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Searching for new targets for treatment of pediatric epilepsy

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Introduction

Gregory L. Holmes, MD

Abstract

The highest incidence of seizures in humans occurs during the first year of life. The high susceptibility to seizures in neonates and infants is paralleled by animal studies showing a high propensity to seizures during early life. The immature brain is highly susceptible to seizures because of an imbalance of excitation to inhibition. Factors such as depolarizing GABA due to high intracellular concentrations of chloride, delayed GABA_B development, high input resistances of neurons and an over-exuberance of excitatory synapses, result in a highly excitable network prone to seizures. While the primary outcome determinant of early-life seizures is etiology, there is evidence that seizures which are frequent or prolonged can result in long-term adverse consequences and there is a consensus that recurrent early-life seizures should be treated. Unfortunately, despite the introduction of many new antiepileptic drugs, many children with early-life seizures remain refractory to therapy. There is a pressing need for new seizure drugs as well as antiepileptic targets in children. This section highlights the work of two young investigators who are studying mechanisms of seizure, including early-life seizures and who have innovative ideas regarding novel molecular targets.

Children during the first months of life are at particularly high risk for seizures with the largest number of new-onset seizure disorders occurring during this time [1, 2]. There is considerable evidence that the immature brain is more susceptible to seizures than the

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mature brain. For example, children under the age of six years are at risk for febrile seizures whereas older children and adults rarely have seizures caused by fever alone. This increased propensity for seizures in humans has also been demonstrated in a wide variety of experimental models, including kainic acid [3, 4], electrical stimulation [5], hypoxia [6], penicillin [7], picrotoxin [8], GABA_B receptor antagonists [9], hyperthermia [10, 11] and increased extracellular potassium [12, 13].

Scientists have identified a number of reasons why the immature brain is highly susceptible to seizures [13, 14]. During the early postnatal period γ -aminobutyric acid (GABA), which in the adult brain is the primary inhibitory neurotransmitter, exerts a paradoxical excitatory action [12, 13]. GABA is initially excitatory because of a larger intracellular concentration of Cl^- in immature neurons than mature ones resulting in E_{GABA} that is depolarized [15-17]. The E_{GABA} shift from depolarizing to hyperpolarizing occurs over an extended period depending on the age and developmental stage of the structure [18]. The shift is mediated by an active $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC1) that facilitates the accumulation of chloride in neurons and a delayed expression of a K^+/Cl^- co-transporter (KCC2) that extrudes Cl^- to establish adult concentrations of intracellular chloride [19]. The depolarization by GABA of immature neurons is sufficient to generate Na^+ action potentials and to remove the voltage dependent Mg^{2+} blockade of NMDA channels and activate voltage-dependent Ca^{2+} channels, leading to a large influx of Ca^{2+} that in turn triggers long term changes of synaptic efficacy. The synergistic action of GABA with NMDA and calcium channels is unique to the developing brain and has many consequences on the impact of GABAergic synapses on the network. In addition, agents that interfere with the transport of Cl^- into the cell exert an anti-epileptogenic action [19]. With maturation there is increasing function of KCC2 and decreasing function of NKCC1, resulting in lower levels of intracellular Cl^- . The lack of an efficient time-locked GABA_A inhibition and the delayed maturation of postsynaptic GABA_B mediated currents place the immature brain in a vulnerable position. One of the few modes of inhibition in the young brain is K^+ channels. Outflow of K^+ ions serve to hyperpolarize the membrane and limit action potentials.

In addition to lack of GABA inhibition, during the first few weeks of life there is enhanced excitation due to an overabundance of N-methyl-D-Aspartate (NMDA) and α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors [21, 22]. High input resistance of immature neurons also facilitate the generation of action potentials and synchronized activities [9, 18, 20].

Unfortunately, the treatment of seizures in young children is suboptimal. In the case of neonatal seizures, little has changed over the past six decades with most physicians using phenobarbital or phenytoin as first line therapies [21, 22] despite well-conducted studies showing both drugs are incompletely effective [23]. In addition to poor efficacy, animal data indicates that both drugs can lead to apoptosis when administered to developing animals [24]. In addition, phenobarbital following prolonged seizures in immature rats impairs spatial learning when the animals are evaluated as adults [25].

In the case of infants and toddlers effective therapies also remain limited. Twelve second-generation antiepileptic drugs have been approved in the US for use in epilepsy over the past 15 years: felbamate, gabapentin, lamotrigine, topiramate, tiagabine, levetiracetam, oxcarbazepine, zonisamide, pregabalin, clobazam, vigabatrin and ezogabine. Their use in children is fairly widespread, despite most of these agents not having US FDA indications for use. Clobazam is approved for children over two years and rufinamide for children over age four years. Only vigabatrin is approved for younger children and here the indication is restricted to infantile spasms. Yet medically intractable epilepsy is common in children and the long-term consequences of recurrent seizures in the developing brain are tragic [26, 27].

Young children have been terribly under-studied in regards to antiepileptic drug efficacy, tolerability and safety. Clearly there is a great need for new molecular targets for treatment of early-life seizures. In this section, two young investigators Yoav Noam and Yogendra Raol provide compelling information to indicate that two novel molecular targets may be important targets of antiepileptic drugs.

Dr. Noam discusses hyperpolarization-activated, cyclic nucleotide-gated channels (HCN), a class of molecules that are open at sub-threshold potentials and therefore are ideally suited for the fine-tuning of intrinsic excitability. As he notes, dysregulation of HCN channels in epilepsy occurs at multiple levels and at different time-scales [28]. Transcriptional regulation of HCN channel protein has been observed in early-life seizure models resulting in altered I_h amplitude and gating properties, and modified neuronal excitability [29, 30]. Understanding the molecular and cellular mechanisms that underlie HCN channel dysregulation in epilepsy could contribute significantly to our understanding of early-life seizures and may provide an important target for rational design of therapeutic strategies [31]. Thus targeting these channels may open an entirely new avenue of treatment.

Dr. Raol discusses potassium channels as a therapeutic target in neonatal seizures. There is increasing evidence that K^+ channels are important in the generation of early-life seizures. Newborns with benign familial neonatal convulsions (BFNC) typically begin having seizures between days 2-8 of life and remit by 16 months [32, 33], with very little residual cognitive impairment. This syndrome is due to mutations in *KCNQ2* and *KCNQ3* which encode the voltage-gated K^+ channels Kv7.2 and Kv7.3. Both *KCNQ2* and *KCNQ3* are expressed in the brain where the gene products form heteromultimeric channels that mediate the M-current, a slowly activating, non-inactivating potassium current that serves to inhibit neuronal firing. Mouse models for human *KCNQ2* and *KCNQ3* mutations demonstrate early-life seizures and reduced amplitude and increased deactivation kinetics of the M current. Recently Weckhuysen et al, [34] showed that a K^+ channel mutation may not be so quite benign. The authors studied a cohort of 80 patients with unexplained neonatal or early infantile epileptic encephalopathies for mutations in *KCNQ2* and *KCNQ3*. *KCNQ2* mutations occurred in 10% of patients. All of the patients had onset of seizures within the first three months of life followed by slowing of psychomotor development. In view of the important role of inhibition of K^+ channels in inhibition in the developing brain, targeting this channel in early-life seizures may be an attractive therapeutic option.

Potassium channel modulators for treatment of neonatal seizures

Yogendra H. Raol, PhD

Abstract

The neonatal brain is different from mature brain anatomically and neurochemically, which can affect how the immature brain responds to both injury and treatment as compared to mature brain. Therefore, to find the most efficacious treatment for early childhood diseases, it is imperative to target age-specific mechanisms and test new therapies in neonatal disease models. Hypoxia-ischemia is a common cause of neonatal seizures and brain injury. Survivors of hypoxia-ischemia often experience neurological problems such as epilepsy and intellectual disability in later life. Studies suggest that seizures contribute to brain injury and effect long-term neurological outcomes. First-line drugs such as phenobarbital, which act by augmenting GABA_A receptor activity, are not fully effective in treating neonatal seizures and are associated with side effects. Potassium channels play a uniquely important role in controlling brain excitability in early life. Our recent study showed that unlike diazepam and phenobarbital, flupirtine, a potassium channel opener, effectively treated chemoconvulsant-induced neonatal seizures. Currently my laboratory is examining the efficacy of flupirtine to

treat hypoxia-ischemia induced neonatal seizures in an animal model. Our future studies will determine if treatment of early-life seizures can alter long-term neurological outcomes.

Although seizures can occur at any age, the risk is high in the neonatal period. In the United States, the occurrence of neonatal seizures is estimated at 1.8 to 3.5 per 1000 live births [35, 36]. This increased risk can be attributed to a relative increase in excitability of developing brain due to age specific mechanisms such as overexpression of glutamate receptors and delayed development of GABA inhibition [37, 38]. There is a broad range of insults that can cause seizures in neonates, but neonatal seizures are most commonly associated with perinatal hypoxic-ischemic encephalopathy [39]. Studies of human neonates and animal models suggest that seizures themselves may independently contribute to brain injury and poor neurological outcome [40-42]. Neonatal seizures can cause excessive fluctuations in brain oxygenation and blood perfusion which may amplify hypoxic-ischemic cerebral injury [9]. Bjorkman and colleagues [43] observed that newborn piglets subjected to hypoxia and had seizures had greater brain injury compared to piglets without seizures. In rats, a single episode of neonatal seizures causes impairment in working memory in adulthood [44]. A recent clinical study found a strong correlation between hypoxia-ischemia induced seizure duration and severity of brain injury [45]. Legido and colleagues [46] found that in patients with hypoxic-ischemic encephalopathy, 100% of patients with a seizure frequency of greater than five/hour had developmental delay and 86% of patients developed epilepsy. In a study of infants with hypoxic-ischemic encephalopathy, it was found that independent of the severity of brain injury, neonatal seizures had worse motor and cognitive outcomes than those without seizures [47]. However, distinguishing between the adverse effects of the etiology of the seizures versus the etiology of the seizures is challenging. For example a recent study of the effects of early-life seizures on spatial cognition using a model of cortical dysplasia showed that the major factor responsible for the cognitive impairment in the rats was the underlying brain substrate, not seizures [48]. In a study using data of infants with hypoxic-ischemic encephalopathy, neonatal seizures were not associated with death, or moderate or severe disability, or lower Bayley Mental Development Index scores at 18 months of life [49]. Despite these controversies most clinicians treat neonatal seizures. However, there are currently no effective pharmacological intervention strategies that consistently stop neonatal seizures and alter long-term neurological outcome.

Lack of good treatment options

Throughout the world, phenobarbital is the most commonly used drugs to treat neonatal seizures. Recent studies found that more than 70% of physicians worldwide used phenobarbital as the first treatment option to stop neonatal seizures [50, 51]. However, in a study of 59 infants with neonatal seizures confirmed by EEG randomized to either phenobarbital or phenytoin seizures were controlled in only 13 of the 30 neonates assigned to phenobarbital (43%) [23]. This low efficacy of phenobarbital in neonatal seizures has been shown by other authors (reviewed by Foster and Lewis [52]). All of the currently available antiepileptic drugs, including phenobarbital, were developed using adult animal models and tested clinically in adult patients. There are many differences between the developing and mature brain, therefore, the immature brain may respond very differently than the adult brain to both injury and treatment. For example, the immature brain reported to have a depolarizing GABAergic system [16], fewer GABA_A receptors [53], lower GABA-mediated currents [54] and is less sensitive to benzodiazepine augmentation [54, 55]. However, the concept of a depolarizing effects of GABA in early-life has been challenged based on recent *in vitro* studies and earlier *in vivo* observations suggesting inhibitory activity of GABA in early-life [56, 57](for reviews see [58-60]).

These properties of GABAergic neurotransmission may explain the lower efficacy of phenobarbital and diazepam (which act by augmenting GABA_A receptor activity) in treating

neonatal seizures. Moreover, clinical and animal research studies show that treatment of the developing brain with antiepileptic drugs can have adverse effects on the brain development. Farwell and colleagues [61] observed that children with febrile seizures that were treated with phenobarbital had lower IQs than the placebo treated children, although the study has serious limitations. Treatment of normal neonatal rats with antiepileptic drugs, such as phenobarbital and diazepam, can increase apoptosis in the brain [62]. A recent study demonstrated that a single dose of phenobarbital given to normal neonatal rats is sufficient to induce schizophrenia like behavior in adulthood [62]. We observed that diazepam and phenobarbital treatment given to the rats during development causes long-term alterations in the expression of GABA receptor subunits, GABA transporters, and GABA synthesis enzymes in the hippocampus [63]. This suggests that exposure of the developing brain to the drugs that modulate GABAergic neurotransmission may cause permanent alterations in the GABA machinery. But it is important to note that all early-life antiepileptic drug treatment studies mentioned above were conducted on normal rats. It will be interesting to find out if these drugs cause similar side-effects in neonatal rats with seizures or brain-injury. The suboptimal efficacy of currently available drugs to treat neonatal seizures, combined with the possibility of adverse effects, suggest a clear and urgent need for development of safe and effective antiepileptic drugs. This can be achieved by designing drugs targeted against an age-specific mechanism. For the reasons described below, my current research work focuses on studying the efficacy of flupirtine, a potassium channel modulator, to treat neonatal seizures.

Potassium channel modulators for the treatment of neonatal seizures

Potassium channels play a critical inhibitory role, especially in the developing brain because of reduced levels of GABAergic inhibition. Because KCNQ (KV7) gene expression begins before birth [64, 65], KCNQ channels are available to dampen the excitatory activity in the brain when GABA-mediated inhibition is weak. KCNQ channels (KCNQ1-5) are voltage-gated, depolarization activated potassium channels and are expressed in the nervous system [66]. KCNQ2-5 subunits form potassium channels that underlie the M-current that regulates brain excitability [66, 67]. Mutations in genes encoding two KCNQ channel subunits (*KCNQ2* and *KCNQ3*) cause benign familial neonatal convulsions (BFNC), an idiopathic epilepsy syndrome [32]. One of the important characteristics of the BFNC is that the seizures begin in the first week of life and spontaneously remit after a few weeks or months. This suggests that potassium channels play a particularly important role in controlling hyperexcitability during the neonatal period and early infancy. This idea is further supported by basic science research. Peters and colleagues [68] observed that blockade of KCNQ2/3 channel activity throughout development in mice resulted in abnormal hippocampal morphology, epilepsy and profound behavioral hyperactivity. In contrast, if the blockade was limited to adulthood, none of these abnormalities occurred. These observations suggest that a potassium channel opener can be an effective way to enhance inhibition and treat neonatal seizures. To test this hypothesis, we studied the efficacy of flupirtine to treat neonatal seizures in animal models.

Flupirtine (ethyl-N-[2-amino-6-(4-fluorophenylmethyl-amino) pyridin-3-yl]carbamate) is a derivative of triaminopyridines and has been in clinical use in Europe since the 1980s as a non-opioid analgesic. Flupirtine causes a hyperpolarizing shift in the voltage dependence of activation of KCNQ channels that mediate the M-current and thus results in an increased threshold for generating neuronal action potentials [69, 70]. A recent study found that flupirtine also shifts the gating of GABA_A receptors to lower GABA concentrations [71]. This mechanism of flupirtine was more pronounced in dorsal root ganglion and dorsal horn neurons than in hippocampal neurons. Effects of flupirtine on G-protein-regulated inwardly rectifying potassium channels are not clear [72, 73]. We first evaluated the efficacy of

flupirtine to treat neonatal seizures, using two established rodent chemoconvulsant models [71, 73]. The efficacy of flupirtine was compared with phenobarbital and diazepam, two drugs widely used for treatment of neonatal seizures. We found that of the three drugs, only flupirtine completely blocked kainic acid and flurothyl induced seizures in 10-day-old rats. To determine the efficacy of these three drugs to stop seizures, the drugs were injected 15 minutes after continuous kainic acid induced electrographic and behavioral seizures were observed. Unlike phenobarbital and diazepam, flupirtine treatment resulted in a rapid, complete and sustained suppression of seizure activity.

Although the models used in our previous study (kainic acid and flurothyl models) replicate some of the long-term outcomes following seizures in neonates, the cause of seizures does not resemble the clinical situation. Therefore, our next step is to determine the efficacy of flupirtine to treat seizures induced by hypoxia-ischemia, one of the most common causes of symptomatic neonatal seizures in humans. Our initial studies, carried out in two well-established animal models of hypoxic-ischemic encephalopathy, suggest that flupirtine pretreatment effectively blocks the development of behavioral seizures during hypoxia. Studies determining the effects of flupirtine on hypoxia-ischemia induced electroclinical seizures (electrographic seizures with behavioral correlates) are ongoing in our lab.

Summary

Recent investigations are leading to an increased understanding of age specific mechanisms of epileptogenicity. This understanding is creating novel opportunities for interventions that can treat or prevent seizures during early development. Our studies show that a novel therapy involving the administration of flupirtine, a potassium channel opener, to treat neonatal seizures in animal models is effective. Although this shows promise in the acute setting of injury, the effects on long-term neurological outcome is still uncertain. This is especially pertinent because plasticity is at its greatest during early development. Our future studies will investigate the effects of administration of flupirtine on chronic neurological outcome measures such as the development of epilepsy.

Trafficking and membrane-expression of HCN channels: Underlying mechanisms and relevance to epilepsy

Yoav Noam, PhD

Abstract

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are a unique class of molecules that play multiple roles in regulating neuronal excitability. HCN channel dysregulation (“channelopathy”) has been observed in many genetic and experimental animal models of epilepsy, as well as in human patients, and is likely to contribute to the abnormal excitability in the epileptic brain. Yet, despite its potential importance, our knowledge of the molecular and cellular pathways that govern these alterations is only partial. In this review, recent advancements in our understanding of HCN channel regulation will be discussed. A special focus will be given to processes of channel trafficking and surface expression, which are critical for the dynamic regulation of excitability. Obtaining a detailed picture of the discrete cellular processes that target HCN channels to specific sub-cellular compartments and control their abundance within the neuronal membrane is not only pivotal for the fundamental understanding of neuronal excitability, but may also open the way for novel therapeutic strategies in epilepsy.

HCN channels: basic properties and physiological significance in health and disease

Brain activity is mediated through highly intricate networks, where populations of neurons communicate with each other by constantly receiving signals and responding to them. In order to enable proper functioning in the face of changing physiological contexts, a robust homeostatic system of checks and balances acts to constrain neuronal excitability within safe limits and to prevent it from reaching extreme hyper- or hypo-excitability states. In epilepsy, failure (or weakening) of such constraining mechanisms results in poor control of excitability, which can eventually lead to the spontaneous occurrence of seizures [28, 74, 75].

Considering the above, the fundamental study of the molecular and cellular mechanisms that regulate neuronal excitability is critical for better understanding epilepsy as well as for improving its treatment. Among the different components that regulate excitability, voltage-gated ion channels play key roles by conducting various types of ionic currents across the neuronal membrane [76]. HCN channels are class of membrane-spanning proteins that possess unique biophysical properties. Unlike other ion channels, HCN channels open upon hyper-polarization of the neuronal membrane, giving rise to the non-selective, non-inactivating cationic current best known as I_h . Because I_h is open at sub-threshold potentials (i.e. when the neuron is not firing an action-potential), it is well suited for the fine-tuning of intrinsic excitability. The exact biophysical properties of I_h (namely, its activation kinetics, voltage-dependence, conductance, and modulation by other molecules) may vary considerably across different brain regions, cell populations, and subcellular compartments. This functional diversity is largely attributed to variability in the molecular composition of the channels: four subunit-isoforms (HCN1-4) assemble in different combinations to yield either homo- or hetero-tetrameric HCN channels, each with its distinct activation kinetics and gating properties. Other factors that influence HCN channel properties include interaction with modulatory molecules (such as cyclic AMP) and auxiliary proteins [77, 78].

How is neuronal excitability regulated by I_h /HCN channels? Active at sub-threshold potentials, I_h contributes a depolarizing drive to the resting membrane potential (bringing it closer to firing threshold) but also reduces the input resistance of the membrane (rendering it less responsive to incoming inputs). The synergistic action of these two I_h -mediated effects may be non-linear and can influence excitability in a highly context-dependent manner [79-82]. Accordingly, rather than serving one canonical role, HCN channels regulate a wide array of physiological phenomena in different brain regions and sub-cellular compartments. These include temporal summation of excitatory synaptic potentials (EPSPs) in dendrites of hippocampal and cortical neurons [83, 84], dendritic calcium signalling [85], synaptic release from axonal terminals [86, 87], theta resonance [88-90], and oscillatory activity in thalamocortical neurons [91, 92].

The involvement of HCN channels in epilepsy has been the subject of intense research for over a decade. Strong evidence from experimental and genetic rodent models of epilepsy as well as from human patients suggests aberrant regulation of HCN channel number, location and biophysical properties [28]. Recent genetic screenings in humans have identified mutations in the *HCN2* gene in patients with genetic epilepsy with febrile seizures plus (GEFS+) and in a patient with idiopathic generalized epilepsy [93, 94]. Notably, in both cases the mutation altered the conductive properties of the channel. Thus, dissecting the molecular and cellular mechanisms that underlie HCN channel dysregulation in epilepsy should contribute significantly to our understanding of this disease and may provide important clues for rational design of therapeutic strategies [31].

Emerging roles for trafficking and surface expression of HCN channels

Dysregulation of HCN channels in epilepsy occurs at multiple levels and at different time-scales [28]. For example, transcriptional regulation of HCN channel protein has been observed in several experimental models of epilepsy [29, 95-97] resulting in altered I_h amplitude and gating properties, and modified neuronal excitability.

Because transcriptional control involves the synthesis, folding and transport of novel protein molecules, this type of regulation is primarily suited for controlling channel number in relatively slow time frames of hours to days. In shorter time-scales, however, control of HCN channel trafficking and surface expression may be an alternative, potent mechanism for I_h modulation. Membrane insertion and internalization of ion channels can change current amplitude (and other current properties) within minutes by influencing the number and composition of functional channels on the cell membrane. Furthermore, trafficking of ion channels governs their preferential targeting to specific subcellular neuronal compartments, where the channels fulfil distinct functions [98].

In accord, converging evidence suggests that the sub-cellular distribution of HCN channels in neurons is tightly regulated and coupled to the physiological role of the channels. For example, the inhomogeneous distribution of HCN1 channels along the somato-dendritic axis of hippocampal CA1 pyramidal neurons regulates the integration of excitatory and inhibitory signals [83, 84, 99]. In other neuronal populations, HCN1 channels are targeted to pre-synaptic terminals where they constrain synaptic release [86] and may serve additional functions in development [87, 100]. Importantly, the targeting of HCN channels to subcellular domains is altered in epilepsy: HCN1 channels are redistributed along the somato-dendritic axis of pyramidal, CA1 hippocampal neurons in the kainic acid rat model of epilepsy [101], as well as in granular cells in the dentate gyrus of epileptic patients [102].

In addition to the preferential subcellular localization of the channels, several lines of evidence imply dynamic control of the number of HCN channels on the cell membrane. For example, activity-dependent regulation of I_h amplitude occurs within minutes following stimulation [103-107] (a time-course which corresponds to that of membrane insertion and internalization events). Furthermore, membrane-expression levels of HCN channels were found altered in chemoconvulsant rat models of epilepsy and these changes were accompanied by concurrent modulation of I_h properties [101, 108]. Despite the potential physiological significance, until recently little was known about the cellular mechanisms that underlie HCN channel trafficking and surface expression. Recent efforts from several groups (including our own) have produced some exciting data that help promote our basic understanding of these processes and may hold promise for epilepsy research. These will be discussed below.

Activity-dependent and subunit-specific regulation of HCN channel trafficking: lessons from live-imaging studies

Faced with the challenge of studying the dynamics of HCN channel trafficking in neurons, we have elected to take a direct approach by visualizing live hippocampal neurons that express fluorophore-tagged HCN channels. Using time-lapse techniques, we were able to witness and monitor for the first time the transport of HCN containing organelles along dendrites of live neurons [109]. Quantitative analysis of discrete trafficking events resulted in several interesting insights; first, focusing on the most abundant HCN channel isoform in the adult hippocampus (HCN1), we observed a highly dynamic vesicular transport in neurons, where many HCN1-containing organelles were travelling back and forth along dendrites. To explore the modulation of HCN channel trafficking by neuronal activity, neurons were exposed to elevated levels of the excitatory neurotransmitter glutamate. To our

surprise, this manipulation lead to the arrest of dendritic HCN1 channel trafficking within minutes, an effect that was reversible following the withdrawal of glutamate from the bath solution. Importantly, the glutamate-dependent arrest of HCN1 channels was accompanied by augmented I_h and increased surface expression of the channels [109]. Activity induced augmentation of I_h is thought to serve an important negative-feedback loop to control neuronal excitability: following excitation, the up-regulated I_h dampens the membrane input resistance and renders the neuron less responsive to incoming signals [103, 104]. Our live imaging observations provide a possible mechanistic explanation for the rapid, activity-dependent up-regulation of I_h : upon excitation, intracellular pools of HCN1 channels are immobilized and incorporated within the membrane, and the resulting up-regulated I_h functions to rapidly adapt neuronal excitability to a new set point, preventing the neuron from excessive firing.

Although HCN1 channels are the predominant HCN channel isoform in the adult hippocampus, HCN2 channels provide an additional contribution to I_h properties in these neuronal populations [110-112]. Curiously, we found substantial differences in the trafficking properties of the two channel isoforms: in contrast to the highly dynamic transport of HCN1 channels, HCN2 channels were distributed in a less punctate pattern along dendrites of hippocampal neurons, and were less mobile than the HCN1 isoform. This isoform-specific behavior may have implications to epilepsy, where isoform-specific regulation of HCN channel transcription has been previously observed [30, 97, 108, 113-115]. Because HCN channel trafficking is isoform-specific, an imbalance between the expression levels of different HCN channel isoforms (as occurs in epilepsy) may also alter the responsiveness of HCN channel trafficking to neuronal excitation. For example, increased contribution of HCN2 channels to I_h in the epileptic brain [30, 96, 97] may hamper activity-dependent up-regulation of I_h and therefore diminish the ability of neurons to adapt to changes in neuronal input. Imbalance in HCN1/HCN2 ratio may also result in increased formation of heteromeric channels [30], which may have different trafficking dynamics than homomeric channels and therefore might respond differently to neuronal excitation. Because the relation between HCN channels and network activity is complex and context-dependent, such hypotheses are clearly not exclusive and will require careful testing. Future studies into the discrete trafficking properties of specific HCN channel isoforms (including heteromeric channels) in epilepsy will be highly beneficial in this regard.

Macromolecular complexes and HCN trafficking

Trafficking and anchoring of ion channels within the cell membrane is non-trivial and requires elaborate cellular machinery. A major focus of our work is to elucidate the macromolecular complexes that control HCN channel dynamics within the neuronal membrane. To date, of the several proteins that have been identified to associate with HCN channels [78], the most extensively studied is TRIP8b. TRIP8b (tetratricopeptide repeat-containing Rab8b-interacting protein) is an intracellular protein, which is homologous to the PEX5p peroxisomal import protein [116]. In 2004, Santoro and colleagues [117] found that direct binding of TRIP8b to HCN can strongly influence the biophysical properties and surface expression of the channel. Further work revealed an elaborate family of TRIP8b splice variants that can either up- or down-regulate HCN channel surface expression, depending on the precise splice isoform [118, 119]. Notably, the most abundant TRIP8b splice isoforms in the brain were found to up-regulate surface expression of HCN channels, leading to augmented I_h amplitude [118, 119]. Recent work in TRIP8b knockout mice indicated a role for specific TRIP8b isoforms in controlling HCN channel targeting to dendrites and axons, as well as in lysosomal degradation of the channels [120-122]. It remains to be seen whether TRIP8b regulates the subcellular distribution of HCN channels in other neuronal populations [or whether alternative pathways exist.

As well as the effects that various TRIP8b isoforms exert on the surface expression of the channels, they also modulate the gating properties of the current by slowing its kinetics and shifting the voltage-activation curve to more hyperpolarized potentials [118, 119]. To tease out the molecular foundations for these different effects of TRIP8b on HCN channel gating and surface expression, we, along with our collaborators (as well as the work of an independent group) have recently demonstrated that the interaction between TRIP8b and HCN1 channels involves two separate molecular interfaces that differentially influence channel gating and surface expression [123, 124]. The recent resolving of HCN2-TRIP8b stoichiometry and crystal structure [125] further refined the molecular details of these interactions and indicated that TRIP8b associates with HCN2 as an obligate tetramer (in a 4:4 subunit ratio).

While the interaction of HCN channels with TRIP8b is likely to be pivotal in controlling channel trafficking and distribution, not all forms of HCN channel regulation can be easily explained by HCN-TRIP8b interactions. For example, because TRIP8b interacts with all HCN channels isoforms [117], it is unclear how TRIP8b would modulate channel trafficking in an isoform-specific manner. An alternative, potential candidate for HCN isoform-specific regulation is filamin A, a structural protein that in addition to its scaffolding functions can regulate the surface expression and intracellular trafficking of several ion channels and receptors [126]. Importantly, filamin A binds HCN1 through a distinct sequence in the channel C' terminus, while not being able to interact with either HCN2 or the HCN4 channel isoform [127]. Interaction of filamin A with HCN1 in melanoma-derived cell lines results in clustering of the channels on the cell surface and reduced current density [127]. Although filamin A is an attractive candidate for isoform-specific regulation of HCN channels (through anchoring of HCN1 to the actin network), it is currently unknown whether such mechanisms apply also to neurons. To start addressing this question, we have systematically analyzed the presence and distribution patterns of filamin A in the adult rat brain using immunohistochemical approaches. This analysis revealed a wide (yet selective) expression of filamin A mRNA and protein in various neuronal populations and subcellular compartments, including regions where HCN1 is abundant (such as hippocampus and cortex) [128]. Our current efforts focus on investigating the potential role of HCN1-filamin interactions in neurons, and the cellular pathways by which this regulation takes place.

Alongside with TRIP8b and filamin A, several other proteins have been reported to interact with HCN channels, including KCNE2, S-SCAM, Mint2, tamalin and KCR1 [77, 78]. The specific function of these proteins in regulating HCN channels are either under debate or unknown [78], and further research is necessary to uncover their implications on HCN channel trafficking. Studies of these molecular complexes will help shed light on how the cellular machinery works in concert to anchor HCN channels to the neuronal membrane and localize the channels to different subcellular compartments.

Future challenges in HCN trafficking research

Our view of HCN channel trafficking and the cellular machinery that guides it has evolved considerably in the past decade: fine regulation of HCN channel sub-cellular distribution has been observed in different neuronal populations [83, 84, 86, 87, 129], HCN-interacting proteins have been identified and characterized [78], and activity-dependent trafficking/surface expression of HCN channels is emerging as a potential mechanism for regulating neuronal function at various time frames [87, 101, 102, 108, 109, 130]. Further research is required to obtain a detailed and comprehensive picture of the molecular pathways that govern HCN channel trafficking, and its relevance to different physiological and pathological states. In particular, harnessing advanced imaging methods to study channel behavior and composition within the cell membrane can be of great value. Additional, functional studies into the local regulation of I_h within restricted sub-cellular regions will be

helpful to better understand the functional implications of these processes at both cellular- and network-levels.

The study of HCN channel trafficking and surface expression is complicated by several technical hurdles. For example, various techniques require the availability of the channel's extracellular domain, including surface-specific antibodies, extracellularly-tagged pH-sensitive fluorophores, and single channel tracking using quantum dots. While such techniques have proven beneficial for the study of many other ion channels and receptors, they may be difficult to apply to HCN channels, where both N' and C' termini of the protein are intracellular. In particular, harnessing alternative advanced imaging approaches to study channel behavior and composition within the cell membrane (such as 'super-resolution' techniques that allow studying membrane proteins at the nano-scale) can be of great value. Additional, functional studies into the local regulation of I_h within restricted sub-cellular regions will be helpful to better understand the functional implications of these processes at both cellular- and network-levels.

Finally, mutations in interacting proteins might emerge that contribute to epilepsy through causing interference with the normal trafficking and membrane expression and distribution of HCN channels.

In summary, a detailed understanding of HCN channel trafficking may open the way for a novel design of therapeutic strategies in epilepsy by allowing fine manipulations of the amount, location and molecular identity of the channels. If proven feasible, such strategies can improve our ability to constrain neuronal excitability in the epileptic brain in a more specific and context-dependent fashion. This will allow amelioration of epilepsy without interference with normal brain function.

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Highlights

- * Current antiepileptic therapy for neonates is suboptimal.
- * Flupirtine is a promising drug for neonatal seizures.
- * Targeting HCN channels may provide novel therapeutics.