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Age-related changes in large-conductance calcium-activated potassium channels in mammalian circadian clock neurons

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ABSTRACT

Aging impairs the function of the suprachiasmatic nucleus (SCN, the central mammalian clock), leading to a decline in the circadian rhythm of many physiological processes, including sleep-wake rhythms. Recent studies have found evidence of age-related changes in the circadian regulation of potassium currents; these changes presumably lead to a decrease in the SCN's electrical rhythm amplitude. Current through large-conductance Ca^{2+}-activated K^+ (BK) channels promote rhythmicity in both SCN neuronal activity and behavior. In many neuron types, changes in BK activity are correlated with changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We performed patch-clamp recordings of SCN neurons in aged mice and observed that the circadian modulation of BK channel activity was lost because of a reduction in BK currents during the night. This reduced current diminished the afterhyperpolarization, depolarized the resting membrane potential, widened the action potential, and increased $[Ca^{2+}]_i$. These data suggest that reduced BK current increases $[Ca^{2+}]_i$ by altering the action potential waveform, possibly contributing to the observed age-related phenotype.

1. Introduction

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus serves as the endogenous biological clock in mammals, controlling a wide range of rhythmic physiological processes and behavior. A loss of SCN rhythmicity is associated with, and in some cases can be causally linked to, disease. For example, rhythm disturbances and decreased rhythm amplitude have been associated with several neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Kudo et al., 2011; Morton et al., 2005; Sterniczuk et al., 2010; Williams et al., 2011; Willison et al., 2013). In aged animals, impaired membrane and synaptic functions reduce the rhythm amplitude in the SCN (Biello, 2009; Farajnia et al., 2012; Nakamura et al., 2011; Satinoff et al., 1993).

The SCN's electrical activity rhythm is characterized by a peak in activity during the day and a trough in activity at night. This rhythm is caused by the modulation of several ionic currents, including fast delayed rectifier (FDR) K^+ currents (Itri et al., 2005), transient A-type (I_A) K^+ currents (Itri et al., 2010), large-conductance calcium-activated K^+ (BK) currents (Meredith et al., 2006), voltage-gated Ca^{2+} currents (Pennartz et al., 2002), and slowly inactivating Na^+ currents (Jackson et al., 2004; Kononenko et al., 2004). FDR and I_A currents increase during the day, thereby maintaining the increased frequency of spontaneously generated action potentials (APs) in SCN neurons. In contrast, currents through BK channels increase at night (Montgomery et al., 2013; Pitts et al., 2006), contributing to a suppression of neural activity in the SCN during the night. BK channel–deficient mice (*Kcnma1*$^{-/-}$) exhibit a reduction in behavioral rhythm amplitude, a slight expansion in the circadian period, and modified, albeit still rhythmic, electrical activity (Meredith et al., 2006). In the course of aging, the period, amplitude, and phase of the circadian rhythm change, resulting in an alteration in overt behavioral activity rhythms (Biello, 2009; Farajnia et al., 2012; Nakamura et al., 2011; Satinoff et al., 1993; Sellix et al., 2012; Van Someren, 2000). We previously reported that aging leads to deterioration of the circadian rhythms of both FDR and I_A currents (Farajnia et al., 2012). Given the circadian behavioral phenotype exhibited by BK-deficient mice, we hypothesized that aging also causes a decline in BK currents in the SCN.

K^+ current through BK channels accelerates repolarization of the membrane during an AP; thus, BK current can influence the duration of the AP and the afterhyperpolarization (AHP) phase (Cloues and Sather, 2003; Faber and Sah, 2002; Lin et al., 2014; Muller et al., 2007; Shao et al., 1999; Womack and Khodakhah, 2002). Decreased BK currents can change the AP waveform, resulting in a significant increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Lin et al., 2014; Miranda et al., 2003; Womack et al., 2009). We,

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therefore, asked whether aging alters BK currents in SCN neurons in aged mice, and we asked whether this change affects $[Ca^{2+}]_i$ in the SCN, given that $[Ca^{2+}]_i$ is elevated in other brain areas in aged animals (Muller et al., 1996; Toescu and Vreugdenhil, 2010).

To address these questions, we performed patch-clamp recordings and Ca^{2+} imaging experiments to measure age-related changes in BK currents and $[Ca^{2+}]_i$, respectively, in the SCN of aged mice (≥23 months of age). We observed that BK currents were selectively reduced during the night in aged mice, leading to a widening of the AP waveform and a reduction in AHP amplitude. Moreover, $[Ca^{2+}]_i$ was increased at night but not in the day in aged SCN neurons, compared with young SCN neurons. These data indicate that age-related changes in BK currents alter the AP waveform and may contribute to the age-related increase in basal $[Ca^{2+}]_i$ in SCN neurons at night.

2. Materials and methods

2.1. Animals and animal husbandry

Young (2–4 months of age, $n = 36$) and old (23–33 months of age, $n = 28$) C57BL/6 mice were maintained in climate-controlled cabinets under a 12-hour light-dark cycle, with ad libitum access to standard food and water. All experimental procedures were performed in accordance with the Dutch Committee on Animal Health and Care (protocol no. 10,079).

2.2. Slice preparation

Slices were prepared either 1 hour after the start of the light phase (zeitgeber time [ZT] 0–1) or at the end of the day (ZT 11–12) as previously described (Farajnia et al., 2012). We have previously shown that preparation of SCN slices during these times did not affect the phase of the circadian rhythm in SCN electrical activity (vanderLeest et al., 2009). In brief, the animals were decapitated under anesthesia (inhalation with 2% isoflurane), the brains were immediately removed, and brain slices (250-μm-thick coronal slices) containing the SCN were prepared in the light in cold, oxygenated artificial cerebrospinal fluid (ACSF) containing high $MgCl_2$ (4 mM) and low $CaCl_2$ (1 mM); the slices were cut using a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany). The slices were then transferred to regular oxygenated (95% O_2 and 2% CO_2) ACSF containing (in mM) 116.4 NaCl, 5.4 KCl, 1 NaH_2PO_4, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 23.8 $NaHCO_3$, and 15.1 glucose (pH 7.2–7.4); the slices were warmed to 37 °C for 20–30 minutes. Before recording, the slices were maintained in room temperature for at least 1 hour.

2.3. Whole-cell patch-clamp recordings

Slices were transferred to a recording chamber (RC-26G; Warner Instruments, Hamden, CT, USA) mounted on an upright microscope (Axioskop 2FS plus; Carl Zeiss Microimaging, Oberkochen, Germany). Cells were visualized using infrared (IR) illumination (IR-differential interference contrast) and an IR camera (VX45; Optronis, Kehl, Germany). The patch-clamp recordings were performed as described previously (Farajnia et al., 2014) during the day (ZT 3–7) and at night (ZT 15–19). In brief, patch pipettes were pulled from borosilicate glass (B150F-4; WPI, Sarasota, FL, USA) using a commercial electrode puller (PC-10; Narishige, Tokyo, Japan). The pipettes were filled with a solution containing (in mM) 112.5 K-gluconate, 1 EGTA, 10 Na-HEPES, 5 MgATP, 1 GTP, 0.1 leupeptin, 10 phosphocreatine, 4 NaCl, 17.5 KCl, 0.5 $CaCl_2$, and 1 $MgCl_2$ (pH, 7.1–77.25; 290–300 mOsm). After obtaining the whole-cell configuration, BK currents were recorded in voltage-clamp mode. Iberiotoxin (IbTX, 100 nM) was used to selectively block BK channels (to isolate BK currents), and tetrodotoxin (0.5 μM) was included to block voltage-dependent fast Na^+ currents. A series of voltage steps (from −50 to +60 mV in 10-mV increments and 180-ms duration) was applied after a 50-ms prepulse to −60 mV; the holding potential was −70 mV. This series was applied in control solution, and ≥10 minutes after IbTX was applied. The IbTX-sensitive current, that is, the BK current, was measured by subtracting the outward currents in the presence of the blocker from the respective control currents. Series resistance was monitored repeatedly, and recordings that exceeded 20 MΩ or changed by >20% during a recording were excluded from the analysis.

2.4. Perforated-patch recordings

APs were recorded in the current-clamp mode using the amphotericin-perforated–patch technique to measure AP parameters in cells with "normal" intracellular Ca^{2+} levels (e.g., D'Ambrosio, 2002). The pipette was filled with a solution containing (in mM) 112.5 K-gluconate, 4 NaCl, 17.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH, 7.2; 290–300 mOsm). Amphotericin B (240 μg/mL) was prepared fresh and added to the pipette solution. To facilitate formation of a gigaohm seal, the pipette tip (5–7 MΩ) was filled with amphotericin B-free solution, and then the shaft of the pipette was backfilled with amphotericin B–containing solution. Intracellular access was established within 5–10 minutes of forming the gigaohm seal, with series resistance reaching 40–100 MΩ, which is sufficient for performing current-clamp recordings. APs were recorded in slices from aged and young animals prepared during the day and during the night.

To estimate the contribution of BK channels to the AP waveform, the duration of the AP, the amplitude of the AP, and the AHP amplitude were measured. AP amplitude was calculated as the difference between resting membrane potential (RMP) and the peak of the AP; RMP was defined as membrane potential 40 ms before the AP peak. AP width was measured at 50% of the AP amplitude. AHP amplitude was calculated as the difference between the membrane potential at the trough of the AHP (within 100 ms of the peak) and RMP.

2.5. Ca^{2+} imaging

SCN neurons were preloaded with the ratiometric fluorescent Ca^{2+} indicator dye fura-2 acetoxymethyl ester (fura-2 AM) as described by Irwin and Allen (2007) because brain slices from older animals (>70 days of age) are generally more difficult to load with acetoxymethyl esters. Brain slices containing the SCN were first treated with a concentrated fura-2 AM solution (1 mM) for 1 minute and then continued loading for 1 hour at room temperature in oxygenated ACSF containing a lower concentration of fura-2 AM (7 μM). The slices were rinsed with ACSF at least 10 minutes before recording and then transferred to the recording chamber.

Quantitative Ca^{2+} measurements were performed as described previously (Farajnia et al., 2014). In brief, a 50-ms pulse of excitation light (340 and 380 nm) was applied using a monochromator (Polychrome V; FEI, Hillsboro, OR, USA) coupled via an optic fiber to the fluorescent port of an upright microscope. Image pairs of the emitted light (505 nm) were acquired using a cooled CCD camera (Sensicam; FEI); images were acquired at 6-second intervals with 4×4 binning to minimize photobleaching. Background-subtracted ratio images (340/380 nm) were generated, and the $[Ca^{2+}]_i$ at defined regions of interest was calculated according to the method of Grynkiewicz et al. (1985). Cells showing $[Ca^{2+}]_i$ >600 nM were considered unhealthy and excluded from further analysis. Experiments were controlled using the TILLvisION software program (FEI).

To block BK channels, IbTX (100 nM) was added to the bath for 15–20 minutes. The effect of IbTX on $[Ca^{2+}]_i$ was not reversible, even after a 15-minute washout period. To determine baseline $[Ca^{2+}]_i$ levels in the neurons, images were acquired for at least 2 minutes.

2.6. Chemicals

Except where indicated otherwise, all chemicals were obtained from Sigma-Aldrich (Munich, Germany). Tetrodotoxin was obtained from Tocris Bioscience (Bristol, UK), IbTX was obtained from Tebu Bio BV (Heerhugowaard, The Netherlands), and fura-2 AM was obtained from Teflabs (Austin, TX, USA).

2.7. Data analyses

The data were analyzed using Fitmaster (HEKA, Lambrecht/Pfalz, Germany), TILLvisION (FEI), Igor Pro (Wavemetrics, Portland, OR, USA), and MiniAnalysis (Synaptosoft, Fort Lee, NJ, USA). Statistical analyses were performed using SPSS (IBM, Armonk, NY, USA). All group values obtained from patch-clamp recordings and Ca^{2+} imaging experiments were tested for significance using the unpaired or paired Student t test and/or equivalent nonparametric test (Mann-Whitney U or Wilcoxon signed-rank test; n, representing the number of recorded neurons). Differences with $p < 0.05$ were considered to be significant. The distributions of IbTX-induced responses were analyzed using the χ^2 test (2 tailed).

3. Results

Our first step was to confirm our previous findings regarding neuronal excitability in SCN cells from aged mice (Farajnia et al., 2012). AP frequency measured during the day was lower in aged SCN neurons (1.95 ± 0.39 Hz, $n = 22$) compared with young SCN neurons (3.39 ± 0.61 Hz, $n = 17$; $p = 0.04$, unpaired Student t test). No age-dependent difference in frequency was observed between young and old SCN neurons when measured at night. We also confirmed that the resting membrane potential at night was more positive in aged cells (−37.36 ± 0.96 mV) than in young cells (−41.73 ± 0.73 mV; $p = 0.001$, unpaired Student t test).

3.1. The rhythm of BK currents diminishes with aging

Consistent with the previous results (Pitts et al., 2006), we observed a daily rhythm in the amplitude of BK currents in the SCN of young animals (Fig. 1A). Specifically, BK currents were larger at night (369.14 ± 43.9 pA) than during the day (202.7 ± 24.8 pA; $p = 0.004$, unpaired Student t test). In contrast, this daily rhythm of BK currents was absent in aged mice; currents recorded at night (232.34 ± 33.71 pA) did not differ significantly from currents recorded during the day (223.3 ± 26.57 pA; $p = 0.83$, unpaired Student t test, Fig. 1B). To account for this loss of rhythm, the currents measured at night in aged animals were significantly lower than currents measured in young animals ($p = 0.024$, unpaired Student t test, Fig. 1C and D), whereas the currents recorded during

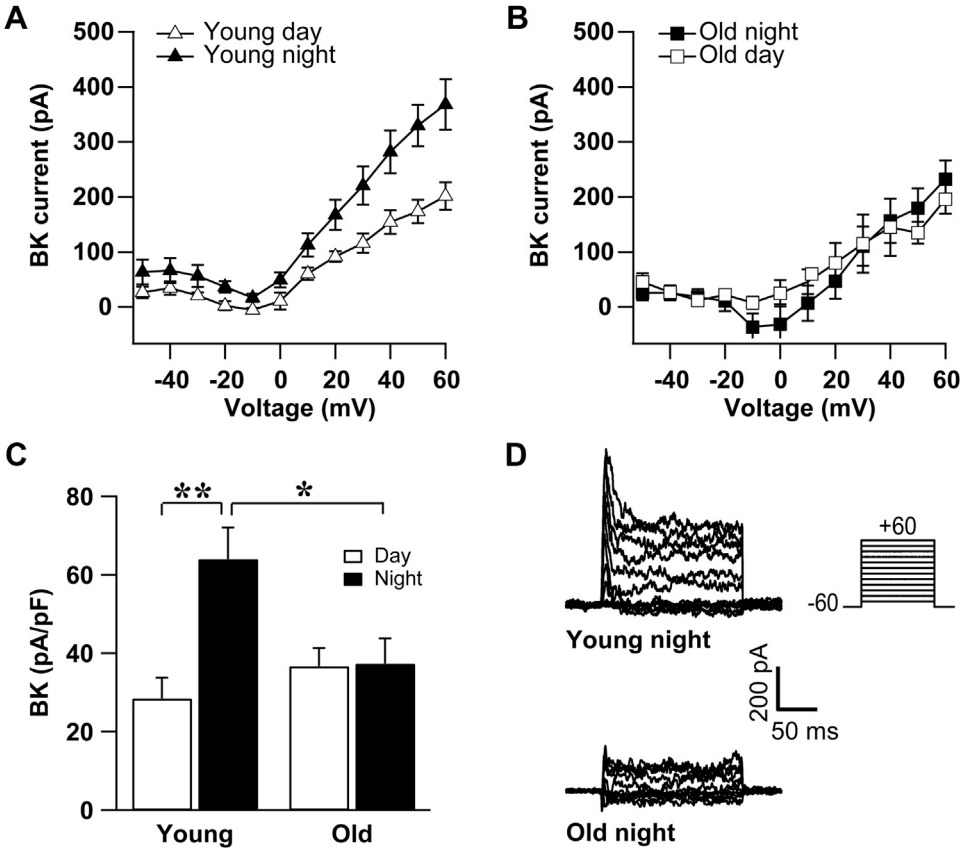

Fig. 1. Large-conductance Ca^{2+}-activated K^+ (BK) current amplitude is modified in aged suprachiasmatic nucleus (SCN) neurons. (A, B) Current-voltage plots of BK currents recorded in young (A) and aged (B) mice. (B) BK currents in aged neurons did not differ significantly between day and night ($p = 0.83$). (C) Bar graph summarizing mean (±standard error of the mean) BK currents recorded at +60 mV. Current density during the day did not differ significantly between young ($n = 7$) and aged neurons ($n = 9$, $p = 0.26$). $^*p \leq 0.05$ and $^{**}p \leq 0.01$. (D) Example BK currents recorded at night from young (top, $n = 13$) and aged (bottom, $n = 7$) SCN neurons; currents were evoked by applying a 180-ms depolarization ranging from −50 mV through +60 mV (in 10-mV increments) after a 50-ms prepulse to −60 mV.

the day were similar between aged and young animals ($p = 0.57$, unpaired Student t test). These data suggest that aging selectively affects BK currents at night.

3.2. The AP waveform in aged animals is modified selectively at night

Because BK currents contribute to both the repolarization and AHP phases of the AP, we performed perforated-patch current-clamp recordings to measure the shape of APs in the SCN of young and aged mice. Consistent with the observed change in BK currents, the magnitude of the AHP measured at night was significantly smaller in aged mice (-3.50 ± 0.6 mV) compared with young animals (-5.11 ± 0.41 mV; $p = 0.031$, unpaired Student t test). In contrast, we found no difference in AHP amplitude between aged (-4.11 ± 0.54 mV) and young animals (-4.48 ± 0.6 mV; $p = 0.65$, unpaired Student t test, Fig. 2A, B, and E) when measured during the day.

Because BK currents also play a role in the repolarization phase of APs, we measured the duration of APs in SCN neurons. When measured at night, the AP waveforms were wider in aged (5.45 ± 0.84 ms) than in young cells (3.03 ± 0.2 ms; $p = 0.018$, unpaired Student t test, Fig. 2B and F). As with AHP amplitude, we found no difference in AP duration between aged (3.62 ± 0.68 ms) and young animals (3.66 ± 0.56 ms; $p = 0.96$, unpaired Student t test, Fig. 2A and F) when measured during the day.

When measured at night, the amplitude of the APs was significantly smaller in aged (43.32 ± 4.58 mV) than in young animals (57.88 ± 3.27 mV; $p = 0.01$, unpaired Student t test, Fig. 2B and G). In contrast, no difference in AP amplitude was found between aged (59.55 ± 4.87 mV) and young mice (50.77 ± 4.53 mV; $p = 0.19$, unpaired Student t test, Fig. 2A and G) when measured during the day. Taken together, these results show that aging affects APs in SCN neurons during the night but not during the day.

3.3. Adjusting the resting membrane potential in aged cells does not restore the AHP at night

We found that at night, RMP of aged SCN cells was more positive than in young cells (Fig. 2D and H, see also Farajnia et al., 2012). To determine whether the age-related changes in AP waveform are dependent on RMP, we hyperpolarized the membranes of aged cells by injecting current to adjust RMP to values recorded from young neurons at night (Fig. 2D). Adjusting RMP in aged neurons reverted both the duration and amplitude of APs to values similar to young neurons ($p = 0.427$ and $p = 0.75$, respectively, unpaired Student t test, Fig. 2D, F, and G). Moreover, we found a negative correlation between AP amplitude and RMP ($p = 0.039$, $r = -0.51$, Pearson correlation coefficient, 2 tailed). In contrast, adjusting RMP had no effect on AHP amplitude; thus, AHP remained significantly different between aged and young neurons, despite having similar RMP values ($p = 0.05$, unpaired Student t test, Fig. 2D and E).

These data suggest that the changes in AP duration and amplitude are related to membrane depolarization, whereas the reduction in AHP amplitude is because of the age-related decrease in BK current at night.

3.4. Applying a BK channel blocker to young neurons mimics the age-associated phenotype

We next determined whether the age-related effect of reducing BK channel activity on the AP waveform can be recapitulated in young SCN neurons. Thus, we applied the BK channel–specific blocker IbTX to slices from young mice at night and recorded their APs (Fig. 2C). Similar to the age-related phenotype, treating young cells with IbTX significantly reduced the amplitude of the AHP (control, -5.11 ± 0.41 mV; IbTX, -2.92 ± 0.28 mV; $p = 0.003$, paired Student t test; Fig. 2E). Interestingly, IbTX also increased the

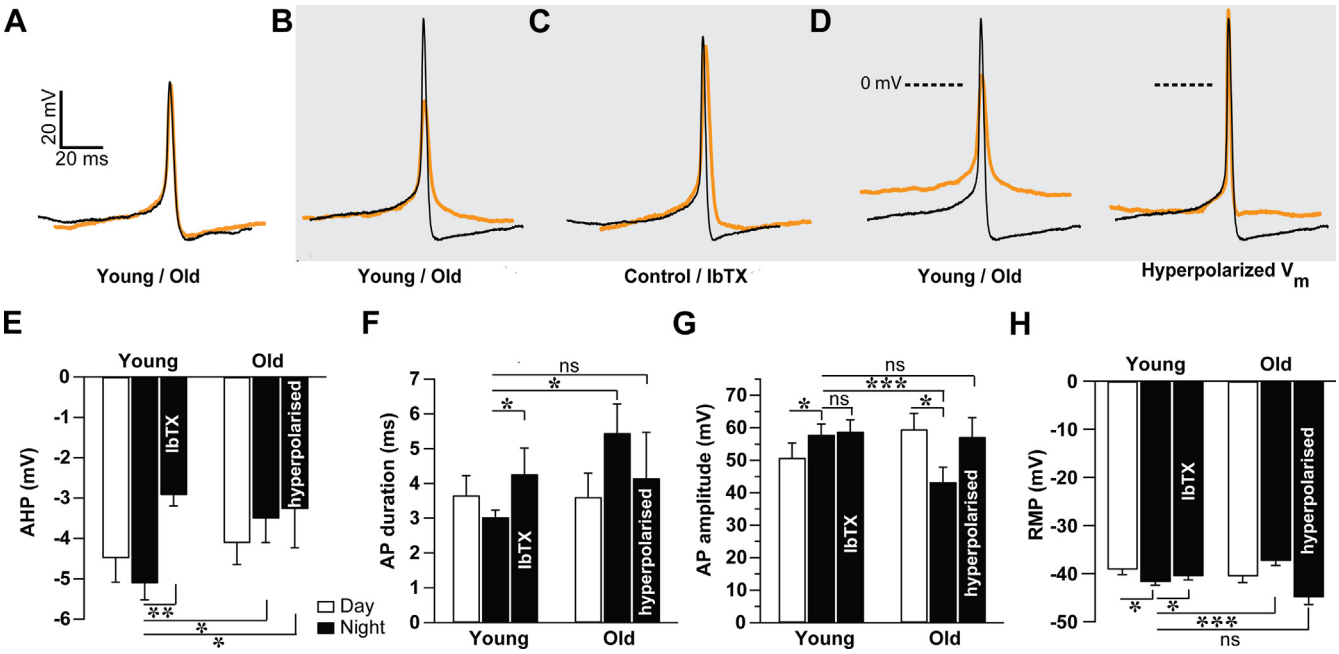

Fig. 2. The properties of the action potential (AP) waveform are altered at night in aged mice because of decreased large-conductance Ca^{2+}-activated K^+ currents. (A–D) Example traces of APs recorded under the indicated conditions. In each pair of traces, the black trace shows a young suprachiasmatic nucleus (SCN) neuron (A, B, and D) or control (pre-iberiotoxin [pre-IbTX]) young neuron (C); the yellow and/or gray trace shows an aged SCN neuron (A, B, and D), IbTX-treated young neuron (C), or hyperpolarized aged neuron (D, right). White background indicates recordings during the day (A), and gray background indicates recordings at night (B–D). (E, F) Summary of afterhyperpolarization amplitude (E), AP duration (F), AP amplitude (G), and resting membrane potential (RMP, H) under the indicated conditions. Values are reported as mean ± standard error of the mean from 7 to 19 neurons per condition. Where indicated, the membrane potential (V_m) of neurons were hyperpolarized by injecting current or treated to IbTX. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and ns = nonsignificant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

duration of APs in young SCN neurons (control, 3.03 ± 0.2 ms; IbTX, 4.28 ± 0.74 ms; $p = 0.017$, paired Student t test; Fig. 2F) and depolarized the RMP (control, −41.73 ± 0.73 mV; IbTX, −40.5 ± 0.8 mV; $p = 0.03$, paired Student t test; Fig. 2H). In contrast, IbTX had no effect on firing frequency ($n = 9$; $p = 0.382$, paired Student t test) or AP amplitude ($p = 0.32$, paired Student t test; Fig. 2G).

Taken together, the data suggest that acutely blocking BK channels with IbTX alters AP duration, AHP amplitude, and membrane potential. Moreover, because the AP waveform in IbTX-treated young neurons was similar to APs recorded in aged SCN neurons, blocking BK currents in young SCN neurons appears to be sufficient to induce the aged phenotype.

3.5. Blocking BK channels increases intracellular Ca^{2+} concentration

As shown earlier, the reduction in BK currents in aged SCN neurons at night caused a change in the AP waveform properties, and blocking BK channels in young mice had the same effect. Because the properties (i.e., shape) of the AP regulate Ca^{2+} influx in neurons (Womack et al., 2009), we reasoned that acutely reducing BK currents in young SCN neurons would alter $[Ca^{2+}]_i$. To test this hypothesis, we blocked BK channels in young SCN neurons using IbTX and measured $[Ca^{2+}]_i$ using ratiometric fluorescence Ca^{2+} imaging. Application of IbTX to young neurons increased mean $[Ca^{2+}]_i$ both during the day ($\Delta [Ca^{2+}]_i = 16.07$ nM, $p = 0.001$, Wilcoxon signed-rank test, 2 tailed) and at night ($\Delta [Ca^{2+}]_i = 25.47$ nM, $p = 3.44 \times 10^{-17}$, Wilcoxon signed-rank test, 2 tailed). This suggests that altering the AP waveform by blocking BK currents increases $[Ca^{2+}]_i$.

Interestingly, although average $[Ca^{2+}]_i$ was increased after IbTX treatment regardless of whether it was applied in the day or at night, IbTX did not affect all neurons similarly; some cells responded with an increase in $[Ca^{2+}]_i$, some responded with a decrease, and some cells failed to respond at all (i.e., nonresponders) (Fig. 3). Most cells responded to IbTX application with an increase in $[Ca^{2+}]_i$. However, the percentage of cells that responded with an increase was higher at night (70%, 144 of 205 neurons) than during the day (55%, 41 of 75; $p = 0.015$, χ^2 test, 2 tailed; Fig. 3B). In contrast, the percentage of cells that responded to IbTX with a decrease in $[Ca^{2+}]_i$ was similar in the day (25%, 19 out of 75) and at night (20%, 40 out of 205; $p = 0.29$, χ^2 test, 2 tailed). A significantly higher percentage of cells measured during the day were nonresponders (20%, 15 out of 75) compared with cells measured in the night (10%, 21 out of 205, $p = 0.031$, χ^2 test, 2 tailed). We also found that baseline (i.e., pre-IbTX) $[Ca^{2+}]_i$ was lower in the subpopulation of cells that responded to IbTX application with an increase in $[Ca^{2+}]_i$ compared with cells that responded with a decrease in $[Ca^{2+}]_i$. During the day, mean baseline $[Ca^{2+}]_i$ was 73.08 ± 4.82 and 149.15 ± 19.81 nM in the cells that responded with an increase in $[Ca^{2+}]_i$ and cells that responded with a decrease, respectively ($p = 0.0003$, 1-way analysis of variance, Bonferroni post hoc); at night, mean baseline $[Ca^{2+}]_i$ was 121.54 ± 3.05 and 141.68 ± 6.3 nM, respectively ($p = 0.009$, 1-way analysis of variance, Bonferroni post hoc).

Thus, IbTX treatment increased $[Ca^{2+}]_i$ in most SCN neurons, regardless of the time of day, and primarily in cells with low baseline $[Ca^{2+}]_i$. Because many cells have lower baseline $[Ca^{2+}]_i$ at night, a higher percentage of cells responded to IbTX with an increase in $[Ca^{2+}]_i$ at night compared with during the day.

3.6. Intracellular Ca^{2+} concentration is higher in aged cells at night but not during the day

To determine whether the age-related reduction in BK currents observed in aged animals at night affects $[Ca^{2+}]_i$, we measured

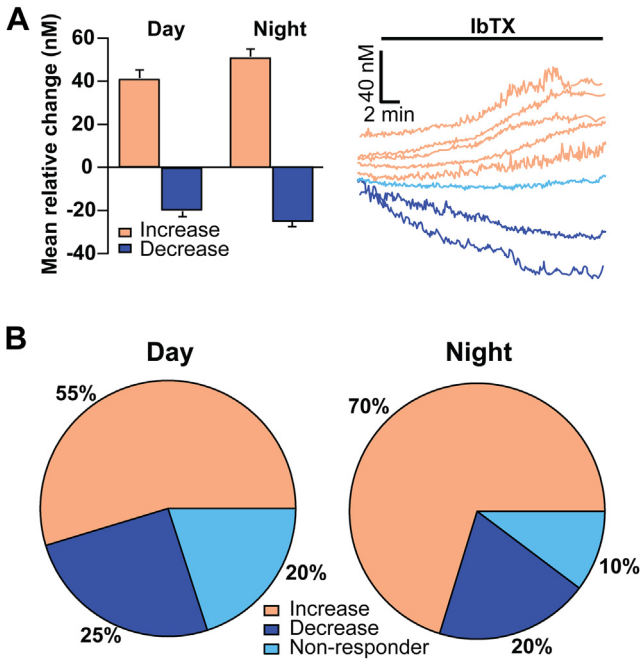

Fig. 3. Applying the large-conductance Ca^{2+}-activated K^+ channel–specific blocker iberiotoxin (IbTX) modulates intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. (A) Left, summary of the mean (±standard error of the mean) increase or decrease in $[Ca^{2+}]_i$. Right, example $[Ca^{2+}]_i$ traces before and during the application of IbTX. Cells that responded with an increase or decrease in $[Ca^{2+}]_i$ are shown in orange and/or gray and dark blue and/or black, respectively; cells that had a <5% change in $[Ca^{2+}]_i$ after IbTX application were considered to be nonresponders and are shown in light blue and/or light gray. (B) Pie charts summarizing the relative proportions of responding cell populations after IbTX application in the day (left, $n = 75$) and at night (right, $n = 205$). The number of cells that responded with an increase in $[Ca^{2+}]_i$ was significantly higher at night than during the day ($p = 0.015$, χ^2 test, 2 tailed). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

baseline $[Ca^{2+}]_i$ in both young and old animals using Ca^{2+} imaging. In young SCN neurons, $[Ca^{2+}]_i$ was higher during the day (154.46 ± 5.85 nM) than at night (125.28 ± 2.6 nM; $p = 7.55 \times 10^{-6}$, unpaired Student t test, Fig. 4); similar results were reported previously (Colwell, 2000). In contrast, in aged animals, $[Ca^{2+}]_i$ was higher at night (214.14 ± 9.81 nM) than during the day (157.63 ± 5.08 nM; $p = 5.5 \times 10^{-7}$, unpaired Student t test, Fig. 4). In comparison with young neurons, $[Ca^{2+}]_i$ was higher at night in aged neurons ($p = 3.93 \times 10^{16}$, unpaired Student t test). Thus, the circadian rhythm in $[Ca^{2+}]_i$ is reversed in aged SCN neurons. These results suggest a link between BK currents and $[Ca^{2+}]_i$, given that $[Ca^{2+}]_i$ is increased only at night in aged SCN neurons, when we also see an age-related reduction in the amplitude of BK currents (Table 1).

4. Discussion

In the SCN, ionic currents are important elements of both intracellular and intercellular communications, and they play an essential role in the accurate functioning of the central circadian clock in mammals. During the aging process, the daily regulation of certain ion channels is eliminated in SCN neurons (Farajnia et al., 2012). Age-related dysregulation of rhythmic ion currents contributes to circadian clock dysfunction, with a resulting detrimental effect on health. Here, we report that the circadian rhythm of BK currents is impaired in SCN neurons during aging. Specifically, the magnitude of BK currents recorded at night in aged SCN neurons was smaller than in young SCN neurons. It is important to point out that BK current is not deteriorated or nonfunctional in aging, but

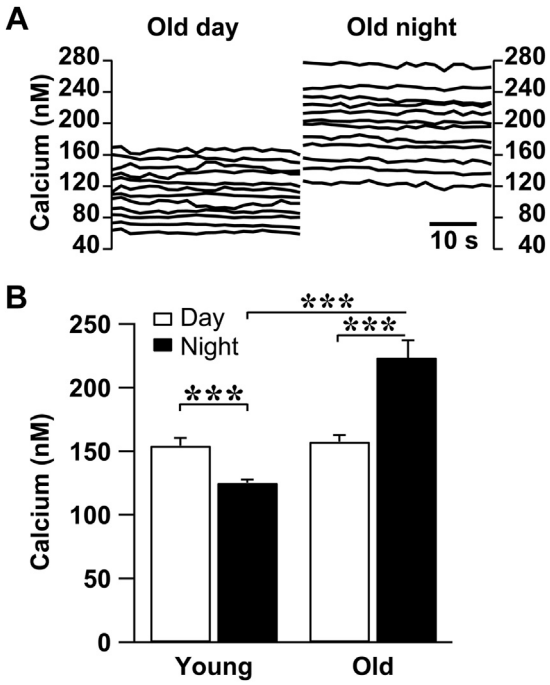

Fig. 4. Intracellular Ca^{2+} concentration in suprachiasmatic nucleus (SCN) neurons is altered in aging. (A) Example traces of baseline intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measured in an aged SCN neuron during the day (left) and in an aged SCN neuron at night (right). (B) Bar graph summarizing baseline $[Ca^{2+}]_i$ measured in young (day, $n = 205$; night, $n = 208$) and aged SCN neurons (day, $n = 168$; night, $n = 207$). Note that baseline $[Ca^{2+}]_i$ decreased at night in young neurons, whereas $[Ca^{2+}]_i$ increased at night in aged neurons. $^{***}p < 0.001$.

rather the circadian modulation of BK activity is disturbed in SCN neurons. The mechanisms for circadian modulation of the BK channels are not known yet. In the SCN, BK channels play a role in suppressing electrical activity at night, and BK-deficient animals have clear changes in SCN physiology and locomotor behavior (Kent and Meredith, 2008; Meredith et al., 2006; Pitts et al., 2006). Therefore, the age-related decrease in BK currents in the SCN at night may underlie the behavioral and physiological phenotypes observed in both aged animals and elderly humans.

Table 1
Relationship between BK current and $[Ca^{2+}]_i$ in young and aged SCN neurons measured during the day and at night

	BK current	$[Ca^{2+}]_i$
Young		
Day	↓	↑
Night	↑	↓
Old		
Day	↓	↑
Night	↓	↑

Note that regardless of age or time of day, a decrease in BK current is accompanied by an increase in $[Ca^{2+}]_i$ (and vice versa).
Key: BK, large-conductance Ca^{2+}-activated K^+; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SCN, suprachiasmatic nucleus; ↑, increase in current or $[Ca^{2+}]_i$; ↓, decrease in current or $[Ca^{2+}]_i$.

4.1. The role of BK current in cell excitability and AP generation

In many neurons, including SCN neurons, BK currents play a key role in membrane repolarization during an AP and/or the AHP. Both the AHP and the duration of the AP control the amount of Ca^{2+} that enters the neuron, and they play an essential role in modulating firing frequency (Cloues and Sather, 2003; Faber and Sah, 2002; Lin et al., 2014; Shao et al., 1999; Womack and Khodakhah, 2002). Thus, BK channels are an essential physiological link between cellular Ca^{2+} signaling and electrical output. We observed significant changes in AP waveforms recorded from aged neurons at night, the time at which BK currents are reduced in these cells; these changes included increased AP duration, decreased AP amplitude, and decreased AHP amplitude. In young neurons, the BK channel–specific blocker IbTX also reduced the magnitude of the AHP and prolonged the AP. On the other hand, firing frequency at night was not affected in either aged SCN neurons or IbTX-treated young SCN neurons.

BK currents regulate firing frequency in many brain regions (Lovell and McCobb, 2001; Smith et al., 2002; Tabak et al., 2011; Womack et al., 2009). In the SCN, however, the role of BK channels in modulating firing rate is unclear, and studies have yielded contradictory results. For example, BK-deficient mice have increased electrical activity in the SCN at night (Kent and Meredith, 2008; Meredith et al., 2006). On the other hand, acutely blocking BK channels in wild-type animals had no significant effect on firing rate at night (Pitts et al., 2006). These latter findings are consistent with our results, as we observed no change in firing frequency after blocking BK channels in young SCN. In addition, although aged SCN neurons had reduced BK currents at night, their firing rate was not affected.

Recently, several groups reported that BK channels can have bidirectional effects on electrical activity in some brain regions (Gu et al., 2007; Ly et al., 2011; Sausbier et al., 2004; Van Goor et al., 2001), including the SCN (Montgomery and Meredith, 2012). These bidirectional effects correlate with opposing roles for BK channels in terms of either decreasing or increasing neuronal excitability. In our experiments, we observed a bidirectional effect of blocking BK channels on $[Ca^{2+}]_i$, in each individual neuron, IbTX either increased or decreased $[Ca^{2+}]_i$, and the relative proportion of cells that responded to IbTX differed between night and day. In essence, acutely blocking BK channels, or reduction of BK currents as a result of aging, may exert a dual effect on electrical activity and AP waveform, as these channels can either have an excitatory or inhibitory function under different physiological conditions. However, a complete loss of BK current (e.g., in BK-deficient mice) eliminates this potential bidirectional effect on electrical activity, explaining the difference between results obtained from BK-deficient mice and our results.

4.2. BK currents and membrane potential

An unidentified K^+ current has been reported to regulate membrane potential in SCN neurons (Kuhlman and McMahon, 2004). Moreover, these K^+ currents appear to be impaired in aging, as RMP is more positive in aged SCN neurons at night, as we reported previously (Farajnia et al., 2012; see also Fig. 2H). In the present study, blocking BK channels induced a small but significant depolarization in young SCN neurons, suggesting that BK currents can affect RMP in SCN neurons. A similar membrane depolarization was observed at night in BK-deficient mice (Montgomery et al., 2013). Thus, the age-related decrease in BK currents at night likely contributes to the depolarization observed in aged SCN neurons.

Changes in the AP waveform in deep cerebellar neurons have been reported to arise from the depolarizing effect of BK channels (Pedroarena, 2011). Thus, the changes that we observed in the AP waveform in aged SCN cells might be explained by the more

positive membrane potential of aged neurons, as we found an inverse correlation between AP amplitude and membrane potential. Therefore, we injected current to drive the RMP of aged neurons to approximately the same RMP as young SCN neurons. Shifting RMP to a more negative value restored AP amplitude and duration to values similar to young neurons but did not restore AHP amplitude. This finding confirms that the age-related reduction in BK currents reduces the AHP component of APs directly.

4.3. BK currents and calcium regulation

The specific shape of an AP determines the amount of Ca^{2+} that will enter the cell via voltage-gated Ca^{2+} channels. A prolonged AP and/or a reduced AHP, as seen in aged SCN neurons and IbTX-treated young SCN neurons, leads to increased $[Ca^{2+}]_i$ (Lin et al., 2014; Miranda et al., 2003; Muller et al., 2007; Womack et al., 2009). Our data suggest that blocking BK channels in young SCN neurons increases $[Ca^{2+}]_i$ in most cells regardless of the time of day. The decrease in BK current at night in aged neurons may, therefore, alter $[Ca^{2+}]_i$ in these cells. Indeed, at night, we observed significantly higher baseline $[Ca^{2+}]_i$ in aged neurons compared with young neurons, whereas we found no difference between aged and young neurons during the day. Thus, in SCN neurons (and regardless of age and time of day), BK current is inversely correlated with $[Ca^{2+}]_i$ (Table 1).

Several circadian-controlled ion channels (e.g., voltage-gated Ca^{2+} channels in the cell membrane and ryanodine-sensitive channels in intracellular membranes) play a role in Ca^{2+} homeostasis within the SCN (Diaz-Munoz et al., 1999; Pennartz et al., 2002). Our data suggest that decreasing BK currents increases $[Ca^{2+}]_i$, perhaps via elevated Ca^{2+} influx through voltage-gated Ca^{2+} channels. Slowing the repolarization phase during an AP can increase Ca^{2+} entry by prolonging the activation of voltage-gated Ca^{2+} channels. Likewise, reducing the AHP can delay deactivation of voltage-gated Ca^{2+} channels, also increasing integrated Ca^{2+} influx (Womack et al., 2009). Interestingly, blocking nuclear BK channels in hippocampal neurons increases nucleoplasmic $[Ca^{2+}]$ via nuclear ryanodine-sensitive channels, thereby regulating gene expression (Li et al., 2014). Our data suggest that impairment of plasma membrane BK channels leads to an increase in cytosolic $[Ca^{2+}]$ in aged SCN neurons.

Given calcium's essential role both in rhythm generation (Harrisingh and Nitabach, 2008) and in intracellular signaling pathways associated with major diseases (Berridge, 2012), the increase in $[Ca^{2+}]_i$ because of age-related reductions in BK currents is expected to have detrimental consequences on both clock function and health. Our findings underscore the notion that the cellular pathways activated by increased $[Ca^{2+}]_i$ are potential causative factors underlying many age-related disorders in the SCN, similar to other brain regions (Duncan et al., 2010; Foster, 2007; Hermes et al., 2010; Kumar et al., 2009). Moreover, restoring baseline $[Ca^{2+}]_i$ levels in aged SCN neurons may help alleviate many age-related problems (Disterhoft and Oh, 2006; Duncan et al., 2010). BK channels can be considered a novel candidate target for restoring Ca^{2+} signaling in aged SCN neurons.

Disclosure statement

The authors have no conflicts of interest to disclose.

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