Heterogeneity of membrane properties in sympathetic preganglionic neurons of neonatal mice: Evidence of four subpopulations in the intermediolateral nucleus

Amanda Zimmerman¹, Shawn Hochman²*

¹Department of Biomedical Engineering, Emory University/Georgia Institute of Technology
²Department of Physiology, Emory University

*Correspondence: Shawn Hochman, Ph.D.
Whitehead Biomedical Research Building, Room 644
Emory University School of Medicine
615 Michael St., Atlanta GA 30322 U.S.A.
Ph: (404) 712-3131 Fax (404) 727-2648
Email: shawn.hochman@emory.edu

Keywords: Hb9, autonomic, IML, spinal cord
Abstract

Spinal cord sympathetic preganglionic neurons (SPNs) integrate activity from descending and sensory systems to determine the final central output of the sympathetic nervous system. The intermediolateral column (IML) has the highest number and density of SPNs, and within this region SPN somas are found in distinct clusters within thoracic and upper lumbar spinal segments. While SPNs exhibit a rostrocaudal gradient of end-target projections, individual clusters contain SPNs with diverse functional roles. Here we explored diversity in the electrophysiological properties observed in Hb9- eGFP-identified SPNs in the IML of neonatal mice.

Overall, mouse SPN intrinsic membrane properties were comparable to those seen in other species. A wide range of values was obtained for all measured properties (up to a 10 fold difference), suggesting that IML neurons are highly differentiated. Using linear regression we found strong correlations between many cellular properties, including input resistance, rheobase, time constant, action potential shape, and degree of spike accommodation. The best predictor of cell function was rheobase, which correlated well with firing frequency – injected current (f-I) slopes as well as other passive and active membrane properties. The range in rheobase suggests that IML neurons have a recruitment order with stronger synaptic drives required for maximal recruitment. Using cluster analysis, we identified at least four subpopulations of SPNs, including one with a long time constant, low rheobase, and high f-I gain. We propose, therefore, that the IML contains populations of neurons that are differentiable by their membrane properties and hypothesize they represent diverse functional classes.
**Introduction**

Sympathetic preganglionic neurons (SPNs) integrate activity from descending and sensory systems to determine the final central output of the sympathetic nervous system. The ILp (also known as the intermediolateral column or nucleus (IML)) has the highest number and density of SPNs (Petras and Cummings 1972; Rando et al. 1981), and within this region SPN somas are found in distinct clusters in each spinal segment. Their dendrites are mainly oriented rostrocaudally within the lateral funiculus and to a lesser extent medially within the grey matter toward the central autonomic area in lamina X, thus forming a ladder-like distribution symmetric around the central canal (Anderson et al. 1989; Sah and McLachlan 1995). SPNs are segmentally organized and exhibit a rostrocaudal gradient of end-target projections, yet individual clusters contain SPNs with diverse functional roles (Forehand et al. 1994).

The cellular physiological properties of SPNs that lie in the IML have been investigated in rats, guinea pigs, and cats to some extent (Dembowsky et al. 1985; Gilbey and Stein 1991; Inokuchi et al. 1993; Pickering et al. 1991; Sah and McLachlan 1995; Spanswick and Logan 1990b), largely using thick transverse (400-500 μm) slices in vitro. Action potentials are notable for long afterhyperpolarizations mediated largely by Ca$^{2+}$ dependent transient- and sustained K$^+$ conductances. Other conductances observed include: a fast 4-AP-sensitive and slower Ba$^{2+}$-sensitive transient outward rectifier (A- and D- type respectively), an atypical K$^+$- mediated sustained outward rectifier with insensitivity to Cs$^+$ and TEA, an anomalous inward rectifier, and a low-voltage activated T-type Ca$^{2+}$ conductance (Miyazaki et al. 1996; Sah and McLachlan 1995; Wilson et al. 2002). While IML SPNs are traditionally treated as a homogenous group, there are some notable electrophysiological differences. Spontaneous activity has been observed in a subset of SPNs in the neonatal rat and adult guinea pig, and is sometimes rhythmic
(Spanswick and Logan 1990a). Additionally, strong electrical interactions have been observed in a subpopulation of SPNs, resulting in a low input resistance in these neurons (Logan et al. 1996). Lastly, a number of investigators report mixed actions of the monoamines on SPNs (Gilbey and Stein 1991; Gladwell and Coote 1999; Yoshimura and Nishi 1982; Yoshimura et al. 1987c; d), suggesting different populations may have different receptor configurations.

Recently, an enhanced green fluorescent protein (eGFP) labeled transgenic mouse (JAX laboratories) has been generated that identifies SPNs based on coupled expression to the HB9 homeodomain protein (Wilson et al. 2005), greatly facilitating ease of identification for electrophysiological and histochemical analyses. The current study represents the first characterization of membrane properties of SPNs in this mouse model, and provides the first detailed appraisal of SPN repetitive firing properties. Lastly, we propose a novel classification scheme to differentiate SPN populations based on their electrophysiological properties.

A portion of this data was previously reported in abstract form (Zimmerman and Hochman 2008).
Materials and Methods

All procedures described here comply with the principles of The Care and Use of Animals outlined by the American Physiological Society and were approved by the Emory University Institutional Animal Care and Use Committee.

Electrophysiology and Slice Preparation

All experiments were performed in transgenic mice expressing HB9-eGFP (JAX laboratories; known to label SPNs), postnatal day 3-9. Animals over age p6 were anesthetized with 10% urethane (2mg/kg ip) and placed on ice to slow the heart rate. All animals were decapitated, eviscerated, and the spinal cords removed. The T8-L2 section of the spinal cord was isolated and sliced into thick transverse (400 μm) and thin horizontal (200 μm) sections using a vibrating blade microtome (Leica VT1000 S). Initial removal of the spinal cord and slicing was performed in cooled (4 °C), oxygenated (95% O₂, 5% CO₂) solution containing (in mM) 250 sucrose, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 1.25 NaH₂PO₄, and 26 NaHCO₃, pH 7.4. Slices were left to recover for at least 1 hour.

The recording chamber was continuously perfused with oxygenated ACSF (in mM: 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 25 D-glucose, 1.25 NaH₂PO₄, and 26 NaHCO₃; pH 7.4) at a rate of ~2ml/ minute. Patch clamp recordings were made from fluorescently-identified SPNs with patch pipettes of resistance 4-8 MΩ. The standard intracellular recording solution contained (in mM): K-gluconate, 140; EGTA, 0.2; HEPES, 10; Mg-ATP, 4; tris-GTP, 1; pH, 7.3. GTP and ATP were included in pipettes to prevent rundown of evoked currents. When assessing the effects of intracellular Cs⁺, the intracellular solutions contained 140 CsF, 11 EGTA, 35 KOH, 10 HEPES, 1 CaCl₂, pH 7.3.
Whole cell patch-clamp recordings were undertaken at room temperature using the Multiclamp amplifier (Molecular Devices, Sunnyvale, CA). eGFP⁺ SPNs were identified using epifluorescent illumination and their location in the intermediolateral column using differential-interference contrast optics (DIC). Voltage- and current-clamp data were acquired on the computer using pClamp 10 acquisition software (Molecular Devices).

Quantification of membrane properties

Immediately after rupture of the cell membrane (in voltage clamp at –90 mV), the current-clamp recording configuration was used to determine resting membrane potential. Junction potential was corrected for after recording, experimentally derived previously to be 10mV (MacLean et al. 1997). In current clamp configuration, electrode resistance was compensated for, and ranged from 8-15 MΩ. Unless otherwise noted, cells were brought to -70 mV holding potential by injecting bias current, and a series of hyperpolarizing and depolarizing current steps 1 second in duration were applied. The membrane time constant (τ_m) was found by fitting the first 500ms of the membrane charging response to small hyperpolarizing current steps with one or two exponentials. In cases where the data was better fit with two exponentials, the longest exponential was used as τ_m as suggested by Rall (Rall 1969) and previously calculated in SPNs (Pickering et al. 1991; Wilson et al. 2002). Additionally in these cases, the equivalent cylinder electrotonic length L was estimated using the formula: 

\[ L = \pi \left( \frac{\tau_0}{\tau_1} - 1 \right)^{-1/2} \]

and τ_1 refers to the first equalizing time constant (Rall 1969). τ_m was averaged for hyperpolarizing current steps causing a change in membrane potential less than 20mV. Current-voltage (I-V) plots were generated from voltage clamp recordings. Electrode series resistance was uncompensated in voltage clamp recordings but was subtracted in current clamp recordings. Series resistance values ranged between 15-38 MΩ. As these values are one to two orders of
magnitude less that measured membrane resistance, the uncompensated voltage drop across the
electrode should not introduce significant error. In voltage clamp, cells were held at -90 mV and
a series of voltage steps (-140 mV to 0 mV, 500 ms duration) were applied. The input resistance
\( R_{in} \) was calculated by fitting a portion of the steady state I-V curve slightly negative to resting
membrane potential (-70 to -90mV) with a straight line. Membrane capacitance \( C_m \) was
determined from Multiclamp Commander automatically, by fitting the capacitive transient to a
brief 10mV voltage step and using the formula: \( \tau_m = \frac{1}{R_{in} \times C_m} \). Peak inward current was
measured as the maximal transient inward current obtained following voltage steps.

Active properties were averaged for all spikes at the lowest spike triggering current step.
The threshold voltage \( V_{th} \) was determined by detecting the maximum 2\(^{nd}\) derivative in phase
space (dVm/dt versus \( V_m \)) for each spike (Sekerli et al. 2004). The spike amplitude and
afterhyperpolarization (AHP) magnitude was taken from this voltage threshold, and the spike
overshoot calculated as the portion of the spike above zero mV. Duration of the action potential
was measured as the time above one-third of the amplitude (Wilson et al. 2002). Duration of the
AHP was measured as the time below one-tenth of the AHP magnitude. Rheobase was the
minimum current injection required to elicit a spike. For frequency-current (\( f-I \)) analyses, both
mean frequency and instantaneous frequency (based on the interspike interval between the first
two spikes) were found. Data from neurons not showing electrode compensation in current
clamp were discarded.

**Statistical Analysis**

All parameter values are reported as mean ± S.D. Matlab software was used to compute
correlation coefficients between membrane properties, and to determine p-values for each
correlation. Unless otherwise noted, only those with p<0.05 were used. For some parameters
with statistically significant correlations, linear regression with a least-squares fit was computed for either a straight line, \(y=mx+b\), or logarithmic line, \(y=bm^x\).

Cluster analysis was performed using a Partition Around Medoids (PAM) method (Kaufman and Rousseeuw 1990) from Libra: a MATLAB Library for Robust Analysis. In short, the PAM method minimizes the sum of dissimilarities between data points, to partition data into \(k\) clusters. This algorithm was run on \(k=2-10\) clusters, with each parameter normalized and centered. Both the Calinski-Harabasz (Calinski and Harabasz 1974) and Silhouette (Kaufman and Rousseeuw 1990) indices were calculated for each cluster number, each giving a weighted comparison between intra- and inter-cluster differences. These indices were maximized to determine the optimal number of data clusters.
Results

General membrane properties

No significant difference in membrane properties was seen between thin horizontal and thick transverse slices, so the data was combined. Data were obtained from 39 neurons and membrane properties quantified as described in the methods section. Their properties are summarized in Table 1. The mean resting potential ($V_{\text{rest}}$) was -60 ± 7 mV, ranging between -44 and -85 mV. The input resistance ($R_{\text{in}}$) was 1.1 ± 0.6 GΩ, ranging from 260 MΩ to 2.6 GΩ with an approximately normal distribution but with a greater spread in high resistance values (not shown). The mean membrane time constant ($\tau_m$) was 92 ± 44 ms, ranging between 36 to 184 ms, with an apparent bimodal distribution (not shown). Frequently, voltage responses to large current pulses were well fit with single exponentials, yet smaller current steps were better fit with double exponentials, also seen in the neonatal rat (Pickering et al. 1991). For those charging curves where double exponential fits were easily distinguished, electrotonic length $L$ was estimated to be 1.83 ± 0.27 (n=17). $R_m$ correlated well with both $\tau_m$ ($\rho = 0.65$, $p=0.001$) and $C_m$ ($\rho = -0.50$, $p=0.01$) indicating that variations in both membrane resistivity and cell size account for much of the range of resistances seen.

The relationships among and between active and passive membrane properties were quantified (Figure 1). A color-coded correlation matrix compared significance of correlations among the membrane properties measured. The best predictor of cell function was rheobase, which accounted for 10.5% of the variance seen. Rheobase was positively correlated with threshold voltage ($V_{\text{th}}$), negatively correlated with $R_{\text{in}}$, $\tau_m$, and peak inward current ($I_{\text{peak}}$), and weakly negatively correlated with both mean and instantaneous firing frequency – injected current ($f$-$I$) slopes, which fell just shy of statistical significance ($\rho=-0.33$, $p=0.08$ for both). The
early peak inward current (presumably Na\(^+\) dominated) contributed greatly to the cell’s active properties, as \(I_{\text{peak}}\) was inversely correlated to both \(V_{\text{th}}\) and rheobase. Additionally, action potential (AP) height was inversely correlated to AP width and directly correlated to \(V_{\text{th}}\), further highlighting the important role of Na\(^+\) channel kinetics in SPN behavior. Lastly, the pronounced AHP magnitude was directly correlated to both mean and instantaneous \(f-I\) slopes, suggesting a strong modulatory role of the underlying currents on SPN excitability.

While not shown in the figure, age of mouse used was also a factor, showing a strong positive correlation with \(I_{\text{peak}}\) (\(\rho=0.73, \ p=3\times10^{-5}\)), a weaker positive correlation with AHP magnitude (\(\rho=0.43, \ p=0.05\)), and a negative correlation with \(\tau_m\) (\(\rho=-0.40, \ p=0.03\)). This would suggest that as the mouse ages, the density of voltage gated Na\(^+\) and K\(^+\) channels increases and membrane resistivity decreases, a pattern supported by motoneuron research (O’Dowd et al. 1988; Viana et al. 1994).

**Anomalous Inward Rectification**

We next examined evidence of voltage-gated channels observed in these neurons compared to those reported previously in guinea pig and rat. In current clamp mode, a number of SPNs exhibited an inward rectification or a fall in input resistance in response to larger hyperpolarizing current steps. This rectification was further explored and quantified in voltage clamp. In response to 500 ms voltage steps (-130 to 0 mV, 10 mV steps), 24/38 SPNs (63%) exhibited an increased conductance (mean change 492 pS) at membrane potentials less than -80mV (Figure 2B). This conductance was instantaneous and sustained, and consistent with that seen in the neonatal rat (Wilson et al. 2002).
Transient Outward Rectification

As seen in the guinea pig and rat (Inokuchi et al. 1993; Miyazaki et al. 1996; Wilson et al. 2002), all neurons displayed a transient outward rectification. This could be seen in current clamp as either a delayed return to resting membrane potential from hyperpolarizing current steps (10-40pA, 1 s duration; Fig 2A), or as a delay in time to fire the first action potential with depolarizing current steps from a hyperpolarized membrane potential of -90 mV (Fig 2Aii). This was further investigated in voltage clamp configuration, where voltage steps (500 ms, 10 mV steps) were applied from a hyperpolarized holding potential (-90 mV). An outward transient current was observed with mean onset of -50.0 ± 6.7 mV, and always with a lower threshold than the sodium spike (Fig 2B). Decay was best fit by double exponentials, with the longest tau at onset of 160 ± 52 ms (Fig 2C). At least a portion of this transient outward current noticeably persisted when Cs⁺ replaced K⁺ in the intracellular solution (n=6; inset Fig 2B).

Repetitive Firing

In current clamp from a -70mV holding potential, 90% (39/43) of SPNs fired repetitively over a wide range of current injections, with the remaining 4 displaying an initial burst or single spike phenotype. Frequency-current (f-I) relationships were measured for both instantaneous and mean firing frequency at each current step. SPN f-I instantaneous slopes had a mean value of 0.228 ± 0.125 Hz/pA with lower values for mean f-I slope (0.196 ± 0.106 Hz/pA). Peak firing frequencies in individual neurons reached up to 28 Hz before depolarization block occurred.

Of SPNs firing repetitively, 70% (21/30) displayed spike frequency adaptation (SFA), or a slowing of the firing rate with long current steps (Fig 3A). SFA was best fit by a logarithmic linear regression (Fig 3B) and only cells with established SFA are shown (fits significantly different from no correlation, p<0.05). The averaged slope (m) of the logarithmic fit at each
current step had a mean value of 0.95 ± .02. This slope was inversely correlated with input resistance, i.e. the greater \( R_{\text{in}} \), the more rapidly spike frequency declined (Fig 3C).

### Persistent Inward Current

When intracellular K\(^+\) was replaced with Cs\(^+\) to block most voltage-gated K\(^+\) conductances, the steady state current-voltage plot revealed a region of negative slope conductance (Fig 4A). This negative slope region indicates the presence of a persistent inward current (PIC) (Harvey et al. 2006). Net inwards currents were absent in several neurons at least partly due to the presence of an outward leak conductance, but could be easily calculated as a deviation from linear leak slope (see Fig 4B). The persistent inward current in the presence of Cs\(^+\) had an average onset of -76 ± 5 mV and peak magnitude 21.6 ±13.5 pA (n=8). In comparison, with K-gluconate intracellular solution, the effects of the PIC were largely hidden by the dominating contribution of activated outward currents during voltage steps, but could be detected during a slow voltage ramp (8mV/s), as a slight deviation from the linear leak slope (Fig 4C, n=2/2).

### Cluster Analysis

Given the wide range of membrane properties recorded, we wondered whether SPNs could be classified into electrophysiological clusters. Using cluster analysis of the parameters measured for each cell and the maximum of the silhouette and Calinski-Harabasz indices, data was best sorted into four clusters (Fig 5A). A one-way ANOVA was performed on each parameter, resulting in statistically significant differences between clusters in \( \tau \), rheobase, \( f-I \) slopes, AP width, and \( I_{\text{peak}} \) (Fig 5B). The mean values are summarized in Table 2. The four groups are as follows: Group 2 and 3 SPNs are recruited first (have lower rheobases), have relatively long \( \tau_{\text{mS}} \) and mid-range \( I_{\text{peak}} \) values. Group 2 neurons have lower \( f-I \) gains and longer
AP durations, while Group 3 SPNs have higher f-I gains and shorter AP durations. Group 1 and 4 SPNs are then sequentially recruited, with group 1 SPNs having the largest and group 4 having the smallest I_{peak} values of all groups.
Discussion

Using eGFP-HB9-transgenics the present study undertook the first characterization of membrane electrical properties of sympathetic preganglionic neurons in the thoracolumbar intermediolateral nucleus of mouse. Studies were undertaken in either thicker transverse or thinner horizontal slices and membrane properties in these populations were indistinguishable. Given the strong rostrocaudal and mediolateral orientation of SPN IML dendrites, horizontal sections would be predicted to provide neurons with largely intact architecture. However, while mediolateral dendrites are strongly present in utero (Phelps et al. 1993), rostrocaudal projections have a relatively later maturation, (2 weeks postnatal; Ezerman and Forehand 1996; Markham et al. 1991), perhaps minimizing the level of dendrotomy in transverse slices at this age. Consequently, it is likely that the SPNs recorded in transverse sections retained considerable rostrocaudal dendrites.

A correlation matrix was used to identify relationships between active and passive membrane properties. One important observation was that rheobase - the amount of current required to recruit a neuron - was the best predictor of cell group and correlated with several other membrane properties. Given the obvious importance of SPN membrane excitability as the ‘final common CNS output’ of sympathetic neural activity, we also undertook a detailed examination of their firing properties. SPNs consistently demonstrated spike-frequency adaptation. In addition, the relation of firing frequencies to magnitude of current injection (f-I relations) generated slopes that varied considerably across the SPN population, indicating that SPNs represent a highly differentiated class of neurons. Indeed, cluster analysis subdivided this nucleus into four subpopulations. The rostrocaudal range (T8-L2) sampled from potentially includes SPNs with five different end-target innervations (adrenal medulla, celiac ganglion,
aorticorenal ganglion, superior mesenteric ganglion, and inferior mesenteric ganglion; Strack et al. 1988). While outside the scope of the current study, it is possible this electrophysiological classification is influenced by end target differentiation, and warrants further investigation. Regardless, the overall conclusion is that this population of output neurons constitutes a heterogeneous population, differentiated by their electrophysiological properties, with complex recruitment properties.

Comparison to membrane properties reported in other species

Membrane properties measured here compare well with those reported previously in other mammalian species. Resting membrane potential values were similar to those reported in the neonatal rat, guinea pig, and adult cat (Dembowsky et al. 1986; Inokuchi et al. 1993; Pickering et al. 1991; Yoshimura and Nishi 1982). Since impalement-induced leak conductance with sharp microelectrodes alters passive membrane properties (Staley et al. 1992), our whole-cell patch recordings can only be compared to patch-clamp recordings as reported in the neonatal rat (Miyazaki et al. 1996; Pickering et al. 1991; Wilson et al. 2002). Similar input resistance ($R_{in}$), membrane capacitance ($C_m$), and membrane time constant ($\tau_m$) values were observed [(Miyazaki et al. 1996; Pickering et al. 1991; Wilson et al. 2002); but see (Wilson et al. 2002) for temperature-dependent differences in $R_{in}$]. The presence of multiple exponentials in the membrane charging curves in a number of neurons here was also reported in the neonatal rat (Pickering et al. 1991; Wilson et al. 2002). Multiple exponential responses are indicative of initial non-uniform distribution of membrane potential, likely due to a complex dendritic tree. Values obtained for $L$ are much greater than those found in patch-clamp recordings from CA3 pyramidal neurons (Major et al. 1994) and even somatic motoneurons (Thurbon et al. 1998), known to have a very extensive dendritic arbor (Rekling et al. 2000). This suggests SPNs are not
as electrically compact and their ability to integrate synaptic input from distal dendrites may be comparatively weak. The functional consequences of this are currently unknown.

The use of neonatal animals in the current study may be subject to criticism, as the sympathetic nervous system of rodents is still maturing at this young age (Black 1978). While end-target responses to central sympathetic activation are not present until after the first week postnatal, level of tonic SPN activity and response to asphyxia and hypoglycemia in the neonate (1-2 days postnatal) were comparable to those in the adult rat (Smith et al. 1982). Additionally in the rat, as early as embryonic day 14.5 SPNs are already positioned in the IML, central autonomic region, and areas in between (Kubasak et al. 2004; Phelps et al. 1993). At birth, SPNs in the IML have the characteristic ladder-like rostrocaudal and mediolateral projecting dendritic arbor, with the rostrocaudal dendrites elongating and cluster separation increasing during the first two weeks (Ezerman and Forehand 1996; Markham et al. 1991; Phelps et al. 1984). Since biochemical markers of synaptic activity and synaptic connections in sympathetic ganglia in the mouse greatly increase during this period (Black et al. 1971), electrophysiological differentiation may play a role in forming appropriate synaptic connections. Interestingly, the similarity of the above mentioned electrophysiological properties in the neonatal mouse to adult cats and more mature rats suggests that while development may affect the size of SPNs and magnitude of conductances, the overall functional aspects of SPNs are largely in place in the neonate. Indeed, while this study used animals with an overlapping age range as undertaken in rat (as young as P7), we also include even younger animals (P3) to demonstrate that SPN membrane properties are specified at a very early age. Moreover, cluster analysis was able to separate the IML neurons into at least 4 discrete groups irrespective of age. This is consistent with anatomical
findings that morphology is also highly differentiated at birth (Phelps et al. 1984; Pyner and Coote 1994), supporting an early maturation of the IML SPN neuronal phenotype.

**Active conductances**

A transient outward rectification was present in virtually all SPNs as seen previously (Dembowsky et al. 1986; Inokuchi et al. 1993; Miyazaki et al. 1996; Pickering et al. 1991; Sah and McLachlan 1995; Wilson et al. 2002). This transient conductance was partially insensitive to intracellular Cs\(^+\), inactive at resting membrane potential, and only released from inactivation with membrane hyperpolarization. In voltage clamp, decay was best fit with double exponential decay, consistent with the dual component A-type K\(^+\) conductances noted by Wilson et al (Wilson et al. 2002). In neonatal rat SPNs this current acts to regulate firing frequency and contributes to spike repolarization and the afterhyperpolarization (Miyazaki et al. 1996). Hyperpolarizations from resting membrane potential evoked inward rectification in most SPNs, and were sensitive to intracellular Cs\(^+\). The conductance is similar to the anomalous rectification recorded in other SPNs (Inokuchi et al. 1993; Miyazaki et al. 1996; Pickering et al. 1991), and may act to return SPNs to an excitable membrane potential after large inhibitory input.

Blockade of most K\(^+\) conductances with Cs\(^+\) revealed the presence of a persistent inward current (PIC). In somatic motoneurons, PICs are thought to be responsible for repetitive firing and membrane bistability (Kuo et al. 2006; Lee and Heckman 1998). In our acute spinalized mouse SPNs, the PIC magnitude was usually small enough to be largely masked by outward K\(^+\) conductances with a K-gluconate based intracellular solution. This may be due to the loss of descending monoaminergic input, which greatly facilitates PICs in motoneurons (Hounsgaard and Kiehn 1989; Lee and Heckman 1999). Given the strong descending monoaminergic
projections to the IML, it is therefore possible that SPNs also possess the ability for bistable
membrane behavior.

Repetitive firing and spike frequency adaptation

Neonatal mouse SPNs showed repetitive firing over a wide range of current injections. Compared to intracellular recordings in guinea pigs and cats, instantaneous firing rates and \( f-I \) slopes were much greater (Dembowsky et al. 1986; Inokuchi et al. 1993). This is likely at least partly due to a reduced leak conductance in patch clamp recordings as compared to conventional sharp intracellular recordings (Staley et al. 1992). Compared to somatic motoneurons, SPN \( f-I \) gain exceeded that in both the primary and secondary firing range by 10 fold (Brownstone 2006) but this is also likely a reflection of markedly increased input resistance observed with patch recordings. Indeed, in patch clamp recordings in putative mouse motoneurons in culture, \( f-I \) gains and variability were remarkably similar (Kuo et al. 2006). The strong correlation between the AHP magnitude and \( f-I \) gain found in the present study supports a functional role of the AHP (and underlying conductances) in controlling cellular excitability. Modulation of the AHP, such as that in response to noradrenaline in both the cat and rat (Sah and McLachlan 1995; Yoshimura et al. 1987a) and caffeine in the rat (Shen et al. 1994), could lead to direct changes in SPN response to synaptic input.

In a majority of neonatal mouse SPNs, we describe a pronounced spike frequency adaptation (SFA). SFA in SPNs has been reported previously (Dembowsky et al. 1986; Sah and McLachlan 1995), but has not been rigorously explored. In contrast, the mechanisms serving SFA have been detailed in mouse motoneurons (Miles et al. 2005). In this study, modeling and patch clamp studies suggest that slow inactivation of the fast inactivating Na\(^+\) conductance is a key factor in SFA (Miles et al. 2005). This work contrasts previous notions on the primary
importance of the AHP (see Discussion in Miles et al. 2005). The physiological relevance of SFA in motoneurons has been interpreted in relation to initial versus sustained force generation in muscle (Stein and Parmiggiani 1979). Analogously, the relevance of SFA in SPNs may relate to the recruitment of postganglionic neurons. In our neonatal mouse SPNs, SFA decay was inversely related to $R_{in}$. Thus, the smallest conductance neurons underwent the greatest SFA. Whether these neurons innervate a different population of postganglionic neurons or have differences in synaptic transmission remains to be determined.

The range of rheobase, $R_{in}$, and degree of SFA observed in SPNs could signify an organizational principle of recruitment with functional significance. For example, somatic motoneurons exhibit a well-defined order of recruitment via the size principle, whereby motoneurons are recruited with increasing size, conduction velocity, and motor unit fatigability (Henneman 1985; Rekling et al. 2000). In fact, lumbar SPNs in the adult cat have distinct differences in conduction velocity, responses to afferent stimuli, and voltage intensity for axonal recruitment based on their end-target innervations (Jänig and Szulczyk 1981).

SPNs normally fire at low frequencies (e.g. usually <1 Hz with peaks of 10 Hz; see McLachlan 2003) so it is worth questioning whether $f$-$I$ curves are physiologically relevant at the higher range of firing frequencies. Peak firing frequencies observed here clearly exceed these steady state values, and many SPNs were not driven to their maximum firing potential. One possibility is that higher firing frequencies are reached during ischemia, drops in blood pressure and states of higher arousal such as the ‘fight or flight’ response. SPNs receive dense modulatory inputs from both brainstem and hypothalamic autonomic circuits (Anderson et al. 1989; Björklund and Skagerberg 1979; Fleetwood-Walker and Coote 1981; Loewy 1981), many of which could greatly increase their excitability on a systematic level. Indeed, addition of
norepinephrine and serotonin has been shown to increase spontaneous discharge in SPNs in the neonatal rat (Lewis et al. 1993; Marks et al. 1990; Shen et al. 1994) and adult cat (Gilbey and Stein 1991; Yoshimura et al. 1987b), often in a bursting rhythm with intraburst frequencies greatly exceeding steady state values.

Individual SPNs project to many postganglionic neurons (1:15 ratio in rat) and postganglionic neurons are innervated by multiple SPNs. The existence of both convergent and divergent synaptic inputs forming the postganglionic ‘autonomic motor unit’ indicates the importance of synaptic integration in their recruitment. Hence as a population, SPN firing properties will be critical in the temporal and spatial summation necessary to activate postganglionics. There appear to be two populations of SPNs based on synaptic strength on postganglionics - strong and weak - with strong synapses lacking P type Ca^{2+} channels and evoking currents individually capable of recruiting postganglionics (McLachlan 2003). Thus, recruitment of postganglionics may only require the activity of individual SPNs. Conversely, weak inputs from multiple SPNs may also be used to recruit postganglionic neurons. The relation between synapse strength and membrane properties remains to be determined. However, the initial high frequency firing of SPNs could act to potentiate synaptic transmission of weak synaptic connections while the slower firing induced by SFA could act to maintain potentiation without neurotransmitter depletion (MacLachlan 1975).

In conclusion, the present study suggests that SPNs in the IML are comprised of multiple subtypes, easily distinguished by electrophysiological parameters. We hypothesize that generation of target- and condition- specific responses of the sympathetic nervous system is largely derived from electrophysiological differentiation. The easy visualization of SPNs afforded by their genetic labeling with Hb9-eGFP in transgenic mice allows for coupling future
studies of electrophysiological results with immunohistochemistry, anatomy, and functional genomics for further exploration.
Acknowledgements

We would like to thank Michael Sorenson for readabf, and Kaijun Wang for the Cluster Validity Analysis Platform, Matlab programs used in this analysis.

Grants

This work was supported by National Institutes of Health Grant NS045248.
References


Figure Legends

Figure 1. Membrane property correlations. Figure shows correlation coefficients between membrane properties, with $\rho$ values close to 1 showing strong positive relationships and $\rho$ values close to -1 showing strong negative relationships. $f$-I slope refers to instantaneous firing frequency. *s denote statistically significant correlations ($p<0.05$) and ?s denote correlations with $0.05<p<0.1$.

Figure 2. Transient outward and anomalous rectification. Ai) Sample membrane response to a series of current steps (1s duration, -15 to 10 pA, 5 pA steps), holding current -12.6 pA. * indicates anomalous inward rectification, □ indicates transient outward rectification, seen as a much longer repolarization time to hyperpolarizing current steps. Aii) Sample voltage response to a series of injected current pulses (1s duration, 10pA steps) from a hyperpolarized holding potential. Note delay to first spike, due to transient outward conductance. B) Sample current response to a series of voltage clamp steps (-30 to +40 mV, 10 mV steps) from a hyperpolarized holding potential of -90 mV. * indicates instantaneous increased conductance at hyperpolarized membrane potentials, □ indicates transient outward conductance, here activated at -70 mV and more pronounced at -60 and -50 mV. Next voltage step (-40 mV) produced an inward action current (not shown). Insert shows sample current response of a different cell to a -50 mV voltage step, when Cs+ replaced intracellular K+.

Figure 3. Repetitive firing properties. A) Sample response to 20 pA current injection (1 s duration). Note the slowing of the firing rate with each spike. B) Frequency response of typical SPN showing SFA to multiple current injections, logarithmic scale. M was the natural log of the slopes of the lines shown. C) Correlation between input resistance ($R_{in}$) and SFA slope m, averaged for each cell. Only cells with statistically significant SFA are shown.

Figure 4. Persistent inward currents. A) Average steady-state current response to a series of 500 ms voltage steps with a CsF and K-gluconate based intracellular solution. Arrows denote absence of anomalous rectifier and onset of negative slope conductance in CsF. B) Sample neuron with a PIC resulting in negative conductance value, CsF-based intracellular solution. Arrows denote
PIC onset and peak magnitude. C) PICs were largely masked by K$^+$ conductances in K-glutamate containing patch electrodes, but could be seen as small deviations from linear conductance during a slow voltage ramp (8mV/s).

Figure 5. Cluster Analysis. A) Analysis of cluster validity, using two different indices. Both indexes peak at four clusters, signifying best fit for data set. B) Distribution of cluster membrane properties as a function of rheobase. Bi) Group 2 and 3 have low rheobase values, with group 3 having larger f-I gains. Groups 1 and 4 are sequentially recruited and can be largely distinguished by rheobase values. Bii) Group 3 neurons have statistically larger $\tau_{ms}$ than Group 1 neurons. Biii) Group 1 neurons display statistically larger $I_{peak}$ values than both Group 2 and 3 SPNs, and all have larger values than Group 4 SPNs.
<table>
<thead>
<tr>
<th>Property</th>
<th>mean</th>
<th>± S.D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-59.8</td>
<td>± 7.4</td>
<td>38</td>
</tr>
<tr>
<td>Input resistance (GΩ)</td>
<td>1.14</td>
<td>± 0.60</td>
<td>38</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>92.4</td>
<td>± 43.7</td>
<td>30</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>32.8</td>
<td>± 14.1</td>
<td>25</td>
</tr>
<tr>
<td>Action potential amplitude (mV)</td>
<td>57.1</td>
<td>± 8.9</td>
<td>30</td>
</tr>
<tr>
<td>Action potential overshoot (mV)</td>
<td>11.8</td>
<td>± 9.6</td>
<td>30</td>
</tr>
<tr>
<td>Action potential duration (ms)</td>
<td>6.3</td>
<td>± 1.4</td>
<td>30</td>
</tr>
<tr>
<td>Threshold voltage (mV)</td>
<td>-45.3</td>
<td>± 5.8</td>
<td>30</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>32.7</td>
<td>± 21.4</td>
<td>30</td>
</tr>
<tr>
<td>Afterhyperpolarization magnitude (mV)</td>
<td>15.2</td>
<td>± 3.6</td>
<td>30</td>
</tr>
<tr>
<td>Afterhyperpolarization duration (ms)</td>
<td>253.3</td>
<td>± 124.5</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 2. Comparison of statistically significant parameter differences between clusters.

<table>
<thead>
<tr>
<th>Group</th>
<th>$\tau_m$ (ms)</th>
<th>Rheobase (pA)</th>
<th>$f$-I slope (Hz/pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56.7 ± 23.1\textsuperscript{3}</td>
<td>36.3 ± 13.8\textsuperscript{3,4}</td>
<td>0.15 ± 0.07\textsuperscript{3}</td>
</tr>
<tr>
<td>2</td>
<td>112.9 ± 53.1</td>
<td>20.0 ± 0.0\textsuperscript{4}</td>
<td>0.17 ± 0.03\textsuperscript{3}</td>
</tr>
<tr>
<td>3</td>
<td>115.5 ± 43.8\textsuperscript{1}</td>
<td>17.9 ± 8.5\textsuperscript{1}</td>
<td>0.36 ± 0.13\textsuperscript{1,2,4}</td>
</tr>
<tr>
<td>4</td>
<td>83.1 ± 28.2</td>
<td>63.3 ± 23.4\textsuperscript{1,2}</td>
<td>0.19 ± 0.08\textsuperscript{3}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>I\textsubscript{peak} (pA)</th>
<th>AP width (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2157.8 ± 270.3\textsuperscript{2,3,4}</td>
<td>5.3 ± 0.7\textsuperscript{2}</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>1553.9 ± 363.4\textsuperscript{1,4}</td>
<td>8.1 ± 1.6\textsuperscript{1,1}</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1608.0 ± 473.7\textsuperscript{1,4}</td>
<td>5.9 ± 1\textsuperscript{2}</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>837.3 ± 426.8\textsuperscript{1,2,3}</td>
<td>6.7 ± 0.6</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: superscripted numbers indicate statistically significant differences (p<0.05) from group noted