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Subcellular control of membrane excitability in the axon

Scott A Alpizar, In Ha Cho and Michael B Hoppa

Ion channels are microscopic pore proteins in the membrane that open and close in response to chemical and electrical stimuli. This simple concept underlies rapid electrical signaling in the brain as well as several important aspects of neural plasticity. Although the soma accounts for less than 1% of many neurons by membrane area, it has been the major site of measuring ion channel function. However, the axon is one of the longest processes found in cellular biology and hosts a multitude of critical signaling functions in the brain. Not only does the axon initiate and rapidly propagate action potentials (APs) across the brain but it also forms the presynaptic terminals that convert these electrical inputs into chemical outputs. Here, we review recent advances in the physiological role of ion channels within the diverse landscape of the axon and presynaptic terminals.

Address
Dartmouth College, Department of Biological Sciences, Hanover, NH, United States

Corresponding author:
Hoppa, Michael B (Michael.B.Hoppa@dartmouth.edu)

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Introduction

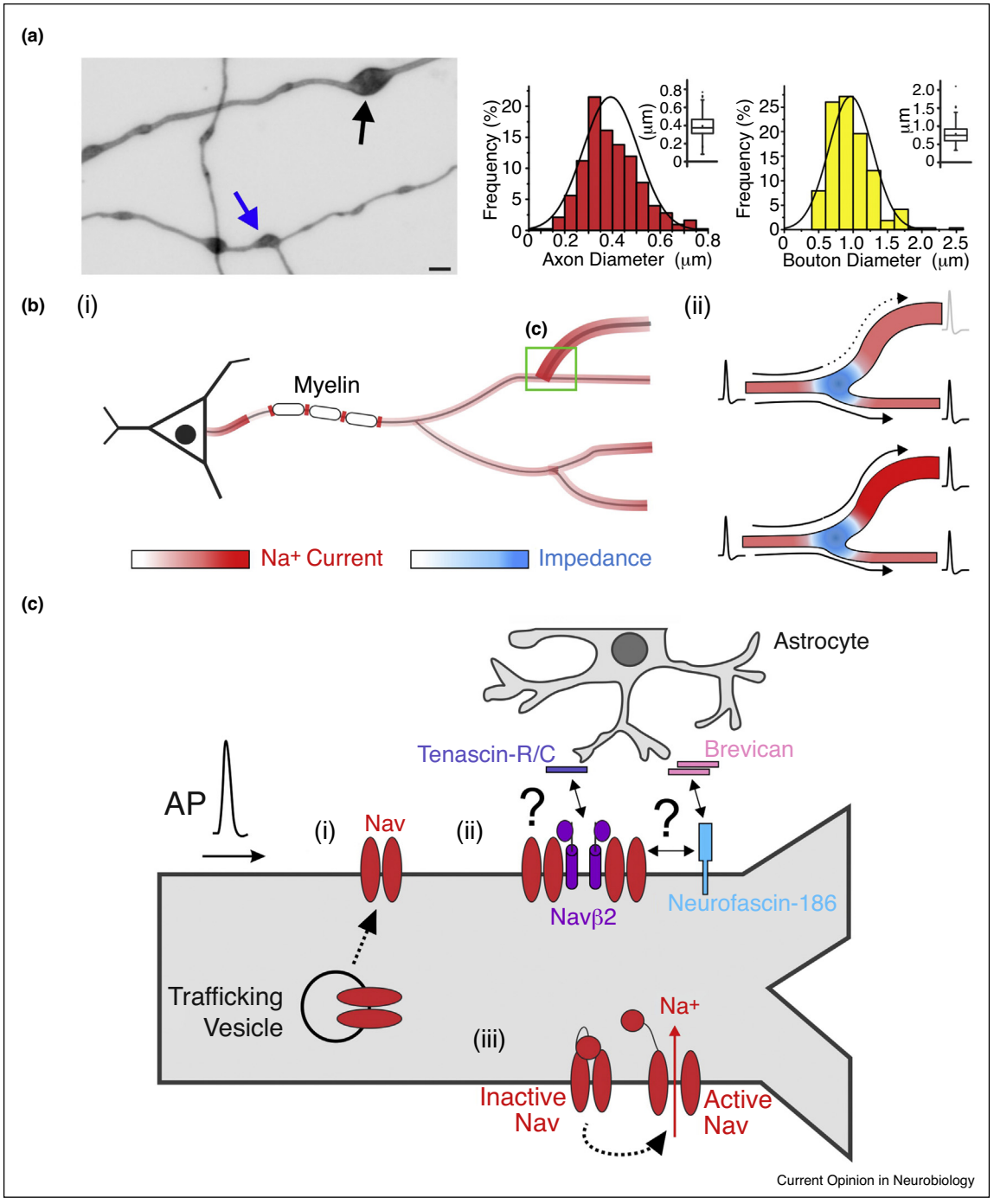
An oft used phrase to summarize Hebbian principles of neurobiology is that neurons that fire together wire together. It is, therefore, enticing to simply consider axons as biological wires that rapidly and reliably transmit electrical spikes to distal synapses. However, morphologically, axons are far from simple. A single axon of a rat hippocampal neuron has a total length of 150–300 mm, is likely to contain 100–200 branch points, and forms 30 000–60 000 synapses [1]. Axon diameter also shows considerable variability (0.4–3 μm) and contains numerous swellings from the formation of *en passant* boutons or 'beads-on-a-string' presynaptic terminals (diameters ~1 μm; Figure 1a) [1,2••]. Recent super resolution studies have even indicated that axon diameter can change acutely based on activity levels [3•]. This geometrical complexity can diminish the amplitude of propagating APs below the necessary voltage amplitude to continue propagation [1,4,5]. However, recorded propagation failures are rare and measurements with recently developed microelectrode arrays [6] demonstrate how axons routinely act to faithfully transmit APs across the arborization despite these biophysical barriers. The simplest explanation as to why axons are so robust despite their morphological complexity is that axon physiology is dynamic, and regions of the axon membrane contain non-uniform conductance properties across subcellular compartments. Furthermore, this non-uniform conductance leads to variability in the AP, even between synaptic terminals of the same axon [7]. Unlike an electrical wire where current is uniformly carried by electrons, axons carry current along their membrane using the flow of ions through precisely gated Na^+ and K^+ channels. The gating of these channels is modulated by voltage, ligands such as Ca^{2+}, and subunits or binding partners specific to subcellular location [8,9]. We will discuss how various combinations of channels influence AP propagation and local membrane excitability across the axon and highlight research demonstrating how they also modulate synaptic transmission.

Subcellular excitability in the axon

Functional compartmentalization of ion channels

Non-uniform distribution of ion channels in the axon was first observed at nodes of Ranvier, but a series of recent experiments have revealed that unmyelinated sections of axon that carry *en passant* synapses also contain heterogeneous distributions of ion channel activity. Subcellular patch clamp recordings throughout the axon of inhibitory basket cells revealed a heterogeneity in voltage-gated Na^+ channel (Nav) densities, particularly in distal processes rich with presynaptic boutons and branch points [10] (Figure 1b). However, patch clamp recording of Nav densities does not account for the distribution of active channels at a given moment. Most Nav are inactivated within axons, but acute hyperpolarization is known to relieve inactivation and amplify the overshoot of subsequent APs [11]. According to the long-standing view of signal propagation in myelinated axons, ion channels are clustered for faithful signal propagation, such as Nav clustering at nodes of Ranvier and voltage-gated potassium channels (Kv) at juxtaparanodal zones [12]. Additionally, Kv7 at nodes of Ranvier in neocortical pyramidal cells [13] and potassium intermediate/small conductance calcium-activated channels (Kca3.1) in cerebellar Purkinkje neurons [8] drive hyperpolarizing currents that

Figure 1



Heterogeneity of axon morphology and ion channel distribution.
(a) Left; AiryScan imaging of rat hippocampal axon expressing QuasAr to illuminate the plasmalemma. AiryScan imaging reveals the variability in the axon diameter and extreme morphology that occurs at brach points (blue arrow) and en passant synaptic terminals (black arrow). Quantification of axon diameters (red; middle panel) and terminal diameters (yellow; right panel) from several hippocampal neurons using a fluorescent tag of synaptic vesicles to identify presynaptic terminals (not shown). Images adapted from Ref. [2••].
(b) (i) Diagram of neuron illustrating both myelinated and unmyelinated axons with branch points and herogeneous daughter branch diameters throughout the arborization, summarizing heterogeneities reported in effective sodium currents found at the AIS, nodes of Ranvier, branch points and distal branches of the axon (red). (ii) Illustration of axonal branch points that contain daughter branches with larger diameters that impose impendance mismatches (blue) [83] that can slow and or halt propagation that assume equal sodium channel activity (top) or heterogeneous sodium channel activity (bottom).
(c) Possible mechanisms to regulate heterogeneous sodium currents in umyelinated branches of an axon as illustrated by green box in Figure 1b.

increase excitability by relieving inactivation of Nav, ensuring signal propagation (Figure 1d). Thus, factors that control both the density and activity of ion channels can dramatically change the excitability within the axonal arborization (Summary in Figure 1c).

Heterogeneous excitability in the axonal arborization

"Evidence is building in support of the AP itself not being a uniform signal, but a plastic waveform that encodes important information for modulating excitability [2••,10,14,15••,16••]. Subcellular recordings throughout the arborization of Purkinje cells showed significant Nav activity differences between presynaptic terminals and regions of adjacent axon [15••]. Additionally, optical measurements of the AP within the axons of cerebellar stellate cells found large heterogeneities in Kv3 activity corresponding to AP width and GABA release, especially between synapses on different axonal branches [16••]. This striking difference between axonal branches was also observed in excitatory hippocampal neurons and correlated with Navβ2 expression independent of Nav function [2••]. Physiological relevance of AP heterogeneity was exemplified *in vivo* with optical recordings of voltage across single axonal arbors in the Drosophila visual system. Differences in the waveform were dependent on the cell type layer that the axon innervated and were strongly correlated with differences in presynaptic Ca^{2+}, indicative of an important role in sensory physiology [17••]. Thus, axonal branch point modulation occurs in physiological contexts.

Glial regulation of axonal excitability

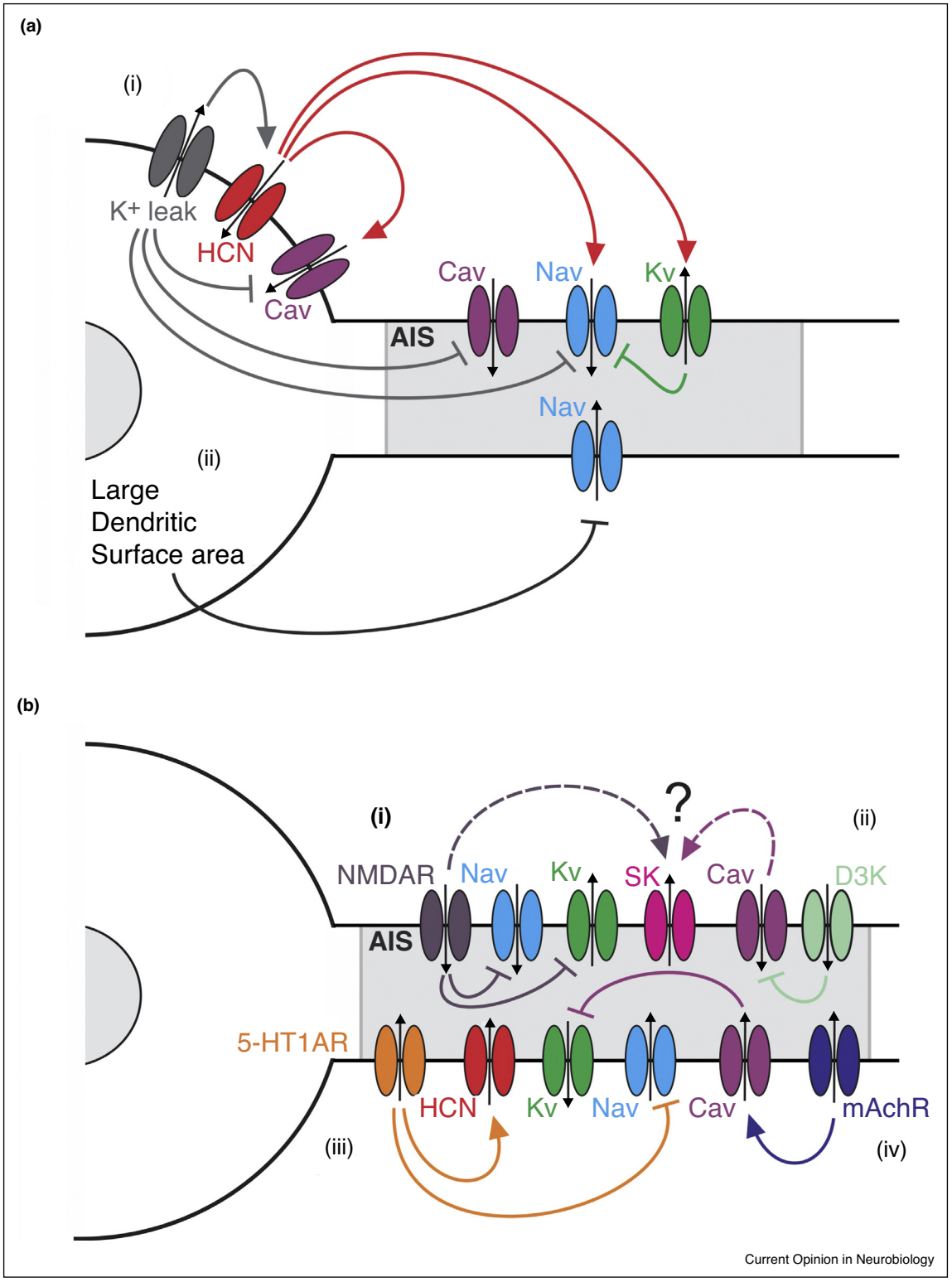
The interplay of neurons and non-neural cells called glia is critical for signal propagation and neurotransmission. Axon-glia interactions were mainly considered restricted to myelin-producing oligodendrocytes, shielding axons and enabling faster conduction velocity [18]. For example, in experimental demyelination models, myelin deficits broadened the AP and initiated propagation failure during high frequency stimulation [19]. Extrinsic signals from astrocytes may also control the AP and influence synaptic strength in unmyelinated axons. Glutamatergic neurons grown without astrocytes showed that EPSCs were distorted by an unexpected change in AP arrival and shape [20]. Additionally, astrocytes have been shown to broaden APs with AMPA receptor activation, facilitating synaptic transmission in CA3 pyramidal neurons [21,22]. Therefore, non-neuronal cells can modulate the propagating waveform locally in myelinated and unmyelinated axons.

Somato-dendritic regulation AIS excitability

While the axon propagates APs, these electrical signals are first generated at the axon initial segment (AIS), a highly specialized cellular compartment connecting the soma and axon that is enriched with voltage-gated ion channels. Among these are Nav, which localize to the AIS based on subtype: Nav1.1/1.2 proximally and Nav1.6 distally [23]. Several Kv subtypes are also enriched within the AIS. K_v7.2/7.3 and K_v1 are distally localized [13,24–27], while K_v2.1 localize along the entire AIS, though the phosphorylation state of these channels differs between the proximal and distal regions [28,29]. Several recent studies have highlighted mechanisms regulating enrichment of Nav and Kv at the AIS through interactions with ankyrin G modulated by intracellular proteins such as fibroblast growth factors [30,31] and membrane proteins such as neurofascin-186 [32]. However, a recently identified factor controlling cellular excitability is the effective ratio of active and inactive Nav and Kv, which is in part regulated by the resting membrane potential (RMP) and can often involve complex combinations of ion channel conductances. Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) localized in the soma of cortical neurons alter the RMP in the AIS and axon relative to somatodendritic regions [33••]. This altered RMP results from a basally enhanced K^+ current in the axon and HCN current in the soma, producing a more negative (∼−3 mV) RMP in the AIS. The strength of this localized somatic HCN current on excitability was demonstrated by blocking HCN, which produced a depolarization in the AIS similar to blocking Nav, Kv7, and voltage-gated calcium channels (Cav), specifically Cav3, combined. This indicates that somatic HCN drive the RMP and excitability of cortical neurons (Figure 2a). The contribution of currents at the soma is likely dependent on cell types; however, somatic morphology can also directly influence the excitability of Nav depending on their location within the axon. A distal AIS location leads to increased input resistance and further isolation from the capacitive load of the somatodendritic compartment (Figure 2a) [34], which itself has recently been shown to influence excitability at the AIS through its morphology [35,36•]. A major caveat of many studies regarding the excitability of the AIS is that they heavily rely on computational modeling. While these studies consider neuron morphology, electrical properties, and contributions of ion currents, detailed experimental studies of molecular mechanisms controlling RMP throughout the axon with complex morphology would greatly increase our understanding of excitability and plasticity, but less invasive

(Figure 1 Legend Continued) This heterogeneity can be caused by directly trafficking more sodium channels to specific regions of the plasmalemma (i). Enrichment of sodium channels can also be caused by extracellular cues from surrounding tissues through interactions with Nav subunits or membrane binding partners that recognize extracellular molecular signals such as tenascin or brevican (ii). A third possibility is that changes in local membrane pontential or binding partners alter the local percentage of inactivated sodium channels throughout the axon through interactions with binding partners or acute signals that alter local membrane potential (iii).

Figure 2



Regulation of excitability at the axon initial segment.
(a) Diagram exploring the factors influencing AIS excitability from the somatodendritic compartments. (i) The resting membrane potential of the AIS is set by a variety of voltage-gated ion channels localized within the soma. Potassium leak channels (K^+ leak, gray) will act to inhibit the conductance of sodium (Nav, cyan) and calcium (Cav, purple) channels, while increasing the conductance of HCN channels (HCN, red). The depolarizing HCN current from somatic channels is critical for setting the resting membrane potential at the AIS (∼−3 mV lower than in the soma)

methods to measure membrane potential are necessary to achieve this aim.

Neurotransmitter regulation of AIS excitability

Neurotransmitter receptors can also alter AIS excitability, typically through the modulation of other ion channel activity within the AIS. Serotonin, through 5-HT1A receptors, inhibits Na^+ current at the AIS in cortical pyramidal neurons [37], 5-HT1A receptors additionally modulate HCN channels at the AIS of the medial superior olive by hyperpolarizing their activation range and lowering spike threshold [38]. Furthermore, activating post-synaptic muscarinic acetylcholine receptors enhanced the activity of Cav3.2 in hippocampal granule cells, resulting in Kv7 inhibition and decreased spike threshold [39]. Ca^{2+} entry through AIS NMDA receptors during increased activity results in Nav and Kv7 endocytosis [40] and along with other Ca^{2+} sources may also activate SK channels recently observed at the AIS of hippocampal neurons [41]. Finally, dopamine can inhibit Cav3 at the AIS of cartwheel cells, lowering excitability and suppressing burst output [42] (Summary in Figure 2b).

Presynaptic terminals and calcium

While initiation and delivery of APs are critical to discussing excitability, the translation of these electrical signals into chemical outputs at presynaptic terminals is of equal importance. A discussion of presynaptic function must address Ca^{2+}, the essential linchpin that allows an AP to catalyze vesicle fusion [43,44]. Not only is it essential that Ca^{2+} occupy binding sites on calcium sensors to initiate vesicle fusion, but the relationship between Ca^{2+} entry and exocytosis magnitude is highly non-linear and best explained by a 3rd–5th order power law [45–48]. Therefore, the probability that an AP will elicit vesicle fusion (Pv), is highly dependent on the intracellular calcium concentration [49,50]. Several mechanisms that control the density of Cav within the active zone and the coupling of these channels to vesicle release sites have proven important to understanding the molecular regulators of synaptic strength [51–56]. It has been postulated that differences in Cav density can explain the variability in Pv and Ca^{2+} influx between presynaptic terminals of a single axon [57]. However, combined approaches of 2-photon Ca^{2+} imaging and detailed 3-D electron microscopy in hippocampal neurons found a disconnect between synapses with higher Pv and Ca^{2+} entry/channel arrangement within the active zone [58••]. These findings suggest that Cav enrichment is not solely responsible for regulating presynaptic strength.

Presynaptic K+ channels modulate neurotransmission

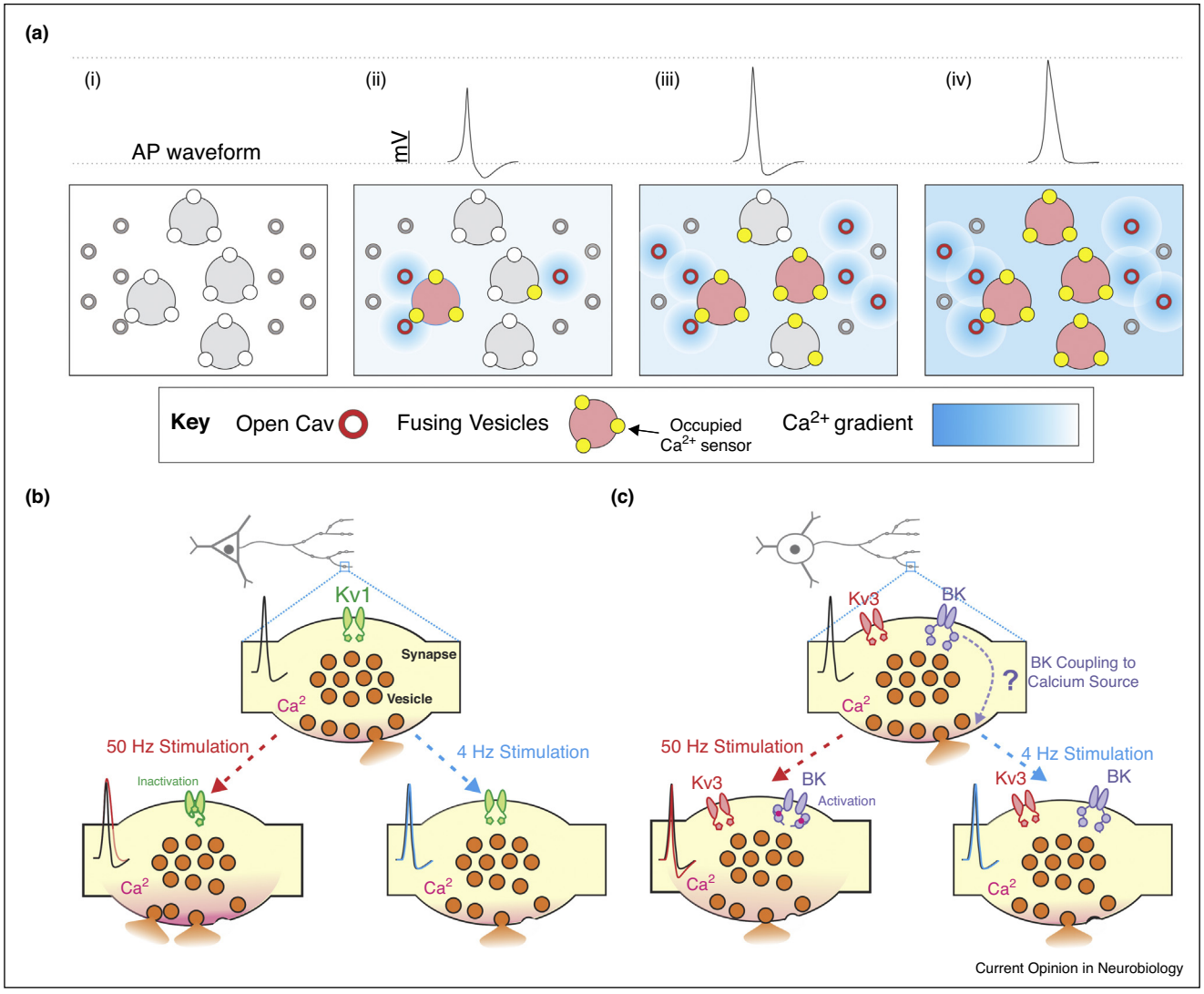
While many possibilities exist to modulate the ability of Cav to initiate vesicle fusion other than coupling channels to release sites, an often-overlooked regulator of Ca^{2+} entry the presynaptic membrane potential. The primary sites of Ca^{2+} entry in a presynaptic terminal are the Cav [59]. Membrane potential not only regulates Cav open probability, but also dictates the driving force for Ca^{2+} entry [60]. Thus, the AP encodes information that controls both the percentage of Cav that will open within a terminal and the temporal details of Ca^{2+} entry, resulting in microdomains that form around the pore of the channel [61] (Figure 3a). Suggesting the AP is a critical modulator of presynaptic Ca^{2+}, and vesicle fusion is not novel. The invertebrate presynaptic terminal has several examples showing that changes in the AP impart large changes on synaptic transmission. In the case of Aplysia, serotonin (5-HT) blockade of a presynaptic K^+ current leads to AP broadening and increased neurotransmitter release [62]. Similarly, AP broadening has been observed in nerve terminals of vertebrate peptide-secreting cells in the neurohypophysis [63,64]. The faster subsequent APs arrive at the terminals, the fewer Kv there are to repolarize the AP, causing a broadening of the AP and increased Ca^{2+} influx.

What about classic GABA and glutamate secreting nerve terminals? This question been difficult to approach due to the submicron size of *en passant* terminals that preclude the use of whole-cell patch clamp for single terminal measurements [65]. A variety of voltage-sensitive and Ca^{2+}-sensitive channels exist with subcellular localization, but functional roles within nerve terminals are still underexplored [66,67]. Certain neurons in the CNS, such as the calyx of Held and mossy fiber neurons of the

(Figure 2 Legend Continued) and for the activation of voltage-dependent conductances within the AIS. Adapted from Ref. [33••] (ii) The surface area of the somatodendritic region also influences AIS excitability, where a larger somatodendritic area creates a larger capacitive load, effectively hyperpolarizing the proximal axonal membrane.

(b) Diagram exploring local neurotransmitter regulation of AIS excitability. (i) NMDA receptors (NMDAR, dark purple) decrease the conductance of sodium (Nav, cyan) and potassium (Kv, green) channels at the AIS by decreasing their surface expression. The calcium entering the AIS from NMDARs and potentially from calcium channels (Cav, purple) may also activate small conductance calcium-activated K^+ channels (SK, orange) depending on their coupling to calcium sources, leading to a hyperpolarization of the AIS, though this has yet to be experimentally confirmed (dashed lines). (ii) Dopamine D3 receptors (D3R, light green) decrease excitability by hyperpolarizing the activation range of Cav at the AIS. (iii) Serotonin receptors inhibit Nav conductance at the AIS by producing a depolarizing shift in their activation curve and facilitating slow inactivation of Na^+ currents, decreasing excitability. Additionally, serotonin hyperpolarizes the activation range of HCN channels (HCN, red) within the AIS, increasing their conductance and thereby reducing excitability. (iv) Muscarinic acetylcholine receptors increase Cav activity as rest, resulting in an elevated intracellular calcium concentration within the AIS. This elevated calcium leads to a subsequent suppression of the M current through Kv, increasing excitability at the AIS.

Figure 3



Presynaptic AP modulation of synaptic transmission and its local regulation by Kv isoforms.
(a) Diagram exploring activation of Cav channels (closed grey) and vesicle fusion in a top down view of the presynaptic active zone from baseline (i) and by three different AP waveforms: (ii) normal, (iii) higher overshoot, and (iv) higher overshoot and broadened width. Impacts of calcium within microdomains around open channels (red) and diffuse calcium are shown in blue. An arbitrary number of calcium sensors promoting vesicle fusion when fully occupied are shown in yellow. Small changes in amplitude will alter the probability of Cav opening such as compared in (ii) and (iii), moving from 30% to 50% of Cavs activated. Changes in width of the AP can have more complex changes in calcium microdomains but generally result in more diffuse build-up within the cytoplasm near the active zone (brighter blue seen in subpanel iv).
(b) Top; Illustration of a single AP evoking calcium entry and vesicle fusion where the falling or hyperpolarizing phase of the waveform is controlled by Kv1 channels. Bottom; If a subsequent AP arrives in 20 ms (50 Hz) versus 250 ms (4 Hz) a larger percentage of Kv1 channels would still be inactivated at 50 Hz stimulating frequency, thus broadening the AP and causing a facilitation of vesicle fusion.
(c) Top; Illustration of a single AP evoking calcium entry and vesicle fusion where the falling or hyperpolarizing phase of the waveform is controlled by Kv3 and/or BK channels. Bottom; If a subsequent AP arrives in 20 ms (50 Hz) versus 250 ms (4 Hz) a larger percentage of BK channels would activate at 50 Hz stimulating frequency, thus narrowing the AP and restricting calcium build-up and reducing vesicle facilitation. Factors that localize the BK channel to the calcium source will strongly influence AP narrowing.

dentate gyrus, offer large nerve terminals (>3 μm) that are amenable to patch clamp. These terminals have widely variable presynaptic APs. At the calyx, the AP is remarkably stereotyped where amplitude and width are consistent in paired-pulse recordings and even at stimulation frequencies above 200 Hz [14]. This appears partially due to an enrichment of Nav1.6 and Kv3 in the presynaptic terminal [14,68]. However, in mossy fiber boutons, there is significant AP broadening associated with stimulation frequency. This broadening is due to

presynaptic terminals dominated by Kv1 that are prone to inactivation [69]. Thus, understanding the presynaptic localization of Kv isoforms across cell types may be informative to understanding synaptic strength and plasticity.

The recent advent of voltage sensitive dyes (VSDs) and genetically encoded voltage indicators (GEVIs) has enabled measurements even in *en passant* terminals and axons [70]. A series of optical recordings in cerebellar stellate interneurons identified heteromeric Kv3.1/3.4 in regulating the presynaptic AP on a synapse by synapse basis [16••,71,72•]. These studies found that the Kv3 heteromers did not inactivate during high frequency stimulation but were highly sensitive to inactivation from subthreshold depolarizations [72•]. Interestingly, optical voltage recordings in presynaptic terminals of excitatory cortical [73] and hippocampal neurons [74] have shown larger contributions of Kv1, which, in agreement with previous work [75], dominate the repolarization of APs. This selective enrichment of Kv1 in several excitatory *en passant* terminals may leave them vulnerable to frequency-dependent AP broadening, which may be relevant to understanding synaptic facilitation. Convincing evidence has demonstrated that synaptotagmin 7 (syt7) is essential for synaptic facilitation in many neuron types [76•]. In syt7 null mice, all types failed to exhibit facilitation except mossy fiber cells, which showed a partial retention attributed to AP broadening [76•]. However, the possibility remains that AP broadening might help elicit the type of Ca^{2+} influx that preferentially engages syt7 in some cell types and play an important role in synaptic facilitation. Indeed, paired pulse facilitation was blocked in basket cells that carry a Kv1 mutation that prevents inactivation and AP broadening in synaptic terminals specifically enriched with this Kv isoform. ([77,78•]). The combinations of Kv present in synaptic terminals are still underexplored and are undoubtedly controlled by different families of Kv. In neocortical somatostatin expressing inhibitory neuron terminals, a combination of Kv1 and BK channels have been identified with Kv1 ([79]). The expression of either Kv3 or BK channels would likely make fast firing inhibitory neurons less prone to broadening and perhaps more susceptible to amplitude depression and even narrowing. Taken together, recent findings of various complements of Kv in presynaptic terminals creates two possible scenarios for local modulation of the AP during high frequency firing in terminals (Figure 3c). However, further investigations into Kv binding partners within specific cell types and subcellular regions will be critical to understanding the kinetics of activation and inactivation beyond typical stereotypes.

Final thoughts on local control of membrane voltage at synaptic terminals

Many questions remain concerning the membrane potential at presynaptic terminals. Primary questions involve the scaffolds that modulate Kv enrichment and gating in presynaptic terminals. A recent screen in yeast combined with synaptic physiology and genetic knockout revealed a novel mechanism to couple BK channels to the active zone via RIM-BPs [80•]. This could significantly alter the presynaptic AP during high frequency stimulation if localized proximal to Cavs within the synapse (Figure 3c). Similar to methods discussed to elucidate subcellular excitability within the axon, the use of VSDs and GEVIs will prove critical in clarifying the mechanisms controlling voltage in presynaptic terminals. Finally, Kv function is dependent upon location and context. In one role, Kv dampen excitability by hyperpolarizing the membrane while in another they increase excitability by preventing Nav inactivation. An exciting advance is the improvement of K^+ indicators [81], as well as single neuron labeling of endogenous ion channels using CRISPR [82]. These advances can ignite further research into the role of these channels and others in controlling axon physiology and synaptic transmission.

Conflict of interest statement

Nothing declared.

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