. Here we have demonstrated that the inhibitory effect predominates during the first postnatal week, while the facilitatory effect of calcium current by $GABA_B$ receptor activation predominates during the second postnatal week. Carter and Mynlieff (2004) have demonstrated that both effects can be present within the same cell, which suggests different mechanisms are involved with the modulation of the different channel types by $GABA_B$ receptors. L-type calcium channels contain several consensus phosphorylation sites for several kinases, including PKC and PKA, while N-type calcium channels are primarily modulated through direct interaction by G-proteins associated with the $GABA_B$ receptor(for review see Catterall, 2000). The signal transduction mechanism involved in the facilitation of L-type current by $GABA_B$ receptors is an area of interest in the laboratory and will be discussed in Chapter 5.

CHAPTER 4

The influx of calcium through L-type calcium channels is necessary for the upregulation of KCC2, but not NKCC1 during development in the rat hippocampus

INTRODUCTION

Early in neuronal development the neurotransmitter GABA exerts an excitatory rather than inhibitory effect due to a high concentration of intracellular chloride ions (Cherubini et al., 1991). During the first two postnatal weeks in the hippocampus of rats the internal concentration of chloride ions decreases. This causes a change in the reversal potential of chloride and thus, a shift from an excitatory effect of $GABA_A$ receptor activation to an inhibitory effect (Ben-Ari et al., 1989; Leinekugel et al., 1995, 1997, 1998; Khazipov et al., 1997; Rivera et al., 1999; Ganguly et al., 2001). Evidence suggests that the upregulation of the K^+Cl^- co-transporter (KCC2) and the downregulation of the $Na^+K^+2Cl^-$ co-transporter (NKCC1) are responsible for shifting the chloride reversal potential (Plotkin et al., 1997; Lu et al., 1999; Rivera et al., 1999; Hübner et al., 2001a). NKCC1 expression predominates in immature neurons and mediates chloride influx, while KCC2 expression predominates in mature neurons and mediates chloride efflux (for review see Delpire, 2000; Payne et al., 2003). Due to the high intracellular chloride concentration in immature neurons the activation of $GABA_A$ receptors depolarizes the cell, which subsequently activates voltage-dependent calcium channels, particularly L-type calcium channels (Yuste and Katz, 1991; Leinekugel et al., 1995; Khazipov et al., 1997; Ganguly et al., 2001). This GABAergic excitation is important for proper neuronal development (for review see Ben-Ari, 2002; Owens and Kriegstein, 2002; Fiumelli and Woodin, 2007; Galanopoulou, 2008; Kahle et al., 2008; Blaesse et al., 2009). As the brain matures, the number of neurons that are excitatory in response to GABA decreases and thus, the magnitude of calcium influx with GABA receptor activation decreases. Once neurons have fully developed, GABA responses are

hyperpolarizing and inhibit the cell from reaching threshold. Additionally, the subunit composition of the $GABA_A$ receptor changes during development (Kanaumi et al., 2006; Liu and Wong-Riley, 2006; Rissman et al., 2006; Yu et al., 2006). This change in subunit composition should not affect the reversal potential directly since that is dependent on the internal and external chloride concentrations, but it does affect the response to various modulators such as zinc and benzodiazepines.

Treatment of embryonic rat hippocampal cultures with L-type calcium channel antagonists prohibits the shift in the chloride reversal potential, suggesting that calcium influx through L-type calcium channels is involved in the changes of chloride transporter expression (Ganguly et al., 2001). However, Ganguly and co-workers did not directly look at the effect of calcium influx through L-type channels on chloride transporter expression. Previous experiments in our laboratory have demonstrated facilitation of L-type calcium current by activation of the metabotropic $GABA_B$ receptor in acutely cultured hippocampal neurons isolated from 5-7 day old rat pups (Carter and Mynlieff, 2004). The data presented in Chapter 3 suggest that the facilitation of L-type calcium current is maximal in the second postnatal week, a time period in which many genes including chloride transporters and neuronal L-type calcium channels are changing their expression as the neurons mature (Nuñez and McCarthy, 2007).

The present study explores the potential connection between chloride transporter expression and calcium influx through L-type calcium channels in the early neonatal period. Although changes in reversal potential have been shown to be dependent on calcium influx through L-type calcium channels, this is the first study to directly investigate the effect of calcium influx on the chloride transporter protein levels in

hippocampal neurons. Since calcium influx is enhanced in a subset of neonatal hippocampal neurons by activation of $GABA_B$ receptors, activation of these receptors may also alter KCC2 and NKCC1 transporter expression. The KCC2 and NKCC1 expression levels throughout early postnatal development were determined by Western blot analysis in the presence and absence of an L-type channel antagonist and a $GABA_B$ receptor agonist.

RESULTS

Developmental Regulation of NKCC1 Protein during the Early Postnatal Period

The expression levels of both KCC2 and NKCC1 were determined by Western blot analysis in hippocampal neurons cultured for various time periods and were compared to that from fresh tissue of equivalent ages. KCC2 is neuron specific, whereas NKCC1 is expressed in neurons and also glial cells (Payne et al., 1996; Lu et al. 1999; Kanaka et al., 2001; Hübner et al., 2001b). Whole hippocampal tissue protein was isolated from rats ranging in ages 1 day to 42 days old and cultured hippocampal neurons that were isolated from 0 day old rat pups and kept in culture for 1 to 15 days. Other investigators have demonstrated that embryonic hippocampal neurons in culture exhibit spontaneous activity and form synaptic connections within a few days following dissociation (Siebler et al., 1993; Bi and Poo, 1998; Vicario-Abejón et al., 1998). In addition, postnatal cultured hippocampal neurons in the Mynlieff laboratory demonstrate excitatory postsynaptic currents if allowed to remain in culture for more than 24 hours (unpublished observations). Anti-NKCC1 antibodies labeled a single band of 143 kDa on Western blots of proteins isolated from either cultured neurons or whole hippocampus (Figure 4.1). Steady state NKCC1 protein levels were determined at each time point studied by dividing the IOD for the 143 kDa band by the IOD for a band labeled with β-tubulin antibodies to control for variations in loading. The internal control was necessary

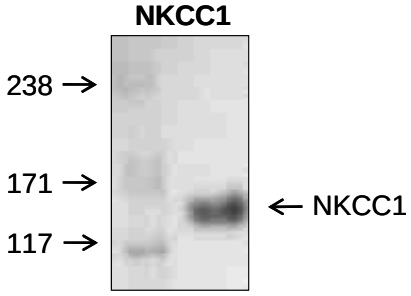

Figure 4.1: Using Western blot analysis, anti-NKCC1 antibodies label a single band with a molecular weight of 143 kDa in protein preparations extracted from whole hippocampus or cultured hippocampal neurons (right lane). The left lane was loaded with MagicMark Western standard markers.

since the amount of protein extracted from cultured neurons was so low that the protein assay results were not accurate. In contrast to KCC2 protein levels (see below), there was little change in the steady state protein levels of NKCC1 during the first two postnatal weeks of development both in fresh tissue and in cultured hippocampal neurons (Figure 4.2; $P = 0.278$ using Two Way ANOVA). Fresh tissue that was analyzed from rats of one month or more in age demonstrated up to a ten-fold increase in the steady state expression level of NKCC1 compared to D1 protein levels (D42, 13.17 ± 4.32).

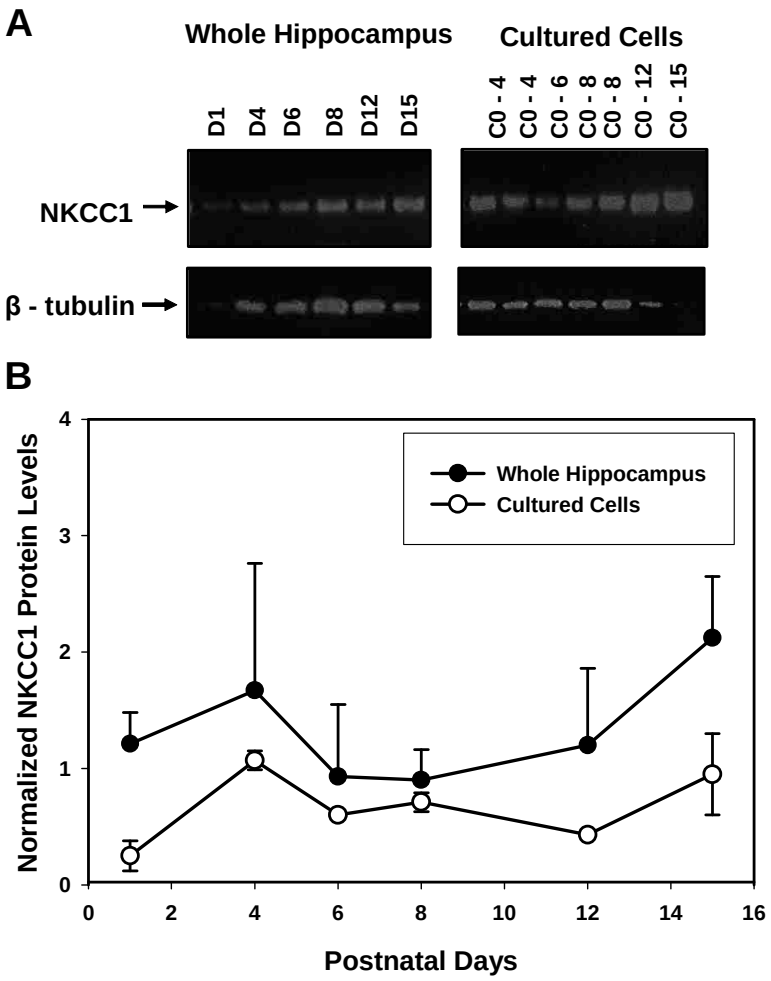

Figure 4.2: Developmentally regulated expression of NKCC1 protein in rat hippocampus and cultured hippocampal neurons. (A) Representative Western blot analysis of proteins extracted from whole hippocampus (left panel; postnatal day 1 to day 15) and cultured hippocampal neurons (right panel). Cultured neurons were obtained from day 0 rats and were kept in culture for 1 to 15 days before protein isolation (C0-1 to C0-15). (B) Summary data comparing the steady state NKCC1 protein levels isolated from whole hippocampal protein extracts (solid circles; N = 3) and cultured hippocampal neurons (open circles; N = 3 – 4, except C0-6 where N = 2). The IOD of the band labeled with NKCC1 antibodies (143 kDa) was divided by the IOD of the band labeled with β-tubulin antibodies (55 kDa) for normalization. In the first two weeks there was no difference in NKCC1 protein levels in the different ages across both tissue types (P = 0.278 using a Two Way ANOVA).

Developmental Regulation of KCC2 Protein during the Early Postnatal Period

Functional KCC2 proteins form oligomers that have a molecular mass higher than the monomeric protein (~125 kDa). Several studies have demonstrated KCC2 dimers along with monomers when performing Western blot analysis (Lu et al., 1999; Blaesse et al., 2006; Zhang et al., 2006). KCC2 dimers are detected on Western blots, because they are resistant to both non-ionic detergents such as Triton X-100 and also the anionic detergent SDS (Blaesse et al., 2006). In our study, antibodies to KCC2 labeled three bands of 248 kDa, 125 kDa, and 46 kDa representing a dimer, a monomer, and a splice variant (Figure 4.3). As the neurons matured there were increasing amounts of the dimer in relation to the monomer in both whole hippocampal protein extracts as well as cultured protein extracts. Steady state KCC2 protein levels were determined at each time point studied by adding the IOD for the band corresponding to the monomer plus the IOD for the band corresponding to the dimer and this value was further divided by the IOD for a band labeled with β-actin antibodies as a control for variations in loading. Since the intensity of the band corresponding to the KCC2 splice variant was a minor component in most preparations and the fact that the splice variant is not considered as an active form of the KCC2 protein, it was not included in the data analysis. Previous studies showed that KCC2 mRNA levels within the rat hippocampus are low following birth and rapidly

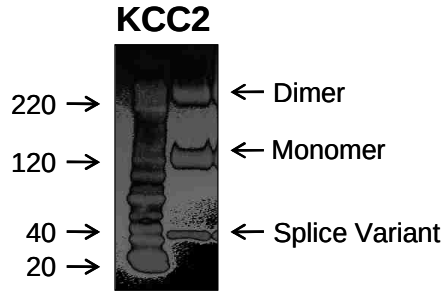

Figure 4.3: This sample Western blot demonstrates the three bands labeled with anti-KCC2 antibodies with molecular weights of 248 kDa, 125 kDa, and 46kDa in protein preparations extracted from whole hippocampus or cultured hippocampal neurons (right lane). The left lane was loaded with MagicMark Western standard markers.

rise in the first few postnatal weeks (Rivera et al., 1999; Balakrishnan et al., 2003). Our protein data is consistent with the mRNA data in that both fresh tissue and cultured neurons demonstrated low KCC2 protein levels during the first postnatal week, but levels rapidly rise during the second postnatal week ((Figure 4.4; $P < 0.001$ using a Two Way ANOVA). In fresh tissue the protein level tripled between day 15 and day 24 (D24, 11.86 ± 4.2), at which point it plateaued through adulthood (D33, 9.31 ± 0.66; D42 9.60 ± 2.76). The increase in KCC2 protein levels during the second postnatal week in development occurred both *in vivo* and in cultured hippocampal cells, supporting the use of cultured hippocampal neurons to study the developmental expression of KCC2. In addition, the results presented in Chapter 3 demonstrate that in hippocampal cell cultures obtained from rats of various ages L-type current facilitation peaks at approximately the same developmental time point as KCC2 expression levels begin to rise. This supports the hypothesis that facilitation of L-type calcium current by $GABA_B$ receptor activation within the hippocampus may play a role in the regulation of KCC2 during the first two postnatal weeks of development.

Other investigators have demonstrated that the internal chloride concentration decreases during the early postnatal period in rat hippocampal neurons. This could be due to a concurrent increase in KCC2 with a decrease in NKCC1, since these transporters move chloride in the opposite direction. However, our data suggest that only the KCC2 transporter levels are changing in the first two postnatal weeks of hippocampal development and that it is the ratio of the two transporters that is important in determining the intracellular concentration of chloride and thus, the reversal potential for

the $GABA_A$ response. Our data demonstrate a large increase in the KCC2 to NKCC1 ratio between postnatal day 8 and 12 (Figure 4.5; P = 0.005 using an ANOVA).

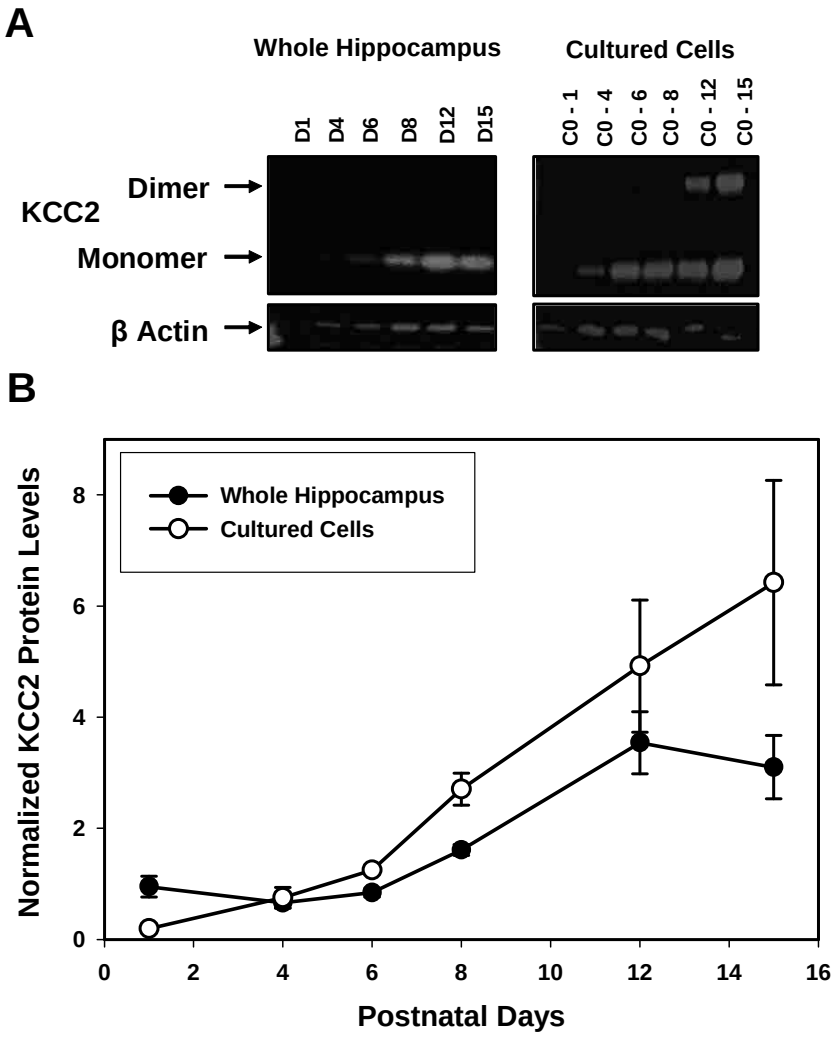

Figure 4.4: Developmentally regulated expression of KCC2 protein in rat hippocampus and cultured hippocampal neurons. (A) Representative Western blot analysis of proteins extracted from whole hippocampus (left panel; postnatal day 1 to day 15) and cultured hippocampal neurons (right panel). Cultured neurons were obtained from day 0 rats and were kept in culture for 1 to 15 days before protein isolation (C0-1 to C0-15). (B) Summary data comparing the steady state KCC2 protein levels isolated from whole hippocampal protein extracts (solid circles; N = 3) and cultured hippocampal neurons (open circles; N = 3 – 4, except C0-6 where N = 2). The IOD of the band corresponding to the monomer (125 kDa) and the IOD of the band corresponding to the dimer (248 kDa) were added together and divided by the IOD for the band labeled with β-actin antibodies (45 kDa). There is a significant difference in KCC2 protein levels at the different ages across both tissue types (P < 0.001 using a Two Way ANOVA).

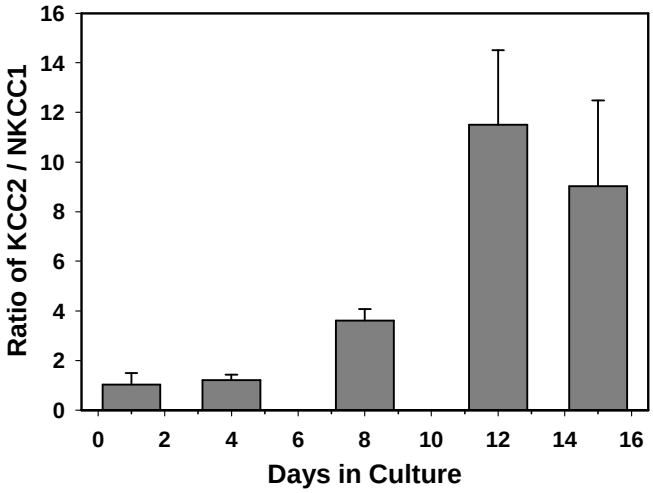

Figure 4.5: KCC2 to NKCC1 ratio. The ratio of KCC2 to NKCC1 was determined using the normalized values for the cultured hippocampal neurons for each date that a preparation was isolated. An average of three to four preparations was determined for each time point. The ratio of KCC2 to NKCC1 is higher at 12 and 15 days in culture ($P = 0.005$ using an ANOVA).

Role of L-type Calcium Channels and $GABA_B$ Receptors in Modulating Chloride Transporter Expression

To determine whether calcium influx through L-type calcium channels and $GABA_B$ receptor activation affect the protein level of KCC2 and NKCC1 during the early neonatal period, hippocampal cultures isolated from 0 day rats were treated daily with the $GABA_B$ receptor agonist baclofen (10 μM) or the L-type calcium channel antagonist nimodipine (5 μM) for one week. Previous studies treating cultures with the vehicles for these drugs demonstrated only a small shift in pH (<0.1) and no effect on cell viability or protein levels (data not shown). Steady state NKCC1 protein levels were determined by Western blot analysis and the values were determined by dividing the IOD for the band corresponding to NKCC1 by the IOD for a band corresponding to β-tubulin. This value was further normalized by dividing it by the value obtained for the control culture grown in the absence of any drugs along side each set of cultures treated with drugs and exposed to the same film and multiplied by 100%. NKCC1 protein levels during the first postnatal week were not modified by treatment with the $GABA_B$ agonist baclofen (101.50 ± 8.90%; N = 4 individual cultures, $P = 0.8772$ using a one sample t-test; Figure

4.6A). In addition, the L-type calcium channel antagonist nimodipine did not affect the steady state protein levels of NKCC1 during the first postnatal week of development (93.50 ± 6.90%; N = 4 individual cultures, P = 0.4177 using a one sample t-test; Figure 4.6A). Therefore, NKCC1 protein levels in hippocampal cultures are not affected by one week treatment of the $GABA_B$ agonist, baclofen or the L-type antagonist, nimodipine.

Steady state KCC2 levels were analyzed by Western blot and the values were determined by adding the IOD for the band corresponding to the monomer plus the IOD of the band corresponding to the dimer and dividing it by the IOD for a band labeled by β-actin antibodies. This value was further normalized by dividing it by the normalized IOD obtained from the control culture that was run on the same gel and exposed to the same film and multiplied by 100%. A total of 8 cultures were treated with each drug. The steady state protein levels of KCC2 after a week in culture were not significantly altered by treatment with the $GABA_B$ agonist baclofen (108.38 ± 12.03%; N = 8, P = 0.5357 using a one sample t-test; Figure 4.6B). The steady state protein levels of KCC2 were greatly reduced by treatment with the L-type calcium channel antagonist nimodipine (46.38 ± 5.37%; N = 8, P = 0.0001 using a one sample t-test; Figure 4.6B). Thus, the upregulation of steady state KCC2 expression that normally occurs during the first postnatal week in culture appears to be dependent, at least in part, on calcium influx through L-type channels.

In addition to baclofen and nimodipine, a small number of cultures were also treated with 10 μM 2-OH-saclofen, a $GABA_B$ antagonist. 2-OH-saclofen (10 μM) did not affect the protein levels of KCC2 during the first postnatal week in culture (data not shown). These results were similar to the data collected from cultures treated with the

$GABA_B$ agonist baclofen, which also did not affect KCC2 protein levels during the first postnatal week in culture. Therefore, additional experiments were not performed using the $GABA_B$ antagonist 2-OH-saclofen.

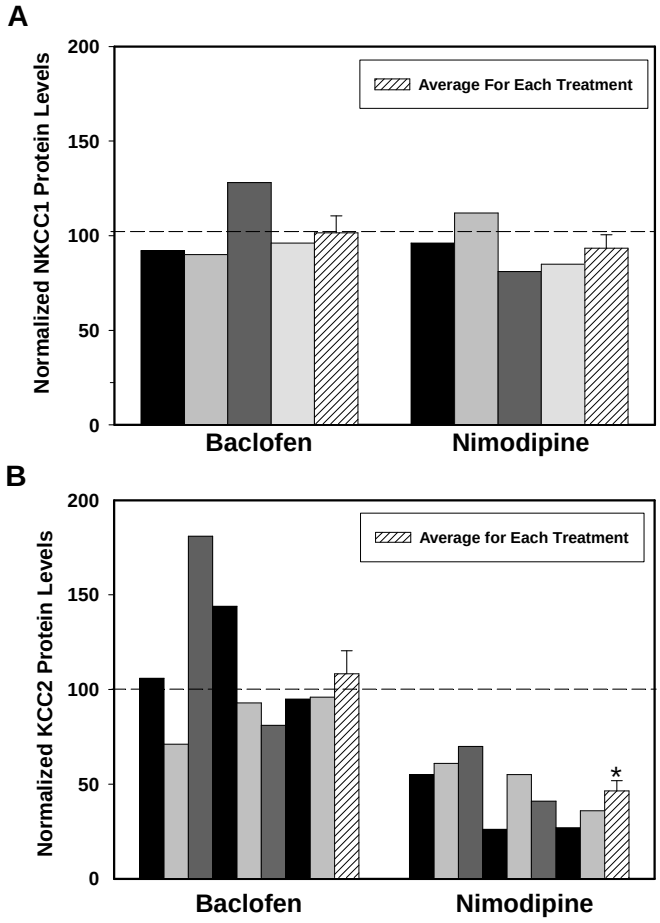

Figure 4.6: Effects of baclofen and nimodipine on chloride transporter expression using Western blot analysis. Hippocampal cultures were obtained from day 0 rats and either 10 μM baclofen, a $GABA_B$ agonist, or 5 μM nimodipine, an L-type calcium channel antagonist were added daily to the cultures for one week. Each bar represents one protein preparation that was isolated from a set of cultures treated with either drug and an average (hatched bar) was determined for both drug treatments. (A) NKCC1 steady state protein levels following treatment with a $GABA_B$ agonist or an L-type calcium channel antagonist. The IOD of the band labeled with NKCC1 antibodies was divided by the IOD of the band labeled with β-tubulin antibodies. This value was further normalized by dividing it by the value obtained for the control culture for each set of cultures and multiplied by 100%. (B) KCC2 steady state protein levels following treatment with a $GABA_B$ agonist or an L-type calcium channel antagonist. The IOD of the band corresponding to the monomer and the IOD of the band corresponding to the dimer of KCC2 were added together and divided by the IOD of the band labeled with β-actin antibodies. This value was further normalized by dividing it by the value obtained for the control culture for each set of cultures and multiplied by 100%. (*P = 0.0001 using a one sample t-test).

DISCUSSION

GABA is the main inhibitory neurotransmitter within the adult brain. However, activation of $GABA_A$ receptors has an excitatory effect in immature neurons due to the high intracellular chloride levels (Cherubini et al., 1991). Later in development the activation of $GABA_A$ receptors has an inhibitory effect that persists into adulthood. Changes in chloride transporter expression levels during the first few postnatal weeks of development are thought to underlie this developmental switch (Plotkin et al., 1997; Lu et al., 1999; Rivera et al., 1999; Hübner et al., 2001a). The mechanisms involved in signaling the shift in transporter expression are not known. It has been demonstrated that blockade of $GABA_A$ receptors or L-type calcium channels delays the change in the reversal potential of chloride in developing hippocampal neurons (Ganguly et al., 2001). Prior to the switch in reversal potential for the $GABA_A$ receptor, activation of these receptors causes calcium influx through VDCCs due to the depolarizing nature of the response. These data from Ganguly and co-workers (2001) suggest that L-type calcium current may play a role in the changes in chloride transporter expression that occurs in the rat hippocampus during the first two postnatal weeks of development. Our laboratory has demonstrated that activation of the $GABA_B$ metabotropic receptor can enhance calcium influx through L-type calcium channels and attenuate calcium influx through N-type calcium channels during the early neonatal period (Carter and Mynlieff, 2004). The attenuation of N-type calcium current by $GABA_B$ receptors is known to persist into adulthood in the hippocampus and is likely to modulate neurotransmitter release from the presynaptic terminal (Dutar and Nicoll, 1988; Harrison, 1990; Lambert et al., 1991; Thompson and Gähwiler, 1992; Davies and Collingridge, 1993; Pfrieger et al., 1994; Wu

and Saggau, 1995). The functional role of L-type calcium channel enhancement is less clear. The present study demonstrated that influx of calcium through L-type calcium channels is necessary for the upregulation of KCC2 protein levels in the first postnatal week within the hippocampus. Thus, these findings support the hypothesis that calcium influx through L-type channels is involved in the developmental switch of the $GABA_A$ response by affecting chloride transporter expression.

Several investigators have described conflicting expression patterns of NKCC1 throughout hippocampal development. Therefore, it was necessary to perform a timecourse of NKCC1 protein level changes in cultured hippocampal neurons. Our developmental timecourse of NKCC1 protein levels is consistent with previous studies looking at NKCC1 mRNA and protein within rat hippocampal neurons (Yan et al., 2001; Wang et al., 2002). These studies observed an upregulation of both NKCC1 mRNA and protein in the developing rat hippocampus with a peak level of expression within the adult. The Western blot analysis of NKCC1 by Yan et al. (2001) demonstrated a trend for an increase in the first three postnatal weeks but only in the adult was the increase statistically significant, which is very similar to the data presented here. The preparations from fresh hippocampal protein also contain glial cells, which are one of the main cell types that express NKCC1 protein in adult hippocampus (Kanaka et al., 2001; Yan et al., 2001; Wang et al., 2002). The fact that whole hippocampal preparations contain glial cells could explain why NKCC1 protein expression levels are higher in the preparations isolated from adult tissue versus tissue isolated from neonates. However, some studies have seen peak expression of NKCC1 mRNA and protein within the rat hippocampus at postnatal day 7 with very little expression in the adult (Plotkin et al., 1997). Our cultures

are strictly neuronal and do not support glial cells (Mynlieff, 1997). Fresh tissue consistently demonstrated higher NKCC1 levels than age matched cultures, which is likely due to the lack of glial cells in the cultures. Therefore, all NKCC1 protein levels measured in culture reflect expression within the first two postnatal weeks exclusively in neurons.

In whole hippocampal protein extracts from 1, 4, and 6 day old rats and in cultures isolated on day 0 and kept in culture for 1, 4, and 6 days, KCC2 protein levels remain relatively low. However, in fresh hippocampal extracts as well as in cells kept in culture, the slope of the KCC2 protein levels drastically increases between day 6 and day 8, correlating well with the peak in the enhancement of L-type calcium current by $GABA_B$ receptor activation during the second postnatal week (Figure 4.7). KCC2 protein levels continue to rise throughout the first few weeks of development and plateau by three weeks of age. This trend in KCC2 expression *in vivo* was similar to what was seen in our cultured hippocampal neurons as well as by others studying expression of KCC2 by Western blot analysis or mRNA levels (Rivera et al., 1999; Lu et al., 1999; Wang et al., 2002; Stein et al., 2004; Nuñez and McCarthy, 2007). The fact that there are not dramatic changes in NKCC1 transporter protein levels during the first two postnatal weeks suggest that the ratio of KCC2 to NKCC1, versus the downregulation of NKCC1 protein levels may be responsible for the change in the reversal potential of chloride and thus, the developmental switch of the $GABA_A$ response from excitatory to inhibitory.

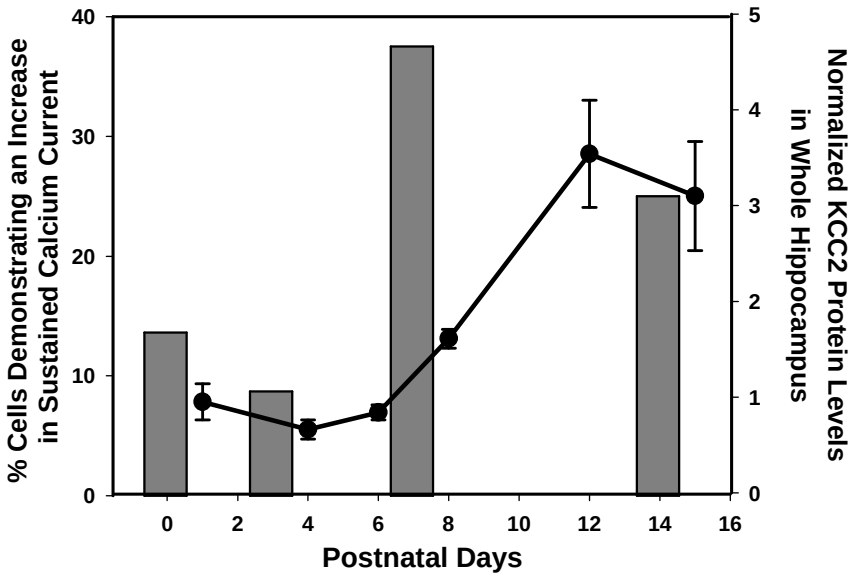

Figure 4.7 Comparison of cell percentage demonstrating L-type current facilitation and KCC2 steady state levels in whole hippocampal protein extracts. The bar graph represents the percent of cells that exhibited L-type calcium current facilitation in response to $GABA_B$ receptor activation at different developmental ages. The line graph represents the steady state KCC2 levels in whole hippocampal protein extracts determined by Western blot analysis.

NKCC1 expression in hippocampal cultures was not affected by either baclofen or nimodipine treatment. While these data suggest that neither $GABA_B$ receptor activation nor L-type calcium current is directly involved in developmental changes of NKCC1 protein levels, the involvement of L-type calcium current in KCC2 expression would still cause a change in the KCC2/NKCC1 ratio. Ultimately, it is the ratio of these two transporters that determines the internal chloride concentration since they carry chloride in opposite directions. Thus, a change in just one transporter will alter the ratio and affect the chloride reversal potential. Relatively little change in the NKCC1 protein levels during the first two weeks in culture is offset by the large increase in KCC2 protein levels.

Treatment of hippocampal cultures with the L-type calcium channel blocker nimodipine significantly blocked the upregulation of KCC2 protein levels compared to the cultures not treated with the blocker. Calcium influx through L-type calcium channels appears necessary to signal a pathway involved in the upregulation of KCC2. These findings support the model that the elevation of intracellular calcium levels through VDCCs promotes the developmental switch of the $GABA_A$ response from excitatory to inhibitory. This role of L-type calcium channels in regulating KCC2 protein levels also suggests that L-type calcium current facilitation by $GABA_B$ receptor activation could enhance the upregulation of KCC2 during the early neonatal period within the rat hippocampus. However, when cultures were treated with the $GABA_B$ agonist baclofen there was no significant change in KCC2 protein levels compared to the untreated cultures. There are a number of reasons that enhancement of L-type calcium current by activation of $GABA_B$ receptors may be important in this developmental switch without demonstrating an effect in the current study. Typically, only about 30% of the neurons demonstrate an enhancement of the L-type calcium current with $GABA_B$ receptor activation. The effect of baclofen may have been quite significant on the KCC2 expression in these individual cells, but when the tissue was pooled for a Western blot analysis it would not be sufficient to see a significant change in the protein levels. Ideally, one would need to perform experiments such as single cell RT-PCR to determine if the chloride transporters are affected by $GABA_B$ receptor activation differently in distinct cells. In addition, there is still a large amount of L-type calcium current in the absence of $GABA_B$ receptor activation. The activation of the $GABA_B$ receptors merely "enhances" the whole cell current that is already present. To see a difference in protein

levels by Western blot analysis one needs a very robust change such as that seen with nimodipine. Perhaps the "enhancement" seen with baclofen was not sufficiently large to analyzed by this method.

In conclusion, the activation of VDCCs following depolarizing $GABA_A$ responses aids in signaling the developmental switch of the $GABA_A$ response from excitatory to inhibitory. Here we have demonstrated that L-type calcium current plays a role in the upregulation of KCC2 protein levels during the first few weeks of hippocampal development without causing a significant change in the NKCC1 protein levels.

CHAPTER 5

The signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptor activation in the neonatal rat hippocampus

INTRODUCTION

The regulation of N-type as well as P/Q-type calcium channels has been well studied in neurons. Less is known about the regulation of L-type calcium channels in neurons. L-type calcium channels play a large role in the function of both cardiac and skeletal muscle. Thus, many investigators have concentrated on studying the regulation of L-type calcium channels in muscle tissue, particularly cardiac muscle. Neuronal activity is commonly regulated by GABA, which can activate metabotropic $GABA_B$ receptors. Upon $GABA_B$ receptor activation, neuronal calcium channels can be directly regulated by either $G\alpha$ or $G\beta\gamma$ subunits or they can be indirectly regulated by G proteins through second messenger systems (for review see Catterall, 2000; Dolphin, 2003; Tedford and Zamponi, 2006).

It has been demonstrated that $GABA_B$ receptors predominantly couple to $G_{i/o}$ proteins (Morishita et al., 1990; Campbell et al., 1993; Menon-Johansson et al., 1993; Greif et al., 2000). One of the most common mechanisms of channel modulation by $GABA_B$ receptor activation occurs through direct modulation by $G\beta\gamma$ subunits associated with $G\alpha_{i/o}$ proteins (for review see Bettler et al., 2004). In the hippocampus, inhibition of N-type and P/Q-type calcium channels by presynaptic $GABA_B$ receptor activation occurs through direct modulation by $G\beta\gamma$ subunits (Kajikawa et al., 2001). Binding of $G\beta\gamma$ subunits to the channel inhibits its activity by making it more difficult for the channel to open (Bean, 1989b; Boland and Bean, 1993; Herlitze et al., 1996; Ikeda, 1996; Zamponi and Snutch, 1998; Colecraft et al., 2000). GIRK channels are directly activated by $G\beta\gamma$ subunits associated with $G\alpha_{i/o}$ proteins coupled to $GABA_B$ receptors. GIRK channels can also be modulated through kinase activity. PKA increases the activation of GIRK

channels, whereas PKC decreases the activation of GIRK channels (for review see Luján et al., 2009).

Previous experiments have demonstrated that $GABA_B$ receptor activation can lead to an attenuation of N-type calcium current and the enhancement of L-type calcium current in hippocampal cultures obtained from neonatal rat pups of various ages (Carter and Mynlieff, 2004; Bray and Mynlieff, 2009). It is well known that the inhibition of N-type calcium current by $GABA_B$ receptor activation is mediated by a G-protein from the $G_{i/o}$ protein family, which are sensitive to PTX. However, the signal transduction pathway involved in the facilitation of L-type current by $GABA_B$ receptors in mammalian neurons is unknown.

In addition to direct modulation by $G\beta\gamma$, activation of G-proteins can also lead to activation of kinases, which can modulate calcium channel activity as well. L-type calcium channels have several consensus sites for phosphorylation by PKC and PKA. PKC is typically activated through the $G\alpha_{q/11}$ pathway and PLC. PKA activation is dependent on the presence of cAMP. The levels of cAMP are determined by adenylyl cyclase activity, which can either be stimulated by $G\alpha_s$ or inhibited by $G\alpha_i$. $GABA_B$ receptor activation of PKC and PKA has been shown to facilitate L-type calcium current in a subset of salamander retinal neurons (Shen and Slaughter, 1999). It has been demonstrated specifically within the rat hippocampus, that activation of PKC can facilitate L-type calcium current (Doerner and Alger, 1992). However, this response was not directly linked to $GABA_B$ receptors.

This study was designed to elucidate the signal transduction mechanism of $GABA_B$ receptor modulation of L-type calcium channels within the neonatal rat

hippocampus. Although activation of PKC has been shown to enhance L-type calcium current within the hippocampus, this is the first study to directly investigate the potential role of PKC in $GABA_B$ receptor mediated facilitation of L-type calcium current. Electrophysiological experiments were performed on cultured hippocampal neurons in the presence and absence of several PKC and PKA antagonists, as well as agonists for both types of kinases to determine their involvement in the signaling pathway of $GABA_B$ receptor activation and the subsequent enhancement of L-type calcium current. Calcium current facilitation in response to baclofen application has been shown to be most prominent in hippocampal cultures isolated from 7 and 14 day rat pups. Therefore, all hippocampal cultures used in these experiments were isolated from rat pups that were 6 to 8 days old in order to maximize the number of cells exhibiting the facilitatory response.

RESULTS

Since one of the most common mechanisms of channel modulation by $GABA_B$ receptor activation occurs through direct modulation by a $G_{i/o}$ protein, the first experiment performed was overnight treatment of hippocampal cultures obtained from 7 day old rat pups with PTX (200 ng/ml). PTX was added to the cultures two hours after dissociation. Addition of PTX inactivates $G_{i/o}$ proteins by ADP-ribosylation. As described in Chapter 2, calcium currents were elicited by a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV (Figure 5.1). The distribution of the

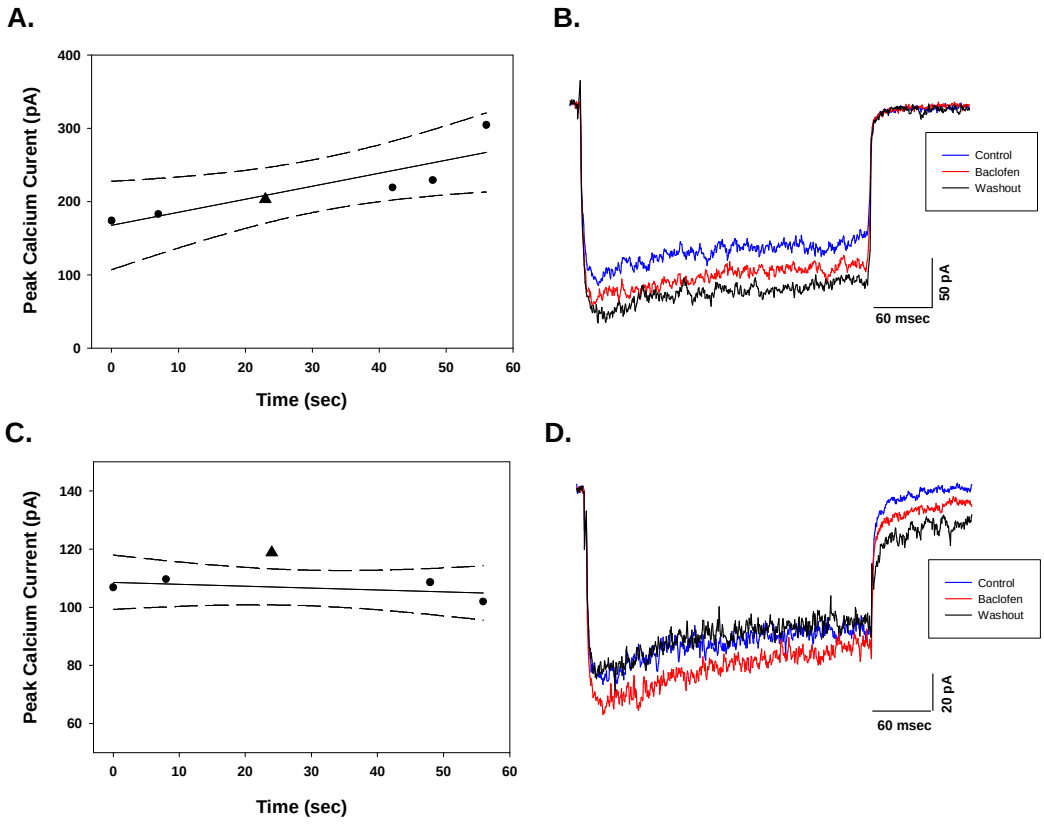

Figure 5.1 Calcium currents in the presence of PTX. Calcium currents were elicited by a 300 msec depolarization to +10 mV from a holding potential of -80 mV. The peak current is measured at the beginning of the 300 msec pulse. The black trace represents the control current, the red trace represents when 10 μM baclofen was applied to the cell, and the blue trace represents the currents after baclofen was washed off of the cell. The cell represented in A and B did not show an effect to baclofen application in the presence of PTX, whereas the cell represented in C and D demonstrated facilitation of peak calcium current in response to baclofen application in the presence of PTX.

responses differed in cultures that were treated with PTX in comparison to non-treated cultures (P = 0.009 using a Chi-square). In cultures that were not treated with PTX, 33.33% of cells demonstrated a decrease, 38.46% of cells demonstrated no change, and 28.21% of cells demonstrated an increase in peak calcium current in response to 10 μM baclofen. In cultures that were treated with PTX, no cells demonstrated a decrease, 70% of cells demonstrated no change, and 30% of cells demonstrated an increase in peak calcium current in response to 10 μM baclofen. Facilitation of calcium current in response to 10 μM baclofen was still seen in cells treated with PTX, which suggests that facilitation of L-type calcium current is not mediated by a PTX sensitive G-protein (Figure 5.2). However, it was demonstrated that the inhibition of N-type calcium current by $GABA_B$ receptors in the neonatal rat hippocampus is mediated through a $G_{i/o}$ protein, because following PTX treatment no cells demonstrated inhibition of their current (P = 0.002 using a Fisher's Exact test for pairwise comparisons).

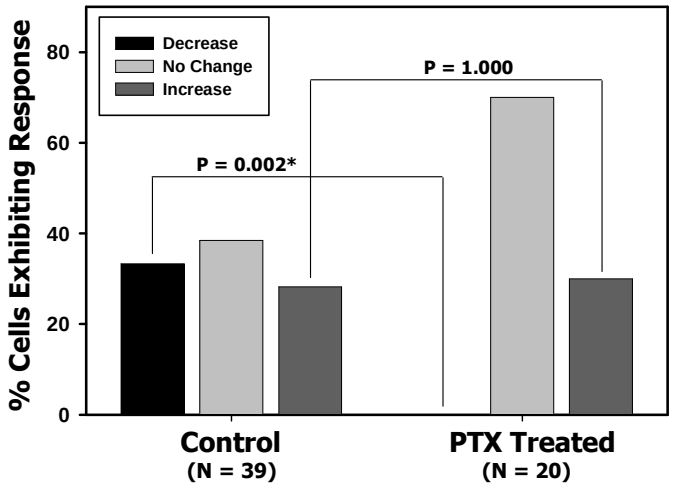

Figure 5.2: Effect of PTX on the baclofen responses in hippocampal neurons. The peak component of calcium current was measured at the beginning of a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen. Data were collected from 39 control cells and 20 cells in the presence of PTX (200 ng/ml, P = 0.009 using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

PKC Inhibitors Block the Ability of Baclofen to Facilitate Sustained Current

The goal of these experiments was to determine whether or not PKC was involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors. Although over 12 different isoforms of PKC have been classified, broad-spectrum PKC inhibitors were used as a first step in determining the involvement of PKC in the signal transduction pathway of the facilitatory response by $GABA_B$ receptor activation. Several PKC inhibitors with different chemical structures were used for verification of the results. Each of the inhibitors used has been shown to be a potent and selective inhibitor of PKC (Table 5).

PKC Inhibitors	PKC Isoforms	References
PKC Fragment 19-36	All isoforms	House and Kemp, 1987
GF-109203X	α, β1, βII, δ, ε, ζ, γ	Toullec et al., 1991; Martiny-Baron et al., 1993
Chelerythrine Chloride	Group A and Group B (α, βI, βII, γ) (δ, ε, ζ, η)	Herbert et al., 1990

Table 5. The PKC inhibitors used were selective for several PKC isoforms.

The first PKC inhibitor tested was the PKC fragment 19-36, which corresponds to a conserved region of the regulatory domain of PKC. This synthetic peptide fragment has been shown to inhibit both autophosphorylation and substrate phosphorylation of PKC by acting as a potent substrate antagonist (House and Kemp, 1987). This peptide was shown to block the facilitation of L-type calcium current by $GABA_B$ receptors in salamander retinal cells (Shen and Slaughter, 1999). Since the PKC fragment 19-36 is not membrane permeable, it was included in the internal pipette solution. For all PKC and PKA antagonists and agonists, the sustained component of the current was analyzed to maximize the contribution of L-type calcium current. It is well documented that

voltage-dependent calcium currents run down or run up in recordings made from cultured cells, which makes the ability to hold a cell through numerous drug applications and washes limited. Therefore, all experiments with kinase inhibitors and activators were carried out as population studies, comparing the distribution of responses in the presence and absence of each compound tested. In addition to the comparison of the percent of cells demonstrating each response to baclofen application, the average percent change in the magnitude of sustained current was also compared back to the average percent change seen in hippocampal cultures isolated from 6 to 8 day old rats that were treated with baclofen in the absence of any kinase inhibitors or activators (Figure 5.3).

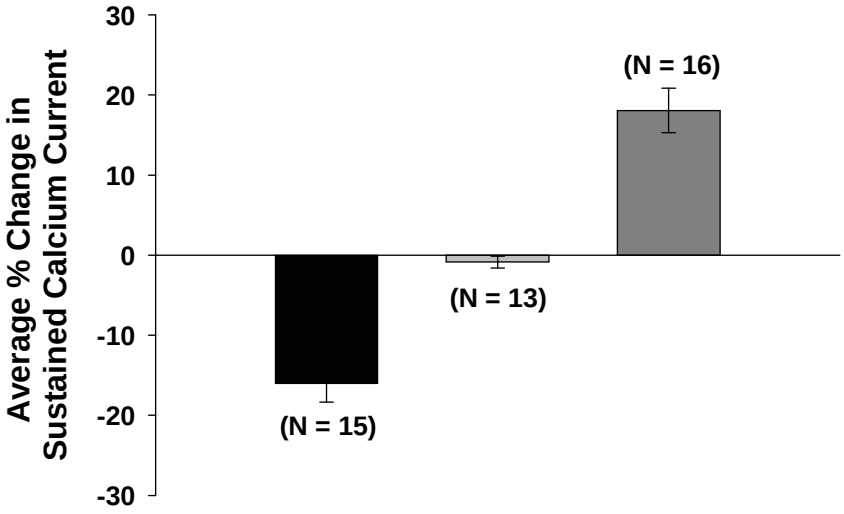

Figure 5.3: Summary of the average percent change in sustained calcium current in response to 10 μM baclofen in control cells isolated from postnatal rats 6 to 8 days old. Each bar represents the mean percent change of sustained calcium current. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen.

The distribution of the responses differed in the presence of the PKC fragment 19-36 in comparison to the control cells (Figure 5.4; $P = 0.003$ using a Chi-square). In control cultures 34.1% of cells demonstrated a decrease in sustained calcium current,

29.5% of cells demonstrated no change in sustained calcium current, and 36.4% demonstrated an increase in sustained calcium current in response to 10 μM baclofen. When the PKC fragment 19-36 (2 μM) was included in the recording pipette, 28.6% of cells demonstrated a decrease in sustained calcium current, 60.7% of cells demonstrated no change in sustained calcium current, and 10.7% of cells demonstrated an increase in sustained calcium current in response to 10 μM baclofen (P = 0.027 using a Fisher's exact test for pairwise comparisons). Data using the PKC pseudosubstrate peptide inhibitor, PKC fragment 19-36, suggest that PKC may be involved in the signaling pathway of L-type calcium current facilitation by $GABA_B$ receptors.

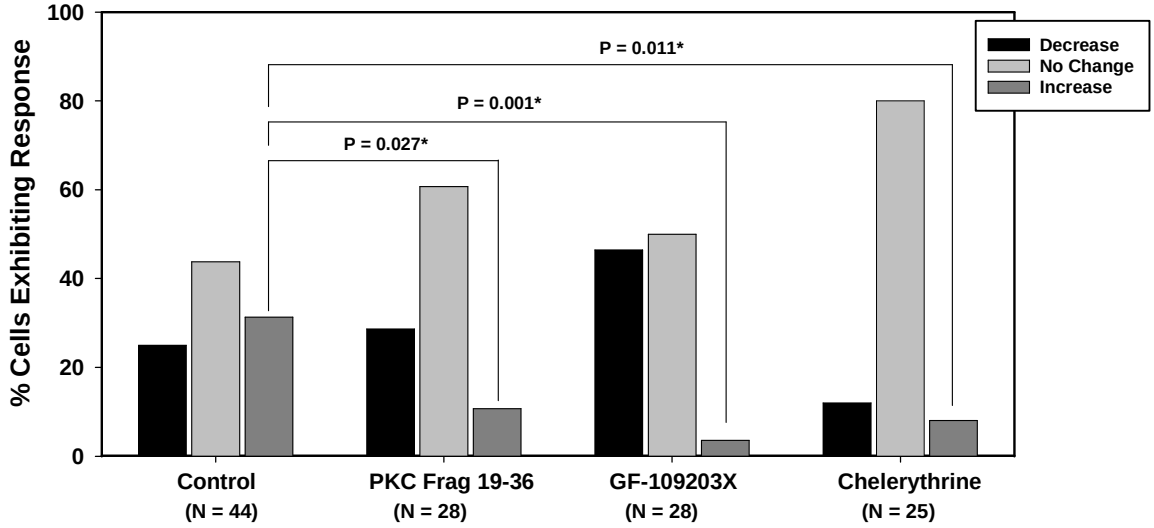

Figure 5.4: Effect of the PKC inhibitors, PKC fragment 19-36, GF-109203X, and chelerythrine chloride on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in sustained calcium current in response to 10 μM baclofen. Data were collected from 44 control cells, 28 cells in the presence of the PKC fragment 19-36 (2 μM), 28 cells in the presence of GF-109203X (500 nM), and 25 cells in the presence of chelerythrine chloride (5 μM). P = 0.003 using a Chi-square when comparing all of the groups together. Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

The PKC inhibitor, GF 109203X (also known as bisindolylmaleimide I and Gö 6850) was also used to determine the involvement of PKC in the signal transduction pathway of $GABA_B$ receptor facilitation of L-type calcium current. This inhibitor was used by Shen and Slaughter (1999) to demonstrate the involvement of PKC in the signal transduction pathway of $GABA_B$ receptor facilitation of L-type calcium current in salamander retinal neurons. GF-109203X is a competitive inhibitor for ATP binding and is highly selective for PKC when compared to several different protein kinases (Toullec et al., 1991; Martiny-Baron et al., 1993). GF-109203X has been shown to inhibit several PKC isoforms in tissue from rat brain including α, βI, ε, δ, and ζ (Martiny-Baron et al., 1993) and α, βI, βII, and γ in bovine brain tissue (Table 5; Toullec et al., 1991). In the study by Toullec and others (1991) GF-109203 inhibited all of the four PKC isoforms tested (α, βI, βII, and γ) with similar potency, whereas Matiny-Baron and others (1993) saw inhibition with a ranked order of potency ($\alpha > \beta I > \varepsilon > \delta > \zeta$).

Since GF-109203X is membrane permeable, the compound was included in both the external bath calcium solution and in the baclofen solution. The distribution of the responses differed in the presence of GF-109203X (500 nM) in comparison to the control cells ($P = 0.003$ using a Chi-square). When GF-109203X (500 nM) was present 46.4% of the cells demonstrated a decrease in sustained calcium current, 50.0% of cells demonstrated no change in sustained calcium current, and 3.6% of cells demonstrated an increase in sustained calcium current in response to 10 μM baclofen (Figure 5.4). Focusing on the facilitatory response, 36.4% of the control cells demonstrated facilitation of sustained calcium current when treated with 10 μM baclofen, while only 3.6% of cells demonstrated facilitation of sustained calcium current treated with 10 μM baclofen when

GF-109203X was included in the external recording solution (P = 0.001 using a Fisher's exact test for pairwise comaprison). Only a single cell out of the 28 cells recorded from demonstrated a 7.51% increase in sustained calcium current with baclofen application when GF-109203X (500 nM) was included in the recording solutions, which is lower than the average percent increase for the 16 control cells demonstrating facilitation (18.07 ± 2.78%, Figure 5.3).

The last PKC inhibitor used to confirm the involvement of PKC in the signal transduction pathway of $GABA_B$ receptor facilitation of L-type calcium current was chelerythrine chloride. Chelerythrine chloride is a benzophenanthridine alkaloid that inhibits PKC by interacting with the catalytic domain (Herbert et al., 1990). This compound has also been shown to affect PKC translocation from the cytosol to the plasma membrane (Chao et al., 1998). Chelerythrine chloride is a selective inhibitor of the conventional PKC isoforms (α, βI, βII, and γ) as well as several of the novel PKC isoforms and atypical PKC isoforms (Herbert et al., 1990; Liu et al., 1994; Chmura et al., 2000; Siomboing et al., 2001). Chelerythrine chloride is membrane permeable and therefore was included in both the external calcium solution and in the baclofen solution. The distribution of the responses differed in the presence of chelerythrine chloride (5 μM) in comparison to the control cells (P = 0.003 using a Chi-square). When chelerythrine chloride was present in the recording solutions, 12.0% of the cells demonstrated a decrease in sustained calcium current, 80.0% of cells demonstrated no change in sustained calcium current, and 8.0% of cells demonstrated an increase in sustained calcium current in response to 10 μM baclofen (Figure 5.4; P = 0.011 using a Fisher's exact test for pairwise comparisons). The 2 cells demonstrating facilitation of sustained

calcium current when treated with 10 μM baclofen in the presence of chelerythrine chloride exhibited an average percent increase of 9.46%, which is lower than the average percent increase of the 16 control cells that demonstrated facilitation in response to 10 μM baclofen (18.07 ± 2.78%, Figure 5.3).

All three of the PKC inhibitors used, PKC fragment 19-36, GF-109203X, and chelerythrine chloride blocked the ability of baclofen to facilitate sustained calcium current in response to baclofen application. These data all support the hypothesis that PKC is involved in the pathway of facilitation of L-type calcium current by $GABA_B$ receptor activation. However, PKC inhibition did not block the ability of baclofen to decrease sustained calcium current, supporting the hypothesis that the decrease of N-type calcium current by $GABA_B$ receptor activation occurs though a different signal transduction mechanism and is not mediated by PKC.

Activation of PKC by PMA Mimics the Effect of Baclofen to Facilitate Sustained Current

If a novel or conventional PKC isoform is involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors, activation of PKC with a phorbol ester should mimic the facilitatory response seen with baclofen application. Like diacylglycerols, phorbol esters activate PKC by binding to the C1 domain of the PKC regulatory region (Burns and Bell, 1991). PMA is a non-selective, general PKC activator and has been shown to activate both the conventional and novel PKC isoforms, but not the atypical PKC isoforms (Riedel et al., 1993a, 1993b; Goode et al., 1994; Shieh et al., 1995, 1996). PMA was applied to the cells the same way baclofen was applied as described in Chapter 2 using a U-tube delivery system made out of PE-10

polyethylene tubing housed in a plexiglass arm. The distribution of the responses differed when PMA was applied to the cells (1 μM) in comparison to the control cells where 10 μM baclofen was applied (P = 0.003 using a Chi-square). As seen with baclofen application, a third of the cells (30.80%) demonstrated facilitation of sustained calcium current when 1 μM PMA was applied to hippocampal cultures isolated from rat pups that were 6 to 8 days old (Figure 5.5). The average percent increase of the 8 cells

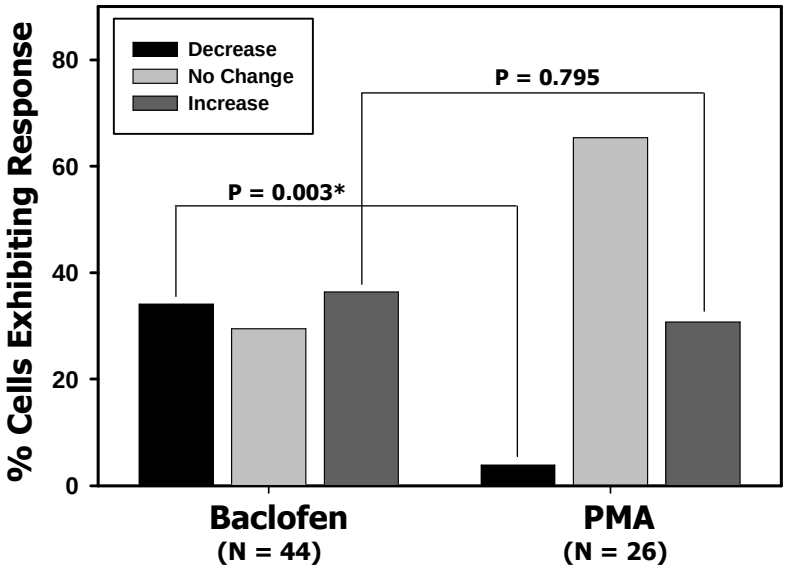

Figure 5.5: Effect of the PKC activator PMA on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen or 1 μM PMA. Data were collected from 44 cells with baclofen and 26 cells with PMA application (P = 0.003 using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

that demonstrated facilitation of sustained calcium current in response to PMA application was 20.11 ± 2.7%, which is consistent with the 16 control cells that demonstrated an 18.07 ± 2.78 % increase with 10 μM baclofen application (Figure 5.2). Only a single cell demonstrated a decrease (8.74%) in response to PMA application, which further suggests that the decrease of calcium current in response to $GABA_B$

receptor activation is not through a PKC mediated pathway. This experiment using the PKC activator PMA supports the hypothesis that PKC is involved in the signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptors.

PKA inhibitors had varying effects on the facilitation of L-type current by $GABA_B$ receptors

In addition to PKC, Shen and Slaughter (1999) showed that the PKA inhibitor, Rp-cAMP (50 μM), was able to suppress L-type current facilitation by $GABA_B$ receptors in the salamander retina. Therefore, two different PKA inhibitors, Rp-cAMPS and H-89, as well as a PKA activator, 8-Br-cAMP, were used to address the involvement of PKA in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptor activation in rat hippocampus.

PKA is a tetrameric structure composed of two regulatory subunits and two catalytic subunits. Inhibition of PKA can be achieved by either inactive cAMP analogues that competitively bind to the cAMP sites on the regulatory subunits or by compounds that block the ATP binding sites on the catalytic subunits. The two PKA inhibitors used to investigate the involvement of PKA, were Rp-cAPMS and H-89. Rp-cAMPS, is a cAMP analogue that has been shown to bind to both type I and type II regulatory subunits of PKA (Rothermel et al., 1984; Van Haastert et al., 1984; Rothermel and Botelho, 1988; Dostmann et al., 1990). Binding of Rp-cAMPS to the cAMP sites prevents the dissociation of the catalytic subunits from the regulatory subunits. Rp-cAMPS has also been shown to be resistant to cyclic nucleotide phosphodiesterases (Erneux et al., 1988). In comparison to Rp-cAMPS, H-89 does not prevent dissociation of the catalytic subunits from the regulatory subunits. Rather, H-89 inhibits the action of PKA by binding to the ATP pocket on the catalytic subunits (Engh et al., 1996). Since both Rp-cAMPS and H-

89 are membrane permeable, these compounds were included in the external calcium solution and in the baclofen solution.

In analyzing both of the PKA inhibitors together and comparing the distribution with the control cells, there is a significant difference in the distribution of the responses to baclofen in the absence and presence of PKA inhibitors (Figure 5.6; P = 0.011 using a Chi-square). However, there were no significant differences in the percentage of cells demonstrating either inhibition or facilitation of sustained calcium current with 10 μM baclofen application when Rp-cAMPS was present (Figure 5.6). In contrast to Rp-cAMP, the percent cells demonstrating an inhibition or facilitation of sustained calcium current in responses to application of baclofen differed significantly in the presence of H-89 (1 μM) in comparison to the control cells (Figure 5.6; decrease, P=0.032; increase, P = 0.001 using a Fisher's exact test for pairwise comparisons). H-89 blocked the ability

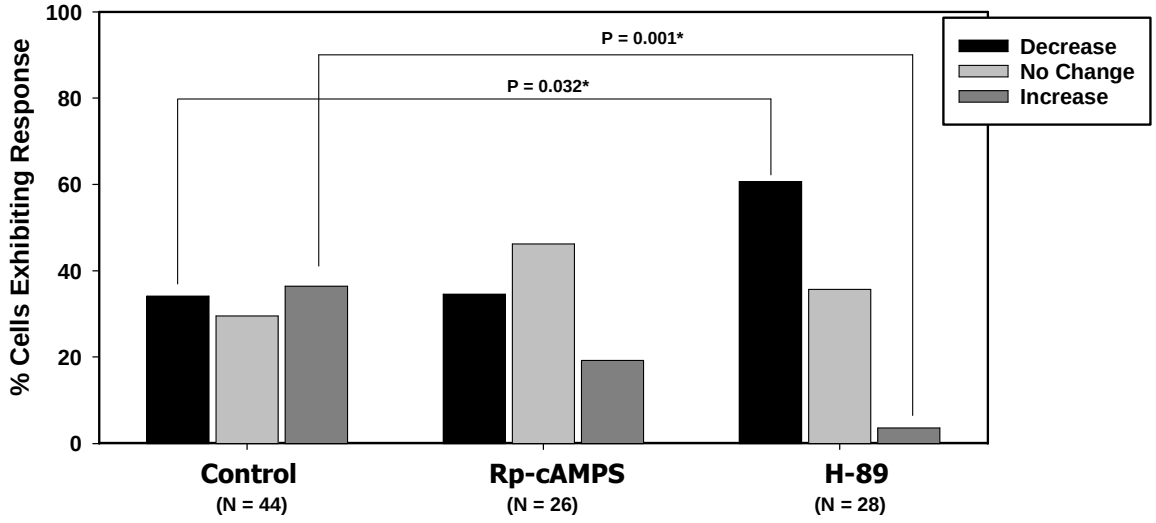

Figure 5.6: Effect of the PKA inhibitors, Rp-cAMPS and H-89, on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen. Data were collected from 44 control cells, 26 cells in the presence of Rp-cAMP (30 μM, P = 0.239 using a Chi-square), and 28 cells in the presence of H-89 (1 μM, P = 0.005). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

of baclofen to facilitate L-type calcium current, but baclofen was still able to facilitate sustained current in the presence of Rp-cAMPS. Data using H-89 suggest that PKA is involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors in the rat hippocampus, whereas data using Rp-cAMPS suggest that PKA is not involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors in the rat hippocampus.

8-Br-cAMP does not Modulate Calcium Currents in the Neonatal Rat Hippocampus

To determine whether PKA activation mimics the modulatory effects of baclofen, the cell-permeable cAMP analogue 8-Br-cAMP (500 μM) was used. 8-Br-cAMP was applied to the cells the same way baclofen was applied as described in Chapter 2. The distribution of the responses differed when 8-Br-cAMP was applied to the cells (500 μM) in comparison to the control cells in which 10 μM baclofen was applied (Figure 5.7; $P < 0.001$ using a Chi-square). Most of the cells (91.7%) demonstrated no effect in response to 500 μM 8-Br-cAMP application, suggesting that PKA is not involved in the signal transduction mechanism of either the facilitation of L-type calcium current or an attenuation of N-type current by $GABA_B$ receptor activation.

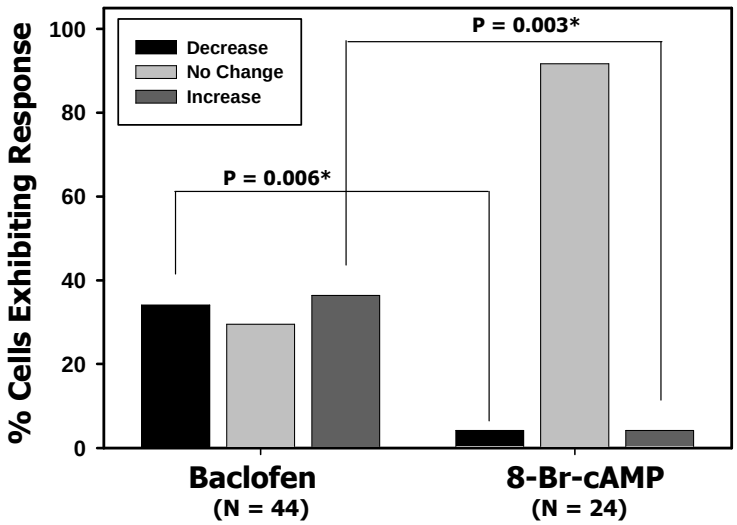

Figure 5.7: Effect of the PKA activator 8-Br-cAMP on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 msec depolarizing pulse to +10 mV from a holding potential of -80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen or 500 μM 8-Br-cAMP. Data were collected from 44 cells with baclofen and 24 cells with 8-Br-cAMP application ($P < 0.001$ using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

DISCUSSION

The importance of calcium influx in neuronal function makes it a prime site for modulation by neurotransmitters and second messengers. Many inhibitory interneuron subtypes within the hippocampus utilize GABA to regulate the overall excitability of the hippocampal region. Our laboratory has demonstrated that activation of $GABA_B$ receptors can enhance calcium influx through L-type calcium channels and attenuate calcium influx through N-type calcium channels during development of the rat hippocampus (Carter and Mynlieff, 2004). $GABA_B$ receptors are metabotropic and often regulate calcium or potassium channels through activation of G-proteins. There are several mechanisms of calcium channel modification through both direct and indirect signaling pathways. One of the most common signaling pathways associated with $GABA_B$ receptor activation is through $G_{i/o}$ proteins (Morishita et al., 1990; Campbell et al., 1993; Menon-Johansson et al., 1993; Greif et al., 2000). Both N-type and P/Q-type calcium current inhibition generally occurs through direct modulation by $G\beta\gamma$ subunits, which are sensitive to PTX suggesting the involvement of $G_{i/o}$ proteins (Kleuss et al., 1991; Herlitze et al., 1996; Ikeda et al., 1996; Furukawa et al., 1998; Zamponi and Snutch, 1998; Kajikawa et al., 2001; Mirshahi et al., 2002). In the current study, treatment with PTX in hippocampal cultures isolated from 7 day old rats blocks the ability of baclofen to decrease calcium current, which has been shown to be through N-type calcium channels (Carter and Mynlieff, 2004). This is consistent with other studies that have demonstrated the involvement of $G_{i/o}$ proteins in the attenuation of N-type calcium current by $GABA_B$ receptor activation in other regions of the rat brain, such as cerebellar granule neurons, cerebrocortical synaptosomes, supraoptic neurons, and

hippocampal pyramidal neurons (Amico et al., 1995; Santos et al., 1995; Harayama et al., 1998; Bertrand et al., 2003). The signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptors in the rat hippocampus is not known. Facilitation of L-type calcium current by $GABA_B$ receptors was still seen in the presence of PTX, which suggests that $G_{i/o}$ proteins are not involved in the signaling pathway of L-type calcium current facilitation by $GABA_B$ receptors and that another mechanism must be involved.

L-type calcium channels have several consensus sites for regulation by protein kinases. Therefore, the involvement of both PKC and PKA in the pathway was investigated using several inhibitors and activators for each kinase. Shen and Slaughter (1999) demonstrated that $GABA_B$ receptor activation can lead to the facilitation of L-type calcium current in a subset of salamander retinal neurons. Approximately 43% of the cells they tested showed a facilitatory response to application of 500 nM baclofen. In their study, the facilitation of L-type calcium current was blocked in the presence of the PKC inhibitors GF-109203X and PKC fragment 19-36. In comparison to the PKC inhibitors, the PKA inhibitor Rp-cAMP only partially suppressed the facilitation of L-type calcium current. Their study suggested that PKC appears to be more important than PKA in the signaling pathway of $GABA_B$ receptor modulation of L-type current in the salamander retina (Shen and Slaughter, 1999).

Our lab was the first to demonstrate the facilitation of L-type calcium current through $GABA_B$ receptor modulation in the mammalian CNS (Carter and Mynlieff, 2004). In the present study, we have demonstrated that PKC is involved in the pathway of L-type calcium current facilitation by $GABA_B$ receptors. Several broad-spectrum PKC

inhibitors were used to verify the involvement of PKC in the pathway. All three PKC inhibitors used, PKC fragment 19-36, GF-109203X, and chelerythrine chloride blocked the ability of baclofen to facilitate L-type calcium current in hippocampal cultures isolated from 6 to 8 day old rats. These PKC inhibitors were chosen based on their specificity to several if not all of the PKC isoforms, their cell permeability, and their varying chemical structures. To further confirm the involvement of PKC, a phorbol ester, PMA, was used to activate PKC and the responses to sustained calcium current were measured. Facilitation was observed with PMA application in 30.80% of the cells, which is consistent with the 36.36% of cells that demonstrated facilitation with baclofen application. All of these data suggest that PKC is involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors in the neonatal rat hippocampus.

Future experiments in the laboratory will explore the involvement of specific PKC isoforms in the pathway. The fact that the facilitatory response is present in hippocampal cultures isolated from postnatal day 7, suggests that some of the PKC isoforms that are expressed later in development may not be involved. The study by Roisin and Barbin (1997), which explored the presence of several PKC isoforms during hippocampal development, suggests that PKCγ is probably not involved in the signaling pathway. PKCγ levels remain relatively low for the first postnatal week and only begin to rise following postnatal day 7 (Roisin and Barbin, 1997). All of the other PKC isoforms tested, α, β, ε, and ζ were highly expressed by postnatal day 7. Therefore, each of these isoforms could be involved in the pathway of $GABA_B$ receptor mediated facilitation of L-type calcium current. Activation of PKC with PMA mimicked the

facilitation normally seen with baclofen application, which suggests that either conventional or novel PKC isoforms are involved in the pathway rather than the atypical isoforms.

Facilitation was still seen in the presence of the PKA inhibitor, Rp-CAMP, but not when the PKA inhibitor H-89 was used. The varying responses seen with the PKA inhibitors could be due to several reasons. Rp-cAMPS and H-89 were chosen, because they are both membrane permeable inhibitors of PKA. However, they inhibit PKA through two very different mechanisms. Rp-cAMPS prevents the dissociation of the catalytic subunit from the regulatory subunit by competitively binding to the cAMP sites on the regulatory subunit. This inhibitor blocks the activation of the catalytic subunit all together and thus dissociation from the regulatory site. In comparison, H-89 associates with the ATP binding site on the catalytic subunit and blocks the phosphorylation processes of PKA. Even though H-89 is a potent inhibitor for PKA (Chijiwa et al., 1990), it has been shown to inhibit several other kinases including p70 ribosomal protein S6 kinase 1 (S6K1; IC_{50} = 80 nM), mitogen- and stress-activated protein kinase (MSK1; IC_{50} = 120 nM), PKG (IC_{50} = 340 nM), CaMKII (IC_{50} = 11 μM), and PKC (IC_{50} = 14 μM; Davies et al., 2000, for review see Lochner and Moolman, 2006). Thus, it is possible that H-89 is inhibiting other kinases, which is causing variation in the calcium current responses seen to baclofen application when compared to the other PKA inhibitor used. Direct activation of PKA with 8-Br-cAMP did not result in the facilitation of L-type calcium current, which suggests that PKA is not involved in the signal transduction pathway of L-type calcium current by $GABA_B$ receptor activation.

In conclusion, during the early neonatal period in the rat hippocampus $GABA_B$ receptor activation can lead to the attenuation of N-type calcium current and the facilitation of L-type calcium current. The current study has demonstrated that the attenuation of N-type calcium current by $GABA_B$ receptor activation is mediated through a PTX sensitive G-protein pathway. Attenuation of N-type calcium current was still observed in the presence of PKC and PKA inhibitors and attenuation of N-type calcium current was not seen with direct activation of PKC and PKA. These experiments suggest that neither PKC nor PKA are involved in the pathway of N-type calcium current modulation by $GABA_B$ receptors. Facilitation of L-type calcium current was still observed in the presence of PTX, which demonstrated that $G_{i/o}$ proteins were not involved in the pathway. Facilitation of L-type calcium current was not seen in the presence of PKC inhibitors and facilitation was observed with direct activation of PKC with a phorbol ester. These experiments suggest that PKC is involved in the pathway of L-type calcium current facilitation by $GABA_B$ receptor activation in the rat hippocampus. Facilitation was still observed in the presence of Rp-cAMP, but not in the presence of another PKA inhibitor H-89. Direct activation of PKA with a cAMP analogue, 8-Br-cAMP, did not result in the facilitation of L-type calcium current. Therefore, the results using Rp-cAMP and 8-Br-cAMP suggest that PKA is not involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors.

CHAPTER 6

GENERAL DISCUSSION

The primary findings presented in these studies demonstrate that the facilitation of L-type calcium current by $GABA_B$ receptor activation is more prominent in the second postnatal week when compared to the first postnatal week of hippocampal development in the rat. In comparison, the inhibition of N-type calcium current was found to be more prominent during the first postnatal week when compared to the second postnatal week of hippocampal development. The facilitation of L-type calcium current was blocked in the presence of PKC inhibitors and the response was mimicked with direct activation of PKC with a phorbol ester. Time course studies using Western blot analysis demonstrated that KCC2 protein levels increase during the first two postnatal weeks, while NKCC1 protein levels remain relatively level in both hippocampal cultures and whole hippocampal protein extracts of rat pups. Calcium influx through L-type calcium channels was shown to be necessary at least in part for the upregulation of KCC2 protein levels.

The hippocampus contains excitatory pyramidal neurons and over 21 different types of inhibitory interneurons. Approximately 30% of the cells in hippocampal cultures isolated from pups 7 to 14 days old demonstrate facilitation of L-type calcium current. Due to the heterogeneity of hippocampal cell cultures, it is possible the facilitatory response of calcium current by $GABA_B$ receptors is only observed in a particular subset of cells. A study by Nuñez and McCarthy (2007), demonstrated peak protein levels of the L-type calcium channel subtype $Ca_v1.2$ in hippocampal protein extracts isolated from 7 day old rat pups. Previous immunocytochemical experiments in the Mynlieff laboratory have demonstrated that only about 30% of the neurons in our

cultures express $Ca_v1.2$, whereas almost all of the neurons express $Ca_v1.3$. Preliminary data using Western blot analysis of membrane proteins isolated from the superior region of hippocampi from rats of varying ages demonstrate that $Ca_v1.2$ levels are high in the first postnatal week, whereas $Ca_v1.3$ protein levels begin low and increase throughout neonatal development (Ingraham, unpublished data). The early expression of $Ca_v1.2$ and its restriction to a subset of cells suggest that $Ca_v1.2$ rather than $Ca_v1.3$ is involved in the facilitatory response of calcium current by $GABA_B$ receptors.

The data presented indicate that PKC is involved in the signaling pathway of L-type calcium current facilitation by $GABA_B$ receptors. Furthermore, this effect is likely to be mediated by a conventional or novel isoform of PKC rather than an atypical form since the effect of baclofen on L-type current was mimicked by the PKC activator, PMA. However, PKC may not directly phosphorylate L-type calcium channels to produce this response. Rather than direct phosphorylation of the calcium channel, PKC may phosphorylate another intermediate effector protein. For example, the enhancement of L-type calcium current has been observed in isolated rat ventricular myocytes by activation of α_{1A} adrenoceptors. This enhancement of L-type calcium current was mediated through a PKC and CaMKII pathway (O-Uchi et al., 2005, 2008). Several other studies in cardiac and smooth muscle myocytes have suggested that CaMKII is involved in a calcium-dependent facilitation of L-type calcium current (McCarron et al., 1992; Anderson et al., 1994; Yuan and Bers, 1994). Thus, it is possible that PKC may phosphorylate and activate CaMKII to facilitate L-type calcium current in the rat hippocampus (Figure 6.1). Additional studies in the laboratory will utilize CaMKII inhibitors, to investigate the

involvement of the kinase in the pathway of L-type calcium current facilitation by $GABA_B$ receptors.

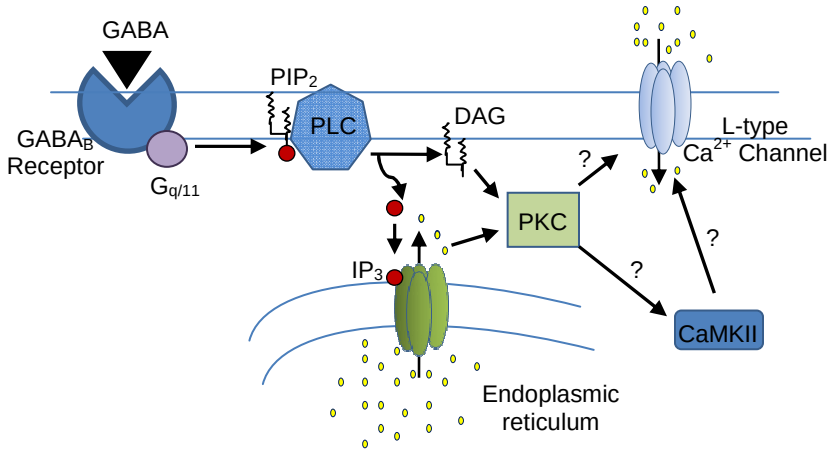

Figure 6.1: Possible signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors. Abbreviations: PIP2, phosphoinositol bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; IP3, inositol trisphosphate; CaMKII, calcium/calmodulin dependent kinase II.

The facilitation of L-type calcium current has been observed in several different cell types, including skeletal muscle, cardiac muscle, smooth muscle, neurons, and adrenal chromaffin cells. The enhancement of L-type calcium current increases the force of contraction during tetanic stimulation in skeletal muscle (Schmid et al., 1985; Arreloa et al., 1987; Sculptoreanu et al., 1993a) and also increases the force of contraction in cardiac muscle during physiological increases in heart rate (Lee, 1987; Zygmunt and Maylie, 1990; Sculptoreanu et al., 1993b; Kamp et al., 2000). In neurons, L-type calcium current facilitation can contribute to changes in neuronal excitability and synaptic plasticity (Kavalali and Plummer, 1996; Parri and Lansman, 1996; Kammermeier and Jones, 1998). Facilitation of L-type calcium current also plays an important role in adrenal chromaffin cells by inducing catecholamine release (Hoshi et al., 1984; Artalejo

et al., 1992). These are just a few examples of the importance L-type calcium current facilitation plays in many native cell types.

Calcium influx through VDCCs and NMDA receptors is crucial in developing neurons. Calcium signaling is involved in the proliferation, migration, and differentiation of neurons (Marty et al., 1996). Calcium influx also plays an important role in synaptogenesis and the integration of new synapses into neuronal networks (Eins et al., 1983; Kneussel and Betz, 2000). An increase in gene expression and DNA synthesis is also observed as a result of the increase in calcium levels (West et al., 2001). It is possible that the enhancement of L-type calcium current in response to $GABA_B$ receptor activation could be playing a very specific and important role in one of these responses, such as signaling changes in gene expression. The occurrence of L-type calcium current facilitation in the early neonatal period suggests that calcium influx plays a functional role in the maturation process of hippocampal neurons.

GABA is the main inhibitory neurotransmitter in mature neurons, but its effects are excitatory in immature neurons. The excitatory actions of GABA during development have been demonstrated in several brain regions, including the hippocampus (Mueller et al., 1984; Ben-Ari et al., 1989; Cherubini et al., 1990; Leinekugel et al., 1995; Khazipov et al., 1997; Ganguly et al., 2001), cerebellum (Eilers et al., 2001), neocortex (Luhmann and Prince, 1991; Yuste and Katz, 1991; Fukuda et al., 1998; Owens et al., 1999), and the hypothalamus (Chen et al., 1996; Obrietan and Van Den Pol, 1999; Gao and Van Den Pol, 2001). Interestingly, GABA and glycine exert excitatory actions in the developing spinal cord as well (Serafini et al., 1995; Wang et al., 1994; Vinay and Clarac, 1999). The excitatory response of GABA during development

plays a critical role in the proliferation, migration, and differentiation of neurons and also in the formation of synapses (for review see Ben-Ari, 2002; Owens, 2002).

The excitatory actions of GABA depend on the location, type, and developmental stage of the neuron. The switch from excitatory-to-inhibitory may occur at different developmental stages in the various brain regions and even occur at different stages for the various cell types found within a single region. For example, in the hippocampus it is suggested that the excitatory-to-inhibitory switch occurs in inhibitory interneurons before pyramidal cells. Studies by Hennou and colleagues (2002) have demonstrated the sequential development of GABAergic and then glutamatergic synapse formation in CA1 hippocampal interneurons in rats. It is possible that the GABAergic switch even within different groups of interneurons can occur at different developmental stages (Hennou et al., 2002). Similarly, GABA synapses precede the formation of glutamatergic synapses in rat hippocampal CA1 pyramidal neurons (Tyzio et al., 1999). In patch clamp recordings from hippocampal slices isolated from postnatal day 0 rats, three different populations of CA1 pyramidal cells were observed (Tyzio et al., 1999). There are pyramidal cells that have functional $GABA_A$ and glutamate receptors, but are silent and do not exhibit excitatory postsynaptic currents (PSCs). There are cells that just exhibit GABA mediated excitatory PSCs, while the last group of cells exhibits both GABA and glutamate mediated excitatory PSCs. The group of cells that exhibit both GABA and glutamate PSCs are more developed than the other two groups and contain both an apical and basal dendrite (Tyzio et al., 1999; for review see Ben-Ari, 2002). The fact that GABAergic synapses are formed on interneurons prior to pyramidal cells, suggests that the excitatory actions of GABA and subsequent influx of calcium may play an important

role in the maturation of interneurons, which subsequently aids in the maturation of pyramidal neurons.

In immature neurons, $GABA_A$ receptor activation can depolarize the membrane and increase intracellular calcium levels through activation of L-type calcium channels (Yuste and Katz, 1991; Leinekugel et al., 1995; Ganguly et al., 2001). Long lasting depolarizing potentials called giant depolarizing potentials (GDPs) are also observed during the first two postnatal weeks in rat hippocampal interneurons and CA3 and CA1 pyramidal neurons and are only present when GABA is excitatory (Ben-Ari et al., 1989; Leinekugel et al., 1995, 1997, 1998; Khazipov et al., 1997). Glutamatergic synapses also contribute to GDPs with the activation of NMDA receptors, which further increase calcium levels in immature neurons. These large oscillations of intracellular calcium levels aid in neuronal growth and synapse formation (for review see Ben-Ari, 2002; Owens and Kriegstein, 2002). Inhibition by $GABA_B$ receptors plays an important role in controlling the duration of the GDPs (Mclean et al., 1996; Caillard et al., 1998).

Whether or not GABA exerts an excitatory response early in development is largely dependent on the reversal potential for chloride. In mature neurons, intracellular chloride levels are relatively low. Therefore, $GABA_A$ receptor activation results in chloride influx and hyperpolarizes the neuron. In immature neurons, intracellular chloride levels are higher than in mature neurons. $GABA_A$ receptor activation in an immature neuron results in chloride efflux and depolarizes the neuron. It is thought that the changes in the intracellular chloride levels are mediated by developmental changes in the expression of the specific chloride cotransporters, NKCC1 and KCC2. NKCC1 predominates in the early neonatal period and mediates chloride influx, while KCC2

expression predominates in mature neurons and mediates chloride efflux. KCC2 protein levels increase during the first two postnatal weeks, while NKCC1 protein levels remain relatively level in both hippocampal cultures and whole hippocampal protein extracts of rat pups. NKCC1 expression is critical during the early neonatal period, however because protein levels remain level throughout the first two weeks of development this cotransporter does not appear to be solely responsible for the GABAergic switch. Our study was the first to compare the protein levels of both NKCC1 and KCC2 throughout hippocampal development to determine a ratio of the two transporters. Rather than the upregualtion of KCC2 and the downregualtion of NKCC1 protein, the data presented here suggest that it is the ratio of KCC2 to NKCC1 that is important for the developmental switch of the $GABA_A$ response.

One of the biggest questions in the developmental regulation of the reversal potential for chloride is what signals are involved in the increase in KCC2 expression during the early neonatal period. Do the excitatory actions of GABA itself trigger the cascade through the increase of intracellular calcium levels? Ganguly et al., (2001) demonstrated that calcium influx is necessary for the excitatory-to-inhibitory switch of $GABA_A$ receptors during the early postnatal period. In addition, they observed a decrease in KCC2 mRNA with the blockade of $GABA_A$ receptors (Ganguly et al., 2001). The current study has demonstrated that calcium influx through L-type calcium channels is necessary for the upregulation of KCC2 protein levels. Inhibition of L-type calcium channels with nimodipine blocked the upregulation of KCC2 protein levels in hippocampal cultures by 50%. Together, these studies demonstrate that $GABA_A$

excitation and calcium influx through L-type calcium channels is necessary for the upregulation of KCC2 and the subsequent switch in the GABAergic response.

Increases in the phosphorylated form of the transcription factor, cAMP response element-binding protein (pCREB), are observed with $GABA_A$ receptor activation during the first postnatal week and this effect is blocked by L-type calcium channel antagonists (Perrot-Sinal et al., 2003; Mantelas et al., 2007; Nuñez and McCarthy, 2007). GABA induced increases in intracellular calcium levels, which activate pCREB, can induce BDNF expression (Berninger et al., 1995; Obrietan et al., 2001; West et al., 2001). BDNF levels are higher in the first two postnatal weeks of development and BDNF may play an important role in the maturation of the GABAergic system. It has been demonstrated that overexpression of BDNF can increase the amount of GABAergic synapses early in development and also increase KCC2 mRNA levels in transgenic embryos (Aguado et al., 2003). Interneurons do not express BDNF. Therefore, it is possible that the release of BDNF from pyramidal cells aids in the upregulation of KCC2 in interneurons. Therefore, BDNF could either increase KCC2 levels in the neuron expressing it or bind to BDNF receptors (trkB) on neighboring interneurons that would lead to an increase of KCC2 expression in these cells. In addition to activation of transcription factors and neurotrophins, L-type calcium current has also been shown to activate kinases such as CaMKII, which could be involved in the phosphorylation of CREB and the increase in BDNF expression (West et al., 2001). Thus, it is possible that activation of neurotrophins, transcription factors, and kinases by calcium influx through L-type calcium channels could all be involved in gene activation and trigger the

upregualtion of KCC2 during the early neonatal period in either pyramidal neurons or interneurons in the hippocampus (Figure 6.2).

The increase in calcium current is also involved in membrane trafficking and posttranslational changes in addition to changes in gene transcription and protein synthesis. In a study by Fiumelli et al., (2005), the shift in the chloride reversal potential was not observed in the presence of Ca^{2+}-dependent PKC inhibitors. Direct phosphorylation of KCC2 by PKC decreases endocytosis of KCC2 proteins, thus enhancing KCC2 protein levels in the membrane (~200% increase, Lee et al., 2007). Therefore, it is possible that activation of PKC by L-type calcium current could be regulating membrane trafficking of the KCC2 transporter (Figure 6.2).

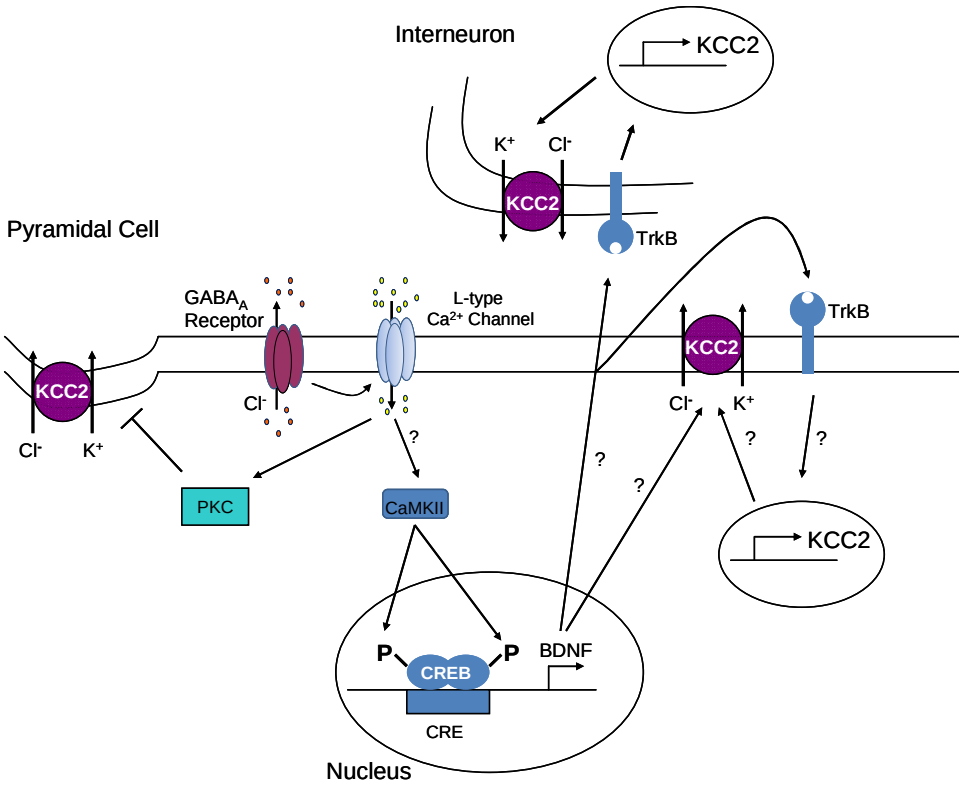

Figure 6.2: Possible signaling events involved in the upregualtion of KCC2. GABA mediated increases in L-type calcium current could activate a signaling cascade involving several steps resulting in the upregualtion of KCC2. In addition, activation of PKC by L-type calcium current can decrease endocytosis thereby decreasing the degradation of KCC2 protein in these neurons.

There are still many questions to be answered regarding the signal transduction mechanism of L-type calcium current facilitation by $GABA_B$ receptors during the early neonatal period in the rat hippocampus. Since PKC is involved in the mechanism of L-type current facilitation it is possible that $GABA_B$ receptors are coupled to $G\alpha_{q/11}$ and PLC in a subset of neurons in the neonatal rat hippocampus. Inhibitors of PLC and the selective $G\alpha_{q/11}$ inhibitor (YM-254890) could be used to investigate the coupling of $GABA_B$ receptors to $G\alpha_{q/11}$ and PLC in neonatal rat hippocampal cultures using electrophysiological recording or calcium imaging. The association of $GABA_B$ receptors with $G\alpha_{q/11}$ would be a novel finding, because $GABA_B$ receptors predominantly couple to $G\alpha_{i/o}$ proteins (Morishita et al., 1990; Campbell et al., 1993; Menon-Johansson et al., 1993; Greif et al., 2000).

Future experimentation could also investigate the involvement of CaMKII in the pathway of L-type calcium current facilitation in the rat hippocampus, since the enhancement of calcium influx through $Ca_v1.2$ channels in cardiac myocytes has been shown to be mediated through a PKC / CaMKII pathway. To determine whether CaMKII is involved in the signal transduction pathway of L-type calcium current facilitation by $GABA_B$ receptors, whole-cell patch clamp recording and calcium imaging in hippocampal neurons could be performed in the presence of CaMKII inhibitors. If facilitation by $GABA_B$ receptors was no longer observed in the presence of CaMKII inhibitors, it would suggest that CaMKII is involved in the facilitatory pathway along with PKC.

In addition, the specific PKC isoforms involved in the signaling mechanism have not been identified. Since the atypical PKC isoforms do not have a DAG binding site and

are not activated by phorbol esters, it is highly unlikely that they are involved in the facilitation of L-type calcium channels by $GABA_B$ receptors in this subset of cells. The involvement of conventional and novel PKC isoforms in the pathway could be investigated using similar electrophysiological experiments that were performed with the general PKC inhibitors presented here, but rather with specific inhibitors of the different PKC isoforms. Calcium imaging, which provides much faster data acquisition than electrophysiological recording, could also be used to test the effects of kinase inhibition on the baclofen responses seen in hippocampal cultures. In addition, Western blot analysis using antibodies against the phosphorylated states of specific PKC isoforms with baclofen treatment could also be used to investigate the PKC isoform involved. Further studies using Western blot analysis could also be used to determine the translocation of the conventional and novel PKC isoforms to the membrane with baclofen treatment.

Several studies have suggested that the $Ca_v1.2$ rather than the $Ca_v1.3$ L-type calcium channel is facilitated by $GABA_B$ receptor activation in neonatal rat hippocampus. Through immunohistochemical experiments and confocal micrscopy in hippocampal slices, it could be determined if $Ca_v1.2$ L-type calcium channels colocalize with $GABA_B$ receptors, and specifically which subtype of the $GABA_B$ receptor is involved: $GABA_{B1a}$ which is located presynaptically or $GABA_{B1b}$ which is primarily located postsynaptically (Vigot et al., 2006). The developmental changes of L-type channels and $GABA_B$ receptor expression could also be observed through immunohistochemical analysis. Using a Zeiss Pascal confocal microscope equipped with two laser lines and fluorescence, the specific subcellular localization of antibody staining could be thoroughly analyzed. Since L-type current facilitation by $GABA_B$ receptor activation is a

developmental phenomenon that predominates during the second postnatal week, it is likely that L-type calcium channels may only be colocalized with $GABA_B$ receptors early in neonatal development and not in adult rats. Further confirmation of the involvement of $Ca_v1.2$ could involve electrophysiological recording in hippocampal cultures in the absence of $Ca_v1.2$ channels. Short hairpin RNA knockdown of the $Ca_v1.2$ α_1 subunit in dorsal horn neurons has been shown to effectively decrease expression of the channel in the membrane (Fossat et al., 2010). If the $Ca_v1.2$ gene is effectively silenced, $GABA_B$ receptors would no longer be able to facilitate L-type calcium current in hippocampal neurons.

Using hippocampal slices, it could be possible to identify the subset of cells in which L-type calcium channels and $GABA_B$ receptors are colocalized. Inhibitory interneurons within the hippocampus are highly diverse, but different types of interneurons can be identified based on morphology. Somatic location within a layer as well as dendritic shape can aid in the identification of an interneuron class. Molecular cell markers such as calcium binding protein, parvalbumin, cholecystokinin, and vesicular glutamate transporter 3 can also be used to identify interneuron classes (Somogyi and Klausberger, 2005; Klausberger and Somogyi, 2008). However, a single molecular marker, such as parvalbumin, could not be used to identify an interneuron class on its own, because many of the interneuron classes can express the same molecular markers. Therefore, both morphological characteristics and antibody labeling would have to be utilized to determine if and which specific class of interneurons $GABA_B$ receptors colocalize with L-type calcium channels. A single hippocampal slice could be labeled with antibodies against $GABA_B$ receptors, $Ca_v1.2$ L-type calcium channels, and

an interneuron marker to distinguish which subset of cells $GABA_B$ receptors and L-type calcium channels are colocalized. It would be interesting to determine the specific subset of cells where $GABA_B$ receptors and L-type calcium channels are colocalized, because it could provide further indications as to the functional significance of L-type calcium current facilitation by $GABA_B$ receptors during development. It is possible that the class of cells in which $GABA_B$ receptors and L-type calcium channels are colocalized may be specifically important in the time course of the maturation of interneurons and pyramidal cells.

Together, the experiments presented here along with the proposed experiments would further our knowledge of the molecular mechanisms that control excitatory and inhibitory synapse maturation in the developing rat hippocampus.

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