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Calcium-activated SK potassium channels are key modulators of the pacemaker frequency in locus coeruleus neurons

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ABSTRACT

The physiological, intrinsic activity of noradrenergic locus coeruleus (LC) neurons is important for the control of sleep/wakefulness, cognition and autonomous body functions. Dysregulations of the LC-noradrenergic network contribute to the pathogenesis of psychiatric disorders and are key findings in early stages of neurodegenerative diseases. Therefore, identifying ion channels mediating the intrinsic pacemaking mechanism of LC neurons, which is in turn directly coupled to Ca^{2+} homeostasis and cell survival signaling pathways, can help to foster our understanding of the vulnerability of these neurons in neurodegenerative diseases. Small-conductance Ca^{2+} -activated K^{+} (SK) channels regulate the intrinsic firing patterns in different central neurons and are essential regulators of the intracellular Ca^{2+} homeostasis. However, the role of SK channels for the intrinsic pacemaking of LC neurons in mice is still unclear. Therefore we performed qPCR expression analysis as well as patch clamp recordings of *in vitro* brainstem slices, for instance testing SK channel blockers and activators like apamin and NS309, respectively. Although we found a transcriptional expression of SK1, SK2 and SK3 channels, SK2 was the predominantly expressed subunit in mouse LC neurons. Using perforated-patch clamp experiments, we found that SK channels are essential regulators of the intrinsic pacemaking of LC neurons, mediating a large fraction of the afterhyperpolarization (AHP) in these cells. Consistent with a previous observation that a concerted action of L- and T-type Cav channels is essential for the pacemaking of LC neurons, we found that SK channel activation, and the respective AHP amplitude, is primarily coupled to Ca^{2+} influx via these types of Ca^{2+} channels. Our study identified SK2 channels as drug targets for the tuning of the pacemaker frequency in disorders involving a dysregulation of the LC.

1. Introduction

The release of noradrenaline (NA) from brainstem locus coeruleus (LC) neurons plays a major role in physiological regulatory networks such as arousal/sleep, cognition or autonomous functions (Szabadi, 2013). Disturbances of the LC-noradrenergic system have been identified in various psychiatric disorders like attention deficit hyperactivity disorder, schizophrenia or posttraumatic stress disorders and represent an early event in neurodegenerative disorders like Alzheimer's (AD) or Parkinson's disease (PD) (Berridge and Waterhouse, 2003; Rommelfanger and Weinshenker, 2007; Benarroch, 2009). As electrical

activity is directly coupled to Ca^{2+} homeostasis and cell survival signaling pathways, it is suggested that ion channels underlying the intrinsic activity of specific neuronal populations can be promising drug targets to prevent neurodegeneration (Surmeier et al., 2011; Dragicevic et al., 2015). Therefore it is of utmost importance to elucidate the mechanisms that regulate the distinct activity patterns of these neurons. The activity pattern of LC neurons *in vivo* depends upon the attention-level of the organism and can be differentiated into either tonic or phasic firing modes. Phasic activity is related to performances that require focused attention (Aston-Jones and Bloom, 1981; Hervé-Minvielle and Sara, 1995), whereas tonic activity is observed during unfocused

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Fig. 1. Electrophysiological characteristics and I_{AHP} in noradrenergic LC neurons of the mouse. (A) Whole-cell current clamp recording of a spontaneously active LC neuron (asterisk) in a brainstem section of a juvenile C57BL/6j mouse. To isolate autonomous spiking ACSF was complemented with GABAergic (CGP, gabazine) and glutamatergic (AP-5, NBQX) blockers. Pacemaking frequency under control conditions was 2.0 ± 0.2 Hz ($n = 13$). Note the pronounced afterhyperpolarization after each action potential. (B) Co-staining of neurobiotin (NB) filled neurons with Alexa488 conjugated streptavidin (green) and anti-Tyrosine hydroxylase (TH)/anti-rabbit Alexa568 (magenta). Scale bar: 20 μ m. (C) Representative whole-cell voltage clamp recording of an I_{AHP} , activated by a two-pulse voltage clamp protocol depicted in the inset. The decay of I_{AHP} followed a bi-exponential time course representing a “medium” and a “slow” component of the I_{AHP} (gray lines). (D) Decay time constants (Tau, τ) were determined by fitting the data to a Boltzmann function. Quantification of the decay time revealed 128 ± 24 ms for τ_{medium} (τ_m) and 967 ± 174 ms for τ_{slow} (τ_s) (mean $\pm$ sem; $n = 22$). Box plots display median and 25/75 percentile, whiskers indicate outliers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alertness and is increased in response to stressful stimuli (Gompf et al., 2010; Takahashi et al., 2010). In freshly prepared slices of rat and mouse brain and under synaptic isolation, LC neurons display pacemaker activity generating regular action potentials (APs) with a frequency of 1–3 Hz (Sanchez-Padilla et al., 2014; Matschke et al., 2015). Electrophysiological studies, mainly conducted in rat LC, have described various potassium currents (I_A , I_K , I_{KATP} , I_{Kir} , I_{KCa}) (Forsythe et al., 1992; Osmanović and Shefner, 1993; Nieber et al., 1995; Murai et al., 1997; Murai and Akaike, 2005; Zhang et al., 2010), inward sodium currents (I_{Na}) (Chiang and Bekkers, 1999) and calcium currents (I_{Ca}) mediated by L-, T-, N- and P/Q/R-type voltage-activated calcium (Cav) channels (Chiang and Bekkers, 1999; Connor et al., 1999; Sanchez-Padilla et al., 2014; Matschke et al., 2015). Nevertheless, there is still a lack of studies analyzing the composition of ion channels driving spontaneous activity of LC neurons in rodents, particularly in mice, which are, however, frequently used in transgenic disease models. Previous studies in the mouse LC suggest that a combination of TTX-sensitive Na^+ and TEA-sensitive K^+ currents (de Oliveira et al., 2010), as well as a combination of L- and T-type Ca^{2+} currents (Matschke et al., 2015) play a role in regulation of the pacemaking mechanism.

Small conductance Ca^{2+} -activated K^+ channels (SK channels) are significant regulators of the Ca^{2+} homeostasis in neurons, as Ca^{2+} -influx mediated activation of SK channels provides a counterbalance for an efficient Ca^{2+} entry (Dolga and Culmsee, 2012). In addition, SK channels modulate the afterhyperpolarization (AHP) and thereby the AP frequency and/or regularity of various central neurons, depending on the expression of different SK channel family members (Sah and Davies, 2000; Deignan et al., 2012). The SK channel family is composed of four members, SK1 to SK4. SK4 is, based on its intermediate conductance, also named IK (Wei et al., 2005). Gating of SK channels is voltage-independent and solely regulated by changes of the intracellular Ca^{2+} concentration (Kuiper et al., 2012). Binding of Ca^{2+} to Calmodulin, which functions as Ca^{2+} sensor constitutively coupled to the C-terminus of these channels, induces conformational changes leading to an opening of the ion conduction pathway in the selectivity filter (Bruening-Wright et al., 2007).

In central neurons SK channels are usually located in close proximity to neurotransmitter receptors (like glutamatergic NMDA- or nicotinic acetylcholine-receptors) and counteract excitatory synaptic potentials, thereby functioning as significant regulators of neuronal excitability (Faber et al., 2005; Dolga and Culmsee, 2012). In addition,

a direct coupling of SK channels to distinct Cav channels has been reported in various types of neurons. For instance, in hippocampal CA1 neurons SK channels are associated with L- and N-type Cav channels (Marrion and Tavalin, 1998; Bowden et al., 2001) whereas in dopaminergic SN neurons coupling of SK channels to T-type Cav channels induces transition into the burst mode (Wolfart and Roeper, 2002).

Osmanovic et al. reported SK channel mediated currents in rat LC neurons and a role of these channels in firing rate accommodation and post stimulus hyperpolarization (PSH) was proposed. However, no significant effects were observed in regulating spontaneous pacemaking (Osmanović et al., 1990; Osmanović and Shefner, 1993). Subsequent studies analyzed the SK conductances in the rat LC, demonstrating a role in the context of ischemic insults (Murai et al., 1997) or as modulators of excitability in response to long depolarizing pulses (Scuvée-Moreau et al., 2004). Despite these early and seminal studies, it is not yet known which types of SK channels are relevant in LC neurons of the mouse, how these channels affect the intrinsic pacemaking mechanism under physiological conditions and whether SK channels couple to distinct Cav channels.

In the present study we aimed to elucidate the molecular identity and a potential role of SK channels in the pacemaking mechanism of murine LC neurons. Using PCR expression analysis, together with slice patch clamp experiments utilizing ion channel blockers and/or activators, we identified the functional expression of distinct SK channel subunits in noradrenergic LC neurons. We observed that in the mouse LC SK channels can be manipulated in a way to either decrease or increase the spontaneous AP frequency and report a coupling of SK channels to distinct Cav channel family members. These novel insights into the role of SK and Cav channels for the intrinsic pacemaking activity of LC neurons provide another important piece of puzzle to foster our understanding of pathological changes during diseases that are associated with dysregulation of Ca^{2+} homeostasis or activity dependent oxidant stress.

2. Results

2.1. Identification of SK channel mediated AHP currents in LC neurons

To investigate whether SK channels play a role in modulating the AHP of murine LC neurons, whole-cell voltage clamp experiments in acute brainstem slices were performed. All recordings were done in the presence of synaptic blockers to isolate the intrinsic firing properties.

Noradrenergic neurons recorded in the LC showed slow spontaneous pacemaker activity (1–3 Hz) with large AHPs following each AP (Fig. 1A), consistent with previous findings in mouse and rat LC (Osmanović et al., 1990; Sanchez-Padilla et al., 2014; Matschke et al., 2015). During whole-cell patch clamp recordings the neurons were filled with neurobiotin (NB) and later co-stained with an anti-Tyrosine hydroxylase (TH) antibody to verify that the analyzed neurons are in fact noradrenergic LC neurons (Fig. 1B).

To examine the AHP currents, a two step voltage clamp protocol was used, similar as in previous studies isolating neuronal AHP current complexes (Storm, 1989; Wolfart et al., 2001). Here, from a holding potential of -70 mV the membrane potential was depolarized to 0 mV for 50 ms followed by a hyperpolarizing step to -50 mV, mimicking a single AP (see inset in Fig. 1C). To preserve physiological Ca^{2+} concentrations, an intracellular solution with low Ca^{2+} buffering (0.1 mM EGTA) was used. AHP outward currents (I_{AHP}) recorded at the step to -50 mV peaked within 10 ms, followed a normal distribution with a mean maximum of 475 ± 22 pA (Fig. 1C; $n = 56$) and were stable throughout the experiment (rundown of $12.4 \pm 6.1\%$ within 10 min; $n = 20$, not illustrated). In the majority of neurons the decay of the I_{AHP} followed a bi-exponential time course with an intermediate decay time constant of 128 ± 24 ms (mI_{AHP}) and a much slower component with a mean time constant of 967 ± 174 ms (sI_{AHP}) (Fig. 1C and D, $n = 22$). However, in only a few neurons an additional I_{AHP} component with ultrafast decay time constants of 23 ± 8 ms could be resolved ($n = 7$, not illustrated). These biophysical characteristics resemble the fast (fI_{AHP}), the medium (mI_{AHP}) and slow (sI_{AHP}) AHP currents, respectively, described in dopaminergic SN, cholinergic vagal or hippocampal pyramidal neurons (Storm, 1987; Sah and McLachlan, 1991; Sah, 1996; Wolfart et al., 2001). In SN and vagal neurons, the respective mI_{AHP} is mostly mediated by SK channels. The currents with medium deactivating kinetics, reflecting the mI_{AHP} in murine LC neurons, were the predominant current component with $75.5 \pm 3.7\%$ of the total I_{AHP} amplitude. To test whether also in LC neurons Ca^{2+} -activated K^+ channels contribute to the I_{AHP} , we removed the extracellular Ca^{2+} from the recording solution. After depletion of extracellular Ca^{2+} the AHP current density was strongly reduced (Fig. 2A and D; control: 8.8 ± 0.6 pA/pF, $n = 20$; 0 Ca^{2+} : 4.7 ± 0.5 pA/pF, $n = 7$), indicating that a major component of the I_{AHP} is indeed mediated by Ca^{2+} -activated K^+ channels. However, the existence of a residual outward current after removal of extracellular Ca^{2+} suggests that a part of the I_{AHP} is independent of the two step protocol mediated Ca^{2+} influx.

To gain closer insights into the K^+ channel subtypes that mediate the Ca^{2+} -sensitive AHP currents in LC neurons, we performed pharmacological experiments using the SK channel activator 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) (Strøbaek et al., 2004) and the SK channel blocker apamin (Lamy et al., 2010). NS309 is an activator of Ca^{2+} -activated K^+ channels with high affinity to both SK and IK channel subunits (Strøbaek et al., 2004). Wash-in of $1 \mu\text{M}$ NS309 significantly increased the AHP peak current densities by a factor of 1.4 to 11.9 ± 1.1 pA/pF (Fig. 2B and D; $n = 6$). In contrast, wash-in of 200 nM of the bee venom apamin, a compound that blocks Ca^{2+} -activated K^+ channels of the SK type in low nanomolar concentrations (Hugues et al., 1982) reduced the AHP peak current densities to the same extent as removal of extracellular Ca^{2+} (Fig. 2C and D; apamin: 5.3 ± 0.4 pA/pF; $n = 9$). Therefore, SK channels predominantly contribute to the Ca^{2+} -sensitive I_{AHP} in LC neurons. In addition, the large amplitude of the apamin-sensitive currents with a rapid decay time of 82 ms (Fig. 2E, gray trace) suggested that SK channels mainly contribute to K^+ outward currents of the mI_{AHP} . To test this hypothesis we again analyzed the biophysical properties of the I_{AHP} and compared the proportion of the medium and slow AHP components before and after wash-in of apamin. Inhibition of SK channels by apamin strongly reduced the large mI_{AHP} component with $75.5 \pm 3.7\%$ of the total I_{AHP} to $34.1 \pm 4.7\%$ (Fig. 2F; $n = 9$), indicating that a major fraction of the mI_{AHP} is mediated by SK channels. In contrast, the slow component of

the AHP currents was not affected by apamin ($127.5 \pm 16.9\%$ residual fitted sI_{AHP} , not illustrated). These results strongly suggest that Ca^{2+} -activated SK channels primarily encode the dominant mI_{AHP} component in LC neurons.

2.2. SK2 is the predominant small conductance Ca^{2+} -activated K^+ channel expressed in LC neurons

Next, we tested which Ca^{2+} -activated K^+ channels are expressed in the LC using qualitative reverse transcriptase (RT)-PCR and quantitative RT-PCR (qPCR) analysis. To this end, 15 – 20 LC neurons were collected (pulled) out of acute brainstem slices with a modified patch pipette and total RNA was subsequently isolated and reverse transcribed (see methods section). To verify that the cDNA samples are in fact derived from LC neurons and to exclude contaminations by interneurons, we first tested for an expression of the marker enzyme of noradrenergic neurons (dopamine-beta-hydroxylase (Dbh)) and the absence of the marker enzyme for GABAergic neurons (glutamic acid decarboxylase (GAD)) (data not shown). Qualitative RT-PCR analysis of these samples revealed expression of SK1, SK2, SK3 and BK channel subunits, with strongest signal for SK2 channels (Fig. 3A, $n = 3$). SK4 was not detected, however, as a positive control SK4 transcripts were amplified from whole brain cDNA of the mouse (Fig. 3B, $n = 3$). Next, we performed qPCR experiments to quantify which SK channel is the predominant subtype in LC neurons. Notably, analysis of the relative expression levels (rE; normalized to GAPDH) revealed a 2.8 -fold higher expression level of SK2 compared to SK1 and SK3 (Fig. 3C and D, $n = 3$). Hence, we propose SK2 as the dominant SK channel in noradrenergic LC neurons, mediating the majority of the Ca^{2+} -sensitive mI_{AHP} component.

2.3. SK channel activity regulates the pacemaker frequency of LC neurons under patch clamp conditions leaving the intracellular Ca^{2+} handling unaffected

Differential expression of SK channels is known to regulate neuronal pacemaker firing patterns. In dopaminergic SN neurons for instance SK2 and SK3 channels regulate pacemaker precision and frequency, respectively (Deignan et al., 2012). In addition, predominant expression of SK2 channels in CA3 stratum radiatum interneurons or cerebellar Purkinje neurons is important for the regulation of the respective intrinsic firing patterns (Stocker, 2004). In rat LC neurons it was shown that apamin-sensitive conductances are functionally important for firing rate accommodation due to prolonged depolarizations. However, in the intracellular recordings performed in this previous study apamin only had an impact on the spontaneous pacemaking in one third of the examined LC neurons (Osmanović et al., 1990).

As we found a strong functional expression of SK2 channel subunits in mouse LC neurons, contributing to the majority of the mI_{AHP} component, and as these channels regulate spontaneous firing in several other neuronal types, we aimed to re-investigate whether SK channels might play a role in regulating the intrinsic firing pattern of these neurons. Therefore we first studied the impact of apamin on spike trains recorded in the whole-cell current clamp configuration with 0.1 mM EGTA intracellular solution. Two minutes after recording spike trains in ACSF with stable frequencies, the solution was switched to another vial containing ACSF complemented with 200 nM apamin. To make sure that effects are not only caused by artifacts of the solution switch, control measurements were performed with a switch to another vial containing control ACSF. The relative change of the intrinsic firing frequency (frequency two minutes after the switch divided by the frequency before the switch of solutions) was assessed in control and in apamin measurements and compared to each other. Using this configuration we detected a mild, but not significant, elevation of spontaneous AP frequency (relative firing rate control: 0.94 ± 0.07 , $n = 8$; 200 nM apamin: 1.14 ± 0.19 , $n = 5$, Fig. 4A), which is consistent with

Fig. 2. Electrophysiological characteristics and I_{AHP} in noradrenergic LC neurons. (A–C) Representative I_{AHP} recordings before and after wash-in of Ca^{2+} free ACSF (A), 1 μ M NS309 (B) or 200 nM apamin (C). (D) I_{AHP} current densities were significantly reduced after depletion of extracellular Ca^{2+} and wash-in of apamin, while NS309 led to increased current densities. Control: 8.8 ± 0.6 pA/pF ($n = 20$); 0 Ca^{2+} : 4.7 ± 0.5 pA/pF ($n = 7$); NS309: 11.9 ± 1.1 pA/pF ($n = 6$); apamin: 5.2 ± 0.6 pA/pF ($n = 9$). (E) Display of a representative difference current (gray) obtained by subtracting AHP current after apamin wash-in from a control recording. (F) Fraction of the medium I_{AHP} component before and after SK channel block with apamin. Apamin significantly reduced the fitted mI_{AHP} from $75.5 \pm 3.7\%$ to $34.1 \pm 4.7\%$ ($n = 9$). Box plots display median and 25/75 percentile, whiskers indicate outliers. *: $P \leq 0.05$; ***: $P \leq 0.001$ using unpaired (D) or paired (F) Student's t -test.

Fig. 3. Mouse LC neurons express distinct types of Ca^{2+} -activated K^+ channels. For expression analysis total RNA was isolated from LC neurons collected out of acute brainstem slices. Intron-spanning primers were designed to generate PCR products of about 110 base pairs. The housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the noradrenergic marker enzyme dopamine-beta-hydroxylase (Dbh) served as positive controls. (A) Reverse transcription (RT-) PCR expression analysis revealed expression of SK1, SK2, SK3 and BK channel subunits, but not of SK4 in LC neurons. (B) Positive control for SK4 using whole brain cDNA. (C) Representative qPCR amplification plots for GAPDH and the SK channel subunits SK1, SK2 and SK3 in LC neurons. (D) Relative expression (rE) levels of SK channel subunits normalized to GAPDH. Note the 2.8-fold higher expression level of SK2 compared to SK1 and SK3. n.c.: no template control. Box plots display median and 25/75 percentile, whiskers indicate outliers. ***: $P \leq 0.001$ using unpaired Student's t -test.

the findings of Osmanovic *et al.*

The open probability of SK channels directly depends upon changes of the intracellular Ca^{2+} concentration, which is delicately regulated by an interplay of buffer systems, pumps and Ca^{2+} channels (Kuiper *et al.*, 2012). It is likely that this interplay is distorted during long-lasting recordings in the whole-cell configuration where the intracellular neuronal milieu is exchanged by the pipette solution. That is why the impact of SK channels on the physiological firing behavior of LC neurons might not be detected in the whole-cell configuration. Therefore,

we subsequently recorded spike trains in the gramicidin-D perforated-patch configuration to warrant a physiological intracellular Ca^{2+} concentration and handling. In this configuration spontaneous APs of the LC neurons spiked with a mean frequency of 2.5 ± 0.7 Hz ($n = 13$) under control conditions (Fig. 4B, left panel). Interestingly, now inhibition of SK channels by apamin led to a consistent increase of the pacemaker frequency in all neurons examined (Fig. 4B; relative firing rate control: 0.96 ± 0.03 , $n = 13$; 200 nM apamin: 1.50 ± 0.13 , $n = 8$). To elucidate whether SK channels might also influence the

regularity of the intrinsic pacemaker, an analysis of the interspike intervals (ISI) was performed. As displayed in the representative ISI histograms of a control cell before and after apamin application, the ISI distributions were well described by Gaussian functions (red lines in Fig. 4C). In accordance to the results obtained by the frequency analysis, the mean ISI was shifted to smaller values after block of SK channels with apamin (Fig. 4C, control: 1.3 ± 0.07 s, $n = 13$; apamin: 0.8 ± 0.01 s, $n = 8$). As a measure for the precision of the pacemaking mechanism, the coefficient of variation (CV) was measured by the standard deviation of the fitted Gaussian function normalized to the mean ISI (Wolfart et al., 2001). Under control conditions the CV of LC

Fig. 4. SK channel activity regulates the AP frequency of spontaneously active LC neurons under conditions of unbiased Ca^{2+} handling. For quantification of changes in firing frequency AP frequency was analyzed at 2 min after application of drug or control solution and normalized to the frequency recorded directly before switching solutions. (A) Example spike trains before (control) and after 200 nM apamin recorded in the whole-cell current clamp configuration (left panel). Quantification of the relative firing rate did not reveal a significant difference due to apamin (right panel). (B) Representative spike trains before (control) and after 200 nM apamin recorded in the gramicidin- π perforated-patch current clamp configuration (left panel). Under these conditions inhibition of SK channels by apamin induced a pronounced acceleration of spontaneous firing (right panel). (C) Representative interspike interval (ISI) histogram of control measurement (upper panel) and after 200 nM apamin (lower panel). ISI distributions were well described by Gaussian functions (red lines). (D) Analysis of the coefficient of variation (CV) as a measure for regularity of the data shown in (C). Note that SK channel inhibition with apamin did not influence pacemaker precision (CV control: $21 \pm 8\%$, $n = 13$; CV apamin: $19 \pm 6\%$, $n = 8$). Box plots display median and 25/75 percentile, whiskers indicate outliers. n.s.: not significant; ***: $P \leq 0.001$ using unpaired Student's t -test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neurons was $21 \pm 8\%$ of the mean ISI, reflecting high precision of the pacemaker mechanism (Fig. 4D, $n = 13$). Application of apamin did not alter the precision of pacemaking in LC neurons, as the CV was not significantly changed (Fig. 4D, CV apamin: $19 \pm 6\%$, $n = 8$).

An increase in firing frequency can result from various alterations in AP shape and duration, including a reduced AHP amplitude. To gain further insights into the mechanism by which SK channels regulate the spontaneous firing frequency, we analyzed the apamin-induced changes of AP parameters in the current clamp mode. Fig. 5A and B illustrate overlays of representative APs of an LC neuron before and after inhibition of SK channels by apamin. For quantification of relative changes of the AP parameters ten APs directly before and ten APs 2 min after the switch of solutions were compared. As described above, control measurements were performed with a switch to another vial containing control ACSF. In all recorded neurons the AHP reached the most negative potentials within 10 ms following the AP. Notably, apamin induced a significant reduction of the AHP amplitude in a time window from 8 ms to about 100 ms following the peak of the spike (Fig. 5A–C), corresponding to the medium AHP (mAHP). However, apamin did not influence the slow AHP (sAHP) or the very fast repolarizing phase (fAHP) (Fig. 5A, B). The maximum reduction of the AHP amplitude was 5.6 ± 1.2 mV (Fig. 5D). Fig. 5E displays zoom-ins of representative APs before and after apamin application to illustrate the analysis of AP amplitude, AP threshold (V_{thresh}) and AP half-width (APD_{50}). AP amplitude was calculated from V_{thresh} to the peak and APD_{50} was defined as the AP duration at 50% of the amplitude. The relative AP amplitude was reduced after SK channel inhibition (Fig. 5F) which might result from an increased number of refractory voltage-dependent Na^+ channels due to the reduced AHP. V_{thresh} was defined as the membrane voltage at which APs at high magnification illustrate an abrupt rise (Bean, 2007). Apamin neither altered the relative V_{thresh} (Fig. 5G) nor the relative APD_{50} (Fig. 5H). Another mechanism how SK channel inhibition could increase firing rate is a shift of the functional membrane potential (MP) to more depolarized potentials, bringing it closer to V_{thresh} . Analysis of the MP revealed a slight apamin-induced depolarization of 1.1 ± 0.7 mV (Fig. 5I) which was, however, not significant compared to control.

To further elucidate the physiological function of SK channels in LC neurons, we also tested the effect of SK channel activation on the intrinsic firing pattern. Hence, current clamp recordings in the gramicidin- π perforated-patch configuration with low ($1 \mu\text{M}$) and higher ($20 \mu\text{M}$) concentrations of NS309 were performed. As we did not detect SK4 (IK) transcripts in the LC (Fig. 3), the effects of NS309 are primarily attributable to the activation of SK1-3 channels. The representative recordings before and after wash-in of NS309 (Fig. 6A, B), as well as the quantification of the relative firing frequencies, revealed a significant and concentration-dependent slowing of AP rates (Fig. 6C, control:

Fig. 5. SK channel-mediated AHP currents shape the AP of LC neurons. (A) Overlay of representative perforated-patch current clamp recordings before (black) and after application of 200 nM apamin (gray). (B) Zoom-in of the recording illustrated in (A) to show details of the afterhyperpolarization (mAHP) amplitude (arrows). (C) Relative apamin-induced change of the AHP amplitude. SK channel block significantly alleviated AHP. (D) Apamin induced an absolute decrease of AHP of 5.6 ± 1.2 mV. (E) Example APs before (black) and after wash-in of apamin to illustrate AP parameters analyzed in F–H. (F) Relative changes of AP amplitude, calculated from the peak to the AP threshold. (G) Wash-in of apamin did not change the AP threshold (V_{thresh}) defined as the membrane voltage at which APs at high resolution illustrate an abrupt rise. (H) Relative change of AP duration at 50% of the amplitude (APD_{50}). (I) Quantification of the apamin-induced change of the functional membrane potential (MP). A–H: control, $n = 13$; apamin, $n = 8$. Box plots display median and 25/75 percentile, whiskers indicate outliers. n.s.: not significant; *: $P \leq 0.05$; **: $P \leq 0.01$ using unpaired Student's t -test.

0.91 ± 0.05 , $n = 8$; $1 \mu\text{M}$ NS309: 0.60 ± 0.10 , $n = 7$; $20 \mu\text{M}$ NS309: 0.29 ± 0.13 , $n = 7$). When exposed to $20 \mu\text{M}$ NS309, two out of the seven neurons examined, even completely stopped firing. In addition, activation of SK channels with NS309 led to a less precise pacemaking, as reflected by an increase of the CV (Fig. 6D, control: $21 \pm 3\%$, $n = 8$; $1 \mu\text{M}$ NS309: $23 \pm 7\%$, $n = 7$; $20 \mu\text{M}$ NS309: $37 \pm 4\%$, $n = 7$). Notably, NS309 induced a significant enlargement of the relative AHP amplitude in both concentrations (Fig. 6E), reflecting an effect opposite

to that obtained with the blocker apamin. The mean AHP increase was 4.0 ± 0.3 mV with $1 \mu\text{M}$ NS309 and 5.2 ± 0.8 mV with $20 \mu\text{M}$ NS309 (Fig. 6F). In addition, the functional MP was slightly shifted to more hyperpolarized potentials by 2.0 ± 0.6 mV with $1 \mu\text{M}$ NS309 and by 3.3 ± 0.5 mV with $20 \mu\text{M}$ NS309 (Fig. 6G). Therefore, the reduced AHP in combination with a slightly more hyperpolarized functional membrane potential most probably provide the explanation for the decreased firing frequency.

Fig. 6. SK channel activation with NS309 decreases the firing frequency of spontaneously active LC neurons. (A–B) Representative spike trains of LC neurons recorded in the perforated-patch current clamp configuration before (control) and after application of $1 \mu\text{M}$ NS309 (A), or $20 \mu\text{M}$ NS309 (B). (C) Quantification of the relative firing frequency revealed a strong NS309 induced decrease of AP-frequency after 4 min, which was concentration dependent. (D) Effect of NS309 on the interspike interval (ISI) coefficient of variation (CV). (Control, $\text{CV} = 21 \pm 3\%$; $1 \mu\text{M}$ NS309, $\text{CV} = 23 \pm 6\%$; $20 \mu\text{M}$ NS309, $\text{CV} = 37 \pm 4\%$). (E) Relative NS309 induced change of AHP. SK channel activation significantly increased AHP amplitude. (F) Absolute increase of AHP by NS309 ($1 \mu\text{M}$ NS309: 4.0 ± 0.3 mV; $20 \mu\text{M}$ NS309: 5.2 ± 0.8 mV). (G) Quantification of relative changes of the functional membrane potential (MP). NS309 led to a slight membrane hyperpolarization of LC neurons. (A–G) control, $n = 8$; $1 \mu\text{M}$ NS309, $n = 7$; $20 \mu\text{M}$ NS309, $n = 7$. Box plots display median and 25/75 percentile, whiskers indicate outliers. n.s.: not significant. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; using unpaired Student's t -test.

Fig. 7. SK channel activation with NS309 is time-dependent and antagonized by apamin. (A) Representative perforated-patch current clamp recording of an LC neuron over 20 min. Following recording of stable action potentials for 5 min first 20 μ M NS309 and subsequently 200 nM apamin were washed in. (B) Quantification of the time-dependent changes of the firing rate due to SK channel activation by NS309 and subsequent block by apamin. Note that apamin could completely restore regular firing frequencies ($n = 5$). *: $P \leq 0.05$; using Wilcoxon signed-rank test.

As NS309 diffuses into the cytosol of the neurons and thus might also modulate SK channels located at the inner mitochondrial membrane (Dolga et al., 2014), we probed whether the NS309-induced AP alterations are mainly caused by activation of plasmalemmal SK channels. Therefore, spike trains were recorded over 20 min in the presence of NS309 and subsequently membrane impermeable apamin was applied. Analysis of the spontaneous activity of the patched neurons revealed that 9 min after application of NS309 (20 μ M) three out of five cells completely stopped firing and two neurons had a significantly decreased firing rate. Strikingly, subsequent wash-in of 200 nM apamin led to a swift re-occurrence of APs within 2 min, and only 3 more minutes later the firing frequency reached control values again (Fig. 7A, B). As the effects of NS309 on LC APs were swiftly and completely antagonized by apamin, it can be postulated that these effects were indeed mediated by a modulation of plasmalemmal SK channels. These data further support our hypothesis that SK channels play a crucial role in regulating the intrinsic pacemaking of LC neurons.

2.4. SK channel mediated AHP currents are activated by L- and T-type Cav channels

Functional coupling of SK channels to L-, P/Q-, N- or T-type Cav channels, with an impact on the AHP, was demonstrated in several previous studies (Sah and Davies, 2000). In a recent report we have demonstrated that a concerted action of L- and T-type Cav channels regulates the pacemaker frequency of murine LC neurons by modulating the AHP amplitude (Matschke et al., 2015). These results, together with the identification of functional SK1-3 channels in LC neurons described in the current study, point towards a functional coupling of Cav to SK channels. Therefore, in a next experiment, we tested whether SK channel-mediated AHP currents are sensitive to Cav channel inhibitors. Using low concentrations of selective inhibitors for Cav1 (200 nM isradipine), Cav3 (1 μ M Z944) or Cav2 (1 μ M ω -agatoxin + 1 μ M ω -conotoxin) channels, we aimed to determine which family of Cav channels are functionally coupled to SK channels in LC neurons (Williams et al., 1992; Teramoto et al., 1993; Ito et al., 1997; Tringham et al., 2012). Two-pulse protocol evoked AHP currents were recorded under control conditions, followed by wash-in of Cav channel blockers. Subsequently, apamin (200 nM) was washed in to identify the complete SK channel mediated current amplitudes. Fig. 8A displays representative AHP current traces during control, isradipine and apamin application. Low nanomolar concentrations of isradipine (200 nM) significantly reduced the peak I_{AHP} . However, subsequent apamin application led to a further, significant reduction of the AHP currents (Fig. 8A, B, control: 7.6 ± 0.7 pA/pF; isradipine: 5.4 ± 0.8 pA/pF; apamin: 3.8 ± 0.7 pA/pF, $n = 9$). AHP currents were blocked to a similar extent by 1 μ M of the Cav3 channel blocker Z944 (Fig. 8C, D, control: 8.3 ± 0.7 pA/pF; Z944: 6.2 ± 0.6 pA/pF;

apamin: 4.6 ± 0.6 pA/pF, $n = 8$). When isradipine and Z944 were applied simultaneously apamin did not further reduce AHP currents (Fig. 8E, F, control: 8.3 ± 0.5 pA/pF; isradipine + Z944: 4.2 ± 0.5 pA/pF; apamin: 3.8 ± 0.6 pA/pF, $n = 5$). In contrast, a blocker cocktail of ω -agatoxin (1 μ M) and ω -conotoxin (1 μ M), to inhibit P/Q- and N-type Cav channels, only slightly reduced the AHP current amplitudes (Fig. 8G, H, control: 9.0 ± 1.9 pA/pF; agatoxin + conotoxin: 8.6 ± 2.1 pA/pF; apamin: 4.0 ± 1.2 pA/pF, $n = 7$). Comparison of the Cav channel blocker- and apamin-sensitive I_{AHP} current densities revealed that the drug-sensitive AHP currents had similar amplitudes for isradipine (2.2 ± 0.4 pA/pF) and Z944 (2.0 ± 0.1 pA/pF), whereas the agatoxin/conotoxin sensitive I_{AHP} component was only 0.5 ± 0.4 pA/pF (Fig. 8I). Thus, the mixture of agatoxin/conotoxin did not significantly influence the apamin-sensitive I_{AHP} . Similar to the results obtained above (Fig. 2D), the apamin-sensitive component of the I_{AHP} had an amplitude of 4.1 ± 0.3 pA/pF (Fig. 8I). Our data indicate that about 50% of the SK channel mediated I_{AHP} is coupled to a Ca^{2+} influx via Cav1 channels (isradipine-sensitive) while the other 50% are related to a coupling to Cav3 channels (Z944-sensitive). This assumption is supported by the finding that simultaneous application of isradipine and Z944 reduced the I_{AHP} to a similar extent as apamin (4.0 ± 0.2 pA/pF drug-sensitive current, Fig. 8I). To further support a functional coupling of SK channels to Cav channels we determined whether the Cav channel blockers exert an effect on the I_{AHP} after apamin has been washed in. In these experiments none of the applied blockers led to a further reduction of the AHP current densities after SK channels were blocked with apamin (Fig. 9).

3. Discussion

Identifying the composition of ion channels underlying the complex pacemaking mechanism of LC neurons can help to understand the vulnerability of these neurons described in neurodegenerative diseases such as AD and PD, which is linked to the intrinsic activity and an imbalance of the intracellular Ca^{2+} homeostasis (Surmeier et al., 2010; Sanchez-Padilla et al., 2014). SK channels are important regulators of the Ca^{2+} homeostasis and regulate the intrinsic firing patterns in various central neurons (Sah, 1996). Although an apamin-sensitive conductance was already described > 20 years ago in LC neurons of the rat (Osmanović et al., 1990), the biophysical properties of SK channel mediated currents, the impact of SK channels on the intrinsic pacemaking of LC neurons, the molecular identity of these channels in the LC and their coupling to distinct types of Cav channels were still unknown. Based on the data presented here, we can draw three main conclusions: first, murine LC neurons express SK1, SK2 and SK3, with SK2 being the predominant small conductance Ca^{2+} -activated K^{+} channel expressed. Second, SK channels mediate a large fraction of the K^{+} currents flowing during the mAHP of LC neurons and thereby,

Fig. 8. SK channel-mediated AHP currents are sensitive to Cav channel inhibitors. Two-step voltage clamp evoked SK channel-mediated AHP currents were recorded under control conditions followed by wash-in of Cav channel blockers and subsequent wash-in of apamin. (A–B) Nanomolar concentrations of the Cav1 channel blocker isradipine (200 nM) significantly inhibited a large fraction of the apamin-sensitive I_{AHP} . (C–D) The Cav3 channel blocker Z944 (1 μ M) reduced the apamin-sensitive I_{AHP} to a similar extent as isradipine. (E–F) Simultaneous application of isradipine and Z944 induced a strong reduction of the I_{AHP} . Subsequent wash-in of apamin did not further reduce current density. (G–H) A blocker cocktail to inhibit Cav2 channels ((1 μ M ω -agatoxin (agat.) + 1 μ M ω -conotoxin (conot.)) had no impact on the peak current density of the I_{AHP} . (I) Comparison of Cav channel blocker- and apamin-sensitive current densities obtained by subtracting peak currents after drug wash-in from those of control measurements (isradipine: 2.2 ± 0.4 pA/pF, $n = 9$; Z944: 2.0 ± 0.1 pA/pF, $n = 8$; isradipine + Z944: 4.2 ± 0.5 pA/pF, $n = 5$; agat. + conot.: 0.5 ± 0.4 pA/pF, $n = 7$; apamin: 4.1 ± 0.3 pA/pF, $n = 24$). Box plots display median and 25/75 percentile, whiskers indicate outliers. n.s.: not significant. **: $P \leq 0.01$; ***: $P \leq 0.001$; using paired Student's t -test.

under conditions of Ca^{2+} handling not biased by the experimenter, regulate the pacemaker frequency of these noradrenergic cells. Third, SK channel activation is coupled to Ca^{2+} influx via L- and T-type Cav channels in LC neurons of the mouse. These results provide important further insights into the complex regulation of the pacemaking of LC neurons.

3.1. SK channels, mainly composed of SK2 subunits, mediate a large fraction of the mI_{AHP} in LC neurons

In agreement with previous studies, LC neurons examined under synaptic isolation in our study generated regular APs with prominent after hyperpolarizations (Osmanović et al., 1990; Matschke et al., 2015). Based on our results, we conclude that LC neurons functionally express SK1, SK2 and SK3 channel subunits that mediate a large fraction of the hyperpolarizing K^+ currents underlying an intermediate

Fig. 9. Sensitivity of the SK channel independent AHP to Cav channel inhibitors. Two-step voltage clamp evoked SK channel-mediated AHP currents were recorded under control conditions followed by wash-in of apamin and subsequent wash-in of Cav channel blockers + apamin. (A–D) Neither 200 nM isradipine (A), nor 1 μ M Z944 (B), nor isradipine + Z944 (C), nor 1 μ M ω -agatoxin + 1 μ M ω -conotoxin (D) induced further reduction of the peak current density of the I_{AHP} after apamin has been washed in (isradipine: $n = 3$; Z944: $n = 3$; isradipine + Z944: $n = 4$; agat. + conot.: $n = 3$). Box plots display median and 25/75 percentile, whiskers indicate outliers. n.s.: not significant. *: $P \leq 0.05$; **: $P \leq 0.01$; using paired Student's t -test.

phase of the AHP (mAHP). Analyzing mRNA from LC neurons, collected out of acute brainstem slices, by RT-PCR and qPCR revealed the expression of all three SK channel subunits with SK2 being the predominant variant (Fig. 3). The advantage of the multi-cell PCR approach performed in this study is the possibility to selectively choose neurons based on morphological criteria. Contamination of the probe with GABAergic interneurons was excluded by testing for GAD1 and GAD2 (see methods). In addition, pooling of 10–15 neurons allows a reproducible detection of transcripts with low abundance like ion channels which is less feasible and reliable using single-cell RT-PCRs. Previous studies analyzing the expression pattern of SK channels in rat and mouse brain are conflicting. Whereas in an immunohistochemical approach in mouse brain by Sailer *et al.* a high immunoreactivity was detected for SK3, but not for SK1 or SK2 in the LC region (Sailer *et al.*, 2004), the *in situ* hybridization study by Stocker and Pedarzani in rat brain shows strong signals for SK3, but also for SK2 (Stocker and Pedarzani, 2000). These contradictions might be caused by the different methodological approaches utilized or might reflect different protein turnover rates and half-life times of the different types of SK channels or the existence of alternative SK1 and SK2 splice variants (Shmukler *et al.*, 2001; Scholl *et al.*, 2014). Hence, even though our data strongly suggest SK2 as the predominant subtype, further studies using knock-out mice will be necessary to unequivocally determine the functional SK channel subtype composition of murine LC neurons.

The findings of our qPCR experiments are supported by the presence of a robust Ca^{2+} -sensitive mI_{AHP} . The biophysical properties of the mI_{AHP} with a fast activation within 10 ms and a mean decay time of 100–150 ms resembles the SK channel mediated mI_{AHP} described in several central neurons (Sah and McLachlan, 1991; Sah, 1996; Wolfart *et al.*, 2001). Depletion of extracellular Ca^{2+} as well as application of apamin led to a significant, but not complete inhibition of the mI_{AHP} . This suggests a major contribution of SK channels to the mI_{AHP} , however other Ca^{2+} independent conductances, that still need to be determined, seem to be involved as well. The increase of peak AHP currents observed with NS309 is in agreement with our observation that SK channels strongly contribute to the AHP current, but also demonstrates that SK channels are not fully activated by Ca^{2+} during these whole-cell recordings. The findings of these I_{AHP} recordings can be transferred to the apamin- and NS309-induced alterations of the AHPs detected in our gramicidin-D perforated-patch current clamp recordings. In accordance with previous studies (Osmanović *et al.*, 1990; Scuvée-Moreau *et al.*, 2004) the AHP of LC neurons had three components. Apamin or NS309

preferentially affected the mAHP, whereas the fAHP and sAHP component were not altered (Figs. 5 and 6). However, the magnitude of the apamin-induced reduction of the AHP amplitude was somewhat larger in our gramicidin-D perforated-patch recordings (~ 6 mV; Fig. 5D) than that in the intracellular recordings of Osmanovic *et al.* (~ 5 mV) or Scuvée-Moreau *et al.* (~ 3 mV). These variable findings might be explained by the fact that previous studies performed intracellular recordings or recordings in the whole-cell configuration which almost inevitably results in different intracellular Ca^{2+} levels due to dialysis of the cells with intracellular pipette solutions.

3.2. SK channels regulate the intrinsic pacemaking of LC neurons

The data presented in our study clearly demonstrate a role of SK channels in regulating the frequency of the intrinsic pacemaking in LC neurons. In our gramicidin-D perforated-patch recordings all neurons examined showed increased tonic firing rates after application of apamin and decreased firing rates after NS309 application (Figs. 4, 5 and 6). Noteworthy, these effects were not present in whole-cell recordings, which is consistent with a previous study by Osmanovic *et al.* using intracellular recordings to analyze the role of Ca^{2+} -activated conductances for the firing patterns of LC neurons. Here, no consistent effect of apamin on tonic firing, but an increase of firing rate induced by long depolarizing pulses, was observed (Osmanović *et al.*, 1990). In a latter study the same group demonstrated a major impact of Ca^{2+} -activated K^{+} currents on the poststimulus hyperpolarization of rat LC neurons (Osmanović and Shefner, 1993). Sharp electrodes used for intracellular recordings possess high resistances of up to 100 M Ω and are prone to cause damage to the cells which might induce leakage currents. Therefore intracellular recordings might not be close enough to the physiological state to allow detection of SK channel mediated changes of spontaneous activity. The same holds true for our whole-cell measurements. A possible explanation of why we did not detect an effect of apamin in our whole-cell recordings is that the intracellular Ca^{2+} concentration was ‘clamped’ non-physiologically and therefore the relevance of SK channels for tonic firing of the LC was not detected. Our hypothesis that SK channels act as important regulators of the intrinsic pacemaking of LC neurons is supported by the fact that the differential expression of SK2 and/or SK3 channels regulate the tonic firing frequency of several other neurons (Stocker, 2004). In dopaminergic SN neurons distinct roles for different SK channels were described, with SK3 channels regulating the frequency and SK2 the

precision of the spontaneous pacemaking (Deignan et al., 2012). Therefore, elucidating the distinct roles of the different SK channels expressed in the LC for the regulation of the spontaneous pacemaking might be worth addressing in future studies.

3.3. Ca^{2+} influx via L- and T-type Cav channels work in concert to activate SK channels in LC neurons

Several studies have shown that a high local concentration of Ca^{2+} is needed to activate Ca^{2+} -activated K^+ channels. Only if Cav and SK channels are located in a tight spatial proximity a functional coupling can take place (Roberts, 1993; Robitaille et al., 1993; Wisgirda and Dryer, 1994). Due to this spatial aspect, it is possible that Ca^{2+} influx mediated by different types of Cav channels activates specific SK channels in a neuron specific manner. Therefore, it is by now well established that SK channels in central neurons are differentially activated by one or the combination of several Cav channels and that this interplay regulates the shape of the AHP (Marrion and Tavalin, 1998; Sah and Davies, 2000; Stocker, 2004). Based on our results with mouse LC neurons, we conclude a preferential functional coupling of SK channels to L- and T-type Cav channels. Although previous studies have shown the presence of various other types of Cav channels in LC neurons (L-, T-, N- and P/Q/R-type) (Chieng and Bekkers, 1999; Connor et al., 1999; Sanchez-Padilla et al., 2014; Matschke et al., 2015), SK channel mediated I_{AHP} currents were not significantly affected by the P/Q- and N-type channel blockers ω -conotoxin and ω -agatoxin, respectively (Williams et al., 1992; Teramoto et al., 1993) (Fig. 8E). However, about 50% of the SK-mediated I_{AHP} currents were blocked by either isradipine or Z944, effects that were observed using concentrations that are likely to selectively block L- and T-type Cav channels, respectively (Ito et al., 1997; Tringham et al., 2012). In addition, when the L- and T-type blockers were applied simultaneously drug-sensitive AHP currents had a similar size as apamin-sensitive AHP currents (Fig. 8). In a recent study we have demonstrated that a combined inhibition of L- and T-type Cav channels leads to an elevated firing rate of LC neurons due to a reduction of the AHP amplitude (Matschke et al., 2015). These effects were highly similar to that obtained with apamin in our present study. Although we cannot exclude that other sources of Ca^{2+} increase, for instance via R-type Cav channels and/or from internal Ca^{2+} stores, also play a role in activation of SK channels, our findings strongly suggest a functional and combined coupling of L- and T-type Cav channels to SK channel activation in LC neurons. This interrelationship between L- and T-type Ca^{2+} currents and SK channel activation was further supported by the result that Cav blockers did not exert additional effects on AHP currents after apamin has been washed in (Fig. 9).

3.4. Potential disease relevance

Dysregulation of the intracellular Ca^{2+} balance is a known common feature of neurodegenerative diseases such as AD or PD. Non-physiologically high intracellular Ca^{2+} levels can lead to dysfunction of various metabolic processes which are directly coupled to cell death signaling pathways (Beal, 1998; Lipton and Nicotera, 1998). One possible reason for an elevation of the intracellular Ca^{2+} level is a permanent increase of the firing frequency in spontaneously active cells. In neuronal subpopulations that are vulnerable in PD, like the SN, the dorsal motor nucleus of the vagus and the LC, it has been postulated that the intrinsic activity, accompanied by Ca^{2+} influx via Cav channels, is ultimately resulting in ‘activity-dependent oxidant stress’ (Sanchez-Padilla et al., 2014; Surmeier et al., 2017). In addition, overstimulation of Ca^{2+} permeable glutamate (NMDA-) receptors, known as excitotoxicity, contributes to the pathogenesis of AD and PD (Beal, 1998; Zádori et al., 2014). Therefore, modulation of the intrinsic firing frequency and the intracellular Ca^{2+} homeostasis via Ca^{2+} -activated K^+ channels is a promising therapeutic approach to protect neurons from Ca^{2+} induced toxicity. In dopaminergic midbrain neurons the SK2/3

channel activator CyPPA decreased the spontaneous firing rate and prevented neuronal cell loss in an AMPA-dependent toxicity model, whereas inhibition of SK channels with NS8953 induced a reduced number of dopaminergic neurons (Benítez et al., 2011). In addition, activation of SK2 channels with NS309 or 1-EBIO regulated Ca^{2+} homeostasis, reduced brain damage area in an ischemia model and protected dopaminergic neurons against glutamate- and rotenone-induced toxicity (Allen et al., 2011; Dolga et al., 2011; Dolga and Culmsee, 2012; Dolga et al., 2014). As we found a strong mRNA expression of SK2 channels, a functional coupling of SK channels to Cav1 and Cav3 and a strong impact of SKs on the tonic activity of the LC, SK channels represent promising novel drug targets to protect these neurons from Ca^{2+} induced pathological changes in neurodegenerative diseases. Future studies might aim to address whether SK channel function in the LC is altered in mouse models of AD and PD and whether SK channel activators might be beneficial to slow or prevent neurodegeneration at an early stage of these diseases.

4. Experimental methods

The investigation conforms to the principles outlined in the Declaration of Helsinki and to the guide for the Care and Use of laboratory Animals (NIH Publication 85-23).

4.1. Brainstem slice preparation

Male C57BL/6j mice (postnatal days 20–30) were kept in groups of three to five on a 12 h day/12 h night cycle with food and water ad libitum. All of the conducted experiments and animal care were in accordance with the guidelines of the German Animal Protection Law (issued in 2006) and were approved by local authorities.

For slice preparation, mice were decapitated under isoflurane anaesthesia and brains were removed rapidly. Coronal sections (180–200 μm thick) were made using a Campden MA752 vibratome (Campden Instruments) in an ice-cold cutting solution composed of (in mM): 2.5 KCl, 1.25 NaH_2PO_4 , 10 MgSO_4 , 20 PIPES, 10 glucose, 200 saccharose, and 0.5 CaCl_2 , pH 7.35 with NaOH. After sectioning, slices were maintained at 30 °C for 30 min submerged in artificial cerebrospinal fluid (ACSF) composed of (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgCl_2 , and 25 glucose, equilibrated to pH 7.4 with 95% O_2 /5% CO_2 . Subsequently, slices were kept at room temperature for up to 5 h. For electrophysiological recordings slices were transferred to a recording chamber mounted on an upright microscope and continuously perfused with ACSF, unless specified otherwise. LC neurons were visualized with a Zeiss AxioCam MRm camera (Carl Zeiss Microscopy, LLC, United States) mounted on a Zeiss Examiner.D1 microscope (Carl Zeiss Microscopy, LLC, United States) equipped with a 40 $\times$ /0.75 phase contrast, water immersion objective. Brainstem slices containing a clearly defined LC at the edge of the fourth ventricle were used for the experiments.

4.2. Slice patch clamp recordings

Patch clamp recordings were performed at room temperature in LC neurons identified by their large somata and pacemaker frequencies between 1 and 5 Hz. Patch pipettes were prepared from borosilicate glass capillaries GB 150TF- 8P (Science Products, Hofheim, Germany), and had tip resistances between 3 and 5 M Ω . Conventional tight seal whole-cell voltage clamp experiments were conducted with ACSF as external solution and an internal solution containing (in mM): 135 K-Glutamate, 5 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl_2 , 0.1 Li_2GTP , 5 Na_2ATP , and adjusted to pH 7.35 with KOH. For perforated-patch clamp experiments the internal solution was supplemented with 100 $\mu\text{g}/\text{ml}$ gramicidin-D. The patch pipette was tip-filled with normal internal solution and filled up with the gramicidin-D containing solution. Successful establishment of the perforated-patch configuration was

assessed by appearance of slow capacitative currents usually 10 min after reaching the on-cell configuration.

All patch clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale CA, USA) and Clampex 10.0 software (Molecular Devices, Sunnyvale CA, USA). Data were digitized at 10 kHz with a Digidata 1440A digitizer (Molecular Devices, Sunnyvale CA, USA) and filtered at 1–5 kHz. Electrode capacitance was compensated and only recordings with a constant series resistant < 15 MΩ for whole-cell measurements and < 30 MΩ for perforated-patch measurements were used for analysis. Data were analyzed with ClampFit10 (Molecular Devices, Sunnyvale CA, USA).

4.3. Immunostaining

For neurobiotin (NB) stainings, LC neurons were filled with NB by adding 0.1% (w/v) NB to the pipette solution. After finishing patch clamp recordings, slices were subsequently fixed in a solution composed of 4% PFA in 0.1 M phosphate buffered saline (PBS), pH 7.4. After fixation for 48 h, slices were stored in 30% sucrose in 0.1 M PBS. Sections were blocked in 10% horse serum followed by overnight incubation with a TH antibody (Calbiochem, catalog number 657012, working dilution 1:1000). Anti-Rabbit Alexa 568 (Invitrogen, catalog number A11011, working dilution 1:750) served as secondary antibody and co-staining was performed with streptavidin conjugated with Alexa 488 (Invitrogen, catalog number S11223, working dilution 1:1000).

Images were acquired with an Observer.Z1 microscope (Carl Zeiss Microscopy, LLC, United States) equipped with standard EGFP filter and Texas red filter sets and an Axio Cam MRm camera (Carl Zeiss Microscopy, LLC, United States). Digital images were processed using ZEN 2.3 (Carl Zeiss Microscopy, LLC, United States).

4.4. PCR analysis

For reverse transcription (RT-) and quantitative (q-) PCR experiments, single LC neurons were collected out of acute brainstem slices as described previously (Matschke et al., 2015). Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen). Reverse transcription (RT) was performed with random hexamers (Roche) and Superscript II reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. Only cDNA pools that were positive for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and dopamine-beta-hydroxylase (Dbh) as well as negative for glutamic acid decarboxylase (GAD) (data not shown) were used for further experiments. Intron-spanning primers were designed to generate PCR products of about 110 base pairs: GAPDH for 5'-ACTTCAACAGCAACTCCCACTCT-3', GAPDH rev 5'-GCTGTAGCCGTATTCATTGTCATA-3'; Dbh for 5'-ACTATGTGCACTACTACCCCCAGA-3', Dbh rev 5'-CTCCTCACTGTGAACCTGTTTAC-3'; GAD for 5'-GGAGCGGATCCTAATACTACCAA-3', GAD rev 5'-GTAAGAAGCCACAGATCTTCAGG-3'; SK1 for 5'-GAGAAACACGTGCACAACTTCAT-3', SK1 rev 5'-CAGCTCTGACACCACTCATATG-3'; SK2 for 5'-GAATGACC AAGCAAATACCCTAGT-3', SK2 rev 5'-GTGACGATCCTTTTCTCAAAGTCT-3'; SK3 for 5'-GAAAGAGAAAGCGACTGAGTGAC-3', SK3 rev 5'-CATGGAATCCTTTGAGTACAAACC-3'; SK4 for 5'-GAACAAGTGAATTCATGGTGAC-3', SK4 rev 5'-AGACCGTCGATTCTCTTCCAG-3'; BK for 5'-CTTGCCAGAGTCAAGATAGAGTCA-3', BK rev 5'-GATGGAGATCACTCTCATGATGTT-3'.

For all primer pairs, the amplification products were confirmed by sequencing. RT-PCRs were performed using 5 U/μl Taq Gold Polymerase (Applied Biosystems) according to the instructions of the manufacturer. Reaction mixtures were preheated at 96 °C for 5 min followed by 40 cycles at 96 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s. PCR products were visualized by gel-electrophoresis on 5% NuSieve agarose gels. All PCR experiments were repeated at least three times and representative agarose gels visualizing the amplification products were illustrated.

qPCRs were performed using the Platinum SYBR Green qPCR

SuperMix-UDG (Invitrogen) according to the instructions of the manufacturer. Reaction mixtures were preheated at 50 °C for 2 min and at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Emitted fluorescence was detected online using a Mx3000P real-time PCR system (Stratagene). For all primer pairs the amplification products were confirmed by sequencing, no template control (n.c.) and dissociation curve analysis. In addition, amplification efficiency was determined by analyzing the slope of a Ct/log (template concentration) plot. For normalization, primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used ($rE^{GAPDH} = 1/2^{\Delta Ct}$). qPCR reactions were performed in duplicate or triplicate, and control experiments in the absence of cDNA (n.c.) were included. Three independent qPCR experiments were performed.

4.5. Drugs

All drugs were obtained from Sigma-Aldrich Co. LLC or Alomone Labs. Drugs were stored as DMSO stocks and final DMSO concentration did not exceed 0.1%.

4.6. Data analyses

Data are reported as means ± S.E.M and were analyzed using Microsoft Excel and OriginPro (OriginLab Corp., Guangzhou, China). Data sets were tested for Normality using the Shapiro Wilk test and for equal variances using the Levene's test. In normally distributed data sets, statistical significance was calculated using paired or unpaired Student's *t*-test. Otherwise non-parametric statistical tests, e.g. the Wilcoxon signed-rank test for paired data or the Mann-Whitney-*U* test for unpaired data were used. All experiments were performed at room temperature (21–24 °C). *, indicates *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Declarations of interest

None.

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