Postnatal Developmental Trends in Membrane Excitability and BK Channel Function in the Rodent Hippocampus

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POSTNATAL DEVELOPMENTAL TRENDS IN MEMBRANE EXCITABILITY AND BK CHANNEL FUNCTION IN THE RODENT HIPPOCAMPUS

By
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A Dissertation Submitted to the Faculty of the Graduate School, Marquette University, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Milwaukee, WI,
May 2023
ABSTRACT

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Michael S. Hunsberger, B.S.

This dissertation outlines the postnatal development of excitability as well as expression and function of BK potassium channels in hippocampal neurons. I used patch clamp electrophysiology to measure how neuronal action potential waveforms and action potential firing frequencies change in early development, and how pharmacological blockade of BK channels affects these properties in hippocampal neurons. I also describe how the protein expression of the BK channel pore-forming α subunit and mRNA expression of different variants of the pore forming α subunit and auxiliary β-4 subunit changes with development.

I demonstrate in both cultured rat hippocampal neurons across the first seven postnatal days and in putative mouse hippocampal pyramidal neurons from postnatal day four to fifteen in acutely prepared slices that maturation brings changes in action potential kinetics and large increases in the capacity for high frequency firing.

In the cultured neurons I demonstrate that during the first postnatal week, the contribution of BK channels to action potential repolarization decreases but the channel's role in maintaining high-frequency firing increases. This change in the timing of BK channel activity in the action potential waveform is accompanied by a slowing of BK current onset measured by a decreasing effect of BK current blockade on potassium current rise time. Additionally, I demonstrated in pyramidal neurons in mouse brain slices that there is a loss of BK channel contribution to action potential repolarization between postnatal day four and fifteen.

These changes in the BK channel's role in neuronal action potential firing are accompanied by large increases in the expression of α subunit protein measured by Western blot and by large increases in mRNA transcript expression, measured by RT-qPCR, of both the α and β-4 subunits in the hippocampus. The rise in β-4 subunit expression can explain the apparent slowing of BK channel activation through development.

I investigated whether BK channel blockade in neonatal brain slices could attenuate hyperexcitability in a model of seizure activity as BK channels in immature neurons share properties of BK channel variants implicated in epilepsy but found no evidence to support this hypothesis.
ACKNOWLEDGMENTS

I would like to thank everyone at Marquette who made this possible: my advisor, Dr. Michelle Mynlieff; my committee, Dr. Thomas Eddinger, Dr. Pinfen Yang, Dr. Robert Wheeler, and Dr. Jim Buchanan for their long-suffering and well-tailored support. I would like to thank the Department of Biological staff for keeping the place running and always being flexible with the deadlines. And not least, I want to thank my fellow graduate students. I am lucky to call you my friends; I truly could not have done it without you.

I also want to thank my family. My parents who always made me feel free to choose my own path. My wife who has been my tireless rock through the process. And my sons, Conrad and Lewis; you may not have made it any easier, but you brought me so much joy along the way.
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LIST OF COMMON ABBREVIATIONS AND GENE NAMES

aCSF: artificial cerebrospinal fluid
Actb: β-actin gene or transcript
AP: Action potential
BK channel: large conductance calcium activated potassium channel
EPSC: Excitatory post-synaptic current
IPSC: Inhibitory post-synaptic current
Kcnma1: BK channel α subunit gene or transcript
Kcnmb4: BK channel β-4 subunit gene or transcript
P: Postnatal day (e.g. P7 is postnatal day seven)
Pax: Paxilline
qPCR: quantitative polymerase chain reaction
RT-qPCR: reverse transcription quantitative polymerase chain reaction
STREX: stress hormone regulated exon containing splice variant of the BK channel
ZERO: Splice variants of the BK channel lacking the STREX exon
Chapter 1: Introduction

1.1 Postnatal Brain Development

The neonatal brain is naïve and plastic, primed to be molded by experience and as such is markedly different from the mature brain [1]. Connectivity of neurons changes dramatically in early development with rapid synaptogenesis beginning prenatally in humans and extending through the first year of life, at which point synapses begin to be eliminated in an experience-dependent manner, or pruned, faster than they form. This process of synaptic pruning refines the connectivity to optimize central nervous system function [2], [3]. This rapid building and refinement of connections in the brain is accompanied by increases in grey matter, the formation and myelination of white matter tracks and gyrification, the formation of the folding patterns of the brain allowing for increases in cortical surface area. These changes occur most rapidly in the prenatal and early postnatal period and in some cases continue into adulthood [4].

In addition to large macroscopic changes in brain structure and connectivity, brain function at the level of the individual neuron also changes rapidly. The normally inhibitory GABAergic system is excitatory in prenatal and very early postnatal development and underlies synchronous giant depolarizing potentials, depolarizations that are important for building functional connections in the maturing brain [5]. Meanwhile the main synaptic machinery of excitatory transmission, the AMPA type glutamate receptor, is not functional in many developing synapses until synapses are activated by NMDA mediated long term potentiation – an event that is often dependent on the giant depolarizing potentials [6]–[8]. GABAergic and glutamatergic receptors have different subunit expression during development which affects the physiology of excitatory and inhibitory synapses [9], [10]. The voltage gated sodium and potassium channels that carry ionic conductance during action potential firing are expressed at low
levels in the prenatal and early postnatal development and rise sharply with the greatest increases in expression occurring at postnatal day four to five in the rodent brain [11], [12].

The net result of the differences between immature and adult brains is that the immature brain is hyperexcitable and more susceptible to seizures, which puts infants at risk of developing epilepsy and other neurological deficits. While infants and young children are more susceptible to seizures there is a shortage of effective treatments for these populations [13]–[15]. Because of this, there is a need to expand our current understanding of how excitability is governed in the neonatal brain. This dissertation examines the changes in expression and function of a specific potassium channel known to contribute to action potential duration and firing rate in adults, the BK potassium channel, throughout early development as it relates to neuronal excitability. These studies help to define its contributions to neuronal physiology in the developing brain and how modulation of its activity may serve to reduce neonatal seizure activity.

1.2 Survey of Kv and KCa Channels and Their Contributions to Action Potential Firing

In their seminal 1950’s work Hodgkin and Huxley reported that the repolarization of the action potential is accomplished by transient, voltage-dependent potassium currents in the squid giant axon [16], [17]. While the next section (1.3) will discuss the physiological role of the BK channel in modulating the repolarization of neuronal action potentials, it is by no means the major potassium current that underlies this process. This section will review the potassium channel currents that repolarize the action potential to contextualize the role of the BK channel among them. For the sake of brevity and relevance to this dissertation I will prioritize discussion to findings in mammalian cell types.

Most of the potassium current passed during action potential firing is carried by
channels from the voltage gated potassium (K_{v}) channel family. This is a large and diverse family of channels comprised of K_{v1.x}-K_{v12.x} channels (the ‘x’ indicates the channel isoform; different isoforms within the same channel type [i.e. K_{v2.1} and K_{v2.2}] may differ somewhat in kinetics but will not be differentiated here). K_{v1}-4 channels underlie the A and D-type delayed rectifier potassium currents – so termed because their activation lags behind that of the voltage-gated sodium channels that underlie membrane depolarization and the current is not symmetrical in both directions. These channels account for the majority of the potassium current responsible for repolarization of the membrane during an action potential [18].

Delayed rectifying potassium currents can be further subdivided into fast and slow delayed rectifying currents. The classical, slow delayed rectifier current described in Hodgkin and Huxley’s experiments is now known to be carried by the K_{v1} and K_{v2} family of channels. Fast delayed rectifying currents are carried by K_{v3} channels and in contrast with the classical slow delayed rectifiers are activated at much higher voltages. K_{v3} channels are fast activating and inactivating channels that are open only during action potential repolarization and are single-handedly responsible for repolarizing some types of fast-spiking cortical and hippocampal interneurons which enables their characteristic high firing frequencies [19]–[21].

A-type potassium currents are the first of the two classically identified inactivating currents; they inactivate and recover from inactivation rapidly with a time course of <100 milliseconds. A-type potassium currents are carried by K_{v4} channels and participate in repolarization of neurons and it has been suggested that their inactivation kinetics and expression in burst-firing cell types are especially suited to modulating firing frequencies of burst-firing neurons [18], [22]–[24].

The D (delay) type potassium current is carried by channels in the K_{v1}/Shaker potassium channel family that inactivate and recover from inactivation slowly (hundreds
of milliseconds to seconds) [18]. D-type currents are named for their ability to delay the onset of action potential firing in response to a stimulus and are probably important in signal integration but also contribute a large amount of the repolarizing potassium current in certain cells such as cortical pyramidal cells [25]–[27].

As for the remainder of the Kv channel superfamily, Kv5,6,8 and 9 do not assemble into functional channels on their own but form heterotetramers with Kv2 channels [28]. Kv7 channels are also known as KCNQ channels and underlie the M-current, a non-inactivating current inhibited by muscarinic cholinergic activation (which is where the “M” comes from) that reduces excitability by hyperpolarizing the resting membrane potential [29], [30]. Kv10-12 are known as the ether-á-go-go family of channels (named for a line of mutant drosophila which reminded the researchers of diminutive go-go dancers when exposed to ether). The exact role of these channels in neurons is difficult to pin down due to a lack of specific antagonists, but can influence resting membrane potential, speed of repolarization, and shape repetitive firing in select neuronal populations [31].

Members of the calcium activated potassium channel (KCa) family are also activated during and contribute to action potential firing. This family consists of the small conductance (SK), the intermediate conductance (IK), and large conductance (BK) calcium activated potassium channels. To put some numbers behind the designations of small, intermediate, and large conductance, SK, IK, and BK have single channel conductances of approximately 10, 40, and up to 300 pS respectively [32]–[34].

The SK channel has three isoforms, SK1-3 which are encoded by the genes KCNN1-3 (in humans) that are differentially expressed in different regions of the central nervous system, all of which are gated by a constitutively associated calmodulin subunit [35]–[37]. SK channels underlie a major part of the medium afterhyperpolarization, a hyperpolarizing event following action potentials that lasts up to 100 ms, reducing
neuronal excitability and controlling spike timing (Fig. 1.1) [38]–[42]. While primarily known for their role in regulating action potential firing, dendritic SK currents also work to blunt excitatory synaptic transmission by repolarizing postsynaptic currents [43].

IK channels are encoded by the gene KCNN4 and are sometimes historically referred to as SK4, but are typically categorized separately because their conductance and physiological roles are so divergent from SK1-3 [44]. IK channels are expressed in neurons in the peripheral and enteric nervous system, but within the central nervous system they seem to be limited to glia where they are implicated in responses to pathological states [45]–[47].

The last member of the K_Ca channel family is the BK channel. It’s structural features, its modulatory subunits, its regulation by divalent cations and other compounds, its functions in shaping action potential firing and other neurophysiological processes and its implications in epilepsy and other disease states will be described extensively in the remainder of this introduction.

1.3 Structural Features and Subunits of Neuronal BK Channels

Calcium activated potassium currents, though previously theorized, were first
observed when the advent of patch-clamp electrophysiology allowed for the ready manipulation of intracellular calcium concentrations [48], [49]. Within a few years several reports were written of a calcium-dependent potassium channel exhibiting unusually large conductance (100-300 pS) for a selective ion channel [32], [50]–[56]. These large calcium activated currents became affectionately known as “big brothers” due to their sometimes vexing propensity to overshadow smaller calcium-dependent events [49].

Over the years this channel acquired several names including slowpoke (thanks to the whimsical naming conventions of the *Drosophila* biologists [57]), slo1, maxi-K, K_{Ca}1.1, and BK. BK, standing for Big Potassium, is currently the most widely used name for the channel and will be used for this dissertation.

The BK potassium channels is a high-voltage and calcium-activated potassium channel that is nearly ubiquitous and highly conserved channel in animals and even has analogs in prokaryotes [58], [59]. This dissertation deals with the role of the BK channel in neuronal excitability; however, BK channels are broadly expressed and play roles in many tissues. Neurons aside, BK channels are probably most broadly studied in muscle, being observed very early in skeletal muscle cultures [32]. They are particularly prominent in smooth muscle where they are important modulators of vascular tone [60] and are expressed in the heart where they modulate rhythmic activity, responding to calcium oscillation but also to changes in tension due to apparent mechanosensitive properties of the channel [61], [62]. Additionally they play roles in the kidneys [63], bone [64] and many other tissues [65]. While this dissertation will primarily treat BK channels as calcium and voltage activated channels as those are the channel’s most potent and important gating mechanisms in the central nervous system, it should be noted that they can be modulated in several ways that reflect their array of roles in many tissue types and their functional diversity. In addition to calcium they can be modulated by other divalent metal cations, particularly magnesium and zinc [66], [67], are intrinsically
chemosensitive to carbon monoxide and protons [68], [69], and are mechanosensitive [70], [71]. This dissertation will also primarily investigate BK channels in the neuronal membrane, but it should be noted that they are also present and important in organelle membranes including the nucleus, mitochondria, and lysosome, and endoplasmic reticulum [72]–[75].

BK channels in mammals are all encoded by one gene, KCNMA1 in humans (Kcnma1 in mice). KCNMA1 transcripts undergo extensive alternative splicing into at least 17 identified isoforms in humans and 26 distinct full-length sequences in mice available on the NCBI database. At least nine distinct splice variants are found in the human brain [76]. Alternate splicing allows for massive functional diversity of BK channels, principally through alteration of the C-terminal calcium binding region such that different splice variants possess varying calcium affinities and gating kinetics [76]–[78]. To illustrate how splicing of BK of channels produces functional diversity, the ability to perceive different pitches of sound depends on alternatively spliced BK channels producing a spectrum of electrical resonance patterns in hair cells along the cochlear tonotopic axis [79]–[81].

Voltage gated potassium channels, as well as the BK channels have a stereotypical 6 $\alpha$-helical transmembrane pass structure that can be subdivided into 2 domains (Fig 1.3A). Helices one through four (S1-S4) compose the voltage sensing domain with the key voltage sensing residues residing on S4. Helices S5-S6 form the pore-gate domain [82], [83].

The BK channel falls into this clade of ion channels but has some divergent structural elements. Rather than the typical six transmembrane helices, BK channels possess a seventh transmembrane $\alpha$-helix, S0 (the transmembrane helices are termed
S0-S6 rather than S1-S7 to preserve the naming of homologous helices with the $K_V$ channel family). The S0 transmembrane helix affects the voltage activation of the channel [84], [85]. In addition, the S0 helix also mediates the $\alpha$-subunit's association with regulatory $\beta$-subunits. The S0 subunit was initially observed in 1996 and determined to be necessary for the association of the channel with the only $\beta$-subunit known at that time ($\beta$-1) [86]. Three additional $\beta$-subunits have subsequently been identified. It was later discovered that S0 is necessary for association of the $\alpha$-subunit with the $\beta$-1, but not the $\beta$-2 subunit suggesting that other domains on the $\alpha$-subunit govern binding with other subunits [87], [88].

As in $K_V$ channels, S1-4 (or rather S0-4 in BK) make up the voltage sensing domain of the channel. In $K_V$ channels S1-S4 all contain some charged residues with a large concentration of positively charged amino acids on S4 or distributed across S3-S4 in what is described as a voltage sensing paddle [89], [90]. While the gating residues are highly conserved in voltage-gated potassium channels, the BK channels have a reduced number of positively charged voltage sensing residues in S4 relative to $K_V$ channels leading to them to have lower gating currents and a much less robust voltage dependency [91], [92]. This relative voltage insensitivity means that the channel must
also depend on calcium binding to achieve an open conformation under physiological conditions [93] (Fig 1.2).

Calcium regulation of the BK channel works through the large, globular intracellular region of the $\alpha$-subunit monomer. Binding of a calcium ion to a calcium binding site at one tetramer allosterically alters the voltage sensing domains of all subunits in the heterotetramer assembly [94]. Conformational changes in the intracellular domain are induced by calcium binding and are transmitted to S6, one of the pore forming helices by a linker region [58], [95] The elucidation of the means by which
calcium regulates BK channel activation has relied both on mutagenesis and the cloning of chimeric BK channels [96] as well as the use the prokaryotic BK channel homolog MthK, which is composed of a two-helix transmembrane region and an intracellular calcium binding region – essentially a BK channel without a voltage sensing domain [90]. This channel enables investigation of the structural basis of calcium binding promoting channel activation without the requirement of concurrent voltage activation that is present in eukaryotic BK channels.

The necessity of the intracellular domain for calcium activation was first demonstrated by creating chimeric channels of mouse and drosophila BK transmembrane and intracellular domains, as drosophila BK channels have much greater calcium sensitivity [97]. After the tail was identified as the calcium binding domain, two high affinity calcium binding RCK (regulator of potassium conductance) sites were identified. The calcium binding site on the C-terminal portion of the channel was identified as a highly conserved aspartate rich sequence that, when deleted, resulted in a large, but incomplete attenuation of the channel’s calcium activation [98]. This site was initially termed the calcium bowl and later termed the RCK2 site. The calcium binding in the RCK2 site of the human channel is coordinated by Q889, a DQDDDDD sequence spanning residues 892-898, E902, and Y904 [99]. The site of the second calcium binding was discovered as homologous site to the RCK region of prokaryotic calcium-activated potassium channels and determined to participate in the BK channel’s activation by calcium [100]. This site has since been termed the RCK1 site. Calcium binding at the RCK1 site is coordinated by a far less consecutive sequence of amino acids, D367, N499, R514, S533, and S600 that are brought into proximity by the secondary and tertiary structure of the protein[99]. There is evidence of cooperativity between the RCK1 and RCK2 sites mediated by crosstalk between R514 of RCK1 and E902 and Y904 of RCK2. However, the extent (and even direction!) of the cooperativity
is unclear with some methodologies indicating negative cooperativity and some indicating positive cooperativity [101]–[103].

In addition to the two calcium binding sites, the BK channel is also activated by magnesium through a third divalent cation binding site chiefly coordinated by E374 and E399 on the intracellular domain as well as D99 and N127 on an adjacent α-subunit monomer. [66], [104], [105]. Although under physiological conditions, magnesium potentiates BK currents, the full scope of magnesium’s interaction with BK channels is somewhat more complicated as magnesium can inactivate the channel at depolarized voltages and can competitively inhibit the calcium binding sites at high concentrations [66]. For a reviews of the discovery of calcium and magnesium regulation (and more) of BK channels see Latorre and Brauchi, 2006 and Sancho and Kyle, 2021 [96], [106].

So far, discussion of the BK channel has been limited to the α subunit, which forms the pore and contains the calcium binding sites and can itself be the target of other regulatory modifications such as phosphorylation and ubiquitination. (These will not be reviewed at length here, but neuronal specific post-translational modifications will be later discussed as they pertain to this dissertation). The α subunit tetramer is a functional channel protein capable of passing current on its own but can associate with modulatory β and γ subunits which modify its properties (Fig 1.3). These regulatory subunits can confer changes in the voltage activation, calcium activation, and pharmacology of the BK channel.

BK channel β subunits (which are encoded by the genes KCNMB1-4) were first discovered in smooth muscle through crosslinking and purification assays of channels labelled with [125I]charybdotoxin when the α subunit of the channels copurified with a separate 39 kDa protein [107]. This newly discovered subunit (β-1) sensitized the channel to calcium, altered the voltage dependence, conferred sensitivity to the agonist
dehydrosoyasaponin, and increased the channel affinity for charybdotoxin [108], [109]. Additionally, association of the α and β-1 subunits promotes surface expression [110].

The β-2 subunit was discovered based on sequence homology with β-1. This broadly expressed subunit provided a molecular basis for a previously observed phenomenon – fast inactivation of BK channels, which was shown to take place by a ball-and-chain mechanism. In addition, like β-1 the β-2 subunit confers sensitivity to dehydrosoyasaponin, but opposite to β-1 it confers relative resistance to charybdotoxin [111], [112]. The β-3 subunit is strongly expressed in the testes, weakly across other non-neuronal tissues and has little to no expression in the brain. β-3 has the most subtle effect on BK channel function, increasing the calcium sensitivity and activation speed [113].

The β-4 subunit is the primary neuronal subunit and has low to absent expression in other tissues [111], [113]. In neurons, unassociated α subunits are termed type-I BK channels while α/β-4 complexes are termed type-II BK channels [114].

Higher volatges than type-1 BK channels provided sufficient calcium is present. However type-II BK channels also have drastically slower activation kinetics and so do not open fast enough to contribute to action potential repolarization as type-1 channels [113]. BK/β-4 complexes are additionally relatively insensitive to blockade with the selective BK channel antagonist iberiotoxin, which provides a convenient way to test for relative contribution of type-I and type-II channels [115].

β subunits share a general structure (cryoEM structures α/β-4 channel complex shown in Fig 1.3D) of two transmembrane helices connected by a large, heavily glycosylated extracellular loop. The N and C-terminus are both on the intracellular side of the membrane and the N terminus of β-2 and β-3 contains a ball and chain inactivation gate [107], [113], [116]–[118]. The inclusion and tissue specific expression of
β subunits represents another means by which BK channels achieve broad functional diversity despite all being encoded by a single gene.

The second class of subunit, the γ subunits, were first observed in reports published the same year that described currents in prostate cancer cell lines and cochlear hair cells that, in all electrical and pharmacological aspects, resembled BK currents with the notable exception that they were active at relatively low voltages in the absence of calcium [119], [120]. Later research into the calcium-independent BK currents of this prostate cancer line revealed a 35 kDa, single pass transmembrane protein (γ-1) that shifted the voltage dependence of the channel by 135-160 mV [121]. Follow up investigations based on sequence homology to γ-1 revealed three additional proteins with a characteristic leucine-rich repeat (LRR) sequence that were capable of modulating BK channel voltage sensitivity. This family is termed LRRC26 (γ-1), LRRC52 (γ-2), LRRC55 (γ-3), and LRRC38 (γ-4). The effect of γ-2-4 subunits is to shift the voltage activation leftward by 100, 50, and 20 respectively [122]. All γ subunits have some detectable expression in brain tissue, but γ-3 appears to be relatively neuronal specific and γ-1 is notably expressed at much higher in the fetal brain than the adult brain, signaling that it may play some developmental role [122].

Association of the α subunit with β and γ subunits is not mutually exclusive. Channel complexes consisting of α, β-2, and γ-1 were observed and characterized in a heterologous expression system [123]. The possibility of α/β/γ channel complex increases the potential functional diversity of BK channels, but this combination has not been shown to happen in tissue in vivo, and all combinations of β and γ subunits may not be possible as β subunits do not all associate with the same domain of the α subunit [124].
1.4 Physiological Roles of Neuronal BK Channels

The earliest investigations of BK channel mRNA expression, protein expression, and localization revealed that the channel is broadly expressed throughout the brain and is chiefly localized in the axons with the exception being the Purkinje cells of the cerebellum where BK channels are primarily located in the soma and dendrites [125]. This indicates that while BK channels in Purkinje cells shape action potentials as in other neuronal types they may play additional roles in synaptic coding and coordinating outputs in the cerebellum [125]–[128]. Subsequent study of BK channel expression using immunofluorescence confirmed that BK channels are primarily expressed in the axons and presynaptic terminals of excitatory neurons in the hippocampus and GABAergic basket cells in the cerebellum – further evidence that BK channels play a unique role in the latter region [129].

Unlike the other members of the K\textsubscript{Ca} channel family, BK channels have a functional voltage sensing domain (see section 1.2) and so are gated by both membrane depolarization as well as rises in intracellular calcium, requiring both to open at physiological membrane potentials [93]. This requirement for both calcium and voltage gating means that neuronal BK channels open during action potential firing when voltage gated sodium channels depolarize the membrane and voltage gated calcium channels (typically) provide the calcium source. Once activated, they contribute to the repolarization phase of the action potential waveform and underlie the fast afterhyperpolarization, a hyperpolarizing event that occurs within 5 ms after an action potential (Fig. 1.2) [130], [131]. By contributing to the repolarization and fast afterhyperpolarization BK channels enable high frequency firing by a few proposed mechanisms: first, prompt termination of depolarization and voltage dependent calcium influx limits the activation of slow delayed rectifier and SK channels that carry the slower components of the afterhyperpolarization thereby reducing their inhibitory effects;
second, fast repolarization ‘de-inactivates’ the voltage dependent inactivation of voltage
gated sodium channels; third, more recent computational evidence suggest that the fast
afterhyperpolarizing currents carried by BK channels can facilitate resurgent voltage
activated sodium currents [114], [132], [133]. While increasing neuronal firing rates is the
best understood and most widely reported role of BK channels it should be noted that
this is not universally their role; under some circumstances BK currents have been
reported to reduce neuronal firing rates [134], [135]. This variability in the role of BK
channels is likely owed to the different configurations of BK channels and the precise
cellular context in which they exist. For example, pairing with β-4 subunits changes the
time course such that BK channels may contribute to the medium afterhyperpolarization
decreasing excitability [114]. As another example, BK currents in neurons of the
suprachiasmatic nucleus alternate calcium sources between extracellular in the day and
sarcoplasmic- reticulum stores at night which leads to a daytime increase in excitability
by BK channel activation and a nighttime decrease [135], [136].

In addition to modulating action potential firing rates in the axon, it was an early
observation in the field that BK channels modulate neurotransmitter release. BK
channels tightly associate with voltage gated calcium channels (necessary for their
function) at the presynaptic terminal and act as a negative feedback control of calcium
influx and subsequent neurotransmitter release at neuromuscular junctions in mouse
and frog models [137], [138]. Since then, studying the role of the BK channel in
regulating neurotransmitter release has yielded mixed results depending on the model
system used. Studies in invertebrate models have shown that loss-of-function mutations
in the BK channel orthologue in C. elegans (SLO-1) increase neurotransmitter release in
C. elegans neuromuscular junctions [139] in concurrence with observations in the
vertebrate models. Alternately, analogous mutations in D. melanogaster BK channel
(slo-1) decrease neurotransmitter release in the larval neuromuscular junctions [140].
Studies of how BK channels affect neurotransmitter release in the central nervous system are limited but one study demonstrates that BK channels decrease neurotransmitter release at local CA3 synapses in cultured mouse hippocampal slices [141]. My own speculation is that whether BK channels increase or decrease neurotransmitter release depends on how the channels primarily affect action potential waveforms and firing. That is, if BK channel inhibition primarily broadens the presynaptic spike with little change in firing frequency, neurotransmitter release will increase whereas if BK channel inhibition decreases the firing rate of presynaptic action potentials, neurotransmitter release will decrease. Whichever effect prevails depends on the splice variant expressed, accessory subunit pairing, and post-translational modification (see section 1.3)

As could be predicted from the fact that they are enriched at the terminals of excitatory neurons in the hippocampus [129], BK channel function has been linked to learning and memory in such a way that learning processes seem to be associated with a downregulation of BK channels. Training in a hippocampal dependent task results in CA1 pyramidal neurons with a diminished fast afterhyperpolarization (BK channel-dependent in pyramidal neurons) and reduced responsiveness to the BK antagonist paxilline [134] suggesting reduced function or expression of BK channels. Additionally, BK channel knockout mice had deficiencies in spatial learning [142]. BK channel inhibition can also reverse cognitive deficits induced by thalidomide – which appears to be partly mediated by increased BK channel surface expression [143]. Beyond their role in classical hippocampal dependent learning, BK channels have also been implicated in fear conditioning in the amygdala where successful conditioning is associated with reduced channel function [144]. Expanding out one’s view to the level of brain activity, BK channel inhibition leads to an increase in the band power of γ frequency oscillations. Γ oscillations are 30-80 Hz brain waves that are thought to represent coordination of
neuronal activity in the hippocampus and underlie memory-related processes and other cognitive function [145], [146]. In summary, downregulation of BK channel activity seems to enable learning and memory formation and can also offset the effects of separate insults that lead to cognitive deficits.

Beyond governing (what this writer considers) more abstract processes like memory and cognition, BK channels are also important governors of neuronal excitability. The importance of BK channels in this regard can be seen in the profound clinical manifestations of BK channel dysfunction. As potassium currents are hyperpolarizing, the simplest conclusion to draw would be that loss-of-function mutations would lead to pathological hyperexcitation as reduced potassium current depolarizes neurons. In reality, BK channels play quite nuanced roles in neuronal and circuit excitability and so both gain and loss of function mutations are implicated in epilepsy [65] (BK channels and epilepsy will be examined more closely in a subsequent section). BK channel mutations are also linked to motor disorders such as ataxia – first described in 2004 and then shown to be the result of mitochondrial BK channel dysfunction [147], [148], and paroxysmal nonekinesigenic dyskinesia (PNKD) [149]. Fragile-X mental retardation is also in part a BK channelopathy; the fragile-X mental retardation protein (FMRP) shifts the calcium gating curve of the BK channel to the left by interacting with the β-4 subunit. This decreases action potential duration and neurotransmitter release [150].

A final consideration that shapes the functional role of the BK channel in neuronal excitability is the question of “where does this calcium activated channel get its calcium?”. BK channels have been demonstrated to be activated by various calcium sources in different cell types and under different conditions. BK channels in hippocampal neurons rely on N-type calcium channels [151]. Cortical neuron BK channels are activated by calcium influx through N and L-type calcium channels. Adrenal
chromaffin cell BK channels are activated by calcium influx through L and Q-type channels [152]. BK channels in neurons from the cervical spinal ganglia and in the suprachiasmatic nucleus are activated by calcium through L-type channel as well as ryanodine receptors for calcium from intracellular stores [136], [153]. The source of the BK channel’s calcium is an important consideration as the different calcium dynamics from different sources have different functional outcomes. When the BK channel relies on calcium from the extracellular environment, the channel contributes to repolarization and the speeding of action potential firing but calcium from intracellular sources results in BK channels contributing to the after-hyperpolarization and the slowing of neuronal firing rates [98], [99]. The difference between effects of intracellular and extracellular calcium sources for BK channels is apparent in the suprachiasmatic nucleus where neurons alternate calcium sources on a circadian basis. When BK channels rely on L-type calcium channels, BK currents exert an excitatory affect however when the channels depend on calcium via ryanodine receptors BK currents exert an inhibitory effect on action potential firing [135], [136].

1.5 Neuronal BK Channels Through Development

This dissertation is concerned with how the BK potassium channel’s role in governing neuronal excitability changes during early postnatal development. Underlying changes in the channel’s functional role are changes in the total amount and the variants of the BK channels expressed. Ionic currents that underlie action potential firing sharply increase at birth in the rodent brain and will rise steadily through early development [11], [12] and the BK channel’s expression patterns match this trend with several-fold increases of expression of both KCNMA1 transcripts across multiple brain regions and α subunit protein expression in the hippocampus [155], [156].

In addition to the rise in total expression of the α subunit, there is a shift in the
predominant splice variant. In rodent embryonic development, channels including the stress hormone related exon (STREX BK) predominate, but by the end of the first postnatal week, variants lacking this exon (ZERO BK) represent the vast majority of neuronal BK channels [155]. STREX BK channels have different effects on action potential wave forms and neuronal excitability, with STREX BK having greater calcium affinity and a higher open probability which results in greater BK channel contributions to hyperexcitability than other channel isoforms [78], [157], [158].

The STREX variant also has profound differences in how the channel is regulated by protein kinases. The STREX isoform introduces different outcomes to phosphorylation of the channel at important regulatory sites by protein kinase A (PKA) and protein kinase C (PKC). Phosphorylation of the BK channel α subunit at serine 879 (S879) is requisite for channel activation and so PKA is a central gatekeeper of BK channel currents, but STREX contains an inhibitory PKA phosphorylation site introducing divergent regulation by PKA in the developmentally dependent isoforms [159]. Phosphorylation of the RCK1-RCK2 linker region at serine 700 (S700) by PKC inhibits BK channel currents, but STREX anchors the linker region to the membrane and restricts PKC access to S700, rendering STREX BK channels insensitive to this form of regulation [160], [161].

Postnatal development is also accompanied by a rise in expression of the β-4 subunit across brain regions (the subunit is virtually absent in embryonic development) meaning that there is likely a shift from predominantly fast-activating type-I to the slower type-II gated BK channels [114], [162]. Adding another wrinkle of complexity, the β-4 subunit has variable effects depending on the α subunit expressed. The β-4 subunit slows activation and reduces conductance for both ZERO BK and STREX BK but while it slows deactivation in ZERO BK channels, it speeds deactivation (in the presence of at
least 5 µM calcium) of STREX BK channels[163]. The interplay of rising β-4 subunit expression and falling STREX BK expression may have interesting effects on neuronal function.

Developmentally dependent roles of the BK channel could also result from the developmental trends in the expression and activity of the channel’s various calcium sources. As mentioned in Section 1.2, BK channels are activated by a variety of voltage gated calcium channels conducting calcium from the extracellular fluid as well as ryanodine receptors which conduct calcium from the endoplasmic reticulum; alteration in the calcium source can significantly alter the BK channel’s role in neuronal function [135], [136], [154]. The two main channels that are functionally coupled to BK channels, L and N-types, undergo spatial redistributions during early postnatal development and at least in the case of the N-type channel, large changes in expression level [164], [165]. Changes in the BK channel’s calcium source represent an avenue by which the BK channel function could change with development without requiring any changes made to the channel itself.

1.6 BK Channels in Epilepsy

As mentioned previously, BK channels are heavily implicated in seizures and epilepsy [65], [166]. The question of how BK channel dysfunction leads to epilepsy resists a straightforward explanation as epilepsy has been linked to both gain-of-function and loss-of function-mutations of the channel [65], [149], [167], [168]. The BK channel’s enigmatic relationship to seizures can be partly understood as a result of the functional diversity BK channels achieved by alternate splicing of the α subunit [76] and different combinations of auxiliary subunits [117]. Mutations to exons that are not present in all splice variants or that disrupt subunit association will affect functionally distinct subpopulations of the BK channels.
BK channel loss of function leading to seizures can be understood in the context of the BK channel's role in neurotransmitter release. BK channels repolarize the action potential at the presynaptic membrane [169], terminating neurotransmitter release and underlie the fAHP [134] which delays subsequent spiking. Therefore BK channel loss-of-function can lead to hyperexcitability of the postsynaptic cell through increased excitatory neurotransmission. Meanwhile, BK channel gain-of-function can be understood by considering BK channels role in enabling high frequency firing [132] - in this case BK channel gain-of-function increases neuronal gain and potentiates transmission in excitatory circuits. The most dramatic possible case - global knockout of the BK channel- leads to spontaneous seizures in mice (in addition to a host of other complications) [168]. The pilocarpine model for chronic epilepsy [170] causes downregulation of BK channel expression throughout the hippocampus [171] and rats selectively bred to be susceptible to audiogenic seizures show blunted calcium activated potassium currents in hippocampal neurons [172]. This could be a mechanism of kindling whereby prior seizures or sub convulsive episodes render the brain more vulnerable to subsequent seizures and ultimately the development of epilepsy [173]. Alternately it could represent a compensatory, neuroprotective mechanism in which the likelihood of a seizure is reduced by reducing the capacity for fast spiking activity in excitatory neurons.

Clinical reports tie both gain-of-function and loss-of-function mutations to epilepsy [65], however, the existing body of experimental evidence points to the case that BK gain-of-function underlies epilepsy and that BK channel inhibition is a promising therapeutic [174], [175]. BK channel blockade with intraperitoneal injections of paxilline is sufficient to prevent seizure induction with an otherwise seizure-inducing dose of the GABA agonist picrotoxin and delays the onset of seizures induced with the cholinergic agonist pilocarpine. Paxilline only inhibited seizures in mice that had previously
undergone seizure induction with picrotoxin or pilocarpine. A possible explanation for why mice with a history of seizures respond to paxilline while those naïve to seizures do not is that seizures induce changes in BK expression and/or activity; this seems to be the case as picrotoxin-induced seizures result in faster action potential repolarization, higher excitability, and faster spontaneous firing rates in cortical pyramidal neuron that could be reversed by paxilline administration [176].

Seizures that occur in these BK channel gain-of-function mutant backgrounds are not a unique product of the kinetics of the mutant channels but are a simple result of more total BK channel current. BK channels surface expression is restricted by ubiquitination via the E3 ubiquitin ligase CRL4ACRB. When this ubiquitination is impaired by mutation or pharmacological antagonism in mice, membrane expression of BK channels increases and treated animals are dramatically more susceptible to seizures [177]; a similar outcome the gain-of-function mutants in spite of the channels having “normal”, wild-type kinetics.

At least 37 variants in the BK channels α subunit have been linked to epilepsy, dyskinesia, and other neurological disorders [166]. I will discuss two of them that have been well characterized and identified as gain-of-function mutations: D434G near the RCK1 region [167] and the more potent mutant N999S in the C terminal region. The D434G mutant BK channel has been studied using heterologous expression in Xenopus oocytes, HEK293 cells, and CHO cells and has even been knocked-in to mice to determine its effect on neuronal physiology [149], [175], [178]–[180]. The N999S mutant has been well characterized in HEK293 cells, but has not yet been characterized in a neuronal model [180], [181]. I will describe these mutants to give the physiological basis for why BK channel gain-of-function can lead to epilepsy and other neurological disorders.

The D434G mutant was described in 2005 through studies of a family in which
roughly half of the members were diagnosed with epilepsy, primary dyskinesia, or both [149]. Aspartate 434 is located in the RCK1 (Fig 1.4) domain and when substituted with glycine the channel increases calcium sensitivity three-to-five fold and shifts voltage activation to the left at any given calcium concentration [149]. The increased calcium sensitivity of this mutant results in much faster activation and greater open probabilities for the channel which results in much larger BK-mediated potassium currents in response to an action potential wave form [149], [167], [178], [179]. This increase in calcium sensitivity is so profound that the activation on the D434G mutant is unaffected by co-expression of the β-4 subunit, which acts to decrease calcium sensitivity and slow activation of the wild-type α subunit [113], [178]. The caveat to this conclusion is that it is unclear whether this effect is due entirely to changes in calcium sensitivity or if the D434G mutation prevents α/β-4 subunit association. In a mouse knock-in model, the D434G mutation produced an absence seizure phenotype with increased spontaneous epileptiform discharges and shorter latency to seizures in a pentylenetetrazole (PTZ) model. Single cell recordings revealed that dentate granule and cortical pyramidal cells in D434G mice had increased BK currents, faster action potential repolarization, and increased gain and maximal firing rates — which were reversed by the BK channel antagonist paxilline [175], [182].

The N999S mutant (also known as N995S and N1053S throughout the literature depending on the reference sequence used [65]) is a point mutation in the more C-terminal RCK2 site that is associated with epilepsy and dyskinesia and, since its identification has become the most common mutation associated the BK channelopathy [166], [181]. While the mutation is located near the RCK2 binding site (Fig 1.4), it does not seem to alter calcium sensing like the D434G mutant, instead increasing voltage sensing and stabilizing the open-conformation [181]. The N999S mutant is an even more
potent gain-of-function variant than the D434G mutant by several measures; it has a current-voltage relationship that is shifted even more to the left than that of D434G, it passes more current in response to an action potential-shaped voltage command than the D434G mutant, and has a notably slower deactivation time [180], [182]. Dentate granule neurons in heterozygous N999S mice have larger increases in intrinsic excitability, and the N999S mice experience seizures very readily in response to PTZ. This mutation produces a more severe phenotype than the D434G mutation; bolstering the notion that this is the more pathogenic mutation is the fact that homozygous D434G mice can be produced, while the homozygous N999S mutation appears to be embryonic lethal as crosses of N999S mice produce no homozygous offspring [182].

There are additional BK channel variants that have been identified in patients with epilepsy, dyskinesia, or other motor disorders that result, at least putatively in gain or loss of function as well as several more of unknown significance [65], [166] but these have not been characterized to the extent of D434G and N999S and so they will not be individually treated here at any length. One potential explanation for why BK channel variants have such an unpredictable relationship with epilepsy and other neurological disorders is, as offered by Griguoli et al.(2016): that BK channels have overlapping roles
with other potassium channels and thus other potassium channels can compensate for loss of function in BK channels [183]. For example BK channels and Kv1.3 (shaker) channels exert similar and overlapping effect on synaptic transmission and either channel can compensate when one is mutated; severe deficits are only observed in double mutants or in single mutant backgrounds when the other channel is pharmacologically inhibited [115], [140], [169], [184]. Under this view, whether a disease state is associated with gain-of-function or loss-of-function BK variants depends on whether, or which, mutations are present in channels with which BK channels have overlapping function.

In addition to being potentially causative in epilepsy it is possible that BK channel dysfunction contributes to the ongoing pathogenesis of epilepsy regardless of the primary cause. It has been established in rodent models that seizures result in changes to the BK channel isoforms and β subunit expression that can contribute to neuronal hyperexcitability. Pilocarpine induced seizures in rats result in a persistent overall decrease in BK channel expression, but an upregulation in the relative expression of the STREX isoform (which has a heightened open probability [157], [158]) in the dentate gyrus that was observed at 10 days after seizure-induction and persisted for months following seizure induction [185]. In addition to changes in the α subunit expression, seizure induction with pilocarpine and kainic acid in mice results in decreased hippocampal expression of the β-4 subunit (the subunit associated with dramatically slowing of BK channel activation [113] and protection against seizures [186], [187]) 48 hours post seizure induction and shifts BK channel gating in hippocampal from type-II iberiotoxin resistant gating to type-I iberiotoxin sensitive gating which is consistent with a loss of β-4 subunit modulation [115], [187]. It should be noted that Whitmire et al. (2017) did not observe a change in relative alpha subunit expression after seizure induction.
hours while Ermolinsky et al. (2011) did \cite{[185],[187]}. If this cannot be attributed to interspecific differences (i.e., rats vs. mice) or differences in protocol (e.g., pharmacological parameters) it may be explained by Ermolinsky et al. monitoring expression over a longer period of time suggesting that the decrease in the alpha subunit is a longer term maladaptive response.

While a growing body of evidence implicates BK channel dysfunction in epilepsy, there are some important considerations in pursuing BK channel antagonism as a therapeutic. BK channels are important in learning and cognition and coordination of motor function \cite{[142],[144],[145],[148]}. For this reason, long term management of seizures with BK channel antagonists may be critically disruptive to normal function. Such a treatment strategy may only be suitable for acute management for seizures that are intractable the GABA\textsubscript{A}-based treatments, or dosing may have to be carefully titrated to return BK channel function to normal levels in the case of gain-of-function mutations.

### 1.7 BK Channels and Epilepsy in the Developing Brain

One of the most translational reasons to study how BK channels affect excitability through postnatal development is to explore BK channel antagonism as an intervention for seizures in infants and young children. Benzodiazepines are allosteric activators of GABA\textsubscript{A} receptors (GABA gated chloride channels) that have been widely and successfully used for over half of a century to reduce seizures \cite{[188],[189]}. However, GABA\textsubscript{A} receptor agonism is not a viable strategy for managing seizures early in development as GABAergic inhibition is not fully established in the early postnatal period. Changes in the expression ratio of the NKCC1 and KCC2 chloride transporters across development mean that mature chloride gradients are not established in early development, neonates express a different complement of GABA\textsubscript{A} receptor subunits, and inhibitory synapses are not fully developed at birth \cite{[190],[192]}. The immature state
of inhibitory mechanisms means that increasing GABA_A currents with benzodiazepines is not sufficiently inhibitory to manage seizures and may even have excitatory effects [5], [193], [194]. Infant brains are more excitable than adult brains and are susceptible to febrile seizures which put the individual at risk for long term neurological problems including chronic epilepsy and sudden death [14], [195]. Furthermore, seizures in infancy can affect neuronal growth and differentiation in ways that seizures later in life do not [196]. Both benzodiazepines and more recently developed alternatives do not provide adequate treatment options for seizures in infants so there is a pressing need to explore new drug targets for this population.

There is a small but growing, and consistent body of work that supports inhibiting BK channels as a treatment option for epilepsy, and that BK channelopathies (particularly arising from gain-of-function mutations) are commonly associated with hereditary epilepsy. As outlined in the previous section, BK channel antagonism appears to most effectively prevent seizure formation in juvenile animals that have had previous seizures which leads to long term upregulation of the STREX splice variant, downregulation the β-4 subunit and a transition from predominantly slower acting type-II BK channels to faster acting type-I BK channels [174], [176], [185], [187].

Intriguingly, the alterations to BK channel expression patterns induced by prior seizure mirror the characteristics of BK channels in the early postnatal period. In the early postnatal period, the STREX splice variant predominates, expression of the β-4 subunit is reduced, and as the data in this dissertation show neonatally expressed BK channels have faster, type-I activation [155], [156]. This suggests to me that neonatal brains may be in a state where BK channel blockade may be an effective early intervention for seizures whereas adult brains require a history of seizures before BK antagonism is a viable option. In addition, the STREX variant is functionally very similar
to the D434G pathogenic BK channel variants in that it raises channel open probability at lower calcium concentrations [149], [158], [179], [181]. The similarity between pathogenic mutants and neonatally expressed splice variants may indicate that the BK channels present partly contribute to seizure susceptibility in neonatal brain.

This dissertation details the changes in BK channel’s expression patterns and physiological roles in rat and mouse hippocampal neurons across early postnatal development and explore the effect of BK channel blockade on a seizure model in an *in vitro* seizure model.
Chapter 2: Developmental Changes in Action Potentials and the Changing Role of BK Potassium Channels in Rat Hippocampal Neurons

2.1 Introduction

A major hallmark of neuronal development is the shortening of the action potential waveform, an increase in the action potential amplitude, and an increase in the intrinsic excitability of cells resulting from establishment of mature patterns of ion channel expression [11], [12]. These patterns have been observed in many neuronal populations including rat cortical pyramidal neurons from postnatal day 1 (P1) to P36 [197], rat spinal motor neurons from embryonic day 15 (E15; full term in rats is 21-22 days) to P3 [11], mouse spinal motor neurons from P2 to P21 with development occurring unevenly throughout the spinal cord in line with spatial patterning of development of mobility [198], fast-spiking cortical interneurons dependent on Kv3 [199], and in cortical somatostatin expressing interneurons [200].

I characterized these changes in action potential waveform and excitability in cultured hippocampal neurons with two main aims: to understand developmental trends in these neurons to contextualize changes in ion channel expression and function, and to determine if acutely cultured neurons exhibit age-appropriate characteristics or if culturing neurons reverts them to an immature state.

I measured the contribution of three calcium dependent potassium channels in regulating action potential repolarization and neuronal excitability using specific channel blocking agents. Small conductance (SK) potassium channels carry the medium afterhyperpolarization, decrease neuronal excitability by transiently hyperpolarizing the membrane after action potential firing thereby increasing interspike intervals, and are blocked by the honeybee venom-derived peptide toxin apamin [33], [40]. Large
conductance (BK) calcium activated potassium channels are somewhat enigmatic in their physiological roles, but typically are understood to enable repetitive firing by ensuring prompt repolarization and are blocked by the scorpion venom-derived iberiotoxin [132], [201]. KCNQ channels are activated by muscarinic-type acetylcholine receptors, decrease excitability by hyperpolarizing the membrane, and are blocked by linopirdine [30], [202]. Unlike SK and BK, KCNQ is not part of the traditional calcium activated potassium channel (KCa) family and does not have intrinsic calcium sensing like BK, nor is it constitutively associated with calmodulin subunit like SK. However, KCNQ exhibits some calcium sensitivity due to association with calmodulin subunits under some conditions [203].

In this chapter I describe how cultured neurons exhibit changes in action potential waveform and increases in excitability that mirror neurodevelopmental trends in acutely prepared brain slices where local circuits are intact. I report that SK, BK and KCNQ channels all have significant effects on action potential repolarization and repetitive spike firing.

Preliminary data showed intriguing changes in the effect of blocking BK channels across the age range studied. These channels control the repolarization and fast afterhyperpolarization of the action potential [131], [132] and modulate neurotransmitter release [129], [141] so changes in BK channel activity are likely to have significant impacts on neuronal function. This project ultimately culminated in an extensive characterization of the changing roles of BK channels in hippocampal neurons cultured from P1-P7 rats. I report that BK channel contributions to repolarization of a single action potential decreases with development despite large rises in BK channel protein expression from P1 to P7. In cultured neurons capable of fast repetitive firing, BK channels play a greater role at P7 but exhibit delayed activation.
2.2 Methods:

Animal care and use: Laboratory animals. All animal protocols were approved by the Marquette University Institutional Animal Care and Use Committee according to the guidelines set forth by the National Research Council in the Guide for Care and Use of Laboratory Animals. Sasco Sprague Dawley rats (Charles River; Wilmington, MA) were housed and bred at Marquette University. Pups were removed from dams just before tissue isolation and anesthetized with CO\textsubscript{2} for dissection.

Neuronal primary cultures: Acute cultures of hippocampal neurons were prepared according to the method established in the Mynlieff lab, which results in glia-free neuronal cultures [204]. Dissections and culturing were performed with autoclaved and UV light sterilized equipment, a positive pressure hood and sterile technique to minimize contamination. Superior regions of the hippocampi from rat pups aged postnatal day 1 through 7 (P1–P7) were dissected in ice cold rodent Ringer’s solution (solutions recipes are included at the end of this section). The tissue was then transferred to a small vial containing 1-2 mL PIPES (piperazine-N-N’-bis-2-ethanesulfonic acid) buffered saline with 0.5% trypsin type XI (Sigma-Aldrich, St. Louis, MO) and 0.01% DNase type I (Worthington, Lakewood, NJ) under continuous oxygen flow. The tissue was held in the PIPES saline (pH 7.0) for 20-30 min at room temperature followed by 60 minutes at 35°C to enzymatically break down the extracellular matrix and enable dissociation of neurons. Following enzymatic breakdown, the tissue was transferred to rodent Ringer’s solution containing trypsin inhibitors (1 mg/mL trypsin inhibitor type II-O: chicken egg white and 1 mg/mL bovine serum albumin; Sigma-Aldrich, St. Louis, MO) to quench protease activity. The tissue was then triturated by repeated aspiration and expulsion with a fire-polished Pasteur pipette to dissociate neurons and dissociated neurons were plated onto poly-L-lysine (30,000 – 70,000; Sigma-Aldrich, St. Louis, MO) coated culture dishes with Gibco neurobasal-A culture medium (ThermoFisher Scientific, Waltham, MA)
fortified with B-27 supplement, 0.5 mM glutamine, and 0.02 mg/mL gentamicin. Plated neurons were placed in a 37°C, 5% CO₂ incubator overnight to allow for viable neurons to establish in culture and re-express membrane proteins that may have been damaged by the enzymatic digestion.

**Slice preparation:** Brains were dissected from rats aged P4-P5 and P7-P8 and immediately immersed them in ice cold HEPES-buffered, high-sucrose aCSF (artificial cerebral spinal fluid) that was continuously bubbled with 100% O₂. The cerebellum was removed and discarded to create a flat caudal surface on the cerebrum which was then mounted onto the cutting platform of a Vibroslice tissue slicer (Campden instruments, Lafayette, IN) caudal end upward. 200-300 μM brains slices containing sections of the hippocampus were cut and reserved in room temperature, oxygenated HEPES buffered aCSF for a minimum of 1 hour before recording to allow for permeation of aCSF into the slice and for the tissue to recover and equilibrate to the new extracellular environment.

**Electrophysiological recordings:** Recordings from cultured neurons were taken the day after culturing. This allows time for channels to be reinserted into the membrane and for surface proteins that were degraded by enzymatic digestion to be replaced. It also minimizes axonal and dendritic growth (which can cause space clamp issues in voltage clamp recordings). Recordings were not done after more than a day in culture to minimize *in vitro* development so that the data more closely reflected developmental stage of the neurons as they existed *in vivo*. Both voltage-clamp and current-clamp recordings were obtained by whole cell patch-clamp recording with a Dagan 3900A patch-clamp amplifier (Dagan Corporation, Minneapolis, MN), Axon Digidata 1322A 16-bit data acquisition system, and pClamp 10.4 data acquisition software (Molecular Devices, San Jose, CA). Borosilicate glass capillaries (Sutter Instruments, Novato, CA; catalog no. BF150-86-10) were pulled to a resistance of 4-8 MΩ. Data were digitized at a rate of 20 kHz; voltage clamp signals were filtered with a 1-kHz low pass filter and
current clamp signals were filtered with a 10-kHz lowpass filter.

For voltage clamp recordings of currents, the culture media was replaced with a bath solution of room temperature physiological saline containing 1 μM tetrodotoxin (Alomone Labs, Jerusalem, Israel) to block voltage-dependent sodium currents. Neurons with smooth exterior surfaces, no visible nuclei (a visible nucleus is an indicator of an unhealthy cell), and no apparent physical contact with neighboring neurons were selected for recordings. I held neurons in whole cell configuration at -80 mV and then briefly stepped the membrane potential down to -90 mV for 100 ms to totally remove inactivation of any potassium currents before depolarizing the membrane from -50 to +50 mV for 100 ms in 10 mV steps.

For current clamp recordings of action potentials, membrane properties (capacitance and resistance) were recorded using the onboard membrane test function in PClamp 10 upon achieving the whole-cell configuration and then current was injected to adjust the resting membrane potential to in between -65 and -70 mV before evoking action potentials in order to standardize recording conditions across all cells. I evoked single action potentials by injecting a 0.1 ms, 8 nA current, which was sufficient to produce action potentials consistently in all cells. Action potential trains were evoked using 100-ms or 1 s depolarizing current injections ranging from 10 to 200 pA to determine a maximal firing rate before the neuron entered depolarizing block.

Contributions of potassium channel function in cultured neurons was determined by bathing neurons with the physiological saline containing 100 nM iberiotoxin (BK channel antagonist), 1 μM apamin (SK channel antagonist), or 10 μM linopirdine (KCNQ channel antagonist), and fast green dye to visualize the antagonist solution in the bath. Drug solutions were perfused from a U shaped length of PE10 polyethylene tubing with a perforation that I placed near the patched neuron. This applicator allowed for faster
and more focal perfusion and more efficient washout than could be accomplished by whole-bath perfusion and allowed for multiple trials from the same culture dish of neurons as the antagonist did not permeate the entirety of the bath solution. Spread of the antagonist-containing medium could be visualized due the inclusion of fast green dye.

Identical instrumentation and stimulus paradigms were used for current clamp recordings in acute slices with the exception of the recording chamber and solution used. Recordings from slices were performed in an open batch chamber under constant lateral-flow perfusion at a rate of 2-4 mL/minute of HEPES buffered aCSF that was continuously bubbled with 100% O₂ to maintain tissue oxygenation. Ascorbic acid was included in the recording solution to offset the formation of reactive oxygen species by the oxygenation.

All membrane potentials reported have been adjusted post recording to account for the liquid junction potential calculated in pClamp 10 (15.4 mV in cultured cell recordings, 17.2 mV in slice recordings). Data were not included in the analysis from cells for which stable membrane properties were not attainable or if the action potential response or currents were not relatively stable over time.

**Data analysis:** Action potential duration was measured as the time interval (in ms) between the rising and falling phase of the spike at one-half of the spike amplitude from baseline (half-width). Fast afterhyperpolarization (fAHP) magnitude was measured at the lowest voltage attained during hyperpolarization subtracted from the resting membrane potential recorded before the stimulus used to induce the action potential. Effects of ion channel blockade during current clamp recordings was reported as the difference between the value recorded during the drug application and the average of a value collected before drug application and after washout to account for effects of prolonged patching and electrical stimulation. Instantaneous frequencies were calculated as the
inverse of the peak-to-peak time interval between two successive action potentials.

There was a large amount of current rundown in voltage-clamp recordings so to account for that, I calculated the effect of the iberiotoxin as the difference between the value recorded during drug application and the mean of two traces collected before drug application and two collected after drug washout.

All statistical tests were performed using SigmaPlot 14 (Systat Software, San Jose, Ca). Much of the data collected was not normally distributed so nonparametric statistical test were used where appropriate. All electrophysiology data was initially separated by sex but no significant difference between male and female pups was observed so it was pooled for the final analysis.

**Protein extraction and Western blotting:** Hippocampi from rats aged 0, 2, 4, 6, and 8 days, homogenized in ice-cold protein extraction buffer with protease inhibitors (0.5 mg/mL Pefabloc, 1 μg/mL leupeptin, and 1 μg/mL pepstatin; Sigma-Aldrich, St. Louis, MO), and centrifuged at 3,622 g for 10 min at 4°C to separate cell membranes. The supernatant was collected from the 3,622 g centrifugation and centrifuged at 35,000 g for 30 minutes at 4°C to pellet the proteins. The supernatant was discarded and the protein pellet was resuspended in fresh extraction buffer. Protein content was determined using a BCA assay kit (Pierce, Rockford, IL) that measured transmittance of 562 nm light through the samples against a standard curve in a Biophotometer spectrophotometer (Eppendorf, Hamburg, Germany); Protein concentration was reported as the average of triplicate readings. After measuring protein concentration, the samples were stored at -80°C in multiple aliquots to avoid excessive freeze-thaw cycles.

To prepare the protein for western blotting 1.5 μg of protein from each animal was denatured in NuPage lithium dodecyl sulfate (LDS) with a reducing agent by incubating it at 70°C for ten minutes. Denatured protein was separated on a NuPage 3-
8% Tris-acetate Novex minigel. Following gel electrophoresis, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane with 0.45 μm pore size in NuPage transfer buffer (all NuPage Western blotting reagents were obtained from Invitrogen, Carlsbad, CA). Following transfer, membranes were washed in phosphate-buffered saline (PBS) and blocked for 1 hour with PBS containing 0.05% TWEEN 20, 5% nonfat dry milk, and 0.1% bovine serum albumen (blocking solution) at room temperature. Membranes were then incubated overnight at 4°C in the previously described blocking solution containing rabbit-anti-BK antibodies (Genetex GTX54874; lot 821503077) at a 1:2,000 dilution and rabbit anti-α/β-tubulin antibodies (Cell Signaling Technology 2148S; lot 6) at a 1:4,000 dilution. Following decoration with primary antibodies, membranes were washed in PBS with 0.05% Tween 20 for 1 hour at room temperature and then incubated in blocking solution with goat-anti-rabbit (Pierce no. 1858415 lot GC95095) horseradish peroxidase (HRP) conjugated antibodies at a 1:1,000 dilution in blocking solution for 90 min. Secondary antibodies were removed by washing the membranes in PBS with 0.05% Tween 20 and finally incubated for 5 minutes in SuperSignal West Dura extended signal substrate (ThermoFisher Scientific, Waltham, MA) to allow for visualization of the protein bands. Labeled membranes were exposed to classic blue autoradiography film (MidSci, St. Louis, MO). Signal was quantified using Labworks 4 imaging and analysis software (UVP, Upland, CA) by comparing the integrated optical density (IOD) of the BK channels band to IOD the α/β-tubulin bands (BK channel expression = $\frac{IOD_{BK}}{IOD_{Tubulin}}$).

Solutions (all values are in mM):

*Rodent Ringer's solution*: 146 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 11 glucose, pH 7.4 (adjusted with NaOH)
**PIPES buffered saline:** 120 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 25 glucose, 20 PIPES, pH 7.0 (adjusted with NaOH)

**High-sucrose artificial cerebral spinal fluid (aCSF; HEPES-buffered):** 129 sucrose, 3 KCl, 1.4 CaCl₂, 1.2 MgSO₄, 10 glucose, 25 HEPES, 0.4 L-ascorbic acid, pH 7.4 (adjusted with NaOH)

**aCSF for slices (HEPES-buffered):** 129 NaCl, 3 KCl, 1.4 CaCl₂, 1.2 MgSO₄, 10 glucose, 25 HEPES, 0.4 L-ascorbic acid, pH 7.4 (adjusted with NaOH) [205]

**Pipette internal solution:** 140 K-gluconate, 0.5 CaCl₂, 2 MgCl₂, 1 EGTA, 2 ATP-Na₂, 0.2 GTP-Na₂, and 10 HEPES, pH 7.2–7.4 (adjusted with KOH), 130 nM free Ca²⁺ (estimated with Maxchelator Ca/Mg/ATP/EGTA Calculator v2.2 [https://somapp.ucdmc.ucdavis.edu/pharmacology/bers[maxchelator/CalMgATPEGTA-NIST-Plot.htm]])

**Physiological saline for electrophysiology in cultured neurons:** 115 NaCl, 25 NaHCO₃, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH of 7.4 (adjusted with NaOH).

**Protein extraction buffer:** 250 sucrose, 10 Tris, 10 HEPES, and EDTA, pH of 7.2 (adjusted with HCl)

**Phosphate-Buffered Saline (PBS):** 134.4 NaCl, 4.36 KCl, 10.56 Na₂HPO₄, 1.66 NaH₂PO₄, pH of 7.4 (adjusted with HCl)

### 2.3 Developmental Changes in AP Waveform and Neuronal Excitability

The aim of this project is to understand how BK potassium channels contribute to action potential firing and neuronal excitability across early development. This section describes the trends in passive electrical properties, neuronal action potential waveform, and excitability across early development to contextualize the changes in BK channel function reported later.

Cultured rat hippocampal neurons had similar resting membrane potentials
regardless of when they were cultured during the first postnatal week. I recorded a mean resting membrane potential of \(-57.9 \pm 1.3\) mV in neurons cultured from P1 rats (72 cells, 11 animals) and \(-56.9 \pm 1.0\) mV in neurons cultured from P7 rats (70 cells, 9 animals, Fig 2.1A). Likewise, cell capacitance, which is a proxy measurement for cell size, did not change between neurons cultured from P1 and P7 neurons with respective capacitances of \(21.2 \pm 0.7\) pF (72 cells, 11 animals) and \(21.9 \pm 0.7\) pF (70 cells, 9 animals, Fig 2.1B). Membrane resistance decreased between P1 and P7 which likely corresponds to increased channel expression in neuronal membranes (P<0.001, one-way ANOVA, P<0.05, Holm-Sidak pairwise comparisons). Neurons from P1 rats had a mean membrane resistance of \(1.93 \pm 0.13\) G\(\Omega\) (80 cells, 11 animals) and neurons from P7 rats had a mean membrane resistance of \(1.03 \pm 0.11\) G\(\Omega\) (70 cells, 9 animals, Fig 2.1C).

Through development the neuronal action potential in cultured rat hippocampal neurons “refines” from long lasting, low amplitude action potential to a more

**Figure 2.1: Passive membrane properties of cultured hippocampal neurons across the first postnatal week.**

A Mean±SEM initial resting membrane potential upon break-in of P1-P7 cultured hippocampal neurons. B Mean±SEM initial capacitance upon break-in of P1-P7 cultured hippocampal neurons. C Mean±SEM initial membrane resistance upon break-in of P1-P7 cultured hippocampal neurons (One-way ANOVA, P < 0.001; Holm-Sidak pairwise comparisons, P < 0.05 for the comparison to P1) Sample size for all graphs is N, n where N is the number of animals and n is the total number of neurons.
depolarizing, briefer spike (Fig 2.2A). The mean amplitude (peak amplitude - resting membrane potential) of an action potential increase from 80.96 ± 1.48 mV in neurons cultured from P1 rat pups (80 cells, 9 animals) to 91.65 ± 1.39 mV in neurons cultured from P7 rat pups (71 cells, 11 animals; one-way ANOVA on ranks, P < 0.001, Dunn’s...
multiple comparisons, P < 0.05; Fig 2.2B,C). At the same time, the duration (width in milliseconds at half-maximum amplitude) decreased from 3.52 ± 0.17 ms in neurons from P1 rats (78 cells, 9 animals) to 2.35 ± 0.11 ms in neurons from P7 rats (68 cells, 11 animals; P < 0.001, Dunn’s multiple comparisons P < 0.01 Fig 2.2D,E).

The action potential waveform was highly variable at all ages which is likely a reflection of the homogenous cell population contained in the neuronal cultures. Action potential amplitudes ranged from 53.65 mV to 107.76 mV in neurons from P1 rats and from 57.71 to 108.81 in neurons from P7 rats (Fig 2.2C). Durations varied from 1.25 ms to 8.95 ms in neurons from P1 rats and from 1.08 ms to 5.21 ms in neurons from P7 rats (Fig 2.2E). The amplitude of the afterhyperpolarization did not change from P1-P7 (Fig 2.2F).

The developmental changes in the action potential waveform were accompanied by an increase in the capacity for repetitive action potential firing; this was measured as the maximum number of action potential fired in response to a 100 ms depolarizing pulse. Neurons cultured from P1 rats fired an average of 2.0 ± 0.1 action potentials per 100 ms while the neurons cultured from P7 rats fired an average of 3.3 ± 0.2 action potential per 100 ms (one-way ANOVA on ranks, P < 0.001, Dunn’s multiple comparisons, P < 0.05; Fig 2.3A,B). As can be noted on the box-and-whisker plot (Fig 2.3C), the median neuron from a P1 rat fired only one action potential; it was typical that these neurons would fire only one action potential before entering depolarizing block, a state where the neuron does not repolarize sufficiently to undo the voltage dependent inactivation on voltage dependent sodium channels and so a subsequent action potential cannot be initiated. This was exhibited by some neurons at all ages but was most characteristic of neurons from P1 rats.

Culturing neurons is a disruptive process. Neuronal processes are sheared
during trituration, membrane proteins are digested by proteases, and the neurons are isolated from their natural context. To test if the age-dependent differences observed in cultured neurons are an artifact of culturing I repeated the measurements of action potential waveforms and repetitive firing in CA1 pyramidal layer neurons in acute hippocampal slices and compared these properties between neurons from rats aged P4-P5 (9 cells, 3 animals for all measurements) and P7-P8 (18 cells, 6 animals for all measurements). Under the conditions used, I could not reliably obtain viable tissue-patched neurons in slices from animals younger than P4. At ages younger than P4, the neurons did not reliably fire action potentials in response to depolarizing stimuli, so these findings do not correspond to the same developmental span as findings in cultured neurons.

Like in the cultured neurons, resting membrane potential did not significantly change with values of -81.2 ± 2.1 mV at P4-P5 (9 cells, 3 animals) and -78.3 ± 2.4 mV

**Figure 2.3 100 ms action potential trains across the first postnatal week:**

- **A** Representative traces of action potential trains evoked by 100 ms depolarizing pulses in P1 and P7 rat hippocampal neurons.
- **B** Mean ± SEM maximum action potentials fired per 100 ms (one-way ANOVA on ranks, P < 0.001, Dunn’s multiple comparisons, P < 0.05; *significant difference from P1-P2, **significant difference from P1-P4).
- **C** Box and whisker plots (box shows 2nd-3rd quartile, whiskers indicate 10th-90th percentile) of maximum action potentials fired per 100 ms in P1 and P7 hippocampal neurons.
Capacitance was also not significantly different at 27.0 ± 2.4 pF at P4-P5 neurons and 24.3 ± 1.8 pF at P7-P8. I did not observe an age dependent change in resistance which I measured at 1.3 ± 0.2 GΩ at P4-P5 and 1.0 ± 0.2 GΩ at P4-P5. An age dependent change in resistance in hippocampal neurons in slices can’t be ruled out as that trend only appeared in the cultured neurons across the entire P1-P7 span and
From P4-P5 and P7-P8 action potential amplitude did not significantly change (76.84 ± 3.63 mV at P1 vs. 77.90 ± 2.83 mV at P7) which matches the data findings in cultured neurons as there were no differences in action potential amplitude between P4 or P5 and P7 in that data set Action potential duration decreased from 6.89 ± 0.99 ms at P4-P5 to 4.5 ± 0.51 ms at P7-P8 (Mann-Whitney rank sum test, P < 0.02; Fig 2.4 D). Capacity for repetitive firing also increased with developmental time in slice recordings. Neurons in brain slices from P4-P5 rates fired an average of 1.89 ± 0.35 action potentials per 100 ms while neurons in brain slices from P7-P8 fired 3.5 ± 0.36 action potentials per 100 ms (student's t-test, P < 0.05; Fig 2.4E). This agrees with the findings in cultured neurons where there was a significant difference in the maximum number of action potential per 100 milliseconds between P4 and P7 (Fig 2.2B).

2.4 The Role of Calcium-Activated Potassium Channels in Determining the Action Potential Waveform and Firing Rate in Cultured Neonatal Neurons Across the First Postnatal Week

In order to determine how calcium dependent potassium channels, in totality and individually, contribute to action potential waveform and repetitive firing in neonatal rats (P1-P7) I recorded the effects of the L-type calcium channel blocker nimodipine (20 µM; IC$_{50}$: 20 nM [206]) which should eliminate the contribution of all calcium dependent channels, the SK channel blocker apamin (1 µM; IC$_{50}$: 480 pM [40]), the BK channel blocker iberiotoxin (100 nM; IC$_{50}$: 27.4 pM [207]), and the KCNQ channel blocker linopirdine (10 µM; IC$_{50}$: 3.6 µM [208]).

All four drugs significantly increased the action potential duration by slowing the repolarization rate (one-sample t-tests, all P < 0.001). Nimodipine increased the action potential half-width by 0.95 ± 0.23 ms, apamin by 0.87 ± 0.22 ms, iberiotoxin by 0.44 ± 0.37 ms, and linopirdine by 0.43 ± 0.08 ms. Nimodipine and apamin both had a
significantly greater effect on action potential duration than iberiotoxin and linopirdine (one way ANOVA P < 0.001, Holm-Sidak pairwise comparisons P < 0.05; Fig 2.5A).

All drugs significantly decreased the magnitude of the fast afterhyperpolarization; nimodipine by 2.50 ± 0.78 mV, apamin by 1.67 ± 0.50 mV, iberiotoxin by 1.96 ± 0.14 mV, and linopirdine by 2.26 (one-sample t-tests, all P < 0.01). There were no significant differences in the magnitude of the effect of different blockers (one-way ANOVA, P = 0.544) which may be due to heterogeneity of the cell population studied and variability of the AHP amplitude within a cell (Fig 2.5B).

All four drugs also significantly decreased the maximum number of action potentials evoked by a 100 ms square depolarizing pulse in neurons that fired at least two action potentials in response to the control stimulus protocol: nimodipine by 2.59 ± 0.34 action potentials, apamin by 0.67 ± 0.14 action potentials, iberiotoxin by 0.27 ± 0.03
action potentials, and linopirdine by 0.43 ± 0.10 action potentials (one sample t-tests, all P < 0.001 for all drugs). Nimodipine had a significantly greater effect than all the other drugs as one would expect since it should block all of the calcium dependent channels simultaneously. Apamin had a greater effect than iberiotoxin (One-way ANOVA P < 0.001, Holm-Sidak post hoc, P < 0.001 for all vs. Nimodipine, P < 0.01 for apamin vs iberiotoxin; Fig 2.5C). The iberiotoxin experiments showed a clear age-dependent trend across the P1-P7 range, and so the BK channels became the focus of the research project (data not shown).

2.5 BK Channel Protein Expression in Rat Hippocampi During the Early Neonatal Period

I measured the BK channel protein expression in the hippocampus by Western blot to contextualize the changes measured in the physiological role of BK channels. I extracted protein from the hippocampi of 3 male and 3 female mice aged P0, P2, P4, P6, P8 and 6 weeks and separated it by SDS-PAGE. I probed the blot for the BK channel α subunit and for α/β-tubulin which served as a loading control and measured BK channel expression as a ratio of BK channel signal to α/β-tubulin signal. The 6-week timepoint was collected to provide a reference of adult channel expression, but adult expression was so high that I was not able to find a combination of antibody concentration and film exposure time that allowed for detection of a signal from the earliest timepoints without overexposing the adult sample, so I ultimately excluded the oldest timepoint and focused the experiment on the young animals.

I separated the data by sex and found that there was a main effect of sex on the channel expression with the mean signal ratio being higher at all timepoints in the hippocampi of female rats. The sex difference was relatively moderate and there were no significant pairwise differences at any given age (two-way ANOVA main effect of sex
Both sexes showed an age dependent increase in channel expression and both sexes showed an inflection in expression levels at P4 so sexes were pooled to test age dependent changes in expression.

BK channel expression was steady from P0 to P2, significantly increased above the P0-P2 levels at P4 and P6, and expression at P8 was significantly higher than all previous timepoints (One-way ANOVA, P < 0.001; Holm-Sidak multiple comparisons, P = 0.05; Fig 2.6C). BK channel expression ultimately increased roughly three-fold between P0 and P8 which is in line with the increase in KCNMA1 (the gene encoding the BK channel α subunit) mRNA expression across a similar developmental window (embryonic day 18 to P7) in the mouse hippocampus [155].

The expression data reported here represent protein levels in whole tissue homogenate of the hippocampus and so it should be noted that the data do not solely
describe changes in neuronal BK channel expression as BK channels are expressed in many cell and tissue types including blood vessels which could account for a non-trivial amount of the BK channel expression in the central nervous system [60].

2.6 Age Dependent Changes in BK Channel Roles in Action Potential Firing

To test the role of BK channels in the action potential waveform and repetitive firing, I perfused neurons with the BK channel antagonist 100 nM iberiotoxin and evoked both single action potentials and trains of action potentials evoked by a 100 ms square depolarizing pulse. I compared the action potential half-width, afterhyperpolarization amplitude, and maximum number of action potentials evoked under BK channel blockade with the average value of measurements taken before and after blockade for each parameter.

BK channel blockade with iberiotoxin increased action potential duration more in neurons from P1 rats than in neurons from P6 and P7 rats (one way ANOVA, P = 0.001; Dunn’s multiple comparisons, P < 0.05; P7 was also significantly different than P4). BK channel blockade increased action potential duration by 0.63 ± 0.10 ms in neurons from P1 rats (64 cells, 6 animals); at P7, BK channel blockade increased action potential duration by 0.24 ± 0.08 ms (45 cells, 6 animals; Fig 2.7B). This decreased contribution to action potential duration seems to contradict the dramatic rise in channel expression (section 2.6) but could also suggest a difference in activation timing of the BK channels present in neurons across early postnatal development.

A second explanation is that the effect is simply due to age dependent changes in the action potential waveform, i.e. the longer waveform of younger neurons results in more calcium influx and greater channel activation. To test if this is the case, I analyzed the subset of neurons with action potential between two and three ms in duration in neurons from P1-P2 rats (27 cells, 12 animals) and neurons from P6-P7 rats (# cells, #
animals). Iberiotoxin increased the action potential duration by $0.46 \pm 0.12$ ms in the P1-P2 group and by $0.17 \pm 0.05$ in the P6-P7 group (Mann-Whitney rank sum test, $P = \ldots$).
Because I still found decreased contribution of BK channels to action potential duration with age in a subset of neurons with comparable duration, I can conclude that the decreased contribution is not simply a function of the changing action potential waveform.

Effects on Iberiotoxin on the other parameters were less conclusive. Iberiotoxin diminished the afterhyperpolarization following a single action potential to a greater degree in neurons from P6 rats than P3 rats (One-way ANOVA, P < 0.01, Holm-Sidak multiple comparisons, P < 0.01; Fig 2.7D) but there were no significant differences between any other timepoints, and no trend was apparent in the data. The lack of conclusive findings regarding afterhyperpolarizations may be the result of the highly heterogeneous nature of the neuronal cultures.

Testing the role of BK channels in repetitive firing yielded similarly inconclusive results. The only significant finding was that iberiotoxin decreased repetitive firing to a greater extent at the P7 timepoint (-0.53 ± 0.11 action potentials/100 ms, 45 cells, 6 animals) than the P3 time point (-0.05 ± 0.06 action potentials/100 ms, 42 cells, 6 animals; one-way ANOVA, P < 0.01, Dunn’s multiple comparisons, P < 0.05; Fig 2.7E). This significant difference did not seem to be part of a broader trend. A major confounding factor when looking at repetitive firing was that neurons from rats early in the first postnatal week have a much lower capacity for repetitive firing than neurons cultured later in the first postnatal week and the variability in how many action potentials could be evoked per 100 ms was a highly variable at all timepoints.

In order to further examine the role of BK channels in enabling high frequency action potential firing (a key physiological role of BK channels [132]) in hippocampal neurons, I evoked trains of action potentials with a series of one second, 10-100 pA square depolarizing pulses in 10 pA increments in neurons before and during BK
channel blockade with Iberiotoxin. I restricted this experiment to analysis of neurons that fired at least 10 action potentials in one second with a minimum initial firing frequency of 20 Hz; these criteria ensured that I was examining the role of BK channels in governing repetitive firing in neurons that were capable of repetitive firing in the first place.

Comparing the current input/action potential output relationship for neurons from P1 (20 cells, 8 male animals) and P7 rats (13 cells, 4 male animals) revealed age dependent differences in how BK channels govern high frequency firing. BK channel blockade decreased the number of action potentials evoked by the series of depolarizations (2-way ANOVA, main effect of iberiotoxin P < 0.001 at both ages; Fig. 2.8B). Something interesting also happens with the shape of the input/output relationship. The neurons from P1 rats reached a maximum output at 50 pA and the number of action potentials decreased with increasing current inputs. Application of 100 nM iberiotoxin decreased the action potential output (2-way ANOVA, P < 0.001) but did not change the shape of the relationship; firing rates still peaked at 50 pA current injections and decreased at higher inputs. In the neurons from P7 rats, increased current evoked more action potentials for all current steps from 10-100 pA. However, when I applied iberiotoxin, action potential outputs peaked at 50 pA and declined with further increasing current injections. This suggests that BK channels are important for enabling high frequency firing by preventing depolarizing block in the more mature neurons (Fig 2.8B).

To understand how BK channels control spike timing during repetitive firing I examined the effect of BK channel blockade on instantaneous action potential frequencies \( \frac{1}{\text{interspike interval}} \) for the first 20 pairs of action potentials. For this analysis I used the same recordings as were used to compare changes in fAHP amplitude. The first thing I measured was how BK channel blockade with iberiotoxin affected spike-
frequency adaptation, the successive slowing of action potential frequency with continued action potential firing. Fig. 2.8C illustrates the individual spike frequency adaptations and shows that while the starting frequency differed from cell to cell, the overall shape of the adaptation curve is similar across cells. To standardize this measure across cells with variable firing rates, I set the first instantaneous frequency to zero and plotted each subsequent instantaneous frequency as change from the initial, so that a negative value indicated a decrease in the instantaneous firing rate. In the neurons from P1 rats, BK channel blockade with iberiotoxin introduced a significant amount of additional spike frequency adaptation (2-way ANOVA, main effect of iberiotoxin P < 0.001) but the effect size was too small to detect any pairwise differences between individual spike pairs. BK channel blockade with Iberiotoxin introduced a greater degree of spike frequency adaptation (2-way ANOVA, main effect of iberiotoxin, P < 0.001,
interaction of iberiotoxin and spike pair #, P = 0.02) and significant decreases in the instantaneous firing frequency were detected at spike pairs 4-20 (Holm-Sidak pairwise comparison, P < 0.05; \textbf{Fig 2.8D}).

In addition to measuring how BK channel blockade introduced more spike frequency accommodation, I also wanted to examine how it affected instantaneous frequency at each pair of successive action potentials (spike pairs). To measure this, I subtracted the control instantaneous frequency from the iberiotoxin treated instantaneous frequency of each spike pair for the first 20 spike pairs. The effect of BK channel blockade was significantly different between spike pairs 1-5 and spike pairs 15-18, with a greater decrease in instantaneous frequency in the P1 cohort at pairs 1-5 and a greater decrease in the P7 cohort at spike pairs 15-18 (effect size was similar at spike pairs 19-20, but statistical power was reduced at later spike pairs as not all neurons fired 20 action potentials under both conditions [two-way ANOVA, P < 0.001, Holm-Sidak multiple comparisons, P < 0.05; \textbf{Fig 2.8E}]). In neurons from P1 rats, BK channel blockade caused a moderate decrease in instantaneous frequency at all spike pairs. Interestingly in neurons from P7 rats, BK channel blockade increased instantaneous frequency initially, but this effect was transient and was followed by a decrease in instantaneous frequency as spike accommodation began to occur.

\textbf{Figure 2.9 BK channel blockade initially increases instantaneous frequency in P7 fast firing neurons:} Expanded view of the first 5 spikes of each action potential train pictured in figure 2.8 A. Note the decrease in the first interspike interval with iberiotoxin in the P7 traces.
frequency for the initial spike pair (speeding initial action potential firing; see Fig 2.9 for inset), but as action potential firing progressed it the effect transitioned to decreased instantaneous frequencies. The greater effect of BK blockade achieved with continuous firing in neurons from P7 rats is consistent with the greater expression measured by western blot. The shift in the effect of BK blockade in neurons from P7 rats from increasing firing rate early in action potential trains to depressing firing rate as firing continues is intriguing and might suggest that different BK channel subtypes contribute at different phases of repetitive firing or that BK channel subtypes present in P7 neurons require levels of calcium achieved by continuous action potential firing to be completely activated.

Fast afterhyperpolarization (fAHP) carried by BK channels is important for enabling high frequency firing [133]. I measured the extent to which the fast-firing neurons in P1 and P7 were able to repolarize after each spike for the first 20 action potentials in a train. I compared trains of action potentials before and after iberiotoxin application that were evoked by a by the same current injection -ideally an injection that produced high firing rates under both conditions. I normalized the amplitude of the fAHP to zero and reported each subsequent fAHP as a deviation from the initial fAHP so that a positive value indicates a diminished degree of afterhyperpolarization. In spike trains in neurons from P1 rats, there was a similar diminution of the fast afterhyperpolarization with successive action potential firing with and without iberiotoxin. For control recordings in neurons from P7 rats, there was little diminution of the fAHP with successive firing, but fAHP magnitude did diminish with successive firing significantly more with iberiotoxin application (2-way ANOVA, main effect of iberiotoxin P < 0.001; Fig 2.8F). From this we can conclude that as hippocampal neurons mature, BK channels play an important role in maintaining repetitive firing by carrying the fast afterhyperpolarization.
2.7 Age Dependent Changes in BK Channel Currents

To further explore differences in BK channel timing across early development I measured the effect of BK channel blockade on whole-cell potassium currents. I recorded whole cell potassium currents from 33 cultured neurons from four P1 rats and 42 neurons from P7 rats. A small number of cells did not hold stable patches across the entire recording protocol and were removed so that I was left with a final sample size of 32 P1 neurons and 39 P7 neurons for examining the effects of BK channel blockade. Between the two ages, peak whole-cell potassium currents increased from 2091.13 ± 116.19 pA to 3176.99 ± 147.42 pA (Mann-Whitney rank sum test, P < 0.001; Fig 2.10C).

BK channel blockade with iberiotoxin reduced peak current amplitude by 57.79 ± 14.48 pA in neurons from P1 rats and by 13.21 ± 34.87 pA in neurons from P7 rats which was not significantly different, but this may have been due to the variability of potassium current amplitude (Fig 2.10D). When the data was normalized so that the difference in peak current between the BK-blocked current and the control was expressed as a percent of the control peak potassium current there was a significant difference. Iberiotoxin blocked 3.62 ± 1.14% of the total potassium current in neurons from P1 rats and 0.32 ± 1.11% of the total potassium current in neurons from P7 rats (Mann-Whitney rank sum test, P < 0.05; Fig 2.10E). Though significant, this effect is relatively small and unlikely to account for physiological differences, although it’s possible that some of the effect may have been obscured by the method by which I accounted for current rundown.

Current clamp recordings suggested differences in the timing of BK channel activity between P1 and P7, so I compared how BK channel blockade with iberiotoxin affected the onset rate of potassium currents. BK channels play a greater role in the onset phase of potassium currents in neurons from P1 rats than in neurons from P7 rats.
At P1 iberiotoxin decreased the maximal rise slope of the total whole-cell potassium current by $12.01 \pm 1.91\%$ but only by $1.94 \pm 1.70\%$ at P7 (Mann-Whitney rank sum test, $P < 0.001$; **Fig 2.10F**). This difference in activation speed may be due to BK channels with faster kinetics expressed in P1.
I calculated the peak conductance of the total BK current at each holding potential (-50 mV to +50 mV) and expressed it as a fraction of the peak conductance at the +50 mV holding potential (G/G_{max}). The conductance curve for neurons from P1 was shifted to the left relative to the curve for neurons from P7 rats, indicating that total potassium conductance in P1 rat neurons has lower voltage dependency than those in P7 rat neurons. The P1 rat neurons achieved a greater portion of the total conductance at the +20 mV through the +30 mV voltage steps (three-way ANOVA, P < 0.001 for the interaction of age and voltage step; Holm-Sidak pairwise comparisons, P < 0.01; Fig. 2.10G). BK channel blockade with Iberiotoxin did not alter the voltage-conductance relationship at either age, so the differences in voltage dependence do not appear to be due to differences in BK channels between the two ages (Fig. 2.10H-I).

### 2.8 Conclusions

In my recordings from acutely cultured hippocampal neurons, I observed the hallmarks of neuronal development that have been shown in a wide variety of neuronal types and tissue preparations: large decreases in membrane resistance, a sharpening of the action potential waveform and a rise in the capacity of neurons to fire multiple action potentials.
potentials [11], [12], [197]–[200], and Figs 2.2-2.3. This data mirrored the trends observed in the acute slice model (Fig 2.4) This was important to demonstrate because I wanted to confirm that acutely culturing neurons at successive postnatal timepoints accurately reflects in vivo development. With regards to changes in intrinsic excitability, this appears to be a valid model system.

Blockade of L-type calcium channels with nimodipine affected the both the waveform of action potentials and their firing rates suggesting the involvement of calcium dependent channels in these processes. Channel blockade with the selective potassium channel antagonists apamin, iberiotoxin, and linopirdine show that SK, BK, and KCNQ potassium channels all are present and affect neuronal physiology in the early developmental period. What I found is that blockade of all three channels has distinct effects of increasing action potential duration, decreasing afterhyperpolarization and reducing the capacity for repetitive firing. This effect is consistent with the generally reported role of BK channels in neurons [131], but stands in contrast to the canonical roles of SK and KCNQ channels. SK channels, being voltage insensitive and coupled to calmodulin are generally too slowly activating to contribute to repolarization and the fast afterhyperpolarization, but carry the medium afterhyperpolarization and decrease firing rates [36], [39]. KCNQ currents canonically hyperpolarize the membrane and reduce excitability (although if they are conducting current during evoked action potential firing they will doubtlessly affect the action potential waveform) [202]. Some of this apparent non-canonical functioning of SK and KCNQ channels could be due to two factors. First, action potential durations in these neonatal neurons are extraordinarily long relative the action potential in a neuron from a mature brain – while a current may activate too slowly to affect the action potential of a mature neuron, it may affect the slower action potential of a neonatal neuron. Second, a channel’s function may result from its subcellular localization. As these recordings were take approximately 24 hours after culturing, the
neurons were essentially all soma with minimal processes and channels expressed outside of their canonical subcellular environment may play noncanonical roles.

What drew me to focus this study on the BK channel was the enigmatic decrease in effect of channel blockade on action potential repolarization at a time when expression should be rising. This preliminary observation was confirmed as I expanded the data set from a small preliminary sample to > 45 neurons from at least 6 animals at each age from P1-P7 (Fig 2.6). This result indicated that, although fewer in number, BK channels activate more readily in immature neurons in response to a single depolarizing event and that their activation slows as development progresses. This was also indicated by a much larger decrease in the onset rate of potassium currents by BK channel blockade with iberiotoxin in P1 neurons than P7 neurons Fig 2.10. These findings align well with the differences between STREX BK, which predominates at P1 and ZERO BK (channels lacking STREX), which predominates at P7. STREX BK has a fast onset time during action potential firing due to its higher calcium sensitivity, while ZERO BK may activate too slowly to be a significant player in repolarizing a single action potential [77], [158].

Recordings from selected cultured fast-firing neurons revealed the physiological implications of decreasing BK channel activation speed and increasing expression. In neurons from P7 rats, BK channel blockade with iberiotoxin produced a consistent depression of firing rates throughout 1 second of evoked firing. In the P7 neurons, there is less depression of firing rates early in the action potential train (and even an increase in firing over the control!), but as firing progresses iberiotoxin slows firing in the P7 neurons to a greater extent than the P1 neurons by the 15th consecutive action potential pair. This suggests a model where in immature neurons, BK channels respond readily at the beginning of firing as the depolarization and the early rise in intracellular calcium are sufficient to activate the channel, but in the P7 neurons there is insufficient calcium in the cell to activate BK channels in time to contribute to repolarization. This could be due to a
combination of the rapid speed of the more mature action potential so that less calcium enters as well as changes in the calcium sensitivity of the BK channel as the isoform switches from STREX to ZERO. As firing progresses and intracellular calcium rises, the channels are fully activated by depolarization and achieve a greater effect than in P1 neurons as would be suggested by the higher expression at P7 (Fig. 2.8). The reason for the initial increase in spike firing is unclear but it may be due to BK currents at early spike pairs activating too late to repolarize the neuron but contribute to the afterhyperpolarization – iberiotoxin reduces this afterhyperpolarization and decreases the interval before the next spike.

The voltage clamp recordings also support the presence of faster acting, more calcium sensitive BK channel isoforms at earlier ages. Higher BK channel contribution to early onset potassium currents in hippocampal neurons could be due to more calcium sensitive BK channel subtypes present at P1. A less calcium sensitive subtype would need prolonged depolarization before sufficient calcium enters to activate the channel, which is why, under this proposed model there is little contribution to the rising current at P7. A more calcium sensitive subtype would require much shorter and lower depolarization to completely activate and can open very quickly once the membrane is depolarized which is why there is significantly more contribution to the rising phase.

That there was a sex difference in the protein expression but not in the electrophysiological measures raises some questions. In addition, there are reported sex differences in calcium handling in neurons in the early postnatal period. Resting levels of intracellular calcium are higher in cultured female hippocampal neurons than in male neurons and male neurons have higher levels of calcium buffering proteins in early postnatal time [209]–[211]. Increased intracellular calcium and reduced capacity for calcium buffering along with higher BK channel expression in females would predict greater BK channels activation in female neurons and a larger contribution to action
potential repolarization. That no sex-dependent difference was observed may have simply been due a small effect size and the contribution of multiple additional potassium channels to action potential repolarization.

So far in this discussion I have not considered the effects of β-4 subunit expression. BK channels that express the β-4 subunit are insensitive to iberiotoxin and so it could be concluded that the decrease in effect of iberiotoxin on action potential repolarization represents a transition from iberiotoxin-sensitive to iberiotoxin-insensitive isoforms [115]. This is possible, but as iberiotoxin has a larger effect on repetitive firing at P7 than P1 it can be concluded that there is still a significant portion of iberiotoxin-sensitive (β-4 unassociated) BK channels at P7 and that the age dependent effects observed stem from differences in the activation properties of the α subunit.
Chapter 3: Development of Excitability and the Role of BK Channels in Regulating Action Potential Firing and Modulating Seizure Activity in Mouse Hippocampal Neurons

3.1 Introduction

The findings in the previous chapter describe the role of BK channels in the excitability of cultured rat hippocampal neurons across the first postnatal week. The present chapter continues that theme but transitions model systems to an acute mouse hippocampal slice model. The use of mice also allows for comparisons to future studies in transgenic mice. The hippocampal slice model allows a broader range of developmental time to be studied as we observed a steep drop in viability when attempting to culture neurons from rats older than 7 days; meanwhile viable slices can theoretically be prepared from animals of any age. Slices additionally retain synaptic connectivity and even retain rhythmic brain activity that occurs in vivo [212]. This makes the slice model not only suitable for studying intrinsic cellular properties but also for synaptic physiology and organ-level disease states such as epilepsy. A final advantage of the slice model is that it allows for identification of cell type based on location in addition to electrical properties; this chapter focuses on hippocampal pyramidal neurons as BK channel expression within the hippocampus is concentrated in the axons and presynaptic terminals of the pyramidal neurons [129].

In the previous chapter I demonstrated that BK channels in cultured neurons isolated from newborn rats contribute to action potential firing in a way consistent with type-I BK channel kinetics as they have fast activation and open in time to contribute to repolarization of a single action potential – kinetics associated with a lack of β subunits. As development progresses through the first postnatal week there is a transition to type-II kinetics, which are associated with α/β-4 BK channel assemblies, where channels
have slower activation and are more significant modulators of repetitive firing [114], [156].

Here I tested whether this developmental trend in BK channel activity was also present in mouse hippocampal pyramidal neurons across postnatal development using the in vitro slice preparation where local circuits remain intact and neurons are not subjected to enzymatic digestion. Instead of restricting the investigation to the first postnatal week I studied mice at three timepoints from postnatal day 4 to postnatal day 15 (P4-P5, P9-P10, and P14-15). These timepoints in a mouse approximately correspond a developmental window that spans prenatal development to childhood in humans, although perfect parallels between rodent and human brain development cannot be drawn [213]. The younger ages are largely uninvestigated in the literature, but the oldest timepoint is commonly used for electrophysiology studies including investigations into the BK channels [176].

In chapter 2, I measured steady state protein expression of the BK channel α subunit from postnatal day zero to eight using Western blots. The aim in the present study was to examine differential expression of α subunit isoforms; analysis of protein expression at this time is constrained by an absence of commercially available antibodies specific to different BK channel α subunit isoforms. I opted to examine the expression pattern of BK channel mRNA to see if changes in the pattern of BK channel α subunit isoform and abundance of β subunits correspond to changes in the electrophysiology. The STREX isoform of the BK channels contains an exon that increases the calcium sensitivity of the BK channels and allows it to open faster in response to depolarization [77], [158]. This isoform has been shown to be the predominant form of the BK channels in the prenatal rodent brain and given its activation kinetics may underlie the type I kinetics previously observed in early postnatal neurons. By the end of the first postnatal week, BK channels lacking the STREX exon
predominate [155]. In addition to expression patterns of the α subunit, changes in the β subunit contribute to changes in BK channel kinetics [117]. The β-4 subunit may also underlie a transition from fast type I to slower type II BK channel kinetics [118].

Finally, I investigated whether BK channel blockade represents a viable treatment strategy for epilepsy treatment in the early postnatal period. BK channel blockade has shown some promise as a treatment for epilepsy but only under specific conditions. Previously, the BK channel antagonist paxilline has been demonstrated to attenuate seizures in a gain-of-function Kcnma1 mutant mouse as well as in mice that had previously induced seizures [174], [175]. Prior seizure sensitizes the brain to subsequent seizures in a phenomenon known as kindling; BK channels may play a role in kindling as seizures cause an upregulation of the STREX isoform and downregulation of the β-4 subunit causing epileptic neurons to display type I kinetics [185], [187]. As early postnatal neurons are characterized by type I rather than type II BK channel activity, I hypothesized that BK channel blockade with paxilline can attenuate seizure activity in brain slices from young mice with no history of seizures and that efficacy will decline as postnatal age increases.

Blockade of BK channels could modulate seizure activity in several ways. The first factor to consider is how BK channels will affect neurotransmitter release based on their physiological role. If BK channels are principally acting in the axons to maintain high firing frequencies, then BK channel blockade will reduce neuronal firing rates and reduce neurotransmitter release [132]. In this first case, BK channel blockade will dampen circuit excitability and counteract seizures. Alternately, if BK channels are primarily acting to terminate the presynaptic action potential and limit neurotransmitter release, then blocking BK channels with increase neurotransmission drive circuit excitability – not a desirable characteristic in an anti-seize drug [183]. These proposed mechanisms only consider BK channels in the excitatory pyramidal neurons of
hippocampal circuits; if we broaden consideration to the effects of blocking BK channels on inhibitory interneurons the effects on circuit excitability may be reversed and the picture becomes much murkier.

It appears from the limited number of studies available that the effect of BK channel blockade dampens excitability – consistent with BK channels enabling high firing frequencies in excitatory cell types [174], [175]. The interneurons are unlikely to mediate any anti-seizure effects of BK channel blockade in the hippocampus as BK channels in that brain structure are primarily found in the pyramidal neurons. In fact, the parvalbumin expressing interneurons, an inhibitory neuronal class type highly implicated in regulating seizures relies on KV₃ exclusively for action potential repolarization [20], [214]. The final part of this chapter will investigate the effects of BK channel blockade in a zero magnesium model of inducing in vitro seizure activity. The effect of BK channel blockade on action potential firing frequency and synaptic currents will be examined to determine the mechanism through which it might modulate seizure activity.

3.2 Methods

Animal care and use: All animal protocols were approved by the Marquette University Institutional Animal Care and Use Committee according to the guidelines set forth by the National Research Council in the Guide for Care and Use of Laboratory Animals. Wild type male C57Bl6/J mice (Jackson Laboratories Bar Harbor, ME, USA) were bred with female C57BL/6-Tg(Pvalb-tdTomato)15Gfng/J mice (Strain #:027395, Jackson Laboratories, Bar Harbor, ME). These mice were used because the original iteration of this project involved investigations of parvalbumin expressing interneurons, but this aim was discarded as low parvalbumin expression in neonatal mice made positive identification of parvalbumin expressing neurons unreliable. Pups were separated from dams just prior to experiments and rapidly decapitated for experimental use.
Slice preparation: After mice were decapitated, the brains were transferred to a high magnesium, sucrose based artificial cerebrospinal fluid (sucrose based aCSF) frozen to an ice slurry consistency and bubbled with 95% O₂/5% CO₂. All solution recipes are given at the end of the methods section. Brains were then sliced in the same solution to a thickness of 300 μm using a Leica VT1000S vibratome (Wetzlar, Germany). Whole brain slices containing hippocampus were transferred to warm (35-37°C) aCSF that was continuously bubbled with 95% O₂/5% CO₂ and incubated for at least one hour to allow slices to recover from temperature and any osmotic stress incurred during slicing and to allow the aCSF to diffuse into tissue slices and replace the native extracellular fluid. During incubation the aCSF was allowed to return to room temperature.

Electrophysiology: For recording, slices were transferred to a lateral flow recording chamber perfused with 30-33°C aCSF continuously bubbled with 95% O₂/5% CO₂ aCSF. Filamented borosilicate glass pipettes (Sutter Instruments, Novato, CA; Cat# BF-150-860-10) were pulled to a resistance of 4-8 MΩ. Both voltage-clamp and current-clamp recordings were obtained by whole cell patch-clamp recording with a Dagan 3900A patch-clamp amplifier (Dagan Corporation, Minneapolis, MN), Axon Digidata 1322A 16-bit data acquisition system, and pClamp 10.4 data acquisition software (Molecular Devices, San Jose, CA). Data were digitized at a rate of 20 kHz; voltage clamp signals were filtered with a 1-kHz low pass filter and current clamp signals were filtered with a 10-kHz lowpass filter.

All cells used for recordings were putative CA1 region pyramidal neurons. Putative pyramidal neurons were identified by their presence in the pyramidal layer where pyramidal neurons greatly outnumber other neuronal types [215], presence of sag voltage in response to hyperpolarizing current injections which is a trait of, though not exclusive to pyramidal neurons [216], [217], and an absence of the very brief spikes and
pronounced fast afterhyperpolarizations characteristic of interneurons [218]. The location and electrophysiological properties of the neurons included in the study provide relative confidence that they are glutamatergic pyramidal neurons and not local GABAergic, inhibitory interneurons, but subsequent histological or molecular characterization was not performed so the cells included in the study are putative pyramidal neurons. Furthermore, glutamatergic pyramidal neurons are not a homogenous population of cells as they have been classically perceived but a diverse cell populations with genetically and molecularly distinct subpopulations [219], [220]. Recording location and examples of records with and without sag voltages are shown in Fig 3.1.

Upon break-in, neurons were clamped at a membrane potential of -80 mV and capacitance was measured using the membrane test function in pClamp. After capacitance was acquired the holding current was adjusted to -60 mV for recording of synaptic currents or the configuration was changed to current clamp for voltage

Fig 3.1: Identification of putative pyramidal neurons in slices A schematic hippocampal slice showing recording location. B Sample traces from a putative pyramidal neuron (left) displaying a prominent overshoot of steady state voltage, or sag, in response a hyperpolarizing voltage injection and from a putative fast-spiking interneuron (left) lacking a voltage sag.
Effects of BK channel blockade on action potentials: Once current clamp mode was achieved resistance was measured and action potential firing was assessed to confirm putative pyramidal cell’s identity by a series of one-second square current injections from -100 to +80 pA in 20 pA steps. After the initial recording, patched neurons were held at rest for 10 minutes to ensure a viable patch and full dialysis of the internal solution into the neuron. This ensured that differences between baseline and drug treated traces weren’t attributable to changing cytosolic composition. After allowing the pipette solution to dialyze, membrane resistance was again determined by the series of one-second current injections from -100 to +80. Next, single action potentials were evoked for waveform analysis using rheobase current injections – a one-second current injection of the minimum amplitude (to the nearest pA) required to evoke an action potential. Next a series of currents from 20-200 pA in 20 pA steps was injected to generate a current input-to-action potential output curve and data for spike train analysis. After the baseline measures were taken, the slices were perfused for 5 minutes in aCSF containing 1 μM paxilline to block BK channels. The series of recording protocols was repeated with BK channel blockade by 1 μM paxilline. Throughout the experiment resting membrane potential was monitored and current was injected as necessary to maintain resting membrane potential at -65 to -70 mV. All analyses were performed using Easy Electrophysiology version 2.5 (Easy Electrophysiology Ltd, London, UK).

Seizure protocol for membrane potential recordings: For the monitoring of single cell membrane potential during a seizure induction protocol, pyramidal layer cells were patched and identified as before and the series of one-second current injections from -100 to +80 was performed to determine membrane resistance and confirm the putative pyramidal cell identity. After this membrane potential was continuously recorded in current clamp for five minutes in the absence of any stimuli to determine resting
membrane potential and excitability. Following a five minute baseline recording, magnesium free aCSF was perfused onto the slice for 15 minutes to induce a hyperexcitable state, presumably by removing the magnesium block on NMDA type glutamate receptors thus amplifying neuronal responsiveness to glutamatergic inputs [221]. Magnesium free aCSF was applied for 15 minutes or until cells became irreversibly depolarized by the treatment. Control slices were treated with only magnesium free aCSF. Slices in the experimental group were treated with magnesium free aCSF containing 1 μM paxilline.

Analysis of membrane potential recordings consisted of onset time of seizure-like activity, overall frequency of action potentials throughout the recording, and intra-burst frequency during the magnesium free wash. Onset time was measured as the time of the first action potential in seconds from the start of the magnesium free wash. Recordings with an onset time of less than 60 seconds were excluded from this analysis as the perfusion rate was approximately 2 mL/minute and the volume of tubing joining the reservoir and recording chamber was approximately 1.5 mL so activity occurring within 60s of the beginning of magnesium free aCSF perfusion occurred before a meaningful amount of standard aCSF was replaced with magnesium free aCSF and that activity could not be attributed to the seizure initiation protocol.

Overall frequency was the frequency (in Hz) of action potentials during the entire baseline condition and during the zero magnesium wash beginning five minutes after zero-magnesium aCSF perfusion began (to ensure total replacement with zero magnesium aCSF) until either the recording was terminated, or the membrane potential was irreversibly depolarized above the action potential threshold without action potential firing.

Intra-burst frequency was measured as the average instantaneous frequency of action potentials within a burst; bursts were defined as successive action potentials firing
at frequencies greater than 1 Hz. The end of a burst was defined once an inter-spike interval reached or exceeded one second. The burst frequency for a neuron reported was the mean bursting frequency of all bursts evoked by the magnesium free wash. 

*Seizure protocol for synaptic current recording:* The recording paradigm for the voltage clamp recording was identical to that used in the membrane potential recordings (above) with the difference that recordings were performed in voltage clamp mode with a holding potential of -60 mV to distinguish excitatory postsynaptic currents (EPSCs) from inhibitory postsynaptic currents (IPSCs). Raw current recordings were manually adjusted in pClamp 10 to straighten the baseline so that events could be detected by a linear threshold.

EPSC detection was performed in Easy Electrophysiology using a template search. A template was generated from a representative EPSC from recorded data. This event was used to search current recordings for EPSCs that matched the template with a correlation cutoff of 0.70 and exceeded -20 pA in amplitude; the template was then refined to individual recordings by aligning the template to the rise-time kinetics of the average detected event to optimize detection for each recording. The amplitude, half-width, and frequency of EPSCs was recorded. EPSC frequency was defined as the average instantaneous frequency based on inter-event intervals. IPSCs were not analyzed as they were not present or detectable in many recordings, whether due to lack of GABAergic synapses or due to currents being small due to a weak electrochemical gradient for chloride in immature neurons.

The baseline values were measured across the entire five minute baseline recording period. Values for the magnesium free wash were measured for the portion of the recording beginning five minutes after the magnesium free aCSF perfusion started. This delay allowed for total replacement on the aCSF bath solution with magnesium free solution.
**RNA isolation and RT-qPCR:** RNA was isolated from mice aged 0, 5, 10, and 15 postnatal days using ZymoResearch Quick-RNA™ miniprep kits with DNase treatment and quantified by nanodrop. RNA was then frozen at -70 °C until reverse transcription. 1 μg of isolated RNA was reverse transcribed to cDNA with 1 μL BioRad iScript™ reverse transcriptase, 4 μL iScript reaction mix (Biorad, Hercules, Ca), and RNase/DNase free water to a 20 μL total reaction volume. Reverse transcription reactions were performed on a BioRad T100 Thermal Cycler with a protocol consisting of a five minute step at 25 °C, 20 minutes at 46 °C, one minute at 95 °C, followed by an infinite hold at 4 °C. cDNA was then diluted five-fold in TE buffer and frozen at -20 °C until qPCR was performed. Each qPCR reaction consisted of 10 μL PowerTrack™ SYBR green master mix (ThermoFisher, Waltham, MA), 1 μL of combined forward and reverse primer stock to a final concentration of 500 nM primer, 5 ng cDNA (2 μL of cDNA stock), 0.5 μg of PowerTrack™ yellow sample buffer, and 6.5 μL of RNase/DNase free water. qPCR was run on a BioRad CFX Connect Real-Time PCR detection system and data was acquired and analyzed with BioRad CFX Maestro software. Amplification conditions consisted of three minute hot-start step at 95 °C followed by 60 cycles of a 15 second melt step at 95 °C, a 30 second annealing step at 55 °C, and a 30 second plate read step at 72 °C. Following amplification, a melt curve from 65 °C to 95 °C was measured to ensure each reaction generated a single amplicon. Each sample was run in triplicate for each target at the reported CT value is the mean CT value of the triplicate measures. If the CT values triplicates varied by >0.5 cycles, that sample was rerun.

**Primer sequences:** all ordered from Integrated DNA Technologies (Coralville, IA):

**Total Kcnma:** IDT PrimeTime® qPCR Primer assay Mm.PT.58.9795337

**F:** 5'CGAAAGTGTCCATATTGCT3'  
**R:** 5'CCTTGTCCTGAAGCGAAGTATC3'  

**STREX:** Intron spanning primer from McDonald e. al. 2006[155]
F: $^{5}$TTTGATTGCGGACGTTCTGA$^{3'}$
R: $^{5}$TCTCTCAAGGGTGTCACGTTAC$^{3'}$

ZERO: Intron spanning primer from McDonald e. al. 2006[155]

F: $^{5}$GCCAAAGAAGTTAAAAAGGCATT$^{3'}$
R: $^{5}$CGGCTGCTCATCTTCAAGC$^{3'}$

Kcnmb4: IDT PrimeTime® qPCR Primer assay Mm.PT.58.9107809

F: $^{5}$GGCAGCAGTATTGGAAGATG$^{3'}$
R: $^{5}$GAGCAATCTCGTGTGTGT$^{3'}$

Actb: IDT PrimeTime® qPCR Primer assay Mm.PT.39a.22214843.g

F: $^{5}$GATTACTGCTCTGGCTCTAG$^{3'}$
R: $^{5}$GACTCATCGTACTCCTGCTTG$^{3'}$

Solutions (All values are in mM):

- **Pipette internal solution:** 140 K-gluconate, 0.5 CaCl$_2$, 2 MgCl$_2$, 1 EGTA, 2 ATP-Na$_2$, 0.2 GTP-Na$_2$, and 10 HEPES, pH 7.2–7.4 (adjusted with KOH)
- **aCSF:** 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 10 glucose, 0.4 L-ascorbic acid, 1.3 MgCl$_2$, 2 CaCl$_2$; pH was 7.4 when at 30-33 °C and bubbled with 95% O$_2$/5% CO$_2$
- **Sucrose based aCSF:** 230 sucrose, 1.9 KCl, 1.2 Na$_2$HPO$_4$, 33 NaHCO$_3$, 10 glucose, 0.4 L-ascorbic acid, 6 MgCl$_2$, 0.5 CaCl$_2$; pH was 7.4 when at 30-33 °C and bubbled with 95% O$_2$/5% CO$_2$
- **0 Mg$_2$, aCSF:** 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 10 glucose, 0.4 L-ascorbic acid, 2 CaCl$_2$; pH was 7.4 when at 30-33 °C and bubbled with 95% O$_2$/5% CO$_2$

3.3 Passive Properties of Putative Pyramidal Neurons in Acute Hippocampal Slices

Neuronal physiology changes drastically during postnatal development. Some of these changes are a product of changes in the passive electrical properties of neurons.
Membrane resistance was calculated using a series hyperpolarizing current injections (Fig 3.2A, B) of -100, -80, -60, -40, -20, and 0 pA (depolarizing current injections of 20, 40, 60, and 80 were also included in the protocol to confirm the neuronal identity of the patched cell). Membrane resistance decreases with age (Fig 3.2D) from 442.57 ± 20.11 MΩ at P4-P5 (53 cells, 26 animals) to 291.59 ± 11.11 MΩ at P9-P10 (45 cells, 19 animals) and to 225.19 ± 12.84 MΩ at P14-P15 (37 cells, 19 animals; one-way ANOVA P < 0.001, Holm-Sidak multiple comparisons p< 0.001 for P4-P5 vs. P14-P15, p < 0.001 for P4-P5 vs P9-P10, p = 0.007 for P9-P10 vs P14-P15). Membrane resistance is determined by the presence of open channels conducting charge at rest, or leak channels (resistance is the inverse of conductance so more open channels results in lower membrane resistance)[222]. The observation that resistance decreases with age agrees with broadly reported observations that ion channel expression increases during postnatal development [11], [12], [156], [200]. The age dependent change in resistance can also be observed in the trace above where the P4 neuron is far more hyperpolarized in response to a -100 pA current injection than the P14 neuron 3.2A.

The capacitance of a neuron, which can serve as a rough proxy for cell size as it is a product of the specific capacity of biological lipid bilayers (although it can vary independently of size between different classes of cells and can be affected by factors aside from membrane surface area [223][224], [225]) was determined with the on-board membrane test function in pClamp 10. Capacitance increases with age (Fig 3.2E). The capacitance of putative hippocampal pyramidal neurons is 38.83 ± 1.54 pF in neurons from animals at postnatal age four to five days (P4-P5; 53 cells, 25 animals), 47.27 ± 1.67 pF in P9-P10 neurons (40 cells from 19 animals), and 51.68 ± 3.30 pF in P14-P15 neurons (30 cells from 17 animals). Capacitance in P9-P10 and P14-P15 is significantly greater than in P4-P5 (One-way ANOVA, p < 0.001, Holm-Sidak multiple comparisons, p
A neuron’s membrane resistance and capacitance both affect the speed at which a neuron’s membrane potential responds to currents, which is defined by the neuron’s time constant ($\tau$). The theoretical time constant is calculated as the product of resistance
and capacitance $[\tau(s) = R_m \text{ (Ohms)} \times C_m \text{(Farads)}]$ [226]. The experimental time constant of putative hippocampal pyramidal neurons is significantly faster at each successive postnatal age measured; it is $16.41 \pm 0.71$ ms at P4-P5, to $13.36 \pm 0.59$ ms from P9-P10, and to $11.16 \pm 0.62$ ms at P14-P15 (one-way ANOVA, $p < 0.001$; Holm-Sidak pairwise comparisons, $p < 0.05$). While the moderate increases in capacitance would predict a moderately longer time constant, large drops in resistance ultimately lead to a shortened time constant with age. This does not preclude the age-dependent change in capacitance having significant effects on synaptic integration as resistance and capacitance affect electrical events in a neuron (as opposed to the idealized cylinders of cable theory) in frequency dependent manners and the attenuation of fast electrical events in the dendrite (i.e. post synaptic currents) is relatively resistant to changes in membrane resistance [223].

Resting membrane potential was measured as the mean voltage at the beginning of the protocol prior to the first current injection. Initial resting membrane potential was not significantly different across all three ages (Fig. 3.2G); $-66.26 \pm 0.86$ mV at P4-P5,
-65.29 ± 0.84 mV at P9-P10, and -65.04 ± 0.91 mV at P14-P15

3.4 Effects of Postnatal Age and BK Channel Blockade on the Action Potential Waveform

As a voltage and calcium activated channel, the BK channel is activated during action potential firing and modifies the action potential waveform. BK channels have been shown to modify repolarization rates, increasing the duration of the action potential or altering the afterhyperpolarization; one report found that BK channel blockade altered action potential threshold, but the basis for this is unclear as neuronal BK channels are not thought to be activated at the low depolarizations of action potential initiation [187]. To examine the effect of developmental time and BK channels on action potentials in mouse hippocampal pyramidal neurons I evoked action potentials using one second depolarization in increments of one pA to determine a precise rheobase current. Rheobase in the minimum stimulus intensity required to reach threshold given an indefinite depolarization (in practice >300 ms). The action potential waveform changes considerably with postnatal time. The duration of the action potential, measured as the width of the spike at half the amplitude (threshold-to-peak) decreases from 2.73 ± 0.24 ms at P4-P5(14 cells, 7 animals) to 1.44 ± 0.08 ms at P9-P10 (10 cells, 5 animals), and to 1.08 ± 0.06 ms at P14-P15 (12 cells, 6 animals; two-way RM ANOVA, p < 0.001 for the main effect of age, action potentials in P9-P10 and P14-P15 hippocampal neurons are significantly shorter in duration than in P4-P5 neurons according to Bonferroni multiple comparisons, p < 0.001, Fig. 3.3A, B). The action potential threshold, measured as the point at which the first derivative of the rising phase of the action potential reaches 20 mV/ms, decreases with age (Fig 3.3C). Action potential threshold hyperpolarizes from -35.75 ± 1.48 mV in P4-P5 neurons (14 cells, 7 animals) to -45.78 ± 1.58 mV in P9-P10 neurons, and to -49.77 ± 1.14 mV in P14-P15 neurons (12 neurons,
6 animals; two-way RM ANOVA, p < 0.001 for the main effect of age; action potential thresholds in P9-P10 and P14-P15 hippocampal pyramidal neurons are more hyperpolarized than P4-P5 according to Bonferroni multiple comparisons, p < 0.001).

When measuring action potential afterhyperpolarization (AHP) as the difference between the threshold and the lowest voltage within 100 ms after the threshold the magnitude of the AHP decreases with age (Fig 3.3D). This effect is driven by age dependent differences in action potential threshold and so I adjusted the AHP value for each cell by the difference between the mean threshold for that cell’s age group and the mean threshold of the P14-15 age group; in other words, the raw AHP value was adjusted by 14.02 mV for the P4-P5 age group, by 3.99 for the P9-P10 age group, and the P14-15 age group was unadjusted. When the AHP values were normalized to account for
Changes in threshold, there was an age dependent increase in AHP magnitude (Fig 3.3E). Adjusted AHP magnitude was -3.18 ± 1.04 mV in P4-P5 neurons (14 cells, 7 animals), -7.63 mV ± 1.12 mV in P9-P10 neurons (10 cells, 5 animals), and -7.98 ± 0.80 mV in P14-P15 neurons (12 cells, 6 animals; two-way RM ANOVA, p < 0.01 for the main effect of age; P4-P5 is significantly different than P9-P10 and P14-P15 according to Bonferroni multiple comparisons p < 0.05 and p < 0.01, respectively).

After recording action potentials in normal aCSF I perfused the neurons with aCSF containing the BK channel blocker paxilline at a 1 μM working concentration. Paxilline is a selective BK channel inhibitor with a high affinity for BK channels in the closed state (IC50 10 nM) but is a less effective antagonist against BK channels in the open conformation (IC50 10 μM; [227]). While the working concentration of paxilline is too low to block BK channels in all conformations but neuronal BK channels have very low open probability at resting membrane potentials (approximately -60 to -70 mV for the cell population studied) so BK channels in these experiments would be nearly universally in the closed conformation at the time of paxilline application.
BK channel blockade selectively increased action potential duration in the P4-P5 putative pyramidal neurons (Fig 3.3B). Action potential half-width increased from \(2.73 \pm 0.24\) ms to \(3.11 \pm 0.23\) ms (two-way RM ANOVA \(p < 0.001\) for the main effect of paxilline and \(< 0.05\) for the interaction of age and paxilline. There is a significant pairwise difference between the P4-P5 control and paxilline condition according to Bonferroni multiple comparisons \(p < 0.001\). There was no significant effect of BK channel blockade on action potential duration in P9-P10 and P14-P15 neurons. BK channel blockade did not significantly affect the action potential threshold or afterhyperpolarization (Fig 3.3 C-E).

3.5 Effects of Age and BK Channel Blockade on Repetitive Firing

A widely studied role of BK channels in neurons is regulating repetitive firing. However, depending on the cellular context BK channels have been reported to alternately decrease or increase neuronal firing rates[132]–[135]. In chapter two of this dissertation, I report that BK channel blockade has an age-dependent effect on repetitive firing in cultured rat hippocampal neurons with a small significant effect decrease in firing frequency at P1 and a significantly larger effect at P7. To examining how repetitive firing was affected by BK channel blockade in neonatal mouse hippocampal pyramidal neurons, I evoked action potential trains with a series of increasing one second current injections from 20 to 200 pA at 20 pA intervals (Fig 3.4A bottom) before and after BK channel blockade with 1 \(\mu\)M paxilline.

I monitored membrane resistance throughout the recording and found that while resistance did change with age as it did in the larger dataset (Fig 3.2), it did not significantly vary between the control recording and the recording during paxilline treatment (two-way repeated measure ANOVA, \(p < 0.001\) for the main effect of age, \(p > 0.05\) for the main effect of paxilline; Bonferroni multiple comparisons \(p < 0.01\) for the
comparisons of P4-P5 vs P9-P10 and P14-P15, Fig 3.4B). BK channels represent a small percentage of the overall ion channel population and have a negligible open probability at the hyperpolarized voltages used to determine membrane resistance so it unlikely that BK channel blockade would result in measurable change in input resistance. Because membrane resistance did not change over the course on the experiment, any differences observed between the control and treatment recordings were not attributable to the same depolarizing current producing a different depolarization at different times in the experiment.

While there was a high degree of heterogeneity, there was a clear trend of increased capacity for high frequency firing with neuronal maturation. More mature
neurons fired more action potentials than the P4-P5 group with current injections of 100 pA or greater (two-way RM ANOVA p < 0.01 for the main effect of age and p < 0.001 for the interaction of age and current injection; Bonferroni’s multiple comparisons, p < 0.05 Fig 3.4D). At larger current injections (typically >100 pA), P4-P5 neurons displayed some depolarizing block that appears as a sustained depolarization in response to the current without continued action potential firing. For illustration, the beginning of depolarizing block can be seen in Fig. 3.4A; in the sample paxilline-treated trace the final action potentials do not completely repolarize and the membrane remains depolarized without the initiation of another action potential. As voltage gated ion channel expression increases with age the neuron can repolarize in the face of stronger stimuli and most P9-P10 and P14-P15 neurons do not enter depolarizing block during the stimulus protocol used. When BK channels were blocked with paxilline and the current injection protocol was repeated, I observed no significant effect of the channel blockade on the number of action potentials evoked at any level of current injection Fig 3.4C.

To test how BK channel blockade affects progressive firing rates within a train of action potentials, I analyzed the action potential output for one current injection for each cell, selecting a current injection that produced the highest firing rates a neuron achieved while exhibiting minimal depolarizing block. This range was highly variable, some neurons, especially from younger animals entered depolarizing block with within the first two current injections, while some (even some P4-P5 neurons) did not enter depolarizing block in response to any current injections up to 200 pA. In P4-P5 neurons this trace was not typically produced by the largest injection as these neurons entered depolarizing block at the upper ranges of the protocol but around the 100 pA step (Fig 3.4D); some P4-P5 neurons achieved a maximal response at even lower in injections and some display increases in action potential outputs throughout the stimulus protocol. In the
neurons from older animals the trace used for frequency analysis was typically the one evoked by the largest current injection. I compared the instantaneous frequency of each successive pair of action potentials (spike pair) for the first 20 action potentials in a train to see in BK channel blockade altered firing frequencies of patterns of spike-frequency accommodation (Fig 3.4E). When comparing the difference in instantaneous frequency caused by BK channel blockade (a subtraction of the black and gray lines in Fig 3.3E) the P14-P15 group is significantly different than both the P4-5 and P9-10 group with an apparent modest elevation of instantaneous frequency at each spike pair for the P14-P15 trace (two-way ANOVA, p < 0.001 for the main effect of age; Bonferroni multiple comparisons p < 0.001). No significant effects were observed on the difference of instantaneous firing frequencies with paxilline at each age (two-way RM ANOVAs on the levels of drug treatment and spike pair; Fig. 3.4F). There appears to be an elevation in instantaneous frequency with paxilline at each spike pair in P14-P15 neurons; While this is not significant (2-way RM ANOVA, p=0.08 for the main effect of treatment), it is likely the effect that is driving the age dependent significant difference seen in Fig 3.4F.

The effects of age and BK channel blockade on spike-frequency accommodation was also measured by calculating spike frequency accommodation across an entire recording. Spike-frequency accommodation index was measured as the ratio of the first inter-spike interval to the last inter-spike interval; a smaller number indicates a greater degree of spike-frequency accommodation. I compared spike-frequency accommodation across all ages before and after BK channel blockade and found that the degree of spike frequency accommodation increased with age with the P14-P15 group having significantly more spike frequency accommodation (two-way repeated measure ANOVA, p < 0.01 for the main effect of age, Bonferroni multiple comparisons p > 0.01; Fig 3.4G). BK channel blockade increased the degree of spike frequency adaptation in the P4-P5 group but did not alter spike frequency accommodation by this measure in the P9-P10 or
P14-P15 age group (Bonferroni multiple comparisons, p > 0.01).

The distribution of spike-frequency accommodation was not normal (Shapiro-Wilk normality test p < 0.05). To confirm the significant effect, I analyzed the difference in spike frequency accommodation between the control and the paxilline treated recording using a one-way ANOVA on ranks and confirmed that BK channel blockade significantly and selectively alters spike-frequency adaptation index in the P4-P5 group (p < 0.05, Dunn’s multiple comparisons p < 0.05).

3.6 Expression Patterns of BK Channel mRNA Across Early Development

To understand what underlies the difference in physiological effects of BK channel blockade – the selective effect on action potential duration in the more immature neurons and the different effect on firing rates between more and less mature neurons, I extracted RNA from the hippocampi of three male mice aged P0, P5, P10, and P15 and measured the total level of BK channel mRNA transcripts (Kcnma1), two developmentally regulated BK channel isoforms (STREX and ZERO), and the neuronal BK channel β-4 subunit (Kcnmb4) by RT-qPCR. I analyzed expression of these targets at timepoints corresponding to the ages for which I have electrophysiology data, P5, P10, and P15 as well as newborn P0 pups. Transcript expression of for β-actin (Actb) was used as the loading control in these experiments. The organization and localization of cytoskeletal actin is highly dynamic as neurons grow and develop [228] and so I compared the expression of Actb in my samples across developmental time to ensure that it was a valid loading control. The CT value, or number of amplification cycles necessary to produce a fluorescent signal detectable above background, did not change between P0-P15, which validated Actb as a loading control for age-dependent
Figure 3.5: BK channel mRNA expression patterns in postnatal development

A
Mean + SEM fold change value for Kcnma1 transcript expression. One-way ANOVA, p < 0.001; Holm-Sidak multiple comparisons, p < 0.01. *Significantly greater than P0; **Significantly greater than P5.

B
Mean + SEM fold change for STREX splice variant transcript expression.

C
Mean + SEM fold change for ZERO splice variant transcript expression. One-way ANOVA, p < 0.05. #Significant difference of means across age groups.

D
Mean + SEM normalized expression of the STREX and ZERO variants as a percentage of the normalized expression of total Kcnam1 transcripts. Two-way ANOVA p < 0.001 for the main effect of variant; p < 0.01 for pairwise comparisons at each age.

E
Mean + SEM fold change value for Kcnmb4 transcript expression. One-way ANOVA, p < 0.05; Holm-Sidak multiple comparisons, p < 0.05. *Significantly greater than P0; **Significantly greater than P5.

F
Mean + SEM CT values for Actb transcript expression.
experiments (Fig 3.5F). Expression of all targets is expressed as the change in the amplification threshold relative to expression of Actb normalized to P0 ($2^{-\Delta CT}$; see methods for full calculation).

Kcnma1 mRNA expression increases over three-fold from P0 to P15 with significantly higher expression at P10 than P0 and higher expression at P15 than P0 and P5 (one-way ANOVA, $p < 0.001$; Holm-Sidak multiple comparisons $p < 0.01$, Fig 3.5A). When I analyzed the expression levels of the STREX and ZERO isoforms, I found that he expression of STREX is relatively stable across the first two postnatal weeks (Fig 3.5B) while the expression of ZERO – a splice variant lacking the STREX exon – significantly increases during this time (one-way ANOVA, $p < 0.05$ Fig 3.5C).

I used Biorad CFX-Maestro™ to estimate the percentage of each variant as a percentage of the notal BK mRNA expression. I found that the STREX variant accounts for $15 \pm 3.16\%$, $11.76 \pm 3.54\%$, $6.27 \pm 1.24\%$, and $5.67 \pm 1.72\%$ of total Kcnma1 transcripts at P0, P5, P10, and P15 respectively. Meanwhile the ZERO variant accounts for $52.58 \pm 1.94\%$, $53.64 \pm 1.25\%$, $78.13 \pm 24.18\%$, and $53.45 \pm 3.79\%$ of total Kcnma1 transcripts at P0, P5, P10, and P15 respectively. Relative expression of individual isoforms does not significantly change across ages, but at all timepoints STREX accounts for a smaller proportion of Kcnma1 transcripts than the ZERO isoform (two-way ANOVA, $p < 0.001$ for the main effect of age; Holm-Sidak comparisons $p < 0.01$ for all comparisons of STREX% vs. ZERO %). The inclusion of the P0 timepoint demonstrates that the predominance of the STREX variant at early developmental time observed by MacDonald et al. (2006) must be reversed between E18 and E20 as ZERO is the predominant variant at all postnatal timepoints ([155], Fig 3.5D).

Another source of functional diversity in BK channels is whether or not channels are coupled to β subunits [5]. Expression of the β-4 subunit transcripts, Kcnmb4, which
is the predominant neuronal subunit was measured. Other β subunits are present and functional in some neuronal populations but in general are detectable at very low levels in neuronal tissue [113], [229], [230]. Expression of Kcnmb4 transcripts increase with age; P15 hippocampi had higher expression than P0 and P5 (one-way ANOVA, p < 0.05; Holm-Sidak multiple comparisons, p < 0.05, Fig 3.4E).

3.7 Induced Seizure Activity in a Hippocampal Slice Model

To examine how developmental time and BK channels affect seizure activity I induced seizure-like activity in acute brain slices while recording from pyramidal-layer neurons in whole cell configuration. Seizure activity was induced by replacing the standard aCSF recording solution with magnesium-free aCSF; this method induces a hyperexcitable state by removing the magnesium block on NMDA receptors thus increasing glutamatergic transmission[221]. This recording method of monitoring seizure-activity with single-cell recording obscures population-level activity of neurons and so it may fail to detect seizure activity if an individual cell is not active during a seizure-like event. My lab has previously used field recording to measure zero magnesium evoked seizure-like-activity in a slice model, but this technique needs further validation in our hands. It also cannot necessarily be assumed that hyperexcitability in single neuron is part of a large, synchronous population event that models a seizure.

<table>
<thead>
<tr>
<th>Table 3.1 Sample sizes for seizure model recordings</th>
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<tr>
<td></td>
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<tr>
<td>All Cells</td>
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<tr>
<td>Onset Time</td>
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<tr>
<td>Burst Frequency</td>
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<td>EPSC properties</td>
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N,n; N = number of cells, n = number of animals
Despite this weakness it offers the benefit of allowing for direct measurements of how different experimental conditions affect action-potential firing and synaptic transmission during a seizure-like event.

For voltage recordings, I patched cells in whole-cell configuration with a holding potential of -80 mV and recorded the capacitance using the membrane test function of pClamp 10. I then switched to current clamp mode and measured resistance using a series of hyperpolarizing pulses (see section 3.3). After that I recorded membrane potentials in standard aCSF for 5 minutes to assess baseline conditions and ensure a stable seal. After the baseline conditions were measured, I began perfusion with magnesium free aCSF to induce seizure-like activity (Fig 3.6A). For experimental neurons, 1 μM paxilline was included in the magnesium free aCSF to assess whether BK channel blockade prevented the incidence or modulated the intensity of seizure-like activity.

I tested whether the proportion of putative pyramidal neurons in which the magnesium-free wash evoked action potential firing varied across age or with BK channel blockade with paxilline. Proportions and relative cell counts for cells with action potential firing induced by the magnesium free aCSF can be found in Table 3.2. I analyzed whether either paxilline or age altered the proportion of cells that fired action potentials during magnesium free conditions by building a binary logistic regression model. I found that neither age (p=0.358), nor BK channel blockade (p=0.211) contributed to the statistical model so that in this model of seizure activity there is no

<table>
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<th>Table 3.2</th>
<th>Proportion of cells active during magnesium free wash</th>
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<tr>
<td></td>
<td>P4-P5</td>
</tr>
<tr>
<td>Control</td>
<td>0.62 (8/13)</td>
</tr>
<tr>
<td>Paxilline</td>
<td>0.77 (10/13)</td>
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change in susceptibility with age and BK channel blockade does not reduce the incidence of seizure-like activity in an acute slice model.

The zero magnesium model did increase action potential firing frequency in individual neurons treated in hippocampal slices. When the overall firing frequency before and during magnesium-free conditions was analyzed in a pairwise fashion, there was a main effect of zero magnesium aCSF on frequency (two-way repeated measure ANOVA, p < 0.05 for the main effect of zero magnesium; Bonferroni multiple comparisons, p < 0.05 for the pairwise comparisons of baseline and zero magnesium and for the comparison of baseline and zero magnesium aCSF within the P14-P15 age group Fig 3.6B). I also compared the mean action potential frequency in all conditions, in this case the data from control and paxilline treated cells for the baseline aCSF condition was pooled as there was no difference in the baseline condition for the two treatments. There was a significant effect of the seizure protocol on action potential firing. Magnesium free aCSF, but not the magnesium free aCSF with paxilline, induced significantly higher action potential frequencies than in the standard aCSF (two-way ANOVA, p < 0.001 for the main effect of aCSF condition; Holm-Sidak multiple comparisons, p < 0.01 Fig 3.6C). However, when the overall action potential frequency before and after applying zero magnesium aCSF by treatment was compared by age and paxilline treatment there was no significant effect of either level (two-way ANOVA, p > 0.05 for all levels Fig 3.6B). While zero magnesium, but not the zero magnesium with paxilline, had a significant effect over the standard aCSF there was no significant difference between the two zero magnesium conditions. One factor that may have contributed to the lack of statistical significance between some treatments (particularly the aCSF and the zero magnesium aCSF with paxilline) is the high variance in the data. While the zero magnesium conditions consistently increased excitability, the degree to which they increased excitability varied across orders of magnitude.
I then analyzed whether BK channels blockade with paxilline was able to modulate the onset and action potential firing burst characteristics of seizure-like activity induced by magnesium free conditions. BK channel blockade did not alter the onset time to zero magnesium the first action potentials fired in the zero magnesium (two-way ANOVA on the levels of age and paxilline treatment, p = 0.446 for the main effect of age; 0.808 for the main effect of treatment; 0.458 for the interaction of age and treatment Fig 3.6D). It also had no effect on the frequency of action potentials within bursts evoked by zero magnesium aCSF (two-way ANOVA on the levels of age and paxilline treatment, p = 0.36 for the main effect of age; 0.67 for the main effect of treatment; 0.62 for the interaction of the two levels Fig 3.6E).

One of the key advantages of measuring seizures by single cell recordings as opposed to recording population level activity is that synaptic transmission can be directly measured. To assess the effects of age and BK channel blockade on synaptic transmission during a seizure induction protocol, I used the same protocol as used for current clamp recordings of seizure-like activity, but after breaking in and measuring...
capacitance I continued to record in voltage clamp, adjusting the holding potential to -60 mV so that EPSCs (excitatory post-synaptic currents) and IPSCs (inhibitory postsynaptic currents) could be distinguished from one another. A sample recording can be seen in Fig 3.7A. Although recordings were performed to enable analysis of both EPSCs and IPSCs only EPSCs were analyzed. IPSCs were not detectable in a large portion of the
recordings, potentially due to the delayed integration of inhibitory interneurons into the hippocampal microcircuits which has been shown to occur in the second postnatal week across multiple regions of the rodent brain [231], [232].

As with the voltage recording, I did not find any significant effect of age or BK channel blockade on excitatory transmission induced by zero magnesium. Rises in mean EPSC frequency (frequency based on the average inter-event interval) did not significantly vary with age or paxilline treatment when comparing firing before and after seizure induction for each cell (two-way repeated measure ANOVA, p = 0.08 for the main effect of age; p = 0.86 for the main effect of treatment; p = 0.19 for the interaction of the two levels; Fig 3.7C). The zero magnesium did increase mean EPSC frequencies
relative to the baseline recordings regardless of whether the zero magnesium solution contained paxilline or not with the rise in frequency being particularly pronounced in the youngest age group (two-way ANOVA, p < 0.001 for the main effect of solution; Holm-Sidak multiple comparisons p < 0.01 for the effect of both zero magnesium conditions in the P4-P5 group Fig 3.7D) There was no effect of age or BK channel blockade on the EPSC amplitude (two-way repeated measure ANOVA, p = 0.81 for the main effect of age; p = 0.30 for the main effect of treatment; Fig 3.7E-F). EPSC duration, as measured as the width of the current waveform at half the amplitude, was not significantly affected by paxilline or seizure induction at any age (two-way repeated measure ANOVA, p = 0.81 for the main effect of age; 0.30 for the main effect of treatment; p= 0.75 for the interaction of the two levels Fig 3.7G). EPSC duration did show an age dependent increase (two-way ANOVA, p < 0.001 for the main effect of age; Holm-Sidak multiple comparisons p < 0.05 for both comparison of P14-P15 versus the other age groups (Fig 3.7H). Representative excitatory postsynaptic currents are shown in Fig. 3.7B.

A possible phenomenon underlying the lack of effect observed on EPSCs is that seizure induction had a variable effect on EPSC waveform within individual cells. In some cells, zero magnesium resulted in more frequent, larger unitary EPSCs than were observed in the control trace. In other cells, EPSCs were more frequent but smaller in amplitude, with the average EPSC amplitude hovering around the detection threshold. This latter case could represent a desynchronization of synaptic inputs onto pyramidal cells.

3.8 Conclusions

Postnatal development is associated with changes in the passive and active properties of neurons consistent with a general increase in ion channel expression. Decreases in membrane resistance, a faster action potential waveform, and increases in
neuronal gain—all of which support the functional needs of mature neurons [11], [12], [197]–[200]. In chapter two I demonstrated that these developmental changes occur in cultured rat hippocampal neurons from P1-P7. In this chapter, I demonstrate that maturation of the neuronal electrical properties brings changes to neuronal action potential firing. Between P4-P5 and P14-P15 the threshold potential for action potential initiation becomes more hyperpolarized, the waveform of the action potential duration significantly shortens, and the magnitude of afterhyperpolarization increases.

With age, neurons also gain an increased capacity for repetitive firing between P4-P5 and later ages examined. Putative pyramidal neurons at all ages had similar action potential outputs across the lower range of depolarizing current injections (20-80 pA) but at higher current injections the P4-P5 neurons outputs plateaued while the P9-P10 and P14-P15 groups showed increased firing. Action potential outputs were not significantly different between the P9-P10 and P14-P15 group at any current injection. This does not preclude the possibility that P14-P15 neurons have higher capacity for action potential firing than P9-P10 neurons as the P14-P15 neurons were less depolarized than the P9-P10 group due to their lower membrane resistance. I also did not measure maximal action potential outputs in all cells as many cells, especially in the older age groups, continued to display increased firing in response to all current steps and would likely show continued increase with depolarizing current injections greater than 200 pA.

Additionally, I demonstrate that membrane capacitance increases with age. This observed difference is most likely due to differences in the tissue preparation method. Membrane capacitance is a product of the phospholipid bilayer and capacitance can be used as a proxy measurement for cell surface area. Cultured cells used in chapter two are triturated which shears axons and dendrites of neurons and recordings were performed before neuronal processes regrew in vitro— in short recordings from cultured
neurons in chapter two were essential recordings from isolated somata. In contrast, neurons in acute slices are relatively intact and retain cell processes such as the extensive dendritic arbors of hippocampal pyramidal cells. The increase in capacitance is likely a reflection of growth and increased complexity of the dendritic compartment [196] and not somatic size. This may also represent a difference between rats and mice or be a product of the different developmental interval studied (P4-P15 vs. P1-P7).

I also demonstrated an age dependent decrease in the contribution of BK channels to the action potential waveform in mouse hippocampal neurons. BK channel blockade modestly delays the repolarization of P4-P5 neurons but has no effect on repolarization of action potentials in older age groups. This agrees with my data in chapter 2 using isolated and cultured rat hippocampal neurons and a previous report that action potential repolarization is unaffected by BK channel blockade in neurons from P13-P16 mice [176].

There was, however, very little detected effect of BK channel blockade on action potential firing rates when comparing action potential counts in response to current injection or comparing instantaneous frequencies across successive spike firing. Both modeling and previous experimental data support the role of BK channels in modulating action potential firing rates although experimental data has shown that under different conditions BK channel blockade can either increase or decrease neuronal firing [132]–[135], [156]. I did observe, most notably in the P14-P15 age group that BK channel blockade markedly increased action potentials output in some cells and decreased it in others, which prevented a statistical determination of an effect of BK channel blockade.

In spite of BK channel blockade having no discernable effect on firing frequency, there was an age dependent of BK channel blockade on spike frequency accommodation, the continued decreases of frequency with sustained action potential firing. By one measure of spike frequency accommodation which compares the first and
last inter-spike interval in an action potential train, BK channel blockade in P4-P5 neurons did introduce a greater degree of spike-frequency accommodation than in the older age groups, suggesting that BK channels may play a greater role in maintaining action potential frequency in P4-P5 neurons.

The variability in the effect of BK channel blockade may stem from the fact that the cell population studied, putative hippocampal glutamatergic pyramidal neurons, does not represent a molecularly or physiologically unified class of neurons [219], [220], [233]. It is also important to keep in mind that there is an important distinction between neurons derived from animals of the same age and neurons of the same age as neurons are not synchronously generated in development and neurons with different birth dates can have unique physiological properties [234]. This presents an added layer of difficulty to interpreting findings from early developmental periods.

I had originally hypothesized that age dependent change in BK channel activity, namely the loss of type-I BK channel kinetics, was due to changes in the relative expression of α subunit isoforms. This was based on a previous report that between E18 and P7, the predominantly expressed isoform shifted from STREX (which is associated with type-I kinetics) to ZERO (which is associated with type-II kinetics) [155]. If type-I BK channel kinetics early in postnatal development are due to a predominance of the STREX variant then the STREX variant must be detectable as the major isoform, or at least a significant proportion of the total BK channel expression at some point in early postnatal development. In contrast to this, the STREX isoform only represents a small proportion of total BK channel expression in the newborn mouse hippocampus. It is unlikely that type-1 BK channel kinetics in immature neurons results from STREX isoform expression. Instead, the mRNA expression data here suggest that it is the product of low expression on the β-4 subunit, which does significantly increase through the first two weeks postnatally in the mouse hippocampus. Rising expression of the β-4
subunit and increasing inclusion of it in BK channel assemblies will slow the kinetics of BK channels so that they no longer open in response to the depolarization associated with a single action potential.

Unexpectedly low expression of the STREX isoform in the early postnatal period may also be why I could find no evidence to support my hypothesis that BK channel blockade could attenuate seizure activity in the early postnatal period. This hypothesis was informed by evidence that BK channel blockade only attenuated seizures in mice with gain-of-function BK channel mutations (which are analogous to the STREX variant) or in animals with prior seizures which upregulates STREX isoform expression [175], [176], [185]. According to this hypothesis, high levels of STREX expression are not present in the healthy postnatal rodent brain, so BK channel blockade has too small of an effect on the action potential firing and cannot modulate seizure activity.

At this point, I find no evidence for BK channel blockade as a potential therapeutic for seizures in very young individuals – at least in the absence of a gain-of-function mutation. My single-cell recording experiments of seizure-like activity, particularly the recordings of synaptic currents, had small sample sizes and were probably only sufficiently powered to detect large effects. The caveats of a single-cell recording seizure model, mainly that single-cell recordings may obscure or misrepresent what is happening on an organ level. It would be worthwhile to repeat the seizure induction experiments using a techniques that captures a broader picture of neuronal activity during seizure induction such as recordings of field potentials, optical measures that would capture network activity in the entire slice using voltage or calcium sensing dyes, or directly measuring glutamate transmission [235], [236].
Chapter 4: Discussion

4.1 Summary of Noteworthy Findings

This dissertation makes several contributions to a complete understanding of the early postnatal development of hippocampal neurons and the role of BK channels in this period. First, it characterizes the postnatal development of passive and active electrical properties of neurons and draws comparisons between an acute slice model and a primary culture model. It establishes that neurons in short term primary cultures developmentally appropriate electrophysiological properties. Relative to neurons in acutely prepared slices, cultured neurons show similar developmental trajectories in membrane resistance and action potential firing, reflecting maturing patterns of ion channel expression. Whereas dissociated primary cell cultures may not have advantages over slice preparations for studying action potential firing, they have the advantage of better voltage control when studying ionic currents. This dissertation establishes that electrical properties observed in cultured neurons recapitulate the electrical properties of developing neurons and acute culture preparations can be used to study functional changes in ion channels across development.

This dissertation also provides more detailed time-courses of electrophysiological properties and BK channel expression than have been previously published. I show through electrophysiology as well as mRNA and protein expression data that, rather than gradual development during the postnatal period, there is a punctuated rise in neuronal excitability and BK channel expression at postnatal day four to postnatal day five. This time may represent a major critical period in the rodent brain.

I report a shift in the role of BK channels in action potential firing during the first two postnatal weeks. There is a transition from fast, type-I BK channel kinetics where BK channels contribute to action potential repolarization to slower type-II kinetics where BK
channel require more prolonged stimulus than a singular action potential to open. I demonstrate that this shift to slower kinetics is accompanied by rising expression of the β-4 subunit. This shift of the kinetics underlies a transition from BK channels that contribute to action potential repolarization to BK channels that enable repetitive firing and limit spike-frequency-accommodation.

4.2: BK Channels and Development

How excitability is governed in the immature brains is markedly different from the adult brain in ways that are necessary for development but render the brain more susceptible to seizures [5], [237]–[239]. This dissertation has been an exploration of the role of BK channels in the excitability of developing hippocampal neurons. Despite this channel being an important player in central nervous system physiology as well as in many other tissues and organ systems, its role through development has been relatively understudied. Previously it was understood that the expression of the BK channel α subunit increased along with the density of BK channel currents [240]. Meanwhile the dominant isoform of the pore forming α-subunit switches from a more calcium sensitive isoform (STREX) to a less calcium sensitive splice variant (ZERO) between embryonic day 18 and postnatal day seven in the hippocampus which slows the activation time during action potential firing as the ZERO variant requires higher voltages to open [78], [155], [158]. Additionally, the neuron-specific β-4 subunit enhances BK channel calcium responsiveness, shifts the activation voltage to more hyperpolarized potentials and slows activation kinetics [162]. These changes predict an overall increase in BK channel current, but that the kinetics of the current. Therefore the physiological role of BK channels may change. These changes in BK channels do not necessarily occur at the same developmental time point in all neuron types so that investigating this in different cell types is critical to understanding how BK channels contribute to excitability. Slowing
kinetics as BK channel subunit assembly and isoforms change predicts an age-
dependent transition from type-I BK channels, which activate fast enough to contribute to
the repolarization of a single action potential to type II channels which are activated with
continuous firing of action potentials [114]. My work demonstrates the physiological
differences in action potential firing that result from development and parses out what is
due to the developmental patterns of BK channel expression.

4.3 Postnatal Development of Excitability in Rat and Mouse Hippocampal
Neurons

There is a broadly observed pattern during postnatal development that, across
neuronal types, action potential duration decreases and excitability of neurons increases
in the rodent central nervous system. [11], [12], [197], [198], [200]. The work presented
here represents a characterization of these changes in the first postnatal week of
hippocampal development. Studies in chapter two were carried out in isolated rat
neurons, which allows maximal control over the recording environment as well as good
space clamp control for current measurements. For the studies in chapter 3, a switch
was made to in vitro brain slices from mice. The switch to slices allowed for recording
from neurons that are integrated into intact hippocampal circuits. The switch to mice
allows for future comparisons to studies performed with transgenic mice. Domesticated
rats and mice have a strikingly similar timeline of developmental landmarks with similar
gestation times, age at which eyes open, weaning age, and age at which sexual maturity
is reached [241], [242]. For this reason, I believe that there is at least some basis to
compare neurodevelopmental timelines across the two species. However, the two model
systems did not support investigating the same developmental ages. Neuronal survival
and viability were very good in the primary culturing system but dropped off beyond the
first postnatal week; decreases in the viability of cultured are widely observed and most
likely stem from the fact that plasticity and regeneration becomes more constrained as neurons mature [243]. The acute slice model enabled studying neurons from older animals than in the culture model but when I patched neurons from animals younger than P4 a large proportion fired no action potential in responses to stimuli. This may have been because immature neurons have very different energy substrate utilization than mature neurons and the aCSF I used included glucose as the only energy substrate. It is possible that a different formulation of aCSF would have enabled studying neurons in slices from younger animals [244]–[246].

From previous investigations of other neuron populations, there is an inflection in ion channel expression and developmental increases in neuronal gain around postnatal day four [11], [12]. By recording every day across the first postnatal week in cultured hippocampal neurons I confirm that this uptick in excitability and BK channel expression occurs at postnatal day four Fig 2.6, Fig 2.8, Fig 3.3, Fig 3.5.

Cultured rat hippocampal neurons and mouse hippocampal neurons in acutely prepared brain slices displayed the same developmental patterns of passive and active electrical properties. In both models there is an age-dependent decrease in membrane resistance that likely reflects an increasing in the resting leak current [222] Only the slice model displayed an age dependent increase in capacitance, but this is probably explained by developmental increases in capacitance being a product of dendritic growth and the cultured cells had cellular processes sheared off during the culturing process [196].

Action potential duration also decreased in both preparations across developmental time with most of the effect occurring within the first postnatal week – or at least the first 10 days as the cultured neurons showed significant decreases during the first week and the slice system showed significant changes from P4-P5 to P9-P10, but not from P9-P10 and P14-P15. It is not possible to compare the exact values of
action potential half-width between the two systems due to differences in the stimulus paradigm by which action potentials were evoked and the way that action potential properties were calculated (see the chapters two and three methods for full detail. The neurons in the slice model exhibited an increase in the magnitude of afterhyperpolarization whereas the cultured neurons did not; this may have been a result of the different age interval surveyed, differences in the cell populations studied (randomly selected neurons in heterogenous cultures vs putative pyramidal neurons in slices), or simply differences in how afterhyperpolarization was measured in the two studies.

Both isolated rat neurons and neurons in acute mouse brain slices displayed rises in excitability from day one to at least postnatal day 10 with a punctuated rise around P4 observed in the rat neurons. My data do not demonstrate an increase in excitability from P9-P10 to P14-15 in hippocampal neurons from slices, but neither does it exclude that excitability increases. I only measured excitability via neuronal responses to depolarizing current injections of 20-200 pA. P14-P15 neurons have lower membrane resistance than P9-P10 neurons and therefore displayed similar excitability to P9-P10 neurons in response to less depolarizing voltage. If an actual maximum firing rate was determined for all neurons, it is likely that P14-P15 neurons would have displayed a higher maximum firing frequencies than P9-P10 neurons.

The increase in excitability, measured as the capacity to fire action potentials more frequently, was accompanied by an increased resistance to depolarizing block. Neurons from younger animals were more likely to enter depolarizing block, a failure to fire action potentials during prolonged stimulation, during stimulation paradigms relative to more mature neurons. This is likely due to overall increases expression of K_, channels as well as increased BK channels expression, as repolarization and fast afterhyperpolarizations by these channels ensure the removal of sodium channel
inactivation allowing the next action potential to be generated [131], [132]

4.4 Postnatal Trends in Contribution of BK Channels to Spike Waveform and Repetitive Action Potential Firing in Rat and Mouse Hippocampal Neurons

In both cultured rat hippocampal neurons and mouse hippocampal neurons in acute slices there was a loss of BK channel contribution to single-evoked action potentials with development. BK channel blockade significantly lengthened action potential duration in cultured neurons from P1-P7 rats but the magnitude of the effect declined with age. In the mouse acute slice model, I measured a significant effect of BK channel blockade on action potential duration at P4-P5, but not at P9-10 and P14-15. These data suggest that the proportion of type-I BK channels declines steadily from P1-P7 and are completely replaced by type-II BK channels by P9-P10 as the type-II channels would activate too slowly to affect the kinetics of a single action potential.

The use of two different toxins for BK channel blockade was also illuminating. Iberiotoxin and paxilline block BK channels by distinct mechanisms; iberiotoxin blocks BK channels by occluding the pore from the extracellular surface while paxilline works by occluding the central cavity [247], [248]. Because of this difference in blockade mechanism, BK channel assemblies that include the β-4 subunit are resistant to blockade by iberiotoxin but not paxilline, likely because the β-4 subunit occludes the extracellular face of the α-subunit where iberiotoxin binds [115], [249], [250] (see fig 1.3 for illustration). One possible interpretation of the loss of effect of BK channel blockade with iberiotoxin on the action potential waveform in rat neurons with development is that BK channels continue to contribute to action potentials, but it is undetected due to the loss of efficacy of the antagonist as β4 subunit expression increases. Confirming the age dependent loss of type-I channel kinetics with paxilline in mouse hippocampal slices strengthened the case that there is an age dependent loss of BK channel contribution to single spikes and excluded the possibility that BK channel contribution to single spikes
remains constant while expression of the β-4 subunit increases.

Comparing the effects of BK channel blockade on firing frequency across the two models leads to somewhat less unified conclusions. BK channel blockade with iberiotoxin yielded results that fit nicely within the model of BK channels with type-I kinetics being developmentally replaced by BK channels with type-II kinetics (Fig 2.8). In cultured P1 neurons, BK channel blockade with iberiotoxin consistently slowed instantaneous firing frequencies across one second of continuous firing, consistent with a model of BK channels contributing to the repolarization of each action potential within a train. In P7 neurons, BK channel blockade had the paradoxical effect of speeding instantaneous firing frequencies early in sustained firing but reducing frequencies as successive firing continued. This fits in a model of type-II BK channel kinetics where BK channels do not activate in time to repolarize early action potentials but instead reduce the degree of afterhyperpolarization which speeds the initiation of the following spike. As firing progresses and intracellular calcium rises BK channels are open during the repolarization of later action potentials and contribute to spike repolarization (Fig 4.1). The age-dependent increase in BK channel expression is also apparent in this data.

Figure 4.1: Proposed timing of BK channel activity in P1 and P7 cultured hippocampal neurons. Fast activating BK channels in P1 Neurons open readily in response to depolarization. Slower variants in P7 neurons do not open in time to repolarize early action potentials but will open more readily with successive firing as intracellular calcium rises. Traces are the first five spikes of an action potentials train evoked by a 1s depolarization in cultured neurons. The full traces are shown in fig 2.8A. Red bars indicate the proposed timing of BK channel activity.
set as, by the end of one second of successive action potential firing, BK channel blockade achieved greater decreases in firing frequency in the P7 neurons.

While the results from cultured rat hippocampal neurons fit a model of type-I BK channels being replaced by type-II BK channels, the results from the mouse neurons in acute slices are less definitive. There was a small age-dependent effect of BK channel blockade on instantaneous frequencies of action potential firing, but the effect was not strong enough that pairwise comparisons revealed an increase or decrease in firing with BK channel blockade in one age relative to another. Had the data followed the trend with increased sampling, pairwise comparisons may have revealed that BK channel blockade in P15 mouse hippocampal pyramidal neurons moderately elevated firing. This is an age that was not sampled in isolated rat neurons due to the difficulty in obtaining viable neurons at this age. One potential confound is that there seemed to be a bimodal effect of BK channel blockade in mice with some cells responding with decreases and others with increase in action potential outputs and firing frequency. Paradoxically, there was more variance observed in the putative pyramidal neurons identified by electrical characteristics and location in mice than in the recordings from heterogeneous cultured neurons.

Classical understanding of the hippocampal function is that the structure consists of repeating “lamella”, planar organizations of homogenous glutamatergic pyramidal cells in a trisynaptic circuit (dentate gyrus → Ca3 → Ca1) governed by simple, stereotypical inhibitory local inhibitory interneuron circuitry that function in parallel and largely independent of adjacent – and certainly independent of more distal – lamella [251]. This model soon failed to account for the complexity observed within the hippocampus and this model has been replaced with one of a complex, interconnected brain structure with integral roles and subregional specifications in regulating learning and memory, spatial navigation, cognition, and affective states [233], [252], [253]. As this
understanding grew along with our ability to interrogate the molecular biology of individual cells, the hippocampal pyramidal neurons were redefined from a relatively homogenous class of glutamatergic cells to a transcriptomically and physiologically diverse superclass of neurons that imparts on the hippocampus a level of sophistication and complexity that mirrors and rivals that of the cortex [219], [220]. In line with this, studies of hippocampal pyramidal neurons may require very large sample sizes to either achieve statistical significance in the face of a high degree of heterogeneity or allow for subdivision of the dataset by unifying physiological, transcriptional, regional, morphological or other properties. The failure of this study to discern significant, or even consistent directionality of the effect of BK channel blockade may have arisen from treating putative hippocampal pyramidal neurons as a single distinct class. Future work should employ single-cell qPCR to allow for more precise identification of pyramidal neuron subtypes. This may reveal specific electrophysiology signatures of transcriptomically unique neuronal types and differences in the importance and roles of BK channels.

4.5 Postnatal Expression Patterns of BK Channels in the Hippocampus and Their Implications for Neuronal Physiology

Underlying the shift from type-I to type-II kinetics of BK channels are changes in the expression patterns of neuronal BK channel isoforms and auxiliary subunits. While I demonstrated that the total protein expression of the BK channel α subunit increases approximately three-fold in the rat hippocampus from P0 to P8 with an inflection point at P4 and the α subunit mRNA expression increases to approximately the same degree in the developing mouse hippocampus from P0 to P15 with an inflection point shortly after P5 (Fig 2.6, 3.5). While the protein expression in the rat hippocampus and the mRNA expression in the mouse hippocampus show the same general trend, you can’t estimate protein expression from mRNA expression as there could be differences in the rate of
translation of the dynamic of protein degradation. This data does not allow for direct comparisons of BK channel expression between rats and mice.

The initial explanation I gravitated to for explaining the differences in BK channel kinetics was a switch in the dominant α subunit isoform that exhibits different activation kinetics. For this model to be correct, the transition of the dominant α-subunit variant that occurs between E18 to P7 [155] must occur at a steady rate, so that there is a postnatal time when the STREX isoform predominates (Fig 4.2A). After measuring mRNA expression of the STREX and ZERO isoform as well as the β-4 subunit I found that, contrary to my hypothesis STREX mRNA transcripts account for a minority of the total transcripts of α subunits even at P0 and therefore cannot explain postnatal shifts in BK channel kinetics. My data , along with that of MacDonald et al. (2006) [155], support a model where the reversal of the STREX:ZERO ratio occurs rapidly between embryonic day 18 (E18) and P0 (Fig 4.2B). The expression of β-4 mRNA continues to increase from P0 to P15. Both a loss of the STREX isoform and the rise in β-4 subunit expression can explain the transition from type-I to Type-II BK channel kinetics as it slows the activation kinetics in BK channels [78], [118]. Since predominance of the ZERO isoform is established prenatally or perinatally the most likely explanation for loss of BK channel
involvement in controlling the action potential duration with postnatal development is the 
increase in $\beta$-4 subunit expression as it, unlike the STREX isoform, undergoes 
significant postnatal changes in expression that correspond to the timing of the shift 
from type-I to type-II BK channel kinetics (Fig 3.5).

It is worth mentioning that while BK channels are expressed in neurons, they are 
also broadly expressed in other tissues, such as the glia and vasculature [60], [254], 
[255] which are present in the whole hippocampal tissue preparations from which protein 
and mRNA were isolated. A single-cell transcriptomics approach such as patch-seq 
would provide a more precise quantification of neuronal expression trends when the 
protein or transcript of interest has overlapping expression in multiple tissue types 
present in the sample. I would also allow patterns in BK channel expression to correlated 
to transcriptomically defined neuronal subtypes.

4.6 Effect of BK Channel Blockade in Zero Magnesium Induced Hyperexcitability

BK channels are heavily implicated in familial epilepsy and blockade of BK 
channels has, under certain conditions been demonstrated to be a viable intervention for 
seizures. Both gain-of-function and loss-of-function mutations in the KCNMA1 gene have 
been linked to seizures and other neuropathologies [65], [167], [180]. There is some 
question as to if and which BK channels mutations lead to epilepsy alone or if a BK 
channel mutation in concert with other mutation and pathological states is required for 
epilepsy to develop; this combinatorial effect with other mutations could explain why both 
increased and decreased conductance of the same channel can produce the same 
disease state.

A growing body of evidence is now indicating that the gain-of-function mutations 
in the KCNMA1 gene are independently sufficient to produce epilepsy; notably these 
gain-of-function mutants increase calcium sensitivity and produce more conductance in
response to a single action potential waveform [175], [182]. Furthermore it is apparently only in the background of these mutations, and pathological remodeling of BK channel splice variants and β subunit assemblies that BK channel blockade is able to attenuate seizures. This pathological remodeling of BK channels in response to seizures includes a reversion to type-I BK channel kinetics, upregulation of the STREX variant, and downregulation of the β-4 subunit, (the subunit itself being protective against seizures [186]) [176], [185], [187]). As BK channel blockade only attenuates seizures in adult and juvenile rodents in a state where their neuronal BK channel properties overlap with early postnatal neurons (although I have since identified that high STREX expression is not a feature or early postnatal neurons) I hypothesized that BK channel blockade may be a viable intervention in a seizure model employing wild-type, non-epileptic early postnatal rodents.

The seizure model I chose to employ was depletion of extracellular magnesium which induces hyper-excitability by increasing NMDA receptor mediated glutamatergic transmission [221]. I selected this model as it is less likely to be confounded by development than other models of inducing seizure activity and differences. The most common seizure induction model is GABA_A antagonism which is inappropriate due to developmental changes in chloride gradients and GABA_A receptor expression and subunit composition [5], [191]. Another common pharmacological method of inducing seizures, cholinergic agonism with pilocarpine, depends on the development of long-range inputs to the hippocampus as well as the establishment of functional inhibitory networks; both of these factors are highly variable across developmental time [170], [256]. The zero magnesium model has the advantage of directly increasing the excitability of pyramidal neurons and although there are developmental differences in glutamate receptors, it minimizes the developmentally variable confounding factors present in other models [221], [257].
I monitored for hyperexcitability by whole cell recording of individual pyramidal neurons during zero magnesium-induced hyperexcitability. This cannot properly be called a recording of seizure activity as a seizure is a by definition a population level event – not a single cell event and thus this method runs the risk of “missing the forest for the trees” when trying to assess seizure activity. Despite this weakness it does offer some strengths, namely the ability to measure effects of seizure induction and pharmacological treatment on action potential firing frequencies and synaptic transmission.

I did not observe any effect of BK channel blockade on zero magnesium induced hyperexcitability, and only saw a variable, but significant effect of BK channel blockade on synaptic current half width. The simplest and most straightforward conclusion is that, like in more mature brains, BK channel blockade will not impede seizure activity in a wild-type, seizure naïve state, and my hypothesis that BK channel blockade is an effective therapeutic in some conditions is due to the neonate-like state of BK channels (whether in the gain-of-function mutants or the expression changes induced by seizures) was incorrect. The neonatal brain is not analogous to the epileptic brain; while the epileptic brain may have some neonatal characteristics it still retains adult characteristics. Not least among these adult characteristics is synaptic connectivity – notably the integration of inhibitory interneurons into local hippocampal circuits which occurs during postnatal development [231]. Activation of local inhibitory interneurons is able to terminate seizures [214], [259] and so it may be that brains must be in a state where both BK channel blockade can more effectively dampen excitatory transmission due to type-I BK channel kinetics and inhibitory circuits are established.

It is still possible that BK channel blockade is a viable intervention for neonatal seizures, but I did not use a model system that captures the effect. As seizure are events that occur on the level of local neuronal populations or entire brain regions, single
cell recordings may not have captured some effects of channel blockade on hyperexcitability. The proposal for this dissertation included plans to assess the effect of BK channels blockade on seizure activity by extracellular recordings in the hippocampal layer; this aim was pursued by undergraduate researchers that I mentored but there were ongoing problems with validating the model. Other models used to assess seizures in the works cited here include behavioral scoring of seizures [174], [185], [185], and electroencephalography [175], [187], both of which fall outside of the expertise of this lab. The single cells recording model could also have been refined to generate more specific conclusions by concurrent whole cell recordings and extracellular recordings in the same region to confirm that activation of a single cell represented participation in a

**Figure 4.3: Three models of simultaneous recording**

A Simultaneous recording of a pyramidal neuron (blue triangular cell) by whole-cell recording and population activity by extracellular recording with a large diameter pipette. B Simultaneous whole-cell recordings of local, synaptically connected pyramidal neurons. C Simultaneous whole-cell recordings of a pyramidal neuron and a local, synaptically connected inhibitory interneuron (red circular cell).
seizure-like event (Fig 4.3A). Simultaneous whole-cell recordings of synaptically
connected cells during seizure induction could also be used to study how age and drug
treatment affect microcircuit activity during seizure activity (Fig 4.3 B). In this
configuration paired recordings of pyramidal neurons could be used to determine how
excitatory transmission is affected by different experimental conditions. Paired
recordings of pyramidal neurons and local interneurons could be used to study how
participation of GABAergic interneurons during seizure-like activity varies with
development and interventions (Fig 4.3C). These multiple recording techniques have not
been done previously in this lab but could be valuable in decoding the roles of neuronal
subpopulation whether performed by traditional electrophysiology or by newer, optical
techniques [235], [236], [260].

One final consideration I have is that the seizure model employed may affect
outcomes. While magnesium depletion is a reliable way to induce seizure activity in a
slice model it may be too robust of a means of inducing hyperexcitability. Such a “blunt
force” instrument for inducing seizures may provide little latitude in which a putative
intervention can operate. Other common methods of inducing seizures, whether in in
vitro or in vivo models involve inhibiting GABAergic transmission with GABA$_A$ receptor
antagonists such as pentylenetetrazol, picrotoxin, or bicuculline [261] or by disrupting
neuromodulators such as in the pilocarpine model which disrupts cholinergic
transmission and dysregulates hippocampal circuits [170], [262]. It is possible that some
models of seizure induction are so robust that they mask the potential effects of a
candidate intervention. I have no compelling reason to believe that the magnesium free
model was the wrong intervention to test my hypothesis, but in cannot be excluded that
different results may have been achieved with a different model.
4.7 Concluding Remarks

The work in this dissertation expands the understanding of how neuronal excitability develops in the postnatal rodent brain. Previous investigations did not report day-by-day resolution of this process, but only reported developmental changes across broader intervals. Comparing development across the span of a week may seem like a relatively granular interval in human terms, but that week may represent years of development in the rodent brain. I report that development of excitability occurs gradually across the first postnatal week with the exception of a punctuated increase around postnatal day four to five.

This work is also the first description the functional consequences of changing patterns of BK channel expression. I have reported that developmental expression patterns, likely the rise in the β-4 subunit underlie a shift from type-I to type-II kinetics and alters how BK channels currents contribute to action potential firing.

This last finding informed a hypothesis that BK channel blockade may be an effective intervention for epilepsy in the developing rodent brain. While my experiments did not demonstrate this I believe it was a worthwhile hypothesis to test and I cannot eliminate the possibility that experiments that measure hyperexcitability or synaptic transmission on a more global level may show more conclusive results.
BIBLIOGRAPHY


channel: Activation by Ca2+ and voltage,” *Biol. Res.*, vol. 39, no. 3, 2006,

KCa channels determined by a separable domain,” *Neuron*, vol. 13, no. 3,

channel,” *Biophysical Journal*, vol. 73, no. 3, pp. 1355–1363, Sep. 1997,

validation of Ca2+-binding residues from the crystal structure of the BK ion
channel,” *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1860,

of the RCK Domain from the E. coli K+ Channel and Demonstration of Its
Presence in the Human BK Channel,” *Neuron*, vol. 29, no. 3, pp. 593–601,

Activation of BK Channels by Ca2+,” *Journal of General Physiology*, vol.

[102] T.-B. Sweet and D. H. Cox, “Measurements of the BKCa Channel’s High-
Affinity Ca2+ Binding Constants: Effects of Membrane Voltage,” *Journal of
General Physiology*, vol. 132, no. 5, pp. 491–505, Nov. 2008, doi:
10.1085/jgp.200810094.

[103] Y. Zhou, H. Yang, J. Cui, and C. J. Lingle, “Threading the biophysics of
mammalian Slo1 channels onto structures of an invertebrate Slo1 channel,”
*Journal of General Physiology*, vol. 149, no. 11, pp. 985–1007, Nov. 2017,

[104] J. Shi et al., “Mechanism of magnesium activation of calcium-activated
doi: 10.1038/nature00941.

Slo1 BK channels by Mg2+ coordinated between the voltage sensor and
RCK1 domains,” *Nat Struct Mol Biol*, vol. 15, no. 11, pp. 1152–1159, Nov.
2008, doi: 10.1038/nsmb.1507.


