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

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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, backpropagation (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  $\alpha$ -subunits. The goals of the experiments presented here were to test this hypothesis and to determine the molecular identities of the Kv channel  $\alpha$ -subunits that generate  $I_A$  in cortical pyramidal neurons. Combining genetic disruption of individual Kv  $\alpha$ -subunit genes with pharmacological approaches to block Kv currents selectively, the experiments here revealed that Kv1.4, Kv4.2, and Kv4.3  $\alpha$ -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  $\alpha$ -subunits encoding components of  $I_A$  results in electrical remodeling that is Kv  $\alpha$ -subunit specific.

## Introduction

Voltage-gated  $K^+$  (Kv) currents play distinct roles in controlling neuronal action potential waveforms, repetitive firing patterns (Crill and Schwindt, 1983), responses to synaptic inputs (Kole et al., 2007), neurotransmitter release (Ishikawa et al., 2003), and synaptic plasticity (Schrader et al., 2002). Consistent with these diverse roles, multiple types of Kv currents with distinct time- and voltage-dependent properties are coexpressed in most neurons. The functional diversity of neuronal Kv currents is generated, in part, through the expression of multiple Kv channel pore-forming ( $\alpha$ ) subunits. In cortical pyramidal neurons, for example, multiple Kv channel  $\alpha$ -subunits from different subfamilies are coexpressed (Guan et al., 2006). The macroscopic Kv currents in these cells can be separated into four components based on differing time constants ( $\tau$ ) of inactivation:  $I_{A1}$ , which inactivates rapidly ( $\tau \approx 25$  ms);  $I_{D1}$ , characterized by an intermediate rate of inactivation ( $\tau \approx 250$  ms);  $I_{K1}$ , which inactivates slowly ( $\tau \approx 2$  s); and  $I_{SS}$ , which is non-inactivating (Locke and Nerbonne, 1997a). Previous studies suggest that these kinetically

distinct current components are encoded by molecularly distinct populations of channels (Yuan et al., 2005).

The rapidly activating and rapidly inactivating Kv current,  $I_A$ , which is widely expressed in central and peripheral neurons (Rogawski, 1985), regulates multiple neuronal processes, including action potential repolarization, repetitive firing (Yuan et al., 2005), synaptic integration, and the backpropagation (into dendrites) of action potentials (Cai et al., 2004). 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 (Bernard et al., 2004). Additionally, a mutation in Kv4.2 has been identified in a patient with temporal lobe epilepsy (Singh et al., 2006). Other studies using experimental models of epilepsy have described alterations in the expression and the subcellular localization of Kv4.2 and Kv4.3 in the hippocampus (Lugo et al., 2008; Monaghan et al., 2008).

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 (Nerbonne et al., 2008), hippocampal pyramidal neurons (Chen et al., 2006), and neurons from the dorsal horn of the spinal cord (Hu et al., 2006). In each of these studies however, rapidly activating and inactivating currents similar to  $I_A$  in wild-

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type (WT) 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  $\alpha$ -subunits, to identify the Kv channel  $\alpha$ -subunits responsible for the generation of  $I_A$  in cortical pyramidal neurons.

## Materials and Methods

**Isolation and maintenance of cortical pyramidal neurons.** WT C57BL/6 mice and mice harboring targeted genetic disruption of the *Kcna4* (*Kv1.4*<sup>-/-</sup>) (London et al., 1998), *Kcnd2* (*Kv4.2*<sup>-/-</sup>) (Nerbonne et al., 2008), or *Kcnd3* (*Kv4.3*<sup>-/-</sup>) (Niwa et al., 2008) locus in the C57BL/6 background were used in the experiments presented here. Neurons were isolated from the primary visual cortices of postnatal day 6–8 animals of both genders using previously described methods (Huettner and Baughman, 1986; Locke and Nerbonne, 1997a,b; Nerbonne et al., 2008). Briefly, mice were first anesthetized with halothane and then decapitated. After dissection of the visual cortex, the tissue, which contained all cortical layers, was chopped into small pieces and incubated in Neurobasal medium containing papain (Worthington Biochemicals) under 95% oxygen/5% CO<sub>2</sub> at 37°C for 30 min. After the incubation, the tissue pieces were triturated using fire-polished Pasteur pipettes. Isolated neurons were recovered by centrifugation through a bovine serum albumin gradient. Cells were resuspended in Neurobasal media (Invitrogen) and plated on previously prepared monolayers of cortical astrocytes (Huettner and Baughman, 1986). One hour after plating, the media was replaced with Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum and 0.14 mM L-glutamine. Cultures were maintained in a 5% CO<sub>2</sub>, 37°C incubator.

**Electrophysiological recordings.** Whole-cell recordings were obtained from isolated cortical pyramidal neurons at room temperature (22–23°C). Recordings were obtained from pyramidal-shaped neurons on the first and second day in culture before the elaboration of extensive processes to ensure adequate voltage-clamp control (see analysis below). Data were collected using an Axon 1D amplifier (Molecular Devices) controlled through a Digidata 1322 analog/digital interface (Molecular Devices). Pipettes were fabricated from borosilicate glass (WPI) with a Sutter Instruments model P-87 horizontal puller. Using a standard pipette solution (see below), pipette resistances were between 2 and 4 M $\Omega$ . For recordings, bath solution contained the following (in mM): 140 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 5 glucose, 0.001 TTX, and 0.1 CdCl<sub>2</sub>, pH 7.4 (300 mOsm). The recording pipette solution contained the following (in mM): 135 KCl, 10 HEPES, 5 glucose, 1.1 CaCl<sub>2</sub>, and 2.5 BAPTA, and 3 MgATP and 0.5 NaGTP were added the day of recording, pH 7.4 (300 mOsm). The calculated free Ca<sup>2+</sup> in this BAPTA-buffered pipette solution was 100 nM (MAXCHELATOR) (Patton et al., 2004). The K<sup>+</sup> channel blockers used, tetraethylammonium (TEA), 4-aminopyridine (4-AP), heteropodatoxin-2 (Hptx-2),  $\alpha$ -dendrotoxin ( $\alpha$ -Dtx) (Alomone Labs), and Ba<sup>2+</sup>, were added to the bath solution immediately before recordings. All reagents were from Sigma unless otherwise noted.

In all experiments, junction potentials were zeroed before forming pipette–membrane seals. Signals were low-pass filtered at 10 kHz and sampled at 100 kHz. Whole-cell Kv currents were routinely evoked in response to 4 s depolarizing voltage steps to potentials between -40 and +40 mV (in 10 mV increments) from a holding potential of -70 mV. In parallel experiments, a prepulse paradigm was used to facilitate the isolation of the rapidly inactivating currents in each cell. In this case, currents evoked at test potentials from -40 to +40 mV (in 10 mV increments) after a brief (60 ms) step to -10 mV were recorded. Offline subtraction of the currents evoked after the prepulse from the currents evoked without the prepulse allowed the isolation of the rapidly inactivating outward K<sup>+</sup> currents (see Fig. 1).

**Data analysis.** Data were compiled and analyzed using ClampFit (Molecular Devices), Microsoft Excel, and Prism (GraphPad Software). Only data from cells with input resistances greater than 300 M $\Omega$  and access resistances <15 M $\Omega$  were included in the analyses. Membrane capaci-

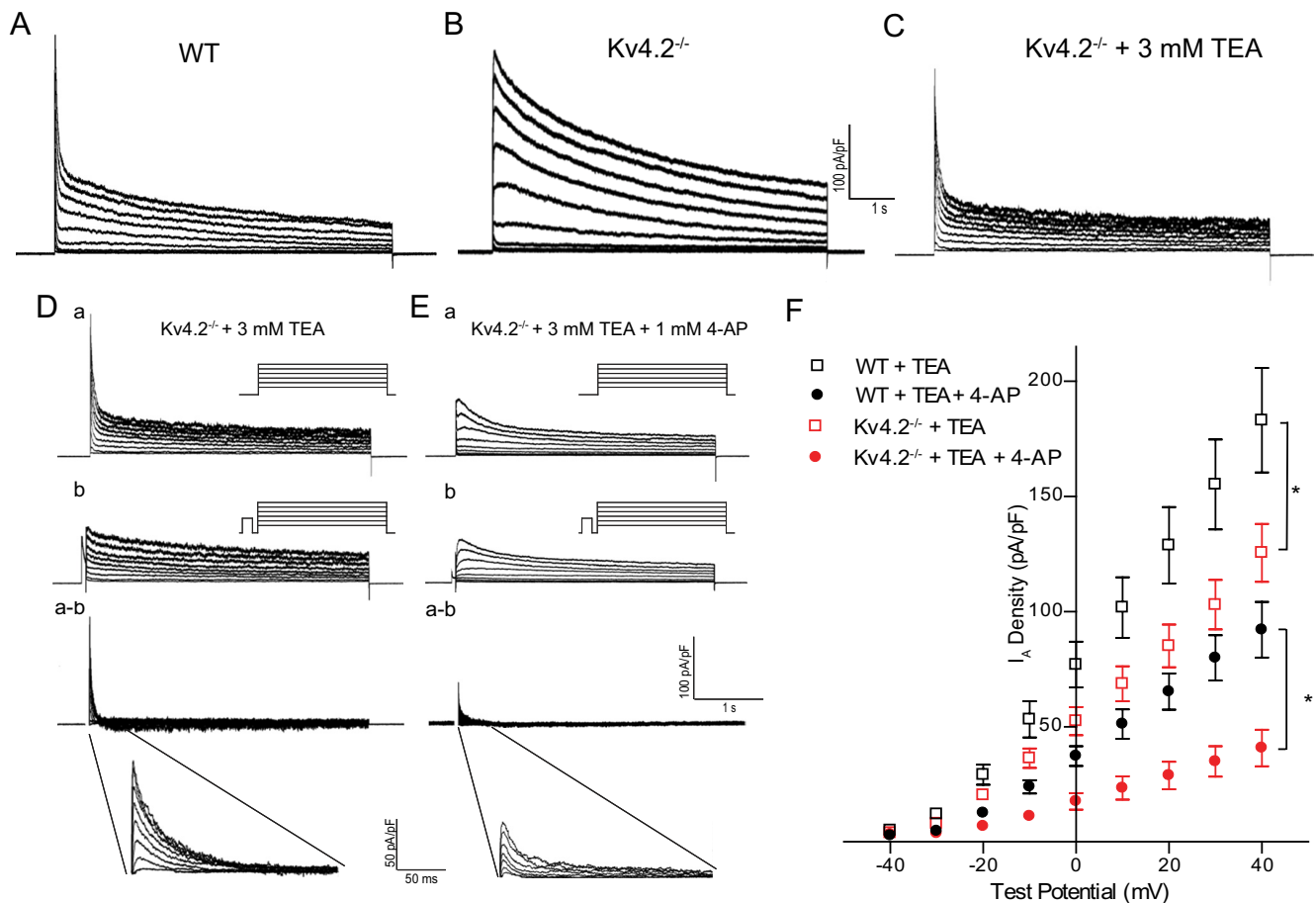
ties were determined by analyzing the decays of capacitive currents elicited by short (25 ms) voltage steps ( $\pm 10$  mV) from the holding potential (-70 mV). Whole-cell membrane capacitances ( $C_m$ ) were calculated for each cell by dividing the integrated capacitive transients by the voltage. Consistent with the short time in culture and lack of extensive processes, the capacitive transients of recorded cells had single-exponential decay phases. Input resistances were calculated from the steady-state currents elicited by the same  $\pm 10$  mV steps (from the holding potential). For each cell, the series resistance was calculated by dividing the time constant of the decay of the capacitive transient (fit by a single exponential) by the  $C_m$ ; the mean  $\pm$  SEM series resistance was  $5.4 \pm 0.1$  M $\Omega$  ( $n = 222$ ). Series resistances were compensated electronically by >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  $y = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3} + C$ , where  $A_1$  ( $I_A$ ),  $A_2$  ( $I_D$ ), and  $A_3$  ( $I_K$ ) 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. For statistical analyses, current–voltage ( $I$ - $V$ ) plots were compared using repeated-measures ANOVA. The statistical significance of the differences between individual  $I$ - $V$  plots were, subsequently, calculated using Tukey's multiple comparisons *post hoc* test, and  $p$  values are reported in the text or in the figure legends.

**Western blots.** For biochemical experiments, mice were deeply anesthetized with halothane and decapitated, and the brains were rapidly removed. Posterior (~1 mm) cortices from four animals of each genotype (WT, *Kv1.4*<sup>-/-</sup>, *Kv4.2*<sup>-/-</sup>, and *Kv4.3*<sup>-/-</sup>) were dissected and flash frozen in liquid nitrogen. Protein lysates were prepared from the posterior cortices using previously described methods (Brunet et al., 2004). The protein concentration in each sample was determined using a Bio-Rad protein assay kit, following the instructions of the manufacturer. Equal amounts of proteins were fractionated on 7.5% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, incubated in blocking buffer at room temperature for 1 h, followed by incubation with primary antibodies against the individual Kv  $\alpha$ -subunits overnight at 4°C. The monoclonal anti-Kv4.2 and anti-Kv4.3 antibodies were obtained from the University of California, Davis (UCD)/National Institutes of Health (NIH) NeuroMab Facility, supported by NIH Grant U24NS050606 and maintained by the Department of Neurobiology, Physiology, and Behavior, College of Biological Sciences at UCD. The polyclonal anti-Kv1.4 antibody used was obtained from Millipore Bioscience Research Reagents. Bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-mouse IgG (Bethyl Labs) or goat anti-rabbit IgG (GE Healthcare) and the Dura West chemiluminescence reagent (Pierce). Signals were detected and quantified using the Bio-Rad ChemiDoc system and Quantity One software (Bio-Rad). Blots were reprobbed with primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam) to verify equal loading of lanes. For quantification, the anti-GAPDH signals were used to normalize the Kv  $\alpha$ -subunit signals measured from the same blot.

## Results

### Distinct component of $I_A$ evident in *Kv4.2*<sup>-/-</sup> pyramidal neurons

Previously, we reported that the rapidly activating and rapidly inactivating Kv current,  $I_A$ , which is prominent in WT cortical pyramidal neurons (Fig. 1A), was not evident in most (80%) cortical pyramidal neurons (Fig. 1B) isolated from mice (*Kv4.2*<sup>-/-</sup>) harboring a targeted disruption of the *Kcnd2* (*Kv4.2*) locus (Nerbonne et al., 2008), findings consistent with previous suggestions that Kv4.2 is the critical Kv  $\alpha$ -subunit encoding  $I_A$  channels in cortical (and hippocampal) pyramidal neurons (Korngreen and Sakmann, 2000; Kim et al., 2005; Chen et al., 2006). In a small subset (~20%) of *Kv4.2*<sup>-/-</sup> cells, however, a rapidly inactivating current was observed (Nerbonne et al., 2008), suggesting the expression of a molecularly distinct (non-Kv4.2-encoded)  $I_A$  in this subset of cortical pyramidal neurons.



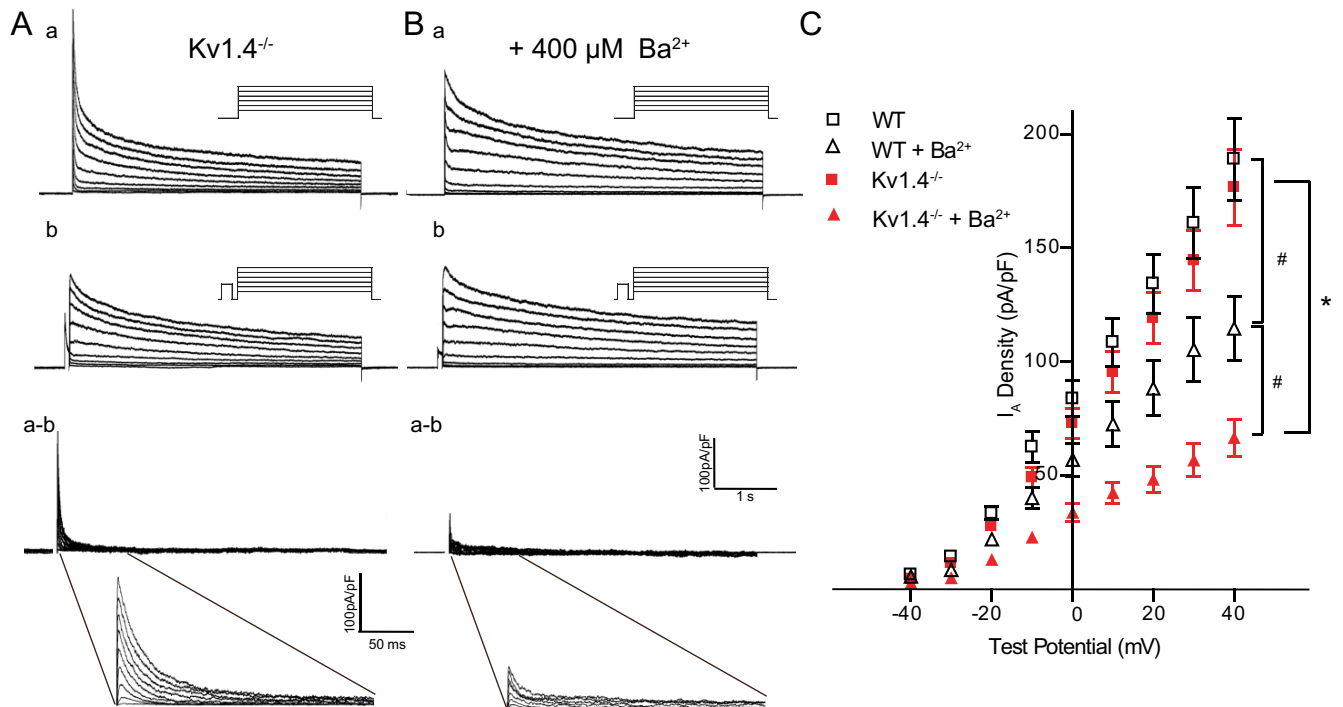
**Figure 1.** A component of  $I_A$ , sensitive to 1 mM 4-AP, is present in all  $Kv4.2^{-/-}$  cortical pyramidal neurons. Representative outward Kv current waveforms, elicited by 4 s voltage steps to test potentials between  $-40$  and  $+40$  mV (in 10 mV increments) from a holding potential of  $-70$  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  $\pm$  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  $\pm$  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**).

The marked differences in the waveforms of the Kv currents in the vast majority (80%) of  $Kv4.2^{-/-}$  cortical pyramidal neurons (Fig. 1B) and those recorded from WT cells (Fig. 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 TEA. As illustrated in Figure 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 (Fig. 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 60 ms prepulse to  $-10$  mV was used to inactivate  $I_A$  (Fig. 1Db). Offline subtraction of the current records obtained with the prepulse from the control records in the same cell (Fig. 1Da) allowed the isolation  $I_A$  (Fig. 1Da–b). The peak amplitudes of the subtracted current records were measured and normalized to cell capacitance to yield  $I_A$  densities. The mean  $\pm$  SEM density of  $I_A$  in  $Kv4.2^{-/-}$  neurons was significantly ( $p < 0.01$ ) lower than in WT cells (Fig. 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 (Burkhalter et al., 2006; Nerbonne et al., 2008). Thus, the observation of a reduction in, but not the elimination of,  $I_A$  in  $Kv4.2^{-/-}$  neurons indicates that other Kv

$\alpha$ -subunits (in addition to Kv4.2) contribute to the generation of functional  $I_A$  channels in cortical pyramidal neurons.

#### Pharmacologic characterization of $I_A$ in $Kv4.2^{-/-}$ neurons

Subsequent experiments were focused on determining the molecular identity of the Kv  $\alpha$ -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 (Blair et al., 1991; Ruppertsberg et al., 1991; Rettig et al., 1994; Trudeau et al., 1999). Previous studies (Camerino et al., 2007) also suggest that the channels encoded by the various Kv  $\alpha$ -subunits can be distinguished by using selective pharmacologic blockers. In control experiments, 3 mM TEA, which effectively blocks Kv3 channels (Rudy and McBain, 2001; Gutman et al., 2003), 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-AP. Previous studies have shown, for example, that Kv1 channels are blocked effectively by submillimolar concentrations of 4-AP (Grissmer et al., 1994; Rasmusson et al., 1995; Gutman et al., 2003), whereas



**Figure 2.** Use of  $Ba^{2+}$  to block Kv4-encoded currents reveals a role for Kv1.4 in the generation of  $I_A$  in cortical pyramidal neurons. Outward Kv currents were evoked and  $I_A$  was measured in  $Kv1.4^{-/-}$  neurons under control conditions (**A**) and in the presence of  $400 \mu M Ba^{2+}$  (**B**) using the subtraction protocol described in the legend to Figure 1. Similar experiments were performed on WT neurons (data not illustrated). The mean  $\pm$  SEM densities of  $I_A$  in WT ( $n = 22$ ) and  $Kv1.4^{-/-}$  ( $n = 13$ ) neurons were similar under control conditions. In the presence of  $400 \mu M Ba^{2+}$ , however, the mean  $\pm$  SEM  $I_A$  density in  $Kv1.4^{-/-}$  neurons ( $n = 22$ ) was significantly ( $\#p < 0.05$ ) lower than in WT neurons ( $n = 14$ ) (**C**). The presence of  $Ba^{2+}$  significantly ( $*p < 0.01$ ,  $\#p < 0.05$ ) reduced the mean  $\pm$  SEM  $I_A$  density in  $Kv1.4^{-/-}$  and in WT neurons.

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

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

All values are mean  $\pm$  SEM density. Kv currents recorded at  $+30$  mV (from a holding potential of  $-70$  mV) were analyzed.  $*p < 0.01$ , significantly different from those in WT neurons.

Kv4 channels are blocked by 4-AP in the several millimolar range (Serôdio et al., 1996) and Kv12 channels are insensitive to high millimolar concentrations of 4-AP (Trudeau et al., 1999; Gutman et al., 2003).

To explore the possible role of Kv1 channels, recordings were obtained from  $Kv4.2^{-/-}$  and WT neurons exposed to  $1$  mM 4-AP (in the presence of  $3$  mM TEA) (Fig. 1E). Addition of  $1$  mM 4-AP to the bath solution markedly reduced  $I_A$  in both  $Kv4.2^{-/-}$  and WT neurons (Fig. 1F), demonstrating the presence of a  $1$  mM 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 (Serôdio et al., 1996; Gutman et al., 2003). 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  $Kv4.2^{-/-}$  and WT neurons (Fig. 1F). The mean  $\pm$  SEM density of the 4-AP-resistant  $I_A$ , however, was significantly ( $p < 0.01$ ) lower in  $Kv4.2^{-/-}$  than in WT cells (Fig. 1C,D), consistent with the elimination of the Kv4.2-encoded component of  $I_A$  in  $Kv4.2^{-/-}$  neurons. In recordings obtained from 10 of 12  $Kv4.2^{-/-}$  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  $Kv4.2^{-/-}$  cells examined (see Discussion).

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

The 4-AP sensitivity of  $I_A$  (Fig. 1) suggests that one component of  $I_A$  in WT and  $Kv4.2^{-/-}$  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 (Po et al., 1993) and that other Kv1 subfamily members can generate rapidly inactivating currents through heteromeric assembly with Kv1.4 or by combining with accessory Kv $\beta$  subunits, specifically Kv $\beta$ 1 or Kv $\beta$ 3 (Po et al., 1993; Heinemann et al., 1995; Leicher et al., 1998). 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 ( $Kv1.4^{-/-}$ ) harboring a targeted disruption of the *Kcna4* (Kv1.4) locus (London et al., 1998). The waveforms of the Kv currents in  $Kv1.4^{-/-}$  neurons (Fig. 2A) were similar to those in WT neurons (Fig. 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  $\pm$  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

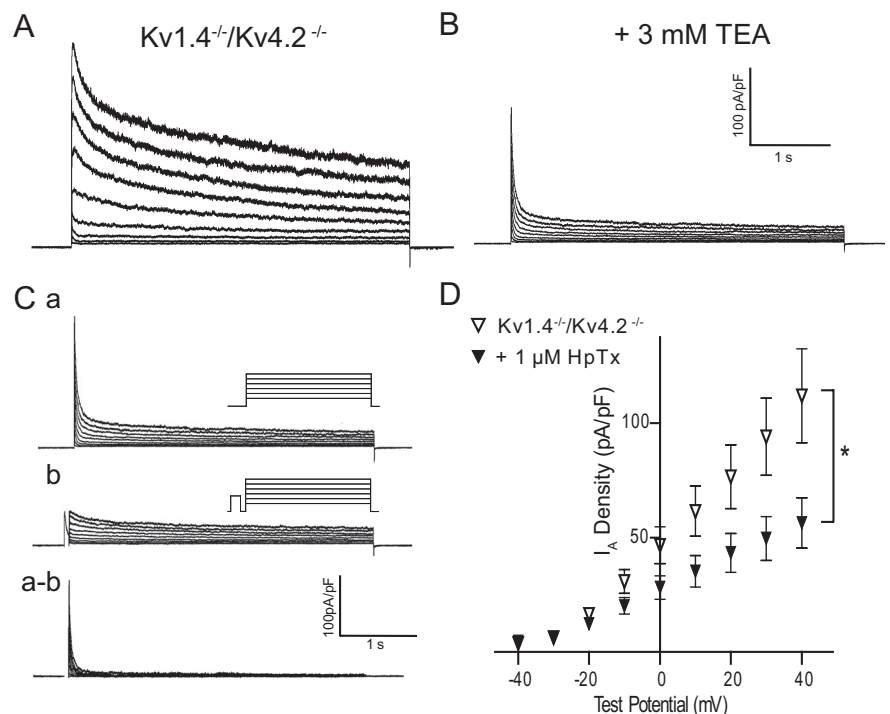
( $p < 0.01$ ), difference in  $I_{SS}$  densities measured in  $Kv1.4^{-/-}$  and WT neurons (Table 1). The observation that mean  $\pm$  SEM  $I_A$  densities are similar in  $Kv1.4^{-/-}$  and WT neurons may indicate that Kv1.4 does not contribute to  $I_A$  in WT cells or, alternatively, that other  $I_A$  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,  $Ba^{2+}$  in the hundred micromolar range has been shown to reduce  $I_A$  selectively (Gasparini et al., 2007; Andrásfalvy et al., 2008). In addition, the effect of  $Ba^{2+}$  on  $I_A$  was reportedly reduced in  $Kv4.2^{-/-}$  hippocampal neurons (Andrásfalvy et al., 2008), 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 (Schoots et al., 1996; Hughes et al., 2000). Control experiments revealed that the effect of 400  $\mu M$   $Ba^{2+}$  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  $\pm$  SEM density of the  $Ba^{2+}$ -sensitive component of  $I_A$  was  $75 \pm 4$  pA/pF in WT neurons and  $42 \pm 3$  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  $\mu M$   $Ba^{2+}$  (Fig. 2). As illustrated in Figure 2, addition of 400  $\mu M$   $Ba^{2+}$  to the bath significantly ( $p < 0.01$ ) reduced the density of  $I_A$  in  $Kv1.4^{-/-}$  neurons (Fig. 2C). In addition, the mean  $\pm$  SEM density of the  $Ba^{2+}$ -resistant component of  $I_A$  was significantly ( $p < 0.05$ ) larger in WT than in  $Kv1.4^{-/-}$  neurons (Fig. 2C), indicating a role for Kv1.4 in the generation of  $I_A$  in WT neurons. This observation, together with the finding that mean  $\pm$  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.

#### Residual $I_A$ is present in neurons lacking both Kv4.2 and Kv1.4

The results of the experiments described above using 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 of  $I_A$  current in cortical pyramidal neurons. To determine whether 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 (Fig. 1B), marked increases in the delayed rectifier currents ( $I_K$  and  $I_{SS}$ ) were evident in records obtained from  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons (Fig. 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 (Fig. 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  $\alpha$ -subunits in the generation of the

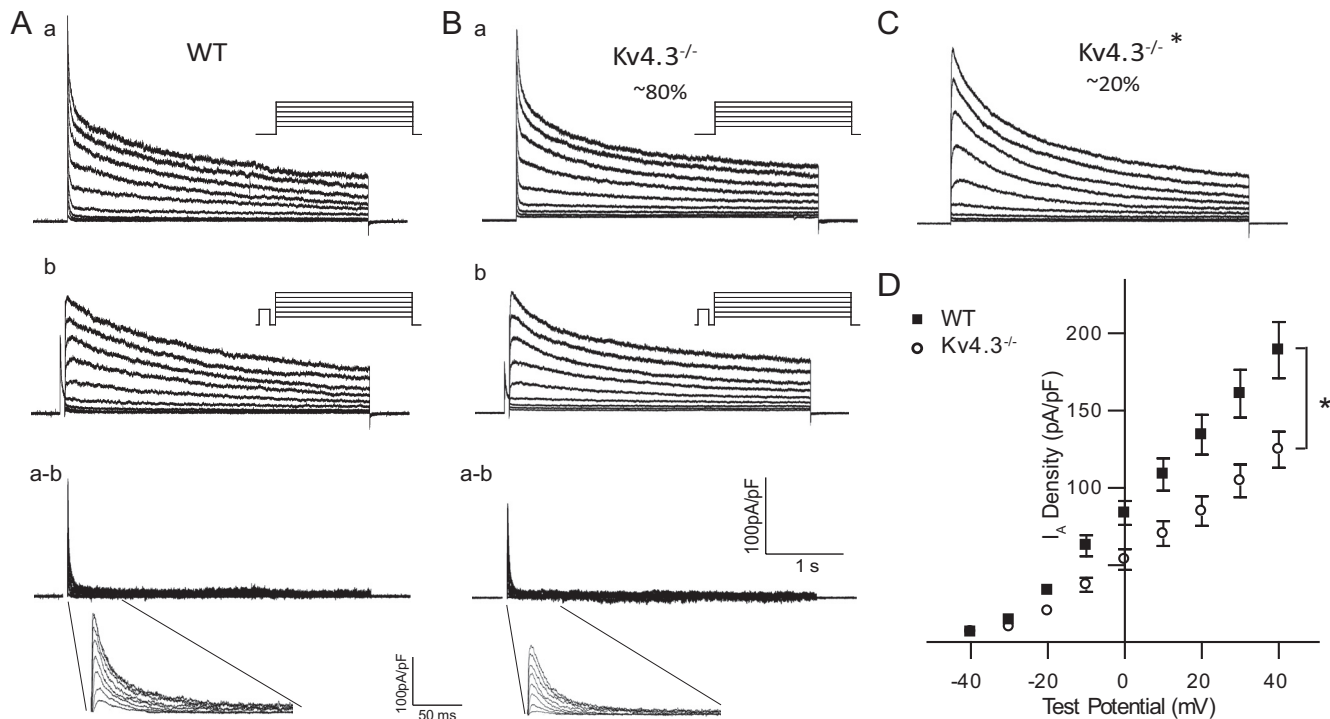


**Figure 3.** A heteropodatoxin-2-sensitive component of  $I_A$  is present in  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons. Similar to the findings in  $Kv4.2^{-/-}$  neurons (Fig. 1), large delayed rectifier currents ( $I_K$  and  $I_{SS}$ ) were evident in recordings obtained from  $Kv1.4^{-/-}/Kv4.2^{-/-}$  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  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons examined. The amplitude of the  $I_A$  component remaining in  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons was quantified (C) using the same subtraction protocol as used in experiments on  $Kv4.2^{-/-}$  (Fig. 1) and  $Kv1.4^{-/-}$  (Fig. 2) neurons and as described in the legend to Figure 1. The mean  $\pm$  SEM density of the  $I_A$  component remaining in  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons ( $n = 20$ ) was reduced significantly ( $*p < 0.01$ ) by addition of 1  $\mu M$  Hptx-2 ( $n = 14$ ) to the bath (D).

component of  $I_A$  remaining in  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons (Fig. 3). In addition to Kv1.4, several Kv1  $\alpha$ -subunits, including Kv1.1, Kv1.2, and Kv1.6, are reportedly expressed in cortical pyramidal neurons (Guan et al., 2006). Initial experiments were performed using the peptide toxin  $\alpha$ -Dtx, which has been reported to block heterologously expressed Kv1.1-, 1.2-, and 1.6-encoded currents and has been used previously to examine the roles of Kv1 channels in cortical neurons (Harvey and Robertson, 2004; Guan et al., 2006; Kole et al., 2007). As was done in the experiments on  $Kv4.2^{-/-}$  neurons (Fig. 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 (Fig. 3A). The further addition of  $\alpha$ -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 (data not illustrated). Similar experiments were performed using Hptx-2, which is specific for Kv4 channels (Zarayskiy et al., 2005). In contrast to  $\alpha$ -Dtx, addition of Hptx-2 (at 1  $\mu M$ ) significantly ( $p < 0.01$ ) reduced  $I_A$  amplitudes of  $Kv1.4^{-/-}/Kv4.2^{-/-}$  neurons (Fig. 3D), revealing an additional role for Kv4  $\alpha$ -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 (Serôdio and Rudy, 1998; Burkhalter et al., 2006), suggesting a role for Kv4.3.

#### Targeted disruption of *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 *Kcnd3* ( $Kv4.3^{-/-}$ ) locus (Niwa et al., 2008). In most (20 of 24)



**Figure 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 ( $Kv4.3^{-/-*}$ ) (**C**), large delayed rectifier currents ( $I_K$  and  $I_{SS}$ ) were evident, reminiscent of the Kv current waveforms observed in  $Kv4.2^{-/-}$  neurons (Fig. 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  $\pm$  SEM  $I_A$  density in  $Kv4.3^{-/-}$  ( $n = 24$ ) neurons was significantly ( $*p < 0.01$ ) lower than in WT ( $n = 22$ ) neurons (**D**).

of the  $Kv4.3^{-/-}$  neurons studied, the waveforms of the Kv currents (Fig. 4B) were similar to those recorded from WT neurons (Fig. 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 (Fig. 4C), however, were distinct and resembled the current waveforms seen in most  $Kv4.2^{-/-}$  neurons (Fig. 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  $\pm$  SEM density of  $I_A$  was significantly ( $p < 0.01$ ) lower in  $Kv4.3^{-/-}$  neurons ( $n = 24$ ) than in WT cells ( $n = 22$ ) (Fig. 4D).

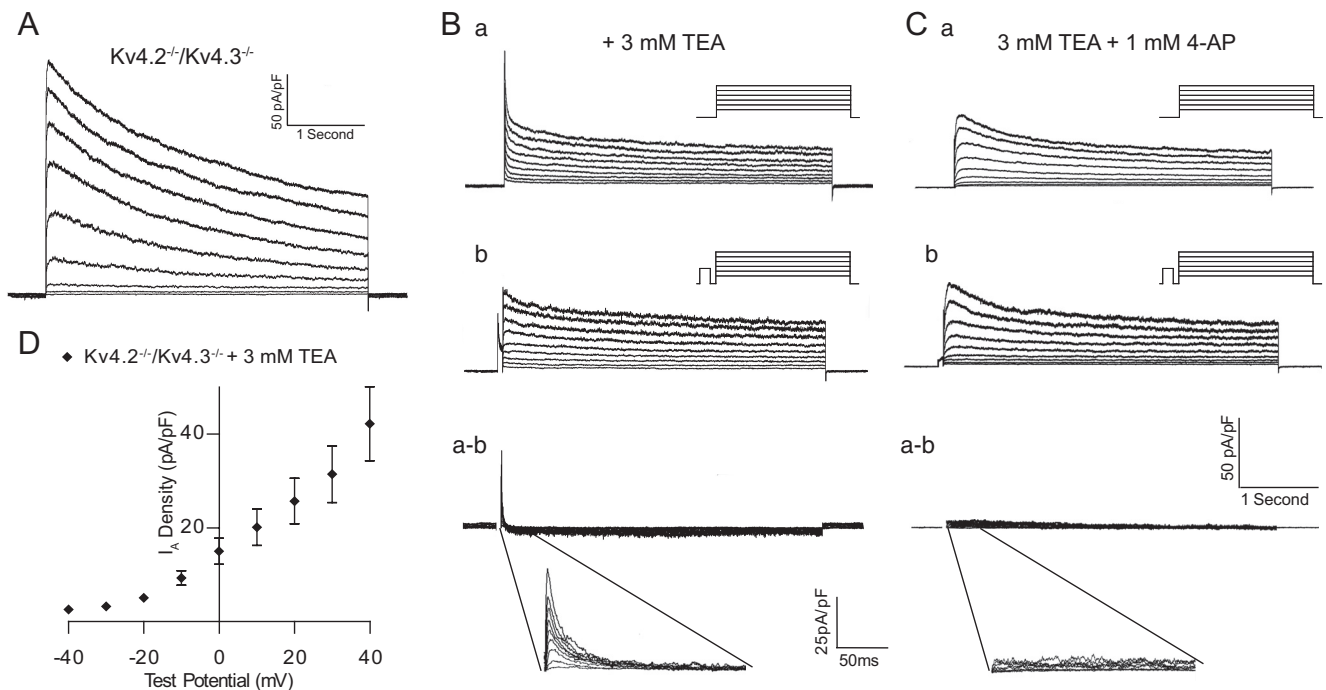
#### 4-AP-sensitive $I_A$ component remains in $Kv4.2^{-/-}/Kv4.3^{-/-}$ cortical pyramidal neurons

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 1 mM 4-AP. This hypothesis was tested directly by obtaining recordings from neurons isolated from mice lacking both Kv4.2 and Kv4.3 ( $Kv4.2^{-/-}/Kv4.3^{-/-}$ ), generated by crossing the  $Kv4.2^{-/-}$  and  $Kv4.3^{-/-}$  animals. The waveforms of the Kv currents in  $Kv4.2^{-/-}/Kv4.3^{-/-}$  cortical pyramidal neurons (Fig. 5A) resembled those in

$Kv4.2^{-/-}$  neurons (Fig. 1B) with large delayed rectifier currents and without a prominent rapidly inactivating current component. Inclusion of 3 mM TEA in the bath unmasked a rapidly inactivating current in all ( $n = 18$ )  $Kv4.2^{-/-}/Kv4.3^{-/-}$  neurons (Fig. 5B). Interestingly, the mean  $\pm$  SEM peak density of the  $I_A$  remaining in  $Kv4.2^{-/-}/Kv4.3^{-/-}$  neurons is similar in magnitude to the  $I_A$  component eliminated in  $Kv1.4^{-/-}$  neurons (Fig. 2B). In addition, as illustrated in Figure 5C, no rapidly inactivating currents remained in  $Kv4.2^{-/-}/Kv4.3^{-/-}$  neurons ( $n = 13$ ) in the presence of 1 mM 4-AP (Fig. 5C).

#### 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  $\alpha$ -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 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  $\alpha$ -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  $\alpha$ -subunit-specific antibody signals in each lane. Quantification of the normalized Kv  $\alpha$ -subunit-specific antibody signals revealed that the expression levels of Kv1.4 and Kv4.3 in samples

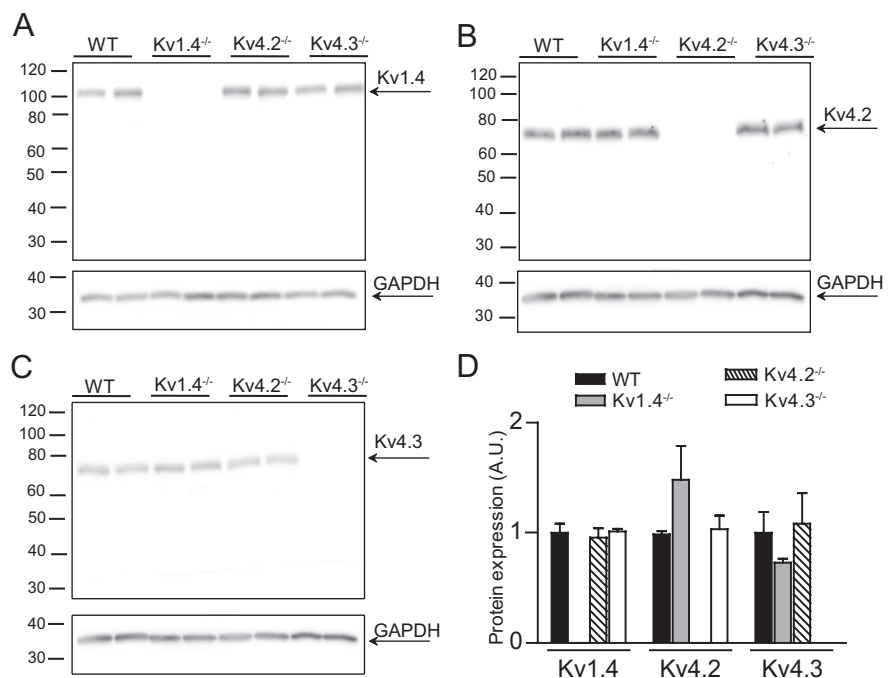


**Figure 5.**  $I_A$  in  $Kv4.2^{-/-}/Kv4.3^{-/-}$  neurons is blocked completely by 1 mM 4-AP. Similar to the findings in  $Kv4.2^{-/-}$  neurons (Fig. 1), large delayed rectifier currents ( $I_K$  and  $I_{Ks}$ ) were seen in all records from  $Kv4.2^{-/-}/Kv4.3^{-/-}$  neurons (**A**). Addition of 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**, mean  $\pm$  SEM data shown in **D**). In contrast to WT and  $Kv4.2^{-/-}$  neurons (Fig. 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**).

from mice with targeted disruption of the other Kv  $\alpha$ -subunits were similar to those in WT cortices (Fig. 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) (Fig. 6D). These results indicate that the total protein expression levels of the Kv  $\alpha$ -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  $\alpha$ -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 used to explore this possibility.

#### Genetic and pharmacologic dissection of $I_A$

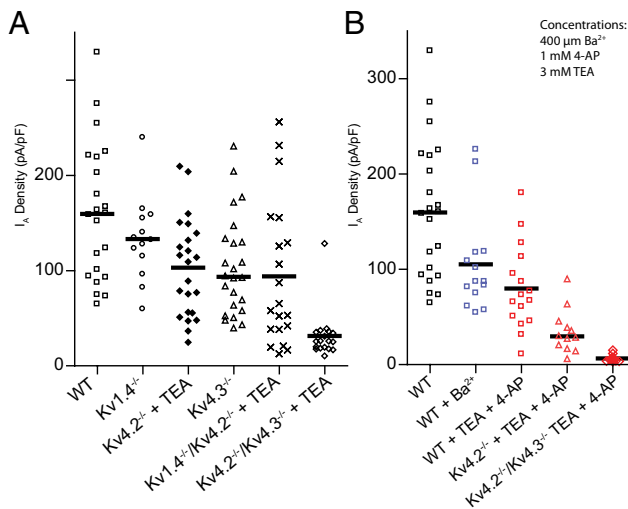
The contributions of Kv1.4, Kv4.2, and Kv4.3 to the generation of the total macroscopic  $I_A$  in cortical pyramidal neurons is revealed in direct comparisons of  $I_A$  densities in cortical pyramidal neurons of the various genotypes and examined under different pharmacologic conditions (Fig. 7). As 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



**Figure 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,  $Kv1.4^{-/-}$ ,  $Kv4.2^{-/-}$ , and  $Kv4.3^{-/-}$  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  $\pm$  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) toward increased Kv4.2 expression and a decreased Kv4.3 expression in the  $Kv1.4^{-/-}$  samples.

currents ( $I_K$ ) that are present in  $Kv4.2^{-/-}$  neurons (Fig. 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





**Figure 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  $\pm$  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 toward 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  $\alpha$ -subunits are plotted. Mean  $I_A$  density was reduced in WT cells by 400  $\mu$ M  $Ba^{2+}$  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.

heterogeneity in peak  $I_A$  densities among WT cortical pyramidal neurons (Fig. 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 (Fig. 7A).

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  $I_A$ . Furthermore, 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  $I_A$ .

## Discussion

### Multiple components of $I_A$ in cortical pyramidal neurons

A systematic experimental approach, using genetic disruption of Kv  $\alpha$ -subunit expression paired with pharmacology, was used to identify the Kv channel  $\alpha$ -subunits responsible for the generation of macroscopic  $I_A$  in cortical pyramidal neurons. The results pre-

sented 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  $\alpha$ -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  $\alpha$ -Dtx, indicating that Kv1  $\alpha$ -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  $\alpha$ -subunits. The limitations of pharmacology can be seen in Figure 7 where the reduction of  $I_A$  density in WT neurons attributable to 1 mM 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 (Marder and Goillard, 2006; Van Wart and Matthews, 2006; Nerbonne et al., 2008). The summary data (Fig. 7) indicate that Kv4.2 and Kv4.3  $\alpha$ -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 (Fig. 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 (Fig. 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 (McCormick and Prince, 1987; Kasper et al., 1994). It seems reasonable to suggest, therefore, that Kv1.4, Kv4.2, and Kv4.3 all contribute to  $I_A$  in cortical pyramidal neurons throughout postnatal development. As neurons mature, however, the subcellular distribution patterns and/or functional roles of individual channel types may change. Additional experiments will be necessary to explore these questions directly.

### Remodeling of Kv currents in response to the loss of $I_A$ encoding $\alpha$ -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 ( $\sim 80\%$ ) of  $Kv4.2^{-/-}$  neurons, marked differences in Kv current waveforms

were evident, reflecting increased TEA-sensitive delayed rectifier currents (Fig. 1) (Nerbonne et al., 2008). Surprisingly, only 20% of  $Kv4.3^{-/-}$  neurons displayed a similar remodeling (Fig. 4C) despite similar decreases in mean  $I_A$  density in  $Kv4.2^{-/-}$  and  $Kv4.3^{-/-}$  neurons (Fig. 7A). These observations illustrate the complexity of ascertaining the functional roles of individual channel  $\alpha$ -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 (Andrásfalvy et al., 2008), suggesting that widespread, compensatory changes in neuronal properties and excitability can occur in response to alterations in Kv  $\alpha$ -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 (Guo et al., 2005). 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 coimmunoprecipitated from brain, consistent with the presence of heteromultimeric Kv4.2/Kv4.3 channels (Marionneau et al., 2009). 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 (Burkhalter et al., 2006). 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 coexpression of numerous accessory subunits, such as the  $K^+$  channel interacting proteins KChIPs and dipeptidyl peptidases DPP6 and DPP10 (Schrader et al., 2002; Jerng et al., 2005; Maffie and Rudy, 2008).

### 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 (Lai and Jan, 2006; Dai et al., 2009). Identification of the Kv  $\alpha$ -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  $\alpha$ -subunits 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.

Despite 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 (Jerng et al., 2005). A recent study examining the effects of dis-

rupting 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 (Kim et al., 2008). 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  $\alpha$ -subunit expression (Guo et al., 2005; Nerbonne et al., 2008). Interestingly, several channel accessory subunits have been suggested to interact with and differentially regulate multiple types of ion channels (Li et al., 2005; Nerbonne and Kass, 2005; Solé et al., 2009; Thomsen et al., 2009), 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  $\alpha$ -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.

### References

- Andrásfalvy BK, Makara JK, Johnston D, Magee JC (2008) Altered synaptic and non-synaptic properties of CA1 pyramidal neurons in Kv4.2 knock-out mice. *J Physiol* 586:3881–3892.
- Bernard C, Anderson A, Becker A, Poolos NP, Beck H, Johnston D (2004) Acquired dendritic channelopathy in temporal lobe epilepsy. *Science* 305:532–535.
- Blair TA, Roberds SL, Tamkun MM, Hartshorne RP (1991) Functional characterization of RK5, a voltage-gated  $K^+$  channel cloned from the rat cardiovascular system. *FEBS Lett* 295:211–213.
- Brunet S, Aimond F, Li H, Guo W, Eldstrom J, Fedida D, Yamada KA, Nerbonne JM (2004) Heterogeneous expression of repolarizing, voltage-gated  $K^+$  currents in adult mouse ventricles. *J Physiol* 559:103–120.
- Burkhalter A, Gonchar Y, Mellor RL, Nerbonne JM (2006) Differential expression of  $I_A$  channel subunits Kv4.2 and Kv4.3 in mouse visual cortical neurons and synapses. *J Neurosci* 26:12274–12282.
- Cai X, Liang CW, Muralidharan S, Kao JP, Tang CM, Thompson SM (2004) Unique roles of SK and Kv4.2 potassium channels in dendritic integration. *Neuron* 44:351–364.
- Camerino DC, Tricarico D, Desaphy JF (2007) Ion channel pharmacology. *Neurotherapeutics* 4:184–198.
- Chen X, Yuan LL, Zhao C, Birnbaum SG, Frick A, Jung WE, Schwarz TL, Sweatt JD, Johnston D (2006) Deletion of Kv4.2 gene eliminates dendritic A-type  $K^+$  current and enhances induction of long-term potentiation in hippocampal CA1 pyramidal neurons. *J Neurosci* 26:12143–12151.
- Crill WE, Schwandt PC (1983) Active currents in mammalian central neurons. *Trends Neurosci* 6:236–240.
- Dai S, Hall DD, Hell JW (2009) Supramolecular assemblies and localized regulation of voltage-gated ion channels. *Physiol Rev* 89:411–452.
- Gasparini S, Losonczy A, Chen X, Johnston D, Magee JC (2007) Associative pairing enhances action potential back-propagation in radial oblique branches of CA1 pyramidal neurons. *J Physiol* 580:787–800.
- Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, Karmilowicz MJ, Auperin DD, Chandy KG (1994) Pharmacological characterization of five cloned voltage-gated  $K^+$  channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol Pharmacol* 45:1227–1234.
- Guo W, Lee JC, Tkatch T, Surmeier DJ, Armstrong WE, Foehring RC (2006) Expression and biophysical properties of Kv1 channels in supragranular neocortical pyramidal neurons. *J Physiol* 571:371–389.
- Guo W, Jung WE, Marionneau C, Aimond F, Xu H, Yamada KA, Schwarz TL, Demolombe S, Nerbonne JM (2005) Targeted deletion of Kv4.2 eliminates  $I_{(to,f)}$  and results in electrical and molecular remodeling, with no evidence of ventricular hypertrophy or myocardial dysfunction. *Circ Res* 97:1342–1350.
- Gutman GA, Chandy KG, Adelman JP, Aiyar J, Bayliss DA, Clapham DE, Covarrubias M, Desir GV, Furuichi K, Ganetzky B, Garcia ML, Grissmer S, Jan LY, Karschin A, Kim D, Kupersmidt S, Kurachi Y, Lazdunski M,

- Lesage F, Lester HA, McKinnon D, Nichols CG, O'Kelly I, Robbins J, Robertson GA, Rudy B, Sanguinetti M, Seino S, Stuehmer W, Tamkun MM, Vandenberg CA, Wei A, Wulff H, Wymore RS; International Union of Pharmacology (2003) International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: potassium channels. *Pharmacol Rev* 55:583–586.
- Harvey AL, Robertson B (2004) Dendrotoxins: structure-activity relationships and effects on potassium ion channels. *Curr Med Chem* 11:3065–3072.
- Heinemann SH, Rettig J, Wunder F, Pongs O (1995) Molecular and functional characterization of a rat brain Kv beta 3 potassium channel subunit. *FEBS Lett* 377:383–389.
- Hu HJ, Carrasquillo Y, Karim F, Jung WE, Nerbonne JM, Schwarz TL, Gereau RW 4th (2006) The kv4.2 potassium channel subunit is required for pain plasticity. *Neuron* 50:89–100.
- Huettnner JE, Baughman RW (1986) Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci* 6:3044–3060.
- Hughes BA, Kumar G, Yuan Y, Swaminathan A, Yan D, Sharma A, Plumley L, Yang-Feng TL, Swaroop A (2000) Cloning and functional expression of human retinal kir2.4, a pH-sensitive inwardly rectifying  $K^+$  channel. *Am J Physiol Cell Physiol* 279:C771–C784.
- Ishikawa T, Nakamura Y, Saitoh N, Li WB, Iwasaki S, Takahashi T (2003) Distinct roles of Kv1 and Kv3 potassium channels at the calyx of Held presynaptic terminal. *J Neurosci* 23:10445–10453.
- Jerng HH, Kunjilwar K, Pfaffinger PJ (2005) Multiprotein assembly of Kv4.2, KChIP3 and DPP10 produces ternary channel complexes with ISA-like properties. *J Physiol* 568:767–788.
- Kasper EM, Larkman AU, Lübke J, Blakemore C (1994) Pyramidal neurons in layer 5 of the rat visual cortex. II. Development of electrophysiological properties. *J Comp Neurol* 339:475–494.
- Kim J, Wei DS, Hoffman DA (2005) Kv4 potassium channel subunits control action potential repolarization and frequency-dependent broadening in rat hippocampal CA1 pyramidal neurons. *J Physiol* 569:41–57.
- Kim J, Nadal MS, Clemens AM, Baron M, Jung SC, Misumi Y, Rudy B, Hoffman DA (2008) Kv4 accessory protein DPPX (DPP6) is a critical regulator of membrane excitability in hippocampal CA1 pyramidal neurons. *J Neurophysiol* 100:1835–1847.
- Kole MH, Letzkus JJ, Stuart GJ (2007) Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. *Neuron* 55:633–647.
- Korngreen A, Sakmann B (2000) Voltage-gated  $K^+$  channels in layer 5 neocortical pyramidal neurons from young rats: subtypes and gradients. *J Physiol* 525:621–639.
- Lai HC, Jan LY (2006) The distribution and targeting of neuronal voltage-gated ion channels. *Nat Rev Neurosci* 7:548–562.
- Leicher T, Bähring R, Isbrandt D, Pongs O (1998) Coexpression of the KCNA3B gene product with Kv1.5 leads to a novel A-type potassium channel. *J Biol Chem* 273:35095–35101.
- Li H, Guo W, Mellor RL, Nerbonne JM (2005) KChIP2 modulates the cell surface expression of Kv 1.5-encoded  $K^+$  channels. *J Mol Cell Cardiol* 39:121–132.
- Locke RE, Nerbonne JM (1997a) Three kinetically distinct  $Ca^{2+}$ -independent depolarization-activated  $K^+$  currents in callosal-projecting rat visual cortical neurons. *J Neurophysiol* 78:2309–2320.
- Locke RE, Nerbonne JM (1997b) Role of voltage-gated  $K^+$  currents in mediating the regular-spiking phenotype of callosal-projecting rat visual cortical neurons. *J Neurophysiol* 78:2321–2335.
- London B, Wang DW, Hill JA, Bennett PB (1998) The transient outward current in mice lacking the potassium channel gene Kv1.4. *J Physiol* 509:171–182.
- Lugo JN, Barnwell LF, Ren Y, Lee WL, Johnston LD, Kim R, Hrachovy RA, Sweatt JD, Anderson AE (2008) Altered phosphorylation and localization of the A-type channel, Kv4.2 in status epilepticus. *J Neurochem* 106:1929–1940.
- Maffie J, Rudy B (2008) Weighing the evidence for a ternary protein complex mediating A-type  $K^+$  currents in neurons. *J Physiol* 586:5609–5623.
- Marder E, Goaillard JM (2006) Variability, compensation and homeostasis in neuron and network function. *Nat Rev Neurosci* 7:563–574.
- Marionneau C, Leduc RD, Rohrs HW, Link AJ, Townsend RR, Nerbonne JM (2009) Proteomic analyses of native brain  $K_v4.2$  channel complexes. *Channels (Austin)* 3:284–294.
- McCormick DA, Prince DA (1987) Post-natal development of electrophysiological properties of rat cerebral cortical pyramidal neurones. *J Physiol* 393:743–762.
- Monaghan MM, Menegola M, Vacher H, Rhodes KJ, Trimmer JS (2008) Altered expression and localization of hippocampal A-type potassium channel subunits in the pilocarpine-induced model of temporal lobe epilepsy. *Neuroscience* 156:550–562.
- Nerbonne JM, Kass RS (2005) Molecular physiology of cardiac repolarization. *Physiol Rev* 85:1205–1253.
- Nerbonne JM, Gerber BR, Norris A, Burkhalter A (2008) Electrical remodeling maintains firing properties in cortical pyramidal neurons lacking KCND2-encoded A-type  $K^+$  currents. *J Physiol* 586:1565–1579.
- Niwa N, Wang W, Sha Q, Marionneau C, Nerbonne JM (2008) Kv4.3 is not required for the generation of functional Ito,f channels in adult mouse ventricles. *J Mol Cell Cardiol* 44:95–104.
- Patton C, Thompson S, Epel D (2004) Some precautions in using chelators to buffer metals in biological solutions. *Cell Calcium* 35:427–431.
- Po S, Roberds S, Snyders DJ, Tamkun MM, Bennett PB (1993) Heteromultimeric assembly of human potassium channels. Molecular basis of a transient outward current? *Circ Res* 72:1326–1336.
- Rasmusson RL, Zhang Y, Campbell DL, Comer MB, Castellino RC, Liu S, Strauss HC (1995) Bi-stable block by 4-aminopyridine of a transient  $K^+$  channel (Kv1.4) cloned from ferret ventricle and expressed in *Xenopus* oocytes. *J Physiol* 485:59–71.
- Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Dolly JO, Pongs O (1994) Inactivation properties of voltage-gated  $K^+$  channels altered by presence of beta-subunit. *Nature* 369:289–294.
- Rogawski MA (1985) The a-current: how ubiquitous a feature of excitable cells is it. *Trends Neurosci* 8:214–219.
- Rudy B, McBain CJ (2001) Kv3 channels: voltage-gated  $K^+$  channels designed for high-frequency repetitive firing. *Trends Neurosci* 24:517–526.
- Ruppersberg JP, Frank R, Pongs O, Stocker M (1991) Cloned neuronal IK(A) channels reopen during recovery from inactivation. *Nature* 353:657–660.
- Schoots O, Yue KT, MacDonald JF, Hampson DR, Nobrega JN, Dixon LM, Van Tol HH (1996) Cloning of a G protein-activated inwardly rectifying potassium channel from human cerebellum. *Brain Res Mol Brain Res* 39:23–30.
- Schrader LA, Anderson AE, Varga AW, Levy M, Sweatt JD (2002) The other half of Hebb:  $K^+$  channels and the regulation of neuronal excitability in the hippocampus. *Mol Neurobiol* 25:51–66.
- Seródio P, Rudy B (1998) Differential expression of Kv4  $K^+$  channel subunits mediating subthreshold transient  $K^+$  (A-type) currents in rat brain. *J Neurophysiol* 79:1081–1091.
- Seródio P, Vega-Saenz de Miera E, Rudy B (1996) Cloning of a novel component of A-type  $K^+$  channels operating at subthreshold potentials with unique expression in heart and brain. *J Neurophysiol* 75:2174–2179.
- Singh B, Ogiwara I, Kaneda M, Tokonami N, Mazaki E, Baba K, Matsuda K, Inoue Y, Yamakawa K (2006) A Kv4.2 truncation mutation in a patient with temporal lobe epilepsy. *Neurobiol Dis* 24:245–253.
- Solé L, Roura-Ferrer M, Pérez-Verdaguer M, Oliveras A, Calvo M, Fernández-Fernández JM, Felipe A (2009) KCNE4 suppresses Kv1.3 currents by modulating trafficking, surface expression and channel gating. *J Cell Sci* 122:3738–3748.
- Thomsen MB, Wang C, Ozgen N, Wang HG, Rosen MR, Pitt GS (2009) Accessory subunit KChIP2 modulates the cardiac L-type calcium current. *Circ Res* 104:1382–1389.
- Trudeau MC, Titus SA, Branchaw JL, Ganetzky B, Robertson GA (1999) Functional analysis of a mouse brain Elk-type  $K^+$  channel. *J Neurosci* 19:2906–2918.
- Van Wart A, Matthews G (2006) Impaired firing and cell-specific compensation in neurons lacking nav1.6 sodium channels. *J Neurosci* 26:7172–7180.
- Yuan W, Burkhalter A, Nerbonne JM (2005) Functional role of the fast transient outward  $K^+$  current  $I_A$  in pyramidal neurons in (rat) primary visual cortex. *J Neurosci* 25:9185–9194.
- Zarayskiy VV, Balasubramanian G, Bondarenko VE, Morales MJ (2005) Heteropoda toxin 2 is a gating modifier toxin specific for voltage-gated  $K^+$  channels of the Kv4 family. *Toxicol* 45:431–442.