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Review

HCN channels: Structure, cellular regulation and physiological function

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Abstract. Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels belong to the superfamily of voltage-gated pore loop channels. HCN channels are unique among vertebrate voltage-gated ion channels, in that they have a reverse voltage-dependence that leads to activation upon hyperpolarization. In addition, voltage-dependent opening of these channels is directly regulated by the binding of cAMP. HCN channels are encoded by four genes (HCN1 – 4) and are widely expressed throughout the heart and the central nervous system. The current flowing through HCN channels, designated I_h or I_f, plays a key role in the control of cardiac and neuronal rhythmicity ("pacemaker current"). In addition, I_h contributes to several other neuronal processes, including determination of resting membrane potential, dendritic integration and synaptic transmission. In this review we give an overview on structure, function and regulation of HCN channels. Particular emphasis will be laid on the complex roles of these channels for neuronal function and cardiac rhythmicity.

Keywords. HCN channels, pacemaker channels, I_h, dendritic integration, sinoatrial node.

Introduction

In various types of neurons a voltage-activated Na^+/K^+ current designated I_h has been identified. Unlike the vast majority of cellular conductances, I_h is activated by membrane hyperpolarization (I_h = hyperpolarization-activated current) rather than by depolarization. Moreover, activation of I_h is facilitated by cAMP in a direct, protein kinase A (i.e. phosphorylation) independent fashion. A current corresponding to I_h is also present in cardiac pacemaker cells where it was named "funny current" (I_f). Many functional roles have been attributed to I_h/I_f. First of all, it was assumed that this current plays a key role in the initiation and regulation of the heart beat ("pacemaker current"). An involvement of I_h in the control of rhythmic activity in neuronal circuits (e.g. in the thalamus) was also postulated. In addition, I_h was claimed to contribute to several other neuronal processes, including determination of resting membrane potential, dendritic integration and synaptic transmission. The ion channels underlying I_h/I_f were discovered about a decade ago. With reference to their complex dual activation mode these proteins were termed hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels. Currents obtained after heterologous expression of the cDNAs of HCN channels revealed the principal features of native I_h confirming that HCN channels indeed represent the molecular correlate of I_h. In this review we provide an

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overview of the structure, function and regulation of HCN channels. Particular emphasis will be laid on the roles of these channels for neuronal function and cardiac rhythmicity. To this end we will also describe recent results from the analysis of HCN channel knockout or knock in mice and discuss data from human HCN4 channelopathies.

Structure-function relation of HCN channel domains

Together with cyclic nucleotide-gated (CNG) channels and the Eag-like K^+ channels [1–3] HCN channels form the subgroup of cyclic nucleotide-regulated cation channels within the large superfamily of the pore-loop cation channels [4]. HCN channels have been cloned from vertebrates and several invertebrates [5–15] but are missing in *C. elegans*, yeast and prokaryotes. The core unit of HCN channels has a similar architecture to that of many other pore loop channels. It consists of four subunits that are arranged around the centrally located pore (Fig. 1). In mammals, four homologous HCN channel subunits (HCN1–4) exist. These subunits form four different homotetramers with distinct biophysical properties. There is evidence that the number of potential HCN channel types is increased *in vivo* by the formation of heterotetramers [16–22]. In addition, as in many other ion channels, alternative splicing was reported for HCN channel transcripts in spiny lobster, *Drosophila* and *Apis mellifera* [23–25]. Each HCN channel subunit consists of two major structural modules, the transmembrane core and the cytosolic C-terminal domain. The transmembrane core harbours the gating machinery and the ion conducting pore, the cytosolic C-terminal domain confers modulation by cyclic nucleotides. Both modules allosterically cooperate with each other during channel activation.

Transmembrane segments and voltage sensor. The transmembrane channel core of HCN channels consists of six α-helical segments (S1–S6) and an ion-conducting pore loop between S5 and S6 (Fig. 1). A highly conserved asparagine residue in the extracellular loop between S5 and the pore loop is glycosylated (N380 in murine HCN2) and it was shown that this post-translational channel modification is crucial for normal cell surface expression [16]. The voltage sensor of HCN channels is formed by a charged S4-helix carrying nine arginine or lysine residues regularly spaced at every third position [26, 27]. Positively charged S4 segments are found in all voltage-dependent members of the pore-loop cation channel superfamily [28]. However, inward movement of S4 charges through the plane of the cell membrane leads to

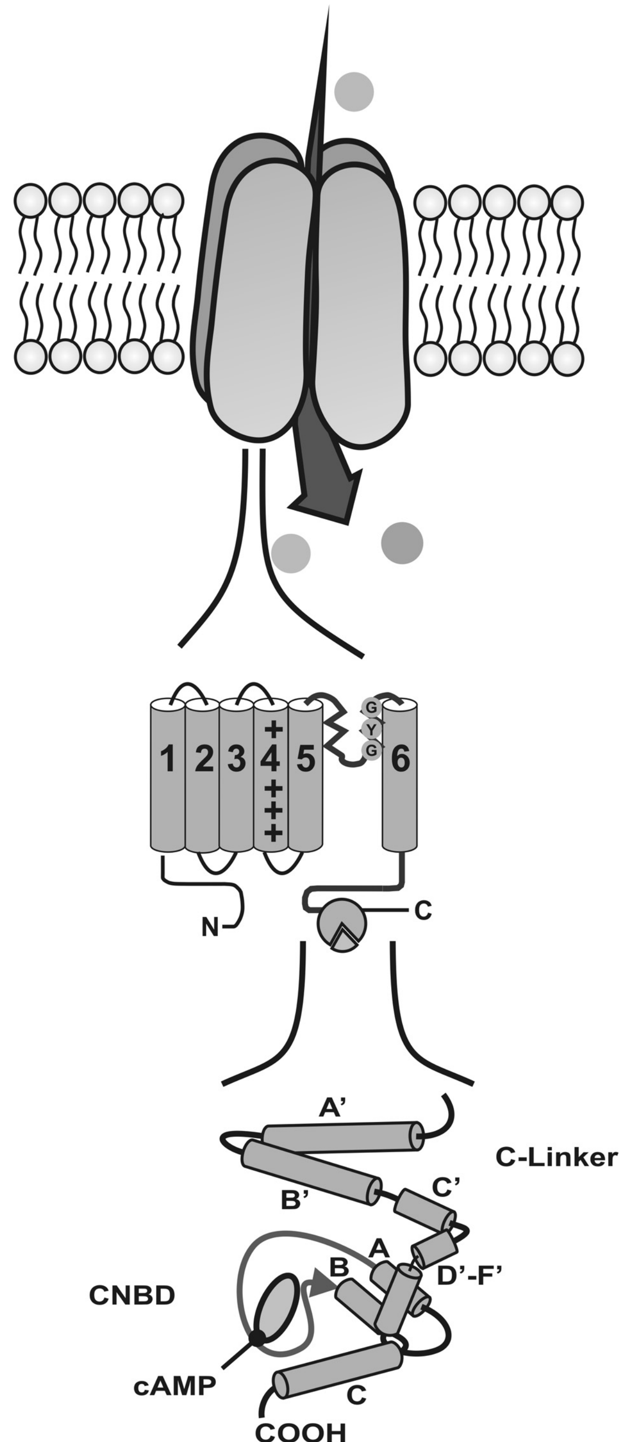

Figure 1. Structure of HCN channels. Top: HCN channels are tetramers. One monomer is composed of six transmembrane segments including the voltage sensor (S4), the selectivity filter and the pore region between S5 and S6. The C-terminal channel domain contains the cyclic nucleotide-binding domain (CNBD; middle). Bottom: the C-terminal channel domain is composed of two domains. The C-linker domain consists of six α-helices, designated A' to F'. The CNBD follows the C-linker domain and consists of α-helices A–C with a β-roll between the A- and B-helices (arrow).

opening of HCN channels while it triggers the closure of depolarization-activated channels such as the Kv channels [29]. The molecular determinants underlying the different polarity of the gating mechanism of HCN and depolarization-gated channels remain to be determined. However, there is initial evidence that the loop connecting the S4 with the S5 segment plays a crucial role in conferring the differential response to voltage [30–32].

Pore loop and selectivity filter. HCN channels conduct Na^+ and K^+ with permeability ratios of about 1:4 and are blocked by millimolar concentrations of Cs^+ [33]. Despite this preference for K^+ conductance, HCN channels carry an inward Na^+ current under physiological conditions. HCN channels also seem to display a small permeability for Ca^{2+} [34, 35]. At 2.5 mM external Ca^{2+}, the fractional Ca^{2+} current of HCN2 and HCN4 is about 0.5 %. The functional relevance of Ca^{2+} entry through HCN channels is not clear at the moment. Surprisingly, the sequence of the HCN channel pore loop is closely related to that of highly selective K^+ channels [28]. In particular, although HCN channels conduct Na^+ under physiological conditions they contain a glycine-tyrosine-glycine (GYG) motif that in K^+ channels forms the selectivity filter for K^+ ions. So far, efforts to solve this structural conundrum have been unsuccessful. However, one may speculate that in the tetrameric HCN channel complex the GYG motif is coordinated in a less rigid fashion than in K^+ channels, allowing the entrance of cations of different size.

C-linker and cyclic nucleotide-binding domain. The sensitivity of HCN channels to cAMP is mediated by the proximal portion of the cytosolic C-terminus [36] (Fig. 1). This part of the channel contains a cyclic nucleotide-binding domain composed of about 120 amino acids (CNBD) and an 80 amino acid long C-linker region that connects the CNBD with the S6 segment of the channel core. Binding of cAMP to the CNBD speeds up channel opening and shifts the voltage-dependence of activation to more positive voltages [37] (Fig. 1, Fig. 8B). Thus, at a given voltage the current flowing through HCN channels is larger in the presence than in the absence of cAMP. Several studies have suggested that cAMP increases channel activity by removing tonic channel inhibition that is conferred by the C-linker-CNBD [38, 39]. The determination of the crystal structure of the C-linker-CNBD of HCN2 has provided important insights into the molecular determinants of cAMP-dependent channel modulation [36]. The CNBD shows a highly conserved fold, consisting of an initial α-helix (A-helix), followed by an eight-stranded antiparallel β-roll (β1–β8), a short B-helix and a long C-helix. The C-linker consists of six α-helices (A'–F'). Four C-linker-CNBDs are assembled to a complex with a fourfold axis of symmetry which is consistent with the tetrameric architecture of pore-loop channels. Most of the subunit-subunit interactions in the tetramer are mediated by amino acid residues in the C-linker. The binding pocket for cAMP is formed by a number of residues at the interface between the β-roll and the C-helix [36, 40, 41]. Within the binding pocket of HCN2, a group of seven amino acids interacts with ligand [40]. Three of these residues are located in the β-roll (R591, T592 and E582), and four in the C-helix (R632, R635, I636, and K638). Among these, only one key residue of the C-helix (R632) was identified that controlls the efficacy by which cyclic nucleotides enhance channel opening [40]. Four residues in the C-helix (e.g. R632, R635, I636 and K638) contribute to the higher selectivity of HCN2 channels for cAMP than for cGMP [40]. However, it is not clear how these residues generate cAMP selectivity. It was suggested that cAMP selectivity at least partially is not due to its preferential contacts with the protein, but rather reflects the greater hydration energy of cGMP relative to cAMP. This could result in a greater energetic cost for cGMP binding to the channel [42]. So far, only limited information has become available on the dynamics of the allosteric process that couples cAMP binding with channel opening. Addressing this issue is complicated by the fact that, in the available crystal structure, the C-linker seems to be caught in the resting conformation (i.e. the conformation that is occupied in the absence of cAMP) while the CNBD is in its active (i.e. cAMP-bound) conformation [2]. According to a recent model [2], in the absence of cAMP the C-linker is thought to be in a "compact" conformation that produces an inhibitory effect on channel opening. Binding of cAMP in turn induces a conformational change in the CNBD involving the C-helix. The conformational change in the C-helix is then coupled to the C-linker that occupies a more "loose" conformation leading to an alteration of the intersubunit interface between helices of neighboring subunits. The resulting change in the quaternary conformation removes the inhibition of the C-terminus, destabilizes the closed state, thus promoting the opening of the channel. One would expect that the described activation process comes along with large translocations of subdomains in the C-linker-CNBD. However, recent data suggest that this may not necessarily be the case. Fluorescent resonance energy transfer (FRET) measurements in a cyclic nucleotide-gated (CNG) channel, a close relative of HCN channels, suggested that movements within the C-

linker are probably subtle, involving only limited rearrangements [43].

Basic biophysical properties of HCN channel types

Native I_h is a cAMP-modulated mixed Na^+/K^+ current that activates upon hyperpolarization with a characteristic sigmoidal time course (Fig. 8B). Unlike most other voltage-gated currents I_h does not inactivate in a voltage-dependent fashion. All four HCN channel types (HCN1–4) display the principal biophysical properties of native I_h but do differ from each other with respect to their voltage dependence, activation time constants (τ) and the extent of the cAMP-dependent modulation.

Voltage-dependent activation. The voltage-dependent activation differs quantitatively between the HCN channel subtypes. Typical values for the midpoint of activation (V0.5) are –70 mV, –95 mV, –77 mV to –95 mV and –100 mV for HCN1, HCN2, HCN3 and HCN4 respectively [20, 44, 45]. Several conditions may alter these values significantly, giving rise to a broad range of V0.5 observed by different laboratories [46] (see below). Recently, an interesting new aspect of voltage-dependent activation of HCN channels was discovered in spHCN [13], an HCN channel from the sea urchin sperm that displays voltage-dependent inactivation in the absence of cAMP. In the presence of cAMP spHCN currents resemble the waveform of mammalian HCN currents [13, 41, 47, 48]. It was shown that the voltage-dependent activation of the sea urchin sperm channel HCN channel (spHCN) can shift between two modes depending on the previous activity [49–51]. In mode I, gating charge movement and channel opening occur at very negative potentials, while in mode II, both processes are shifted to > 50 mV more positive potentials [50]. The transition from mode I to mode II is favored in the open state, while the transition from mode II to mode I preferentially occurs in the closed state. The shift between these two modes leads to a voltage hysteresis of the ionic currents and also affects the kinetics of the channel activation and deactivation. This interesting gating behavior of spHCN is probably the result of a slow conformational change attributable to lateral movement of S4 that stabilizes the inward position of S4 upon hyperpolarization [29, 49–53]. For HCN1 similar findings have been observed as for spHCN channels [51]. By contrast, for HCN2 the voltage hysteresis is less pronounced, but the effects of the mode shift on the deactivation kinetics are present. For HCN4, only the changes in deactivation kinetics are seen under certain conditions using high K^+ concentrations in the extracellular recording solution. It has been suggested that the mode shift is important for short-term, activity-dependent memory in HCN channels [49].

Activation kinetics. The kinetics for voltage-dependent activation also differs between the HCN channel subtypes. HCN1 is the fastest channel (with a range of τ from 25–300 ms depending on the voltage [7, 54]), while HCN4 is the slowest channel [8, 10, 12], displaying τ values between a few hundred milliseconds at strongly hyperpolarized voltages (–140 mV) up to several seconds at normal resting potential (-70 mV). HCN2 and HCN3 activate with kinetics in between HCN1 and HCN4 [8, 44, 55].

Modulation by cAMP. Although all four HCN channels carry a highly conserved C-linker-CNBD they are modulated quantitatively differently by cAMP. Cyclic AMP shifts the activation curves of HCN2 and HCN4 by about +10 to 25 mV [8, 10, 12, 20, 36, 38, 39, 56, 57] and speeds up the opening kinetics. For HCN2 the K_a value has been reported to be 0.5 μM for cAMP and 6 μM for cGMP [6]. By contrast, HCN1 and HCN3 are, if at all, only weakly affected by cAMP [20, 38, 39, 44, 57]. Interestingly, when the CNBD of HCN4 is replaced by the corresponding domain of HCN3, cAMP sensitivity is fully maintained, suggesting that the CNBD of HCN3 is principally able to bind cAMP and to mediate cAMP-dependent gating [44]. Thus, within the HCN3 channel, the CNBD may be functionally silenced by a structural change in channel domains that communicate cAMP binding with channel gating. A similar mechanism may explain the lack of cAMP sensitivity in members of the Erg K^+ channel family that also contain a CNBD [36, 58, 59].
The activation time constants and voltage-dependence of HCN channels are strongly influenced by experimental parameters (e.g. pulse protocol, temperature) and the intracellular milieu (e.g. pH, concentration of modulatory factors) [33, 54, 60]. This intrinsic sensitivity may explain some of the variability of biophysical parameters that have been found for the same HCN channel by different laboratories [54].

Model for dual channel activation. The dual activation of HCN channels by voltage and by cyclic nucleotides has been described by a cyclic allosteric model [61, 62]. According to this model, each of the four subunits of the tetrameric channel are independently gated by voltage. Every time a voltage sensor switches to the activated state the probability for channel opening increases. The opening/closing reactions occur allosterically and involve concerted transitions of all four subunits. This transition occurs if the channel is

unliganded, partially liganded or fully liganded and is energetically stabilized by a constant amount for each cAMP bound. The model further assumes that cAMP has a higher binding affinity to open than to closed channels [61–63]. The model reproduces the characteristic features of HCN channel gating like the voltage-dependence and the activation by cyclic nucleotides. In addition, several kinetic features are well described by the model, including the delay in current activation and deactivation. However, the model also has several limitations. For example, in the original model, the mode shift and the voltage hysteresis cannot be explained. To solve this issue the model has been extended [51]. Moreover, there is evidence [64] that the final allosteric open/close transition is not voltage-dependent as suggested previously [61]. Recently a modified cyclic allosteric model has been suggested to explain the efficacy of cyclic nucleotide binding to HCN channels [40].

Single channel conductance. There is an ongoing controversy on the size of the single channel conductance of I_h. Originally, single channel conductance was found to be very low, being in the range of around 1 pS [65]. This estimate is in good agreement with very recent data [66]. However, single channel conductances that are 10–30 times higher have been reported for cloned HCN channels [67] as well as for native cardiac [67] and neuronal I_h [68]. An explanation for this major discrepancy is not known, but may stem from the different cell preparations or patch configurations (i.e. cell-attached *versus* inside-out). Furthermore, single channel conductance as well as other biophysical parameters may be dynamically regulated by modulatory factors and proteins assembled with HCN channels *in vivo*. The regulatory network in which HCN channels are embedded may profoundly vary from cell type to cell type and, hence, could substantially contribute to the diversity of biophysical parameters reported for native I_h. However, extreme care must be taken before a final conclusion is drawn on this issue. It will be necessary to rigorously prove that the recorded currents are indeed HCN channel currents. For example, one problem with single channel recording reported by Michels et al. [67] is that the ensemble records of single channel activity poorly match known kinetic properties of HCN channel current based on whole cell recordings. This issue needs to be clarified in the future.

Regulation of HCN channels

HCN channels are tightly regulated by interacting proteins as well as by low molecular factors (e.g. PIP_2, protons, chloride ions) in the cytosol and the extracellular space. These molecules control the functional properties of the channels in the plasma membrane, regulate their cell surface expression (i.e. the number of functional channels in the membrane) and control their targeting to defined cellular compartments. In the following section we will give an overview on HCN channel regulators that have been identified in the last couple of years.

Regulation by PIP_2. As in many ion channels [69], voltage-dependent gating of HCN channels is allosterically regulated by membrane phosphoinositides, including phosophatidylinositol-4,5-bisphosphate (PIP_2) [70, 71]. These membrane lipids act as allosteric ligands that shift the voltage-dependent activation of HCN channels about 20 mV toward positive potentials independent of the action of cyclic nucleotides. As a result, phosphoinositides adjust HCN channel opening to a voltage range relevant for the physiological role of I_h-channels. Washout of PIP_2 could substantially contribute to the pronounced run-down of HCN currents in excised patches or during prolonged whole-cell recordings that result in an extensive hyperpolarizing shift of the steady-state activation. PIP_2-mediated regulation of HCN channels may be of physiological significance for the function in neuronal circuits, as enzymatic degradation of phospholipids reduces channel activation and slows down firing frequency of neurons. Different levels of PIP_2 may also explain the profound variations in the half-maximal activation voltages of I_h in cardiac cells of different developmental stage or distinct regional distribution [72–74]. The molecular determinants conferring the effect of PIP_2 on HCN channel gating are not yet known. Most likely, HCN channels are activated by an electrostatic interaction between the negatively charged head groups of phosphoinositides and the channel protein. A similar mechanism for PIP_2 modulation of gating was identified in voltage-gated and inwardly-rectifying K^+ channels [75–78]. It was proposed that the interaction between PIP_2 and I_h-channels relieves an inhibition of channel opening conferred by an inhibitory channel domain. This "PIP_2 responsive" domain is clearly different from the CNBD because the PIP_2 effect is still present in channels lacking the CNBD.

Regulation by protons. The activity of HCN channels depends on both intracellular [79, 80] and extracellular [81] concentrations of protons. Intracellular protons shift the voltage-dependence of channel activation to more hyperpolarized potentials and slow down the speed of channel opening. In murine HCN2 a protonable histidine residue (His321) local-

ized at the boundary between the voltage-sensing S4-helix and the cytoplasmic S4-S5 linker has been identified that confers intracellular pH (pH_i) sensitivity [79]. At acidic (pH_i: 6.0) and alkaline pH_i (pH_i: 9.0) the midpoint potential of HCN2 activation is shifted by about 10 mV to more hyperpolarized and depolarized potentials, respectively, compared to physiological pH_i (pH: 7.4) [79]. The effect of intracellular protons on HCN channels may have an important physiological impact for the modulation of HCN channel activity in the brain [80], for example for the regulation of thalamic oscillations and the respiratory frequency. The protective action of carbonic anhydrase inhibitors in generalized seizures has been attributed to the high sensitivity of HCN channels to intracellular pH [82]. Inhibition of the carbonic anhydrase causes an increase in pH_i and augments I_h in thalamocortical neurons. As a result, these neurons are depolarized and their engagement in synchronized paroxysmal discharges is reduced. Inhibition of HCN channels by intracellular acidosis could also be pathophysiologically relevant during cardiac ischemia and heart failure.
Acidic extracellular pH ($pH_e < 5.0$) activates I_h by profoundly shifting the voltage-dependence of HCN channels to more positive voltages. This mechanism was shown to contribute to sour taste transduction in a subset of rat taste cells [81].

Regulation by chloride. The steady-state conductance of I_h is regulated by extracellular chloride [83]. This effect of Cl^- was found to be pronounced for HCN2 and HCN4 while it is rather weak for HCN1 [83]. A single amino acid residue in the pore region was identified as a molecular determinant of extracellular Cl^- sensitivity [83]. Channels with high Cl^- sensitivity (HCN2 and HCN4) carry an arginine residue at this position (R405 in HCN2; R483 in HCN4), while HCN1 carries an alanine (A352). The regulation of HCN channels by Cl^- is probably relevant for heart (patho)physiology. A reduction of the amplitude of sinoatrial I_h that is carried by HCN4 and HCN2 could be involved in the generation of arrhythmias observed in hypochloremia.
Cl^- regulates HCN channel function also from the intracellular site. In a recent study it was shown that intracellular Cl^- acts as a physiological suppressor of the instantaneous component of I_h (I_{INS}) [84]. An increase of intracellular Cl^- from physiological concentrations (10 mM) to high concentrations (140 mM) almost completely abolished I_{INS} while it had no significant effect on the steady-state component of I_h. The physiological relevance of this regulation remains to be determined.

Regulation by tyrosine phosphorylation. Yeast two-hybrid and GST pulldown assays indicate that the non-receptor tyrosine kinase c-Src constitutively binds via its SH3 domain to the C-linker-CNBD of HCN2 and leads to a phosphorylation of the C-linker region of this channel [85]. As a result the activation kinetic of the channel is enhanced. Inhibition of Src by pharmacological blockers or cotransfection of a negative-dominant Src mutant slows down channel kinetics probably via dephosphorylation by not yet defined cellular phosphatases. The residue conferring Src modulation (Y476) has been identified in HCN2 by mass spectrometry. The residue is localized in the B'-helix of the C-linker and is conserved in all HCN channel isoforms. Replacement of this residue by phenylalanine renders the kinetics of HCN2 or HCN4 insensitive to Src. A recent study identified an additional tyrosine residue in HCN4 (Y531 in A'-helix of the C-linker) that may also be involved in Src-mediated channel regulation [86]. At this point the mechanism of Src-mediated regulation of HCN channel gating remains speculative. However, one could imagine that the presence of negatively charged phosphate groups affects the structure and, thereby, the allosteric movement of the C-linker region during channel activation.
Regulation of I_h by tyrosine phosphorylation through Src kinase has been demonstrated under physiological conditions in sinoatrial pacemaker cells in the murine [85] and rat heart [87, 88] as well as in neurons [85]. These results support the notion that the control of the phosphorylation status is indeed an important regulatory mechanism to adjust the properties of I_h to the specific requirements of different types of neurons and heart cells.

Regulation by p38-MAP (mitogen activated protein)-kinase. In addition to tyrosine kinases, HCN channels are also regulated by the serine/treonine kinase, p38-MAP kinase [89]. In hippocampal pyramidal neurons, activation of p38-MAP kinase significantly shifts the voltage-dependent activation towards more positive potentials. This regulation may functionally affect temporal summation and neuronal excitability. It is not clear whether p38-MAP kinase induces the observed effects by direct phosphorylation of the HCN channel protein or by phosphorylation of another protein interacting with these channels.

Regulation by interacting proteins. There is growing evidence that ion channels usually are macromolecular protein complexes that, in addition to the principal pore-forming subunits, contain auxiliary proteins that are required for the fine-tuning of electrophysiological properties, the functional coupling to signaling

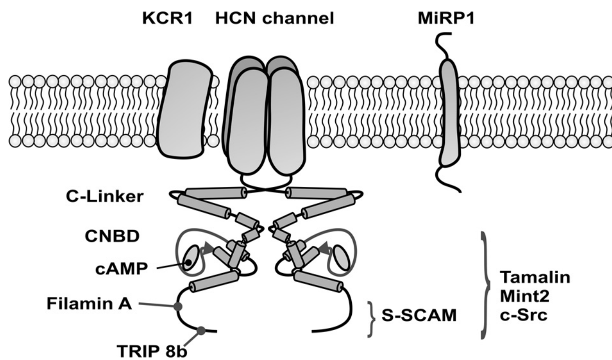

Figure 2. Regulation of HCN channels by interacting proteins. Only two C-termini of the tetrameric channel complex are shown. Proteins interacting with HCN channels are indicated. KCR1, MiRP1, Filamin A, TRIP8b, S-SCAM, Tamalin, Mint2 and c-Src interact with different channel portions as indicated.

pathways, and trafficking to specific cellular compartments. HCN channels make no exception to this rule. In the last years several interacting proteins have been described in the literature (Fig. 2).

The MinK-related protein MiRP1 (encoded by the gene KCNE2) was reported to interact with several HCN channel types [90–92]. MiRP1 is a member of a family of single transmembrane-spanning proteins and is an established auxiliary subunit of the HERG delayed rectifier K^+ channel [93–95]. MiRP1 was found to interact with HCN2 in rat neonatal cardiomyocytes and canine sinoatrial node tissue [92]. Overexpression studies in Xenopus oocytes [91] and neonatal rat cardiomyoctes [92] showed that MiRP1 increases current densities and accelerates activation kinetics of HCN2. By contrast, MiRP1 did not affect voltage-dependence of activation. In overexpression systems, MiRP1 was also found to interact with HCN1 [91] and HCN4 [90]. MiRP1 increased current densities of HCN4 but, unlike in coexpression experiments with HCN1 or HCN2, it slowed down kinetics and induced a negative shift of the activation curve of this channel [90]. Collectively, these results may suggest that MiRP1 is an auxiliary subunit of HCN channels. However, extreme care should be taken before a final decision on this issue is made. It will be necessary to verify the interaction between MiRP1 and HCN channels, including its functional implications in native tissues. Furthermore, the specificity of antibodies used for immunoprecipitations must be confirmed in adequate knockout models. Very recently, another transmembrane protein (KCR1) was reported to interact with HCN2 and native cardiac I_h-channels [96]. KCR1 is a plasma membrane-associated protein with 12 putative transmembrane regions that, like MiRP1, can associate with the HERG K^+ channel [97, 98]. Upon overexpression in CHO cells KCR1 reduces HCN2 current densities and affects single channel current parameters of this channel. Overexpression in rat cardiomyocytes also reduced current densities of native I_h and suppressed spontaneous action potential activity of these cells. From these experiments it was concluded that KCR1 is an inhibitory auxiliary subunit of HCN channels and serves as a regulator of cardiac automaticity. As discussed for MiRP1, further experiments will be required to verify this hypothesis.

Several scaffold proteins interact with the C-terminus of HCN channels. These proteins were mainly identified in neurons and may regulate channel targeting to distinct subcellular compartments (e.g. dendrites or synapses). A brain specific protein termed TRIP8b (TPR-containing Rab8b interacting protein) interacts through a conserved tripeptide sequence in the C-terminus of HCN channels [99]. This protein is thought to play an important role in the trafficking of vesicles to their final targets [100]. TRIP8b colocalizes with HCN1 in dendrites of cortical and hippocampal pyramidal cells. Functional coexpression of TRIP8b with HCN protein, either in native cells or heterologous systems, results in a strong downregulation of HCN channels in the plasma membrane. Based on these experiments and on the analysis of HCN1 knockout mice it was speculated that TRIP8 is involved in the generation of somatodendritic HCN1 channel gradients in cortical layer V pyramidal neurons [99].

HCN2, but not other HCN channel types, interacts with the neuronal scaffold protein tamalin, mostly through a PDZ-like-binding domain [101]. In addition, HCN2 interacts with the scaffold proteins Mint2 and S-SCAM through distinct protein-binding domains at the carboxy-terminal tail. In COS-7 cells, HCN2 levels were increased upon coexpression with Mint2, suggesting that this protein is a positive regulator of cell surface expression of HCN channels [101].

HCN1, but not HCN2 or HCN4, was found to bind filamin A via a 22 amino acid sequence downstream of the CNBD [102]. Filamin A is a putative cytoplasmic scaffold protein that binds actin and thereby links transmembrane proteins, among these are the K^+ channels Kv4.2 and Kir2.1, to the actin cytoskeleton [103, 104]. Based on heterologous overexpression in filamin A-expressing and filamin A-deficient cell lines, it was proposed that filamin causes clustering and slows down activation and deactivation kinetics of HCN1 [102].

I_h-channels localize to membrane lipid rafts in sinoatrial myocytes and in HEK293 cells expressing HCN4, the major HCN channel isoform contributing to native sinoatrial I_h [105]. Coimmunoprecipitation experiments indicate an interaction between HCN4

and caveolin-3, which is a marker protein for so-called caveolae [106]. Caveolae represent a morphologically distinct type of lipid rafts. In cardiomyocytes and pacemaker cells several elements of the β-adrenergic signaling pathway, including β_2 adrenoceptors that regulate HCN channel activity, are localized in caveolae. It was proposed that clustering of HCN4 in caveolae is essential for normal function and regulation of this channel. Indeed, disruption of lipid rafts by cholestrol-depletion caused a redistribution of HCN channels within the membrane and modified their kinetic properties [105].

Tissue expression of HCN channels

HCN channels are mainly expressed in the nervous system and the heart. The expression pattern of all HCN channels has been extensively studied on the protein and mRNA level by several groups [5, 11, 54, 107–112] and will not be summarized here in detail. Briefly, in the brain HCN1 is expressed in the neocortex, hippocampus, cerebellar cortex and in the brainstem [5, 54, 107, 108, 113]. In addition, HCN1 expression was reported in the spinal cord [113]. HCN2 is distributed nearly ubiquitously throughout most brain regions with the highest expression in the thalamus and brainstem nuclei [54, 107, 108]. By contrast, HCN3 is sparsely distributed at very low levels in the central nervous system. Moderate to high expression has only been detected in the olfactory bulb and in some hypothalamic nuclei [107, 108]. HCN4 is strongly expressed in some parts of the brain, e.g. in various thalamic nuclei and in the mitral cell layer of the olfactory bulb [54, 107, 108]. In other brain regions the expression of HCN4 is much lower. In the peripheral nervous system all four HCN subtypes have been reported in the dorsal root ganglion, with HCN1 being the most abundant one [114].
All four HCN channel isoforms have been detected in heart. The expression levels of these isoforms strongly depend on the cardiac region and, in addition, seem to vary between species. In the sinoatrial node, in all species analyzed so far (e.g. rabbit, guinea pig, mouse and dog) HCN4 is the major isoform, accounting for about 80 % of I_h [11, 109]. The remaining fraction of this current is species-dependent. In rabbit this fraction of I_h is dominated by HCN1 [11] while in mice, HCN2 accounts for this fraction [110]. So far, no data on the expression of HCN channels in human sinoatrial node are available.
In other parts of the cardiac conduction system, HCN4 is also the major isoform with expression in the atrioventricular node [111] and the Purkinje fibers [112]. HCN3 is expressed only at very low levels in the conduction system. HCN channels are also present in atrial and ventricular myocytes. In these cells, HCN2 is the dominant isoform displaying a rather ubiquitous distribution. Transcripts of HCN1, HCN3 and HCN4 have also been detected in heart muscle. In general, expression levels of HCN channels are low in normal heart muscle compared to cells of the conduction system. However, upregulation of HCN channels may occur during heart diseases [115–118]. For example, in cardiac hypertrophy and heart failure, HCN2 and HCN4 are upregulated in the atrial and ventricular myocardium [115–121]. Thus, I_h overexpression could well be an important trigger of arrhythmogenic activity in the hypertrophied heart [115–118, 120, 121].

Functional properties of I_h

Steady state and dynamic properties of I_h near the resting membrane potential

In this section, the role of I_h for the regulation of the resting membrane potential is discussed. Firstly, I_h channels are open at rest and set and stabilize the membrane potential in many cells [33, 110, 122–128]. In dendrites of CA1 pyramidal neurons this function of I_h influences the kinetics and the amplitude of propagated excitatory postsynaptic potentials (EPSPs) [129–131]. Secondly, the dynamic voltage-dependent gating of I_h near the resting membrane potential actively opposes changes in membrane potential and thus stabilizes the membrane potential [122, 132–134]. These basic functions provide the leitmotif for the more specific functions of I_h discussed in the following sections.
In sinoatrial pacemaker cells of the heart as well as in many neurons of the central nervous system I_h-channels are constitutively open at voltages near the resting membrane potential [110, 122–124], pass a depolarizing non-inactivating inward current and set the membrane potential to more depolarized voltages. In addition, constitutively open I_h-channels *per se* stabilize the resting membrane potential by lowering the membrane resistance R_m [110, 122–124]. Therefore, in the presence of I_h any given input current evokes a smaller change in membrane potential (ΔV) than in the absence of I_h (Fig. 3A). Constitutively open I_h-channels seem to function as a slow "voltage clamp" [122] tending to stabilize the membrane potential by opposing depolarizing or hyperpolarizing inputs [135]. This effect suppresses low frequency fluctuations in membrane potential [122]. In dendrites, constitutively open I_h-channels influence the passive propagation of excitatory postsynaptic potentials (EPSPs) [129–131]. In the absence of active voltage-

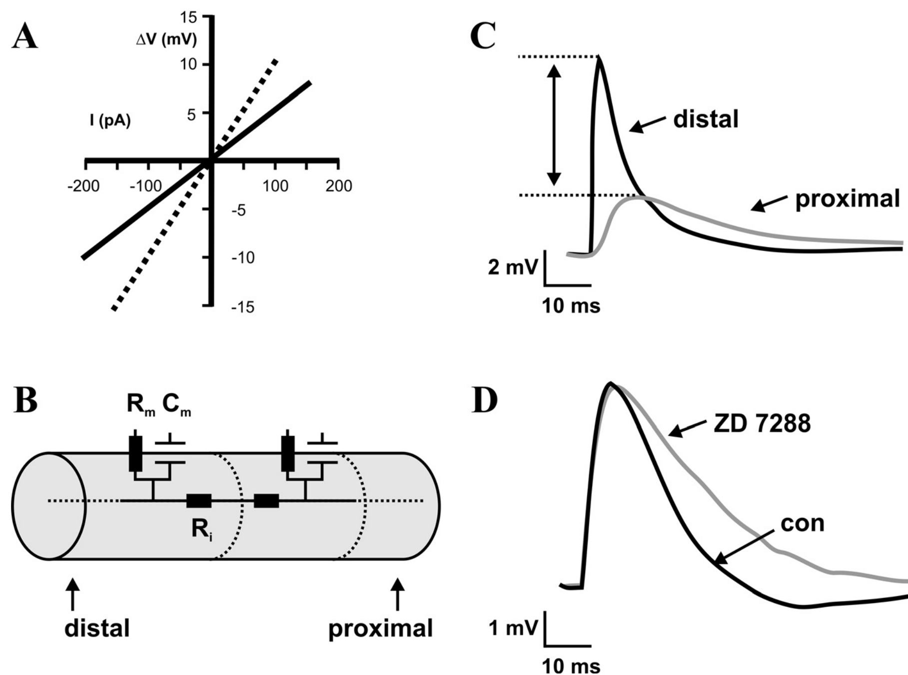

Figure 3. Basic properties of I_h. (*A*) The effect of I_h on the input resistance R_{in}, given by the slope of the current voltage relation. The presence of I_h (solid line) decreases R_{in}. After blockade of I_h by Cs^+ (dotted line) the resistance increases. (*B*) Equivalent electrical circuit of an idealized cylindrical segment of a dendrite. Three electrical components are responsible for the passive features of a dendrite: the specific membrane resistance (R_m), the specific membrane capacitance (C_m), and the intracellular resistance (R_i). For simplicity the resistance of the extracellular space has been neglected. (*C*) The passive properties of a dendrite attenuate the amplitude (arrow) of an EPSP as it spreads from dendrites to the soma (EPSP at the distal synapse: black line, the same EPSP after propagation to the soma: grey line). (*D*) EPSP recorded at the soma after propagation from a distal dendrite before (con) and after blockade of I_h with ZD 7288. In the presence of I_h (black line) the EPSP rise and decay faster than after blockade of I_h with ZD 7288 (grey line).

dependent ion channels, the passive properties of a dendrite attenuate the amplitude and slow down the kinetics of an EPSP as it spreads from its site of origin in the dendrites to the soma (Fig. 3B–D). This process is comparable to a wave travelling across water. Just as a wave moving across water widens and diminishes in height over distance, EPSP signals can degrade with distance along the neuronal membrane (Fig. 3C). The presence of I_h in dendrites lowers R_m and thereby further increases the amplitude attenuation of EPSPs. In addition, I_h counteracts kinetic filtering of propagating EPSP also by lowering R_m. Therefore, the EPSP upstroke and decline are faster (Fig. 3D). In the absence of I_h (e.g. after blockade of I_h by a specific I_h blocker ZD 7288 or after genetic deletion) somatic EPSPs rise and decay more slowly if they are generated in distal dendrites compared to proximal dendrites [129–131, 136] (Fig. 3B).

An important part of the stabilizing effect of I_h results from its dynamic voltage-dependent gating. Voltage-dependent activation and deactivation of I_h actively oppose deviations of the membrane potential away from the resting membrane potential [122, 132–134]. This property is a consequence of the unusual relation between the activation curve and the reversal potential of I_h. In contrast to voltage-gated Ca^{2+} and Na^+ channels (Fig. 4B right, black line), the reversal potential of I_h falls close to the base of its activation curve [137] (Fig. 4B left, black line). If membrane hyperpolarization increases the fraction of open I_h-channels the depolarizing inward current drives the membrane potential back towards the reversal potential of I_h and thereby to the initial value. This important feature can be demonstrated in a simple current clamp experiment (Fig. 4A). A negative current step causes a membrane hyperpolarization that activates I_h. As a result, a slowly depolarizing inward current develops that partially counteracts the hyperpolarization, drives the membrane voltage towards the reversal potential of I_h and thereby back close to its initial resting potential (Fig. 4A left). This characteristic effect is called "depolarizing voltage sag" [33, 138]. At the end of the current step, a brief rebound afterdepolarization occurs before the membrane potential returns to rest, reflecting an inward current through deactivating I_h-channels. A depolarizing current step deactivates I_h, reducing the inward current, which then reverses the depolarization. This partial hyperpolarization is called "hyperpolarizing voltage sag" [33, 138] (Fig. 4A right). At the end of the current step, a brief rebound afterhyperpolarization occurs. This results from a high fraction of initially

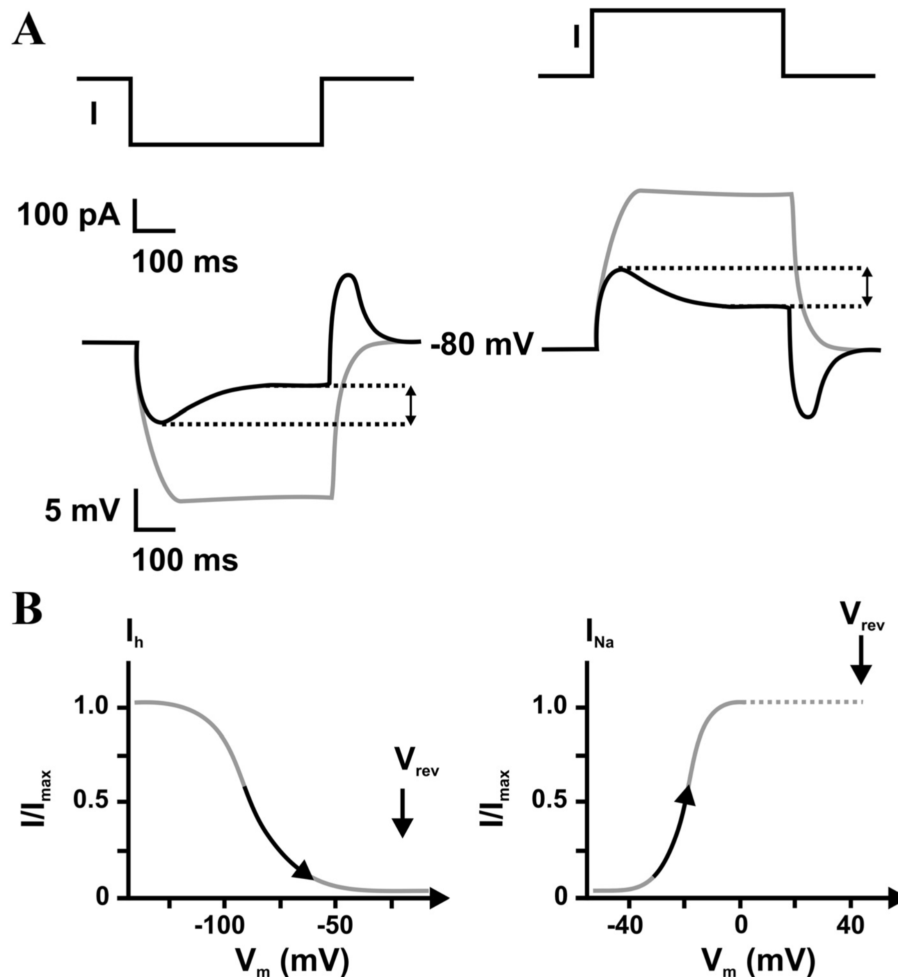

Figure 4. (*A*) I_h actively opposes changes in membrane voltage. Cartoon of a current clamp experiment in a neuron. In the presence of I_h, a hyperpolarizing current step (-200 pA) induces a depolarizing voltage sag (arrow, left panel), a depolarizing current step (200 pA) induces a hyperpolarizing voltage sag (arrow, right panel). Blockade of I_h by Cs^+ eliminates the sag (grey traces). (*B*) The reversal potential (V_{rev}) of I_h falls near the base of its activation curve. Therefore I_h actively opposes changes in membrane voltage. Partial activation of the current drives the membrane potential towards the reversal potential on the base of the activation curve (left, arrow in the activation curve) and thus is self limiting and stabilizing for the membrane potential. By contrast, voltage-gated currents whose reversal potential falls near the top of its activation curve (e.g. I_{Na}; right) are destabilizing and self regenerative. Partial activation drives the membrane potential towards the reversal potential at the top of the activation curve (right, arrow in the activation curve).

deactivated I_h-channels before the activation of I_h drives the membrane potential back to the resting level. As will be described below the voltage sag and voltage rebound are a prerequisite for resonance [137].

Involvement of I_h in the control of neuronal functions

I_h is involved in several important neuronal functions (Fig. 5). Six functions of I_h are prominent and are discussed in the following section: The role of I_h for dendritic integration (1), for the control of working memory (2), for long term potentiation (3), for synaptic transmission (4), for resonance and oscillations (5), as well as for motor learning (6).

I_h in dendritic integration

I_h is involved in the process of dendritic integration [129–131, 136]. Dendritic integration has been extensively studied in CA1 hippocampal and neocortical pyramidal neurons. This process is crucial in most neurons, since single EPSPs are too small to bridge the gap between the resting membrane potential and the action potential threshold. Therefore, at the soma multiple synaptic inputs must integrate and sum up to produce action potential firing. In this context I_h and most notably the component encoded by HCN1 channels has an important role [129–131, 136, 139]. The way incoming EPSPs are summed up in time largely depends on kinetic filtering by passive cable properties of the dendrites. As mentioned above, dendritic filtering slows down the time course of EPSPs resulting in somatic EPSPs that rise and decay more slowly if they are generated in distal dendrites compared to proximal dendrites (Fig. 3B, C). As a consequence of filtering one would expect that repetitive EPSPs arising from more distal synapses should summate at the soma to a greater extent and over a longer time course than EPSPs generated in more proximal dendrites. However, in many neurons, e.g. CA1 [129, 130] and neocortical layer 5 pyramidal

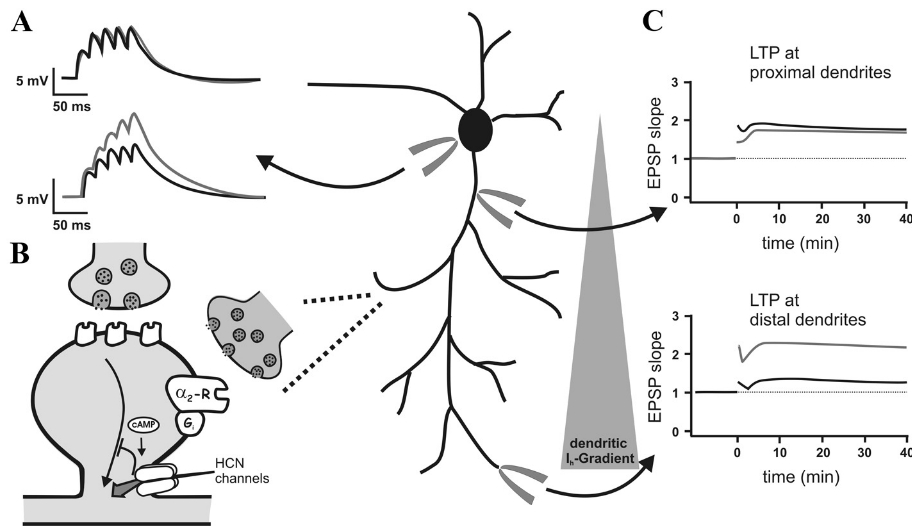

Figure 5. Proposed roles of I_h in various neuronal functions. (*A*) The somato-dendritic gradient of I_h effectively counteracts kinetic filtering by dendrites and normalizes the localization dependence of temporal summation. EPSP from proximal (black trace) and distal (grey trace) dendrites recorded at the soma after propagation. The voltage recordings are shown before (upper panel) and after (lower panel) inhibition of I_h by the selective I_h blocker ZD 7288. (*B*) Hypothetical model of α_2-adrenoceptor-cAMP-HCN regulation in prefrontal cortex neuron networks involved in the control of spatial working memory. For details see text. α_2-R: α_2-adrenoceptors; G_i: G_i-protein. (*C*) HCN1 constrains LTP in perforant path synapses on distal dendrites of CA1 neurons (lower panel, grey line: HCN1 deficient mice, black line: WT mice), but not in Schaffer collateral synapses on proximal dendrites (upper panel, grey line: HCN1 deficient mice, black line: WT mice).

cells [136, 140, 141], this localization dependence of temporal summation is not observed. This discrepancy is probably explained by a gradient of I_h whose density rises progressively by more than six-fold with distance from the soma [129–131, 136, 142] (Fig. 5). This gradient efficiently counteracts dendritic filtering by lowering R_m (see above). In addition, during the rising phase of an EPSP I_h-channels rapidly deactivate. Turning off the inward current carried by I_h leaves an effective net outward current that hyperpolarizes the plasma membrane and accelerates the decay of each EPSP (Fig. 3C, D). Because of the density gradient of I_h along the dendrite, distal EPSPs decay faster and are therefore shorter (Fig. 3C, D). As a consequence, after propagation to the soma the temporal summation is more dampened for distal than for proximal inputs. Therefore, the temporal summation of all inputs reaching the soma is about equal (Fig. 5A). However, the normalization of somatic EPSP time course by I_h comes at a cost. The activation of I_h increases the dendro-somatic attenuation of EPSP amplitude and thus the dependence of somatic EPSP amplitude on synapse location (Fig. 3C). As a result, I_h-channels act as a spatial filter that preferentially dampens distal inputs.

Collectively these results may suggest that the proposed role of I_h in dendritic integration critically depends on an increasing somato-dendritic I_h gradient. However, there are studies indicating that there is a shallower I_h gradient or even a reversed I_h distribution in a subset of CA1 pyramidal cells [143, 144]. Interestingly, in these cells the dendritic integration and temporal summation are similar as in pyramidal cells with a distal enrichment of I_h. This controversy indicates that normalization of temporal summation may occur in a variety of I_h subcellular distribution patterns and that further studies are needed to clarify this issue.

Besides I_h-channels, other voltage-gated ion channels also have important roles in dendritic integration. For example, a gradient of A-type potassium channels rising from proximal to distal dendrites has been reported [140, 145–147]. These potassium channels counteract dendritic depolarization and thereby modulate dendritic integration. In addition, it has been shown that the density of AMPA glutamate receptors is increased in distal dendrites of several neurons, increasing the strength of distal synapses [148–150]. The relative importance of individual ion channels and receptors to the complex process of dendritic integration may vary among different neurons.

A complementary or possibly alternative role in HCN channels in distal dendrites has been suggested recently [139]. In this study it was suggested that I_h in distal dendrites controls Ca^{2+} spiking by setting the resting membrane potential (see below, section "I_h constrains LTP in CA1 neurons"). It will be an interesting challenge for future research to determine which, if any, of these proposed roles are functionally important.

I_h controls working memory

A recently discovered role of I_h is the control of the spatial working memory [151]. This form of memory depends on prefrontal cortical networks that have the unique ability to represent information that is no longer present in the environment and to use this "representational knowledge" to guide behavior. In monkeys performing a spatial working memory task prefrontal cortical neurons increase firing in response to visual stimuli presented in the preferred direction, while they decrease firing when stimuli are presented in the nonpreferred direction. This property is called spatial tuning. Spatial tuning is regulated by the opposing effects of adrenoceptors and D1 receptor on intracellular cAMP signaling [152, 153]. It has been suggested that HCN channels are important downstream targets for cAMP in this signaling pathway [151]. It was shown that HCN channels colocalize and functionally interact with α_{2A}-adrenoceptors on dendritic spines of prefrontal neurons. The functional interaction was explained according to the following model [151]: α_{2A}-adrenoceptors are activated by relatively low levels of norepinephrine released during alert and wakefulness [154]. The activation of α_{2A}-adrenoceptors inhibits the production of cAMP via G_i signaling and thus reduces the open probability of HCN channels. As a result, R_m, the efficiency of synaptic input and the network activity of these neurons increase. Based on this model it was speculated that the reduced HCN channel activity on spines receiving inputs from neurons with similar spatial properties increases the firing to preferred spatial directions and thereby augments relevant information [152]. By contrast, activation of D1 receptors increases the production of cAMP via G_s signaling. As a result, HCN channels open, reduce the membrane resistance and, thereby, decrease inputs to the spines (Fig. 5B). This could selectively disconnect spinous inputs to the dendrites. Thus, activation of D1 receptors on spines receiving inputs from neurons with dissimilar spatial properties could suppress irrelevant information ("noise") from the non preferred direction [152, 153, 155]. So far, the regulation of HCN channels by D1 receptors has not directly been demonstrated and therefore remains hypothetical [152]. During stress, higher concentrations of norepinephrine may be present and activate low affinity α_1- and very low affinity β_1-adrenoceptors, giving rise to increased cAMP levels [154]. In this situation cAMP levels could significantly increase the open probability of HCN channels and functionally disconnect spinous inputs to the dendrites [152]. Thus, connections of the prefrontal cortex would be functionally cut off.

An alternative modulation of HCN channels by α_2-adrenoceptors in prefrontal neurons has been proposed by a different group [156]. Carr et al. suggest that α_2-adrenoceptor activation suppresses HCN channels in prefrontal neurons through a signalling pathway that does not appear to involve cAMP, but appears to be mediated by PLC-PKC signalling. This is not entirely consistent with the model proposed by Arnsten et al. indicating that future experiments are required to clarify this issue.

I_h constrains LTP in CA1 neurons

The role of I_h encoded by HCN1 for long term synaptic plasticity (LTP) and dendritic excitability has recently been determined for CA1 pyramidal neurons [123, 139]. Mice in which the HCN1 gene has been selectively deleted from the forebrain, show an increase in spatial learning in behavioral experiments. For example, these mice show an enhancement in their ability to learn to navigate to a hidden platform in a water maze. In the hippocampal CA1 region the cellular basis of the observed behavioral effects was also characterized. The major finding of this study is that these mice show an increase in temporal integration and long-term potentiation of EPSPs generated at synapses contacting the distal dendrites of pyramidal neurons from the CA1 region of the hippocampus (Fig. 5C). These neurons provide the major output of the hippocampus and receive two major sources of excitatory synaptic input (Fig. 6A). One set of inputs, the Schaffer collaterals, comes from hippocampal CA3 pyramidal neurons and terminates on regions of CA1 neuron dendrites that are relatively close to the cell body, an area with only moderate HCN1 expression. The second set of inputs, the perforant path, represents a direct connection from layer III neurons of the entorhinal cortex and terminates on the distal dendritic regions of the CA1 neurons, an area where HCN1 expression is very high. At the more proximal Schaffer collateral pathway both synaptic transmission and long-term plasticity are relatively unaffected by the deletion of HCN1, consistent with the relatively low expression of HCN1 in this dendritic region. By contrast, at distal perforant path synapses a significant enhancement in the integration of synaptic potentials and an increase in LTP was observed, consistent with the relatively high expression of HCN1 in this region. From these results it was concluded, that HCN1 channels exert an inhibitory constraint on dendritic integration and synaptic long term plasticity at the perforant path inputs to CA1 pyramidal neurons. Consistent with this conclusion, in wild type mice

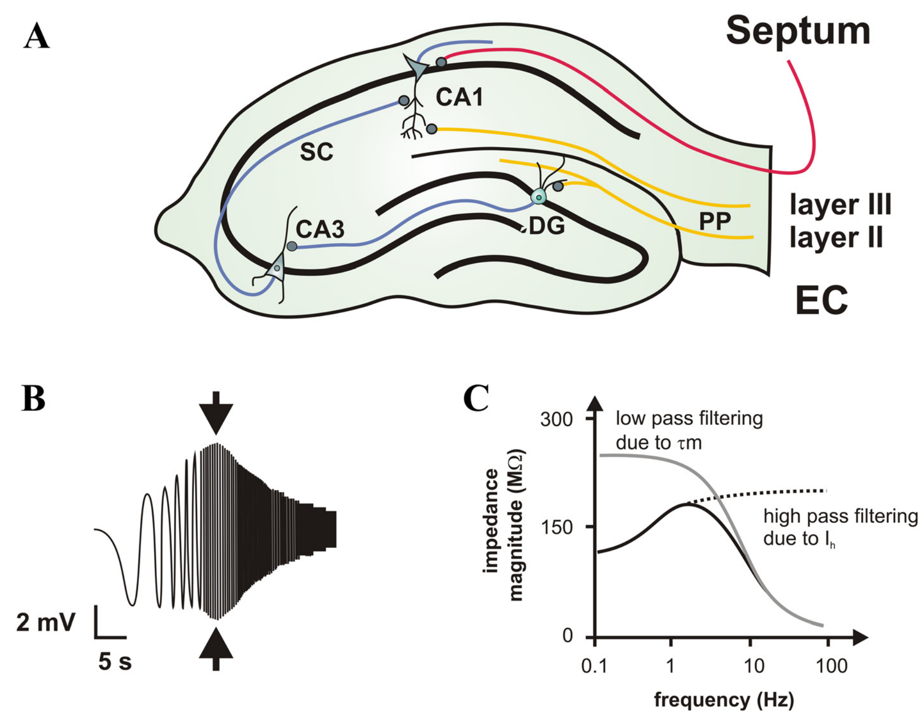

Figure 6. Proposed roles of I_h in resonance and oscillation. (*A*) Anatomy of the hippocampal formation. For details see text. EC: entorhinal cortex; CA1: CA1 pyramidal neurons; PP: perforant path input; DG: dendate gyrus; CA3: CA3 pyramidal neurons; SC: Schaffer collaterals. (*B*) Resonance is formed by the interaction of active and passive properties in a neuron. The response to a ZAP current (current with linearly increasing frequency) shows a prominent resonant peak (arrows). (*C*) I_h and passive membrane properties of the membrane act in concert to produce resonance. The broken line shows the contribution of I_h, the grey line the contribution of the passive membrane properties (τ_m) to the total impedance. The bell shaped curve is the resulting impedance curve for the combined system (black line).

hippocampal-dependent spatial learning was impaired [123]. An alternative explanation could be that learning was not impaired in wild-type mice, but appeared to be enhanced in the knockout mice. The mechanism behind these findings was suggested recently [139]. HCN1 channels limit LTP by inhibiting Ca^{2+} spikes at distal dendrites of CA1 pyramidal neurons. Such spikes have been suggested to be important for the induction of LTP at the perforant path synapses, since somatic action potentials fail to back-propagate into the distal CA1 dendrites [157, 158]. The distal Ca^{2+} spikes in CA1 neurons are triggered by synaptic activation of AMPA and NMDA receptors. The activation of these glutamate receptors generates an initial depolarization that most likely activates T-type (Cav3.3) and N-type voltage-dependent Ca^{2+} channels. The inhibitory effect of HCN channels has been explained by their effect on the resting membrane potential. The activation of I_h makes the resting membrane potential more positive and thereby shifts voltage-gated T- and N-type channel to their respective inactivated states. Thus, the availability of these channels to participate in an action potential is decreased and Ca^{2+} influx during the distal Ca^{2+} spikes is reduced. In other words, HCN1 can be considered a partial brake that constrains dendritic Ca^{2+} spikes. Releasing the brake in knockout mice leads to an enhancement of dendritic excitability.

The role of I_h in motor learning

The role of I_h encoded by HCN1 for motor learning has recently become clear in mice with a global deletion of HCN1 [124]. Deletion of HCN1 causes profound learning and memory deficits in behavioral tests which require complex and repeated coordination of motor output by the cerebellum (e.g. in visible platform water maze and rotarod tasks). In contrast, the deletion of HCN1 does not modify acquisition or extinction of eyelid conditioning, a discrete motor behavior that also involves cerebellar synaptic plasticity. The actions of HCN1 were tested in cerebellar Purkinje cells, the key component of the cerebellar circuit required for learning of correctly timed movements [124]. In these spontaneously spiking neurons, HCN1 mediates a depolarizing inward current that counteracts inputs that hyperpolarize the membrane below the threshold for spontaneous spiking. By stabilizing the integrative properties of Purkinje cells, HCN1 enables the Purkinje cells to maintain an input-output relation that is independent of the neuron's previous history of activity. This function of HCN1 is required for reliable encoding of information and ensures accurate decoding of input patterns.

I_h and synaptic transmission

Electrophysiological recordings have demonstrated the presence of I_h in several presynaptic terminals,

including the crustacean neuromuscular junction [159], avian ciliary ganglion [160], cerebellar basket cells, and the calyx of held in the auditory brain stem [161]. It was suggested that an important role of these presynaptic I_h-channels consists of controlling synaptic transmission. In support of this hypothesis, long-term facilitation of synaptic transmission in crustacean motor terminals was shown to be conferred by cAMP-dependent upregulation of I_h [159, 162]. The downstream pathway coupling I_h activation to synaptic release is not yet known. However, there is initial evidence that I_h-channels may directly interact with the release machinery, perhaps mediated by the cytoskeleton [159, 162]. In vertebrates, the functional relevance of I_h in regulating synaptic transmission is currently disputed. Mellor et al. reported that presynaptic I_h is involved in the generation of LTP in hippocampal mossy fiber synapses that terminate on CA3 pyramidal cells [163]. However, this finding was challenged by another study that, in the same system, showed that LTP is independent of I_h [164].

The role of I_h in resonance and oscillations in single neurons and neuronal networks

The term "resonance" describes a property of a neuron to selectively respond to inputs at a preferred frequency [137]. In electrophysiological experiments, resonance can be induced by applying a current stimulus with linear increasing frequency (ZAP current). In many neurons the voltage answer shows a prominent resonant peak at the natural frequency or Eigenfrequency of the neuron (Fig. 6B arrow). To generate resonance a neuron needs to have properties of a low pass filter combined with properties of a high pass filter [137, 165, 166]. The low pass filtering is caused by the membrane time constant τ_m. The high pass filtering is caused by the action of several voltage-gated currents including I_h. To act as a high-pass filter, these currents firstly need to be able to actively oppose changes in membrane voltage (Fig. 4A, B). Secondly, these currents have to activate slowly relative to the membrane time constant. Both requirements are met for I_h. At low frequencies the I_h-channels have time to activate and actively oppose changes in membrane potential. At high frequencies there is not enough time for the I_h-channels to open. As a result, high frequency changes are not suppressed, rendering the neuron responsive for fast trains of spikes. Resonance arises at intermediate frequencies where input induced voltage changes are too high to be opposed by I_h and too low to be filtered by τ_m. The importance of I_h for the generation of membrane resonance has been demonstrated for several neurons, for example in neocortical pyramidal cells of the rat [167], in subicular pyramidal neurons of the rat [168] and in neurons from the sensorimotor cortex of juvenile rats [169].

Sustained rhythmic oscillations are a hallmark feature of neuronal circuits in various brain regions [166, 170]. Oscillations arise from synchronized activity of neurons and are thought to play an essential role in information processing in neuronal networks. There are several types of oscillations in different frequency bands, called delta, theta, gamma and fast "ripple" oscillations [166, 170]. One oscillation, the theta frequency oscillation (4–12 Hz) is prominent in all areas of the hippocampal formation. These oscillations may be important for various cognitive processes including processing, encoding and storing of spatial information [170] as well as for memory formation and retrieval [171, 172]. Hippocampal theta oscillations have been proposed to originate from reciprocal interactions between rhythmic inputs from the medial septum-diagonal band of Broca, the entorhinal cortex and other subcortical structures (Fig. 6A; [173–175]). The inputs from septal neurons to the hippocampus seem to be mainly responsible for the generation of the theta rhythm [170, 173]. In this process the perforant path and various other brain regions are also involved [176, 177].

In mice with a forebrain-restricted deletion of HCN1 changes in hippocampal-dependent network oscillation have been described [123]. While low- and high-frequency network oscillations appeared to be unchanged in the knockout mice, there was a significant enhancement in the theta frequency band (4–12 Hz) during REM sleep and during wheel running [123]. Consistent with these results, in CA1 neurons responses to low-frequency inputs at the theta range are preferentially increased in HCN1 deficient mice. HCN1 channels can therefore be thought of as a partial brake that contributes to resonance by preferentially dampening low-frequency components of input waveforms at frequencies below the theta range. Releasing the brake in knockout animals causes a general enhancement in the voltage response to low-frequency oscillatory currents.

Besides the important role of I_h in the generation of theta oscillation, I_h is also involved in the generation of other types of oscillations. There are several studies that have demonstrated the importance of I_h for γ-oscillations in the hippocampus [178, 179], synchronized oscillations in the inferior olive [180], and subthreshold oscillations in the entorhinal cortex [181, 182]. Moreover, I_h has been suggested to influence network oscillations that play an important role in learning and memory [126, 135, 178, 183].

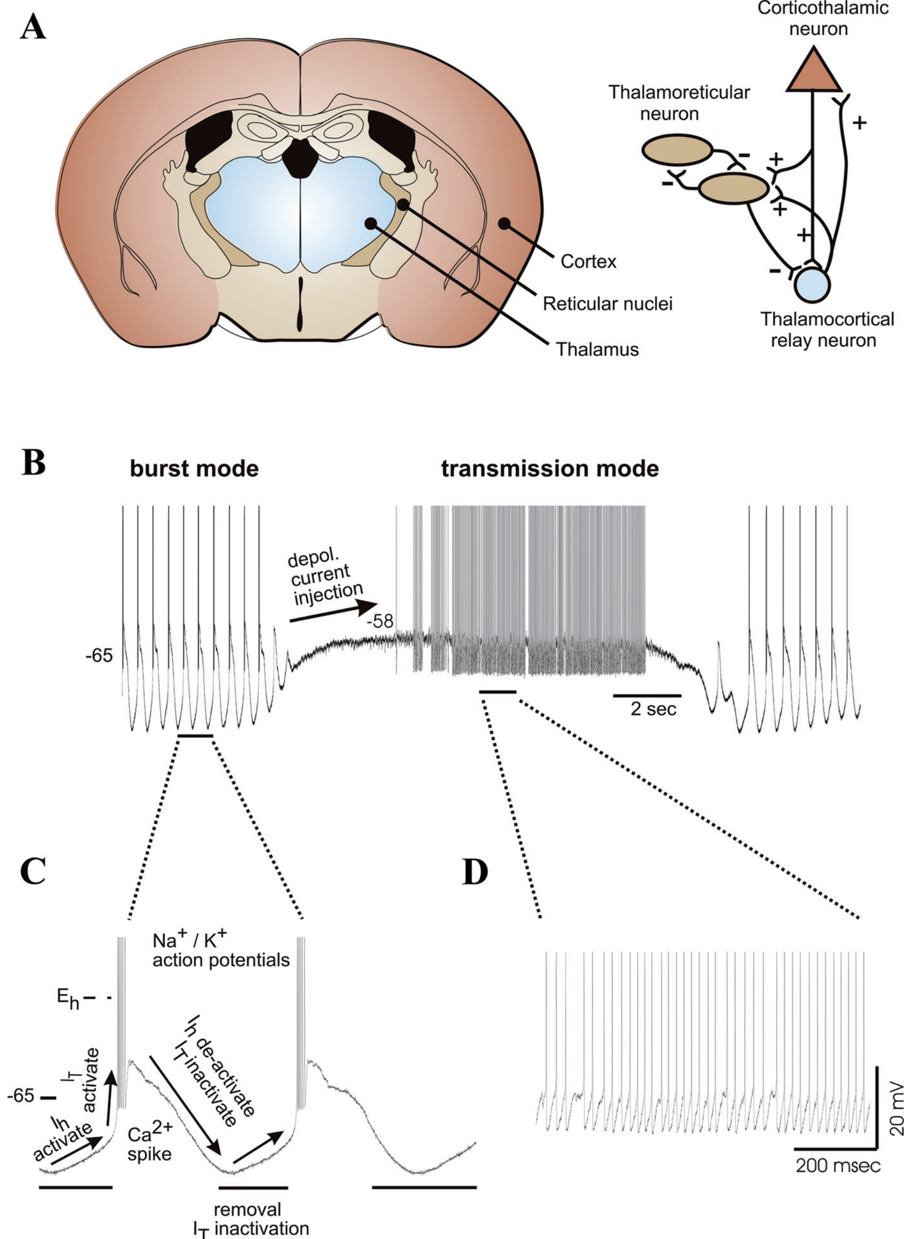

Figure 7. The role of I_h for the generation of thalamic oscillations. (*A*) Left: coronal section through the mouse brain. The cortex, the reticular thalamic nuclei and the thalamus are indicated. Right: Schematic diagram of the thalamocortical loop. "+" indicates excitatory glutamatergic synaptic contacts, "-" indicates inhibitory GABAergic contacts. For detailed information see text. (*B*) Firing modes of thalamocortical neurons. (*C*), (*D*) higher temporal resolution of sections shown in B. Modified from [184].

The role of I_h in the generation of thalamic rhythms by single neuron and network oscillations

Synchronized neuronal oscillations are produced in thalamocortical networks during sleep [184–187], sensory processing [188] and seizures [189]. The generation and synchronization of many oscillations (spindle waves and slow waves) require extensive synaptic interaction within the thalamocortical network [190]. By contrast, a delta frequency rhythm can be generated in single cells [184, 190, 191]. We first discuss the role of I_h in the generation of characteristic firing patterns that arise in single thalamocortical cells and then discuss the role of I_h in oscillations that require more extensive synaptic interactions. In our discussion we will focus on data from *in vitro* slice preparations due to the current paucity of *in vivo* data.

Single cell oscillations in thalamic relay neurons

In vitro [192–198] and *in vivo* [185, 198–202] studies have shown that thalamocortical neurons fire in two distinct firing modes called transmission mode and burst mode [192–198] (Fig. 7B). During wakefulness and REM sleep thalamocortical neurons are depolarized by afferent inputs and switch to the transmission- or "single spike"- mode [203–205]. During this mode

information is gated through the thalamus and forwarded to the cortex [184, 191]. The transmission mode is characterized by the generation of repetitive single Na^+ spikes at depolarized potentials where T-Type Ca^{2+} channels are inactivated (Fig. 7D). During this mode, sufficient excitation repetitively discharges thalamocortical neurons. The output-frequency of these neurons increases with increasing depolarization induced by afferent excitatory inputs [206].

During the burst mode, thalamic networks display a monotonous repetitive firing pattern at hyperpolarized membrane potentials [203, 204, 207–209]. This firing mode is characteristically seen during non-REM sleep or epileptic discharges and has been considered to decrease transfer of sensory information to the cortex [207]. *In vitro* studies have demonstrated that thalamic neurons fire in the "burst mode" through the interaction of a low threshold Ca^{2+} current (I_T) and I_h (Fig. 7B, C) [33, 184]. The following model has been proposed: The activation of I_h by hyperpolarization beyond −65 mV slowly depolarizes the membrane potential until a rebound low-threshold Ca^{2+} spike is generated by the activation of I_T at more depolarized potentials. Because these "slow spikes" are depolarizing and last for tens of milliseconds, typically a series of Na^+ spikes rides on it. The inactivation of I_T terminates the low threshold Ca^{2+} spike. During the spike I_h is deactivated. As a result, the termination of the spike is followed by a hyperpolarizing "overshoot". In turn, I_h is activated, the cycle repeats and continuous rhythmic burst firing is sustained. The intrinsic interplay of these currents promotes delta rhythmicity in single thalamocortical neurons [210–215]. These single cell oscillations are synchronized in large neuronal circuits and can be recorded in the EEG as delta waves during non-rapid eye movement (non-REM) sleep [191, 212, 216, 217].

Network oscillations

I_h is involved in the generation of a number of rhythmic oscillations in thalamocortical networks. During the early state of non-REM sleep synchronized oscillations are generated in the thalamocortical network that give rise to spindle waves in the surface EEG [185, 191, 193]. Spindle waves are characterized by a crescendo-decrescendo type of oscillation at 6–14 Hz that last for 1–4 seconds followed by a refractory period of 5–20 seconds that terminates the oscillations [185, 191, 218, 219]. These waves are assumed to have an essential functional role in synaptic plasticity of cortical and thalamic neurons [207]. Spindle waves have been investigated in *in vivo* studies [186, 199, 220–223] and after the discovery of slice preparations that spontaneously generate spindle waves [224] *in vitro*. These studies indicate that spindle waves are generated by a cyclic interaction between excitatory thalamocortical cells and inhibitory thalamic reticular neurons [185, 191, 192, 198, 224] (Fig. 7A). Thalamic reticular cells are the pacemakers for the generation of spindle wave oscillations [225, 226]. In these neurons rhythmic bursts are generated by low-threshold Ca^{2+} spikes. Burst firing in thalamic reticular neurons induces rhythmic IPSPs in thalamocortical cells. These IPSPs hyperpolarize thalamocortical cells remove inactivation of the low-threshold Ca^{2+} current and at the same time activate I_h. The resulting depolarization triggers a rebound Ca^{2+} spike and a burst of action potentials (Fig. 7C). These bursts re-excite the thalamic reticular neurons and also stimulate cortical pyramidal cells. The nearly simultaneous occurrence of spindle waves over widespread cortical territories is produced by network synchronization involving the cortex and the thalamus [227]. This synchronized activity can be recorded in EEG as spindle waves.

The silent period between spindle waves is largely determined by persistent I_h activation in thalamocortical cells [185, 191, 193]. This persistent activation of I_h results from an increase in intracellular Ca^{2+} primarily triggered by the rebound low-threshold Ca^{2+} bursts that occurred during the spindle wave generation. The Ca^{2+} ions most likely activate a Ca^{2+} sensitive isoform of adenylyl cyclase, producing an increase in cAMP synthesis that enhances I_h [196]. The persistent activation in turn suppresses the next spindle wave until I_h is slowly decayed. Spindle waves disappear during wakening or REM sleep. The transition to these states is regulated by several neurotransmitters such as norepinephrine and serotonin that both increase intracellular cAMP and lead to a consecutive upregulation of I_h [184].

In absence epilepsy, normal sleep spindle oscillations observed during sleep or drowsiness may develop into paroxysmal synchronization that gives rise to spike-and-wave discharges in the EEG [189, 226, 228–232]. Although absence epilepsies are mainly generated within the cortex, the thalamus is also involved [189, 232]. There is evidence that dysregulation or loss of I_h in the thalamus can lead to absence epilepsy [110]. In thalamocortical cells of mice with a global deletion of HCN2 [110] I_h is almost completely lost, resulting in a pronounced hyperpolarizing shift of the resting membrane potential. This hyperpolarizing shift partially removes inactivation of T-Type Ca^{2+} channels. Thus, thalamocortical cells mainly fire in the burst mode. Interestingly, the mice suffer from absence epilepsy with characteristic spike-and-wave discharges in the EEG and periods of behavioural arrest. In human the

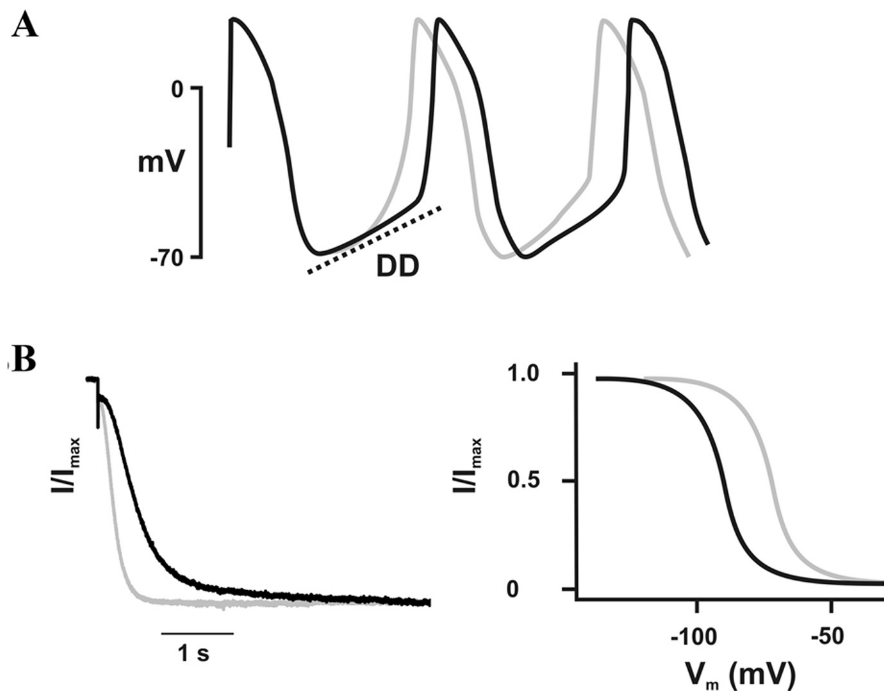

Figure 8. The role of I_h for cardiac automaticity. (*A*) Pacemaker potentials in the absence (black) and presence (grey) of adrenergic stimulation. DD: diastolic depolarization. (*B*) Left: sinoatrial I_h activates faster in the presence of cAMP (grey) than in the absence (black) of cAMP at maximal activation voltage (–140 mV). Right: activation curve of I_h in the absence (black) and the presence (grey) of cAMP. The activation curve of I_h is shifted to the right in the presence of cAMP.

role of I_h in the generation of absence epilepsy remains to be defined.

Role of I_h in cardiac rhythmicity

The heart beat originates from specialized pacemaker cells in the sinoatrial (SA) node region of the right atrium. These cells generate a special kind of action potential ("pacemaker potential") that is characterized by the presence of a progressive diastolic depolarization (DD) in the voltage range between –65 to –45 mV (Fig. 8A). After the repolarization phase, it is the DD that drives the membrane potential of an SA node cell back toward the threshold of calcium channel activation, thereby maintaining firing. The DD is generated by the concerted action of several currents, among which I_h is considered to play a prominent role because it is activated at negative potentials and, therefore, potentially could serve as primary initiator of the DD. In addition to I_h, other inward currents, including calcium currents (I_{CaT} and I_{CaL}) [233], as well as the sustained inward current (I_{st}) [234], whose molecular correlate is not yet known, may contribute to the DD. Furthermore, it was proposed that the DD may be initiated by the decay of outward rectifier K^+ currents (I_{Kr} and I_{Ks}) or by mechanisms involving intracellular Ca^{2+} release through ryanodine [235–237] or IP_3 receptors [238]. I_h is not only involved in principal rhythm generation, it also plays a key role in heart rate regulation by the autonomic nervous system. Sympathetic stimulation activates I_h and, hence, accelerates heart rate via β-adrenoceptor-triggered cAMP production (Fig. 8B) while vagal stimulation lowers heart rate via inhibition of cAMP synthesis and an ensuing inhibition of I_h activity.

Recent genetic mouse models have made it possible to evaluate the proposed roles of I_h in cardiac rhythmicity under *in vivo* conditions. As mentioned above, HCN4 makes up about 80 % of SA node I_h and has been considered to be crucial for the generation of the heart beat [239–241]. In support of this notion, mice with global- or heart-specific disruption of the HCN4 gene die *in utero* between embryonic days 10 and 11.5 [241]. The embryonic hearts of HCN4-deficient mice analyzed before embryonic day 10 show a reduction of the beating frequency of about 40 %. Importantly, these hearts do not respond to β-adrenergic stimulation. Thus, one may conclude that in the embryonic heart HCN4 is not required for principal heart beat, at least not before embryonic day 11.5, but that this channel is absolutely crucial for autonomous heart rate regulation [241]. In support of this conclusion, hearts of mice carrying a mutation in the CNBD that abolishes high-affinity cAMP binding ($HCN4^{R669Q}$) also fail to respond to β-adrenergic stimulation [240]. Further analysis revealed that HCN4-deficient mice probably die from a developmental defect of the SA node [241]. The mice develop normal embryonic pacemaker cells that produce primitive pacemaker potentials but fail to generate adult-type pacemaker cells. Obviously, the latter cells are crucially required

to drive the heart beat after embryonic day 11. Interestingly HCN4^{R669Q} mice are able to generate this kind of pacemaker cell but, like HCN4-deficient mice, also die around embryonic day 11.5 [240]. Together, the mouse studies indicate that HCN4 protein is required (1) for the formation of adult pacemaker cells during embryonic heart development and (2) for confering cAMP-dependent up-regulation of embryonic heart rate. Both HCN4-mediated functions are dispensable in early heart function and early embryonic heart development but are needed for the transition to late embryonic stages.

Given its vital role in embryonic heart, it came as a big surprise that mice in which HCN4 was deleted in adult SA node using a temporally controlled knock-out approach were viable and displayed a rather mild cardiac phenotype [239]. As in embryonic pacemaker cells of global HCN4-deficient mice, I_h was reduced by about 80 % in SA node cells of the adult HCN4-deficient mice [239]. However, the lack of this current component led neither to a major impairment of pacemaker potential generation nor to interference with β-adrenergic regulation of heart rate. Unlike global knockout mice, adult HCN4 knockout mice revealed a normal basal heart rate and normal sympathetic and vagal heart rate modulation. However, adult HCN4-deficient mice displayed a cardiac arrhythmia characterized by recurrent sinus pauses. Pacemaker cells of adult HCN4 knockout mice were hyperpolarized by about 8 mV and in most cases did not fire spontaneously under basal conditions. However, this functional impairment could be compensated by β-adrenergic stimulation. In conclusion, these findings suggest that in the adult SA node, HCN4 may serve as a kind of stabilizer of the pacemaker potentials. In most situations, and particularly during sympathetic stimulation, HCN4 does not seem to be required to promote stable pacemaking. However, after an increase in repolarizing currents (e.g. vagal stimulation or transition from activated to basal cardiac state), HCN4 is activated and provides a depolarizing current, keeping the system well-balanced. The loss of this "depolarization reserve" may explain the induction of recurrent sinus pauses in HCN4 knockout mice.

With respect to the stabilizing function, HCN2 that makes up about 20 % of SA node I_h in mice may serve as a complementary channel to HCN4 [110]. HCN2-deficient mice also reveal a sinus dysrhythmia that is characterized by varying peak-peak intervals in the electrocardiogram [110]. Other parameters of the sinus rhythm, including autonomous heart rate regulation, are normal in these mice. As with HCN4, the maximal diastolic potential (MDP) of HCN2-deficient SA node cells is slightly hyperpolarized. It is important to note that HCN2 obviously is not crucial for the development of cardiac conduction system since HCN2-deficient mice do not display increased embryonic lethality.

In conclusion, genetic mouse studies indicate that the two components of SA node I_h carried by HCN4 and HCN2 are required for maintaining a stable cardiac rhythm. However, unlike predicted, neither HCN2 nor HCN4 is needed for principal pacemaking and for autonomous rate regulation in the adult heart. Further experiments (e.g. analysis of HCN2/4 double knockout mice) will be required to solidify these conclusions. Importantly, it remains to be determined why heart rate regulation in early embryos requires HCN4 but is independent of this channel in adult animals. In this context, it remains to be determined which current(s), if not I_h, are the downstream target(s) of cAMP-signaling in adult SA node cells. Finally, it should be considered that the importance of HCN channels for cardiac rhythm initiation and frequency modulation may be different between species.

The analysis of human HCN4 channelopathies supports this notion [242–245]. So far, four different heterozygous HCN4 mutations have been identified in humans [242–245]. The mutations lead to loss of cAMP-dependent modulation (HCN4-573X) [242], hyperpolarizing shift of the activation curve (S672R and G480R) [243, 245] or a severe reduction of cell surface expression (D553R) [244]. Interestingly, all patients suffering from these mutations have in common that they display a more or less severe bradycardia, a clinical phenotype that is not observed in HCN-channel deficient mouse models. Another issue, that is difficult to explain at this moment is the effect of bradycardic agents, such as cilobradine, ivabradine and zatebradine [246] as well as clonidine [247]. It was proposed that these agents lower heart rate by blocking I_h-channels and reducing the firing frequency of SA node cells. In agreement with this hypothesis, cilobradine lowers heart rate in wild-type mice but does not so in adult HCN4 knockouts. On the other hand, given that HCN4 confers the heart-rate lowering effect of cilobradine and related substances, why then do HCN4 deficient mice (which corresponds to a 100 % pharmacological block) display no basal bradycardia? At present, there is no satisfying answer to this conundrum; however, solving this issue will undoubtedly profoundly advance our understanding of the molecular basis of rhythmicity in normal and diseased heart.

Summary and future directions

Ten years after cloning of HCN channels many principles of HCN channel function and regulation are known. Insights from biophysical studies, from genetic mouse models and first reports of HCN-channelopathies in human have extended our knowledge of these proteins. Moreover, first insight into the structural biology of HCN channels has been provided by the crystallization of the C-terminus of HCN2. One important goal for the future will be to obtain structural data about the pore region of the voltage sensing domain. The next step then will be to gain insight into the 3D-structure of the whole channel homo- or heteromer or the whole channel protein in complex with known HCN channel subunits. These data are very important to understand the structural basis of HCN channel regulation by voltage and cAMP. A second important goal will be to characterize the system properties of single channel components in the context of cell signaling networks. For example it would be very interesting to learn more about how HCN channels are regulated within neuronal circuits and how these channels fulfill their complex functions. The third challenge is to understand how HCN channel plasticity is produced *in vivo.* In this context, a very interesting question is to find out how these channels are up- and down-regulated due to changes in neuronal or cardiac activity. It will be very exciting to obtain the answers to these open questions in the different fields of ion channel research in the future.

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