Developmental regulation of membrane excitability in rat spinal lamina I projection neurons

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ABSTRACT

It is now universally recognized that neonates can experience considerable pain. While spinal lamina I neurons projecting to the brain contribute to the generation of hyperalgesia, nothing is known about their electrophysiological properties during early life. Here we have used in vitro whole-cell patch clamp recordings in rat spinal cord slices to determine whether the intrinsic membrane properties of lamina I projection neurons, as well as their synaptic inputs, are developmentally regulated during the early postnatal period. Projection neurons were identified via retrograde transport of Dil injected into the parabrachial nucleus (PB) or periaqueductal gray (PAG) and characterized at postnatal days (P) 2-5, P10-12, P19-23 and P30-32. Both spino-PB and spino-PAG neurons demonstrated an age-dependent reduction in spike threshold and duration at room temperature, which was accompanied by a developmental increase in the frequency of miniature excitatory and inhibitory postsynaptic currents. Notably, in both groups, age-dependent changes in the passive membrane properties or rheobase only occurred after the third postnatal week. However, spontaneous activity was significantly more prevalent within the developing spino-PB population and was dominated by an irregular pattern of discharge. In addition, while the instantaneous firing frequency remained unaltered in spino-PB neurons during the first weeks of life, spino-PAG cells fired at a higher rate at P19-23 compared to younger groups, suggesting that the gain of parallel ascending nociceptive pathways may be independently regulated during development. Overall, these results demonstrate that intrinsic membrane excitability is modulated in a cell-type specific manner within developing spinal nociceptive circuits.
INTRODUCTION

Growing awareness that even the youngest infants can experience pain has driven increased efforts to understand the maturation of nociceptive pathways at a cellular and molecular level. As a result, it is now clear that pain networks in the CNS undergo considerable reorganization during the early postnatal period. In the superficial dorsal horn (SDH) of the rodent spinal cord, this includes age-dependent alterations in the pattern and strength of primary afferent input (Beggs et al. 2002; Baccei et al. 2003) as well as significant changes in the properties of local synaptic inhibition (Keller et al. 2001; Baccei and Fitzgerald 2004; Cordero-Erausquin et al. 2005; Bremner and Fitzgerald 2008). In addition, the morphology (Bicknell, Jr. and Beal 1984) and intrinsic membrane properties (Walsh et al. 2009) of SDH neurons are known to be developmentally regulated. However, nociceptive processing within the immature SDH cannot be fully understood without specific knowledge of how the excitability of ascending projection neurons, which are responsible for the output of the spinal pain circuit, is modulated during early life.

Unfortunately, little is known about the electrophysiological properties of these projection neurons during the neonatal period, as numerous lines of evidence suggest that the information gained from prior studies of the general population of lamina I-II neurons cannot be extrapolated to immature projection neurons. First, since the vast majority of SDH neurons consist of propriospinal or local circuit interneurons (Bice and Beal 1997a, 1997b) and ascending projection neurons comprise only ~5% of all lamina I cells (Spike et al. 2003), projection neurons undoubtedly represent an extremely small fraction of the sampled neurons when recording from unidentified cells in the developing SDH. In addition, examination of identified lamina I projection neurons in the young adult rat has demonstrated that these cells possess distinct passive and active membrane properties compared to adjacent, unidentified lamina I neurons, including unique intrinsic firing patterns (Ruscheweyh et al. 2004), greater levels of
spontaneous synaptic input (Dahlhaus et al. 2005) and an enhanced propensity for activity-dependent synaptic plasticity (Ikeda et al. 2003).

Lamina I projection neurons target multiple supraspinal sites including the parabrachial nucleus (PB), periaqueductal gray (PAG), caudal ventrolateral medulla (CVLM) and thalamus, with the majority of neurons that project to the PAG, CVLM and thalamus also projecting to the PB (Hylden et al. 1989; Spike et al. 2003). Despite this anatomical overlap, recent studies have demonstrated clear differences in the spino-PB and spino-PAG populations based on their firing patterns (Ruscheweyh et al. 2004), synaptic inputs (Dahlhaus et al. 2005) and characteristics of long-term potentiation (Ikeda et al. 2006). Our previous work suggests that functional differences between these groups may also exist during the neonatal period, as spino-PB neurons exhibited a significantly higher prevalence of spontaneous firing compared to the spino-PAG group (Li and Baccei 2011). Nonetheless, the extent to which the intrinsic membrane properties of these different populations change during early postnatal development remains unknown. These properties, along with the level of excitatory and inhibitory synaptic input to these cells, will clearly impact signal integration within lamina I projection neurons.

Given that an estimated 80-85% of all lamina I projection neurons target the PB (Hylden et al. 1989; Spike et al. 2003) and that spino-PB and spino-PAG cells are known to exhibit important functional differences later in life (Ruscheweyh et al. 2004; Ikeda et al. 2006), the goal of the present study was to characterize the intrinsic excitability of these two populations during early postnatal development. The results demonstrate that although lamina I neurons projecting to the PB and PAG exhibit similar developmental changes in many of their active membrane properties, spino-PB neurons exhibit higher levels of spontaneous activity throughout the postnatal period and only spino-PAG neurons undergo an age-dependent increase in firing frequency. Interestingly, the passive membrane properties of both groups remained stable over the first three weeks of life, while a significant increase in both excitatory and inhibitory synaptic input occurred over this period. Collectively, these changes are expected to shape the
transmission of nociceptive signals within ascending pain pathways during postnatal
development.

MATERIALS AND METHODS

Ethical approval

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

DiI injections

Sprague-Dawley rats were anesthetized via i.p. injection of a mixture of ketamine (90
mg/kg) and xylazine (10 mg/kg) on postnatal days (P)0-1 and placed in a plaster body mold
which was secured in a stereotaxic apparatus (World Precision Instruments, Sarasota, FL) as
described previously (Hoorneman 1985). The scalp was incised and a small hole was made in
the skull using a 30 gauge needle. The pup received a single injection (50-100 nl) of FAST DiI
oil (2.5 mg/ml; Invitrogen, Carlsbad, CA) into either the parabrachial nucleus (PB) or the
periaqueductal gray (PAG) using a Hamilton micro-syringe (62RN) equipped with a 28 gauge
needle. Stereotaxic coordinates were based on an atlas of the E22 (i.e. P0) rat brain (Altman
and Bayer 1995) as follows: (in mm; relative to lambda): PB: 2.7 caudal, 1.0 lateral and 3.3
ventral; PAG: 1.9 caudal, 0.60 lateral and 2.9 ventral. The skin was closed with Vetbond and
the pups returned to the home cage until the beginning of the electrophysiological experiments
or the age of weaning. Following euthanasia, the brain was harvested, immersed in 4%
paraformaldehyde, and 30 μm coronal sections were cut on a cryostat and examined using a
light microscope in order to verify the accuracy of the injection site. Preparation of spinal cord slices

Rats (at P2-5, P10-12, P19-23 or P30-32) were deeply anesthetized with sodium
pentobarbital (30 mg/kg; i.p.), perfused with ice-cold dissection solution consisting of (in mM):
250 sucrose, 2.5 KCl, 25 NaHCO$_3$, 1.0 NaH$_2$PO$_4$, 6 MgCl$_2$, 0.5 CaCl$_2$, and 25 glucose
continuously bubbled with 95% O$_2$ / 5% CO$_2$, and decapitated. The lumbar spinal cord was
isolated and immersed in low-melting-point agarose (3% in above solution; Invitrogen) and
parasagittal slices (350–400 µm) were cut from the contralateral side 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
aCSF solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.0 NaH$_2$PO$_4$,
1.0 MgCl$_2$, 2.0 CaCl$_2$, and 25 glucose 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) which was equipped with fluorescence to allow for the
identification of Dil-labeled neurons. Slices were then perfused at room temperature with
oxygenated aCSF at a rate of 1.5-6 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, Novato, CA). Pipette resistances ranged from 4 to 6 MΩ and seal
resistances were >1 GΩ. For current-clamp 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 Na$_2$GTP, pH 7.2 (305 mOsm). Voltage-clamp recordings
used an intracellular solution containing the following (in mM): 130 Cs-gluconate, 10 CsCl, 10
HEPES, 11 EGTA, 1.0 CaCl$_2$, and 2.0 MgATP, pH 7.2 (300-305 mOsm).

Projection neurons were located under epi-fluorescence (Fig. 1), visualized with infrared
differential interference contrast optics, and patch clamp recordings were obtained using a
Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). 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 ($V_{\text{rest}}$). Membrane capacitance was calculated using the built-in pClamp membrane test (applied at 33.3 Hz), while membrane resistance was measured using the hyperpolarization produced by a -20 pA current injection from $V_{\text{rest}}$. To characterize the properties of evoked action potential (AP) discharge, intracellular current injections (from -10 to +70 pA in 5 pA increments; 800 ms duration) were applied from $V_{\text{rest}}$. AP amplitude was measured as the difference between AP threshold and the peak amplitude, while the spike duration at 50% of the peak amplitude was used to calculate AP half-width. Instantaneous firing frequency (IF) was calculated as $1 / \text{interspike interval (ISI)}$, while the degree of spike frequency adaptation was measured as the following ratio: $(\text{IF using last ISI}) / (\text{IF using first ISI})$. Rheobase was defined as the minimum current step (delivered in 2.5 pA increments at 50 ms duration) which evoked AP discharge.

Voltage-clamp experiments included the bath application of 500 nM TTX, in order to record miniature excitatory postsynaptic currents (mEPSCs) from a holding potential of -70 mV and miniature inhibitory postsynaptic currents (mIPSCs) from a holding potential of 0 mV in the same lamina I projection neurons.

Membrane voltages were adjusted for liquid junction potentials 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 10.0 software; Molecular Devices).

**Data analysis and statistics**

Miniature postsynaptic currents (mPSCs) were analyzed via visual inspection using MiniAnalysis (version 6.0.3; Synaptosoft, Decatur, GA) while AP properties were analyzed using
Clampfit (Molecular Devices) software. The threshold for mPSC detection was set at twice the mean amplitude of the background noise. Nonparametric statistical tests (Mann–Whitney test for two groups; Kruskal-Wallis test for >2 groups; Prism 5.0 software; GraphPad Software, La Jolla, CA) were used in cases in which the distribution of data failed the D'Agostino & Pearson normality test or when the number of observations was insufficient ($n < 24$) to definitively conclude that data were distributed in a Gaussian manner. Where parametric tests were appropriate, one-way ANOVAs (with Tukey post-tests) or two-way ANOVAs (with Bonferroni post-tests) were used unless otherwise stated. The $\chi^2$ test was used to determine if the fraction of neurons exhibiting spontaneous activity or afterdepolarizations changed significantly with age. $n$ refers to the number of neurons sampled in a given group. Data are expressed as means ± SEM.

RESULTS

Passive membrane properties of lamina I projection neurons remain stable during early postnatal development

Lamina I projection neurons were identified via the retrograde transport of Dil injected into either the parabrachial nucleus (PB) or periaqueductal gray (PAG) at birth (Fig. 1). Spinal cord slices were subsequently prepared at P2-5, P10-12, P19-23 or P30-32 and whole-cell patch clamp recordings were obtained from spino-PB ($n = 116$) or spino-PAG ($n = 97$) neurons. As illustrated in Table 1, we failed to observe significant changes in the resting membrane potential ($V_{\text{rest}}$), membrane capacitance ($C_m$) or membrane resistance ($R_m$) in either the spino-PB or spino-PAG populations during the first three postnatal weeks ($p>0.05$; one-way ANOVA). However, $V_{\text{rest}}$ did become significantly more negative in both groups between P19-23 and P30-32 (Table 1).
Spontaneous and evoked firing in developing projection neurons

We have previously classified the spontaneous firing patterns of newborn lamina I neurons as “silent”, “irregular”, “tonic” or “bursting” and demonstrated that neither spino-PB nor spino-PAG projection neurons exhibit rhythmic burst-firing during the first days of life (Li and Baccei 2011). To determine if these patterns of spontaneous activity (SA) are developmentally regulated in ascending projection neurons, we recorded spontaneous action potential (AP) discharge in these cells from V_{rest} (Fig. 2A) in the four postnatal age groups. Within the spino-PB population, the majority of neurons fired APs in an irregular manner regardless of age, with the remainder of neurons generally failing to show SA (Fig. 2B). Interestingly, the percentage of spino-PB neurons which exhibited some form of SA was not significantly different between age groups (p = 0.325; $\chi^2$ test). Although spino-PAG neurons were more likely to be silent at V_{rest} (Fig. 2C; p = 0.006 compared to spino-PB cells; Fisher’s exact test), the overall prevalence of SA in this population was similarly independent of postnatal age (p = 0.514; $\chi^2$ test). In addition, while we again failed to observe spontaneous burst-firing in newborn lamina I projection neurons, a small number of spino-PB (n = 2) and spino-PAG (n = 4) cells generated rhythmic bursting at later ages (Fig. 2B, 2C).

To examine the patterns of evoked AP discharge in developing projection neurons, intracellular current injections (800 ms) of increasing intensity were applied from V_{rest} via the patch electrode. Previous examinations of rat lamina I neurons (Prescott and De Koninck 2002; Ruscheweyh et al. 2004) have identified up to seven patterns of evoked firing (tonic, delayed, initial bursting, single spike, phasic, gap firing and bursting firing). However, the present study (Fig. 3A-D) classified evoked discharge into four categories (tonic, phasic, delayed and bursting) since we failed to observe single spike, initial bursting or gap firing patterns in the sampled populations. As illustrated in Fig. 3E-F, tonic firing predominated throughout the first three
postnatal weeks in spino-PB and spino-PAG neurons, although in both groups the prevalence of tonic discharge appeared to decrease with age and a more even distribution of firing patterns was observed by P30-32. Phasic and delayed firing were notably absent in the newborn spino-PB group and rarely seen in spino-PAG cells at the same time point. In addition, despite the lack of spontaneous burst-firing in neonatal lamina I projection neurons, a small percentage of both the spino-PB and spino-PAG populations exhibited bursting in response to intracellular current injection at P2-5. This fraction did not change significantly with postnatal development in either the spino-PB (p = 0.606; $\chi^2$ test) or spino-PAG (p = 0.103) group.

Lamina I interneurons which exhibit spontaneous bursting are characterized by a significantly lower $C_m$ and higher $R_m$ compared to adjacent, non-bursting neurons (Li and Baccei 2011). To determine if spinal projection neurons showing evoked burst-firing (Fig. 3D) are similarly distinguished by their passive membrane properties, we compared $C_m$, $R_m$ and $V_{rest}$ between bursting and non-bursting neurons with projections to the PB or PAG (pooled across ages). Surprisingly, spino-PAG neurons which demonstrated bursting in response to current injection ($n = 24$) had a significantly higher $C_m$ compared to spino-PAG cells which lacked burst-firing ($n = 73$; p = 0.0002; Mann-Whitney test; Fig. 4A, right), as well as a lower $R_m$ ($p<0.0001$; Mann-Whitney; Fig. 4B, right) and a more hyperpolarized $V_{rest}$ ($p = 0.0009$; unpaired t-test; Fig. 4C, right). A decreased $R_m$ in bursting ($n = 12$) relative to non-bursting ($n = 101$) cells was also observed within the spino-PB population ($p = 0.0008$; Fig. 4B, left). In addition, rheobase levels were significantly higher in bursting neurons compared to cells exhibiting other patterns of evoked discharge in both the spino-PB (Bursting: $48.8 \pm 5.2$ pA; Non-bursting: $29.7 \pm 2.4$ pA; $p = 0.0015$; Mann-Whitney test) and spino-PAG (Bursting: $47.8 \pm 4.9$ pA; Non-bursting: $32.3 \pm 2.7$ pA; $p = 0.006$; data not shown) groups.

We next examined the relationship between spontaneous AP discharge and evoked firing patterns within individual spino-PB and spino-PAG lamina I neurons. In the spino-PB
group, a significantly higher fraction of cells exhibiting irregular spontaneous activity (SA) fired
tonically in response to direct current injection (63/75; 84%) compared to neurons which were
silent at rest (18/38; 47%; \( p = 0.001 \); Fisher’s exact test; Table 2). A similar relationship was
observed in the spino-PAG population, as 77% (33/43) of irregular neurons and 46% (23/50) of
silent cells (\( p = 0.003 \)) demonstrated tonic firing in response to intracellular current injection.
Strikingly, the vast majority of cells which were capable of burst-firing during current injection
lacked SA in both the spino-PB (10/12) and spino-PAG (18/24) populations within lamina I.

Parallel developmental changes in action potential properties in spino-PB and spino-PAG
lamina I neurons

To investigate whether the different populations of lamina I projection neurons exhibit
distinct age-dependent changes in their active membrane properties, we first measured the
rheobase in spino-PB and spino-PAG neurons at various times during postnatal development.
Rheobase levels did not significantly change over the first three postnatal weeks in either the
spino-PB (\( n = 27-32 \) in each age group; \( p > 0.05 \); Kruskal-Wallis test with Dunn’s Multiple
Comparison test; Fig. 5A) or spino-PAG population (\( n = 17-28 \); Fig. 5B), although significantly
higher rheobase levels were observed in the P30-32 group (\( p < 0.05 \)). Meanwhile, action
potential (AP) threshold significantly decreased between P2-5 and P19-23 in spino-PAG
neurons (\( p < 0.01 \); one-way ANOVA with Tukey’s Multiple Comparison test; Fig. 5D) and was
also lower in spino-PB cells at P30-32 compared to younger ages (\( p < 0.05 \); Kruskal-Wallis test
with Dunn’s Multiple Comparison test; Fig. 5C). The spike duration (as measured by AP half-
width) significantly decreased during postnatal development in both populations of projection
neurons (Fig. 5E and 5F), while AP amplitude depended on age in spino-PB neurons (P2-5:
\( 75.3 \pm 2.0 \text{ mV}; \) P10-12: \( 73.3 \pm 1.8 \text{ mV}; \) P19-23: \( 79.4 \pm 1.7 \text{ mV}; \) P30-32: \( 82.2 \pm 1.7 \text{ mV}; \) \( p < 0.05 \)
for P2-5 vs. P30-32; \( p < 0.01 \) for P10-12 vs. P30-32; one-way ANOVA) but not in the spino-PAG
group (P2-5: 70.6 ± 1.7 mV; P10-12: 76.8 ± 1.6 mV; P19-23: 75.2 ± 1.8 mV; P30-32: 77.9 ± 2.4 mV; p>0.05; data not shown). Finally, in both the spino-PB and spino-PAG populations, afterdepolarizations following an AP (see Fig. 3D, inset) were rarely observed in the first days of life and were significantly up-regulated during postnatal development (p<0.0001; $\chi^2$ test, Fig. 5G-H).

**Target-specific changes in firing frequency in developing lamina I projection neurons**

To examine whether the ability of lamina I projection neurons to fire repetitively was significantly altered during early postnatal development, we measured the mean instantaneous firing frequency (see Methods) in response to current injections of increasing intensity (-10 to +70 pA) in tonically-firing spino-PB and spino-PAG neurons at different ages. We failed to observe significant age-related changes in the stimulus-response relationship within the spino-PB group (p>0.05; two-way ANOVA with Bonferroni post-test; Fig. 6A). However, spino-PAG neurons increased their rate of AP discharge after the second postnatal week (Fig. 6B), as the mean instantaneous firing frequency of the P19-23 group was significantly higher than at the younger ages (p<0.05; two-way ANOVA with Bonferroni post-test). Despite this developmental increase in average firing frequency, the degree of spike frequency adaptation (SFA) in spino-PAG neurons did not change significantly with age (p>0.05; two-way ANOVA; Fig. 6D). The population of spino-PB neurons also exhibited a similar degree of SFA throughout development (Fig. 6C).

**Strengthening of excitatory and inhibitory synaptic input onto ascending projection neurons during early postnatal development**
We have previously reported a developmental increase in both spontaneous excitatory and inhibitory transmission onto lamina II neurons in the rat spinal cord (Baccei and Fitzgerald 2004; Li et al. 2009). To determine if the efficacy of synaptic inputs to lamina I projection neurons undergoes a similar modulation during the first three weeks of life, we recorded mEPSCs (Fig. 7A) and mIPSCs (Fig. 7B) in spino-PB and spino-PAG cells at P2-5 and P19-23.

As illustrated in Fig. 7C, spino-PB neurons showed a clear age-dependent increase in the frequency of mEPSCs ($n = 18-22$ at each age; $p = 0.0003$; Mann-Whitney test; left), while mEPSC amplitude was not significantly altered ($p = 0.059$; right). Likewise, mIPSC frequency markedly increased with age in the same neurons ($p<0.0001$; Mann-Whitney; Fig. 7E, left) without statistically significant changes in mIPSC amplitude ($p = 0.066$; Fig. 7E, right). A similar developmental trend was observed in the spino-PAG group, as the frequency of both mEPSCs ($p = 0.0013$; Mann-Whitney; Fig. 7D, left) and mIPSCs ($p = 0.0002$; Fig. 7F, left) were significantly elevated by the end of the third postnatal week with no accompanying changes in the mean amplitude of these currents ($n = 20-26$ at each age; Figs. 7D and 7F, right).

**DISCUSSION**

The present study characterizes, for the first time, the electrophysiological properties of identified lamina I projection neurons during early postnatal development. When considered alongside prior investigations of the developing superficial dorsal horn (SDH), our results suggest that the maturation of intrinsic membrane properties within ascending projection neurons may occur with a distinct time course compared to the surrounding population of interneurons within the SDH. For example, neither the spino-PB nor spino-PAG groups showed age-dependent shifts in resting membrane potential ($V_{\text{rest}}$; Table 1), membrane resistance ($R_m$), membrane capacitance ($C_m$) or rheobase (Fig. 5) during the first three weeks of life. Interestingly, significant changes in resting potential and rheobase were observed after P23.
(Fig. 5 and Table 1), suggesting that a subset of electrophysiological properties may be slow to mature within the population of lamina I projection neurons. In contrast, previous studies have documented an age-dependent hyperpolarization of $V_{\text{rest}}$, decrease in $R_m$, and elevation in rheobase across the general population of lamina I (Li and Baccei 2011) and lamina II (Walsh et al. 2009) neurons in the rodent spinal cord during the first three postnatal weeks. In addition, while the prevalence of spontaneous activity (SA) in unidentified lamina I neurons (the vast majority of which correspond to interneurons) clearly decreases during early life (Li and Baccei 2011), the level of SA in both the spino-PB and spino-PAG populations remained unchanged during the course of development. Finally, while the firing frequency of unidentified SDH neurons does not appear to depend on age (Baccei and Fitzgerald 2005), lamina I neurons projecting to the PAG (but not PB) discharged action potentials at a significantly higher rate after the second postnatal week (Fig. 6A,B). The selective effect of age on the firing rate of spino-PAG cells suggests that the gain of ascending nociceptive pathways may be differentially regulated during the postnatal period. Collectively, the available evidence strongly suggests that neuronal excitability may be modulated in a cell-type specific manner within the developing SDH.

Given their dependence on the surface area of the membrane (Hille 1992), the passive membrane properties (such as $R_m$ and $C_m$) of a neuron will be significantly influenced by their morphological properties including the relative size and complexity of the dendritic compartment (Rall and Rinzel 1973). As a result, it is notable that the anatomical maturation of SDH neurons occurs in two distinct phases. Supraspinal projection neurons are generated before local circuit interneurons in the embryonic dorsal horn (Nandi et al. 1993), regardless of their target in the brain (Bicknell, Jr. and Beal 1984; Bice and Beal 1997a) or whether they project ipsilaterally or contralaterally (Nandi et al. 1991). Importantly, their axonal and dendritic development predominantly occurs prior to birth (Bicknell, Jr. and Beal 1984). Meanwhile, presumptive
interneurons within the SDH undergo a dramatic growth and reorganization of their dendritic
trees during the early postnatal period (Bicknell, Jr. and Beal 1984). This may explain the
observations that the passive membrane properties of ascending projection neurons remain
stable during the first three postnatal weeks (Table 1) while those of unidentified SDH neurons
(i.e. interneurons) exhibit significant developmental changes (Walsh et al. 2009; Li and Baccei
2011). However, the potential mechanisms which contribute to the delayed changes in $V_{\text{rest}}$ and
rheobase within projection neurons remain unclear.

Despite the fact that the morphological development of lamina I projection neurons
occurs before birth, our results clearly demonstrate that the maturation of excitatory and
inhibitory synaptic inputs onto these cells continues throughout the first three postnatal weeks
(Fig. 7). The selective elevation in the frequency of the miniature postsynaptic currents predicts
a developmental increase in the number of synapses onto ascending projection neurons and/or
an enhanced probability of transmitter release at these synapses. However, we cannot
completely exclude the possibility that the age-related facilitation in mEPSC frequency reflects a
conversion from 'silent' (i.e. pure NMDAR-only) to functional glutamatergic synapses via the
insertion of AMPARs into the postsynaptic membrane (Li and Zhuo 1998; Bardoni et al. 1998;
Torsney 2011). It is also presently unclear if the changes in glutamatergic input onto projection
neurons reflect an increased efficacy of synapses formed by primary afferents (Todd 2002),
local excitatory interneurons (Cordero-Erausquin et al. 2009) or other lamina I projection
neurons (Szucs et al. 2010; Luz et al. 2010).

As a result, it will be important to characterize the nature of primary afferent synaptic
input to spino-PB and spino-PAG neurons at various stages of early postnatal development.
Previous studies have demonstrated that low-threshold Aβ fiber input to the SDH is more
pronounced during the neonatal period (Jennings and Fitzgerald 1996; Beggs et al. 2002;
Daniele and MacDermott 2009) and becomes subject to tight inhibitory control at later ages
(Torsney and MacDermott 2006). However, since primary afferents may target subtypes of SDH neurons in a highly selective manner (Lu and Perl 2003, 2005; Zheng et al. 2010), it remains to be determined if newborn spinal projection neurons do in fact receive enhanced low-threshold sensory input. It should also be noted that low-threshold input to adult lamina I projection neurons can also arise from C-fiber mechanoreceptors (Andrew 2010).

Overall, our results also suggest that spino-PB and spino-PAG neurons exhibit many similar developmental changes in their active membrane properties. Both groups demonstrate an age-dependent reduction in action potential threshold, which would be predicted to facilitate synaptic potential-spike coupling (Andersen et al. 1980; Sharifullina et al. 2004) and thus enhance the effectiveness of nociceptive inputs to these neurons. Spike duration also decreased with age in both groups (Fig. 4), which may allow developing projection neurons to respond with high fidelity to a progressively higher frequency of presynaptic input. In addition, neither population exhibited significant developmental changes in the degree of spike frequency adaptation (Fig. 6) or the distribution of spontaneous firing patterns, as irregular neurons represented the majority of spontaneously active cells throughout the postnatal period (Fig. 2B-C). Interestingly, spontaneous burst-firing was observed in a small percentage of spino-PB and spino-PAG neurons at later ages. Future experiments are required to determine if this rhythmic bursting represents the emergence of intrinsic “pacemaker” activity, as has been documented in lamina I interneurons during the neonatal period (Li and Baccei 2011), or is instead driven by synaptic transmission within the SDH network. A larger number of projection neurons appear capable of bursting following intracellular current injection (Fig. 3D-F), but the vast majority of these neurons are silent at their resting membrane potential (Table 2). Notably, bursting spino-PAG neurons exhibit significantly lower $R_m$, larger $C_m$ and a more hyperpolarized $V_{rest}$ compared to other spino-PAG cells which fail to show evoked burst-firing (Fig. 4). Since pacemaker neurons within lamina I appear distinguished by their high $R_m$ (Li and Baccei 2011), the passive
membrane properties of developing projection neurons may suppress spontaneous burst-firing by making it more difficult to achieve sufficient membrane depolarization to activate the voltage-gated conductances (such as persistent Na\(^+\) and high-threshold Ca\(^{2+}\) currents) which drive burst generation. Clearly, it will also be important to characterize in detail the expression of the membrane currents which underlie the observed firing patterns within developing lamina I projection neurons.

At first glance, the patterns of evoked AP discharge reported here (tonic, phasic, delayed, and bursting) differ from a previous study of rat spino-PB and spino-PAG lamina I neurons at ~3-4 weeks of age (Ruscheweyh et al. 2004). For example, we failed to observe gap firing (characterized by a long first interspike interval followed by tonic discharge) in projection neurons at any stage of postnatal development (Fig. 3), while Ruscheweyh et al. (2004) found that ~75% of spino-PAG and ~45% of spino-PB neurons exhibited this pattern at room temperature. Instead, we observed a high incidence of tonic firing in both the spino-PB and spino-PAG populations throughout development. This apparent discrepancy is likely explained by differences in experimental approach between the two studies. Specifically, we classified evoked firing patterns from \(V_{\text{rest}}\), which averaged approximately -70 mV after correction for liquid junction potentials (see Methods). Meanwhile, Ruscheweyh et al. (2004) employed multiple holding potentials, including voltages that were significantly more negative (< -80 mV without correcting for liquid junction potentials) than the ones used in the present study. This is noteworthy because gap firing is only observed at holding potentials more negative than -75 mV, due to the need to remove the steady-state inactivation of a slow, A-type K\(^+\) current (Ruscheweyh et al. 2004). Indeed, the authors reported that the use of a more depolarized holding potential could convert neurons from gap firing to tonic firing. Similar reasons could explain why we alone observed delayed firing in projection neurons, as this pattern only requires holding potentials below -60 mV and reflects the voltage-dependent properties of a fast A-type
K⁺ current (Ruscheweyh and Sandkühler 2002; Ruscheweyh et al. 2004). Meanwhile, our results (Fig. 3) are in general agreement with previous findings that lamina I projection neurons do not exhibit the initial burst pattern of discharge and are rarely single-spiking (Ruscheweyh et al. 2004).

Different firing patterns may be observed within projection neurons using an elevated temperature, which has been reported to decrease input resistance and reduce the excitability of subsets of SDH neurons (Graham et al. 2008). Thus it is possible that the use of higher recording temperatures may reveal a decreased prevalence of spontaneous activity (Fig. 2) and the appearance of single-spiking (or “reluctant firing”) within projection neurons, although it should be noted that the prevalence of tonic firing cells was unaltered by changes in temperature (Graham et al. 2008). Importantly, these patterns of AP discharge may correlate with distinct types of signal processing within lamina I neurons, as tonic and delayed neurons have been proposed to act as integrators of sensory information while phasic and single-spiking neurons are well-suited to function as coincidence detectors (Prescott and De Koninck 2002).

Therefore, the predominance of tonic firing in lamina I projection neurons throughout early life (Fig. 3) may reflect their responsibility for integrating signals from a complex interneuronal network in order to govern the output of the spinal nociceptive circuit.

In conclusion, the present study provides the first electrophysiological characterization of spinal lamina I projection neurons during early life, which has important functional implications for nociceptive processing in the neonate. For example, the fact that these neurons possess relatively stable intrinsic membrane properties during the neonatal period may facilitate the faithful transmission of noxious stimuli to the immature brain, as nociceptive-specific cortical potentials have been observed as early as 25 gestational weeks in humans (Slater et al. 2006), thus leading to an appropriate behavioral response which alerts a caregiver that a potential injury has occurred. Meanwhile, the persistence of spontaneous activity within lamina I
projection neurons throughout the first weeks of life could provide an endogenous excitatory
drive to ascending pain pathways, thereby promoting the maturation of supraspinal nociceptive
circuits as well as descending modulatory pathways from the brainstem, which appear to
develop gradually during the postnatal period (Fitzgerald and Koltzenburg 1986; Hathway et al.
2009). Finally, since lamina I projection neurons are known to be involved in the generation of
chronic pain states (Mantyh et al. 1997; Nichols et al. 1999; Suzuki et al. 2002), a more
complete understanding of how membrane excitability is regulated within this population may
yield insight into novel, age-specific strategies to modulate the ascending flow of noxious
information, and thus pain perception, in infants and children.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Retrograde labeling of developing lamina I neurons which project to the parabrachial nucleus (PB) or periaqueductal gray (PAG).  A, Section of rat brain at postnatal day (P)3 illustrating the site where DiI was injected into the PB at birth.  SC, superior colliculus; IC, inferior colliculus; CB, cerebellum; v4i, fourth ventricle (isthmal).  Scale bar = 400 μm.  B, Sagittal spinal cord section illustrating a narrow band of retrogradely labeled neurons within lamina I at P21 following the injection of DiI into the PB at birth.  Orientation arrows indicate dorsal (D), ventral (V), rostral (R) and caudal (C) axes.  C, Higher magnification of boxed region in panel B.  D, P3 brain section demonstrating the location of DiI injection into the PAG at birth.  dPAG, dorsal PAG; vPAG, ventral PAG; va, aqueduct.  Scale bar = 400 μm.  E, Example of lamina I neurons fluorescently labeled at P21 following injections of DiI into the PAG at birth.  Same orientation as in panel B.  F, Higher magnification of the same section as in E (see boxed region).

Figure 2. Spontaneous firing patterns in developing lamina I projection neurons.  A, Spino-PB and spino-PAG neurons located in lamina I of the rat spinal cord were classified as irregular (exhibiting intermittent spike activity; top), tonic (continuous firing at a relatively constant frequency; middle), bursting (demonstrating rhythmic burst-firing; bottom) or silent (lack of action potential discharge; not shown).  B, C, Patterns of spontaneous activity (SA) in spino-PB (B) and spino-PAG (C) neurons at different postnatal ages, illustrating the predominance of irregular spike discharge in both groups throughout development and the greater overall prevalence of SA in the spino-PB population.  Data for spino-PB group:  P2-5: n = 33 cells from 3 rats; P10-12: n = 29 cells from 3 rats; P19-23: n = 27 cells from 4 rats; P30-32: n = 28 cells.
from 4 rats. Data for spino-PAG group: P2-5: \( n = 27 \) cells from 4 rats; P10-12: \( n = 28 \) cells from 4 rats; P19-23: \( n = 27 \) cells from 5 rats; P30-32: \( n = 17 \) cells from 3 rats.

**Figure 3. Evoked action potential discharge in ascending projection neurons during the early postnatal period.** Direct current injection through the patch electrode at increasing intensities (*bottom to top*) revealed four firing patterns in developing spino-PB and spino-PAG lamina I neurons. **A**, Tonic neurons fired action potentials (APs) throughout the 800 ms depolarizations. All traces in panel originate from the same lamina I projection neuron. **B**, Phasic neurons exhibited APs at the beginning of the current step but did not discharge spikes throughout the prolonged depolarization, as irregular gaps in their firing were evident at many stimulus intensities (*arrows*). **C**, Delayed neurons were distinguished by a long latency to the first spike which varied with stimulus intensity. **D**, Bursting neurons were identified by their slow plateau potentials with superimposed bursts of high-frequency AP discharge. *Inset*, Example of spike afterdepolarization (*see boxed region*). **E**, When APs were evoked by intracellular current injection from the resting membrane potential, the majority of both spino-PB (*E*) and spino-PAG (*F*) neurons exhibited tonic firing during the first three postnatal weeks, while a more even distribution of firing patterns was evident by P30-32. Sample sizes were the same as described in Fig. 2.

**Figure 4. Distinct passive membrane properties of ascending projection neurons which demonstrate evoked burst-firing.** **A**, The average membrane capacitance of bursting (*black*) spino-PAG neurons was higher compared to non-bursting cells (*white*) in this population (**p = 0.0002**; Mann-Whitney test; *right*), while no significant differences were noted in the spino-PB group (**p = 0.141**; *left*). **B**, Bursting neurons (*black*) exhibited significantly lower membrane
resistance compared to other projection neurons (white) in both the spino-PB (**p = 0.0008; Mann-Whitney test; left) and spino-PAG (**p<0.0001; right) populations. C, The resting membrane potential was more hyperpolarized in spino-PAG neurons showing burst-firing in response to current injection compared to spino-PAG cells showing other patterns of evoked discharge (**p = 0.0009; right). Data on bursting neurons originate from 5 rats for the spino-PB group and 12 rats for the spino-PAG population.

Figure 5. Age-dependent modulation of membrane excitability in developing spinal projection neurons. A, B, The minimum current needed to evoke an AP (i.e. rheobase) did not change significantly during early postnatal development in either the spino-PB (A) or spino-PAG (B) population of lamina I neurons, although higher rheobase levels were seen in the P30-32 group (*p<0.05; Kruskal-Wallis test with Dunn’s Multiple Comparison test). However, an age-related reduction in AP threshold was observed in both spino-PB (C; *p<0.05, ***p<0.001; Kruskal-Wallis test with Dunn’s post-test) and spino-PAG (D, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA with Tukey’s Multiple Comparison test) neurons. (E, F) Both groups of projection neurons exhibited a developmental decrease in spike duration (*p<0.05, **p<0.01, ***p<0.001; Kruskal-Wallis test with Dunn’s post-test). Spino-PB (G) and spino-PAG (H) cells also demonstrated an increased prevalence of spike afterdepolarizations (ADP) with age (p<0.0001; χ² test). Sample sizes were the same as described in Fig. 2.

Figure 6. The firing rate of spino-PAG, but not spino-PB, projection neurons accelerates during postnatal development. A, Plot of mean instantaneous firing frequency as a function of stimulus intensity in spino-PB neurons reveals no significant differences in repetitive firing between age groups (p>0.05; two-way ANOVA; n = 12-30 in each group). B, In contrast, P19-
23 spino-PAG neurons fired at a significantly higher frequency compared to younger ages (*p<0.05, **p<0.01 compared to P10-12; &p<0.05 compared to P2-5; **p<0.01, ***p<0.001 compared to both ages; two-way ANOVA with Bonferroni post-tests; n = 14-20 in each group).

Spino-PAG neurons were not analyzed at P30-32 due to the low number (n = 3) of tonically-firing neurons observed at this age. C, D, The degree of spike frequency adaptation across a range of stimulus intensities did not change significantly with age in either population of lamina I projection neurons (p>0.05; two-way ANOVA). The numbers of animals used were the same as described in Fig. 2.

Figure 7. Developmental increase in the efficacy of spontaneous excitatory and inhibitory synaptic transmission onto spinal projection neurons. A, Example of mEPSCs isolated at a holding potential of -70 mV in an immature lamina I projection neuron identified by retrograde transport of Dil. B, Example of mIPSCs recorded in the same neuron from a holding potential of 0 mV. C, D, The frequency of mEPSCs increased with age in both spino-PB (**p = 0.0003; Mann-Whitney test; C, left) and spino-PAG (***p = .0013; D, left) neurons without a significant change in mEPSC amplitude (right). Similarly, mIPSC frequency was significantly higher at P19-23 compared to P2-5 in the same populations of spino-PB (**p<0.0001; Mann-Whitney; E, left) and spino-PAG neurons (***p = 0.0002; F, left) while mIPSC amplitude was unaltered (right). Data for spino-PB group are derived from 3 rats at P2-5 and 4 rats at P19-23. Data for spino-PAG group are derived from 3 rats at P2-5 and 4 rats at P19-23.
Table 1. Passive membrane properties of developing lamina I projection neurons

<table>
<thead>
<tr>
<th></th>
<th>P2-5</th>
<th>P10-12</th>
<th>P19-23</th>
<th>P30-32</th>
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<tbody>
<tr>
<td><strong>Spino-PB (n = 27-33):</strong></td>
<td></td>
<td></td>
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<tr>
<td>Resting Potential (mV)</td>
<td>-69.4 ± 1.1</td>
<td>-67.5 ± 1.4</td>
<td>-71.5 ± 1.3</td>
<td>-80.1 ± 1.4***</td>
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<td>Membrane Capacitance (pF)</td>
<td>72.0 ± 3.3</td>
<td>72.6 ± 3.3</td>
<td>83.3 ± 5.5</td>
<td>64.6 ± 3.9**</td>
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<td>Membrane Resistance (MΩ)</td>
<td>1012 ± 69</td>
<td>1083 ± 82</td>
<td>892 ± 52</td>
<td>845 ± 55</td>
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<td>3</td>
<td>4</td>
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<tr>
<td><strong>Spino-PAG (n = 17-28):</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Resting Potential (mV)</td>
<td>-68.4 ± 1.4</td>
<td>-71.6 ± 1.4</td>
<td>-70.1 ± 1.3</td>
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<td>Membrane Capacitance (pF)</td>
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</table>

Values are means ± SEM. **p<0.01 compared to P19-23, ***p<0.001 compared to all other ages; ††p<0.01 compared to P10-12; †††p<0.001 compared to P2-5 and P20-23; one-way ANOVA with Tukey’s Multiple Comparison test. Data were obtained using the potassium gluconate-based intracellular solution. See METHODS for calculations.
Table 2. *Spontaneous vs. evoked firing patterns in developing projection neurons*

<table>
<thead>
<tr>
<th>Evoked Discharge</th>
<th>Spino-PB Spontaneous Discharge</th>
<th>Spino-PAG Spontaneous Discharge</th>
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<tbody>
<tr>
<td>Silent</td>
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</tr>
<tr>
<td>Irregular</td>
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<td>33</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Bursting</td>
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<td>0</td>
</tr>
<tr>
<td>Phasic</td>
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<td>2</td>
</tr>
<tr>
<td>Delayed</td>
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<td>0</td>
</tr>
<tr>
<td>Burst</td>
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<tr>
<td>Burst</td>
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</table>

Values represent the number of neurons exhibiting the indicated pattern of action potential discharge.