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Differential regulation of HCN channel isoform expression in thalamic neurons of epileptic and non-epileptic rat strains

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Abstract

Hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels represent the molecular substrate of the hyperpolarization-activated inward current (I_h). Although these channels act as pacemakers for the generation of rhythmic activity in the thalamocortical network during sleep and epilepsy, their developmental profile in the thalamus is not yet fully understood. Here we combined electrophysiological, immunohistochemical, and mathematical modeling techniques to examine HCN gene expression and I_h properties in thalamocortical relay (TC) neurons of the dorsal part of the lateral geniculate nucleus (dLGN) in an epileptic (WAG/Rij) compared to a non-epileptic (ACI) rat strain. Recordings of TC neurons between postnatal day (P) 7 and P90 in both rat strains revealed that I_h was characterized by higher current density, more hyperpolarized voltage dependence, faster activation kinetics, and reduced cAMP-sensitivity in epileptic animals. All four HCN channel isoforms (HCN1–4) were detected in dLGN, and quantitative analyses revealed a developmental increase of protein expression of HCN1, HCN2, and HCN4 but a decrease of HCN3. HCN1 was expressed at higher levels in WAG/Rij rats, a finding that was correlated with increased expression of the interacting proteins filamin A (FILA) and tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b). Analysis of a simplified computer model of the thalamic network revealed that the alterations of I_h found in WAG/Rij rats compensate each other in a way that leaves I_h availability constant, an effect that ensures unaltered cellular burst activity and thalamic oscillations. These data indicate that during postnatal developmental the hyperpolarizing shift in voltage dependency (resulting in less current availability) is compensated by an increase in current density in WAG/Rij thereby possibly limiting the impact of I_h on epileptogenesis. Because HCN3 is expressed higher in young versus older animals, HCN3 likely does not contribute to alterations in I_h in older animals.

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Keywords

h-current; thalamocortical relay neurons; absence epilepsy; thalamic dysfunction; computer modeling

Introduction

A number of brain rhythms are controlled by HCN channels, the molecular substrate of the pacemaker current, I_h (Biel et al., 2009; Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003; Santoro and Baram, 2003). The HCN gene family is comprised of four pore-forming subunits (HCN1–4) that assemble as homo- or heteromers thereby forming functional channels (Brewster et al., 2005; Chen et al., 2005; Much et al., 2003). Cyclic AMP (cAMP) rapidly regulates channel opening of HCN2, HCN4, and, to a lesser extent, HCN1 (Biel et al., 2009; Robinson and Siegelbaum, 2003; Wainger et al., 2001). The HCN3 isoform appears to be inhibited by cAMP (Mistrik et al., 2005; Stieber et al., 2005). The expression pattern of HCN isoforms in the mammalian brain displays activity-, age-, region-, and species-dependent differences (Bender and Baram, 2008; Bender et al., 2001; Fan et al., 2005; Kanyshkova et al., 2009; Monteggia et al., 2000; Moosmang et al., 1999; Narayanan et al., 2010; Noam et al., 2010; Notomi and Shigemoto, 2004; Shin and Chetkovich, 2007; van Welie et al., 2004). Epileptic activity results in altered electrophysiological properties of I_h and influences the ratio of HCN1 and HCN2 expression in the hippocampus and entorhinal cortex (Bender et al., 2003; Brewster et al., 2002; Chen et al., 2001; Shah et al., 2004). Furthermore, β -subunits seem to influence the surface expression and electrophysiological properties of HCN channels (Lai and Jan, 2006; Pongs and Schwarz, 2010). Along these lines, the K^+ channel ancillary subunit Mink-related peptide 1 (MirP1), also termed KCNE2, enhances expression and speeds activation of HCN1, HCN2, and HCN4 (Decher et al., 2003; Yu et al., 2001). The cytoplasmic scaffolding protein filamin A (FilA) interacts with HCN1 and influences its membrane expression and localization (Gravante et al., 2004). Recent evidence indicates a central role for TRIP8b in the complex regulation of HCN channels in the brain (for review see: Braun, 2009). All TRIP8b isoforms produce a hyperpolarizing shift in voltage-dependency and antagonize the cAMP-induced enhancement of HCN channels, while splice variants produce either an increase or a decrease in the cell surface expression of HCN channels (Lewis et al., 2009; Santoro et al., 2009; Zolles et al., 2009).

In the thalamocortical system slow rhythmic synchronized activity during slow-wave sleep and absence epilepsy depends on HCN channel activation, and regulation of the voltage-dependence of I_h through the cAMP system is one important mechanism for the control of this activity mode (Lüthi and McCormick, 1998; Pape, 1996; Pape et al., 2005). TC neurons of the dLGN, a thalamic area known to be critically involved in physiological sleep (McCormick and Pape, 1990) and pathological epileptic rhythms (Guyon et al., 1993), have been frequently investigated as prototypical sensory relay cells. Alterations in the postnatal HCN isoform expression profiles in dLGN were accompanied by changes in I_h properties in TC neurons and the maturation of sleep-related slow oscillations (Kanyshkova et al., 2009). Evidence suggests a role for thalamic and cortical HCN channel dysfunction (for review see: Poolos, 2006) in the generation of epileptic discharges in the thalamocortical system. Epileptic activity appears as spike-and-wave discharges (SWD) which are initiated in cortical areas (Crunelli and Leresche, 2002; Pinault and O'Brien, 2007; van Luijtelaa et al., 2011). Studies in rat models of absence epilepsy, the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) and Wistar Albino Glaxo rats from Rijswijk (WAG/Rij), reveal abnormal regulation of I_h . In both epileptic strains, TC neurons in different sensory thalamic nuclei exhibit reduced responsiveness to cAMP, associated with a selective increase in the

expression of the relatively cAMP-insensitive HCN1 isoform (Budde et al., 2005; Kuisle et al., 2006). Based on the parallel early postnatal maturation of I_h and sleep oscillations (Kanyshkova et al., 2009), the gradual manifestation of SWD during ontogeny (Depaulis and Van Luijckelaar, 2006), and the observation that SWD are preceded by physiological sleep oscillations (van Luijckelaar et al., 2011), we hypothesize that alterations in the normal developmental expression profiles of HCN channels contribute to the occurrence of abnormal oscillatory behavior in the thalamocortical system. Therefore we conducted a quantitative study of the expression of all HCN channel isoforms, and selective HCN channel interacting proteins in the thalamus of epileptic WAG/Rij in comparison to non-epileptic ACI rats. We utilized a combination of electrophysiological, immunofluorescence and biochemical studies to correlate I_h current density, voltage-dependence and cAMP-sensitivity with HCN channel subunit expression. Mathematical modeling demonstrated the importance of I_h for rhythmic activity in the thalamic network in epileptic and non-epileptic animals.

Materials and Methods

Preparation

All animal work has been approved by local authorities (review board institution: Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; approval ID: 8.87-51.05.2010.117, 8.87-51.04.2010.A322). Rats (P7-P90) were sacrificed and in rapid sequence, after surgically removing a skull cap caudal to the bregma, a block of brain tissue containing the thalamus was removed from the cranial vault and submerged in ice-cold aerated (O_2) saline containing (in mM): sucrose, 200; PIPES, 20; KCl, 2.5; NaH_2PO_4 , 1.25; $MgSO_4$, 10; $CaCl_2$, 0.5; dextrose, 10; pH 7.35, with NaOH. Thalamic slices were prepared as coronal sections on a vibratome. Before recording, slices were kept submerged in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4 , 1.25; $NaHCO_3$, 24; $MgSO_4$, 2; $CaCl_2$, 2; dextrose, 10; pH adjusted to 7.35 by bubbling with carbogen (95% O_2 and 5% CO_2).

Whole-cell patch clamp

Recordings were performed on TC neurons of the dLGN at controlled room temperature ($22 \pm 1^\circ C$) in a solution containing (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4 , 1.25; HEPES, 30; $MgSO_4$, 2; $CaCl_2$, 2; dextrose, 10; pH 7.25 was adjusted with HCl. In some experiments the temperature of the recording chamber was set to controlled values of $30^\circ C$ and $35^\circ C$ (In-Line Solution Heater, Harvard Apparatus, March, Germany). Individual cells were visually identified by infrared differential interference contrast videomicroscopy (DIC-IR). Inspection of dLGN slices with DIC-IR videomicroscopy readily revealed two morphologically distinct types of neurons: neurons possessing a multipolar cell body (15–25 μm in diameter) giving rise to three to six dendrites, and smaller neurons (about 10 μm in diameter) of a fusiform or oval shape with dendrites emerging from opposite poles of the soma which represent TC neurons and interneurons, respectively (Munsch et al., 1997). TC neurons from different rat strains labeled with 50 μM Alexa Fluor® 594 (Fig. S1A–C) revealed no differences in the number of primary dendrites (Fig. S1D) and the diameter of the soma (Fig. S1E). In addition TC neurons are characterized by robust burst firing, a feature that is less pronounced or lacking in interneurons (Broicher et al., 2007). Membrane currents were measured with glass microelectrodes pulled from borosilicate glass capillaries (GC150T-10; Clark Electromedical Instruments, Pangbourne, UK) and filled with (in mM): K-gluconate, 95; K_3 -citrate, 20; NaCl, 10; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; BAPTA, 3; Mg -ATP, 3; Na_2 -GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolality of 295 mOsm/kg. The electrodes were connected to an EPC-10 amplifier (HEKA Elektronik, Lamprecht, Germany) with a chlorided silver wire. Electrode resistances were in

the range of 2–3 MΩ, with access resistances in the range of 5–20 MΩ. Series resistance compensation of >30% was routinely applied. Voltage-clamp experiments were controlled by the software Pulse or PatchMaster (HEKA Elektronik) operating on an IBM-compatible personal computer. Measurements were corrected for a liquid junction potential of 10 mV.

The protocol used to assess I_h was designed in order to increase the stability of whole-cell recordings and account for increasingly fast activation kinetics of I_h (see Fig. 1A left inset). Therefore the pulse length was shortened by 1500 ms with increasing hyperpolarization (3.5 s pulse length at –130 mV) (Kanyshkova et al., 2009). Including an additional step to –140 mV (2 s pulse length) did not produce further activation. Steady-state activation of I_h , $p(V)$, was estimated by normalizing the mean tail current amplitudes (I) 50–100 ms (see Fig. 1A, right inset) after stepping to a constant potential from a variable amplitude step using the following equation:

$$p(V) = (I - I_{\min}) / (I_{\max} - I_{\min}),$$

with $I_{\max}$ being the tail current amplitude for the voltage step from –130 mV to –100 mV and $I_{\min}$ for the voltage step from –40 mV to –100 mV, respectively. I_h activation was well accounted for by a Boltzmann equation of the following form:

$$p(V) = 1 / (1 + \exp((V - V_h) / k)),$$

where V_h is the voltage of half-maximal activation and k the slope factor. The amplitude of I_h was calculated by subtracting the instantaneous current amplitude from the steady-state current. The density of I_h was calculated by dividing the I_h current amplitude at –130 mV by the membrane capacitance obtained during whole-cell recordings.

The time course of I_h activation in TC neurons at room temperature was best approximated by the following single-exponential equation:

$$I_h(t) = A_0 + A_1 \times e^{-t/\tau},$$

where $I_h(t)$ was the current amplitude at time t , and A_0 , A_1 and τ are axis intercept, amplitude coefficient, and time constant, respectively. Biexponential fitting of the time course of activation was inconsistent at room temperature but adequate at increased recording temperatures (30°C, 35°C).

Drugs

During experiments with 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP; water soluble; Sigma, Taufkirchen, Germany) added to the recording pipette, properties of I_h were determined 10–15 min after obtaining the whole-cell configuration.

Immunofluorescence

Rats (P30) were deeply anesthetized using pentobarbital (50 mg/kg body weight) and transcardially perfused with PBS, followed by an ice-cold 4% PFA/PBS for 35–40 min. Brains were removed, postfixed for 4 h in 4% PFA/PBS and cryoprotected with 25% sucrose. Coronal sections (40 μm) were cut at the level of the dLGN, collected in Tris-buffered saline (TBS). After several washings with TBS, sections were blocked with 10% normal horse serum (NHS), 2% BSA, 0.3% Triton X-100 in TBS for 2 h to minimize nonspecific binding before incubation of slices with primary antibodies in 2% NHS, 2%

BSA, 0.3% Triton X-100 in TBS at 4°C for 16–18 h. The following antibodies were used: rabbit anti-HCN1 (1:500, Alomone Labs, Jerusalem, Israel), mouse anti-HCN1 (1:300, UC Davis / NIH NeuroMab Facility), rabbit anti-HCN2 (1:250, Alomone Labs), mouse anti-HCN2 (1:300, NeuroMab), rabbit anti-HCN3 (1:500, Alomone Labs), mouse anti-HCN3 (1:300, NeuroMab), rabbit anti-HCN4 (1:100, Alomone Labs), mouse anti-HCN4 (1:200, NeuroMab), mouse anti-NeuN (1:200, Chemicon, Schwalbach, Germany), and guinea pig anti-MAP2 (1:300, Synaptic Systems). After washing (3 × 10 min with TBS), sections were exposed to Cy2- or Cy3-conjugated donkey-IgG (1:300, Dianova, Germany) or AlexaFluor405-conjugated goat IgG for 1.5 h, washed again and cover slipped with Immumount. For negative controls occlusion of the primary antibody from the staining procedure was routinely performed with no positive immunological signal detected.

Western blotting

ACI and WAG/Rij rats of different ages (P7, P14, P30, and P90) were rapidly decapitated. Thereafter dLGNs were quickly dissected and frozen immediately in liquid nitrogen. Tissue was then homogenized in ice-cold buffer containing 50 mM HEPES, pH 7.6, 1 mM EDTA and 25 mM sucrose. Protease inhibitor cocktail (Roche, Mannheim, Germany) was added according to the manufacturer's guidelines. Protein samples were centrifuged at 2500g for 10 min at 4°C, and pellets were discarded. The resulting supernatants were separated by 12% SDS-PAGE. Proteins were transferred in 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol onto Hybond ECL nitrocellulose membranes at 20 V overnight at 4°C. Thereafter, membranes were blocked with 5% nonfat milk in TBS-0.1% Tween for 1h and incubated with primary antibodies for 2 h at room temperature. The following antibodies were used: rabbit anti-HCN1, rabbit anti-HCN2, rabbit anti-HCN4 (1:300 for each antibody, Alomone Labs), mouse anti-HCN3 (1:300, NeuroMab), monoclonal mouse anti-β-actin (1:4000, Abcam), mouse anti-MirP1 (1:500, Abnova), and mouse anti-FilA (1:200, Sigma). Following washing (3 × 10 min in TBS-0.1% Tween) blots were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (1:2000 for each antibody, Dako A/S) for 1 h at room temperature. Washing was performed as before and blots were developed using an ECL Plus detection kit (Amersham Biosciences) according to the manufacturer's guidelines. For quantification of HCN1–4 expression in epileptic and non-epileptic rat strains two sets of analysis were performed. First, an age-dependent expression profile was evaluated for each isoform and rat strain on separate gels (e.g., ACI/HCN1: P7, P14, P30, P90). Second, HCN1–4 expression was compared for each isoform separately using WAG/Rij and ACI protein probes obtained from the same age on the same gel (e.g., P7/HCN1: ACI, WAG/Rij). In all sets of experiments the protein content was normalized using β-actin as an internal and loading standard. Quantification of HCN expression was achieved by the densitometer analysis of corresponding protein bands (Quantity One 4.2.2. software, ChemiDoc, Bio-Rad).

For TRIP8b detection a custom made antibody was used (Fig. S2). cDNA encoding amino acids 272–615 of mouse TRIP8b (Accession Number: NM_021483) was generated by PCR using primers (5'-CGCGAATTCACCACCATGCAGGCAGAATGGGAGG and 3'-CGCGCGCTCGAGCCCGGTCAAGGATCCAAATTGAAAG), followed by subcloning the PCR product into the *EcoRI* / *XhoI* sites of the glutathione-S-transferase-producing vector, pGEX-4T1 (Pharmacia, Piscataway, NJ, USA). The resulting GST-TRIP8b (272–615) fusion protein was expressed in BL21 bacteria (Stratagene, La Jolla, CA) and purified by Glutathionesepharose affinity chromatography according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ). Mice were immunized and hybridomas prepared and screened by indirect ELISA with the GST-TRIP8b(272–615) by the Northwestern University Monoclonal Antibody Facility (Chicago, IL). One of several monoclonal hybridoma lines (1A5) was selected for further testing. Culture media prepared from anti-

TRIP8b line 1A5 was tested at 1:10 for Western blotting using methods described previously (Shin et al., 2008).

Statistics

All results are presented as mean $\pm$ SEM. By default statistical significance was tested using the nonparametric Mann-Whitney test (Graph Pad Prism software; Graph Pad, San Diego, CA). For V_h a normal distribution of measured values was found. Therefore the parametric *t*-test was used for this parameter (OriginLab software, Additive GmbH, Friedrichsdorf, Germany). For multiple comparisons (age-dependent profiles; comparison of multiple strains) ANOVA testing (Graph Pad Prism software) was used. Differences were considered statistically significant if $p < 0.05$. For the analysis of age-groups data points represent animals of the stated age ± 2 days.

Computer modeling

Simulations have been conducted within the NEURON simulation environment (Hines and Carnevale, 2001) at temperatures of 23°C and 36°C lasting 6 s and 2 s, respectively. All simulations were based on a modified version of a previously published thalamocortical network comprising two TC and two reticular thalamic (RE) neurons (Fig. 7B; the original model can be downloaded at the SenseLab ModelDB website: <http://senselab.med.yale.edu/ModelDB/>. Model accession number: 3343, delta oscillations, short run). In contrast to the original model (Destexhe et al., 1996) both TC cells have been equally parameterized (I_h conductance: $ghbar_iar = 1e-5$ S/cm², potassium leak conductance: $kl.gmax = 0.005$ μ S) to function as pace makers (Kanyshkova et al., 2009). All other parameters remained unaffected in the control condition. For I_h basal parameters were set as follows: $ghbar_iar = 1e-5$ S/cm²; $V_h = -74.7$ mV; maximum τ_m value at -80 mV = 1010 ms. The Q_{10} value of I_h was set to 3 (Pena et al., 2006). The accuracy of the mathematical description of I_h with respect to currents recorded at physiological temperature has been demonstrated before (Huguenard and McCormick, 1992). I_h properties were altered in a stepwise manner: activation speed was increased by 20% (maximum τ_m value at -80 mV = 808 ms), the activation curve was shifted by 6 mV in the hyperpolarizing direction ($shift_iar = -6$ mV), and I_h conductance was increased by 19% ($ghbar_iar = 1.19 e-5$ S/cm²). In an additional step the T-type Ca²⁺ channel conductance was increased for both cell types (TC neurons: +20%, $gcabar_it = 0.0024$ S/cm²; RE neurons: +15%, $gcabar_it2 = 0.00345$ S/cm²). Interburst (rate of low threshold Ca²⁺ spike (LTS) generation) and intraburst frequency (rate of Na⁺/K⁺ action potential generation) were determined based on the evaluation of the last three bursts of the voltage traces simulated for TC1. Calculation of the interburst frequency was achieved by measuring the time interval between the peaks of the first action potentials of two consecutive LTS. The intraburst frequency was obtained by measuring the time interval between the first and the second Na⁺/K⁺ action potential in a burst.

Results

Basic properties of I_h in developing TC neurons

Data from visually and electrophysiologically identified dLGN TC neurons of ACI (Fig. 1A) and WAG/Rij (Fig. 1B) rats of different postnatal ages (P7, P14, P30, and P90) were analyzed. I_h was activated in the presence of Ba²⁺ (1 mM) from a holding potential of -40 mV by using hyperpolarizing voltage steps of increasing ($\Delta V = -10$ mV) amplitude and decreasing ($\Delta t = -1500$ ms) duration followed by a constant step to -100 mV (the left inset in Fig. 1A shows a scheme of the voltage protocol). As revealed by analyzing the deactivating (tail) currents (expended tail currents are shown in the right inset of Fig. 1A), there was an inverse developmental change in the voltage-dependency of the I_h activation

curves in the two rat strains (Fig. 1C). While half-maximal I_h activation (V_h) became increasingly hyperpolarized throughout development in WAG/Rij rats (P7: -87.6 ± 1.1 mV, $n = 8$; P14: -89.7 ± 0.8 , $n = 18$; P21: -94.3 ± 0.8 , $n = 24$; P30: -92.8 ± 1.0 , $n = 8$; P90: -95.7 ± 0.6 mV, $n = 9$), a depolarization of V_h was observed in ACI rats (P7: -93.2 ± 0.6 mV, $n = 9$; P14: -92.6 ± 0.9 , $n = 14$; P21: -88.4 ± 0.9 , $n = 28$; P30: -86.8 ± 0.9 , $n = 12$; P90: -87.8 ± 0.9 mV, $n = 14$; Fig. 2A). At all developmental stages V_h was significantly different between the two rat strains ($p < 0.001$). Adult values were reached at about P17.

Since it has been observed that nonconvulsive spontaneous thalamocortical SWD occur in many laboratory rat strains (Gralewicz, 1999), we analyzed the I_h voltage-dependency in one additional epileptic rat strain (GAERS) and three additional control strains, namely non-epileptic control rats (NEC), Sprague Dawley (SD) rats, and Long Evans (LE) rats. While WAG/Rij-ACI and GAERS-NEC represent well established corresponding pairs of epileptic and epilepsy-free strains (Depaulis and Van Luijckelaar, 2006), the epileptic status of SD and LE rats is undefined. Over the developmental range analyzed here, V_h became increasingly hyperpolarized reaching adult values in animals older than about P16 in SD (Kanyshkova et al., 2009) as well as LE rats (Fig. 2B). While the two epileptic strains (WAG/Rij, GAERS) revealed hyperpolarized V_h values (negative to about -93 mV), the four control strains were characterized by significantly ($p < 0.001$) more depolarized V_h values (positive to about -89 mV; data from P19 to P23 were averaged; Fig. 2C).

The amplitude of I_h determined at -130 mV significantly ($p < 0.001$) increased from 95 ± 7 pA ($n = 19$) at P7 to 662 ± 86 pA ($n = 17$) at P90 in WAG/Rij rats and 53 ± 6 pA ($n = 12$) at P7 to 401 ± 39 pA at P90 ($n = 23$) in ACI rats (data not shown). Between strains significant differences ($p < 0.01$) were found for P21 and P90. When absolute amplitude values were normalized for the cell size, the density of I_h measured at the step to -130 mV significantly (WAG/Rij: $p < 0.001$; ACI: $p < 0.01$) increased as a function of postnatal age in both rat strains (Fig. 2D). Current density increased from 1.2 ± 0.2 pA/pF ($n = 17$) at P7 to 5.3 ± 0.5 pA/pF ($n = 15$) at P90 in WAG/Rij rats (Fig. 2D, black bars) and 0.9 ± 0.1 pA/pF ($n = 12$) at P7 to 3.6 ± 0.2 pA/pF at P90 ($n = 19$) in ACI rats. For animals $\geq$ P14 differences between both rat strains were significant ($p < 0.05$).

Since the relative contribution of different HCN isoforms to the properties of I_h is especially evident in the kinetics of I_h activation, we analyzed time constants of activation as a function of age. At room temperature the activation time course was best approximated by a mono-exponential function throughout the age range examined (Fig. 2E). When the two rat strains were compared, time constants ranged between a minimum of $\tau = 398 \pm 32$ ms ($n = 12$) at P7 in ACI rats and a maximum of $\tau = 579 \pm 13$ ms ($n = 31$) at P30 in ACI rats (Fig. 2F). Significantly ($p < 0.01$) faster time-dependent I_h activation was found in WAG/Rij rats for P14 and P30.

Strong modulation by intracellular cAMP is a hallmark of I_h in TC neurons and can reflect the relative contribution of the different HCN isoforms expressed (Kanyshkova et al., 2009). To examine the cAMP effect at different developmental stages, we recorded I_h in TC neurons from P7, P30, and P90 rats while the pipette solution contained 0.1, 1, or 10 μ M 8-bromo-cAMP (Fig. S3). In comparison to cells recorded under control conditions, V_h was significantly ($p < 0.01$) more depolarized for most combinations of age, rat strain, and intracellular 8-bromo-cAMP concentrations. For two conditions no significant depolarizing shifts were found: ACI rats at P90 and WAG/Rij rats at P7 were not different with 0.1 μ M cAMP added to the internal solution. Between rat strains significant ($p < 0.05$) differences were found for P7 and P30 but not for P90 animals. The most depolarized activation curves were found in the presence of 10 μ M intracellular 8-bromo-cAMP in TC neurons taken from P7 ACI rats (-71.8 ± 1.5 mV, $n = 22$).

To verify that the differences in I_h properties between rat strains found at room temperature sustain at more physiological temperature, we tested the effect of increased recording temperature (30°C and 35°C; P18–P23) on the voltage-dependency of I_h (Fig. 3A shows examples at 30°C). With increasing temperature activation curves (Fig. 3B; 30°C) and V_h values (Fig. 3C) shifted to more depolarized potentials. The degree of positive shift in V_h was about 10 mV / 10°C in both rat strains (cf. Yanagida et al., 2000). At all temperatures differences between strains were significant ($p < 0.01$). Furthermore, I_h current amplitudes at –130 mV increased with increasing temperature (about 150 pA / 10°C) and were significantly ($p < 0.05$) higher in epileptic rats (Fig. 3D). Next activation time constants were determined. In contrast to the findings at room temperature, current kinetics at elevated temperature were best approximated by a bi-exponential function. While the fast current component was in the range of about 100–200 ms, the slow component was in the range of about 1–2 s (Fig. 3E). Significant differences between strains ($p < 0.001$) were found for both temperatures.

In summary the electrophysiological analysis of I_h in ACI and WAG/Rij rats revealed a strong increase in current density during development in both rat strains which was accompanied by a depolarizing and a hyperpolarizing shift in V_h in ACI and WAG/Rij rats, respectively. In young animals I_h in WAG/Rij rats is typically less sensitive to cAMP and has the tendency to activate faster. These differences were found at room and physiological temperature.

Expression of HCN channel proteins in dLGN

Next, we quantified protein expression of all HCN isoforms. Protein expression of HCN2 and HCN4 increased from P7 to P90 in both rat strains (Fig. 4C, 4D). Significant increases in protein levels were found for HCN2 (ACI: P7/P90, $p < 0.05$; WAG/Rij: P7/P90, $p < 0.01$) and HCN4 (ACI: P7/P30, $p < 0.05$; WAG/Rij: P7/P14, $p < 0.05$; P7/P30, $p < 0.05$; P7/P90, $p < 0.01$). For the HCN1 isoform (Fig. 4A) a significant increase in protein expression was found for WAG/Rij (P7/P30, $p < 0.05$; P7/P90, $p < 0.01$) and ACI rats (P7/P30, $p < 0.05$). At P30 ($p < 0.01$) and P90 ($p < 0.05$) HCN1 was significantly more expressed in WAG/Rij rats in comparison to ACI rats (Fig. 4A) and SD rats (Fig. S4A). The age dependency of the HCN3 isoform (Fig. 4B) was different from all other subtypes in that expression decreased rather than increased throughout postnatal development (ACI: P14/P90, $p < 0.01$; WAG/Rij: P7/P30, $p < 0.05$; P7/P90, $p < 0.001$). At P7 WAG/Rij rats expressed significantly ($p < 0.05$) more HCN3 protein in comparison to ACI rats.

For comparison HCN1 protein levels were determined in the ventrobasal thalamic complex (VB). In general HCN1 expression levels were lower compared to dLGN (Fig. S4B). Furthermore, the nominal stronger expression level of HCN1 protein (WAG/Rij: 0.05 ± 0.01 , $n = 4$; ACI: 0.02 ± 0.01 , $n = 4$; Fig. S4B) in the VB of WAG/Rij rats was accompanied by significantly more hyperpolarized V_h values in epileptic animals (WAG/Rij: $V_h = -86.2 \pm 1.1$, $n = 23$; ACI: -83.1 ± 0.7 , $n = 20$; Fig. S4C).

To localize the protein expression of HCN channel isoforms in dLGN, immunohistochemical staining was performed (Fig. 5A). The results indicated that at the age of P30 HCN2 and HCN4 were strongly expressed in dLGN. No fluorescence signal was found for HCN3 in either strain. Furthermore, while HCN1 was clearly detectable in slices obtained from WAG/Rij, ACI rats revealed low HCN1 expression. Next the subcellular localization of HCN channels was probed by co-staining with antibodies directed against NeuN and MAP2. While co-staining of HCN1 with the neuronal marker protein NeuN revealed strong overlap there was rather little overlap with the dendritic marker MAP2 (Fig. 5B, upper row). Localization of the other isoforms was different from HCN1 in that HCN2

was found in soma and dendrites (Fig. 5B, lower left images) and HCN4 was mainly expressed in dendrites (Fig. 5B, lower right images).

In summary the expression analysis of HCN channel protein revealed an increase in HCN1, HCN2, and HCN4 during postnatal development with HCN1 more expressed in epileptic rats. The HCN3 isoform on the other hand revealed a strong decrease in expression at early postnatal stages.

Expression of presumed HCN channel interacting proteins in dLGN—HCN channel family members interact with a number of cytoskeletal and scaffolding proteins, some of them influence membrane trafficking and electrophysiological I_h properties (Lewis et al., 2010). In order to explore the possibility that altered expression of TRIP8b, FilA, or MirP1 influence the increased HCN1 expression in WAG/Rij rats, we performed Western blotting of dLGN tissue extracts. For TRIP8b (Fig. 6A) as well as FilA (Fig. 6B) significantly more protein was found in WAG/Rij compared to ACI rats. While MirP1 was expressed in cortical and hippocampal tissue no MirP1-specific band was detected in dLGN (Fig. 6C).

These findings demonstrate that increased HCN channel function is accompanied by stronger expression of two HCN channel interacting proteins in WAG/Rij rats.

Influence of altered I_h properties on thalamic rhythmic activity—To assess the influence of the changes in I_h found in WAG/Rij rats on thalamic rhythmic activity, we used a mathematical modeling approach (Fig. 7, 8). Spontaneous rhythmic burst activity was analyzed in a model network (Fig. 7B) consisting of two TC and two RE neurons which is able to sustain rhythmic delta activity (Destexhe et al., 1996; Kanyshkova et al., 2009). This model seems appropriate since SWD in WAG/Rij rats is preceded by delta precursor activity in the thalamus (Smyk et al., 2011; van Luijckelaar et al., 2011). Thereby, RE neurons reciprocally communicated via GABA_A mediated connections (dashed arrows) and projected to both TC neurons via GABA_A and GABA_B (continuous arrows). The feedback from TC cells was carried by AMPA receptors in both RE cells (lines with circled end). In a first set of simulations the effect of I_h changes was probed at a physiological temperature of 36°C. At a hyperpolarized membrane potential of about -70 mV and I_h properties (channel conductance = $1e-5$ S/cm²; V_h = -74.7 mV; maximum τ_m at -80 mV = 1010 ms) set as in the original model (Destexhe et al., 1996), both TC neurons generated spontaneous burst activity within a frequency range of 4–5 Hz (close to the frequency of delta oscillations) for the modeling period of 2 s (Fig. 7A, 7D). Regarding the entire network, synchronized activity was also observed in both RE neurons (not shown). In the following I_h properties in both TC neurons were changed to mimic the differences found for WAG/Rij rats (τ_m 20% faster; channel conductance increase by 19%; hyperpolarizing shift of V_h by 6 mV) in an additive manner. A faster kinetic of I_h activation increased the frequency of the network oscillation from 4.6 Hz to 5.3 Hz (Fig. 7D) which is closer to the frequency range (7–11 Hz) of SWD generation found in rat absence epilepsy models (Budde et al., 2006). This effect was accompanied by an earlier onset of delta activity after starting the simulation (Fig. 7C) and a decrease in intraburst frequency (Fig. 7E). Next, the I_h conductance (g_h) was increased by 19%. As a result the oscillation frequency further increased to 5.8 Hz (Fig. 7D). When the V_h value of I_h was shifted to a more hyperpolarized value under these conditions, all analyzed parameters returned to control values (Fig. 7C–E).

We have recently described an increase in the expression of T-type Ca²⁺ channels and the corresponding T-type Ca²⁺ currents (I_T) in TC and RE neurons of WAG/Rij rats (Broicher et al., 2008). To also account for these changes, we increased the T-type Ca²⁺ channel conductance by 20% and 15% in TC and RE neurons, respectively (Fig. 7A, 5th panel). This

maneuver accelerated thalamic oscillations to 5.3 Hz (Fig. 7D) and strongly enhanced burst firing as is evident from the fast onset of oscillations (Fig. 7C) and the increased intraburst frequency (Fig. 7E).

To assess whether the network was able to oscillate at room temperature, simulations were run at 23°C in the following (Fig. 8; 6 s simulation periods were analyzed). Changes in I_h and I_T were applied in the same sequence as described for 36°C. As before both TC neurons generated spontaneous burst activity from hyperpolarized potentials under control conditions, yet at a lower frequency of 1.5 Hz (Fig. 8A, 8D). In comparison to 36°C, bursts at room temperature revealed more action potentials (Fig. 8B) and the changes in I_h properties had different qualitative effects on the intraburst frequency (Fig. 8E) due to the distinct temperature-dependency of Ca^{2+} -dependent mechanisms. For the other two analyzed parameters, namely frequency of burst generation (Fig. 8D) and time to the generation of the first action potential (Fig. 8C), changes in I_h properties had qualitatively very similar effects at room temperature in comparison to 36°C.

These findings indicated that the alteration of I_h properties found in TC neurons of WAG/Rij rats compensate each other in a way that keeps rhythmic burst activity in the thalamic network constant. Changes in additional membrane currents, like I_T , seem to be necessary to induce epilepsy-like network activity.

Discussion

The results of the present study can be summarized as follows: (1) I_h current amplitude and density strongly increased from P7 to P90 in TC neurons from ACI and WAG/Rij rats. This increment was associated with increased expression of HCN1, HCN2, and HCN4 subunits and a decrease in HCN3. The relationship between overall HCN protein expression and I_h current density was linear in both rat strains (Fig. S5). (2) HCN1 expression and I_h current density were higher in adult WAG/Rij as compared to ACI rats. (3) The voltage-dependency of activation changed reciprocally and was significantly different between ACI and WAG/Rij rats throughout postnatal development. Current kinetics and sensitivity to modulation by cAMP were significantly different only in young animals. (4) Expression of the HCN channel interacting proteins TRIP8b and FilA was increased in WAG/Rij as compared to ACI rats. Increased TRIP8b expression can explain several of the changed I_h properties in WAG/Rij rats. (5) Incorporation of altered I_h properties (increased current conductance, faster activation kinetic, more hyperpolarized V_h value) into a simplified thalamic network model oscillating in the delta frequency range indicated that the changes in voltage-dependency and current density compensate each other. These findings suggest homeostatic compensation and underscore the importance of thalamic TC neurons as neuronal oscillators in the delta range. (6) In conclusion, our data provide evidence for a dysregulation of HCN channels in TC neurons of epileptic WAG/Rij rats that starts as early as P16 (Budde et al., 2005) and continues into adulthood. Although the resulting alterations of I_h current density, voltage-dependency, current kinetics, and cAMP-dependent modulation are mostly compensated in older animals (Kuisle et al., 2006), early HCN channel dysregulation may contribute to the development of an epileptic phenotype (Blumenfeld et al., 2008; Schridde et al., 2006).

Correlation of HCN expression and I_h properties

The most straight forward correlation between thalamic HCN expression and I_h properties is given by the linear relationship between total HCN protein and I_h current density (Fig. S5).

The four HCN isoforms expressed in dLGN exhibit different voltage-dependency, activation kinetics, and cAMP-sensitivity in heterologous expression systems (Biel et al., 2009;

Robinson and Siegelbaum, 2003). While HCN2 and HCN4 display slow activation kinetics and strong cAMP-sensitivity, HCN1 is characterized by faster voltage-dependent activation and weaker cAMP-dependent modulation. Although HCN3 is nearly identical to HCN2 with respect to voltage-dependency and kinetic properties, homomeric HCN3 channels are negatively modulated by cAMP (Mistrik et al., 2005; Stieber et al., 2005). In TC neurons the combined expression of HCN2 and HCN4 is thought to generate an I_h with a slow activation time course and a relatively high cAMP-sensitivity, with the latter of these properties being fine-tuned by the degree of HCN1 contribution (Budde et al., 2005; Budde et al., 2008; Kanyshkova et al., 2009; Ludwig et al., 2003). During early postnatal stages HCN3 seems to additionally influence I_h properties. We speculate that the high cAMP content of dLGN tissue during early postnatal stages (Kanyshkova et al., 2009) is counterbalanced by high expression levels of HCN3 thereby limiting the depolarizing shift of the basal I_h activation curve. When intracellular cAMP levels decrease later in postnatal development, HCN3 is down regulated.

Functional relevance of I_h properties during development

In developing TC neurons the increase in HCN channel expression enables the thalamic network to generate slow wave sleep-associated delta oscillations as soon as a certain I_h current density is reached (Kanyshkova et al., 2009). HCN channel function in the brain is also influenced by interacting proteins such as TRIP8b (Braun, 2009; Lewis et al., 2010). Although alternative splicing produces TRIP8b isoforms with differential effects on HCN channel function, the most abundant TRIP8b isoforms hyperpolarize the activation voltage of I_h , increase HCN channel surface expression, and block modulation of HCN channels by cAMP (Lewis et al., 2009; Santoro et al., 2009; Santoro et al., 2004; Zolles et al., 2009). Therefore the increased TRIP8b expression in WAG/Rij rats is in agreement with most of the alterations of I_h properties (increased current density, negative V_h values, and reduced cAMP-sensitivity) found in this study. Faster I_h activation kinetics in WAG/Rij rats may be consequently based on the increased influence of the fast activating HCN1 channels on the compound current. Although FilA specifically interacts with HCN1 (Gravante et al., 2004), and we observe increased FilA in adult WAG/Rij rats, it is unknown how FilA affects HCN channel function in the brain. Based on published proteomic data, only TRIP8b co-purified with HCN subunits in native brain extracts, suggesting its central importance as an auxiliary subunit for HCN channels in brain (Zolles et al., 2009).

Developmental regulation of HCN channels and I_h is relevant to childhood absence epilepsy

When assessing the possible involvement of I_h in the generation of SWD the following basal considerations should be taken into account: (1) Physiological (sleep oscillations) and pathophysiological (SWD) activity in the thalamocortical neuronal network is based on the same molecular and cellular substrate (Beenhakker and Huguenard, 2009; Budde and Pape, 2009; Pinault and O'Brien, 2007). (2) Alterations in ion channels and membrane receptors have been suggested to contribute to the transformation of naturally occurring oscillations into pathological oscillations. (3) Developmental changes in HCN channel expression and I_h properties contribute to maturation of sleep oscillations. (4) Therefore it is tempting to assume that the deviations from the normal postnatal developmental of I_h found in WAG/Rij rats in the present study may contribute to the generation of absence epilepsy.

Absence seizures are characterized by SWD, and represent a state of hyper-synchronous activity in the thalamocortical system (Budde and Pape, 2009; Crunelli and Leresche, 2002; Pinault and O'Brien, 2007). Although only a minority of TC neurons recorded in GAERS rats during SWD activity in vivo exhibit robust rhythmic LTS generation (Pinault et al., 1998), I_h plays a critical role in generating burst discharges in TC neurons during seizure-

related SWD in WAG/Rij rats (Budde et al., 2005; van Luijtelaar et al., 2011). Importantly, altered expression of HCN1 channels in TC neurons and cortical pyramidal neurons was found to underlie the SWD phenotype of WAG/Rij and GAERS rats (Budde et al., 2005; Kuisle et al., 2006; Strauss et al., 2004). Moreover we have shown here that hyperpolarized I_h activation curves, which result in less availability of this current at physiological voltages, is a feature of TC neurons in epileptic rats while different control strains exhibit a more depolarized voltage-dependency. Indeed, reduced availability of I_h by hyperpolarizing shifts in the activation curve induced by neuromodulators (Pape, 1992) or partial blockade of this current by drugs promotes rhythmic thalamic activity (Yue and Huguenard, 2001). As indicated by our computer modeling this effect seems to be counteracted by an increase in I_h current density in WAG/Rij rats. Therefore, our findings are in agreement with the functional compensation of weakened thalamic I_h regulation in juvenile animals by Ca^{2+} -dependent mechanisms in adult GAERS rats (Kuisle et al., 2006). This homeostatic regulation seems to be critical for normal neuronal processing and avoiding of pathological conditions (Olypher and Prinz, 2010; van Welie et al., 2004).

Since SWD become manifest in the cortical EEG of WAG/Rij rats at an age of 2 to 3 months, and all animals display SWD at the age of 4 months, the alterations shown here appear well before the onset of epileptic activity (Coenen et al., 1992; Coenen and Van Luijtelaar, 2003). How might these early changes in I_h contribute to the development of epileptic seizures? Environmental manipulations and pharmacological treatment early in postnatal development before SWDs occur (neonates to P21) alter HCN1 expression in somatosensory cortex, I_h , and seizure activity in adult WAG/Rij rats (Blumenfeld et al., 2008; Schridde et al., 2006). Along this line it is of special interest that pharmacological treatment with T-type Ca^{2+} channel blockers that prevent SWD also blocked changes in the expression of HCN1 normally associated with epilepsy in this model (Blumenfeld et al., 2008). Even more important, systemic injection of specific I_h blockers in WAG/Rij rats results in a dose-dependent decrease in SWD (van Luijtelaar et al., 2011). Furthermore, the reduced cAMP-dependency of I_h in WAG/Rij and GAERS rats hinders the ability of TC neurons to leave the burst mode of action potential generation in young animals (Budde et al., 2005; Kuisle et al., 2006). These alterations of intrinsic neuronal properties may - together with other changes in ion channels (e.g. T-type Ca^{2+} channels) and membrane receptors (e.g. GABA_A receptors) - create conditions that switch the thalamic network from natural physiological oscillations (sleep-related or sensorimotor oscillations) to SWD generation (Budde and Pape, 2009; Pinault and O'Brien, 2007). Prolonged and strong burst firing in early life may induce activity-dependent changes in neuronal properties which subsequently lead to the appearance of epileptic seizures in later life (LeMasson et al., 1993; Olypher and Prinz, 2010; Turrigiano et al., 1994).

Conclusion

Together with earlier findings (Budde et al., 2005; Kanyshkova et al., 2009; Pape et al., 2005) the present paper points to a scenario where TC neurons regulate I_h by differential expression of pore-forming HCN subunits and interacting proteins to achieve current properties that sustain physiological firing properties in face of developmental changes of the external and internal environment, especially intracellular cAMP levels (Surges et al., 2006). Deviations from this ontogenetic program may be correlated with disease states like absence epilepsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACI	August Copenhagen Irish rat
ACSF	artificial cerebrospinal fluid
ATP	adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BSA	bovine serum albumin
C-terminal	carboxy-terminal
cAMP	cyclic adenosine monophosphate
cdNA	complementary deoxyribonucleic acid
Cy2/Cy3	cyanine dyes 2/3
DIC-IR	infrared differential interference contrast
dLGN	dorsal part of the lateral geniculate nucleus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FilA	filamin A
GABA	γ -aminobutyric acid
GAERS	Genetic Absence Epilepsy Rats from Strasbourg
g_h	I _h conductance
GTP	guanosine triphosphate
GST	glutathione-S-transferase
HCN	hyperpolarization-activated cyclic nucleotide-gated cation channel
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	immunoglobulin G
I_h	hyperpolarization-activated inward current
I_T	T-type Ca ²⁺ current
LE	Long Evans rat
LTS	low threshold Ca ²⁺ spike
MirP₁	Mink-related peptide 1
NEC	non-epileptic control rat
NHS	normal horse serum
P	postnatal day
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PFA	paraformaldehyde
RE	reticular thalamic
SD	Sprague Dawley rat
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SWD	spike-and-wave discharge
TBS	Tris-buffered saline
TC neuron	thalamocortical relay neuron
TRIP8b	tetratricopeptide repeat-containing Rab8b-interacting Protein
VB	ventrobasal thalamic complex
V_h	half-maximal activation voltage
WAG/Rij	Wistar Albino Glaxo rats from Rijswijk

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Highlights

- (1) I_h was analyzed in non-epileptic ACI and epileptic WAG/Rij rats.
- (2) I_h current density, voltage dependence, and kinetics were altered in WAG/Rij.
- (3) HCN1 and the auxiliary subunit TRIP8b were stronger expressed in WAG/Rij rats.
- (4) Alterations of I_h in WAG/Rij rats counteract to keep I_h availability constant.
- (5) Computer simulations indicate that collectively the changes in I_h may have little impact on epileptogenesis.

Fig. 1.

Increase of I_h amplitudes and change in voltage-dependency during postnatal development in dLGN TC neurons. (A, B) Representative current traces obtained from cells of ACI (A) and WAG/Rij (B) rats at different postnatal ages (as indicated). Currents were elicited by applying the shown voltage clamp protocol (see left inset) starting from -40 mV. The tail current voltage was -100 mV. The duration of each hyperpolarizing step was shortened as command potentials became more negative. The right inset shows tail currents at an expanded time scale. The left and right vertical line indicate the 50 ms and 100 ms time point after stepping to -100 mV, respectively. The mean current value of this range was taken for calculation of activation curves. (C) Mean steady-state activation curves at different postnatal ages (as indicated in A) were obtained by plotting normalized tail current amplitudes against the step potential and fitting them with a Boltzmann function (ACI, gray triangles; WAG/Rij, black circles). The number of averaged cells ranged between 8 and 38 for the different age-groups and rat strains.

Fig. 2.

Change in I_h properties during postnatal development in TC neurons from different rat strains. **(A)** Mean half-maximal activation voltages (V_h) vs. postnatal age reveals a depolarizing and a hyperpolarizing shift in V_h with age in ACI (gray triangles) and WAG/Rij (black circles) rats, respectively. At all developmental stages significantly different results were obtained for the comparison of the two strains. **(B)** Mean half-maximal activation voltages (V_h) vs. postnatal age for Long Evans (LE) and Sprague Dawley (SD) (Kanyshkova et al., 2009) rats ($n = 4-17$ per data point). For comparison a fit through the data from WAG/Rij rats (black line) is shown. **(C)** Mean half-maximal activation voltages (V_h) of different rat strains are shown. Data from animals ranging from P19 to P23 were averaged. Numbers in parenthesis indicate the number of averaged cells. **(D)** Mean current density vs. postnatal age reveals increasing I_h currents. Current densities were calculated by dividing I_h amplitudes at -130 mV by the membrane capacitance obtained during whole-cell recordings. Data from animals $\geq P14$ were significantly different between ACI (gray bars) and WAG/Rij (black bars) rats. The number of averaged cells ranged between 12 and 19 for the different age-groups and rat strains. **(E)** Monoexponential fits (continuous lines) to I_h current traces elicited by a step from -40 to -130 mV in ACI (open triangles) and WAG/Rij (open circles) rats (P30). **(F)** Bar graph representation of the mean activation time constants at a potential of -130 mV vs. postnatal age in ACI (gray bars) and WAG/Rij (black bars) rats (color labeling as in panel D). The number of averaged cells ranged between 12 and 31 for the different age-groups and rat strains.

Fig. 3.

Dependency of the I_h activation curve on the recording temperature. **(A)** Representative current traces obtained from cells of WAG/Rij (left panel) and ACI (right panel) rats at postnatal ages P18 and P19 at a recording temperature of 30°C. Currents were elicited from a holding potential of -40 mV by using a hyperpolarizing protocol reaching a final potential of -130 mV (3.5 s step duration). **(B)** Mean steady-state activation curves for 30°C were obtained by plotting normalized tail current amplitudes against the step potential and fitting them with a Boltzmann function (ACI, gray triangles, $n = 7$; WAG/Rij, black circles, $n = 6$). **(C)** Mean V_h values of activation curves obtained at 22°C, 30°C and 35°C from ACI and WAG/Rij rats (P18–23) as indicated. The number of averaged cells ranged between 6 and 28 for the different temperature-groups and rat strains. **(D)** Mean values of I_h amplitude obtained at different temperatures from ACI and WAG/Rij (P18–23) rats as indicated. The number of averaged cells is as in panel C. **(E)** Bar graph representation of the mean fast (τ_{fast}) and slow (τ_{slow}) time constants of activation at a potential of -130 mV vs. temperature in ACI (gray bars) and WAG/Rij (black bars) rats at 30°C and 35°C. The number of averaged cells ranged between 6 and 9 for the two temperatures and rat strains. (*= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$).

Fig. 4.

Quantitative analyses of postnatal protein expression of HCN channels in dLGN tissue. HCN1 (A), HCN3 (B), HCN2 (C), and HCN4 (D) were detected by means of Western blotting (A = ACI, gray bars; W = WAG/Rij, black bars). Data from 4 independent protein isolations per age-group and strain were averaged (*= $p < 0.05$; **= $p < 0.01$).

Fig 5.

Immunohistochemical characterization of HCN channel localization in dLGN. **(A)** Specific antibodies directed against HCN1 (1st column), HCN2 (2nd column), HCN3 (3rd column), and HCN4 (4th column) were applied in WAG/Rij (upper row) and ACI (lower row) rats. The results reveal the typical expression pattern of thalamic neurons with moderate HCN1 expression, strong HCN2 expression, lack of HCN3 expression, and strong HCN4 expression. Note that HCN1-specific immunoreactivity was stronger in WAG/Rij compared to ACI rats. Scale bars: 100 μ m. In all images the localization of the dLGN is indicated by a dotted line. **(B)** Co-staining of HCN1- (upper right images), HCN2- (lower left images), and HCN4-specific (lower right) antibodies with an antibody directed against the dendritic marker protein MAP2 (as indicated). Dendritic expression of HCN1, HCN2, and HCN4 was low, medium, and strong, respectively. Co-staining of HCN1- and NeuN-specific antibodies revealed strong overlap (upper left images). Scale bars: 25 μ m.

Fig. 6.

Protein expression of HCN channel interacting proteins in rat dLGN at P30. Expression levels of TRIP8b (**A**, upper panel) and Filamin A (**B**, upper panel) in the dLGN at P30 of WAG/Rij (WAG) and ACI rats were evaluated using Western Blot with β-actin as a housekeeping gene. For both TRIP8b and FilA protein expression levels were significantly different between epileptic and control dLGN at P30 samples (**A**, **B**, lower panels). No protein expression was found for MirP1 in dLGN at P30 of both WAG/Rij and ACI (**C**, upper panel) whereas positive signals were detected in cortex of both rat strains (**C**, lower panel). (*= $p < 0.05$; **= $p < 0.01$).

Fig. 7.

Mathematical modeling of thalamic network oscillations at a temperature of 36°C. **(A)** Exemplary voltage traces of the thalamocortical relay neuron TC1 of the network depicted in panel **B**. In the control condition (con) I_h properties set as in the original model (Destexhe et al., 1996) were used, thereby representing the non-epileptic state. In three additional sets of computer simulations, I_h properties were changed in an additive manner: firstly, a 20% faster activation kinetic was implemented ($\Delta\tau$); secondly, a 19% increase in I_h conductance was added ($\Delta\tau + \Delta g_h$); thirdly, a 6 mV hyperpolarizing shift in V_h was added ($\Delta\tau + \Delta g_h + \Delta V_h$). Finally, additional modifications comprising an 20% and 15% increase in I_T conductance in both TC and RE neurons, respectively, was implemented on top ($\Delta\tau + \Delta g_h + \Delta V_h + \Delta g_T$) thereby representing the full epileptic state. Starting potential of all traces was -70 mV. Scale bars: 500 ms, 40 mV. **(B)** Network structure. Both TC and RE neurons were exact clones. **(C, D, E)** Quantitative analysis of oscillation onset **(C)** and the last three bursts of TC1 (shown in panel **A**) under different modeling conditions (as indicated) revealed a change in the interburst **(D)** and intraburst **(E)** frequency. The latter could not be determined for condition Δg_h where only one action potential was present.

Fig. 8. Mathematical modeling of thalamic network oscillations at a temperature of 23°C. **(A)** Exemplary voltage traces of the thalamocortical relay neuron TC1 of the network depicted in Fig. 7B. All changes in I_h and I_T properties were done as in Fig. 7A, but the modeling temperature was set to 23°C. Starting potential of all traces was -70 mV. Scale bars: 1 s, 20 mV. **(B)** Comparison of burst activity at 23°C and 36°C as indicated. Scale bars represent 100 ms and 20 mV. **(C, D, E)** Quantitative analysis of oscillation onset **(C)** and the last three bursts of TC1 under different modeling conditions (as indicated) revealed a change in the interburst **(D)** and intraburst **(E)** frequency.