

# Ca<sup>2+</sup>-dependent large conductance K<sup>+</sup> currents in thalamocortical relay neurons of different rat strains

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**Abstract** Mutations in genes coding for Ca<sup>2+</sup> channels were found in patients with childhood absence epilepsy (CAE) indicating a contribution of Ca<sup>2+</sup>-dependent mechanisms to the generation of spike-wave discharges (SWD) in humans. Since the involvement of Ca<sup>2+</sup> signals remains unclear, the aim of the present study was to elucidate the function of a Ca<sup>2+</sup>-dependent K<sup>+</sup> channel (BK<sub>Ca</sub>) under physiological conditions and in the pathophysiological state of CAE. The activation of BK<sub>Ca</sub> channels is dependent on both voltage and intracellular Ca<sup>2+</sup> concentrations. Moreover, these channels exhibit an outstandingly high level of regulatory heterogeneity that builds the basis for the influence of BK<sub>Ca</sub> channels on different aspects of neuronal activity. Here, we analyse the contribution of BK<sub>Ca</sub> channels to firing of thalamocortical relay neurons, and we test the

hypothesis that BK<sub>Ca</sub> channel activity affects the phenotype of a genetic rat model of CAE. We found that the activation of the β<sub>2</sub>-adrenergic receptor/protein kinase A pathway resulted in BK<sub>Ca</sub> channel inhibition. Furthermore, BK<sub>Ca</sub> channels affect the number of action potentials fired in a burst and produced spike frequency adaptation during tonic activity. The latter result was confirmed by a computer modelling approach. We demonstrate that the β<sub>2</sub>-adrenergic inhibition of BK<sub>Ca</sub> channels prevents spike frequency adaptation and, thus, might significantly support the tonic firing mode of thalamocortical relay neurons. In addition, we show that BK<sub>Ca</sub> channel functioning differs in epileptic WAG/Rij and thereby likely contributes to highly synchronised, epileptic network activity.

**Keywords** BK<sub>Ca</sub> channels · Thalamic firing modes · Computer modelling · Spike frequency adaptation · WAG/Rij rat · Absence epilepsy

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## Introduction

Depending on the state of vigilance, thalamocortical relay (TC) neurons of the mammalian brain operate in two different firing modes. Rhythmic oscillations dominate the TC network during states of slow-wave sleep as well as absence epilepsy [14, 52]. On the cellular level, this state of network activity is characterised by burst-like action potential (AP) discharges and a relative unresponsiveness of TC neurons to peripheral inputs. On the other hand, tonic firing of single APs prevails during wakefulness. In this state, incoming sensory stimuli from the periphery are faithfully transferred to cortical regions for further processing [53]. The switch between burst and tonic firing is partially governed by noradrenaline, released from neurons residing in the locus coeruleus [34]. Prominent targets of the β-adrenergic/cyclic AMP/protein kinase A

(PKA) pathway are the pacemaker current  $I_h$  [41], the L-type  $\text{Ca}^{2+}$  current [40], and the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current ( $I_{\text{KCa}}$ ) [1]. These currents (among others) influence and shape the above-mentioned firing patterns. Former characterisation of whole-cell currents and computer models indicated that  $I_{\text{KCa}}$ , which has the characteristics of a current through  $\text{BK}_{\text{Ca}}$  channels, contributes to tonic firing of TC neurons [1, 9, 26, 35]. However, the exact role of  $\text{BK}_{\text{Ca}}$  channels in the generation of TC activity patterns remains elusive.  $\text{BK}_{\text{Ca}}$  channels (also known as maxi- $\text{K}^+$ ,  $\text{K}_{\text{Ca}}1.1$ , Slo1) are expressed in various regions of the brain. Based on their  $\text{K}^+$  conductance in the range of 200 to 250 pS in symmetrical 150 mM  $\text{K}^+$ , they can be distinguished from the closely related small conductance (SK; 5–20 pS) and intermediate conductance (IK; 20–40 pS)  $\text{K}^+$  channels [18]. These channels are regulated by phosphorylation through diverse kinases, e.g. PKA [47, 58]. Functional  $\text{BK}_{\text{Ca}}$  channels are comprised of four pore-forming  $\alpha$ -subunits encoded by a single gene (Slo1, KCNMA1). Nevertheless, native  $\text{BK}_{\text{Ca}}$  channels exhibit a broad variety of functional properties in different cell types [19, 20] or during altered physiological conditions [29] and undergo developmental modifications [32]. This functional diversity is caused by extensive alternative splicing of the KCNMA1 pre-mRNA [57]. ZERO and STREX represent two splice variants which reveal specific PKA consensus sequence patterns that determine activation or inhibition by phosphorylation [54]. Their sensitivity to increased intracellular  $\text{Ca}^{2+}$  levels, cyclic AMP-induced phosphorylation, as well as depolarised membrane potentials makes these variants unique in that they can integrate three independent signals to promote an adequate neuronal response. This high level of regulatory heterogeneity builds the basis for the influence of  $\text{BK}_{\text{Ca}}$  channels on different aspects of neuronal activity, like firing frequency, AP width as well as early and late phases of frequency adaptation in different cell types [21, 48].

In the context of childhood absence epilepsy (CAE), missense mutations in genes coding for  $\text{Ca}^{2+}$  channels were found in genetic animal models and in CAE patients, indicating a contribution of  $\text{Ca}^{2+}$ -dependent mechanisms to the pathophysiology of this disorder [5, 6, 12]. Moreover, the effect of a highly effective drug in the treatment of absence epilepsy (ethosuximide, ETX [43]) was ascribed to the combined action on three types of ion channels, including  $\text{BK}_{\text{Ca}}$  [6]. The aim of our study was to elucidate the function of  $\text{BK}_{\text{Ca}}$  channels under physiological conditions and in the pathophysiological state of CAE. Therefore, we compared data from the epileptic WAG/Rij strain to results derived from its corresponding control strain August-Copenhagen Irish (ACI) [16, 27]. Animals of the ACI strain have been frequently used as controls to compare molecular and cellular properties of thalamic neurons in epileptic vs. non-epileptic rats [4–6, 8, 28]. By making use of electrophysiological, molecular biological, immunohistochemical and computer modelling approaches, we analysed the  $\beta_2$ -adrenergic receptor-mediated modulation of  $\text{BK}_{\text{Ca}}$  channels and the firing behaviour

of TC neurons of a non-epileptic rat strain, ACI, and we tested the hypothesis that the  $\text{Ca}^{2+}$ -activated and cAMP-modulated  $\text{BK}_{\text{Ca}}$  channels contribute to the occurrence of the CAE pathophysiology in the genetic rat model WAG/Rij [13].

## Materials and methods

### Tissue preparation

All animal work has been approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; approval ID: 8.87-51.05.20.10.117 and 87-51.04.2010.A322). For electrophysiological recordings, thalamic slices were prepared as described earlier [38] from WAG/Rij or ACI rats at an age ranging from postnatal day (P)14 to P25.

### Patch clamp recordings

Whole-cell recordings were performed on visually identified TC neurons of the dorsal part of the lateral geniculate nucleus (dLGN) in acute brain slices at room temperature (~23 °C), using glass microelectrodes pulled from borosilicate glass capillaries (GC150TF-10, Harvard Apparatus) that were connected to an EPC-10 amplifier (HEKA Elektronik). Typical electrode resistance was 2–4 MΩ, while access resistance was 5–15 MΩ. Experiments were controlled by Pulse software (HEKA Elektronik). For voltage-clamp recordings of the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current, the following solutions were used (in mM): NaCl, 140; KCl, 2; HEPES, 10; glucose, 10; MgCl<sub>2</sub>, 3; CaCl<sub>2</sub>, 1; TTX, 5  $\mu$ M; pH 7.35 with NaOH. The drug ZD7288 (30 μM) was routinely added to the perfusion medium to block  $I_h$  currents. The K-gluconate-based internal solution contained (in mM) NaCl, 10; K-gluconate, 95; K<sub>3</sub>-citrate, 20; HEPES, 10; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 0.1; EGTA, 1.1; phosphocreatine, 15; Mg-ATP, 3; Na<sub>2</sub>-GTP, 0.5; QX-314-Cl, 3.35; pH 7.25 with KOH. For current-clamp measurements, the bath medium was artificial cerebrospinal fluid (ACSF) which contained (in mM) NaCl, 120; KCl, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; NaHCO<sub>3</sub>, 22; MgSO<sub>4</sub>, 2; CaCl<sub>2</sub>, 2; glucose, 20; pH 7.35 with 95 % O<sub>2</sub>–5 % CO<sub>2</sub>. The intracellular solution in these experiments contained (in mM) K-gluconate, 88; NaCl, 10; K<sub>3</sub>-citrate, 20; HEPES, 10; BAPTA, 3; phosphocreatine, 15; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 0.5; Mg-ATP, 3; Na<sub>2</sub>-GTP, 0.5; pH 7.25 with KOH. Drugs were purchased from Biotrend (paxilline, iberiotoxin, okadaic acid), from Sigma Aldrich (gramicidine) or from Tocris Bioscience (salmeterol, charybdotoxin, xamoterol hemifumarate, ZD7288). An equimolar concentration of ascorbic acid was routinely added to salmeterol-containing solutions as an antioxidant. For perforated patch clamp, after getting the GΩ-seal with gramicidine (5 nM) in the internal solution, it took 5–20 min until the access to the cell was

visible on the oscilloscope by sudden appearance of capacitance transients.

All values are presented as mean  $\pm$  SEM. Statistical significance of data with Gaussian distribution was evaluated by Student's *t* test, while non-parametric data were tested using the Mann–Whitney test. Values of  $p < 0.05$  were considered statistically significant. For current-clamp data, custom written MatLab routines were used to analyse AP characteristics during burst and tonic firing. Mean values for the number of APs, for interspike intervals (ISI) as well as the adaptation index (AI) were calculated in every depolarising pulse. The time between the release from the hyperpolarisation step and the peak of the first AP crowning the low-threshold burst was taken as time-to-peak (TTP). The adaptation index is defined as follows:

$$\text{AI} = \begin{cases} \frac{\text{mean ISI of last three APs}}{\text{mean ISI of first three APs}}, & \text{if } |\text{APs}| \geq 5 \\ \frac{\text{ISI of last two APs}}{\text{ISI of first two APs}}, & \text{if } |\text{APs}| \in \{3, 4\} \end{cases} \quad (1)$$

For the analysis of voltage-clamp recordings, values for the peak amplitude, the inactivation ratio (IR) and the TTP for the current induced by the voltage step to +60 mV were calculated. OriginPro8G software (OriginLab) was used for data analysis and figure plotting.

#### Immunohistochemistry in brain slices

Rats were deeply anaesthetised using pentobarbital (50 mg/kg body weight) and transcardially perfused with phosphate buffered saline (PBS), followed by ice-cold 4 % paraformaldehyde (PFA)/PBS for 35–40 min. Brains were removed, postfixed for 12 h in 4 % PFA/PBS and cryoprotected with 30 % sucrose. Coronal sections (40  $\mu\text{m}$ ) were cut at the level of the dLGN. After several washing steps, free-floating sections were blocked with 10 % normal horse serum (NHS), 2 % bovine serum albumin (BSA) and 0.05 % Triton X-100 in Tris-buffered saline (TBS) for 2 h. Slices were co-stained with goat anti-KCNMA1 (anti-BK<sub>Ca</sub>) (1:100, Abnova) and rabbit anti- $\beta_2$  adrenoceptor (1:300, Alomone Labs) at 4 °C for 16–18 h and after washing steps thereafter with Cy3-conjugated donkey anti-rabbit IgG (1:300, Dianova) and Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400, Invitrogen) for 1.5 h in 2 % NHS, 2 % BSA and 0.05 % Triton X-100 in TBS. As negative controls, occlusion of the primary antibody from the staining procedure was routinely performed, and no positive immunological signal was detected (not shown). All images were obtained using a confocal laser scanning microscope (Nikon eC1 plus).

#### Polymerase chain reaction

RNA was prepared from freshly dissected tissue by extraction with Trizol reagent according to the manufacturer's

instructions (RNeasy Mini Kit, Qiagen). First-strand cDNA was primed with oligo(dT) from 2.5  $\mu\text{g}$  of mRNA and synthesised using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences). PCR was performed in a 25- $\mu\text{l}$  reaction mixture using 1 U HotStarTaq polymerase (Qiagen) for amplification of BK<sub>Ca</sub> templates; in all cases, the mixture contained 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP and 50 pmol of each primer. Primer sequences were the following: for both STREX and ZERO amplicates, 5'-AGTGCCTTCG TGGGCTGTCCTTC-3' and 5'-CACATTGGAGTCATGT TGT-3' [32] (annealing temperature  $T_{\text{ann}}=55$  °C), ZERO: 5'-GCCAAAGAAGTTAACAGGGCATT-3' and 5'-CGGCTGCTCATCTCAAGC-3' and STREX: 5'-TTTGA TTGCGGACGTTCTGA-3' and 5'-TCTCTCAAGGGTG TCCACGTTAC-3' [33] (both  $T_{\text{ann}}=60$  °C). Cycling protocols were as follows: 15 min at 95 °C, 40 cycles (30 s at 95 °C, 30 s at  $T_{\text{ann}}$ , 1 min at 72 °C); 7 min at 72 °C final elongation;  $\approx 4$  °C. A template-free water control was included in every run. PCR products were visualised by electrophoresis in 2 % agarose gel.

#### Computer simulations

All simulations were processed within the NEURON Simulation Environment [24] and are based on a temperature of 23 and 36 °C, respectively. Length and diameter of the single compartment model were 96  $\mu\text{m}$  each, thereby resulting in a total area of about 29,000  $\mu\text{m}^2$ . The model included  $I_{\text{KCa}}$  (also termed  $I_C$ ) [25],  $I_L$  [25], Hodgkin–Huxley sodium and potassium currents [17], a non-selective, passive leak current [24], as well as a calcium pump [17]. Besides the passive membrane channel, which is part of the NEURON Simulation Environment, all current implementations were gained from the SenseLabModelDB (accession numbers 3808 and 3343). Ion concentrations were set to (extra-/intracellular, in mM) Na<sup>+</sup>=143/10, K<sup>+</sup>=2.5/148 and Ca<sup>2+</sup>=2/0.5.

## Results

### Cyclic AMP-dependent modulation of BK<sub>Ca</sub> channels affects tonic AP firing in TC neurons

We investigated TC neurons in dLGN, representing a prototype of thalamocortical neurons that is involved in both sensory signal transmission and rhythmic-synchronised activity during spike-wave discharges (SWD) [22, 51]. To assess the modulation of the tonic firing mode in TC neurons by  $\beta_2$ -adrenergic stimulation, we performed current-clamp recordings in the presence of the  $I_h$  blocker ZD7288 (30  $\mu\text{M}$ ). Under these conditions, TC neurons of ACI rats revealed stable resting membrane potentials ( $-80.2 \pm 0.8$  mV,  $n=49$ ). To allow tonic firing, cells were depolarised to  $-57.3 \pm 1.4$  mV ( $n=49$ ) by using constant DC current injection. Tonic firing was

elicited by injection of short (800 ms) depolarising DC current pulses (100 to 200 pA) every 30 s. Under control conditions in ACSF (Fig. 1a), TC neurons generated trains of APs that showed slight frequency adaptation ( $AI=1.3\pm0.02$ ) with an increased ISI ( $p<0.01$ ) after 30 and 50 min (Fig. 1a, g; for absolute AP numbers and ISI, see Supplementary Table 1). This effect was accompanied by a significant ( $p<0.01$ ) decrease in the number of APs ( $n=5$ , Fig. 1a, h). The amplitude of the constant DC current injection was not changed throughout the experiment. No changes in membrane potential occurred within the 50-min recording period, indicating overall stable cell viability and recording conditions (Supplementary Fig. 1). We activated the endogenous canonical cyclic AMP producing pathway, involving  $\beta_2$ -adrenergic receptors,  $G\alpha_s$  proteins, adenylyl cyclases and PKA, by applying the  $\beta_2$ -adrenergic agonist salmeterol [37]. When this cascade was activated by salmeterol (10  $\mu$ M) after an initial control period of 10 min, the increase in ISI and the decrease in the number of APs after a recording period of 30 min were no longer significant ( $n=8$ ; Fig. 1b, g, h). Following wash out of salmeterol, the ISI and the number of APs after a recording time of 50 min approached the values seen under control conditions. Next, TC neurons from WAG/Rij were analysed under current-clamp conditions (resting membrane potential  $-81.1\pm1.5$  mV;  $n=9$ ; data not shown). Following DC depolarisation to  $-58.3\pm0.1$  mV, depolarising current pulses induced tonic AP firing with only moderate spike frequency adaptation ( $AI=1.4\pm0.1$ ). As for ACI, application of salmeterol in WAG/Rij prevented the long-term changes in ISI and the number of APs. Since the effect of salmeterol in WAG/Rij was not significantly different in comparison to ACI, the following experiments were solely performed in ACI rats. As  $Ca^{2+}$ -activated  $K^+$  currents possess the ability to induce spike frequency adaptation [21], we applied the BK<sub>Ca</sub>-specific blocker iberiotoxin (100 nM; Fig. 1c). In these experiments, neither significant changes in ISI nor in the numbers of APs were observed ( $n=18$ ; Fig. 1g, h). Co-application of iberiotoxin and salmeterol did not induce any further effect on ISI or the number of APs ( $n=9$ ; Fig. 1d, g, h). For comparison, the potent and specific BK<sub>Ca</sub> channel blockers charybdotoxin (ChTx; 200 nM) and paxilline (Pax; 10  $\mu$ M) were used in combination, since paxilline strongly enhances the binding of ChTx to BK<sub>Ca</sub> channels [44]. The combined application of ChTx and paxilline exerted the strongest suppression of spike frequency adaptation with increasing numbers of APs and reduced ISIs ( $n=9$ ; Fig. 1e, g, h). Inhibition of endogenous kinase activity is able to increase BK<sub>Ca</sub> channel activity approximately threefold [23]. Therefore, we tested tonic firing with a phosphatase inhibitor (okadaic acid, 10 nM;  $n=5$ ) in the internal solution (Fig. 1f–h). Within about 30 min, okadaic acid led to a hyperpolarisation of the membrane and associated dampening of spike firing (Fig. 1f, middle trace). For better comparison of tonic firing under phosphatase inhibition with the other recording conditions, the membrane potential was

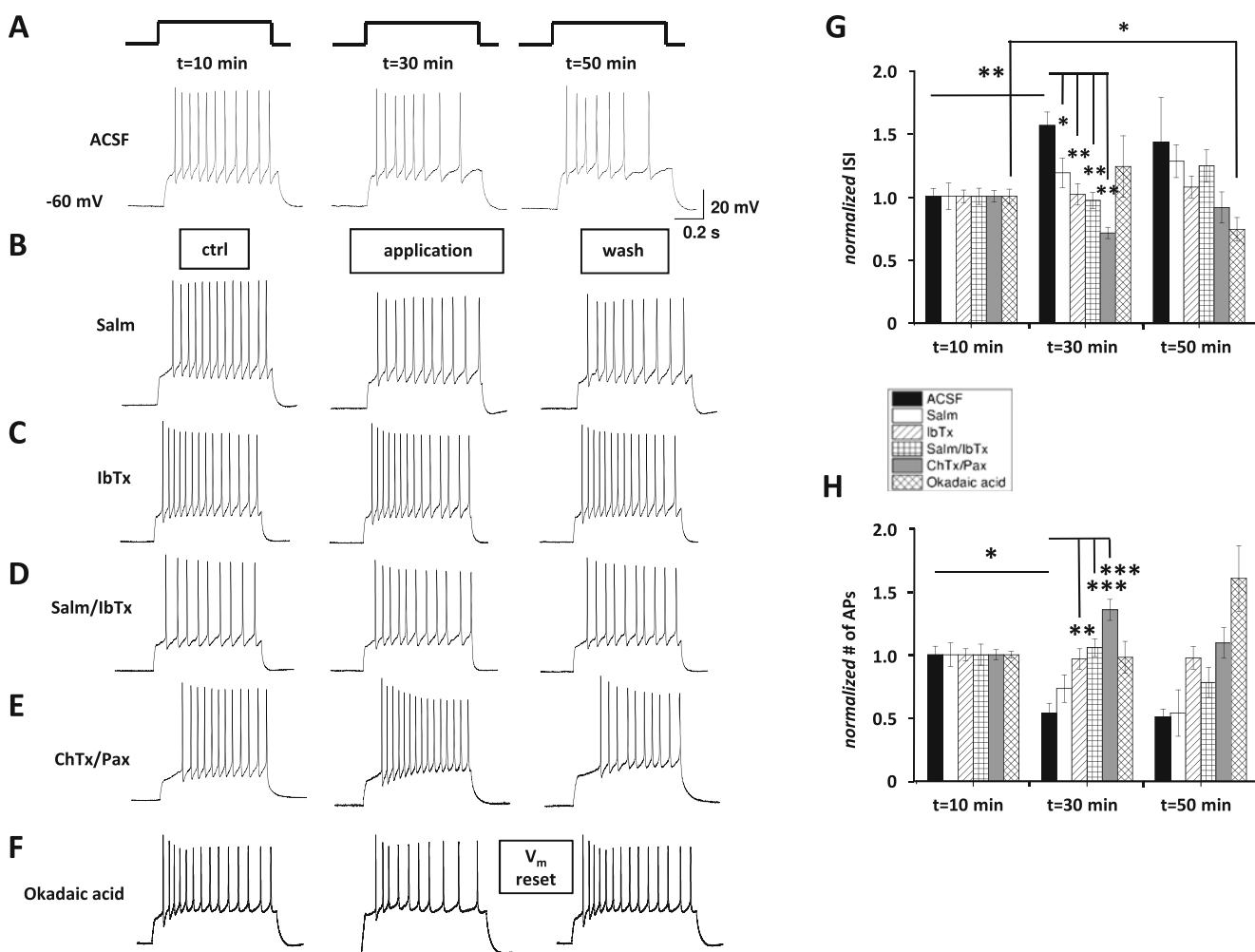
reset to the initial level and action potential trains were analysed. Since more APs were fired and ISIs were decreased compared to control, spike frequency adaptation could not be detected (Fig. 1f, right trace). This indicates that the increased phosphorylation leads to BK<sub>Ca</sub> channel inhibition. Since we have recently detected expression of  $\beta_1$ -adrenergic receptors in TC neurons [42], we tested the effect of the  $\beta_1$ -specific agonist (10  $\mu$ M xamoterol hemifumarate) on tonic firing of dLGN neurons. Although  $\beta_1$ -adrenergic receptors activate cAMP/PKA signalling similar to  $\beta_2$ -adrenergic receptors [45], xamoterol application did not induce alterations in tonic firing behaviour ( $n=7$ , see Supplementary Table 1). As control, recordings in perforated patch mode with and without phosphatase inhibitors in the pipette solution were performed, and the results were found to be similar to those gained in the whole-cell mode (see Supplementary Table 1). Moreover, calculation of the ChTx/Pax-sensitive  $I_{KCa}$  fraction of the total outward current revealed stable current amplitudes after 50 min of whole-cell recordings, indicating constant recording conditions (Supplementary Fig. 2). In sum, these findings indicate a BK<sub>Ca</sub> channel-dependent spike frequency adaptation in TC neurons from ACI and WAG/Rij rats which is prevented by phosphatase inhibition and  $\beta_2$ -receptor stimulation.

#### Analysis of $I_{KCa}$ -mediated effects on burst firing behaviour in TC neurons

To analyse the influence of  $I_{KCa}$  on the burst firing mode of TC neurons, we performed whole-cell current-clamp recordings in acute brain slices. Injection of negative current pulses (100 to 150 pA, 2 s duration) induced a hyperpolarisation of the membrane potential to approximately  $-100$  mV (Fig. 2a, b). Following the release from hyperpolarisation, a low-threshold spike (LTS) crowned by a burst of APs was elicited. Recordings of burst activity for up to 1 h (data not shown) revealed no change in the number of LTS-crowning action potentials in both strains over time. In another set of recordings, the selective BK<sub>Ca</sub> channel blocker combination ChTx/Pax was applied. Under control conditions, the number of APs averaged  $1.5\pm0.2$  ( $n=8$ ) and  $1.6\pm0.2$  ( $n=6$ ) in ACI and WAG/Rij, respectively ( $p=0.73$ ). Application of the BK<sub>Ca</sub> channel blockers significantly raised the number of APs crowning the LTS in ACI dLGN neurons ( $n=6$ ,  $p=0.01$ ), while there was no change in recordings from WAG/Rij neurons ( $n=6$ ,  $p=0.48$ ). In both strains, time-to-peak showed only a nominal increase (Fig. 2c, for absolute TTP values see Supplementary Table 2). The ChTx/Pax-sensitive currents were found to be similar in ACI and WAG/Rij (Figs. 2d and 4b(a)).

#### Detection of BK<sub>Ca</sub> channel transcripts and proteins in dLGN

In the following, immunohistochemical staining and PCR analysis were combined in order to determine the cellular



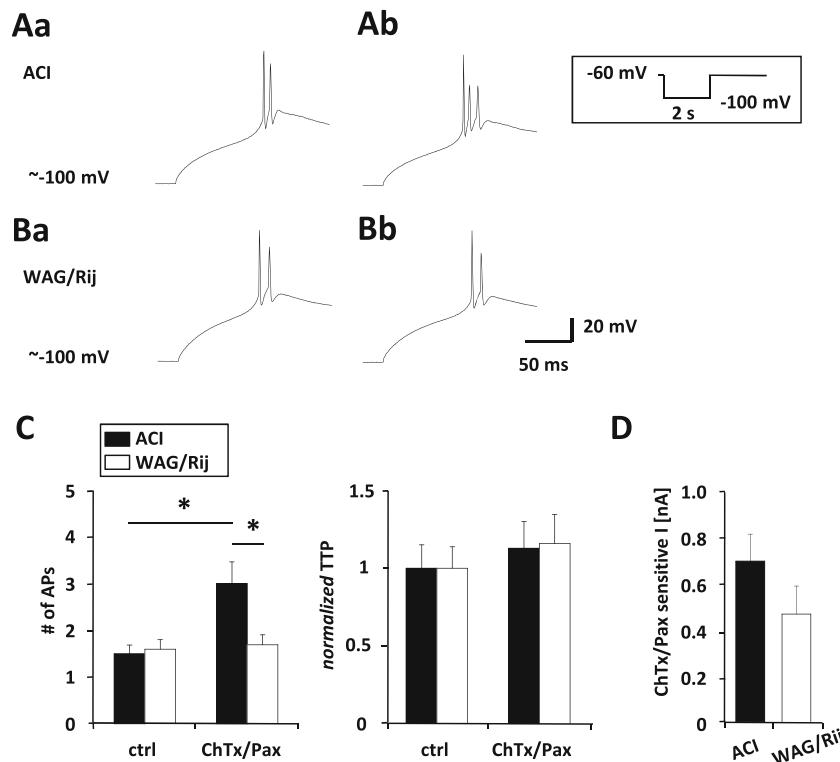
**Fig. 1** Blockade of BK<sub>Ca</sub> channels inhibits time-dependent spike frequency adaptation in thalamic neurons. **a–f** Example traces recorded from ACI neurons. Firing patterns were induced by 800 ms depolarising pulses (100–200 pA) from a potential set to about -60 mV by DC current injection. External solution was either artificial cerebrospinal fluid (ACSF, **a**) or ACSF with either salmeterol (**b**), iberiotoxin (a specific BK<sub>Ca</sub> channel blocker, **c**), a combination of salmeterol and iberiotoxin (**d**) or the BK<sub>Ca</sub> blockers charybdotoxin and paxilline (**e**). Traces in **b–e** show firing patterns under control conditions (10 min), drug application (30 min) and wash out (50 min). Since okadaic acid had to be added to the internal solution (**f**), phosphatases were inhibited during the whole recording period. After 50 min of okadaic acid

recordings, the holding potential was reset to the initial value (as indicated), since application of the phosphatase inhibitor induced a hyperpolarisation of the membrane potential. Calibration bars indicated in **a** account for all traces. **g** Bar graph of normalised interspike intervals (ISI) after 10, 30 and 50 min, respectively. Note significant differences for both blocker and salmeterol application compared to recordings in ACSF alone (black bars). Phosphatase inhibitor, okadaic acid, added to the internal solution reduces ISI values after 50 min compared to control. **h** Bar graph of normalised numbers of action potentials (APs). *Salm* salmeterol, *IbTx* iberiotoxin, *ChTx* charybdotoxin, *Pax* paxilline (significant changes are highlighted as follows: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001)

localisation and expression of splice variants of BK<sub>Ca</sub> channels in the thalamus. The use of a specific antibody revealed positive staining for BK<sub>Ca</sub> channels in all neuronal somata (Fig. 3a). A standard PCR approach demonstrated the presence of the stress-regulated exon splice variant of BK<sub>Ca</sub> channels, termed STREX, as well as the insert less variant, ZERO, in dLGN, the ventrobasal complex of the thalamus (VB), and the hippocampus (HC; Fig. 3b). The detection of the STREX variant points to the possibility of a blockade of BK<sub>Ca</sub> channels after phosphorylation via PKA in dLGN TC neurons.

#### Cyclic AMP-dependent modulation of BK<sub>Ca</sub> channel-mediated currents in TC neurons

Whole-cell patch clamp recordings were performed in acute brain slices, and modulation of  $I_{KCa}$  via the  $\beta_2$ -adrenergic pathway and specific blockers was tested by monitoring the total K<sup>+</sup> outward current in dLGN TC neurons. K<sup>+</sup> currents were evoked by a voltage protocol starting from a holding potential of -70 mV, followed by a hyperpolarisation to -110 mV (1 s), a conditioning pulse to -40 mV (100 ms) and depolarising voltage steps varying bet-



**Fig. 2** Suppression of  $I_{KCa}$  leads to an altered burst firing behaviour in TC neurons of non-epileptic rats. **a, b** Example traces showing burst firing before (*a*) and during ChTx/Pax application (*b*). Thalamocortical relay neurons of ACI (**a**) and WAG/Rij (**b**) were held at about  $-60$  mV and step-hyperpolarised to  $\sim 100$  mV. After the release from hyperpolarisation, these cells showed burst firing. *Calibration bars* are valid for all traces. The *inset* shows the current-clamp stimulation protocol underlying the depicted traces. **c** In cells of non-epileptic ACI (black bars), application of the selective  $BK_{Ca}$  blocker cocktail (200 nM ChTx+10  $\mu$ M Pax) induced a significant increase in the number of LTS associated APs (*left*), while no significant changes were detected in recordings from WAG/Rij neurons (white bars). Time-to-peak values (TTP, *right*) showed nominal increase in both strains. **d** Charybdotoxin/paxilline (ChTx/Pax)-sensitive portions of the total  $K^+$ -current in TC neurons from non-epileptic ACI and the epileptic WAG/Rij rats show nominal differences (\* $p < 0.05$ )

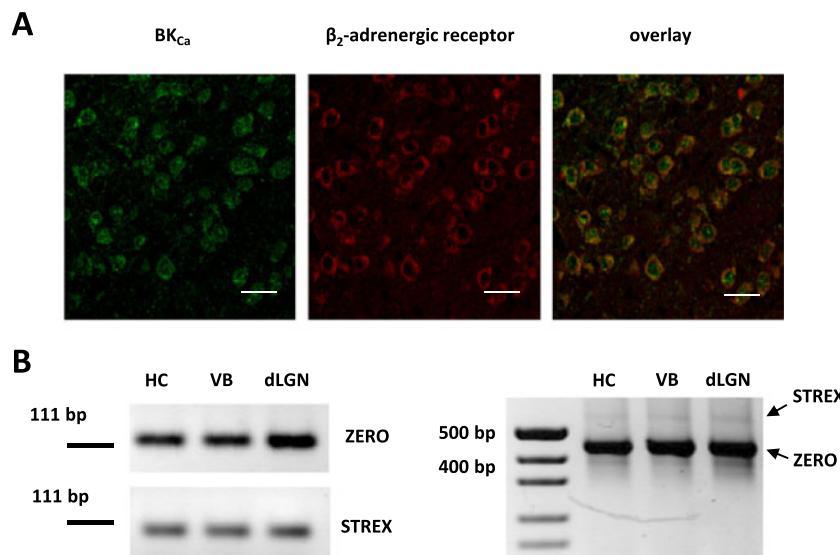
bars), application of the selective  $BK_{Ca}$  blocker cocktail (200 nM ChTx+10  $\mu$ M Pax) induced a significant increase in the number of LTS associated APs (*left*), while no significant changes were detected in recordings from WAG/Rij neurons (white bars). Time-to-peak values (TTP, *right*) showed nominal increase in both strains. **d** Charybdotoxin/paxilline (ChTx/Pax)-sensitive portions of the total  $K^+$ -current in TC neurons from non-epileptic ACI and the epileptic WAG/Rij rats show nominal differences (\* $p < 0.05$ )

ween  $-60$  and  $+90$  mV (2 s, 30 mV increments, Fig. 4d). Due to the conditioning pulse, the fast, transient A-current was inactivated and the currents evoked by the depolarising voltage steps revealed medium fast activation and slow, incomplete inactivation. It has been shown before that a fraction of this current is sensitive to the removal of extracellular  $Ca^{2+}$ , is inhibited by activation of adenylyl cyclases and is mediated by  $BK_{Ca}$  channels [1, 9]. Application of salmeterol significantly ( $p < 0.05$ ) reduced current amplitudes in non-epileptic and epileptic animals (ACI  $-16.1 \pm 3.4\%$ ,  $n=5$ ; WAG/Rij  $-12.5 \pm 3.2\%$ ,  $n=8$ ), but no differences for the inactivation ratios and TTP in both rat strains were found (Fig. 4a, b), data and traces from ACI rats, for absolute values see Supplementary Table 3). Next, the blocker combination ChTx/Pax was tested. In both rat strains, a significant ( $p < 0.005$ ) reduction of  $K^+$  currents was found (ACI  $-15.7 \pm 2.8\%$ ,  $n=5$ ; WAG/Rij  $-12 \pm 2\%$ ,  $n=6$ ), while there were no significant effects on inactivation ratios and TTP (Fig. 4b(a, b), data and traces from ACI rats). Since the results for ACI and WAG/Rij rats were found indistinguishable, the following analysis was performed solely on ACI rats. Activation of the  $\beta_2$ -adrenergic receptor

in the presence of the blocker cocktail (Fig. 4c(a)) did not result in further current inhibition ( $-16.2 \pm 3.8\%$ ;  $n=7$ ,  $p < 0.01$ , Fig. 4c(b)). Again for the IR as well as TTP, no significant differences could be detected between control conditions and in the presence of salmeterol and ChTx/Pax. These findings indicate a  $\beta_2$ -adrenergic receptor-dependent inhibition of  $BK_{Ca}$  channels in dLGN relay neurons of non-epileptic ACI as well as the epileptic WAG/Rij rats.

Single-compartment modelling confirms  $BK_{Ca}$  channel-dependent spike frequency adaptation during tonic firing

Next, the contribution of  $BK_{Ca}$  channels to the characteristics of the tonic firing mode was evaluated in silico by a single-compartment TC neuron model which has been described earlier [56]. The model's resting membrane potential was adjusted to  $-60$  mV. While tonic firing was elicited by the application of depolarising pulses (200 pA, 800 ms) in the current-clamp mode, the intracellular  $Ca^{2+}$  dynamic and temperature-dependent AP generation was monitored in the presence (Fig. 5a, b(a)) and absence (Fig. 5a, b(b)) of  $I_{KCa}$ .



**Fig. 3** Detection of BK<sub>Ca</sub> channel protein and transcripts in dLGN. **a** Immunohistochemical staining of dLGN neurons with specific antibodies against the BK<sub>Ca</sub> channels (green) and β<sub>2</sub>-adrenergic receptors (red) demonstrates co-expression at cell membranes. Scale bar represents 25 μm. **b** Detection of BK<sub>Ca</sub> splice variants in tissue from the hippocampus (HC), the ventrobasal thalamic complex (VB) and dLGN of ACI rats revealed by standard PCR. ZERO (upper panel, left) and

STREX PCR fragments (lower panel, left) were detected in independent reactions in all three tissue samples. The right panel shows the amplification products of both splice variants derived from a single reaction. The expected product lengths for STREX and for ZERO were about 540 and 423 bp, respectively. Horizontal bars mark positions of DNA ladder bands with indicated size. The arrows point to the expected band sizes

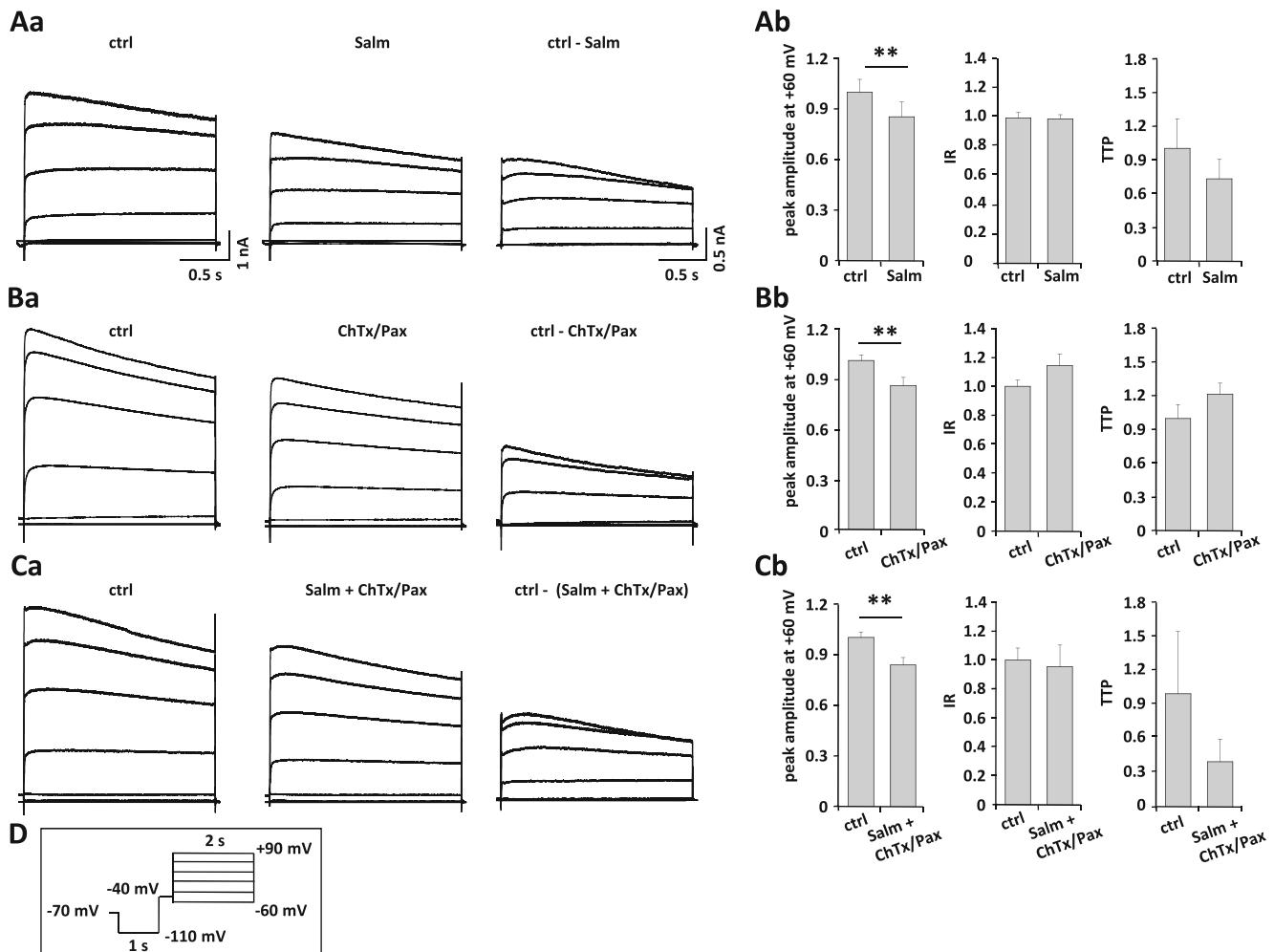
Comparing the ISIs at the beginning with those at the end of the depolarising pulse revealed that BK<sub>Ca</sub> channels led to increasing ISIs and thus influenced the model's overall firing pattern. Tonic AP firing was associated with a slow and stepwise increase in the intracellular Ca<sup>2+</sup> concentration, with each step being correlated to one of the APs (Fig. 5a(a, b)). Removing I<sub>KCa</sub> from the model cell totally abolished spike frequency adaptation. The ISIs were constant throughout the whole pulse, while the intracellular Ca<sup>2+</sup> level increased faster and reached higher amplitudes (Fig. 5a(b)). Since all in vitro experiments were performed at room temperature (~23 °C), the computer modelling approach was used to evaluate the temperature influence on the TC cell's tonic firing mode (Fig. 5b). When the simulation temperature was elevated to body temperature (36 °C), ISIs were increased with an AI of 1.2 and 1.5 at 23 and 36 °C, respectively (Fig. 5b(a)). Removal of I<sub>KCa</sub> from the computer model completely abolished spike frequency adaptation (Fig. 5b(b)) and thus confirmed the in vitro results.

Endogenous protein phosphatases were shown to slowly disinhibit and thus gradually increase the activity of heterologously expressed BK<sub>Ca</sub> channels [23]. In the present study, brain slice recordings pointed to the possibility that BK<sub>Ca</sub> channels get progressively activated during long-lasting patch clamp recordings. In long-term recordings, we found that the I<sub>KCa</sub> fraction of the total outward current after 50 min is larger than 10 min after establishment of the whole-cell configuration (Supplementary Fig. 2). Therefore, the TC cell model was used to assess the influence of increasing BK<sub>Ca</sub> channel

availability on the tonic firing mode (Supplementary Fig. 3). Starting at 0 %, the I<sub>KCa</sub> amplitude was successively increased in 10 % steps and the corresponding spike frequency adaptation was calculated. The resulting graph shows an almost linear relationship between AI and I<sub>KCa</sub> that finally converges to a constant plateau. Example traces (Supplementary Fig. 3Aa, b) of recordings with 80 % and 120 % of I<sub>KCa</sub> show that the increasing BK<sub>Ca</sub> conductance reduces the generated number of APs (depolarising pulse as before) and, thus, perfectly mimics the alterations of tonic firing found in our in vitro experiments. Taken together, these experiments demonstrate that the K<sup>+</sup> current through BK<sub>Ca</sub> channels is able to shape the tonic firing pattern of TC neurons.

## Discussion

The present study was undertaken to assess the contribution of BK<sub>Ca</sub> channels to the firing modes of TC neurons in control and epileptic rats. Therefore, we investigated well-established lines (WAG/Rij vs. ACI) of epileptic and non-epileptic rats [13, 15, 16, 27] and chose a prototypical sensory thalamic nucleus (dLGN) contributing to SWD activity [22, 49, 51]. Since critical pathophysiological mechanisms have been identified at juvenile age in these rats, before SWDs are apparent on the electroencephalogram [3, 8, 28, 30, 46], we investigated animals in this developmental stage. The results can be summarised as follows: (1) In both strains, analysis of tonic firing revealed a slow and time-dependent increase of spike



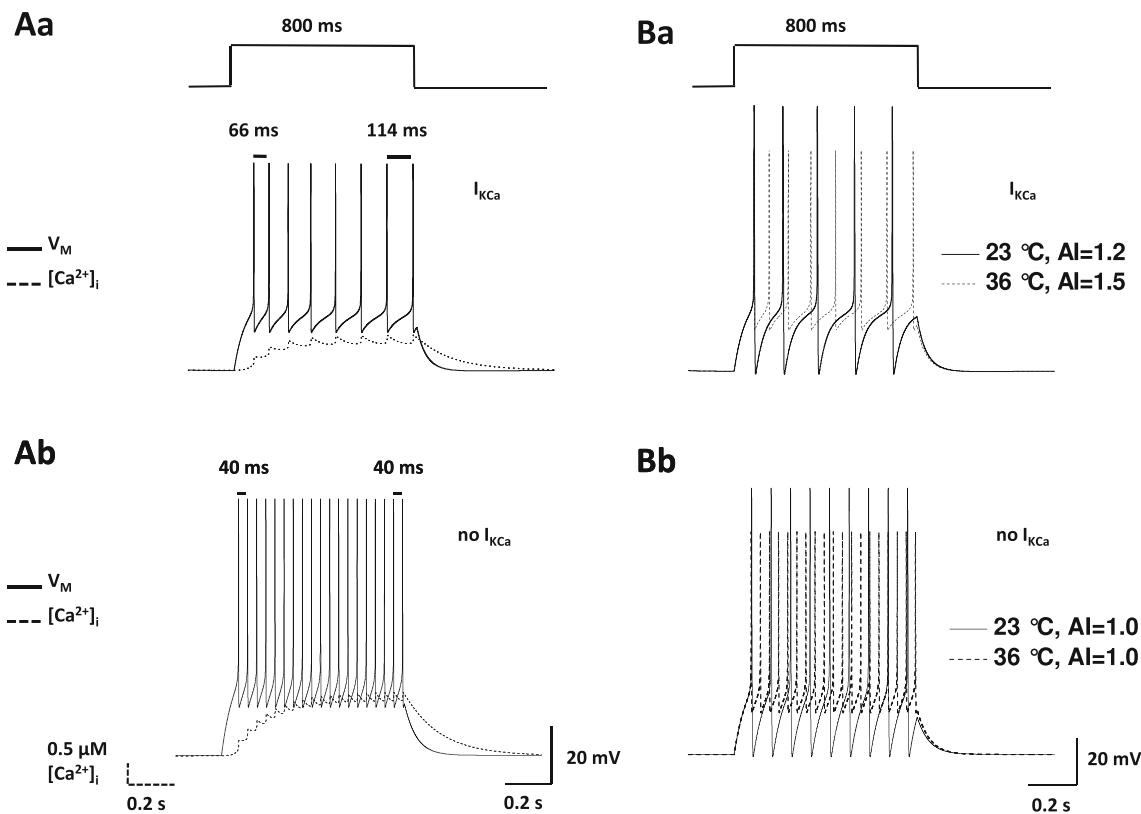
**Fig. 4** Effects of the  $\beta_2$ -adrenergic agonist salmeterol and the  $BK_{Ca}$ -specific blocker cocktail (ChTx/Pax) on  $K^+$  outward currents. Salmeterol (**a**), the  $BK_{Ca}$ -specific blockers charybdotoxin and paxilline (**b**) as well as the combination of all three substances (**c**) were applied to thalamocortical neurons of ACI dLGN. Original traces of the total outward current under control (*left panel*) and substance application conditions (*middle panel*) as well as the sensitive component (ctrl—substance application; *right panel*) are shown in **a(a)**, **b(a)** and **c(a)** and

were obtained from the same neurons, respectively. Calibration bars indicated in **a(a)** at the left account for all left and middle traces, and calibration bars indicated in **a(a)** at the right account for all right traces. Bar graphs in **a(b)**, **b(b)** and **c(b)** indicate the relative values of the reduction of the current peak amplitude, the inactivation ratio (IR) and the time-to-peak (TTP), respectively. **d** Voltage-clamp stimulation protocol underlying the depicted traces (\*\* $p < 0.01$ )

frequency adaptation and a decrease in the number of APs per spike train. This effect was abolished by  $\beta_2$ -adrenergic activation, phosphatase inhibition and direct blocking of  $BK_{Ca}$  channels. (2) Analysis of burst firing at hyperpolarised levels of the membrane potential showed that blockade of  $BK_{Ca}$  channels led to an increase in the number of APs crowning the LTS in neurons of non-epileptic rats, while burst firing behaviour of cells from absence epilepsy rats (WAG/Rij) remained unchanged. (3) The phosphorylation-regulated splice variations of  $BK_{Ca}$  channels—STREX and ZERO—are expressed in ACI dLGN neurons. (4)  $\beta_2$ -adrenergic stimulation-dependent inhibition of  $BK_{Ca}$  channels appears to be complete, since the extent of current reduction was similar with a specific channel blocker combination. (5) Single-cell

computer modelling confirmed an  $I_{KCa}$ -induced spike frequency adaptation in TC neurons.

It is concluded that current through  $BK_{Ca}$  channels contributes to both major firing modes of dLGN TC neurons. This is the first time that  $\beta_2$ -adrenergic stimulation was shown to suppress  $BK_{Ca}$  channel activation in these neurons. Beyond the cellular level, this effect might be of relevance during the behavioural state of wakefulness, since it should promote the faithful transfer of sensory information. However, during the pathophysiological state of epilepsy,  $BK_{Ca}$  channels seemingly contribute to alterations of burst firing that might support the oscillatory, highly synchronised activity of neuronal networks.



**Fig. 5** Reducing  $I_{KCa}$  affects tonic firing in silico. The single TC neuron model was set to rest at a potential of  $-60$  mV and tonic firing was analysed by applying 800 ms lasting depolarising pulses in current-clamp mode (stimulation protocol indicated in **a(a)** and **b(a)**). **a** Typical tonic firing behaviour (solid line) in combination with intracellular  $Ca^{2+}$  dynamics ( $[Ca^{2+}]_i$ ; dashed line) at body temperature with (a) and without (b) the contribution of  $I_{KCa}$ . Note that the interspike interval (ISI) for the first two APs and the last two APs are almost doubled in a, while the ISIs remain stable in b.  $Ca^{2+}$  peaks coincide with APs while the intracellular  $Ca^{2+}$  concentration slowly rises during

the depolarising pulse and finally reaches a plateau. Probably due to the increased firing rate in b, the maximal  $Ca^{2+}$  concentration is higher. Calibration bars are valid for a and b. **b** Comparison of tonic firing behaviour with (a) and without (b)  $I_{KCa}$  at room and body temperature. Note in a that the in vitro condition at room temperature (solid line) even dampens the spike frequency adaptation as the adaptation index (AI) at body temperature (dashed line) is larger (AI=1.5) compared to 23 °C (AI=1.2). Exclusion of  $I_{KCa}$  still abolishes any spike frequency adaptation. Calibration bars are valid for a and b

#### The contribution of $BK_{Ca}$ channels to burst firing patterns of TC neurons

Upon depolarisation from potential levels negative to  $-65$  mV, thalamic neurons fire AP bursts on top of a LTS, both under *in vivo* and *in vitro* conditions. It is well known that this burst firing behaviour dominates the TC network during physiological as well as pathophysiological behavioural states, i.e. slow-wave sleep, deep anaesthesia and absence epileptic seizures. It is also known that  $\beta$ -adrenergic signalling suppresses rhythmic burst firing and induces tonic firing in TC neurons by the modulation of hyperpolarisation-activated, cyclic nucleotide-gated cation (HCN) channels [36, 41].

Long-term recordings revealed no difference between the rat strains in the number of action potentials per burst. However, specific blockade of the current through  $BK_{Ca}$  channels led to a significant increase in the number of LTS-associated APs in ACI TC neurons, while there were

no alterations in WAG/Rij cells. TTP values were only slightly increased in both strains. Moreover, we found a nominally smaller blocker-sensitive current in WAG/Rij neurons (see Fig. 2). It is well known that different membrane currents contribute to LTS generation, among them the low-threshold  $Ca^{2+}$  current ( $I_T$ ) and the pacemaker current  $I_h$ . For both membrane currents, altered electrophysiological properties have been found in TC neurons from ACI compared to WAG/Rij rats [5, 28]. These alterations seem to stabilise LTS generation in TC neurons from WAG/Rij rats in a way that block of  $BK_{Ca}$  channels did not change LTS generation of epileptic rats in the present study. We therefore thought it legitimate to propose that a lack of  $I_{KCa}$  will influence the delicate interplay of ionic currents leading to diverging firing patterns in ACI and WAG/Rij neurons. Although long-term recordings revealed no differences between the strains, these findings suggest that TC neurons of epileptic WAG/Rij rats might possess  $BK_{Ca}$  channels with different pharmacological profiles (i.e. splice variants)

compared to the control group. However, studies concerning the quantitative expression of BK<sub>Ca</sub> channel variants in different rodent strains are still missing. Previous studies have shown that the classical anti-absence drug ETX blocks—besides other ion channels—BK<sub>Ca</sub> channels [6, 31]. Indeed, application of this drug induced a modulation of thalamic burst activity in terms of a delayed LTS onset as well as an increase in the number of LTS-associated APs [6]. In this previous work, we have shown that the block of  $I_T$  induced the delayed onset, whereas a reduction in  $I_{KCa}$  was proposed to have caused the higher number of APs crowning the LTS. This conclusion is verified by the use of highly specific BK<sub>Ca</sub> channel blockers in the present study. On the level of neuronal networks, these BK<sub>Ca</sub>-mediated alterations of burst firing might—like ETX application does—support the suppression of oscillatory, highly synchronised activity.

#### The contribution of BK<sub>Ca</sub> channels to tonic firing of dLGN TC neurons

Here, we show that  $I_{KCa}$  in dLGN TC neurons is modulated by the cyclic AMP/PKA signalling pathway. In addition, STREX mRNA is expressed in the rodent thalamus at all postnatal ages [32] and was detected in dLGN in the present study (see Fig. 3). Inhibition of BK<sub>Ca</sub> channels requires the phosphorylation of only one  $\alpha$ -subunit at a STREX-specific PKA consensus site [11, 32, 54], and previous data from our lab revealed a cyclic AMP-mediated inhibition of BK<sub>Ca</sub> channels in TC neurons of the VB [1]. The STREX variant is labelled by a 174-bp insertion into the C-terminus of the pore-forming subunit that comprises an additional PKA consensus sequence [55]. Furthermore, distinct stoichiometry of BK<sub>Ca</sub> channel tetramer phosphorylation specifies channel activation or inhibition. Phosphorylation of serine 899 (S899) by PKA in all four  $\alpha$ -subunits of the BK<sub>Ca</sub> channel is required to increase channel activity, but PKA phosphorylation within the STREX-insert of a single  $\alpha$ -subunit is sufficient for channel inhibition [11, 32, 54]. Thus, it is reasonable to assume that channel phosphorylation leads to current reduction in dLGN TC neurons. What are the functional consequences of this current decrease for the tonic firing behaviour? During the time course of the whole-cell current-clamp experiments presented here, spike frequency adaptation occurred at recording times exceeding 15–20 min while applying repeated depolarising current steps. This build-up of adaptation was sensitive to application of selective BK<sub>Ca</sub> channel blockers (IbTx; ChTx/Pax), blockade of intracellular phosphatases and pharmacological  $\beta_2$ -adrenergic (not  $\beta_1$ -adrenergic) receptor stimulation. The results were confirmed in perforated patch recordings, suggesting a physiological mechanism, rather than a matter of cell viability. Inhibition of endogenous phosphatases even induces a stronger effect than the activation of the cAMP/PKA pathway after salmeterol application. This is

most probably due to the fact that phosphorylation-induced inhibition of BK<sub>Ca</sub> channel activity cannot only be ascribed to PKA, but also to protein kinase C activity [50]. In sum, these findings suggest that there is a highly regulated kinase–phosphatase balance and that basal and/or stimulus-induced activity of endogenous protein phosphatases causes a slow increase in BK<sub>Ca</sub> channel availability (see Fig. 1a and Supplementary Fig. 2 [23]), so that spike frequency adaptation appears. In silico, a simulation of a slowly increasing  $I_{KCa}$  induced spike frequency adaptation in TC neurons (see Supplementary Fig. 3). Dephosphorylation of PKA consensus sites on STREX-containing BK<sub>Ca</sub> channels is a possible basis for this change in channel function [23, 54]. Stimulation of BK<sub>Ca</sub> channel phosphorylation by salmeterol may abolish this phenomenon during the recordings presented here.

Besides BK<sub>Ca</sub> channels, HVA Ca<sup>2+</sup> channels which are thought to contribute to tonic firing in TC neurons are sensitive to cAMP-dependent modulation [40, 42]. Furthermore, a member of the two pore domain (K<sub>2P</sub>) channel family, termed TREK-1, is known to be inhibited by cyclic AMP and expressed in TC neurons [2, 39]. Indeed, a cyclic AMP-inhibited leak conductance has been described in TC neurons of rat dLGN [7]. Therefore, several ion channels are expected to contribute to the cyclic AMP-dependent modulation of the tonic firing mode of thalamic neurons.

Within a train of APs, BK<sub>Ca</sub> channels only became activated at late phases of the depolarising pulse, when intracellular Ca<sup>2+</sup> levels had slowly built up [10], thereby allowing the voltage-induced channel activation. This conclusion is confirmed by the finding that BK<sub>Ca</sub> channel blockers had no effect on the ISIs of the first few APs (data not shown). This scenario is supported by addressing the functional significance of BK<sub>Ca</sub> channel activation for tonic AP firing in computer simulations. During long depolarising current steps, intermittent AP firing, a deviation from regular tonic firing, was previously modelled by the interaction of inward Ca<sup>2+</sup> currents and a Ca<sup>2+</sup>-activated K<sup>+</sup> current (resembling current through BK<sub>Ca</sub> channels) via an intracellular increase of Ca<sup>2+</sup> [56]. When BK<sub>Ca</sub> channels were removed from the computer model, only full tonic firing without any intermittence was obtained thereby corroborating the results presented here. As ion channel kinetics and intracellular Ca<sup>2+</sup> dynamics are known to be temperature dependent, we additionally used this approach to verify that the results obtained from slice experiments at room temperature were comparable to the results obtained at body temperature.

#### BK<sub>Ca</sub> channels as a fine adjustment mechanism of thalamocortical activity

The findings of the present study demonstrate that BK<sub>Ca</sub> channel-mediated membrane current contributes to the

major firing patterns of dLGN TC neurons, burst and tonic. In the burst mode, BK<sub>Ca</sub> channels affect parameters (number of APs) that may support an anti-oscillatory effect on network activity. Importantly, this influence is reduced in neurons of epileptic WAG/Rij. In tonic firing,  $\beta_2$ -adrenergic modulation of  $I_{KCa}$  will affect the information relay to the cortex. Although the cyclic AMP-dependent increase in L-type and N-type Ca<sup>2+</sup> channels [40] and the release of Ca<sup>2+</sup> from intracellular stores, occurring during tonic firing [10], are generally able to strongly activate BK<sub>Ca</sub> channels during membrane depolarisation, the cyclic AMP-dependent inhibition of STREX-containing channel heteromers seems to outbalance this effect. In terms of information processing, this effect makes perfect sense, since spike frequency adaptation gets prevented and information from the periphery is faithfully transmitted for cortical processing. It is concluded that BK<sub>Ca</sub> channels exert a precisely tuneable influence on thalamic activity modes and, in particular, modulate the 1:1 relay of sensory information through the thalamus.

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