Tripping the HCN Breaker

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Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels regulate neuronal excitability, pacemaking, dendritic integration, and homeostatic plasticity and are culprits in aberrant neuronal activity in certain epilepsies. In this issue of *Neuron* two manuscripts (Santoro et al. and Zolles et al.) report that HCN channel gating and expression are controlled by Trip8b (Pex5R) but with a bidirectional spin.

Anyone who's had to feel their way along the dank walls of a dark basement searching for the circuit breaker knows how home electrical circuits respond to overload; the circuit breaker is tripped and the system shuts down. Simple and effective, but not an option for neuronal circuits that have to maintain uninterrupted output in the face of large fluctuations in excitability. Ion channels that open at negative membrane potentials are part of the solution. These include hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels, one-pore domain inwardly rectifying potassium channels, and two-pore domain leak potassium channels. Collectively, these currents stabilize neuronal membrane potentials, limit abnormal hyperexcitability, and still allow transmission of essential signals/spikes.

First identified as a pacemaking channel in cardiac sinoatrial nodal cells and spontaneously firing neurons, HCN channels are now recognized as being far more versatile, essential for dendritic integration in hippocampal and cortical neurons and homeostatic plasticity in the hippocampus, and culprits of aberrant neuronal activity in certain epilepsies (Wahl-Schott and Biel, 2009; Baruscotti et al., 2005; Robinson and Siegelbaum, 2003). Reflecting their major role in controlling neuronal and cardiac excitability, HCN channels sense and respond to changes in activity and are modulated by neurotransmitters and circulating hormones.

Currents generated by HCN channels, called I_h or I_f, have a unique, some say "funny," property—they activate as the membrane potential is hyperpolarized and deactivate as the membrane potential depolarizes (leak subtractors out there beware!) (Baruscotti et al., 2005). HCN current amplitude, kinetics, and activation thresholds are all adjustable and underlie both beneficial and detrimental changes in neuronal excitability.

How do HCN channels sense changes in neuronal activity, and, on the other hand, how do cells control HCN channel activity to regulate neuronal excitability? As defined in their name, cyclic AMP facilitates HCN channel activity by speeding gating and shifting activation thresholds to more depolarized voltages. Sensitivity to cAMP, however, varies among different HCN channels and in different cell types, suggesting structural and functional heterogeneity. Other molecules, including PIP_2, protons, and kinases, regulate HCN currents in different cells, but the molecular basis underlying up- and downregulation of HCN current densities in hippocampal dendrites that parallel bidirectional changes in synaptic activity is not known (Brager and Johnston, 2007).

Arriving at generally similar conclusions but originating from different locations, three groups show that Trip8b is the gate keeper of HCN channel activity. Trip8b (for tetratricopeptide-repeat containing Rab8b-interacting protein), also called Pex5R (for peroxisomal import protein 5-related protein), specifically interacts with at least three of four HCN channel subtypes (HCN1, -2, and -4) through a conserved sequence in their C-terminal tails. Trip8b strongly influences HCN channel trafficking to the plasma membrane (Lewis et al., 2009; Santoro et al., 2009 [this issue of *Neuron*]) and occludes the stimulatory actions of cAMP on gating (Santoro et al., 2009; Zolles et al., 2009 [this issue of *Neuron*]).

Trip8b was originally identified by Santoro, Siegelbaum, and colleagues as a binding partner of HCN channels by a yeast two-hybrid screen. They showed that Trip8b colocalized with HCN1 channels in dendrites of neurons and in *Xenopus* oocytes and that Trip8b strongly attenuated HCN currents by reducing surface HCN channel expression. In this issue of *Neuron*, Klöcker and colleagues use an unbiased affinity purification screen to establish that Trip8b (Pex5R) is in a 1:1 complex with HCN channels in rat brain. Consistently, manipulations of Trip8b levels in cultured neurons and cardiac cells (Zolles et al., 2009; Lewis et al., 2009) and in vivo (Santoro et al., 2009) collectively demonstrate the functional significance of this partnership.

What does Trip8b do? Well, it depends on which Trip8b you are talking about. Santoro and colleagues show that the brain expresses multiple splice isoforms of Trip8b distinguished by their unique N termini. Most notably, these isoforms have dramatically different effects on HCN channel expression levels as discussed below. N-terminal isoforms of Trip8b originate from the alternative use of two promoters (exons 1a and 1b) and cell-specific inclusion of optional exons (2, 3, and 4) during pre-mRNA processing. By contrast, exons 5 through 16 that encode the rest of the protein are invariant among all Trip8b cDNAs isolated from mammalian brain to date (Figure 1). All Trip8b isoforms slow HCN channel gating and shift channel activation thresholds to more hyperpolarized voltages. Both studies highlighted here present evidence that Trip8b acts by opposing the stimulatory actions of cAMP on HCN channels—although details differ between studies. Specifically, Zolles and colleagues find that Trip8b (Pex5R) influences gating of

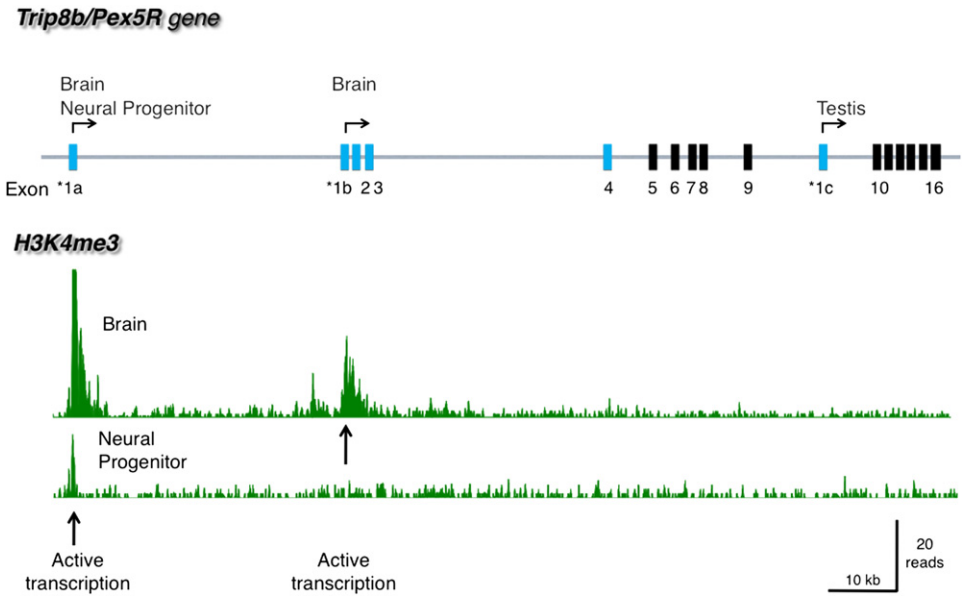

Figure 1. Alternate Promoters and Alternatively Spliced Exons in *Trip8b (Pex5R)*

Epigenetic data predict that brains use both alternate promoters 1a and 1b in *Trip8b (Pex5R)*, but neural progenitor cells use only the first promoter in this gene. Structure of the mouse *Trip8b* (*Pex5R*) gene shown together with chromatin immunoprecipitation—sequence density plots of histone-3 lysine 4 trimethylation (H3K4me3) for whole-brain and neural precursor cells. The *Trip8b (Pex5R)* gene is predicted to contain three promoters 1a, 1b, and 1c. The third 1c promoter is only used in testis, while exons 1a and 1b are used in brain. The H3K4me3 signal is shown at 25 bp resolution across the *Trip8b* (*Pex5R*) gene (Meissner et al., 2008). For illustrative purposes, exons are not drawn to scale. The frequency of sequences for H3K4Me3 predicts the level of transcription activation in brain and neural precursor cells derived from embryonic stem cells. Alternatively spliced exons in *Trip8b* (*Pex5R*) are blue, and constitutive exons are black. The CHIPseq Track was generated at the Broad Institute and in the Bradley E. Bernstein lab at Massachusetts General Hospital/Harvard Medical School and deposited at http://genome.ucsc.edu.

HCN2 and HCN4 subtypes but not HCN1, a finding inconsistent with the studies of Santoro and colleagues. In their report, Santoro and colleagues suggest that some of these discrepancies might be attributed to the transient nature of Trip8b-HCN1 interactions under certain conditions.

Why might it be advantageous for cells to reduce HCN channel activity by occluding cAMP? Decreased cAMP sensitivity would reduce basal HCN channel activity and in general increase neuronal excitability but, perhaps more importantly, also limit the spatial and temporal reach of cAMP. HCN channels in a complex with Trip8b would need to be closer to the source of cAMP to feel its influence. For example, it could be especially important to limit signaling between G protein-coupled receptors and HCN channels within the same synaptic structure.

Using antibodies to immunoprecipitate HCN1 and HCN2 channels, respectively, from mouse and rat brains, the Santoro and Zolles groups conclude that a large fraction of HCN proteins exist in a tight complex with Trip8b (Pex5R). Two intriguing observations suggest that the stability of HCN-Trip8b complexes might differ depending on location. The Zolles and Santoro studies present strong evidence that exogenous Trip8b modulates HCN channel activity as well as its distribution in cultures of dissociated and organotypic slices of hippocampus (similar findings were also reported last month by Lewis and colleagues [Lewis et al., 2009]). However, exogenous Trip8b introduced using viral methods appears unable to influence HCN channel expression patterns in vivo in the brains of wild-type mice as assessed by immuno-localization. Subsequent experiments show that exogenous Trip8b can successfully incorporate into the distal portion of apical dendrites of the hippocampus but only in a HCN1 null background. Moreover, when coexpressed with exogenous HCN1 in vivo in HCN1 knockout mice, Trip8b appeared to form de novo Trip8b-HCN1 complexes. Santoro and colleagues suggest that exogenous Trip8b might not be able to displace endogenousTrip8b from HCN because of the stability of the HCN1-Trip8b complex in vivo. In contrast, their studies in isolated membrane patches from cells expressing exogenous Trip8b and HCN1 channels suggest that Trip8b might dissociate from HCN1 under these conditions.

One might conclude that Trip8b proteins exert a tonic inhibitory control over a large fraction of HCN channels—but altering channel gating is not all Trip8b does. Compared to effects on gating, Trip8b's influence on HCN channel expression is striking in its varied magnitude and direction. In their 2004 study, Santoro and colleagues showed that Trip8b profoundly repressed surface expression of HCN1 channels. They now show that the 1b-2 isoform of Trip8b accounts for only ~10%–15% of the total pool of Trip8b mRNAs in mouse brain. The two most abundant Trip8b mRNAs in brain, 1a and 1a-4, collectively account for up to 70% of the pool. Both isoforms contain the first alternative promoter (1a), lack exons 2 and 3, but are distinguished by the absence and presence of exon 4, which encodes 35 amino acids.

At an unexpected turn in the road, Santoro and colleagues find that the dominant Trip8a isoform in the brain, 1a-4, increases

HCN current amplitudes 6-fold over control levels, again generally consistent with the findings of Lewis and colleagues (Lewis et al., 2009). Isoforms 1b-2 and 1a-4 share no common exon in their variable N-terminal region, and amino acids unique to these isoforms clearly contribute to their distinct effects on HCN channel trafficking (Figure 1). Of note, exon 2 contains a classic internalization/endocytic motif present in a number of membrane proteins, YXXL, which Santoro and colleagues show underlies the major inhibitory actions of Trip8b 1b-2 on HCN expression. However, exon 1a also contains a dileucine internalization motif that contributes to HCN channel repression but only when exon 4 is absent. Exon 4 in contrast appears to encode a sequence that enhances HCN and perhaps trafficking to the plasma membrane. Further, there are complex functional interactions among domains. As discussed by Santoro and colleagues, the presence of these canonical endocytic motifs together with data that show Trip8b interacts with clathrin, and the clathrin adaptor AP-2, point to Trip8b involvement in clathrin-dependent endocytosis of HCN, a theory that needs to be tested.

Using viral infection in vivo, Santoro and colleagues show that exogenous isoforms 1b-2 and 1a-4 support different patterns of subcellular HCN1 expression in hippocampal dendrites, consistent with 1b-2 retaining HCN1 channels in sub-plasma membrane compartments and 1a-4 promoting their surface expression. Lewis and colleagues use short hairpin RNA against all Trip8b isoforms and show that acute knockdown of endogenous Trip8b expression in hippocampal cultures leads to smaller HCN currents in hippocampal cells. This suggests that endogenous Trip8b exerts a tonic stimulatory effect on HCN current density (Lewis et al., 2009). How the enhancing actions of Trip8b on HCN current amplitude combine with their inhibitory actions on HCN channel gating molds the overall activity of HCN in neurons is not known and will require further study.

Despite overwhelming evidence for extensive alternative pre-mRNA splicing in the mammalian nervous system, studies like those described here, which link a specific splice isoform to an identified population of neurons and ultimately to a specific cell function, remain limited (Lipscombe, 2005). Part of the problem arises from the challenges associated with identifying functionally relevant splice isoforms from the larger pool of expressed mRNAs and finding ways to selectively target specific protein isoforms to establish function. Recent genome-wide high-throughput sequencing projects that map epigenetic markers of transcription and that identify and characterize sites of splice factor binding to mammalian genomes are generating extremely exciting data that should lower these technical hurdles (Li et al., 2007; Licatalosi et al., 2008).

As shown by Santoro and colleagues using RT-PCR, neurons use two alternate *Trip8b* promoters and alternative splicing of exons 2 and 4 to generate a number of isoforms with distinct N termini (Figure 1). Depending on the choice of promoter, 1a or 1b, Trip8b mRNA isoforms might also have different mRNA stabilities and translation efficiencies. As a complement to traditional RT-PCR amplification methods (including 5′ RACE), data from genome-wide mapping of epigenetic markers that, for example, locate active transcription initiation depending on cell type, tissue type, and cell state can be highly informative. To illustrate the predictive value of these markers, we analyzed CHIPseq data derived from chromatin-immunoprecipitation and massively parallel DNA sequencing to view sites of histone methylation in the mouse *Trip8b* (*Pex5R*) gene (http://genome.ucsc.edu, Broad Institute CHIPseq Track) (Meissner et al., 2008). We show histone-3 lysine-4 trimethylation (H3K4me3) signals from analyses of brain and neural progenitor cell genomic DNA that predict sites of active transcription, aligned to the *Trip8b* (*Pex5R*) gene (Figure 1). Consistent with RT-PCR analyses (Santoro et al., 2009), two strong H3K4Me3 signals in whole-brain-derived DNA correspond to exons 1a and 1b in the *Trip8b* (*Pex5R*) gene. By comparison, only a single H3K4me3 signal is observed at exon 1a in samples from embryonic stem cells (data not shown), embryonic fibroblasts (data not shown), and neural precursors (Figure 1), suggesting that Trip8b is expressed in these tissues but that all isoforms contain exon 1a. As high-throughput sequencing data are added from analyses of other tissues, brain regions, and different developmental stages, it should become easier to predict when during development and in which brain regions exons 1a and 1b of *Trip8b* are employed.

The studies discussed here offer valuable insights into the functional importance of alternative pre-mRNA splicing in controlling HCN channel activity and ultimately neuronal excitability. There is little evidence that HCN channels themselves are subject to alternative splicing, but there must be advantages for cells to use alternative pre-mRNA splicing of Trip8b to control HCN channel function. Changes in the pattern of alternative pre-mRNA splicing should support relatively rapid and relatively stable changes in protein activity, bypassing the need to alter gene expression. By switching the pattern of exon 1–4 splicing in *Trip8b*, neurons could achieve relatively stable bidirectional changes in HCN current density. Activity-dependent splicing of *Trip8b* pre-mRNAs could underlie bidirectional changes in HCN current density in dendrites of hippocampus that accompany long-term synaptic potentiation and depression. If dendritic nonnuclear splicing and local translation of *Trip8b* also occurs, then alternative splicing could be synapse specific (Miyashiro et al., 2009). Although there are examples of activity-dependent splicing events in neurons, we know little about how cellular splicing factors coordinate alternative splicing events within a single pre-mRNA and across functionally related pre-mRNAs in response to neuronal activity.

Until now, information on splicing factor binding to specific pre-mRNA targets has been limited to a few select genes. New genome-wide analyses of splicing factor-RNA binding events should help identify factors that coordinate exon selection in specific gene products, within defined populations of neurons to modify function (Licatalosi et al., 2008; Zhang et al., 2008). The presence, absence, and/or ratios of cell-specific splicing factors that associate with the pre-mRNA define splicing patterns (Sharp, 2005). In turn, the cellular levels of these splicing factors depend on features such as cell type, developmental stage, and neuronal activity (Li et al., 2007). Splicing factors include both enhancer and repressor proteins, and notably, the same splicing factor can

serve both as enhancer or repressor depending on whether its binding sites are positioned up- or downstream of the target exon—an example of bidirectional regulation at the level of RNA processing.

Alternative pre-mRNA splicing events are predicted to occur in ~95% of multi-exon human genes. Neurons use alternative splicing extensively to tailor protein activity profiles to optimize neuronal tasks and to adapt to physiological demands. Coordinated alternative pre-mRNA splicing across functionally related genes offers a mechanism for cells to orchestrate changes in ion channels to achieve balance. Future studies aimed at identifying cell-specific and activity-dependent splicing factors that coordinate Trip8b exon inclusion and repression could show if and how neuronal excitability is controlled at the molecular level. Furthermore, once the splicing factors are known, their levels could be manipulated to induce changes in the abundance of specific *Trip8b* isoforms to assess their influence on HCN channel activity in neurons.

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Inhibition Acts Globally to Shape Olfactory Cortical Tuning

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Lateral inhibition between near-neighbor neurons has long been thought to be important for narrowing the receptive fields of neurons in many sensory systems. A new study by Poo and Isaacson in this issue of *Neuron* examining olfactory processing finds that "global" inhibition within the primary olfactory cortex might accomplish a similar end.

Within many sensory systems, broadly tuned lateral inhibition has commonly been proposed to narrow the receptive fields of neurons, a function that could be important for contrast enhancement. This mechanism however has come under some question in recent years, based on experiments in which inhibitory and excitatory synaptic activity has been directly recorded in neurons in vivo. Inhibition and excitation in fact often appear to be "balanced," meaning that inhibition is no more ubiquitous or broadly tuned to different stimuli than excitation is. Balanced inhibition and excitation is observed in the primary sensory cortices involved in visual, auditory, and somatosensory processing (Anderson et al., 2000; Wehr and Zador, 2003; Tan et al., 2004; Wilent and Contreras, 2005; Priebe and Ferster, 2008), all structures where lateral inhibition has been thought to have important functions.

Within this issue of *Neuron*, Poo and Isaacson (2009) provide interesting experimental results to add to the discussion, based on their in vivo patch-clamp recordings of synaptic activity within the primary olfactory cortex, specifically the anterior piriform cortex, which is the structure that receives the most direct inputs from olfactory bulb mitral cells. Their basic strategy, analogous to what has been used in studies in other sensory systems, was to record inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs, respectively) in pyramidal cells (PCs) in response to a small panel of monomolecular odors. From these recordings, they derived estimates both of how responsive the synaptic activity of a single PC was to the panel of odors, and of how responsive