# Inward-rectifying potassium (K$_{ir}$) channels regulate pacemaker activity in spinal nociceptive circuits during early life

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## Abstract

Pacemaker neurons in neonatal spinal nociceptive circuits generate intrinsic burst-firing and are distinguished by a lower "leak" membrane conductance compared to adjacent, non-bursting 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 non-pacemakers 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$_{50}$ ~ 10 μM), which suggests the presence of 'classical' K$_{ir}$ (K$_{ir}$2.x) channels in the neonatal SDH. The reduced K$_{ir}$ conductance within pacemakers is unlikely to be explained by an absence of particular K$_{ir}$2.x isoforms, as immunohistochemical analysis revealed the expression of K$_{ir}$2.1, K$_{ir}$2.2 and K$_{ir}$2.3 within spontaneously bursting neurons. Importantly, Ba$^{2+}$ application unmasked rhythmic burst-firing in ~42% of non-bursting 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 path-finding (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; Tazerart et al., 2008), where they are proposed to contribute to rhythmogenesis in locomotor networks (Brocard et al., 2010). Pacemaker neurons were also recently identified within lamina I of the newborn superficial dorsal horn (SDH) (Li and

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Baccei, 2011), which receives direct projections from nociceptive Aδ- 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 to adjacent, non-bursting lamina I neurons (Li and Baccei, 2011). Leak conductance potently 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 majority 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 environment.

## 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 day (P)2–5 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 above solution; Invitrogen, Carlsbad CA) and parasagittal slices (350–400 μm) were cut using a Vibroslice tissue slicer (HA-752; Campden Instruments, Lafayette, IN). The slices were placed in a chamber filled with oxygenated dissection solution for 30 min then allowed to recover in an oxygenated artificial CSF (aCSF) solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.0 $NaH_2PO_4$, 1.0 $MgCl_2$, and 2.0 $CaCl_2$, and 25 glucose, pH 7.2 for    1 hour at room temperature.

### Patch clamp recordings

After recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments, Hamden, CT) and mounted on the stage of an upright microscope

(BX51WI; Olympus, Center Valley, PA). 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, Sarasota FL) using a microelectrode puller (P-97; Sutter Instruments, Novato, CA). Pipette resistances ranged from 4 to 6 MΩ and seal resistances were >1 GΩ. In the majority of experiments, patch electrodes were filled with a solution containing the following (n mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 MgATP, and 0.3 $Na_2$-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 $Na_2$-GTP, 1.45 $K_2$ATP and 2.55 $MgCl_2$ or a "Low $Mg^{2+}$" solution comprised of (in mM): 90 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 0.3 $Na_2$-GTP, 21.6 $K_2$ATP and 1.56 $MgCl_2$. Using a Mg-ATP calculator (1.3; MaxChelator) (Schoenmakers et al., 1992), the solutions were designed to produce free $[Mg^{2+}]_{internal}$ of 1 mM (High $Mg^{2+}$) or 10 μM (Low $Mg^{2+}$), while maintaining a constant $[Mg\text{-}ATP]_{internal}$ of 1.55 mM in order 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, Sunnyvale, CA). 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 $V_{rest}$.

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 μM NBQX, 25 μM AP-5, 10 μM gabazine (GBZ) and 0.5 μM 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 μM–200 μM) 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 ($g_{Ba\text{-}sensitive}$) was calculated as: $g = I / (V_m - E_{rev})$ at two different membrane potentials that were equidistant (25 mV) from the reversal potential (Derjean et al., 2003). To estimate the degree of $K_{ir}$ inward rectification, a ratio of these two conductances was calculated as: $g_{(E-25)}/g_{(E+25)}$. 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 in order to reduce non-specific binding of the peptide to the perfusion tubing. At this concentration, tertiapin-Q was found to partially block the outward currents evoked by the $GABA_B$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 (rom −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 (approximately 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, Cincinnati, OH) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.2 software; Molecular Devices).

## Biocytin staining and immunohistochemistry

Rat pups (P1–5) of either sex were deeply anesthetized with sodium pentobarbital (30 mg/kg ip), 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), non-bursting 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 0.1M PBS overnight. Cords were rapidly frozen, then parasagittal sections cut at 10 μm on a cryostat, mounted onto Superfrost Plus slides (Fisher Scientific; Pittsburgh, PA) and left to dry overnight on a slide warmer at 39°C. Sections were washed 3×5 min in 0.1M PBS, permeabilized with 0.3% Tween-20 in 0.1M PBS for 10 min, and incubated with avidin conjugated to Rhodamine Red (1:1000; Invitrogen) for 2 hours at room temperature. The slides were then washed for an additional 3×5 min in 0.1M PBS and loosely cover-slipped with an excess of Vectashield mounting medium (Vector Labs, Burlingame CA), and biocytin-filled neurons were visualized on a Nikon Microshot-SA fluorescence microscope. Slides containing labeled neurons were then subjected to immunostaining for $K_{ir}2$ channels as described below.

Slides containing labeled cells (or those selected for peptide pre-absorption control experiments) were additionally washed 6×10 min and blocked in 10% Normal Goat Serum/0.3% Tween-20 in 0.1M PBS for one hour. Primary antibodies raised in rabbit against $K_{ir}2.1$ or $K_{ir}2.3$ (1:200; Alomone Labs; Jerusalem, Israel) or $K_{ir}2.2$ (1:250; Epitomics; Burlingame, CA) were applied in dilution buffer containing 2% Normal Goat Serum, 0.06% Tween-20 and 0.1M PBS, and incubated for 48 hours at 4°C. For each antibody used, a pre-absorption control was prepared by incubating undiluted antibody with its corresponding peptide (Alomone Labs) in a 1:5–1:10 ratio for 1 hour prior to antibody dilution. Tissue was then washed 3×5 min in 0.3% Tween-20 in 0.1M PBS, and incubated for 2 hours at room temperature in goat anti-rabbit secondary antibody conjugated to Alexa 488 (1:1000) diluted in 2% Normal Goat Serum in 0.1M PBS. Sections were washed 6×5 min and cover-slipped with Vectashield. Images were acquired on a Zeiss LSM510 inverted confocal microscope with a 63× Real Zoom oil immersion objective and processed in Zen imaging software. Cells were imaged at an optical thickness of 0.5 μm 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 co-localized with avidin-conjugated rhodamine fluorescence.

### Data analysis and statistics

Electrophysiological data were analyzed using Clampfit (Molecular Devices) and Origin (OriginLab Corp., Northampton, MA) software. Neurons were classified as bursting (i.e. pacemakers) if they exhibited slow oscillations in membrane potential which gave rise to multiple action potentials (Li and Baccei, 2011). Nonparametric tests were used in cases in which the distribution of data failed the D'Agostino & Pearson normality test (Prism, GraphPad Software, La Jolla CA) 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 ± SEM.

## Results

### Pacemaker neurons are distinguished by low conductance through $Ba^{2+}$-sensitive, inward-rectifying $K^+$ ($K_{ir}$) 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 postnatal days (P)2–5. The pattern of spontaneous activity at room temperature was classified as 'bursting', 'tonic', 'irregular' (Fig. 1A) or 'silent' (not shown), as described earlier (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 to adjacent, non-bursting 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.

Inward-rectifying $K^+$ ($K_{ir}$) 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. Following the classification of spontaneous firing pattern, voltage clamp experiments were performed in which lamina I neurons were hyperpolarized from −55 mV to −155 mV (at a rate of 0.2 mV/ms) in the presence of a cocktail of antagonists to block fast synaptic transmission in the slice (see Methods). $BaCl_2$ (200 μM) 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 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.

The effects of extracellular $Ba^{2+}$ application on the current evoked by the negative voltage ramp clearly differed between pacemaker and non-pacemaker neurons within lamina I of the neonatal spinal cord. In non-pacemakers, $Ba^{2+}$ blocked a portion of the recorded current

throughout the voltage ramp, thus lowering the overall slope of the observed current (Fig. 2A, 2C). Meanwhile, in pacemaker neurons from the same slices, $Ba^{2+}$ evoked only a minimal block of the current between −55 mV and −85 mV (Fig. 2B, 2D), 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 ($0.039 \pm 0.009$ nS; $n = 10$) compared to non-bursting lamina I neurons ($0.156 \pm 0.036$ 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 $g_{Ba-sensitive}$ which 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 to adjacent, non-bursting neurons within lamina I of the neonatal spinal cord.

Importantly, $g_{Ba-sensitive}$ measured at positive membrane potentials (i.e. $E_{rev} + 25$) 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 if 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 pacemaker and non-pacemaker populations within lamina I, the currents evoked by the negative voltage ramp were highly sensitive to external $Ba^{2+}$ ($IC_{50}$ at $V_m = -140$ mV: Bursting: $8.62 \pm 1.7$ μM; $n = 6$; Non-bursting: $13.9 \pm 2.3$ μM; $n = 8$; $p = 0.109$; 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 G-protein-coupled inward rectifying $K^+$ (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 non-bursting ($n = 4$) cells was unaffected by the bath application of the selective 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 employed to detect the presence of $I_h$ in identified pacemaker neurons within lamina I during early life (see 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 which is characteristic of $I_h$ expression (Fig. 4D). Similarly, the vast majority of non-bursting neurons (59 of 63; $p = 1.0$ compared to bursting cells; Fishers' 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 $K_{ir}2.x$ channels.

The low $K_{ir}$ conductance which distinguishes spinal pacemakers (Fig. 2) could reflect the absence of particular $K_{ir}2.x$ isoforms within this population. To investigate this possibility, we characterized the pattern of $K_{ir}2.x$ 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 $K_{ir}2.x$ channels. $K_{ir}2.1$ immunoreactivity was observed in every lamina I neuron exhibiting spontaneous, rhythmic burst-firing (13 of 13 cells examined; Fig. 5, *top*). Similarly, $K_{ir}2.2$-positive puncta were found in 9 out of 9 pacemaker cells sampled (Fig. 5, *middle*). Finally, the vast majority of bursting neurons (10 of 12) also demonstrated immunoreactivity for $K_{ir}2.3$ (Fig. 5, *bottom*). In all cases, pre-absorption of the $K_{ir}2$ primary antibody with the relevant antigen, or omission of the primary antibody, failed to produce measurable signals within the SDH (data not shown). Collectively, the data suggest that $K_{ir}2.x$ 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 μM – 1 mM) to non-bursting 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 which 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 upon 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 demonstrated that directly depolarizing non-pacemaker neurons via the patch electrode only evoked bursting in ~12% of cells at P2–3 (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 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, in comparison to 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' inward-rectifying potassium ($K_{ir}$) 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 to adjacent, non-bursting 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 if $K_{ir}$ currents are developmentally up-regulated in lamina I neurons.

The available evidence strongly suggests that classical $K_{ir}$ ($K_{ir}2.x$) 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 $K_{ir}2.x$ family (Preisig-Muller 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 $K_{ir}2.1–2.3$ channels are found in immature SDH cells (Fig. 5). In addition, while GIRK ($K_{ir}3.x$) channels are also expressed within lamina I–II (Luscher et al., 1997; Marker et al., 2005; Marker et al., 2006) and 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 non-pacemaker populations are unlikely to explain the lower $K_{ir}$ conductance seen in spontaneously bursting neurons. Instead, the data collectively suggest that although rhythmic burst-firing is suppressed in the majority of immature lamina I neurons due 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 which 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 following 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). While $K_{ir}2.4$ 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 to other $K_{ir}2.x$ channels (Topert 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 to other $K_{ir}2.x$ channels (Pruss 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 to 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-Muller et al., 2002), it is feasible that bursting neurons express $K_{ir}$ channels with a distinct stoichiometry. $K_{ir}2.x$ isoforms which 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) which 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 to 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 non-hormonal 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 non-pacemakers at physiological potentials (Fig. 2) may also reflect the contribution of additional ion channels which 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 which is expressed in the dorsal horn from the first days of life (Kar and Quirion, 1995; Cheunsuang et al., 2002), as substance P is known to regulate pacemaker activity in other regions of the CNS (Pena 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; Zitron et al., 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 which exhibit plateau potentials (Reali 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 which 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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**A**

Bursting



15 mV
1 s

Tonic



15 mV
1 s

Irregular



15 mV
1 s

**B**



**C**
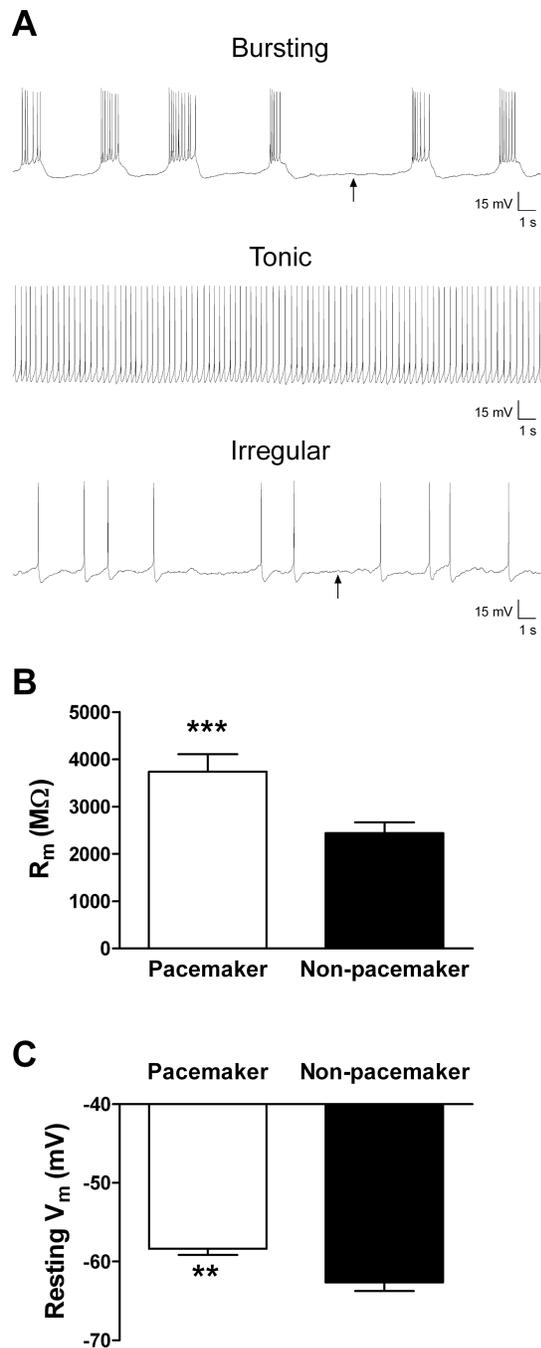


**Figure 1.**
Lamina I pacemaker neurons are distinguished by high membrane resistance and depolarized resting potentials. *A,* Spontaneous firing patterns were classified as rhythmic bursting (*top*), tonic (*middle*), irregular (*bottom*) or silent (*data not shown*). Arrows indicate approximate regions 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 to adjacent, non-bursting neurons (***p = 0.0001; Mann-Whitney test). *C,* Pacemaker neurons also exhibited a significantly more depolarized resting potential ($n = 15$) compared to non-pacemaker neurons ($n = 22$; **p = 0.007; Mann-

Whitney test). Tonically-firing neurons were excluded from the analysis due to difficulties in accurately measuring their resting potential.
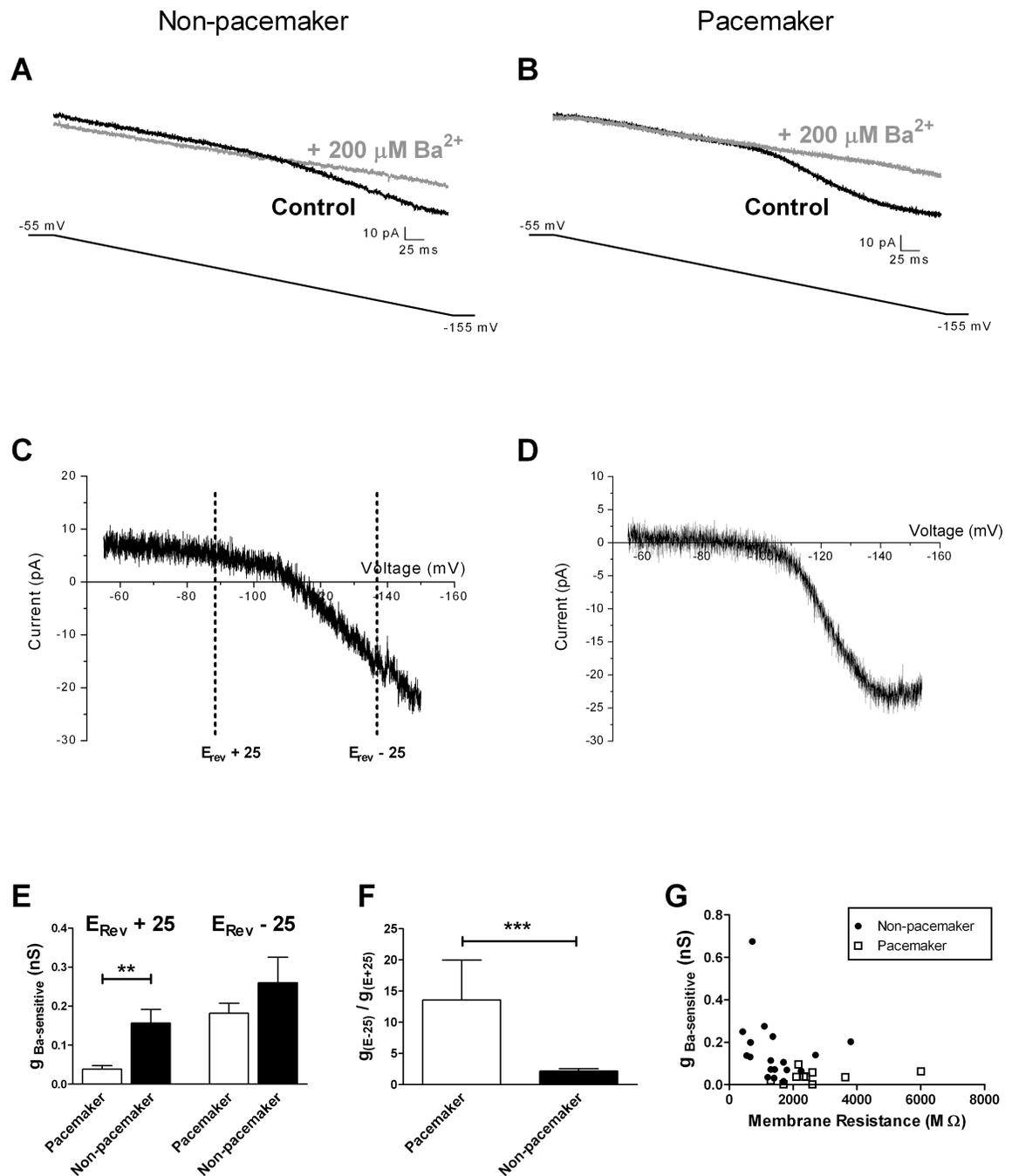
**Figure 2.**
Low $K_{ir}$ conductance in spontaneously bursting neurons within the newborn SDH. **A,** Representative currents recorded in non-pacemaker neurons during negative voltage ramps from a holding potential of −55 mV to −155 mV before (Control; *black*) and after (*gray*) the bath application of 200 μM $BaCl_2$. 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* – *gray*) plotted as a function of membrane voltage for non-

pacemaker (*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 ($E_{rev}$). Panel *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 to adjacent, non-bursting 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 $g_{(Erev\ -25)}/g_{(Erev\ +25)}$) 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.
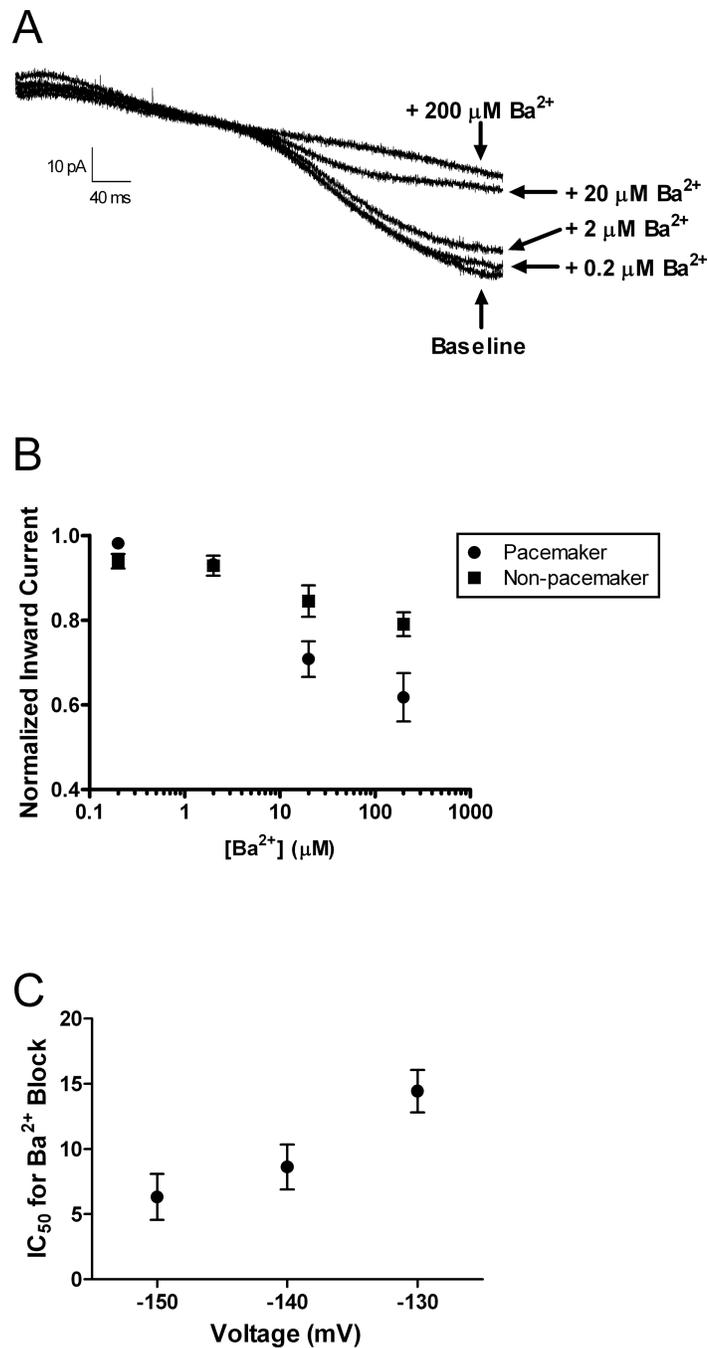
**Figure 3.**
High $Ba^{2+}$-sensitivity of $K_{ir}$ currents in neonatal lamina I neurons. **A,** Representative currents evoked during voltage ramps from $-55$ mV to $-155$ mV in an individual lamina I pacemaker neuron at baseline and in the presence of increasing concentrations of external $BaCl_2$ (0.2 – 200 μM). **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 non-pacemaker 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 non-bursting groups of lamina I cells.
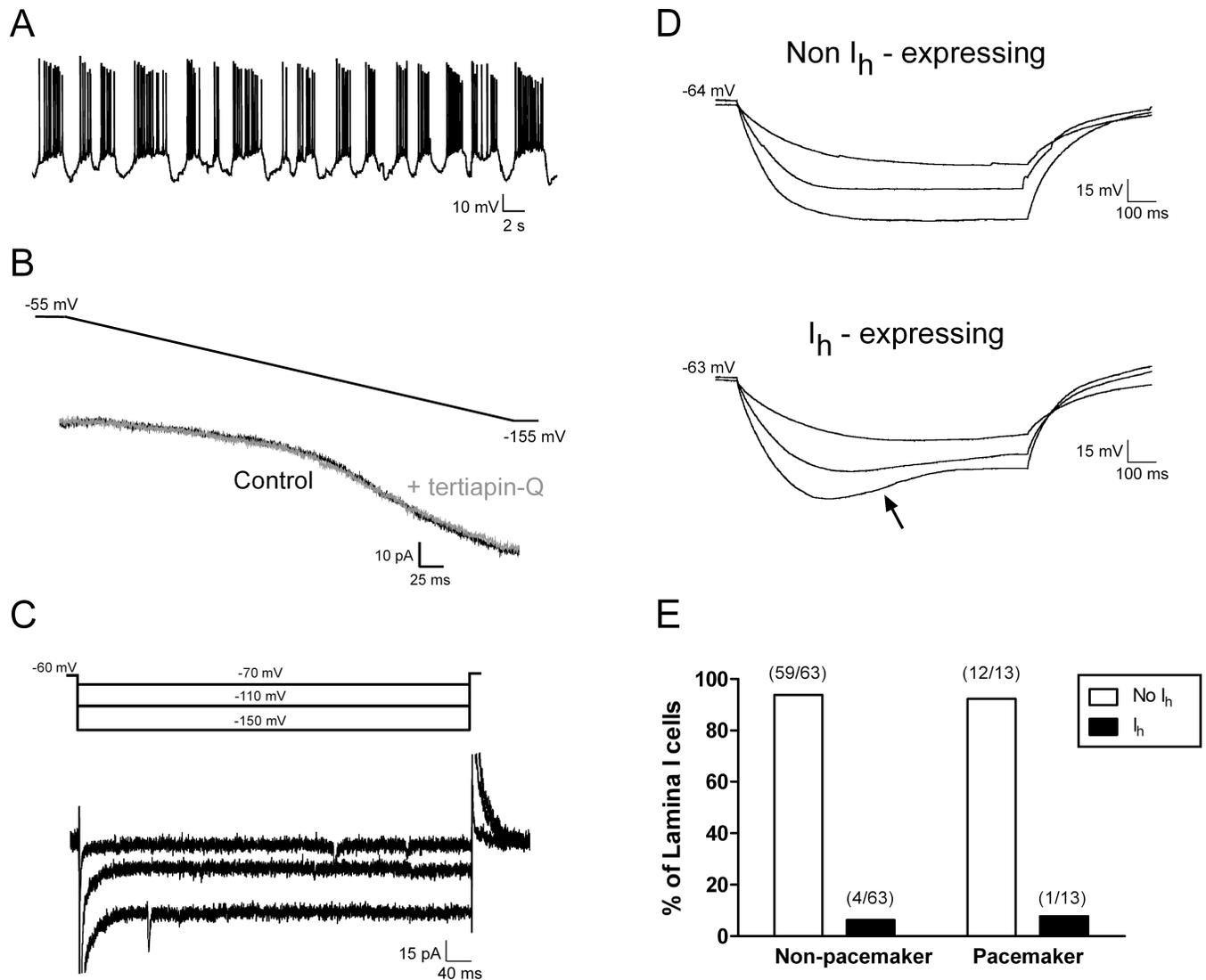
**Figure 4.**

G-protein-coupled $K_{ir}$ channels (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 tertiapin-Q (100 nM). *C,* The majority of pacemaker neurons exhibited no evidence of the time-dependent inward current at hyperpolarized $V_m$ which 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 (*see arrow*) witnessed in a minority of lamina I neurons at the same age (*bottom*). *E,* Group data demonstrating that the majority of pacemaker and non-pacemaker neurons in lamina I of the neonatal spinal cord lack hyperpolarization-activated cation current ($I_h$).
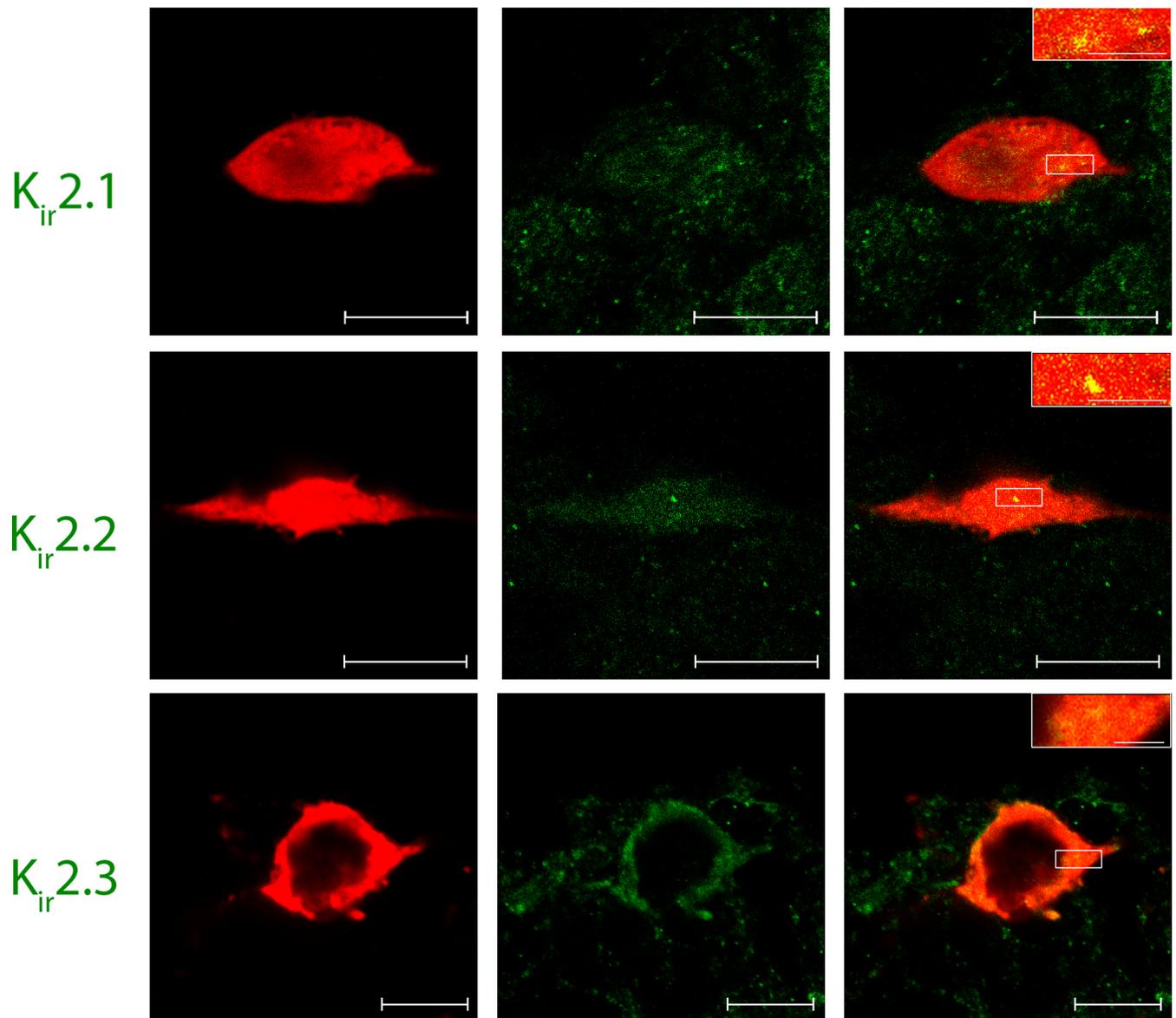
**Figure 5.**
Lamina I pacemaker neurons express classical $K_{ir}2.1–2.3$ channels. Confocal images (0.5 μm optical thickness) of representative biocytin-filled pacemaker neurons (*red*) processed for $K_{ir}$ immunohistochemistry (*green*) with $K_{ir}2.1$ (*top row*), $K_{ir}2.2$ (*middle*) or $K_{ir}2.3$ (*bottom*) antibodies. Merged images demonstrate that immunoreactive puncta for all three $K_{ir}2$ isoforms (*yellow; see inset*) were localized to identified pacemaker neurons within lamina I of the neonatal spinal cord (*right column*). Scale bars = 10 μm in main panels and 2 μm in inset.

**A**

Baseline

+ 10 μM BaCl$_2$

15 mV
2 s

15 mV
2 s

**B**

+ BaCl$_2$

10 mV
10 s

V$_m$

I

0 pA

-1 pA

**C**

Baseline

+ 1 mM BaCl$_2$

Rinse

15 mV
2 s

15 mV
4 s

15 mV
2 s
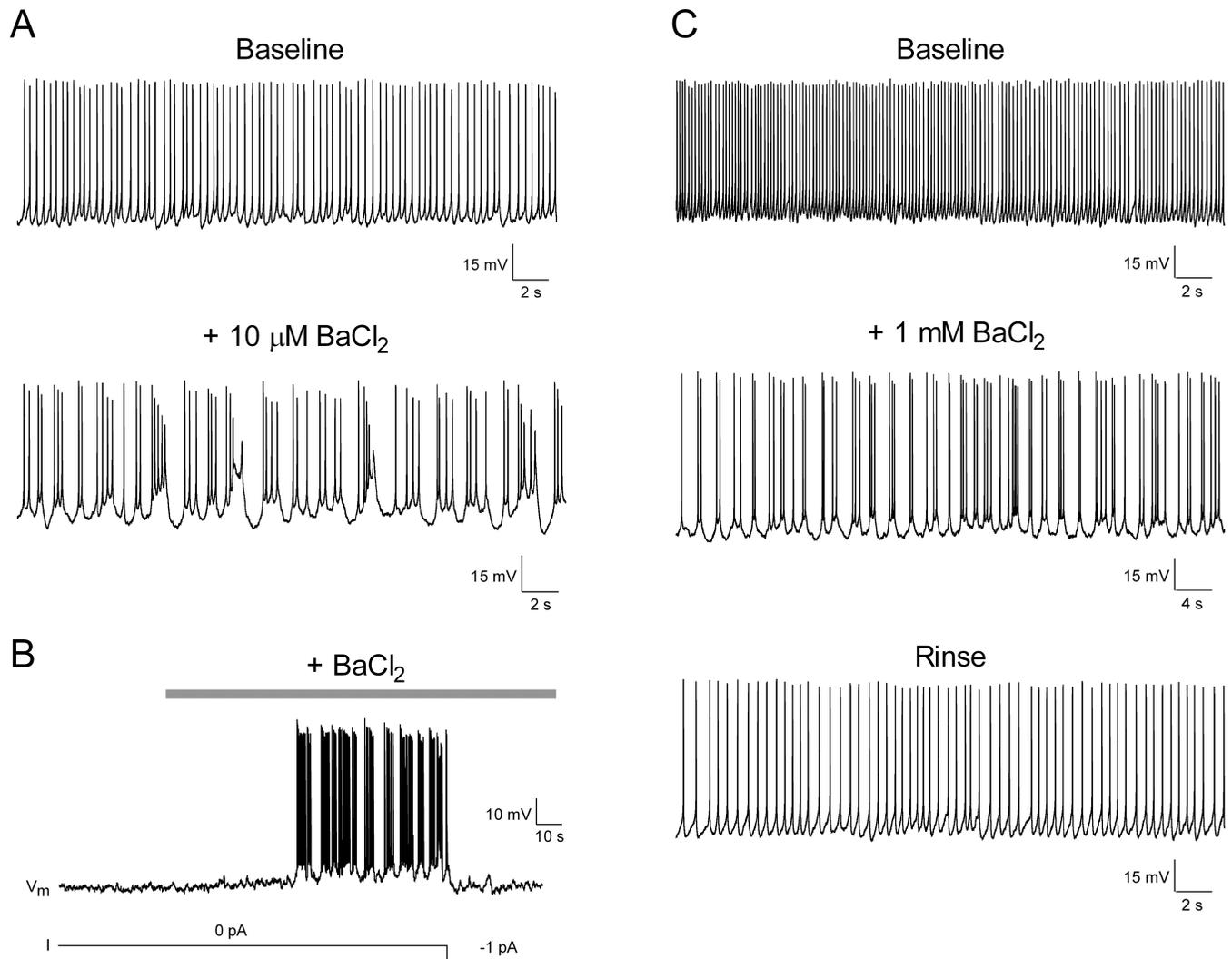
**Figure 6.**

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 μM) revealed the ability of this neuron to generate burst-firing. *B,* In a 'silent' lamina I cell, external Ba$^{2+}$ application (200 μM) 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 which switched to rhythmic burst-firing in the presence of external Ba$^{2+}$ and then reverted back to a tonic mode of action potential discharge upon washout.
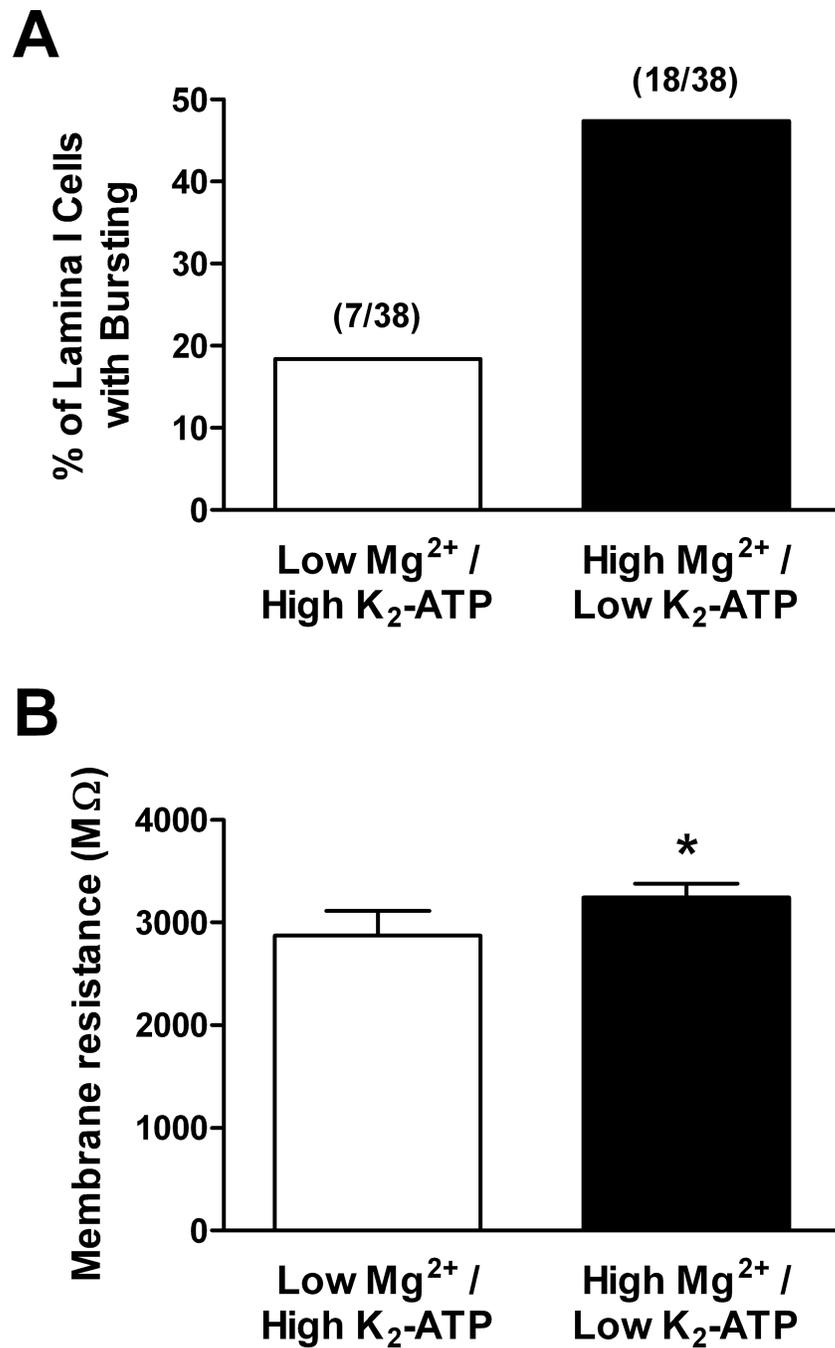
**Figure 7.**
Prevalence of rhythmic burst-firing is regulated by intracellular cation levels within neonatal lamina I neurons. *A,* Elevating the intracellular levels of free $Mg^{2+}$ (to ~1 mM) significantly increased the percentage of lamina I neurons demonstrating pacemaker activity during early 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 to the use of a patch solution containing low internal $Mg^{2+}$ within the same slices.