PACEMAKER CHANNEL FUNCTION AND REGULATION IN THE HEALTHY AND EPILEPTIC THALAMUS

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1 SUMMARY

The control of electrical activity in the brain is important for all brain-related behavior, including attention, arousal, action, but also drowsiness and sleep. Voltage-gated ion channels are central to all these aspects of neuronal excitability. Accordingly, malfunction of ion channels, be it inherited or acquired, tremendously compromises brain function, and leads to states of epilepsy, movement disorders, sensory deficits and neuropsychiatric disease.

Voltage-gated ion channels form a large class of pore-forming, transmembrane proteins, and include channels selective for the major ions Na^+, Ca^{2+}, K^+, Cl^-, and HCO_3^-. This family also includes ion channels permeable for several ions. Amongst these, the hyperpolarization-activated cyclic nucleotide-gated cation-nonselective (HCN) channels occupy a unique position. First, being hyperpolarization-, not depolarization-activated, these channels possess the capacity to function as pacemakers. Second, in addition to the voltage-gating, HCN channels are directly modulated by intracellular cAMP levels. Third, the channels show the greatest sensitivity to brief periods of abnormal neuronal activity documented so far that manifests as a change in expression and function after periods of hours to days following abnormal electrical activity. This unique sensitivity has prompted an interest into how HCN channels may underlie the transformation of well-balanced neuronal circuits into hyperexcitable networks typically observed after an epileptic insult or after injury.

The wealth of novel information about the molecular and regulatory properties of HCN channels accumulated over the past years raised a series of questions related to the function of this unique ion channel in neuronal cells and networks, including those in the intact animal.

1. At the level of the neuronal network: How does abnormal HCN channel expression and function causally relate to the emergence of pathological neuronal activity?
2. At the level of the neuron: Are there, and if yes, which are the cell-type specific modes of cAMP-dependent regulation of the channels?

In my thesis, I have addressed these questions by combining electrophysiological, imaging, and molecular biological techniques in healthy animals and a rat model of epilepsy.

1. We have used the GAERS model to investigate the properties of HCN channel regulation in both pre-epileptic and chronically epileptic stages. This approach has allowed us to address the temporal relation between abnormal HCN channel function and the emergence of epilepsy. The findings imply that pacemaker currents undergo an abnormal regulation in the cause of epileptogenesis, but remain unaffected in chronic epilepsy. Interestingly, thalamic cells overcome these deficits by developing compensatory changes that stabilize HCN channel function.

2. Neurotransmitter-mediated cAMP synthesis and subsequent enhancement of HCN-currents is a well-established mechanism that controls thalamic relay functions. The maintenance of arousal and wakefulness is connected with tonic activity of the noradrenergic locus coeruleus in the thalamocortical system. How and whether prolonged noradrenergic input modulates HCN channels in thalamic nuclei is subject of the second part of my thesis. Furthermore, a differential β-adrenergic subtype expression pattern in functionally distinct thalamic nuclei suggests that there could be a nucleus-specific component in the control of waking and sleep homeostasis. The results of my study indeed reveal a distinct β-adrenergic regulation of HCN channels within the thalamus. A strong β-adrenergic regulation of HCN-currents appears to be pronounced in those portions being involved in sensory relay, while they may not be associated with general arousal functions.

2 ZUSAMMENFASSUNG

Sämtliche Gehirnfunktionen, einschliesslich solcher wie Aufmerksamkeit, Aufwachen, aber auch Müdigkeit und Schlaf stehen unter der Kontrolle von elektrischer Aktivität im Gehirn. Diese wird entscheidend von spannungsabhängigen Ionenkanälen beeinflusst. Demzufolge kann eine abnormale Funktion von Ionenkänalen, sei sie angeboren oder erworben, zu enormen Einschränkungen der Gehirnfunktionen führen, und dabei Zustände wie Epilepsie, Bewegungsstörungen, sensorischen Störungen oder neuropsychiatrische Krankheiten hervorrufen.

Spannungsabhängige Ionenkanäle bilden eine grosse Klasse von transmembranären Proteinen, mit Kanälen selektiv für die Haupt-Ionen Na^+, Ca^{2+}, K^+, Cl^- und HCO_3^-. Dazu gehören auch Ionenkanäle, die permeabel für verschiedene Ionen sind. Unter diesen nehmen die hyperpolarisations-aktivierten, zyklisch Nukleotid-gesteuerten, nicht-selektiven Kation (HCN) Kanäle eine besondere Stellung ein. Erstens, da die Kanäle durch Hyper- und nicht durch Depolarisation aktivieren, können sie Schrittmacherfunktion übernehmen. Zweitens werden HCN Kanäle, zusätzlich zur Spannungsabhängigkeit, direkt durch den intrazellulären cAMP-Spiegel moduliert. Drittens zeigen HCN Kanäle die bislang stärkste bekannte Empfindlichkeit auf kurze Episoden von abnormaler neuronaler Aktivität. Diese kann über eine Zeitdauer von Stunden bis zu Tagen nach abnormaler elektrischer Aktivität zu Veränderungen in Expressionsmuster und Funktionalität führen. Durch diese einzigartige Sensitivität auf neuronale Aktivität stellt sich die Frage, ob und wie HCN Kanäle in der Transformation vom ausgeglichenen zum hyperaktiven, neuronalen Netzwerk involviert sind, das typischerweise während einer Epilepsie beobachtet wird.

In den letzen Jahren hat sich eine Menge an zusätzlichen Erkenntnissen über die molekularen und regulativen Eigenschaften der HCN Kanäle angesammelt. Diese werfen eine Reihe von neuen Fragen auf, die sich mit der Funktion dieses einzigartigen Ionenkanals in neuronalen und zellulären Netzwerken beschäftigen.

1. Auf der Ebene des neuronalen Netzwerkes: Welches sind die kausalen Zusammenhänge zwischen abnormaler HCN Kanal Expression und Funktion zum Auftreten von pathologischer neuronalen Aktivität?
2. Auf der Ebene des Neuronen: Welche zell-spezifischen Mechanismen der cAMP abhängigen Regulation der HCN Kanäle gibt es?

In meiner Doktorarbeit habe ich mich mit diesen Fragestellungen beschäftigt. Dabei habe ich elektrophysiologische, bildgebende und molekularbiologische Techniken benutzt, und diese sowohl in gesunden Tieren wie auch den GAERS, einem durch Inzucht entstandenen Ratten-Modell für Epilepsie angewendet.

1. Die GAERS dienten als Modell um die Eigenschaften der HCN Kanal-Regulation im pre-epileptischen wie auch im chronisch epileptischen Zustand zu erforschen. Diese Annäherung hat es uns erlaubt, eine zeitliche Relation zwischen abnormer HCN Kanal-Funktion und dem Auftreten von Epilepsie herzustellen. Die Ergebnisse zeigen, dass während der Pathogenese der Epilepsie eine abnormale Regulation der Schrittmacher-Ströme auftritt. Interessanterweise entwickelten thalamische Zellen einen kompensatorischen Mechanismus, der die HCN Kanal-Funktion im erwachsenen Tier stabilisiert.

2. Die Kontrolle thalamischer Schaltfunktionen durch Neurotransmitter (wie z.B. Noradrenalin) erfolgt unter anderem über cAMP-vermittelte Regulation der HCN-Ströme. Gleichzeitig sind Wachzustand und Wachsein mit einer tonischen Aktivität des noradrenergen Locus coeruleus im thalamocorticalen System verbunden. Wie und ob sich dieser lang anhaltende noradrenerge Einfluss auf die HCN Kanäle in verschienen thalamischen Kernen auswirkt ist Thema des zweiten Teils meiner Doktorarbeit. Ein unterschiedliches ß-adrenerges Expressionsmuster in thalamischen Kernen lässt eine Verbindung vermuten zu den unterschiedlichen Rollen thalamischer Kerne in der Regulation von Wachzustand und Schlaf. Die Ergebnisse meiner Studie zeigen tatsächlich eine unterschiedliche ß-adrenerge Regulation von HCN Kanälen innerhalb des Thalamus. Eine starke ß-adrenerge Regulation der HCN-Ströme scheint vor allem verstärkt dort aufzutreten, wo sensorische Information weitergeleitet wird. Gleichzeitig sieht man kaum ß-adrenerge Regulation von HCN Kanälen in thalamischen Kernen, die zuständig für die allgemeine Wachfunktion sind.

3 INTRODUCTION: PACEMAKER CHANNELS

3.1 Introduction to the ion channel family

The hyperpolarisation-activated cyclic nucleotide-gated cation (HCN) currents were identified in the early 1980s as the pacemaker currents driving the autonomous rhythmic discharges of the sinoatrial node (SAN) cells in the heart. These currents have been variously designated as I_h or I_q in the brain ('h' and 'q' stand for 'hyperpolarisation' and 'queer', respectively), or I_f for 'funny' current in the heart. This naming reflects that these currents behave opposite to most other ion currents: they gate upon membrane hyperpolarisation, not depolarisation (Figure 1). Historically, the pacemaking properties have dominated the physiological profile of I_{HCN} for almost twenty years. Recently, the views on the role of I_{HCN} have broadened significantly, largely due to the cloning of the genes for HCN channels. They now cover aspects in synaptic function, dendritic integration, plasticity, learning and pathological neuronal and cardiac states. This chapter reviews these clear and straight modern developments of a historically funny and queer current (which I refer to soberly as I_{HCN}).

Strong evidence for the multiple involvement of HCN channels in neuronal functions comes from several sources: first, HCN channel proteins show organized expression patterns throughout the brain, including in particular areas involved in cognitive functions, such as learning and memory. Second, they are localized not only in somatic compartments, but also in dendrites, presynaptic zones and axonal elements, thus being instrumental for synaptic integration and transmission. Third, HCN channels belong to a small subgroup of ion channels whose expression is regulated in an activity-dependent manner. As a consequence, changes in HCN subunit expression accompany plasticity-promoting stimuli. Moreover, aberrant electrical activity, such as that found during epilepsy or cardiopathy, may persistently alter their expression levels, implicating these ion channels in the pathology of excitable systems.

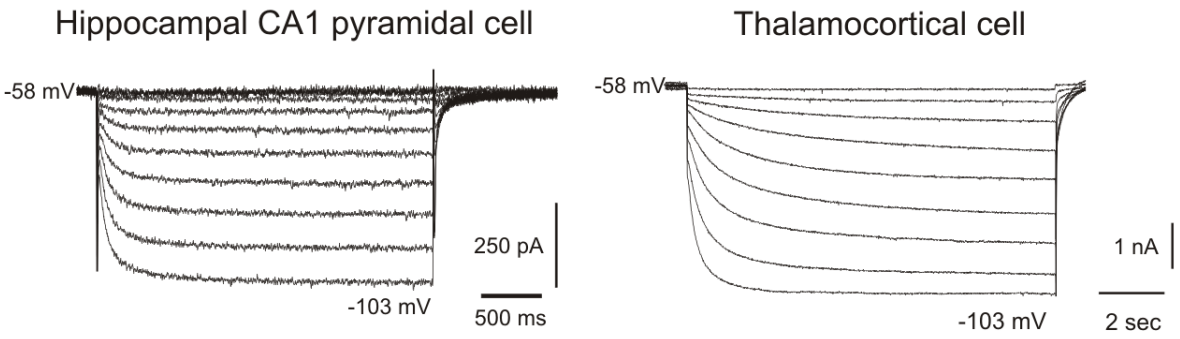

Figure 1. Hyperpolarization-activated currents in neurones. Families of current responses in voltage-clamp conditions were obtained by stepping the membrane voltage to increasingly negative potentials in 5 mV steps (most negative value reached, -103 mV, is indicated next to the current traces). Note the different time scales over which current activation proceeds in hippocampal pyramidal cells (left) and in thalamocortical cells (right). In hippocampal pyramidal cells, expression of HCN1 and HCN2 predominates, whereas HCN2 and HCN4 are the major channel isoforms in thalamocortical cells.

3.2 Molecular characterization of the ion channel family

3.2.1 Gene products and primary structure of HCN proteins

The late 1990s brought the breakthrough in the identification of the molecular basis of hyperpolarisation-activated cation channels (Santoro *et al.*, 1997; Gauss *et al.*, 1998; Ludwig *et al.*, 1998; Santoro *et al.*, 1998). To date, a family of up to four genes is known for mammals, including mouse (Ludwig *et al.*, 1998; Santoro *et al.*, 1998), rat (Monteggia et al., 2000), rabbit (Ishii *et al.*, 1999; Shi *et al.*, 1999), and human (Ludwig *et al.*, 1999; Seifert *et al.*, 1999; Vaccari *et al.*, 1999) (for review, see (Kaupp and Seifert, 2001)). The adapted nomenclature for the mammalian gene family is HCN1-HCN4, standing for hyperpolarisation-activated cation-nonselective channels. For a detailed list of old and standard nomenclature, see Table 1 in (Kaupp and Seifert, 2001). The chromosomal location of the *HCN* channel genes has been determined for a) human *HCN2*: 19p13.3 (Ludwig *et al.*, 1999; Vaccari *et al.*, 1999) and b) human *HCN4*: 15q24-q25 (Seifert *et al.*, 1999).

Two genes were reported for sea urchin sperm flagella (SpHCN1-2) (Gauss *et al.*, 1998; Galindo *et al.*, 2005), and one each for *Drosophila melanogaster* (DmHCN) (Marx *et al.*, 1999), the silkmoth *Heliothis virescens* (HvHCN) (Krieger *et al.*, 1999) the honeybee *Apis mellifera* (AMIH) (Gisselmann *et al.*, 2004) and the lobster *Panulirus argus* (PAIH) (Gisselmann *et al.*, 2005).

The nucleotide sequence encodes for transcripts of ~780-1200 amino acids, with a predicted primary structure typical for voltage-gated K^+ channels, including six transmembrane-spanning domains S1-S6 that are highly conserved (80-90%) within the gene family, and a positively charged S4 domain. Notably, the pore region between S5 and S6 contains the selectivity filter motif GYG that is a hallmark for voltage-gated K^+-selective channels. However, adjacent amino acids differ for the HCN channel genes, perhaps contributing to the lack of strong K^+ selectivity in I_{HCN}. In addition, all HCN genes show a highly conserved C-terminal cyclic nucleotide-binding domain (CNBD) that is 120 amino acids in length and linked to S6 by an 80 amino acid C-linker region. The CNBD is homologous to those found in cyclic nucleotide-dependent kinases, in the bacterial cAMP binding protein catabolite gene activator protein, and in cyclic nucleotide-gated ion channels of olfactory sensory and photoreceptor cells. The CNBD shows uniquely positioned amino acids that help explain its selectivity for cAMP over cGMP (Kaupp and Seifert, 2001; Craven and Zagotta, 2006). The *HCN* genes are poorly conserved and variable in length within the sequences in the distal N- and C-termini, with the HCN4 protein showing an N-terminus and

C-terminus longer by ~80-120 and ~300-400 amino acids, respectively, compared to HCN1-HCN3.

3.2.2 Human diseases related to the genetic locus of HCN channels

Three patients with idiopathic sinus node disease, characterized by marked bradycardia and life-threatening cardiac arrhythmia, were found to carry a mutation in one of the two *HCN4* genes. In two cases, the mutation co-segregated with the disease in the pedigree of the family in a dominant fashion (Ueda *et al.*, 2004; Milanesi *et al.*, 2006). Mutated genes either encode a channel subunit with a deleted CNBD (Schulze-Bahr *et al.*, 2003) or cause poor expression (Ueda *et al.*, 2004). In the third case, mutated channels show a negatively shifted activation range (Milanesi *et al.*, 2006). Mutated channel subunits showed dominant-negative effects on the expression of the wild-type channels. Additional human diseases were linked to the chromosomal locus 15q24, such as a syndrome of mental retardation (Mitchell *et al.*, 1998), and an autosomal dominant nocturnal frontal-lobe epilepsy (Phillips *et al.*, 1998), although the involvement of mutated *HCN* genes remains to be demonstrated. As discussed in Section 3.7, abnormal mRNA expression for HCN channels is found in cardiac and neuronal pathologies.

3.2.3 Heterologous expression of HCN channels

All four mammalian genes, as well as the invertebrate genes, give rise to hyperpolarisation-activated cation currents when expressed heterologously in *Xenopus* oocytes or in mammalian cell lines (Gauss *et al.*, 1998; Ludwig *et al.*, 1998; Santoro *et al.*, 1998; Ludwig *et al.*, 1999; Seifert *et al.*, 1999). These currents show characteristic similarities with native I_{HCN} (Figure 1), in particular with respect to a) their activation by membrane hyperpolarisation below ~-60 mV b) their complex activation kinetics c) their permeability to both Na^+ and K^+ ions d) the regulation by cyclic nucleotides, with a preference for cAMP over cGMP, e) their lack of inactivation (with the exception of spHCN) and f) their blockade by millimolar concentrations of extracellular Cs^+, but not by traditional K^+ current blockers (tetraethylammonium, 4-aminopyridine). The current generated by HCN1 is the poorly cAMP-sensitive, rapidly activating current isoform, whereas HCN2 and HCN4 give rise to highly cAMP-sensitive currents that activate more slowly. In recordings from intact cells, HCN1-mediated currents showed a half-activation voltage ($V_{1/2}$) around -70 mV, whereas

HCN2- and HCN4-mediated currents gated half-maximally around -80 to -90 mV. In cell-free recordings, these values shifted by 40 to 60 mV in the hyperpolarising direction for both HCN1 and HCN2 channels (Chen *et al.*, 2001c). The reason for the hyperpolarising shift in $V_{1/2}$ with patch excision is unknown, although widely observed (DiFrancesco and Mangoni, 1994). Recent studies reveal a role for phosphoinositides, such as phosphaditylinositol-4,5-bisphosphate (PIP_2) in HCN channel gating (see Section 3.2.6, (Pian et al., 2006; Zolles et al., 2006)). Application of exogenous PIP_2, together with cAMP, restored channel activation in excised patches (Pian et al., 2006; Zolles et al., 2006).

Activation occurs after an initial lag of tens of ms, and proceeds with a complex time course that is typically best described by a biexponential function and accelerates with increased membrane hyperpolarisation. At 35°C, around the half-maximal voltage, time constants of activation for HCN1 amount to ~10 ms and ~100 ms, whereas activation of HCN2 and HCN4 is best described by time constants in the order of hundreds of milliseconds and seconds, respectively. In the case of HCN2 and HCN4, cAMP shifts the activation range by ~15-25 mV towards more positive potentials (Ludwig *et al.*, 1999; Chen *et al.*, 2001c; Stieber *et al.*, 2005), whereas this shift amounts to 2-6 mV in the case of HCN1 (Santoro *et al.*, 1998; Chen *et al.*, 2001c; Stieber *et al.*, 2005). The half-maximal concentration for cAMP is < 1 μM (60-800 nM) (Gauss *et al.*, 1998; Ludwig *et al.*, 1998; Chen *et al.*, 2001c; Zagotta *et al.*, 2003), whereas that for cGMP is ~6-8 μM (Ludwig *et al.*, 1998; Zagotta *et al.*, 2003). The HCN3-mediated current is weakly modulated by cAMP, yet shows a voltage dependence similar to HCN2-mediated currents (Mistrík *et al.*, 2005; Stieber *et al.*, 2005).

3.2.4 Formation of heteromers between HCN channel subunits

Evidence for the heteromerization of HCN subunits was first provided by identifying interactions between N-termini of HCN1 and HCN2 (Proenza *et al.*, 2002b) and by showing that co-expressed dominant-negative pore mutants of HCN1 inhibited HCN2-mediated currents (Xue *et al.*, 2002). With the exception of HCN2 and HCN3, all dual combinations of channel subunits express and colocalize at the plasma membrane in heteromeric complexes (Much *et al.*, 2003). Co-expression of two of each HCN1, HCN2 and HCN4 produce currents that incorporate properties of both isoforms in ways that do not correspond to those expected from the linear interpolation of homomers (Chen et al., 2001c; Ulens and Tytgat, 2001; Altomare et al., 2003; Michels et al., 2005). The most direct evidence for heteromerization is found in the distinct properties of single-channel events arising from co-expressed HCN2 and

HCN4 (Michels *et al.*, 2005). The voltage dependence of HCN1-HCN2 heteromeric currents is close to that of HCN2-mediated currents, but currents show a decreased cAMP sensitivity at submaximal cAMP concentrations. This property could have implications for disease, since HCN1 protein is sensitively regulated by abnormal neuronal activity and tends to form heteromers (see Section 3.7.1). Co-expression of HCN1 or HCN2 with HCN4 produces channels with properties approximating those of HCN4 homomers (Altomare *et al.*, 2003; Michels *et al.*, 2005).

3.2.5 Control of HCN channel expression

Biochemical and functional studies indicate a role for both N-terminal and C-terminal sequences in the assembly and trafficking of functional channels to the surface (Proenza *et al.*, 2002b; Tran *et al.*, 2002). A stretch of 52 amino acids (positions 131-182 in HCN2) located N-terminally of the S1 segment is > 90% conserved amongst the HCN isoforms and deletion of this region in HCN2 results in intracellular retention of the protein. In addition, an intact CNBD is required for surface expression of HCN2 in CHO cells (Proenza *et al.*, 2002b), although the C-linker is sufficient in *Xenopus* oocytes (Wainger *et al.*, 2001). In contrast to the critical N-terminal sequences, however, not every subunit of a channel needs to contain a CNBD to generate a functional channel. Finally, *N*-glycosylation at asparagine 380 of HCN2, lying between S5 and the pore region, is important for subunit expression, although again not required for every channel subunit (Much *et al.*, 2003). The physiological relevance of *N*-glycosylation is underscored by the immunoblot analysis of native subunits, which are about 20 kDa larger than predicted by the primary sequence. Pharmacological removal of *N*-glycosylation restored the predicted molecular weight (Santoro *et al.*, 1997; Much *et al.*, 2003).

3.2.6 Accessory and regulatory proteins of HCN channels

The list of proteins that physically interact with HCN channels and regulate their properties and expression is currently growing (see Figure 2 for an overview). A widely expressed ß subunit for Kv channels, the single transmembrane MinK related protein (MiRP1 or KCNE2 (McCrossan and Abbott, 2004)) enhances the amplitude of I_{HCN} and affects activation kinetics, although effects vary depending on expression systems and HCN isoforms (Yu *et al.*, 2001; Proenza *et al.*, 2002a; Altomare *et al.*, 2003; Decher *et al.*, 2003; Qu *et al.*,

2004). MiRP1 and HCN2 are highly expressed in SAN, and MiRP1 co-immunoprecipitated with HCN2 in cultured neonatal cardiac myocytes overexpressing both proteins (Qu *et al.*, 2004).

The C-terminal sequence, including the CNBD and the less conserved regions at the distal C-terminal end, mediate important protein-protein interactions. The extreme C-terminal tripeptide (SNL in HCN1, HCN2 and HCN4, ANM in HCN3) tightly binds to the protein TRIP8b (for tetratricopeptide repeat-containing Rab8b interacting protein), which colocalizes with endosomal markers and could be involved in the endocytosis of HCN channels (Santoro *et al.*, 2004). This protein closely matches HCN1 expression in the dendrites of CA1 hippocampal pyramidal and cortical layer V cells and its overexpression reduces the density of native channels. Alternatively spliced isoforms of Trip8b exist, suggesting that the significance of these proteins in controlling HCN channel function may further diversify in the near future. In particular, it will be important to see how the two apparently incongruent observations, colocalization of TRIP8b and HCN1 in a parallel gradient within the apical dendrites on the one hand, but negative regulation of channel expression by TRIP8b on the other hand, can be reconciled. C-terminal sequences also interact with scaffolding proteins that may mediate binding to the cytoskeleton and to postsynaptic elements (Kimura *et al.*, 2004). In HCN1 protein, a 22-amino acid sequence downstream of the CNBD binds to filamin A, a protein involved in the organization of functional complexes involving receptors, ion channels and cytoskeletal elements (Gravante *et al.*, 2004). In SAN cells, HCN4 protein and components of the β-adrenergic signaling pathway are found in membrane fractions containing structurally specialized portions of the lipid membrane, the caveolae (Barbuti *et al.*, 2004).

Membrane phospholipids such as PIP_2 control HCN channel gating and produce a right-shift of the steady-state activation (Pian et al., 2006; Zolles et al., 2006). Mechanistically, the negatively charged headgroup of PIP_2 could electrostatically interact with the channel protein and, as a consequence, facilitate the voltage-dependent activation of HCN channels (Zolles et al., 2006).

Protein kinases, predominantly protein kinase A (PKA) and tyrosine kinases, regulate I_{HCN} properties in a number of cell types (for review, see (Frère *et al.*, 2004)). Additional studies now indicate that kinases may be physically associated with the channels. The C-linker domain, which mediates intersubunit interactions required for channel gating (see Section 3.3.6), contains a tyrosine residue (Y476 in HCN2, Y554 in HCN4) that is phosphorylated by the non-receptor protein tyrosine-kinase Src. This kinase was used as a bait

in the first identification of the *HCN* genes (Santoro *et al.*, 1997) and is now known to bind to the C-terminus (Zong *et al.*, 2005; Arinsburg *et al.*, 2006). Inhibition of Src phosphorylation in HCN2/HCN4-expressing human embryonic kidney (HEK) 293 cells, in SAN cells and in dorsal root ganglia decelerates current activation.

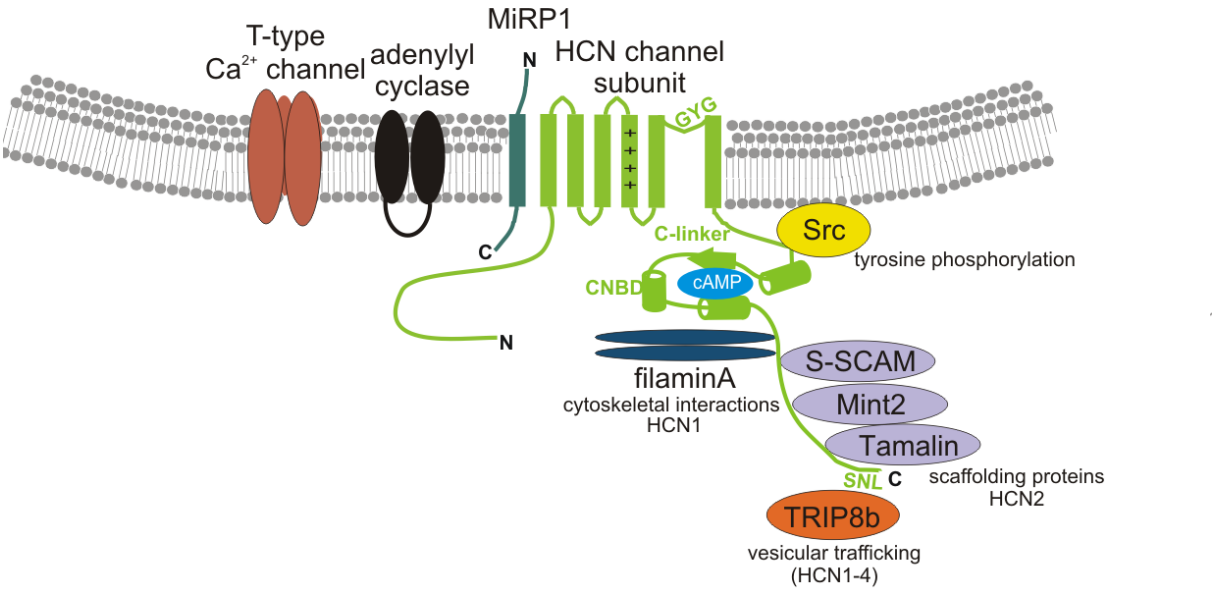

Figure 2. Schematic view of accessory and regulatory proteins. The cartoon comprises those proteins for which a close colocalization with HCN channels has been demonstrated functionally (T-type Ca^{2+} channels, adenylyl cyclase, MiRP1) or for which binding to HCN channels has been demonstrated by co-immunoprecipitation from brain tissue (Src, filamin, synaptic scaffolding molecules S-SCAM, Mint2, tamalin) or both co-immunoprecipitation and colocalization in neuronal cells (Trip8b).

3.3 Biophysical properties

The unique biophysical characteristics of I_{HCN} are presented in excellent reviews (Pape, 1996; Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003; Baruscotti and DiFrancesco, 2004; Cohen and Robinson, 2006; Craven and Zagotta, 2006). Recent research has focused, on the one hand, on identifying additional current properties, such as the single-channel characteristics and instantaneous forms of current activation. On the other hand, the molecular understanding of the events underlying channel gating and its regulation by cyclic nucleotides has advanced. These studies have also demonstrated that HCN channels are composed of a tetrameric arrangement of HCN subunits (Xue et al., 2002; Ulens and Siegelbaum, 2003; Zagotta et al., 2003), in agreement with their evolutionary relationship to the tetrameric voltage-gated K^+ channels. This section briefly describes the main molecular underpinnings of HCN channel properties, and refers to the most relevant reviews for further details and for comparison to additional members of the voltage-gated ion channel family.

3.3.1 Prototypical biophysical properties

The three prototypical biophysical properties of native I_{HCN} are highlighted here that make them unique amongst the family of voltage-gated ionic currents.

First, I_{HCN} typically activates upon membrane hyperpolarisation (below ~-60 mV) rather than depolarisation, just opposite to most voltage-gated ionic currents that are involved in shaping neuronal excitability (Figure 1). This unusual voltage window of activation is reflected in the standard denomination I_h, where "h" stands for hyperpolarisation. Upon hyperpolarisation, the conductance activated is fairly selective for both Na^+ and K^+ ions (permeability ratio Na^+ : K^+ = 0.2-0.3) ((DiFrancesco, 1981; Wollmuth and Hille, 1992; Gauss et al., 1998) for a review, see (Pape, 1996)). The current is carried mainly by Na^+ ions at the membrane voltages within its activation range and produces an elevation in the intracellular Na^+ concentration (Knöpfel *et al.*, 1998). In addition to contributing to ion flux, extracellular K^+ ions determine the channel conductance for Na^+ (for a detailed review, see (Pape, 1996)). More recently, a small permeability to Ca^{2+} ions has also been identified for heterologously expressed HCN4 channels (0.6% of the inward current evoked at -120 mV (Yu *et al.*, 2004)) and presynaptically expressed I_{HCN} in crayfish (Zhong *et al.*, 2004). The reversal potential of I_{HCN} lies between -40 to -25 mV, leading to depolarising currents around resting membrane potentials. The resulting depolarising drive at subthreshold potentials is the

basis for the multifunctionality of I_{HCN}, in particular its pacemaking properties and its control of excitability and synaptic integration.

Second, activation of the current is generally slow, with activation time constants ranging between hundreds of milliseconds and seconds, even at strongly hyperpolarised voltages around -100 mV (for an overview of activation kinetics in different cell types, see Table 2 in (Santoro and Tibbs, 1999)). Few exceptions include pyramidal neurones from hippocampus and cortex, as well as amygdalar and cerebellar neurones, in which activation is complete within tens of milliseconds. Once activated, the current does not inactivate, such that a steadily activated ('standing') I_{HCN} contributes to the resting membrane potential in many neurones, often by opposing the action of tonic outward currents (see Section 3.6.3).

Third, I_{HCN} is, in most cases, sensitive to the presence of intracellular cyclic nucleotides. The cyclic nucleotides cAMP and cGMP bind directly to the channels (DiFrancesco and Tortora, 1991; Pedarzani and Storm, 1995), accelerate the kinetics of activation, and shift the voltage dependence of activation towards more depolarised values. In the presence of these ligands, the extent and duration of current activation within the intermediate voltage range is increased (Robinson and Siegelbaum, 2003). This facilitatory effect of cyclic nucleotides can give rise to persistently activated forms of current activation that outlast the presence of available free cAMP (Lüthi and McCormick, 1999b; Wang et al., 2002). HCN-currents are also regulated by extra- and intracellular changes in pH. Extracellular pH decreases augment I_{HCN}, whereas intracellular acidification decreases I_{HCN}. The extracellular pH sensitivity has been implicated in the transduction of sour (pH 3-5) stimuli in taste receptor cells (Stevens *et al.*, 2001), whereas the intracellular pH sensitivity may be involved in regulating the discharge of neuronal networks in response to moderate pH changes (< 1 unit), such as those occurring during intense neuronal activity (Munsch and Pape, 1999b, a). In HCN2 channels, intracellular pH sensitivity is mediated by direct protonation of a histidine residue (H321) located at the boundary between S4 and the S4-S5 linker (Zong *et al.*, 2001).

Two, most recently identified biophysical characteristics include the single-channel properties and instantaneous forms of channel gating.

3.3.2 Single-channel properties

The first resolution of putative single channel events underlying I_{HCN} was achieved in rabbit SAN (DiFrancesco, 1986) and revealed an unusually low single-channel conductance

< 1 pS (DiFrancesco and Mangoni, 1994). This low single-channel conductance (~0.68 pS) was recently determined for I_{HCN} found in the apical dendrites of cortical layer V pyramidal cells using fluctuation analysis of membrane current noise (Kole *et al.*, 2006). These dendrites express I_{HCN} at densities up to 550 channels/μm^2, which is about 1000-fold higher than the average channel density on a typical I_{HCN}-expressing cell. In spite of their low conductance, this high density renders HCN channels important determinants of membrane current noise in these cells. Single-channel conductances of heterologously expressed channels are considerably larger (2.5-35 pS) (Johnson and Zagotta, 2005; Michels et al., 2005). Furthermore, somatic HCN channels recorded from dissociated hippocampal neurones show a higher conductance (~10 pS) (Simeone *et al.*, 2005). A high single-channel conductance of ~30 pS has also been reported for the HvHCN channels (Krieger *et al.*, 1999).

3.3.3 Instantaneous HCN-currents

The slowly developing inward current is the predominant manifestation of I_{HCN} activation by voltage. However, a small, voltage-independent leakage current accompanies the gating of HCN2 channels expressed in CHO cells (Proenza *et al.*, 2002a) and of spHCN channels expressed in HEK 293 cells (Proenza and Yellen, 2006). This current persisted when mutations were introduced into the channel that abrogated voltage-sensitive current components (Macri *et al.*, 2002; Proenza *et al.*, 2002a). The amplitude of this current correlates with that of the time-dependent current and shows a similar reversal potential (Proenza et al., 2002a; Macri and Accili, 2004), indicating that it is dependent on the expression of HCN2 channels. However, both K^+ and Na^+ ions are independently moving charge carriers (Macri *et al.*, 2002), in contrast to the voltage-gated channel (see Section 3.3.1). Moreover, the instantaneous current component of HCN2-mediated currents is insensitive to extracellular Cs^+ ions and slightly reduced by intracellular cAMP (Proenza *et al.*, 2002a). Finally, ZD7288 (see Section 3.5.2) more rapidly blocked instantaneous than the time-dependent currents. This observation is consistent with a model in which the instantaneous current is generated by an independent channel subgroup that is not in rapid equilibrium with voltage-gating channels (Proenza and Yellen, 2006). To what extent native currents contain instantaneous components remains to be examined.

3.3.4 Additional biophysical properties

More recent studies have identified novel properties of expressed channels. Most interestingly, currents carried by spHCN or HCN1 channels show a hysteresis of voltage dependence, evident as a more positive voltage dependence of the current when measured starting from a hyperpolarising potential (Mannikko *et al.*, 2005). Such hysteresis may contribute to the regularity of action potential discharge, since it helps prolonging the interspike interval, while facilitating repolarisation. HCN2- and HCN4-mediated currents also show a dependence on extracellular and intracellular Cl^- (Wahl-Schott *et al.*, 2005; Mistrik *et al.*, 2006).

A number of biophysical characteristics of I_{HCN} have been investigated at the molecular level. In Figure 3, the amino acids implicated in these functions are indicated.

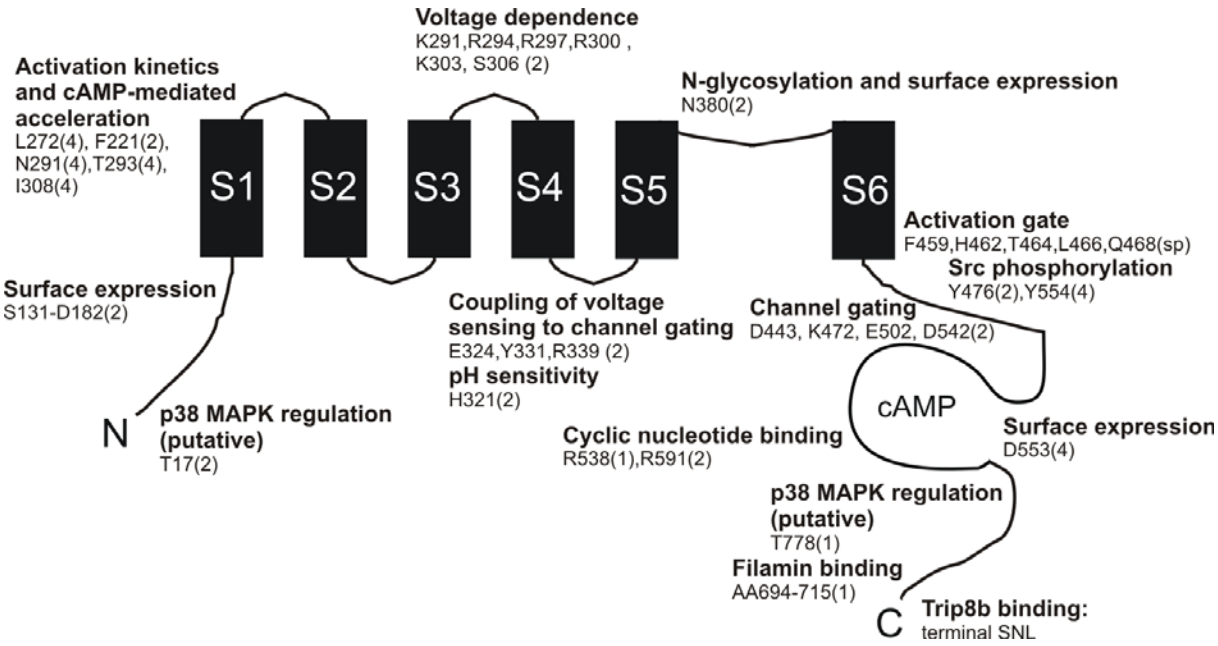

Figure 3. Putative transmembrane topology of the HCN channel and functional roles of the best characterized amino acids and amino acid sequences. The primary structure of the HCN channel, including its N- and C-terminus (N, C), as well as the six transmembrane-domain sequences S1-S6, are depicted schematically. Position and types of amino acids are indicated below a keyword description of their roles, see text for more details. Numbers and letters indicated in parenthesis refer to the HCN channel subunit in which these amino acids were studied. In most cases, these amino acids are conserved in equivalent positions of other HCN isoforms. Amino acids included here are those for which mutations were shown to abrogate or alter their respective functions, or for sequences with which yeast two-hybrid screens for performed to study protein-protein interactions (filamin, Trip8b). The amino acid D553 was found in an inherited form of sinus node disease. The putative phosphorylation sites for p38 MAPK were determined in a screen for consensus sequences, but are not yet validated by mutational analysis. AA stands for amino acids in cases in which the full sequence is not written out.

3.3.5 Molecular elements controlling voltage dependence

The steps leading to channel gating show similarities to the activation of voltage-gated channels and of cyclic nucleotide-gated channels (Chen et al., 2002; Robinson and Siegelbaum, 2003; Horn, 2004; Rosenbaum and Gordon, 2004; Craven and Zagotta, 2006). Thus, the positively charged amino acids in S4 are essential for voltage sensing (Chen *et al.*, 2000; Vaca *et al.*, 2000). Neutralizing each of the first four of these produces negative shifts in current voltage dependence of ~-20 mV/residue. Cysteine-substituted amino acids in S4 show a differential sensitivity to thiol reagents, depending on whether spHCN channels are opened or closed. From these experiments, it could be shown that the S4 domain moves towards the extracellular surface of the membrane upon depolarisation, while it moves inward upon hyperpolarisation (Mannikko *et al.*, 2002). This direction of S4 translocation is thus that of the canonical model of charge movement in six-transmembrane-domain voltage-gated ion channels, although the detailed picture of conformational changes differs in HCN channels (Bell *et al.*, 2004; Horn, 2004; Vemana *et al.*, 2004). Movement of the S4 domain is dependent on the S3-S4 linker (Tsang *et al.*, 2004) and coupled to channel pore opening via unique amino acids within the S4-S5 linker domain (Chen et al., 2001a; Macri and Accili, 2004). Particularly interesting is the requirement for an aromatic residue in this linker (Y331 in HCN2), because it suggests that aromatic interactions in the vicinity of the inner pore region are important for coupling voltage changes to gating. Indeed, an activation gate in spHCN localizes to the pore-forming regions of S6 (Figure 3), and the accessibility of this gate to modulating or blocking agents is dependent on channel opening (Shin *et al.*, 2001; Rothberg *et al.*, 2002; Rothberg *et al.*, 2003). The closure of this activation gate also determines the peculiar inactivation properties of spHCN channels (Shin *et al.*, 2004).

Altogether, the role of protein domains essential for voltage sensing and channel gating are remarkably conserved between depolarisation- and hyperpolarisation-activated ion channels (see also (Robinson and Siegelbaum, 2003)). This similarity, however, still leaves the puzzling question of how channel gating is oppositely coupled to voltage changes in HCN channels. Notably, a recent study has identified a residue (D443) within the S4-S5 linker that stabilizes the closed channel state and that interacts via salt bridges with the unique C-linker domain (Decher *et al.*, 2004). It will be of great interest to see whether these electrostatic interactions may be controlled by voltage-induced conformational arrangements within S4, thereby helping to explain channel opening upon hyperpolarisation.

3.3.6 Molecular elements controlling cyclic nucleotide regulation

The regulation by cyclic nucleotides is a most striking property of HCN channels and affects current properties in a dual manner. First, it shifts the voltage dependence of the current into the more depolarised voltage range; second, it accelerates activation kinetics (Pape, 1996; Robinson and Siegelbaum, 2003). Numerous studies have reported cAMP-dependent actions on native currents, from which it became clear that cAMP-dependent effects are mediated by direct binding to the channel, rather than by PKA (DiFrancesco and Tortora, 1991; Pedarzani and Storm, 1995) (for review, see (Frère *et al.*, 2004)). The cAMP-effects are mediated by a cytosolic CNBD within the C-terminus (Barbuti *et al.*, 1999; Wainger *et al.*, 2001; Zagotta *et al.*, 2003). Removal of the CNBD abolishes cyclic nucleotide sensitivity (Wainger *et al.*, 2001), and mutation of a conserved arginine residue within the CNBD (R538 for HCN1, R591 for HCN2) largely abolishes cAMP modulation (Chen *et al.*, 2001c). All CNBDs of each subunit must be bound to cAMP to achieve a maximal effect on the voltage dependence (Ulens and Siegelbaum, 2003).

How can a C-terminally located cytosolic portion of a channel steer voltage-gating and activation kinetics that are determined by the core transmembrane regions of the channel? Although the full picture of the interaction between the C-terminus and the core regions is not yet elaborated, important insights in the cAMP-induced structural arrangements within the CNBD and the C-terminus have been achieved. Cyclic allosteric gating models, containing unliganded and liganded open and closed channel states in a cyclical arrangement, were first used to explain the dual modulation of HCN channels by voltage and ligand (DiFrancesco, 1999; Wang *et al.*, 2001). These studies assumed that the channel subunits undergo a voltage-dependent transition between a closed and an open state, but that the affinity of cAMP for the open state is greater by a factor of ~40-80 compared to the closed state (for review, see (Frère *et al.*, 2004)). In their simplified form, these allosteric models capture the essential consequences of some of the mechanistic steps underlying the cAMP-binding to HCN channels. Thus, these models predict that the ligand-free CNBD inhibits channel gating, while cAMP-binding relieves this inhibition and stabilizes the channel in the open state. Indeed, HCN channels with a truncated CNBD activate about as rapidly as an intact channel exposed to a maximal cAMP concentration (Barbuti *et al.*, 1999; Wainger *et al.*, 2001). An essential role for this inhibitory action is played by the C-linker domain that connects the CNBD to S6. The C-linker of each subunit is required for normal cAMP modulation (Ulens and Siegelbaum, 2003) and salt bridges both between C-linkers and between C-linkers and CNBDs underlie the coupling of ligand binding to channel opening (Craven and Zagotta,

2004).

Crystallization and analytical ultracentrifugation of the C-terminal portion of HCN2, including the C-linker and the CNBD, has dramatically shaped the molecular understanding of HCN channel gating. Cyclic nucleotide binding greatly favours the formation of a tetramer of C-terminal fragments, while monomeric and dimeric configurations prevail in the absence of the cyclic nucleotide (Zagotta *et al.*, 2003). These tetramers form a gating ring with a four-fold rotational symmetry, with the major portion of intersubunit interactions mediated by the C-linkers. Elegant support for the tetramerization of CNBDs as a prerequisite for HCN channel gating comes from electrophysiological studies on tandem dimers or tetramers of HCN channels, in which mutated CNBDs were introduced in two subunits lying adjacent or diagonal to each other (Ulens and Siegelbaum, 2003). Cyclic AMP gated those channels more effectively in which functional CNBDs were arranged diagonally to each other. This dependence on the symmetry of CNBD arrangement is best explained in a model whereby a cAMP-induced dimerization of CNBDs is followed by the dimerization of these dimers into a tetramer. Thus, cAMP gating of HCN channels can be visualized as a series of structural rearrangements within the C-terminus. These involve the formation of cAMP-induced dimers of CNBDs that promote the interaction between C-linkers and the generation of a tetramer. The tetramer forms a bulky ring-like structure beneath the channel pore that extends by ~5 nm into the cytosol. By an as yet unknown mechanism, the conformational changes induced in the C-linkers are then transmitted to the transmembrane gating domains, thereby facilitating channel opening. This coupling appears to involve the S1-S2 linker and the S2 domain (Stieber et al., 2003a), and additional unknown channel portions (Stieber *et al.*, 2005). A detailed review further describes and compares the currently proposed activation mechanisms of HCN channels by cAMP (Craven and Zagotta, 2006).

3.3.7 Molecular elements controlling isoform-specific properties

Some of the molecular elements underlying the striking differences in basal activation kinetics between HCN1- and HCN4-currents (Ishii *et al.*, 2001) and HCN2- and HCN4-currents were determined (Stieber et al., 2003a). In both cases, exchanging S1 and S1-S2 linker domain interconverted the basal activation properties of the currents (Ishii et al., 2001; Stieber et al., 2003a). In addition, in HCN1 channels, the C-terminal intracellular portion may contribute to the rapid activation kinetics (Ishii *et al.*, 2001). A particularly important role for the difference between HCN2 and HCN4 is played by the leucine at position 272 within S1,

because its mutation to phenylalanine, the respective residue in HCN2, transfers both the basal activation kinetics of HCN2, as well as cAMP-induced changes in current kinetics typical for HCN2. However, when leucine 272 was replaced with methionine, the equivalent amino acid found in HCN1, activation kinetics were not accelerated. This supports the notion that more complex structural differences exist between the rapidly activating HCN1- and the more slowly activating, and structurally tightly related, HCN2- and HCN4-currents.

The potency of modulation by cAMP is strikingly low for HCN1-mediated currents (Santoro *et al.*, 1998), while it is high for HCN2- and HCN4-mediated currents (Ludwig *et al.*, 1999; Seifert *et al.*, 1999). Chimeric channels, composed of domains derived from HCN1 and HCN2 protein, were used to examine the molecular basis for this difference (Wang *et al.*, 2001). The difference in cAMP modulation can be largely attributed to differences in the C-linker domains between the two channel isoforms, whereas differences in basal voltage dependence are determined by the C-terminal portions and by core transmembrane regions.

3.4 Cellular and sub-cellular localization

This chapter is devoted to the molecular expression patterns of HCN subunits in the brain and in the heart, and briefly summarizes the detailed studies carried out at the mRNA and protein levels. Moreover, it asks about how the molecular expression correlates with the properties of electrophysiologically characterized currents. Finally, it highlights recent studies that analyze the subcellular localizations of HCN channels, showing that these confer to neurones a compartmentalized control of their intrinsic excitability.

3.4.1 Molecular expression patterns of HCN channel subunits in brain and heart

All four HCN channel isoforms are expressed in the rodent brain. At the mRNA level, HCN channel subunits show distinct expression patterns that are largely consistent between studies on rat and mouse (Moosmang et al., 1999; Monteggia et al., 2000; Santoro et al., 2000). A detailed map of HCN protein localization at cellular and subcellular levels in the rat brain was established (Notomi and Shigemoto, 2004). Immunohistochemical expression largely confirmed and further refined mRNA expression studies. HCN1 expression shows a predominance in cortical structures, with distinct expression in dendritic fields of hippocampus, cerebral cortex and superior colliculus. In addition, Purkinje cells of the cerebellum and brainstem motor nuclei show high HCN1 mRNA levels, although immunoreactivity in Purkinje cells was comparatively moderate. In contrast, HCN2 expression is found more uniformly throughout the brain, with strong expression in olfactory bulb, in thalamus, and in brainstem regions. HCN2 expression often overlaps with other HCN channel subunits, most notably with the gradient of HCN1 expression in cortical and hippocampal apical dendrites, but accounts for most of the HCN expression in nucleus reticularis thalami and the subthalamic nucleus. Notably, a subpopulation of oligodendrocytes that occupy perineuronal space throughout the brain, shows HCN2-like immunoreactivity. HCN3 generally shows the weakest expression, but is present in olfactory bulb, hypothalamus and the substantia nigra pars compacta. Finally, HCN4 is found in regions in which I_{HCN} functions as pacemaker, such as the thalamus, but is also present in olfactory bulb and in hypothalamus.

Messenger RNA expressed in the heart is predominantly found for HCN1, HCN2 and

HCN4, but isoform expression varies among species and cardiac tissue. The SAN exhibits the highest expression levels of HCN channels, with HCN4 accounting for ~80% of the total HCN message (Ishii *et al.*, 1999; Ludwig *et al.*, 1999; Shi *et al.*, 1999) in all species investigated (rabbit, mouse, dog). Species-dependent differences for HCN2 and HCN1 were seen for the remaining 20% (Moosmang et al., 2001; Marionneau et al., 2005; Zicha et al., 2005). Expression of HCN channel subunits is also detected outside the sinus node region, albeit at lower levels. Both HCN1 and HCN4 message decrement in expression from atrial ventricular node to the ventricles (Marionneau *et al.*, 2005).

3.4.2 Molecular expression patterns of HCN channel subunits in sensory systems and spinal chord

HCN channels are prominently expressed in diverse types of sensory neurones, including neurones of the dorsal root ganglia (Mayer and Westbrook, 1983; Moosmang et al., 2001; Chaplan et al., 2003; Tu et al., 2004) and the nodose ganglia (Doan *et al.*, 2004). High levels of HCN1 mRNA have been detected in mouse photoreceptor cells (Moosmang *et al.*, 2001; Demontis *et al.*, 2002; Müller *et al.*, 2003). The cell-type specific distribution of HCN1 channels has been described for the main olfactory bulb (Holderith *et al.*, 2003), and for spinal cord and medulla oblongata (Milligan *et al.*, 2006).

3.4.3 Correlation of expression patterns with native current properties

The expression patterns of HCN subunits are remarkably predictive, at least in a semiquantitative manner, for some of the basic properties of expressed currents, in particular their activation kinetics and their cAMP sensitivity. Typically, rapidly activating I_{HCN} is found in HCN1-expressing tissue, while slow currents predominate in regions with HCN2 and HCN4 (Santoro *et al.*, 2000). This correlation was established more quantitatively for HCN1. Current properties correlate with HCN1 mRNA in single-cells (Franz *et al.*, 2000) and gradients of current amplitudes in dendrites of cortical layer V pyramidal neurones are proportional to gradients of HCN1 subunit expression (Lörincz *et al.*, 2002; Kole *et al.*, 2006).

Notably, in the nucleus laminaris of the chick, HCN1 is expressed in a gradient, along which current properties gradually change correspondingly (Yamada *et al.*, 2005). Studies in animal models of epilepsy indicate that slight imbalances in HCN1 expression, be it up- or

downregulation, alter current properties accordingly in different cell types (Strauss *et al.*, 2004; Budde *et al.*, 2005; Kuisle *et al.*, 2006). Finally, developmental studies show that expression of HCN1 leads to an acceleration of current kinetics (Vasilyev and Barish, 2002), while in ventricular myocytes a decreased expression of HCN1 and HCN4 mRNA is paralleled by a decrease in I_{HCN} (Cerbai et al., 1999b; Shi et al., 1999; Yasui et al., 2001).

There are also a number of cases, in which a correlation between the native current properties and expressed isoforms could only be partially achieved. In SAN, native current properties are not fully congruent with those of HCN1-HCN4 heteromers (Altomare *et al.*, 2003), but currents generated by HCN2-HCN4 heteromers fit well with the properties of atrial I_{HCN}. In nucleus reticularis cells, HCN2 is expressed strongly, but current amplitudes are comparatively small (Santoro et al., 2000; Rateau and Ropert, 2006).

3.4.4 Subcellular localization

Two prominent cases point to a highly regulated subcellular expression of HCN channels. The first is the gradient of HCN1 and HCN2 expression of pyramidal cells in layer V cortical neurones and in hippocampal CA1 neurones (Santoro *et al.*, 1997; Lörincz *et al.*, 2002). Immunogold labeling shows that this labeling is predominantly dendritic, and largely absent from glutamatergic synapses (Notomi and Shigemoto, 2004). This increase in HCN channel density correlates with an increasing I_{HCN} amplitude (Magee, 1998; Williams and Stuart, 2000; Kole et al., 2006) and is of great relevance in the dendritic computational properties of these cells (see Section 3.6). How the subcellular trafficking of HCN1 protein gives rise to this steep gradient is unclear, but a first protein regulating channel expression has been identified (Santoro *et al.*, 2004) (see Section 3.2.6). Labeling in the pyramidal cell layer is mostly due to presynaptic labeling of basket cell terminals.

Recordings from presynaptic terminals revealed the presence of I_{HCN}. These include chick ciliary ganglion neurones (Fletcher and Chiappinelli, 1992), crayfish neuromuscular junction (Beaumont and Zucker, 2000), cerebellar basket cells (Southan *et al.*, 2000) and the calyx of Held in auditory brainstem (Cuttle *et al.*, 2001). HCN1 protein is expressed in basket cell terminals (Santoro *et al.*, 1997), and colocalizes with GAD65 and synaptophysin, suggesting that HCN1-currents are involved in inhibitory synaptic transmission (Luján *et al.*, 2005). Indeed, in some neurones, pharmacologically reducing I_{HCN} with ZD7288 reduces GABAergic transmission in a manner consistent with a presynaptic mechanism (Southan *et al.*, 2000; Aponte *et al.*, 2006). Presynaptic expression of HCN channel protein was also

found in retinal bipolar cells and in photoreceptors (Müller *et al.*, 2003).

HCN channels may be localized in discrete microdomains of the cellular membrane, in conjunction with other ion channels and regulatory molecules. HCN4 channels are found in membrane fractions containing structural proteins of lipid rafts ('caveolae') in HEK and SAN cells, and disruption of lipid rafts altered current-voltage dependence (Barbuti *et al.*, 2004). Additionally, HCN channels appear to be colocalized with T-type, low-threshold Ca^{2+} channels in dendrites of thalamocortical neurones (Stuart and Williams, 2000), perhaps explaining the regulation of I_{HCN} by Ca^{2+} in these cells (Lüthi and McCormick, 1998a).

3.4.5 Developmental expression patterns

Differential up- and downregulation of individual HCN channel subunits occurs during development (for review see (Frère *et al.*, 2004), as shown in most detail for hippocampal CA1 and CA3 principal cells and interneurones (Bender et al., 2001; Vasilyev and Barish, 2002; Bender et al., 2005; Brewster et al., 2006; Surges et al., 2006). The temporal profile of HCN channel expression and current density matches that of synchronized electrical activity appearing during circuit maturation (Bender *et al.*, 2005; Brewster *et al.*, 2006). Activation rates increase with development, in agreement with a strong increase of HCN1 channel expression in comparison to HCN2 and HCN4 channels.

In embryonic ventricular myocytes, an initially large I_{HCN} gradually decays until the adult stage (Robinson et al., 1997; Cerbai et al., 1999b), being accompanied perinatally by the loss of the ability to generate spontaneous activity (Cerbai et al., 1999b; Shi et al., 1999; Yasui et al., 2001). This decrement is paralleled by a gradually reduced expression of HCN1 and HCN4 mRNA, until HCN2 becomes predominant in adult cardiac myocytes (Shi *et al.*, 1999).

3.5 Pharmacology

The sensitivity of I_{HCN} to millimolar concentrations of extracellular Cs^+, and its relative insensitivity to Ba^{2+}, were crucial for its original isolation as a cationic current in cardiac cells (Fain et al., 1978; Bader et al., 1979; DiFrancesco, 1981; Wollmuth and Hille, 1992). The mechanism of action of these ions involves binding within a multi-ion conducting pore that competes with the fully permeant ions Na^+ and K^+ (Pape, 1996). The pharmacological profile of I_{HCN} has diversified in recent years. In addition to bradycardiac agents, volatile and general anaesthetics interfere with HCN channel gating, while the antiepileptic agent lamotrigine potentiates hippocampal I_{HCN}. However, a common characteristics of all I_{HCN} blockers, including the most recently described ones, is the lack of full specificity for HCN channels. Furthermore, the pharmacology of I_{HCN} awaits the development of isoform-specific compounds that would allow to define subunit-specific roles in discrete brain regions.

3.5.1 Extracellular cations

Millimolar concentrations of Cs^+ ions, and, to a much weaker extent, Rb^+ ions, are fast blockers of I_{HCN}, acting from the extracellular side of the channel and penetrating deep into the pore to bind to an inner 'blocking site' (DiFrancesco, 1982; Gauss *et al.*, 1998). The block is voltage-dependent, being greater at hyperpolarised potentials, and vanishing at more depolarised potentials (for review, see (Pape, 1996)). The disadvantage of the experimental use of Cs^+ is its limited specificity: it also blocks neuronal K^+ channels (Constanti and Galvan, 1983) and interferes with K^+ uptake in glial cells (Janigro *et al.*, 1997).

Extracellular Ba^{2+} ions, principally known as blockers of inward rectifier K^+ currents, fairly strongly (~55%) reduce I_{HCN} in photoreceptor cells (Wollmuth, 1995). In contrast, Ba^{2+} effects are minor for hippocampal I_{HCN} and for heterologously expressed HCN1 and HCN2 channels (~20-40%) (van Welie et al., 2005). In general, the use of Ba^{2+} ions is well established to distinguish (even small) I_{HCN} components from large inward rectifier currents (see e.g. (Rateau and Ropert, 2006)) and to isolate cyclic-nucleotide-dependent current modulation via G_i-coupled neurotransmitter receptors that also target inward rectifier K^+ currents (Frère and Lüthi, 2004).

3.5.2 Bradycardiac agents

The important role of HCN channels on pacing in the heart is strongly supported by a class of heart rate-reducing agents that powerfully block the cardiac isoform of I_{HCN} (for review see (Baruscotti *et al.*, 2005) and (DiFrancesco, 2005)). These agents are termed bradycardiacs, because they selectively slow heart rate without interfering with other cardiovascular functions. Alinindine (ST567), was the first member of the family but its mechanism of action was not limited to I_{HCN} (Snyders and Van Bogaert, 1987).

Today the most widely used bradycardiac agent in I_{HCN} pharmacology is ZD7288 (originally named ICI D7288), which exhibits greatest specificity (Briggs *et al.*, 1994). ZD7288 blocks I_{HCN} in neurones and photoreceptor cells at concentrations between 10-100 μM (Harris and Constanti, 1995; Gasparini and DiFrancesco, 1997; Williams et al., 1997; Lüthi et al., 1998; Satoh and Yamada, 2000). Additionally, ZD7288 induces a negative shift of the I_{HCN} activation curve at a concentration of 0.3 μM *in vitro* in SAN cells (BoSmith et al., 1993). Distinctive characteristics of ZD7288 actions on whole-cell currents involve a) its slow kinetics, ranging in the order of minutes b) its poor reversibility c) and its apparent lack of use dependence in moderate voltage ranges, although its actions are relieved by strong hyperpolarisations. These properties are consistent with the localization of ZD7288 binding in the vicinity of the intracellular portions of the channel pore (Shin *et al.*, 2001). In spite of the remarkable selectivity of ZD7288 for HCN channels, it should be considered that ZD7288 induces a downregulation of synaptic transmission that is independent of I_{HCN} (Chevaleyre and Castillo, 2002) and could involve a direct action on ionotropic glutamate receptors (Chen, 2004). This renders the usage of ZD7288 particularly delicate when assessing potential roles for I_{HCN} in synaptic transmission (Mellor *et al.*, 2002).

In addition to ZD7288, zatebradine (UL-FS 49) (Pape, 1994) and its more potent derivative cilobradine (DK AH 268) (Pape, 1994; Van Bogaert and Pittoors, 2003) block I_{HCN} in a concentration range of 10-100 μM. In contrast to ZD7288, however, these agents are "open channel blockers" and their effects are strictly use-dependent.

The member of the family of bradycardiac agents with greatest specificity for cardiac I_{HCN} is ivabradine [Corlentor®]. It is clinically used to reduce heart rate in the treatment of stable angina pectoris (Bucchi *et al.*, 2002; Bucchi *et al.*, 2006). Like zatebradine, ivabradine blocks I_{HCN} in a use-dependent manner from the intracellular side in a low micromolar concentration range. Unlike zatebradine, the action of ivabradine is dependent on ion flow across the channel pore (Bucchi *et al.*, 2002).

Currently available studies on bradycardiac agents reveal no specificity for HCN channel isoforms, although the mechanisms of block may differ for HCN1 and HCN4 (Bucchi *et al.*, 2006; Stieber *et al.*, 2006).

3.5.3 Anaesthetic agents

Volatile anaesthetics have been predominantly known to act as powerful modulators of the two pore domain K^+ channels (for review see (Franks and Lieb, 1988)). However, studies on both native and cloned HCN channels have revealed a strong inhibitory action of volatile anaesthetics at clinically relevant concentrations (Sirois et al., 1998; Sirois et al., 2002; Chen et al., 2005b). These studies reveal the first evidence for an isoform-specific blockade of HCN channels. Thus, halothane [Fluothane®] primarily shifts the voltage dependence of HCN1-currents towards the more hyperpolarised range, while reducing the maximal conductance of HCN2-currents (Chen *et al.*, 2005b). These effects correlate strongly with the molecular make-up of the two channel isoforms: the CNBD and the C-linker of HCN1 channels mediate the halothane-induced shift in voltage dependence, while core transmembrane domains of HCN2 mediate the inhibition of conductance. The differential actions of halothane on isoforms are lost when cAMP is bound, implying a role for the CNBD, and its inhibitory effects on channel gating, in the isoform specificity of halothane.

The general anaesthetic propofol [Diprivan®] inhibits I_{HCN} in addition to its modulatory action on GABAergic inhibition (Jurd *et al.*, 2003) and on small conductance (SK)-type K^+ channels (Ying and Goldstein, 2005). Both in cortical pyramidal neurones (Chen *et al.*, 2005a) and in thalamocortical cells (Ying *et al.*, 2006) strong negative shifts of about 10 mV were induced by propofol at clinically relevant concentrations of 3-5 μM. Propofol also inhibits expressed channels in an isoform-specific manner, with the most pronounced actions on HCN1-currents, and more moderate effects on HCN2- and HCN4-currents (Cacheaux *et al.*, 2005; Chen *et al.*, 2005a). Given the strong expression of HCN2 and HCN4 protein in thalamus, it remains unclear which native channel isoform in thalamocortical cells confers high propofol sensitivity. Interestingly, propofol also induces a decrement of HCN2 and HCN4 protein expression 3-24 hours after a single propofol injection (Ying *et al.*, 2006).

3.5.4 Antiepileptic agents

Commonly used antiepileptic drugs are powerful potentiators of HCN channels (Poolos

et al., 2002; Berger *et al.*, 2003; Surges *et al.*, 2003). In hippocampal principal cells, lamotrigine [Lamictal®] acutely causes a several fold upregulation of dendritic I_{HCN} amplitude around resting membrane potentials and an 11 mV positive shift in current voltage-dependence (Poolos et al., 2002; Berger and Luscher, 2004) at concentrations of 50-100 μM *in vitro*. This dendritic current enhancement may attenuate neuronal excitability, either via a decrease in dendritic input resistance or a reduced temporal summation of synaptic inputs (see Section 3.6.5). However, additional effects of lamotrigine on voltage-gated Na^+ channels may explain its neuronal-specific actions (Berger and Luscher, 2004). A potentiating action has also been reported for gabapentine [Neurontin®] (Surges *et al.*, 2003).

3.5.5 Additional blockers

An inhibitory effect on HCN1-mediated currents (Gill et al., 2004), and of hippocampal I_{HCN} (Ray *et al.*, 2003), has been reported for capsazepine, a blocker of vanilloid receptors, with an IC_{50} of 8 μM**.** QX-314, a quaternary derivative of lidocaine and best known as an intracellular blocker of voltage-activated Na^+ channels, completely blocked I_{HCN} in CA1 pyramidal cells (Perkins and Wong, 1995). Additionally, it was proposed that the tyrosine kinase inhibitor genistein (Shibata et al., 1999; Altomare et al., 2006) and the α_2-receptor agonist clonidine (Parkis and Berger, 1997; Knaus et al., 2007) might directly inhibit I_{HCN}.

3.6 Physiology

Previous reviews have excellently summarized the role of HCN-currents in neuronal and cardiac functions (Pape, 1996; Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003; Baruscotti and DiFrancesco, 2004; Stieber et al., 2004; Cohen and Robinson, 2006). This chapter reports mostly on the novel insights into long-recognized functions of I_{HCN}, and then describes some of the most recently recognized functions of this current.

3.6.1 Physiological and behavioral deficiencies in HCN-subunit knockout mice

Animals deficient in HCN-channel subunits illustrate the multiple involvement of HCN channels in neuronal and cardiac functions. The lack of the HCN1 gene in Purkinje and basket cells of cerebellum causes selective deficits in the training of repetitive movements that involve the phasic excitation and inhibition of cerebellar Purkinje cells (Nolan *et al.*, 2003). In these cells, the HCN1-deficiency retards the resumption of action potential discharge after hyperpolarisation, since any voltage deviation induced by transient inhibition is greater than normal and repolarisation is slowed. Thus, by allowing a rapid response to oscillatory inputs, I_{HCN} may facilitate the coincidence detection of pre- and postsynaptic activity that is required for synaptic plasticity of afferents to Purkinje cells. In contrast, the lack of HCN1 in cortical structures facilitates hippocampal-dependent spatial learning, potentiates long-term synaptic plasticity at some hippocampal afferents, and enhances subthreshold neuronal responses to inputs in the theta frequency range (Nolan *et al.*, 2004). Deleting the *HCN2* gene produces striking deficiencies in thalamic and cardiac cells. The resting membrane potential of thalamic neurones is strongly hyperpolarised, the propensity to discharge in bursts increased, and synchronous thalamocortical activity typical for generalized spike-and-wave discharges recorded in the electroenecephalogram (EEG). HCN2-deficient mice show cardiac arrhythmia, while sympathetic stimulation remains intact (Ludwig *et al.*, 2003). Finally, HCN4-deficient mice die at embryonic stages; show a strongly reduced heart rate and insensitivity to sympathetic stimuli. Cardiomyocytes from these animals are virtually devoid of hyperpolarisation-activated currents and pacemaker-like action potentials (Stieber et al., 2003b). Taken together, the deficits observed in HCN-channel subunit deficient animals illustrate, and further strengthen, the long-recognized importance of HCN-currents for some

of the cornerstones of neuronal excitability and of pacemaker functions. However, they also point to an unexpected involvement of I_{HCN} in synaptic integration and long-term synaptic plasticity (Nolan *et al.*, 2003; Nolan *et al.*, 2004).

3.6.2 HCN-currents and pacemaking

The discovery that the diastolic phase of the cardiac action potential is carried predominantly by a cationic hyperpolarisation-activated current has set the ground for the unique profile of I_{HCN} as a pacemaker current (Brown *et al.*, 1979; DiFrancesco, 1981). Detailed reviews on the mechanisms of pacemaking in cardiac and neuronal preparations are found in (Lüthi and McCormick, 1998b; Robinson and Siegelbaum, 2003; Frère et al., 2004; Baruscotti et al., 2005; Cohen and Robinson, 2006). Neuronal pacemaker functions attributed to I_{HCN} figure prominently in rhythms studied extensively in *in vitro* neuronal preparations, including sleep-related oscillations in thalamocortical cells and circuits (see Section 3.6.8 for detail, for review, see (Lüthi and McCormick, 1998b)), γ-oscillations in hippocampus (Fisahn *et al.*, 2002), synchronized oscillations in inferior olive (Bal and McCormick, 1997), and subthreshold oscillations in entorhinal cortex (Dickson *et al.*, 2000). In autonomously firing neurons, a kinetically precisely tuned I_{HCN} sets the regularity and timing of action potential discharge (Chan *et al.*, 2004).

Importantly, I_{HCN} not only serves as a pacemaker by virtue of its voltage-dependent activation at hyperpolarised potentials. The dual allosteric gating by cAMP and voltage also allows for a slow, persistent form of current enhancement that may underlie the recurrence of synchronous oscillations at time scales of once every 10-30 s (Lüthi and McCormick, 1998b; Wang et al., 2002).

Most recent advances concerning the role of HCN channels in pacemaker mechanisms include the molecular identification of the HCN channel isoforms for distinct types and aspects of cardiac and neuronal pacemaking. In cardiac myocytes, overexpression and dominant-negative suppression of HCN2 and HCN4 increase or suppress cardiac beating, respectively, both *in vitro* and *in vivo* (for review, see (Cohen and Robinson, 2006)). Genetic deletion of HCN2 reduces SAN currents by 30%, whereas eliminating HCN4 in embryonic myocytes almost entirely abolished the current. Consistent with these effects, heart rate was more variable, but still present in HCN2-deficient animals, whereas the HCN4-deficient animals are embryonically lethal due to a failure of heart beating.

The fact that HCN2- and HCN4-mediated currents are largely responsible for the

diastolic phase of the cardiac rhythm has prompted the idea that *HCN* genes could be used to re-create a pacemaker for the arrhythmic or failing heart. The initial experiments with viral or cell-based delivery of *HCN* genes into cardiac cells have proven remarkably successful (for review, see (Robinson *et al.*, 2006)). Thus, within three days after injection of HCN2-containing adenovirus in the canine atrium or in the Purkinje fibres, substantial current was expressed that promoted escape rhythms driving the heart after sinus node arrest (Qu *et al.*, 2003; Plotnikov *et al.*, 2004).

3.6.3 HCN-currents and resting membrane potentials

By virtue of its 10-15% activation around resting membrane potential and its non-inactivating properties, standing HCN-currents exert a depolarising action on neuronal resting properties in many different cell types (Pape, 1996). Activation of I_{HCN} around rest stabilizes membrane potential against hyperpolarisation (Ludwig *et al.*, 2003; Nolan *et al.*, 2003), while its deactivation antagonizes membrane depolarisation (Magee, 1999). The importance of shortening transient hyperpolarisation to re-instate cellular excitability is perhaps best demonstrated in bulbospinal neurones involved in rhythmic breathing movements (Dekin, 1993) and in Purkinje cells that are phasically inhibited during motor activity (Nolan *et al.*, 2003). Around rest, the depolarising effect of I_{HCN} is cancelled by hyperpolarising currents to abolish net current flow at the resting membrane potential. In thalamocortical neurons, HCN2-currents and TASK3-currents counterbalance each other (Meuth *et al.*, 2006). Consequently, although both these channel types are highly sensitive to extracellular acidification, thalamocortical cellular resting membrane potential is relatively unaffected by drops in the pH. Conversely, volatile anesthetics, which inhibit HCN- and potentiate TASK-currents, dramatically hyperpolarise resting membrane potential (Sirois et al., 2002). Interactions between HCN channels and K_{ir}/K_{leak} channels are prominent in pyramidal cell dendrites and maintain I_{HCN} activation sufficiently high to allow for its control of temporal summation (Day *et al.*, 2005). A standing I_{HCN} is also crucial for stabilizing the on-going action potential discharge in Purkinje neurons (Williams *et al.*, 2002). Primary sensory neurons exploit the pH and temperature sensitivity of HCN currents to generate sensory receptor potentials (Stevens *et al.*, 2001; Viana *et al.*, 2002).

3.6.4 Regulation by cAMP and novel modulators

A canonical way of I_{HCN} regulation is via the direct binding to cyclic nucleotides, independently of the action of PKA (DiFrancesco and Tortora, 1991; Pedarzani and Storm, 1995). Since then, it has been shown that many G-protein-coupled neurotransmitters, endogenous neuropeptides and inflammatory agents regulate I_{HCN} via altering intracellular cAMP and cGMP levels (for an overview, see (Pape, 1996; Frère *et al.*, 2004)). These compounds principally mediate their cAMP-dependent actions by shifting the activation curve towards more depolarised potentials, while leaving maximal current conductance unaltered. The regulation by cAMP is bidirectional, in that both increases and decreases in standing cAMP levels, mediated by G_s- and $G_{i/o}$-coupled neurotransmitter receptors, respectively, can be detected (for an overview, see (Frère *et al.*, 2004)). In entorhinal cortex, the dopaminergic decrease in neuronal excitability is mediated, at least in part, by cAMP-dependent regulation of HCN channels present in apical dendrites (Rosenkranz and Johnston, 2006). A number of effects of neurotransmitters on I_{HCN} appear, however, unrelated to cAMP. In hippocampus, muscarinic receptor agonists augment I_{HCN} by increasing its maximal conductance, independently of cAMP (Colino and Halliwell, 1993; Fisahn et al., 2002), and serotonin augments I_{HCN} by affecting both maximal conductance and activation curve, an effect that is not fully mimicked by cAMP (Gasparini and DiFrancesco, 1999; Bickmeyer et al., 2002). Furthermore, a κ-opioid receptor agonist augments the maximum conductance of I_{HCN} in brainstem neurones via mobilization of intracellular Ca^{2+}, without shifting the I_{HCN} activation curve (Pan, 2003). In hypothalamic paraventricular neurones, neuromedin-U, best known for its effect on gut smooth muscle, positively shifted the voltage dependence of I_{HCN}, likely via stimulation of phospholipase C pathways (Qiu *et al.*, 2003). Finally, the sleep-promoting peptide cortistatin augments the maximal conductance of I_{HCN} by a cAMP-independent mechanism (Schweitzer *et al.*, 2003). From these available data, it is reasonable to assume that additional modulators of HCN channels exist. Some of these are likely to be found in the messenger pathways involving mobilization of intracellular Ca^{2+} and phosphoinositide pathways, although Ca^{2+} has no direct effect on at least the thalamic isoforms of I_{HCN} (Lüthi and McCormick, 1999b; Fan et al., 2005). Most recently, p38 MAPK was identified as a powerful modulator of the voltage dependence of hippocampal I_{HCN} (Poolos *et al.*, 2006). Inhibition of p38 MAPK negatively shifted the activation curve of hippocampal I_{HCN} by ~25 mV, whereas activation of this kinase induced an 11 mV positive shift, revealing the largest voltage window so far reported for I_{HCN} regulation. The proposed phosphorylation site for p38 MAPK involves T778 on HCN1, located distal to the CNBD, whereas it is T17 for

HCN2 on the N-terminus (Figure 3). The existence of consensus phosphorylation sites for additional kinases (Poolos *et al.*, 2006), and the diverse types of tyrosine kinase modulation of heterologously expressed (see e.g. (Arinsburg *et al.*, 2006)) and native isoforms of I_{HCN} (Thoby-Brisson *et al.*, 2003) strongly suggests that elucidation of the pathways controlling HCN channel phosphorylation may soon lead to additional insights into the physiological roles of this current.

3.6.5 HCN-currents in dendritic integration

The active properties of dendrites play an important role in how synaptic input is shaped and integrated. A notable feature of dendritic HCN1 is its expression along a steep gradient in the apical dendrites of CA1 pyramidal neurons and layer V cortical neurons, with channel density increasing by over 60-fold towards the distal end in the latter, and about 7-fold in the former (see Section 3.4.3, 3.4.4). A standing I_{HCN} contributes up to 11 mV to the resting membrane potential of apical dendrites (Williams and Stuart, 2000). When this current is deactivated partially, as it may occur by small subthreshold depolarisation, it is rapid enough to accelerate the decay of these deviations from resting potential. The deactivation of I_{HCN} is boosted during repetitive depolarisations and produces a net membrane hyperpolarisation that effectively impairs the temporal summation of postsynaptic excitatory potentials, generated by presynaptic firing up to about 100 Hz. These effects are most pronounced for inputs at distal dendrites, where HCN channel density is highest. Notably, the density of HCN channels appears tuned such that it exactly compensates the incrementing electrotonic filtering of the tapering dendritic cables, thereby rendering the temporal summation essentially independent of dendritic location (Williams and Stuart, 2000). By a similar mechanism, dendritic I_{HCN} also antagonizes the summation of backpropagating action potentials into cortical layer V dendrites (Berger *et al.*, 2003). The acceleration, and hence sharpening of excitatory postsynaptic potentials (EPSPs), could be particularly important in auditory processing, in which high temporal precision in the detection of inputs is required (Yamada *et al.*, 2005). The high HCN channel density, however, also imposes a shunting conductance on the hippocampal (Berger *et al.*, 2001) and cortical (Fernandez *et al.*, 2002) neuronal dendrites, and produces a disproportionate attenuation of the amplitude of distal inputs. A higher density of AMPA-type glutamate receptors at distal dendrites reinstates the site independence of EPSP amplitude (Andrasfalvy and Magee, 2001).

The rapid deactivation and reactivation of I_{HCN} shapes the responsiveness of dendrites to

oscillatory input, imparting to them a resonance at frequencies in the theta range (~5 Hz) (Magee, 2001; Hu *et al.*, 2002). Computational models show that stochastic fluctuations of HCN channels, present at high densities in cortical dendrites, are strong enough to help regulate the precision of action potential discharge to periodic inputs (Kole *et al.*, 2006).

3.6.6 HCN-currents in synaptic plasticity

A novel and exciting theme in HCN channel physiology is the recognition that these channels are members of the growing family of ion channels the expression of which is rapidly regulated by neuronal activity. Thus, I_{HCN} is an important determinant of how intrinsic excitability can be modified, thereby influencing how synaptic input is transformed into axonal output (Zhang and Linden, 2003). Modifications in excitability, accompanied by changes in I_{HCN}, have been reported from different groups. In the CA1 area, HCN-mediated currents were enhanced several fold and rapidly, within tens of minutes following exposure to pressure-applied glutamate (van Welie *et al.*, 2004), following synaptic release of glutamate via a theta-burst pairing protocol, or following brief increases in extracellular K^+ levels (Fan et al., 2005). In younger animals, input-specific modifications of I_{HCN}, as well as local changes in dendritic integration were reported (Wang *et al.*, 2003). Long-term plasticity was accompanied by an enhanced expression of HCN1 protein, while HCN2 expression remained unchanged. Entry of Ca^{2+} was required for this effect, with NMDA receptors as the principal source, and CaMKII and the protein synthesis machinery as likely mediators. The increased I_{HCN} decreases neuronal excitability persistently and could hence be involved in the dendritic mechanisms controlling synaptic plasticity. Presynaptically expressed I_{HCN} has also been implicated in mediating presynaptic forms of synaptic plasticity, both in mammalian hippocampus (Mellor *et al.*, 2002) (but see (Chevaleyre and Castillo, 2002)), as well as in invertebrates (Beaumont and Zucker, 2000; Beaumont et al., 2002).

3.6.7 HCN-currents in presynaptic neurotransmitter release

In one of the original cloning studies, it was realized that HCN1 protein was strongly expressed in axon terminals of cerebellar basket cells (Santoro *et al.*, 1997). In these terminals (Southan *et al.*, 2000), as well as in the giant presynaptic terminals of the rat auditory pathway (Cuttle *et al.*, 2001) and the avian ciliary ganglion (Fletcher and Chiappinelli, 1992), substantial I_{HCN} is expressed. However, so far, evidence for an involvement of this current in

presynaptic neurotransmitter release in these terminals is lacking, while presynaptic I_{HCN} in dentate gyrus basket cells (Aponte *et al.*, 2006) and in crustacean terminals was proposed to facilitate neurotransmitter release (Beaumont and Zucker, 2000). In cerebellar basket cells, as well as in stratum oriens interneurones of hippocampus (Lupica *et al.*, 2001), blocking I_{HCN} reduces spontaneous GABAergic transmission, consistent with I_{HCN} controlling somatodendritic excitability of these neurones and axonal propagation. More recently, it was shown that blocking I_{HCN} increases the threshold for axonal action potential initiation (Hiramatsu *et al.*, 2002; Aponte *et al.*, 2006), probably because the membrane hyperpolarisation generated by the electrogenic Na^+/K^+ ATPase (Pachucki *et al.*, 1999) is no longer antagonized by I_{HCN}.

3.6.8 HCN-currents in the thalamus

To specifically highlight the importance of I_{HCN} in the thalamocortical system, the focus of this thesis, the following chapter describes its role as pacemaker current in rhythmic electrical activity on both the level of single neurons as well as on neuronal networks. The chapter summarizes classic work on thalamic I_{HCN}, which was fundamental in recognizing many of its unique properties. In addition, it addresses the issue of how HCN channels help to regulate the firing mode of thalamocortical cells and, by doing so, contribute to the control of arousal states. A correlation of the resting membrane potential, the firing mode of thalamocortical cells and arousal states could be determined *in vivo*. Furthermore, *in vitro* electrophysiological recordings revealed the involvement of HCN-currents in sleep related oscillations, namely delta waves and spindle waves.

Action potential firing mode. A principal property of thalamocortical neurons is to show dual action potential discharge modes (Figure 4). Depending on their membrane potential, these neurons fire in rhythmic burst discharges or, if they are depolarized, display tonic single spike activity ((Jahnsen and Llinas, 1984) for review, see (McCormick and Bal, 1997)). Thus, by contributing to the resting membrane potential both *in vivo* and *in vitro* (see Section 3.6.3; (Ludwig *et al.*, 2003; Meuth *et al.*, 2006)) HCN-currents help to set the firing mode of thalamocortical neurons. HCN channels activated at rest give rise to a tonic inward current. A positive shift in the activation range of HCN channels due to increased cAMP synthesis could increase the amount of this tonic inward current resulting in membrane depolarization. Closure of HCN channels would mean membrane hyperpolarization. Thus, *in vitro*-recordings

showed that modulation of I_{HCN} could result in a change of the action potential firing mode. This applies for blockade of HCN channels by Cs^+ (McCormick and Pape, 1990b), but also for the activation of I_{HCN} after stimulation of G_s-protein-coupled ß-adrenergic and serotoninergic receptors (McCormick and Pape, 1990a) that, together with the reduction of a resting leak K^+ conductance, induced membrane depolarization (for review, see (McCormick and Bal, 1997)). Interestingly, *in vivo*-recordings revealed a correlation between the membrane potential of thalamocortical neurons and the state of arousal. During sleep, thalamocortical neurons showed a hyperpolarized membrane potential and the main discharge pattern observed consisted in action potential bursting. Conversely, during waking or rapid-eye-movement sleep (REM sleep), the membrane potential was depolarized, supporting the tonic action potential firing mode (Hirsch *et al.*, 1983; McCarley *et al.*, 1983; Weyand *et al.*, 2001). In this manner, by their contribution to the resting membrane potential of thalamocortical neurons HCN channels could help to regulate the arousal state. For example, *in vitro*, sleep related oscillations were demonstrated to stop after application of noradrenalin and serotonin (Lee and McCormick, 1996). Increased cAMP synthesis after activation of G-protein coupled receptors (GPCRs) can shift the activation range of HCN channels to more depolarized potentials, thus depolarizing the membrane potential by giving rise to an inward current. These data demonstrate that HCN channels represent one of the cross-points between ascending neurotransmitter systems that activate cAMP generating GPCRs and the firing mode of thalamocortical cells.

Delta waves. In the absence of synaptic input, thalamocortical neurons show a pattern of slow rhythmic burst discharges at a frequency of 0.5-4 Hz. These delta-oscillations are found *in vitro* (McCormick and Pape, 1990b; Leresche et al., 1991; Soltesz et al., 1991; Destexhe et al., 1993; Steriade et al., 1993b; Hughes et al., 1998) and *in vivo* (Steriade *et al.*, 1991) and contribute to the emergence of slow EEG waves during deep sleep phases. The rhythmic bursts are driven through the interaction of a low-threshold Ca^{2+}current (I_T) and HCN-currents (for review, see (Pape, 1996; McCormick and Bal, 1997; Lüthi and McCormick, 1998b; Robinson and Siegelbaum, 2003). Lacking external synaptic influence, thalamocortical cells hyperpolarize around -75 mV. This membrane potential is sufficient to activate HCN-currents that depolarize the cell back towards threshold for I_T, generating a low-threshold Ca^{2+} spike. The peak of the Ca^{2+} spike is often crowned by a burst of high frequency action potentials. In-activation of I_T terminates the Ca^{2+} spike and the following hyperpolarization can activate HCN-currents again.

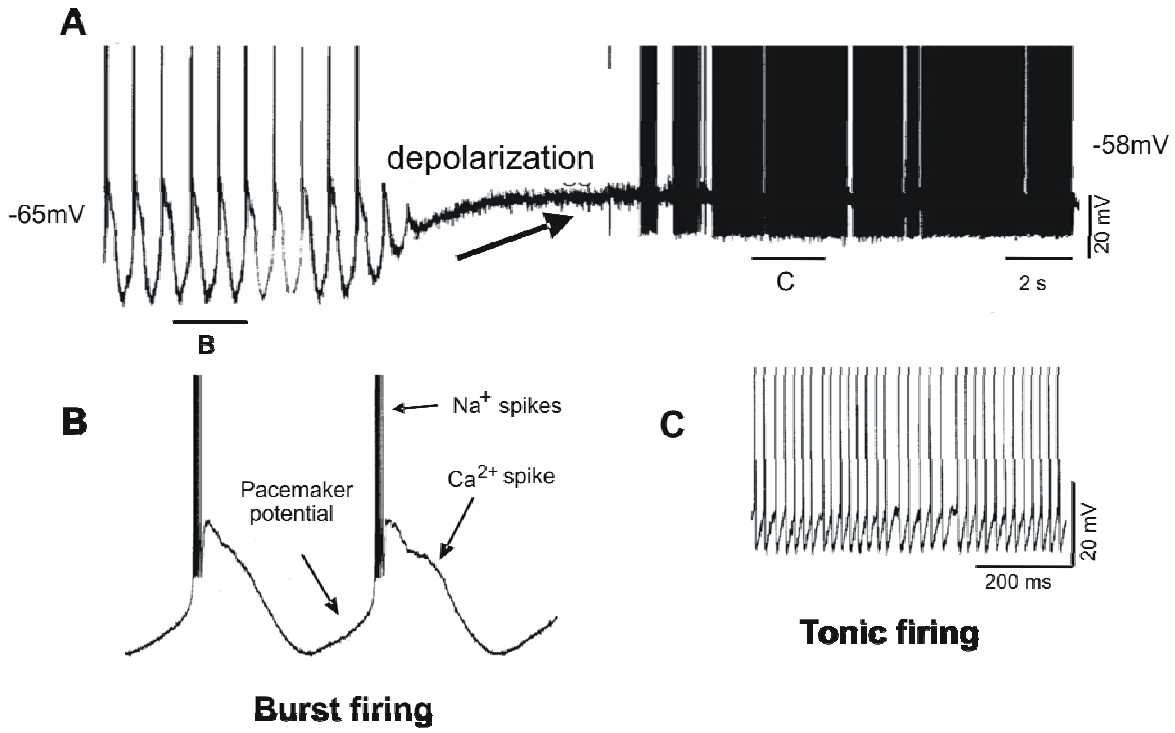

Figure 4. Two different firing modes of action potentials in thalamocortical neurons. *A,* Rhythmic burst firing in a thalamic relay neuron *in vitro* at a membrane potential of -65 mV (left). Depolarization of the cell to -58 mV (by intracellular injection of current) induces a switch to the tonic, or single spike, mode of action potential generation (right). *B*, Expanded trace of oscillatory burst activity that is mediated by an interaction of pacemaker currents and low-threshold Ca^{2+} currents. *C*, Expanded trace of single-spike activity. Adapted from (McCormick and Pape, 1990b).

Spindle waves. HCN-currents not only control oscillatory activity of single cells, but also contribute to network oscillations observed in intact thalamic preparations. During slow wave sleep, but also during some types of epilepsy, the thalamocortical system generates typical slow, synchronized firing patterns, which appear in the EEG as the so called spindle waves (Figure 5*A*). Thalamic neurons fire action potentials at 7-14 Hz that wax and wane over a period of 1-3 s. The phase of rapid spiking is followed by a prolonged period of silence lasting for 5-20 s (for review, see (McCormick and Bal, 1997)). Spindle waves are generated through an reciprocal interaction between the inhibitory, GABAergic neurons of the thalamic reticular nucleus and the excitatory, glutamatergic thalamic relay cells (Figure 5*B*, reviewed by (McCormick and Bal, 1997)). During this interaction, action potential firing in the excitatory thalamocortical relay neurons drives the firing of the reticular neurons. This, in turn, generates a burst of hyperpolarizing inhibitory postsynaptic potentials (IPSPs) in the relay neurons. These IPSPs activate HCN-currents and low-threshold Ca^{2+} spikes in

thalamocortical cells, which then, through the generation of bursts of action potentials, can reexcite the reticular cells. *In vitro*, the waxing of the spindle oscillations is due to a progressive increase in the recruitment of neurons that participate in the oscillation. The waning is induced by a prolonged depolarization of the thalamic relay neurons that results in the inactivation of the low-threshold Ca^{2+}-current, thus preventing the neurons from spiking (Figure 5*C*) This afterdepolarization (ADP) could be triggered by an experimental upregulation of HCN-currents through repetitive current injections, mimicking the arrival of spindle waves (Bal and McCormick, 1996). During the evoked rebound bursts both hyperpolarization activated HCN-currents and an intracellular increase in Ca^{2+} is required. The subsequent activation of Ca^{2+} stimulated adenylyl cyclases leads to elevated cAMP levels that further enhance the activation of HCN-currents (Lüthi and McCormick, 1999b). The persistence of HCN channel activation is also determined by the slow rate of cAMP dissociation from the CNBD at the cytoplasmic tail of the channel (Wang *et al.*, 2002). In this manner the slow periodicity of spindle waves, the re-occurrence of these events every 5-20 s only is governed by the unique properties of HCN channels, being dually gated by hyperpolarization and by cAMP.

Knockout animals. Studies on knockout animals give additional evidence for the involvement of I_T and I_{HCN} in thalamocortical oscillations. Mice lacking HCN2 channels, the HCN-subunit that is mainly expressed in the thalamus, showed increased spontaneous activity in thalamic neurons of brain slices (Ludwig et al., 2003). This oscillatory activity could be blocked by a specific T-type Ca^{2+} channel antagonist and could be mimicked in wild-type slices by treatment with the HCN-current blocker CsCl (Ludwig et al., 2003). Also T-type Ca^{2+} channel knockout mice had defects in sleep waves such as lack of delta oscillations (1-4 Hz) and alteration of sleep spindles (7-15 Hz) (Lee et al., 2004). These deficits were accompanied by sleep disturbances (Lee et al., 2004; Anderson et al., 2005).

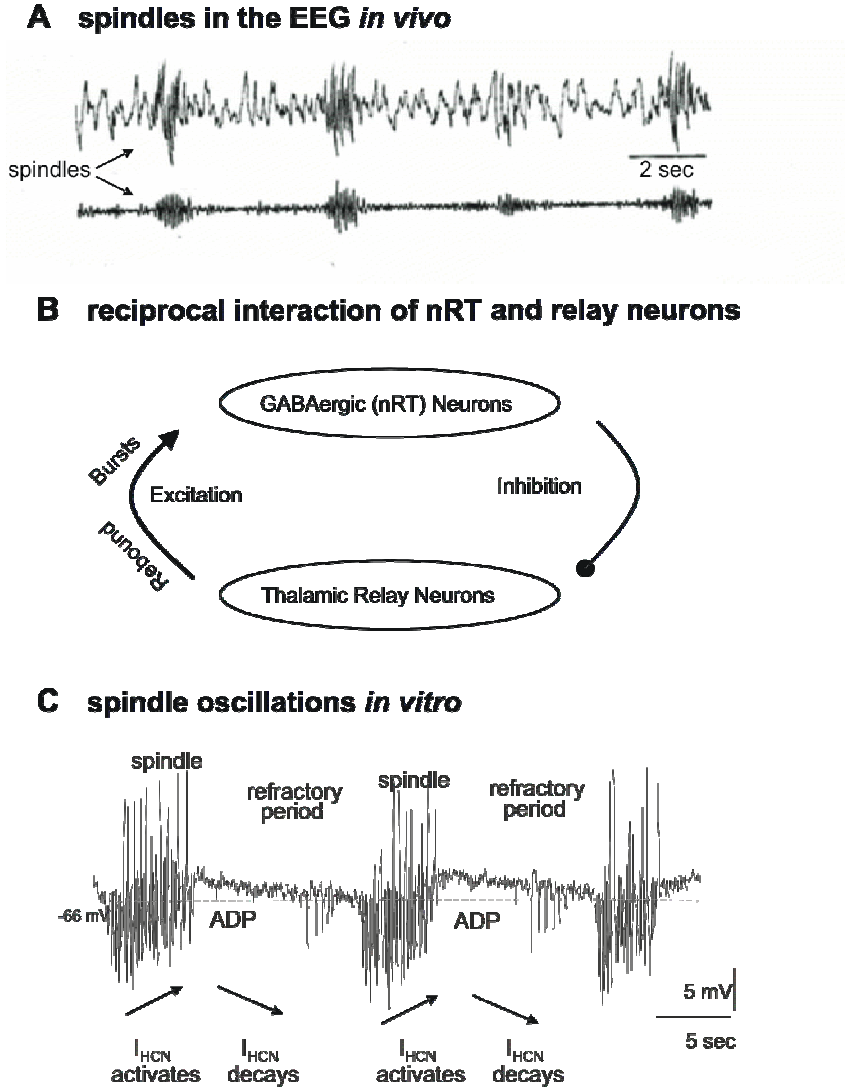

Figure 5. Spindle waves and the interaction between nRT and thalamocortical neurons during spindle wave generation. *A, In vivo* nREM sleep EEG recordings (upper trace) that are filtered for spindle waves (bottom trace) with a waxing and waning appearance. From (Steriade and Llinas, 1988). *B*, Spindle waves are generated *in vitro* by the reciprocal interaction between inhibitory neurons from the nRT and excitatory relay neurons. Inhibitory postsynaptic potentials can activate low-threshold Ca^{2+} rebound burst discharges in relay neurons. These rebound burst discharges can re-activate nRT neurons. *C*, Intracellular recordings from thalamocortical neurons *in vitro* show the slow afterdepolarization (ADP) that occurs in the refractory period following the generation of spindle waves. This ADP originates by upregulated HCN-currents. Adapted from (Bal and McCormick, 1996).

3.7 Relevance to disease states

HCN channels belong to the few ionic channels known to be sensitively altered at the transcriptional level in response to brief periods of aberrant electrical activity (Waxman, 2001). The ensuing abnormal functional expression of HCN channels may produce maladaptive increases in neuronal excitability, such as those typical for epilepsy and neuropathic pain. In the heart, ventricular myocytes show changes in HCN subunit transcription in response to cardiac stress, which may contribute to the arrhythmias that are seen in cardiac disease.

3.7.1 Epilepsies

Changes in HCN expression were found in a developmental form of epilepsy, in temporal lobe epilepsy and in generalized epilepsy (Santoro and Baram, 2003; Poolos, 2004). In animal models of developmental and temporal lobe epilepsy, HCN channel expression is disturbed following single seizure episodes and changes may persist for weeks, implicating them in the development of chronic epilepsy. Animal models of generalized epilepsy show abnormal HCN expression prior to the onset of the seizures that persists unaltered in the chronic phase, suggesting a genetically determined defect in HCN channel function that accompanies epilepsy. HCN channels appear thus involved in both inherited, as well as in acquired forms of epilepsy, and their rapid and high susceptibility to single phases of hyperexcitation could render them important causative factors in the processes of epileptogenesis.

Generalized epilepsies. Perhaps the most extensive characterization of HCN channel expression and properties is now available for models of absence epilepsy, a generalized form of absence epilepsy characterized by spike-and-wave discharges (SWDs) in the electroencephalogram (Waxman, 2001; Meeren *et al.*, 2005). These discharges arise from a hypersynchronous oscillation in reverberating thalamocortical loops, and both thalamic, as well as cortical mechanisms underlie these pathological oscillations. Remarkably, the two independent rat models of absence epilepsy, the WAG/Rij (Strauss *et al.*, 2004; Budde *et al.*, 2005) and the GAERS (Kuisle *et al.*, 2006) as well as the stargazer mouse model (Di Pasquale *et al.*, 1997), show altered I_{HCN} properties. Thalamocortical neurones of both WAG/Rij and GAERS show an increased expression of the cAMP-insensitive HCN1 channel isoform that is accompanied by a weakened cAMP sensitivity of HCN channels. The enhanced HCN1

expression persists throughout chronic stages, and may lead to an enhanced heteromer formation with HCN2, thereby reducing the sensitivity of the channels to cAMP transients typical for normal sleep-related oscillations (Kuisle *et al.*, 2006). In cortex of the WAG/Rij model, HCN1 expression and function was downregulated in layers II/III, producing an increase in the excitability of these neurones (Strauss *et al.*, 2004). Conversely, the stargazer mouse model shows an enhanced current amplitude in layer V principal neurones (Di Pasquale *et al.*, 1997).

Febrile seizures. Seizures induced by fever are the most common type of seizure in the developing brain and affect up to 5% of children younger than five years. In young rats, febrile seizures can be induced by briefly exposing the brain to a single (~20 min) period of hyperthermia. Such animals reliably (> 98%) show seizures and an increased tendency to develop epilepsy in adulthood (Toth *et al.*, 1998), suggesting that early life seizures may predispose to later epileptic susceptibility (Walker and Kullmann, 1999; Baram et al., 2002). Febrile seizures are accompanied by a number of persistent modifications of neuronal excitability in hippocampal neurones, amongst which changes in HCN mRNA expression figure prominently (Brewster *et al.*, 2002; Brewster *et al.*, 2005). A single experimental febrile seizure induced a persistent decrease in HCN1 mRNA and an increase in HCN2 mRNA in hippocampal CA1 pyramidal cells (Brewster *et al.*, 2002; Brewster *et al.*, 2005), that were accompanied by a depolarising shift in the current activation curve (Chen *et al.*, 2001b). These altered expression patterns were not found when animals were given antiepileptics prior to induction of hyperthermia, indicating that seizure activity, and not the elevated brain temperature, triggered changes in subunit expression. Notably, the changes in HCN mRNA expression levels persisted for up to 3 months, suggesting a long-lasting perturbation of the developmental program of HCN channel expression. In addition to the altered expression, HCN1 and HCN2 proteins increasingly associated in heteromers following the seizures, further promoting the functional expression of I_{HCN} with altered biophysical properties (Brewster *et al.*, 2005).

Temporal lobe epilepsy. The kainate-model model of temporal lobe epilepsy is produced by a single injection of the convulsant agent kainic acid, which leads to chronic epilepsy after a latency period of a few weeks (White, 2002). During this latent phase, which lacks behaviorally overt epileptic activity, HCN1 and HCN2 proteins in entorhinal cortex, the major component of the temporoammonic pathway into the hippocampus, are markedly downregulated within 24 hrs, and current amplitude is reduced for up to one week (Shah *et al.*, 2004). This downregulation of I_{HCN} causes enhanced excitability of entorhinal cortex layer

III neurones and increased temporal summation of excitatory input. It is widely believed that such modifications may represent a first step in the processes leading from the latent, seizure-free episode to chronic epilepsy.

In contrast, during advanced stages of chronic temporal lobe epilepsies, the expression of HCN protein in surviving neurones is altered in a manner that suggests a neuroprotective effect. Thus, in surviving dentate granule cells of sclerotic human hippocampus, HCN1 is markedly increased compared to the non-sclerotic case. Such current enhancements in surviving cells may help to counteract the excessive excitation and the associated excitotoxic cell death (Bender *et al.*, 2003).

These currently available studies document that, in at least some instances, an altered HCN subunit expression in neurones may be a defining characteristic of neuronal networks prone to develop an epileptic phenotype. The detailed patterns of modified channel expression are determined not only by the type of seizures, but also by the cell type and the developmental stage. In addition, HCN subunit expression, in particular HCN1 and HCN2, shows a high vulnerability to single periods of enhanced neuronal activity. The influences during developmental stages appear to be particularly multifactorial, given that single febrile seizures produce persistent current enhancements (Chen *et al.*, 2001b), but can be influenced by neonatal behavioural experience (Schridde *et al.*, 2006). The mechanisms translating seizures into altered HCN channel expression remain, so far, unexplored, but could range from acute influences, such as synaptic activity (see e.g. (van Welie *et al.*, 2004)) to long-term, chronic modulation of channel expression, for example via hormones and inflammatory processes (see e.g. (Armoundas et al., 2001; Vasilyev and Barish, 2004)).

3.7.2 Cardiopathies

Cardiac myocytes undergo electrical and structural remodeling to adapt to external stressful factors, such as pressure overload (e.g. hypertension), inflammation and infarction (for review, see (Armoundas *et al.*, 2001)). A number of cardiovascular diseases are accompanied by abnormal expression of HCN mRNA, notably also in areas in which this channel is poorly expressed under healthy conditions. Most prominently, cardiac ventricular myocytes, which are not normally involved in cardiac pacemaking, show increases in I_{HCN} magnitude or shifts in voltage dependence, rendering them potentially rhythmogenic (Cerbai and Mugelli, 2006).

An unexpected presence of I_{HCN} was first observed in ventricular myocytes of

spontaneously hypertensive rats that was accompanied by a small diastolic depolarisation (Cerbai *et al.*, 1994). The degree of rat myocardial hypertrophy is positively correlated with an increase in the density of I_{HCN}. This correlation indicates that the mechanisms underlying cardiac hypertrophy such as alteration in action potential properties and response to cellular stress steer HCN subunit expression (Cerbai *et al.*, 1996). At the molecular level, an upregulation of the HCN2 and HCN4 mRNA (Hiramatsu *et al.*, 2002; Fernandez-Velasco *et al.*, 2003) and HCN2 protein expression (Fernandez-Velasco *et al.*, 2006) the predominant isoforms underlying ventricular I_{HCN} (Shi *et al.*, 1999), were described. Again, the change in expression levels was most pronounced in those cardiac regions that had highest pressure overload (Fernandez-Velasco *et al.*, 2003; Sartiani *et al.*, 2006). These changes are fairly specific to HCN proteins amongst the family of currents involved in cardiac diastolic potentials (Fernandez-Velasco *et al.*, 2006). Cardiac remodelling also alters β-adrenergic regulation of HCN currents in ventricular myocytes (Cerbai et al., 1999a; Sartiani et al., 2006). Qualitatively similar observations have also been reported for the failing heart in humans, including larger current densities and a positive correlation between the severity of hypertrophy and increased current density (Hoppe *et al.*, 1998; Cerbai *et al.*, 2001). Additionally, gene micorarray analysis points to an upregulated HCN4 gene expression in the failing human ventricle (Borlak and Thum, 2003).

Signalling pathways switching on/off HCN channel expression in non-pacemaker cells may involve the renin-angiotensin system. Chronic administration of the type I angiotensin receptor-blocker losartan to old hypertensive rats not only reduces cardiac hypertrophy, but also reverses I_{HCN} upregulation and overexpression of HCN2 and HCN4 mRNA (Cerbai et al., 2000; Hiramatsu et al., 2002). G-protein coupled receptors also seem to play a role in HCN channel expression. The β2-adrenergic receptor overexpressing mice show a five times higher ventricular I_{HCN} than normal animals, and a preferential upregulation of HCN4 compared with HCN2 (Graf *et al.*, 2001). Furthermore, HCN mRNA is correlated to thyroid hormone and/or thyroid hormone receptor level (Pachucki *et al.*, 1999; Gloss *et al.*, 2001).

HCN channel dysregulation not only plays a role in ventricular cells, but also in the pacing regions of the heart, further increasing the risk for arrhythmic impulses. For example, in the rabbit failing heart, SAN cell automaticity is impaired by downregulation of I_{HCN} (Verkerk *et al.*, 2003), and a reduced expression of HCN2 and HCN4 subunits was described in a dog model of congestive heart failure (Zicha et al., 2005).

Taken together HCN expression patterns in cardiac myocytes appear strongly correlated with the degree of cardiac overload and are thus electrical markers of cardiac remodelling.

Given the similarities in the expression profile of fetal and hypertrophied myocytes, it has been speculated that cardiac hypertrophy involves a recapitulation of gene expression patterns typical for neonatal stages and a re-entry of cells into a juvenile program.

3.7.3 Injuries

Peripheral nerve injury is often accompanied by syndromes of neuropathic pain such as allodynia (strong sensation evoked by light mechanical stimuli) and spontaneous painful sensations. Sensory pathways and a misrepresentation of sensory information are primarily thought to contribute to the clinical symptoms of neuropathic pain. This manifests in a hyperexcitability of dorsal root ganglion cells bodies that give rise to large, myelinated Aβ/δ-fibres, which are normally not involved in the transmission of pain (Shir and Seltzer, 1990; Chaplan et al., 2003). Rat models of spinal cord injury, such as axotomy (Black *et al.*, 1999), chronic constriction (Dib-Hajj *et al.*, 1999) or ligation of spinal nerves (Kim et al., 2001a) show that an altered expression of several voltage-gated Na^+ channel subunits contributes to the persistence of neuronal firing in injured cells (for review see (Waxman, 2001)). However, administration of ZD7288 reduced allodynia in rat models of neuropathic pain (Chaplan et al., 2003; Dalle and Eisenach, 2005; Lee et al., 2005) and reversed the spontaneous discharges in injured large myelinated fibres (Chaplan *et al.*, 2003). In this latter model of spinal nerve ligation (Chaplan *et al.*, 2003) as well as in a model of chronic compression (Yao *et al.*, 2003) the maximal I_{HCN} density was enhanced 1.5-2-fold compared to control, with variable effects Ī□□□□□□□□e dependence and kinetics. These findings establish I_{HCN} upregulation, resulting from nerve injury, as an essential factor leading to neuropathic pain. The molecular identity of the HCN channels that contribute to these changes remains to determined but appears to involve a decrease in the amount of HCN1 and HCN2 mRNA and protein in the case of nerve ligation (Chaplan *et al.*, 2003).

Besides neuronal injury, lesions in excitatory input can also cause an altered expression of HCN channels. Functional changes of I_{HCN} and HCN channel expression are described for a lesion of the enthorhinal cortex (provides most of the synaptic inputs to the hippocampus), where a decreased expression of HCN1 channels was accompanied by a negative shift of I_h activation and a faster kinetic in several neuronal cell types of the hippocampus (Bräuer *et al.*, 2001). These changes were partly reversed following reactive sprouting and replacement of entorhinal input by septal and associational afferents.

HCN channel activity is also modified after other forms of insults, such as hypoxia

(Erdemli and Crunelli, 1998) and inflammation (Ingram and Williams, 1996; Linden et al., 2003).

3.8 Concluding remarks

The HCN channels have seen a paradigmatic change in their physiological standing within the voltage-gated ion channel family. Funny and queer in earlier times, these channels have evolved to belong to the best characterized ion channels today. Particularly remarkable within this ion channel family is that comparatively minor differences amongst channel isoforms, such as their kinetics, voltage dependence and cAMP sensitivity, render them implicated in the most diverse neuronal functions. Two of the numerous insights into HCN channel structure and function are expected to have a great impact in the physiological and pathological situation of these channels. First, HCN channel expression in hippocampus is regulated rapidly by glutamatergic excitatory activity. This implicates these channels in the major control pathways regulating neuronal excitability, plasticity and homeostasis. It is thus reasonable to expect that, in the near future, a major pathway in the HCN channel field will consist in the elucidation of the activity-dependent co-assembly, trafficking and regulation of HCN channels in excitatory neuronal networks.

Second, accruing evidence indicates that dysregulated HCN channel function and abnormal expression is related to cardiopathies and central nervous system diseases, and some of the rules by which this happens are currently being established. On the one hand, dysfunctional HCN channels in thalamus precede the onset of epilepsy in rat models, suggesting that a common genetically or developmentally determined defect in I_{HCN} may predispose to epilepsy. On the other hand, brief periods of epileptic activity or neuronal injury persistently alter hippocampal I_{HCN}, pointing to a high susceptibility of the mechanisms controlling HCN channel expression to unbalanced activity. Elucidating the genetic and activity-dependent mechanisms underlying these disturbances will prove crucial for therapeutic approaches for neuronal and cardiac diseases, including those aiming at the *de novo* creation of biological pacemakers as a substitute for electronic pacemakers.

In conclusion, by merging the voltage- and ligand-gating modules into a single ion channel, it is probably fair to speculate that nature has generated a masterpiece of flexibility that it may not have previously anticipated.

4 AIM OF THIS THESIS

The growing information about expression and function of ion channels in the nervous system is not only accompanied by the cognition of the tremendous importance of pacemaker channels to fulfill fundamental neuronal tasks, but gives an insight about their involvement in malfunction and disease. This thesis addresses two major open questions that remain in the field of HCN channels. First, does a disease characterized by aberrant electrical activity, like absence epilepsy, involve altered functionality of the channels that are known to show greatest sensitivity to abnormal excitability? Second, how is the channel modulated by the neurotransmitter of one of the most important arousal system in a cell-type specific manner? The experiments target function and regulation of pacemaker channels in the epileptic and healthy thalamus. The thalamus is the part of the brain that not only relays incoming information from the periphery to the cortex, but also has a tremendous influence on our behavioral state, be it sleeping or waking, unconsciousness (like in absence epileptic seizures) or highly alert.

Thus, the aim of this work is to further investigate the pathological situation and physiological pathways of HCN channel regulation in the thalamic system, where HCN channels are known to have an important influence on neuronal electrophysiological properties.

5 RESULTS

PAPER I: FUNCTIONAL STABILIZATION OF WEAKENED THALAMIC PACEMAKER CHANNEL REGULATION IN ABSENCE EPILEPSY

Introduction to paper I:

Absence epilepsy

Epilepsy is a common chronic neurological condition that is characterized by recurrent unprovoked epileptic seizures. It affects approximately 50 million people worldwide (World Health Organization). During an epileptic seizure, abnormal, typically excessive, electrical activity in the brain prevents interpreting and processing incoming sensory signals. Also the control of muscles gets lost. As a result of this, an epileptic patient may fall down and show the characteristic twitching.

Types of epilepsy. There are several types of epilepsy that are classified into generalized and partial seizures. Each type has different behavioral effects and is treated with different methods.

1. During a generalized seizure uncontrollable discharge of neurons appear on both sides of the brain. It is the most common type of epilepsy. The seizure starts on one area of the brain and spreads across both brain hemispheres. These seizures produce muscle twitches, convulsions and loss of consciousness. People with this type of epilepsy do not remember having a seizure. Generalized seizures are tonic-clonic (“grand-mal”) seizures, absence (“petit-mal”) seizures, atonic seizures and status epilepticus.

2. Partial seizures are seizures where only a small part of the brain is involved in the abnormal electrical activity. Sometimes a partial seizure can spread to the whole brain. Two types of partial seizures are simple (“focal”) seizures and complex partial (psychomotor) seizures.

Absence ("petit mal") seizures. Absence seizures are a generalized, nonconvulsive form

of epilepsy and characterized by behavioral arrest and abrupt cessation of consciousness. A person having an absence becomes unaware of his or her surroundings and may stare off in space or freeze. A seizure lasts 5-30 s and, in human, is typical for children starting between 3 to 8 years. In the electroencephalogram (EEG) absence seizures are characterized by bilaterally spreading of 3-4 Hz rhythmic large amplitude spike-and-wave discharges (SWDs). The two crucial structures in the generation and maintenance of absence seizures are the thalamus and the cortex (for review, see (Blumenfeld, 2005; Pinault and O`Brien, 2007)). Chemical ablation of either of these structures abolished SWDs in the GAERS model of absence epilepsy (Vergnes and Marescaux, 1992). There is, however, an ongoing debate about the source of the oscillations (Blumenfeld, 2005; Meeren *et al.*, 2005). A large body of experimental results point to a critical role for the thalamus in absence seizure generation. In respect to this, absence seizures could be induced by the disturbances of intrinsic thalamic neuronal properties or synaptic interactions between thalamic circuits (Seidenbecher et al., 1998; Crunelli and Leresche, 2002; Ludwig et al., 2003; Budde et al., 2005). Conversely, other studies show strong evidence that the cortex exerts a leading role in the initial phase of seizure onset (Meeren et al., 2002; Steriade and Amzica, 2003; Pinault et al., 2006).

The pharmacological response profile distinguishes absence seizures from other, particularly focal, seizure types. Valproate and ethosuximide are the first choice for most (80%) of the patients. However, several other anti-epileptic drugs that are effective in focal seizures, including carbamezepine, and drugs that enhance GABA activity in the brain, such as vigabatrin and tiagabine, are either ineffective or aggravate absence seizures in humans and animal models (for review, see (Pinault and O`Brien, 2007)).

Loss of consciousness. The involvement of the thalamocortical network in absence epilepsy manifests in the loss of consciousness during the seizures. The definition of consciousness or perception might be more philosophical than scientific as “episodes of unresponsiveness or decreased responsiveness, which are not caused by motor alterations” (Kostopoulos, 2001). However, research interest in mechanisms underlying consciousness all include some role for thalamocortical circuits. The hypothesis is that the generation of a functional state that characterize cognition is based on the temporal coincidence of specific, first-order and non-specific, intralaminar thalamic activity (Llinás et al., 1998). If the cortex is ready to fire with each thalamic input as seems to be the case during SWDs, such ability would be lost.

Spike-and-wave discharges. SWDs are driven by the hypersynchronized discharging of reciprocally connected corticothalamic and thalamocortical circuits (Crunelli and Leresche, 2002; Meeren et al., 2002; Steriade and Amzica, 2003; Pinault et al., 2006). Several neurophysiological studies support the hypothesis that SWDs are sustained by a functional alteration of the same synchronizing mechanism that promotes sleep spindle oscillations in the thalamocortical system (for spindle oscillations see Section 3.6.8 of this thesis). Thus, in a model of feline generalized penicillin epilepsy after systemic penicillin injection spindle oscillations were transformed into SWDs, as a result of an increase in excitability of cortical neurons (Kostopoulos *et al.*, 1981). Additionally, an *in vitro* study on thalamic slices showed that the isolated thalamo-reticular-thalamic circuit responsible for spindle generation is also able to generate rhythmic oscillations in the frequency range of absence-like SWDs (von Krosigk *et al.*, 1993). The theory that SWDs are a perversion of sleep spindles is further corroborated by the fact that these two oscillatory modes share a) the need of the thalamo-cortical network, b) the dependence on GABAergic mechanisms and c) both confer unawareness (for review, see (McCormick and Contreras, 2001)).

Ionic and synaptic mechanisms underlying SWDs. The cellular mechanisms of SWD generation appear to be related to those for the generation of sleep spindle oscillations. These involve reciprocal excitatory and inhibitory connections in the cortex and thalamus (see chapter 3.6.8 of this thesis). Changes in neuronal activity in one part of the thalamocortical network could transform the rhythmic behavior of the entire system. Because many ion channels and receptors contribute to this balance, there are numerous ways how this fundamental circuit could be induced to generate abnormal oscillations. Amongst these alterations in HCN channels, Ca^{2+} channels and GABA receptors seem to be particularly important for the generation of absence epilepsy.

1. HCN channels: The prominent role of HCN channels in absence epilepsy is discussed below.

2. Ca^{2+} channels: Beside HCN-currents, low-threshold Ca^{2+} currents (I_T) are crucial for the generation of rhythmic oscillations in thalamic neurons. Consistently, animals deficient in the α_{1G} subunit of the T-type Ca^{2+} channel show abnormal oscillatory activity, lacking the thalamic burst firing mode. These animals were specifically resistant to the generation of SWDs in response to $GABA_B$ receptor activation (Kim et al., 2001b). Additionally, an enhanced low-threshold Ca^{2+} current expression was reported in several animal models of SWDs (Guyon et al., 1993; Talley et al., 2000; Zhang et al., 2002) and an increased I_T is

observed before seizure onset in the GABAergic nucleus reticularis thalami (nRT) (Tsakiridou et al., 1995). Ethosuximide, a known antiepileptic drug effective in the treatment of absence seizures was shown to block I_T in thalamocortical neurons (Coulter et al., 1989). Spontaneous absence seizures have also been observed in mice with mutation in various subunits of P/Q-type Ca^{2+} channels (for review see (Shin, 2006)). However, these mutants required α_{1G} T-type Ca^{2+} channels for the generation of SWDs as SWDs were suppressed by the deletion of the α_{1G} gene (Song et al., 2004).

3. GABA receptors: The GABAergic projections coming from the reticular thalamic nucleus are considered to play a crucial role in the generation of SWDs. The release of GABA from neurons of the nRT results in the membrane hyperpolarization in thalamocortical neurons mediated by $GABA_B$ receptors, removing inactivation from T-type Ca^{2+} currents and causing rebound burst discharges (for review see (Crunelli and Leresche, 1991)). Recent investigations in rodent models revealed that activation of $GABA_B$ receptors in thalamic relay nuclei is important for the generation of SWDs (Hosford et al., 1992; Snead, 1992). Application of $GABA_B$ receptor agonists, like baclofen or γ-hydroxybutyrate induced absence seizures in mice. These seizures were characterized by bilaterally synchronous SWDs on EEGs associated with behavioral arrest (Kim et al., 2001b). $GABA_A$ and $GABA_B$ mediated inhibition seem to be essential for the generation of spindle and absence-like oscillations in thalamocortical neurons in an *in vitro* network that contained thalamocortical and nRT neurons (Bal et al., 2000; Blumenfeld and McCormick, 2000). However, in the WAG/Rij genetic model, the $GABA_A$ receptor makes an essential contribution to SWD-related activity, while $GABA_B$ receptor have minor relevance (Staak and Pape, 2001). The pharmacological block of $GABA_A$ receptors with bicuculline *in vitro* resulted in the transformation from spindle to SWD oscillations via a disinhibition of neurons in the nRT (Bal et al., 1995). Subtle, nucleus-specific, $GABA_A$ receptor abnormalities underlying SWDs of typical absence seizures rather than a full block of these receptors across the whole thalamocortical network occurred prior to seizure onset in GAERS suggesting epileptogenic significance (Bessaih et al., 2006).

Human genetic studies have so far elucidated mutations in only a few families or individuals with absence seizure. Mutations in genes associated with absence epilepsy have been found for the $GABA_A$ receptor and for the α_{1A} subunit of P/Q type Ca^{2+}channels (for review see (Crunelli and Leresche, 2002; Blumenfeld, 2005).

Animal models. Absence seizures accompanied by SWDs have been studied in several

animal models (for review see (Snead *et al.*, 1999)). Pharmacological models include the feline generalized epilepsy with penicillin and the γ-hydroxy-butyrate model. Here, seizures are induced in otherwise non-epileptic animals by administration of a chemical stimulus that results in SWDs accompanied by behavioral arrest. Genetic models include several mutant mice (for review see (Noebels, 1999; Steinlein and Noebels, 2000; Crunelli and Leresche, 2002)) like the *tottering* mouse (Kostopoulos, 1992) or the *stargazer* (Noebels *et al.*, 1990). In these animals mutations in subunits of voltage-gated Ca^{2+} channels have been found (Steinlein and Noebels, 2000; Crunelli and Leresche, 2002).

By far the most extensively studied rat genetic models are the GAERS (genetic absence epilepsy rat from Strasbourg (Danober *et al.*, 1998)) and WAG/Rij (Wistar albino Glaxo from Rijswijk (Coenen *et al.*, 1992)), which were independently derived by inbreeding of normal Wistar rats. The EEG and behavioral manifestations of these animal models seem to be similar to those of typical human absence seizures, rendering them suitable models. These include the unresponsiveness to mild stimuli, the spontaneity of SWDs and associated behavioural arrest and the pharmacological responses to anti-absence drugs (Coenen *et al.*, 1992; Danober *et al.*, 1998). Compared with the human phenotype, the main differences are the higher frequency (7-11 Hz) of SWDs and the late development and persistence into adulthood of both the EEG and behavioral components of the seizures in these rats (Coenen et al., 1992; Danober et al., 1998; Crunelli and Leresche, 2002).

HCN channels and absence seizures

To investigate the involvement of HCN channels in absence epilepsy was indicated by two observations: First, expression and function of HCN channels show greatest sensitivity to aberrant neuronal activity with particular relationship to hyperexcitable epileptic systems (see chapter 3.7.1 of this thesis). Second, the contribution of HCN channels in generating and terminating spindle oscillations (see chapter 3.6.8 of this thesis) suggests a role in SWDs, as SWDs are considered to be a hypersynchronized form of spindling.

Moreover, the diversity of viewpoints on the role of HCN channels in epilepsy is currently the subject of much debate (Poolos, 2004). Recent studies reveal either a positive or negative relationship between HCN-current density and epileptic activity, depending on the type of seizure and the investigated type of cells. It is possible that different types of seizures respond differently to the modulation of I_{HCN}. For example, enhancing HCN-currents might help to terminate seizure activity in the thalamus. There, an increase in I_{HCN} prevents low-

threshold Ca^{2+} channels from generating rhythmic burst activity (Lüthi and McCormick, 1999b). In contrast, it appears that decreasing I_{HCN} has a net anti-epileptic effect in cortical and hippocampal structures. For example pharmacological blockade of I_{HCN} has been shown to abolish hyperexcitability after febrile seizures (Chen et al., 2001b) and also to increase the threshold for paroxysmal discharges evoked by high-frequency electrical stimulation in the hippocampus *in vivo* (Kitayama et al., 2003).

Even within one type of epilepsy conflicting results were obtained concerning a seizure-promoting or neuroprotective effect of HCN channels. In absence seizures these effects were different across different brain areas. In the cortex of the WAG/Rij rat, the HCN1 channel subunit was found to be reduced (Strauss *et al.*, 2004; Kole *et al.*, 2007), while in the thalamus, an increase of HCN1 was associated with diminished cAMP sensitivity of HCN channels before the onset of the seizures (Budde *et al.*, 2005). That reduced HCN-currents promote absence seizures in the rat model is consistent with the HCN2 knockout mice that show an absence epileptic phenotype (Ludwig *et al.*, 2003). However, there are experimental (Di Pasquale *et al.*, 1997) and computational (Timofeev *et al.*, 2002) studies that associate SWDs with increased HCN-currents. Altogehter, the data suggests a very specific and organized regulation of HCN channels with individual influence on neuronal and network excitability. The net impact of a given change in HCN channel function is likely to be determined by other factors such as other ionic conductances present in the cell, the nature and spatial distribution of synaptic inputs, and the specific molecular composition and subcellular localization of HCN channels.

Conclusions about a causal relationship between HCN channel function and epilepsy are complicated by reports that changes in HCN channel isoforms can be acquired in models where focal epileptic seizures are generated by kainic acid or hyperthermia (Chen et al., 2001b; Shah et al., 2004; Brewster et al., 2005), indicating that activity-dependent HCN modifications can also evolve secondarily to seizure activity.

Thus, it is still unclear in what way HCN channels contribute to epileptogenesis and chronic epilepsy. The novelty of our study is, to investigate the animals during both pre-epileptic and adult, chronically epileptic stages to answer this question. We focused on that part of the thalamus that is known to be primarily involved in SWDs.

Functional stabilization of weakened thalamic pacemaker channel regulation in absence epilepsy

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Abstract

Aberrant function of pacemaker currents (I_h), carried by hyperpolarization-activated cation-nonselective (HCN) channels, affects neuronal excitability and accompanies epilepsy, but its distinct roles in epileptogenesis and chronic epilepsy are unclear. We probed I_h function and subunit composition during both pre- and chronically epileptic stages in thalamocortical (TC) neurones of the Genetic Absence Epilepsy Rat from Strasbourg (GAERS). Voltage-gating of I_h was unaltered in mature somatosensory TC cells, both i*n vivo* and *in vitro*. However, the enhancement of I_h by phasic, near-physiological, cAMP pulses was diminished by ~40% and the half-maximal cAMP concentration increased by ~5-fold. This decreased responsiveness of I_h to its major cellular modulator preceded epilepsy onset in GAERS, persisted throughout the chronic state, and was accompanied by an enhanced expression of the poorly cAMP-sensitive HCN1 channel mRNA (> 50%), without changes in the mRNA levels of HCN2 and HCN4. To assess for alterations in TC cell excitability, we monitored the slow upregulation of I_h that is induced by Ca^{2+}-triggered cAMP synthesis and important for terminating *in vitro* synchronised oscillations. Remarkably, repetitive rebound Ca^{2+} spikes evoked normal slow I_h upregulation in mature GAERS-neurones that sufficed to attenuate spontaneous rhythmic burst discharges. These adaptive mechanisms occurred upstream of cAMP turnover and involved enhanced intracellular Ca^{2+} accumulation upon repetitive low-threshold Ca^{2+} discharges. Therefore, HCN channels appear to play a dual role in epilepsy. Weakened cAMP binding to HCN channels precedes, and likely promotes, epileptogenesis in GAERS, whereas compensatory mechanisms stabilizing I_h function contribute to the termination of spike-and-wave discharges in chronic epilepsy.

Introduction

The hyperpolarization-activated cation non-selective (HCN) channels are emerging as important targets in neurological diseases, including epilepsy (Santoro and Baram, 2003; Bender et al., 2004; Frère et al., 2004; Poolos, 2004). HCN channels give rise to hyperpolarization-activated inward currents (I_h) that are sensitive to intracellular cAMP levels (Pape, 1996; Robinson and Siegelbaum, 2003). The dual gating by voltage and cAMP allows I_h to widely control neuronal and network excitability (Santoro and Baram, 2003; Frère et al., 2004). Furthermore, I_h is regulated by neuronal activity, including seizures: Hippocampal I_h is acutely increased by synaptically released glutamate (van Welie *et al.*, 2004), and abnormal regulation of I_h and of HCN channel subunit expression occurs in brain regions involved in seizure generation (Di Pasquale *et al.*, 1997; Chen *et al.*, 2001b; Brewster *et al.*, 2002; Bender *et al.*, 2003; Shah *et al.*, 2004; Strauss *et al.*, 2004; Budde *et al.*, 2005), suggesting that modified HCN channel function may contribute to hyperexcitability.

The recognition of an involvement of I_h in epileptic processes has prompted much interest into how aberrant I_h function is causally linked to the initiation and the maintenance of seizures. Both inherited deficits in HCN channel function, present before epilepsy onset, and acquired modifications resulting from seizure activity, could contribute to the epileptic phenotype. However, the role of I_h in epilepsy was mostly studied in either pre-epileptic (Shah *et al.*, 2004; Budde *et al.*, 2005) or chronic epileptic conditions (Chen *et al.*, 2001b; Strauss *et al.*, 2004), leaving the relative importance of inherited and acquired channel alterations undetermined (Poolos, 2004). Furthermore, epileptic models showing perturbations in I_h are often accompanied by additional changes in ion channel or synaptic function (Guyon et al., 1993; Tsakiridou et al., 1995; Di Pasquale et al., 1997; Chen et al., 2001b; Zhang et al., 2002; Klein et al., 2004; Holter et al., 2005), thereby modifying I_h-dependent changes in excitability. Finally, HCN channel subunit composition strongly determines the gating by cAMP (Chen *et al.*, 2001c; Altomare *et al.*, 2003; Budde *et al.*, 2005), but whether altered isoform expression affects the efficacy of cAMP transients produced during neuronal activity is not clear.

Abnormal expression and regulation of HCN channels in thalamus and cortex was found in rodent models of generalized absence epilepsy, recognized by spike-and-wave discharges (SWDs) in the EEG (Di Pasquale *et al.*, 1997; Strauss *et al.*, 2004; Budde *et al.*, 2005). A principal way by which HCN-channel-mediated currents may be gated during SWDs is via a transient cAMP synthesis that is triggered, to a large extent, by Ca^{2+} entry

through the low-threshold (LT) Ca^{2+} spikes (Lüthi and McCormick, 1999b; Wang et al., 2002). The resulting slow afterdepolarization (ADP) terminates synchronised thalamic oscillations *in vitro* (Bal and McCormick, 1996; Lüthi et al., 1998). Here, we identified a diminished cAMP-dependent I_h regulation that was apparent before seizure onset in thalamocortical (TC) cells of the Genetic Absence Epilepsy Rat from Strasbourg (GAERS), a well-recognized genetic model of absence epilepsy, and persisted throughout the chronic epileptic state. However, adaptive mechanisms upstream of cAMP turnover, involving enhanced intracellular Ca^{2+} accumulation during repetitive LT Ca^{2+} spiking, restored the activity-dependent current upregulation in mature TC cells, thereby contributing to the termination of SWDs.

Methods

All experiments were performed according to the guidelines of the institutions and the Veterinary Offices (Comité Régional d'Ethique en Matière d'Expérimentation Animale, Strasbourg, France; Veterinäramt Basel-Stadt, Switzerland; UCI Animal Care committee), and conformed to NIH guidelines.

In vivo electrophysiological recordings

Experiments were conducted in inbred, adult male Wistar rats (71 GAERS and 56 control non-epileptic (NE) rats, 3–6 months). All surgical procedures were made under deep general anaesthesia (pentobarbital: 40 mg kg^{-1}, I.P., and ketamine: 50 mg kg^{-1}, I.M.). A tracheotomy and a catheterization of the penile vein were performed, and the animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A stabilizing craniotomy-duratomy technique was systematically applied to improve the success rate of single-cell electrophysiology experiments, to increase the precision to reach stereotaxically single neurons in a target region, and to eliminate undesirable non-neuronal rhythms (heart and respiratory movements) during intracellular recordings (Pinault, 2005). The rat's rectal temperature was maintained at 37ºC with a thermoregulated blanket (Fine Science Tools, Heidelberg, Germany).

The neuroleptanalgesia was initiated before the end of the general pentobarbital-ketamine anaesthesia, then maintained by an intravenous injection of a mixture containing D-tubocurarine chloride, fentanyl, haldol, and glucose (Pinault, 2003). All rats were artificially ventilated in the pressure mode (SAR-830, CWE, Ardmore, PA; 8-12 cm H_2O; 60-65 breaths

min^{-1}) using an O_2-enriched gas mixture (70-50% air / 30-50% O_2). The EEG, which displayed spontaneously synchronised slow oscillations (Pinault, 2003), and heart rate were also continuously monitored to maintain a constant depth of anaesthesia by adjusting the injection rate of the anaesthetic solution. Subcutaneous infiltrations of xylocaine (2%) were applied every 3 h at all surgical sites.

Glass micropipettes (30-70 MΩ) were filled with a solution containing 1.5% N-(2 amino ethyl) biotin amide hydrochloride (Neurobiotin®) dissolved in 1 M KAcetate. It was then lowered with a stepping micro-driver (Burleigh, Fishers, NY) into the somatosensory thalamus to reach a single TC neurone (Fig. 1*A*), which was extracellularly and/or intracellularly recorded simultaneously with the EEG of the frontoparietal cortex.

Electrophysiological data were processed with band passes of 0.1-1200 Hz for the EEG, and of 0-6 kHz for cellular activity (Cyber-Amp 380, Molecular Devices, Foster City, CA). Signals were digitized at a sampling rate > 18 kHz. During the intracellular recording session, a current pulse in the range from -0.2 to -0.5 nA was applied every 2 s to keep the Wheatstone bridge balanced. Using square wave current pulses (range of ± 3 nA), input membrane resistance and intrinsic firing patterns of thalamic neurones could be assessed.

At the end of the recording session, some of the units were individually labelled using the intracellular tracer microiontophoresis technique for standard histological identification (Fig. 1*A2*). After a survival time of at least 30 min, animals were killed with an intravenous overdose of pentobarbital, transcardially perfused with 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM phosphate buffer saline, and the brain tissue was processed using standard histological techniques for retrieving the tracer-filled neurones.

Electrophysiological recordings were analysed with the pClamp 7.01. software (Molecular Devices), and the tracer-filled neurones were examined with a light microscope (E600, Nikon, Champigny-sur-Marne, France). Some of the neurones were reconstructed using the Neurolucida system (Microbrigthfield, Colchester, VT) (Fig. 1*A3*). The location of marked cells was ascertained by consulting a stereotaxic atlas (Paxinos and Watson, 1998).

In vitro electrophysiological recordings

Slices were prepared from mature, chronically epileptic rats (3-8 months) or young pre-epileptic animals (19-24 days) and age-matched NE control animals. The majority of the experiments were carried out blind to the colony from which the animals were derived. Rats were gas-anaesthetized with isoflurane and decapitated. Coronal slices (300 μm) containing the somatosensory ventrobasal nuclear complex were prepared on a vibratome (VT1000S,

Leica, Glattbrugg, Switzerland) in an ice-cold oxygenated solution containing (in mM): 63 NaCl, 107 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 18 dextrose, 1.7 L(+)-ascorbic acid. The slices were allowed to recover for 5 min in a home-made interface-type chamber at 35.0°C in the cutting solution, before being transferred to a sucrose-free solution containing 126 mM NaCl instead and cation concentrations were altered to 2 mM $CaCl_2$ and 4 mM $MgCl_2$. After an additional 30 min, slices were incubated at room temperature for 1–2 h and then transferred individually into the recording chamber. Pretreatment of slices with the adenylyl cyclase inhibitor SQ22,536 started ~30 min after incubation at room temperature. Slices were transferred for at least 2 h to a small interface chamber containing 10 ml of 0.6 mM SQ22,536 before recordings commenced in SQ22,536-free bathing solution. SQ22,536-treated slices were used for maximally 1 h in the recording chamber.

Whole-cell recordings were obtained from TC neurones under visual control using differential interference contrast microscopy via upright microscopes (Olympus BX50WI and BX51WI, Volketswil, Switzerland) at 33.5-35°C. The location of the ventroposterior medial nucleus (Vpm) of the somatosensory thalamus was determined before pipette positioning using a 10x objective, and was clearly recognizable based on its apposition to the ventroposterior lateral nucleus (Vpl), which has a striated appearance. Slices were not used when the Vpm could not be clearly delineated. Patch pipettes were pulled from borosilicate glass tubing (TW150F-4, OD 1.5 mm, WPI, Berlin, Germany) on a vertical two-step puller (PP-83, Narishige, Tokyo, Japan) and filled with the following solution (in mM): 130 KGluconate, 10 KCl, 10 HEPES, 2 $MgCl_2$, 2 Na_2ATP, 0.2 NaGTP, 10 phosphocreatine, adjusted to 290 mOsm with sucrose, pH 7.25. This solution was found previously to yield ADPs with kinetics and amplitudes similar to those reported from microelectrode recordings (Bal and McCormick, 1996), indicating that it minimally perturbed the cytosolic components required for the generation of the ADP. GTP was freshly added daily from stocks (100-fold concentrated). The resistance of the electrodes was 2.5-4 MΩ and yielded series resistances in the range between 7-19 MΩ. If series resistance changed by more than 10%, the experiment was not included in the analysis. A liquid junction potential of 10 mV measured as described (Neher, 1992) was taken into account for all data. The bath was constantly perfused with fresh medium at a rate of 3 ml min^{-1} throughout the recording and contained (in mM): 126 NaCl; 2.5 KCl; 1.25 NaH_2PO_4; 2 $MgCl_2$; 2 $CaCl_2$; 26 $NaHCO_3$, 18 dextrose, 1.7 L(+)-ascorbic acid. Data from voltage- and current-clamp recordings were collected through an Axopatch 200B amplifier (Molecular Devices), filtered at 2 kHz and acquired at 5 kHz using pClamp 9.2.

software. Caged cAMP (P^1-(2-Nitrophenyl)ethylester, 100 μM) was added to the patch solution from a 100-fold concentrated stock solution in DMSO immediately before the experiment and the pipette solution was kept on ice and protected from light. A minimal time of ~3-5 min was allowed for the perfusion of caged cAMP and 8Br-cAMP (0.1-10 μM) into the cell. Flashes were applied with a ultraviolet (UV) lamp attached to the epifluorescence pathway of the microscope and discharged via the capacitive discharges of the FlashMic (80% of maximal capacitive charge, Rapp Optoelectronics, Hamburg, Germany). This procedure permits the generation of cAMP transients in a reproducible manner and was previously used to establish dose-response curves (Lüthi and McCormick, 1999b). Current amplitudes were measured with 1.5 s voltage steps from -60 to -90 mV at 4 s intervals and quantified by averaging responses of two successive flashes applied at intervals > 1 min. Amplitudes of ADPs were quantified 2 s after termination of the last current injection. For the data included in the concentration-response curve between 8Br-cAMP and $V_{1/2}$ (Fig. 2*G*), the average series resistances did not differ between the groups (12.0 ± 0.4 MΩ, n = 32, and 11.5 ± 0.3 MΩ, n = 31, for cells derived from NE animals and GAERS, respectively, $p > 0.05$). The values for $V_{1/2}$ were derived from the average of 1-4 activation curves obtained within a recording time of 5-20 min after gaining whole-cell access. Slices preincubated with SQ22,536 showed an attenuated amplitude of the ADP (2.5 ± 0.4 mV in control, n = 6, 1.4 ± 0.2 mV in SQ22,536, n = 4, $p < 0.05$), indicating that stimulation of adenylyl cyclases was selectively reduced, but a hyperpolarization-induced sag potential was unchanged, reflecting unaffected voltage-gating of I_h around –80 mV (5.8 ± 0.3 mV, n = 45 in control, 6.0 ± 0.3 mV, n = 77 in SQ22,536, $p > 0.05$).

Amplitudes of currents and ADPs were measured in ClampFit (v. 9.0.). Tail current analysis was used for determining activation curves of I_h. Igor v. 4.0.8 was used for fitting Boltzman and Hill equations to I_h activation curves and 8Br-cAMP concentration- response curves, respectively. Hill coefficients were fixed at values between 1 and 1.4 (Lüthi and McCormick, 1999b; Chen et al., 2001c), yielding half-maximal concentrations that differed by < 5%.

Fluorescent imaging of intracellular Ca^{2+} concentrations

Relative fluorescent changes (ΔF/F) of the long-wavelength Ca^{2+} indicator Oregon Green 488 BAPTA-2 (75 μM; K_d = 580 nM) were used to compare intracellular Ca^{2+} levels reached after single or repetitive rebound Ca^{2+} spikes (up to 16 pulses, 4 Hz, -0.9 nA current injection for 125 ms) in adult NE animals and GAERS (4-8 months). The dye was added to

the intracellular solution (see above) and the cells were perfused via the patch pipette for 10-25 min, until proximal dendrites of TC cells could be visualised. Intracellular perfusion with the dye did not alter resting membrane potentials (-62.9 ± 0.7 mV, n = 10 and -62.0 ± 1.1 mV, n = 7, for NE and GAERS cells, respectively, $p > 0.05$) nor amplitudes of evoked ADPs (2.1 ± 0.2 mV, n = 10, and 2.3 ± 0.4 mV, n = 7 for NE and GAERS cells, respectively, $p > 0.05$). Excitation occurred at 488 nm using a Polychrome IV (TILL Photonics, Gräfelfing, Germany), and the light emitted at 510 nm was collected via a cooled CCD camera (Imago VGA, 480 x 640 pixels) after passing through a 40x objective and an Olympus fluorescein isothiocyanate filter set. Images were acquired for 150 ms using a binning of 8 at 0.2 s intervals during and at 0.5 s intervals before and after stimulation. The image acquisition was synchronised with the electrophysiological recordings using the Sync output of the imaging control unit, and data were analysed using Till Vision Imaging Software (v. 4.01) and SigmaPlot (v. 8.0) for fitting the time course of decay. Average fluorescence was determined for regions of interest (typically 80-400 μm^2) over the soma (avoiding the nucleus) or a stem dendrite, and the average background fluorescence of a region away from the filled cell, with the threshold fluorescence level set to 10, was subtracted. Relative fluorescence ($\Delta F/F$ in %) was calculated for each image [(average fluorescence - average baseline fluorescence) * 100 / average baseline fluorescence]. Baseline fluorescence was determined by the average of 7 images before application of repetitive current injections. Ca^{2+} signals elicited by the protocols used for evoking ADPs were obtained at periods of 25 sec and 5 successive sweeps were averaged in each cell. Minimal photobleaching was observed and the fluorescence change for each individual sweep was calculated with respect to the baseline signal prior to cell stimulation.

Quantitative in situ hybridization

For *in situ* hybridization procedures, rats were quickly decapitated (4-5 rats per group), brains dissected and placed on powdered dry ice as described (Brewster *et al.*, 2002; Brewster *et al.*, 2005). Quantitative analyses of thalamic HCN isoform mRNA levels were accomplished using antisense ^{35}S-cRNA probes synthetized by *in vitro* transcription from cDNAs containing specific gene regions of mouse HCN1 and HCN2 channels (Brewster *et al.*, 2002; Bender *et al.*, 2003). Briefly, 20-µm thick brain sections were cut, mounted on gel-coated slides and fixed in 4% paraformaldehyde. Following a graded ethanol treatment, sections were exposed to acetic anhydride-triethanolamine, then dehydrated through 70-100% ethanol. Sections were then pre-incubated in hybridization solution (50% formamide, 5X

SET, 0.2% sodium dodecyl sulfate, 5X Denhardt's solution, 0.5 mg ml^{-1} salmon sperm sheared DNA, 250 g L^{-1} yeast tRNA, 100 mM DTT, 10% dextran sulfate) and probed overnight at 55ºC with antisense ^{35}S-CTP radiolabeled HCN probes (0.5-1x10^6cpm / 30 µl / per section). The specific activity of the probes was 1.67-5.2 x10^8 cpm μg^{-1}. On the following day, sections were washed in decreasing concentrations of saline sodium citrate (SSC) solutions, with the most stringent wash at 0.03 x SSC for 60 minutes at 62^{o}C. Following dehydration in increasing alcohol concentrations, sections were apposed against Kodak Biomax films. Optimal exposure time was monitored using ^{14}C standards to maintain signal linearity.

All analyses were performed by investigators unaware of the origin (GAERS *vs.* NE animals) of all samples. Data acquisition and quantification of *in situ* hybridization signals were carried out on sections run concurrently by measuring optical density of incorporated radioactivity in CA1 using the image analysis program ImageTool (v. 1.25; University of Texas Health Science Center, San Antonio, TX) (Brewster *et al.*, 2002). Optical density measured over the corpus callosum was used as background signal. Linearity of hybridization signal was ascertained using ^{14}C standards (American Radiolabeled Chemicals Inc., St. Louis, MO). Statistical analyses for *in situ* hybridization data were performed using GraphPad software (PRISM, San Diego, Ca, USA).

Anaesthetics, Chemicals and Reagents

Pentobarbital was purchased from Sanofi, Libourne, France, and Ketamine from Merial, Lyon, France. Fentanyl and Haldol were obtained from Janssen, Boulogne-Billancourt, France; Xylocaine from Astra, Rueil-Malmaison, France; Neurobiotin® from Vector Labs, Burlingame, CA. Caged cAMP was obtained from Calbiochem and Oregon Green 488 BAPTA-2 from Molecular Probes. D-Tubocurarine chloride, salts and chemicals were purchased from SIGMA-Aldrich.

Statistical analysis

Data were evaluated for statistical significance using paired- or unpaired Student's t-test and ANOVA as appropriate, unless otherwise indicated. Significance level was set to 0.05. Data are presented as means ± s.e.

Results

Unaltered hyperpolarization-activated depolarizing sag potentials in TC neurons in adult GAERS *in vivo*

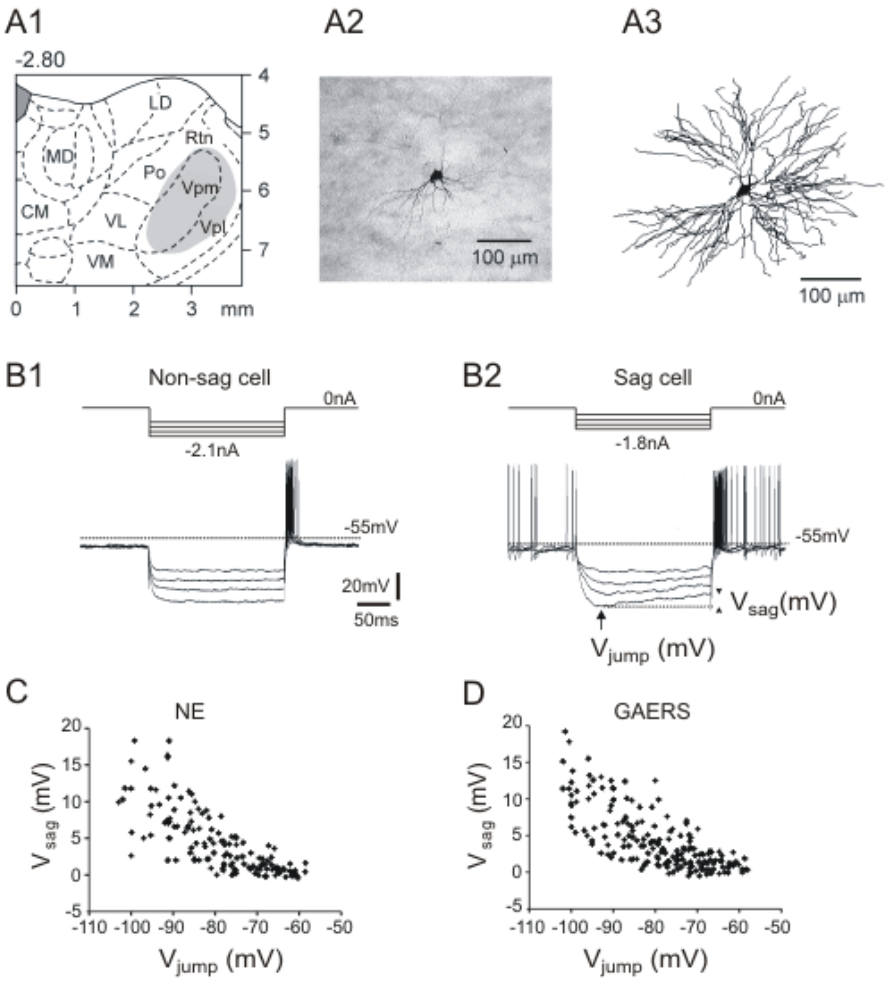

Figure 1. Hyperpolarization-induced depolarizing sags in thalamocortical (TC) neurones *in vivo*. *A1*, Stereotaxic location (2.8 ± 0.2 mm posterior to bregma) of the intracellularly recorded TC neurones (*gray area*). *A2*, *A3*, Photomicrograph and 3D-reconstruction of a typical TC neurone intracellularly labelled with Neurobiotin in the Vpm nucleus (frontal section). *B1*, *B2*, Four voltage responses to 200 ms hyperpolarizing current pulses of increasing intensity in a neurone without (*left,* "non-sag cell") and with (*right,* "sag cell") a sag. The sag amplitude (V_{sag}) was measured as indicated in *B2* (*arrowheads*). *C*, *D*, Plot of V_{sag} amplitudes *vs.* V_{jump} values in TC neurones of GAERS (*C*; n = 28 cells with at least 5 values per cell) and of control non-epileptic (NE) rats (*D*; n = 22 cells with at least 5 values per cell). CM = central medial; LD = lateral dorsal; MD = medial dorsal; Po=posterior thalamic nuclear group; VL = ventral lateral; VM = ventral medial; Vpl = ventral posterolateral; Vpm = ventral posteromedial; Rtn = nucleus reticularis thalami.

The h-current functions as a pacemaker for thalamic neuronal and network oscillations, and alterations in its voltage dependence strongly affect the propensity of TC cells to generate

oscillatory burst discharges (Pape, 1996; Lüthi and McCormick, 1998b). To determine whether voltage gating of I_h was affected in GAERS, intracellular recordings were performed *in vivo* from 73 and 61 TC neurones in GAERS and NE animals, respectively. The data reported here are based, respectively, on 55 and 45 recordings, which fullfilled the following three criteria: 1) a stable resting membrane potential without holding polarizing current; 2) a firing pattern similar to that recorded extracellularly in the same or in other TC neurones; and 3) an overshooting of the action potentials. The recordings were performed in the Vpl and Vpm nuclei, the nuclei known to be primarily involved in SWDs in GAERS (Vergnes *et al.*, 1990; Seidenbecher *et al.*, 1998; Manning *et al.*, 2004). The location of recorded neurones was ascertained on the basis of their stereotaxic location, their receptive field, and/or their labelling (Fig. 1*A*). Impaled neurones were assessed for resting membrane potential, input resistance, and a depolarizing sag potential, the physiological correlate of I_h voltage-gating. The basal membrane properties of the TC neurones are summarized in Table 1. No significant differences in passive and active membrane properties were found between TC neurones of Vpl and Vpm nuclei. Two principal types of TC neurones were distinguished based on their ability to generate a measurable hyperpolarization-induced depolarizing sag potential (maximal sag amplitude ≥ 1 mV at a membrane potential < -75 mV) (Fig. 1*B*). The proportion of sag-displaying TC neurones ("sag cells") recorded in GAERS and NE rats was comparable (76.4% *vs.* 62.2%, $\chi^2 = 2.357$, df = 1, $p > 0.05$). Moreover, in both GAERS and NE rats, the amplitude of the sag increased similarly as a function of membrane polarization, with values reaching 1.3 ± 0.2 mV and 1.5 ± 0.3 mV with V_{jump} between -65 and -70 mV, 2.6 ± 0.5 mV and 3.2 ± 0.6 mV between -75 and -80 mV, and 9.9 ± 1.5 mV and 9.1 ± 0.8 mV between -95 and -100 mV for NE (n = 22) and GAERS (n = 28) cells, respectively ($p > 0.05$; Fig. 1*C* and *D*). Neurones generating sags had, on average, a higher input resistance than neurones without a sag ("non-sag cells") and a more depolarized mean resting membrane potential (Table 1). Taken together, the relative number of I_h-expressing cells, as well as the amplitude of the sag potential, the physiological correlate of rapid voltage gating of I_h, was unaffected in the epileptic rat strain.

Table 1. Values of membrane properties of sag and non-sag TC neurones recorded in vivo in GAERS and NE rats.

	Resting MP (mV)	peak IR (MΩ)
Sag cells		
GAERS (n = 42)	-59.5 ± 0.6 GAERS/NE, p > 0.05 sag/non-sag, p < 0.01	25.1 ± 1.1 GAERS/NE, p > 0.05 sag/non-sag, p < 0.01
NE (n = 28)	-58.5 ± 0.6 sag/non-sag, p < 0.05	23.5 ± 1.3 sag/non-sag, p < 0.01
Non-sag cells		
GAERS (n = 13)	-65.2 ± 1.7 *GAERS/NE, p > 0.05*	18.2 ± 1.8 *GAERS/NE, p > 0.05*
NE (n = 17)	-63.7 ± 1.8	18.2 ± 1.3

IR=input resistance; MP=membrane potential.

Reduced sensitivity of I_h to near-physiological cAMP pulses in both pre-epileptic and mature GAERS

The voltage dependence of I_h is regulated by cAMP, and the high cAMP sensitivity of thalamic I_h is essential for its multiple roles in thalamic oscillatory behavior (Lüthi and McCormick, 1998b). Therefore, we next investigated cAMP-dependent modulation of I_h in whole-cell patch-clamp recordings in thalamic slice preparations *in vitro* from adult NE rats and GAERS. Slices used for these experiments were pretreated with SQ22,536 (0.6 mM), an adenylyl cyclase inhibitor, to minimize binding of endogenous cAMP to the channels (see Methods). Values of basic cellular properties and the quality of electrical access during recordings were similar for both strains (resting membrane potential -59.0 ± 3.0 mV for NE, n = 8, -60.4 ± 1.6 mV for GAERS, n = 7, p > 0.05; input resistance 223 ± 56 MΩ for NE, n = 8, 156 ± 27 MΩ for GAERS, n = 7, p > 0.05; series resistance 11.8 ± 0.6 MΩ for NE, n = 8, 12.0 ± 0.9 MΩ for GAERS, n = 7, p > 0.05). To probe the sensitivity of I_h to cAMP in mature NE rats and GAERS, we initially bath-applied the non-hydrolysable analogue of cAMP, 8Br-cAMP (1 mM), to TC cells located in the Vpm (Fig. 2*A* and *B*). Gradual wash-in of 8Br-cAMP (1 mM) enhanced current amplitude at -90 mV by a similar extent; from -370 ± 40 pA to -463 ± 36 pA (n = 8, p < 0.001) in NE, and from -329 ± 47 pA to -462 ± 58 pA in GAERS (n = 7, p < 0.025; Fig. 2*C*). Half-activation voltages ($V_{1/2}$) shifted from -89.6 ± 0.6 mV to -81.5 ± 0.9 mV (n = 7, p < 0.001; Fig. 2*B* and *C*) in GAERS, with the slope values remaining unchanged (from 8.5 ± 0.7 mV to 9.7 ± 0.7 mV, p > 0.05). Cells from mature NE rats yielded a shift from -87.5 ± 0.9 mV to -78.6 ± 1.6 mV (n = 8, p < 0.001; Fig. 2*A* and *C*) and the slope values were 8.3 ± 0.5 mV and 9.2 ± 0.8 mV (p > 0.05 compared to

corresponding values from GAERS), respectively. These data suggest that, when exposed to high steady levels of 8Br-cAMP, the sensitivity of thalamic I_h to the cyclic nucleotide is indistinguishable between GAERS and NE animals.

We next sought to investigate whether I_h in GAERS and NE animals differed in cAMP sensitivity during cAMP signals approximating physiological situations. Indeed, activity-dependent alterations in cAMP levels occurring during spontaneous thalamic oscillations *in vitro* are phasic and only partially shift the voltage dependence of I_h (Lüthi and McCormick, 1998a). We found previously that, by using photolytic release of cAMP, we induced a dose-dependent, submaximal enhancement of I_h, which is occluded by Ca^{2+}-dependent I_h upregulation (Lüthi and McCormick, 1999b). Therefore, we applied single UV-light flashes to cells filled with caged cAMP (100 μM; see Methods) (Fig. 2*D* and *E*). Photolytic release of cAMP induced an increase to 122.5 ± 2.4% of control amplitude in cells from NE animals ($n = 8$, $p < 0.0001$; Fig. 2*E*), reflecting a sub-maximal cAMP stimulation of the channel (Lüthi and McCormick, 1999b). In contrast, current amplitudes in GAERS increased to only 113.8 ± 1.3% ($n = 6$, $p < 0.025$ compared to NE; Fig. 2*E*) after photolysis of caged cAMP. These values are ~40% smaller compared to NE and suggest that the sensitivity of HCN channels in GAERS to physiologically relevant, phasic cAMP transients is weakened.

To assess whether or not this difference reflected acquired alterations in channel function due to chronic epilepsy, we repeated the flash photolysis experiment in young animals (P19-24) which have not yet developed SWDs. Photolytic release of cAMP induced an increase to 118.5 ± 2.9% of control current in cells from the NE strain ($n = 12$, $p < 0.0001$), whereas the increase was only 110.8 ± 1.4% in cells from GAERS ($n = 12$, $p < 0.05$ compared to NE; Fig. 2*D* and *E*). Moreover, cells from young animals (both the GAERS and the NE strain) showed similar maximal shifts in current voltage dependence when 1 mM 8Br-cAMP was bath-applied (data not shown).

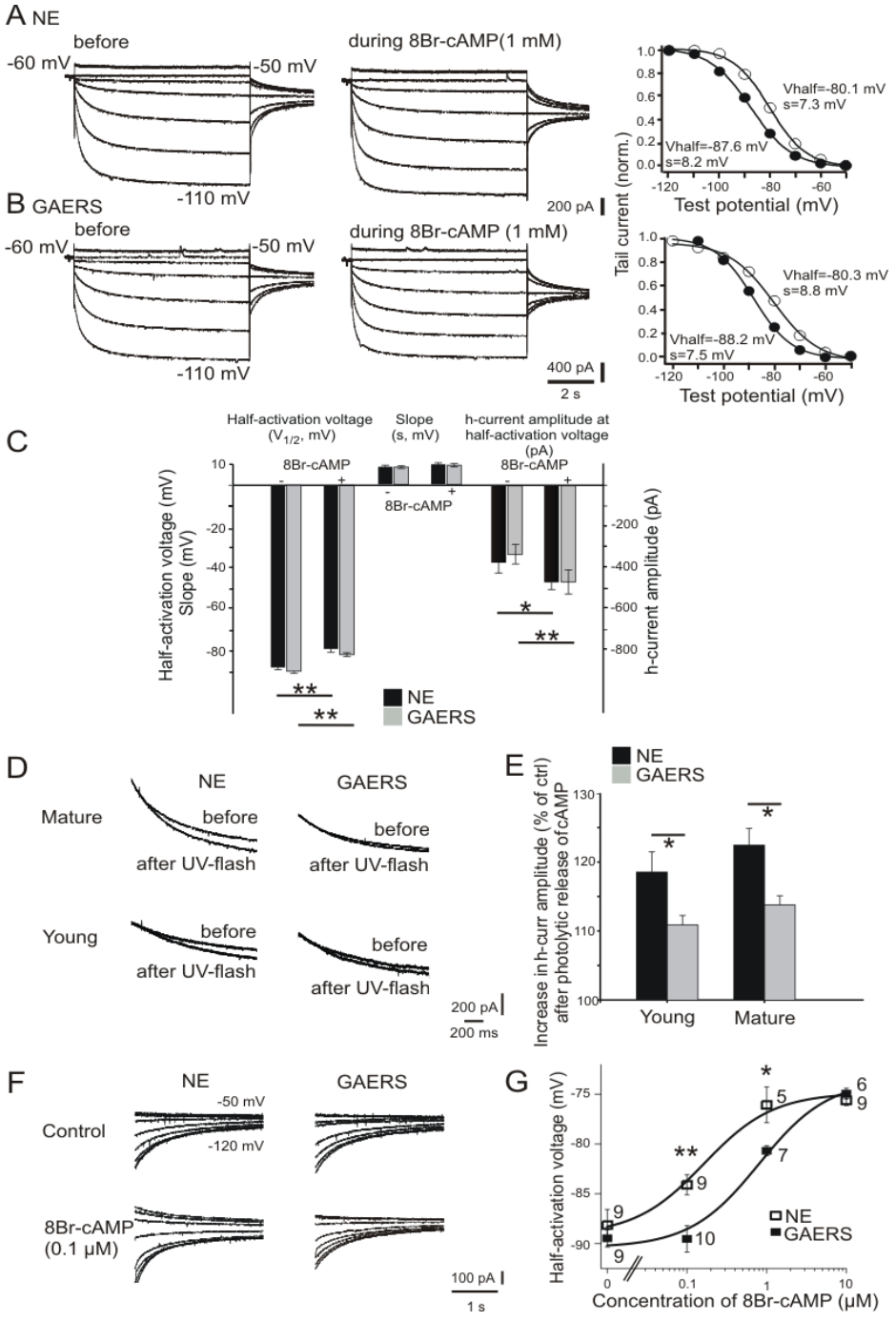

Figure 2. The I_h of GAERS TC cells shows a diminished sensitivity to sub-maximal, near-physiological cAMP pulses, but not to saturating cAMP concentrations. *A, B*, Current responses of mature TC cells from a NE rat (*A*) and a GAERS (*B*) to increasing negative test voltages (test voltages -50 mV and -110 mV are indicated next to the traces) before, and in the continuous presence of, 8Br-cAMP at a saturating concentration of 1 mM. Corresponding activation curves, constructed from tail currents evoked at -80 mV (see Methods), are shown to the *right*. Thick lines represent the optimal fit of a Boltzmann curve, with the resulting values for the half-activation voltage ($V_{1/2}$) and the slope (s) indicated next to the traces. *Filled* and *open circles* represent values before and during 8Br-cAMP application, respectively. *C*, Histogram showing the pooled data for $V_{1/2}$, for s, and for the current amplitude at -90 mV before, and in the continuous presence of 8Br-cAMP (1 mM). Except for s, 8Br-cAMP significantly altered all control values. The changes in all three parameters were indistinguishable between mature NE (n = 8) and GAERS (n = 7) TC cells. *D*, Responses of I_h to photolytic

release of caged cAMP in mature *(top row*) and young (*bottom row*) NE animals and GAERS. Overlay of current responses to 30 mV hyperpolarizing voltage steps before and after application of a UV-flash in cells perfused with caged cAMP (see Methods). Holding potential was -60 mV. For clarity, only current relaxations during the hyperpolarizing voltage step are shown, passive responses to the step voltage were blanked. *E*, Histogram showing the percentage increase in current response in young and mature NE animals (young: n = 12; mature: n = 8) and GAERS (young: n = 12; mature: n = 12). *F*, Representative tail currents obtained from TC cells in the absence of (control) or during perfusion of the cellular interior with 0.1 μM 8Br-cAMP. The same voltage protocol as in panel *A* was used, voltage steps applied prior to evoking tail currents are indicated next to the traces, and tails were evoked at – 75 mV. *G*, Concentration-response curve for the effect of 8Br-cAMP on $V_{1/2}$. The number of recorded cells is indicated next to the symbols. Fitting of the Hill equation was achieved by fixing the Hill coefficient to 1 (see Methods), yielding a ~5-fold increase of half maximal concentration of 8Br-cAMP. * denotes $p < 0.05$, ** $p < 0.01$.

Flash photolysis of caged cAMP does not allow determining the free cAMP concentrations reached. Therefore, we next assessed cAMP regulation of I_h quantitatively by constructing a concentration-response curve between 8Br-cAMP, included at defined concentrations in the patch pipette, and $V_{1/2}$ values in TC cells in mature animals. Activation curves were constructed after allowing for equilibration of 8Br-cAMP within the cell (see Materials and Methods). Notably, in TC cells from GAERS, the shifts induced in the concentration range between ~ 0.1 μM - 1 μM were significantly smaller than those in cells from NE animals (Fig. 2*F* and *G*). For example, including 0.1 μM 8Br-cAMP yielded values of $V_{1/2}$ = -84.1 ± 1.0 mV in NE TC cells (n = 9), while GAERS TC cells had a $V_{1/2}$ = -89.9 ± 1.4 mV (n = 9, $p < 0.01$), indicating that the voltage dependence of I_h was not appreciably affected in GAERS. Furthermore, whereas 1 μM 8Br-cAMP produced a near-maximal shift of $V_{1/2}$ in NE TC cells (-76.1 ± 1.8 mV, n = 5), the voltage dependence of GAERS TC cells was only partially shifted ($V_{1/2}$ = -80.7 ± 0.5 mV, n = 7, $p < 0.05$). Fitting a Hill equation to the concentration-response curve yielded a half-maximal concentration of 0.16 ± 0.07 μM 8Br-cAMP for NE cells, whereas a similar shift in GAERS required 0.81 ± 0.38 μM. These experiments reveal that I_h in TC cells from GAERS shows a distinctly reduced sensitivity to submaximal steady concentrations of cAMP, consistent with the results obtained via flash photolysis and with a recent report on pre-epileptic TC cells in the dorsal lateral geniculate nucleus of the WAG/Rij strain (Budde *et al.*, 2005). Moreover, the reduced I_h sensitivity to non-saturating cAMP concentrations precedes the onset of the seizures. This supports a causal role of this defect in SWD generation, and excludes the possibility that these changes are compensatory or secondary to the seizures.

Altered expression of HCN channel subunit mRNA in thalamocortical neurones of GAERS

The decreased sensitivity of I_h to cAMP in GAERS raises the possibility that the subunit composition of the channel was altered, such that weakly cAMP-sensitive isoforms were expressed at relatively higher levels. To test this hypothesis, we examined the expression of the three HCN channel isoforms present in thalamus in mature GAERS and NE controls. Messenger RNA levels of the cAMP-sensitive HCN channel subunit most abundant in thalamus, HCN2, did not differ between GAERS and NE animals in either the Vpm or in the reticular nucleus (Rtn) (Fig. 3, top). For example, in Vpm, mRNA levels were 71 ± 0.4 and 70 ± 0.4 nCi g^{-1} in NE rats and GAERS, respectively. Similarly, no difference was found in the expression of the other abundant, cAMP-sensitive HCN channel isoform, HCN4 (Fig. 3, bottom). However, a significant, 58.6% increase in the expression levels of the relatively cAMP-insensitive HCN1 channel isoform was found in the GAERS Vpm (34.1 ± 3.3 nCi g^{-1}) compared with mature NE rats (21.5 ± 0.79 nCi g^{-1}, $p < 0.025$ [t-test with Welch's correction]) (Fig. 4). Similar changes were found also in the Rtn (31.17 ± 1.3 *vs.* 24.33 ± 1.47 nCi g^{-1}, $p < 0.01$). Note that the increased expression of HCN1 channels was specific to thalamic nuclei: mRNA expression levels of this isoform were similar in somatosensory cortical layer V of GAERS and NE control rats (61.8 ± 5.2 and 64.2 ± 5.0 nCi g^{-1} in controls and GAERS, respectively, $p > 0.05$). The increased HCN1 isoform expression in select thalamic nuclei, with maintained levels of HCN2, is consistent with the overall reduced sensitivity of the resulting complement of cellular HCN channels to cAMP.

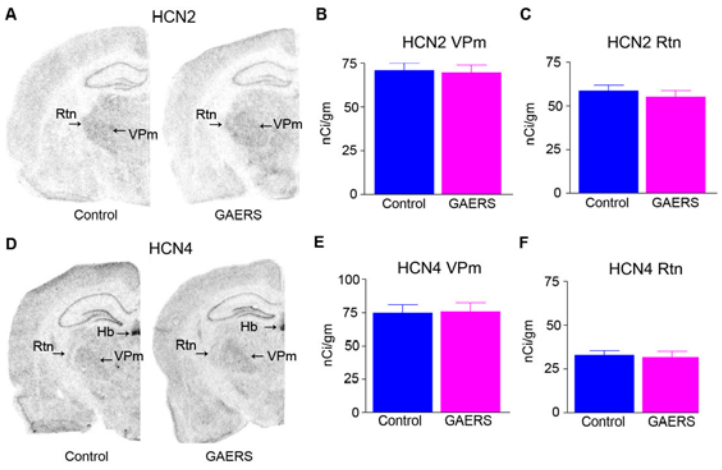

Figure 3. Expression of HCN2 (*top row*) and HCN4 (*bottom row*) mRNA in selected thalamic nuclei does not differ in GAERS and NE controls. *A*, Representative coronal brain sections at the level of the thalamus, that have been subjected to quantitative *in situ* hybridization for the HCN2 channel isoform. The preferential

expression of this isoform in thalamus (compared to hippocampus) is apparent. *B*, *C,* Quantitative analysis of mRNA expression levels of HCN2 channels comparing GAERS to NE rats. The strains are not distinguishable in the expression of this isoform. *D,* Representative sections of GAERS and NE rat brain for HCN4 mRNA expression. Note the relatively lower expression of this isoform in the Rtn, and the typical signal of HCN4 in the habenula, supporting the specificity of the probes. *E, F*, Quantitative analyses of HCN4 channel mRNA expression indicate the absence of significant differences in GAERS *vs.* controls. Ha = habenula; Rtn = reticular nucleus of the thalamus.

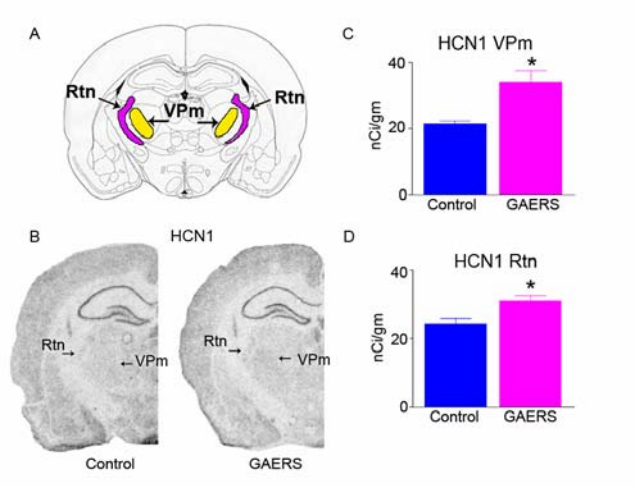

Figure 4. Quantitative analysis of the expression of the HCN1 channel mRNA in thalamic neurones of GAERS and NE rats. *A*, A schematic showing the level where coronal sections from brains of GAERS and NE control rats were obtained for *in situ* hybridization. *B*, Representative sections from GAERS and NE rats. Although HCN1 channel expression in the thalamus is significantly lower than that of the HCN2 isoform, the darker *in situ* hybridization signal over the Vpm and Rtn is apparent in the GAERS brain. Note the robust expression of HCN1 in the principal cell layers of the hippocampus of both strains. *C*, Quantitative analysis reveals a 58% increase in HCN1 mRNA levels in GAERs Vpm. *D*, Quantitative analysis shows a more modest increase of HCN1 mRNA in the Rtn (n = 5 per group). * denotes a significance level of $p < 0.05$ (see Methods).

Maintained I_h-mediated afterdepolarizations in fully epileptic mature GAERS

We next queried whether the decreased cAMP sensitivity of I_h in TC cells from GAERS translates to an altered endogenous regulation of I_h, thereby furthering the propensity of this rat strain to generate long-lasting SWDs. We focused here on a slow, activity-dependent upregulation of I_h that is particularly crucial for the cessation of synchronised oscillations and leads to a persistent ADP induced by repetitive LT Ca^{2+} spikes (Bal and McCormick, 1996). This ADP reflects the combined influence of Ca^{2+} entry through voltage-gated Ca^{2+} channels, Ca^{2+}-dependent cAMP synthesis, HCN channel voltage gating and binding to cAMP, and the degradation of cAMP by phosphodiesterases (Lüthi and McCormick, 1999b; Wang et al.,

2002). The reduced sensitivity of I_h to cAMP in GAERS predicted smaller ADPs following repetitive rebound Ca^{2+} spike generation.

Cells were held between -58 and -64 mV, close to their resting membrane potentials (-65.7 ± 0.7 mV for NE, n = 25; -63.5 ± 1.2 mV for GAERS, n = 24; $p > 0.05$), and 16 negative current pulses (-0.3 to -0.9 nA), each lasting 120 ms, were injected at 4 Hz (Fig. 5*A* and *D*, bottom), a protocol producing maximal ADPs (Lüthi and McCormick, 1999a). The resulting ADP amplitude was measured relative to baseline 2 s after termination of these current injections to minimize the contribution of a rapidly decaying depolarization immediately after the end of the pulses (Fig. 5*A*). These ADPs, albeit small, efficiently attenuated spontaneous δ-oscillations that were observed in some recordings (n = 2; Fig. 5*B*), documenting the efficacy of small membrane depolarizations in preventing or terminating burst discharges in TC cells. In mature NE animals, ADP amplitudes were 2.3 ± 0.2 mV (n = 24; Fig. 5*A* and *F*) and these were reduced > 80% by extracellular application of the I_h blocker Cs^+ (3 mM, n = 4, $p < 0.05$; Fig. 5*C*). Interestingly, amplitudes of ADPs in mature GAERS did not differ from those in age-matched controls (2.8 ± 0.2 mV, n = 24; Fig. 5*D* and *F*), sufficed to dampen intrinsic rhythmic activity (n = 4; Fig. 5*E*) and were largely blocked by extracellular Cs^+ (n = 3, $p < 0.05$, data not shown).

In contrast to cells from mature animals, ADPs elicited in cells from pre-epileptic GAERS and young NE animals were too small for further analysis (1.7 ± 0.2 mV, n = 8; for both NE and GAERS, $p < 0.001$ compared to adults), indicating insufficient maturation of the functional interaction between Ca^{2+} spikes and I_h before the onset of SWDs. These data suggest that both NE animals and GAERS go through a developmental enhancement of Ca^{2+}-induced persistent I_h upregulation, and that, in GAERS, this occurs in the face of enduring reduction of cAMP regulation of I_h.

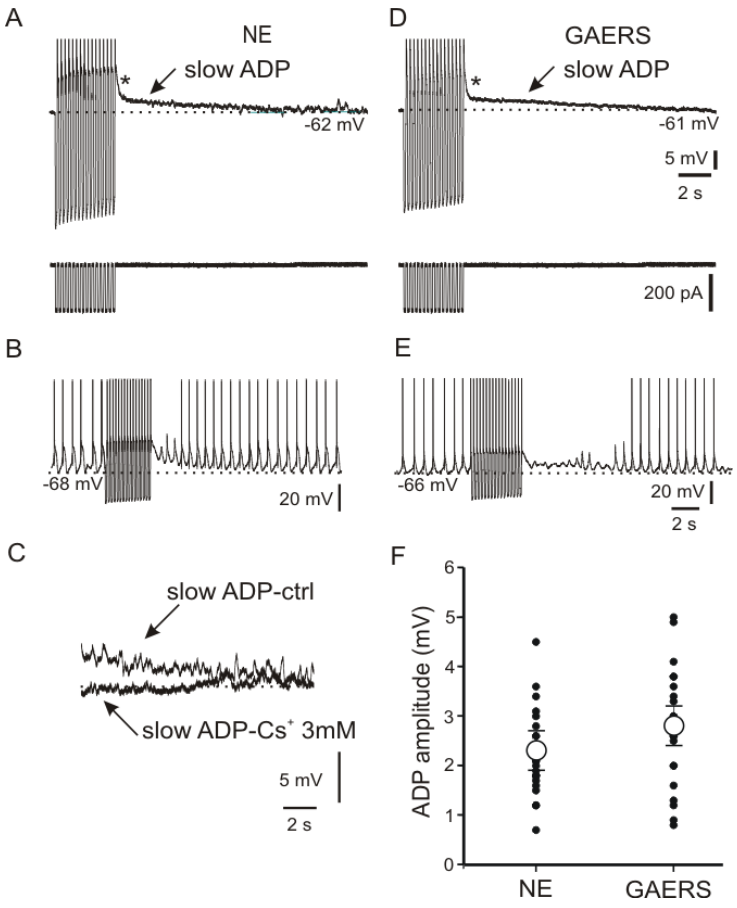

Figure 5. Mature GAERS TC cells generate unaltered afterdepolarizations (ADPs) *A, D*, Representative ADPs (*arrow*) found in TC cells from mature NE rats (*A*) and GAERS (*D*). The star (*) denotes a fast ADP that is not mediated by I_h. The current injection protocols used to elicit the ADPs in *A* and *D* are shown below the traces. *B, E,* Cells showing spontaneous clock-like δ-oscillations in a TC cell of a NE animal (B) and a GAERS (*E*). Note the attenuation of these oscillations during the ADP and their gradual reappearance. *C,* The slow ADP, shown here for a NE control (*slow ADP-ctrl*), is strongly reduced in the presence of 3 mM Cs^+ in the superfusing solution (*slow ADP- Cs^+ 3 mM*). *F,* Pooled values of ADP amplitudes for all experiments (n = 24 for both strains). *Filled circles* denote results of individual experiments, and are sometimes superimposed on each other. Average values (*open circles*) were not significantly different ($p > 0.05$).

Unaltered cAMP turnover in TC neurones of mature GAERS

We next aimed to determine the mechanisms in the mature GAERS that could overcome the functional deficits of HCN channels and facilitate the generation of robust ADPs. A potential mechanism yielding higher cAMP levels in the vicinity of the HCN channels in GAERS could be a reduced degradation of cAMP.

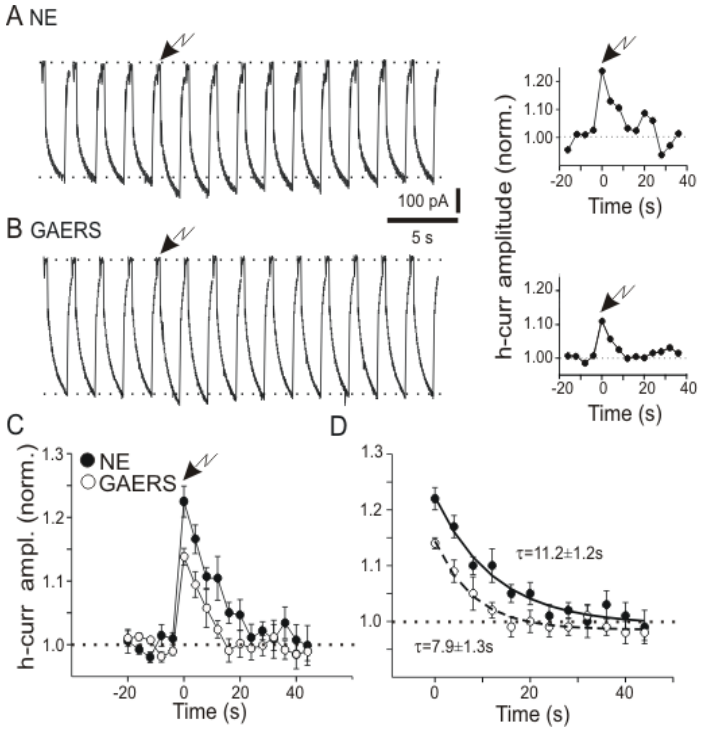

Figure 6. Unaltered decay time course of current enhancement following photolytic release of caged cAMP. *A, B,* Representative responses in cells derived from mature NE animals and GAERS on the *left*, with normalized plot of current amplitude presented to the *right*. Voltage protocol involved a -30 mV hyperpolarizing step from -60 mV, flash application occurred after 4 control responses (*arrow*, time 0 in the graphs). *C*, Normalized, averaged responses in NE (*closed circles*, n = 7) and GAERS (*open circles*, n = 6) to repetitive 30 mV hyperpolarizing steps, UV-flash application occurred at time 0 (*arrow*). Note decreased response in cells from GAERS ($p < 0.02$ at the peak of the cAMP-induced effect). *D*, Monoexponential fitting of the decay time course of the current responses after photolysis of caged cAMP. The time constant values indicated next to the traces are the average ± sem values obtained by exponential fitting of the traces from individual cells (see Methods), and these values were not significantly different ($p > 0.05$).

To obtain a measure of cAMP turnover, including phosphodiesterase activity, we examined the time course of the photolytic responses in mature GAERS and NE controls. In both cases, responses to the UV-light flash peaked within the first voltage step after flashing (see Methods) and then gradually decayed back to baseline levels. This decay reflects the superposition of cAMP dissociation from the channels and its subsequent degradation (Lüthi and McCormick, 1999b). In both NE and GAERS TC neurones, recovery was complete within 6 current responses obtained at 4 s intervals after flash application, characterized by a time constant of 11.2 ± 1.2 s for NE and 7.9 ± 1.3 s for GAERS (n = 7 for NE, n = 6 for GAERS, $p > 0.05$; Fig. 6), similar to previous observations (Lüthi and McCormick, 1999b). These data indicate that unbinding and/or diffusion/degradation of cAMP are not altered in

mature GAERS neurones, and therefore cannot account for the preserved ADP amplitudes.

Enhanced intracellular Ca^{2+} accumulation in GAERS neurones during repetitive low-threshold Ca^{2+} spikes

Molecular analyses have shown that expression of Ca^{2+} channel subunits in rat decreases by ~25% between 2 weeks and 2-3 months of age, but this reduction is less pronounced in GAERS (Guyon et al., 1993; Talley et al., 2000). In addition, increased amplitudes of LT Ca^{2+} currents in TC neurones were reported in several mouse models of SWDs (Zhang et al., 2002; Song et al., 2004). Therefore, Ca^{2+} entry via these channels might be altered in GAERS, and contribute to the normalization of the ADPs. To test this possibility, we performed simultaneous electrophysiological and Ca^{2+} imaging in cells filled with the Ca^{2+} indicator dye Oregon Green Bapta-2 (75 μM, see Methods). Changes in fluorescence were monitored following rebound Ca^{2+} spikes evoked by repetitive hyperpolarizing pulses. These Ca^{2+} spikes induced a progressive increase in the fluorescence signal that summated to reach a maximal average value of 115 ± 3 % of baseline after about 8 to 16 rebound bursts (n = 8; Fig. 7*A*, *B* and *D*). At the end of the stimulation protocol, the fluorescence signal returned to baseline within seconds (τ = 2.6 ± 0.5 s, n = 8). These results are in agreement with observations from Budde and colleagues (Budde *et al.*, 2000). To confirm that our stimulation protocol did not saturate the Ca^{2+} indicator and permitted recording larger fluorescence changes, positive current injections (+0.5 nA) were used to depolarize cells to suprathreshold voltages. Under these conditions, the increases in fluorescence recorded were about 2-fold larger ($\Delta F/F$ = 132 ± 2 %, $p < 0.005$; Fig. 7*C* and *D*), but decayed on a similar time scale (τ = 2.5 ± 0.5 s, n = 8, $p > 0.05$). These data indicate that repetitive LT Ca^{2+} spikes did not saturate the indicator dye, permitting comparison of Ca^{2+} rises triggered by LT spikes in GAERS *vs.* controls.

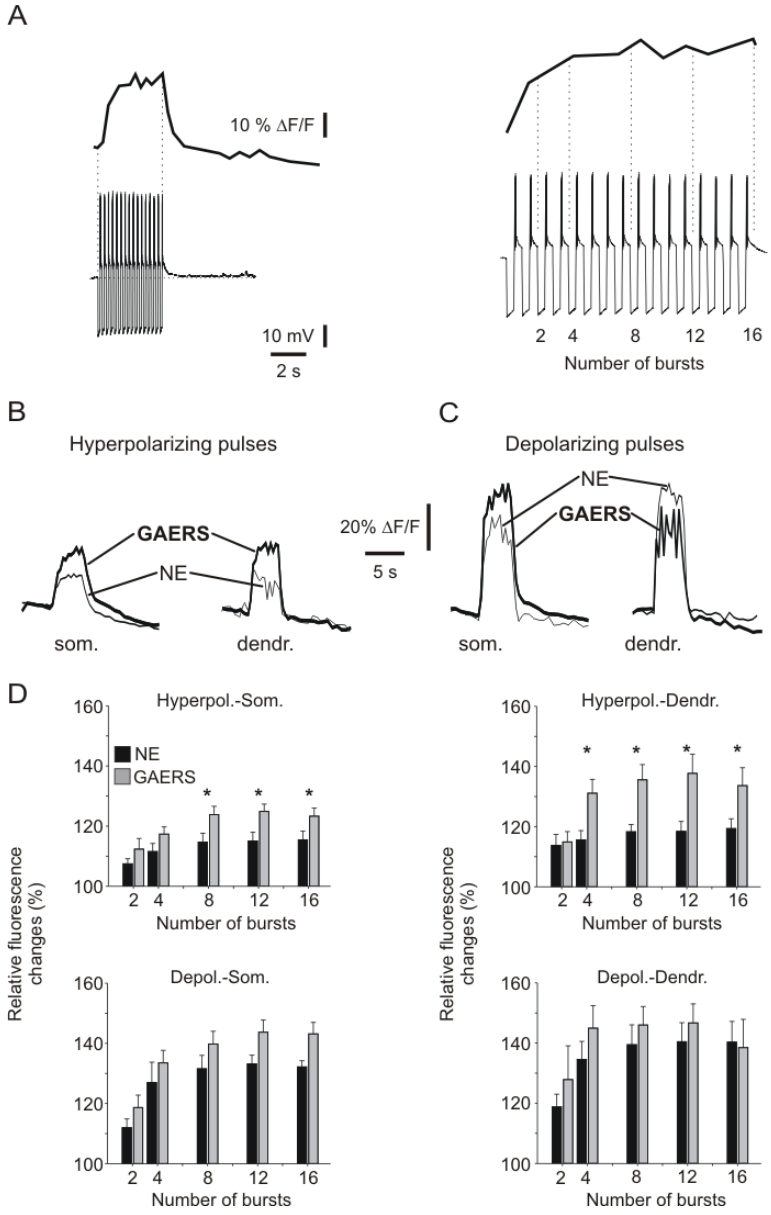

Figure 7. Repetitive hyperpolarizing current pulses evoke greater intracellular Ca^{2+} increases in GAERS neurones. *A*, *Left*, Representative sweeps showing the relative fluorescence changes evoked after repetitive rebound Ca^{2+} bursting in a cell from a NE rat. *Right*, Expanded portion of the trace illustrating the time sections (*vertical dotted lines*) within which Ca^{2+} signals were averaged and compared. *B*, Ca^{2+} transients recorded in somatic (som.) and dendritic (dendr.) regions after somatic injection of hyperpolarizing currents to induce rebound bursting. Transients are averages of 5 successive sweeps for cells from a NE animal (*thin line*) and a GAERS (*thick line*). *C*, As in *B*, but depolarizing current pulses were injected. *D*, *Top*, pooled data showing the relative changes in fluorescence evoked after repetitive hyperpolarizing pulses and recorded from somatic (Hyperpol.-Som., n = 8 for both NE and GAERS) or dendritic (Hyperpol.-Dendr., n = 6 for NE, n = 5 for GAERS) regions. *Bottom*, as top but for depolarizing somatic pulses recorded in soma (Depol.-Som., n = 6 for NE, n = 5 for GAERS) or in dendrites (Depol.-Dendr., n = 4 for both NE and GAERS). The stars (*) indicate $p < 0.05$.

We obtained fluorescence data from both somatic and proximal dendritic compartments, where T-type Ca^{2+} channels are expressed and colocalized with HCN channels (Stuart and Williams, 2000). Signals evoked by hyperpolarizing pulses were comparable in amplitude at the end of the train in both regions ($\Delta F/F = 115 \pm 3$ % in the soma, n = 8, $\Delta F/F = 119 \pm 3$ % in dendrites, n = 6, $p > 0.05$; Fig. 7*B* and *D*), but dendritic signals reached a plateau (Fig. 7*B* and *D*) and decayed more rapidly ($\tau = 1.4 \pm 0.1$ s, n = 6, $p < 0.05$). Depolarizing pulses evoked comparable fluorescent signals in the two compartments (Fig. 7*C* and *D*), with dendritic signals again decaying slightly faster ($\tau = 1.5 \pm 0.20$ s, n = 4, $p < 0.05$).

Ca^{2+} levels were markedly elevated in somata and dendrites of GAERS compared with NE cells when 8 or more hyperpolarizing pulses were injected ($\Delta F/F = 123$ - 125 %, n = 8, $\Delta F/F = 134$ - 138 %, n = 5, in somata and dendrites, respectively, $p < 0.05$ compared to NE; Fig. 7*B* and *D*), while time constants of decay obtained by monoexponential ($\tau = 1.7 \pm 0.1$ s and 1.2 ± 0.04 s in somata and dendrites, respectively, $p > 0.05$) or biexponential fitting (data not shown, $p > 0.05$) were unaltered. GAERS and NE cells did not differ in intracellular Ca^{2+} concentrations after depolarizing pulses (n = 6 for somatic, n = 4 for dendritic recordings, $p > 0.05$; Fig. 7*C* and *D*). These data indicate that the summation of Ca^{2+} transients induced by LT Ca^{2+} spikes in GAERS cells is selectively augmented, and can potentiate Ca^{2+}-dependent I_h upregulation, thus contributing to ADP normalization.

Discussion

In this study, we addressed the role of I_h during both epileptogenesis and the chronically epileptic state of the GAERS. Importantly, we focused on I_h in the somatosensory thalamus that constitutes the primary thalamic area generating SWDs in rat models of absence epilepsy (Vergnes et al., 1990; Seidenbecher et al., 1998; Renier and Coenen, 2000; Manning et al., 2004; Nersesyan et al., 2004; Budde et al., 2005). This approach allowed us to evaluate the contribution of inherited (or developmentally determined), compared with acquired changes in current properties to the pathogenesis of generalized epilepsy.

The principal findings presented here are: (1) basal electrophysiological characteristics, including resting membrane potential and voltage-gating of I_h, were unaltered in TC cells of adult GAERS, both *in vivo* and *in vitro*; (2) during both pre-epileptic and chronic epileptic stages, I_h was less sensitive to cAMP when the nucleotide was delivered at non-saturating doses; (3) a marked increase in mRNA levels of the relatively cAMP-insensitive HCN1 isoform occurred in the GAERS thalamus, without significant changes in cAMP-sensitive

HCN2 and HCN4; (4) ADP amplitudes were normal in mature GAERS, despite the enduring, reduced cAMP sensitivity of I_h and the upregulation of HCN1 subunit expression; (5) compensatory mechanisms, restoring ADP amplitude, involved enhanced Ca^{2+} accumulation in mature GAERS TC cells resulting from LT Ca^{2+} spikes. In summary, I_h may play a dual role in the GAERS epilepsy. Abnormal regulation of this current appears not only to be involved in the pathogenesis of absence epilepsy, but to trigger adaptive responses to antagonize the functional impact of these deficits, thereby restoring I_h functions that are important for the termination of synchronous network activity.

Our work further highlights that neuronal networks are exquisitely sensitive to imbalances in I_h regulation, consistent with the multiple roles of I_h in thalamic and cortical cell excitability (Santoro and Baram, 2003; Frère et al., 2004). Indeed, prior studies have identified comparatively small changes in current amplitude around resting membrane potentials (Di Pasquale *et al.*, 1997) or current voltage dependence (Chen *et al.*, 2001b; Budde *et al.*, 2005) in rodent models of epilepsy, yet these were accompanied by major increases in the propensity of neuronal networks to generate seizures.

Role of altered I_h function in the development of absence epilepsy

We identified a lowered sensitivity of I_h to near-physiological, phasic cAMP pulses in pre-epileptic TC cells of GAERS. In addition, consistent with a recent study on pre-epileptic WAG/Rij rats (Budde *et al.*, 2005), we also reveal an altered cAMP sensitivity of the I_h voltage-gating. Therefore, although we did not quantify HCN mRNA levels in the pre-epileptic state, our findings strongly suggest that abnormal cAMP sensitivity of HCN channels in thalamus may be a common denominator of rodent absence epilepsy during pre-epileptic stages. Cellular activity typical for SWDs in GAERS TC neurones involves occasional LT Ca^{2+} spikes superimposed on a tonic membrane hyperpolarization that is more pronounced compared to that in normal, sleep-related oscillations in these networks (Pinault *et al.*, 1998; Seidenbecher *et al.*, 1998; Pinault, 2003). The I_h is a key factor controlling membrane potential in TC neurons. Thus, genetic deletion of HCN2 reduces thalamic I_h by > 90% (Ludwig *et al.*, 2003) and provokes strongly hyperpolarized resting membrane potentials, burst discharges, and SWDs. In pre-epileptic GAERS, the gating of I_h by cAMP levels, but not its voltage dependence, was affected, leading to an attenuated depolarizing drive of cAMP-dependent I_h gating. This more subtle weakening of I_h–dependent membrane potential shifts likely contributes to the facilitation of burst discharge in pre-epileptic TC cells

(Budde *et al.*, 2005), to the synchronization of TC networks, and to the eventual appearance of SWDs. The decreased efficacy of cAMP could result from a genetically determined predisposition of the GAERS strain (Rudolf *et al.*, 2004), but might also be a consequence of an abnormal development of the TC network. For example, levels of neuronal and glial enzymes involved in the turnover of glutamate are reduced in TC networks during pre-epileptic stages (Dutuit *et al.*, 2000; Dutuit *et al.*, 2002), promoting elevated concentrations of ambient glutamate and, therefore, altered regulation of I_h (van Welie *et al.*, 2004).

Of note, a number of recent studies have highlighted changes in functional and molecular properties of cortical I_h, particularly a decreased HCN1 expression (Di Pasquale *et al.*, 1997; Strauss *et al.*, 2004). These changes enhance temporal summation of excitatory synaptic responses and may hence critically contribute to the hyperexcitability of TC circuits and promote SWDs (Meeren *et al.*, 2005). Our molecular analysis failed to demonstrate altered HCN1 or HCN2 channel expression in somatosensory cortex and thus does not support a major contribution of abnormal cortical I_h in the pathological condition of GAERS. Although potential functional changes in selected cortical neurons cannot be excluded, the observation that HCN2- (Ludwig *et al.*, 2003), but not HCN1-deficient (Nolan *et al.*, 2004) mice show spontaneous SWDs points to a major role of thalamic I_h isoforms in the susceptibility to SWD development.

Molecular composition of thalamic HCN channels in GAERS

The mRNA expression of HCN1, the subunit with the weakest sensitivity to cAMP, was augmented in adult GAERS, raising the question of how increased HCN1 protein modifies cAMP sensitivity of native HCN channels, without altering resting membrane potential and basal current amplitudes. Assuming that protein expression of HCN1 correlates quantitatively with the mRNA levels (Brewster *et al.*, 2005; Brewster *et al.*, 2006), the contribution of HCN1 to the total complement of HCN channels in Vpm is 12.5% in controls and ~20% in GAERS. In addition, voltage-gating of I_h around resting potentials is small (~10%, see Fig. 2*A*). Therefore, although activation of heterologously expressed HCN1 homomers occurs at more depolarized potentials than that of HCN2 homomers (Chen *et al.*, 2001c), the subtle change in HCN1 expression levels is expected to have minimal effects on cellular properties characterizing resting states. Furthermore, genetic deletion of HCN2 virtually abrogated I_h in TC neurones, despite preserved HCN1 protein expression (Ludwig *et al.*, 2003), indicating that HCN1 protein generates only a small component of thalamic I_h on its own, whereas the

HCN2 channel isoform carries the majority of voltage-gated current. However, a selective reduction of cAMP sensitivity in GAERS HCN channels may derive from enhanced heteromerization of the channels, driven by increased relative HCN1 subunit abundance (Brewster *et al*. 2005). HCN1-HCN2 heteromers and HCN2 homomers have a cAMP concentration-response curve with a similar maximum, yet heteromers show a weaker shift at subsaturing concentrations (Chen *et al.*, 2001c), thereby producing channels with properties approximating those found in GAERS. Taken together, our data are consistent with reduced contribution of HCN2 homomeric channels to whole-cell I_h of GAERS TC cells, coupled with increased contribution of heteromeric HCN1/HCN2 channels. The findings fit less well with HCN1-HCN4 heteromerization, because the maximal shift of HCN1-HCN4 heteromers is markedly smaller than that of HCN4 homomers (Altomare *et al.*, 2003), and HCN4 significantly contributes to thalamic I_h (Seifert *et al.*, 1999). Thus, the impact of HCN1-containing heteromers on cAMP sensitivity of whole-cell currents may depend on the type and amount of heteromers generated, as well as be influenced by a number of proteins interacting with HCN channels (Yu et al., 2001; Decher et al., 2003; Gravante et al., 2004; Santoro et al., 2004; Vasilyev and Barish, 2004). Altered expression or function of these (or other) proteins might influence, and perhaps disproportionately strengthen, the function and localization of HCN1-containing channels in GAERS TC cells.

Role of altered I_h function in chronic epilepsy

In mature GAERS, TC cells recorded *in vivo* had normal resting membrane and I_h sag potentials. In addition, the fraction of cells expressing a measurable sag potential was unchanged, indicating that chronic epilepsy in mature animals did not modify the relative proportions of TC cell subtypes described *in vivo* (Pinault, 2003). However, the reduced cAMP sensitivity of I_h, accompanied by an elevated HCN1 mRNA expression, persisted in the chronic state of epilepsy. Thus, the changes in thalamic I_h function, already present during pre-epileptic stages, appear to be unaltered by the onset of epileptic activity. This is in contrast to other experimental models, in which a single seizure episode markedly and persistently affects current properties and subunit expression (Chen *et al.*, 2001b; Brewster *et al.*, 2002; Shah *et al.*, 2004). However, chronic epilepsy is accompanied by compensatory adjustments of a regulatory mode of I_h that specifically controls network function and helps antagonizing the pronounced hyperpolarization of TC cells typical for SWDs. An attractive putative mechanism for such a compensatory effect involves augmented Ca^{2+} currents. In

addition to enhanced LT current expression in animal models of SWDs (Guyon et al., 1993; Talley et al., 2000), T-type channel expression is persistently enhanced by pathological hyperactivity, either due to experimental seizures (Beck et al., 1998; Su et al., 2002; Zhang et al., 2002), or to genetic interference with Ca^{2+} channel activity or neurotransmitter release (Song et al., 2004; Zhang et al., 2004). Here, we found a greater temporal summation of Ca^{2+} signals evoked by repetitive opening of the T-type Ca^{2+} channels in GAERS at frequencies approximating those in SWDs. However, no overt changes occurred after single or few spikes. This suggests that, rather than the expression of T-channels, the intracellular clearance of Ca^{2+} ions entering through these channels was diminished in GAERS, for example through reduced expression of Ca^{2+}-binding proteins (Montpied *et al.*, 1995) known to be expressed in TC cells (Meuth *et al.*, 2005). Although our analysis did not reveal differences in the decay kinetics of the Ca^{2+} transients, an indicator for changes in endogenous Ca^{2+} buffering or extrusion mechanisms, further analysis of the expression patterns and functional roles of endogenous Ca^{2+}-binding proteins is required to elucidate the mechanisms underlying enhanced Ca^{2+} accumulation in GAERS TC cells.

Enhanced Ca^{2+} influx through T-channels has been implicated in augmented transcription factor activity in mouse models of SWDs (Ishige *et al.*, 2001), suggesting an involvement of T-current-mediated Ca^{2+} signaling in the molecular mechanisms underlying SWDs. In GAERS, elevated levels of free Ca^{2+} in the vicinity of T-type Ca^{2+} channels should facilitate the stimulation of Ca^{2+}-sensitive adenylyl cyclases, characterized by a steep Ca^{2+} dependence in the ~100 nM - 1 μM concentration range (Cooper, 2003), which contribute to slow I_h upregulation and ADPs (Lüthi and McCormick, 1999b). We excluded decreased cAMP degradation as a mechanism stabilizing ADPs, although the possibility remains that adenylyl cyclases activated by Ca^{2+} are increasingly expressed or more active in GAERS.

The present results further substantiate the critical role of thalamic HCN channels in the involvement of thalamic networks in SWDs (Pinault *et al.*, 1998; Seidenbecher *et al.*, 1998; Pinault, 2003; Meeren *et al.*, 2005). We demonstrate that activity-dependent alterations in HCN channel function are required for epileptogenesis, while basal current properties are minimally affected. In addition, we show that imbalances in HCN channel transcription accompany chronic epilepsy, rendering HCN channels candidates for the growing family of ion channels underlying transcriptional channelopathies (Waxman, 2001). A reduced HCN1 protein was previously found in cortical neurones in the WAG/Rij absence model (Strauss *et al.*, 2004). Thus, the construction of functional HCN channels may be affected at multiple levels and in a cell-type specific manner in TC networks generating SWDs. Moreover, while

thalamic neurones use homeostatic mechanisms to compensate for deficits at the channel level, cortical abnormalities persist through adulthood (Strauss *et al.*, 2004). This differential adaptation may explain the persistence of SWDs in adult rodent models, where cortical hyperactivity and exacerbated burst discharges of Rtn cells initiate SWDs (Slaght *et al.*, 2002; Manning *et al.*, 2004; Meeren *et al.*, 2005).

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PAPER II: ß-ADRENERGIC MODULATION OF HCN CHANNELS IN DIFFERENT THALAMIC NUCLEI

Introduction to paper II

First-order and intralaminar thalamic nuclei

In this part of my thesis I address the question if there are cell-type specific modes of cAMP-dependent regulation of HCN channels. As described in chapter 3.6.8, HCN channels are particularly important for the electrophysiological properties of neurons in the thalamus, consisting of distinct nuclei.

First-order thalamic nuclei are considered to be the "classical thalamic relays". In the tonic firing mode during waking, neurons of these "specific" first-order thalamic nuclei transmit the incoming information relatively linear to the cortex (Sherman and Guillery, 2005). To fulfill this function, it is crucial for the resting membrane potential of the cells to be depolarized sufficiently. This depolarization is under the control of ion channels that are modulated by G-protein-coupled receptor (GPCR)-activating neurotransmitters (McCormick, 1992). Here, the regulation of HCN channels by β-adrenergic receptors (ß-ARs) plays a pivotal role (McCormick and Pape, 1990a).

In contrast, the "non-specific" intralaminar nuclei rather provide the necessary arousal of cortical and subcortical regions (for review, see (Van der Werf *et al.*, 2002)). Thus, high frequency stimulation *in vivo* in the intralaminar thalamic region leads to desynchronized cortical activity related to high levels of wakefulness and vigilance (Shirvalkar *et al.*, 2006). Low-frequency stimulation causes slow-wave activity in the entire cortical mantle accompanied by somnolence (Van der Werf et al., 2002). These actions can be achieved by the widespread projection of intralaminar thalamic nuclei to cortical areas (Groenewegen and Berendse, 1994). Neurons of intralaminar thalamic nuclei can exert tonic or bursting firing properties like first-order thalamic neurons. However, these neurons discharge in bursts not only during sleep, but also during waking (Glenn and Steriade, 1982; Steriade et al., 1993a), contrary to neurons of first-order thalamic nuclei. Thus, the resting membrane potential seems to be influenced by a different regulation of ion channels. This is accompanied by a distinct expression pattern of GPCRs (Mansour *et al.*, 1987; Mansour *et al.*, 1994; Vizuete *et al.*,

1997; Marcus *et al.*, 2001), including ß-ARs (Rainbow et al., 1984; Nicholas et al., 1993).

The noradrenergic system and locus coeruleus

The adrenergic system is an essential regulator of neuronal, endocrine, cardiovascular, vegetative, and metabolic functions. Activation induces the "fight-or-flight" response, facilitating immediate physical reactions associated with danger or stressful situations, respectively. These include acceleration of heart and lung action, dilation of blood vessels in muscle tissue, and inhibition of stomach and intestinal action.

In the nervous system, the neurotransmitter noradrenalin (NA), also named norepinephrine, is released by stressful events that require high attention. This is partly caused by discharges of an area of the brain stem called the locus coeruleus (LC). There, a majority of brain noradrenergic neurons are concentrated and supply NA throughout the central nervous system by a widespread ascending projection system (for review, see (Berridge and Waterhouse, 2003)). Increasing tonic LC output activates the forebrain, which could be shown by EEG (Berridge and Foote, 1991). It is correlated with arousal levels (Aston-Jones and Bloom, 1981), and progression from drowsy low-attention states to alert, highly vigilant states (Usher *et al.*, 1999). Additionally, it was shown that locally applied NA or LC stimulation can modulate the responsiveness of neurons, including those in the thalamus, to non-monoaminergic synaptic inputs (Berridge and Waterhouse, 2003). In the thalamus of waking rats the output from LC-firing both modulates the responsiveness of single thalamic neurons to synaptic input, and the representation of sensory information across ensembles of thalamic neurons (Devilbiss *et al.*, 2006). These findings provide evidence that the noradrenergic pathway controls thalamic responses to sensory driven synaptic input.

Adrenergic receptors

The neurotransmitter NA activates GPCRs to transmit the signal across the plasma membrane. These adrenergic receptors (ARs) can be divided into three different groups: the α1-receptors (α1A, α1B, α1D), α2-receptors (α2A, α2B, α2C), and β-receptors (β1, β2, β3). Upon binding of NA, the receptors undergo a conformational change that leads to the activation of heterotrimeric GTP-binding proteins (G-proteins). The three groups of ARs couple to and activate only certain G-proteins, thus leading to specific intracellular signals. α1-ARs are coupled to G_q-mediated pathways, which increase intracellular inositiol-

trisphosphate (IP_3) and Ca^{2+} concentrations. Activation of the presynaptic α2-ARs leads to the inhibition of adenylyl cyclases via G-proteins of the $G_{i/o}$ family. ß-ARs are known to couple to G_s-proteins and activation stimulates adenylyl cyclases and results in increased intracellular cAMP levels.

ß-Adrenergic receptor signaling. In 1986, the ß2-AR was the first GPCR to be cloned (Dixon *et al.*, 1986). Since then a large array of cellular signaling mechanism has been identified *in vitro*. The initially linear signaling cascade of a receptor - G-protein - second messenger - effector coupling has evolved into a complex network of receptor activated signaling molecules. For some of these pathways the molecular mechanisms of signaling have been described in great detail. For example, ß-AR activation also initiates the process of receptor desensitization, an adaptive response used by cells to arrest G-protein signaling. This desensitization process can be induced by phosphorylation of the receptor by G-protein coupled receptor serine/threonine kinases (GRKs) or by protein kinase A (PKA) dependent phosphorylation (for review, see (Kohout and Lefkowitz, 2003)). The desensitization can initiate a redistribution of the receptor away from the cell surface by endocytosis, also known as internalization or sequestration. Internalization can, however, positively regulate receptor signaling and promote receptor resensitization (Kohout and Lefkowitz, 2003). Additional mechanisms can contribute to the dampening of receptor signaling after prolonged stimulation. These include receptor degradation or down-regulation, which is often carried out in lysosomes, a process generally operating over longer periods of time (hours) than receptor phosphorylation (Pierce et al., 2002; Kohout and Lefkowitz, 2003). Furthermore, in particular β2-ARs are known to show a switch from the well-established G_s-protein coupled pathway to the activation of G_i-proteins (Daaka et al., 1997). Specific scaffolding proteins like the ß-arrestins have been identified as multifunctional adaptors that recruit a broad spectrum of signaling molecules to the receptors (Pierce et al., 2002; Lefkowitz and Whalen, 2004). Thus, an individual ß-AR can dynamically couple to multiple signaling proteins in a temporally and spatially regulated manner.

For cardiac myocytes, recent data about subtype-specific function and the signaling mechanism of ß-ARs has improved the understanding of the causal relationship between ß-adrenergic stimulation and heart failure. ß1-AR-activated cAMP signaling is widely broadcast within cardiomyocytes, while ß2-AR-stimulated cAMP signaling is spatially and functionally compartmentalized (for review, see (Xiao *et al.*, 2006)).

However, despite the wealth of information regarding the signaling pathways engaged

by ß-adrenergic stimulation, a gap exists between *in vitro* heterologous expression systems and the *in vivo* situation. Thus, detailed mechanisms of adrenergic signaling in neurons remain open.

ß-Adrenergic receptors in neurons. ß-ARs are mostly known for their role in the regulation of cardiovascular, airway, uterine and metabolic functions. However, the brain is densely innervated by NA-containing neurons and ß-ARs have been identified in rat brains by *in situ* hybridization (Nicholas *et al.*, 1993) and an antibody-binding study (Rainbow *et al.*, 1984). The adrenergic system plays a central role in regulating numerous functions of the central nervous system, such as learning and memory, mood, and the state of arousal.

For example, the circadian rhythm of melatonin synthesis is controlled via ß-ARs by NA released from sympathetic nerves in the pineal gland (for review, see (Simonneaux and Ribelayga, 2003)). The involvement of the adrenergic system in memory functions has been studied in mice lacking ß-AR subtypes or dopamine ß-hydroxylase, the enzyme responsible for the synthesis of NA (Winder et al., 1999; Murchison et al., 2004). NA acting at ß1-ARs has been found to be essential for the retrieval of contextual and spatial memory but does not seem to be necessary for the retrieval of emotional memories (Murchison et al., 2004). A model of synaptic changes required for learning is hippocampal long term potentiation (LTP). LTP induced by prolonged theta stimulation has been found to be facilitated by activation of ß1-ARs (Winder et al., 1999). Also associative LTP was shown to be increased after stimulation of ß-ARs in hippocampal slices (Lin et al., 2003). The memory strengthening effects of emotion is thought to be induced by adrenergic action on ß-ARs in the amygdaloid complex (McGaugh and Roozendaal, 2002).

Several recent studies on ß-adrenergic modulation of L-Type Ca^{2+} channels provide evidence for a highly organized ß-adrenergic signaling system in neurons. A localized action to Ca^{2+} channels after ß2-AR stimulation has been reported in the hippocampus, that ensures rapid cell signaling (Davare et al., 2001; Hoogland and Saggau, 2004). In rat adrenal chromaffin cells an opposite action of ß1- and ß2-ARs on Ca^{2+} channels has been reported. Here, the ß1-AR pathway develops slowly according to its diffusive characteristic, while ß2-ARs signaling is fast (Cesetti et al., 2003).

In first-order thalamic nuclei, activation of ß-ARs is known to upregulate HCN-currents via cAMP (McCormick and Pape, 1990a). Following the coupling of pacemaker channels to cAMP by monitoring HCN-currents provides an opportunity to investigate ß-AR signaling. For example, (Frère and Lüthi, 2004) could demonstrate a cross-talk between ß-adrenergic

and $GABA_B$-receptors in first-order thalamic neurons. The enhancement of HCN-currents by stimulation of ß-ARs was increased when simultaneously an activation of $GABA_B$-receptors occurred. Importantly, the dynamics of HCN channel modulation by GPCR-agonists reflected the time course of intracellular cAMP concentrations (Frère and Lüthi, 2004). Thus, by studying the ß-adrenergic modulation of HCN-currents, my thesis not only investigates the regulation of a particularly important (see Section 3.6.8 of this thesis) ion channel, the pacemaker, in neurons of distinct thalamic nuclei. It will further contribute to the field of subtype specific GPCR-signaling and to resolve the complex signaling cascades following GPCR activation.

Neuronal ß-adrenergic receptors in pharmacotherapy. In humans, many drugs are currently used that interact with neuronal ß-ARs, including ß-adrenergic antagonists (ß-blockers), such as propranolol, metoprolol or timolol. In pharmacotherapy, central ß-blockers are used to treat chronic migraine, essential tremor and certain anxiety disorders (for review, see (Emilien and Maloteaux, 1998)). Of these, in both, chronic migraine (Shields and Goadsby, 2005) as well as essential tremor (Hua *et al.*, 1998) the thalamus seems to be involved. However, the exact mechanism of drug action in these conditions has not been identified yet. To uncover the physiological and pharmacological significance of ß-ARs in a subtype specific manner will be helpful in developing drugs with minimized side-effects.

ß-adrenergic modulation of pacemaker currents in different thalamic nuclei

Unpublished data

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Abstract

The thalamus is the most important subcortical station allowing for relay and coordination of sensory information and is broadly classified into first-order (FOn), higher-order and intralaminar/midline (IMn) nuclei. Neurotransmitter-mediated cAMP synthesis and enhancement of the hyperpolarization-activated cation current (I_h) are well-established ionic mechanisms that control relay functions in FOn. In FOn, β1-adrenergic receptor expression is predominant, whereas β2-receptor expression outweighs β1-receptors in IMn nuclei. To address differential β-adrenergic regulation of I_h in thalamic nuclei, whole-cell I_h was recorded in visually identified mouse thalamic cells in the ventroposterior medial (Vpm), a FOn, and in the centrolateral nucleus (Cln), an IMn.

I_h amplitudes were strikingly smaller in Cln compared to Vpm throughout the entire voltage activation range, but were equally modulated by cAMP. Bath-application of the non-specific β-adrenergic agonist isoprenaline (Iso) induced a concentration-dependent enhancement of I_h amplitude in Vpm, described by an EC_{50} of 0.44 µM and a maximal potentiation after 1 min that fully decayed after 6 min. This desensitization could be prevented in the presence of the phosphodiesterase (PDE) inhibitor IBMX (100 µM), indicating an essential requirement of PDEs, while proteinkinase A-activity seems not to be involved. In contrast, application of Iso in the Cln induced a minor, not significant increase of I_h amplitude. The Iso response in the Vpm was mediated by β1-adrenergic receptors, because it remained unaltered in the presence of the β2-antagonist ICI118,551 (100 µM), and was mimicked by the β1-agonist xamoterol. Selective β2-receptor activation produced no response, neither in the Vpm, nor in the Cln. Additionally, β2-adrenergic modulation could not be rescued by preventing β-adrenergic desensitization during cutting and incubation of the thalamic slices in the presence of the β-adrenergic antagonist propranolol. Increasing the apparent affinity for Iso to β-adrenergic receptors by lowering the temperature increased β1 mediated I_h responses in both Vpm and Cln.

Strong β-adrenergic regulation of I_h appears thus to be limited to portions of the thalamus involved in sensory relay, while not being involved in general arousal function.

Introduction

Hyperpolarization-activated cation (HCN) channels are widely expressed in the nervous system and often function as "pacemaker" for rhythmic electrical behavior (for review, see (Pape, 1996; Santoro and Tibbs, 1999; Robinson and Siegelbaum, 2003). These channels open in response to membrane hyperpolarization and give rise to a cationic current (I_h) that, under physiological conditions, is depolarizing. The voltage-dependence of HCN channels is influenced by the second messenger cAMP that directly binds to the channel and shifts the activation-range to more depolarized potentials (Wainger *et al.*, 2001). Thus, HCN channels can be modulated by activation of the G-protein-coupled receptors (GPCRs) linked to cAMP synthesis, such as the ß-adrenergic receptors (ß-ARs) ((McCormick and Pape, 1990a; Frère and Lüthi, 2004) for review, see (McCormick, 1992; Frère *et al.*, 2004)). In recent years, the palette of physiological functions of HCN-currents has increased dramatically, and now covers aspects of synaptic function, dendritic integration, plasticity, learning and pathological neuronal and cardiac states (for review, see (Robinson and Siegelbaum, 2003; Frère et al., 2004)).

In view of this functional diversity, understanding in detail the native pathways leading to cAMP synthesis and hence, HCN channel upregulation, is important. Numerous studies have documented in detail that GPCRs coupled to the stimulatory G-protein (G_s) can upregulate I_h via cAMP (for review, see (Frère *et al.*, 2004)). Nevertheless, elementary questions remain open. First, how do receptor subtypes control cAMP synthesis? This question arises in particular for the ß-ARs, as the ß1- and ß2-AR subtypes are well-known for showing a differential signaling and functionality in cardiac myocytes (for review, see (Xiao *et al.*, 2006)). Thus, ß2-ARs are not only coupled to G_s-proteins, but switch to activate pertussis toxin (PTX)-sensitive inhibitory G-proteins (G_i) after prolonged receptor stimulation (Xiao et al., 1995; Daaka et al., 1997). Second, it has been shown that complex interactions between GPCRs exist (Pedarzani and Storm, 1996; Frère and Lüthi, 2004). And third, it will also be important to determine, whether HCN channels may be components involved in macromolecular complexes of local cAMP signaling, such as that described for Ca^{2+} channels (Jurevicius and Fischmeister, 1996; Davare et al., 2001), or whether they are cAMP sensors for the average cAMP activity in a cell.

The pathway from GPCR activation to ion channel upregulation is well characterized for the ß-adrenergic regulation of HCN channels. In particular in the thalamus, noradrenergically mediated cAMP synthesis and subsequent enhancement of I_h is a well-

established mechanism that helps to control relay function (McCormick and Pape, 1990a). The firing mode of thalamic neurons, bursting or tonic, is strongly voltage-dependent and hence largely determined by their membrane potential. The resting membrane potential is in turn, controlled by the opening and closure of several ion channels (Hille, 2001), amongst which HCN channels play an important role in thalamic neurons (Ludwig *et al.*, 2003; Meuth *et al.*, 2006). Activated upon ß-AR stimulation, the current flowing through these channels provokes membrane depolarization and brings the membrane potential out of the bursting range, thereby promoting the tonic firing mode that is typical for waking. This effect is thought to essentially contribute to the maintenance of arousal, which is accompanied by tonic activity of the noradrenergic locus coeruleus in the thalamocortical system (Aston-Jones and Bloom, 1981; Berridge and Waterhouse, 2003).

The thalamus is the most important subcortical station allowing for relay and coordination of sensory information (Sherman and Guillery, 2005). Far from being a pool of similar nuclei, it is now clear that it is composed of a highly differentiated set of nuclei that can be broadly classified into first-order, higher-order and intralaminar thalamic nuclei based on a number of functional, network, cellular and molecular differences (for review, see (Groenewegen and Berendse, 1994; Sherman and Guillery, 1996; Deschenes et al., 1998; Van der Werf et al., 2002)). First-order neurons are recognized as the neurons that relay incoming information from the periphery to the cortex, higher-order neurons are thought to serve as a link in cortico-thalamo-cortical pathways that continue to process these information streams, while intralaminar neurons have been implicated in general arousal functions. A principal source of noradrenergic receptor activation innervates the anterior intralaminar thalamic nuclei (including the centrolateral nucleus, Cln) (Krout *et al.*, 2002). Therefore, the intralaminar nuclei link the brainstem arousal system to the cortical network crucial for the organization of wakeful behavior.

An additional notable characteristic of thalamic subdivision is a differential expression of GPCR subtypes. This has been demonstrated for serotoninergic (Vizuete *et al.*, 1997), orexinergic (Marcus *et al.*, 2001) and opioid receptors (Mansour *et al.*, 1987; Mansour *et al.*, 1994), all of which show differential expression of receptor subtypes across the three classes of thalamic nuclei. A few recent studies have shown an action of several neurotransmitters and neuropeptides on intralaminar thalamic neurons. Orexin and dopamine application can induce a depolarization, possibly via K_{leak} not via I_h (Govindaiah and Cox, 2006), while μ-opiods were shown to inhibit neurons of the Cln (Brunton and Charpak, 1998), and serotonin to suppress a slow AHP in intralaminar and midline nuclei (Goaillard and Vincent,

2002). Intralaminar and first-order thalamic nuclei also show a differential subtype expression pattern of ß-ARs. ß1-ARs were found to be predominant in the first-order ventroposterior medial (Vpm) complex, whereas ß2-ARs outweigh ß1-ARs in the Cln, an intralaminar nucleus (Rainbow *et al.*, 1984; Nicholas *et al.*, 1993).

We take these differences in the expression pattern to estimate the roles of ß-AR subtypes in modulating HCN-currents, hence in controlling membrane potential and function of thalamic cells. By following the coupling of HCN channels to prolonged ß-AR stimulation, we are studying the differential effects of noradrenalin in two distinct thalamic nuclei. Moreover, we see a specificity of GPCR function associated with the phenomenon of compartmentalized and local cAMP signalling that was not described for thalamic neurons before.

Methods

All experiments were performed according to the guidelines of the Veterinary institute of the Canton Basel-Stadt, Switzerland.

Slice preparation

C57BL/6-mice of either sex between 17-24 days were deeply anesthetized with isoflurane vapor in a mobile anesthesia station (Provet, Switzerland) and immediately decapitated. Coronal slices (300 µm) containing the Vpm and Cln and horizontal slices containing the Vpm were prepared on a vibratome in an ice-cold oxygenated solution containing (in mM): 63 NaCl, 107 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 18 dextrose, 1.7 L(+)-ascorbic acid. The slices were allowed to recover for 5 min in a home-made chamber at 35°C in cutting solution, before being transferred to a sucrose-free solution containing 126 mM NaCl instead and cation concentrations were altered to 2 mM $CaCl_2$ and 4 mM $MgCl_2$. After an additional 30 min, slices were incubated at room temperature for at least 1 h and then transferred individually into the recording chamber.

Electrophysiological recordings

Whole-cell recordings were obtained from visually identified neurons in the Vpm and Cln of the thalamus (Olympus BX50WI and BX51WI, Volketswil, Switzerland) at 33-35°C. The Vpm of the somatosensory thalamus was determined before pipette positioning using a 10x objective, and was clearly recognizable based on its apposition to the ventroposterior

lateral nucleus, which has a striated appearance. Neurons in the Cln were identified as belonging to a band of longitudinally oriented cells running from dorsal to lateral between the habenula/midline thalamic nuclei and the posterior thalamic group, curving towards the central nucleus. Slices were not used when the cells could not be clearly delineated. Patch pipettes were pulled from borosilicate glass tubing (TW150F-4, OD 1.5 mm, WPI, Berlin, Germany) on a vertical two-step puller (PP-83, Narishige, Tokyo, Japan) and filled with the following solution (in mM): 130 KGluconate, 10 KCl, 10 HEPES, 2 $MgCl_2$, 2 Na_2ATP, 0.2 NaGTP, 10 phosphocreatine, adjusted to 290 mOsm with sucrose, pH 7.25. GTP was freshly added daily from stocks (100-fold concentrated). The resistance of the electrodes was 2.5-4 MΩ and yielded series resistances in the range between 8-20 MΩ. If series resistance changed by more than 20%, the experiment was discarded. A liquid junction potential of 10 mV measured as described (Neher, 1992) was taken into account for all data. The bath was constantly perfused with fresh medium at a rate of 3 ml min^{-1} throughout the recording and contained (in mM): 126 NaCl; 2.5 KCl; 1.25 NaH_2PO_4; 2 $MgCl_2$; 2 $CaCl_2$; 26 $NaHCO_3$, 18 dextrose, 1.7 L(+)-ascorbic acid. Data from voltage- and current-clamp recordings were collected through an Axopatch 200B amplifier (Molecular Devices), filtered at 2 kHz and acquired at 5 kHz using pClamp 9.2 software.

Discharge properties of thalamic neurons were investigated in the current-clamp mode by applying increasing negative or positive current steps (-500 to +500 pA) lasting 70 ms for negative and 600 ms for positive current steps. The membrane potential was held at -60 mV prior to current injection. Action potential discharge frequencies were measured in the time from 300 to 500 ms after the onset of the positive current step.

HCN-currents were activated in the voltage-clamp mode by applying 2.5 s hyperpolarizing voltage commands from a holding potential of -60 mV at an interstimulus interval of 12 s. Amplitudes were determined by measuring the steady-state current and subtracting from the instantaneous current at the onset of the voltage step (see Fig 2B). HCN-currents were nornalized to the average of the 5 current amplitudes before application of the drug. Activation curves were determined by applying 10 mV increasing hyperpolarizing voltage steps, tail currents were measured at a potential of -75 mV and normalized with respect to the maximal tail current amplitude. The analysis program Igor v. 4.0.8 was used for fitting Boltzmann equations with $I/I_{max} = (1 + exp [(V - V_{half})/s])^{-1}$ to determine the half-maximal activation voltage (V_{half}) and *s* the slope factor (*s*). Isoprenaline (Iso) dose-response curve was fit with the Hill-Equation $I/I_{max} = [1 + (c/c_0)^p]^{-1}$ with I/I_{max} representing the normalized HCN-current increase, *c* the concentration of Iso and *p* for the rate.

Monoexponential decay time constants (τ_{decay}) were obtained by using the Chebychev routine in ClampFit to approximate voltage or current response from their peak (Ca^{2+} spike, HCN-current desensitization, respectively) to baseline.

8-Bromo-cAMP (8Br-cAMP) was applied via the patch-pipette and a time of ~3-5 min was sufficient to perfuse the cells and record stable effects on the current. All other drugs were applied through the bath (Iso, 3-isobutyl-1-methyl-xanthine (IBMX), ICI118,551, atenolol, salbutamol, xamoterol). For blockade of proteinkinase A (PKA), slices were incubated with the inhibitor (H-89, KT5720) for at least 2 hours prior to recordings. Experiments with blocked ß-ARs were performed by adding propranolol in all solutions used to cut, prepare and store slices.

Data are presented as mean ± S.E.M. Paired or unpaired t-test as appropriate were used for statistical analysis and value of $p < 0.05$ was considered statistically significant.

Results

Distinct electrophysiological properties of Vpm and Cn neurons

To study the β-adrenergic regulation of HCN-currents, whole-cell current-clamp and voltage-clamp recordings were obtained from visually identified neurons in the Vpm and Cln of the thalamus. Resting membrane potentials were similar in both thalamic nuclei (-74.4 ± 1.5 mV in Cln, n = 16 and -74.0 ± 1.1 mV in Vpm, n = 23; $p > 0.5$), but the Cln neurons showed a higher input resistance (317 ± 25 MΩ in Cln, n = 16 and 202 ± 17 MΩ in the Vpm, n = 25, $p < 0.005$). To further distinguish the neurons in the Cln and Vpm, we investigated neuronal discharge properties by applying incremental depolarizing and hyperpolarizing current steps from a holding potential of -60 ± 2 mV. Upon hyperpolarization, both thalamic neuronal types showed the typical rebound low-threshold Ca^{2+} spikes crowned by 2 to 5 action potentials (Brunton and Charpak, 1998; Bayer et al., 2002; Goaillard and Vincent, 2002; Sherman and Guillery, 2005; Govindaiah and Cox, 2006). The Ca^{2+} spike appeared with a delay after the offset of the hyperpolarizing step (Fig. 1*A*). On average, the delay from the offset of the hyperpolarizing current pulse to the peak of the Ca^{2+} spike lasted 112 ± 13 ms in the Cln (n = 8) and 53 ± 8 ms in the Vpm (n = 9; $p < 0.005$; Fig. 1*B*) after current injections of -500 pA. Moreover, once activated, the low-threshold bursts decayed with a time constant of 57 ± 12 ms in the Cln (n = 5) and 28 ± 2 ms in the Vpm (n = 7, $p < 0.05$).

When depolarized, neurons displayed tonic action potential discharges. Neurons in Cln

showed a more pronounced increase in action potential discharge frequency with increasing depolarizing current injections (100, 150, 200 and 300 pA; Fig. 1*C,D*), pointing to a greater excitability in the Cln than in the Vpm. After a 200 pA positive current injection for 70 ms, Cln cells fired action potentials with a mean frequency of 39 ± 8 Hz (n = 8), while Vpm neurons were significantly less excitable, firing action potentials with a frequency of 8 ± 4 Hz (n = 11, $p < 0.005$). However, the discharge frequency eventually leveled off for both Cln and Vpm neurons around 70-80 Hz (for 500 pA: 80 ± 4 Hz in Cln, n = 6 and 72 ± 15 Hz in Vpm, n = 8, $p > 0.05$). Altogether, these data indicate that Cln neurons show a greater responsiveness to depolarizing stimuli, but are more resistant to burst generation, and, in part, seem to lack the intrinsic conductances, such as the sag potential, that facilitate bursts.

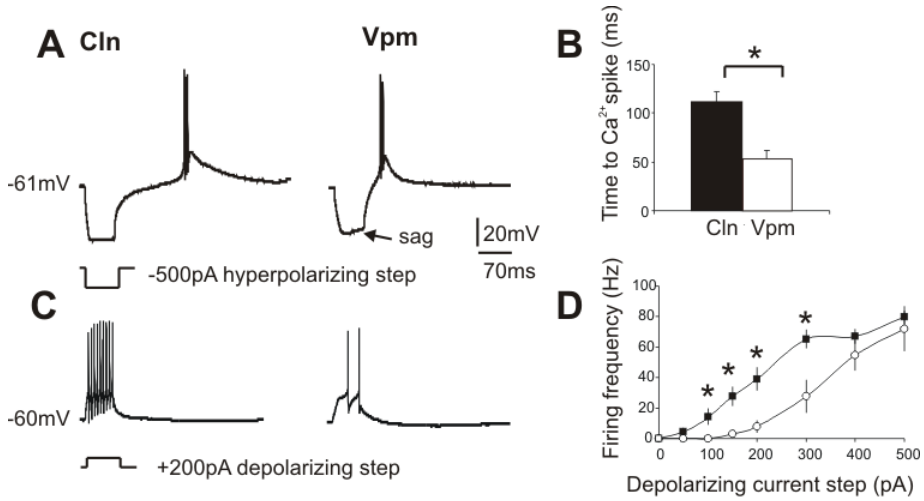

Figure 1. Vpm and Cln neurons show distinct discharge properties. *A*, Representative current-clamp recordings show the typical rebound burst response of a Cln (left) and a Vpm (right) cell to a -500 pA current injection for 70 ms. The current injection protocol is shown below the traces, membrane potential was set to -60 ± 2 mV before applying the protocol. Note the pronounced 'sag' potential (arrow) in Vpm cells. *B*, Histogram showing the averaged delay to the peak of the Ca^{2+} spike, measured from the offset of the hyperpolarizing current pulse (500 pA) for Cln (n = 8) and Vpm (n = 9) cells. *C*, Positive current injection provoking tonic action potential discharge in the two cell types. The protocol is shown below, a 200 pA depolarizing step was applied for 70 ms in both, from a holding potential of -60 ± 2 mV. *D*, Firing rate vs. current injection curve ('f-I curve') for Cln (n = 8, black squares) and Vpm neurons (n = 11, white circles) obtained from recordings as presented in *C*. *denotes $p < 0.05$ in B,D

HCN-currents in Vpm and Cln neurons

We next used voltage-clamp recordings to carry out a comparative analysis of the properties and the cAMP regulation of HCN-currents in Vpm and Cln cells. Previous reports

found a predominance of the highly cAMP sensitive HCN2 and HCN4 subunits in both nuclei, albeit at weaker levels for Cln (Notomi and Shigemoto, 2004). Consistent with this observation, membrane hyperpolarization (-30 mV negative voltage-step, holding potential -60 mV), yielded smaller current amplitudes in the Cln (-85.3 ± 6.3 pA, n = 16, Fig. 2*A*) than in the Vpm (-390.3 ± 37.0 pA, n = 25; $p < 0.005$, Fig. 2*B*). Significantly smaller current amplitudes in the Cln were found throughout the whole activation-range (Fig. 2*C*).

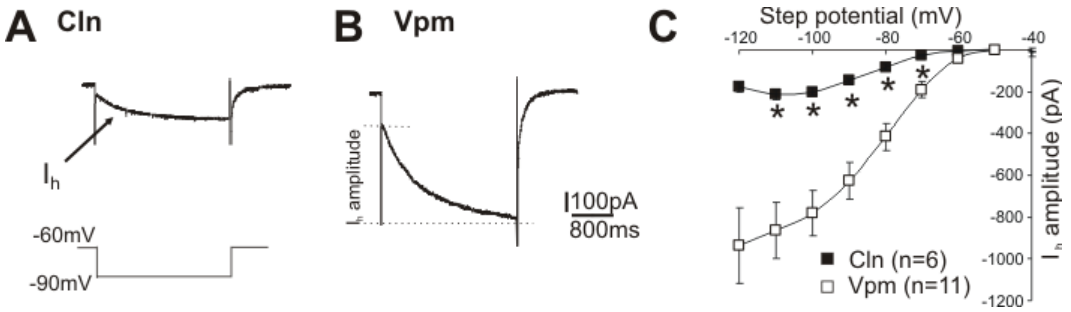

Figure 2. HCN channels generate smaller current amplitudes in Cln cells. *A, B*, Representative current responses to a hyperpolarizing voltage step in Cln (A) and Vpm (*B*). Voltage clamp protocol is shown below the traces. Dotted lines indicate I_h amplitude, measured at steady-state, the instantaneous current amplitude at the onset of the voltage step was subtracted *C,* The full current-voltage-relationship for I_h amplitudes in Cln (n = 6, black) and Vpm (n = 11, white). Here, the hyperpolarizing voltage steps were applied for a duration of 8 s to guaranty full steady-state I_h activation. *denotes $p < 0.05$.

cAMP sensitivity of HCN-currents

The cAMP sensitivity of HCN-currents in the two thalamic nuclei were assessed using two well-established procedures (see e.g. (Kuisle et al., 2006)). First, we determined the activation curve with and without 8Br-cAMP (10 µM) in the patch-pipette (Fig. 3*A,B*). Tail current analysis revealed a positive shift of the half-maximal activation voltage (V_{half}) in both thalamic nuclei Cln (control V_{half} = -91.0 ± 2.9 mV, n = 6; 8Br-cAMP V_{half} = -71.3 ± 2.4 mV, n = 5, $p < 0.05$, Fig. 3*A,C*) and Vpm (V_{half} = - 82.4 ± 0.9 mV, n = 10 and V_{half} = -73.3 ± 0.9 mV, n = 6, $p < 0.05$, Fig. 3*B,D*). This shift in the voltage dependence reflects a strong modulation of thalamic I_h by exogenous cAMP, consistent with previous descriptions (Lüthi and McCormick, 1999b). Slope values were similar in Vpm and Cln (control Vpm 7.9 ± 0.4 mV and Cln 9.3 ± 0.7 mV $p > 0.05$; 8Br-cAMP Vpm 6.7 ± 0.3 mV and Cln 7.1 ± 0.5 mV $p > 0.05$). Second, we increased endogenous cAMP levels with the phosphodiesterase-inhibitor IBMX (100 µM). Bath-application of this compound augmented HCN-current amplitudes at -90 mV to 130.1 ± 6.6% of control in Cln (n = 7, Fig. 4*A,B*) and to

124.6 ± 4.5% of control in Vpm cells (n = 12, Fig. 4*C,D*).

Taken together, these results demonstrate that, in both thalamic nuclei, HCN-currents were expressed and highly sensitive to cAMP, which renders them well-suited to compare modulation of the currents by GPCRs, like the β-ARs.

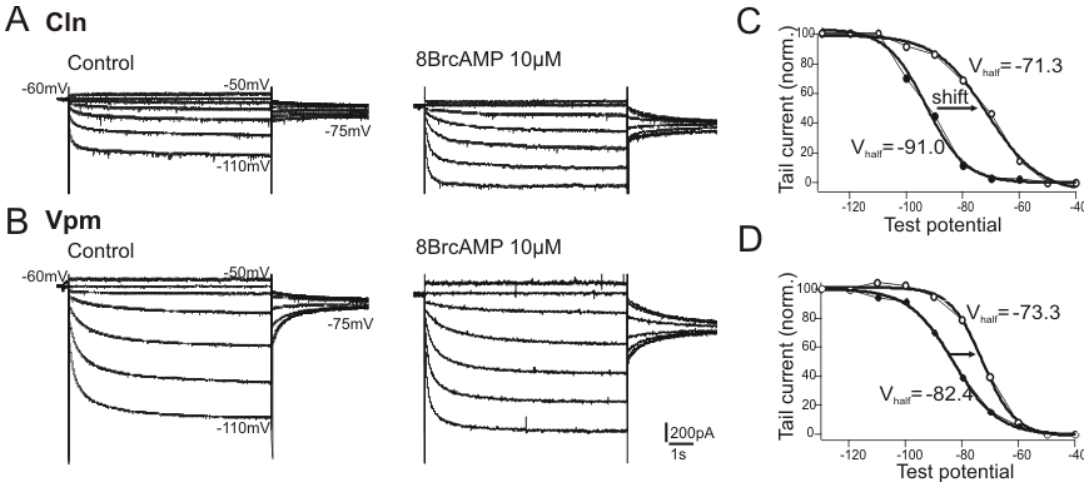

Figure 3. HCN channels show comparable cAMP sensitivity in Cln and Vpm. *A ,B*, Representative current responses of a Cln (*A*) and a Vpm (*B*) cell to increasing hyperpolarizing voltage steps as indicated at the end of each response, in control (left) and with 8Br-cAMP (10 µM, right) in the patch-pipette. *C, D*, Activation curves of representative cells, constructed from tail current analysis and normalized to the maximal current under control conditions. A positive shift of V_{half} was observed after application of 8Br-cAMP (10 µM, open circles) in both Cln (*C*) and Vpm (*D*).

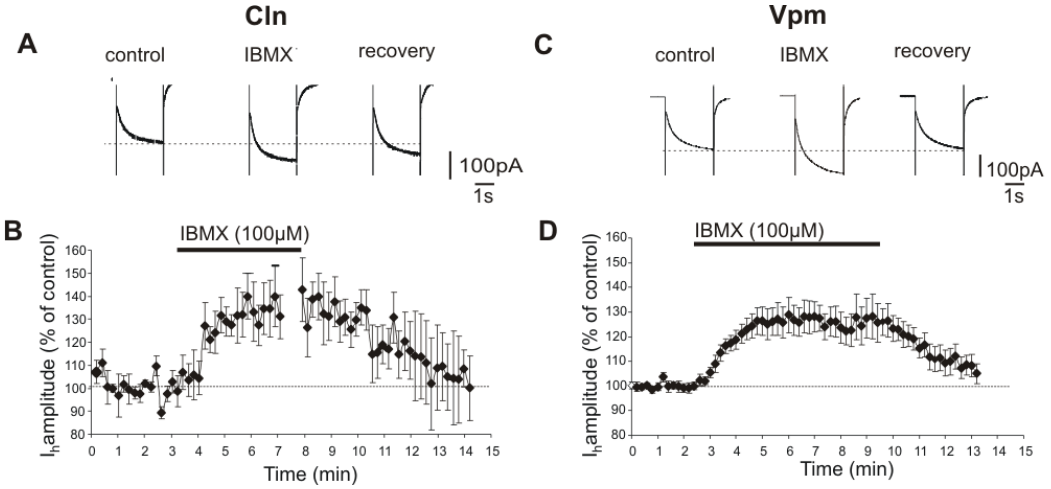

Figure 4. Pacemaker currents in Vpm and Cln neurons are upregulated by endogenously increased cAMP levels. *A*, Representative voltage-clamp recordings showing an increased HCN current amplitude at -90 mV in response to bath-application of the phosphodiesterase inhibitor IBMX (100 µM) in a Cln neuron. *B*, Average time course of normalized HCN-current amplitude during IBMX application in the Cln (n = 7). *C, D*, Corresponding data for the Vpm (n = 12).

β-adrenergic modulation of HCN-currents in Vpm and Cln

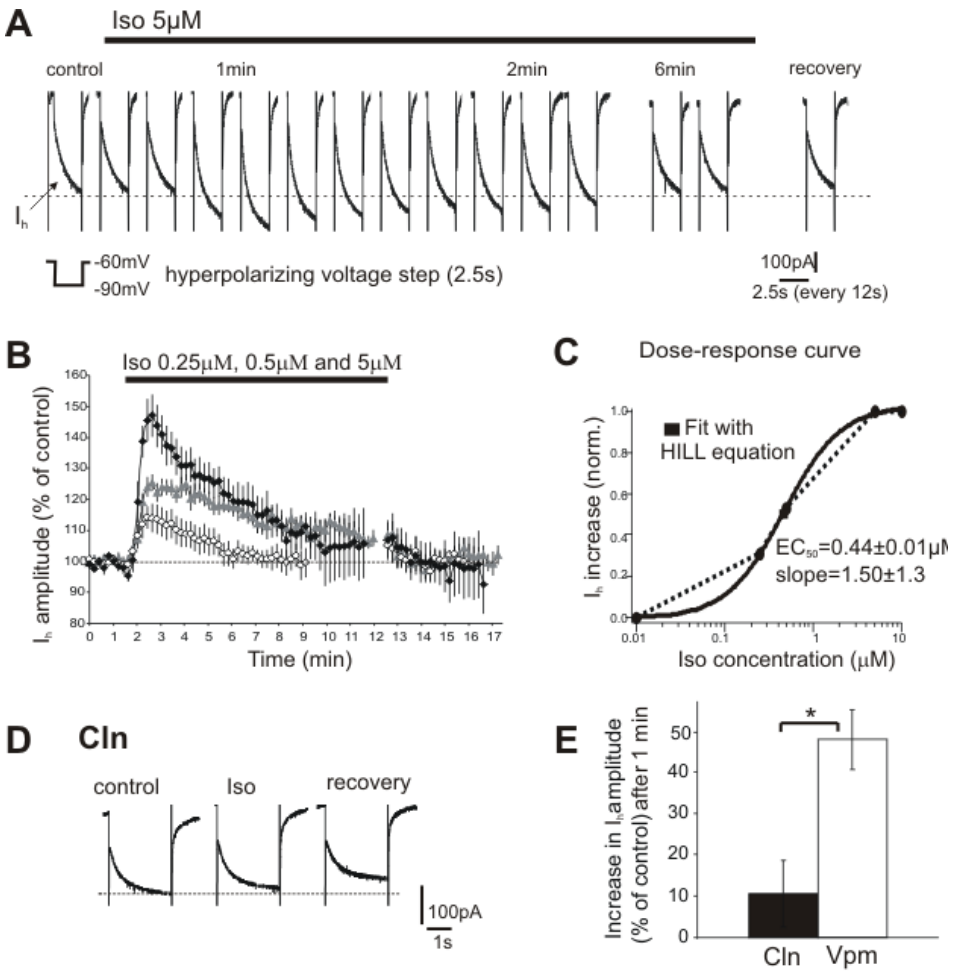

Figure 5. Stimulation of ß-adrenergic receptors in Vpm and Cln. *A*, Representative recordings during prolonged Iso application (5 µM) in the Vpm. Time points for which recordings are presented are shown above the traces. Currents were evoked with a 2.5 s lasting hyperpolarizing voltage-step as indicated below the first current trace. Dotted line denotes level of steady-state current amplitude before Iso application. *B*, Averaged time course of HCN-current upregulation and desensitization for Iso 0.25 µM (white circles), 0.5 µM (grey triangles) and 5 µM (black squares)(n = 8-9 for each). Iso produced a dose-dependent increase of the current amplitude with a maximal potentiation of 45 ± 6.7% after 1 min. Complete desensitization occurred at all concentrations without significant differences in time course ($p < 0.05$ to peak after 4 min for all concentrations). *C*, Dose-response curve for HCN-current increase after application of Iso measured at the peak of its effect (dotted line). Normalized current increases were fitted with the HILL-equation (thick line), yielding a regression coefficient of 1.5. *D*, A representative voltage-clamp recording of a Cln cell shows no change in the HCN-current amplitude at -90 mV with bath-application of Iso (5 µM). *E*, Histogram comparing the average increase of I_h amplitude after 1 min of Iso (5 µM) in neurons of the Cln (filled bar, n = 7) and the Vpm (open bar, n = 8, $p < 0.05$).

In agreement with previous reports (McCormick and Pape, 1990a; Pedarzani and Storm, 1995; Saitow and Konishi, 2000; Frère and Lüthi, 2004), bath-application of the non-selective β-adrenergic-agonist Iso (5 μM) induced an enhancement of I_h at -90 mV to 145.4 ± 6.7% of control in Vpm cells (n = 8; Fig. 5*A,B*). The increase in current amplitude reached a maximum after 1 min of application, but prolonged application of the agonist led to a desensitization of this potentation that occurred at all concentrations without significant differences in time course (τ_{decay} = 2.4 ± 0.6 min for 0.25 μM, τ_{decay} = 3.9 ± 0.7 min for 0.5 μM, τ_{decay} = 4.3 ± 1.3 min for 5 μM; p > 0.05). The HCN-current enhancement, measured at the peak of responses, showed a dose-dependence with an EC_{50} of 0.44 ± 0.02 μM (Fig. 5*B,C*).

In striking contrast to HCN-currents in first-order thalamic neurons, in the Cln HCN-currents did not show a significant increase in their amplitude upon bath application of Iso (5 μM). When measured at -90 mV, close to the half-maximal activation, current responses remained unaltered compared to control amplitude (110.5 ± 8.1%, n = 7, p > 0.05, Fig. 5*D*). Furthermore, Iso did not affect the holding current at -60 mV (18.7 ± 11.8 pA before and 17.3 ± 14.6 pA after 1 min Iso application, p > 0.5), and the input resistance (316 ± 43 MΩ before and 288 ± 40 MΩ after 1 min Iso application, p > 0.05, data not shown). These data strongly suggest that β-AR activation has minor functional consequences on currents active within the resting membrane voltage range in Cln neurons, in particular HCN-current.

To test how the different effects of Iso on HCN-currents correlated with the differential expression pattern of β-AR subtypes in the Cln and Vpm, we further investigated the modulation of I_h by applying selective β1- and β2-adrenergic agonists. Indeed, in the Vpm, where mainly β1-ARs are expressed, selective activation of these receptors using pharmacological strategies previously shown in studies using acute preparations (Skeberdis et al., 1997; Viard et al., 2001; Karle et al., 2002; Cesetti et al., 2003), induced current changes similar to those observed with Iso. First, bath-application of Iso (5 μM) in the continuous presence of the selective β2-antagonist ICI118,551 (0.1 μM) increased HCN-current amplitudes at -90 mV to 136.2 ± 10.4% (n = 10, p < 0.01 to control amplitude, Fig. 6*A*). Second, wash-in of the selective β1–agonist xamoterol (10 μM) augmented HCN-current amplitudes to 120.6 ± 6.3% (n = 7; p < 0.05 to control amplitude, data not shown). Conversely, selective stimulation of β2-ARs with salbutamol (80 μM) did not affect current amplitudes in the Vpm (105.1 ± 2.4%, n = 9, p > 0.05 to control amplitude; Fig. 6*B*). Therefore, the strong β-adrenergic modulation of HCN-currents in the Vpm seemed to be mediated predominantly, if not exclusively, by β1–ARs. Furthermore, we could not observe a

modulation of HCN-currents upon bath-application of salbutamol in the Cln (100.6 ± 4.7%, n = 5, p > 0.05; Fig. 6*D*). These data reveal a lack of I_h modulion by ß-ARs in the Cln.

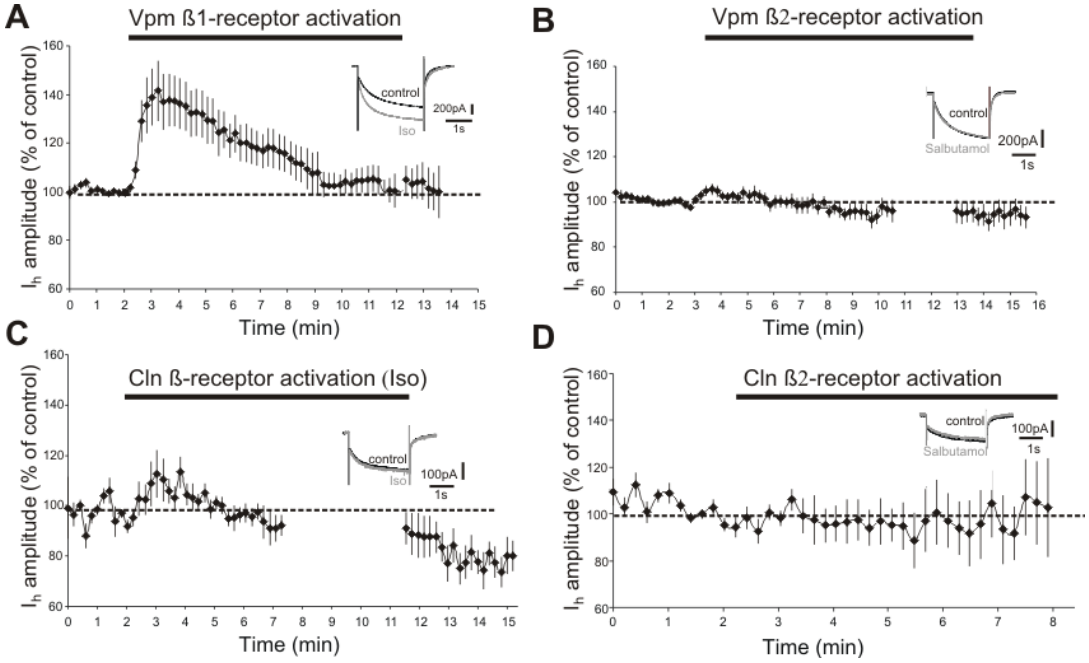

Figure 6. The ß-adrenergic response is mediated by ß1-ARs, while ß2-ARs show no coupling, neither in Vpm nor in Cln. *A*, Time course of averaged HCN current responses to Iso (5 µM) in the continuous presence of the ß2-adrenergic antagonist ICI118,551 (0.1 µM, n = 10, p > 0.05 for Iso with/without ICI118,551 after 1, 4 and 8 min) in the Vpm. *B*, Averaged current responses during application of the selective ß2-receptor agonist salbutamol (80µM) in the presence of the ß1-antagonist atenolol (10 µM, n = 9, p > 0.05) in the Vpm. *C, D*, Time course of averaged HCN-current responses in the Cln. Neither Iso application (*C*, n = 7) nor selective ß2-AR activation (*D*, n = 5) affected HCN-current amplitudes (p > 0.05). For A-D, insets show an overlay of I_h activated during a voltage step from −60 to −90 mV in control (black) and after 1 min application of the ß-adrenergic agonist (grey), respectively.

Mechanisms underlying the lack of β-adrenergic modulation of HCN-currents in the Cln

The lack of HCN-current regulation by ß2-AR activation could be caused for a number of reasons. First, β2-ARs, in spite of increasing cAMP levels, may simply not couple to HCN channels. In support of this possibility, a selective coupling of these receptor subtypes to ion channels in native cells has been described previously (Davare *et al.*, 2001; Balijepalli *et al.*, 2006). Second, β2-ARs may not form functional receptors at Cln neuronal membranes, but could be internalized in endocytic compartments. However, a quantitative autoradiography with selective antagonists for labeled ß-ARs suggests membrane-bound ß2-ARs in the Cln

(Rainbow et al., 1984). Third, β2-ARs may be functionally present, but could be in a desensitized state, as has been described previously (for review see (Kohout and Lefkowitz, 2003; Lefkowitz and Whalen, 2004)). Pronounced desensitization has been described in detail for ß2-ARs.

In a first step, we aimed at testing a possible receptor desensitization by adding the non-selective ß-adrenergic antagonist propranolol (10 µM) in all solutions used for preparing and storing slices. Before preparing brain slices, animals may be in a highly aroused state due to the exposure to novel environments (e.g. anesthesia chamber), which could lead to noradrenaline release in the brain (Berridge and Waterhouse, 2003). During brain slicing, mechanical stress of the tissue may also stimulate release from severed nerve endings. We reasoned that by adding propranolol during slicing, we would prevent β–AR activation at all stages from brain isolation throughout cutting and storing slices and hence minimize desensitization. Furthermore, this treatment should allow for resensitization of internalized ß-ARs (Yu et al., 1993; Gardner et al., 2006). Propranolol was washed-out in the recording chamber for at least 15 min prior to recordings (Bennett *et al.*, 1998). Under these conditions, bath-application of Iso (5 µM) evoked HCN-current increases that were similar to those found without propranolol incubation. HCN-currents increased up to 120.3 ± 6.1% in Vpm cells ($n = 5$, $p < 0.05$ to control amplitude, $p > 0.05$ for max. increase with/without propranolol incubation, Fig. 7*A*), confirming full wash-out of propranolol and recovery of β1-AR function. However, propranolol treatment failed to unravel an Iso effect on I_h in Cln neurons (106.0 ± 5.2%, $n = 4$, $p > 0.05$; Fig. 7*B*). We conclude that receptor desensitization induced by excessive noradrenalin is unlikely to play a major role in the distinct effects of Iso on Vpm and Cln.

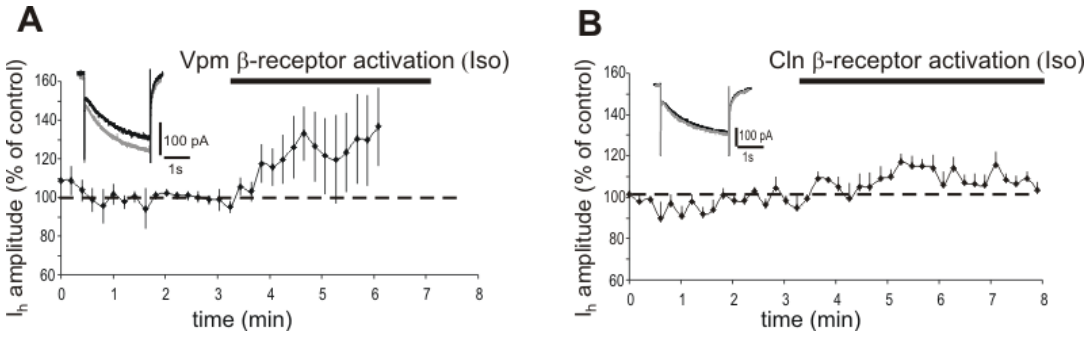

Figure 7. Stimulation of ß-adrenergic receptors in Vpm and Cln is unaltered after incubation with propranolol. *A, B*, Time course of averaged responses to Iso (5 µM) after incubation with propranolol (10 µM) shows current increases similar to those induced without incubation. While in the Vpm I_h amplitudes increased significantly ($n = 3$, *A*), there was no change in the Cln ($n = 4$, *B*)

We explored additional strategies to potentate GPCR function. High temperatures are known to decrease the affinity of Iso to β-ARs and to promote the low affinity, desensitized state of the receptor (Weiland *et al.*, 1979; Scarpace *et al.*, 1986). Thus, we tested the temperature dependence of the β-adrenergic responses in thalamic cells. Notably, in Vpm cells, we found that the response of I_h to Iso was strongly increased when the recordings were performed at room temperature (RT, 25°C) instead of at 35°C. At RT, a low concentration of Iso (250 nM) augmented I_h amplitudes at -90 mV to 157 ± 7.9 % of control (n = 7), which is a more than 4-fold enhancement compared to physiological temperature (113.9 ± 4.2 % of control, n = 9, $p < 0.05$; Fig. 8*A*). Additionally, at RT, current responses showed only a minor desensitization, but increases persisted as long as Iso was present. Under these conditions, we HCN-current modulation by Iso (5 µM) was clearly present in neurons of the Cln. I_h amplitudes at -90 mV were increased up to 121.8 ± 4.6% (n = 5, $p < 0.05$ to control amplitude, Fig. 8*B*), in contrast to the recordings at 35°C (see Fig. 4*D,E*). However, contrary to our expectation, this effect of Iso could not be mimicked by stimulation of ß2-ARs with salbutamol (80 µM), which did not affect HCN-currents (96.1 ± 4.4 %, n = 5, $p > 0.05$ to control; Fig. 8*D*). Instead, in the continuous presence of the β2-antagonist ICI 118,551, Iso induced current responses similar to those without this antagonist (120.9 ± 7.4%, n = 3, $p > 0.05$ with/without ß2-antagonist; Fig. 8*C*). Accordingly, the stimulation of ß1-ARs could increase I_h-amplitudes under low temperature conditions, which is characterized by the prominent lack of a slow form of receptor desensitization. These data show that temperature-dependent receptor desenstitization is prominent in our brain slices. Moreover, they establish ß1-ARs as the primary ß-AR subtype targeting HCN-currents in thalamic cells.

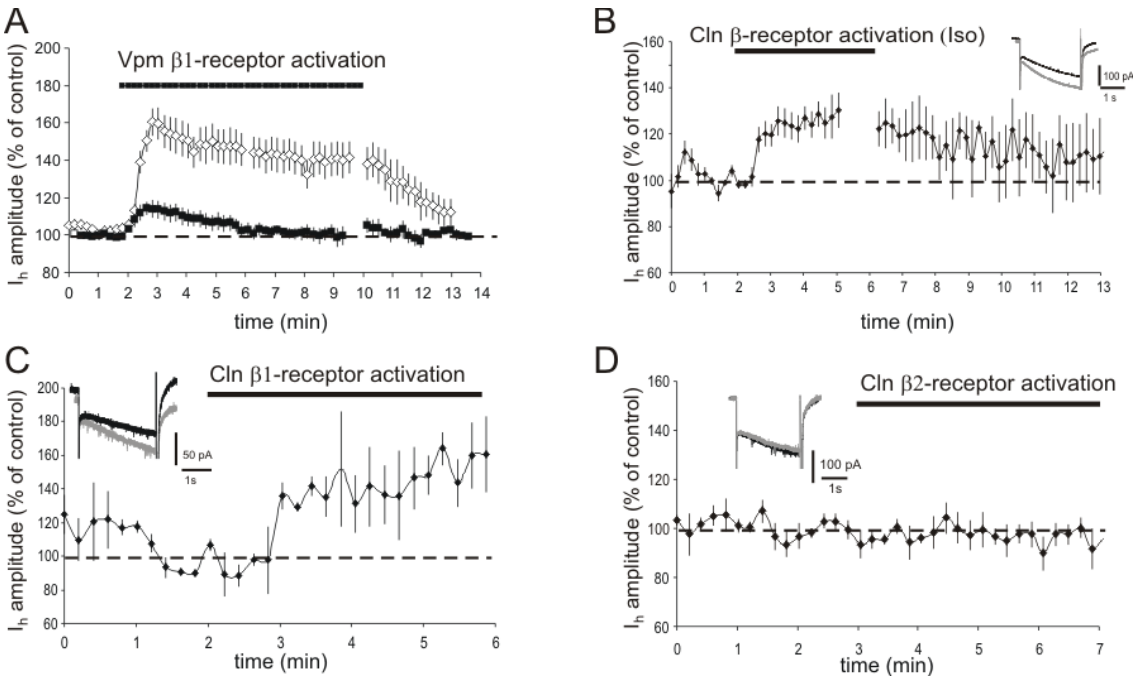

Figure 8. Stimulation of ß-adrenergic receptors in Vpm and Cln potentates HCN-currents at room temperature. *A*, Time course of averaged responses to a low concentration of Iso (250 nM) in the Vpm shows more pronounced HCN-current increases when recordings were performed at lower temperature (n = 7, white circles) in comparison to 35°C (n = 9, black squares *B,* In the Cln, HCN current increased after application of Iso (5 µM) at room temperature (n = 5). *C, D*, Selective ß1-AR stimulation with Iso in the presence of the ß2-antagonist ICI118,551 (100 µM) showed similar responses than Iso alone (n = 3, *C*), while selective ß2-AR activation with salbutamol (80 µM) did not change I_h amplitude (n = 5, *D*).

Further description of the β-adrenergic modulation of HCN currents and its desensitization in the Vpm

Desensitization of β1-AR has not been described in the thalamus before. We reasoned that a prerequisite for further understanding the distinct functional roles of β-ARs in thalamic nuclei was to understand the cellular mechanisms underlying slow desensitization. Since HCN-current potentation desensitizes with Iso, but not with IBMX (see Fig. 4*D*), desensitization must occur upstream of the cAMP-HCN channel interaction. This conclusion was further supported by the observation that a second application of Iso after a 10-min exposure within 10-25 min did not increase HCN-current amplitudes (98.3 ± 1.9%, n = 8, $p > 0.05$ to control amplitudes, data not shown), whereas the effects of IBMX were independent of prior Iso treatment (117.7 ± 4.3%, n=6, $p > 0.05$ before/after Iso application, data not shown). This indicates that recovery of Iso desensitization takes tens of minutes to start.

The selective degradation of cAMP seems to play a pivotal role in controlling not only precisely localized but also exactly timed cAMP signaling (for review see (Houslay and Adams, 2003; Baillie and Houslay, 2005). Therefore, we investigated the role of PDEs in β1-adrenergic desensitization by applying Iso (0.5 µM) in the continuous presence of the PDE-inhibitor IBMX (100 µM). Blocking PDEs completely prevented desensitization, and I_h amplitude still remained elevated to 136.4 ± 5.8% after 4 min of Iso application (n = 15, $p > 0.05$ to peak, Fig. 9*A,B*). To verify that this effect was really mediated by PDEs we applied Iso (5 µM) in the continuous presence of a low concentration (0.1 µM) of 8Br-cAMP. In this manner we rule out that increased cAMP levels (after blockade of its degradation with IBMX) per se interfere with desensitization. HCN-current increases were smaller than without 8Br-cAMP (105.2 ± 1.7%, n=10, $p<0.05$ to control amplitude, Fig. 9*C,D*) probably due to saturating effects. However, we saw a clear desensitization of the answer (98.3 ± 2.7% after 4 min Iso application, n = 7, $p > 0.5$ to control amplitude, τ_{decay} = 4 min, Fig. 9*C,D*), similar to responses without 8Br-cAMP ($p > 0.05$). Thus, we demonstrated a desensitization occurring independent of increased cAMP levels and depending on PDE activity that was likely recruited when receptor stimulation was initiated.

Short term regulation of PDE4, a PDE subtype recruited during ß-AR desensitization, is known to involve proteinkinase A (PKA)-dependent phosphorylation (for review see (Houslay and Adams, 2003). Thus, we tested the role of PKA in ß1-AR desensitization in the Vpm. Slices were incubated with the PKA-inhibitor H-89 (1 µM), which was shown previously to attenuate PKA activation effectively (Lüthi and McCormick, 1999b). Contrary to our expectations, desensitization was not prevented in these experiments. The HCN-current increases after 1 min of Iso-application were rather diminished when PKA was inhibited (120.6 ± 4.7%, n= 11, $p < 0.05$ to control amplitude, Fig. 9*E,F*), but declined to control values after 4 min (107.4 ± 3.9%, n = 11, $p > 0.05$ to control amplitude) with a τ_{decay} of 3 min, similar to that without PKA-inhibition ($p > 0.05$). Comparable results were obtained when slices were incubated with another PKA-inhibitor KT5720 (1 µM). I_h-increased to 118.1 ± 9.6% after 1 min Iso application (n = 6) and desensitized back to 96.5 ± 11.6% after 4 min (data not shown).

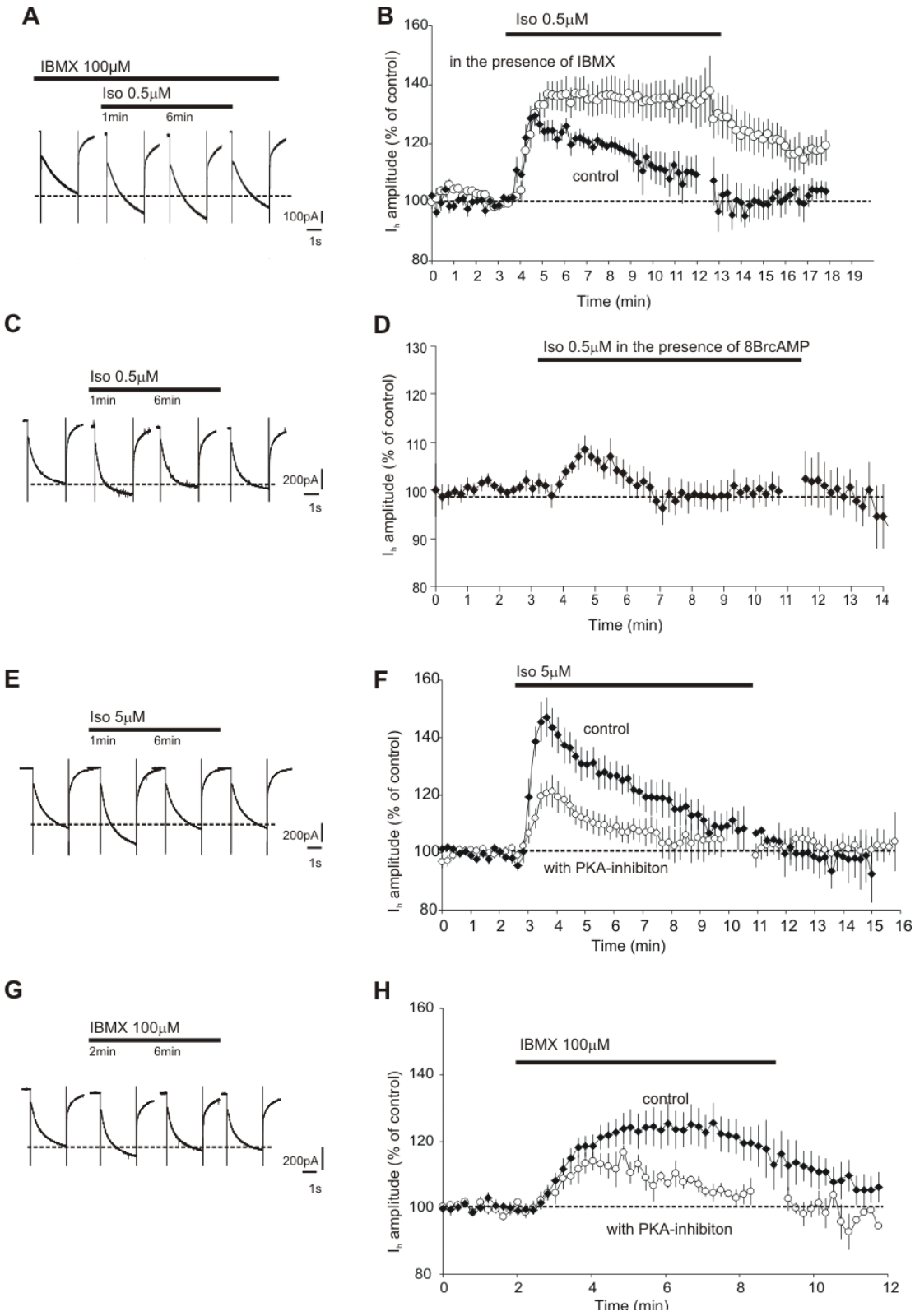

Figure 9. Desensitization of the ß-adrenergic receptors in the Vpm is dependent on the recruitment of phosphodiesterases and independent of PKA. *A*, Representative current recordings of I_h activated with a voltage step from -60 mV to -90 mV. Application of Iso (0.5 µM) in the continuous presence of IBMX (100 µM), showing a HCN-current increase that persisted as long as the drug was present. Current traces represent conditions before, during short (1 min), during prolonged (6 min) and after Iso application. *B*, Time course of averaged data shows that the presence of IBMX prevented ß-adrenergic desensitization ($p > 0.05$ to

peak after 8 min). *C, D*, Representative recordings (*C*) and average time course (n = 10, *D*) of current responses after Iso (0.5 µM) application in the presence of a low concentration of 8Br-cAMP (0.1 µM) in the patch-pipette. *E, F*, Desensitization did not depend on PKA, as it was still apparent when PKA was inhibited after incubation with H89. Representative recordings in E, and average time course in F, n = 11. *G, H*, PKA inhibition changed I_h responses upon IBMX application (100 µM). Current increases were significantly smaller and not persistent as long as IBMX was present. Representative recordings in *G*, and average time course in *H* (n = 10).

Although incubation of slices with PKA-inhibitors is widely used (Pedarzani and Storm, 1995; Lüthi and McCormick, 1999b; Mellor et al., 2002), we wanted to exclude that we did not induce other basic changes in the recorded cells. HCN-current amplitudes were unchanged after slice incubation with H-89 (-390 ± 37 pA in control, n = 25, -459 ± 41 pA with H-89, n = 17, $p > 0.05$; data not shown). Also the cAMP sensitivity was preserved as we tested by flash-photolytic release of caged cAMP (HCN-current increase to 119.2 ± 0.6% in control, n = 8, 121.7 ± 4.2% in H-89, n = 10; $p > 0.05$; data not shown). HCN-current responses to IBMX application were significantly reduced with H-89 PKA-inhibition. I_h increased only to 110.3 ± 3.1% (n = 10, Fig. 9*G,H*) on its maximum after 2 min, significantly less than without H-89 (121.6 ± 3.3%, n = 10, $p < 0.05$; Fig. 4*D*), proving the efficacy of PKA-inhibition.

Discussion

Using *in vitro* electrophysiological recording techniques, we describe β-AR modulation of HCN channels in first-order and intralaminar thalamic nuclei. The main result of this work revealed a non-uniform HCN channel regulation in the thalamus. In the Vpm, a first-order thalamic nucleus, a strong coupling of HCN channels was mediated by β1-ARs that desensitized within minutes. This desensitization occurred independently of PKA activity, but seemed to involve the recruitment of PDEs. In contrast, in the Cln, an intralaminar thalamic nucleus stimulation of β-ARs did not change HCN-current amplitudes.

Overall, these findings indicate that the functional subdivision of the thalamus is accompanied by a subdivision with respect to one of the most important ascending arousal system. As such, our work could have implications for the role of different thalamic nuclei in the control of sleep and attentive states.

Firing properties and HCN-currents

Firing properties of thalamic neurons in Cln and Vpm were similar to those reported elsewhere, in particular the capability to discharge in both tonic and burst firing modes (for Cln see (Brunton and Charpak, 1998; Bayer et al., 2002; Goaillard and Vincent, 2002; Govindaiah and Cox, 2006), for Vpm see (Sherman and Guillery, 2005)). Neurons of the Cln showed a greater action potential discharge frequency to depolarizing stimuli compared to Vpm neurons, but a delayed rebound excitation after hyperpolarization. Although neuronal discharge properties are typically determined by a combination of intrinsic properties and geometric characteristics, the differences between Cln and Vpm excitability can be most parsimoniously explained by the different I_h amplitudes. HCN channels give rise to a depolarizing current upon hyperpolarization that can be seen as the so called “sag” in the current-clamp protocol. This current depolarizes a cell back to potentials necessary to activate low-threshold Ca^{2+} channels generating a rebound Ca^{2+} spike (Robinson and Siegelbaum, 2003). Reduced I_h, and a concomitant weakening of this depolarizing influence, could thus explain the delayed rebound excitation after hyperpolarization.

Recently, the contribution of I_h to the resting membrane potential of first-order thalamocortical neurons was determined (Ludwig *et al.*, 2003; Meuth *et al.*, 2006). By closing upon depolarization, thus reducing a tonic inward current, HCN channels could counterbalance excitation analogous to what is described for temporal integration of synaptic

input in the hippocampus (Magee, 1998). In this manner, the larger I_h amplitudes in first-order thalamic neurons could dampen excitable inputs, explaining reduced tonic action potential firing frequency in these neurons.

Molecular expression patterns of HCN subunits

HCN channels are composed of four different subunits HCN1 to 4. The immunohistochemical localization study by (Notomi and Shigemoto, 2004) shows mainly HCN2 and HCN4 subunits in both Vpm and Cln. However, absolute expression levels are weaker in the Cln than in the Vpm (Notomi and Shigemoto, 2004)**.** The different magnitudes of I_h amplitudes are in good agreement with this molecular expression pattern of HCN channel subunits, suggesting a proportionality between available protein and functional current. This correlation between molecular and functional expression patterns is in line with repeated observations that protein and current levels show a strong correlation in the HCN channel family (see e.g. (Santoro *et al.*, 2000)). Furthermore, the similar cAMP sensitivity of HCN channels in the two distinct parts of the thalamus is also consistent with the HCN channel expression pattern. HCN2 and HCN4 subunits are known to be the most cAMP sensitive isoforms (for review, see (Frère *et al.*, 2004). Thus, the predominance of these two isoforms, although to a weaker extend in the Cln, explains the high sensitivity to cAMP in both parts of the thalamus.

Coupling of ß-adrenergic receptors to HCN channels in first-order nuclei

A principal property of thalamic neurons is to show dual action potential discharge modes. Depending on their membrane potential, these neurons fire in rhythmic burst discharges or, with depolarization, display tonic single spike activity (for review, see (McCormick and Bal, 1997). During sleep, thalamocortical neurons are hyperpolarized and the main discharge pattern consists in action potential bursting. Conversely, during waking or rapid-eye-movement sleep (REM sleep), the membrane potential is depolarized, supporting the tonic firing mode (Hirsch *et al.*, 1983). Thus thalamic activity is correlated with the arousal state. Interestingly, the maintenance of arousal is in turn connected to increased activity of ascending brainstem fibers, in particular of the locus coeruleus (Aston-Jones and Bloom, 1981). The release of neurotransmitters from the brainstem terminals can modulate the firing properties. Amongst other neurotransmitters (acetylcholine, serotonin), noradrenalin plays a pivotal role in setting the resting membrane potentials in thalamocortical neurons. One

important action is the activation of ß-ARs, via which cAMP is produced and the activation range of HCN channels is shifted to more positive potentials. Upregulated I_h produces a greater standing current around resting membrane potentials and induces a depolarization of thalamic first-order neurons (McCormick and Pape, 1990a). We now show that this effect is mediated by the ß1-AR subtype. Selective β1-AR activation produced similar responses than Iso, while the β2-AR agonist salbutamol failed to modulate HCN-currents. This finding is in agreement with expression studies showing a predominantly ß1-AR expression in the Vpm (Rainbow *et al.*, 1984; Nicholas *et al.*, 1993). Notably, a recent *in vivo*-electrophysiological study investigated the possible mechanism of ß-blockers in migraine. Only ß1-, not ß2-AR antagonists could inhibit activity in the Vpm in response to stimulation of the superior sagittal sinus, a known pain-producing structure. The authors suggest the inhibitory action of ß1-blockers could be mediated by reduced HCN-currents (Shields and Goadsby, 2005). Thus, it would be interesting to examine whether also HCN cannel antagonists could be possible candidates in therapy of migraine.

Coupling of ß-adrenergic receptors to HCN channels in intralaminar nuclei

Intralaminar thalamic nuclei that are considered to be important for general arousal function also receive noradrenergic input (Krout *et al.*, 2002). The ß-adrenergic subtype expression pattern in the Cln is different than in the Vpm, showing mainly ß2-ARs (Rainbow *et al.*, 1984; Nicholas *et al.*, 1993) that are known to tightly regulate ion channels (Davare *et al.*, 2001; Lavine *et al.*, 2002; Jurevicius *et al.*, 2003). However, so far little is known about neurotransmitter mediated regulation of ionic currents by these receptors in thalamic neurons. Surprisingly, the non-specific ß-adrenergic agonist Iso did not change I_h amplitudes when experiments were performed at physiological temperatures, nor could we observe any coupling of HCN channels to ß2-ARs in the Cln. Currently, there are multiple explanations for this result, and further experimental tests are required to distinguish between these.

a) ß2-ARs are known to be not only coupled to stimulatory G_s-proteins, but also to PTX-sensitive inhibitory G_i–proteins (Daaka et al., 1997). This uncoupling of β2-ARs from G_s- to G_i-proteins could prevent HCN channel activation. A recent study in cardiac ventricular myocytes has shown that specific ß1-AR stimulation causes a larger positive shift of the I_h activation curve than specific activation of ß2-ARs. Although this difference could be attenuated by incubation with PTX, ß2-AR stimulation never reached the amplitude of ß1-AR activation (Cerbai et al., 1999a). Thus it is tempting to speculate that the increase in average

cAMP levels produced by ß2-AR stimulation is smaller than that of ß1-AR stimulation.

b) A decreased average cAMP synthesis could be consistent with a greater subcellular localization of cAMP signals produced by ß2-ARs. Indeed, in PTX-treated cardiac myocytes a ß2-adrenergic effect on L-type Ca^{2+} channels could be eliminated by disruption of caveolar lipid rafts (Balijepalli *et al.*, 2006). Also HCN4 channels were shown to be localized in lipid rafts (Barbuti *et al.*, 2004). In cardiac sinoatrial myocytes a specific ß2-adrenergic effect on HCN channels was lost by causing a disorganization of lipid rafts (Barbuti *et al.*, 2007). Not only in the heart, but also in the brain a tight co-localization of ß2-ARs and ion channels was demonstrated. A macromolecular complex localized to dendritic spines of CA1 pyramidal neurons was described recently, which provides a direct functional coupling between ß-ARs and L-type Ca^{2+} channels (Davare *et al.*, 2001). Selective activation of ß2-ARs resulted in a preferential enhancement of spinuous compared to dendritic Ca^{2+} transients (Hoogland 2004). The distinct localization of ß2-ARs with respect to HCN channels could prevent an effective functional coupling of receptor to channel. This would mean that ß2-ARs activate membrane delimited pathways, in contrast to ß1-AR stimulation, which is diffusive and involves more distal effectors.

Interestingly, also activation of other GPCRs, such as orexinergic-receptors failed to alter I_h in the Cln (Govindaiah and Cox, 2006). This further emphasizes a tight regulation of receptor- and ion channel activation in neurons, but still leaves the question about role and function of modulation of HCN channels in intralaminar thalamic nuclei.

c) Given ample evidence for β2-AR desensitiziation, we made every attempt to reduce ß2-AR desensitization during our experimental procedure. This could arise from increased release of noradrenalin due to arousal and stress before cutting, or due to constitutive release of noradrenalin during the incubation period. Therefore, we cut and incubated the slices with solutions supplemented with the ß-AR antagonist propranolol. However, contrary to our concerns, propranolol incubation for at least 1.5 hours did not apparently alter HCN-current responses to Iso-application, neither in the Vpm nor in the Cln. The incubation time should be sufficient for ß-AR resensitization, as the time course for resensitization was reported to be in the order of minutes ($t_{1/2}$~3min for ß2-ARs in CHO (Yu *et al.*, 1993), $t_{1/2}$~15min for ß1-ARs in HEK293 (Gardner *et al.*, 2006)). We conclude that already before cutting, HCN channels are largely uncoupled from putative ß2-AR signaling in the Cln.

With these experiments, a number of additional events limiting β2-AR function cannot be avoided. Thus, we cannot rule out receptor down-regulation, as in the latter there is an actual decrease in the total cellular receptor number and resensitization cannot take place

within minutes. However, down-regulation is only apparent after prolonged (>1 hour) exposure to the agonist (Bohm *et al.*, 1997) and thus is unlikely to be caused by our experimental procedure. There could be physiological receptor down-regulation, due to the prolonged noradrenergic input the thalamus receives during waking (Aston-Jones and Bloom, 1981; Berridge and Waterhouse, 2003). Inhibition of this noradrenergic input (e.g. via prolonged application of ß-blockers to the living animal or via lesions in the noradrenergic system) could give an idea of receptor down-regulation *in vivo*.

Desensitization of ß1-ARs in first-order thalamic neurons

In addition to G-protein coupling, activation of a GPCR by its ligand also initiates the process of desensitization, preventing potentially harmful effects that could result from persistent receptor activation. Locus coeruleus neurons fire tonically during waking (Aston-Jones and Bloom, 1981). Thus, we wanted to verify, whether this sustained noradrenergic input would lead to desensitization of β-ARs in thalamocortical neurons. By applying ß-adrenergic agonists for prolonged periods of time (10 min) we followed the coupling of ß-ARs to HCN channels. In the Vpm, we found a desensitization mediated by ß1-ARs that occurred at all concentrations with a τ_{decay} around 3 min. Interestingly the desensitization involved a temperature-dependent step, as it was not apparent when recordings were performed at lower temperatures. Additionally, HCN-current increases with Iso were much more pronounced under these conditions and made an enhancement of HCN-currents mediated by ß1-ARs possible also in the Cln. Thus, at the more physiological temperature of 35°C a temperature-dependent step seemed to limit the ß1-adrenergic effect on HCN-currents in the Cln, at least to an extent that we cannot measure any increases in I_h amplitude.

The mechanism of phosphorylation, desensitization and internalization of GPCR have been well described in heterologously expressed systems and in cardiac myocytes (for review, see (Kohout and Lefkowitz, 2003)). However, still little is known about the physiological conditions leading to desensitization in neurons. Thus, to further characterize the mechanism mediating the desensitization of these receptors in the Vpm, we first concentrated on the role of PDEs. PDEs are the sole means of degrading cAMP and play a pivotal role in shaping and controlling intracellular cAMP gradients ((Baillie *et al.*, 2003; Jurevicius *et al.*, 2003), for review, see (Houslay and Adams, 2003; Baillie and Houslay, 2005)). PDEs can be recruited to β-ARs by interaction with β-arrestins upon receptor activation (Perry *et al.*, 2002), thus bringing the cAMP-degrading enzymes close to the point of cAMP production in an agonist-

dependent manner (for review, see (Baillie and Houslay, 2005)). Consequently, the selective degradation of cAMP seems to play an important role in controlling not only precise localized but also exactly timed cAMP signaling (Houslay and Adams, 2003; Baillie and Houslay, 2005). Indeed, we could prevent desensitization with the non-selective PDE-blocker IBMX in our experiments, indicating that desenstitization of ß1-ARs in first-order thalamic neurons involves the recruitment of PDEs.

PKA is known to be implicated in PDE regulation (for review, see (Houslay and Adams, 2003). For example, in cardiac myocytes PKA activation of PDE negatively regulated cAMP increases triggered by ß-ARs (Rochais *et al.*, 2004). Therefore, we investigated the role of PKA in ß1-adrenergic desensitization in the Vpm. The prolonged desensitized state of β1-ARs suggests an additional mechanism to increased PDE recruitment, especially because IBMX application before and after desensitization induced similar HCN-current responses. However, our experiments revealed a β-adrenergic desensitization independent of PKA-activity that occurred still after inhibition of PKAs. Thus, PDEs do not necessarily need to be activated by PKA to induce desensitization, similar to what is described for β2-ARs mediated PDE recruitment in frog ventricular myocytes (Jurevicius *et al.*, 2003).

Moreover a recently described switch of G_s to G_i coupling mediated by PKA (Daaka et al., 1997; Martin et al., 2004) seems not to be the reason for β-AR desensitization in the Vpm. Also the so called *heterologous* or non-agonist–specific desensitization that is mediated by PKA and that was described besides the *homologous* desensitization mediated by G-protein receptor kinases (GRKs) (for review, see (Gainetdinov *et al.*, 2004)) seems not to apply for the desensitization we observe here. Although PKA phosphorylation contributes to β1-AR desensitization described in heterologously systems (Rapacciuolo *et al.*, 2003), it appears not to be involved in β1-adrenergic desensitization in the Vpm.

Altogether, for the first time, we describe a desensitization of ß1-ARs in thalamocortical neurons *in vitro*, the time course of which can be followed by the coupling of ß-ARs to HCN channels in the Vpm. HCN channels were not modulated upon activation of ß2-ARs, neither in the Vpm, nor in the mainly ß2-ARs expressing cells of the Cln.

Functional implications of differential HCN channel regulation

Although HCN channels showed similar cAMP sensitivity, strong ß-AR modulation appeared to be pronounced in portions of the thalamus involved in sensory relay, but may not be involved in general arousal functions. Interestingly, synaptically evoked bursts are

characteristic for the hyperpolarized state of thalamic first-order neurons during sleep (Weyand *et al.*, 2001), but were observed during wakefulness as well in cells of the Cln (Glenn and Steriade, 1982). This suggests that the ascending neurotransmitter systems active during waking are modulating ion channels not sufficiently enough to depolarize the membrane potential to leave the burst firing mode in the Cln. In contrast to other thalamic regions, intralaminar nuclei are also innervated by additional arousal related regions, such as the hypothalamus, and a depolarizing action of wake-related hormone orexin was found only in this part of the thalamus (Govindaiah and Cox, 2006). Thus, a specific regulation of ion channels seems to provide a cellular mechanism to organize the activity of different thalamic nuclei in a manner that they can fulfill their specific function in the control of sleep and wakefulness.

6 GENERAL DISCUSSION

The results of my thesis have substantiated the sensitivity of neuronal networks and neurons to imbalances of pacemaker channel regulation. In this respect it could be shown that the pathogenesis of absence epilepsy, a disease characterized by abnormal neuronal activity, is accompanied by an altered modulation of HCN channels. In the thalamus of a rat model of absence epilepsy (GAERS), a decreased responsiveness to cAMP was associated with a changed expression pattern of HCN channel subunits. These deficits were, however, compensated by a mechanism that stabilized HCN channel function in the adult, epileptic animal. Thus, pacemaker channels seem to play a dual role in absence epilepsy: on the one hand abnormal HCN channel regulation is involved in epileptogenesis, while it on the other hand appears to trigger adaptive changes to restore HCN channel function in the thalamus.

The second part of the thesis shows a cell-type specific pattern of cAMP dependent regulation of HCN channels. Throughout the thalamus, pacemaker channels were non-uniform modulated by ß-adrenergic receptors (ß-ARs). A tight coupling of ß1-ARs in first-order thalamic neurons was contrasted by a lack of ß2-AR modulation. ß2-ARs are highly expressed in intralaminar thalamic nuclei (Rainbow et al., 1984; Nicholas et al., 1993) where activation of ß-ARs did not modulate HCN-currents. This specific coupling of GPCRs to HCN channels suggests a compartmentalized and local cAMP signaling in thalamic neurons. Moreover, as the ß-ARs mediate the action of noradrenalin, the neurotransmitter of one of the most important ascending arousal systems, the differential regulation of HCN channels could contribute to the different roles of thalamic nuclei in the control of sleep and arousal.

In this chapter some of these major findings are discussed in more detail. They are presented in a more general context and described in respect to the most obvious functional implications. Finally, I will also reason about this work in the light of GPCR- and cAMP-signaling. I used HCN channels to uncover a subtype specific β-AR signaling in different thalamic cells, making a contribution to a more detailed understanding of neuronal β-AR function. Moreover, based on these results, I established HCN channels as cAMP on-line sensors for GPCR-signaling in a heterologous expression system.

6.1 Discussion of paper I:

My work about "Functional stabilization of weakened thalamic pacemaker channel regulation in rat absence epilepsy" contributes to the debate about the role of HCN channels in epilepsies with respect to a) the thalamic or cortical focus theory in absence epilepsy after onset of seizure activity and b) modulated ion channels as possible epilepsy-promoting factors.

6.1.1 Cortical focus-theory in absence epilepsy

It is well known that SWDs require the bi-lateral network activity of reciprocally connected thalamic and cortical structures. However, the site of initiation of the hypersynchronized activity is still unclear (Blumenfeld, 2005; Meeren *et al.*, 2005). This controversy over the components of the thalamocortical circuits required for the generation of the seizures get a fundamental issue regarding treatment of absence epilepsy. Thus, pharmacotherapy should target the appropriate epileptogenic region. Initially, the thalamus was considered to be the centre for the generation of absence seizures. In an early study, SWDs were found to start first in the thalamus (Williams, 1953). In addition, electrical stimulation of the thalamus in cats produced bilaterally synchronous EEG discharges that resembled the classic absence pattern (Jasper and Droogleever-Fortuyn, 1947). In contrast, recent recordings at high temporal resolution of cortical and thalamic activity during SWDs have found that the first ~0.5 sec are dominated by cortical activity (Seidenbecher *et al.*, 1998; Meeren *et al.*, 2002; Pinault, 2003; Pinault *et al.*, 2006). In our study, we reveal that mature thalamic cells developed a compensatory mechanism that restored HCN channel function necessary to terminate SWD-like activity. In cortical cells, changed HCN channel expression does not seem to trigger adaptive responses (Strauss *et al.*, 2004; Kole *et al.*, 2007), suggesting a role of cortical HCN channel dysfunction in the maintenance of SWDs in adult epileptic animals. Thus, with our results, we further push the discussion in the direction of the cortical focus-theory. In the cortex the loss of HCN1 channels could not be compensated, while in the thalamus cells overcame HCN channel dysfunction.

6.1.2 Role of HCN channels in epileptogenesis

Interestingly, the most direct evidence for a role of decreased HCN-current in absence

epilepsy exists for the thalamus (Ludwig *et al.*, 2003), and not for the cortex (Nolan *et al.*, 2004). Thus, animals deficient in thalamic HCN2 channel subunits do show generalized absence seizures (Ludwig *et al.*, 2003), whereas animals lacking the mainly cortical expressed HCN1 subunit do not (Nolan *et al.*, 2004). This suggests that deficits in thalamic cells may be important for the generation of epileptogenesis.

Epileptogenesis is referred to be the process by which the brain is altered in a manner that there is a propensity for recurrent spontaneous seizures. To understand the cellular and molecular mechanism involved in the progression of the epileptic disease is especially important, as currently available antiepileptic drugs do not seem to be antiepileptogenic, rather being used for symptomatical treatment. We found that epileptogenesis in the GAERS was accompanied by a changed cAMP-dependent modulation of HCN channels in the thalamus. Not only seem HCN channels to be sensitive to aberrant neuronal activity as it was reported previously (Chen *et al.*, 2001b; Shah *et al.*, 2004; van Welie *et al.*, 2004; Brewster *et al.*, 2005), but also can relatively small changes in HCN channel regulation have an influence on neuronal network activity (Di Pasquale *et al.*, 1997; Budde *et al.*, 2005). In future studies, it would be interesting to test, whether restored HCN channel function during the pre-epileptic phase could prevent seizure onset in the GAERS. The precise mechanism involved in changing HCN channel expression still remains unclear. A feasible way for its investigation could be the use of substances that upregulate HCN channels like lamotrigine (Poolos *et al.*, 2002) see chapter 3.5.4 of this thesis). Interestingly, lamotrigine is already licensed for the treatment of absence seizures in children. It is tempting to speculate that substances targeting HCN channels provide a promising approach in the search for new antiepileptic drugs. In particular, because these substances might give an opportunity to obtain therapeutic targets that could counteract the development of epilepsy.

6.2 Discussion of paper II:

The second part of my thesis about “ß-adrenergic modulation of pacemaker currents in different thalamic nuclei” not only shows a non-uniform regulation of an ion channel within the thalamus, but also might this non-uniform regulation have an effect on the distinct roles of thalamic nuclei in controlling arousal states. Furthermore, by following the coupling of ß-ARs to HCN channels we could assess ß-AR signaling in thalamic cells. For the first time we describe a desensitization of ß1-ARs in thalamic cells. The missing coupling of ß2-ARs to HCN channels suggests a tight organization of signaling pathways in these cells.

6.2.1 Differential role of thalamic nuclei in control of sleep and waking

The different roles of thalamic nuclei in the control of sleep and wakefulness were reflected in a differential regulation of HCN channels by one of the most important arousal systems. Such, our work could have implications for how we see the role of the thalamus in the control of sleep and attentive states. It contributes to understand the cellular mechanisms that help to organize the activity of different thalamic nuclei necessary to maintain the diverse arousal states.

The transition from sleep to the waking state is associated with depolarization of first-order thalamocortical neurons and a conversion from rhythmic burst firing to the tonic mode of action potential generation (for review, see (McCormick and Bal, 1997)). Here, the regulation of HCN channels by β-adrenergic receptors (ß-ARs) plays a pivotal role (McCormick and Pape, 1990a). The results of this thesis revealed that this regulation is mediated by the ß1-AR subtype. Immediate stimulation of ß1-ARs activated HCN-currents, while prolonged exposure of ß1-adrenergic agonists induced receptor desensitization. Possibly, this desensitization could also occur *in vivo* when thalamic cells receive prolonged noradrenergic input during waking (Aston-Jones and Bloom, 1981). The sustained depolarization of the resting membrane potential of thalamic cells could be provided by the reduction of a resting leak K^+ conductance that is also induced by neurotransmitters released from ascending systems (for review, see (McCormick, 1992; McCormick and Bal, 1997)).

Neurons of intralaminar thalamic nuclei can exert tonic or burst firing properties like first-order neurons. However, neurons of the intralaminar nuclei discharge in bursts not only during sleep, but also during waking (Glenn and Steriade, 1982; Steriade et al., 1993a), contrary to neurons of first-order thalamic nuclei. Thus, the resting membrane potential seems to be influenced by a different regulation of ion channels. Accordingly, HCN channel expression is weaker (Notomi and Shigemoto, 2004), and we found smaller HCN-currents that were not coupled to ß-ARs in centrolateral thalamic neurons. This suggests that the increased activity of the locus coeruleus during waking (Aston-Jones and Bloom, 1981) cannot activate HCN-currents and induce depolarization via the ß2-AR-pathway in neurons of intralaminar thalamic nuclei.

6.2.2 ß-adrenergic receptors and compartmentalized cAMP signaling

With our experiments, we show a strong coupling of β1-ARs to HCN channels in first-

order thalamic neurons. In neurons of the intralaminar thalamic nuclei, β1-adrenergic affinity to its agonist Iso had to be increased to see β-adrenergic regulation of HCN channels, probably because of the lower expression density of these receptors (Rainbow *et al.*, 1984). However, this is still leaving the question, what the function of β2-ARs could be in intralaminar thalamic neurons. We could neither observe a change in the resting membrane potential nor was there an alteration of the input resistance of these cells after application of the β-adrenergic agonist. The intrinsic agonist and neurotransmitter NA plays an important role in the control of cAMP-dependent gene expression during states of arousal in the thalamocortical system (Cirelli et al., 1996; Cirelli and Tononi, 2000). These effects cannot be monitored with our electrophysiological approach. Another possibility would be that ACs in intralaminar thalamic nuclei need the coincident activation of different GPCRs to sufficiently increase cAMP levels to activate HCN channels. A complex interaction of β-ARs with other GPCRs was already shown for neurons in other parts of the brain (Andrade, 1993; Pedarzani and Storm, 1996; Frère and Lüthi, 2004). The intralaminar thalamic nuclei are known to be innervated by different ascending arousal systems (Krout *et al.*, 2002). Thus, it could be possible that a synergistic activation of different GPCRs is necessary to activate ACs sufficiently enough to see a change in HCN channel gating.

However, the fact that activation of β2-ARs did not couple to HCN channels points to a spatially and/or functionally compartmentalized signaling of these receptors in thalamic neurons, similar to what is known about these receptors in cardiac myocytes (Xiao *et al.*, 2006). In this manner, my work not only describes the modulation of a specific ion channel, the pacemaker, in different parts of the thalamus, but furthers the idea of a very complex and organized GPCR signaling in neurons. To understand these mechanisms in a receptor-subtype specific manner in neurons could have implications on the pharmacology and physiology of neuronal β-ARs. Subtype specific β-adrenergic antagonists could increase potency and minimize negative side effects of drugs acting on central β-ARs.

6.2.3 HCN channels as cAMP sensors to monitor GPCR signaling

Based on the experiments in thalamic cells that demonstrated HCN channels as reliable on-line sensors for cAMP-signaling, we decided to further investigate HCN channel modulation under the control of GPCRs in a heterologous expression system. We expressed the cAMP sensitive HCN2 channel subunit in Chinese hamster ovary K1 (CHO-K1) cells that stably express $G_{i/o}$-coupled $GABA_B$-receptors. Alterations in the amplitude of cAMP-

sensitive currents following activation of $G_{i/o}$-coupled neurotransmitter receptors have been associated with an inhibition of either basal or forskolin-stimulated AC activity (Pape, 1992; Ingram and Williams, 1993; Svoboda and Lupica, 1998). Thus, we applied baclofen (40 µM), an agonist for $GABA_B$-receptors to the CHO cells transfected with HCN2 channels and monitored HCN-current amplitudes in whole-cell voltage clamp recordings at -100 mV, activated from a holding potential of -60 mV. Currents were decreased to 84.2 ± 5.1% of control 3 min after baclofen was washed in the bath (n = 6, $p < 0.05$, Fig. 6*A*). Furthermore, we tested whether prior increases in AC activity could strengthen the inhibiting effect of the $GABA_B$-agonist on HCN-currents. Indeed, when forskolin, an activator of AC was applied previous to baclofen, amplitudes were then decreased to 57.7 ± 11.0% of control level (n = 3, $p < 0.05$ baclofen-effect with/without forskolin, Fig. 6*B*). These results show that a) activation of $GABA_B$-receptors has an inhibiting effect on cAMP synthesis in CHO cells and b) HCN channel modulation by GPCR-dependent alteration of cAMP turnover can be reconstituted and recorded in a mammalian cell line.

With these experiments we show that HCN channels could serve as on-line cAMP sensors in heterologous expression systems and provide a model to further investigate GPCR-signaling.

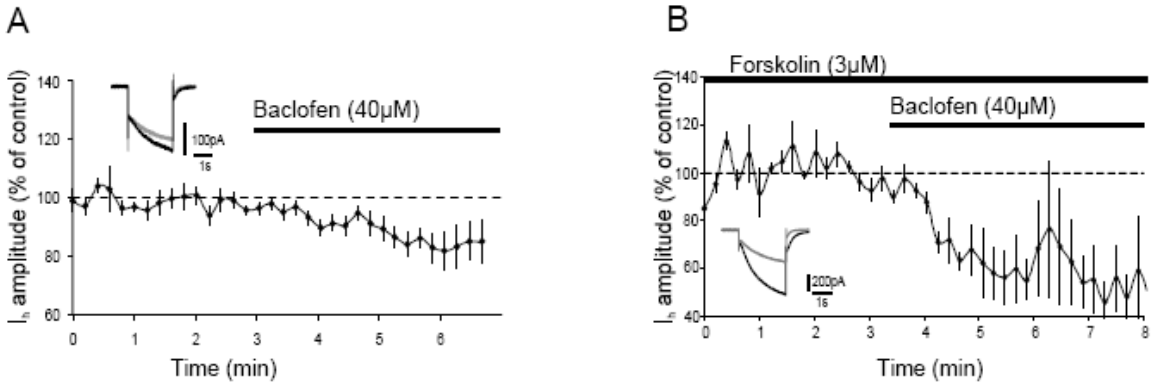

Figure 6. Regulation of HCN2-currents by activation of co-expressed GPCRs in a heterologous system. *A*, Time course of averaged responses of HCN-currents to the activation of $GABA_B$-receptors by the application of baclofen (40 µm) shows the decrease of the normalized current amplitude at -100 mV to 84.2 ± 5.1% (n = 6, $p < 0.05$). The black bar indicates baclofen application. Inset show representative current recordings before (black) and during (grey) baclofen application. *B*, Similar as in A, stimulation of AC with forskolin previous to baclofen application increased inhibiting effect of the $GABA_B$-agonist (57.7 ± 11.0% of control amplitude, n = 3, $p < 0.05$ with/without forskolin).

7 CONCLUSIONS AND OUTLOOK

The experiments carried out for my thesis have predominantly dealt with the investigation of pathological and physiological pathways of HCN channel regulation in the thalamus. An abnormal HCN channel expression and function was causally related to the emergence of absence epilepsy which implies that neuronal networks react highly sensitive to aberrant HCN channel activity. Additionally a differential regulation of pacemaker channels in different thalamic nuclei was observed. This suggests that the difference mediated by the neurotransmitter of one of the most important arousal systems could contribute to the different roles of thalamic nuclei in the control of wakefulness and attentive states. In summary the results once more demonstrate the complexity of neurons and neuronal networks being tremendously influenced by small changes and differences in the regulation and function of pacemaker channels. Therefore it is most reasonable that a decreased cAMP sensitivity was involved in the pathogenesis of absence epilepsy and a different ß-adrenergic modulation contributed to different electrophysiological properties of thalamic cells, respectively.

These results, however, have raised new questions about the regulation of pacemaker channels in the thalamus. For the understanding of the pathological pathways leading to absence epilepsy it would be important to know how the changed HCN channel expression pattern was induced. Future studies should also focus on the question whether epileptogenesis could be prevented by drugs acting on HCN channels. The answer would definitely prove a causal relationship of HCN channel dysfunction and epilepsy. This would require compounds restoring HCN channel function before seizure onset. To address this issue, however, GAERS are not an appropriate model system since HCN channels are not simply up- or downregulated. Nevertheless, if the trigger for restoring HCN channel function in the thalamus of adult epileptic animals could be identified, this would shed new light on the development of adaptive mechanisms in thalamic neurons.

Finally, it would be of interest to further investigate the significance of distinct HCN channel regulation for the control of sleep and consciousness *in vivo*, since the mechanism of sedation by central acting ß-blockers is still unclear. Is this effect mediated via HCN channels in the thalamus? Is there a difference between selective antagonists for ß1- and ß2-ARs? Although the answers to these questions are highly relevant for the exploration of new drug targets, up to now, GPCR and ß-AR signaling in intralaminar thalamic nuclei is rarely investigated. Just as little is known about the regulation of HCN channels by other GPCR

activating neurotransmitters. Anyway, while working through these issues the role for the pacemaker channel in intralaminar thalamic nuclei might be defined, similar to what had previously been described for first-order thalamic neurons.

The results of this thesis already provide new information about HCN channels. Together with the results of future projects my work might also contribute to a better understanding of the role of voltage-gated ion channels in thalamic function.

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9 LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ADP	Afterdepolarization
AHP	Afterhyperpolarization
AP	Action potential
AR	Adrenergic receptor
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5'-guanosine monophosphate
CHO cells	Chinese Hamster ovary cells
Cln	Centrolateral thalamic nucleus
CNBD	Cyclic nucleotide binding domain
EEG	Electroencephalogramm/graphic
EPSP	Excitatory postsynaptic potential
FOn	First-order thalamic nucleus
GABA	γ-aminobutyric acid
$GABA_A$-receptor	γ-aminobutyric acid receptor type A
$GABA_B$-receptor	γ-aminobutyric acid receptor type B
GAERS	genetic absence epilepsy rat from Strasbourg
GPCR	G-protein coupled receptor
G-protein	GTP binding protein
GRK	G-protein receptor serine/threonine kinase
GTP	Guanosine-tri-phosphate
HCN	Hyperpolarization-activated cationic non-selective
HEK cells	Human embryionic kidney cells
IBMX	3-isobutyl-1-methyl-xanthine (PDE-inhibitor)
IMn	Intralaminar/midline thalamic nuclei
I_h / I_{HCN}	Hyperpolarization-activated cationic current
I_{inst}	Instantaneous current
IP_3	Inositol-3-phosphat

IPSP	Inhibitory postsynaptic potential
I_T	Low-threshold calcium current
Iso	Isoproterenol (β-agonist)
K_{ir}	Inward rectifier K^+-channel
LC	locus coeruleus
LTP	Longterm potentiation
LTS	Low-threshold spike
PDE	Phosphodiesterase
PKA	Protein kinase A
PTX	Pertussis toxin
NA	noradrenaline
NE	non-epileptic
nRt/RTn	Nucleus reticularis thalami
PIP_2	Phosphatidylinositol-4,5-bisphosphate
REM	sleep rapid eye movement
RT	Room temperature
SAN	Sinoatrial node
SWD	Spike-and-wave discharge
TASK	TWIK-related acid-sensitive K^+ channel
TC	Thalamocortical
$V_{1/2,}$ V_{half}	half-activation voltage
Vpm	Ventroposterior medial thalamic nucleus
WAG/Rij	Wistar Albino Glaxo rat, bred in Rijswijk

10 CURRICULUM VITAE

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