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Function and Mechanism of Axonal Targeting of Voltage-sensitive Potassium Channels

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Abstract

Precise localization of various ion channels into proper subcellular compartments is crucial for neuronal excitability and synaptic transmission. Axonal K⁺ channels that are activated by depolarization of the membrane potential participate in the repolarizing phase of the action potential, and hence regulate action potential firing patterns, which encode output signals. Moreover, some of these channels can directly control neurotransmitter release at axonal terminals by constraining local membrane excitability and limiting Ca²⁺ influx. K⁺ channels differ not only in biophysical and pharmacological properties, but in expression and subcellular distribution as well. Importantly, proper targeting of channel proteins is a prerequisite for electrical and chemical functions of axons. In this review, we first highlight recent studies that demonstrate different roles of axonal K⁺ channels in the local regulation of axonal excitability. Next, we focus on research progress in identifying axonal targeting motifs and machinery of several different types of K⁺ channels present in axons. Regulation of K⁺ channel targeting and activity may underlie a novel form of neuronal plasticity. This research field can contribute to generating novel therapeutic strategies through manipulating neuronal excitability in treating neurological diseases, such as multiple sclerosis, neuropathic pain, and Alzheimer's disease.

Keywords

axonal targeting; action potential; neurotransmitter release; voltage-gated potassium channel; axon initial segment; neuronal polarity

1. Introduction

Neurons are highly polarized cells, typically with multiple dendrites and a long axon, important for inputs and outputs of signals, respectively. In the mammalian central nervous system (CNS), neuronal dendrites often receive and integrate thousands of synaptic inputs. Then the synaptic potentials are converted into action potentials (APs) at the axon initial segment (AIS). APs are conveyed along axons and trigger neurotransmitter release at axonal

terminals. Released neurotransmitters activate their receptors located on postsynaptic membranes of the target cells.

Output functions of axons require concerted actions of various types of ion channels, including voltage-gated sodium (Nav) channels, voltage-gated potassium (Kv) channels, and voltage-gated calcium (Cav) channels. Different types of neurons in nervous systems differ in their axon/dendrite morphology and AP firing patterns. The number and activity of these channels at specific axonal locations are fundamental to the axonal functions for various neurons. The biophysical properties of most of these channels have been extensively characterized in native cells and expression systems. Besides critical roles in synaptic transmission, the importance of axonal ion channel targeting in axonal electrical computation was proposed by the studies using computer simulations (Debanne, 2004; Li and Ascoli, 2006). Given the complex branching patterns of dendrites and axons of central neurons, especially interneurons, precise prediction by simulation is very difficult, if not impossible, when the targeting patterns of key ion channels are not known. Therefore, it is important to understand the mechanisms underlying the precise targeting of channel proteins and how they affect neuronal excitability, such as the input-output relationship. Elucidating the modulation of neuronal excitability, a new form of neuronal plasticity, is an essential step for understanding the dynamics and function of neural circuits.

Recently, progress has been made in understanding the local function of axonal ion channels, and identifying the targeting motifs and interacting proteins critical for targeting channel proteins into axons and at specific axonal sites. These studies have been carried out with contemporary patch clamp recording, molecular and cell biology, and protein biochemistry techniques. In this review, we focus on the function and mechanism of four types of K⁺ channels that exist in axons and are activated by membrane potentials. When activated, these channel complexes selectively allow K⁺ ions to flux out of the cell following the electrochemical gradient of K⁺ ion and hence hyperpolarize the membrane potential back to the resting level (towards K⁺ equilibrium). Therefore, they are involved in regulating AP initiation, waveform, frequency and uni-directional propagation, and neurotransmitter release.

2. Potassium channel superfamily

K⁺ channels are probably the most diverse family of ion channels. K⁺ channel superfamily contains approximately 80 different genes encoding various pore-forming subunits. Based on the secondary structure of channel subunits, they can be grouped into three major families (Fig. 1A). Kv channels belong to the first family. Each subunit of this family contains six to seven transmembrane (TM) segments and one pore-forming loop between the last two TM segments. Each channel complex contains four such pore-forming subunits. In this family, there are also members that are activated by both the membrane potential and intracellular chemical signals. We collectively call them, “voltage-sensitive K⁺ channels”. Inwardly rectifying (conducting current more efficiently in the inward direction) K⁺ channels belong to the second family. The members of this family have the simplest secondary structure, two TM domains and one pore-forming loop. They also form tetramers and often co-assemble with other membrane proteins. Two-pore domain K⁺ channels belong to the third family. Each subunit contains four TM segments and two pore-forming loops. In this family, two subunits form a channel complex. Most members of this third family generate currents with little voltage or time dependence, creating leak K⁺ channels.

2.1. Voltage-sensitive potassium channels

Kv channels form a large and diverse family, which can be further divided into many subfamilies. We focus on the voltage-sensing and pore-forming α subunits. The prototypical

Kv channels are divided into four subfamilies based on the similarity to their homologues in fruit fly *Drosophila*, Kv1 (Shaker; KCNA), Kv2 (Shab; KCNB), Kv3 (Shaw; KCNC), and Kv4 (Shal; KCND) (Fig. 1B). Since the initial cloning of these four subfamilies, eight other subfamilies (Kv5 to Kv12) have been identified to date (Chandy, 1991; Gutman *et al.*, 2005). Among all subfamilies, Kv5 (KCNF), Kv6 (KCNG), Kv8 (KCNV), and Kv9 (KCNS) α subunits have not been shown to yield functional channels by themselves in expression systems, but can modulate other functional Kv channels (Gutman *et al.*, 2005; Vacher *et al.*, 2008).

The four subfamilies of Kv1 to Kv4 express extensively in nervous systems. Their channel activities, pharmacological properties, and expression and distribution patterns have been extensively studied. The Kv7 (KCNQ) subfamily was identified relatively more recently (Fig. 1B). It appears to be a very important subfamily. Mutations in four out of five KCNQ genes (KCNQ1 to KCNQ5) cause diseases in humans including cardiac arrhythmias, deafness and epilepsy (Jentsch, 2000). The Kv10-Kv12 channels are categorized into the KCNH subfamily. Members of this subfamily are also expressed in nervous systems and play important roles in regulating neuronal excitability of nervous systems.

There are two other groups of K^+ channels that can be categorized into this family, calcium-activated K^+ channels, although they are sometimes assigned to a separate group. One is the large conductance Ca^{2+} -regulated, “big” K^+ (BK) channels encoded by the Slo1 gene (a name derived from the conserved gene that encodes this channel, first cloned from fruit fly *Drosophila*) family, which is among the largest and most complex of the K^+ channel superfamily (Fig. 1B). The BK channel is gated by both voltage and intracellular Ca^{2+} . Its α subunit contains seven TM segments and one pore-forming loop. The BK and related channels resemble Kv channels, but possess a large conserved carboxyl terminus, which allows channel gating to be altered in response to the direct sensing of several different intracellular ions, and by other second messenger systems (Salkoff *et al.*, 2006). In this subfamily, there are three other members, Slo2.1 (Slick), Slo2.2 (Slack), and Slo3. They differ with the BK channel in gating properties and Ca^{2+} regulation (Salkoff *et al.*, 2006). There are four beta subunits for Slo1, $\beta 1$ to $\beta 4$ (KCNMB1 to KCNMB4) (Table I). They have two membrane-spanning segments, with N and C termini located on the cytoplasmic side. They regulate Slo1 activity and trafficking. The second group includes small- and intermediate-conductance calcium-regulated K^+ channels. Their subunits contain six TM segments and one pore-forming loop, structurally similar to Kv channels, but their activation is regulated by intracellular Ca^{2+} and insensitive to the membrane potential (Weatherall *et al.*, 2010).

2.2. Inward-rectifying potassium channels

In contrast to Kv channels, inwardly rectifying K^+ channels (Kir; gene symbol: KCNJ) allow K^+ ions to more efficiently flow into, rather than out of, the cell. Each Kir subunit contains two TM segments and one pore-forming loop and each Kir channel complex contains four such subunits. To date, fifteen Kir subunit genes have been identified and grouped into seven subfamilies (Kir1.x to Kir7.x). These subfamilies can be further categorized into four functional groups: (1) classical Kir channels (Kir2.x); (2) G protein-gated Kir channels (Kir3.x); (3) ATP-sensitive K^+ channels (Kir6.x); (4) K^+ -transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x) (Hibino *et al.*, 2010). Kir channels not only control the passive and active electrical properties of cells, but they are also involved in G protein-coupled receptor signaling, and they may link cellular metabolic state and membrane excitability *in vivo* (see more extensive reviews about Kir channels (Bichet *et al.*, 2003; Hibino *et al.*, 2010; Loussouarn *et al.*, 2002; Luscher and Slesinger, 2010)). The trafficking and function of Kir channels in nervous systems is beyond the scope of this review.

2.3. Two-pore domain potassium channels

Leak K^+ channels contribute to the resting membrane potential and are involved in regulating neuronal excitability. The first channel, TWIK (tandem of pore domains in a weak inward rectifying K^+ channel, now called TWIK-1), was identified in 1996 (Lesage *et al.*, 1996). Currently, there are fifteen members in the KCNK family in human genome (KCNK1 to KCNK18, but KCNK8, KCNK11, and KCNK14 do not exist). These two-pore domain K^+ channels can be grouped into six subfamilies, TWIK, THIK, TREK, TASK, TALK, and TRESK. These channels can be modulated by a host of different endogenous and clinical compounds such as neurotransmitters and anesthetics, and by other voltage-independent factors such as temperature, pH, oxygen tension, and osmolarity. Many of these channels are expressed in mammalian brains and participate in homeostatic regulation of physicochemical processes (see more extensive reviews about two-pore domain channels (Enyedi and Czirjak, 2010; Goldstein *et al.*, 2001; Talley *et al.*, 2003)). The axon-dendrite targeting of this channel family has not yet been established.

This review focuses on the function and mechanism of four subfamilies of axonal K^+ channels that are directly involved in regulating neuronal excitability and synaptic transmission. They include Kv1, Kv3, KCNQ and BK channel subfamilies (Table I). Among them, Kv3 channels have the highest activation threshold of membrane potentials (~ -10 mV), fastest activation (activation constant, the time for half-maximal conductance, < 1 ms) and deactivation (deactivation constant, ~ 2 ms) kinetics, the biophysical features thought to be critical to enable rapid spiking of some central neurons (Jonas *et al.*, 2004; Rudy and McBain, 2001). In contrast, KCNQ and BK channels operate at much slower kinetics (activation constant, > 100 ms; deactivation is very slow) and are subject to the regulation of various intracellular factors (Salkoff *et al.*, 2006). The activation and deactivation kinetics of Kv1 are intermediate between the two groups (activation constant, $\sim 5 - 10$ ms; deactivation constant, $\sim 10 - 40$ ms). The activation threshold of Kv1 and KCNQ channels is more negative (~ -40 mV) and close to the resting membrane potential. Therefore, the biophysical properties of these channels differentially impact AP initiation, waveform, frequency, and invasion into axonal terminals. Their precise localization along axons allows them to profoundly regulate the electrical signal computation in axons.

3. Physiological functions of axonal targeting of voltage-sensitive potassium channels

3.1. Axonal compartments and ion channel functions at the axon initial segment

An axon is usually a long and slender projection of a neuron that conducts electrical impulses away from neuronal soma. It contains several structurally distinct regions that play key roles in axonal electrical and chemical functions. The AIS, adjacent to the axon hillock, is the site where APs are initiated in most neurons due to the presence of high-density Nav channels. Its position varies between neurons and is subject to regulation. Nodes of Ranvier are the gaps (~ 1 μ m in length) between the myelin sheaths formed along myelinated axons, where Nav channels are also highly concentrated. The nodal region is actively involved in the saltatory conduction of APs. Axonal terminals are the sites where neurotransmitters are released upon activation. AISs, nodes of Ranvier, axonal branching points, and axonal terminals are strategic sites for electrical signal computation of axons (Debanne, 2004). Axonal localization of various ion channels profoundly affects AP initiation, propagation and invasion to nerve terminals, and neurotransmitter release.

The AIS is not only the AP initiation site but also the gate where the entry of axonal proteins and organelles is controlled. Analysis of the structure and function of the AIS attracted much attention lately. Localization of AISs including key molecular components can vary in

different cell types (Lorincz and Nusser, 2008) and be regulated by neuronal activity, which may constitute a novel form of neuronal plasticity (Grubb and Burrone, 2010; Kuba *et al.*, 2010). Different types of ion channels are localized at the AIS and carry out different functions. For instance, the differential targeting of Nav1.2 and Nav1.6 at the AIS controls uni-direction propagation of APs (Boiko *et al.*, 2003; Hu *et al.*, 2009), and the T- and R-type Cav channels at the AIS shape spike bursts (Bender and Trussell, 2009). Kv channels also play critical roles at the AIS.

3.2. Regulation of action potential firing by voltage-sensitive potassium channels

Kv1 channels at the AIS of pyramidal neurons in brain contribute to a low threshold, slowly inactivating, outward current during the AP generation, which shortens the AP duration (Kole *et al.*, 2007; Shu *et al.*, 2007) (Fig. 2A–D). Local application of the specific blocker of Kv1 channels, α -dendrotoxin, at the AIS but not the soma broadened the APs (Kole *et al.*, 2007; Shu *et al.*, 2007) (Fig. 2C,D). In rapid-spiking GABAergic interneurons of the neocortex, Kv1 channels at the AIS control the AP threshold so that the cells respond preferentially to large inputs (Goldberg *et al.*, 2008). Blocking Kv1 channels with α -dendrotoxin shortens the delay of the first spike, and hence converts the delay-type spiking of the cells to continuous spiking without influencing the high-frequency firing (Goldberg *et al.*, 2008) (Fig. 2E–K). KCNQ channels at the AIS of hippocampal pyramidal neurons regulate the inherent spontaneous firing via controlling axonal resting membrane potential and AP threshold (Shah *et al.*, 2008). Kv2.1 and Kv2.2 channels are present at the AIS (Johnston *et al.*, 2008; Sarmiere *et al.*, 2008). Kv2.2 may play a crucial role in maintaining AP amplitude by regulating the interspike potential during high frequency firing in medial nucleus of the trapezoid body neurons (Johnston *et al.*, 2008). Currently, it remains unclear whether Kv3 and Slo1 channels play an important role at AISs. In the neurons of spinal cord, it is shown that Kv3.1b is concentrated at nodes of Ranvier but not at AISs (Devaux *et al.*, 2003).

K⁺ channels along the axonal shaft regulate AP waveform, frequency, and uni-directional propagation. In particular, axonal Kv3 may play a critical role in rapid spiking of APs in some GABAergic interneurons (Jonas *et al.*, 2004; Rudy and McBain, 2001). The unique channel properties of Kv3 channels, high activation threshold, fast activation and deactivation kinetics (Coetzee *et al.*, 1999; Rudy and McBain, 2001), allow neurons to fire APs at high frequency, up to several hundred Hz. In contrast, BK channels operate with much slower kinetics and contribute to the formation of afterhyperpolarization (AHP), which lasts several tens of milliseconds. BK channels also play important roles in spike frequency adaptation. During repetitive AP firing, increased Ca²⁺ entry results in greater activation of BK channels and hence reduction of AP firing. Low-threshold, slowly inactivating K⁺ currents, containing Kv1.2, play a key role in the flexible properties of intracortical axons and may contribute significantly to intracortical processing (Shu *et al.*, 2007).

In myelinated axons, Kv1 channels are concealed under the myelin sheath, thus they may contribute to maintaining the AP fidelity by preventing repetitive and aberrant firing. In contrast, Kv3.1b and KCNQ channels are found at nodes of Ranvier and may be directly involved in the saltatory conduction of APs. Moreover, localized ion channels may play critical roles in axonal branch points during AP failure and frequency change (Debanne, 2004). However, the exact roles of ion channels in axonal branching points have not yet been experimentally established.

3.3. Regulation of neurotransmitter release by voltage-sensitive potassium channels

All axonal voltage-sensitive K^+ channels can regulate neurotransmitter release. The action can be either directly through regulating AP invasion into axonal terminals and limiting Ca^{2+} influx via Cav channels by hyperpolarizing axonal membranes, indirectly via regulating AP propagation, or both.

Kv1 channels regulate neurotransmitter release in various neurons. Kv1.1 channels regulate neurotransmitter release at motor nerve terminals. Deleting Kv1.1 produces a striking temperature-sensitive excitability change at nerve terminals (Zhou *et al.*, 1999; Zhou *et al.*, 1998). Here, Kv1.1 channels most likely exert their function by regulating AP propagation rather than directly regulating neurotransmitter release, since Kv1.1 channels are not present in terminal membranes (Zhou *et al.*, 1998). On the other hand, Kv1 channels directly regulate neurotransmitter release at the cerebellar basket cell terminals, since they are highly concentrated there (McNamara *et al.*, 1993; Rhodes *et al.*, 1995; Rhodes *et al.*, 1997; Wang *et al.*, 1993; Wang *et al.*, 1994). A recent study shows that axonal Kv1 channels are found to regulate synaptic strength at proximal axonal terminals of layer 5 pyramidal neurons (Kole *et al.*, 2007). Furthermore, in hippocampal mossy fiber boutons fast inactivating K^+ channels dynamically regulate presynaptic Ca^{2+} influx, contributing to the control of efficacy of a glutamatergic synapse in the cortex (Geiger and Jonas, 2000). Kv1 channels suppress terminal hyperexcitability during the depolarizing after-potential (Dodson *et al.*, 2002; Dodson *et al.*, 2003).

Kv3 channels are directly involved in neurotransmitter release in nerve terminals of various neurons, including rapid-spiking GABAergic interneurons (Goldberg *et al.*, 2005), cerebellar basket cell nerve terminals (Southan and Robertson, 2000), the calyx of Held terminals (a synapse required for the binaural processing of sound-source localization) (Elezgarai *et al.*, 2003; Ishikawa *et al.*, 2003), and motor nerve terminals (Brooke *et al.*, 2004a; Brooke *et al.*, 2004b). At the calyx of Held terminal, Kv3 channels (including Kv3.1 and Kv3.4) directly regulate evoked transmitter release, whereas Kv1 channels reduce nerve terminal excitability, thereby preventing aberrant transmitter release (Ishikawa *et al.*, 2003).

KCNQ channels give rise to the non-inactivating M-current in neurons (Brown and Adams, 1980). Presynaptic M-channels on hippocampal CA3 neurons regulate axonal excitability and neurotransmitter release (Vervaeke *et al.*, 2006). M-channels act pre- and postsynaptically to dampen neuronal excitability (Peretz *et al.*, 2007). Presynaptic M-channels (KCNQ2) inhibit transmitter release by reducing Ca^{2+} entry through P/Q-type voltage-gated Ca^{2+} channels in the cortex (Luisi *et al.*, 2009), and modulate neurotransmitter release from rat hippocampal nerve terminals (Martire *et al.*, 2004).

In axonal terminals, BK channels are activated by both Ca^{2+} entry and depolarization of membrane potentials during AP invasion. This results in acceleration of the repolarization of the terminal membrane, and thereby exert negative feedback on the release process (Berkefeld and Fakler, 2008; Berkefeld *et al.*, 2006; Grimes *et al.*, 2009; Petersen and Maruyama, 1984; Raffaelli *et al.*, 2004; Robitaille *et al.*, 1993).

4. Axonal targeting of Kv1 (Shaker) channels

4.1. Axonal targeting of endogenous Kv1 channels

Among the eight α subunits of Kv1 channels (Kv1.1 to Kv1.8), Kv1.4 is the only one carrying transient (inactivating, or A type) current when expressed alone. Kv1.1, Kv1.2, and Kv1.4, the three most abundant Kv1 α subunits in mammalian brain, primarily localize in axons and nerve terminals (Trimmer and Rhodes, 2004). Kv1.1 and Kv1.2 colocalize at AISs of various central neurons (Dodson *et al.*, 2002; Inda *et al.*, 2006; Van Wart *et al.*,

2007), the juxtaparanodal (JXP) regions of myelinated axons in spinal cord and cortex (Rasband, 2004; Rasband *et al.*, 2002; Rasband and Trimmer, 2001; Rasband *et al.*, 1999; Rasband *et al.*, 1998; Rhodes *et al.*, 1995; Rhodes *et al.*, 1997; Vabnick *et al.*, 1999; Wang *et al.*, 1993; Wang *et al.*, 1994), and axonal terminals of cerebellar basket cells (McNamara *et al.*, 1993; Rhodes *et al.*, 1995; Rhodes *et al.*, 1997; Wang *et al.*, 1993; Wang *et al.*, 1994) and other neurons (Dodson *et al.*, 2003) (Fig. 3). Kv1.4 colocalizes with Kv1.1 in mossy fiber axons in the hippocampus (Cooper *et al.*, 1998; Sheng *et al.*, 1992; Wang *et al.*, 1993; Wang *et al.*, 1994). Kv1.4 also localizes in small diameter axonal fibers of dorsal root ganglion neurons, which are involved in pain sensing (Rasband *et al.*, 2001).

Kv1.3 and Kv1.6 are less abundant in neurons. Kv1.3 is highly expressed in the parallel fiber axons of cerebellar granule cells (Koch *et al.*, 1997; Veh *et al.*, 1995), and localizes in presynaptic terminals of brainstem auditory neurons (Gazula *et al.*, 2010). Kv1.6 is found primarily in interneurons (Rhodes *et al.*, 1997). Other Kv1 channels, including Kv1.5, Kv1.7 and Kv1.8, are either not present in neurons or not known (Chittajallu *et al.*, 2002; Kalman *et al.*, 1998; Pannasch *et al.*, 2006; Vacher *et al.*, 2008). It is important to note that dendritic staining of some Kv1 channels, albeit less abundant, is seen in the brain, which may play an important role in dendrite integration. The functions of various Kv channels in dendrites are beyond the scope of this review.

Kv1 α subunits can coassemble into either homotetramers or heterotetramers with distinct channel properties. Furthermore, assembly with auxiliary (Kv β 1 to Kv β 3) subunits can markedly affect expression, targeting, and activity of the channel complex (Li *et al.*, 2006; Pongs *et al.*, 1999; Pongs and Schwarz, 2010). For instance, the presence of Kv β 1.1 can convert the Kv1.1 or Kv1.2-containing channel complexes from carrying sustained currents to carrying transient currents (Rettig *et al.*, 1994). Among three isoforms of Kv1 auxiliary subunits, Kv β 1 and Kv β 2 are extensively expressed in the brain, and generally coexpressed and colocalized with Kv1 α subunits (Monaghan *et al.*, 2001; Rettig *et al.*, 1994; Rhodes *et al.*, 1995; Rhodes *et al.*, 1996; Rhodes *et al.*, 1997). In particular, Kv β 2 appears to colocalize with most Kv1-containing channel complexes. Immunostaining for Kv β 2 shows it is present in almost every location where immunoreactivity for Kv1 α subunits is observed, including AISs, the JXP regions, and cerebellar basket cell terminals (Rasband *et al.*, 1999; Rhodes *et al.*, 1996; Rhodes *et al.*, 1997). However, there is also extensive immunostaining for Kv β 2 in soma and dendrites of neurons.

Owing to complex neuronal structure and other surrounding cells in the brain, it is not always easy to unequivocally determine the amount and localization of Kv1 channels using immunohistochemistry, especially in the hippocampus. Using an excitotoxin lesion strategy combined with immunohistochemistry, it was confirmed that Kv1 channel complexes predominantly localize on axons and nerve terminals in the hippocampus (Monaghan *et al.*, 2001). Axonal localization of Kv1 channels has also been confirmed with cultured hippocampal neurons (Grosse *et al.*, 2000; Gu *et al.*, 2003) (Fig. 3D).

4.2. Mechanisms underlying intrinsic Kv1 axonal targeting

Kv1 T1 domains have been identified as the axonal targeting domain for Kv1 channels. When over-expressed in cultured hippocampal neurons, the HA-tagged Kv1.2 is enriched on axonal membranes (Gu *et al.*, 2003). A non-polarized reporter protein, CD4, can be enriched on axonal membranes when fused with the Kv1.2 T1 domain but not with the Kv1.2 C-terminus (Gu *et al.*, 2003). In addition, fusing the T1 domains from Kv1.1, Kv1.2, Kv1.3 or Kv1.4, but not from Kv4.2, to a dendritic reporter protein, the transferrin receptor, results in axonal localization of the fusion protein (Gu *et al.*, 2003; Rivera *et al.*, 2005). Owing to the high homology of T1 domains within the Kv1 subfamily members, the evidence is compelling that the Kv1 T1 domain is the axonal targeting domain.

Kv β subunits likely play a critical role in mediating the axonal targeting of Kv1 channels. Most importantly, Kv β directly binds to the Kv1 T1 domains. Co-expression of Kv β 2 markedly enhances axonal targeting of Kv1.2 (Gu *et al.*, 2006). Using live cell imaging technique, Kv β 2 was found to markedly enhance the velocity of anterograde transport of YFP-tagged Kv1.2 (Gu and Gu, 2010). Surprisingly, coexpressed Kv β 2 also enhanced the retrograde transport of YFP-Kv1.2 (Gu and Gu, 2010). Overall, the effect of Kv β 2 is more biased towards the anterograde direction and hence promotes Kv1.2 axonal targeting. The effect of Kv β 2 may act via the functions of a plus end tracking protein EB1 and KIF3A/kinesin II, both of which associate with Kv β 2 (Gu *et al.*, 2006). Disrupting the function of either EB1 or KIF3A impairs both the axonal targeting and anterograde transport of Kv1 channels (Gu *et al.*, 2006; Gu and Gu, 2010) (Fig. 4A).

KIF5B/kinesin I is also implicated in the axonal targeting of Kv1.3 channels (Rivera *et al.*, 2007) and in forward trafficking of cardiac Kv1.5 channels (Zadeh *et al.*, 2009). How these channels are loaded onto the KIF5B motor remains unclear. The association and transport of different cargos of even the same KIF5 motor can be differentially regulated (Hirokawa *et al.*, 2009; Hirokawa and Takemura, 2005). Moreover, electron microscopy shows multiple tiny bridges linking an intracellular vesicle to microtubules, suggesting that a vesicle cargo can be transported by more than one type of kinesin motor (Hirokawa and Takemura, 2005). Therefore, it is possible that a Kv channel complex can be transported by different motors simultaneously, or by different types of motors in different types of cells, developmental stages and activity states (Fig. 4A).

Members of the MAGUK (membrane-associated guanylate kinase) family, including postsynaptic density protein 95 (PSD-95) and postsynaptic density protein 93 (PSD-93), also play important roles in Kv1 channel trafficking along axons. The interaction between Kv1.4 and PSD-95 was proposed to be critical for the axonal targeting of Kv1.4 channels (Arnold and Clapham, 1999). PSD-93 clusters Kv1 channels at AISs independently of contactin-associated protein 2 (Caspr2) (Ogawa *et al.*, 2008).

4.3. Regulation of Kv1 channel axonal targeting

Targeting of axonal Kv1 channels can be further regulated by various factors. In myelinated axons of both CNS and peripheral nervous system (PNS), Kv1 channels are clustered into JXP regions, controlling the fidelity of AP propagation (Hille, 2001; Salzer, 2003; Wang *et al.*, 1993; Zhou *et al.*, 1998). In the knockout mice of Caspr2 or transient axonal glycoprotein-1 (TAG-1), a glycosylphosphatidylinositol (GPI)-anchored membrane protein, the JXP targeting of Kv1 channels was largely eliminated. Therefore, it was hypothesized that Caspr2 interacts with TAG-1 and clusters Kv1 channels via a PDZ domain-containing protein (Poliak *et al.*, 2003; Traka *et al.*, 2003). Whereas Caspr2 is expressed only in neurons, TAG-1 is expressed in both neurons and myelin cells (Poliak *et al.*, 1999; Traka *et al.*, 2002; Traka *et al.*, 2003). The function of Caspr2/TAG-1 interaction is somewhat analogous to that of Caspr/contactin interaction required for the paranodal formation (Traka *et al.*, 2003) (Fig. 4B). Interestingly, Caspr2 but not Caspr has a PDZ domain ligand at the C-terminus (Poliak *et al.*, 1999; Poliak and Peles, 2003; Salzer, 2003), while both interact with protein 4.1B, which may link the paranodal and JXP adhesion complexes to the axonal cytoskeleton (Horresh *et al.*, 2010). A recent study suggests that the expression of TAG-1 in myelin cells is sufficient to rescue the JXP complex and phenotypes of TAG-1 knockout mice (Savvaki *et al.*, 2010). Thus, in addition to the regulation of axonal and dendritic bundling (Barry *et al.*, 2010), polarized targeting of cell adhesion molecules can regulate the localization of ion channel proteins in response to alteration of cellular environment.

Deleting other proteins with different functions in mice also disrupted precise Kv1 JXP clustering (Bhat *et al.*, 2001; Dupree *et al.*, 1999; Ishibashi *et al.*, 2002; Schaeren-Wiemers

et al., 2004; Tanaka *et al.*, 2009; Wang *et al.*, 1995). This suggests their involvement in the Kv1 JXP targeting. Furthermore, a recent study shows that a disintegrin and metalloproteinase 22 (ADAM22) is an axonal component of the Kv1 channel complex that recruits PSD-93 and PSD-95 to juxtaparanodes (Ogawa *et al.*, 2010). However, deleting either ADAM22, PSD-93, or PSD-95 in mice had no effect on the Kv1 JXP targeting (Horresh *et al.*, 2008; Ogawa *et al.*, 2010). The mechanism underlying Kv1 clustering in the JXP regions remains to be fully elucidated.

A recent study shows that cyclin-dependent kinase (Cdk)-mediated phosphorylation of the Kv β 2 subunit regulates Kv1 channel axonal targeting via disrupting the Kv β 2/EB1 interaction (Vacher *et al.*, 2011). Kv1 channel trafficking may also be regulated by other interacting proteins, although whether these regulations occur in mammalian axons remains to be determined in future studies. Axonal Kv1 channels can potentially be regulated by G-protein-coupled receptor initiated signaling. Activation of muscarinic acetylcholine receptors activates tyrosine kinase, which phosphorylates Kv1.2 and induces channel endocytosis mediated by cortactin and dynamin, to suppress the channel function (Cachero *et al.*, 1998; Hattan *et al.*, 2002; Nesti *et al.*, 2004; Tsai *et al.*, 1997). Calnexin, an endoplasmic reticulum chaperone, promotes the surface expression of Kv1.2, but not Kv1.1 or Kv1.6, likely through the same forward-trafficking pathway that is facilitated by Kv β subunits (Manganas and Trimmer, 2004). The leucine-rich glioma inactivated gene 1, recently identified as an associated protein of Kv1.1 channel complex and involved in lateral temporal lobe epilepsy, selectively prevents N-type inactivation mediated by the Kv β 1 subunit (Schulte *et al.*, 2006). Another recent study shows that a GPI-anchored protein, SLEEPLESS (a Ly-6.neurotoxin family member), plays important roles in regulating the levels, localization and activity of *Drosophila* Shaker channels (Koh *et al.*, 2008; Wu *et al.*, 2010).

5. Axonal targeting of Kv3 (Shaw) channels

5.1. Axonal targeting of endogenous Kv3 channels

The Kv3 subfamily consists of four genes, Kv3.1, Kv3.2, Kv3.3, and Kv3.4. The Kv3.1 and Kv3.2 channels carry sustained currents, whereas the Kv3.3 and Kv3.4 channels carry transient currents (Rudy *et al.*, 1999). They can also form heterotetramers with intermediate gating characteristics (Rudy *et al.*, 1999). Furthermore, Kv3 mRNAs are subjected to extensive alternative splicing, which exclusively gives rise to different C-termini (Luneau *et al.*, 1991). The alternative splicing usually does not affect channel biophysical properties, but affects channel protein targeting.

Unlike Kv1 channels, Kv3 channels have prominent presence in both axons and dendrites of neurons in the brain. The axonal and dendritic targeting of Kv3 channels appears to rely on the neuronal type, channel isoform, and alternative splicing. Kv3.1b is found in proximal dendrites, soma, axons, and axonal terminals (Chow *et al.*, 1999; Elezgarai *et al.*, 2003; Goldberg *et al.*, 2005; Ishikawa *et al.*, 2003; Sekirnjak *et al.*, 1997; Weiser *et al.*, 1995). In axons, Kv3.1b is found at some nodes of Ranvier in the CNS but not in the AIS in spinal cord (Devaux *et al.*, 2003) (Fig. 5A). In contrast, Kv3.1a proteins are predominantly expressed in the axons of some of the same neurons expressing Kv3.1b (Ozaita *et al.*, 2002). The shorter form, Kv3.1a, is less abundant, and is down-regulated and replaced gradually by Kv3.1b channels in adults (Gan and Kaczmarek, 1998; Perney *et al.*, 1992).

Kv3.2 is found in non-fast-spiking, somatostatin- and calbindin-containing interneurons (Chow *et al.*, 1999). Kv3.2 is also found in the terminals of basket cells that synapse onto Purkinje cell AISs (Boiko *et al.*, 2003). In a study using Madin-Darby canine kidney cells as a model system, the targeting of Kv3.2 splice variants has been examined (Ponce *et al.*, 1997). Whereas Kv3.2b and Kv3.2c are expressed predominantly in the apical membrane

(corresponding to the axonal compartment (Dotti and Simons, 1990)), Kv3.2a localizes to the basolateral membrane (corresponding to the somatodendritic membrane) (Ponce *et al.*, 1997). Therefore, similar to Kv3.1, alternative splicing of Kv3.2 generates functionally similar variants of the same channel with altered subcellular distribution.

Co-localization of different Kv3 subunits in different neurons further generates Kv3 heterotetramer channel complexes with distinct channel properties and targeting patterns. Kv3.3 coexpresses with Kv3.1b in parvalbumin-positive interneurons and is also found in mossy fiber axons of hippocampal dentate granule cells (Chang *et al.*, 2007). Kv3.3 not only exists in axons of cerebellar Purkinje cells and motor nerve terminals (Fig. 5B), but also coexpresses and co-localizes with Kv3.4 in the somatodendritic regions of Purkinje cells to regulate large depolarization events (Chang *et al.*, 2007; Martina *et al.*, 2003) (Fig. 5C). At the calyx of Held terminal, a synapse required for the binaural processing of sound-source localization, Kv3.4 and Kv3.1 channels directly regulate evoked transmitter release (Ishikawa *et al.*, 2003). Kv3.4 is located in nerve terminals of other principal neurons (Brooke *et al.*, 2004a; Brooke *et al.*, 2004b; Rettig *et al.*, 1992; Weiser *et al.*, 1994).

5.2. Mechanisms underlying intrinsic Kv3 axonal targeting

Compared to Kv1 channels, the trafficking mechanism for Kv3 channels is less studied. The C-terminal PDZ domain of Kv3.3 is required for Kv3.3 targeting into distal dendrites of pyramidal neurons of *Apteronotus leptorhynchus* (Deng *et al.*, 2005). Given the divergence of Kv3 C-terminus, it is possible that other motifs are involved in axonal targeting of Kv3 channel protein complex. By combining hippocampal neuron culture, mutagenesis, and protein biochemistry, we have identified a conditional axonal targeting motif (ATM) in the Kv3.1 C-terminus near the 6th TM segment (Xu *et al.*, 2007). In cultured hippocampal neurons, although the two splice variants of Kv3.1, Kv3.1a and Kv3.1b, are differentially targeted to the somatodendritic and axonal membrane, respectively. They surprisingly share the same lysine-rich and conditional ATM. The ATM directly binds to the T1 domain in a Zn²⁺-dependent manner, and binds to the ankyrin-repeat domain of ankyrin G. Interestingly, the full-length channel proteins of Kv3.1b display stronger association to ankyrin G than those of Kv3.1a, suggesting that the unique splice domain at Kv3.1b C terminus influences ATM binding to T1 and ankyrin G (Xu *et al.*, 2007).

Because ankyrin G resides at the AIS, where all axonal proteins have to pass through, we propose that ankyrin G may function as a conditional barrier for axon-dendrite targeting of Kv3.1 channels (Xu *et al.*, 2007). Ankyrin G, a critical adaptor protein localized at the AIS, contains 24 N-terminal ankyrin repeats (Bennett and Baines, 2001; Bennett and Chen, 2001; Bouzidi *et al.*, 2002), which are 33-residue motifs involved in protein recognition. Since these ankyrin repeats bind to membrane proteins, this region of ankyrin G is also called the membrane-binding (MB) domain. The ankyrin repeats are followed by a spectrin-binding domain, providing a link to the actin cytoskeleton (Bennett and Chen, 2001). Thus, ankyrin G links the spectrin-based membrane skeleton to integral membrane proteins, including the anion exchanger, the Na⁺/Ca²⁺ exchanger, the Na⁺/K⁺ ATPase, members of L1-CAM family, Nav channels, and KCNQ channels (Boiko *et al.*, 2003; Davis *et al.*, 1992; Garrido *et al.*, 2003; Jenkins and Bennett, 2001; Pan *et al.*, 2006). Ankyrin G also interacts with Kv3.1b channels. Kv3.1b channels and ankyrin G co-immunoprecipitate from rat brain lysates and co-localize at nodes of Ranvier in the CNS (Devaux *et al.*, 2003).

Consistent with our hypothesis that ankyrin G plays an important role in differential targeting of Kv3.1 splice variants, disrupting ankyrin G function either by over-expressing a dominant-negative mutant or by RNAi knockdown decreases polarized axon-dendrite targeting of both Kv3.1a and Kv3.1b (Xu *et al.*, 2007). Indeed, recent studies show that ankyrin G may generally play a critical role in the maintenance of neuronal axon-dendrite

polarity (Hedstrom *et al.*, 2008; Sobotzik *et al.*, 2009; Song *et al.*, 2009). Knocking down endogenous ankyrin G with siRNA in cultured hippocampal neurons allows dendritic proteins to traffic into axons and makes axons adopt some dendritic features including some dendritic spine-like structures (Hedstrom *et al.*, 2008; Song *et al.*, 2009). When ankyrin G is deleted in mouse cerebellum, some Purkinje cell axons develop spine-like structures, which even form asymmetric synapses with other presynaptic axons revealed by electron microscopy (Sobotzik *et al.*, 2009). Therefore, there is compelling evidence supporting our hypothesis that the conditional ATM masked by the T1 domain in Kv3.1a is exposed by the splice domain in Kv3.1b, and is subsequently recognized by ankyrin G to target Kv3.1b into axons (Xu *et al.*, 2007).

The next important question is how Kv3.1b channels are transported down the axon. There are 45 members in the mammalian kinesin superfamily. Conventional Kinesin I, a major anterograde motor in axons, is composed of a heavy chain (KIF5) dimer and two light chains (KLC). The heavy chains, including KIF5A, KIF5B and KIF5C, have an N-terminal motor domain, followed by a stalk domain responsible for dimerization through coiled-coil regions, and a C-terminal tail domain containing cargo-binding sites (Asbury *et al.*, 2003; Gennerich and Vale, 2009; Goldstein, 2001; Hirokawa and Noda, 2008). KLCs directly bind to a region close to the junction of the stalk and tail domains, mediating the transport of many cargos in axons (Glater *et al.*, 2006; Hirokawa and Takemura, 2005; Setou *et al.*, 2002). In a recent study, we show that KIF5/kinesin I transports Kv3.1 channels through the AIS via direct binding (Xu *et al.*, 2010). We have found that the Kv3.1 N-terminal T1 domain directly binds to a conserved region in KIF5 tail domains. Proper T1 tetramerization is required for the binding. Over-expression of the KIF5B tail domains or suppression of the endogenous KIF5B level by RNA interference markedly reduced Kv3.1b axonal levels. Interestingly, co-expression of YFP-tagged KIF5B assists dendritic Kv3.1a and even mutants with a disrupted ATM to penetrate the AIS. Finally, fluorescently tagged Kv3.1 channels co-localize and co-move with KIF5B along axons revealed by two-color time-lapse imaging. Our data suggest that the binding to KIF5 ensures transport of properly assembled and functioning Kv3.1 channels into axons (Xu *et al.*, 2010).

Because of the high sequence homology, the direct interactions between Kv3.1 C-terminal region and ankyrin G (Xu *et al.*, 2007), and between Kv3.1 T1 domain and the 70-residue region in KIF5 tail (Xu *et al.*, 2010), are likely evolutionarily conserved. Moreover, by interacting with both ankyrin G at the AIS (Xu *et al.*, 2007) and the KIF5 motor, Kv3.1 channels are in a unique position to provide insights into the collaboration of the AIS filter and kinesin motors (Fig. 6). Although Kv3.1 is the only known protein interacting with both ankyrin G and kinesin, the targeting strategy may be shared by many other axonal proteins. The transport efficacy of the motor-cargo complex, but not the cargo or the motor alone, was suggested to determine the axonal entry (Song *et al.*, 2009). The cargo binding to the motor may change the conformation of the motor and affect the recognition of microtubules and the direction of transport (Hirokawa and Takemura, 2005; Setou *et al.*, 2002). Therefore, the binding between the Kv3.1 C-terminus and ankyrin G may build enough tension to change the conformation and hence the activity of KIF5 motors (Fig. 6). The following two interesting questions remain to be determined in future investigation. How does Kv3.1 shift between actin- and microtubule-based trafficking? How might the N/C-terminal interaction of Kv3.1 be involved in this process?

5.3. Regulation of Kv3 channel axonal targeting

How the axonal targeting of Kv3 channels is regulated remains unclear. However, there are some clues regarding the regulation of Kv3 channel trafficking. The next important step is to determine whether these regulations influence Kv3 channel axonal targeting. Protein phosphorylation has been shown to regulate the activity and trafficking of Kv3 channels.

Whether protein phosphorylation can regulate Kv3 axonal targeting is an intriguing question for future studies. Protein kinase C (PKC) has been implicated in modulating the firing patterns of auditory neurons to adjust to the acoustic environment (Macica *et al.*, 2003; Song *et al.*, 2005; Strumbos *et al.*, 2010). Casein kinase 2 has been shown to alter the voltage dependence of Kv3.1, but the specific residue(s) phosphorylated remains unknown (Macica and Kaczmarek, 2001). Protein kinase A (PKA) has been implicated in histamine-dependent regulation of high-frequency oscillation of hippocampal interneurons (Atzori *et al.*, 2000). The PKC phosphorylation site in Kv3.1b C-terminus is S503 (Macica *et al.*, 2003). A putative PKA phosphorylation site in Kv3.2 channels has also been identified (Rudy and McBain, 2001). Interestingly, a recent study suggested that oscillation of Kv3.1b channel protein levels may alter the AP firing pattern in pacemaker neurons of the suprachiasmatic nucleus (SCN) across the circadian cycle (Itri *et al.*, 2005). During day-to-night transition, Kv3.1b levels decrease, resulting in decreased firing rate of SCN neurons; the decrease may involve endocytosis of channel proteins, leading to altered targeting and possibly degradation of channel proteins. During night-to-day transition, Kv3.1b levels increase, resulting in faster firing of SCN neurons; the increase may involve channel protein synthesis and forward trafficking to the plasma membrane (Itri *et al.*, 2005).

Kv3.1b channel axonal targeting can be further regulated by myelin. It has been shown that Kv3.1b co-localizes with ankyrin G at nodes of Ranvier of central neurons (Devaux *et al.*, 2003). Ankyrin G clusters several proteins, including Nav channels, neurofascin, etc. It has been shown that phosphorylation of Nav channels by casein kinase regulates the interaction with ankyrin G (Brecht *et al.*, 2008). How ankyrin G clusters Kv3.1b channels in response to myelin remains unclear. Taken together, there are several interesting and important questions that remain to be resolved in future studies.

6. Difference and similarity of axonal targeting of Kv1 and Kv3 channels

Kv1 channels are broadly expressed in different cells of both CNS and PNS. Kv3 channels are mainly expressed in GABAergic interneurons as well as some principal neurons. Kv1 and Kv3 are coexpressed in some neurons, such as interneurons, cerebellar Purkinje neurons, auditory neurons, motor neurons, etc. The patterns of their polarized axon-dendrite targeting are different. Kv1 channels are predominantly localized in axons. The presence of Kv1 channels in dendrites is usually restricted to a few particular neurons and at a lower level. In contrast, Kv3 channels are localized in both dendrites and axons, where they play critical roles.

Recent studies suggest that the mechanisms governing Kv1 and Kv3 their axonal transport are likely very different. Whereas EB1 and KIF3A are involved in transporting Kv1 channels, KIF5 appears to be the major anterograde motor transporting Kv3.1 channels (Gu *et al.*, 2006; Gu and Gu, 2010; Xu *et al.*, 2007; Xu *et al.*, 2010). Despite their differences, Kv1 and Kv3 channels do share a common strategy. They are linked to kinesin motor proteins via their T1 domains for long-distance and microtubule-based trafficking, in which proper T1 tetramerization is required (Gu *et al.*, 2003; Gu *et al.*, 2006; Xu *et al.*, 2010). The important role of the N-terminal T1 domains of Kv channels in long-distance trafficking is consistent with the notion that Kv channel T1 domains can form tetramers right after emerging from the ribosomal exiting tunnel once synthesis of the T1-S1 linker is completed (Kosolapov *et al.*, 2004). Their C-terminal domains are linked to local retention or trafficking, either through ankyrin G and the actin cytoskeleton (Xu *et al.*, 2007), or through a tyrosine-based endocytic motif and a PDZ-domain ligand (Lai and Jan, 2006). In contrast, dendritic ion channels are more likely linked to different kinesin motors via their C-termini (Chu *et al.*, 2006; Setou *et al.*, 2000; Setou *et al.*, 2002).

7. Axonal targeting of KCNQ (Kv7) channels

7.1. Axonal targeting of endogenous KCNQ channels

All five members of KCNQ channel family can form homotetramer channels *in vitro*, but the formation of heterotetramers appears to be restricted to certain combinations (Kubisch *et al.*, 1999; Lerche *et al.*, 2000; Schroeder *et al.*, 2000; Schroeder *et al.*, 1998). The major KCNQ subunits expressed in mammalian brain include KCNQ2, KCNQ3, and KCNQ5. KCNQ4 is found only in a few nuclei and tracts mainly in the brainstem (Kharkovets *et al.*, 2000). KCNQ1 is restricted to the heart, peripheral epithelial and smooth muscle cells.

KCNQ2 and KCNQ3 predominantly localize in axons as revealed by immunohistochemistry studies. Previous studies show that they mainly localize in mossy fiber axons but not in dentate granule cell somas in rodent hippocampus (Cooper *et al.*, 2001; Roche *et al.*, 2002). A subset of GABAergic interneurons in hippocampus and some neurons in basal ganglia also express KCNQ2 (Cooper *et al.*, 2001; Roche *et al.*, 2002). KCNQ2 is found at AISs of hippocampal and cortical pyramidal neurons, and at nodes of Ranvier in spinal cord and optic nerves (Devaux *et al.*, 2004; Rasmussen *et al.*, 2007) (Fig. 7). However, in the cerebellum, KCNQ2 appears to localize in Purkinje cell soma and dendrites (Cooper *et al.*, 2001).

KCNQ3 displays similar localization patterns to KCNQ2, which is consistent with the notion that KCNQ2 and KCNQ3 form heterotetramers as M channel complexes. KCNQ3 also localizes at AISs and nodes of Ranvier (Devaux *et al.*, 2004; Pan *et al.*, 2006; Rasmussen *et al.*, 2007).

KCNQ5 is widely distributed in the brain, including the hippocampus (Jensen *et al.*, 2005; Lerche *et al.*, 2000; Schroeder *et al.*, 2000; Tzingounis *et al.*, 2010). KCNQ5 is found in presynaptic terminals of the auditory nerve, as well as in dendrites of rat auditory system (Camino *et al.*, 2007). KCNQ5 also localizes in synaptic endings in auditory brainstem (Garcia-Pino *et al.*, 2010).

7.2. Mechanisms underlying intrinsic KCNQ axonal targeting

A short motif, common to both KCNQ2 and KCNQ3, which mediates ankyrin G interaction and retention of KCNQ2/3 at the AIS, has been identified (Pan *et al.*, 2006). This motif contains several acidic residues and is nearly identical to the sequence in Nav α subunits binding to ankyrin G (Pan *et al.*, 2006). This common ankyrin G-based mechanism, which retains Nav and KCNQ channels at the AIS, was shown to have evolved separately, first Nav, then KCNQ (Hill *et al.*, 2008). Deletion of the ankyrin G-binding motif of KCNQ2 and KCNQ3 disrupts the channel AIS localization (Rasmussen *et al.*, 2007). In the KCNQ2/KCNQ3 heterotetramer channels, the ankyrin G-binding motif of KCNQ3 appears to be dominant over that of KCNQ2 (Rasmussen *et al.*, 2007).

Interestingly, using hippocampal neuron culture, another study shows that KCNQ2/3 channel complexes are preferentially localized to axonal membranes at both proximal and distal axons (Chung *et al.*, 2006). While the AIS targeting of surface KCNQ channels is mediated by the ankyrin G binding motifs of KCNQ2 and KCNQ3, sequences mediating targeting to more distal portion of the axon reside in the membrane proximal and A domains of the KCNQ2 C-terminal tail (Chung *et al.*, 2006) (Fig. 8).

7.3. Regulation of KCNQ axonal targeting

Much less is known regarding the potential regulation of the axonal targeting of KCNQ channels. They may share the regulatory mechanisms for other channel proteins. For

instance, like Nav channels, KCNQ channels localize at the nodes of Ranvier, suggesting that myelin potentially regulates the distribution of KCNQ channels via ankyrin G. Protein phosphorylation has been shown to regulate the interaction between ankyrin G and Nav channels (Brechet *et al.*, 2008). Whether protein phosphorylation also regulates KCNQ channel localization remains to be determined. Many other proteins have been identified as regulators of KCNQ channel activity and trafficking, such as calmodulin, phosphatidylinositol 4,5-bisphosphate, the A-kinase anchor proteins 150 and Yotiao, src tyrosine kinase (Dai *et al.*, 2009; Delmas and Brown, 2005). Their potential roles in regulating the channel targeting and functions in axons remain unknown. This may be an interesting topic for future investigation.

8. Axonal targeting of large conductance Ca^{2+} -regulated potassium (BK) channels

8.1. Axonal targeting of endogenous BK channels

BK channels are widely distributed in the nervous systems, where they are localized to neuronal somas, dendrites and presynaptic terminals (Knaus *et al.*, 1996; Marrion and Tavalin, 1998) (Fig. 9). They are powerful regulators of synaptic transmission in central neurons. Deletion of postsynaptic BK channels leads to cerebellar ataxia (abnormal locomotion and motor incoordination) along with Purkinje cell dysfunction in mice (Sausbier *et al.*, 2004). This is most likely due to depolarization-induced inactivation of APs, since BK channels provide the main negative feedback mechanism for timing the bursts of Ca^{2+} APs, such as those generated in the dendrites of cerebellar Purkinje neurons (Swensen and Bean, 2003; Womack and Khodakhah, 2004). BK channels contribute to the AHP in the soma of pyramidal neurons of the hippocampal CA1 region (Faber and Sah, 2003; Lancaster and Nicoll, 1987). The AHP is a main determinant of the refractory period and hence the overall firing rate of a neuron. In presynaptic terminals, BK channels provide a homeostatic mechanism for regulating synaptic transmission by limiting the influx of extracellular Ca^{2+} through presynaptic voltage-gated Ca^{2+} channels (Hu *et al.*, 2001; Raffaelli *et al.*, 2004; Robitaille *et al.*, 1993; Sun *et al.*, 2004; Wang *et al.*, 2001). Moreover, BK channels are also found to modulate pre- and postsynaptic signaling at reciprocal synapses in retina (Grimes *et al.*, 2009).

The existence of BK channels at presynaptic terminals has also been confirmed by immunocytochemistry studies. BK channels are found enriched in axons and nerve terminals in rat brain (Knaus *et al.*, 1996). Using cultured hippocampal neurons and combined with electron microscopy, BK channel staining is found in both presynaptic terminals in axons and postsynaptic sites in dendrites (Sailer *et al.*, 2006). BK channels are associated with glutamatergic synapses in hippocampus and GABAergic synapses in cerebellum (Misonou *et al.*, 2006) (Fig. 9).

8.2. Potential mechanism underlying BK channel axonal targeting

The molecular mechanism underlying the axonal and presynaptic targeting of BK channels remains unknown. With the advance of the research field in channel axonal targeting, we expect that the mystery should be resolved in the near future. Among four types of β subunits ($\beta 1$ - $\beta 4$), $\beta 4$ subunits are almost exclusively expressed in the brain (Brenner *et al.*, 2000). $\beta 2$ and $\beta 3$ subunits are expressed in the brain and other tissues (Brenner *et al.*, 2000; Wallner *et al.*, 1996), whereas the $\beta 1$ subunit is primarily localized in smooth muscle cells (Jiang *et al.*, 1999). β subunits not only regulate BK channel activity, including kinetic behavior, voltage dependence, calcium sensitivity, and sensitivity to channel blockers (such as iberiotoxin and charybdotoxin) (Dworetzky *et al.*, 1996; Lippiat *et al.*, 2003), but also regulate channel protein trafficking (Torres *et al.*, 2007). It would be of interest in future

studies to determine whether β subunits play a role in targeting BK channels into axons. Furthermore, extensive alternative splicing occurs in BK channels (Fodor and Aldrich, 2009). Whether alternative splicing plays an important role in BK channel targeting is also an interesting question for future studies.

To fully elucidate the basic mechanism governing BK channel axonal targeting, two major questions need to be addressed. First, how are BK channels transported over long distance along axons? The potential motor protein carrying BK channels should be identified first. It would be interesting to determine whether BK channels share the same kinesin motors with Kv1 or Kv3 channels. Second, how are BK channels localized in presynaptic terminals? In this case, potential cytoskeleton associating proteins may be involved. Several such proteins have been identified to interact with BK channels, such as ankyrin-repeat family A protein and microtubule associated protein 1A (Lim and Park, 2005; Park *et al.*, 2004). Whether these interactions are critical for BK channel presynaptic targeting remains unknown. Furthermore, since BK channels are functionally coupled with presynaptic Cav channels, it would be interesting to determine whether BK channels share similar mechanisms with Cav channels in axonal targeting. Once the basic mechanism of BK channel axonal targeting is established, it would be of interest to understand how the channel targeting is regulated in response to neuronal activity.

Many other proteins have been identified to regulate BK channel activity and trafficking, such as membrane-associated guanylate kinase with inverted orientation protein-1, nephrin, the $\beta 1$ subunit of Na^+/K^+ -ATPase, caveolin-1, canonical transient receptor potential channels, c-Src tyrosine kinase, PKC adaptor protein RACK1, leucine-rich repeat-containing protein 26, PKA, etc (Alioua *et al.*, 2008; Dai *et al.*, 2009; Jha and Dryer, 2009; Kim *et al.*, 2009; Kim *et al.*, 2008; Lim and Park, 2005; Park *et al.*, 2004; Ridgway *et al.*, 2009; Yan and Aldrich, 2010). Whether they play an important role in BK channel axonal targeting remains unknown, but they are good candidates for initial investigation.

9. Future perspective

Despite much progress, an immediate key question in this field is how axonal distribution of various voltage-gated ion channels exactly contributes to electrical signal computation, such as input-output relationship, AP initiation, frequency and failure at branch points, and AP invasion of nerve terminals to trigger neurotransmitter release. Different types of neurons express a distinct set of channel proteins and target them to a different set of subcellular compartments. Therefore, the electrical function of channel targeting might be specific for a particular neural circuit. Unfortunately, this research endeavor is hindered by major technical challenges. Most axons are too small for electrophysiological recording. Optic imaging techniques developed for recording rapid electrical events demand fluorescent probes with high temporal resolution and signal-to-noise ratio. With the rapid advance of electrical and optic recording techniques, this question should become attainable in the near future.

The second major question is to understand how each channel protein is precisely targeted into distinct locations along axons. New mechanistic insights into the axonal targeting of Kv1 and Kv3 channels that have been provided in recent studies (Gu *et al.*, 2003; Gu *et al.*, 2006; Rivera *et al.*, 2007; Rivera *et al.*, 2005; Xu *et al.*, 2007; Gu and Gu, 2010; Xu *et al.*, 2010) may serve as a framework for studies of the mechanism and function of other key axonal ion channels, in understanding long-distance transport and local trafficking/retention. The answer to this question is highly attainable. Over the next ten years, we should have enough information to categorize axonal ion channels and receptors into different groups in terms of the axonal targeting mechanisms utilized. The third question is how the targeting

can be regulated during neural development, in an activity-dependent manner, and during pathogenic processes of various neurodegenerative disorders. The long-term impact of this research field may be its contribution to the efforts in drug discovery for curing neurological diseases via modulating axonal electrical and chemical functions.

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Abbreviations

K⁺	potassium ions
Ca²⁺	calcium ions
CNS	central nervous system
AP	action potential
AIS	axon initial segment
Nav channel	voltage-gated sodium channel
Kv channel	voltage-gated potassium channel
Cav channel	voltage-gated calcium channel
TM	transmembrane
BK	large conductance calcium-regulated potassium channel
Kir channel	inwardly rectifying potassium channel
AHP	afterhyperpolarization
JXP	juxtaparanodal
MAGUK	membrane-associated guanylate kinase
PSD-95	postsynaptic density protein 95
PSD-93	postsynaptic density protein 93
Caspr2	contactin-associated protein 2
PNS	peripheral nervous system
TAG-1	transient axonal glycoprotein-1
ADAM22	a disintegrin and metalloproteinase 22
ATM	axonal targeting motif
KLC	kinesin light chain
PKC	protein kinase C
PKA	protein kinase A
SCN	suprachiasmatic nucleus
KIF3	kinesin family member 3
KIF5	kinesin family member 5

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Fig. 1. Structural diagram of potassium channel α subunits

(A) Structure diagram of the α subunits of three major groups in the potassium channel superfamily. The α subunits in the group I contain six to seven transmembrane (TM) segments and one pore-forming loop. The α subunits in the group II contain two TM segments and one pore-forming loop. The α subunits in the group III contain four TM segments and two pore-forming loops. "N" and "C" indicate the amino and carboxyl termini of the protein, respectively. (B) Structure diagram of the α subunits of voltage-gated K^+ (Kv) channels (Kv1 to Kv4), KCNQ (Kv7) channels, and the BK (Slol) channel.

Fig. 2. Regulation of action potential (AP) firing by Kv1 channels localized at axon initial segments

(A) AP is initiated at the AIS of pyramidal neurons. Photograph of a biocytin-filled layer 5 pyramidal neuron indicates the soma, the unmyelinated AIS, a myelinated axonal region, and an axonal bleb site accessible for whole-cell recording (left). Example traces show APs evoked by somatic current injection, and recorded at the AIS 38 μm from the axon hillock (red) and at the soma (black) (top), and recorded at an axon bleb 620 μm from the hillock (bottom). (B) Mean amplitude of slowly inactivating K^+ currents evoked by voltage steps from -110 to $+45$ mV recorded in cell-attached patches from the soma, proximal AIS (5–30 mm from hillock), distal AIS (35–55 mm), and axonal sites (70–400 mm). Asterisks indicate significant increases in K^+ peak amplitudes compared to the soma (***) $p < 0.001$ and ** $p < 0.01$). Traces show examples of slow-inactivating K^+ current at the indicated locations (averages of four to six patches). (C) Outside-out recordings of K^+ currents from AIS or axonal blebs before and after puffs with 500 nM DTX-1. Subtracted traces show the DTX-sensitive current. (D) Impact of bath application of 50–100 nM DTX-1 on somatic (top) and axonal (bottom) APs before (black) and several minutes after DTX-1 (red) ((A)–(D) modified from Kole *et al.*, 2007 with copyright permission from Neuron). (E) Discharge pattern of a fast-spiking (FS) neuron in layer 2/3 barrel cortex. Shown are voltage responses to 600 ms current injections of, from bottom to top, -100 , 100 , 345 , 360 , and 440 pA. Gray bars highlight delay to first spike. Inset in the topmost trace shows the first two spikes on an expanded timescale (Scale bar, 10 ms). (F) Firing pattern of an adjacent pyramidal neuron. (G) f – I curves for a subset of representative FS cells (blue) and PCs (red), with the cells in (E) and (F) indicated by dots. (H) Summary data illustrating initial firing frequency and the slope of the f – I relation for FS cells and PCs. (I) Firing pattern of an FS cell in layer 2/3 barrel cortex to current injections. (J) Same cell as in (I), after bath application of 50 nM DTX-1. (K) Summary data. DTX-1 produces no change in R_m or f_i but decreased I_{TH} and shifted the voltage threshold for AP generation to hyperpolarized potentials ((E)–(K) modified from Goldberg *et al.*, 2008 with copyright permission from Neuron).

Fig. 3. Kv1 channels are mainly localized in axon initial segments, juxtaparanodal regions, axonal terminals in nervous systems

(A) Distribution of Nav and Kv1.2 channels in the AIS of pyramidal neurons and their relationship with chandelier cell axonal terminals (modified from Inda *et al.*, 2006 with copyright permission from Proc. Natl. Acad. Sci. U.S.A.) (B) Double labeling by using anti-Kv1.2 (green) and anti-Nav channel (red) antibodies in myelinated rat spinal cord (modified from Rasband and Trimmer, 2001 with copyright permission from J. Comp. Neurol.). Scale bar, 10 μm. (C) Localization of Kv1.1 in mouse cerebellar basket cell terminals. General distribution of Kv1.1 in mouse cerebellum (top) and higher magnification of the boxed region (bottom) (modified from Wang *et al.*, 1994 with copyright permission from the J. Neurosci.) (D) Double labeling reveals segregation of endogenous Kv1.2 and Kv4.2 in mature hippocampal neurons (21 DIV) (modified from Gu *et al.*, 2003 with copyright permission from Science). Immunofluorescence intensity profiles, along a 40-μm line (black, lower left) were measured from the original 16-bit TIFF images with NIH Image J (lower right). AU, arbitrary unit.

Fig. 4. Mechanisms underlying Kv1 channel targeting in axons

(A) Axonal transport of Kv1 channels. The study using live cell imaging on hippocampal neuron axons expressing YFP-Kv1.2 suggests that Kv1/Kv β channel complexes reside in small round vesicles moving anterogradely along axons. These vesicles are carried by KIF3A/kinesin II motors, where plus end tracking protein EB1 also plays a critical role. It is also possible that KIF5B might transport Kv1 channels anterogradely under some conditions. Kv1 channel-containing vesicles can also be transported retrogradely, which is potentially mediated by dynein complex. How Kv1 channel complex is linked to dynein remains unknown. (B) Targeting of Kv1 channels into the JXP regions in myelinated axons. TAG-1 expressed on myelinating glial and axonal membranes can form homomultimers in *trans*. TAG-1 can bind to Caspr2 on axonal membranes in *cis*. Since both Kv1 channels and Caspr2 have PDZ-binding ligand at their C-termini, PDZ domain-containing proteins, such as PSD-95 and PSD-93, may cluster Kv1 channel complexes and Caspr2 together. Therefore, Kv1, Caspr2 and TAG-1 co-cluster in the juxta-paranodal regions of myelinated axons.

Fig. 5. Axon-dendrite targeting of Kv3 channels

(A) Differential localization of Kv3.1b and Kv1.2 in axons of central nervous systems. These are images of longitudinal sections of the ventral column (vc) of unfixed lumbar spinal cord from adult mice. In the spinal cord, the narrow band of nodal Kv3.1b staining (top) is flanked by two broader regions of JXP Kv1.2 staining (middle). The merged image is at the bottom. Note the relative lack of labeling for Kv3.1b in the nodes of peripheral nervous systems (double arrowheads) of the ventral rootlet (vr) (modified from Devaux *et al.*, 2003 with copyright permission from J. Neurosci.). (B) Kv3.3 (flexor digitorum brevis) (top) and Kv3.4 (transverses abdominus)(bottom) localize in presynaptic motor nerve terminals revealed by double labeling with the postsynaptic nicotinic acetylcholine receptors by FITC- α bungarotoxin (left). The presynaptic localization of Kv3.3 and Kv3.4 was further confirmed by electron microscopy studies (modified from Brooke *et al.*, 2004 with copyright permission from Eur. J. Neurosci.). (C) Presence of Kv3.3 in soma and dendrites of Purkinje neurons in rat cerebellum (modified from Martina *et al.*, 2003 with copyright permission from J. Neurosci.).

Fig. 6. Local trafficking and long-distance transport of axonal Kv3.1 channels

A proposed model for the potential interplay of KIF5 and ankyrin G during transportation of Kv3.1 channels through the AIS. (A) A KIF5 motor directly binds to the Kv3.1 T1 tetramer via its C-terminal tail and transports the channel tetramer. Kv3.1 channel may or may not also bind to ankyrin G through its C-terminus assembled together with the T1 tetramer. For clarification, only one C-terminal tail is shown here. (B) Disassembly or conformational change of the T1 tetramer dissociates the channel from KIF5 and allows a stronger binding of ankyrin G to the C-terminus. The process may involve more intermediate states besides the two shown here (modified from Xu *et al.*, 2010 with copyright permission from J. Neurosci.).

Fig. 7. Axonal localization of KCNQ channels

(A)–(D) Colocalization of KCNQ2 and KCNQ3 in AISs in cortex. These are images of horizontal sections of unfixed mouse brain immunolabeled for KCNQ2, KCNQ3, and ankyrin G; DAPI was used as a nuclear counterstain in (A) and (B). In the CA3 region of the hippocampus, many AISs in the stratum pyramidale (sp), as well as the mossy fibers of the stratum lucidum (sl), are strongly KCNQ2 positive (A). The stratum radiatum (sr) and stratum oriens (so) are indicated. KCNQ3 was found with KCNQ2 in the AISs of some pyramidal cells in CA3 but also in the mossy fibers (B). KCNQ3 colocalized with both ankyrin G and KCNQ2 in the AIS of neurons from the CA1 (C) and temporal neocortex (D). The asterisk in (B) marks a KCNQ3-positive blood vessel. (E) KCNQ2 localized to CNS nodes and initial segments. Anti-KCNQ2 (red), anti-panNav (green, top), and anti-Kv1.2 (green, bottom) antibodies were used to stain rat spinal cord. (F) KCNQ2 is localized to PNS nodes of Ranvier. The staining was performed on unfixed teased fibers from adult rat sciatic nerves (modified from Devaux *et al.*, 2004 with copyright permission from J. Neurosci.).

Fig. 8. Motifs of KCNQ channels responsible for axonal targeting

A structure diagram of KCNQ subunit shows the important trafficking motifs in the C-terminus. MP domain, membrane proximal domain (amino acids 323–500); A domain, amino acids 501–579; SID, subunit interaction domain (amino acids 580–623); Ankyrin G-binding domain, amino acids 624–844.

Fig. 9. Axon-dendrite targeting of BK channels

(A) Immunolocalization of BK channels in hippocampus. The monoclonal anti-BK antibody (L6/60) (red) was used to stain the hippocampal sections from wild type mouse (top) and from BK-knockout mouse (bottom). The anti-Kv2.1 staining (green) was used as control. (B)–(D) In rat cerebellar Purkinje cell layer, BK channels are localized in Kv1.2-positive basket cell terminals (B), around the neurofilament-positive AISs (C), and in calbindin-positive Purkinje cell soma and dendrites (D). (E) BK channel staining associates with axons and dendrites of cultured hippocampal neurons (modified from Misonou *et al.*, 2006 with copyright permission from J. Comp. Neurol.).

Table I

Four subfamilies of voltage-sensitive K⁺ channels

Subfamily	Channel	Gene	Chromosomal localization	Blockers	Openers	Auxiliary subunits
Kv1 (Shaker)	Kv1.1*	KCNA1	12p13.3	DTX, TEA, 4-AP		Kvβ1 (KCNAB1) Kvβ2 (KCNAB2) Kvβ3 (KCNAB3)
	Kv1.2	KCNA2	1p13			
	Kv1.3	KCNA3	1p13.3	TEA, 4-AP		
	Kv1.4	KCNA4	1p14.3-15.2	4-AP		
	Kv1.5*	KCNA5	12p13.3	4-AP		
	Kv1.6	KCNA6	12p13.3	DTX, TEA, 4-AP		
	Kv1.7	KCNA7	19q13.3	4-AP		
	Kv1.8	KCNA10	1p13.3-22.1			
Kv3 (Shaw)	Kv3.1	KCNC1	1p14.3-15.2	TEA, 4-AP		
	Kv3.2	KCNC2	19q13.3-13.4			
	Kv3.3*	KCNC3	19q13.3			
	Kv3.4	KCNC4	1p21			
	Kv7.1*	KCNQ1	11p15.5	Linopirdine, XE991,	Flupirtine retigabine	
KCNQ (Kv7)	Kv7.2*	KCNQ2	20q13.3			
	Kv7.3*	KCNQ3	8q24.22-q24.3			
	Kv7.4*	KCNQ4	1p34			
	Kv7.5	KCNQ5	6q14			
	KCa1.1*	KCNMA1	10q22	Iberitoxin, charybdotoxin, (+)-tubocurarin e, kalitoxin, penitram A, TEA	NS004 NS1619 DHS1	
BK (Slol)						β1(KCNMB1) β2(KCNMB2) β3(KCNMB3) β4(KCNMB4)

* Mutations have been identified to cause diseases in humans.