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Suppression of HCN channel function in thalamocortical neurons prevents genetically determined and pharmacologically induced absence seizures

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Author contributions

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ABSTRACT

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and the I_h current they generate contribute to the pathophysiological mechanisms of absence seizures (ASs), but their precise role in neocortical and thalamic neuronal populations, the main components of the network underlying AS generation remains controversial. In diverse genetic AS models, I_h amplitude is smaller in neocortical neurons and either larger or unchanged in thalamocortical (TC) neurons compared to non-epileptic strains. A lower expression of neocortical HCN subtype 1 channels is present in genetic AS-prone rats and HCN2 Knock-Out mice exhibit ASs. Furthermore, whereas many studies have characterized I_h contribution to "absence-like" paroxysmal activity *in vitro*, no data is available on the specific role of cortical and thalamic HCN channels in behavioural seizures. Here, we show that the pharmacological block of HCN channels with the antagonist ZD7288 applied via reverse microdialysis in the ventrobasal thalamus (VB) of freely moving male Genetic Absence Epilepsy Rats from Strasbourg decreases TC neuron firing and abolishes spontaneous ASs. A similar effect is observed on γ-hydroxybutyric acid-elicited ASs in normal male Wistar rats. Moreover, thalamic knockdown of HCN channels via virally-delivered shRNA into the VB of male Stargazer mice, another genetic AS model, decreases spontaneous ASs and I_h-dependent electrophysiological properties of VB TC neurons. These findings provide the first evidence that block of TC neuron HCN channels prevents ASs and suggest that any potential anti-absence therapy that targets HCN channels should carefully consider the opposite role for cortical and thalamic I_h in the modulation of absence seizures.

87 **Significance statement**

88 Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels play critical roles in the
89 fine tuning of cellular and network excitability and have been suggested to be a key element of
90 the pathophysiological mechanism underlying absence seizures. However, the precise
91 contribution of HCN channels in neocortical and thalamic neuronal populations to these non-
92 convulsive seizures is still controversial. In the present study, pharmacological block and genetic
93 suppression of HCN channels in thalamocortical neurons in the ventrobasal thalamic nucleus
94 leads to a marked reduction of absence seizures in one pharmacological and two genetic rodent
95 models of absence seizures. These results provide the first evidence that block of TC neuron
96 HCN channels prevents ASs.

INTRODUCTION

Absence seizures (ASs), which consist of relatively brief periods of lack of consciousness accompanied by spike-and-wave discharges (SWDs) in the EEG, are a feature of many genetic generalized epilepsies and believed to be generated by abnormal neuronal activity in reciprocally connected neocortical and thalamic territories (Crunelli and Leresche, 2002; Blumenfeld, 2005). Among the different voltage-dependent channels that may be involved in the pathophysiological mechanisms of these non-convulsive seizures, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which are present in the vast majority of cortical and thalamic neurons, have been extensively investigated (Huang et al., 2009; Noam et al., 2011; Reid et al., 2012). However, the selective contribution of cortical versus thalamic HCN channels in ASs is still not fully understood (Noam et al., 2011).

Several studies in humans have reported an association between HCN channel mutations and genetic epilepsies: in particular, mutations in HCN subtype 1 (HCN1) and HCN2 were reported in patients with genetic generalized epilepsies (Tang et al., 2008; DiFrancesco et al., 2011), including febrile seizures and early infantile epileptic encephalopathy (Nakamura et al., 2013; Nava et al., 2014). However, it is difficult to draw any firm conclusion from these human studies since ASs are not the only phenotype present in these diverse forms of epilepsy.

As far as cellular effects are concerned, *in vitro* studies have shown that blocking the I_h current that HCN channel generate in thalamocortical (TC) neurons enhances bicuculline-elicited synchronized thalamic activity resembling absence paroxysms by increasing burst firing in TC neurons (Bal and McCormick, 1996). The observation that mice with spontaneous or induced genetic ablation of HCN subtype 2 (HCN2) channels exhibit ASs (Ludwig et al., 2003; Chung et al., 2009; Heuermann et al., 2016) has been interpreted as providing support to this view.

120 However, since HCN2 channels are highly expressed in both cortical and thalamic neurons
121 (Notomi and Shigemoto, 2004), these *in vivo* data cannot be used to draw any firm conclusion on
122 a pro-absence role of thalamic HCN channels. Indeed, in genetic AS models I_h of neocortical
123 neurons is smaller (Strauss et al., 2004; Kole et al., 2007) resulting in increased temporal
124 summation of EPSPs and enhanced burst firing (Strauss et al., 2004), whereas in TC neurons I_h
125 has been reported to be either larger or unchanged compared to non-epileptic strains (Kuisle et
126 al., 2006; Kanyshkova et al., 2012; Cain et al., 2014) and the ability of burst firing is decreased
127 (Cain et al., 2014). More importantly, the precise influence of HCN channels of thalamic versus
128 cortical neurons on behavioral seizures has never been investigated. This, together with the
129 complexity of the diverse cellular and synaptic effects that I_h can exert under normal conditions
130 and their consequences on paroxysmal network excitability (Huang et al., 2009; Noam et al.,
131 2011; Reid et al., 2012), makes it difficult to draw causal links between HCN channel function
132 and ASs.
133 Here we directly investigated the role of thalamic HCN channels in ASs using both
134 pharmacological and genetic tools to selectively suppress HCN channel function in TC neurons
135 in rodent models of absence epilepsy under freely moving conditions. We report that bilateral
136 reverse microdialysis application of the HCN blocker ZD7288 into the ventrobasal thalamic
137 nucleus (VB) blocks ASs in two well-established absence models, the Genetic Absence Epilepsy
138 Rats from Strasbourg (GAERS) (Depaulis et al., 2015) and the γ-hydroxybutyric acid (GHB)-
139 injected Wistar rats (Venzi et al., 2015), and decreases tonic, but not burst, firing in TC neurons
140 of freely moving GAERS. Furthermore, silencing thalamic HCN gene expression with shRNA in
141 the VB nucleus of Stargazer mice, another genetic absence epilepsy model (Fletcher and Frankel,
142 1999), is effective in reducing spontaneous ASs. Thus, in contrast to inferences from previous *in*

143 *vitro* studies in thalamic slices (Kuisle et al., 2006; Kanyshkova et al., 2012) and *in vivo*
144 investigations using brain-wide HCN channel manipulations (Ludwig et al., 2003; Chung et al.,
145 2009), block of TC neuron HCN channels prevents ASs.

MATERIALS AND METHODS

All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedure) Act, 1986, and local ethics committee and expert group guidelines (Lidster et al., 2015). All efforts were made to minimize animal suffering and the number of animals used. Experiments were performed on adult (2-5 month old) male Wistar (Harlan Laboratories) and GAERS (School of Bioscience, Cardiff, UK) rats, and Stargazer mice (School of Bioscience, Cardiff, UK), which were maintained on a normal diet and under an 8.00am-8.00pm light-on/light-off regime.

Surgical procedures for recordings under anesthesia

Implantation of microdialysis and silicone probes for recording under ketamine/xylazine anesthesia rats were carried out as described in David et al., (2013) and Taylor et al., (2014). In brief, initial dose of anesthetics (ketamine, 120 mg/kg, and xylazine, 20 mg/kg) and maintenance dose (ketamine, 42 mg/kg/h, and xylazine 7 mg/kg/h) were injected intraperitoneally (i.p). Body temperature was maintained at 37°C with a heating pad and measured with a rectal probe. A microdialysis probe (CMA 12 Elite), with 2 mm dialysis membrane length, was slowly (500µm every 5 min) inserted unilaterally into the ventrobasal (VB) thalamus (AP -3.2 mm, ML 5.3 mm, DV -7 mm) (Paxinos and Watson, 2008) at a 16° angle with respect to the vertical axis such that its final position would rest between 0.05-1 mm away from the tip of the silicone probe, which was subsequently inserted. Artificial cerebrospinal fluid (aCSF) alone or containing ZD7288 (500 µM in the inlet tube) was then delivered through the dialysis at a constant flow rate of 1 µL per minute. A 32-channel silicone probe with four shanks (Buzsaki32L-CM32, NeuroNexus Technologies) was then slowly lowered into the VB (AP -3.2 mm, ML 2.8 mm, DV -4.5 mm)

and the full-band signal including unit activity was recorded during 40 minutes of aCSF and 1 hour of ZD7288 reverse microdialysis injection.

Surgical procedures for EEG recordings in freely moving rats

Rats under isoflurane anesthesia were implanted bilaterally with guide cannulas for microdialysis probes so that their tips rested just above the VB (AP -3.2 mm, ML ±2.8 mm, DV -4.5 mm). Frontal (AP +2.0 mm, ML ±2.0 mm) and parietal (AP -1.8 mm, ML ± 5.0 mm) EEG screws were then implanted and the rats were allowed to recover for at least 5 days. Twenty-four hours before each experiment, microdialysis probes with 2 mm dialysis membrane were inserted into the VB guide cannulas. On the day of recording, the rat was connected to the recording apparatus to habituate to the recording cage for one hour. While habituating, aCSF was delivered via the inlet tube of the dialysis probes at 1 µL/min to allow stabilization of the surrounding tissue. For GAERS, the recording session consisted of one hour of aCSF injection followed by 100 min of administration of either aCSF or ZD7288 (1 - 500 µM in the inlet tube) solutions, while recording the EEG continuously throughout the recording session. For recording in GHB-injected rats, the one hour habituation was followed by a 40 min period where either aCSF or ZD7288 (500 µM in the inlet tube) solutions were delivered through the inlet tubing. Then, either saline or gamma-butyrolactone (GBL), a GHB pro-drug, was injected i.p (100 mg/kg) and the EEG was recorded for one hour. Rats and mice were randomly assigned to receive either aCSF or ZD7288 first, and then followed by the other solution a week later. No animal was treated more than twice.

Neuronal recordings in freely moving rats

When microdialysis was combined with unit recordings in freely moving conditions, procedures similar to those described in Taylor et al., (2014) were used. First, one guide cannula was implanted with the silicone probe mounted on a microdrive and its tip placed above the VB. On the day of the experiment, the dialysis probe delivering aCSF was inserted into the guide cannula, and the microdrive was advanced until suitable thalamic units were found. A control period of 20 min was always allotted before delivering ZD7288 (500 μM in the inlet dialysis tube). Note that unless otherwise indicated the concentration of ZD7288 is always expressed in the test and figures as that of the solution perfused in the dialysis inlet tube. The corresponding tissue concentration can be deduced considering the general dialysis recovery of 5-10% (Chan and Chan, 1999; David et al., 2013; Montandon and Horner, 2013).

HCN-targeting and non-targeting shRNA

The shRNA design is similar to that in our previously published papers (Chávez et al., 2014; Neuner et al., 2015). In brief, the HCN-targeting shRNA sequence (CAGGAGAAGTACAAGCAAGTAGA) was chosen to target a conserved region within the open reading frame of mouse and rat HCN1–4. A non-targeting shRNA (GAGGATCAAATTGATAGTAAACC), which showed no homology to any known genes was used as a control. Both sequences were screened for sequence homology to other genes with NCBI-BLAST (www.ncbi.nlm.nih.gov/BLAST) and did not contain known immune response inducing motifs (GTCCTTCAA, CTGAATT, TGTGT, GTTGTGT) (Hornung et al., 2005; Judge et al., 2005; Robbins et al., 2009). In addition, both sequences follow rational designs developed for siRNAs (Amarzguioui and Prydz, 2004; Hsieh et al., 2004; Reynolds et al., 2004; Takasaki et

al., 2004; Ui-Tei et al., 2004; Huesken et al., 2005; Vert et al., 2006; Ichihara et al., 2007; Katoh and Suzuki, 2007).

Desalted shRNA oligos containing a modified miR155 loop (GTTTTGGCCACTGACTGAC) and overhangs complementary to BamHI and XhoI restriction sites were custom synthesized (Life Technologies), resuspended using Duplex Buffer (Integrated DNA Technologies), and cloned into a "CreOff" adeno-associated virus (AAV) vector with a floxed cassette that contains a U6 polymerase III promoter to drive shRNA expression and a CMV promoter to drive eGFP expression for identification of transduced neurons. Constructs were cloned into pFB-adeno-associated virus (AAV) shuttle plasmids to allow for a baculovirus expression system-based AAV production. AAV constructs were maintained and propagated with Stbl3 competent cells (Life Technologies). Strict attention was paid to the integrity of the vector inverted terminal repeats in plasmid preparations. All AAV plasmids were verified by diagnostic enzyme digestions. High titer AAVs with serotype 9 were commercially produced by Virovek (Hayward, CA), and included the green fluorescent protein eGFP under a CMV promoter (Chávez et al., 2014; Neuner et al., 2015) to label infected cells (Fig. 7A).

Viral injection

Eighteen Stargazer mice were implanted with epidural fronto-parietal stainless steel EEG screws under isoflurane anesthesia, as described earlier for rats. A craniotomy was performed above the VB (AP -1.8 mm, ML 1.5 mm) (Paxinos and Watson, 2008) and a A10_l Gastight Hamilton syringe with a 34 GA needle that was filled with mineral oil and viral vector (see below) was inserted vertically. Needles were then lowered slowly into the thalamus (DV: -3.0 mm from the pia) and left in place for 10 min. The viral vector was diluted to a final titer of 2.18×10^{13} vg/ml

(Control, non-targeting, shRNA; vg: viral genome copy) and 1.145 x 10^{13} vg/ml (HCN-shRNA) and injected bilaterally (2 x 500 nL) at a rate of 100 nl/min using a programmable micro-pump (UMP3–1, WPI) and allowed to disperse for a further 10 min before the needle was slowly retracted.

Normal (3-month old) male C57BL/6J mice were injected with HCN-targeting (n=6) and non-targeting (n=7) shRNA (as described above) into the VB for investigating the effect of these shRNAs on the *in vitro* electrophysiological properties of TC neurons. Since the results from these normal mice were similar to those obtained from Stargazer mice, the electrophysiological data from the two strains were pooled.

Thalamic slice preparation, in vitro whole-cell recording and data analysis

Thirty-two to thirty-six days following the viral injection, a modified method optimized for adult mice was used to prepare thalamic slices containing the VB (Ting et al., 2014). Briefly, mice were deeply anesthetized with Ketamine/Xylazine (80/8 mg/kg) and transcardially perfused with 20-25 ml cold (4°C) ACSF containing (in mM): 93 N-methyl-D-glucamine, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 N-acetyl-L-cysteine, 5 Na-ascorbate, 3 Na-pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$. The brains were then quickly removed from the skull, blocked and sliced (320 µm thickness) in the coronal plane. After a short (12 min) recovery in a warmed (35°C) NMDG ACSF, the slices were incubated at room temperature (20 °C) in HEPES holding ACSF containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.3 $NaHCO_3$, 20 HEPES, 25 glucose, 5 N-acetyl-L-cysteine, 5 Na-ascorbate, 3 Na-pyruvate, 3 $CaCl_2$ and 1.5 $MgSO_4$. For recording, slices were submerged in a chamber perfused with a warmed (35°C) continuously

oxygenated (95% O_2, 5% CO_2) ACSF containing (in mM): 130 NaCl, 3.5 KCl, 1 KH_2PO_4, 24 $NaHCO_3$, 1.5 $MgSO_4$, 3 $CaCl_2$, and 10 glucose.

Whole-cell patch-clamp recordings of TC neurons located in the VB were performed using a Heka EPC9 amplifier (Heka Elektronik). Patch pipettes (tip resistance: 4–5 MΩ) were filled with an internal solution containing the following (in mM): 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na_2, 10 HEPES, 10 kreatin-phosphate, 8 Biocytin, pH 7.25, osmolarity 275 mOsm. The liquid junction potential (-13 mV) was corrected offline. Access and series resistances were constantly monitored, and data from neurons with a >20% change from the initial value were discarded. The ratio of the input resistance at the peak (R_{peak}) and that at the end of the 1 sec long voltage step (R_{ss}) (as illustrated in Fig. 5) was taken as a measurement of the depolarizing "sag" elicited by HCN channel activation. Action potential amplitude was measured from threshold (20 mV/ms on the first derivative of the membrane potential) to the peak of the action potential. Analysis of these whole-cell data was performed using custom routines written in Igor.

In vivo data acquisition and analysis

Spike sorting. For unit recordings, signals were digitized with 64 channel integrated recording system (Plexon Version 2.3.0, 2006) at 20 kHz with 16-bit resolution. EEG data were low-pass filtered with a windowed sinc filter at 100 Hz and downsampled to 200 Hz. Spike sorting and data preprocessing were performed with the Klusters, Neuroscope, NDManager and Klustakwik software suites (Harris et al., 2000; Hazan et al., 2006). A typical high frequency bursts of action potentials of TC neurons was defined as a group of spikes that were separated by less than 7 ms, was preceded by a 100 ms period of electrical silence, and showed the characteristic decelerando pattern within a burst (Domich et al., 1986).

Spike and wave discharge analysis. The EEG was recorded using an SBA4-v6 BioAmp amplifier (SuperTech), digitized at 1 kHz (Micro3 D.130, Cambridge Electronic Design) and analyzed with CED Spike2 v7.3 and Matlab (R2011b, MathWorks). Spike-and-wave discharges (SWDs) that accompanied behavioural ASs were detected semi-automatically with the aid of the SeizureDetect script (kindly provided by Dr. Steven Clifford (Cambridge Electronic Design) in Spike2 v7.3 as described in detail in Venzi et al. (2016). For analysis of GAERS SWDs, data were normalized in two steps: first, all values were measured as percentage variation compared to the average values of the control periods, then all individual percentage values were recalculated as percentage change compared to the average value at each time-point of the control group (set to 0% change). Only the second step of this calculation was applied to the SWDs of GHB and Stargazer data for which no control period exists. The time-frequency representation of SWDs was performed with a wavelet transform of SWD, as described in (David et al., 2013). The frequency of SWDs was estimated from the distribution of the intervals that separate each spike-and-wave complex (SWC) extracted with the SeizureDetect programme (Cambridge Electronic Design).

Histology

To examine the relative position of the tracks of the microdialysis and silicone probes, methods similar to those described in David et al (2013) and Taylor et al. (2014) were used. Data were excluded from analysis if either the dialysis or the silicone probes were misplaced.

For HCN2 immunohistochemistry, brains were perfused with 4% PFA then stored in 0,1 M PB with 0.05% sodium azide at 4°C prior to slicing at 40 µm on a vibratome (VT1000S, Leica Microsystems). After 1 hour in 5% normal Horse serum (NHS) blocking solution, the sections

were incubated in the primary antibodies: Rabbit anti-HCN2 (Alomone Labs, 1:200), diluted in 0.1 M Tris-buffered saline (TBS, pH 7.4) /0.1% Triton-X 100 (Sigma) + 3% followed by the secondary antibody Cy3 Donkey anti-Rabbit (Jackson Immunoresearch, 1:500) and DAPI staining (Millipore, 1:200) and then mounted in Vectashield (Vector Labs) prior to imaging with a confocal microscope (FW 1000, Olympus). Quantitative analysis of HCN and GFP expression levels were performed with the ImageJ software. Zones of interest of neuronal cell bodies were delimited manually and the intensity was measured in the respective spectra (green: λ= 594 nm for HCN, red: λ=488 nm for GFP). GFP green fluorescence intensity (in arbitrary unit(au)) was taken as an indicator of viral infection in a cell and correlated with the anti-HCN antibody red fluorescence intensity (see Fig. 6D).

Experimental Design and Statistical Analysis

Experiments with reverse microdialysis on thalamocortical unit activity (Figs. 1 and 2), were designed so that at least 5 neurons could be recorded per data point (David et al, 2013; McCafferty et al, 2018). Experiments involving SWD measurement involved a minimum of 6 to 11 animals, which in previous similar studies allowed statistical significance to be detected (Cope et al 2009). Immunohistological procedures were performed on 3 animals per treatment group in order to collect enough thalamic slices (Cope et al., 2009).

Group comparisons were performed using the Wilcoxon signed rank test and the Wilcoxon rank-sum test were used for paired or unpaired datasets. A logistic regression of the dose-dependent effect of ZD7288 on GAERS SWDs was performed with Sigma-plot. Linear regressions were performed for correlating the HCN-related fluorescence intensity to the GFP-related fluorescence intensity. Circular statistics was performed using the Kuiper 2-sample test. All quantitative data

328 in the text and figures are expressed as mean ± standard error of the mean (SEM). Values were
329 defined as outliers if they were larger than $q_3 + w(q_3 - q_1)$ or smaller than $q_1 - w(q_3 - q_1)$,
330 where q_1 and q_3 are the 25th and 75th percentiles, respectively, and w is 1.5 which corresponds to
331 ±2.7 standard deviations for normally distributed data (as defined in Matlab, Mathworks).

RESULTS

Time-course and diffusion of microdialysis-applied ZD7288

We first characterized the time-course and diffusion of the I_h antagonist ZD7288 applied via reverse microdialysis into the centre of the VB, the thalamic nucleus somatotopic with the cortical "initiation site" of ASs in genetic rat models (Meeren et al., 2002; Polack et al., 2007). To this end, we measured the firing rate of TC neurons (the only neuronal population present in this thalamic nucleus) using a silicone probe closely positioned to a dialysis probe in ketamine/xylazine anesthetized Wistar rats (n=21) (Fig. 1A). Under this condition, the EEG mostly expressed sleep slow waves and TC neurons preferentially fired high frequency bursts of action potentials (Fig. 1B). Unilateral application of 500 μM ZD7288 in the inlet dialysis tube, corresponding to a tissue concentration of 25-50 μM for a standard dialysis recovery of 5-10% (Chan and Chan, 1999; David et al., 2013; Montandon and Horner, 2013), led to a maximum and sustained firing reduction of about 50% within 40 min from the start of the injection (Fig. 1C). This action was apparent in neurons located <600 μm from the dialysis probe but was absent in those located ≥600 μm away from the dialysis probe (Fig. 1D). As it has been previously reported in anesthetized rats during ZD7288 iontophoretic application (Budde et al., 2005), bursts recorded in the continuing presence of dialysis-applied ZD7288 were characterized by a significantly increased number of action potentials ($p=6.9.10^{-5}$, n=45 neurons) (Fig. 1E). Thus, in view of the dimensions of the rat VB thalamic nucleus (Paxinos and Watson, 2008), ZD7288 applied via a microdialysis probe placed in the middle of the VB is able to affect TC neuron firing in almost the entirety of this thalamic nucleus (red circled, striped area in Fig. 1A) and largely sparing the NRT, as we reported previously for a similarly applied Ca^{2+} channel blocker (David et al., 2013; Taylor et al., 2014).

Neuronal effects of microdialysis-applied ZD7288 during ASs and interictal periods

No study so far has investigated the effect of I_h on TC neuron firing under natural conditions (i.e. in non-anesthetized animals), probably because of technical difficulties. Thus, having established the time-course and diffusion of ZD7288, we then applied this antagonist by unilateral microdialysis into the VB while simultaneously recording firing activity of single TC neurons in a freely moving AS model, the GAERS (n=3), with a close-by positioned silicone probe (Fig. 2A,B). In contrast with the increase observed in the same neuronal type *in vitro* (Lüthi et al., 1998), analysis of the activity of TC neurons (n=7) showed that ZD7288 significantly decreased the total firing by about 60 and 40% interictally and ictally, respectively (Fig. 2C). When different types of firing were analyzed individually, tonic firing was significantly reduced both ictally and interictally by ZD7288 (Fig. 2D) whereas burst firing was not (Fig. 2E). Importantly, in contrast to the results obtained under anesthesia (Fig. 1E), the number of spikes per burst in TC neurons recorded in freely moving rats was not significantly affected by ZD7288 (Fig. 2F). Finally, the time-distribution of the extracellularly recorded action potentials with respect to the SWC (analyzed with circular statistics) was different between SWDs recorded during ACSF application and those during ZD7288 injection (ACSF mean angle: -2.2°; ZD7288 mean angle: 3.4°, p=0.001, Kuiper test) (Fig. 2G, left and top right plots), with the maximal difference between these two experimental conditions occurring just before 0 degree (Fig. 2G, bottom right plot).

Pharmacological block of HCN channels in VB TC neurons impairs the expression of ASs

We next investigated the effect of blocking I_h in VB TC neurons on spontaneous genetically determined ASs in freely moving GAERS (Fig. 3A). Application of ZD7288 by bilateral reverse microdialysis in the VB produced a marked and concentration-dependent (EC_{50}: 29 µM) decrease of the total time spent in seizures, with 500 µM almost abolishing ASs (82±3%, $p=4.10^{-4}$, n=6), while no significant effect was observed with 1 µM (n=8) (Fig. 3B-C). These effects were mostly driven by a marked reduction (75±4%, $p=4.10^{-4}$) in the number of seizures (Fig. 3E), though a small decrease in the length of individual seizures (34±13%, p=0.025) was also observed (Fig. 3D).

Since genetically determined and pharmacologically induced ASs may depend on different cellular and network mechanisms (Crunelli and Leresche, 2002; Blumenfeld, 2005), the action of ZD7288 was then investigated in ASs elicited by systemic injection of a GHB pro-drug, GBL (hereafter referred to as GHB) (Venzi et al., 2015), in Wistar rats implanted with bilateral dialysis probes in the VB (Fig. 4A). Well-separated ASs mainly occur up to 20-30 minutes following GBL administration (Fig. 4B) (Venzi et al., 2015). Therefore, GHB was injected 40 min after the start of 500 µM ZD7288 microdialysis application, i.e. at a time when the effect of ZD7288 throughout the VB has reached steady-state (cf. Fig. 1C). As observed in GAERS, ZD7288 significantly decreased (58±9%, $p=9.4.10^{-4}$, n=11) the total time spent in seizures in the first 20 min after GHB injection (Fig. 4C). However, the ZD7288-elicited reduction was smaller than that observed in GAERS and was mainly due to a reduction in the length of individual seizures (40±7%, p=0.016) (Fig. 4D) with no statistically significant effect on the number of seizures (Fig. 4E). No effect of ZD7288 on GHB-elicited ASs was observed beyond 20 min after GHB injection (data not shown). Thus, the pharmacological block of I_h in VB TC neurons by

ZD7288 decreases both genetically determined and pharmacologically elicited ASs in freely moving animal models.

Cellular effects of the HCN-targeting shRNA

In addition to the pharmacological block, we investigated whether reducing the expression of HCN channels in the VB using shRNA could also suppress ASs. First, we assessed the functional effect of this genetic approach by monitoring the electrophysiological properties of VB TC neurons in slices taken from mice previously (32-36 days) injected with either HCN-targeting or non-targeting shRNA in this thalamic nucleus (see Methods). Only TC neurons that showed eGFP fluorescence were patch-clamped in slices from HCN-targeting shRNA mice. The resting membrane potential of TC neurons in slices from animals injected with HCN shRNA (-68±6 mV, n=18) was more hyperpolarized than in mice that had received the non-targeting shRNA (-63±7 mV, n=18, p=0.032) (Fig. 5D). Moreover, the depolarizing "sag" of hyperpolarizing voltage steps was almost abolished in VB TC neurons infected with HCN-targeting shRNA compared to non-targeting shRNA (Fig. 5A,B), resulting in a similar input resistance at steady-state (R_{in}-ss) in the two groups (217±75 MΩ, n=18, and 186±73 MΩ, n=24, respectively, p=0.56) (Fig. 5F). Moreover, the steady-state and peak input resistance ratio (R_{in}-ss/R_{in}-peak) was significantly larger in neurons from HCN-targeting than non-targeting shRNA (0.94±0.08 and 0.82±0.01, n=18 and 24, respectively, $p=6.2.10^{-5}$) (Fig. 5E), indicating that the "sag" difference is not a consequence of a difference in R_{in}. Application of ZD7288 (10 μM) to 5 TC neurons transfected with HCN-targeting shRNA abolished the small remaining "sag" (where present) but had no effect on the resting membrane potential (not shown). In contrast, action potential properties were not affected (threshold: -45±6 vs -48±5 mV; amplitude:

82±2 mV vs 80±2 mV, both n=15 and p=0.17 and p=0.46 respectively) (Fig. 5G,H). These data demonstrate that our HCN-targeting shRNA does selectively affect I_h-dependent membrane properties of VB TC neurons without altering other neuronal properties.

Genetic ablation of HCN channels reduces ASs

Having established the functional effect of the HCN-targeting shRNA on TC neurons membrane properties, we next assessed the effect of this genetic suppression of I_h on ASs in 9 Stargazer mice, a monogenic mouse model of ASs (Fletcher and Frankel, 1999), which had received bilateral injection of viral construct into the VB. Another group (n=9) of Stargazer mice was bilaterally injected with a non-targeting shRNA. ASs were then monitored every four days for over a month. A statistically significant reduction of the total time spent in seizures (57±12 and 45±9%, p=0.036 and p=0.029, n=9) and the average length of individual seizures (38±7 and 31±6%, both p=0.035 and p=0.043) was observed in HCN-targeting compared to non-targeting shRNA injected mice at 28 and 32 days post-injection, respectively (Fig. 6A-C). The reduction in the average number of seizures was not significant at both days (35±14 and 8±12%, respectively, p=0.056 and p=0.42) (Fig. 6D).

At the end of the behavioural experiment (i.e. day 32 post-injection), the brain of the Stargazer and wild-type mice, that had been injected, were harvested to measure GFP and HCN expression in thalamic and cortical slices (Fig. 7B). Triple labeling of VB TC neurons showed the co-localization of GFP, HCN2 and DAPI in all mice (Fig. 7C). As shown in Fig. 7C,D, in HCN shRNA-infected mice TC neurons that were immuno-positive for GFP had a low HCN immunoreactivity compared to non-targeting shRNA-infected animals. Indeed, a negative correlation was observed between HCN and GFP immune-staining in 5 out of the 6 slices that

had received the HCN shRNA (Fig. 7D, bottom, while no correlation was observed in all 6 mice injected with the missense RNA (Fig. 7D, top) (linear regression R^2=0.12, p=3.82.10^{-9} versus R^2=0.0004, p=0.77 when pooling all data points together). Notably, the expression of the virus was restricted to the VB, as indicated by the data showing that i) the GFP expression remained restricted to the thalamus and only projecting fibers were visible in the neocortex (Fig. 7B,C), and ii) cortical expression of HCN immunofluorescence was still prominent in the neocortex (Fig. 7C, bottom).

Effect of thalamic I_h block on SWD parameters

Finally, we compared some SWD parameters between control animals and those with a pharmacological or genetic suppression of thalamic HCN channel function. The time-frequency representation of SWDs indicated a decrease of the first harmonic (~14Hz) in the presence of ZD7288 in GAERS (Fig. 8A). To quantify this change, we calculated the averaged power spectra and found that the main frequency component of the SWDs at 7Hz had a significantly increased power while the harmonic at ~14 Hz was significantly smaller during the seizures that remained in the presence of ZD7288 in GAERS (Wilcoxon rank-sum test, control: n=142, ZD7288: n=45 seizures p=2.4.10^{-6}) (not shown). However, these changes were not observed following suppression of HCN channels with ZD7288 during GHB-elicited seizures and with shRNA in Stargazer mice. Moreover, the frequency of SWDs (estimated from the peak of interSWC-spike probability density (Fig. 8B-D, left panels) was not significantly different between control conditions and during the block of thalamic I_h for both spontaneous ASs in GAERS (control: 7.0±0.1 Hz, n=9; ZD7288: 6.8±0.1 Hz, n=6, p=0.11) and in Stargazer mice (control; 6.4±0.2 Hz,

469 n=7, shRNA: 6.1±0.2 Hz, n=8, p=0.44) (Fig. 8 B,D right panels) as well as for GHB-elicited ASs

470 (control: 6.8±0.5 Hz, n=6; ZD7288 7.0±0.5 Hz, n=8, p=0.82) (Fig. 8C, right panel).

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DISCUSSION

This study provides the first demonstration that i) a reduction of I_h function in TC neurons of three animal models of absence epilepsy does reduce ASs, and ii) the overall effect of blocking TC neuron HCN channels is a marked reduction in their firing rate both ictally and interictally. Therefore, in contrast to previous *in vitro* investigations and *in vivo* studies under anesthetic/neuroleptic regimes (Kuisle et al., 2006; Kanyshkova et al., 2012; Cain et al., 2014), these results demonstrate that I_h of TC neurons positively modulates the expression of ASs and support the view that the increased HCN channel function reported in TC neurons of genetic absence epilepsy models does contribute to and/or aggravate ASs and is not simply a seizure-related compensatory mechanism.

Action of ZD7288 in freely moving animals

Before discussing the implications of our findings for ASs, it is important to consider some issues related to ZD7288 action. First, since ZD7288 concentration in the neuronal tissue is about one order of magnitude smaller than that in the inlet tube of the microdialysis probe (Chan and Chan, 1999), we are confident that the tissue concentrations achieved in our study are similar to those reported by us and others as selective for I_h (Harris and Constanti, 1995; Hughes et al., 1998; Blethyn et al., 2006). Indeed, we observed a significant effect on ASs at ZD7288 tissue concentrations as low as 10μM. Moreover, the sigmoid shape of the ZD7288 concentration-response curve on GAERS ASs (Fig. 3C) speaks against an action on two different cellular targets under the freely moving conditions of this study. Indeed, in view of the standard 5-10% recovery rate of dialysis membranes, the EC50 (29 μM) of ZD7288 found here *in vivo* on the total time spent in seizures is similar to the 2 μM EC50 observed *in vitro* on I_h (Harris and

Constanti, 1995). It is also unlikely that ZD7288 effect on ASs is mediated by an unselective action on Na^+ channels since under the same microdialysis conditions ZD7288 decreases tonic, but not burst, firing of TC neurons in freely moving GAERS. Finally, the similarity in the effect on ASs with either the shRNA-elicited or ZD7288-mediated reduction of HCN channels in Stargazer or GAERS and GHB models, respectively, indicates that ZD7288 action under our experimental freely moving conditions is selective for I_h.

Second, the ability of ZD7288 to affect GHB-elicited ASs only in the first 20 minutes after GHB administration should not be surprising since we recently showed that it is only in this initial period following injection that GHB elicits well-separated *"bona fide"* ASs (with their clear behavioral and EEG components) while subsequent activity is characterized by a behavior more consistent with sedation/hypnosis and is accompanied by continuous low-frequency waves in the EEG (Venzi et al., 2015).

Third, a presynaptic, non-I_h-mediated action of ZD7288, that is present at concentrations known to affect I_h, was reported at hippocampal synapses (Chevaleyre and Castillo, 2002; Mellor et al., 2002). However, this ZD7288 effect is absent at neuromuscular junctions (Beaumont and Zucker, 2000; Beaumont et al., 2002) and has not been investigated at TC neuron synapses. Moreover, all the above data were obtained *in vitro* and thus it is not known whether this presynaptic, non-I_h-mediated action of ZD7288 occurs *in vivo* in freely moving animals (as those used in the present study), a condition where due to the more depolarized membrane potential than in *in vitro* experiments the voltage-dependent K^+ current(s) that might underlie this ZD7288 effect (Chevaleyre and Castillo, 2002) may not be operative. Indeed, the similarity of the action of ZD7288 and the HCN-targeting shRNA support the view that the observed effect of ZD7288

517 on genetically determined and pharmacologically induced ASs occur via this drug action on I_h of
518 TC neurons.
519
520 **I_h modulation of TC neuron ictal firing**
521 Microdialysis application of ZD7288 in the GAERS VB increased the burst duration in TC
522 neurons during ketamine/xylazine anesthesia, as shown previously in WAG/Rij rats under
523 pentobarbital or neuroleptic regime (Budde et al., 2005). In contrast, in freely moving GAERS
524 ZD7288 did not affect interictal and ictal burst firing and burst duration, while total and tonic
525 firing were decreased both in between and during ASs. This differential action of ZD7288 on the
526 two patterns of TC neuron firing is intriguing: it may be that the removal of the depolarizing
527 influence of I_h has little effect on burst firing as TC neurons are relatively depolarized during
528 ASs (Pinault et al., 1998), while it easily affects tonic firing. Alternatively, the somatodendritic
529 distribution of HCN channel subtypes in TC neurons (Abbas et al., 2006) may contribute
530 differently to the generation of tonic and burst firing (Connelly et al., 2015, 2016). Lastly, the
531 increase in tonic $GABA_A$ current that is present in TC neurons of the GAERS, Stargazer and
532 GHB models (Cope et al., 2009) may differently offset the action of a decreased I_h on the
533 summation of ictal corticothalamic EPSPs in these neurons (Ying et al., 2007), as it has been
534 shown in cortical pyramidal neurons (Chen et al., 2010).
535 The recent characterization of the firing dynamics of thalamic neurons in freely moving GAERS
536 and GHB models show that during ASs single TC neurons are mostly electrically silent or fire
537 single action potentials, with T-type Ca^{2+} channel-mediated bursts of action potentials occurring
538 rarely (McCafferty et al., 2018). Moreover, block of T-type Ca^{2+} channels of TC neurons does
539 not affect behavioral ASs and the synchrony of the ictal thalamic output to the neocortex

(McCafferty et al., 2018). These data, together with i) the ZD7288-induced reduction of tonic but not burst firing (Fig. 2), and ii) the block of behavioral ASs following the pharmacological or genetic suppression of TC neuron HCN channels (Figs. 3,4 and 6), suggest that the most likely role for HCN channels of TC neurons in ASs is a contribution to the membrane potential: thus, the block of HCN channels of TC neurons will hyperpolarize these neurons, decreasing the synchronized thalamic output to the neocortex, thus compromising the re-engagement of the cortical network during on-going seizures and ultimately being responsible for the reduction of ASs. Importantly, although the hyperpolarization induced by the block of I_h may increase T-type Ca^{2+} channel availability and thus the generation of a low threshold spike, as observed in thalamic slices and in the whole animal under anesthesia/neurolept regime (Fig. 1) (Budde et al., 2005), burst firing itself does not increase during ictal activity in the presence of ZD7288 in freely moving animals (Fig. 2), probably because of the less negative membrane potential in the latter than in the former vigilance state.

Opposite role for cortical and thalamic I_h in ASs

In the WAG/Rij and GAERS models, different, and at time contrasting, results have been reported on I_h of TC neurons (in either VB or dorsal lateral geniculate nucleus), including a clear increase in amplitude (Cain et al., 2014), an increased channel density but a hyperpolarized $V_{½}$ (Kanyshkova et al., 2012) or no apparent alteration in amplitude but an altered response to cAMP (Kuisle et al., 2006). The increased I_h of GAERS TC neurons has been suggested to be responsible for the reduced burst firing *in vitro* (Cain et al., 2014). In contrast, spontaneous or induced ablation of HCN2 channels lead to ASs and an enhanced ability to generate burst firing in TC neurons *in vitro* (Ludwig et al., 2003; Chung et al., 2009; Heuermann et al., 2016).

Our present results provide direct evidence that a pharmacological or genetic block of HCN channels in TC neurons reduces behavioral ASs in three freely moving absence epilepsy models. Though all these data may appear controversial, their apparent disagreement may originate from the "thalamocentric" interpretation of *in vivo* data obtained from brain-wide genetic manipulations that had explained these results on ASs by almost exclusive effects on thalamic network activity discarding any contribution by cortical HCN channels. Thus, in view of our results, it is more likely that the pro-absence effect of global HCN2 knock-out in normal mice (Ludwig et al., 2003) results from a cortical I_h loss-of-function. Similarly, a developmental decrease of HCN1 (but not HCN2) channels that leads to an I_h loss-of-function in the apical dendrites of layers 5 pyramidal neurons has been reported in the WAG/Rij absence model (Kole et al., 2007). In contrast, global HCN1 knock-out mice do not show an absence phenotype (Chen et al., 2009; Zhou et al., 2013) and I_h is increased in the soma of GAERS cortical layer 5/6 neurons (Williams et al., 2016). Whether these contradictory cortical data stems from compensatory changes in KO mice or are simply a reflection of opposite changes in cortical I_h in diverse models (Di Pasquale et al, 1997; Strauss et al., 2004) remains to be investigated.

In conclusion, using a pharmacological and a genetic approach to selectively suppress HCN channel function in TC neurons of three well-established AS models, this study provides conclusive evidence on the long-standing controversial role for thalamic I_h in ASs by demonstrating that HCN channels of TC neurons have a pro-absence effect.

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779

780 **FIGURE LEGENDS**

781 **Figure 1. Temporal and spatial dynamics of the effect of ZD7288 applied by reverse**
782 **microdialysis in the VB of ketamine/xylazine-anesthetized Wistar rats.**

783 **A)** Position of the unilateral 4-shank silicone probe (four thin orange lines) and microdialysis
784 probe (black thick oblique line) on a rat brain schematic drawing at the level of the VB (modified
785 from Paxinos and Watson, 2008). The red-circled, striped area indicates the diffusion of ZD7288
786 as measured in D. **B)** Extracellular high-pass filtered traces from 5 adjacent contact points of a
787 silicone probe show high-frequency bursts of action potentials of 3 clustered (color-coded) TC
788 neurons during aCSF (left) and ZD7288 (right) microdialysis application. On the right:
789 enlargement of bursts from the same TC neuron before (top) and during (bottom) ZD7288
790 dialysis. Same y-scale for all traces. **C)** Time-course of total firing of TC neurons during aCSF
791 (black) and ZD7288 (green) microdialysis injection (500 µM in the inlet tube). Data are shown
792 as percentage firing relative to that during aCSF (solid lines and shadows: mean ± SEM). Red
793 vertical line (at time 0) indicates the start of ZD7288 application. Data from 87 and 45 neurons
794 for aCSF and ZD7288, respectively, from 21 Wistar rats (see Methods for further details). **D)**
795 Distance-profile of the ZD7288 effect (green) on total firing compared to ACSF (black) (same
796 number of neurons as in C). Horizontal bars indicate electrode position standard deviations
797 relative to the dialysis membrane and calculated in 250 µm space bins, vertical bars indicate
798 SEM of ZD7288 effect. **E)** ZD7288-elicited increase in the number of spikes per burst (n=45
799 neurons) (solid line and shadows: mean ± SEM). Time is centred on the half-time of the effect of
800 ZD7288 estimated by a logistic function fit on the total firing rate variation after ZD7288
801 application. Box plot indicates median (red), upper and lower quartiles (box edges), extreme
802 points (whiskers) and outliers (red crosses) (see Statistical Analysis and Experimental Design

803 section in Material and Methods). Median post-drug (3.75 spike/burst) is significantly higher
804 than pre-dug (3.02 spikes/burst) (*p=6.9 10^{-5}, Wilcoxon signed rank test.).
805
806 **Figure 2. Effect of ZD7288 microdialysis injection in the VB on TC neuron firing in freely**
807 **moving GAERS.**
808 **A)** Position of the unilateral 4-shank silicone probe (four thin orange lines) and microdialysis
809 probe (black thick oblique line) on a rat brain schematic drawing at the level of the VB (modified
810 from Paxinos and Watson, 2008). The red-circled, striped area indicates the diffusion of ZD7288
811 as measured in Fig. 1D. **B)** Extracellular low-pass filtered traces from the silicone probe show
812 ictal periods (with SWDs) and interictal periods, with below raster of clustered (color-coded)
813 spikes of 3 TC neuron during ACSF (top traces) and ZD7288 application (bottom traces). Note
814 the drastic change of firing between ictal and interictal periods. **C-D)** Time-course of total (C)
815 and tonic firing (D) (solid line and shadows: mean ± SEM) during interictal periods (left) and
816 during ASs (right) recorded during ACSF (data to the left of red horizontal line) and ZD7288
817 (data to the right of the red horizontal line) microdialysis application. Red vertical lines (at time
818 0) indicates the start of ZD7288 injection. The change in activity is illustrated by the inset plots
819 that show total and tonic firing rate (Hz) for individual neurons during aCSF (pre-drug) versus
820 ZD7288 (post-drug) (with black dashed line indicating equal pre- and post-drug values) (*:
821 significant p-values from left to right are 0.016, 0.039, 0.023, 0.016, Wilcoxon signed rank test,
822 n=7 neurons). **E-F**) Plots, as inset plot in C-D, showing the non-significant (n.s) changes in burst
823 firing and number of spikes per burst induced by ZD7288 microdialysis during interictal and
824 ictal periods p-values from left to right are 0.078, 0.19, 0.11, 0.5, Wilcoxon signed rank test, n=7
825 neurons). **G)** Left: circular distribution plot of action potentials with respect to the SWC

826 indicates a significant different distribution before (black line) and during ZD7288 application
827 (red line) (p=0.001, Kuiper 2-sample test, n=58.8 10^3 vs n=40.6 10^3action potentials). Right: the
828 maximal difference in the time-distribution of action potentials between ACSF (black line, top
829 plot) and ZD7288 (red line, top plot) occurs just before 0 degree (defined as the peak of the
830 SWC), as highlighted by the subtraction of these two curves (gray line, bottom plot).
831
832 **Figure 3. Effect of bilateral microdialysis injection of ZD7288 in the VB on ASs in freely**
833 **moving GAERS.**
834 **A)** Position of the bilateral microdialysis probes (black thick lines) and diffusion areas (red
835 circled, striped areas) of ZD7288 are depicted on a rat brain schematic drawing at the level of the
836 VB (modified from Paxinos and Watson, 2008). **B)** Representative EEG traces showing
837 spontaneous SWDs during aCSF and ZD7288 (500 μM in the inlet tube) microdialysis
838 application. **C)** Time-course (left) and concentration-response curve (right) of ZD7288 effect
839 (solid line and shadows: mean±SEM) on the total time spent in seizure normalized to aCSF
840 values (see Methods for further details). Illustrated concentration color-code refers to the
841 ZD7288 concentration in the dialysis inlet tube. Time 0 indicates the start of ZD7288 dialysis.
842 Number of animals: 9 (aCSF), 4 (1 μM), 8 (10 μM), 7 (100 μM), 2 (250 μM), 6 (500 μM) (left
843 *p<0.05; **p<0.01, p=0.023 (100 μM) and p=4.10^{-4} (500 μM) Wilcoxon rank sum test on
844 averages between ACSF and 40-80 min data from the start of ZD7288 application (left)).
845 Absolute aCSF values (mean±SEM) for the 6 reported time points are: 295.4±51.6, 308.8±54.5,
846 276.7±53.8, 275.2±48.0, 219.1±45.5, and 246.3±52.5 sec. A logistic fit of the concentration-
847 response curve of ZD7288 indicate an EC_{50} of 29 μM. **D)** Same as C for the length of individual
848 seizures. Absolute ACSF values (mean±SEM) are: 7.91±1.29, 7.64±1.33, 8.24±1.28, 7.96±1.33,

7.29±1.25 and 6.13±0.83 sec (left *p=0.025 Wilcoxon rank sum test). **E)** Same as C for the number of seizures. Absolute aCSF values (mean±SEM) are: 56.9±10.4, 46.9±8.6, 43.4±9.1, 51.0±11.7, 43.9±11.2 and 45.6±10.3 seizures (left *p=0.011 **p=4.10^{-4} Wilcoxon rank sum test).

Figure 4. Effect of bilateral microdialysis administration of ZD7288 in the VB on GHB-elicited ASs in freely moving Wistar rats.

A) Position of the bilateral microdialysis probes (black thick lines) and diffusion areas of ZD7288 (red circled, striped area) are depicted on a rat brain schematic drawing at the level of the VB (modified from Paxinos and Watson, 2008). **B)** Representative EEG traces showing GHB-elicited SWDs during aCSF and ZD7288 (500 µM in the inlet tube) administration (**p=9.4 10^{-4}, *p=0.013, Wilcoxon rank sum test, n=7 and 11 animals in each group). **C)** Left: effect (mean ± SEM) of ZD7288 (500 µM, black bars, n=11 rats) versus aCSF (white bars, n=7) on total time spent in seizures illustrated for 10 min bins. ZD7288 dialysis started 40 min before GHB injection (see Methods for further details). Right: individual data points (aCSF: white; ZD7288: black) for the 0-10 and 10-20 min time bins after GHB injection are normalized to data recorded during aCSF injection. **D-E)** Similar bar-graphs (left) and scatter plots (right) as in C for average length of individual seizures (D) (*p=0.016, **p=0.0082, Wilcoxon rank sum test, n=7 and 11 animals in each group) and number of seizures (E). (p=0.1 and p=0.34, Wilcoxon rank sum test, n=7 and 11 animals in each group).

Figure 5: Effect of the HCN-targeting shRNA on the membrane properties of VB TC neurons *in vitro*.

872 **A)** Representative voltage responses of VB TC neurons to a hyperpolarizing and depolarizing
873 current step (-100 and 50 pA, respectively) from non-targeting (Control, black traces) and HCN-
874 targeting shRNA-injected (shRNA, red traces) mice (membrane potential: -60 mV for both).
875 Triangles and squares indicate the time of measurement of peak and steady-state input resistance
876 (R_{in}-peak and R_{in}-ss, respectively, in the other panels of this figure). Note the lack of a
877 depolarizing "sag" in the hyperpolarizing response of the HCN-targeting shRNA injected
878 neuron. **B)** Averaged hyperpolarizing voltage responses (solid line: mean; shadow: ±SEM) in all
879 recorded neurons show the marked reduction in the depolarizing "sag" in neurons injected with
880 HCN-targeting shRNA (n=18) compared to control (n=18). **C)** Voltage-current plots from all
881 neurons show the lack of inward rectification in HCN-targeting shRNA injected mice (triangles
882 and squares: amplitude of hyperpolarizing pulse at peak and steady-state, respectively, cf. **A** and
883 **B**). **D-H)** Resting membrane potential (**D**), ratio of R_{in}-ss and R_{in}-peak (**E**), R_{in}-ss (**F**), and action
884 potential (AP) threshold (**G**) and amplitude (**H**) for neurons treated with non-targeting (control,
885 black squares) and HCN-targeting shRNA (shRNA, red squares) (large symbols indicate
886 mean±SEM; * and ** indicate statistical significance; n.s.: not significant; p values are: 0.032
887 (**D**), 6.2 10^{-5} (**E**), 0.56 (**F**), 0.17 (**G**) and 0.44 (**H**); Wilcoxon rank sum test).
888
889 **Figure 6. Effect of bilateral injection in the VB of an HCN shRNA on ASs in freely moving**
890 **Stargazer mice.**
891 **A)** Representative EEG traces showing spontaneous SWDs in a non-targeting, control shRNA
892 (top) and an HCN shRNA-injected Stargazer mouse. **B-D)** Left: effect of shRNA injection (solid
893 line and shadows: mean±SEM) (red line, n=9) on total time spent in seizure (B), length of
894 individual seizures (C) and number of seizures (D) compared to non-targeting, control shRNA

(black line, n=9) measured at the indicated days after shRNA injection (day 0). Values are normalized to control group mean (black line) for each time point. Right: histograms of absolute values of total time, length of individual seizures and number of seizures for test day 28 and 32 (3-hour recordings. *indicate statistical significance; p values are: 0.036 and 0.029 (A), 0.035and 0.043 (B), and 0.056 and 0.42 (C) (Wilcoxon rank sum test). Absolute values for the control group in all other test days were not different from those of day 28 and 32.

Figure 7. Genetic suppression of thalamic HCN channels decreases their expression in VB. **A)** AAV construct includes eGFP and shRNA targeting HCN subunits or non-targeting HCN subunits. **B)** Composite image showing GFP fluorescence restricted to thalamic nuclei and their projection to somatotopic cortical areas. Note barrels in somatosensory cortex. **C)** Top row: confocal images showing GFP and intrinsic HCN2 expression in DAPI-positive VB TC neurons from a Stargazer mouse injected with the non-targeting control shRNA. Middle row: same for a mouse injected with the shRNA targeting the HCN sequence. Note the low level (or absence) of HCN fluorescence in those TC neurons that express a high level of GFP signal (white arrow heads) compared to cells expressing a low level of GFP (white arrows). Bottom row: same for a cortical area of the same mouse. Note the absence of GFP-positive soma in the cortical section and the low-level of HCN signal in the thalamic section (scale bar: 10μm). **D)** Quantifications of GFP and HCN expression in thalamic sections of Stargazer mice which received the non-targeting, control shRNA (top) and the HCN targeting shRNA (bottom) for each neuron (symbols). Each line corresponds to the linear regression between the green (GFP) and the red (HCN) fluorescence of neurons from a single section. Same color line or symbols indicate cells of the same section**.** None of the correlations for the non-targeting, control shRNA was

significant (p=0.31, 0.14, 0.23, 0.67, 0.051, 0.59), whereas 5 out of 6 sections from mice injected with the shRNA had a significant negative correlation (p=0.041, 0.0006, 0.001, 0.03, 0.17, 0.0001). Red dashed lines indicate the linear regression for the entire population of neurons (top: p=0.77; bottom: $p=3.82\ 10^{-9}$).

Figure 8. Effect of pharmacological and genetic suppression of I_h on SWD properties.

A) Representative examples of wavelet transform (top plots) of SWDs (bottom traces) before (ACSF) and during ZD7288 application. A clear loss of power is visible towards the end of the SWD recorded during ZD7288 application. A decrease of the first harmonic indicates a reduction of the spike component and an increase of the wave component of the SWD. **B-D)** Frequency analysis of SWDs in the GAERS (**B**) (p=0.11, n=9, n=6) and GHB (**C**) models injected with ZD7288 (p=0.83, n=6, n=8) and in Stargazer mice treated with HCN-targeting shRNA (**D**) (p=0.44, n=8, n=7) (left: probability density plots of intervals between spike of SWCs; right: scatter plots of peak frequency for individual animals with mean±SEM) (Wilcoxon rank-sum test).