

Inward-Rectifying Potassium (K_{ir}) Channels Regulate Pacemaker Activity in Spinal Nociceptive Circuits during Early Life

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Pacemaker neurons in neonatal spinal nociceptive circuits generate intrinsic burst firing and are distinguished by a lower “leak” membrane conductance compared with adjacent nonbursting neurons. However, little is known about which subtypes of leak channels regulate the level of pacemaker activity within the developing rat superficial dorsal horn (SDH). Here we demonstrate that a hallmark feature of lamina I pacemaker neurons is a reduced conductance through inward-rectifying potassium (K_{ir}) channels at physiological membrane potentials. Differences in the strength of inward rectification between pacemakers and nonpacemakers indicate the presence of functionally distinct K_{ir} currents in these two populations at room temperature. However, K_{ir} currents in both groups showed high sensitivity to block by extracellular Ba^{2+} ($IC_{50} \sim 10 \mu M$), which suggests the presence of “classical” K_{ir} ($K_{ir2.x}$) channels in the neonatal SDH. The reduced K_{ir} conductance within pacemakers is unlikely to be explained by an absence of particular $K_{ir2.x}$ isoforms, as immunohistochemical analysis revealed the expression of $K_{ir2.1}$, $K_{ir2.2}$, and $K_{ir2.3}$ within spontaneously bursting neurons. Importantly, Ba^{2+} application unmasked rhythmic burst firing in $\sim 42\%$ of nonbursting lamina I neurons, suggesting that pacemaker activity is a latent property of a sizeable population of SDH cells during early life. In addition, the prevalence of spontaneous burst firing within lamina I was enhanced in the presence of high internal concentrations of free Mg^{2+} , consistent with its documented ability to block K_{ir} channels from the intracellular side. Collectively, the results indicate that K_{ir} channels are key modulators of pacemaker activity in newborn central pain networks.

Introduction

Spontaneous activity is essential for the proper maturation of neuronal circuits in the CNS, via its wide-ranging effects on neurotransmitter phenotype (Borodinsky et al., 2004), axonal pathfinding (Hanson et al., 2008), synapse formation (Gonzalez-Islas and Wenner, 2006), and the synchronization of firing across networks (Tritsch et al., 2007). Spontaneous network activity during early development can reflect a variety of underlying mechanisms, such as transient synaptic connections, gap junction coupling, GABAergic depolarizations, and the presence of pacemaker neurons (Blankenship and Feller, 2010), which have been previously defined as cells exhibiting intrinsic, oscillatory burst firing (Ramirez et al., 2004).

Within the neonatal spinal cord, pacemaker-like cells have been described in the ventral horn (Tazerart et al., 2007, 2008), where they are proposed to contribute to rhythmogenesis in lo-

comotor networks (Brocard et al., 2010). Pacemaker neurons were also recently identified within lamina I of the newborn superficial dorsal horn (SDH) (Li and Baccei, 2011), which receives direct projections from nociceptive A δ - and C-fiber sensory neurons (Light and Perl, 1979; Sugiura et al., 1986) and represents a key component of the ascending pain pathway. Pacemakers constitute ~ 25 – 30% of the lamina I population during the first days of life (Li and Baccei, 2011) and are similar to intrinsically bursting neurons in other regions of the CNS (Del Negro et al., 2002) in that they are distinguished by a high ratio of persistent, voltage-gated Na^{+} conductance to “leak” membrane conductance. This has been attributed to a significantly higher membrane resistance compared with adjacent nonbursting lamina I neurons (Li and Baccei, 2011). Leak conductance potentially regulates neuronal excitability across the CNS and can result from any channel being open at the resting membrane potential (Goldstein et al., 2001). This raises the possibility that altered leak channel function and/or expression may predispose a subset of neurons to function as pacemakers within the immature SDH.

Inward-rectifying potassium (K_{ir}) channels are strong candidates to modulate intrinsic burst firing within the newborn spinal cord. Low K_{ir} conductance in neurons can drive spontaneous firing by depolarizing the membrane potential above the threshold to recruit persistent voltage-gated Na^{+} currents (Leao et al., 2012), which are essential for rhythmic bursting within lamina I (Li and Baccei, 2011). In addition, the block of K_{ir} currents by metabotropic inputs unmasks endogenous burst firing in the ma-

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jority of deep dorsal horn neurons from the adult spinal cord (Derjean et al., 2003). Unfortunately, little is known about the underlying basis for leak membrane conductance in developing SDH neurons. As a result, the degree to which K_{ir} channels regulate spontaneous pacemaker activity within newborn spinal pain circuits remains unknown.

Here we demonstrate that a reduced conductance through “classical” K_{ir} channels near physiological potentials is a critical determinant of intrinsic burst firing in spinal lamina I neurons during early life. This implies that the number of functional pacemaker neurons within the developing SDH network is not constant. It is instead dependent on K_{ir} function, which may be modulated by both the extracellular and the intracellular environments.

Materials and Methods

All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee.

Preparation of spinal cord slices. Sprague Dawley rats of either sex were deeply anesthetized with sodium pentobarbital (30 mg/kg) at postnatal days 2 (P2) to P5 and perfused with ice-cold dissection solution consisting of (in mM) 250 sucrose, 2.5 KCl, 25 NaHCO_3 , 1.0 NaH_2PO_4 , 6 MgCl_2 , 0.5 CaCl_2 , and 25 glucose, continuously bubbled with 95% O_2 /5% CO_2 . The lumbar spinal cord (L2–L6) was isolated and immersed in low-melting-point agarose (3% in the above solution; Invitrogen), and parasagittal slices (350–400 μm) were cut using a Vibroslice tissue slicer (HA-752; Campden Instruments). The slices were placed in a chamber filled with oxygenated dissection solution for 30 min and allowed to recover in an oxygenated artificial CSF (aCSF) solution containing (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO_3 , 1.0 NaH_2PO_4 , 1.0 MgCl_2 , 2.0 CaCl_2 , and 25 glucose, pH 7.2, for ≥ 1 h at room temperature.

Patch-clamp recordings. After recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments) and mounted on the stage of an upright microscope (BX51WI; Olympus). Slices were then perfused at room temperature with oxygenated aCSF at a rate of 1.5–3 ml/min.

Patch electrodes were constructed from thin-walled, single-filamented borosilicate glass (1.5 mm outer diameter; World Precision Instruments) using a microelectrode puller (P-97; Sutter Instruments). Pipette resistances ranged from 4 to 6 $\text{M}\Omega$, and seal resistances were >1 $\text{G}\Omega$. In the majority of experiments, patch electrodes were filled with a solution containing the following (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 MgATP , and 0.3 $\text{Na}_2\text{-GTP}$, pH 7.2 (305 mOsm). To determine the extent to which bursting activity within lamina I depended on the intracellular levels of free Mg^{2+} , some experiments used either a “high- Mg^{2+} ” internal solution [consisting of (in mM) 130 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 0.3 $\text{Na}_2\text{-GTP}$, 1.45 K_2ATP , and 2.55 MgCl_2] or a “low- Mg^{2+} ” solution [composed of (in mM) 90 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 0.3 $\text{Na}_2\text{-GTP}$, 21.6 K_2ATP , and 1.56 MgCl_2]. Using a Mg-ATP calculator (version 1.3; MaxChelator) (Schoenmakers et al., 1992), the solutions were designed to produce free $[\text{Mg}^{2+}]_{\text{internal}}$ of 1 mM (high Mg^{2+}) or 10 μM (low Mg^{2+}), while maintaining a constant $[\text{Mg-ATP}]_{\text{internal}}$ of 1.55 mM to minimize potential differences in ion channel phosphorylation and “rundown” between intracellular solutions (Pearson and Dolphin, 1993).

Dorsal horn neurons were visualized with infrared differential interference contrast, and patch-clamp recordings were obtained from L4–L5 cells located across the mediolateral extent of the spinal cord using a Multiclamp 700B amplifier (Molecular Devices). Sampled cells were categorized as lamina I neurons if they resided within 40 μm of the edge of the dorsal white matter (Lorenzo et al., 2008). Approximately 1 min after establishment of the whole-cell configuration, the spontaneous firing patterns of dorsal horn neurons were classified at the resting membrane potential (V_{rest}) as “silent,” “irregular,” “tonic,” or “bursting” (Li and Baccei, 2011). Membrane resistance (R_m) was measured using the hyperpolarization produced by negative current injections (5–10 pA) from V_{rest} .

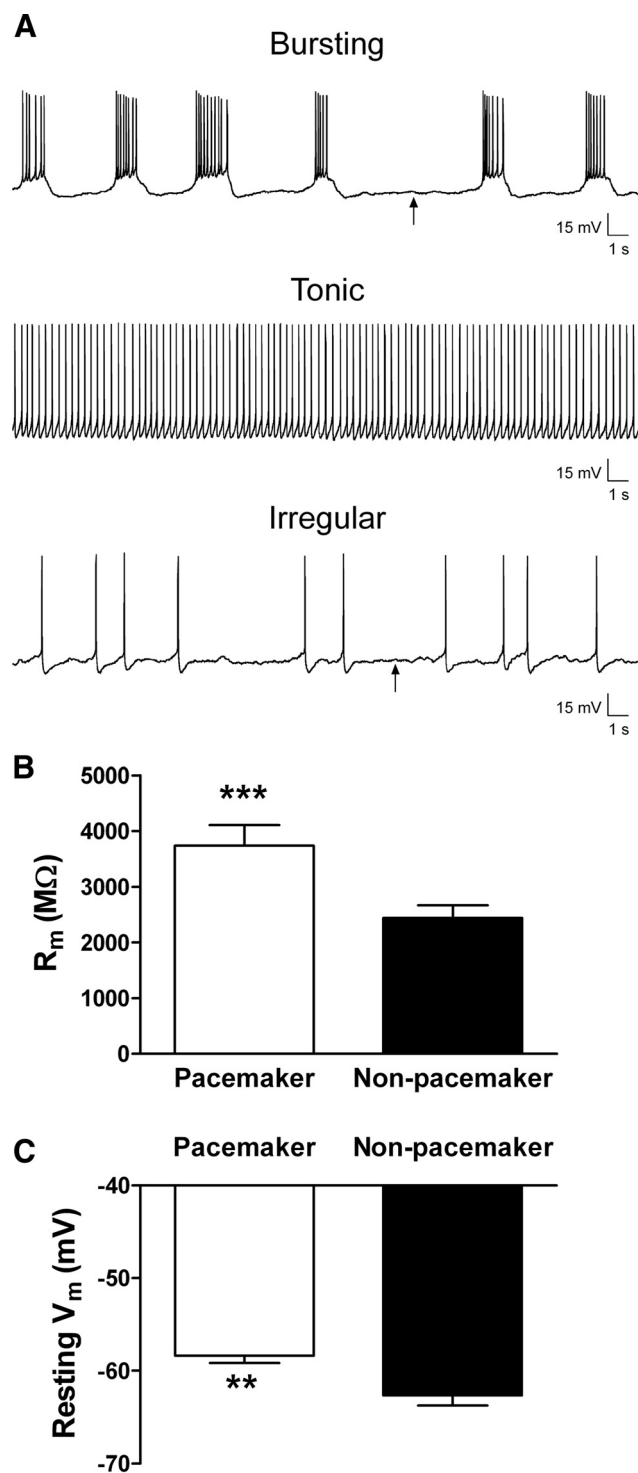


Figure 1. Lamina I pacemaker neurons are distinguished by high membrane resistance and depolarized resting potentials. *A*, Spontaneous firing patterns were classified as rhythmic bursting (top), tonic (middle), irregular (bottom), or silent (data not shown). Arrows indicate approximate regions used to measure the resting membrane potential in a spontaneously active neuron. *B*, Membrane resistance (R_m) was significantly higher in the pacemaker population of lamina I neurons compared with adjacent nonbursting neurons ($***p = 0.0001$, Mann–Whitney test). *C*, Pacemaker neurons also exhibited a significantly more depolarized resting potential ($n = 15$) compared with nonpacemaker neurons ($n = 22$; $**p = 0.007$, Mann–Whitney test). Tonic firing neurons were excluded from the analysis because of difficulties in accurately measuring their resting potential.

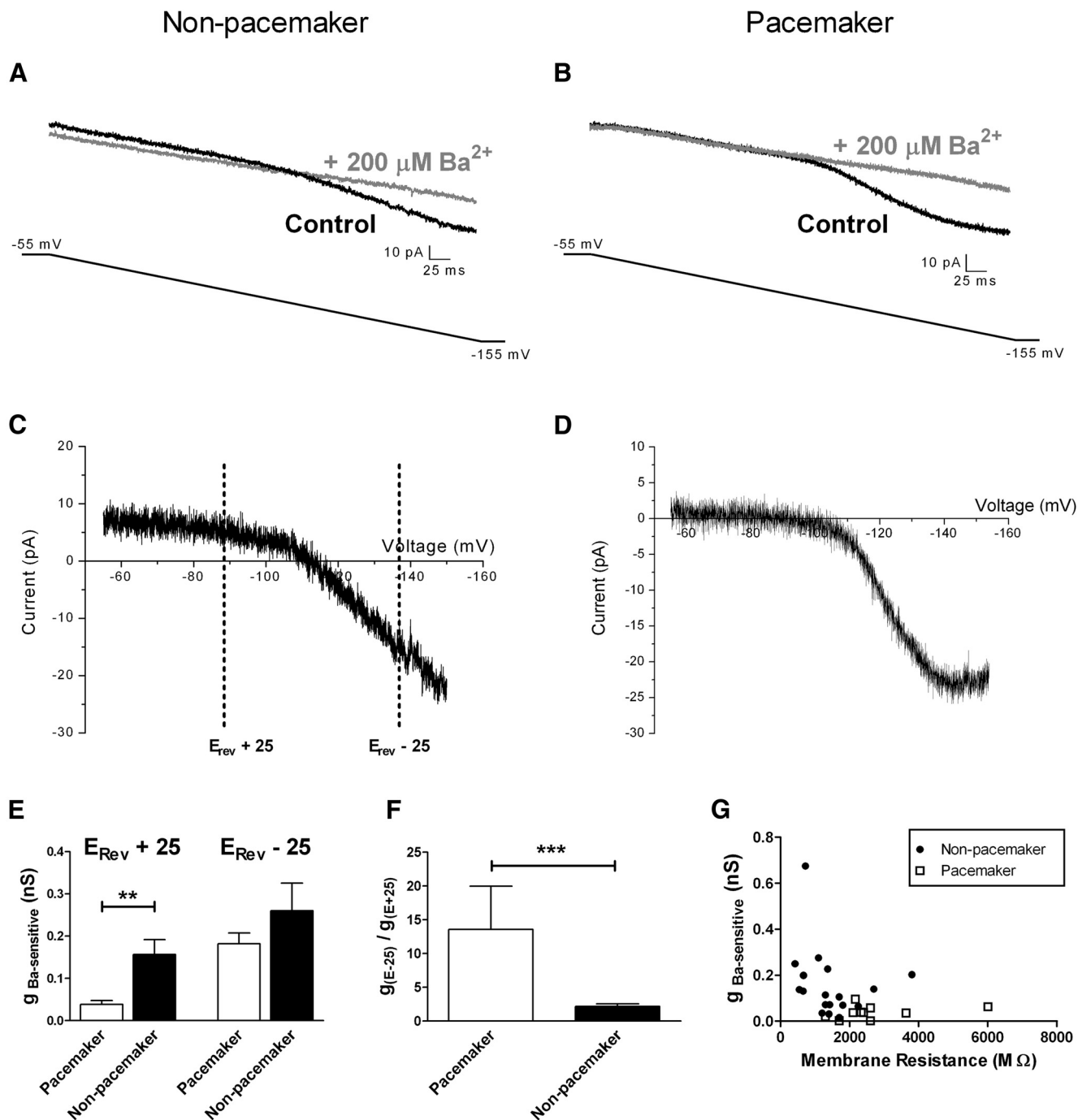


Figure 2. Low K_{ir} conductance in spontaneously bursting neurons within the newborn SDH. **A**, Representative currents recorded in nonpacemaker neurons during negative voltage ramps from a holding potential of -55 to -155 mV before (control; black) and after (gray) the bath application of 200 μ M $BaCl_2$. The illustrated example corresponds to a silent lamina I neuron at P3. **B**, Representative traces obtained using the same protocol as in **A**, administered to a lamina I pacemaker neuron. Note the overlap in traces during the early portion of the negative voltage ramp. **C**, **D**, Example of Ba^{2+} -sensitive currents obtained by electronic subtraction (black minus gray) plotted as a function of membrane voltage for nonpacemaker (**C**) and pacemaker (**D**) neurons within lamina I of the neonatal spinal cord. Inward rectification was observed in all cases as the slope was greater at potentials negative to the observed reversal potential (E_{rev}). **C** includes representative voltages (equidistant from E_{rev}) used to calculate the Ba^{2+} -sensitive conductance ($g_{Ba-sensitive}$). **E**, Bursting lamina I neurons possessed significantly lower $g_{Ba-sensitive}$ compared with adjacent nonbursting cells at potentials positive to E_{rev} (** $p = 0.002$, Mann–Whitney test; left) but not at potentials more negative than E_{rev} ($p = 0.981$; right). **F**, The degree of inward rectification of Ba^{2+} -sensitive K^+ currents [measured as $g(E_{rev} - 25) / g(E_{rev} + 25)$] was significantly greater in the population of pacemaker neurons within lamina I (*** $p = 0.0007$, Mann–Whitney test). **G**, Across the general population of neonatal lamina I neurons, there was a significant inverse correlation between the level of Ba^{2+} -sensitive conductance and the membrane resistance ($r = -0.398$; $p = 0.036$; Spearman's test), suggesting an important contribution of $g_{Ba-sensitive}$ to resting leak conductance within these cells.

Inward-rectifying K^+ (K_{ir}) currents were isolated as described previously (Derjean et al., 2003). Briefly, neurons were voltage clamped at -55 mV in the presence of 10 μ M NBQX, 25 μ M AP-5, 10 μ M gabazine, and 0.5 μ M strychnine to block fast synaptic transmission in the slice. Negative voltage ramps (from -55 to -155 mV) were applied at a rate of 0.2

mV/ms. $BaCl_2$ (0.2–200 μ M) was bath applied to block K_{ir} (Coetzee et al., 1999), and the Ba^{2+} -sensitive component of the current was subsequently isolated via electronic subtraction (see Fig. 2). Conductance ($g_{Ba-sensitive}$) was calculated as $g = I / (V_m - E_{rev})$, at two different membrane potentials that were equidistant (25 mV) from the reversal poten-

tial (Derjean et al., 2003). To estimate the degree of K_{ir} inward rectification, a ratio of these two conductances was calculated as follows: $g_{(E-25)}/g_{(E+25)}$. The potential contribution of constitutively active G-protein-coupled K_{ir} channels (GIRKs) to the above ramp currents was investigated by bath-applying the selective GIRK antagonist tertiapin-Q (100 nM). Bovine serum albumin (0.1 mg/ml) was included in the solution to reduce nonspecific binding of the peptide to the perfusion tubing. At this concentration, tertiapin-Q was found to partially block the outward currents evoked by the $GABA_B$ receptor ($GABA_B$ R) agonist baclofen (data not shown), confirming that the peptide adequately penetrated the slice under our experimental conditions. To examine the expression of hyperpolarization-activated cation currents (I_h) in lamina I neurons, hyperpolarizing voltage steps (from -70 to -150 mV in 10 mV increments) were delivered from a holding potential of -60 mV. Alternatively, in current-clamp mode, hyperpolarizing current steps (0–20 pA) were administered from the resting potential to detect the presence of a depolarizing “sag” in the membrane potential region, which is characteristic of I_h expression (Yoshimura and Jessell, 1989).

Membrane voltages were adjusted for liquid junction potentials (~ 14 mV) calculated using JPCalc software (P. Barry, University of New South Wales, Sydney, Australia; modified for Molecular Devices) unless otherwise specified. Currents were filtered at 4–6 kHz through a -3 dB, four-pole low-pass Bessel filter; digitally sampled at 20 kHz; and stored on a personal computer (ICT) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.2 software; Molecular Devices).

Biocytin staining and immunohistochemistry. Rat pups (P1–P5) of either sex were deeply anesthetized with sodium pentobarbital (30 mg/kg, i.p.), perfused with aCSF solution, and decapitated. The lumbar spinal cord was isolated with dura and pia matter removed from the dorsal surface. The intact cord was transferred to the recording chamber and perfused at room temperature with oxygenated aCSF at 1.5–3 ml/min. Whole-cell patch-clamp recordings were obtained from lamina I neurons under infrared LED illumination (Safronov et al., 2007; Szucs et al., 2009) using the standard K-gluconate intracellular solution (see above) with the addition of 0.3% biocytin. Following the classification of spontaneous firing pattern (Fig. 1), nonbursting neurons were removed from the spinal cord under visual control via strong negative pressure applied to the electrode, thus ensuring that cells later identified with biocytin staining corresponded to pacemakers. Meanwhile, neurons exhibiting spontaneous burst firing were dialyzed with biocytin for 30–40 min. Approximately two to six pacemaker neurons were labeled in this manner per spinal cord.

The spinal cords were then fixed overnight at 4°C in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose in 0.1 M PBS overnight. Cords were rapidly frozen, and parasagittal sections were cut at 10 μm on a cryostat, mounted onto Superfrost Plus slides (Fisher Scientific), and left to dry overnight on a slide warmer at 39°C . Sections were washed three times for 5 min in 0.1 M PBS, permeabilized with 0.3% Tween 20 in 0.1 M PBS for 10 min, and incubated with avidin conjugated to Rhodamine Red (1:1000; Invitrogen) for 2 h at room temperature. The slides were then washed an additional three times for 5 min in 0.1 M PBS and loosely coverslipped with an excess of Vectashield mounting medium (Vector Laboratories), and biocytin-filled neurons were visualized on a Microshot-SA fluorescence microscope (Nikon). Slides containing labeled neurons were then subjected to immunostaining for K_{ir} channels as described below.

Slides containing labeled cells (or those selected for peptide preabsorption control experiments) were additionally washed six times for 10 min and blocked in 10% normal goat serum/0.3% Tween 20 in 0.1 M PBS for 1 h. Primary antibodies raised in rabbit against $K_{ir}2.1$ or $K_{ir}2.3$ (1:200; Alomone Labs) or $K_{ir}2.2$ (1:250; Epitomics) were applied in dilution buffer containing 2% normal goat serum, 0.06% Tween 20, and 0.1 M PBS and incubated for 48 h at 4°C . For each antibody used, a preabsorption control was prepared by incubating an undiluted antibody with its corresponding peptide (Alomone Labs) in a 1:5 to 1:10 ratio for 1 h before antibody dilution. Tissue was then washed three times for 5 min in 0.3% Tween 20 in 0.1 M PBS and incubated for 2 h at room temperature in a goat anti-rabbit secondary antibody conjugated to Alexa 488 (1:

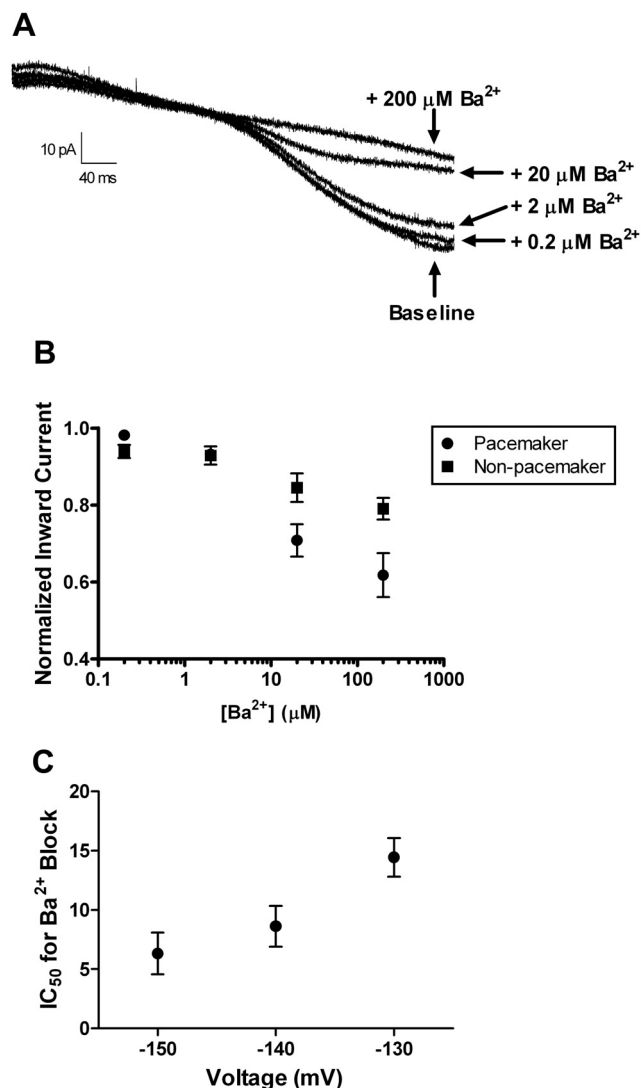


Figure 3. High Ba^{2+} sensitivity of K_{ir} currents in neonatal lamina I neurons. **A**, Representative currents evoked during voltage ramps from -55 to -155 mV in an individual lamina I pacemaker neuron at baseline and in the presence of increasing concentrations of external $BaCl_2$ (0.2 – $200 \mu\text{M}$). **B**, Dose–response relationships suggest that a fraction of the recorded current (measured at -140 mV) was highly sensitive to Ba^{2+} block in both pacemaker and nonpacemaker populations within lamina I of the immature spinal cord. **C**, The measured IC_{50} for the block by external Ba^{2+} was voltage dependent, consistent with the presence of classical $K_{ir}2.x$ channels. Data are pooled from bursting and nonbursting groups of lamina I cells.

1000) diluted in 2% normal goat serum in 0.1 M PBS. Sections were washed six times for 5 min and coverslipped with Vectashield. Images were acquired on a LSM510 inverted confocal microscope (Zeiss) with a $63\times$ Real Zoom oil-immersion objective and processed in Zen imaging software. Cells were imaged at an optical thickness of $0.5 \mu\text{m}$ to visualize the presence of K_{ir} protein located on the cell surface. Cells were determined to express $K_{ir}2.1$, $K_{ir}2.2$, or $K_{ir}2.3$ protein if Alexa 488-positive puncta colocalized with avidin-conjugated rhodamine fluorescence.

Data analysis and statistics. Electrophysiological data were analyzed using Clampfit (Molecular Devices) and Origin (OriginLab Corporation) software. Neurons were classified as bursting (i.e., pacemakers) if they exhibited slow oscillations in membrane potential that gave rise to multiple action potentials (Li and Bacceti, 2011). Nonparametric tests were used in cases in which the distribution of data failed the D’Agostino & Pearson normality test (Prism; GraphPad Software) or when the number of observations was insufficient ($n < 24$) to definitively conclude that data were distributed in a Gaussian manner. $p < 0.05$ was considered

significant. n refers to the number of neurons sampled in a given group. Data are expressed as means \pm SEM.

Results

Pacemaker neurons are distinguished by low conductance through Ba^{2+} -sensitive K_{ir} channels

To identify which subtypes of leak conductance are important for regulating pacemaker activity in the newborn superficial dorsal horn, we obtained *in vitro* whole-cell patch-clamp recordings from lamina I neurons in rat spinal cord slices prepared at P2–P5. The pattern of spontaneous activity at room temperature was classified as bursting, tonic, irregular (Fig. 1A) or silent (data not shown), as described previously (Li and Baccei, 2011). The observed rhythmic burst firing was previously shown to reflect the intrinsic membrane properties of the lamina I neuron, thus constituting “pacemaker” activity within the superficial dorsal horn (Li and Baccei, 2011). Pacemaker neurons exhibited a significantly higher membrane resistance (i.e., lower resting leak conductance) compared with adjacent nonbursting lamina I neurons ($n = 33$ –40 in each group; $p = 0.0001$, Mann–Whitney test; Fig. 1B). This was accompanied by a significantly more depolarized resting potential (V_{rest}) within the pacemaker population ($p = 0.007$, Mann–Whitney test; Fig. 1C). Since membrane resistance in SDH neurons decreases with elevated temperature (Graham et al., 2008), we obtained additional recordings from neonatal lamina I neurons at 32–34°C and found that 9 of the 20 neurons sampled (45%) exhibited spontaneous pacemaker activity. Critically, this demonstrates that the intrinsic burst firing is not an artifact related to recording at room temperature.

K_{ir} channels are known to be important for the maintenance of V_{rest} in both neurons and muscle through their contribution to the leak membrane conductance (Hibino et al., 2010). Therefore, we hypothesized that the distinct passive membrane properties (Fig. 1B,C) of pacemaker neurons reflect, at least in part, a reduced K_{ir} conductance near the resting potential. After the classification of the spontaneous firing pattern, voltage-clamp experiments were performed in which lamina I neurons were hyperpolarized from -55 to -155 mV (at a rate of 0.2 mV/ms) in the presence of a mixture of antagonists to block fast synaptic transmission in the slice (see Materials and Methods). $BaCl_2$ (200 μ M) was subsequently added to the bath. Electronic subtraction of the Ba^{2+} -sensitive component revealed a current with a mean reversal potential (E_{rev}) of -97.7 ± 2.5 mV ($n = 28$), which was close to the predicted equilibrium potential for K^+ ions under our experimental conditions (-101.4 mV). Measurements of conductance (see Materials and Methods) at two potentials equidistant from E_{rev} (Fig. 2C) (Derjean et al., 2003) consistently demonstrated inward rectification, as evidenced by a higher conductance at more negative membrane potentials (V_m), which is consistent with the isolation of K_{ir} current using this protocol.

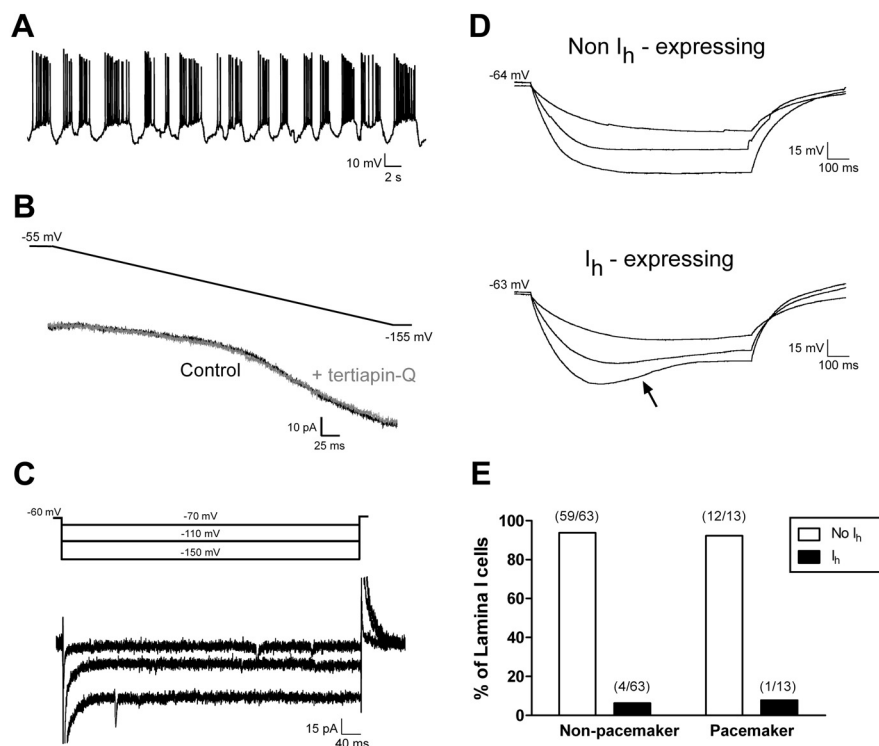


Figure 4. GIRKs and hyperpolarization-activated cation channels do not contribute to resting leak conductance within immature SDH neurons. **A**, Example of spontaneous, rhythmic burst firing in a neonatal lamina I cell. **B**, Current evoked by a negative voltage ramp in the absence (black) or presence (gray) of the selective GIRK antagonist tertipin-Q (100 nM). **C**, The majority of pacemaker neurons exhibited no evidence of the time-dependent inward current at hyperpolarized V_m that would indicate the presence of hyperpolarization-activated cation currents (I_h ; same cell as in **A**). **D**, Current-clamp recordings from a representative pacemaker neuron showing an absence of the depolarizing sag in V_m in response to hyperpolarizing current injections (top), in contrast to the clear sag (arrow) witnessed in a minority of lamina I neurons at the same age (bottom). **E**, Group data demonstrating that the majority of pacemaker and nonpacemaker neurons in lamina I of the neonatal spinal cord lack hyperpolarization-activated cation current (I_h).

The effects of extracellular Ba^{2+} application on the current evoked by the negative voltage ramp clearly differed between pacemaker and nonpacemaker neurons within lamina I of the neonatal spinal cord. In nonpacemakers, Ba^{2+} blocked a portion of the recorded current throughout the voltage ramp, thus lowering the overall slope of the observed current (Fig. 2A,C). Meanwhile, in pacemaker neurons from the same slices, Ba^{2+} evoked only a minimal block of the current between -55 and -85 mV (Fig. 2B,D), suggesting a reduced K_{ir} conductance in this population at these physiological membrane potentials. Indeed, the conductance of the Ba^{2+} -sensitive component ($g_{Ba-sensitive}$) was significantly lower in the bursting group (0.039 ± 0.009 nS; $n = 10$) compared with nonbursting lamina I neurons (0.156 ± 0.036 nS; $n = 18$; $p = 0.002$, Mann–Whitney test; Fig. 2E, left) at V_m positive to the reversal potential (i.e., $E_{rev} + 25$). However, the level of $g_{Ba-sensitive}$ was not statistically different between groups when measured at more negative potentials (i.e., $E_{rev} - 25$; $p = 0.981$; Fig. 2E, right). As a result, K_{ir} currents exhibited a greater degree of inward rectification within the pacemaker population, as the ratio of $g_{Ba-sensitive}$ measured at the more negative V_m relative to that measured at the positive V_m was significantly elevated in the bursting neurons ($p = 0.0007$, Mann–Whitney; Fig. 2F). These data indicate that the lower $g_{Ba-sensitive}$ that characterizes pacemakers at physiological membrane potentials cannot be simply explained by a lower number of K_{ir} channels and in turn suggests that bursting neurons express distinct K_{ir} currents compared with adjacent nonbursting neurons within lamina I of the neonatal spinal cord.

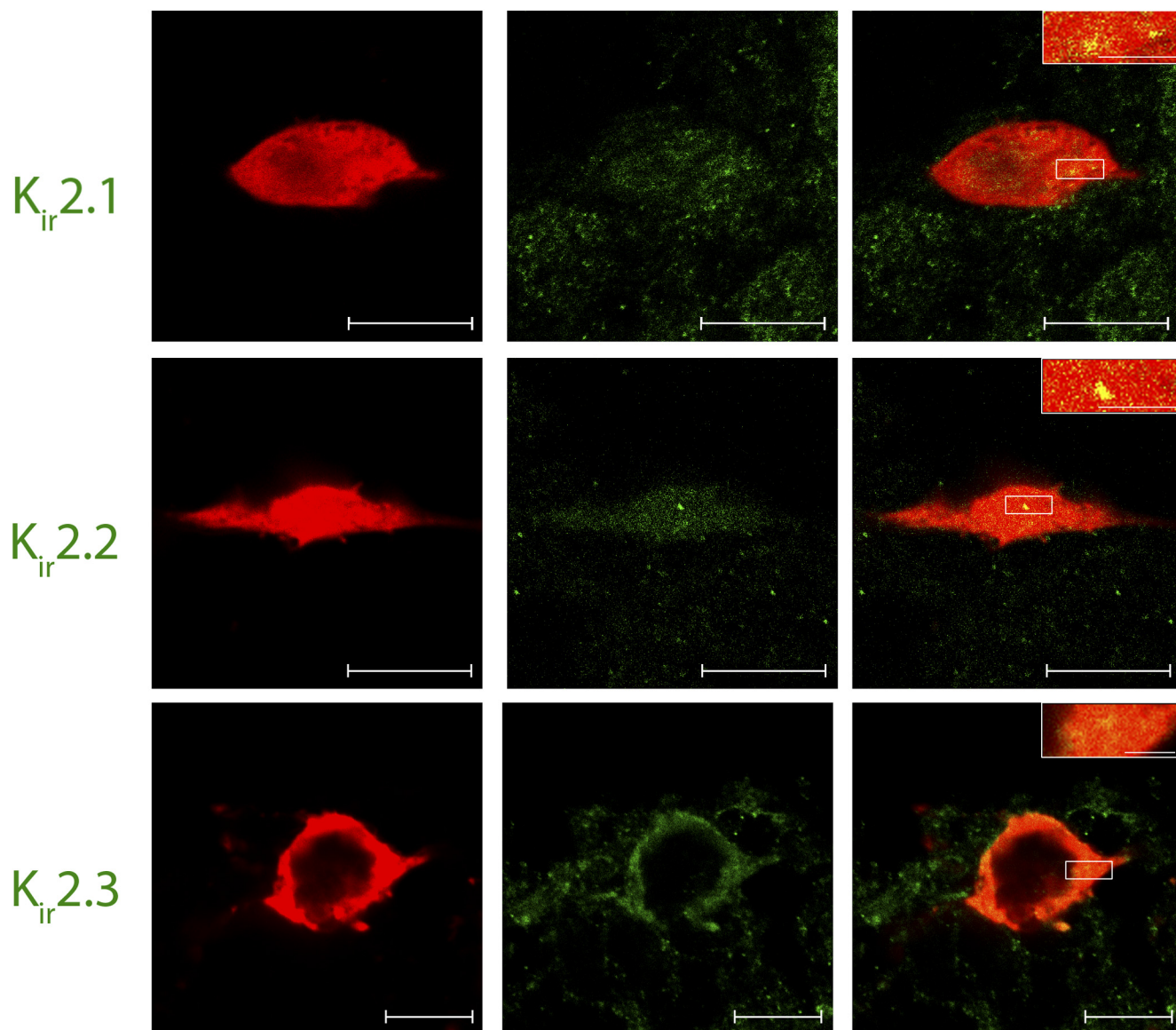


Figure 5. Lamina I pacemaker neurons express classical $K_{ir}2.1$ – 2.3 channels. Confocal images ($0.5\ \mu\text{m}$ optical thickness) of representative biocytin-filled pacemaker neurons (red) processed for K_{ir} immunohistochemistry (green) with $K_{ir}2.1$ (top row), $K_{ir}2.2$ (middle row), or $K_{ir}2.3$ (bottom row) antibodies. Merged images demonstrate that immunoreactive puncta for all three $K_{ir}2$ isoforms (yellow; inset) were localized to identified pacemaker neurons within lamina I of the neonatal spinal cord (right). Scale bars: $10\ \mu\text{m}$; inset, $2\ \mu\text{m}$.

Importantly, $g_{\text{Ba-sensitive}}$ measured at positive membrane potentials (i.e., $E_{\text{rev}} + 25$) was inversely correlated with membrane resistance (R_m) across the lamina I population ($r = -0.398$; $n = 28$; $p = 0.036$; Spearman's test; Fig. 2G). This supports the notion that Ba^{2+} -sensitive leak conductance is an important determinant of R_m in the developing superficial dorsal horn.

Bursting lamina I neurons express classical K_{ir} channels during early life

Classical K_{ir} channels ($K_{ir}2.x$) are known to be constitutively active near the resting potential, exhibit strong inward rectification, and are highly sensitive to block by external Ba^{2+} (Hibino et al., 2010). However, it remains unclear whether functional $K_{ir}2.x$ channels are expressed within the developing dorsal horn. To better characterize the K_{ir} channels found in neonatal lamina I neurons, we examined the sensitivity of K_{ir} currents to increasing concentrations of extracellular Ba^{2+} (Fig. 3A). In both pace-

maker and nonpacemaker populations within lamina I, the currents evoked by the negative voltage ramp were highly sensitive to external Ba^{2+} (IC_{50} at $V_m = -140\ \text{mV}$; bursting: $8.62 \pm 1.7\ \mu\text{M}$, $n = 6$; nonbursting: $13.9 \pm 2.3\ \mu\text{M}$, $n = 8$; $p = 0.109$, unpaired t test; Fig. 3B). In addition, the sensitivity to Ba^{2+} block was lower at more depolarized potentials (Fig. 3C), which is consistent with the known properties of classical $K_{ir}2.x$ channels (Liu et al., 2001; Young et al., 2009).

However, it should be noted that GIRK channels ($K_{ir}3.x$) also exhibit strong inward rectification and sensitivity to external Ba^{2+} (Coetzee et al., 1999). GIRK channels can be constitutively active within some types of central neurons (Takigawa and Alzheimer, 2002; Chen and Johnston, 2005) and thus modulate their resting leak conductance. Importantly, we observed no evidence of tonic GIRK channel activity in neonatal lamina I neurons regardless of the pattern of spontaneous activity, as leak conductance in both bursting ($n = 6$; Fig. 4A, B) and nonbursting ($n = 4$) cells was unaffected by the bath application of the selec-

tive GIRK antagonist tertiapin-Q (Jin and Lu, 1998). This strongly suggests that GIRK channels do not contribute to resting leak conductance within the immature SDH and therefore are not responsible for the Ba^{2+} -sensitive K^+ currents isolated in our experiments (Fig. 2).

Alternatively, the strong inward rectification observed in pacemaker neurons during the negative voltage ramp (Fig. 2D) could potentially reflect the onset of hyperpolarization-activated cation currents (I_h), which have been documented in the rodent superficial dorsal horn (Grudt and Perl, 2002). However, this seems unlikely given the high sensitivity of the observed currents to external Ba^{2+} (Fig. 3A,B), since I_h is reported to be insensitive to Ba^{2+} at submillimolar concentrations (Robinson and Siegelbaum, 2003). Nonetheless, to further address this issue, voltage-clamp and current-clamp protocols were used to detect the presence of I_h in identified pacemaker neurons within lamina I during early life (see Materials and Methods). In 12 of 13 pacemaker neurons examined, negative voltage steps between -70 and -150 mV failed to evoke a slowly activating inward current (Fig. 4C). In addition, injections of hyperpolarizing current into bursting neurons did not produce the depolarizing sag in the membrane potential that is characteristic of I_h expression (Fig. 4D). Similarly, the vast majority of nonbursting neurons (59 of 63; $p = 1.0$ compared with bursting cells, Fisher's exact test) also failed to show evidence of I_h expression (Fig. 4E), suggesting that I_h is not an important contributor to leak conductance in lamina I during early life. Overall, these results further support the idea that the strongly rectifying, highly Ba^{2+} -sensitive currents in newborn lamina I cells correspond to K^+ flux through $K_{ir2.x}$ channels.

The low K_{ir} conductance that distinguishes spinal pacemakers (Fig. 2) could reflect the absence of particular $K_{ir2.x}$ isoforms within this population. To investigate this possibility, we characterized the pattern of $K_{ir2.x}$ expression within identified pacemaker neurons using immunohistochemistry. Whole-cell patch-clamp recordings (with biocytin included in the intracellular solution) were obtained from bursting lamina I neurons in an intact spinal cord preparation (Safronov et al., 2007; Szucs et al., 2009), which has the advantages of better preserving anatomical structures within the dorsal horn and facilitating tissue sectioning for subsequent immunohistological analysis of the recorded neuron. The results clearly indicate that the population of lamina I pacemaker neurons expresses multiple subtypes of classical $K_{ir2.x}$ channels. $K_{ir2.1}$ immunoreactivity was observed in every lamina I neuron exhibiting spontaneous, rhythmic burst firing (13 of 13 cells examined; Fig. 5, top). Similarly, $K_{ir2.2}$ -positive puncta were found in nine of nine pacemaker cells sampled (Fig. 5, middle). Finally, the vast majority of bursting neurons (10 of 12) also demonstrated immunoreactivity for $K_{ir2.3}$ (Fig. 5, bottom). In all cases, preabsorption of the K_{ir2} primary antibody with the relevant antigen, or omission of the

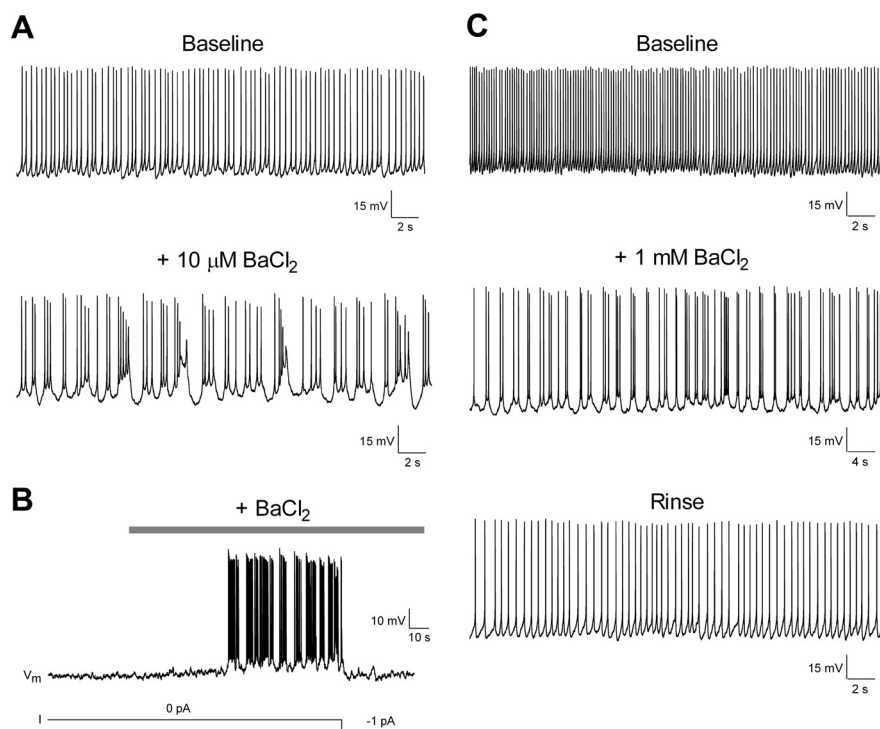


Figure 6. Block of K_{ir} channels can recruit new pacemaker neurons within newborn spinal pain circuits. **A**, Example of neonatal lamina I neuron exhibiting a tonic pattern of spontaneous firing in the presence of aCSF (baseline). Bath application of a low concentration of Ba^{2+} ($10 \mu M$) revealed the ability of this neuron to generate burst firing. **B**, In a silent lamina I cell, external Ba^{2+} application ($200 \mu M$) evoked a slow depolarizing shift in membrane potential, which eventually triggered pacemaker activity. The injection of a small hyperpolarizing current through the patch electrode (bottom) was sufficient to silence this rhythmic burst firing. **C**, Example of a lamina I neuron that switched to rhythmic burst firing in the presence of external Ba^{2+} and reverted back to a tonic mode of action potential discharge after washout.

primary antibody, failed to produce measurable signals within the SDH (data not shown). Collectively, the data suggest that $K_{ir2.x}$ channels are well positioned to regulate the firing of lamina I neurons within the newborn spinal cord.

Block of K_{ir} channels unmasks rhythmic burst firing in neonatal lamina I neurons

The observation that lamina I pacemaker neurons are distinguished by their high membrane resistance (Fig. 1B) and reduced $g_{Ba-sensitive}$ near the resting membrane potential (Fig. 2E) suggests K_{ir} channels modulate the generation of pacemaker activity within the developing SDH. To further examine this possibility, $BaCl_2$ was bath applied ($10 \mu M$ to $1 mM$) to nonbursting neurons within lamina I of the neonatal spinal cord. External Ba^{2+} evoked a depolarizing shift in membrane potential in these cells (3.04 ± 0.73 mV; $n = 33$). More importantly, in a significant proportion of neurons that initially showed either a tonic (3 of 7 cells examined) or irregular (5 of 14 cells) pattern of spontaneous activity, the administration of extracellular Ba^{2+} promoted a switch to rhythmic burst firing that was often reversible after washout (Fig. 6A,C). In addition, in 6 of the 12 silent lamina I neurons sampled, Ba^{2+} application evoked a slow membrane depolarization with subsequent generation of spontaneous bursting (Fig. 6B). In many cases, this burst firing could be silenced by the injections of small amounts (≤ 2 pA) of hyperpolarizing current through the patch electrode (Fig. 6B), indicating that even minor shifts in $g_{Ba-sensitive}$ may have profound consequences for the pattern of spontaneous activity within this population. The recruitment of burst firing is unlikely to be solely explained by the depolarization produced by Ba^{2+} , as our previous work demon-

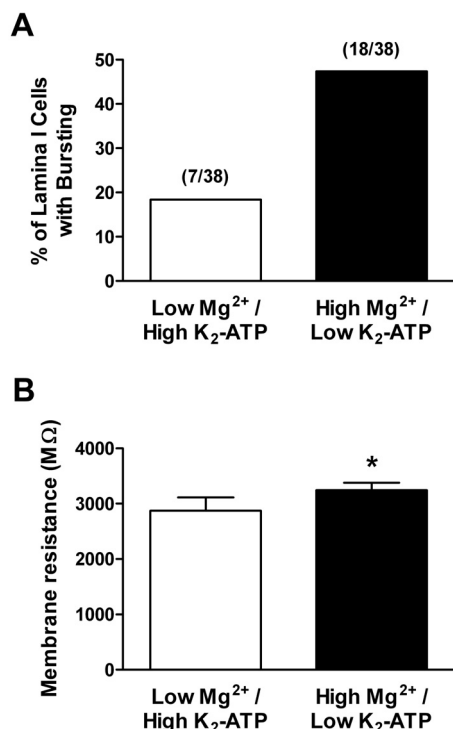


Figure 7. Prevalence of rhythmic burst firing is regulated by intracellular cation levels within neonatal lamina I neurons. **A**, Elevating the intracellular levels of free Mg^{2+} (to ~ 1 mM) significantly increased the percentage of lamina I neurons demonstrating pacemaker activity during early life ($p = 0.014$, Fisher's exact test). **B**, Across the general population of lamina I neurons, a significantly greater membrane resistance was observed with the use of the high- Mg^{2+} intracellular solution ($*p = 0.013$, Mann–Whitney test) compared with the use of a patch solution containing low internal Mg^{2+} within the same slices.

strated that directly depolarizing nonpacemaker neurons via the patch electrode only evoked bursting in $\sim 12\%$ of cells at P2–P3 (Li and Baccei, 2011). Collectively, these results suggest that the propensity of a given lamina I neuron to generate pacemaker activity is tightly controlled by the level of leak conductance through Ba^{2+} -sensitive K_{ir} channels.

Strong inward rectification is not an innate property of K_{ir} channels, but rather arises from the voltage-dependent block of the channel pore by intracellular free Mg^{2+} (Matsuda et al., 1987; Lu and MacKinnon, 1994) and internal free polyamines (Lopatin et al., 1994; Fakler et al., 1995). Therefore, if rhythmic bursting in lamina I neurons is indeed regulated by K_{ir} channels, one would predict that the prevalence of pacemaker activity within the neonatal SDH could be significantly altered by manipulating the intracellular concentrations of these free cations. To further investigate this issue, whole-cell patch-clamp recordings from neonatal lamina I neurons were alternately obtained using intracellular recording solutions designed to contain either low (~ 10 μM) or high (~ 1 mM) levels of free Mg^{2+} via manipulations of the internal ATP concentration (see Materials and Methods). Although our intracellular recording solution did not contain polyamines, endogenous polyamines can be very slow to “wash out” during dialysis in the whole-cell configuration (Shin et al., 2005; Fleidervish et al., 2008). In addition, since ATP also strongly binds to polyamines (Watanabe et al., 1991), altering the internal levels of ATP in our sampled neurons will also modify the intracellular concentration of free polyamines, such that the high $[Mg^{2+}]_{free}$ solution should also contain elevated levels of free polyamines. Importantly, a significantly higher percentage

(18 of 38; 47%) of lamina I neurons exhibited rhythmic burst firing when the high $[Mg^{2+}]_{free}$ internal solution was used, compared with neurons from the same spinal cord slices dialyzed with the low internal $[Mg^{2+}]_{free}$ solution (7 of 38; 18%; $p = 0.014$, Fisher's exact test; Fig. 7A). This was accompanied by a significantly higher membrane resistance under high $[Mg^{2+}]_{free}$ conditions ($p = 0.013$, Mann–Whitney test; Fig. 7B). It should be noted that these experiments cannot conclusively determine whether the increased prevalence of bursting seen with the high- Mg^{2+} / low-ATP internal solution was attributable to elevations in free Mg^{2+} or free polyamines, as the intracellular polyamine concentration remains unknown. Nonetheless, these data provide further support for the notion that K_{ir} channels are key modulators of intrinsic burst firing within developing spinal nociceptive circuits.

Discussion

These results provide the first evidence that classical K_{ir} channels serve as important regulators of pacemaker activity within the developing spinal cord. Neonatal lamina I neurons exhibiting spontaneous burst firing were characterized by higher membrane resistance (R_m) and more depolarized resting potentials (V_{rest}) compared with adjacent nonbursting neurons, which was associated with a significantly lower K_{ir} conductance near V_{rest} within the bursting population. Importantly, block of K_{ir} channels unmasked rhythmic burst firing within newborn spinal pain circuits, suggesting that the ability to generate pacemaker activity may be a latent property of a significant number of SDH neurons during early life.

K_{ir} channels modulate passive membrane properties in neonatal SDH neurons

Neurons with a relatively large K_{ir} conductance are predicted to exhibit more negative resting potentials, lower R_m , and minimal spontaneous activity (Hibino et al., 2010). As in other types of central neurons (Day et al., 2005; Young et al., 2009), K_{ir} channels make a significant contribution to the resting leak conductance within newborn SDH neurons, as the level of Ba^{2+} -sensitive K^+ conductance was inversely correlated with R_m (Fig. 2G) and blocking K_{ir} channels significantly elevated R_m in this population (Fig. 7B). As a result, the higher R_m and more depolarized V_{rest} seen in lamina I pacemaker neurons (Fig. 1B, C) likely reflects, at least in part, a reduced conductance through Ba^{2+} -sensitive K_{ir} channels (Fig. 2E). Given that the prevalence of pacemaker activity significantly decreases after the first postnatal week (Li and Baccei, 2011), it will be interesting to determine whether K_{ir} currents are developmentally upregulated in lamina I neurons.

The available evidence strongly suggests that classical K_{ir} ($K_{ir2.x}$) channels underlie the Ba^{2+} -sensitive K^+ currents observed in the neonatal SDH. First, the strong inward rectification (Fig. 2) and high Ba^{2+} sensitivity (Fig. 3) of the K_{ir} currents point to the involvement of the $K_{ir2.x}$ family (Preisig-Müller et al., 2002; Schram et al., 2002) and are inconsistent with the described properties of other rectifying K^+ channels (Coetzee et al., 1999). Indeed, our results clearly demonstrate that $K_{ir2.1-2.3}$ channels are found in immature SDH cells (Fig. 5). In addition, whereas GIRK ($K_{ir3.x}$) channels are also expressed within lamina I–II (Lüscher et al., 1997; Marker et al., 2005, 2006) and are highly sensitive to Ba^{2+} (Owen et al., 1999; Lancaster et al., 2000), we found no evidence that these channels were constitutively active in SDH neurons at this age. This implies that differences in GIRK expression between the pacemaker and nonpacemaker populations are unlikely to explain the lower K_{ir} conductance seen in spontane-

ously bursting neurons. Instead, the data collectively suggest that although rhythmic burst firing is suppressed in the majority of immature lamina I neurons, attributable in part to $K_{ir,2.x}$ channel activity, a subset of these cells possesses minimal $K_{ir,2.x}$ conductance near V_{rest} and thus becomes endowed with the ability to generate pacemaker activity. Interestingly, genetic inhibition of $K_{ir,2.1}$ currents unleashes pacemaker activity in ventricular myocytes that were previously quiescent (Miake et al., 2002). However, it should be noted that low $K_{ir,2.x}$ conductance by itself is insufficient to generate rhythmic burst firing, as other channels such as persistent voltage-gated Na^+ channels and high-threshold voltage-gated Ca^{2+} channels are critically involved (Li and Baccei, 2011). Lamina I neurons that failed to exhibit pacemaker activity after extracellular Ba^{2+} application may lack sufficient levels of these other ionic conductances.

Potential mechanisms underlying low K_{ir} conductance in spinal pacemaker neurons

The mechanistic basis for the reduced K_{ir} conductance within the pacemaker population remains to be determined. However, the data suggest that it does not result from an absence of particular $K_{ir,2.x}$ isoforms, as $K_{ir,2.1}$, $K_{ir,2.2}$, and $K_{ir,2.3}$ were expressed in lamina I neurons exhibiting spontaneous burst firing (Fig. 5). Although $K_{ir,2.4}$ expression was not examined, the high Ba^{2+} sensitivity of the isolated K_{ir} currents (Fig. 3) argues against a dominant expression of this isoform within the immature SDH, as $K_{ir,2.4}$ is reported to show significantly lower Ba^{2+} sensitivity compared with other $K_{ir,2.x}$ channels (Töpert et al., 1998; Coetzee et al., 1999). In fact, previous studies in the adult rat SDH suggest $K_{ir,2.4}$ expression is weaker compared with other $K_{ir,2.x}$ channels (Prüss et al., 2005).

The low K_{ir} conductance in pacemaker neurons is also unlikely to be explained by a lower number of $K_{ir,2.x}$ channels in the somatodendritic membrane compared with other lamina I cells, since the conductance was similar between the two groups at more negative potentials (Fig. 2E, right). Alternatively, since K_{ir} subunits can form heteromultimers (Fink et al., 1996; Preisig-Müller et al., 2002), it is feasible that bursting neurons express K_{ir} channels with a distinct stoichiometry. $K_{ir,2.x}$ isoforms that show similarly high sensitivity to Ba^{2+} may nonetheless exhibit varying degrees of inward rectification and possess different susceptibilities to block by intracellular polyamines (Dhamoon et al., 2004; Ishihara and Yan, 2007), which are normally present in cells at up to millimolar concentrations (Tabor and Tabor, 1984; Watanabe et al., 1991). Therefore, the higher R_m (Fig. 1B), more depolarized V_{rest} (Fig. 1C), and greater K_{ir} inward rectification (Fig. 2F) that characterize pacemaker neurons could reflect the preferential expression of K_{ir} isoforms that are more sensitive to internal polyamine block. One potential candidate is $K_{ir,2.2}$, which has been associated with both extremely strong inward rectification and increased polyamine sensitivity compared with other $K_{ir,2.x}$ channels (Panama and Lopatin, 2006; Ishihara and Yan, 2007).

Another possibility is that elevated intracellular levels of polyamines predispose a subset of SDH neurons to exhibit the high R_m required for the generation of rhythmic burst firing. The polyamines spermine and spermidine block K_{ir} channels with a much higher (10- to 100-fold) potency than Mg^{2+} (Lopatin et al., 1994; Fakler et al., 1995) and are highly expressed within lamina I of the spinal cord (Laube et al., 2002), where they are synthesized from the amino acids arginine and methionine via the activation of ornithine decarboxylase (Tabor and Tabor, 1984). Interestingly, ornithine decarboxylase expression in the rat brain is highest at birth and declines rapidly during the first week of life (Pujic et al.,

1995). In addition, polyamine levels in the CNS can be modulated by sensory experience during development (Aizenman et al., 2002). Alternatively, it is also feasible that pacemaker neurons are distinguished by differences in Mg^{2+} transport or buffering, which result in elevated intracellular concentrations of free Mg^{2+} . Large changes in internal Mg^{2+} levels can occur in response to both hormonal and nonhormonal stimuli (Romani and Scarpa, 2000; Shindo et al., 2011), including alterations in cAMP levels or PKC activity (for review, see Romani, 2007). This raises the possibility that the enhanced rectification seen in pacemakers is explained by a greater block of K_{ir} channels by intracellular Mg^{2+} at membrane potentials positive to E_K .

Nonetheless, the pronounced difference in K^+ conductance between pacemakers and nonpacemakers at physiological potentials (Fig. 2) may also reflect the contribution of additional ion channels that act preferentially at potentials positive to E_K . For example, some subtypes of voltage-gated K^+ channels, including members of the $K_v3.x$ and $K_v7.x$ (KCNQ) families, can be open near V_{rest} (Schroeder et al., 2000; Abbott et al., 2001). Therefore, the higher R_m within the pacemaker population (Fig. 1) could be partially explained by a lower resting conductance through constitutively active voltage-gated K^+ channels.

K_{ir} channels as putative targets of neuromodulators within newborn spinal nociceptive circuits

Although we failed to observe constitutive activation of GIRK ($K_{ir,3.x}$) channels in the neonatal SDH *in vitro*, previous work suggests that GIRK channels can be opened by metabotropic receptors such as the GABA_BR from birth (Baccei and Fitzgerald, 2004). Thus, the level of endogenous burst firing within the immature SDH may, under certain conditions, be under tight metabotropic control as previously reported in the deep dorsal horn of the adult (Derjean et al., 2003). One prominent candidate is the NK1 receptor that is expressed in the dorsal horn from the first days of life (Kar and Quirion, 1995; Cheun-suang et al., 2002), as substance P is known to regulate pacemaker activity in other regions of the CNS (Peña and Ramirez, 2004).

It should be noted that classical $K_{ir,2.x}$ channels are also influenced by intracellular signaling cascades. For example, PKC and src kinase significantly inhibit $K_{ir,2.2}$ channels (Zitron et al., 2004, 2008). Notably, both PKC and src activity are elevated in the dorsal horn under pathological conditions (Guo et al., 2004; Kawasaki et al., 2004; Liu et al., 2008), suggesting that the prevalence of pacemaker activity within developing spinal nociceptive circuits might be modulated by nerve or tissue injury. Indeed, peripheral nerve damage during adulthood significantly increases the percentage of deep dorsal horn neurons that exhibit plateau potentials (Real et al., 2011). An excessive number of pacemakers after injury could result in abnormal synchronization of firing within the SDH network, thereby significantly facilitating the output of the spinal pain pathway.

Further elucidation of the molecular properties that distinguish intrinsically bursting neurons within the newborn SDH will yield insight into potential strategies to selectively manipulate their excitability *in vivo*, which may then reveal the precise role of spinal pacemaker activity in the maturation of central nociceptive circuits.

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