Differential Inhibition of Ca$^{2+}$ Channels by $\alpha_2$-adrenoceptors in
Three Functional Subclasses of Rat Sympathetic Neurons

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Running head: $\alpha$-adrenergic modulation of identified SCG neurons

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Abstract

A comparison of identified sympathetic neurons in the isolated intact superior cervical ganglion (SCG) revealed that secretomotor, pilomotor and vasoconstrictor cells differ in their action potential mechanisms and in their postsynaptic α2-adrenergic responses to 10 µM norepinephrine (NE). In normal saline, the half-width of the spike afterhyperpolarization (AHP) in secretomotor neurons (103.5 ± 6.2 ms) was double that recorded in vasoconstrictor neurons (47.7 ± 2.9 ms) and 1.5 times that in pilomotor neurons (71.4 ± 10.3 ms). Bath-applied NE reversibly inhibited the action potential repolarization shoulder, AHP amplitude and AHP duration in secretomotor and pilomotor neurons to a similar extent, but had no effect on vasoconstrictor neurons. The insensitivity of vasomotor neurons to NE was not an artifact produced by microelectrode recording because all three cell groups were similar in terms of resting potential and input resistance. Moreover, NE insensitivity was not a natural consequence of briefer AHP duration in vasoconstrictor cells. Adding 10 mM TEA+ caused marked accentuation of the shoulder and AHP duration in vasoconstrictor neurons and comparable changes in the other two cell types, but did not unmask any sign of NE sensitivity in the vasoconstrictors. However, the spike shoulder and AHP in vasoconstrictors were Cd2+-sensitive, blocked by ω-conotoxin, an N-type calcium channel antagonist, and inhibited by oxotremorine-M, a muscarinic receptor agonist. These data show that NE can differentially modulate functional subsets of mammalian sympathetic neurons and that NE insensitivity can serve as a practical experimental criterion for identification of vasomotor neurons in the isolated ganglion.
Introduction

Catecholamines exert a powerful modulatory influence upon the excitability of sympathetic neurons by binding to $\alpha_2$-adrenergic receptors and inhibiting inward $\text{Ca}^{2+}$ currents through N-type $\text{Ca}^{2+}$ channels ($\text{Cav}2.2$) (Galvan and Adams 1982; Lin et al. 1997; Mathie et al. 1992; Schofield 1990). This causes a reduction in the $\text{Ca}^{2+}$-dependent shoulder during action potential repolarization followed by inhibition of the spike afterhypopolarization (AHP) (Horn and McAfee 1980; 1979). The transduction pathway connecting $\alpha_2$-adrenoreceptor activation to $\text{Ca}^{2+}$ channel inhibition in sympathetic neurons begins with the dissociation of pertussis toxin-sensitive G proteins (i/o subtypes) and proceeds through voltage-dependent binding of $\beta\gamma$ subunits to $\text{Ca}^{2+}$ channels (Bean 1989; Beech et al. 1992; Delmas et al. 1999; Herlitze et al. 1996; Ikeda 1996). Reduced calcium entry during the action potential leads to less activation of the outward current through apamin-sensitive, small conductance $\text{Ca}^{2+}$-dependent $\text{K}^+$ channels (SK type) that normally mediates the AHP (Horn and McAfee 1979; Kawai and Watanabe 1986; McAfee and Yarowsky 1979; Sacchi et al. 1995). This fundamental mechanism for modulating $\text{Ca}^{2+}$ influx operates in many neural systems to inhibit neurotransmitter release from nerve terminals (Bean 1989; Dunlap and Fischbach 1978; Herlitze et al. 1996; Hille 1994; Ikeda 1996; Lipscombe et al. 1989). In this way, $\alpha_2$-adrenergic autoreceptors allow norepinephrine to inhibit its own release from postganglionic sympathetic nerve terminals in peripheral end-organs (Langer 2008; Starke 2001; Stephens and Mochida 2005). In addition, the ionic currents regulated by somatic $\alpha_2$-adrenoceptors may influence how sympathetic neurons integrate fast nicotinic excitatory postsynaptic potentials (epsps) evoked by preganglionic neurons (Karila and Horn 2000; Sacchi et al. 1995). To understand how adrenergic modulation at these different
sites interacts to shape circuit function, one must first determine whether such mechanisms are expressed uniformly or differentially within the sympathetic system.

The present experiments were designed to examine the cellular distribution of somatic $\alpha_2$-adrenoceptor-mediated inhibition of the action potential in sympathetic neurons. Previous studies of the superior cervical ganglion (SCG) and other paravertebral chain ganglia indicate that although variable in magnitude, $\alpha$-adrenergic modulation of excitability is robust and expressed by many and perhaps all functional subclasses of sympathetic neurons (Chen and Schofield 1993; Horn and McAfee 1980). However, the design of earlier experiments did not assess whether multiple cell types were sampled in the analysis. These limitations have now been overcome with a method for identification of secretomotor, pilomotor and vasoconstrictor neurons in isolated physiological preparations of the intact rat SCG (Li and Horn 2006).

Functional subsets of sympathetic neurons selectively innervate distinct peripheral end-organs, thus allowing for their differential control during specific autonomic behaviors (Gibbins 2004; Jänig 2006; McLachlan 1995). In accord with this principle, subsets of neurons in the SCG control distinct targets including salivary glands, blood vessels, piloerector hairs, the iris, and the pineal gland. Cells belonging to these neuronal groups can be identified by their projection pathways, electrophysiological properties, cell size and the expression of neuropeptide Y (NPY). By noting such features, one can identify three major cell groups in the caudal portion of the rat SCG whose postganglionic axons project to their targets by way of the external carotid nerve (Li and Horn 2006). Secretomotor neurons controlling the salivary glands are NPY-negative, have relatively large cell bodies and are innervated by preganglionic fibers with low stimulus thresholds. This contrasts with pilomotor and vasoconstrictor neurons in the caudal SCG, which are relatively small and have preganglionic inputs with high stimulus thresholds.
The two groups of high threshold cells can be distinguished by the fact that only vasoconstrictor neurons express NPY. In mice these three cell groups account for approximately 85% of all SCG neurons (Gibbins 1991). By comparing identified cells in the rat SCG, we now report that the spike AHP differs in functional subsets of paravertebral sympathetic neurons and that the somatic $\alpha_2$-adrenergic modulation of excitability is restricted to the secretomotor and pilomotor cell groups.

**Methods**

**Preparation of ganglia.** Male Sprague-Dawley rats (180 - 250g) were euthanized by CO$_2$ inhalation using a protocol approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. The SCG was dissected together with the preganglionic cervical sympathetic trunk (CST) and the postganglionic internal and external carotid nerves (ICN, ECN). After transfer to a small Sylgard-lined recording chamber, the connective tissue sheath surrounding the ganglion was split open and used to stretch and pin down the preparation. Tight fitting suction electrodes were applied to the CST, ECN and ICN for extracellular stimulation and recording. The recording chamber was then mounted on the stage of an upright fixed-stage microscope (Zeiss Axioskop) with 40x Nomarski immersion optics. A more detailed description of the dissection and the methods for recording and histochemistry has already appeared (Li and Horn 2006).

**Extracellular recording and intracellular recording.** Nerves were stimulated with brief (0.1 ms) isolated current pulses (A300/360, WPI, Sarasota, FL) with polarity adjusted for minimum response latency. A simple switching arrangement allowed for electrodes on the ICN and ECN
to be used either for antidromic stimulation or recording. Extracellular compound action potentials (CAPs) were recorded by connecting the ICN and ECN electrodes to AC-coupled differential amplifiers (Grass P15, West Warwick, RI). Intracellular recordings from the somata of postganglionic neurons were made with glass microelectrodes (60 – 100 MΩ) filled with 3M potassium acetate and 0.5% neurobiotin (Vector Labs, Burlingame, CA). Membrane potential ($V_m$) and current ($I_m$) were recorded with an AxoClamp 2B amplifier (Molecular Devices, Union City, CA) set to current-clamp bridge mode. All recording amplifiers were connected to a common Ag-AgCl bath ground. Extracellular and intracellular recordings were monitored on an oscilloscope and digitized at 10 kHz using a Digidata 1440A computer interface and pClamp 10 software (Molecular Devices, Union City, CA).

Experiments were conducted at room temperature (23°C). Throughout dissection and recording, the SCG was bathed in carbogen-equilibrated mammalian Ringer solution composed of (in mM): 146 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 0.6 MgSO$_4$, 1.6 NaHCO$_3$, 0.13 NaH$_2$PO$_4$, 20 HEPES acid and 7.8 glucose (pH 7.3). During recording, the preparation was superfused at 1 – 2 ml/min with a peristaltic pump (bath volume ~ 1 ml). Drugs and sources were: L-(−)-norepinephrine (+)-bitartrate (NE, Sigma), tetraethylammonium chloride (TEA$^+$, Eastman Kodak), oxotremorine-M (Oxo-M, Sigma), CdCl$_2$ (Fisher Scientific), ω-conotoxin GVIA (ω-ctx, Alomone Labs, Israel), (-)-propranolol hydrochloride (Sigma) and (-)-isoproterenol hydrochloride (Sigma).

**Neuronal fills and immunocytochemistry.** After successful electrophysiological study, neurons were injected with neurobiotin by passing 500 pA, 150 ms depolarizing current pulses at 1 Hz for 3 minutes. One to 3 cells were filled per experiment and then the SCG was immersed in
fixative containing 2% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.3) for 1 hr at room temperature, then overnight at 4°C. To visualize neurobiotin-filled neurons for subsequent identification, the ganglion was washed the next day in phosphate buffered saline (PBS) and processed as a whole mount with streptavidin-conjugated CY3 (1:200 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA). After imaging the filled cells (Olympus Fluoview FV1000), the ganglion was washed in PBS, dehydrated through graded ethanol, infiltrated with polyethylene glycol (PEG 1000) and embedded in molten PEG (MW 1450). Then the embedded tissue was cut into serial 10 µm sections on a Leica RM 2165 microtome. For immunocytochemistry, floating sections were rinsed in PBS, treated for 2 hours at room temperature in PBS containing 1.5% donkey serum (Jackson ImmunoResearch Laboratories) and 0.3% triton-X100 (Sigma), incubated overnight at 4 °C in rabbit anti-NPY (1:2000; Peninsula, San Carlos, CA), followed by secondary incubation at room temperature in 1:200 donkey anti-rabbit CY2 (Jackson ImmunoResearch Laboratories). Stained sections were then rinsed, mounted on slides and viewed. Physiologically characterized cells were identified by their neurobiotin-CY3 fluorescence, scored for NPY-immunofluorescence (Zeiss Axioskop 2) and photographed (Zeiss Axiocam HrC and Axiovision 4.2 software).

**Analysis and statistics.** Resting membrane potential ($V_{\text{rest}}$) was noted at the end of each recording as the change in potential upon withdrawal of the microelectrode from a cell. Only cells with $V_{\text{rest}} < -45$ mV and action potential (AP) amplitude $>80$ mV in amplitude were included in the analysis. AP half-width was measured at the potential half way between $V_{\text{rest}}$ and the action potential peak. AHP amplitude was measured as the maximum negative voltage deflection relative to $V_{\text{rest}}$. AHP half-width was measured as the width at half-amplitude.
Grouped data are presented as the mean ± standard error. Individual effects of NE upon resting membrane properties and action potentials were compared using paired t-tests. Statistical differences between the three functional cell groups were examined using a one-way ANOVA, followed by Tukey’s test for multiple comparisons (Prism 4.0, GraphPad, San Diego, CA). $P < 0.05$ was the criterion for significance. Figures were prepared using Igor 5.0 (Wavemetrics, Lake Oswego, OR), Adobe Photoshop CS and Adobe Illustrator CS.

Results

**NE modulates the action potential in secretomotor and pilomotor neurons, but not in vasoconstrictor neurons**

The neuromodulatory actions of NE were studied in three groups of neurons (Fig 1, left column) whose projections into the ECN were confirmed by antidromic activation during intracellular recording. As in previous observations (Li and Horn 2006), the extracellular compound action potential (CAP) recorded from the ECN had two peaks with distinct presynaptic stimulus thresholds. Individual cells were classified as low or high threshold by correlating recruitment of the two extracellular CAP peaks with the presynaptic stimulus strength needed to elicit an intracellular suprathreshold synaptic response (Fig. 1, middle column). Sixteen low threshold, ECN projecting cells were all NPY-negative and thus identified as secretomotor neurons innervating salivary glands (Fig. 1A, middle and right columns). Fifteen NPY-negative cells with high presynaptic stimulus thresholds were identified as pilomotor neurons (Fig. 1B). This contrasted with 14 NPY-positive, high threshold neurons, which were identified as vasoconstrictors (Fig. 1C). The three groups of identified cells were
indistinguishable by their resting potentials (~ -55 mV) or input resistances (125 – 143 MΩ), and these properties were unaffected by bath application of 10 μM NE (Table 1).

To assess the modulation of excitability, action potentials were elicited by injecting 300 pA current pulses whose duration was adjusted to be slightly above threshold. In 16 of 16 secretomotor neurons, bath-applied 10 μM NE decreased AP half-width by 6% ($P < 0.05$), lowered peak AHP amplitude by 35% ($P < 0.001$) and shortened AHP half-width by 61% ($P < 0.001$) (Figs. 1A left column, 2). These effects occurred within less than 6 minutes of NE exposure, were readily reversible with 15 minutes of washing in normal Ringer solution and did not desensitize during repeated applications. NE produced similar actions in 14 of 15 pilomotor neurons, where it reversibly reduced AP half-width by 9% ($P < 0.01$), peak AHP by 39% ($P < 0.001$) and AHP half-width by 63% ($P < 0.001$) (Figs. 1B, 2). Different results were observed in 14 vasoconstrictor neurons, whose action potentials and AHPs were uniformly insensitive to NE (Figs. 1C, 2).

**Cell-specific differences in AHP duration**

Comparing action potentials from individual neurons (Fig. 1) and groups (Fig. 2) showed that the three sympathetic cell types differed, not only in their responses to NE, but also in normal AHP duration. In normal Ringer, the AHP in secretomotor neurons was significantly longer than in pilomotor and vasoconstrictor neurons (Fig. 2C).

**TEA+ accentuates NE responses**

To determine whether the NE insensitivity of vasoconstrictor neurons was simply a case of low sensitivity, additional experiments were done using bath-applied 10 mM TEA+ to
accentuate the AP shoulder and AHP (Horn and McAfee 1980). Before adding the TEA+, neurons were first classified as low or high threshold and tested for NE responsiveness in normal Ringer solution. Then in TEA+ the effects of NE upon AP shape were re-examined. Finally, the cell was injected with neurobiotin and processed for NPY-immunoreactivity to complete the cell identification process. These measurements revealed that TEA+ lengthened the AP and AHP duration in all three cell types, but had no effect on AHP amplitude (Figs. 3 left column, 4) or upon the cellular specificity of NE responsiveness (Figs 3, 5).

In 6 secretomotor neurons, 10 mM TEA+ increased AP half-width to 24.2 ± 3.2 ms, and AHP half-width to 173.3 ± 31.3 ms. In TEA+, 10 μM NE reversibly reduced AP half-width by 33% \((P < 0.01)\), AHP amplitude by 39% \((P < 0.01)\) and AHP half-width by 58% \((P < 0.01)\) (Figs. 3A, 5). Similarly, in 5 pilomotor neurons, TEA+ increased AP half-width to 20.6 ± 2.2 ms and AHP half-width to 91.6 ± 12.9 ms, and NE reversibly inhibited AP half-width by 43% \((P < 0.01)\), AHP amplitude by 25% \((P < 0.01)\) and AHP half-width by 42% \((P < 0.01)\) (Figs. 3B, 5). Once again vasoconstrictor neurons behaved differently. In 5 vasoconstrictor neurons, TEA+ increased AP half-width to 27.1 ± 5.7 ms and AHP half-width to 114.2 ± 28.1 ms, but NE still had no effect on the action potential (Figs. 3C, 5).

A 10 μM concentration of NE was chosen for these experiments because it produces maximal Ca\(^{2+}\) channel inhibition without desensitization (Diverse-Pierluissi et al. 1996; Horn and McAfee 1980). However, since NE also acts on β-adrenoceptors, we ran additional controls to rule out possible confounding effects. For these experiments, TEA-treated vasoconstrictor neurons were exposed to 10 μM NE after 20 minutes of pretreatment with 1 μM propanolol, a β-antagonist, and to 1 μM isoproterenol, a selective β-agonist. Neither experiment revealed any evidence of β-adrenergic modulation (Table 2).
Vasoconstrictor neurons express N-type calcium channels

To examine the possibility that vasoconstrictor neurons were unresponsive to NE because they express different Ca\(^{2+}\) channels than other sympathetic neurons, we conducted experiments using Cd\(^{2+}\), a non-selective Ca\(^{2+}\) channel blocker and \(\omega\)-conotoxin GVIA (\(\omega\)-Ctx), a selective blocker of N-type Ca\(^{2+}\) channels (Plummer et al. 1989). In identified vasoconstrictor neurons treated with 10 mM TEA\(^+\) to make the Ca\(^{2+}\)-dependent components more prominent, 100 \(\mu\)M Cd\(^{2+}\) inhibited action potential duration, AHP amplitude and AHP duration (Fig. 6A, Table 2). However, even in normal Ringer, the addition of 5 \(\mu\)M \(\omega\)-Ctx was sufficient to inhibit the AP repolarization shoulder and the AHP in vasoconstrictors (Fig. 6B, Table 2).

Muscarinic inhibition of the action potential in vasoconstrictor neurons.

Apart from the \(\alpha_2\)-adrenergic pathway, muscarinic agonists can inhibit N-type Ca\(^{2+}\) channels in rat SCG neurons by acting through m\(_1\) receptors coupled to the G\(_{q/11}\) protein and m\(_2\) and m\(_4\) receptors coupled to the G\(_{i/o}\) protein (Beech et al. 1992; Bernheim et al. 1992; Fernandez-Fernandez et al. 1999; Liu and Rittenhouse 2003; Mathie et al. 1992). To determine whether either of these pathways operates in vasoconstrictor neurons, Oxo-M was applied to high threshold NE insensitive cells in the presence of 10 mM TEA\(^+\). In 3 of 3 cells, 10 \(\mu\)M Oxo-M inhibited action potential duration, AHP amplitude and AHP duration (Fig. 7, Table 2).
Discussion

Finding that three functional subclasses of sympathetic neurons in the caudal SCG differ in terms of AHP duration and that $\alpha_2$-adrenergic inhibition of $\text{Ca}^{2+}$-dependent action potential components is restricted to two of these cell groups has implications for ganglionic physiology and for the practice of future experiments using preparations of the isolated intact ganglion. The results also pose a paradox because vasoconstrictor neurons are known to express functional presynaptic $\alpha_2$-adrenergic receptors at neurovascular synapses (Langer 2008; Starke 2001).

Functional implications

Inhibition of activity-dependent $\text{Ca}^{2+}$ entry into sympathetic neurons may have important short-term consequences for synaptic integration and long-term consequences for protein phosphorylation, channel function and gene expression. Through membrane hyperpolarization and shunting effects, the increase in $\text{Ca}^{2+}$-dependent $\text{K}^+$ conductance that produces the AHP may normally serve to dampen excitability and make it more difficult for summation of subthreshold nicotinic epsps to stimulate postsynaptic action potentials. Because $\alpha_2$-adrenergic receptor activation inhibits the AHP, it could lead to more effective summation of epsps and thereby enhance postganglionic activity in secretomotor and pilomotor neurons (Karila and Horn 2000; Sacchi et al. 1995; Wheeler et al. 2004).

In addition to effects upon $\text{K}^+$ channel gating (Belluzzi and Sacchi 1990; Kawai and Watanabe 1986; Marsh and Brown 1991), $\text{Ca}^{2+}$ entry into neurons may also produce long term physiological effects by influencing kinases, protein translocation and gene expression (Merrill et al. 2005; West et al. 2002). In this way, the activity-dependent phosphorylation of the cyclic AMP response element-binding protein (CREB) enhances expression of immediate early genes
such as c-fos and the subsequent transcriptional regulation of other genes. L-type Ca^{2+} channels may be especially important in such signaling because activity-dependent Ca^{2+} entry can release a proteolytic fragment from the CaV1.2 type channel, which then acts as a transcription factor (Gomez-Ospina et al. 2006; Hell et al. 1996).

What then about Ca^{2+} entry through N-type Ca^{2+} channels? In rat sensory neurons from the petrosal ganglion (Brosenitsch and Katz 2001) and in sympathetic neurons from the SCG (Zhao et al. 2007), Ca^{2+} entry through N-type as well as L-type channels can trigger CREB phosphorylation, leading to upregulation of c-fos and tyrosine hydroxylase. Interestingly, both studies show that activation of L-type channels is favored by chronic depolarization in high K^{+} solutions, while demonstrating a role for N-type channels requires patterned electrical stimulation. This implies that regulation of gene expression may be encoded by different patterns of natural physiological activity. In this way activation of somatic α2-adrenergic receptors may add another layer to the activity-dependent regulatory control of gene expression, but not in vasoconstrictor neurons.

**In vivo sources of catecholamines**

The experiments in this paper address the actions of catecholamines, but not their physiological origins. One possibility is that epinephrine and norepinephrine released by adrenal chromaffin cells, together with norepinephrine spillover from sympathetic nerve endings, reach sympathetic ganglia through the circulation. Measurements in human subjects and experimental animals indicate that plasma catecholamine concentrations are in the low nanomolar range and rise by less than ten-fold during exercise, stress and disease states such as hypertension and metabolic syndrome (Buhler et al. 1978; Jänig 2006; Mancia et al. 2007). This indicates that
circulating catecholamines straddle the concentration threshold for pharmacological modulation of the action potential in sympathetic neurons (Horn and McAfee 1980). A second possible source of norepinephrine is local release within the ganglion, either from cell bodies, dendrites or axonal varicosities (Bulbring 1944; Kawai et al. 1993; Kiraly et al. 1989; Kondo et al. 1980; Noon et al. 1975; Reinert 1963). One might expect local release to produce higher extracellular concentrations of norepinephrine within sympathetic ganglia and thereby cause greater α₂-adrenergic inhibition of Ca²⁺ currents than circulating catecholamines.

**Simplification of practical criteria for cell identification**

The analysis and comparison of synaptic integration in functional subsets of sympathetic neurons requires criteria for identifying the cell types. In this paper, we relied on the presence of NPY immunoreactivity as the essential marker for identifying vasoconstrictor neurons (Li and Horn 2006). Finding that vasoconstrictors are uniquely insensitive to norepinephrine provides an alternative identification criterion that is much simpler than marking cells with neurobiotin and then processing them for immunocytochemistry.

Our data also show for the first time that secretomotor, pilomotor and vasoconstrictor neurons in the SCG differ in terms of their AHP durations. Although these differences may influence synaptic integration of subthreshold nicotinic epsps, they are not sufficiently distinct to allow for unambiguous cell identification.

**The paradox posed by vasoconstrictor insensitivity to norepinephrine**

In blood vessels it is well established that presynaptic α₂-adrenoreceptors mediate the autoinhibition of norepinephrine release from sympathetic nerve terminals (Langer 2008; Starke
To investigate the mechanistic basis for such inhibition, numerous studies have relied on intracellular recordings from neuronal cell bodies (Bean 1989; Dunlap and Fischbach 1978; Herlitze et al. 1996; Hille 1994; Ikeda 1996; Lipscombe et al. 1989). An elegant recent example of this approach draws the connection between $\alpha_2$-adrenergic inhibition of $\text{Ca}^{2+}$ currents, $\beta\gamma$ subunits of G proteins and the inhibition of transmitter release using dissociated rat SCG neurons grown under conditions where they express a cholinergic phenotype and form nicotinic synapses (Stephens and Mochida 2005). It was therefore very surprising to discover that the cell bodies of vasoconstrictor neurons in the acutely isolated intact SCG were insensitive to norepinephrine. Several mechanisms could explain how vasoconstrictors limit $\alpha_2$-adrenergic inhibition to nerve terminals. First, vasoconstrictors may express the receptors in terminals, but not cell bodies. Immunocytochemical localization indicates $\alpha_{2A}$-adrenergic receptors are present in most rat SCG neurons (Gold et al. 1997), but this study did not distinguish cell surface receptors from the intracellular pool. A second mechanism might arise from alternative splicing of mRNA for N-type $\text{Ca}^{2+}$ channels (CaV2.2) in the SCG (Lin et al. 1997). Recent studies have identified exons that control the binding of $\beta\gamma$ G-protein subunits to $\text{Ca}^{2+}$ channels (Raingo et al. 2007) and that target the channels to nerve terminals (Maximov and Bezprozvanny 2002). In this way, one could postulate that alternative splicing targets $G_{\beta\gamma}$-sensitive channels to terminals and $G_{\beta\gamma}$-insensitive channels to cell bodies in vasoconstrictor neurons, but not in other sympathetic neurons. However, it is important to note that the known properties of CaV2.2 splice variants in sensory neurons (Raingo et al. 2007) cannot account for the complete NE insensitivity that we observed in vasoconstrictor sympathetic neurons.

Why did previous studies fail to observe norepinephrine-insensitive SCG neurons?
Although this type of question can never be answered with certainty, possible explanations include sampling bias, a focus on other issues and neuronal plasticity. In the earliest studies of noradrenergic modulation, which used microelectrodes (Horn and McAfee 1980; 1979), there was almost certainly a bias towards recording from larger cells because they are less easily damaged and against sampling vasoconstrictor neurons because they are the smallest SCG neurons (Li and Horn 2006). When subsequent work transitioned to patch-electrode recordings in culture, the focus shifted to voltage-clamp analysis and questions of signal transduction. Again, sampling bias may have occurred because when cells in a signaling experiment do not respond to an agonist, they are quickly rejected as damaged. However, unpublished anecdotal evidence suggests that under some culture conditions most sympathetic are NE-sensitive. This might arise because the phenotype properties of sympathetic neurons in culture are very sensitive to age, axotomy and growth conditions (Boeshore et al. 2004; Furshpan et al. 1986; MacPhedran and Hall 2001). Alternatively, acute dissociation might disrupt an unknown mechanism that normally decouples somatic $\alpha_2$-adrenoreceptors from Ca$^{2+}$ channels in vasoconstrictor neurons. Resolving these issues will require more information about the intact SCG and the properties of cultured sympathetic neurons. For example, it would be very useful to have a optical method for sampling NE sensitivity in large numbers of SCG neurons and thereby circumvent the sampling limitations imposed by microelectrode recording. Perhaps more important will be to follow the electrophysiological specialization described in this paper from the acutely isolated intact ganglion to a defined culture system where molecular methods can be more easily applied.
Conclusions

Previous studies have defined in detail the mechanism for $\alpha_2$-adrenergic inhibition of N-type Ca$^{2+}$ channels in rat sympathetic neurons and the consequences for action potential shape. By studying identified neurons in the intact SCG, we discovered that secretomotor, pilomotor and vasomotor neurons differ in terms of their normal AHP duration and that $\alpha_2$-adrenergic inhibition of the action potential is restricted to secretomotor and pilomotor cells. From these findings, one can predict that measuring the summation of subthreshold nicotinic epsps in identified neurons will reveal differences between functional subclasses of sympathetic neurons in terms of their synaptic integration and its noradrenergic modulation.

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Disclosures

None.
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## Tables

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**Table 1.** Resting membrane potentials (V<sub>rest</sub>), and input resistances (R<sub>in</sub>) were indistinguishable in three classes of identified sympathetic neurons and unaltered by bath-applied 10 μm NE. Input resistances were measured from steady-state I – V curves constructed using families of constant current pulses.
Table 2. Pharmacology of action potential modulation in vasoconstrictor neurons.

Vasoconstrictor cells were identified as high threshold, NPY-immunoreactive, ECN-projecting neurons. In cells whose action potential shoulder had been accentuated with TEA⁺, further treatment with the β-adrenergic blocker propranolol did not unmask any response to NE. Such cells were similarly unresponsive to the β-adrenergic agonist isoproterenol. Ca²⁺-dependent components of the action potential were detected in TEA⁺-treated vasoconstrictor neurons using
Cd²⁺ and in normal Ringer using ω-Ctx. The muscarinic agonist Oxo-M inhibited the AP shoulder and AHP in identified vasoconstrictor neurons. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**Figure Legends**

**Figure 1.** Norepinephrine modulates the action potential in secretomotor (A) and pilomotor neurons (B), but not in a vasoconstrictor neuron (C). Intracellular responses to bath-applied 10 \(\mu\)M NE (left column) in ECN-projecting neurons that were identified by electrophysiological characterization of their synaptic response as low or high threshold (middle column) and by the expression of NPY immunoreactivity (right column). Action potentials (left column) were elicited by depolarizing current pulses (300 pA, 10 ms) in normal Ringer solution (black) and after 10 min in NE (red). In secretomotor (A) and pilomotor (B) cells, NE inhibited the action potential repolarization shoulder and the slower AHP that followed. Note the control AHP appears longest in the secretomotor neuron. Individual neurons (intracellular recording) were classified as low or high threshold by determining whether the presynaptic stimulus required to evoke an action potential correlated with recruitment of peak 1 (low threshold) or peak 2 (high threshold) in the postsynaptic compound action potential recorded from the external carotid nerve. Stars (*) mark traces elicited by stimuli just above threshold. At the end of each recording, neurons were filled with neurobiotin (red) and then processed for immunocytochemistry (right column). Vasoconstrictor cells (C), identified by their high stimulus thresholds and NPY-immunoreactivity (green that turns yellow when merged with the neurobiotin image), were insensitive to NE.

**Figure 2.** Summary of noradrenergic modulation of the action potential in identified sympathetic neurons. Bath applied 10 \(\mu\)M NE inhibited action potential half-width (A), peak AHP amplitude (B) and AHP half-width (C) in secretomotor and pilomotor, but not
vasoconstrictor neurons. Grouped data from cells like those illustrated in Fig. 1. Comparison of control responses in the three cell types revealed that AHP half-width was longer in secretomotor neuron than in either pilomotor or vasoconstrictor neurons. Panel C denotes the numbers of cells in each group. *, P <0.05; **, P<0.01; ***, P<0.001.

**Figure 3.** TEA⁺ accentuates noradrenergic modulation of the action potential. The synaptic and NE responses of neurons were assessed in control Ringer. Then they were bathed in 10 mM TEA⁺, re-tested in 10 μM NE and ultimately identified after establishing their NPY-immunoreactivity (Fig. 1). TEA⁺ caused marked spike broadening and it increased AHP duration (left column) in secretomotor (A), pilomotor (B) and vasoconstrictor (C) cells. Under these conditions NE (middle column) continued to inhibit spike duration and the AHP in secretomotor (A) and pilomotor (B) neurons, but still had no effect on vasoconstrictor neurons (C). These effects of NE were reversed by 15 minutes of washing in TEA⁺ Ringer (right column). Action potentials were elicited by 300 pA intracellular current pulses whose durations denoted by a bar below the recording. Arrows mark AHP half-width.

**Figure 4.** Summary of TEA⁺ effects upon action potential duration (A), peak AHP amplitude (B) and AHP half-width (C). Grouped data shows that 10 mM TEA⁺ produced spike broadening and increased AHP duration in all three subclasses of identified sympathetic neurons. Panel C contains the numbers of cells in each group. *, P <0.05; **, P<0.01; ***, P<0.001.
Figure 5. Phenotypic restriction of noradrenergic modulation of the action potential remains unchanged in TEA+. Grouped data from identified neurons like those illustrated in Figure 3 show that treatment with TEA+ to accentuate Ca²⁺-dependent components of the action potential did not reveal any change in the cellular specificity of NE effects. In 10 mM TEA+, bath-applied 10 μM NE inhibited AP half-width (A), peak AHP amplitude (B) and AHP half-width in secretomotor and pilomotor neurons, but had no effect upon vasoconstrictor cells. Panel C indicates how many cells were in each group. **, P<0.01

Figure 6. Ca²⁺ channel antagonists alter the action potential in vasoconstrictor neurons.

(A) Action potential and AHP durations in a TEA+-treated, identified vasoconstrictor neuron were insensitive to 10 μM NE (10 min), but inhibited by 100 μM Cd²⁺ (10 min).

(B) In an identified vasoconstrictor neuron in normal Ringer, 10 μM NE had no effect, while 5 μM ω-ctx inhibited the Ca²⁺-dependent action potential shoulder and AHP. Arrows mark AHP half-widths.

Figure 7. Oxotremorine-M inhibits the AP shoulder and AHP in a vasoconstrictor neuron.

Although insensitive to NE, both the AP shoulder and AHP duration were inhibited in an identified, TEA+-treated, vasoconstrictor neuron after bath-application of 10 μM Oxo-M. Arrows mark AHP half-widths.
A Secretomotor neuron

Action potential

AHP

300 pA

40 mV

50 ms

5 mV

40 mV

20 ms

1 mV

Synaptic responses

intracellular

extracellular

low threshold

Peak 1

Peak 2

Histochemistry

Neurobiotin

NPY

Merge

20 μm

B Pilomotor neuron

Pilocarpine neuron

high threshold

C Vasoconstrictor neuron

300 pA

high threshold