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Subcellular localization of K⁺ channels in mammalian brain neurons: remarkable precision in the midst of extraordinary complexity

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Abstract

Potassium channels (KChs) are the most diverse ion channels, in part due to extensive combinatorial assembly of a large number of principal and auxiliary subunits into an assortment of KCh complexes. This structural and functional diversity allows KChs to play diverse roles in neuronal function. Localization of KChs within specialized neuronal compartments defines their physiological role, and also fundamentally impacts their activity, due to localized exposure to diverse cellular determinants of channel function. Recent studies in mammalian brain reveal an exquisite refinement of KCh subcellular localization. This includes axonal KChs at the initial segment, and near/within nodes of Ranvier and presynaptic terminals, dendritic KChs found at sites reflecting specific synaptic input, and KChs defining novel compartments. Painting the remarkable diversity of KChs onto the complex architecture of mammalian neurons creates an elegant picture of electrical signal processing underlying the sophisticated function of individual neuronal compartments, and ultimately neurotransmission and behavior.

Introduction

Mammalian brain neurons are distinguished from other cells by extreme molecular and structural complexity that is intimately linked to the array of intra- and inter-cellular signaling events that underlie brain function. Integral to the functional complexity of neurons is the array of proteins they express (estimated to encompass the products of two-thirds of the genome), a complexity markedly enhanced by compartmentalization of specific proteins and their functions at highly restricted sites within the neuron's complex structure. Moreover, dynamic changes in the subcellular localization of these proteins, in the absence of any changes in overall expression level, can confer functional plasticity to neurons. While this structural and molecular complexity provides neurons with a deep diversity and flexibility of function, it also creates challenges for understanding mechanisms controlling

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neuronal function at the proteomic level. As a prominent example of this molecular diversity is the expression of a large superfamily of plasma membrane ion channels that carry out the bulk of intercellular (*via* ionotropic receptors) and intracellular (*via* non-receptor ion channels) neurotransmission. Among the large and diverse set of non-receptor ion channels, which together comprise the third largest set of mammalian signaling proteins [after GPCRs and protein kinases; (Yu and Catterall, 2004)], is the subset defined by their selective passage of K⁺ ions, referred to generically as K⁺ channels (KChs). KChs are by far the most diverse group of mammalian ion channels, starting with the 80 paralogous genes encoding the principal transmembrane ion conducting or α subunits (<http://www.genenames.org/genefamilies/KCN>) and the dozen or so additional genes that encode auxiliary subunits, defined as stably-associated non-conducting or modulatory components of KCh complexes. These genes are expressed in distinct cellular expression patterns throughout the brain, such that particular neurons express specific combinations of KCh α and auxiliary subunits. However, the proteomic complexity of KChs is much greater, as KChs exist as multisubunit complexes formed by coassembly of multiple (typically four, but in the case of two pore KChs only two) α subunits, plus a variable number of auxiliary subunits. Co-assembly of different α and auxiliary subunits in a wide variety of combinations yields a huge diversity in KChs with distinct subunit composition and functional characteristics (Jan and Jan, 2012).

In spite of the challenges presented by the combined molecular complexity of KChs, and structural complexity of the mammalian brain, tremendous progress has been made in our understanding of the roles of specific brain KChs. The cloning of KChs has allowed for generation of a reliable set of subtype-specific antibodies against KCh α and auxiliary subunits, some specific for distinct functional states, that, when combined with advances in sample preparation, labeling and imaging techniques, have allowed for numerous insights into the diversity of KCh subcellular localization, as reviewed here, that rivals that of any other family of mammalian proteins. In contrast to excitatory ionotropic receptors located primarily near the site of their neurotransmitter stimuli at synapses, triggers for gated KCh activity are generated at many different sites in neurons, such that KChs can, and do, operate at many different sites. KCh subcellular localization delineates their role in neuronal physiology, through their impact on local electrical signaling events that play specific roles in intra- and inter-cellular neurotransmission. Moreover, at these sites, specific and functionally distinct KCh complexes sense and respond to local changes in levels of their activating stimuli, associate with other cellular proteins in macromolecular complexes, and are modulated by a wide array of local activity-dependent and modulatory signaling pathways. These patterns of KCh subcellular localization map at the first level to the basic plasma membrane compartments of neurons (dendrite, cell body, axon), and then to distinct subcompartments (*e.g.*, dendritic spines, axon initial segment (AIS), nodes of Ranvier (NoRs), presynaptic terminals, etc.). While each of these sites contains a unique repertoire of ion channels associated with the primary events in neurotransmission (*e.g.*, ionotropic glutamate receptors at the excitatory post-synapse; voltage-gated Na⁺ (Nav) channels at the AIS; voltage-gated Ca²⁺ (Cav) channels in the presynaptic terminal, etc.), we now appreciate that each also harbors a distinct population of KChs, playing crucial roles as both determinants and modulators of these events. Moreover, the subcellular localization of certain KChs has defined novel neuronal plasma membrane subcompartments, whose

function in neurotransmission is less well established, but that likely impact significant aspects of neuronal function, as the observed patterns of subcellular distributions are highly conserved across diverse mammalian species.

Molecular relationships among KChs

KCh principal or α subunits are multipass transmembrane polypeptides that contain the K^+ selective pore, as well as the domains that allow them to respond to diverse stimuli. The 80 genes encoding mammalian KCh α subunits are segregated into four main branches (Figure 1) and numerous sub-branches, based on sequence similarity and function. The four main branches correspond to: constitutively open or “leak” KChs of the tandem pore or K2P branch (15 members); constitutively active, and G protein and ATP regulated inwardly rectifying KChs of the Kir branch (15 members); Ca^{2+} and Na^+ activated KChs of the KCa branch (8 members); and voltage-gated KChs of the Kv branch (41 members). It should be noted that some KChs respond to more than one of these stimuli (*e.g.*, KCa1.1 channels are calcium- and voltage-dependent), acting as coincidence detectors for chemical and electrical signaling events. Two pore KCh α subunits assemble into dimers to function, and all of the others into tetramers, and branches and then sub-branches provide strong inferences as to which KCh α subunits can efficiently co-assemble with one another to form homo- or hetero-meric channels [*e.g.*, Kv1 α subunits can co-assemble with one another, but not with Kv2s or Kv3s, etc., and *vice-versa*; (Jan and Jan, 2012; Papazian, 1999)]. Auxiliary subunits generally co-assemble with these tetrameric α subunit complexes in a sub-branch-specific manner (*e.g.*, Kv β subunits with Kv1s, AMIGO with Kv2s, KChIPs and DPPs with Kv4s, etc.). The subunit composition of KChs defines their intrinsic functional properties, as well as diverse aspects of expression, localization and modulation. As such, defining the subunit composition of KChs found at specific sites in mammalian brain neurons is crucial to understanding the basic tenants of their function. Given that subunit composition determines pharmacology, accurately defining the subunit composition of KChs and recapitulating them in a format that allows for high-throughput screening of libraries of compounds and biologicals is a requisite component of drug discovery research (Rhodes and Trimmer, 2008). This has and will continue to achieve increased importance as we enter a new era of enhanced access of therapeutic antibodies and other biologicals to the brain (Niewoehner et al., 2014; Watts and Dennis, 2013).

Analyses of expression patterns of endogenous KCh transcripts in mammalian brain by *in situ* hybridization, including efforts focused on individual KChs [reviewed in (Vacher et al., 2008)] as well as large-scale (*i.e.*, genome-wide) endeavors such as Genepaint (Geffers et al., 2012; Visel et al., 2004) and the Allen Brain Atlas (Lein et al., 2007; Sunkin et al., 2013), have provided a wealth of information as to the cellular expression patterns of individual KCh subunits. However, while the rules for KCh assembly are relatively simple (Papazian, 1999), the determinants defining which subunit combinations traffic through the endomembrane system and appear in the plasma membrane are more complex [*e.g.*, (Manganas and Trimmer, 2000; Zerangue et al., 1999), etc.], such that subunit composition of native KChs is determined both by rules governing restrictive subunit assembly, and by the selective trafficking of KCh complexes containing specific subunit combinations. Thus, the expression levels of a particular KCh subunit in the plasma membrane do not necessarily

reflect its mRNA levels. Furthermore, when considering subcellular localization of KChs, there exist considerations that do not hold for proteins such as transcription factors and chromatin components that, whether expressed in a cell that is simple and small, or (like many brain neurons) large and complex, these proteins are reliably found near the site of their mRNA within or near the nucleus. However, as KChs function in the plasma membrane, by definition they will be found at sites quite distinct from their encoding mRNAs. In many non-neuronal cell types, the distance between the nucleus and the cell boundary can be measured in microns or tens of microns, but in the case of vertebrate neurons, with their extensive dendritic and axonal processes, the boundary membrane can be millimeters, centimeters and even meters away. As such, understanding KCh protein expression, in terms of both the subunit composition of native KChs, and their subcellular localization cannot be resolved or accurately predicted from studies of mRNA expression, but need to be addressed at the proteomic level.

It remains a practical reality that how much we know of any particular protein is determined to a great extent by whether specific probes exist to study it (Edwards et al., 2011). Given the complexity of KCh subunit composition, and the complexity of mammalian brain neuron structure, it is clear that specific probes for efficiently and specifically labeling native, endogenously expressed KCh α and auxiliary subunits are especially crucial to understanding their diverse functional roles. Unlike the facile and straightforward design and synthesis of complementary nucleotide probes to label individual KCh mRNAs by *in situ* hybridization, current technology does not allow for the directed synthesis of probes for labeling KCh polypeptides. Given the diversity of KChs, it is not feasible to approach systematic analyses of subcellular localization in mammalian brain neurons using genetically encoded tagged KCh subunits, as one can do in yeast or *C. elegans*, even if one could be assured of accurate incorporation of the exogenous subunit into normal oligomeric complexes, and obviously this approach is impossible in human brain. As such we are left with the need for high quality probes that efficiently and specifically label endogenous KCh subunit proteins *in situ*.

The cloning of KCh subunits and determination of their deduced amino acid sequences, combined with advances in synthetic peptide synthesis and recombinant protein expression, has allowed for production of designer immunogens that can be used to generate antibodies against individual KCh subunits. Some of these anti-KCh antibodies were generated as singular efforts in individual research laboratories, others from commercial endeavors, and others from systematic NIH-funded efforts, for example at the UC Davis/NIH NeuroMab Facility (neuromab.ucdavis.edu). While much thought and effort has gone into generating and validating anti-KCh antibodies, monospecific antibodies for each KCh family member remains an unmet need. There is a documented propensity (the Harlow-Knapp effect) to focus research efforts on the same small subset of proteins about which we already know quite a bit (in many cases because reliable probes do exist), and to mostly ignore the rest (Fedorov et al., 2010; Grueneberg et al., 2008). Consistent with this, the availability of rigorously validated antibodies against certain KCh α and auxiliary subunits has led to an extensive knowledge base regarding their subunit composition and subcellular localization in mammalian brain, while hardly any or no validated probes exist for most others, about

which we know little or nothing at all (Table 1, Figure 1). Recognizing the universal need for high quality protein-specific probes, government agencies have initiated large scale efforts to generate high affinity “binders”, in most cases antibodies, for (ultimately) every human protein (Taussig et al., 2013). However, we are still a long way from achieving this goal.

While how much we know of the subcellular localization of KChs in mammalian brain is biased toward a select subset based on probe availability, it is also heavily weighted towards particular neurons and/or brain regions. We know a tremendous amount about the situation in hippocampus and cerebellum, in large part due to the relatively simple neuroanatomical organization of these regions, as well as a few other neurons with exceptional characteristics (*e.g.*, globular bushy neurons in the anterior ventral cochlear nucleus or AVCN that give rise to the extraordinary calyx of Held nerve terminals), and much less about most other brain regions. We also know more about KChs localized within subcellular compartments that we have learned to visualize at the level of light microscopy (*e.g.*, the AIS, NoRs, and surrounding regions), than in many others that remain to be characterized, due to their size, density and/or complexity (*e.g.*, neocortical dendritic spines and presynaptic terminals). Lastly, among mammals we know the most about KCh subcellular localization in rat and mouse brain, and less so in human and non-human primates.

Having a collection of antibodies in hand is necessary but not sufficient; as to obtain reliable information on expression and subcellular localization of brain KChs these antibodies then need to be used in careful immunohistochemistry experiments. This involves using brain sections that have been prepared to preserve anatomical structure and immunoreactivity (Lorincz and Nusser, 2008b), labeled with optimal levels of high quality and rigorously validated primary (Rhodes and Trimmer, 2006) and secondary (Manning et al., 2012) antibodies. The latter issue deserves additional mention, due to the underutilization of high quality and widely available secondary antibodies specific for individual mouse IgG subclasses (IgG1, IgG2a, IgG2b being the most common) that allow for easy and reliable multiplex labeling by mixing and matching mouse monoclonal antibodies of different subclasses. This achieves increased importance given the availability of a large and growing number of high quality mouse monoclonal antibodies against KCh subunits and many other neuronal proteins, and that multiplex immunofluorescence labeling, especially when combined with optical sectioning, array tomography, or super-resolution imaging, allows for insights into subcellular colocalization well beyond what any single label experiment could provide. The labeling obtained then needs to be appropriately imaged, and the images correctly processed and analyzed to represent its true nature and extent.

Careful experimental design including attention to controls aimed at determining whether each of these goals was achieved underlies the validity of the results (Lorincz and Nusser, 2008b; Rhodes and Trimmer, 2006; Saper, 2005; Saper and Sawchenko, 2003). As pointed out astutely in a valuable toolbox article (Lorincz and Nusser, 2008b), the conditions used for sample preparation can fundamentally impact the results, such that an antibody validated for specificity under one set of experimental conditions can end up yielding incomplete or incorrect information under another set of conditions. While this is especially relevant when making big leaps, such as from immunoblots to immunohistochemistry, it also holds for

immunohistochemistry performed under different conditions, for example “conventional conditions” used for preparing lightly fixed and detergent permeabilized brain sections for light microscope level analyses of immunoperoxidase/diaminobenzidine (LM-DAB) and immunofluorescence (LM-IF) labeling, *versus* non-permeabilized sections for pre-embedding immunoperoxidase/DAB (EM-DAB) or immunogold (EM-IG) labeling for electron microscopic analysis, *versus* the conditions used for more heavily fixed post-embedding immunogold labeling, or the exceptional requirements for SDS digested freeze fracture replica immunogold labeling (SDS-FRL) for electron microscopy (Masugi-Tokita and Shigemoto, 2007). However, as many questions regarding KCh expression and localization in brain neurons require using more than one of these approaches, it is important to have a reliable antibody, or set of antibodies, that together have been validated for efficacy and specificity under each condition used.

Two techniques deserve special mention. Recent advances in super-resolution imaging microscopy (Evanko, 2009), primarily applied to thin samples such as monolayer cell cultures, but more recently in relatively thick (*i.e.*, 10–70 μm) brain sections [*e.g.*, (Dani et al., 2010)], have allowed for insights into details of the subcellular localization of KChs. For example, structured illumination microscopy or SIM imaging of brain sections multiplex labeled with mouse monoclonal antibodies of different IgG subclasses allowed for the precise localization of clustered Kv2.1 channels at sites on the AIS deficient in expression of ankyrin-G (AnkG) defining these sites as unique ion channel clustering domains (King et al., 2014), and at sites over ryanodine receptors in striatal medium spiny neurons (Mandikian et al., 2014). The general application of superresolution imaging, using SIM and higher resolution methods (Evanko, 2009), to future studies of KCh subcellular localization will lead to additional insights into distinct neuronal domains and associated molecular components. The use of EM-IG labeling of SDS-FRL samples (Masugi-Tokita and Shigemoto, 2007) has recently emerged as a powerful approach for quantitative ultrastructural analyses of subcellular localization of K⁺ channels. This technique, while currently practiced in only a handful of laboratories, has yielded remarkable insights into KCh localization, and promises to play a major role in defining at exquisite detail quantitative aspects of subcellular localization with exquisite detail, as it has for ionotropic receptor ion channels at synapses [*e.g.*, (Antal et al., 2008; Tarusawa et al., 2009)], and Nav channels on dendrites (Lorincz and Nusser, 2010).

Leitmotifs of KCh subcellular localization

Recent reviews have provided comprehensive treatment of cellular and subcellular expression of ion channels, including KChs in mammalian brain neurons (Lujan, 2010; Nusser, 2012; Vacher et al., 2008), and mechanisms underlying such localization (Gu and Barry, 2011; Jensen et al., 2011; Vacher and Trimmer, 2012). In addition, recent studies have revealed details of KCh compartmentalized subcellular localization, and striking similarities between KChs distantly related in molecular and functional characteristics (Table 1, Figure 1), and which has shaped our thinking of the role of specific subunits and combinations thereof in neuronal function. I emphasize that given the diversity of neuron classes in mammalian brain, it is not surprising that there will be certain neurons that have KChs localized in subcellular compartments distinct from those summarized in Table 1 and

Figure 1. Prominent examples of neuron classes that seem to do things differently include local inhibitory interneurons, olfactory bulb neurons, and cerebellar Purkinje cells, among others, which appear to have distinct rules for subcellular localization (Vacher et al., 2008). However, these summaries, while an oversimplification of all mammalian species, all brain neuron classes, and even more strikingly, all developmental stages, are a useful summary of the most detailed studies in the literature. Here I provide some salient examples of these themes focusing on findings obtained from a set of specific neurons and/or specific neuronal compartments.

Dendritic KChs: input-specific localization in hippocampal CA1 pyramidal cell apical dendrites

Diverse populations of KChs and dynamic modulation of their activity are major determinants of the electrical compartmentalization of dendrites, from distinct proximal to distal gradients (Hoffman et al., 1997; Korngreen and Sakmann, 2000; Schaefer et al., 2007), to differences between oblique dendrites that yield branch-specific differences in dendritic excitability (Losonczy et al., 2008). Additionally, local activity of KChs at or near spines impacts maturation (Kim and Hoffman, 2012) and function (Bloodgood and Sabatini, 2007; Sala and Segal, 2014; Yuste, 2013) of individual synapses. Given these important roles, it is not surprising that disruption of the normal patterns of expression, localization and function of dendritic KChs in brain neurons has emerged as the likely pathophysiological basis for a number of neurological and psychiatric disorders (Poolos and Johnston, 2012). Much of what we know of dendritic KChs is from studies in the apical dendrites of hippocampal CA1 pyramidal neurons, in part due to the focus of many neurophysiological studies on the Schaffer collateral CA3:CA1 synapses located here, but also due to the large size of the primary apical dendrite that is amenable to direct patch clamp analyses (Chen and Johnston, 2006).

Transient A-type Kv4 channels, small conductance Ca²⁺-activated KCa2/SK and G protein-coupled inwardly rectifying Kir3/GIRK channels are consistently found in dendrites (Table 1, Figure 1). In many neurons, these KChs exhibit input-specific compartmentalization, being enriched at specific subcompartments of the dendritic membrane that correspond to sites of distinct synaptic input. Kv4.2 immunolabeling in the apical dendrites of CA1 pyramidal neurons (Figure 2B, 2C, 3D) has a non-uniform input-specific distribution (Rhodes et al., 2004; Sheng et al., 1992), being more robust in *stratum radiatum* (*sr*), the site of Schaffer collateral input from hippocampal CA3, than in *stratum lacunosum moleculare* (*slm*), the site of lateral perforant path input into CA1 from entorhinal cortex. Cytoplasmic KChIP2 and KChIP4 (Rhodes et al., 2004) auxiliary subunits exquisitely co-localize with Kv4.2 in *sr* of CA1 (Figure 2C), while DPP6 auxiliary subunits are expressed uniformly across *sr* and *slm*, perhaps suggesting a function in distal dendrites distinct from acting as a Kv4.2 auxiliary subunit (Clark et al., 2008). In contrast to labeling for Kv4.2, light microscope level immunolabeling for small conductance calcium activated KCa2.2 α subunits labeling is sparse in *sr* of CA1, and robust in *slm* (Sailer et al., 2002; Sailer et al., 2004). Labeling for Kir3.1 and Kir3.2 G protein coupled KCh α subunits is also prominent in *slm*, as well as distal *sr* (Figure 3A) (Drake et al., 1997; Kirizis et al., 2014; Liao et al.,

1996; Miyashita and Kubo, 1997). This suggests input-specific requirements for specific KChs on CA1 apical dendrites.

Recent studies employing quantitative EM-IG labeling of SDS digested freeze fracture replicas (SDS-FRL) of CA1 apical dendrite plasma membrane KChs consistent with these light microscopy analyses. The highest levels of Kv4.2 EM-IG labeling are observed in CA1 *sr* (Kerti et al., 2012), and Kir3.2 labeling in CA1 *slm* (Kirizis et al., 2014). Patch clamp mapping studies show that in CA1 *sr*, the density of Kv4.2 mediated A-type channels on CA1 apical dendrites is approximately five-fold higher in distal versus proximal regions (Hoffman et al., 1997). This gradient is crucial for distance-dependent scaling of Schaffer collateral inputs on CA1 dendrites, as the normal increases in mEPSC amplitude with increasing distance from the soma (Andrasfalvy and Magee, 2001) are absent in Kv4.2 knockout mice (Andrasfalvy et al., 2008). However, consistent with the light microscope level studies of expression of both Kv4.2 and KChIPs (Figure 2B, 2C, 3D) (Kerti et al., 2012; Rhodes et al., 2004), the quantitative mapping studies of plasma membrane Kv4.2 immunogold particles in SDS-FRL samples (Figure 2D) suggest only a small if any gradient of labeling along the CA1 apical dendritic arbor, and within radial oblique branches (Kerti et al., 2012). This suggests that either the subset of Kv4.2 α and auxiliary subunits with enhanced expression in distal dendrites are refractory to detection with antibodies, or that not all Kv4.2 channels in the plasma membrane are active, with an enhanced representation of “silent” channels in proximal versus distal dendrites. The former scenario seems unlikely, as similar results on the lack of a gradient have been obtained in multiple laboratories using a wide variety of labeling techniques and employing a large collection of independent antibodies. However, Kv4.2 channel activity is modulated by phosphorylation (Jerng et al., 2004) and there are suggestions of compartment specific labeling with certain anti-Kv4.2 phospho-specific antibodies has been observed (Varga et al., 2000).

Subpopulations of each of these dendritic KChs are found within spines, both within the PSD itself, and in extra-synaptic regions of the spine head membrane, as seen for Kv4.2 (Figure 2D) (Kerti et al., 2012), KCa2.2 (Figure 3B) (Allen et al., 2011; Ballesteros-Merino et al., 2012) and Kir3.2 (Figure 3C) (Drake et al., 1997; Fernandez-Alacid et al., 2011; Kirizis et al., 2014; Koyrakh et al., 2005). It should be noted that in general the PSD itself has been shown to be difficult to access with antibodies (Fukaya and Watanabe, 2000), such that the use of pre-embedding methods [LM-DAB, LM-IF and pre-embedding EM-DAB and EM-IG; (Masugi-Tokita and Shigemoto, 2007)], could yield an underestimate of true levels of expression within the PSD. In cultured neurons, whether Kv4.2 is in spines versus dendritic shafts is dynamically regulated through changes in phosphorylation state and protein-protein interactions (Hoffman, 2013). KCa2.2 mRNA is alternatively spliced to generate short and long forms that have similar functional characteristics when expressed in heterologous cells (Strassmaier et al., 2005), but that exhibit distinct compartmentalization within dendritic spines (Allen et al., 2011). Transgenic mice that express only the short form of KCa2.2 (*i.e.*, long form-specific knockouts) have selective loss of KCa2.2 EM-IG labeling in the PSD, while labeling in the extrasynaptic spine is unaffected. These mice have aberrant synaptically evoked EPSPs and defects in learning (Allen et al., 2011), and the synaptic defects are rescued by reintroduction of exogenous KCa2.2 long form (Allen et al.,

2011). This suggests a crucial and distinct role for precise targeting of KCa2.2 to the PSD in normal synaptic function. Finally, Kir3.2 colocalizes with components of G protein signaling pathways (GABA-B1 receptors, RGS7, G β 5) in dendritic spines, both within and around the PSD (Figure 3C), and less so in dendritic shafts (Fajardo-Serrano et al., 2013; Kulik et al., 2006). In cultured neurons, whether Kir3.2 is in dendritic spines *versus* shafts is dynamic and is associated with changes in Kir3.2 phosphorylation state (Chung et al., 2009).

Input-specific compartmentalization of dendritic KChs in other neurons

Input-specific compartmentalization of Kv4 α and auxiliary subunits, KCa2.2 and Kir3.2 is also seen in other neurons. CA3 pyramidal neurons and dentate granule cells express high levels of both Kv4.2 and Kv4.3, as well as KChIP auxiliary subunits, in prominent and restricted sites in their apical dendrites. In CA3, pronounced labeling is seen in distal dendrites in *sr*, but little or no labeling for these subunits is observed in the more proximal *stratum lucidum*, the site of massive dentate granule cell mossy fiber input into CA3, or in cell bodies. In dentate granule cells, little immunolabeling for these Kv4 subunits is present in cell bodies, or in the inner third of the molecular layer, the site of commissural/associational input, while robust labeling is seen in the middle and outer thirds of the molecular layer, the sites of perforant path input to dentate gyrus (Menegola and Trimmer, 2006; Monaghan et al., 2008; Rhodes et al., 2004). This uneven input-specific compartmentalization of Kv4.2 localization is exacerbated after seizures, with an accumulation resembling a barrier at the boundary between the inner and middle third of the molecular layer (Monaghan et al., 2008), presumably in response to the recurrent input resulting from seizure-induced mossy fiber sprouting, and to prevent overstimulation of distal dendrites by this ectopic input, and/or homeostatically suppress input from the medial perforant path. Kir3.1 and Kir3.2 also exhibit enhanced expression in the outer two-thirds of the dentate molecular layer (Fernandez-Alacid et al., 2011), suggesting a requirement for a diverse collection of KChs at the specific sites of perforant path input on dentate granule cell dendrites.

Kv2 channels: unique localization in proximal dendrites

In contrast to the KCh described above that exhibit enhanced expression in distal dendrites, delayed rectifier Kv2 α subunits in neurons across the brain are distinguished restricted high level expression in proximal dendrites. This is consistent with prominent expression of sustained or delayed rectifier Kv channels in proximal but not distal regions of apical dendrites of neocortical layer 5 pyramidal neurons (Korngreen and Sakmann, 2000; Schaefer et al., 2007) that defines the distal dendrite as a low threshold region for synaptic signal amplification. This is very apparent for Kv2.1 expressed in hippocampal CA1 and CA3 pyramidal neurons (Figures 2C, 3D, 5A) (Maletic-Savatic et al., 1995; Mandikian et al., 2014; Rhodes et al., 2004; Rhodes et al., 1995; Sarmiere et al., 2008; Speca et al., 2014) as well as for Kv2.2 (Speca et al., 2014). Quantitative immunogold labeling of SDS-FRL samples from adult rat CA1 reveals substantial Kv2.1 labeling on the proximal dendrites, and no detectable labeling at any site distal to this (Kirizis et al., 2014). Live cell imaging of cultured neurons reveals that Kv2.1 is carried in transport vesicles distinct from those containing distally directed Kv4.2, and that selective interaction of Kv2.1-containing

vesicles with myosin II is crucial to their proximal localization, perhaps through interaction with a distinct pool of randomly oriented actin filaments present in proximal but not distal dendrites (Jensen et al., 2014). Kv2.1 (Figure 3E) (Trimmer, 1991) and Kv2.2 (Kihira et al., 2010) are prominently expressed in large clusters on somata that define a novel neuronal plasma membrane domain located over subsurface cisternae (Du et al., 1998; Mandikian et al., 2014), sites of intracellular Ca^{2+} release, that in certain neurons contain high densities of ryanodine receptor release channels (Figure 3E) that are juxtaposed to plasma membrane Kv2.1 labeling (Antonucci et al., 2001; Mandikian et al., 2014; Misonou et al., 2005).

KChs in Axons and Presynaptic Terminals

KChs play a prominent role in repolarizing axons after action potential initiation at the AIS, regulating propagation of action potentials in unmyelinated and myelinated axons, determining how action potentials invade nerve terminals to trigger neurotransmitter release, and in subsequent repolarization of the preterminal membrane after an excitatory event (Debanne et al., 2011). Accordingly, the molecular heterogeneity of axonal KChs, and the restricted subcompartmentalization of different isoforms within axons, is extensive (Table 1, Figure 1). Disruption of these defined patterns of subcellular localization is associated with a variety of neurological pathologies (Child and Benarroch, 2014), and axonal KChs have both established utility (*e.g.*, retigabine, dalfampridine) and future potential (Judge et al., 2007) as drug targets.

KChs at the axon initial segment

KChs at the AIS play a prominent and diverse role in regulating initiation of action potentials, both in the anterograde direction down the axon, and retrograde direction back through the cell body and into dendrites (Bender and Trussell, 2012; Kole and Stuart, 2012). Precise compartmentalization of ion channels within distinct AIS subcompartments defines the properties of spike initiation (Brette, 2013). Moreover, the KChs that are highly concentrated at the AIS are dynamically regulated to yield distinct local regulation of ion channel function (Bender and Trussell, 2012; Kole and Stuart, 2012). While this unique neuronal subcompartment makes up only a tiny fraction of the neuronal plasma membrane surface area, it is emerging as a “hot spot” for disease, with a preponderance of mutations associated with inherited epilepsies in genes encoding proteins, including KChs, selectively expressed at the AIS (Child and Benarroch, 2014; Wimmer et al., 2010). The localization of KChs and other AIS proteins is also altered in response to injury and disease, and in numerous neurological and psychiatric disorders (Buffington and Rasband, 2011; Buttermore et al., 2013),

Within the AIS, low threshold delayed rectifier Kv1.1 and Kv1.2 α subunits are selectively expressed in the distal region (Figure 4B) (Goldberg et al., 2008; King et al., 2014; Lorincz and Nusser, 2008a; Ogawa et al., 2008). While in some neurons this localization can be observed in samples prepared using conventional fixation conditions (Dodson et al., 2002; Inda et al., 2006; Van Wart et al., 2007), in many brain neurons, antigen retrieval or altered fixation conditions are needed to see Kv1 labeling at the AIS (Lorincz and Nusser, 2008a, b). Kv1 channels at the distal AIS play prominent roles in determining the properties of action potentials, and in repolarizing the distal AIS, the site of spike initiation, after an

action potential has been fired (Kole et al., 2007; Rowan et al., 2014). Kv7.2 and Kv7.3 are subunits of neuronal M-type KChs (Wang et al., 1998), and that are also found at the distal AIS (Figure 4C) of many mammalian brain neurons (Battefeld et al., 2014; Devaux et al., 2004; Klingner et al., 2011; Pan et al., 2006). Immunolabeling for Kv7 subunits at the AIS can be observed in frozen unfixed but not conventionally fixed samples. Kv7.2 and Kv7.3 contain a consensus AnkG binding motif that directs their localization to the AIS (Pan et al., 2006), while Kv1 α subunits lack such motifs, and may localize to distal regions of the AIS through a mechanism involving MAGUKs (Ogawa et al., 2008). That Kv7 channels are compartmentalized in more distal AIS (Battefeld et al., 2014; Pan et al., 2006), while AnkG is uniformly expressed in both proximal and distal AIS suggests that Kv7 localization within the AIS is regulated by factors other than the simple presence of the AnkG binding motif. This is also the case for Nav channels, which all contain virtually identical AnkG binding motifs (Lemaitte et al., 2003), yet different Nav channel α subunit isoforms exhibit compartmentalized localization within the AIS (Hu et al., 2009; Lorincz and Nusser, 2008a; Van Wart et al., 2007), contributing to their distinct roles in anterograde (*e.g.*, distal Nav1.6; Figure 4B) and retrograde (*e.g.*, proximal Nav1.1, Nav1.2) action potential initiation (Hu et al., 2009), and that is crucial to the sharpness of spike initiation (Brette, 2013). AnkG interaction and overall targeting of Nav channels to the AIS is influenced by phosphorylation (Brecht et al., 2008), raising the possibility that localization of Kv7 (and Nav) channels within specific AIS domains could be determined by phosphorylation or other post-translational modification or protein-protein interactions that are compartmentalized within the AIS. Perhaps consistent with this, the protein kinase casein kinase 2 is concentrated in the AIS, where it plays a role in stabilizing Nav channel: ankyrin G interaction (Brecht et al., 2008), and also interacts with and regulates Kv7.2/Kv7.3 channels (Kang et al., 2014), and both Kv7.2 and Kv7.3 are extensively phosphorylated but at sites in the primary sequence distinct from the AnkG binding motif [reviewed in (Trimmer and Misonou, 2014)]. Calmodulin binding to a region distinct from the AnkG binding motif promotes formation of Kv7.2/Kv7.3 heteromers (Liu and Devaux, 2014), and as heteromeric assembly of Kv7.2 with Kv7.3 promotes its targeting to the AIS, *via* the stronger AnkG binding motif in Kv7.3 (Chung et al., 2006; Rasmussen et al., 2007), calmodulin binding promotes AIS targeting (Liu and Devaux, 2014). The involvement of calmodulin may link Ca^{2+} signaling to dynamic regulation of Kv7 channel expression in the AIS. I note that in contrast to the discordant results discussed above that show gradients of dendritic Kv4.2 expression by electrophysiology but not by immunolabeling, combined electrophysiology and immunostaining results reveal similar patterns of Kv7 channel expression and function in proximal versus distal AIS (Battefeld et al., 2014). The genes encoding Kv7.2 (Biervert et al., 1998; Singh et al., 1998) and Kv7.3 (Charlier et al., 1998) were initially identified as harboring partial loss of function mutations in genetic analyses of benign familial neonatal convulsions, and many more mutations have been identified in these patients [reviewed in (Wimmer et al., 2010)], as well as recently identified dominant-negative *de novo* mutations that yield a syndrome of epilepsy, global developmental delay, and autism (Orhan et al., 2014; Weckhuysen et al., 2012). Consistent with the crucial role of AIS KChs in neuronal excitability (Kole and Stuart, 2012). Kv7.2 and Kv7.3 are targets for the anti-epileptic drug retigabine (Main et al., 2000), a first in class positive allosteric modulator of KCh activity (Stafstrom et al., 2011) that yields reduced intrinsic excitability,

presumably at least in part through modulation of AIS Kv7 channels (Brown and Passmore, 2009). A recent report suggests epistasis between Kv7 and AnkG in bipolar disorder (Judy et al., 2013), consistent with a key role for correct subcellular localization of AIS (or NoR, see below) Kv7 channels in normal neuronal function. Kv7 channels remain prime targets for development of therapeutics for a variety of neurological and psychiatric disorders (Grunnet et al., 2014).

When labeling for primarily somatodendritic Kv2.1 is combined with that for AnkG to mark the AIS, a very minor population of Kv2.1 at the AIS can be detected (King et al., 2014; Sarmiere et al., 2008). Unlike the other AIS KChs discussed above, Kv2.1 is distinct in both being present along the entire length (*i.e.*, in both proximal and distal regions) of the AIS (King et al., 2014; Sarmiere et al., 2008), and by not being expressed elsewhere on axons (Du et al., 1998; Kirizis et al., 2014; Rhodes et al., 2004; Rhodes et al., 1995; Trimmer, 1991). Multiplex LM-IF labeling and super-resolution imaging reveal Kv2.1 has a clustered subcellular localization in a non-canonical AIS membrane compartment devoid of underlying AnkG, and distinct from Kv1 and Nav channels (Figures 4D, 4E), but adjacent to axo-axonal GABAergic synapses, and overlying intracellular cisternal organelles (King et al., 2014). Together these studies show that the AIS, while representing a miniscule fraction of the total neuronal plasma membrane area, contains high densities of a specific set of KChs (and other ion channels) precisely segregated into distinct membrane subcompartments, forming a mosaic of ion channel expression and function that is a dynamic determinant of neuronal excitability.

KChs at the nodes of Ranvier of myelinated axons

A number of KChs exhibit highly enriched and specific subcellular localization at nodes of Ranvier (NoRs) of myelinated axons, including within the node itself, and also at the associated juxtaparanode (JPN) domains [recently reviewed in (Buttermore et al., 2013; Chang and Rasband, 2013)]. Kv1.1 and Kv1.2 (Figure 4F, 4G) are found at high levels at JPNs, and their discovery here (Wang et al., 1993; Wang et al., 1994) provided the first evidence for a molecular distinction between this compartment and the nearby node, and flanking paranode and internode domains. Kv1.1 and Kv1.2, together with the auxiliary Kv β 2 subunit (Rhodes et al., 1997; Shi et al., 1996) are seen consistently at JPNs of myelinated axons throughout the vertebrate nervous system (Rasband, 2004), including in humans (Figure 4G) (Howell et al., 2006; Howell et al., 2010), although there are less frequent examples of transient or A-type Kv1.4 subunits at JPNs (Rasband et al., 2001). Kv1 channels, which at JPNs are located underneath the myelin, are thought to act to prevent repetitive firing and contribute to intermodal resting potential (Faivre-Sarrailh and Devaux, 2013), and their exposure during development, or due to demyelination in disease or injury, leads to conduction failure (Susuki, 2013). The Kv1 channels at JPNs are targets for therapeutics (*e.g.*, dalfampridine) that improve function in patients with demyelinating disorders such as multiple sclerosis (Goodman and Stone, 2013). Clustering of Kv1 channels at JPNs is one of the latest events in the formation of NoRs (Vabnick et al., 1999), and their presence is dynamic and dependent on formation and maintenance of intact myelin (Dupree et al., 1999; Rasband et al., 1999; Rasband et al., 1998), such that Kv1 localization at JPNs is a sensitive diagnostic readout that has been used to define myelination state in animal

studies of developmental NoR formation (Susuki et al., 2013), neuropathic disease (Golan et al., 2013), aging (Hinman et al., 2006), injury (Choo et al., 2009), and *in vivo* models of remyelination by stem cells (Eftekharpour et al., 2007; Ruff et al., 2013; Uchida et al., 2012), and in *post mortem* analyses of donors with human demyelinating diseases such as multiple sclerosis (Figure 4G) (Howell et al., 2006). The molecular basis of the labile nature of Kv1 channel localization at JPNs, including mechanisms to sense and respond to changes in myelination state has not been established. Kv1 α subunits contain C-terminal PDZ binding motifs and interact directly with MAGUKs such as PSD-95 *in vitro* (Kim et al., 1995), and are clustered by MAGUKs in heterologous cells (Kim et al., 1995; Tiffany et al., 2000). Kv1 channels and MAGUKs copurify from brain (Schulte et al., 2011) and Kv1 subunits colocalize with PSD-95 at JPNs (Baba et al., 1999; Rasband and Trimmer, 2001). However, Kv1 channel localization at JPNs remains intact in the absence of PSD-95 (Rasband et al., 2002), although other MAGUKs, such as PSD-93, are also expressed at JPNs (Ogawa et al., 2010) and could potentially compensate. Kv1.2 is phosphorylated at numerous C-terminal sites, and while all known sites are in regions distinct in primary structure from the PDZ binding motif (Trimmer and Misonou, 2014; Yang et al., 2007), it is possible that altered phosphorylation underlies the sensitivity of Kv1 localization at JPNs to myelination state. The role of MAGUKs in localizing Kv1 channels, and other JPN proteins with PDZ binding motifs such as Caspr2 (Poliak et al., 1999) and ADAM22 (Ogawa et al., 2010) remains elusive (Chang and Rasband, 2013).

Immunolabeling for the high threshold Kv3.1b α subunit is found within the NoR of a subset of myelinated axons in unfixed but not conventionally fixed brain sections (Devaux et al., 2003). Kv3.1b and AnkG coimmunoprecipitate from brain (Devaux et al., 2003), and interact directly through an AnkG binding motif present on Kv3.1b that is distinct from that found on Nav and Kv7 channels (Xu et al., 2007). Kv3.1b is also distinct from other AnkG binding ion channels (e.g., Kv7.2, Kv7.3 and Nav channels) in not being present at the AIS (Devaux et al., 2003), suggesting unimpeded transport of NoR-destined Kv3.1b containing vesicles through the AnkG-rich AIS without binding, a process that may involve direct Kv3.1b binding to kinesin I (Xu et al., 2010). Kv7.2 and Kv7.3 are also found at the NoR in frozen samples (Devaux et al., 2004), or in samples prepared with low pH fixation (Figure 4F) [see (Lorincz and Nusser, 2008b) for method], and consistent with the canonical AnkG binding motif on these subunits (Pan et al., 2006). These heteromeric Kv7.2/Kv7.3 channels at the NoR stabilize the membrane potential and increase Nav channel availability and action potential amplitude (Battfeld et al., 2014). Much remains to be learned of the mechanisms involved in generating precise localization of KChs at specific sites in myelinated axons, the sensing mechanisms for maintaining the appropriate levels of particular KChs at these sites in healthy adult axons, and the pathways that lead to their disruption in response to injury and disease.

KChs within/near presynaptic terminals

A diverse set of KChs also plays a key role in regulating neurotransmitter release from nerve terminals. The subunits that comprise these KChs are important targets for physiological neuromodulation to confer plasticity to transmitter release, and for its therapeutic modulation. In this category are KChs at the preterminal segment of the axon, acting as

filters and/or gatekeepers regulating access of axonal electrical signals to the presynaptic terminal, and those within the nerve terminal itself, acting to regulate the presynaptic membrane potential and activity of the Cav channels that underlie release (Debanne, 2004).

Mossy fiber (MFs) axons of dentate granule cells make a diverse set of glutamatergic excitatory synapse types onto multiple classes of target neurons in dentate gyrus hilus and CA3 *stratum lucidum*, and MF synapses exhibit target-specific differences in structure (Rollenhagen and Lubke, 2010), function (Toth and McBain, 2000) and plasticity (Scott et al., 2008). MF axons and/or terminals exhibit prominent immunolabeling for Kv1.1 (Figure 5B) (Wang et al., 1993) and Kv1.4 (Rettig et al., 1992; Sheng et al., 1992), as well Kv β 1 auxiliary subunits (Rhodes et al., 1997). Electron microscopy studies show immunolabeling for Kv1.1 (Wang et al., 1994) and Kv1.4 (Cooper et al., 1998) in preterminal segments of MF axons (Figure 5C), similar to the metabotropic glutamate receptor mGluR2 (Shigemoto et al., 1997; Yokoi et al., 1996), raising the possibility of efficient coupling of metabotropic signaling to modulation of Kv1 channel function. The localization is distinct from that proposed for the Nav1.2 channel, which is highly expressed in MFs (Figure 5B) (Gong et al., 1999; Westenbroek et al., 1989), but which electrophysiology experiments suggest is present within the terminal itself and where it confers somewhat unique excitability to MF terminals (Engel and Jonas, 2005). Fast inactivating DTX-sensitive KChs, presumably Kv1.1/Kv1.4/Kv β 1 complexes, are modulated by CamKII (Roeper et al., 1997) and opioid signaling (Simmons and Chavkin, 1996), and play an important role in dynamic control of Ca²⁺ influx into MF terminals (Geiger and Jonas, 2000). MFs undergo a dramatic reorganization upon MF sprouting that occurs in animal models of mesial temporal lobe epilepsy (MTLE) (Jones et al., 1992) and MTLE patients (Spencer and Spencer, 1994), leading to the formation of ectopic synapses, most prominent in the inner molecular layer of the dentate gyrus, and that contain immunolabeling for Kv1.4 (Monaghan et al., 2008). This suggests that unlike other MF KChs that are not found to these ectopic sites (see below), Kv1.4-containing channels have potential as therapeutic targets for regulating activity of this aberrant recurrent circuit in MTLE patients.

Kv1 channels are also present in axon preterminal segments of other neurons, most prominently studied in the exceptionally large GABAergic terminals of cerebellar basket cells (BCTs), and of AVCN neurons in auditory brainstem that form calyx of Held terminals. Ultrastructural studies of Kv1.1 and Kv1.2 in BCTs show compartmentalization within the preterminal axon plexus and at septate junctions that form between adjacent basket cell axon branches, but not in the terminals themselves (Laube et al., 1996; McNamara et al., 1996; Wang et al., 1994). Immunolabeling for Kv1 α subunits at these septate junctions colocalizes with labeling for PSD-95 (Laube et al., 1996), as predicted from LM-IF colocalization (Kim et al., 1995). However, PSD-95 is not required for localization of Kv1 channels in BCTs, as immunolabeling for these K⁺ channels remains intact in mice lacking PSD-95 (Rasband et al., 2002). Kv1.1 knockout mice have an increase in spontaneous GABA release from BCTs and increased GABA inhibition of Purkinje neuron output (Zhang et al., 1999) that presumably underlies the ataxic phenotype seen in these mice. Loss-of-function mutations in Kv1.1 underlie episodic ataxia type 1, and disruption of the normal function of BCTs is presumed to contribute to the ataxia seen in

these patients (Kullmann and Waxman, 2010), as supported by results from transgenic mice expressing a human episodic ataxia type 1 Kv1.1 mutation, which exhibit both enhanced GABA release onto cerebellar Purkinje neurons, and ataxia (Herson et al., 2003).

Kv1.1 and Kv1.2 α subunits underlie the low threshold currents in the preterminal segment or transition zone between the axon and calyx of Held terminal, and are not found within the terminals themselves (Dodson et al., 2003). This preterminal zone of the axon, also called the heminode (as AVCN axons are myelinated), also contains high levels of immunolabeling for Nav1.6 (Leao et al., 2005), suggesting some similarities between this final segment of the axon and the AIS. Pharmacological dissection of Kv1 currents in the preterminal segment reveals coexistence of homomeric Kv1.2 channels and Kv1.1/Kv1.2 heteromers that is not apparent in light microscope level immunolabeling (Dodson et al., 2003), showing that two different Kv1 channels of distinct but related subunit composition are targeted to the same small subcompartment of these axons. Presumably, the homomeric Kv1.3 channels recently found in calyx of Held terminals (Gazula et al., 2010) bypass this zone to be specifically retained in the terminal. That a distinct Kv1 channel (a Kv1.1/Kv1.2/Kv1.6 heteromer) is present on somata of AVCN globular bushy cells (Dodson et al., 2003) that give rise to calyx of Held terminals implies that these coexpressed Kv1 α subunits can selectively partition into tetrameric channels with distinct subunit compositions, or that these distinct KChs arise from non-overlapping pulses of Kv1 α subunit gene expression. The distinct polarized expression (*i.e.*, somatodendritic versus axonal), and compartmentalized subcellular localization (pre-terminal segment versus terminal) of these highly similar Kv1 channels at the calyx of Held is a remarkable example of precision in subcellular localization of highly related KChs in distinct neuronal compartments.

Kv7.2 and Kv7.3 subunits are also present in mossy fibers (Cooper et al., 2001; Devaux et al., 2004; Klingner et al., 2011), although their localization in specific subcompartments, such as terminals or preterminal segments of axons, has not been determined, nor has their specific role in mossy fiber function. Kv7.5 immunolabeling is present in calyx of Held nerve terminals (Caminos et al., 2007). Kv7.5 expressed in calyx of Held nerve terminals is an important determinant of the resting membrane potential, and as such plays a key role in determining glutamate release probability (Huang and Trussell, 2011).

High threshold Kv3 channels play a critical role in repolarization of MF axons and terminals (Alle et al., 2011; Riazanski et al., 2001), and immunolabeling for Kv3.4 (Rettig et al., 1992; Veh et al., 1995) and Kv3.3 (Chang et al., 2007) is prominent in hippocampal MFs, although ultrastructural studies to determine whether the Kv3 channels are in the MF boutons themselves, or in axons and preterminal segments, have not been performed. Kv3.4 immunolabeling in MFs is reduced in the pilocarpine model of MTLTLE (Pacheco Otalora et al., 2011), perhaps contributing to network hyperexcitability associated with this model. In BCTs, Kv3.4 is found uniformly distributed over the pinceau (Laube et al., 1996), demonstrating distinct local compartmentalization of Kv1 and Kv3 channels within BCTs following delivery from their site of synthesis in the basket cell body. EM-IG labeling reveals Kv3.2 is also present in pinceau in a pattern similar to that observed for Kv3.4 (Bobik et al., 2004). The high threshold current in the calyx of Held is from Kv3.1-containing channels (Dodson et al., 2003), with potential contributions from Kv3.3 (Korber

et al., 2014; Weiser et al., 1994), and/or Kv3.4 α subunits (Ishikawa et al., 2003). Distinct from preterminal segment Kv1 KChs, immunolabeling for Kv3.1 is found within the calyx of Held nerve terminal itself (Dodson et al., 2003), albeit on the non-synaptic (*i.e.*, back) side of the terminal (Elezgarai et al., 2003), presumably to reduce the potential for transynaptic effects on membrane potential (Schneggenburger and Forsythe, 2006). Based on a combination of their distinct functional properties and subcellular localizations, the Kv1 and Kv3 channels at the calyx of Held, like those in MFs and BCTs, have distinct roles, with preterminal Kv1.1/Kv1.2 channels reducing nerve terminal excitability and preventing aberrant neurotransmitter release, and Kv3 channels within the terminal shortening the presynaptic action potential to regulate neurotransmitter release (Ishikawa et al., 2003). A recent study suggests Kv1 and Kv3 channels near/at MF terminals are substrates for a novel form of plasticity, whereby arachidonic acid released as a retrograde messenger from CA3 dendrites/spines inhibits these KChs, resulting in a broadening of action potentials and an increase in glutamate release (Carta et al., 2014). In heterologous cells, arachidonic acid enhances inactivation to suppress activity of Kv1 and Kv3 channels, while PIP2 removes inactivation and enhances their activity (Oliver et al., 2004). Future studies will determine whether this form of plasticity occurs in other central synapses that have Kv1 and Kv3 channels near/at presynaptic terminals.

Immunolabeling for the KCa1.1 α subunit of BK channels is also present at high levels in MFs (Figure 5D) (Knaus et al., 1996; Misonou et al., 2006a; Sailer et al., 2006). No EM level studies have been published to define this labeling as being in axons, preterminal segments or terminals, although KCa1.1 immunolabeling is present in the active zone of glutamatergic Schaffer collateral nerve terminals located in CA1 *sr* (Hu et al., 2001; Sailer et al., 2006). KCa1.1 in MF axons/terminals is downregulated in the pilocarpine model of MTLE (Pacheco Otalora et al., 2008). Unlike Kv1.4 which is present in the ectopic MFs in the inner molecular layer of the dentate gyrus (Monaghan et al., 2008), the residual KCa1.1 remains in MFs within hilar and CA3 regions, and is not observed in the ectopic MFs (Pacheco Otalora et al., 2008). This suggests selective targeting to/retention in normal regions of MFs, and/or exclusion from ectopic regions, and presumably exacerbating the impact of MF sprouting on network hyperexcitability.

Coupling function of diverse ion channels through subcellular colocalization

Given the functional interplay between different classes of ion channels in the electrical events that define neurotransmission, it is not surprising that the subcellular localization of KChs appears to be coordinated with that of other ion channels. KChs play diverse roles in determining the membrane environment that controls Nav channel function, influencing the amplitude and duration of the Nav channel based action potential, and the Nav channel refractory period that impacts the interspike interval. There exist numerous prominent examples where KChs are precisely localized at/near sites of clustered Nav channels, including at the distal AIS, where microclusters of Kv1 and Kv7 channels are interspersed with Nav channels in a fine mosaic of coexpression, which also includes segregated clusters of Kv2.1. At the NoR, Kv3 and Kv7 channels co-cluster with Nav channels at high densities

within the node itself, while Kv1 channels are segregated into the neighboring JPN. Kv7 and Kv3 α subunits contain AnkG binding motifs, as do Nav channels, and presumably directing their colocalization. In certain axons (*e.g.*, at the calyx of Held), the high concentration of Kv1 channels in preterminal segments of axons is paired with a high density of Nav channels, and is separated from the Kv3 and Kv7 channels in the terminals, which lack Nav channels. In other axons and terminals (*e.g.*, MFs) Kv1 channels are at preterminal sites distinct from the nerve terminal themselves, which contain Nav channels. The mechanisms for generating and maintaining this diverse array of associated yet distinct patterns of subcellular localization of KChs and Nav channels on axons/terminals in a neuron-specific manner is not completely understood, and remains a major focus of much research activity.

There also exist numerous examples of KChs that exhibit precise subcellular colocalization, and in some cases physical association, with components of diverse neuronal Ca^{2+} signaling microdomains. These include KChs that are intrinsically Ca^{2+} -sensitive, for which changes in intracellular Ca^{2+} levels are the primary determinant of their activity, whether it be through direct binding, as for KCa1.1 α subunits of BK channels, or through constitutively associated calmodulin, as for KCa2 α subunits of SK channels. It is not surprising to see the precise colocalization (and in certain cases direct intermolecular association) of such KChs with Ca^{2+} channels, both plasma membrane Cav channels and/or intracellular Ca^{2+} release channels, as close association underlies effective coupling of Ca^{2+} channel-mediated Ca^{2+} flux to activation of these Ca^{2+} -sensitive KChs, including intimate coupling of Ca^{2+} flux through individual Cav channels to individual molecules of physically associated KCa1.1 channels (Fakler and Adelman, 2008). An emerging theme is the colocalization, association and functional coupling of Ca^{2+} channels with KChs whose α subunits are not themselves intrinsically Ca^{2+} -sensitive (*i.e.*, those outside the KCa family). These includes Kv4 channels that acquire Ca^{2+} -sensitivity and associate with Cav channels in macromolecular complexes through their EF hand containing/ Ca^{2+} -binding KChIP auxiliary subunits (Turner and Zamponi, 2014), and Kv2 channels, found at/near sites of intracellular Ca^{2+} release (Antonucci et al., 2001; King et al., 2014; Mandikian et al., 2014), which may influence their modulation by Ca^{2+} -regulated protein kinases and phosphatases (Misonou et al., 2006b). For Kv2.1 channels it is clear that their colocalization with intracellular Ca^{2+} release machinery is dynamic and reversible, and regulated by activity-dependent changes in Kv2.1 phosphorylation state (Misonou et al., 2005). It is likely, given the large number of KChs expressed in neurons, that there will be additional examples that colocalize and perhaps even directly associate with ion channels and other signaling proteins in specific compartments.

Overall considerations and future directions

The overall picture that has emerged from the studies detailed above, and many others not covered here, is that individual KCh types are present at precise and diverse locations on neuronal somata, axons and dendrites. As the neuronal plasma membrane surface area can be up to 10,000 times larger than that of a typical mammalian cell (Gonzalez and Couve, 2014), it is striking that the area in which any particular KCh is found can be so tiny. For example, in axons of human neocortical Betz cells, a KCh localized at the thousand or so NoRs in the meter long axon is concentrated in an area representing $\approx 0.1\%$ of the axonal membrane area (and one within the AIS only 0.005%!), percentages that become even

smaller when the extensive dendritic arbor area is included. To so strategically traffic KChs to such exact locations, and then faithfully maintain them there at precise levels, represents a remarkable feat of cell biology whose underlying mechanisms are for the most part not yet clear. Given what we know about the complexity of the molecular machinery underlying the organization of dendritic spines (Sala and Segal, 2014), it is somewhat intimidating to contemplate defining the molecular mechanisms that generate, maintain, and dynamically regulate the diverse array of restricted plasma membrane domains populated, and in some cases defined, by specific KChs. Such efforts remain important, as in many regards, subcellular localization defines native KCh function, in terms of its impact on neuronal physiology, but also how the function and regulation of the KCh is impacted by local environment.

As is clear from the details above, there is much known of the subcellular localization of many different KChs, and this has provided important insights into the specific roles of these important determinants of neurotransmission. However, there remain many KChs for which little information exists, including many members of entire KCh classes, such as K2P leak KChs, electrically silent/modulatory Kv5, Kv6, Kv8 and Kv9 α subunits, and Kv10, Kv11 and Kv12 EAG and EAG-related KChs, whose subcellular localization in mammalian brain has not been described (Table 1, Figure 1). Whether the lack of such information is sufficient justification for a “KCh-ome-wide” initiative to generate validated “binders” for each of the 90 or so KCh α and auxiliary subunits is not clear. Such a prospect is confounded by the fact that it is oftentimes not enough to generate a single probe and “check off the box”. To start, it is important to obtain similar results with at least two independent antibodies with distinct binding sites (Rhodes and Trimmer, 2006). Moreover, certain KCh mRNAs are subjected to alternative splicing, which in some cases leads to changes in subcellular localization in neurons [*e.g.*, Kv3.1 (Ozaita et al., 2002), KCa2.2 (Allen et al., 2011), etc.], and in other cases [*e.g.*, KCa1.1 (Fodor and Aldrich, 2009)] generates a diversity of subunits that compromises the practical reality or utility of obtaining probes for each variant. Clearly, some combination of concerted and individual efforts will continue to provide the probes that will allow for insights into those KChs who are poorly understood, not out of lack of importance, but from a simple lack of tools.

State-specific probes for KChs, both intentional and unintentional

While it remains an unmet challenge to obtain general (*i.e.*, state-independent) probes for each KCh subunit, it is important to recognize the utility of applying state-dependent or -specific antibodies in the study of KChs. As a prominent example, antibodies that recognize specific phosphorylation states of a given KCh are useful reagents for defining both the regulation of that KCh, but also the activity/signaling state of the neuron and/or specific neuronal subcellular compartment in which it resides, as exemplified by studies of activity-dependent regulation of Kv4.2 (Varga et al., 2000), Kv3.1 (Song et al., 2005) and Kv2.1 (Misonou et al., 2006b). Rigorously defined state-specific antibodies, whether specific for phosphosites, other post-translational modifications, or for KChs of specific folding state or subunit composition, have substantial value. However, that it is possible to generate state-specific antibodies intentionally raises the specter that they could be also generated inadvertently. As virtually all “general” anti-KCh antibodies are made against “naked” (*i.e.*,

unmodified) fragments of KCh subunits, one can imagine antibodies that selectively recognize only a subset of specific states in which a KCh can exist, each of which is arguably distinct from the naked fragment used for immunization. These include antibodies whose epitope is occluded in states intrinsic to the target KCh subunit itself (*e.g.*, specific conformational states assumed by the full length subunit, or the presence/absence of post-translational modifications), and by states conferred by environment (co-assembled subunits, interacting proteins, overall protein environment/density). Using such antibodies in studies aimed at subcellular localization of KChs would presumably have the potential to label specific but undefined subsets of target KCh subunits. Examples were detailed above of antibodies that label KChs at certain subcellular locations only when specific sample preparation conditions are used (*e.g.*, Kv7 channels at the AIS and NoR; Kv3 at the NoR; Kv1 at the AIS), but label other compartments (*e.g.*, Kv7 and Kv1 unmyelinated axons and terminals, Kv1 at JPNs, etc.) under most if not all conditions. Presumably, the difficulty in labeling proteins at the AIS and NoR is a reflection of local environment, distinguished by an extremely dense meshwork of interacting proteins (Rasband, 2011), similar to the difficulty of labeling proteins within the PSD (Fukaya and Watanabe, 2000), although other targets at the AIS and NoR (*e.g.*, AnkG and Nav channels) can be effectively labeled mostly regardless of conditions. These considerations and examples underscore the need to carefully validate the specificity of antibodies under the conditions under which they will be used (Lorincz and Nusser, 2008b).

Distinguishing distinct compartments

Higher resolution analyses for KChs, including multiplex LM-IF combined with superresolution microscopy and/or array tomography, and EM-IG labeling on conventional thin sections and SDS-FRL samples, are needed to better define the specific subcompartment harboring KChs. Such analyses to date have provided crucial details on the distribution of certain KCh subunits in specific neuronal subcompartments, but additional analyses using these approaches are needed to fully appreciate the repertoire of KChs in the different functional compartments of neurons, especially in compartments too small to distinguish at the level of the light microscope such as presynaptic terminals and dendritic spines. It remains a challenge that many antibodies that work well for conventional labeling by LM-DAB or LM-IF do not work for labeling samples prepared for array tomography (Micheva et al., 2010) or for SDS-FRL (Masugi-Tokita and Shigemoto, 2007). In general, EM-based analyses, while providing exquisite ultrastructural detail and the opportunity for molecular quantitation, are difficult to perform on large populations of neurons, such as a systematic analysis of KCh across populations of neocortical synapses, a subject that could be addressed with array tomography (O'Rourke et al., 2012) Application of these techniques to the study of the subcellular localization of KChs in mammalian brain will add to the existing knowledge base and provide new insights into these important regulators of neuronal activity.

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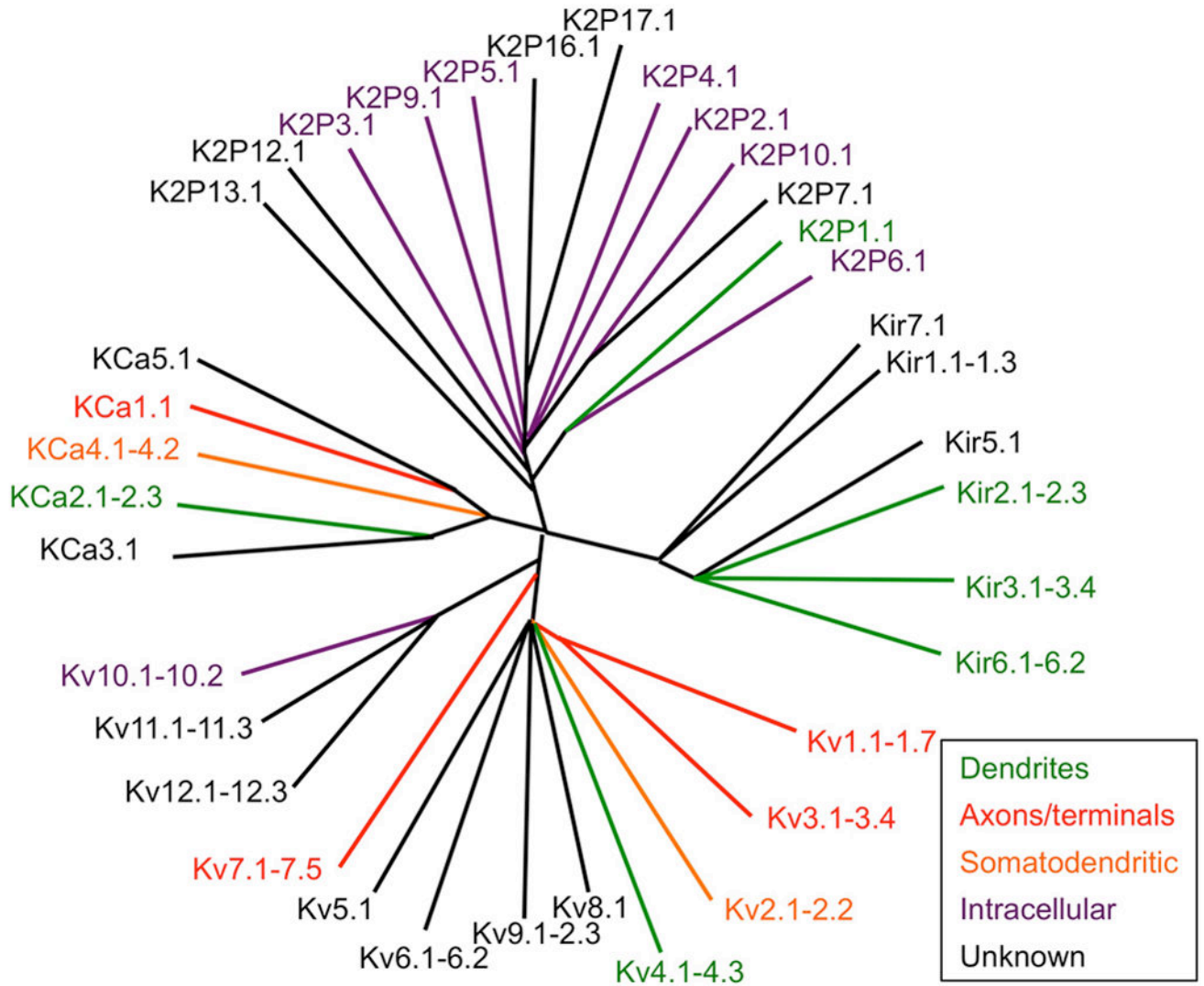


Figure 1. Simplified overview of subcellular localization of KChs
 Dendrogram format derived from that available as an online resource courtesy of Fabrice Duprat (<https://www.ipmc.cnrs.fr/~duprat/ipmc/nomenclature.htm>), and is not meant to convey precise evolutionary relationships. Subcellular localization is based on certain brain neuron classes as detailed in text, and is meant as a general simplified overview and does not universally apply to all brain regions and neuron types.

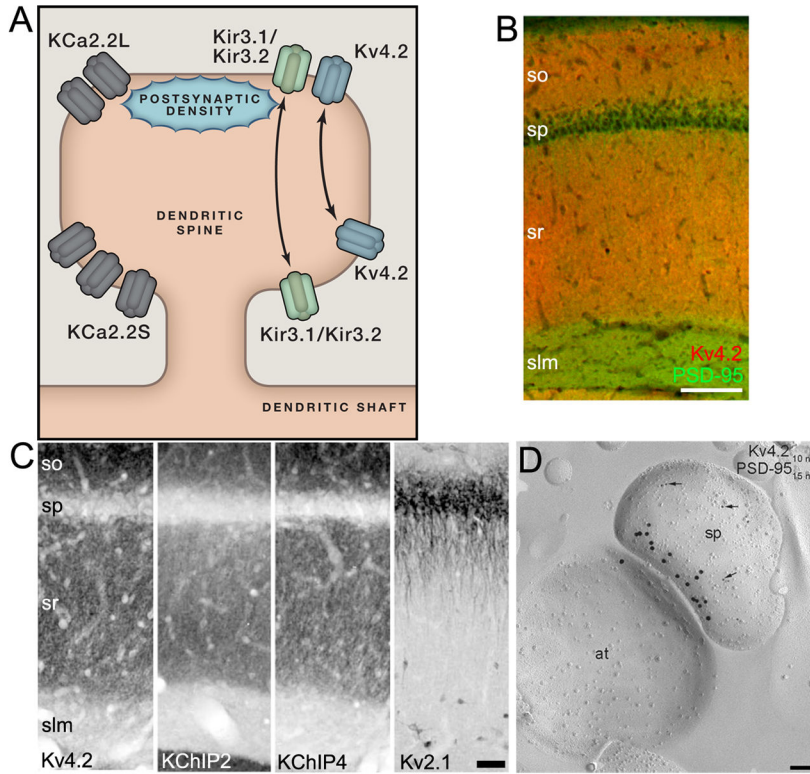


Figure 2. K⁺ channel localization in hippocampal CA1 apical dendrites

A. Cartoon showing localization of KChs in dendritic spines. See text for details. B. Double LM-IF labeling for Kv4.2 (red) and PSD-95 (green) in rat CA1 showing robust labeling for Kv4.2 in *sr* versus *slm*. Scale bar: 100 μm. C. LM-DAB labeling for Kv4.2 (far left), KChIP2 (middle left), KChIP4 (middle right) and Kv2.1 (far right) in rat CA1. Scale bar: 50 μm. Modified from (Rhodes et al., 2004). D. EM-IG labeling for Kv4.2 (small particles) and PSD-95 (large particles) in a SDS-FRL sample from rat CA1 showing a single excitatory synapse (at: axon terminal; sp: spine). Scale bar: 0.1 μm. From (Kerti et al., 2012). Anatomical labels for panels B, C: CA1: so: *stratum oriens*; sp: *stratum pyramidale*; sr: *stratum radiatum*; slm: *stratum lacunosum moleculare*; and for dentate gyrus: ml: molecular layer.

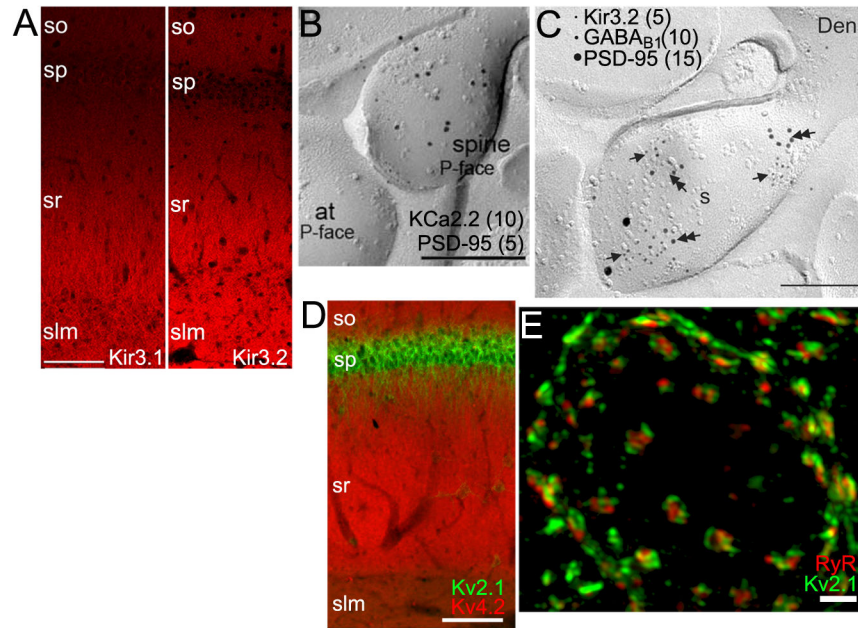


Figure 3. K⁺ channel localization in dendrites and somata

A. LM-IF labeling for Kir3.1 (left) and Kir3.2 (right) in rat CA1. From (Kirizis et al., 2014). Scale bar: 100 μ m. B. EM-IG labeling for KCa2.2 (large particles, 10 nm) and PSD-95 (small particles, 5 nm, at arrow) showing extrasynaptic KCa2.2 within a dendritic spine (sp) at an excitatory synapse (at: axon terminal) in a SDS-FRL sample from mouse CA1. Scale bar: 0.2 μ m. From (Ballesteros-Merino et al., 2012). C. EM-IG labeling of Kir3.2, GABA-B1 receptors, and PSD-95 in a SDS-FRL sample from rat CA1 showing colocalization of Kir3.2, GABA-B1 receptors in the extrasynaptic membrane of a dendritic spine (s) emerging from a dendrite (Den). Scale bar: 0.2 μ m. From (Kulik et al., 2006). D. Double LM-IF labeling for Kv4.2 (red) and Kv2.1 (green) in rat CA1 showing precise compartmentalization of these KChs across different hippocampal strata. Scale bar: 100 μ m. E. Super-resolution (SIM) image of a double label LM-IF of Kv2.1 (green) and ryanodine receptor (red) in a mouse striatal medium spiny neuron. Scale bar: 2 μ m. From (Mandikian et al., 2014). Anatomical labels for panels A, D as in Figure 2.

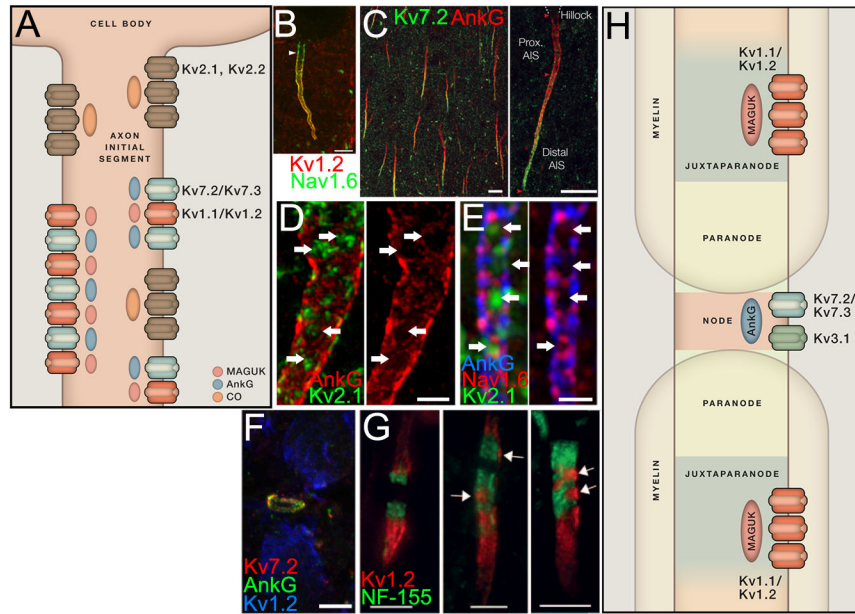


Figure 4. K⁺ channel localization in the axon initial segment

A. Cartoon showing localization of KChs in the AIS. See text for details. B. Double LM-IF labeling for Kv1.2 (red) and Nav1.6 (green) in a pyramidal neuron in rat neocortex layer 5 showing distal compartmentalization of Kv1.2. So: soma. Scale bar: 5 μ m. From (Lorincz and Nusser, 2008a). C. Double LM-IF labeling for Kv7.2 (green) and AnkG (red) in a field of pyramidal neurons in rat neocortex layer 5 (right panel) and a single neuron (left panel) showing distal compartmentalization of Kv7.2. Scale bars: 10 μ m. From (Battfeld et al., 2014). D. Super-resolution (SIM) image of double label LM-IF of Kv2.1 (green), and AnkG (red) showing the localization of Kv2.1 in “holes” in the AnkG matrix within the AIS of a rat neocortical pyramidal neuron. Right panel is same image without Kv2.1. Scale bar: 2 μ m. From (King et al., 2014). E. Super-resolution (SIM) image of triple label LM-IF of Kv2.1 (green), Nav1.6 (red) and AnkG (blue), showing the mosaic of expression of these proteins within the AIS of a rat neocortical pyramidal neuron. Right panel is same image without Kv2.1, showing sites of Kv2.1 labeling are in holes of AnkG and Nav1.6 labeling. Scale bar: 1 μ m. F. Triple label LM-IF of nodal Kv7.2 (red) and AnkG (green), and Kv1.2 (blue) at JPNs in rat brainstem, sample fixed at pH 6.0. Scale bar: 5 μ m. G. Kv1.2 (red) at JPNs and neurofascin-155 (green, to mark paranodes) from multiple sclerosis patients. Images shows labeling at JPNs of normal appearing white matter (far left), and two examples of demyelinated white matter in which Kv1.2 labeling is disrupted. Scale bars: 5 μ m. From (Howell et al., 2006). H. Cartoon showing overall expression of axonal KChs in domains at/near the node of Ranvier. See text for details.

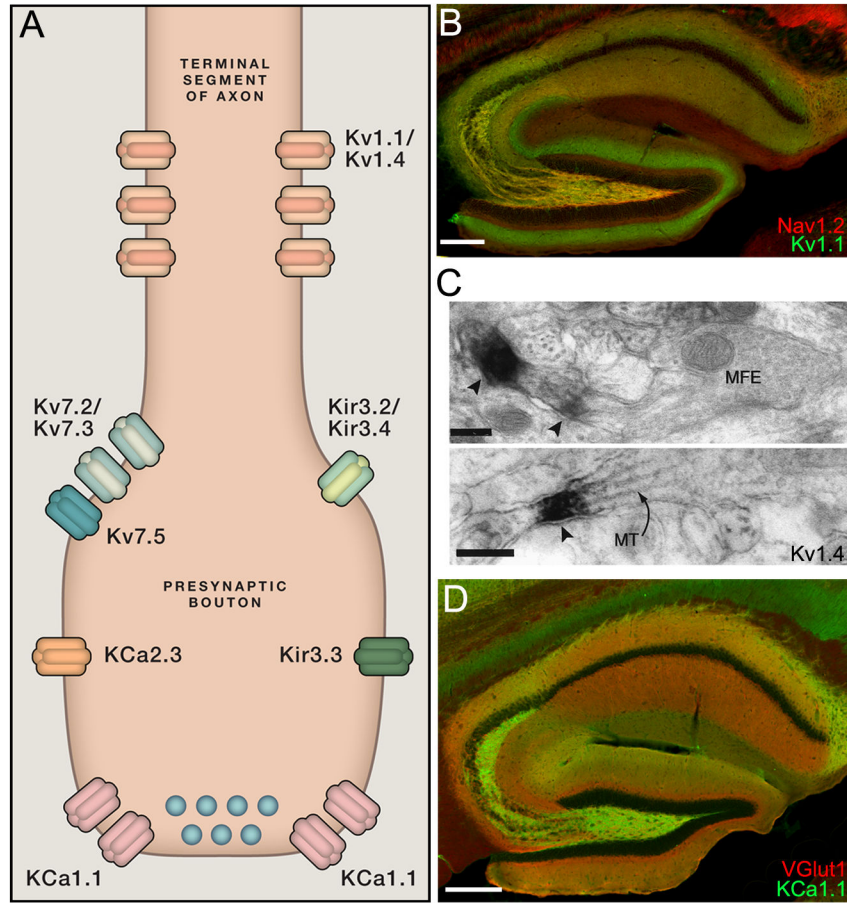


Figure 5. K⁺ channel localization in hippocampal mossy fibers

A. Cartoon showing localization of KChs in presynaptic terminals. See text for details. B. Double label LM-IF labeling for Kv1.1 (green) and Nav1.2 (red) in mouse hippocampus, showing localization in MFs. Scale bar: 200 μ m. C. EM-DAB labeling for Kv1.4 in *stratum lucidum* of CA3 in rat. From (Cooper et al., 1998). MFE = mossy fiber expansion. MT = microtubules. Scale bars: 0.2 μ m. D. Double LM-IF labeling for KCa1.1 (green) and VGlut1 (red) in mouse hippocampus, showing localization in MFs. Scale bar: 200 μ m.

Table 1

Overview of KCh Subcellular Localization in Mammalian Brain

Subunit	Gene	AKA	Predominant localization in brain
KCa1.1	KCNMA1	BK, Slo1	Axons, terminals
KCa2.1	KCNN1	SK1	Dendrites
KCa2.2	KCNN2	SK2	Dendrites/spines
KCa2.3	KCNN3	SK3	Axons, terminals
KCa3.1	KCNN4	IKCA1, SK4	Somata, dendrites
KCa4.1	KCNT1	Slack, Slo2.2	Somatodendritic
KCa4.2	KCNT2	Slick, Slo2.1	Somatodendritic
Kir2.2	KCNJ12	IRK2	Dendritic
Kir2.3	KCNJ4	IRK3	Axons/terminals
Kir3.1	KCNJ3	GIRK1	Dendrites/spines
Kir3.2	KCNJ6	GIRK2	Dendrites/spines
Kir3.3	KCNJ9	GIRK3	Axons/terminals
Kir6.2	KCNJ11	KATP	Dendrites
Kv1.1	KCNA1		AIS, axon preterminals, JPN
Kv1.2	KCNA2		AIS, axon preterminals, JPN
Kv1.3	KCNA3		CoH terminals, microglia
Kv1.4	KCNA4		Axon preterminals
Kv1.6	KCNA6		Somatodendritic
Kv2.1	KCNB1		Somata, proximal dendrites, AIS
Kv2.2	KCNB2		Somata, proximal dendrites, AIS
Kv3.1	KCNC1		Terminals, NoR
Kv3.2	KCNC2		Terminals
Kv3.3	KCNC3		Terminals
Kv3.4	KCNC4		Terminals
Kv4.2	KCND2		Dendrites/spines
Kv4.3	KCND3		Dendrites/spines
Kv7.2	KCNQ2	KCNQ2	AIS, NoR, axons
Kv7.3	KCNQ3	KCNQ3	AIS, NoR, axons
Kv7.5	KCNQ5	KCNQ5	Terminals CoH
Non-neuronal cells	K2p3.1, Kir4.1, Kir6.1, Kv1.5		
Intracellular	K2p1.1, K2p2.1, K2p4.1, K2p5.1, K2p6.1, K2p9.1, K2p10.1, K2p18.1, Kir2.4, Kir3.4, Kv4.1, Kv10.1, Kv10.1, Kv11.1, Kv11.2		
No reports	K2p7.1, K2p12.1, K2p13.1, K2p15.1, K2p16.1, K2p17.1, KCa5.1, Kir1.1, Kir4.2, Kir5.1, Kir7.1, Kv1.7, Kv1.8, Kv5.1, Kv6.1, Kv6.2, Kv6.3, Kv6.4, Kv7.1, Kv7.4, Kv8.1, Kv8.2, Kv9.1, Kv9.2, Kv9.3, Kv12.1, Kv12.2, Kv12.3		