Molecular Dissection of IA Channels in Cortical Pyramidal Neurons

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Molecular Dissection of I_A Channels in Cortical Pyramidal Neurons

by

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Chapter 1

Introduction
Diversity of neuronal $K^+$ currents

Although the classical function of $K^+$ currents in the nervous system is to repolarize action potentials, it is now quite clear that the physiological roles of $K^+$ currents extend to a wide array of neuronal processes including controlling spontaneous firing rates [1-3], determining resting membrane potentials [4], regulating the back propagation (into dendrites) of action potentials [5], modulating neurotransmitter release [6], altering synaptic integration [7, 8], localizing activity dependent synaptic plasticity [9, 10] and even apoptosis [11, 12]. In addition, changes in the properties and expression of $K^+$ currents are potentially important mechanisms allowing neurons to respond to a variety of signals. In fact, $K^+$ channel functioning varies directly in response to multiple types of stimuli including changes in membrane voltage, changes in intracellular $\text{Ca}^{2+}$ and membrane stretch [1]. The generation of $K^+$ currents sufficient to fill these many diverse functional roles is accomplished through the expression of multiple molecularly distinct types of $K^+$ channels. To date, five distinct families of $K^+$ selective pore-forming (α) subunits have been identified: inwardly rectifying $K^+$ channel, two-pore (K2P) $K^+$ channel, voltage-gated $K^+$ (Kv) channel, large conductance $\text{Ca}^{2+}$ and voltage-gated (BK) channel and small conductance $\text{Ca}^{2+}$ gated (SK) channel α-subunits (Figure 1.1A). Further diversity exists within each family, as each family of $K^+$ channel α-subunits has many members. The Kv family of channels, the focus of the studies presented here, is the largest of the human $K^+$ channel families with 40 different Kv α-subunit encoding genes [13]. Functional Kv (and many other $K^+$) channels are formed by the tetrameric assembly of α-subunits and each α-subunit contributes to the formation of the ion conducting, $K^+$
selective pore (Figure 1.1B). Further contributing to the diversity of functional $K^+$ currents, heteromultimers (between select subfamily members) of $\alpha$-subunits can form functional channels that encode currents with distinct properties from those generated by homomeric tetramers [14, 15].
Figure 1.1 K⁺ channel subunits (A) Schematic representation of the transmembrane and pore-forming regions of α-subunits of inward rectifier (Kir), two-pore (K2P), voltage-gated (Kv) and Ca⁺ and voltage-gated large conductance (BK) K⁺ channels. (B) Surface representation of a tetramer of Kv α-subunits viewed from the side (top panel) and from the extracellular side of the membrane with a central K⁺ selective pore region (lower panel) (adapted from the crystal structure of the Kv1.2 channel (Long, Tao et al. 2007)) (C) Schematic representations of the DPP6, Sleepless and Kvβ Kv channel accessory subunits. Each DPP accessory subunit has a single transmembrane domain and a large extracellular domain that resembles other peptidases, but is not catalytically active. Sleepless is a small GPI anchored peptide, identified in *Drosophila*, that resembles small peptide neurotoxins. The accessory Kvβ subunits are intracellular proteins. Each Kvβ subunit has an active catalytic domain that binds NADP⁺ as a co-factor (yellow) and display oxidoreductase activity (Leicher, T. et al. 1996 Tipparaju, S. M. et al, 2008).
**Functional diversity of Kv currents**

Kv channels open in response to depolarization and hyperpolarize the membrane potential by conducting outward K\(^+\) currents. Thus, Kv currents are uniquely positioned to play central roles in regulating the excitability of neurons. Accordingly, Kv currents are critical for many aspects of neuronal function including repolarization of action potentials [16, 17], regulating the firing of action potentials in responses to synaptic inputs [18] and modulating synaptic signaling [5, 19]. Kv channels are also important targets for modulation by neurotransmitters and second messengers that modify neuronal properties and functioning [20]. Consistent with the many important functional roles of Kv currents in neurons, mutations in genes encoding Kv channel subunits and alterations in Kv channel functioning or expression are increasingly associated with pathologies [21-24]. Epilepsy, in particular, has been linked to mutations in genes encoding several Kv channel α-subunits [25].

The responses of individual neurons to synaptic and direct sensory inputs are determined by the types and balance of ionic currents expressed by the cell [26]. Most neurons express multiple Kv currents with distinct time- and voltage-dependent properties. The specific types and densities of the Kv currents expressed are important in determining the intrinsic membrane properties of neurons and in controlling neuronal firing properties and responses to synaptic inputs. Fast spiking cortical interneurons, for example, fire action potentials at sustained high rates in response to depolarizing stimuli. The high frequency firing rates of these neurons are due, in large part, to high expression
levels of Kv currents that activate rapidly at relatively depolarized membrane voltages (above action potential threshold), do not inactivate and deactivate quickly at negative membrane voltages [27]. Expression of high levels of Kv currents with these properties insures rapid repolarization of action potentials. Because these Kv currents activate and deactivate rapidly at relatively depolarized potentials, however, activation does not inhibit the firing of subsequent action potentials [28]. Disrupting the expression of one of the Kv channel α-subunits (Kv3.2) that encodes this current resulted in broader action potentials and slowed firing rates in cortical interneurons, demonstrating the importance of high levels of expression of this Kv current for normal functioning of cortical interneurons [29]. In contrast, many neurons including hippocampal [30], dopaminergic midbrain [31] and sympathetic ganglion [32] neurons express M-currents, which activate slowly at voltages below the action potential threshold and deactivate slowly [33]. As a result, M-currents function to limit the firing of action potentials [34, 35] by activating in response to initial depolarization and remaining activated for prolonged periods, thereby opposing the firing of subsequent action potentials [34]. Interestingly, the term “M” was coined because these currents are inhibited, via second messengers, by the activation of muscarinic acetylcholine receptors [32, 33]. The functional effects of M-current activation can be clearly observed in the marked increase in the firing of action potentials in response to depolarizing stimuli following application of a muscarinic agonist, which inhibits the currents [33]. Reductions in the functional expression of M-currents due to mutations in or the loss of expression of the Kv channel α-subunits that encode these currents can cause many neurologic disorders, including epilepsy [36]. As illustrated by
these two examples, the detailed biophysical properties and relative expression levels of Kv currents in individual neurons are critical in determining the intrinsic excitability of individual neurons and how these neurons function within neural circuits.

**Molecular diversity of Kv channels**

It is now quite clear that the functional diversity of neuronal Kv currents reflects the expression of a molecularly diverse group of proteins that assemble to generate Kv channels. At the core of functional Kv channels are the pore-forming α-subunits. Based on primary sequence homology, 12 subfamilies of Kv channel α-subunits have been identified (Figure 1.2), the largest of which is the *Shaker* (Kv1) subfamily with 8 members [37]. The expression of diverse Kv currents in neurons is generated by the simultaneous expression of multiple types of Kv channel α-subunits in the same cell. Determining the relationship between the Kv channel α-subunits present in a neuron and the Kv currents expressed is a critical step in delineating the molecular mechanisms responsible for regulating neuronal excitability. Substantial progress has been made on this front, in part, through experiments using heterologous cells to express individual Kv channel α-subunits and comparing the properties of the currents generated to native neuronal Kv currents [38-41]. When heterologously expressed, however, different Kv α-subunits are able to generate currents with similar properties [42, 43], hindering determination of the relationship(s) of individual α-subunits to specific Kv currents. In addition, many of the detailed properties of native neuronal Kv currents are absent when Kv α-subunits are expressed in heterologous cells [44]. It seems certain that these
differences reflect the molecular complexity of functional Kv channels and the fact that Kv channel α-subunits can interact with multiple types of accessory and other regulatory proteins. The specific molecular components of the Kv channels, as well as the stoichiometries of the various subunits, in native cells are likely to be numerous and diverse and, as a result, difficult to recapitulate in heterologous expression systems. The studies presented in Chapter 3 combined molecular genetic, electrophysiological and pharmacological tools to determine directly the roles of individual Kv channel α-subunits in the generation of the rapidly activating and inactivating, A-type, Kv currents in (visual) cortical pyramidal neurons, the primary excitatory cells in cortex.
Figure 1.2 Kv α-subunit gene subfamilies. Dendrogram of Kv α-subunit gene subfamilies is illustrated. Red circles indicate the Kv subfamilies that have at least one member that can generate rapidly inactivating currents when expressed in heterologous cells.
**Kv currents in cortical pyramidal neurons**

All available evidence suggests that neurons express many different types of Kv currents (and channels) simultaneously. Macroscopic neuronal Kv currents are routinely examined using whole-cell voltage clamp methods [45]. With the voltage-gated \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) currents pharmacologically blocked, Kv currents can be elicited by stepping the membrane voltage to depolarized potentials, and the amount of current required to hold the membrane voltage at the specified potential can be measured directly [26]. Whole-cell voltage clamp studies focused on examining Kv currents in visual cortical pyramidal neurons have identified four kinetically and pharmacologically distinct Kv current components: the rapidly activating and inactivating current, \( I_A \) (tau inactivation \( \sim 25 \) ms); \( I_D \) which inactivates more slowly than \( I_A \) (tau inactivation \( \sim 250 \) ms); the very slowly inactivating current, \( I_K \) (tau inactivation \( \sim 2 \) s); and the non-inactivating (steady state) current, \( I_{SS} \) (Figure 1.3). The current components are also pharmacologically distinct. \( I_K \), for example, can be blocked by tetraethylammonium (TEA) in the low millimolar range and \( I_A \) can be blocked by (several) millimolar concentrations of 4-aminopyridine [46-49]. The studies presented in the chapters that follow were focused on defining the molecular determinants of the rapidly activating and inactivating current, \( I_A \), which is widely expressed in both central and peripheral neurons [50].
Figure 1.3 Whole-cell Kv current recordings from isolated cortical pyramidal neurons. (A) An isolated cortical pyramidal neuron with a recording pipette and channels (red) in the membrane is illustrated. A tight (gigaohm) seal is first formed between the recording pipette and the cell membrane. Rupturing of the cell membrane achieves the whole cell configuration. The interior of the pipette is continuous with the interior of the cell following rupture of the patch of membrane beneath the opening of the pipette. In this configuration, electrical potentials between the electrode in the bath and the electrode on the cell can be controlled and current flow can be measured. Kv currents are isolated by blocking voltage-gated Na⁺ and Ca²⁺ currents. (B) Representative whole-cell (macroscopic) Kv currents, elicited by voltage steps to depolarizing potentials (-40 mV to +40 mV in 10 mV increments) from a holding potential of -70 mV, recorded from an isolated cortical pyramidal neuron at room temperature (~22-25 °C) are illustrated. In visual cortical pyramidal neurons, four kinetically and pharmacologically distinct Kv current components have been distinguished: the rapidly activating and inactivating current, Iₐ (tau inactivation ~25 ms); Iᵻ, which inactivates more slowly than Iₐ (tau inactivation ~250 ms); the very slowly inactivating current, Iₖ (tau inactivation ~2 s); and the non-inactivating (steady state) current, Iₜ. (see text for further details).
Physiologic functions of $I_A$ in neurons

$I_A$ has long been recognized to play important roles in determining neuronal firing properties [51, 52] and in regulating neuronal excitability under normal [53] and pathologic conditions, such as epilepsy [54]. Because of the unique properties of $I_A$, it plays important roles in controlling intrinsic excitability, synaptic responses and neuronal computations. Importantly, $I_A$ activates at voltages below the action potential threshold in most neurons and, as a results, $I_A$ activation opposes depolarization and the firing of action potentials. A temporal limit, however, is placed on the hyperpolarizing effect of $I_A$ activation because the channels also inactivate rapidly. Sustained depolarizing stimuli are thus able to evoke repetitive firing of action potentials (as $I_A$ channels inactivate). In addition, $I_A$ recovers from inactivation quickly (tau recovery $\sim$100 ms) at hyperpolarized potentials. Following the hyperpolarization phase of an action potential, therefore, $I_A$ channels recover rapidly and are available to oppose subsequent depolarization. In this manner, $I_A$ plays a critical role in regulating the frequency of repetitive firing of action potentials in response to depolarizing stimuli of different intensities, a fundamental mechanism for encoding information in the nervous system [1, 51, 52, 55].

Recent studies have also demonstrated that the expression of $I_A$ in dendritic compartments is important for regulating the back propagation (into dendrites) of action potentials, synaptic potentiation and dendritic integration [10, 56]. Additional recent studies also suggest that $I_A$ expression is dynamically (on a time course of minutes) regulated within dendritic compartments, and that this represents a fundamental
mechanism for determining relative synaptic strengths [9, 10, 56-58]. Recognition of the many important roles that $I_A$ plays in neuronal processing has focused considerable effort on understanding the molecular mechanisms that determine the functional expression of neuronal $I_A$ channels. The studies presented in Chapter 3 have resulted in the identification of three distinct components of $I_A$ in cortical pyramidal neurons. Combining molecular genetic, pharmacological and electrophysiological approaches, these studies have revealed the molecular identities of the $\alpha$-subunits (Kv4.2, Kv4.3 and Kv1.4) that encode three distinct components of $I_A$ in mouse visual cortical pyramidal neurons.

**Kv channel accessory subunits**

The results of a number of recent studies clearly indicate that native neuronal Kv channels, like other types of voltage-gated ion channels, function in macromolecular complexes composed of $\alpha$-subunits, a variety of cytosolic and transmembrane accessory subunits and other regulatory proteins that bind both directly and indirectly to $\alpha$-subunits [59-61]. A number of different types of Kv channel accessory subunits, regulatory molecules and scaffolding proteins have been identified [61, 62]. The Kv channel accessory subunits are molecularly heterogeneous, ranging from transmembrane proteins homologous to peptidases [63], to small peptides resembling neurotoxins [64] (Figure 1.1C). The association of Kv $\alpha$-subunits with accessory subunits is thought to be important for determining the properties, expression levels and the targeting of native neuronal Kv channels, although relatively little is currently known about the roles of
accessory subunits in the generation of native neuronal Kv channels [65]. The presence and functioning of multiple molecularly diverse proteins in Kv channel complexes potentially expands the functional diversity of neuronal Kv channels greatly. In addition, the presence of multiple accessory and regulatory proteins further extends the number of possible mechanisms available to regulate channel expression and function and to modulate neuronal excitability and computation. These factors have combined to motivate considerable interest in defining the roles of Kv channel accessory and regulatory proteins.

**Kv4 channel accessory subunits**

Results of a number of recent studies suggest important roles for the Kv4 α subunits in the generation of Iₐ in central and peripheral neurons [49, 66-68]. Interestingly, members of three families of Kv accessory subunits have been identified as putative Kv4 channel accessory subunits: the Kvβ subunits [69], the dipeptidyl aminopeptidase (DPP) like proteins (DPP6 and DPP10) [63, 70] and the K⁺ Channel Interacting Proteins (KChIPs: KChIP1, KChIP2, KChIP3 and KChIP4) [71, 72]. In addition, members of these subunit families have been co-immunoprecipitated with Kv4 α-subunits from brain [60]. Heterologous coexpression of any one of these accessory subunits with Kv4 α-subunits results in changes in the expression and properties of the resulting Kv4-encoded currents compared to currents generated by the expression the α-subunits alone. Coexpression of Kvβ subunits with Kv4 α-subunits, for example, confers oxygen sensitivity to Kv4-encoded currents [73]. Coexpression of Kv4.2 with DPP6 or
DPP10 results in increased expression of Kv4 encoded currents and the currents inactivate more rapidly and recover more quickly than currents generated by Kv4 α-subunits expressed alone [63, 70, 74]. Similarly, coexpression of KChIPs with Kv4 α-subunits generally results in Kv4-encoded currents that are expressed at higher densities. With the KChIPs, however, Kv4 currents typically inactivate more slowly and recover from inactivation more quickly than Kv4 alone. The individual KChIPs (and splice variants) have been reported to have differential effects on Kv4 currents in heterologous cells [71, 75]. Interestingly, when DPP6 or DPP10 is additionally expressed with Kv4.2 and one of the KChIPs, the effect of accelerating inactivation conferred by the DPP subunit prevails over the slowing effect conferred by the KChIP subunit [76]. The resulting currents, therefore, recapitulate many of the time- and voltage-dependent properties of native I_A, observations interpreted as suggesting that native I_A channel are tripartite complexes of Kv4, KChIP and DPP subunits [44].

Many of the properties of the KChIPs make them of particular interest in neuronal functioning. The KChIPs, for example, are members of the Neuronal Ca^{2+} Sensor protein family and bind Ca^{2+} via EF-hand domains, observations suggesting that KChIPs may serve to modulate Kv4 channel function and expression in response to changes in Ca^{2+} concentrations. In mice (and humans) there are four genes (*Kcnip1, 2, 3 and 4*) encoding KChIPs (KChIP1, 2, 3 and 4) and for each gene multiple splice variants have been described [72, 77-79]. KChIPs bind to the amino-termini of Kv4 α-subunits and complexes containing Kv4 amino termini bound to KChIPs have been crystallized [80, 81]. In the crystal structure, the KChIPs and Kv4 amino termini form an octameric
complex (1 to 1 ratio of KChIPs and α-subunits) [80]. Interestingly, each KChIP is also bound to 2 Ca$^{2+}$ ions and one EF-hand motif is disrupted by the binding of the Kv4 α-subunit [80, 81]. In addition, the KChIPs are found to co-localize with Kv4 α-subunits in many neuronal cell types and have been hypothesized to play critical roles in regulating the expression and trafficking of native neuronal Kv4 channels [75, 82]. Little is known, however, about the functional roles KChIPs play in the generation and functioning of native neuronal Kv4 channels. In the studies presented here (Chapter 4), the functional roles of KChIPs in the generation of native Kv4-encoded channels were examined directly in cortical pyramidal neurons. In the rodent brain, KChIP1 expression has been suggested to be restricted to interneurons [75, 79]. Accordingly, the studies presented in Chapter 4 focused on KChIP2, KChIP3 and KChIP4. Importantly, consistent with previous reports examining the expression of KChIPs in the rodent brain in other cortical areas [75, 79], the studies completed here demonstrated that all three KChIPs are expressed in mouse visual cortex. Combining electrophysiological, molecular genetic and biochemical approaches, the studies detailed in Chapter 4 were focused on the functional roles of the KChIP2, KChIP3 and KChIP4 in the generation of native neuronal Kv4 channels in cortical pyramidal neurons.
Chapter 2

Materials and Methods
DNA constructs

Plasmids containing the coding sequences for mouse Kv4.2, KChIP2, KChIP3 and KChIP4 were obtained from Open Biosystems. The KChIP4 clone used here encodes the same amino acid sequence as the variant termed KChIP4b [83].

Plasmids encoding human microRNA30 (mir30) with substitutions made in the targeting sequence region (miRNA) and a fluorescent protein (see below) were constructed following a previously described approach [84]. Briefly, the mir30 sequence was placed between splice donor and acceptor sequences in an artificial intron. This arrangement allows the miRNA sequence to be spliced away from the transcript, so that a single transcript can be processed to mediate RNA interference (RNAi) and be used for translation to generate the encoded fluorescent protein. The miRNA intron plasmid was assembled by combining the human miR30 sequence from the pPrime system described by Stegmeier et al [85] with the chimeric intronic sequence taken from the Promega PCI-Neo vector. The miR30 sequence was placed in the branch region between the 5’ donor site and the 3’ acceptor site of the first intron of the human beta-globin gene [86, 87]. The sequence containing miR30, as well as the exon and intron components was synthesized to order by CelTek and subsequently cloned into the multicloning site of Clontech N-1 vectors, encoding either the enhanced yellow florescent protein (YFP) or the enhanced cyan florescent protein (CFP), at the NheI and HindIII sites. To generate a red fluorescent version of the vector, the coding sequence was replaced with the sequence coding for the red fluorescent protein tdTomato [88]. Individual targeting sequences
specific for KChIP2, 3 or 4 were obtained from Open Biosystems in pSM2C vectors or were designed using the RNAI Codex algorithm and shRNA designer tool [89] and synthesized (Sigma). Specific targeting hairpins were subsequently cloned into the XhoI and EcoRI sites in the miR30. Multiple targeting sequences for KChIP2, KChIP3 and KChIP4 were screened for effectiveness in reducing the expression of co-transfected target (mouse KChIP2, 3 or 4) when transfected at ratios of 1:1 in HEK-293 cells.

The targeting sequences that proved effective in reducing the expression of the targets were then used in subsequent experiments in neurons. The targeting 22’mer sequences used were as follows: for KChIP2 ATCCATGCAACTCTTTGATAAT, for KChIP3 TCCATGCAGCTGTTTGAAAC, and for KChIP4 CCCAGAGGAAATTCACCAAGGA. BLAST searches confirmed that none of the targeting sequences for individual KChIPs had sequence homology to the other KChIPs or other known genes. The targeting sequence used for KChIP4 is complementary to both the KChIP4a and 4b splice variants. For control experiments, non-targeting (not complementary to any cDNA sequences in the mouse genome) hairpin sequences were used in the intron miRNA vector in place of the specific targeting hairpins. One control sequence targeted luciferase [85] and the other was a scrambled sequence. For experiments, equal amounts of DNA for non-targeting and targeting vectors were transfected into neurons.
Isolation, maintenance and transfection of cortical pyramidal neurons

Neurons were isolated from the primary visual cortices of postnatal day 6-8 mice using previously described methods [48, 90]. Briefly, each animal was anesthetized with isoflurane and rapidly decapitated. The brain was then removed and the posterior cortex dissected. The tissue containing the full thickness of the visual cortex was then dissected, chopped into small pieces and incubated at 37°C in Neurobasal medium (Invitrogen) containing papain (66 Units/ml) (Worthington) under 95%:5% O₂:CO₂ for 30 minutes. Subsequent to the enzyme treatment, tissues pieces were triturated using fire-polished pasteur pipettes. Isolated neurons were recovered by centrifugation for 15 minutes through a bovine serum albumin gradient. Cells were resuspended in neurobasal medium and plated on previously prepared monolayers of rat cortical astrocytes [90]. One hour after plating, Neurobasal medium was replaced with Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.3% glucose and 0.14 mM L-glutamine. Cell cultures were maintained in an incubator with 5% CO₂ at 37°C.

Neurons were transfected using the Amaxa Nucleofector II and the Amaxa mouse hippocampal kit (Lonza) according the directions from the manufacture. For transfections, isolated neurons obtained by centrifugation through a bovine serum albumin gradient were resuspended in the solution included in the nucleofection kit plus the miRNA plasmids, subjected to electroporation, and resuspended in medium immediately prior to plating. Based on fluorescent protein expression 48 hours after transfections approximately 10-30% of cells were transfected (data not shown).
HEK-293 cell culture and transfection

Human embryonic kidney-293 (HEK-293) cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum, 5% horse serum and penicillin/streptomycin (Invitrogen) in a 5% CO₂ 37°C incubator. HEK-293 cells were transfected using lipofectamine 2000 (Invitrogen) and were maintained in Opti-mem (Invitrogen) during the 8 hour transfection period.

Electrophysiological recordings

Recordings of whole-cell Kv currents were obtained from pyramidal shaped neurons on the second and third day in culture (approximately 48-72 hours after plating) at room temperature (22-23°C). Process growth was limited during the first three days in culture, thereby allowing for adequate voltage clamp. Pyramidal shaped neurons expressing the microRNA constructs, as determined by fluorescent protein expression, were selected for recordings. Data were collected using an Axon 1D amplifier (Molecular Devices) interfaced to a personal computer (Dell), using a Digidata 1322 (Molecular Devices) analogue to digital converter. Pipettes were fabricated from borosilicate glass (WPI) with a Sutter model P-87 horizontal puller (Sutter Instruments). For recordings, the bath solution routinely contained (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5 glucose, 0.001 TTX and 0.1 CdCl₂, (pH7.4 and 300mOsM). The recording pipette solution contained (in mM): 135 KCl, 10 HEPES, 5 glucose, 1.1 CaCl₂, 2.5 BAPTA, 3 MgATP and 0.5 NaGTP (pH7.4; 300mOsM). The calculated free Ca²⁺ in this BAPTA buffered pipette solution was 100 nM (MAXCHELATOR:[91]. Using this
pipette solution, pipette resistances were between 2 and 4 MΩ. The K\(^+\) channel blockers used, tetraethylammonium (TEA), 4-aminopyridine (4-AP), Heteropodatoxin-2, α-Dendrotoxin (Alomone Labs) and Ba\(^{2+}\) were added to the bath solution immediately prior to recordings. All reagents were from Sigma unless otherwise noted. All reagents were from Sigma unless otherwise noted.

For all experiments, junction potentials were zeroed prior to forming pipette-membrane seals. Signals were sampled at 100 kHz and low pass filtered at 10 kHz. Whole-cell Kv currents were evoked in response to 4 second depolarizing voltage steps to potentials between -40 mV and +40 mV (in 10 mV increments) from a holding potential of -70 mV. Also, a prepulse paradigm, which included a 60 ms step to -10 mV prior to steps to test potentials from -40 to +40 mV (in 10 mV increments), was used to facilitate the isolation of the rapidly inactivating currents in each cell. Subsequent offline subtraction of the current records obtained with the prepulse from the current records obtained without the prepulse (in the same cell) allowed the isolation of the rapidly inactivating outward K\(^+\) currents (see Figure 3.1).

\textit{Data Analysis}

Data were compiled and analyzed using ClampFit (Molecular Devices), Microsoft Excel, and Prism (Graphpad). Only data from cells with input resistances greater than 300 MΩ and access resistances less than 15 MΩ were included in the analyses. Membrane capacitances were determined by analyzing the decay phases of capacitive currents elicited by short (25ms) voltage steps (± 10 mV ) from the holding potential (-70 mV).
Consistent with the limited outgrowth of processes during the first three days in culture, a single exponential was sufficient to describe the decay phases of the capacitive transients. Whole-cell membrane capacitances (Cm) were calculated for each cell by dividing the integrated capacitive transients by the voltage. Input resistances were calculated from the steady-state currents elicited by the same ± 10 mV steps (from the holding potential of -70 mV). For each cell, the series resistance was calculated by dividing the time constant of the decay of the capacitive transient by the Cm; the mean (± SEM) series resistance was 5.4 MΩ (± 0.1) (n=222) for the experiments in Chapter 3 and 5.8 ± 0.1 MΩ (n=155) for the experiments in Chapter 4. Series resistances were compensated electronically by greater than 80% in all cells. Voltage errors resulting from uncompensated series resistances, therefore, were small (<2 mV) and were not corrected. The inactivation phases of the Kv currents were analyzed using the equation $y=A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3} + C$ where $A_1$, $A_2$, $A_3$ are the amplitudes of individual current components (see text), each with a characteristic time constant of decay ($\tau_1$, $\tau_2$, and $\tau_3$), and $C$ is the non-inactivating component ($I_{SS}$) of the total Kv current [47]. Statistical analyses were conducted using Prism. The statistical significance of observed differences in current-voltage plots (IV plots) was calculated using repeated measurement ANOVA. The Mann Whitney test was used to examine the statistical significance of the differences of the mean ± SEM of results from Western Blot and QRT-PCR data. The column t test was used for statistical analyses of results obtained in the experiments that examined the expression levels of heterologously expressed Kv4.2 and KChIPs.
Immunoprecipitation and Western Blots

For the isolation of tissue for biochemical analyses, mice were anaesthetized with isoflurane, rapidly decapitated and the brains removed. The posterior (~ 1 mm) cortex, which contains visual areas, was dissected and flash frozen in liquid nitrogen. Tissue samples were collected from wild type (WT) C57BL/6 mice and mice (Kv4.2<sup>+/−</sup>, Kv4.3<sup>+/−</sup>, KChIP2<sup>+/−</sup> and KChIP3<sup>+/−</sup>) harboring targeted disruptions of the genes encoding Kv4.2 (Kcnd2) [48, 92], Kv4.3 (Kcnd3) [93], KChIP2 (Kcnip2) [94] and KChIP3 (Kcnip3) [95]. Also, samples from mice (Kv4.2<sup>+/−</sup>/Kv4.3<sup>+/−</sup>) generated by breeding Kv4.2<sup>+/−</sup> and Kv4.3<sup>+/−</sup> animals were used [96].

For experiments focused on examining the expression of the KChIP and Kv4 proteins, total protein samples from posterior cortices collected from adult (WT, Kv1.4<sup>−/−</sup>-Kv4.2<sup>+/−</sup>, Kv4.2<sup>−/−</sup>, Kv4.3<sup>−/−</sup>/Kv4..3<sup>+/−</sup>, KChIP2<sup>+/−</sup> and KChIP3<sup>+/−</sup>) mice were prepared using previously described methods [97]. Protein concentrations were determined for each sample using a Bio-Rad protein assay kit (Bio-Rad) following the directions from the manufacturer. Equal amounts of proteins were then fractionated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. For immunoblotting, PVDF membranes with bound proteins were incubated in blocking buffer (PBS, 1% Tween and 5% dry milk) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against Kv1.4, Kv4.2, Kv4.3 or the individual KChIPs (KChIP2, KChIP3 and KChIP4). All primary antibodies were from the UC Davis/NIH NeuroMab Facility with the exceptions of the goat anti-KChIP4 antibody from Santa
Cruz Biotechnology and the polyclonal anti-Kv1.4 antibody from Chemicon. Bound primary antibodies were detected using horseradish peroxidase conjugated rabbit anti-mouse IgG (GE Healthcare) or rabbit anti-goat IgG (Bethly Labs) and the Durawest chemiluminescence reagent (Pierce). Signals were detected and quantified using the BioRad Chemidoc system and the Quantity One software (Bio-Rad). Blots were then reprobed with primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam), transferrin receptor (Invitrogen) or β-tubulin (Sigma) to verify equal protein loading in each lane. For quantification, the signals from the anti-GAPDH, the anti-transferrin receptor or the anti-β-tubulin antibodies were used to normalize the anti-Kv4 or anti-KChIP signals for each lane on the same blot, as described in the text.

For immunoprecipitations, posterior cortical tissue samples from adult WT and Kv4.2−/− mice were processed and immunoprecipitations conducted using previously described methods [98]. Briefly, the tissue pieces were homogenized in ice-cold lysis buffer: phosphate buffered saline (containing in mM: NaCl 136, KCl 2.6, NaH2PO4 10, KH2PO4 1.7 (pH 7.4)) plus one protease inhibitor cocktail tablet (Roche, USA) and Triton X-100 (1%). After 15 minutes rotation at 4°C, 4 mg of the soluble protein fractions from the WT and Kv4.2−/− brains were used for immunoprecipitations (IP) with 5 μg of an anti-Kv4.2 rabbit polyclonal antibody (Millipore). Protein A-magnetic beads (Invitrogen) were incubated with the protein samples and antibodies for 2 hours at 4°C. Magnetic beads and bound antibodies were then collected, washed four times with ice-cold lysis buffer, and isolated protein complexes were eluted from the beads in 1X
NuPAGE LDS sample buffer (Invitrogen). Samples were then fractionated on SDS-PAGE gels and transferred to PVDF membranes for immunoblotting as described above.

Protein lysates were prepared from HEK-293 cells harvested 24 hours after transfections using ice cold phosphate buffered saline containing 1% Triton and protease inhibitor (Complete mini EDTA free protease inhibitor cocktail tablet from Roche) and processed for Western Blot analysis as described above for cortical samples.

Quantitative real time PCR

For Quantitative Real Time PCR (QRT-PCR) experiments, posterior cortical samples were collected from WT, Kv4.2−/−, Kv4.3−/−, Kv4.2−/−/Kv4.3−/− and KChIP3−/− mice as described above. RNA was isolated from cortical samples and cDNA was produced using standard methods [99]. Briefly, RNA was isolated by using the RNeasy mini kit from Qiagen according to the directions from the manufacturer, and RNA concentrations were determined by optical density measurements. Single stranded cDNA was produced from 2 μg of total isolated RNA using the High Capacity cDNA Archive Kit from Applied Biosystems. The expression levels of KChIP2, 3 and 4 were determined using sequence specific primers (see below) and SYBR green (Applied Biosystems) for QRT-PCR; experiments were conducted on a 7900HT Fast Real Time PCR System (Applied Biosystems). Data were analyzed using the threshold cycle relative quantification method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. Primer sequences used to detect KChIP expression were as follows: KChIP2-
forward: GGCTGTATCAGGAAGGAGA, reverse: CCGTCTTTGTTCTGTCATC; KChIP3- forward: GGAGATCCTGGGCGCATAC, reverse: GTGAACCGTGGCCTTTGC; and KChIP4- forward: TGATCGTCATTGTGCTTTTTGTT, reverse: GCTGTCTTCTAAACCTGCTTCAATC.

*Principal contributions to the work as a whole*

Aaron Norris in collaboration with Jeanne Nerbonne designed of these studies and Aaron carried the out experiments presented here with two exceptions. The experiments presented in figures 4.2 and 4.8 were carried out by Nick Foeger. Expert technical assistance was received from Ms. Amy Huntley (the preparation and maintenance of glia cultures), Ms. Rebecca Mellor (assistance with quantitative PCR) and Mr. Rick Wilson (the maintenance and screening of the mouse lines used in this study).
Chapter 3

Molecular Dissection of $I_A$ in Cortical Pyramidal Neurons Reveals Three Distinct Components Encoded by Kv4.2, Kv4.3, and Kv1.4 $\alpha$-subunits
Abstract

The rapidly activating and inactivating voltage-gated $K^+$ (Kv) current, $I_A$, is broadly expressed in neurons and is a key regulator of action potential repolarization, repetitive firing, back propagation (into dendrites) of action potentials, and responses to synaptic inputs. Interestingly, results from previous studies on a number of neuronal cell types, including hippocampal, cortical and spinal neurons, suggest that macroscopic $I_A$ is composed of multiple components and that each component is likely encoded by distinct Kv channel α-subunits. The goals of the experiments presented here were to test this hypothesis and to determine the molecular identities of the Kv channel α-subunits that generate $I_A$ in cortical pyramidal neurons. Combining genetic disruption of individual Kv α-subunit genes with pharmacological approaches to block Kv currents selectively, the experiments here revealed that Kv1.4, Kv4.2 and Kv4.3 α-subunits encode distinct components of $I_A$ that together underlie the macroscopic $I_A$ in mouse (male and female) cortical pyramidal neurons. Recordings from neurons lacking both Kv4.2 and Kv4.3 (Kv4.2−/−/Kv4.3−/−) revealed that, although Kv1.4 encodes a minor component of $I_A$, the Kv1.4-encoded current was found in all the Kv4.2−/−/Kv4.3−/− cortical pyramidal neurons examined. Of the cortical pyramidal neurons lacking both Kv4.2 and Kv1.4, 90% expressed a Kv4.3-encoded $I_A$ larger in amplitude than the Kv1.4-encoded component. The experimental findings also demonstrate that the targeted deletion of the individual Kv α-subunits encoding components of $I_A$ results in electrical remodeling that is Kv α-subunit specific.
Introduction

Voltage-gated K\(^+\) (K\(_v\)) currents play distinct roles in controlling neuronal action potential waveforms, repetitive firing patterns [100], responses to synaptic inputs [18], neurotransmitter release [6] and synaptic plasticity [8]. Consistent with these diverse roles, multiple types of K\(_v\) currents with distinct time- and voltage-dependent properties are co-expressed in most neurons. The functional diversity of neuronal K\(_v\) currents is generated, in part, through the expression of multiple K\(_v\) channel pore forming (\(\alpha\)) subunits. In cortical pyramidal neurons, for example, multiple K\(_v\) channel \(\alpha\)-subunits from different subfamilies are co-expressed [101]. The macroscopic K\(_v\) currents in these cells can be separated into four components based on differing time constants (\(\tau\)) of inactivation: I\(_{A}\), which inactivates rapidly (\(\tau\approx25\) ms); I\(_{D}\), characterized by an intermediate rate of inactivation (\(\tau\approx250\) ms); I\(_{K}\), which inactivates slowly (\(\tau\approx2\) seconds); and I\(_{SS}\), that is non-inactivating [47]. Previous studies suggest that these kinetically distinct current components are encoded by molecularly distinct populations of channels [49].

The rapidly activating and rapidly inactivating K\(_v\) current, I\(_{A}\), which is widely expressed in central and peripheral neurons [50], regulates multiple neuronal processes, including action potential repolarization, repetitive firing [49], synaptic integration and the back propagation (into dendrites) of action potentials [10]. Considerable, evidence also suggests that alterations in I\(_{A}\) expression and/or function are associated with neuropathology. For example, I\(_{A}\) availability is decreased in a mouse model of temporal
lobe epilepsy [54]. Additionally, a mutation in Kv4.2 has been identified in a patient with temporal lobe epilepsy [102]. Other studies utilizing experimental models of epilepsy have described alterations in the expression and the subcellular localization of Kv4.2 and Kv4.3 in the hippocampus [103, 104].

To understand the molecular mechanisms that regulate the expression, properties and functioning of $I_A$ in normal and pathological states, the pore forming and accessory subunits underlying the generation of $I_A$ channels have to be identified. Previous studies on neurons from mice (Kv4.2$^{-/-}$) in which the Kcnd2 (Kv4.2) locus was disrupted revealed that Kv4.2 contributes importantly to the generation of $I_A$ in cortical pyramidal neurons [48], hippocampal pyramidal neurons [105] and in neurons from the dorsal horn of the spinal cord [67]. In each of these studies however, rapidly activating and inactivating currents similar to $I_A$ in wild type cells were observed suggesting that additional components of $I_A$ (i.e. not encoded by Kv4.2) are expressed in these cells. The studies presented here exploit pharmacology, in combination with genetic tools to disrupt the expression of individual Kv channel α-subunits, to identify the Kv channel α-subunits responsible for the generation of $I_A$ in cortical pyramidal neurons.
Results

Distinct component of $I_A$ evident in Kv4.2$^{-/-}$ pyramidal neurons

Previously we reported that the rapidly activating and rapidly inactivating Kv current, $I_A$, that is prominent in wild type (WT) cortical pyramidal neurons (Figure 3.3.1A), was not evident in most (80%) cortical pyramidal neurons (Figure 3.1B) isolated from mice (Kv4.2$^{-/-}$) harboring a targeted disruption of the Kcnd2(Kv4.2) locus [48], findings consistent with previous suggestions that Kv4.2 is the critical Kv $\alpha$-subunit encoding $I_A$ channels in cortical (and hippocampal) pyramidal neurons [68, 105, 106]. In a small subset (~20%) of Kv4.2$^{-/-}$ cells, however, a rapidly inactivating current was observed [48], suggesting the expression of a molecularly distinct (non-Kv4.2 encoded) $I_A$ in this subset of cortical pyramidal neurons.

The marked differences in the waveforms of the Kv currents in the vast majority (80%) of Kv4.2$^{-/-}$ cortical pyramidal neurons (Figure 3.1B) and those recorded from WT cells (Figure 3.1A) reflects the upregulation of delayed rectifier currents ($I_K$ and $I_{SS}$), as evidenced by the sensitivity of the upregulated currents to the $K^+$ channel blocker tetraethylammonium (TEA). As illustrated in Figure 3.1C, addition of 3 mM TEA to the bath reduced $I_K$ and $I_{SS}$ revealing a rapidly inactivating current component similar to $I_A$ in WT neurons (Figure 3.1C). Similar results were obtained in all (n=23) Kv4.2$^{-/-}$ neurons examined in the presence of TEA. To quantify the amplitude of $I_A$ in these (Kv4.2$^{-/-}$) cells, a 60ms prepulse to -10mV was used to inactivate $I_A$ (Figure 3.1Db). Offline subtraction of the current records obtained with the prepulse from the control records in
the same cell (Figure 3.1Da) allowed the isolation of $I_A$ (Figure 3.1Da-b). The peak amplitudes of the subtracted current records were measured and normalized to cell capacitance to yield $I_A$ densities. The mean ± SEM density of $I_A$ in Kv4.2$^{-/-}$ neurons was significantly ($p<0.01$) lower than in WT cells (Figure 3.1F), an observation that might be interpreted as suggesting the presence of residual Kv4.2 encoded channels in Kv4.2$^{-/-}$ cells. Previous studies, however, have shown that no Kv4.2 protein or transcript is detectable in the cortices of Kv4.2$^{-/-}$ mice [48, 107]. Thus, the observation of a reduction in, but not the elimination of, $I_A$ in Kv4.2$^{-/-}$ neurons indicates that other K\text{v} \alpha$-subunits (in addition to Kv4.2) contribute to the generation of functional $I_A$ channels in cortical pyramidal neurons.
Figure 3.1 A component of $I_A$ sensitive to 1 mM 4-AP, is present in all Kv4.2$^{-/-}$ cortical pyramidal neurons. Representative outward Kv current waveforms, elicited by 4 s voltage steps to test potentials between -40 and +40 mV (in 10 mV increments) from a holding potential of -70 mV, in WT neurons (A), Kv4.2$^{-/-}$ neurons (B) and Kv4.2$^{-/-}$ neurons in bath containing 3 mM TEA (C), are illustrated. (D-E) The amplitudes of $I_A$ in individual cells were determined from subtracted records (a-b) of the Kv currents elicited by depolarizing steps preceded by a (60 ms) prepulse to -10 mV (b) from the Kv currents evoked by identical voltage steps without the prepulse (a). The mean ± SEM density of $I_A$ in Kv4.2$^{-/-}$ (n=23) neurons was significantly (*p<0.01) lower than in WT (n=23) neurons (F). Addition of 1 mM 4-AP to the bath reduced $I_A$ density in Kv4.2$^{-/-}$ and WT neurons (E), although the mean ± SEM density of the 4-AP-resistant component of $I_A$ was significantly (*p<0.01) lower in Kv4.2$^{-/-}$ neurons (n=12) than in WT neurons (n=18) (F).
Pharmacologic characterization of $I_A$ in Kv4.2$^{-/-}$ neurons

Subsequent experiments were focused on determining the molecular identity of the Kv α-subunit(s) underlying the non-Kv4.2-encoded component(s) of $I_A$ in cortical pyramidal neurons. In heterologous expression systems, subunits of the Kv1, Kv3, Kv4 and Kv12 subfamilies can generate rapidly activating and inactivating (A-type) Kv currents [108-111]. Previous studies [112] also suggest that the channels encoded by the various Kv α-subunits can be distinguished by using selective pharmacologic blockers. In control experiments, 3 mM TEA, which effectively blocks Kv3 channels [28, 37], had no measurable effects on $I_A$ in WT neurons, indicating that Kv3 channels do not contribute to the generation of $I_A$ in mouse visual cortical pyramidal neurons (data not shown). The remaining Kv subfamilies, Kv1, Kv4 and Kv12, can be, at least partially, separated based on differential sensitivities to 4-aminopyridine (4-AP). Previous studies have shown, for example, that Kv1 channels are blocked effectively by submillimolar concentrations of 4-AP [37, 113, 114], whereas Kv4 channels are blocked by 4-AP in the several millimolar range [115] and Kv12 channels are insensitive to high millimolar concentrations of 4-AP [37, 108].

To explore the possible role of Kv1 channels, recordings were obtained from Kv4.2$^{-/-}$ and WT neurons exposed to 1mM 4-AP (in the presence of 3 mM TEA) (Figure 3.1E). Addition of 1 mM 4-AP to the bath solution markedly reduced $I_A$ in both Kv4.2$^{-/-}$ and WT neurons (Figure 3.1F), demonstrating the presence of a 1mM 4-AP-sensitive (Kv1 encoded?) component of $I_A$ in neurons of both (WT and Kv4.2$^{-/-}$) genotypes. Although this concentration of 4-AP was selected to facilitate selective block of Kv1-
encoded channels, Kv4-encoded channels might also be affected, albeit to a lesser extent [37, 115]. Consistent with the suggestion that Kv4.2 channels are not effectively blocked by 1 mM 4-AP, the magnitude of the reduction in $I_A$ density caused by 1 mM 4-AP was similar in Kv4.2<sup>−/−</sup> and WT neurons (Figure 3.1F). The mean ± SEM density of the 4-AP-resistant $I_A$, however, was significantly (p<0.01) lower in Kv4.2<sup>−/−</sup>, than in WT, cells (Figure 3.1C-D), consistent with the elimination of the Kv4.2-encoded component of $I_A$ in Kv4.2<sup>−/−</sup> neurons. In recordings obtained from 10 (of 12) Kv4.2<sup>−/−</sup> neurons in 1 mM 4-AP a rapidly inactivating current remained, suggesting the presence of an additional, non-Kv1-encoded component of $I_A$. There was no detectable 1 mM 4-AP-insensitive component of $I_A$ in the other 2 (of 12) Kv4.2<sup>−/−</sup> cells examined (see Discussion).

**Kv1.4 encodes a component of $I_A$ in cortical pyramidal neurons**

The 4-AP sensitivity of $I_A$ (Figure 3.1) suggests that one component of $I_A$, in WT and Kv4.2<sup>−/−</sup> neurons is likely encoded by Kv1 channels. Previous studies in heterologous cells have shown that Kv1.4 generates rapidly activating and inactivating currents when expressed alone [116] and that other Kv1 subfamily members can generate rapidly inactivating currents through heteromeric assembly with Kv1.4 or by combining with accessory Kvβ subunits, specifically Kvβ1 or Kvβ3 [116-118]. To explore directly the hypothesis that Kv1.4 encodes a component of $I_A$, whole-cell Kv currents were examined in cortical neurons isolated from mice (Kv1.4<sup>−/−</sup>) harboring a targeted disruption of the Kcn4 (Kv1.4) locus [119]. The waveforms of the Kv currents in Kv1.4<sup>−/−</sup> neurons (Figure 3.2A) were similar to those in WT neurons (Figure 3.1A) and the inactivation
phases of the currents were also well described by the sum of three exponentials. Analysis of the Kv current waveforms revealed that the mean ± SEM density of the peak Kv current as well as $I_A$, $I_D$, and $I_K$ densities were similar in $Kv1.4^{-/-}$ neurons ($n=15$) to those determined in WT cells ($n=28$). There was, however, a small, but statistically significant ($P<0.01$) difference, in $I_{SS}$ densities measured in $Kv1.4^{-/-}$ and WT neurons (Table 1). The observation that mean ± SEM $I_A$ densities are similar in $Kv1.4^{-/-}$ and WT neurons may indicate that $Kv1.4$ does not contribute to $I_A$ in WT cells or, alternatively, that other $I_A$ components are upregulated in $Kv1.4^{-/-}$ neurons masking the loss of the $Kv1.4$-encoded current. To explore these possibilities, pharmacological experiments were conducted.

In hippocampal pyramidal neurons barium ($Ba^{2+}$) in the hundred micromolar range has been shown to reduce $I_A$ selectively [120, 121]. In addition, the effect of $Ba^{2+}$ on $I_A$ was reportedly reduced in $Kv4.2^{-/-}$ hippocampal neurons [121] suggesting that, of the channels that may encode $I_A$, $Ba^{2+}$ is selective for $Kv4$-encoded channels, although block of inward rectifier channels has also been reported [122, 123]. Control experiments revealed that the effect of 400 μM $Ba^{2+}$ on $I_A$ amplitudes in $Kv4.2^{-/-}$ neurons ($n=18$) was significantly ($p<0.01$) smaller than in WT cells ($n=14$). At +40 mV, for example, the calculated mean ± SEM density of the $Ba^{2+}$ sensitive component of $I_A$ was 75 ± 4 pA/pF in WT neurons and 42 ± 3 pA/pF in $Kv4.2^{-/-}$ neurons. In subsequent experiments, therefore, Kv currents in $Kv1.4^{-/-}$ and WT neurons were examined in control bath solution and in the presence of 400 μM $Ba^{2+}$ (Figure 3.2). As illustrated in Figure 3.2, addition of 400 μM $Ba^{2+}$ to the bath significantly ($p<0.01$) reduced the density of $I_A$ in $Kv1.4^{-/-}$
neurons (Figure 3.2C). In addition, the mean ± SEM density of the Ba\(^{2+}\)-resistant component of \(I_A\) was significantly (p<0.05) larger in WT than in Kv1.4\(^{-/-}\) neurons (Figure 3.2C), indicating a role for Kv1.4 in the generation of \(I_A\) in WT neurons. This observation, together with the finding that mean ± SEM peak \(I_A\) densities are similar in Kv1.4\(^{-/-}\) and WT neurons, suggests the Kv4-encoded component of \(I_A\) is upregulated in Kv1.4\(^{-/-}\) neurons.

Table 1. Kv current densities in Kv1.4\(^{-/-}\), Kv4.3\(^{-/-}\), and WT cortical pyramidal neurons

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>(I_{peak}) Density(pA/pF)</th>
<th>(I_A) (pA/pF)</th>
<th>(I_D) (pA/pF)</th>
<th>(I_K) (pA/pF)</th>
<th>(I_{SS}) (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>23</td>
<td>250.4 ±19.1</td>
<td>98.3 ±11.7</td>
<td>40.9 ± 3.2</td>
<td>77.0 ±7.2</td>
<td>32.8 ±2.2</td>
</tr>
<tr>
<td>Kv1.4(^{-/-})</td>
<td>14</td>
<td>264.3 ±32.4</td>
<td>86.1 ±11.9</td>
<td>55.0 ±8.2</td>
<td>99.6 ±13.3</td>
<td>41.7 ±2.9*</td>
</tr>
<tr>
<td>Kv4.3(^{-/-})</td>
<td>20</td>
<td>231.6 ±21.8</td>
<td>53.7 ±7.8*</td>
<td>40.8 ± 5.1</td>
<td>81.9 ±10.6</td>
<td>43.7 ± 4*</td>
</tr>
</tbody>
</table>

All values are mean ± SEM density. Kv currents recorded at +30 mV (from a holding potential of -70mV) were analyzed. * Indicated values are significantly (p<0.01) different from those in WT neurons.
Figure 3.2. Use of Ba\textsuperscript{2+} to block Kv4 encoded currents reveals a role for Kv1.4 in the generation of I\textsubscript{A} in cortical pyramidal neurons. Outward Kv currents were evoked and I\textsubscript{A} was measured in Kv1.4\textsuperscript{-/-} neurons under control conditions (A) and in the presence of 400 μM Ba\textsuperscript{2+} (B) using the subtraction protocol described in the legend to Figure 1. Similar experiments were carried out on WT neurons (not illustrated). The mean ± SEM densities of I\textsubscript{A} in WT (n=22) and Kv1.4\textsuperscript{-/-} (n=13) neurons were similar under control conditions. In the presence of 400 μM Ba\textsuperscript{2+}, however, the mean ± SEM I\textsubscript{A} density in Kv1.4\textsuperscript{-/-} neurons (n=22) was significantly (# p<0.05) lower than in WT neurons (n= 14) (C). The presence of Ba\textsuperscript{2+} significantly (*p<0.01, #p<0.05) reduced the mean ± SEM I\textsubscript{A} density in Kv1.4\textsuperscript{-/-} and in WT neurons.
Residual $I_A$ is present in neurons lacking both Kv4.2 and Kv1.4

The results of the experiments described above utilizing pharmacology in combination with the targeted disruption of Kv4.2 or Kv1.4 revealed roles for both of these subunits (Kv4.2 and Kv1.4) in the generation $I_A$ current in cortical pyramidal neurons. To determine if there are additional Kv channels that contribute to the generation of macroscopic $I_A$ in cortical pyramidal neurons, we generated mice lacking both Kv1.4 and Kv4.2 (Kv1.4$^{-/-}$/Kv4.2$^{-/-}$) and examined Kv currents in cells isolated from these animals. Similar to the Kv currents in Kv4.2$^{-/-}$ neurons (Figure 3.1B), marked increases in the delayed rectifier currents ($I_K$ and $I_{SS}$) were evident in records obtained from Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ neurons (Figure 3.3A). Also similar to the Kv4.2$^{-/-}$ neurons, addition of TEA to the bath blocked the delayed rectifier currents and unmasked a residual component of $I_A$ in 18 of 20 Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ cells examined (Figure 3.3B-C). In 2 (of the 20) Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ neurons studied, no rapidly inactivating currents were detected in the presence of TEA.

Subsequent experiments were focused on exploring the possible roles of Kv1 and Kv4 α-subunits in the generation of the component of $I_A$ remaining in Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ neurons (Figure 3.3). In addition to Kv1.4, several Kv1 α-subunits including Kv1.1, Kv1.2 and Kv1.6 are reportedly expressed in cortical pyramidal neurons [101]. Initial experiments were performed using the peptide toxin α-Dendrotoxin (α-Dtx), which has been reported to block heterologously expressed Kv1.1-, 1.2-, and 1.6-encoded currents and has previously been used to examine the roles of Kv1 channels in cortical neurons.
As was done in the experiments on Kv4.2−/− neurons (Figure 3.1), TEA was added to the bath to block the large delayed rectifier currents (I_K and I_SS) in Kv1.4−/−/Kv4.2−/− neurons (Figure 3.3A). The further addition of α-Dtx to the bath solution at a concentration of 100 nM had no significant effect on I_A currents in Kv1.4−/−/Kv4.2−/− neurons (not illustrated). Similar experiments were carried out using Heteropodatoxin-2 (Hptx-2), which is specific for Kv4 channels [125]. In contrast to α-Dtx, addition of Hptx-2 (at 1 μM) significantly (p<0.01) reduced I_A amplitudes of in Kv1.4−/−/Kv4.2−/− neurons (Figure 3.3D), revealing a further role for Kv4 α-subunits (i.e. in addition to Kv4.2) in the generation of I_A in cortical pyramidal neurons. Interestingly, previous reports demonstrated that Kv4.3 is expressed in cortical pyramidal neurons [66, 107], suggesting a role for Kv4.3.
Figure 3.3 A Heteropodatoxin-2 sensitive component of $I_A$ is present in 
Kv1.4$^{+/+}$/Kv4.2$^{+/+}$ neurons. Similar to the findings in Kv4.2$^{+-}$ neurons (Figure 1), large 
delayed rectifier currents ($I_K$ and $I_{SS}$) were evident in recordings obtained from Kv1.4$^{+/+}$/4.2 $^{+-}$ neurons (A). Addition of 3 mM TEA to the bath solution (to block the delayed rectifier 
currents), however, revealed a rapidly inactivating current (B) in 18 of 20 Kv1.4$^{+/+}$/4.2 $^{+-}$ 
neurons examined. The amplitude of the $I_A$ component remaining in Kv1.4$^{+/+}$/4.2 $^{+-}$ neurons 
was quantified using the same subtraction protocol as employed in experiments on Kv4.2$^{+/+}$ 
neurons (Figure 1) and Kv1.4$^{+/+}$ neurons (Figure 2) and as described in the legend to Figure 1. The 
mean ± SEM density of the $I_A$ component remaining in Kv1.4$^{+/+}$/4.2$^{+-}$ neurons (n=20) was 
reduced significantly (*p<0.01) by addition of 1 μM Hptx-2 (n=14) to the bath (D).
Targeted disruption of \textit{Kcnd3} reveals a role for Kv4.3 in the generation of I$_A$ in cortical pyramidal neurons

To explore directly the role of Kv4.3 in the generation of I$_A$ in cortical pyramidal neurons, whole-cell Kv current recordings were obtained from neurons isolated from animals harboring a targeted disruption of the \textit{Kcnd3} (Kv4.3$^{-/-}$) locus [93]. In most (20 of 24) of the Kv4.3$^{-/-}$ neurons studied, the waveforms of the Kv currents (Figure 3.4B) were similar to those recorded from WT neurons (Figure 3.4A) with a prominent rapidly inactivating I$_A$ component. Similar to the Kv currents in WT neurons, the inactivation phases of the Kv currents in these (20 of 24) Kv4.3$^{-/-}$ neurons were well described by the sum of three exponentials, consistent with the expression of I$_A$, I$_D$, I$_K$ and I$_{SS}$ (Table 1). The waveforms of the Kv currents in the remaining (4 of 24) Kv4.3$^{-/-}$ neurons (Figure 3.4C), however were distinct, and resembled the current waveforms seen in most Kv4.2$^{-/-}$ neurons (Figure 3.1B) with large delayed rectifier currents and without a prominent rapidly inactivating current component. The prepulse paradigm described previously was used to isolate (and allow the quantification of) I$_A$ in Kv4.3$^{-/-}$ neurons. These experiments revealed that the mean ± SEM density of I$_A$ was significantly (p<0.01) lower in Kv4.3$^{-/-}$ neurons (n=24) than in WT cells (n=22) (Figure 3.4D).
Figure 3.4. Genetic disruption of Kv4.3 reduces $I_A$ density in cortical pyramidal neurons. Whole-cell Kv current recordings revealed heterogeneity in the waveforms of the currents recorded in Kv4.3−/− neurons, specifically in the inactivation phases of currents. In the vast majority (20 of 24) of the Kv4.3−/− neurons (B), the Kv currents were similar to those recorded from WT neurons (A) with a prominent rapidly inactivating current. In the remaining (4 of 24) Kv4.3−/− neurons (Kv4.3−/−*), large delayed rectifier currents ($I_k$ and $I_{ss}$) were evident, reminiscent of the Kv current waveforms observed in Kv4.2−/− neurons (Figure 1), suggesting that marked remodeling of Kv currents also occurs in this subset of Kv4.3−/−* cortical pyramidal neurons. In all Kv4.3−/− neurons (n=24), however, $I_A$ was present and subsequently quantified using the subtraction protocol described in the legend to Figure 1. These analyses revealed that the mean ± SEM $I_A$ density in Kv4.3−/− (n=24) neurons was significantly (*p<0.01) lower than in WT (n=22) neurons (D).
4-AP-sensitive $I_A$ component remains in $\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$ cortical pyramidal neurons

The results of the experiments presented above indicate that in addition to Kv4.2, Kv1.4 and Kv4.3 also encode $I_A$ channels and contribute to the generation of the macroscopic $I_A$ recorded in cortical pyramidal neurons. Accordingly, disruption of both Kv4.2 and Kv4.3 should leave only the Kv1.4-encoded component of $I_A$, a current that would be expected to be blocked completely by 1mM 4-AP. This hypothesis was tested directly by obtaining recordings from neurons isolated from mice lacking both Kv4.2 and Kv4.3 ($\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$), generated by crossing the $\text{Kv4.2}^{-/-}$ and $\text{Kv4.3}^{-/-}$ animals. The waveforms of the Kv currents in $\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$ cortical pyramidal neurons (Figure 3.5A) resembled those in $\text{Kv4.2}^{-/-}$ neurons (Figure 1B) with large delayed rectifier currents and without a prominent rapidly inactivating current component. Inclusion of 3mM TEA in the bath unmasked a rapidly inactivating current in all ($n=18$) $\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$ neurons (Figure 3.5B). Interestingly, the mean ± SEM peak density of the $I_A$ remaining in $\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$ neurons is similar in magnitude to the $I_A$ component eliminated in $\text{Kv1.4}^{-/-}$ neurons (Figure 3.2B). In addition, as illustrated in Figure 3.5C, no rapidly inactivating currents remained in $\text{Kv4.2}^{-/-}/\text{Kv4.3}^{-/-}$ neurons ($n=13$) in the presence of 1 mM 4-AP (Figure 3.5C).
Figure 3.5. $I_A$ in Kv4.2−/Kv4.3− neurons is blocked completely by 1 mM 4-AP. Similar to the findings in Kv4.2−/− neurons (Figure 1), large delayed rectifier currents ($I_K$ and $I_{SS}$) were seen in all records from Kv4.2−/Kv4.3− neurons (A). Addition of 3 mM TEA to the bath solution, however, revealed a rapidly inactivating current component in all Kv4.2−/Kv4.3− neurons (n=18) examined (B). In contrast to WT and Kv4.2−/− neurons (Figure 1), however, the rapidly inactivating current was blocked completely in all Kv4.2−/Kv4.3− (n=13) neurons by addition of 1 mM 4-AP to the bath (C).
The expression of Kv1.4, Kv4.2 and Kv4.3 proteins is independent in the cortex

To examine the relative expression levels of the Kv1.4, Kv4.2, and Kv4.3 proteins in cortex and the impact of the targeted deletion of individual α-subunits, Western blots were conducted on lysates prepared from posterior cortices (containing visual cortex) dissected from WT, Kv1.4\(^{+/−}\), Kv4.2\(^{+/−}\) and Kv4.3\(^{+/−}\) animals (n=4 for each genotype). As illustrated in Figure 3.6A-C, the Kv1.4, Kv4.2 and Kv4.3 proteins were readily detected in the cortical lysates from WT mice, demonstrating that all three of these Kv α-subunits are expressed. In addition, in the samples from Kv1.4\(^{+/−}\), Kv4.2\(^{+/−}\) or Kv4.3\(^{+/−}\) animals, no Kv1.4, Kv4.2 or Kv4.3 protein, respectively, was detected, confirming the specificity of each of the antibodies used. Each blot was also probed with an anti-GAPDH antibody to confirm equal protein loading in each lane. The anti-GAPDH signals were used to normalize the Kv α-subunit-specific antibody signals in each lane. Quantification of the normalized Kv α-subunit-specific antibody signals revealed that the expression levels of Kv1.4 and Kv4.3 in samples from mice with targeted disruption of the other Kv α-subunits were similar to those in WT cortices (Figure 3.6D). The expression of Kv4.2 appeared to be slightly increased in samples from Kv1.4\(^{+/−}\) animals relative to those from WT animals, but the difference did not reach statistical significance (p=0.15 by t-test)(Figure 3.6D). These results indicate the total protein expression levels of the Kv α-subunits encoding individual components of I\(_A\) do not undergo appreciable remodeling in response to the genetic disruption of the other I\(_A\) encoding Kv α-subunits. Nevertheless, it is certainly possible that remodeling of the subcellular distribution of Kv subunits in
different neuronal compartments occurs in one or more of the targeted deletion animals. Alternative experimental approaches need to be employed to explore this possibility.

**Genetic and pharmacologic dissection of $I_A$**

The contributions of Kv1.4, Kv4.2 and Kv4.3 to the generation of the total macroscopic $I_A$ in cortical pyramidal neurons is revealed in direct comparisons of $I_A$ densities in cortical pyramidal neurons of the various genotypes and examined under different pharmacologic conditions (Figure 3.7). As discussed previously, in all experiments conducted on Kv4.2−/− (as well as Kv1.4−/−/Kv4.2−/− and Kv4.2−/−/Kv4.3−/−) neurons 3 mM TEA was used to facilitate the isolation of $I_A$ by reducing the large delayed rectifier currents ($I_K$) that are present in Kv4.2−/− neurons (Figure 3.1). In control experiments on WT cells 3 mM TEA had no effect on $I_A$ (data not shown). Examination of the distributions of $I_A$ densities determined in individual cells reveals that there is considerable heterogeneity in peak $I_A$ densities among WT cortical pyramidal neurons (Figure 3.7A). In both Kv4.2−/− and Kv4.3−/− neurons, the distributions of $I_A$ densities in individual cells are similar to WT neurons, although the mean $I_A$ densities are lower. In the Kv1.4−/−/Kv4.2−/− cortical pyramidal neurons the mean $I_A$ density was similar to that measured in Kv4.2−/− neurons, although the distribution of $I_A$ densities in individual neurons is shifted considerably, revealing that more Kv1.4−/−/Kv4.2−/− neurons displayed low $I_A$ densities. In addition, $I_A$ densities in Kv4.2−/−/Kv4.3−/− neurons were low and tightly clustered (Figure 3.7A).
Figure 3.6. Expression of Kv1.4, Kv4.2, and Kv4.3 subunits in mouse cortex. Western blots on fractionated protein lysates prepared from the posterior cortices of WT, Kv1.4\textsuperscript{-/-}, Kv4.2\textsuperscript{-/-}, and Kv4.3\textsuperscript{-/-} mice were probed with antibodies specific for Kv1.4 (A), Kv4.2 (B), or Kv4.3(C). The blots were subsequently probed with an anti-GAPDH antibody for quantification purposes and to verify equal loading of proteins in each lane. The anti-GAPDH signals were used to normalize the anti-Kv \(\alpha\)-subunit signals in the same lane. No significant differences in the mean ± SEM (n=4 for each genotype) expression levels of the Kv1.4, Kv4.2 or Kv4.3 proteins were observed (D), although there appears to be a trend (that did not reach statistical significance) towards increased Kv4.2 expression and a decreased Kv4.3 expression in the Kv1.4\textsuperscript{-/-} samples.
Analyses of results of the experiments conducted using the various pharmacological manipulations yielded similar conclusions. Consistent with roles for Kv4 and Kv1 channels in the generation of I_A, for example, both Ba^{2+} and 4-AP reduced the density of I_A in WT cortical pyramidal neurons. In addition, the 1 mM 4-AP-resistant component of I_A was reduced in Kv4.2^{-/-}, relative to WT, neurons, consistent with the loss of the Kv4.2-encoded component of I_A. Further, the component of I_A remaining in Kv4.2^{-/-}/Kv4.3^{-/-} neurons was eliminated completely by 1 mM 4-AP, indicating that the 4-AP resistant component of I_A in Kv4.2^{-/-} neurons is encoded by Kv4.3. Finally, complete block of the component of I_A remaining in Kv4.2^{-/-}/Kv4.3^{-/-} cells by 1 mM 4-AP is consistent with Kv1.4 containing channels encoding this component of I_A.
**Figure 3.7. Summary of the genetic and pharmacologic dissection of $I_A$ in cortical pyramidal neurons** (A) Peak $I_A$ densities in individual cells of each genotype (at +30 mV) are plotted; mean values are indicated by the horizontal bars. Considerable heterogeneity in $I_A$ densities was evident in WT, Kv1.4−/−, Kv4.2−/− and, Kv4.3−/− neurons. The mean ± SEM $I_A$ densities in Kv4.2−/− and Kv4.3−/− neurons were significantly (p<0.01) lower than in WT neurons. In addition, $I_A$ densities in Kv1.4−/−/Kv4.2−/− neurons were skewed towards lower densities although $I_A$ density was high in a few cells. In Kv4.2−/−/Kv4.3−/− cells, $I_A$ densities were low and closely clustered. (B) $I_A$ densities determined (at +30 mV) in experiments combining pharmacological blockers of individual components of $I_A$ with the targeted genetic disruption of individual Kv channel α-subunits, are plotted. Mean $I_A$ density was reduced in WT cells by 400 µM Ba²⁺ and by 1 mM 4-AP. Elimination of Kv4.2 reduced the density of the 4-AP-resistant component of $I_A$, whereas the combined disruption of Kv4.2 and Kv4.3 expression left only the component of $I_A$ that was blocked completely by 1 mM 4-AP.
Discussion

Multiple components of $I_A$ in cortical pyramidal neurons

A systematic experimental approach, employing genetic disruption of Kv α-subunit expression paired with pharmacology, was utilized to identify the Kv channel α-subunits responsible for the generation of macroscopic $I_A$ in cortical pyramidal neurons. The results presented here demonstrate that Kv1.4, Kv4.2 and Kv4.3 all contribute to macroscopic $I_A$ in (mouse visual) cortical pyramidal neurons. In Kv1.4$^{-/-}$, Kv4.2$^{-/-}$ and Kv4.3$^{-/-}$ neurons, a component of $I_A$ is lost. The components of $I_A$ encoded by Kv1.4 and Kv4.3 were individually isolated in neurons (Kv4.2$^{-/-}$/Kv4.3$^{-/-}$ and Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ neurons, respectively) with combined genetic disruption of the other two Kv α-subunits. In each case, the remaining component of $I_A$ was blocked selectively by 4-AP or Hptx-2 (respectively). In addition, the component of $I_A$ remaining in Kv1.4$^{-/-}$/Kv4.2$^{-/-}$ neurons was sensitive to Hptx-2 but not to α-Dtx, indicating that Kv1 α-subunits do not contribute to $I_A$ in cortical pyramidal neurons in the absence of Kv1.4. To the best of our knowledge, this study represents the first complete molecular dissection of $I_A$ in mammalian neurons and, in addition, provides the first direct demonstration of a native neuronal Kv1.4-encoded current.

Precise determination of the contributions of Kv1.4-, Kv4.2- and Kv4.3-encoded channels to the generation of the macroscopic $I_A$ in individual WT neurons has been limited by the lack of potent blockers specific for channels encoded by these α-subunits. The limitations of pharmacology can be seen in Figure 3.7 where the reduction of $I_A$
density in WT neurons due to 1mM 4-AP is larger than expected if 1 mM 4-AP specifically and selectively blocks only Kv1-encoded channels. The magnitude of the reduction in $I_A$ density by 1 mM 4-AP suggests, probably not surprisingly, some block of Kv4 channels at this (1 mM) concentration. The use of targeted gene disruption is specific, but is hindered by the now well documented electrical remodeling evident in neurons when the expression of the normal channel repertoire is altered [48, 126, 127]. The summary data (Figure 3.7) indicate that Kv4.2 and Kv4.3 α-subunits are the major contributors to macroscopic $I_A$ in cortical pyramidal neurons. The cumulative results also indicate that Kv1.4-encoded channels contribute a minor component of $I_A$, expressed at a lower density in cortical pyramidal neurons than the Kv4-encoded components (Figure 3.7A). The recordings here were obtained from young postnatal cortical pyramidal neurons and it is certainly possible that the relative contributions of Kv1.4, Kv4.2 and Kv4.3 to the total $I_A$ changes during development. Interestingly, the biochemical data revealed the robust expression of Kv1.4, Kv4.2 and Kv4.3 in adult cortex (Figure 3.6). Previous studies suggest that the physiological properties of cortical neurons do change during postnatal development although the major effects appear to be quantitative changes in current densities, rather than qualitative changes in current properties/types [128, 129]. It seems reasonable to suggest, therefore, that Kv1.4, Kv4.2 and Kv4.3 all contribute to $I_A$ is cortical pyramidal neurons throughout postnatal development. As neurons mature, however, the subcellular distribution patterns and/or functional roles of individual channel types may change. Further experiments will be necessary to explore these questions directly.
Remodeling of Kv currents in response to the loss of I_A encoding α-subunits

The experiments here also revealed that Kv current remodeling is evident in Kv1.4\(^{-/-}\), Kv4.2\(^{-/-}\), and Kv4.3\(^{-/-}\) neurons. The characteristics of the Kv current remodeling, however, were different in each case. In Kv1.4\(^{-/-}\) neurons, a small increase in I\(_{SS}\) was seen in conjunction with an increase in a Ba\(^{2+}\) sensitive (Kv4-encoded) rapidly inactivating current. In the majority (~80%) of Kv4.2\(^{-/-}\) neurons, marked differences in Kv current waveforms were evident, reflecting increased TEA-sensitive delayed rectifier currents (Figure 3.1) [48]. Surprisingly, only 20% of Kv4.3\(^{-/-}\) neurons displayed a similar remodeling (Figure 3.4C) despite similar decreases in mean I_A density in Kv4.2\(^{-/-}\) and Kv4.3\(^{-/-}\) neurons (Figure 3.7A). These observations illustrate the complexity of ascertaining the functional roles of individual channel α-subunits by using genetic disruption alone. Interestingly, other studies have reported changes occurring at the circuit level in Kv4.2\(^{-/-}\) mice. Specifically, experiments on acute brain slices from Kv4.2\(^{-/-}\) mice revealed increased inhibition of hippocampal pyramidal neurons mediated by an increase in tonic GABA currents [121], suggesting that widespread, compensatory changes in neuronal properties and excitability can occur in response to alterations in Kv α-subunit expression.

Molecular diversity of Kv4 channels

The results presented here demonstrate that both Kv4.2 and Kv4.3 can form functional channels in cortical pyramidal neurons independent of each other. Finding functional Kv4.3 channels independent of Kv4.2 expression in cells that normally express
both is somewhat surprising given that cardiac myocytes from Kv4.2−/− mice have no remaining Kv4-encoded current despite the expression of Kv4.3 [92]. Although the experiments here demonstrate that Kv4.2 and Kv4.3 can function independently, previous studies have shown that Kv4.2 and Kv4.3 can be co-immunoprecipitated from brain, consistent with the presence of heteromultimeric Kv4.2/Kv4.3 channels [98]. The formation of functional homomultimeric or heteromultimeric Kv4 channels is also consistent with the partially overlapping subcellular localization of Kv4.2 and Kv4.3 in cortical neurons [107]. The molecular diversity of neuronal Kv4 channels could enable precise and independent modulation of multiple neuronal processes, as well as differential sensitivities to multiple regulatory pathways. The functional diversity of neuronal Kv4 channels is likely further expanded by the co-expression of numerous accessory subunits, such as the K⁺ Channel Interacting Proteins, KChIPs, and Dipeptidyl Peptidases, DPP 6 and 10 [8, 44, 76].

**Implications for future studies on Kv channel macromolecular complexes**

Numerous recent studies suggest that neuronal Kv channels, like other types of ion channels, function as components of macromolecular protein complexes [65, 130]. Identification of the Kv α-subunits responsible for the generation of specific Kv currents is a critical first step in determining the composition of functional Kv channel complexes and the roles individual Kv α- and accessory subunits play in controlling channel properties and in regulating neuronal excitability. Knowing that Kv1.4, Kv4.2 and Kv4.3 encode distinct components of I_A in cortical pyramidal neurons, therefore, provides a
foundation for studies aimed at defining the physiological roles of accessory subunits and other regulatory proteins in the generation of functional I_A channel complexes.

In spite of the many studies in heterologous expression systems, very little is known about the *in situ* functioning of Kv channel accessory subunits and translating findings from heterologous systems to native cells has proven difficult. For example, studies in heterologous cells suggest that DPP6 plays a dominant role in determining the kinetic properties of Kv4-encoded currents [76]. A recent study examining the effects of disrupting DPP6 expression in hippocampal neurons, however, described very small changes in the properties of I_A, although there were marked and unexpected alterations in neuronal excitability [131]. Although the authors interpreted the functional effects in terms of changes in I_A, the experiential observations may, in part, reflect electrical remodeling with knockdown of DPP6, as is evident in response to disruption of Kv channel α-subunit expression [48, 92]. Interestingly, several channel accessory subunits have been suggested to interact with and differentially regulate multiple types of ion channels [132-135], highlighting the possibility that accessory subunits may play complex roles in regulating different types of channels, as well as in orchestrating electrical remodeling. The demonstration here that Kv1.4, Kv4.2 and Kv4.3 each encode a component of I_A in cortical pyramidal neurons and that varied electrical remodeling occurs in response to the disruption of Kv α-subunit expression will facilitate the design and, perhaps most importantly, the interpretation of experiments focused on defining the roles of I_A channel accessory and regulatory proteins.
Chapter 4

Interdependent Roles for the Accessory Subunits KChIP2, KChIP3 and KChIP4 in the Generation of Kv4-encoded $I_A$ Channels in Cortical Pyramidal Neurons
Abstract

The rapidly activating and inactivating voltage-dependent outward K\(^+\) (K\(_v\)) current, \(I_A\), is widely expressed in central and peripheral neurons. \(I_A\) has long been recognized to play important roles in determining neuronal firing properties and regulating neuronal excitability. Previous work demonstrated that Kv4.2 and Kv4.3 \(\alpha\)-subunits are the primary determinants of \(I_A\) in mouse cortical pyramidal neurons.

Accumulating evidence indicates that native neuronal Kv4 channels function in macromolecular protein complexes that contain accessory subunits and other regulatory molecules. The K\(^+\) Channel Interacting Proteins (KChIPs) are among the identified Kv4 channel accessory subunits and are thought to be important for the formation and functioning of neuronal Kv4 channel complexes. Molecular genetic, biochemical and electrophysiological approaches were exploited in the experiments described here to examine directly the roles of KChIPs in the generation of functional Kv4-encoded \(I_A\) channels. These combined experiments revealed that KChIP2, KChIP3 and KChIP4 are robustly expressed in adult mouse posterior (visual) cortex and that all three proteins co-immunoprecipitate with Kv4.2. In addition, in cortical pyramidal neurons from mice lacking KChIP3 (KChIP3\(^{-/-}\)), mean \(I_A\) densities were reduced modestly, whereas in mean \(I_A\) densities in KChIP2\(^{-/-}\) and WT neurons were not significantly different. Interestingly, in both KChIP3\(^{-/-}\) and KChIP2\(^{-/-}\) cortices the expression levels of the other KChIPs (KChIP2 and 4 or KChIP3 and 4, respectively) were increased. In neurons expressing constructs to mediate simultaneous RNA
interference-induced reductions in the expression of KChIP2, 3 and 4, I_A densities were markedly reduced and Kv current remodeling was evident.
**Introduction**

The rapidly activating and inactivating voltage-dependent outward $K^{+}$ ($Kv$) current, $I_A$, is widely expressed in central and peripheral neurons [50]. $I_A$ has long been recognized to play important roles in determining neuronal firing properties [51, 52] and regulating neuronal excitability under normal [53] and pathologic conditions, such as epilepsy [54]. Recent studies have also demonstrated that alterations in the functional expression of $I_A$ in dendritic compartments is important for regulating the back propagation (into dendrites) of action potentials, synaptic potentiation and dendritic integration [10, 56]. Recognition of the many important roles that $I_A$ plays in neuronal processing has focused considerable effort on understanding the mechanisms that determine the functional expression of $I_A$ channels.

Substantial progress has been made in understanding the roles of $Kv$ pore-forming ($\alpha$) subunits in the generation of $I_A$. In hippocampal pyramidal and dorsal horn neurons, $Kv4.2$ $\alpha$-subunits encode $I_A$ [67, 105] whereas, in dorsal root ganglion neurons and hippocampal interneurons, $Kv4.3$ has been suggested to generate $I_A$ [136, 137]. In mouse cortical pyramidal neurons, however, $I_A$ is encoded by $Kv4.2$, $Kv4.3$ and $Kv1.4$ $\alpha$-subunits [96]. Considerable evidence indicates that neuronal $Kv4$ channels function in macromolecular complexes that contain accessory subunits and other regulatory molecules [98, 138, 139]. Multiple putative $Kv4$ accessory subunits have been identified including: $Kv\beta$ subunits [69], Dipeptidyl Peptidase (DPP) family...
members (DPP6 and DPP10) [63, 76], MinK/MiRP family members [140], as well as K^+ Channel Interacting Proteins (KChIP1, KChIP2, KChIP3 and KChIP4) [71, 72].

Several previous studies in heterologous expression systems have demonstrated that coexpression of one or more of these Kv channel accessory subunits dramatically alters the properties and functional expression of Kv4-encoded currents [44, 141, 142]. Coexpression with one of the KChIPs, for example, results in Kv4 currents that inactivate more slowly and recover from inactivation more quickly than the currents generated by Kv4 α-subunits expressed alone [71]. Also, the coexpression of KChIPs markedly increases Kv4-encoded current densities [75]. Interestingly, heterologous coexpression of other accessory subunits, such as the DPPs and Kvβs, with Kv4 α-subunits also alters densities and properties of Kv4-encoded currents [76, 143]. Although the many studies conducted in heterologous cells have informed our understanding of the interactions of accessory subunits with Kv4 α-subunits, little is presently known about the functional roles played by accessory subunits in the generation of native neuronal Kv4 channel complexes. A combination of biochemical and molecular genetic tools was exploited in the studies described here to examine directly the functional roles of the KChIPs in regulating/modulating Kv4-encoded currents in (mouse visual) cortical pyramidal neurons.
Results

Disruption of KChIP3 expression decreases I\textsubscript{A} density in cortical pyramidal neurons

The finding that expression of KChIP3 protein was markedly decreased in the cortices of mice harboring a targeted disruption of the \textit{Kcnid2} (Kv4.2) locus [48] suggested that KChIP3 may play an important role in the generation of functional Kv4-encoded I\textsubscript{A} channels in cortical neurons. To explore this hypothesis, whole-cell Kv currents, evoked in response to steps to depolarized potentials (-40 mV through +40 mV in 10 mV increments) from a holding potential of -70 mV, were examined in cortical pyramidal neurons isolated from WT and KChIP3\textsuperscript{-/-} mice. The waveforms of the Kv currents recorded from WT (Figure 4.1A) and KChIP3\textsuperscript{-/-} (Figure 4.1B) neurons were similar, with prominent rapidly inactivating current components (I\textsubscript{A}). To facilitate the quantification of I\textsubscript{A}, Kv currents were also recorded from each cell using a prepulse paradigm (Figure 4.1Ab and Bb) to inactivate I\textsubscript{A} selectively [96]; an additional 60 ms step to -10 mV was included prior to the depolarizing voltage steps (see paradigm illustrated in Figure 4.1). Offline subtraction of the records obtained using the prepulse paradigm from the control records (obtained from the same cell) allowed the isolation of I\textsubscript{A} (Figure 4.1Aa-b and 1Ba-b). Analysis of I\textsubscript{A} amplitudes in these subtracted records revealed that the mean ± SEM I\textsubscript{A} density was modestly, but significantly (p<0.05), lower in KChIP3\textsuperscript{-/-} (n=24), compared to WT (n=22), cortical pyramidal neurons (Figure 4.1C).
In addition to the rapidly inactivating $I_A$, previous studies [46-49, 96] have identified the presence of additional Kv currents in cortical pyramidal neurons: $I_D$, which inactivates more slowly than $I_A$ (tau inactivation ~250ms); $I_K$, which inactivates very slowly (tau inactivation ~2 s); and the non-inactivating current, $I_{SS}$. Analysis of the decay phases of Kv currents in WT and KChIP3$^{-/-}$ neurons revealed that, unlike $I_A$, the mean ± SEM amplitudes/densities of $I_D$, $I_K$ and $I_{SS}$ were not significantly affected by the loss of KChIP3 (data not shown). The selective reduction of $I_A$ in KChIP3$^{-/-}$ cortical pyramidal neurons suggests that KChIP3 plays an important role in the generation and/or functioning of Kv4 channel complexes. The magnitude of $I_A$ remaining in KChIP3$^{-/-}$ cortical pyramidal neurons (Figure 4.1C) also suggests that other KChIPs likely contribute to the generation of functional Kv4 channels. To examine the possible role of KChIP2, Kv current recordings were obtained from neurons isolated from mice (KChIP2$^{-/-}$) harboring a targeted disruption of the gene ($Kcnip2$) encoding KChIP2 [94]. Analysis of the Kv currents using the prepulse paradigm described above, however, revealed that the mean ± SEM $I_A$ density in KChIP2$^{-/-}$ neurons (n=19) was not significantly different from WT cells (n=22) (not illustrated).
Figure 4.1. $I_A$ density is reduced in KChIP3⁻/⁻ cortical pyramidal neurons. (A, B) Whole-cell Kv current recordings were obtained from cortical pyramidal neurons isolated from wild type (WT) and KChIP3⁻/⁻ mice. Representative recordings from WT (A) and KChIP3⁻/⁻ (B) neurons are illustrated. In each cell, Kv currents were elicited by depolarizing voltage steps ranging from -40 mV to +40 mV in 10 mV increments from a holding potential of -70 mV (a). Recordings were then obtained from the same cell using a prepulse paradigm in which the same depolarizing steps, preceded by a prepulse of 60 ms to -10 mV to selectively inactive $I_A$, were presented (b). The paradigms are illustrated in the insets. For each cell, currents recorded with the prepulse (b) were subtracted offline from the control records (a) to isolated $I_A$ (a-b). Similar recordings were obtained from WT (n=22) and KChIP3⁻/⁻ (n=24) neurons. (C) $I_A$ densities were calculated from the subtracted records and mean ± SEM $I_A$ densities are plotted. Mean ± SEM $I_A$ densities were modestly, but significantly (* p<0.05), lower in KChIP3⁻/⁻, compared to WT, neurons.
Co-regulated expression of KChIP2, 3 and 4 in visual cortex

Previous studies have suggested that expression of KChIP1 is restricted to non-pyramidal interneurons in the cortex [144, 145]. As a result, KChIP1 was not considered in the analysis here. As illustrated in Figure 4.2A, mRNA transcripts encoding KChIP2, KChIP3 and KChIP4 were readily detected in RNA samples collected from the posterior (~1 mm) cortices of WT mice (see Material and Methods) consistent with previous reports examining KChIP expression in rodent brain [79, 144]. Immunoprecipitation experiments using a rabbit anti-Kv4.2 antibody revealed that Kv4.2 was readily detected in fractionated protein samples from WT cortices, but not in samples from Kv4.2<sup>−/−</sup> cortices. Also consistent with previous reports [98, 144, 146], Kv4.3 co-immunoprecipitated with Kv4.2 from WT cortical samples (Figure 4.2B). No Kv4.3, however, was detected (Figure 4.2B) in the samples from Kv4.2<sup>−/−</sup> cortices, indicating that the anti-Kv4.2 antibody does not immunoprecipitate Kv4.3 in the absence of Kv4.2 and does not, therefore, cross react with Kv4.3. KChIP2, KChIP3 and KChIP4 also co-immunoprecipitated with Kv4.2 from WT, but not Kv4.2<sup>−/−</sup>, cortical samples (Figure 4.2C). The finding that KChIP2, KChIP3 and KChIP4 co-immunoprecipitate with Kv4.2 further suggests a role for each of these KChIPs in the generation of Kv4.2 channel complexes in cortical pyramidal neurons.
Figure 4.2. Expression of KChIP2, KChIP3 and KChIP4 in cortex and co-immunoprecipitation with Kv4.2. To examine the expression of KChIP transcripts, RNA was extracted from samples prepared from the posterior (~1 mm) cortex (containing visual cortex) of adult WT mice. (A) Using sequence specific primer pairs and RT-PCR, transcripts coding for KChIP2, KChIP3, and KChIP4 were readily detected. Experiments were also performed to examine KChIP protein expression and association with Kv4.2. Immunoprecipitations using a rabbit anti-Kv4.2 antibody were conducted on lysates prepared from the posterior cortices of WT or Kv4.2-/- mice and blots of fractionated immunoprecipitated samples were probed using subunit specific antibodies. (B) In immunoprecipitated samples from WT cortices, but not in those from Kv4.2-/- cortices, both Kv4.2 and Kv4.3 were readily detected. (C) In immunoprecipitated samples from WT, but not Kv4.2-/- cortices, KChIP2, KChIP3 and KChIP4 were also detected indicating that all three KChIP proteins are expressed and assemble with Kv4.2 in cortex. Molecular masses are indicated on the blots in kDa.
In parallel experiments, fractionated protein samples prepared from WT and KChIP3\(^{-/-}\) cortices (n=6 animals for each genotype) were probed with specific anti-KChIP2, anti-KChIP3 and anti-KChIP4 antibodies (Figure 4.3A). The signals from each anti-KChIP antibody were measured and normalized to the signal from the anti-β tubulin antibody in the same lane. Quantitative analysis of multiple blots revealed that the mean ± SEM expression levels of both KChIP2 and KChIP4 were significantly (p<0.02) higher in the KChIP3\(^{-/-}\), relative to the WT, samples (Figure 4.3B). Parallel experiments on KChIP2\(^{-/-}\) cortical lysates revealed that the mean ± SEM protein levels of both KChIP3 and KChIP4 were significantly (p<0.002) higher in KChIP2\(^{-/-}\), compared to WT, samples (Figure 4.3A, B). To explore the role of transcriptional remodeling, RNA was isolated from the (posterior) cortices of KChIP3\(^{-/-}\) and WT mice (n=6 for each genotype) and KChIP2 and KChIP4 transcript expression levels were examined. As illustrated in Figure 4.3C, the mean ± SEM expression level of the transcript encoding KChIP2 was similar in KChIP3\(^{-/-}\) and WT samples. The mean ± SEM level of KChIP4 transcript expression was significantly (p<0.05) higher in KChIP3\(^{-/-}\), relative to WT cortices, although the magnitude (~ 30%) of the increase was much smaller than the twofold increase in KChIP4 protein expression.

Parallel experiments were completed to examine the expression levels of the Kv4.2 and Kv4.3 proteins in KChIP2\(^{-/-}\) and KChIP3\(^{-/-}\) cortices. In contrast to the marked changes in KChIP protein expression, analyses of Western Blots on fractionated protein lysates prepared from the (posterior) cortices of KChIP2\(^{-/-}\),
KChIP3−/− and WT mice (n=6 for each genotype) probed with specific antibodies against either Kv4.2 or Kv4.3 (Figure 4.4A) revealed that the mean ± SEM expression levels of the Kv4.2 and Kv4.3 proteins were not significantly different in samples from KChIP2−/− or KChIP3−/−, relative to WT, cortices (Figure 4.4B).
Figure 4.3. KChIPs are upregulated in KChIP2⁻/⁻ and KChIP3⁻/⁻ cortices.
Figure 4.3. KChIPs are up regulated in KChIP2\textsuperscript{−/−} and KChIP3\textsuperscript{−/−} cortices. (A) Lysates prepared from the posterior (~1 mm) cortices of WT, KChIP2\textsuperscript{−/−} and KChIP3\textsuperscript{−/−} mice (n=6 animals for each genotype) were fractionated, transferred to PVDF membranes and probed with a specific anti-KChIP2, anti-KChIP3 or anti-KChIP4 antibody. All three KChIPs were detected in samples from WT mice. Confirming the specificities of the anti-KChIP2 and anti-KChIP3 antibodies, no signal was detected with the anti-KChIP2 or the anti-KChIP3 antibody in samples from KChIP2\textsuperscript{−/−} or KChIP3\textsuperscript{−/−} cortices, respectively. Blots were also probed with antibodies against β-tubulin to confirm equal loading of proteins. In each lane, anti-KChIP antibody signals were quantified and normalized to the anti-β-tubulin antibody signals. (B) In KChIP2\textsuperscript{−/−} cortices, the mean ± SEM expression levels of KChIP3 and KChIP4 proteins were significantly (+ p<0.01) higher than in WT cortices. Similarly, the mean ± SEM expression levels of the KChIP2 and KChIP4 proteins were significantly (*p<0.05 and +p<0.01, respectively) higher in KChIP3\textsuperscript{−/−} cortices. (C) QRT-PCR analysis revealed that the mean ± SEM expression level of KChIP2 transcript was not significantly different in WT (n=6) and KChIP3\textsuperscript{−/−} (n=6) cortices, whereas the mean ± SEM expression level of KChIP4 transcript was slightly, but significantly (*p>0.05), higher in cortices from KChIP3\textsuperscript{−/−}, compared with WT, mice. Molecular masses are indicated on the blots in kDa.
Figure 4.4. Maintained expression of Kv4.2 and Kv4.3 proteins in KChIP2<sup>-/-</sup> and KChIP3<sup>-/-</sup> cortices. Lysates were prepared from the posterior (~1 mm) cortices of WT, KChIP2<sup>-/-</sup> and KChIP3<sup>-/-</sup> mice (n=6 of each genotype) and fractionated by SDS-PAGE. Following transfer, membranes were probed with a monoclonal anti-Kv4.2 or anti-Kv4.3 antibody and, subsequently, with an anti-GAPDH antibody, to verify equal loading of proteins in each lane. Signals from the anti-Kv4.2 and anti-Kv4.3 antibodies in each lane were quantified and normalized to signals from the anti-GAPDH antibody in the same lane. Molecular masses are indicated on the blots in kDa. (B) Mean ± SEM levels of Kv4.2 and Kv4.3 proteins are not significantly different in either KChIP2<sup>-/-</sup> or KChIP3<sup>-/-</sup>, compared to WT, cortices.
miRNA mediated RNAi knockdown of KChIP2, 3 and 4 expression

The biochemical experiments presented above suggest that the KChIPs are able to compensate for one another. An RNA interference (RNAi) based strategy was developed, therefore, to allow for the simultaneous knockdown of KChIP2, 3 and 4 expression in cortical pyramidal neurons. Briefly, using a previously described approach [84], plasmids encoding a targeting miRNA and a fluorescent protein (CFP, YFP or tdTomato) were designed such that both components are present in a single transcript that is spliced apart and the components are processed separately (as illustrated in Figure 4.5A). Separation of the miRNA and the fluorescent protein coding sequences by RNA splicing enhances the expression the fluorescent protein because the coding region is not degraded during the processing of the miRNA [84], allowing for robust expression of the fluorophore as a faithful reporter of miRNA expression in transfected neurons. The miRNA plasmids described here allowed for the visual identification of transfected neurons expressing multiple miRNA constructs (as illustrated Figure 4.5B) for subsequent electrophysiological recordings.

Constructs containing individual sequences targeting KChIP2, KChIP3 or KChIP4 were co-transfected into HEK-293 cells with a plasmid encoding the targeted KChIP to identify sequences able to suppress the expression of each of the KChIPs. The effectiveness of each targeting sequence in reducing the expression of the targeted KChIP, compared to non-targeting control sequence, was assayed using Western Blots performed on lysates prepared from transfected (HEK-293) cells. Of
the individual sequences tested, those listed in Material and Methods markedly suppressed the expression of KChIP2, KChIP3 or KChIP4 (Figure 4.5C) and were used in subsequent experiments in neurons.
Figure 4.5. Validation of miRNA constructs to mediate RNAi based knockdown of KChIP2, KChIP3 or KChIP4 in neurons.
Figure 4.5. Validation of miRNA constructs to mediate RNAi based knockdown of KChIP2, KChIP3 or KChIP4 in neurons. As described in Materials and Methods, plasmids encoding human miR30, with substituted targeting sequences and a fluorescent protein (YFP, CFP or tdTomato) on a single transcript, were generated. (A) The miR30 sequence was placed on an intron downstream of the CMV promoter and upstream of the sequence coding for the fluorescent protein (CFP, YFP or tdTomato). (B) Transfections of these plasmids into neurons allowed for visual identification of neurons expressing one or all three of the plasmids for subsequent electrophysiological recording. (C) Specific sequences targeting KChIP2, KChIP3 and KChIP4 were screened in HEK-293 cells. The targeted KChIP (KChIP2, 3 or 4) was co-expressed with either a control (non-targeting) miRNA construct or with a miRNA construct containing sequence complementary to the sequence of the targeted KChIP. Lysates were prepared from transfected HEK-293 cells, fractionated by SDS-PAGE, transferred to membranes and probed for KChIP2, KChIP3 or KChIP4. Blots were also probed with an anti-transferrin receptor (Transferrin R) antibody to verify equal loading of proteins. Targeting sequences found to reduce the expression of each of the targeted KChIPs are illustrated and were used in subsequent experiments, on cortical neurons.
Knockdown of KChIP4 expression in KChIP3\(^{-/-}\) neurons decreased \(I_A\) density

The finding that the KChIP4 protein is upregulated in KChIP3\(^{-/-}\) (and in KChIP2\(^{-/-}\)) neurons (and may compensate for the loss of KChIP3 or KChIP2) was initially surprising because previous reports have suggested that KChIP4 acts to suppress, rather than promote, the surface expression of Kv4 channels [75, 147, 148]. To test directly the hypothesis that KChIP4 plays a role in the expression of functional Kv4-encoded \(I_A\) channels, Kv current recordings were obtained from KChIP3\(^{-/-}\) neurons transfected with a plasmid encoding either the control (non-targeting) miRNA (Figure 4.6A) or the miRNA targeting KChIP4 (Figure 4.6B). Parallel recordings were obtained from neurons expressing one (of the two) control non-targeting plasmids. One of these contained a scrambled targeting sequence (\(n=17\)) and the other contained a control sequence targeting luciferase (\(n=20\)). Analysis of the current records revealed no differences and the results were pooled.

Analysis of subtracted records (Figure 4.6A,B,a-b) from KChIP3\(^{-/-}\) neurons transfected with control (\(n=37\)) or KChIP4 targeting (\(n=16\)) plasmids revealed that mean ± SEM \(I_A\) density was significantly (\(p<0.05\)) lower in neurons expressing the KChIP4 targeting miRNA, compared to cells transfected with control miRNA (Figure 4.6C). As illustrated in Figure 4.6B, the knockdown of KChIP4 in KChIP3\(^{-/-}\) neurons resulted in marked changes in the Kv current waveforms, changes reminiscent of those previously described in Kv4.2\(^{-/-}\) cortical pyramidal neurons [48, 96]. Analysis of the inactivation phases of the Kv currents revealed that the mean ± SEM amplitudes
of the delayed rectifier currents, $I_K$ and $I_{SS}$, were significantly ($p<0.01$) larger in KChIP3$^{-/-}$ neurons expressing the KChIP4 targeting miRNA, compared to KChIP3$^{-/-}$ neurons transfected with control miRNA (Figure 4.6D). In contrast, the mean ±SEM densities of $I_D$ were not significantly different in control and KChIP4 targeting miRNA-expressing cells (Figure 4.6D).
Figure 4.6. Knockdown of KChIP4 in KChIP3−/− cortical pyramidal neurons results in decreased $I_A$ density and upregulation of $I_K$ and $I_{ss}$. 
Figure 4.6. Knockdown of KChIP4 in KChIP3<sup>−/−</sup> cortical pyramidal neurons results in decreased $I_A$ density and upregulation of $I_K$ and $I_{ss}$. (A, B) Whole-cell Kv currents, elicited in response to depolarizing voltage steps, were recorded from transfected cortical pyramidal neurons isolated from KChIP3<sup>−/−</sup> mice. Neurons were transfected by electroporation using the Amaxa Nucleofector system at the time of isolation with a miRNA construct containing either sequence targeting KChIP4 or a control non-targeting sequence; whole-cell recordings were obtained on the second and third days following transfections. $I_A$ was isolated and quantified using the prepulse paradigm and offline subtraction method described in the legend to Figure 1. (C) Analysis of the subtracted records (a-b) revealed that mean ± SEM $I_A$ densities were significantly (*p<0.05) lower in KChIP3<sup>−/−</sup> neurons expressing miRNA targeting KChIP4 (n=16) compared to KChIP3<sup>−/−</sup> neurons expressing control miRNA (n=37). (D) Analysis of the inactivation phases of the Kv currents also revealed that, in neurons expressing KChIP4 targeting miRNA, the mean ± SEM densities of $I_K$ and $I_{ss}$ were significantly (+p<0.01) higher than in control miRNA expressing neurons.
Attenuation of Kv4-encoded currents and remodeling of Kv currents with simultaneous knockdown of KChIP2, 3 and 4 in Kv1.4−/− neurons

The results of the experiments described above suggest that KChIP2, KChIP3 and KChIP4 all contribute to the generation of functional of Kv4 channels and, in addition, that the KChIPs functionally compensate for one another. Experiments were undertaken, therefore, to examine the effects of concurrently knocking down the expression of KChIP2, 3 and 4 on Kv4-encoded IA. These experiments were performed in neurons isolated from mice (Kv1.4−/−) harboring a targeted disruption of the gene (Kcna4) encoding for Kv1.4 to allow for the analysis of effects on Kv4-encoded IA without contamination from the Kv1.4-encoded component of IA [96]. Whole-cell Kv current recordings were obtained from Kv1.4−/− neurons (n=20) visually identified to be expressing the three miRNA constructs targeting KChIP2, 3 and 4 or neurons (n=21) transfected with control plasmids (Figure 4.7A-B). Analyses of the subtracted current records revealed residual Kv4-encoded IA in all Kv1.4−/− neurons expressing the three KChIP targeting miRNA constructs (see Discussion). The mean ± SEM IA density, however, was significantly (p<0.001) lower in Kv1.4−/− neurons expressing all three of KChIP targeting miRNA compared to Kv1.4−/− neurons expressing control constructs (Figure 4.7C).

In addition to the marked reductions in IA densities in the subtracted current records (a-b), the Kv current waveforms in Kv1.4−/− neurons expressing the KChIP targeting miRNAs were quite heterogeneous. In about half (11 of 20) of the Kv1.4−/−
neurons expressing the KChIP targeting miRNAs, no prominent rapidly inactivating current component was evident and delayed rectifier Kv currents were increased (Figure 4.7B) reminiscent of the Kv current waveforms in Kv4.2<sup>-/-</sup> neurons, in which upregulated I<sub>K</sub> and I<sub>SS</sub> mask the residual I<sub>A</sub> [48, 96]. In the remaining Kv1.4<sup>-/-</sup> neurons expressing the KChIP targeting miRNAs (9 of 20), a rapidly inactivating component was clearly evident in the macroscopic Kv current waveforms (not illustrated). The marked alteration in the Kv current waveforms and the masking of the residual I<sub>A</sub> component in many of the Kv1.4<sup>-/-</sup> neurons expressing the KChIP targeting miRNAs (Figure 4.7B) suggests a greater upregulation in the amplitudes of I<sub>K</sub> and I<sub>SS</sub> than in KChIP3<sup>-/-</sup> neurons expressing the KChIP4 targeting miRNA (Figure 4.6D). Consistent with remodeling of I<sub>K</sub> and I<sub>SS</sub>, mean ± SEM peak Kv current densities were not significantly different in neurons expressing the three KChIP targeting miRNA constructs, compared to those expressing control miRNA constructs (Figure 4.7D), despite the marked decrease in mean I<sub>A</sub> densities (Figure 4.7C).
Figure 4.7. Concurrent knockdown of KChIP2, KChIP3 and KChIP4 in Kv1.4⁻/⁻ cortical pyramidal neurons results in marked reductions in Kv4-encoded $I_A$ densities and Kv current remodeling.
Figure 4.7. Concurrent knockdown of KChIP2, KChIP3 and KChIP4 in Kv1.4−/−
cortical pyramidal neurons results in marked reductions in Kv4-encoded IA densities
and KV current remodeling. To examine the combined role(s) of the KChIPs in the
generation of Kv4-encoded IA channels, cortical pyramidal neurons were isolated from
Kv1.4−/− mice and transfected with the validated miRNA constructs targeting KChIP2,
KChIP3 and KChIP4 or with plasmids containing control (non-targeting) sequences.
Because each KChIP miRNA construct also encoded for a distinct fluorescent protein (CFP,
YFP or tdTomato) cells expressing all three KChIP targeting miRNA constructs could be
identified. (A) Recordings were obtained from neurons expressing control plasmids or (B)
all three targeting plasmids. Surprisingly, no prominent rapidly inactivating component was
observed in about half (11 of 20) of the neurons expressing the KChIP targeting miRNA
constructs and delayed rectifier currents were increased. In all cells, IA was isolated and
quantified using the prepulse paradigm described in the legend to Figure 1. Analyses of
subtracted records (a-b) revealed residual Kv4-encoded IA in all neurons expressing the three
KChIP targeting miRNAs simultaneously. The mean ± SEM IA density was significantly
(‡p<0.001) lower (C) in neurons expressing the three KChIP targeting miRNA constructs
(n=20) than in neurons expressing control constructs (n=21). (D) Consistent with the
upregulation of delayed rectifier currents, analysis of the peak current (IPeak) revealed no
significant reduction in mean ± SEM IPeak density in neurons expressing KChIP targeting
miRNA compared to those expressing control constructs, despite the marked reduction in
IA densities (c).
Expression of KChIP2, 3 and 4 proteins depends on the expression of Kv4 α-subunit proteins

The observed decreases in KChIP protein expression in Kv4.2+/− cortices (and other brain regions) suggest that KChIP protein expression is directly linked to Kv4.2 expression [48, 149]. To test this hypothesis directly, HEK-293 cells were transfected with DNA constructs encoding Kv4.2 alone, one of the KChIPs alone or Kv4.2 and one of the KChIPs. As illustrated in Figure 4.8A, coexpression of Kv4.2 with KChIP2 (n=6), KChIP3 (n=9) or KChIP4 (n=6) resulted in significant (p<0.05) increases in mean ± SEM Kv4.2 protein levels (Figure 4.8B) compared to cells (n=9) expressing Kv4.2 alone. In addition, expression of Kv4.2 increased KChIP protein expression (Figure 4.8C), and mean ± SEM KChIP2, KChIP3 and KChIP4 protein levels were significantly (p<0.05) higher with co-expression of Kv4.2 compared with cells expressing each of the KChIPs alone (Figure 4.8D).
Figure 4.8. Co-expression of Kv4.2 with KChIP2, KChIP3 or KChIP4 results in the co-stabilization of both the Kv4.2 and KChIP proteins. HEK-293 cells were transfected with DNA constructs encoding Kv4.2 alone (n=9), one of the KChIPs (KChIP2 n=6, KChIP3 n=9, KChIP4 n=6) alone, or Kv4.2 in combination with KChIP2 (n=6), KChIP3 (n=9) or KChIP4 (n=6). (A) Western Blots on lysates prepared from transfected HEK-293 cells were probed with the monoclonal anti-Kv4.2 antibody. Blots were also probed with anti-transferrin receptor antibody (Transferrin R) to verify equal loading of proteins in each lane. The anti-Kv4.2 antibody signals were measured and normalized to the signals from the anti-transferrin receptor in the same lane. (B) Quantitative analyses revealed a significant (*p<0.05) increase in Kv4.2 protein in cells expressing Kv4.2 plus one of the three KChIPs, compared to cells expressing Kv4.2 alone. (C) Western Blots conducted on HEK-293 cell lysates using the anti-KChIP2, anti-KChIP3 or anti-KChIP4 antibody also revealed that KChIP protein expression was increased in cells coexpressing Kv4.2, compared to cells express KChIP2, 3 or 4 alone. (D) Mean ± SEM levels of KChIP2, 3 and 4 protein expression were significantly (*p<0.05, +p<0.01) higher in cells coexpressing Kv4.2 compared to cells expressing either of the KChIP proteins alone.
To explore the hypothesis that the expression of the KChIP2, KChIP3 and KChIP4 proteins is linked to expression of the Kv4 α-subunit proteins in cortical neurons, Western Blots on fractionated proteins from the posterior cortices of adult WT (n=6), Kv4.2−/− (n=6), Kv4.3−/− (n=6) and Kv4.2−/−/Kv4.3−/− (n=3) mice were probed with antibodies specific for KChIP2, KChIP3 or KChIP4 (Figure 4.9). Blots were subsequently probed with an anti-β-tubulin antibody to confirm equal loading of proteins in each lane. Quantitative analysis revealed that the mean ± SEM expression levels of the KChIP2, KChIP3 and KChIP4 proteins were significantly (p<0.01) lower in Kv4.2−/− samples, compared to WT samples (Figure 4.9B). Also, relative to WT samples, the mean ± SEM expression levels of the KChIP2, KChIP3 and KChIP4 proteins were significantly (p<0.01) lower in Kv4.3−/− samples. In addition, when samples from Kv4.2−/−/Kv4.3−/− animals were examined, the KChIP2, KChIP3 and KChIP4 proteins were barely detectable (Figure 4.9).

In contrast, QRT-PCR analysis of RNA samples from posterior cortices of Kv4.2−/− (n=6), Kv4.3−/− (n=6) and Kv4.2−/−/4.3−/− (n=3) mice revealed that the mean expression levels of the KChIP2, KChIP3 or KChIP4 transcripts were not lower in any of the genotypes relative to WT cortices. In fact, the only significant (p<0.05) changes observed were increased (mean ± SEM) KChIP4 transcript expression in both the Kv4.3−/− and Kv4.2−/−/Kv4.3−/− samples (Figure 4.9C). In contrast to the near complete loss of the KChIP2, KChIP3 and KChIP4 proteins in the Kv4.2−/−/Kv4.3−/− cortices, there were no significant changes in mean ± SEM KChIP2 or KChIP3
transcript expression levels. The dramatic decreases in the expression levels of the KChIP proteins resulting from the disruption of Kv4.2 and/or Kv4.3 without corresponding decreases in mRNA levels are consistent with an important role for post-transcriptional mechanisms in the coupling between the expression of the KChIPs and the expression of the Kv4.2 and Kv4.3 α-subunits.
Figure 4.9. Endogenous KChIP2, KChIP3 and KChIP4 protein expression is dependent on the expression of Kv4 α-subunits.
Figure 4.9. Endogenous KChIP2, KChIP3 and KChIP4 protein expression is dependent on the expression of Kv4 α-subunits. (A) Representative Western Blots of fractionated lysates prepared from posterior (~1 mm) cortices of WT (n=6), Kv4.2−/− (n=6), Kv4.3−/− (n=6) and Kv4.2−/−/Kv4.3−/− (n=3) mice were probed with the anti-KChIP antibodies. KChIP protein levels were differentially affected by the loss of Kv4.2 or Kv4.3, although drastic reductions in all three proteins were evident with the loss of both Kv4.2 and Kv4.3. For quantification, blots were also probed with an anti-β-tubulin antibody to confirm equal protein loading, in each lane, and signals from the anti-KChIP2, 3 or 4 antibodies were normalized against the signals from the anti-β tubulin antibody in the same lane. (B) Analysis of mean (± SEM) normalized data revealed that the expression levels of KChIP2, KChIP3 and KChIP4 proteins in Kv4.2−/− and Kv4.3−/− cortices were significantly (*p<0.05, +p<0.01 or ‡p<0.001) lower than in WT cortices. In Kv4.2−/−/Kv4.3−/− cortices KChIP2, KChIP3 and KChIP4 protein expression levels were extremely low. (C) QRT-PCR of analysis of RNA isolated from the posterior cortices of WT (n=6), Kv4.2−/−(n=6), Kv4.3−/− (n=6) and Kv4.2−/−/Kv4.3−/− (n=3) mice revealed no reductions in KChIP transcripts. The mean ± SEM transcript expression level of KChIP4 was, however, significantly (p<0.05) higher in Kv4.3−/− and in Kv4.2−/−/Kv4.3−/−, compared with WT, cortices.
Discussion

**KChIP2, KChIP3 and KChIP4 are critical components of functional Kv4 channel complexes in cortical pyramidal neurons**

The results of the biochemical, molecular genetic and electrophysiological experiments described here suggest that KChIP2, KChIP3 and KChIP4 are critical for the formation of functional Kv4 channel complexes in (mouse visual) cortical pyramidal neurons. All three KChIPs are robustly expressed in posterior cortex and, in addition, co-immunoprecipitate with Kv4.2 (Figure 4.2). The results of the experiments completed here further indicate that the KChIPs can functionally compensate for one another. Specifically, the protein expression levels of the unperturbed KChIPs were upregulated in the cortices of KChIP2−/− and KChIP3−/− mice (Figure 4.3) and I_A densities were either not (KChIP2−/−) or only modestly (KChIP3−/−) affected (Figure 4.1). Further experiments revealed, however, that the simultaneous RNAi-mediated reduction in the expression of KChIP2, KChIP3 and KChIP4 resulted in marked reductions in Kv4-encoded I_A densities (Figure 4.7). Perhaps not surprisingly, I_A was not completely eliminated in the Kv1.4−/− neurons expressing the three KChIP targeting miRNA constructs, consistent with residual KChIP protein, likely reflecting incomplete knockdown of KChIP2, 3 and/or 4 expression.

**Expression of KChIP proteins is dependent on the expression of Kv4 α-subunits**
The results of the experiments detailed here also revealed that the expression of the KChIP2, KChIP3 and KChIP4 proteins is dependent on the expression of Kv4 α-subunits in cortical tissue. Consistent with previous reports that KChIP2 and KChIP3 expression is decreased in Kv4.2−/− hippocampal neurons [149], the results here demonstrate that protein expression levels of KChIP2, KChIP3 and KChIP4 are decreased in the cortices of Kv4.2−/− mice. Reduced expression of the KChIP2, 3 and 4 proteins was also observed in cortical samples from Kv4.3−/− mice. Additionally, all three KChIP proteins were barely detectable in Kv4.2−/−/Kv4.3−/− cortices (Figure 4.9). Importantly, the marked reductions in the expression levels of KChIP proteins do not reflect changes in transcript levels (Figure 4.9C), suggesting that post-translational mechanisms are responsible for the loss of KChIP proteins in Kv4.2−/−, Kv4.3−/− and Kv4.2−/−/Kv4.3−/− neurons.

Previous reports indicate that the KChIPs interact with Kv4 α-subunits early during channel biogenesis [82, 150]. The results of experiments presented here suggest that when the KChIP and Kv4 α-subunits bind, the proteins are stabilized, leading to increased levels of both the Kv4 α-subunit and accessory KChIP proteins (Figure 4.8). The observed increases in the protein expression levels of the remaining KChIP proteins in KChIP2−/− and KChIP3−/− cortices (Figure 4.3) suggest that, when the expression of an individual KChIP is disrupted, the remaining KChIP proteins are stabilized by binding to available Kv4 α-subunits, resulting in net increases in the non-disrupted KChIP proteins. Interestingly, the co-dependence of the expression of
Kv α- and accessory subunit proteins has been previously reported in studies using *C. elegans* [151] and *Drosophila* [152], suggesting that the mechanisms linking accessory and α-subunits expression are highly conserved.

**Functions of KChIP2, 3 and 4**

Interestingly, results from several recent studies suggest that individual KChIPs may interact with specific signaling molecules (e.g. PKA or PKC) to regulate the modulation of Kv4 channels by specific signaling pathways [147, 153]. Further diversity of KChIP function may originate from splice variants of the individual KChIP genes. Splice variants of KChIP2 and KChIP4, for example, have been described to promote surface expression of heterologously expressed Kv4 α-subunits and other variants have been reported to have inhibitory effects on the surface expression of Kv4 α-subunits [75, 78]. It was also recently reported that KChIP3 (but not other KChIPs) functions as a Ca\(^{2+}\) sensor to modulate the voltage-dependence of inactivation of Kv4-encoded I\(_A\) in response to the entry of Ca\(^{2+}\) through Cav3-encoded voltage-gated Ca\(^{2+}\) channels [154, 155]. The findings presented here raise the interesting possibility that KChIP2 and KChIP4, in addition to KChIP3, also participate in the generation and functioning of distinct Kv4 channel complexes in cortical pyramidal neurons. Future experiments designed to explore this hypothesis are necessary to determine the unique roles of each of the individual KChIPs.
The results presented here, which indicate that multiple KChIPs are concurrently involved in the generation and function of Kv4 channels, highlight the molecular diversity that likely exists in Kv4 channel complexes in neurons. One Kv4-channel complex could, for example, be formed by a heterometric complex of Kv4.2 and Kv4.3 α-subunits bound simultaneously to multiple different KChIPs. In the same cell, another Kv4 channel complex could be formed by a homomultimer of Kv4.2 α-subunits with a single type of KChIP. As suggested previously, considerable evidence supports the further inclusion of DPP6 and/or DPP10 subunits, as well as Kvβ subunits in the generation and/or functioning of Kv4 channel complexes [63, 69, 76, 98], thus exponentially diversifying the possible protein combinations that may be present in native neuronal Kv4 channel complexes. This molecular diversity may provide for high resolution regulatory mechanisms for fine tuning neuronal excitability and neuronal computations.

**Remodeling of Kv currents following the disruption of KChIP expression**

Remodeling of Kv currents was evident in KChIP3\(^{-/-}\) neurons expressing KChIP4 targeting miRNA (Figure 4.6) and in Kv1.4\(^{-/-}\) neurons expressing miRNAs targeting KChIP2, 3, and 4 (Figure 4.7). The upregulation of the I\(_K\) and I\(_SS\) components observed when the expression of multiple KChIPs was simultaneously disrupted is similar to the remodeling evident in Kv4.2\(^{-/-}\), Kv4.3\(^{-/-}\) and Kv4.2\(^{-/-}\)/Kv4.3\(^{-/-}\) cortical pyramidal neurons previously reported [48, 96]. Interestingly, no remodeling of Kv currents was evident in response to the loss of Kv4-encoded I\(_A\) by the
expression of a dominant negative Kv4 construct in (rat) cortical pyramidal neurons [49] suggesting that the mechanism mediating the remodeling of Kv currents is based on protein expression, rather than changes in the electrical properties of the neurons. Taken together, the remodeling of Kv currents evident in response to the disruption of the expression of multiple KChIPs and the finding of decreased expression levels of KChIP proteins in samples from Kv4.2−/− mice suggests that the KChIPs, through direct or indirect effects, may play important roles in balancing the expression of ionic conductances in cortical pyramidal neurons. Clearly, future experiments aimed at exploring this possibility will be of considerable interest.
Chapter 5

Conclusions
I. Molecular diversity of $I_A$ channels in cortical pyramidal neurons

Kv4.2, Kv4.3 and Kv1.4 α-subunits encode distinct components of $I_A$ in cortical pyramidal neurons

The studies described in Chapter 3 combined electrophysiological, molecular genetic and pharmacological approaches to define the molecular determinants of $I_A$ in cortical pyramidal neurons. The results of these experiments revealed that in these cells macroscopic $I_A$ is composed of three molecularly distinct components and specifically, that Kv4.2, Kv4.3 and Kv1.4 α-subunits underlie these three components. The results presented also demonstrated that Kv4.2 and Kv4.3 channels make the major contribution to macroscopic of $I_A$ and Kv1.4 in encodes a smaller but ubiquitously expressed component of $I_A$.

Kv1 heteromultimers may function in the generation of molecularly diverse of $I_A$ channels

The simplest interpretation of the results obtained in experiments in Kv1.4$^{-/-}$ neurons (Chapter 3) is that homomeric Kv1.4 channels encode a component of $I_A$ in visual cortical neurons. The multiplicity of Kv1 α-subunits and the fact that these α-subunits readily co-assemble, suggest that substantial molecular diversity of Kv1-encoded $I_A$ channels exists. Many members of the Kv1 family are expressed in cortex [101] and, when these same subunits are heterologously coexpressed, functional heteromeric channels are formed [156, 157]. With the exception of Kv1.4, the currents generated by the various Kv1 α-subunits expressed alone or together inactivate slowly
When Kv1.4 α-subunits are coexpressed with other Kv1 α-subunits, however, the currents generated inactivate rapidly, similar to currents generated by Kv1.4 α-subunits expressed alone [159]. The formation of heteromultimers containing Kv1.4 α-subunits therefore is a potential source of molecular diversity of neuronal I_A channels. Although not extensively studied, several lines of evidence suggest that the formation of heteromultimers is likely to be of functional significance in neurons. Previous studies, for example, have demonstrated that many Kv1 α-subunits contain a trafficking motif in the pore region that is not present/functional in Kv1.4 α-subunits [160] and the masking of this motif can promote the trafficking of Kv1 α-subunits to the cell surface[161]. Interestingly, in heterologous cells coexpression of Kv1.4 and Kv1.2 α-subunits greatly enhanced the trafficking of the Kv1.2 α-subunits to the cell surface [162]. In addition to altering the trafficking of α-subunits, heteromultimers may be differentially regulated by signaling pathways. The surface expression of Kv1.2 channels, for example, has been shown (at least in heterologous cells) to be dependent on phosphorylation. Mutation of the phosphorylation sites in Kv1.2 disrupted surface expression of Kv1.2 homomeric and Kv1.2/Kv1.4 heteromeric channels [162].

Considerable experimental evidence supports the suggestion that functional Kv1.4/Kv1.x heteromeric channels are expressed in neurons. Previous reports have indicated, for example, that Kv1.4 can be co-immunoprecipitated with other Kv1 α-subunits from the brain [163, 164]. In addition, electrophysiological studies aimed at exploring the functional roles of Kv1-encoded channels using α-Dendrotoxin, which blocks channels containing Kv1.1, Kv1.2 and Kv1.6 α-subunits, but does not affect
Kv1.4 homomeric channels, demonstrated the presence of α-Dendrotoxin sensitive (Kv1-encoded) rapidly inactivating currents in cortical neurons [165]. The results presented here, therefore suggest that Kv1.4 is absolutely required for the functional expression of heteromeric Kv1-encoded Iₐ channels.

It is also worth noting that Kv1 channels are present, and likely have important functional roles, in axons and presynaptic terminals [18, 166, 167]. These electrically isolated compartments are, however, not electrically accessible using whole-cell voltage clamp approaches like those used in the studies presented here [167]. Experiments designed to explore the functional roles of Kv1.4-encoded Iₐ channels in axons and presynaptic terminals will extend the findings presented here describing, for the first time, a functional role for Kv1.4 α-subunits in the generation of Iₐ.

**Kv4 heteromultimers are a source of molecularly diverse Iₐ channels**

The electrophysiological studies presented in Chapter 3 demonstrated that Kv4.2 and Kv4.3 can form functional Iₐ channels independently. The results of the biochemical experiments presented in Chapter 4, however, confirmed previous studies [60] that showed that Kv4.2 and Kv4.3 α-subunits co-immunoprecipitate from cortex suggesting that these subunits interact in neurons *in vivo* to generate heteromeric Kv4-encoded Iₐ channels. Also consistent with the suggestions that heteromeric Kv4 channels are present *in situ*, previous studies have shown that the subcellular distributions Kv4.2 and Kv4.3 α-subunits are similar in cortical pyramidal neurons [107]. Taken together with the studies here, these observations suggest that functional Kv4 channels in cortical pyramidal
neurons reflect the formation of least 3 molecularly distinct configurations: Kv4.2 homomers, Kv4.3 homomers and Kv4.2/Kv4.3 heteromers.

The remaining member of the Kv4 subfamily, Kv4.1, is also expressed in the brain, albeit at much lower levels [66]. Interestingly, Kv4.1 has also been demonstrated to co-immunoprecipitate with Kv4.2 α-subunits from brain lysates [60], suggesting that functional Kv4 channels could also contain Kv4.1 α-subunits. In Kv4.2<sup>-/-</sup>/Kv4.3<sup>-/-</sup> cortical pyramidal neurons, however, no residual Kv4-encoded I<sub>A</sub> is present. Thus, Kv4.1 homomeric channels do not appear to be present in cortical pyramidal neurons. It is also possible to conclude from the results here that Kv4.1 containing channels exist only as heteromeric channels containing other Kv4 α-subunits and, in addition, the Kv4.2 (or Kv4.3) subunits are required for the functional expression of these heteromeric channels. Importantly, the presence of Kv4.1 in some heteromeric Kv4 channels would result in further molecular diversity of functional Kv4-encoded neuronal I<sub>A</sub> channels.

In the context of studies performed on other excitable cells, the findings from the experiments presented in Chapter 3, demonstrating that Kv4.2 and Kv4.3 form functional channels independently, were somewhat surprising. In rodent cardiac myocytes both Kv4.2 and Kv4.3 α-subunits are expressed and these α-subunits co-immunoprecipitate [146, 168]. Disruption of Kv4.2 expression alone, however, was sufficient to eliminate Kv4-encoded currents [92, 93], demonstrating that Kv4.3 α-subunits do not form functional channels independently in these cells. The dependence of Kv4.3 on Kv4.2 for functional expression in mouse cardiac myocytes but not in cortical pyramidal neurons,
clearly suggests that different cells use different mechanisms to regulate the functional expression Kv4 channels.

**Accessory subunits dramatically increase the molecular diversity of I_A channels**

The experimental findings presented in Chapter 4 demonstrated that the Kv channel accessory subunits KChIP2, 3 and 4 interact with Kv4 α-subunits in cortical pyramidal neurons and are critical for the normal expression of Kv4-encoded I_A. Previous structural data suggests that Kv4 channels form octameric complexes containing 4 α-subunits bound to 4 KChIPs [80]. The finding that the various KChIPs can assemble with Kv4.2 and generate functional Kv4 channels suggests that neuronal Kv4 channels could contain distinct combinations of KChIP accessory subunits, further increasing the molecular diversity of Kv4-encoded I_A channels in situ. Kv4 channel complexes in cortical pyramidal neurons, for example, could contain KChIP2, 3 and/or 4 in various combinations to fill the complement of 4 KChIPs per channel. Considerable evidence also supports the further inclusion of additional DPP6, DPP10 and Kvβ2 accessory subunits in neuronal Kv4 channel complexes [44, 63, 76, 169], exponentially increasing the possible molecular combinations that may be present in native Kv4-encoded I_A channels. Although the studies presented here focused on the conserved roles of KChIPs in the expression of functional Kv4 channels, recent studies also suggest the intriguing possibility that each of the KChIPs may have unique functions in the regulation and functioning of Kv4 channels.
Since the original descriptions [71, 72] the KChIPs have been suggested to play important roles in multiple neuronal processes and to link channel function and expression to other cellular events. KChIP3, for example, has also been identified as the Ca\(^{2+}\) dependent downstream regulatory element antagonist modulator (DREAM) [170] and as calseinin [171], which binds presenilin and regulates gamma secretase activity [171, 172]. Accordingly, KChIP3 has been shown to play important roles in regulating gene transcription [170] and the production of amyloid precursor protein [171, 172]. KChIP4 was also independently cloned as part of a yeast two-hybrid screen to identify binding partners of presenilin 2 and as a result has also been termed calseinin related protein (Calp). KChIP4, therefore, may also play functional roles in cellular processes other than the modulation of Kv4-encoded I\(_A\) channels [72].

Recent studies, focused on examining the roles(s) of KChIP3 as a Kv4 channel accessory subunit, have demonstrated that KChIP3 operates as a point of signal integration in the regulation of Kv4.2 channel function through its ability to function as a Ca\(^{2+}\) sensor. Specifically, these studies revealed that KChIP3 regulates the voltage-dependence of inactivation of Kv4-encoded channels in cerebellar stellate cells in response to Ca\(^{2+}\) entry through T-type voltage-gated Ca\(^{2+}\) (Cav) channels encoded by pore-forming subunits of the Cav3 family [155]. In the presence of KChIP3, Ca\(^{2+}\) entry through Cav3 channels induced a depolarizing shift in the voltage-dependence of inactivation thereby, linking the amount of available Kv4-encoded current to local changes in Ca\(^{2+}\) concentration [173]. Importantly, the same study showed that the other KChIPs were not able to modulate Kv4-encoded currents in response to Ca\(^{2+}\) entry.
through Cav3 channels, clearly revealing functional differences between the KChIPs. Experiments in heterologous cells have also shown that coexpression of KChIP3 with Kv4 α-subunits markedly increases the surface expression of Kv4 channels [71, 76]. Consistent with these findings, the studies presented in Chapter 4 demonstrated that KChIP3 expression is important for the generation of functional I_A channels in cortical pyramidal neurons. Together, these studies suggest that KChIP3 may regulate the functional expression of Kv4-encoded currents by both controlling the number of Kv4 channels expressed at the surface and by dynamically altering the functional availability of Kv4-encoded currents in response to local Ca^{2+} entry.

Additional experiments suggest that KChIP3 also modulates the regulation of Kv4-channel complexes by multiple types of kinases [153, 174]. In the case of G protein-coupled receptor kinases (GRK), association of KChIP3 with GRK is dependent on Ca^{2+} binding to KChIP3[175]. Inclusion of KChIP3 in Kv4 channel complexes, therefore, could provide a means to integrate local changes in Ca^{2+} levels with active kinase signaling pathways and result in local/selective alterations in channel expression and/or function. Phosphorylation of Kv4 α-subunits, in turn, has been demonstrated to be closely related to surface expression [67, 176, 177]. The possibility that KChIP3 acts as a point of signal integration to regulate the surface expression of Kv4 channels is intriguing, particularly since dynamic alterations in the expression level of Kv4-encoded currents are important in modulating dendritic integration and synaptic potentiation [9, 105]. Little is currently known about the functions of the other KChIPs in regulating the association of kinases (or other regulatory molecules) with Kv4 channel complexes. The
inclusion of different individual KChIPs in Kv4 channel complexes may, however, provide a means to link subpopulations of Kv4 channels to discrete regulatory pathways. Interestingly, KChIPs may play a wider role in balancing ionic conductances in excitable cells as they have been shown to regulate the expression and properties of Cav channels [134] and other Kv channels [133]. The possibility that the KChIPs function in the regulation of Cav and other Kv channel in neurons, however, has not been explored.

II. Mechanisms governing the balancing of ionic currents and electrical remodeling

Unique firing properties of neurons can be produced by different combinations of ionic channels/currents

Previous studies in invertebrate neurons elegantly demonstrated that neurons express multiple ionic currents at varying levels, yet achieve a consistent target excitability profile [127]. Specifically, the excitability and the functional expression of currents in the same identified neuron in a simple circuit were measured in different animals. Although the firing patterns and the functioning of each identified neuron within the circuit were preserved among animals, the relative expression levels (densities) of individual ionic currents contributing to stereotyped firing patterns were highly variable (over several orders of magnitude) among animals [127, 178-180]. These findings clearly indicate the presence of complex mechanisms in neurons that operate to balance...
the functional expression levels of multiple ionic currents dynamically to achieve a defined excitability profile.

Interestingly, a number of recent reports detailing experiments using genetic tools to manipulate the expression of individual ion channels/currents revealed robust and diverse compensatory changes in other ionic currents, further supporting the presence of mechanisms to regulate neuronal excitability dynamically. Artificial increases in the expression of $I_A$ channels in rhythmically active neurons from the lobster stomatogastric ganglion, for example, led to increased expression of hyperpolarization activated currents ($I_h$) \[179, 180\]. The increased expression of the mixed cation current, $I_h$, was sufficient to maintain the normal intrinsic rhythmic firing properties of these neurons, in spite of the large (several fold) increase in $I_A$ expression. When the reverse experiments were carried out, however, artificial upregulation of $I_h$ did not result in an upregulation of $I_A$ \[180, 181\], suggesting that the mechanisms regulating and linking the expression of these ($I_A$ and $I_h$) ionic conductance pathways are complex. A growing number of studies in mammals have also documented remodeling of ionic currents in response to the disruptions of channel expression and, in many cases, this remodeling maintains normal excitability profiles \[127\]. In mice lacking the voltage-gated Na$^+$ channel (Nav) subunit Nav1.1, for example, another Nav α-subunit, Nav1.6, is upregulated \[182\]. In addition, in Purkinje neurons lacking Nav1.6 channels, Cav currents are upregulated and Ca$^{2+}$-dependent K$^+$ currents are downregulated, resulting in the maintenance of normal burst firing despite a reduction in Na$^+$ currents \[183\]. Taken together, these experimental results demonstrate the potential for alterations in the expression of ionic currents in
response to the disruption of the expression (or the functioning) of individual ion channels. In addition, these studies have revealed that the currents altered in response to the loss of a specific channel are often variable and can be unrelated, at the molecular level, to the disrupted channel.

Consistent with the previous studies discussed above demonstrating that different combinations of current densities can generate similar excitability profiles, the studies presented here revealed that the expression levels of the individual Kv current components ($I_A$, $I_D$, $I_K$ and $I_{SS}$) are highly variable in cortical pyramidal neurons (from the same region) that have similar repetitive firing properties [46, 48, 49, 184]. The findings completed here further demonstrated that different types of Kv current remodeling can occur in response to the disruption of Kv channel α-subunit expression, as well as in response to changes in the expression of Kv channel accessory subunits. In neurons lacking Kv4.2 or both Kv4.2 and Kv4.3 expression, for example, $I_K$ and $I_{SS}$ currents were markedly upregulated. In contrast, the disruption of the expression of Kv1.4, which also encodes a component of $I_A$ in cortical pyramidal neurons, led to upregulation of Kv4-encoded $I_A$, but not to changes in $I_K$ and $I_{SS}$. The experiments presented in Chapter 4 revealed that upregulation of $I_K$ and $I_{SS}$ also occurs following the disruption of expression of the Kv4 channel accessory subunits KChIP2, 3 and 4. Although the studies here focused on Kv currents, other ionic currents may also change in response to the loss of Kv α-subunits or KChIPs. Future experiments designed to explore the impact of the loss of specific Kv channel components and of electrical remodeling on the excitability of cortical pyramidal neurons will be of considerable interest. Analysis of the action
potential waveforms and the repetitive firing properties of cortical pyramidal neurons lacking Kv channel components will provide important insights into the possible changes in the expression of additional, non Kv, ionic currents.

**Protein based mechanism in the dynamic regulation of intrinsic excitability?**

Substantial evidence indicates that the expression levels of different ionic conductances are balanced dynamically in neurons to maintain a specific excitability profile over time and in response to various perturbations [127, 185]. Computer simulations examining the number of possible combinations of ionic current densities able to generate a specific firing pattern, for example, indicated that tens of thousands to millions of different combinations of ion current densities (from a defined set of possible types of conductances) could produce firing patterns similar to those observed *in vivo* [186, 187]. Little is known, however, about the nature of the cell intrinsic homeostatic mechanisms responsible for determining the relative balance of the ionic currents expressed in individual neurons.

It has been hypothesized that the set of ion channel genes expressed by a cell is genetically determined and that the densities of the individual currents (functional channels) are modulated individually or in a coordinated manner to achieve the excitability profile required for the each neuron to function in the neuronal circuit in which the individual neuron is embedded [185]. Consistent with this hypothesis, studies on cortical pyramidal neurons, including those presented here, demonstrated that cortical pyramidal neurons express the same repertoire of Kv currents, although the densities of
the individual currents vary among cells [46, 47, 49, 184]. Many mechanisms are likely involved in balancing the expression of Kv and other ionic currents, ranging from signals generated by changes in Ca\(^{2+}\) levels as a proxy for activity to retrograde synaptic signals [185]. The mechanism responsible for determining the relative expression levels of Kv currents, however, are poorly understood.

Taken together with previous findings [48, 49, 178], the studies presented here reveal differential remodeling of Kv currents with the disruption of the expression of individual Kv channel α-subunits and accessory subunits. These observations clearly suggest that the presence of channel proteins *per se* (independent of channel function) is an important part of the molecular machinery governing the balancing of current densities and the remodeling of Kv currents. These findings suggest that the numbers of specific individual channel subunit molecules expressed in a cell can function as a signal to modulate the expression of other, molecularly distinct channels. For example, both the overexpression of mutant non-functional Kv4 α-subunits, which would not lead to an increase in Kv4-encoded current, and the overexpression of functional Kv4 α-subunits, which markedly increased Kv4-encoded current, resulted in I_h upregulation in rhythmically active lobster neurons [179]. In the experiments presented here, in which Kv currents in cortical pyramidal neurons were examined, reduction of Kv4-encoded I_A by disrupting the expression of Kv4.2 (or Kv4.2 and Kv4.3) α-subunits resulted in upregulation of I_K and I_SS. Previous studies using a mutant Kv4 construct that functions as a dominant negative [49] to reduce the functional expression of Kv4-encoded I_A (without disrupting Kv4 protein expression) in rat cortical pyramidal and sympathetic
ganglion neurons, did not reveal Kv current remodeling in spite of the loss of $I_A$ \cite{49, 188, 189}. The observations are consistent with the hypothesis that expression of channel proteins is important for balancing the expression of ionic conductances. Although both the expression of the Kv4 dominant negative construct and the disruption of Kv4.2/Kv4.3 expression markedly reduced (or eliminated) Kv4-encoded $I_A$ in cortical pyramidal neurons, marked remodeling of Kv currents was only observed when the expression of the Kv4 proteins were reduced (Kv4.2\textsuperscript{-/-}) or eliminated (Kv4.2\textsuperscript{+/-}/Kv4.3\textsuperscript{-/-}).

The experiments conducted using the Kv4 dominant negative construct, however, were done \textit{in vitro}, and Kv currents were assayed just days after transfection of the mutant construct. In Kv4.2\textsuperscript{+/-}/Kv4.3\textsuperscript{+/-} neurons, in contrast, the loss of Kv4-encoded $I_A$ is due to germ line genetic manipulations and the channels are absent throughout development. It might be suggested, therefore, that the acute nature of experiments employing the Kv4.2 dominant negative may account for the lack of Kv current remodeling. The experiments presented in Chapter 4, which examined the effects of the disruption of KChIP2, 3 and 4 expression however, also revealed marked increases in $I_K$ and $I_{SS}$ in parallel with reductions in $I_A$. These experiments were carried out using very similar \textit{in vitro} cultures conditions to those used for the studies examining the effects of expression of the Kv4 dominant negative construct \cite{49}. In addition, the time between transfection and recording (2-3 days) was similar in both sets of experiments. The finding of $I_K$ and $I_{SS}$ upregulation in neurons expressing the KChIP targeting miRNA constructs demonstrates that Kv current remodeling can occur quickly in response to \textit{in vitro} manipulations.
The experiments presented here demonstrating reductions in $I_A$ densities in neurons expressing the KChIP targeting miRNA constructs together with the finding that coexpression of KChIPs with Kv4.2 $\alpha$-subunits in HEK-293 cells results in marked increases in Kv4.2 $\alpha$-subunit protein levels, suggest that expression of Kv4 proteins depends on the expression of KChIPs. These results also suggest that the levels of Kv4 $\alpha$-subunit protein may be decreased in parallel with the reduction in $I_A$ densities in neurons expressing KChIP targeting miRNA constructs. Unfortunately, biochemical experiments to test this hypothesis directly are not feasible owing to the fact that these experiments were carried on dissociated cells in vitro. In mouse cardiac myocytes, which express only one of the KChIPs (KChIP2), however, recent studies showed that the targeted disruption of KChIP2 expression leads to a near complete loss of Kv4.2 protein (Nick Foeger and Jeanne Nerbonne, unpublished data). Interestingly, the upregulation of $I_K$ and $I_{SS}$ evident in neurons in which KChIP2, 3 and 4 expression was disrupted is similar to the Kv current remodeling in Kv4.2$^{-/-}$ neurons. These observations further suggest that the loss of channel subunit proteins (not functional currents) contributes importantly to Kv current remodeling, resulting in balancing the expression of multiple ionic currents and the maintenance of normal firing properties.

The identity of individual channel subunit proteins per se may contribute to the type and extent of current remodeling evident when the expression of an individual channel subunit is disrupted. Under physiological conditions, the expression levels of individual ion channel subunit proteins may also be an important factor in determining the relative densities of multiple, molecularly unrelated, ionic currents in neurons.
Potential mechanisms by which channel proteins could mediate these effects include the regulation of multiple ion channels α-subunits by common accessory subunits, as suggested for KChIP2 [133, 134], and through the function of proteolytically cleaved portions of channel subunits functioning to modulate transcription, as has been suggested for Ca$^{2+}$ channels [190]. Experiments designed to test directly the hypothesis that expression of individual channel subunit proteins, independent of conducting ions, plays important roles in mediating electrical homeostasis and remodeling are needed. Exploiting conditional *in vivo* expression of dominant negative constructs and targeted gene deletions will allow studies focused on determine directly if protein-based mechanisms are important for Kv current remodeling. Experiments aimed at exploring the hypothesis that the expression levels of individual Kv channel α-subunit and/or channel accessory subunit proteins are important in balancing the expression levels of multiple of ionic conductances will be of considerable interest and may reveal novel mechanisms involved in the regulation of ion channel function and neuronal excitability.


