Evidence for a Critical Period in the Development of Excitability and Potassium Currents in Mouse Lumbar Superficial Dorsal Horn Neurons

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Walsh MA, Graham BA, Brichta AM, Callister RJ. Evidence for a critical period in the development of excitability and potassium currents in mouse lumbar superficial dorsal horn neurons. J Neurophysiol 101: 1800–1812, 2009. First published January 28, 2009; doi:10.1152/jn.90755.2008. The output of superficial dorsal horn (SDH; laminae I–II) neurons is critical for processing nociceptive, thermal, and tactile information. Like other neurons, the combined effects of synaptic inputs and intrinsic membrane properties determine their output. It is well established that peripheral synaptic inputs to SDH neurons undergo extensive reorganization during prenatal and postnatal development. It is unclear, however, how membrane properties or the subthreshold whole cell currents that shape SDH neuron output change during this period. Here we assess the intrinsic membrane properties and whole cell currents in mouse SDH neurons during late embryonic and early postnatal development (E15–P25). Transverse slices were prepared from lumbar spinal cord and whole cell recordings were obtained at 32°C. During this developmental period resting membrane potential (RMP) became more hyperpolarized (by ~10 mV, E15–E17 vs. P21–P25) and input resistance decreased (1.074 ± 78 vs. 420 ± 27 MΩ). In addition, action potential (AP) amplitude and AP afterhyperpolarization increased, whereas AP half-width decreased. Before and after birth (E15–P10), AP discharge evoked by intracellular current injection was limited to a single AP at depolarization onset in many neurons (~41%). In older animals (P11–P25) this changed, with AP discharge consisting of brief bursts at current onset (~46% of neurons). Investigation of major subthreshold whole cell currents showed the rapid A-type potassium current (I_A) dominated at all ages examined (90% of neurons at E15–E17, decreasing to ~50% after P10). I_A expression levels, based on peak current amplitude, increased during development. Steady-state inactivation and activation for I_A were slightly less potent in E15–E17 versus P21–P25 neurons at potentials near RMP (~55 mV). Together, our data indicate that intrinsic properties and I_A expression change dramatically in SDH neurons during development, with the greatest alterations occurring on either side of a critical period, P6–P10.

INTRODUCTION

Neurons in the superficial dorsal horn (SDH; laminae I–II) of the spinal cord play an important role in processing noxious, thermal, and light touch stimuli before relaying this information to higher brain centers (Light and Perl 1979; Willis Jr and Coggeshall 2004). There is now increasing evidence that both noxious and innocuous stimuli are processed differently in the spinal cords of embryos, neonates, and adults. For example, premature humans (27-wk gestation) exhibit greater “sensitization” to noxious mechanical stimulation compared with full-term infants (41 wk) (Andrews and Fitzgerald 1994) and neonatal rodents show exaggerated reflex responses to noxious stimuli compared with adults (Fitzgerald 2005; Holmberg and Schouenborg 1996).

Like other neurons, the combined effects of synaptic inputs and intrinsic membrane properties will largely determine the output of SDH neurons (Hille 2001; Lüscher and Clamann 1992). For rodents, our understanding of the development of synaptic connections in the SDH is reasonably advanced, especially for primary afferent termination patterns in the dorsal horn. Anatomical studies show that large-diameter Aβ fibers enter the dorsal horn first, at close to embryonic day 15 (E15), and occupy the entire dorsal horn including laminae I–II until well after birth. At about postnatal day 22 (P22) these large-diameter afferents retract from superficial layers of the dorsal horn (Fitzgerald et al. 1994). The smaller-diameter Aδ and C-fiber nociceptive afferents enter the dorsal horn later than large-diameter afferents, at around E19, but remain restricted to laminae I–II throughout development (Mirnics and Koerber 1995). Electrophysiological studies have also confirmed that extensive reorganization of synaptic connections occurs in the SDH during development. The combined effect of this reorganization is to refine peripheral receptive field size, increase mechanical thresholds, and alter the contribution of large-fiber inputs (Beggs et al. 2002; Jennings and Fitzgerald 1998; Park et al. 1999; Torsney and Fitzgerald 2002).

Surprisingly, little information exists on the concomitant changes in membrane and discharge properties of SDH neurons during late embryonic and early postnatal development. This contrasts with the extensive knowledge of these properties for adult SDH neurons (Graham et al. 2004, 2007c; Lopez-Garcia and King 1994; Prescott and De Koninck 2002; Ruscheweyh and Sandkühler 2002; Ruscheweyh et al. 2004; Thomson et al. 1989). There has only been one study (in young rats) on the development of intrinsic excitability in SDH neurons (Baccei and Fitzgerald 2005). No developmental data are available for the subthreshold conductances that underlie intrinsic excitability in SDH neurons, even though an extensive literature is available for adult animals (Graham et al. 2007c; Grudt and Perl 2002; Ruscheweyh et al. 2004).

In this study we used whole cell voltage- and current-clamp techniques to examine membrane and discharge properties, as well as the major subthreshold whole cell currents, in mouse SDH neurons during late embryonic development (E15–E17)
until approximately 4 wk (P25) after birth. We used the mouse for our investigation because of the increasing contribution of this species to our understanding of pain mechanisms (Graham et al. 2004; Harvey et al. 2004). Our results show significant changes in intrinsic membrane properties, the proportions of action potential (AP) discharge categories, and subthreshold whole cell currents throughout the developmental period examined. We propose a developmental timeline for intrinsic membrane properties and excitability in the SDH with a critical time point for maturation on either side of P6–P10.

**METHODS**

**Preparation of spinal cord slices**

C57Bl/6 mice (aged E15–P25, both sexes) were used and The University of Newcastle Animal Care and Ethics Committee approved all procedures. Transverse spinal cord slices were prepared using previously described techniques (Graham et al. 2003, 2007b). Briefly, mice ≥P6 were anesthetized with ketamine (100 mg/kg, administered intraperitoneally [ip]) and decapitated. The vertebral column and surrounding tissue was rapidly isolated and immersed in ice-cold oxygenated sucrose substituted artificial cerebrospinal fluid (S-ACSF) containing (in mM): 250 sucrose, 25 NaHCO3, 10 glucose, 2.5 KCl, 1 NaH2PO4, 1 MgCl2, and 2.5 CaCl2. The S-ACSF was continually bubbled with 95% O2–5% CO2 to maintain a pH of 7.3. The lumbo-sacral enlargement of the spinal cord was removed and placed on a Styrofoam support block. The block and enlargement were glued to a cutting stage with cyanoacrylate glue (Loctite 454; Loctite, Caringbah, Australia) and transverse slices (300 μm thick) were obtained using a vibratome (Leica VT-1000S; Leica Microsystems, Wetzlar, Germany).

Some of these procedures needed to be modified for smaller animals (<P6). For neonates (P0–P5, body weight 1.2–2.9 g), hypothermic anesthesia was induced by immersing animals in ice prior to decapitation. The entire lumbar vertebral column was removed and mounted against a Styrofoam block as earlier and transverse slices (450 μm thick) containing the spinal cord and surrounding vertebral bodies were cut. The above-cited modification provided improved support for the small spinal cords during slicing. Embryos (E15–E17) were rapidly removed via caesarean section from time-mated pregnant females, anesthetized with ketamine (100 mg/kg, ip). Embryonic slices were then cut using the modified procedures described for neonates. All slices were then transferred to an interface storage chamber containing oxygenated ACSF (118 mM NaCl substituted for sucrose in S-ACSF) and allowed to equilibrate for 1 h at room temperature (22–24°C) before recording commenced.

**Electrophysiology**

Slices were placed in a recording bath (volume 0.4 ml) and continually perfused (4–6 bath volumes/min) with ACSF maintained at near-physiological temperature (32°C). Whole cell patch-clamp recordings were obtained from SDH neurons, visualized using infrared differential interference contrast optics and an infrared-sensitive camera (Hamamatsu C2400-79C, Hamamatsu City, Japan). Recordings were confined to the SDH by targeting neurons that were dorsal to the lamina II–III border (i.e., neurons in laminae I and II). This boundary is readily identified in spinal cord slices older than P6 because lamina II is translucent. For younger animals this translucent band was not apparent because myelination in various laminae of the dorsal horn is not fully established. Thus in younger animals recordings were restricted to within 40–120 μm (mean = 76.4 ± 4.3 μm) and 75–140 μm (mean = 99.5 ± 4.2 μm) from the dorsal surface of the spinal cord for E15–E17 and P0–P5 mice, respectively. These regions correspond to the limits of laminae I–II in Nissl-stained slices from late embryonic and P2 animals (Fig. 1).

Patch pipettes (2–5 MΩ) were filled with a potassium methylsulfate–based internal solution containing (in mM): 135 KCH3SO4, 6 NaCl, 2 MgCl2, 10 HEPES, 0.1 EGTA, 2 MgATP, and 0.3 NaGTP (pH adjusted to 7.3 with KOH). Whole cell patch-clamp recordings were obtained using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The whole cell recording configuration was first established in voltage clamp (holding potential −60 mV), Series resistance (Rsh), range 8–10 MΩ, neuronal input resistance (Rin), and capacitance were measured from the averaged response (five trials) to a 5-mV hyperpolarizing voltage step immediately after obtaining the whole cell recording configuration. These parameters were examined at the beginning and end of each recording session and data were rejected if values changed by >20%.

**FIG. 1.** The superficial dorsal horn (SDH) in embryonic and neonatal spinal cords. A and B: cresyl violet–stained spinal cord cross sections from a late embryonic (E16) and neonatal (P2) mouse. High-power views of the dorsal horn (square on inset) show approximate boundary of lamina I–II (dashed lines). These 2 laminae are easily distinguished by the presence of dark staining, uniformly sized small neurons, and tightly packed neuropil. Scale bars = 100 μm. All recordings from embryonic (A) and neonatal (B) spinal cord slices were made within the area outlined by dashed lines. Insets (bottom right) are low-power views, which outline the extent of the gray matter and location of the central canal (scale bar = 200 μm).
Once whole cell recording was established several protocols were run for each SDH neuron. First, the presence and characteristics of the four major subthreshold whole cell currents previously described in adult SDH neurons were assessed in voltage clamp from a holding potential of ~60 mV (Graham et al. 2007c; Grudt and Perl 2002; Ruscheweyh and Sandkuhler 2002; Ruscheweyh et al. 2004; Yoshimura and Jessell 1989). Subthreshold currents were studied by delivering a hyperpolarizing pulse (to ~90 mV, 1-s duration), immediately followed by a depolarizing step (to ~40 mV, 200-ms duration). This protocol was repeated four times to obtain an average response for analysis. Depolarization was limited to ~40 mV to avoid activation of tetrodotoxin-sensitive Na\(^+\) and delayed rectifier channels (Safronov 1999). We used the P/N subtraction method (an on-line protocol in Axograph 4.6) to eliminate the effects of leakage current on whole cell responses (Sontheimer and Ransom 2002). This approach injects a protocol (P) that is reduced by a factor (N) on multiple trials prior to injection of the full-size protocol. Responses to the reduced protocol trials are then averaged, rescaled to original size, and subtracted from the response recorded from the original protocol.

After assessing subthreshold currents the recording mode was switched to current clamp. The membrane potential recorded about 15 s after this switch was designated as the resting membrane potential (RMP). All subsequent recordings were made from this potential and were corrected for a 10-mV junction potential (Barry and Lynch 1991). Individual AP properties and AP discharge were examined by injecting two current profiles. The first was a series of standard depolarizing step currents (800-ms duration, 20-pA increments, delivered every 8 s). The second was a series of depolarizing “pinch currents.” The pinch current was obtained previously in a separate set of in vivo experiments and approximated the pattern of synaptic currents delivered to the soma of an SDH neuron during noxious mechanical stimuli (1-s pinch = 100 g mm\(^{-2}\)) to the hindpaw (for details see Graham et al. 2007b).

Data capture and analysis

Data were digitized on-line (sampled at 100 or 20 kHz, filtered at 5–10 kHz, respectively) via an ITC-16 computer interface (Instrutech, Long Island, NY) and stored on a Macintosh G4 computer using Axograph v4.6 software (Axon Instruments, Foster City, CA). All data were analyzed off-line using Axograph software. Subthreshold currents were first classified according to previously described characteristics (Graham et al. 2007c). Because the rapid A-type potassium current (I\(_{\text{A}}\)) was the dominant subthreshold current at all developmental stages examined, its features were analyzed in detail. I\(_{\text{A}}\) amplitude was measured by subtracting the amplitude of any steady-state current component (in the last 50 ms of the step to ~40 mV) from the maximal I\(_{\text{A}}\) current peak (Graham et al. 2008; Ruscheweyh and Sandkuhler 2002). The decay phase of the I\(_{\text{A}}\) response was fit with a single exponential (over 20–80% of the falling phase).

Individual action potentials (APs) elicited by step current injection were captured using a derivative threshold method, with the threshold set at the highest value of dV/dt that would detect all APs in the data set (dV/dt values ranged from 10 to 15 V/s in embryos to 18–20 V/s in P21–P25 animals). We have used this method in previously published work on adult mouse SDH neurons (Graham et al. 2004, 2008). Rheobase current was defined as the smallest step current that would elicit at least one AP. All AP properties were assessed on the rheobase response. AP threshold was defined as the inflection point during spike initiation (see Fig. 2G). The amplitude of each AP was measured as the difference between AP threshold and its maximum positive peak. AP half-width was calculated at 50% of AP amplitude. AP afterhyperpolarization (AHP) amplitude was measured as the difference between AP threshold and its maximum negative peak.

Various temporal features of the AP discharge were characterized during both step- and pinch-current injection. Discharge latency was defined as the time between the onset of current injection and the first AP elicited. Discharge duration was measured as the time between the first and last APs discharged during current injection in recordings containing multiple APs. Instantaneous AP frequency was calculated as the reciprocal of the time interval between successive APs. Mean frequency was determined as the average of all instantaneous frequencies. The attenuation ratio was calculated by dividing the amplitudes of the last by the first AP in a train. The adaptation ratio was calculated by dividing the instantaneous frequency of the last AP by the instantaneous frequency of the first AP.

ANOVA was used to compare variables between and within SDH neuron groupings based on age or discharge patterns. Scheffé post hoc tests were used to determine which groups differed (SPSS v10, SPSS, Chicago, IL). G-tests, with Williams’ correction, or chi-square tests were used to determine whether the prevalence of discharge categories, subthreshold currents, and pinch-current responses differed between age groups (Sokal and Rohlif 1981). Statistical significance was set at \(P < 0.05\). All values are presented as means ± SE.

RESULTS

Whole cell patch-clamp recordings were made in 264 SDH neurons from 48 postnatal animals and in 51 neurons from 12 embryos (obtained from 8 pregnant females). Data were separated into six age groups: E15–E17, P0–P5, P6–P10, P11–P15, P16–P20, and P21–P25. Recordings were obtained from 4 to 7 neurons per animal.

Membrane and action potential properties

Results for several passive and active membrane properties in SDH neurons are presented in Table 1 and summarized in Fig. 2. During development, input resistance (R\(_{\text{IN}}\)) decreased dramatically from over 1,000 M\(\Omega\) in E15–E17 animals to around 400 M\(\Omega\) in animals aged P6–P10 and older (Fig. 2A). RMP became more hyperpolarized (by ~10 mV) over the first three age groups and stabilized from P10 onward (Fig. 2B). Rheobase current tended to increase slightly during development, although this was only significant for P16–P20 neurons (Fig. 2C). Rheobase APs were typically overshooting at all ages (83% at E15–E17, 78% at P0–P5, 78% at P6–P10, 88% at P11–P15, 100% at P16–P20, and 98% at P21–P25). Several AP features changed during development. When measured from threshold (arrowhead in Fig. 2G), both AP and AHP amplitude increased (24.9 ± 1.8 mV at E15–E17 and 33.5 ± 1.9 at P0–P5 vs. 43.0 ± 1.6 mV at P21–P25, Fig. 2D; −10.1 ± 1.8 mV at E15–E17 vs. −31.0 ± 0.9 mV for P21–P25, Fig. 2E). In contrast, AP half-width decreased (2.7 ± 0.4 ms at E15–E17 vs. 1.2 ± 0.1 ms at P21–P25, Fig. 2F). Example APs from each of the age groups examined are shown in Fig. 2G. Together, these data indicate several important passive and active membrane properties of SDH neurons change during development with most changes occurring on either side of P6–P10.

Responses to step-current injection

We next compared responses of SDH neurons to depolarizing step-current injections of increasing amplitude across the six age groups (Fig. 3, left panels). Previously, our studies in mice (aged P19–P42) have shown that responses (hereafter termed AP discharge patterns) in SDH neurons can be assigned into one of five categories (Graham et al. 2004, 2007b): I) tonic firers exhibit sustained repetitive AP discharge during
multiple step-current injection; 2) initial bursters discharge brief bursts of APs during current injection with APs confined to current onset; 3) delayed firers respond only to high-intensity current injection and exhibit a substantial delay between current onset and AP discharge; 4) single spikers respond only at high-intensity current injections and discharge one or, occasionally, two APs at current onset; and 5) reluctant firers do not exhibit APs even at high-intensity current injection. We previously described the reluctant firing category in both in vivo and in vitro studies and showed that they are not unhealthy neurons (Graham et al. 2004, 2007b). They have similar RMPs and input resistances and can fire APs when membrane potential is depolarized via sustained bias current injection. All five AP discharge categories were observed in this study, although the prevalence of each varied during development.

The prevalence of the five AP discharge categories is summarized in Fig. 3 (right panels). Tonic firers were present in each of the six age groups examined. Although they were rarely observed in E15–E17 neurons (2/51) their prevalence increased after birth and remained relatively stable in all postnatal groups examined (but never >20% of the sample). Initial bursters were also present in all age groups, with proportions increasing during development, from 18% at E15–E17 (9/51) to 38% at P21–P25 (20/52). Delayed firers were rare in animals P10, with only 1/160 neurons exhibiting delayed firing before P10. The prevalence of this discharge category increased to about 20% by P21–P25. In contrast, single spikers were present in all age groups but decreased dramatically after P10, from 53% in E15–E17 neurons (27/51) to only 14% in the P21–P25 age group (7/52). The presence of reluctant firers also decreased, from 26% at E15–E17 (13/51) to 6% of neurons P21–P25 (3/52). In summary, the proportions of each AP discharge category differ significantly during late embryonic and early postnatal development (G-statistic = 91.3, df = 20, P < 0.01; Fig.
Before P10 single spikers dominate the sample (~40%) and delayed firers are rarely observed. In older animals (P11–P25) initial bursters dominate (~30%) and all five discharge patterns are present.

Membrane and AP properties within discharge categories

We next investigated whether membrane and AP properties within each discharge category changed during development. As documented previously in older mice (P26–P42), some of these properties differ across discharge categories. For example, tonic firing neurons have more depolarized RMPs and smaller rheobase currents than neurons in the other discharge categories (Graham et al. 2007b). In this study we also examined membrane and AP characteristics in neurons from each discharge category during development. It should be noted that the dominance or absence of certain discharge categories at different developmental times precluded detailed comparisons for all discharge categories during development. For example, we observed only one delayed firer before P11–P15 (Fig. 3C).

Membrane and AP characteristics for each discharge category throughout development are presented in Table 2. Values for $R_{IN}$, in each discharge category, were tightly clustered for each age group, indicating that $R_{IN}$ followed the same developmental time course observed in the whole population (see Fig. 2A). Similarly, a conserved developmental time course for each of the discharge categories was observed for AHP amplitude and half-width (see Fig. 2, E and F). In contrast, the clustering of data points for RMP, rheobase, and AP amplitude deviated somewhat from the pattern observed in the whole population (Fig. 2, B, C, and D). These comparisons suggest that the time course for achieving the adult firing patterns differs slightly among the five discharge categories.

We also examined the characteristics specific to each AP discharge pattern and found no differences in duration, instantaneous and mean frequencies, or adaptation ratios for any of the observed five patterns throughout development. However, initial bursters in the E15–E17 age group discharged more APs in response to current step injection compared with the older postnatal groups (≥ P11; data not shown). There was also greater attenuation of AP amplitude in E15–E17 initial bursters compared with the older postnatal groups (≥ P11; data not shown).

Responses to pinch-current injection

We have previously used an alternative to step-current injection to further explore mechanisms underlying AP discharge in SDH neurons (Graham et al. 2007b). Our approach was based on the notion that during noxious peripheral stimulation in vivo, SDH neurons are in fact activated by a barrage of synaptic inputs, not square current steps. These provide the soma of a neuron with a conductance change that is composed of transient current fluctuations (see Fig. 4A for an example pinch-current profile). Injection of pinch- versus step-current profiles into SDH neurons reduced the number of observed discharge categories from five to two (Graham et al. 2007b; Fig. 4B). Neurons that exhibit an increase in AP discharge frequency and duration as pinch-current amplitude is increased are termed robust responders (Fig. 4B, top traces). Neurons that exhibit little or no AP discharge in response to even the...
largest pinch current are termed resistant responders (Fig. 4B, middle traces). We were able to classify 218/264 postnatal and 48/51 embryonic neurons as either robust or resistant responders according to their response to pinch-current injection. The proportions of these two response categories change during development (Fig. 4C). In embryos, only 23% of the neurons were robust responders. After birth, the proportion of robust responders remained relatively constant at about 50%. In summary, injection of pinch currents into SDH neurons shows their sensitivity to rapid transients differs significantly before and after birth.

**Relationship between step- and pinch-current responses during development**

We also examined whether there was any relationship between the square wave and pinch-current responses and whether this changed over time. Because there is an abrupt change in pinch-current responses at P0 (Fig. 4C), we restricted comparisons to E15–E17 and P21–P25 animals. In E15–E17 animals 64% of robust responders were either tonic firers or initial bursters. This increased to 82% in P21–P25 animals. In E15–E17 animals almost all (97%) resistant responders were either single spikers or reluctant firers. In P21–P25 animals...
64% of resistant responders were delayed firers, single spikers, or reluctant firers. Thus the strong relationship we previously observed between pinch-current responses and AP discharge frequency in adult animals (Graham et al. 2007b) is also preserved during development.

**Subthreshold whole cell currents**

The AP discharge patterns described in SDH neurons are shaped by the expression of a number of voltage-gated ion channels (Melnick et al. 2004a, b; Ruscheweyh and Sandkühler 2002; Ruscheweyh et al. 2004; Yoshimura and Jessell 1989). We previously showed that four major subthreshold currents exist in adult mouse SDH neurons (Graham et al. 2007c). These currents can be reliably activated by applying a voltage-step protocol, which consists of hyperpolarizing (to −90 mV, for 1 s) and depolarizing steps (to 0 mV) (see METHODS and Fig. 5). The resulting whole cell currents (Fig. 5, A–D, left panels) are then identified according to three criteria: their direction (inward or outward), their kinetic features, and their pharmacology (see Fig. 4 in Graham et al. 2007c). Two outward currents are activated by depolarization (stepping from −90 to −40 mV) and exhibit features of A-type potassium currents (Ruscheweyh et al. 2004; Yoshimura and Jessell 1989). The first (Fig. 5A, left) has rapid activation and inactivation kinetics, is sensitive to high concentrations of 4-aminopyridine (4AP), and is termed rapid A-current (IAr). The second (Fig. 5B, left) has slower kinetics, is sensitive to low concentrations of 4AP, and is termed slow A-current (IAS) (Ruscheweyh et al. 2004). Two inward currents can also be revealed during the voltage-step protocol. One is activated by depolarization (Fig. 5C, left), features rapid activation and inactivation kinetics, and is sensitive to nickel (Yoshimura and Jessell 1989). This current is termed low-threshold activated “T-type” calcium current (ICaL). The second inward current (Fig. 5D, left) is activated by hyperpolarization (stepping from −60 to −90 mV), exhibits slow activation kinetics, is sensitive to cesium, and is termed ICa (Grudt and Perl 2002; Ruscheweyh and Sandkühler 2002; Yoshimura and Jessell 1989).

We assessed the subthreshold whole cell currents in most of our samples of SDH neurons: 245/264 and 43/51 for postnatal and embryonic neurons, respectively. Notably, in the majority of SDH neurons only one of the four currents was dominant. A small proportion of the postnatal neurons (11%, 27/245) showed either no discernable current, a mixed current, or discharged an AP in response to the protocol. Neurons with mixed currents (21/27) exhibited a rapidly activating inward current (T-current) followed immediately by a rapidly activating outward current (IAr) during the depolarization (−90 to −40 mV). We made no attempt to dissect these mixed-current responses and only responses from SDH neurons that exhibited a single dominant subthreshold current were considered in our analysis (Fig. 5).

The most frequently observed whole cell current in SDH neurons was IAS, accounting for ≥50% of the sample at all ages studied (Fig. 5A, right). In embryonic neurons IAS was present in almost all neurons classified (95%). The presence of IAS gradually decreased during postnatal development and stabilized at about 50% of the sample after P10. IAS was observed

### Table 2. Membrane and AP properties within AP discharge categories during late embryonic and early postnatal development

<table>
<thead>
<tr>
<th>Discharge Category</th>
<th>Age Group</th>
<th>n</th>
<th>Input Resistance, MΩ</th>
<th>RMP, mV</th>
<th>Rheobase Current, pA</th>
<th>AP Amplitude, mV</th>
<th>AP Half-Width, ms</th>
<th>AHP Amplitude, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonic firers</td>
<td>E15–E17</td>
<td>2</td>
<td>1.01 ± 302</td>
<td>−60.4 ± 4.9</td>
<td>20 ± 0</td>
<td>48.3 ± 5.9</td>
<td>2.47 ± 0.3</td>
<td>−20.0 ± 2.6</td>
</tr>
<tr>
<td>P0–P5</td>
<td>9</td>
<td>605 ± 88</td>
<td>−52.0 ± 3.0</td>
<td>26 ± 6</td>
<td>47.9 ± 4.7</td>
<td>1.31 ± 0.2</td>
<td>−20.5 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>P6–P10</td>
<td>10</td>
<td>328 ± 33*</td>
<td>−49.8 ± 2.8</td>
<td>26 ± 8</td>
<td>51.2 ± 3.7</td>
<td>1.01 ± 0.1*</td>
<td>−23.1 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>P11–P15</td>
<td>4</td>
<td>200 ± 29*</td>
<td>−50.8 ± 6.6</td>
<td>10 ± 6</td>
<td>47.8 ± 4.6</td>
<td>0.89 ± 0.1*</td>
<td>−27.3 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>P16–P20</td>
<td>8</td>
<td>377 ± 69*</td>
<td>−49.4 ± 2.7</td>
<td>20 ± 4</td>
<td>49.9 ± 3.5</td>
<td>1.04 ± 0.2*</td>
<td>−31.2 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>P21–P25</td>
<td>9</td>
<td>384 ± 70*</td>
<td>−44.6 ± 1.3</td>
<td>22 ± 13</td>
<td>41.4 ± 4.1</td>
<td>1.19 ± 0.2*</td>
<td>−28.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Initial bursters</td>
<td>E15–E17</td>
<td>9</td>
<td>1.120 ± 185</td>
<td>−48.5 ± 2.1</td>
<td>33 ± 10</td>
<td>33.6 ± 3.8</td>
<td>3.02 ± 0.6</td>
<td>−21.7 ± 4.2</td>
</tr>
<tr>
<td>P0–P5</td>
<td>18</td>
<td>493 ± 41*</td>
<td>−52.2 ± 1.9</td>
<td>59 ± 16</td>
<td>36.6 ± 3.0</td>
<td>1.59 ± 0.1*</td>
<td>−15.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>P6–P10</td>
<td>15</td>
<td>379 ± 40*</td>
<td>−54.6 ± 2.2</td>
<td>76 ± 14</td>
<td>54.0 ± 2.0*#</td>
<td>0.93 ± 0.1*</td>
<td>−22.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>P11–P15</td>
<td>26</td>
<td>342 ± 31*</td>
<td>−50.6 ± 1.7</td>
<td>43 ± 6</td>
<td>43.2 ± 2.9</td>
<td>1.04 ± 0.1</td>
<td>−25.2 ± 1.9#</td>
<td></td>
</tr>
<tr>
<td>P16–P20</td>
<td>16</td>
<td>375 ± 44*</td>
<td>−57.7 ± 2.7</td>
<td>105 ± 29</td>
<td>47.8 ± 2.5</td>
<td>0.91 ± 0.1*</td>
<td>−30.1 ± 1.3*#</td>
<td></td>
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<tr>
<td>P21–P25</td>
<td>20</td>
<td>349 ± 30*</td>
<td>−51.0 ± 2.5</td>
<td>68 ± 16</td>
<td>46.5 ± 2.8</td>
<td>1.15 ± 0.1*</td>
<td>−32.3 ± 1.3*#</td>
<td></td>
</tr>
<tr>
<td>Delayed firers</td>
<td>E15–E17</td>
<td>0</td>
<td>743 ± 25</td>
<td>−56.4</td>
<td>40</td>
<td>29.3</td>
<td>1.82 ± 0.6</td>
<td>−20.6 ± 0.6</td>
</tr>
<tr>
<td>P0–P5</td>
<td>1</td>
<td>418 ± 58</td>
<td>−61.1 ± 2.9</td>
<td>103 ± 21</td>
<td>39.2 ± 4.1</td>
<td>0.95 ± 0.1</td>
<td>−29.2 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>P6–P10</td>
<td>0</td>
<td>800 ± 87</td>
<td>−55.0 ± 2.9</td>
<td>93 ± 38</td>
<td>38.6 ± 4.1</td>
<td>1.43 ± 0.2</td>
<td>−25.2 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Single spikers</td>
<td>E15–E17</td>
<td>12</td>
<td>439 ± 34</td>
<td>−61.1 ± 2.6</td>
<td>92 ± 13</td>
<td>28.5 ± 2.7</td>
<td>1.25 ± 0.1</td>
<td>−32.7 ± 1.9</td>
</tr>
<tr>
<td>P0–P5</td>
<td>23</td>
<td>572 ± 64*</td>
<td>−45.5 ± 2.0</td>
<td>77 ± 10</td>
<td>24.9 ± 1.2</td>
<td>2.69 ± 0.4</td>
<td>−6.01 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>P6–P10</td>
<td>21</td>
<td>358 ± 31*</td>
<td>−48.5 ± 1.9</td>
<td>91 ± 9</td>
<td>29.1 ± 3.4</td>
<td>2.20 ± 0.2</td>
<td>−9.02 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Reluctant firers</td>
<td>E15–E17</td>
<td>13</td>
<td>1.005 ± 117</td>
<td>−43.4 ± 1.8</td>
<td>65 ± 10</td>
<td>20.5 ± 1.2</td>
<td>2.67 ± 0.5</td>
<td>−5.7 ± 1.5</td>
</tr>
<tr>
<td>P0–P5</td>
<td>14</td>
<td>378 ± 58*</td>
<td>−56.0 ± 2.7#</td>
<td>151 ± 25*#</td>
<td>38.7 ± 3.6*#</td>
<td>1.12 ± 0.3</td>
<td>−24.5 ± 3.6*#</td>
<td></td>
</tr>
<tr>
<td>P6–P10</td>
<td>11</td>
<td>337 ± 44*</td>
<td>−54.7 ± 2.2*</td>
<td>145 ± 26*#</td>
<td>41.4 ± 2.2*#</td>
<td>0.75 ± 0.1*</td>
<td>−29.7 ± 2.3*#</td>
<td></td>
</tr>
<tr>
<td>P21–P25</td>
<td>7</td>
<td>531 ± 119</td>
<td>−49.7 ± 2.0</td>
<td>106 ± 19</td>
<td>42.6 ± 2.9*#</td>
<td>1.42 ± 0.1</td>
<td>−27.4 ± 2.6*#</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from E15–E17 (P < 0.05); †significantly different from P0–P5 (P < 0.05); ††significantly different from P6–P10 (P < 0.05).
only after P10 (8% for P11–P15 and P16–P20; 16% for P21–P25; Fig. 5, right). The \( I_{\text{Ca}} \) current was present at all ages studied, but never accounted for \( \geq 25\% \) of the sample (Fig. 5C, right). \( I_{h} \) was rarely identified in SDH neurons, with only three neurons exhibiting this current in the entire sample (1/48 in P11–P15 and 2/46 in P16–P20; Fig. 5D, right).

Since \( I_{\text{Ar}} \) was the dominant subthreshold whole cell current observed in SDH neurons at all ages studied (Fig. 5A), we next examined whether features of this current changed during development (Fig. 6). First, we confirmed the 4AP sensitivity of the \( I_{\text{Ar}} \) current in embryonic and P21–P25 neurons (Fig. 6A). Application of 5 mM 4AP reversibly abolished the amplitude of the \( I_{\text{Ar}} \) current in E15–E17 and P21–P25 neurons (\( n = 7 \) neurons for both ages). We next compared the magnitude and kinetics of \( I_{\text{Ar}} \) during development. Most notably, the peak amplitude of the \( I_{\text{Ar}} \) current increased steadily during development (85.6 ± 12.6 pA at E15–E17 vs. 289.7 ± 36.3 pA at P21–P25; Fig. 6B). Adultlike values were reached by P6–P10.
The decay time constant, fitted to the falling phase of the $I_{A}$ current, was unchanged during development (Fig. 6C). The steady-state current measured at the last 50 ms of the depolarizing step increased from around 5 pA in embryonic neurons and then stabilized at close to 40 pA after P10 (Fig. 6D). Finally, we compared the steady-state inactivation and activation profiles of $I_{A}$ in embryonic and adult neurons (Fig. 6E). The curves for both ages were generally similar, although $I_{A}$ in embryonic neurons was slightly more inactivated at hyperpolarized potentials and activated at more depolarized potentials ($n = 15$ for E15–E17 and $n = 17$ for P21–P25 neurons). Together, these data suggest expression levels of $I_{A}$ potassium channels increase during development, but the properties of these channels do not appreciably change over this period. $I_{A}$ is, however, slightly less potent around RMP in embryonic animals.

### Relationships between $I_{A}$ and AP discharge

To access the role of $I_{A}$ in shaping AP discharge we examined the relationship between the presence of $I_{A}$ and a neuron’s response to step- and pinch-current injection. Not surprisingly, because almost all embryonic neurons have $I_{A}$, there was not a strong relationship between the presence of $I_{A}$ and discharge category (tonic fired, 1/1 neurons; initial bursters, 6/7; single spikers, 24/25; reluctant firers, 9/10). In adults, however, only half the neurons expressed $I_{A}$ and specific relationships emerged between $I_{A}$ and various discharge categories (tonic fired, 5/9; initial bursters, 6/20; delayed firers, 7/12; single spikers, 5/7; reluctant firers, 3/3). These observations suggest $I_{A}$ plays a more prominent role in determining AP discharge in adult neurons. For pinch-current injections almost all embryonic neurons exhibiting robust and resistant responses also exhibited $I_{A}$ (30/31 and 10/11, respectively). In adult neurons, this relationship was weaker, with only 50% of either robust or resistant responders expressing $I_{A}$ (13/23 and 10/22, respectively). These observations suggest the expression of $I_{A}$ does not play an important role in determining the response of a neuron to pinch-current injection.

### DISCUSSION

This study examined a range of electrophysiological properties in mouse SDH neurons that shape and characterize neuronal output during late embryonic and early postnatal development. The study was driven by two considerations. First, significant changes occur in behavioral responses to noxious stimuli, the physiological properties of sensory neurons (DRGs), and in the anatomical pathways that carry or modify nociceptive information into the spinal cord during this period (Holmberg and Schouenborg 1996; Koltzenburg et al. 1997; Mirnics and Koerber 1995; Rajaofetra et al. 1989, 1992). Second, because mice have become increasingly popular for...
exploring the role of genes in pain mechanisms (Graham et al. 2007a; Harvey et al. 2004), it is important to know when the electrophysiological properties of SDH neurons can be considered stable or “adultlike” in this species. Our data show that many electrophysiological properties change in SDH neurons during the developmental period examined (E15–P25). Importantly, the majority of these changes occur on either side of P6–P10. We propose that mouse SDH neurons are embryonic or immature prior to P6, mature over a critical period P6–P10, and after P10 most electrophysiological properties are relatively stable and undergo only minor alterations during subsequent development.

Only one other study has used electrophysiological techniques to examine membrane properties of SDH neurons during postnatal development in rodents (Baccei and Fitzgerald 2005). This study, on rats aged P3, P10, and P21, concluded that some membrane and AP properties and the prevalence of the various discharge categories were stable throughout early postnatal development. The study also suggested tonic firing neurons constituted the major discharge category (>75% on neurons tested) in the three age groups studied. Based on these findings the authors proposed that the intrinsic excitability of SDH neurons did not change during the first 3 wk after birth. These rat data are at odds with those presented here for mice. In our study, a number of passive and active membrane properties changed dramatically during early development (Fig. 2). We also observed changes in the expression of each of the five discharge categories during development and tonic firers never comprised ≥20% of the sample in any age group (Fig. 3A). Moreover, expression of the four major ionic subthreshold whole cell currents known to shape AP discharge in adult SDH neurons also changed during this developmental period (Fig. 5). It is unclear why the differences between the two studies exist. Although these discrepancies may simply reflect differences between rats and mice there are, however, other explanations. For example, the rat study sampled fewer neurons (n = 20 vs. ≥49 for each age group), was undertaken at room temperature (23 vs. 32°C), used perforated patch (vs. whole cell) recording techniques, and recorded from more restricted locations in the SDH (laminae I–IIo vs. laminae I–II).

It is unclear whether these factors can adequately account for the differences between the two studies. Nonetheless, our data are consistent with developmental studies in other neuronal populations in rats, including cerebellar Purkinje cells (McClelland and Turner 2005), cortical neurons (Oswald and Greer 1999), motoneurons (Martin-Cabral and Greer 1999), and autonomic neurons (Anderson et al. 2001). In all these studies significant changes were observed in membrane properties during the first 3 to 4 wk after birth.

Development of synaptic connections and pain circuitry in the SDH

For pain circuits to mature and reach adult connectivity patterns several types of synaptic connections need to be established. These include connections: 1) made by the central processes of primary afferents; 2) between various “local” interneuron populations; and 3) with descending brain stem projections that modulate the SDH output (Millan 2002). Exactly when each of these connections is established will shape subsequent development and output of the SDH. Our data suggest that this connectivity will be established on a population of SDH neurons with changing intrinsic properties.

In mouse and rat, the central processes of both large myelinated A-fibers and small unmyelinated C-fibers reach the SDH well before birth at about E15 and E19, respectively (Munniks and Koerber 1995; Ozaki and Snider 1997). Extracellular recordings from rat SDH neurons show A-fibers establish functional connections and can generate APs in SDH neurons at P3, whereas C-fiber connections are not established until P10 (Jennings and Fitzgerald 1998). Our data, albeit from mice, show that during this period (i.e., before P10) a number of electrophysiological properties change in SDH neurons. Thus if spinal cord development is similar in rats and mice, A- and C-fiber connections on SDH neurons are establishing circuits when the intrinsic properties of their target neurons are changing significantly.

Information on the development of “local” connections between interneurons in the SDH is limited and available only for rats. In neonates (P2–P14) excitatory synaptic connections between SDH neurons are present 2 wk after birth (Bardoni et al. 2000; Li and Zhuo 1998). Evidence also exists for the establishment of inhibitory synaptic connections in the SDH soon after birth (P0–P7) (Baccei and Fitzgerald 2004). Thus it appears local excitatory and inhibitory connections are present or forming at P6–P10, the postnatal period we propose is critical for the development of intrinsic properties of SDH neurons.

Some data are available on the development of descending connections between brain stem and SDH neurons in rats. Serotonergic and noradrenergic terminals appear in the dorsal horn at P0 and P3, respectively, and reach the adult pattern by P21 (Rajaofetra et al. 1989, 1992). Electrophysiological studies have shown that no functional descending inhibition is present, at least in the deep dorsal horn, until P10–P12 (Fitzgerald and Koltzenburg 1986). Although such data are not available for SDH neurons in mice, the above-cited observations suggest connections between descending pathways and SDH neurons are being established, at least initially, on electrophysiologically immature neurons.

AP discharge categories and subthreshold whole cell currents during development

Our data show the distribution of AP discharge categories differ dramatically during late embryonic and early postnatal development. Before P10, single spikers account for >40% of the sample and delayed firers are rarely observed. After P10, initial bursters dominate (~30% of sample) and all five discharge patterns are observed. The proportions of each discharge category present after P10 were not statistically different from those previously reported by our laboratory for both in vitro (mouse aged P22–P59) and in vivo (P26–P42) studies (Graham et al. 2004, 2007b). These comparisons suggest the proportions of the five major discharge categories in mouse SDH neurons are relatively stable after P10.

The functional significance of the change in AP discharge categories during development is unclear. Some have proposed that single spikers, which discharge APs with an extremely short latency from stimulus onset (Fig. 3D), are ideally suited for signaling the onset of noxious peripheral stimuli (Prescott and DeKonick 2002). Thus the high proportion of single
spikers in animals before P10 may easily drive the exaggerated responses to hindpaw pinching previously observed in neonates (Fitzgerald and Gibson 1984; Stelzner 1971). Delayed firers, in contrast, have a substantial latency between stimulus onset and AP discharge (Fig. 3C) and consequently have been described as “integrators” (Ruscheweyh and Sandkühler 2002). As such, delayed firers could signal the presence of additional noxious stimuli in multimodal receptive fields. Our findings show that delayed firers constitute a significant proportion of the SDH neuron population after P10. Interestingly, this corresponds with the emergence of high-threshold C-fiber responses in the dorsal horn of rats (Jennings and Fitzgerald 1998). It is possible that the addition of C-fiber sensory information about skin irritation and inflammation (Fitzgerald and Gibson 1984) requires alternative processing within the SDH, especially by neurons, like delayed firers, which are capable of “integrating” information from different sensory modalities.

To our knowledge, this study is the first to examine the development of major subthreshold currents that shape the various AP discharge patterns in SDH neurons. Our major finding is that the fast A-type potassium channel (IA) is the dominant current in SDH neurons regardless of age (Fig. 5). Over 90% of neurons express this current before birth, but levels never drop to <50% over the period examined. Moreover, the expression levels of IA, potassium channels increase during development (Fig. 6B). The other A-type potassium current, IAS, is also developmentally regulated, but is not expressed before P10. The T-type calcium current is present in 5–20% of neurons at all stages, but appears to be stable over this period. Developmental changes in IAS currents have been observed in other CNS neurons. For example, IA, gradually decreases during development in striatal and hippocampal neurons (Costa et al. 1994; Deng et al. 2004), is only transiently expressed at P4–P9 in abducens motoneurons (Russev et al. 2003), and increases during development of cerebellar granule cells (Wakazono et al. 1997). Thus the specific developmental regulation of IA, differs among neuronal populations and may be related to the final, or adult, discharge properties required of neurons within a given circuit.

In addition, some of the above-cited studies suggest that neurons where IA, is developmentally regulated, the subunit composition, and thus kinetics of IA, channels, also change during development (Costa et al. 1994; Deng et al. 2004). If we assume the IA, decay time constant, which remains stable during development in mouse SDH neurons (Fig. 6C), reflects subunit composition of IA, channels then our data suggest a relatively constant subunit composition of IA, during development. In mouse SDH, a significant proportion of A-type currents is due to expression of the Kv4.2 gene. Modulation or elimination of this current in Kv4.2 knockout mice dramatically alters nociceptive behavior and enhances sensitivity to both thermal and tactile stimuli (Hu et al. 2006). In adult animals IA, delays AP firing and reduces neuron excitability (Ruscheweyh and Sandkühler 2002). Our data suggest that in addition to its important role in processing peripheral stimuli, IA, may also be critical for the initial establishment of SDH circuits.

Our data also show the slow A current (IAS) is developmentally regulated and not present until P10. In adult rat SDH neurons this current is thought to underlie “gap firing,” a form of AP discharge characteristic of projection neurons (Ruscheweyh et al. 2004). Because 5–10% of lamina I neurons are projection neurons (Spiske et al. 2003) and some labeling studies show that projection neurons can in fact lie in lamina II (Al-Khater et al. 2008; Bice and Beal 1997), it is possible that some of our IA,-expressing neurons were projection neurons. If this is so, then the maturation of this important neuronal population that relays SDH output to higher brain centers is not complete until at least P10.

Relating the expression of the major subthreshold currents we observed in SDH neurons with the various AP discharge categories remains a challenge. The dominance of IA, in young SDH neurons, which reportedly underlies delayed firing (Ruscheweyh and Sandkühler 2002), is inconsistent with the later appearance of delayed firing neurons after P10. One explanation might be that a certain level of IA, expression is needed before the delayed firing pattern becomes apparent. Additionally, young neurons maintain a more depolarized membrane potential (Fig. 2B), which would tend to reduce the effectiveness of IA, (Graham et al. 2007c). This is consistent with many factors interacting with subthreshold currents to ultimately determine AP discharge profile.

Conclusion and functional implications

Both behavioral and extracellular analyses suggest the dorsal horn is hyperexcitable during the early stages of postnatal development (Andrews and Fitzgerald 1994; Holmberg and Schouenborg 1996). Some of our observations regarding SDH neurons during development support this view. For example, SDH neurons exhibit higher input resistances and are more depolarized in neonates (Fig. 2). Together, these factors would make SDH neurons more easily excited. Other findings, particularly the abundance of fast A-type potassium currents in neonates (<P10), would in isolation reduce the level of excitability in the SDH. It must be remembered, however, that behavioral responses to nociceptive stimuli are measured as motor responses: i.e., the flexion withdrawal reflex. The exaggerated reflexes observed in neonates may in fact mean nociceptive pathways are initially set up to provide input to motoneurons, whereas connections with upstream pain circuitry are established later in development to provide complex processing of sensory experiences. These considerations emphasize how little is known about the detailed synaptic mechanisms and circuitry in the dorsal horn of the spinal cord (Lu and Perl 2005). Future studies that permit the study of both behavioral and subthreshold synaptic events in the SDH during development using in vivo preparations may help resolve these issues.

GRANTS

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REFERENCES


