THE DISTRIBUTION OF SEROTONERGIC, NORADRENERGIC AND DOPAMINERGIC SYNAPSES ON

FLEXOR MOTONEURONS

By

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Abstract

Serotonin (5-HT) and noradrenalin (NA) increase persistent inward currents mediated by sodium and calcium channels on the dendrites of motoneurons. The ability of 5-HT and NA to modulate these channels depends on the distributions of 5-HT and NA synapses. Recent studies of the distribution of 5-HT and NA synapses on motoneurons innervating the neck muscle splenius reported that these synapses are rare on the somata and have a strong bias to dendrites with small diameters. It is unknown whether this distribution pattern represents a general principle of organization (1) for all motoneuron groups or (2) for all types of modulators. To address the first question, we have examined the distribution of 5-HT and NA synapses on flexor motoneurons, which unlike extensor motoneurons, are not able to generate self-sustained discharges known to involve the activation of persistent inward currents. To answer the second question, we have mapped the distribution dopamine (DA) synapses. The dendrites of motoneurons that innervate the neck flexor rectus capitis anterior (RCA) were stained. Synapses containing 5-HT, NA and DA were identified using immunohistochemical techniques. Observations based on five RCA motoneurons indicate that the average densities of 5-HT and NA contacts are 2.3 and 1.4 times less dense than the average densities of 5-HT and NA contacts on splenius motoneurons, respectively. Moreover, pairs of 5-HT contacts and pairs of NA contacts were found to be 3.0 and 1.8 times closer together on splenius compared to RCA motoneurons, respectively. These observations may reflect the inability of flexor motoneurons to generate self sustained discharges. Similar to splenius motoneurons, 5-HT and NA synapses were found to preferentially innervate dendrites with diameters less than 2 µm. Thus, 5-HT and NA synapses facilitate channels in regions where excitatory or inhibitory signals undergo the largest attenuations. DA synapses on the dendritic tree were sparse (0.2 and 0.1 contacts per 1000 µm²), suggesting that the actions of DA synapses are confined to local regions on the
dendritic tree. These results highlight that motoneurons do not all share the same intrinsic properties, and the distribution of modulatory synapses have a crucial role in determining these properties.
Co-Authorship

Dr. P.K. Rose supervised Robert Maratta throughout the collection of data, interpretation of results and planning of the thesis. The first draft of this thesis was written by Robert Maratta. All subsequent drafts were written with collaboration between P.K. Rose who provided many helpful suggestions and comments.
Acknowledgments

It has been my great pleasure over the last two years to work with fantastic people who have taught me so much both inside the lab and out. First I want to thank my family for supporting me in my decision to attend Queen's and pursue my academic interests. They have always been there for me, and I really appreciate their love and support. When I first arrived at Queen's, I was excited but apprehensive about being in a new place on my own. I want to thank my housemates who welcomed me to Kingston and helped me learn how to live independently. Living with my best friends throughout my time here has been incredible and these memories will last a lifetime. I also want to specifically thank my girlfriend Mary, who has always been there to support me through every bump in the road.

In my time at the Rose lab I met some great scientists. Keith Fenrich showed me the ropes for the first year and a half of my degree. Keith, your thoughts, suggestions and help with other various aspects of my project have been invaluable. I have also learned from your ability to reason scientifically, whether it comes to impaling motoneurons or how to ski telemark in powder. I hope to visit you before the end of your post-doc in France. Ethan “Yeechao” Zhao, we have been a tag team since day one throughout our projects in the Rose lab. Your unparalleled expertise in imaging has helped so many aspects of my project to go forward. You have also been a great friend and I will always remember the good times we had at conferences and backyard barbeques. Anirudh Garg, we only spent a few months together at the beginning of my thesis but your perspectives have stuck with me and I wish you the best of luck with your career in medicine. I also want to acknowledge Steven Montague. Though I did not have the chance to work with Steven for an extended period of time, his hours of effort creating programs and analysis methods for different distributions of contacts helped me accomplish the goals of my project. Monica Neuber-Hess, as acting lab-tech your contributions in surgeries and histology
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<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>5-HTTH</td>
<td>serotonin and tyrosine hydroxylase positive</td>
</tr>
<tr>
<td>ACH</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>COM</td>
<td>center of mass</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DJβH</td>
<td>dopamine beta hydroxylase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated</td>
</tr>
<tr>
<td>I_{Ka}</td>
<td>Slowly inactivating K⁺ current</td>
</tr>
<tr>
<td>I_{NA}</td>
<td>persistent Na⁺ current</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>KPBS</td>
<td>medium-duration postspike</td>
</tr>
<tr>
<td>mAHP</td>
<td>afterhyperpolarization</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenalin</td>
</tr>
<tr>
<td>PIC</td>
<td>persistent inward current</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RD</td>
<td>reference distribution</td>
</tr>
<tr>
<td>SK</td>
<td>small conductance calcium-activated potassium</td>
</tr>
<tr>
<td>TASK-1</td>
<td>tandem of P domain weak</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin releasing</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated</td>
</tr>
<tr>
<td>I_{Ka}</td>
<td>Slowly inactivating K⁺ current</td>
</tr>
<tr>
<td>I_{NA}</td>
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<td>mAHP</td>
<td>afterhyperpolarization</td>
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<td>NA</td>
<td>noradrenalin</td>
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### List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>Ca⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>Eₓ</td>
<td>reversal potential of ion X</td>
</tr>
<tr>
<td>Gₓ</td>
<td>conductance of ion x</td>
</tr>
<tr>
<td>iₛoma</td>
<td>current reaching the soma</td>
</tr>
<tr>
<td>iₛynapse</td>
<td>magnitude of current at an ionotropic synapse</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>MΩ</td>
<td>mega ohm</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>nA</td>
<td>nanoamp</td>
</tr>
<tr>
<td>π</td>
<td>pi</td>
</tr>
<tr>
<td>r</td>
<td>radius</td>
</tr>
<tr>
<td>Ri</td>
<td>specific resistivity of the cytoplasm</td>
</tr>
<tr>
<td>Rm</td>
<td>specific resistivity of the membrane</td>
</tr>
<tr>
<td>ri</td>
<td>resistance of the cytoplasm to current</td>
</tr>
<tr>
<td>rm</td>
<td>resistance of the membrane to current</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>Vm</td>
<td>membrane potential</td>
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</tbody>
</table>
Introductory Statements

Sherrington (1906) referred to motoneurons as “the final common pathway” because they integrate information from many sources and they are the last neuron in all CNS circuits involved in motor control. For many years it was widely accepted that the relationship between the input and output is fixed, and that motoneurons acts as a relay station. However, what has become quite clear is that the input-output relationship is not fixed. Changes to the input-output relationship have been largely attributed to the actions of modulator transmitters such as serotonin (5-HT) and noradrenalin (NA) on motoneurons (Heckmann et al., 2005). Nonetheless, exactly how and why these modulators elicit changes to the motoneuron input-output relationship remains unclear. We have investigated the spatial locations of 5-HT, NA and dopamine (DA) synapses on the dendritic trees of neck flexor motoneurons. The purpose of this introduction is to answer one question: what is the evidence that the location of modulatory synapses maybe a critical factor responsible for the regulation of the input-output properties of motoneurons?
Chapter 1. Introduction Part I: From basic properties to the transfer of current by motoneuron dendrites

1.1 Motoneurons are large and complex cells

The dendritic trees of motoneurons are large and complex. Figure 1.1 shows the reconstructions of motoneurons which innervate three different muscle groups in the neck. The surface areas of motoneurons are commonly greater than 400,000 $\mu$m$^2$, and they can receive in excess of 20,000 total synaptic inputs (Ulfhake and Kellerth, 1983; Rose et al., 1985; Cullheim et al., 1987; Ulfhake and Cullheim, 1988; Rose and Neuber-Hess, 1991). Understanding how tens of thousands of synaptic inputs integrate to produce an output is not trivial. The aim of this first chapter is to describe the basic properties of neurons and how the consequences of these basic properties are applied to the unique architecture of motoneurons.

1.2 Basic properties and the input-output relationship

The basis for all excitable tissue is the presence of a semi-permeable membrane barrier that acts to separate charged ions. In neurons, the controlled flow of four key ions across the membrane is important: sodium (Na$^+$), potassium (K$^+$), calcium (Ca$^{++}$) and chloride (Cl$^-$) (see Figure 1.2A). Each current has a reversal potential ($E_x$), where there is no net flow of ions because the concentration gradient causing the flow is countered by electrical repulsion which builds up on one side of the membrane. The $E_x$ is determined primarily by the concentration of ions outside the cell, compared to the concentration of ions inside the cell. The membrane potential ($V_m$) of a neuron at any time is simply the weighted average of the $E_x$ for Na$^+$, K$^+$, Ca$^{++}$ and Cl$^-$, and is always biased toward whichever type of current is dominating. These principles were originally quantified in the Goldman Equation (Figure 1.2A) (Goldman, 1943). The typical $V_m$ at rest for a neuronal membrane as determined by this equation is roughly -65 mV.
Figure 1.1 The reconstructed dendritic trees of motoneurons innervating the splenius (A) (Montague, 2008), biventer cervicis and complexus (B) (Rose et al, 1995) and trapezius (C) (Meehan, unpublished data) neck muscles. Cells are shown from a horizontal perspective (top) and transverse perspective (bottom).
Figure 1.2 (A) Upper diagram shows the four key ion currents in neurons and their concentration gradients. Na⁺, K⁺, Cl⁻, and Ca²⁺ channels are colour coded as green, cyan, purple and yellow, respectively. Lower diagram shows the goldman equation and the calculation of a typical resting membrane potential. (B) Diagram of a current clamp experiment in which a soma is impaled. A current step brings the membrane potential to threshold for an action potential. (C) Upper trace shows the response of a sympathetic ganglion neuron to a prolonged depolarizing current pulse (Jones, 1985). Lower trace shows the responses of a hypothalamic neuron which fires spontaneously over time (McCormick and Huguenard, 1992). (D) Upper trace shows a train of action potentials generated in a motoneuron when a prolonged current is applied. Bottom trace shows resulting F/I relationship (modified to only show one motoneuron) (Granit et al., 1963)
There are two main factors which determine the permeability of a membrane to current: (1) the density of ion channels that are present at a given location, and (2) whether these ion channels are open or closed. To illustrate how these two factors work together, consider the textbook example of an action potential generated at the initial segment of a neuron. Located at the origin of an axonal process, the initial segment has a high density of voltage-gated Na$^+$ channels that open only when Vm has reached a threshold value (Kandel et al., 2000; Leterrier et al., 2011). In a current clamp experiment (Figure 1.2B), positive current can be injected into the cell body of a neuron so that the Vm shifts from resting at -65 mV to a more positive potential. If the Vm crosses the threshold potential (roughly -55 mV) needed to open voltage-gated Na$^+$ channels the permeability of the membrane to Na$^+$ drastically increases and the Vm spikes towards $E_{\text{Na}^+}$ (roughly +60 mV). At this point, the permeability of voltage gated Na$^+$ decreases due to inactivation of these channels and voltage-gated K$^+$ channels which are sensitive to a positive Vm will open. As a result, the permeability of the membrane to K$^+$ will increase, driving the Vm back towards $E_{\text{K}^+}$ (approximately -90 mV). In this way, a spike is generated and then the Vm is returned to resting levels.

This basic example illustrates that action potentials are simply the product of a population of voltage-gated ion channels responding to a change in current. Hence, neurons with unique compliments of ion channels will generate action potentials under different circumstances and with different temporal patterns (Figure 1.2c). For example, sympathetic ganglion neurons respond to prolonged positive current by eliciting only a single action potential due to the actions of an M-Type K$^+$ channel which remains open after the initial spike (Jones, 1985). On the other hand, some neurons do not require any input current at all to generate a response. So called “bursting” neurons in the hypothalamus fire trains of action potentials at regular intervals through the actions of voltage gated Ca$^{++}$ channels and voltage-gated K$^+$

5
channels that consistently bring the cell to threshold for firing (McCormick and Huguenard, 1992). Granit et al (1963) were the first to describe the response of motoneurons to a prolonged depolarizing current (Figure 1.2D). They found that when positive current was injected into the soma of motoneurons there was a resulting high frequency train of action potentials. In addition, the frequency of the spike train was linearly related to the input current applied. This was the first characterization of the input-output relationship in the motoneuron, and it suggested that the firing response of the motoneuron is directly coupled to the current that is injected into the soma.

1.3 The delivery of current to the soma under physiological conditions

In order to fully understand the input-output relationship of motoneurons it is imperative to realize that current reaching the soma is not physiologically derived from an electrode as in the current clamp experiment. Instead, the current that reaches the soma originates from ionotropic synapses on the dendritic tree. Ionotropic synapses release neurotransmitters that bind to ligand-gated channels. The magnitude of a current produced by a specific set of ionotropic synapses is the product of the conductance change caused by the opening of ligand-gated channels and the “driving potential,” which is the difference in voltage between the membrane potential and the reversal potential of the synaptic current (below).

\[ I_{\text{Synapse}} = G_x(V_m - E_x) \]  \hspace{1cm} (1)

Given that the current received at the soma determines the firing rate of the motoneuron (Figure 1.2D), much effort has gone into understanding how much of the current generated at these ionotropic synapses reaches the soma. As illustrated in Figure 1.3, this depends on three main factors: (I) “passive” membrane properties, (II) ion channels in the motoneuron membrane, and (III) the control of these channels.
Figure 1.3 Factors influencing excitatory current transmission to the soma from dendritic synapses. Ionotropic synapses are colour coded in blue and metabotropic synapses are colour coded in yellow. (B) Current attenuation over a given distance (x) is determined by passive membrane properties. (C) Current can travel a further distance on the dendritic tree with the addition of positive current from voltage gated ion channels. (D) The distance current travels can be changed by the absence or presence of modulators that facilitate nearby voltage gated ion channels.
1.4 (I) Passive membrane properties

Rall (1977) was the first to describe the passive properties of dendrites with respect to their geometry based on “cable theory”. Cable theory describes the attenuation of an electrical signal with distance through an infinitely long cylinder. In this model of a dendrite (Figure 1.3B), the current generated from an ionotropic synapse has two options: it can move along the dendrite through the resistance of the cytoplasm ($r_i$), or leave the dendrite through the resistance of the membrane ($r_m$). $r_i$ for a given length of dendrite is determined by a constant specifying the specific resistivity of the cytoplasm ($R_i$) over the cross-sectional area of a dendrite. Similarly, $r_m$ for a given length of dendrite is calculated based on a constant specifying specific resistivity of the membrane ($R_m$) over the circumference of a dendrite (below).

$$r_i = \frac{R_i}{\pi r^2} \quad r_m = \frac{R_m}{2\pi r} \quad (2)$$

Together, $r_i$ and the $r_m$ describe the length constant ($\lambda$) of the dendrite. When the terms are expanded, it becomes clear that the length constant is proportional to the ratio of $R_m$ to $R_i$ as well as the radius of the dendrite (below).

$$\lambda = \sqrt{\frac{r_m}{r_i}} = \sqrt{\frac{R_m x^2}{R_i x}} \quad (3)$$

Using the length constant, voltage attenuation over a given distance ($x$) along a dendrite can be described (below). Note that because we are assuming unchanging resistances along the infinitely long cylinder in this model, the degree of voltage attenuation is proportional to the degree of current attenuation according to Ohm’s law ($V=IR$).

$$\Delta V(x) = V_0 e^{-x/\lambda} \quad (4)$$
Cable theory provides a firm basis from which we can begin to understand how much of the current generated by synapses on dendrites reaches the soma. One of the most basic consequences of the relationship described in equation 4 is that voltage, and hence current, moving down the dendrite will attenuate with distance in a passive cable. The magnitude of this attenuation is proportional to the specific membrane resistivity ($R_m$) over the specific cytoplasmic resistivity ($R_i$) according to equation 3. It turns out that because the $R_m$ is typically 100-300 times higher than the $R_i$, a significant amount of current produced at synapses located on dendrites takes the path of least resistance through the cytoplasm and reaches the soma (Rall, 1977; G Stuart et al., 1999). Considering this observation, one might conclude that the location of a synapse on a dendritic tree is irrelevant: synapses should influence the soma independent of dendritic location. There is a serious flaw in this approximation. Models based on cable theory alone neglect the fact that multiple synapses are tonically active on the dendritic tree of neurons in vivo (Holmes et al., 1992; Kamondi et al., 1998; London and Segev, 2001). The neurotransmitter released by these synapses opens ligand-dependent channels. Hence, the “effective” membrane resistance for a given length of dendrite is much lower than the specific membrane resistivity ($R_m$) due to the presence of background activity. Consequently, much less current actually reaches the soma from dendritic synapses than would be predicted by using $R_m$ and cable theory. There are two main reasons for this. First, because the membrane is more “leaky” due to background activity, more current escapes through the membrane and current generated by synapses on dendrites attenuates much more en route to the soma. In fact, current attenuations of more than 80% have been described in detailed models incorporating full dendrite reconstructions and background synaptic activity (Korogod et al., 2000; Powers and Binder, 2001; Bui et al., 2003). Second, synaptic activity results in a phenomenon known as non-linear summation. As shown in equation 1, the driving force at a
particular synapse is based on the difference between the reversal potential and membrane potential. Increasing levels of excitatory activity will shift the membrane potential and decrease the driving force for subsequently activated excitatory synapses. This results in a non-linear current response such that the sum of the current produced by co-active synapses is less than the algebraic sum of the current produced by individual activation. Non-linear summation has been shown to cause significant current en-route to the soma in models incorporating realistic numbers of synapses (Cushing et al., 2005).

1.5 (II) Voltage dependent and independent ion channels on motoneuron dendrites

It has been estimated that over 95% of total synapses on motoneurons exist on the dendrites (Rose and Neuber-Hess, 1991). Given the pronounced attenuation of current from the synapses on the dendritic tree, the existence of these terminals seems paradoxical. Based on passive membrane properties alone, the current generated at excitatory dendritic synapses will have little impact on the soma. One potential solution to this problem is the presence of voltage gated ion channels on dendrites which could change the permeability of the membrane. These channels could add positive current and therefore increase the total current that reaches the soma (Figure 1.3C). In fact there is mounting evidence that both voltage gated and non-voltage gated channels exist on motoneuron dendrites.

Although there are over 11 subtypes of voltage dependent and at least 4 voltage independent channels in motoneurons, most of these channels subtypes are located somatically, and only a small number are distributed on the dendritic tree (For a full review see Rekling et al., 2000; Alessandri-Haber et al., 2002). Electrophysiological evidence suggests that L-type Ca\(^{++}\) channels, persistent Na\(^{+}\) channels (Heckmann et al., 2005) and small conductance Ca\(^{++}\) activated K\(^{-}\) (SK) channels (Li and Bennett, 2007) are located on the dendrites of
motoneurons. In addition, the presence of L-type Ca\(^{++}\), Kv2.1 and HCN channels on motoneuron dendrites has been confirmed using immunohistochemical approaches (Muennich and Fyffe, 2004; Ballou et al., 2006; Zhao et al., 2010). As a compliment to immunocytochemistry, computational modeling has been used to further confirm the presence of L-type Ca\(^{++}\) channels on dendrites using the geometry of the dendritic tree and constraining parameters to match existing electrophysiological data (Elbasiouny et al., 2005; Bui et al., 2006; Grande et al., 2007; Shapiro and Lee, 2007). Collectively, these investigations provide strong evidence for the presence of ion channels on the dendrites of motoneurons.

Given that dendritic ion channels can modify how much current reaches the soma, the addition of current from these channels is one possible way that excitatory current can be enhanced en-route to the soma. However, there is a flaw in this conceptual framework. We are assuming the conductance and voltage-dependence of these ion channels are constant. This is a simplistic viewpoint which renders a simplistic view of the input–output relationship: fixed (Figure 1.2D). That is, a given input current faithfully and repeatedly produces the same rate of firing. As is discussed in the following chapter, this is in fact not the case.
Chapter 2. Introduction Part II: Modulators and their effects on motoneurons

2.1 (III) The control of voltage gated and non-voltage gated ion channels: modulators and metabotropic signaling

The final factor influencing current transmission from the dendrites to the soma of motoneurons is the control of ion channels on the dendrites (Figure 1.3D). These channels are influenced by modulators, which are released from metabotropic synapses. Modulators from these synapses bind to receptors in the membrane which are coupled to signaling units known as heterotrimeric guanine nucleotide-binding proteins, or G-proteins. G-proteins interact with effector enzymes which catalyze the production of active second messenger proteins (Figure 2.1). Active second messenger proteins and their downstream effectors are known to be involved in controlling many aspects of cell function including metabolism, cell differentiation, and survival (Gudermann et al., 1996; Lodish et al., 2000). In excitable tissue, second messengers activated by modulators can also change the voltage dependency and activation kinetics of nearby ion channels (Kandel et al., 2000). As a result, the relationship between input and output in the motoneuron is in fact not fixed and can be altered in the presence of modulators. This chapter aims to provide a detailed breakdown of the changes to the input-output relationship of motoneurons that can be elicited by modulators.

2.2 Modulators on motoneurons: origins, projections, and receptor systems on motoneurons

There are eight known transmitters which bind to metabotropic receptors on motoneurons: serotonin (5-HT), noradrenalin (NA), dopamine (DA), thyrotropin releasing hormone (TRH), acetyicholine (ACH), adenosine, glutamate and gamma-aminobutyric acid (GABA) (Rekling et al., 2000). For reasons that will be described in chapter 3, we have chosen to focus on three of these modulators: 5-HT, NA and DA.
Figure 2.1 Schematic of metabotropic G-coupled receptor signaling in the context of ion channel modulation (adapted from Lodish et al., 2000)
2.2.1 5-HT origins, projections and receptor systems

Synaptic terminals that release 5-HT originate from cells in the pallidus obscurus in the brainstem and densely innervate the ventral horn of the spinal cord in mammals (Bjorklund and Skakerberg, 1982). As listed in Table 2.1, there are six types of 5-HT receptors on motoneurons. All 5-HT receptors are metabotropic with the exception of ligand gated 5-HT$_3$ receptors, and the function of 5-HT$_3$ receptors on motoneurons is unknown (Morales et al., 1998; Rende et al., 1999). 5-HT$_{1A}$ receptors are located primarily on the soma and axon hillock of motoneurons, (Kheck et al., 1995; Azmitia et al., 1996) while 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors are located on the dendrites of motoneurons (Hsiao et al., 1997; Fink and Göthert, 2007; Kong et al., 2010). In addition, 5-HT has also recently been shown to work through 5-HT$_7$ receptors on motoneurons (Larkman and Kelly, 1997; Inoue et al., 2002). G-proteins are often classified by their large $\alpha$ subunit. 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors utilize the Gq/11 $\alpha$ subunit which activates a signaling pathway involving the production of inositol triphosphate (IP$_3$) and diacylglycerol (DAG) (Bockaert et al., 2006). The second messenger protein kinase C (PKC) is activated directly by DAG and indirectly through intracellular calcium release controlled by IP$_3$ (Nishizuka, 1995; Bockaert et al., 2006). As a kinase, PKC is capable of phosphorylating nearby proteins such as ion channels, thereby modifying their functional characteristics (eg Hell et al., 1993).

In contrast, 5-HT$_7$ and 5-HT$_{1A}$ receptors utilize the G$\alpha$s and G$\alpha$i, which work to activate and inhibit adenylyl cyclase, respectively (Bockaert et al., 2006). Adenylyl cyclase promotes the production of cyclic adenosine monophosphate (cAMP), which can bind to and activate a second messenger known as protein kinase A (PKA) (Lodish et al., 2000). Like PKC, PKA is a kinase which has the capability of phosphorylating ion channels in order to modify their function (eg Wetzel et al., 2001).
Table 2.1 Table outlining the receptors, G-Proteins, second messengers and channels affected on motoneurons for 5HT, NA, and DA modulators (see text for sources). Note that 5-HT₃ receptors are shown as not applicable because they are ligand gated. Under the heading “channel affected“, the channels are coded in green for a facilitating effect and red for an inhibiting effect.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Receptor</th>
<th>G-Proteins and second messengers</th>
<th>Channel Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-HT₃</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5-HT₂ₐ, 5-HT₂₇, 5-HT₂C</td>
<td>Gₛ/p₁₁-PKC</td>
<td>L-type Ca⁺⁺, Iₙₐ, SK, TASK-1, HCN</td>
</tr>
<tr>
<td></td>
<td>5-HT₇</td>
<td>Gₛ-PKA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-HT₁ₐ</td>
<td>G₁-CAMP_inhibition</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>α₁</td>
<td>Gₛ/p₁₁-PKC</td>
<td>L-type Ca⁺⁺, Iₙₐ, SK, TASK-1</td>
</tr>
<tr>
<td>DA</td>
<td>D₁</td>
<td>Gₛ-PKA</td>
<td>SK, Iₖₐ⁺</td>
</tr>
<tr>
<td></td>
<td>D₂</td>
<td>G₁-CAMP_inhibition</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 NA origins, projections and receptor systems

Synaptic terminals that release NA originate from cells in the locus coeruleus and subcoeruleus adjacent to the floor of the fourth ventricle and project to the ventral horn of the spinal cord (Westlund and Coulter, 1980). NA effects on motoneurons were blocked with the application of an antagonist to the α1 NA receptor, and hence this is likely the receptor responsible for the actions of NA on motoneurons (Lee and Heckman, 1999; Harvey et al., 2006a). Like 5-HT receptors, the α1 NA receptor is linked to the Gq/11 α subunit and activates the same intracellular pathway involving the activation of PKC through IP3 and DAG (Zhong and Minneman, 1999; Rho and Storey, 2001).

2.2.3 DA origins, projections and receptor systems

DA terminals in the spinal cord originate from a nucleus in the brainstem known as the A11 cell group (Bjorklund and Hokfelt, 1984). Like 5-HT and NA, DA terminals also exist in the ventral horn in regions that contain motoneuron pools (Holstege et al., 1996). There is evidence that motoneurons have both D1 and D2 dopamine receptors; however it is unclear whether the major effects of dopamine on motoneurons are exerted through D1 or D2 receptors (Dubois et al., 1986; van Dijken et al., 1996). Traditionally, D1 and D2 receptors have opposing actions mediated through Gαs and Gαi, which can activate or inhibit the effector enzyme adenylyl cyclase, respectively (Missale et al., 1998). By acting through Gαs and Gαi subunits, D1 and D2 receptors have the same intracellular signaling pathways as 5-HT7 and 5-HT1A receptors, respectively, which involve the production and inhibition of cAMP and activation of the second messenger PKA.
2.3 The effects of modulators on motoneurons

Changes in the input-output relationships of motoneurons are controlled by more than one type of modulator, sometimes acting on the same set of ion channels, and sometimes acting on a different set of channels. Thus, the control of motoneuron input-output properties will be perspective of the channels. This approach is designed to emphasize the complexity that is inherent when multiple modulators act in concert to change the excitability of motoneurons.

2.3.1 L-type Ca\(^{++}\) channels and persistent Na\(^+\) channels -> plateau potentials and persistent inward currents

Over 30 years ago Schwindt and Crill (1977) described a slow inactivating net inward current in feline lumbar motoneurons that appeared during slow voltage ramps. They later termed this effect as a persistent inward current (PIC) and they observed that this current could cause a prolonged period of membrane depolarization (Schwindt and Crill, 1980a, 1980b). This prolonged period of depolarization, or plateau potential, can cause the motoneuron to continue firing for seconds after the removal of a stimulus. Later studies found that the PIC on motoneurons is primarily produced by L-type Ca\(^{++}\) channels (Hounsgaard and Kiehn, 1989), and an unknown persistent Na\(^+\) current (I\(_{\text{Na}}\)) (Lee and Heckman, 2001; Li and Bennett, 2003) on motoneuron dendrites. Importantly, the activation of PICs is dependent on the presence of the monoamines 5-HT and NA, which facilitate the Ca\(^{++}\) current Na\(^+\) current (Hounsgaard and Kiehn, 1989; Lee and Heckman, 2000; Harvey et al., 2006b, 2006a). This positive current on motoneuron dendrites is also a source of substantial amplification of synaptic inputs. Lee and Heckman (2000) found that increasing amounts of NA and 5-HT applied to the spinal cord could amplify synaptic inputs from 1a afferents up to 6 fold. Hence, this amplification effect can be graded based on the amount of modulator present.
Some of the first characterizations of the effect on PICs on cell firing rates were completed in a decerebrate cat configuration in which the descending spinal projections remain intact so that modulators are continually released onto motoneurons during the experiment (Hounsgaard et al., 1988). Under these conditions motoneurons were impaled and subject to a triangular current ramp. The resulting firing rate was not simply linear as previously reported, but rather it exhibited a “hysteresis” of the input-output relationship (Figure 2.2A). On the upslope of the current ramp the firing rate increases linearly (i). At a certain point, the slope of this linear relationship increases (ii). This is because the voltage has become depolarized enough to activate L-Type Ca^{2+} channels and voltage gated Na^+ channels on the dendrites which now provide additional current to the soma. On the downslope of the current ramp (iii), the firing rate decreases, but remains higher than it was on the upslope when the same amount of current was injected, due to the additional current provided by the activated voltage gated channels. Finally, when the current injection reaches zero (iv), the motoneuron continues to fire because the current provided from the voltage dependent channels maintains a plateau potential which results in self sustained firing. Hence the motoneuron is said to be “bistable” because it has two firing profiles for the same input conditions; quiescent and self sustained firing (Hounsgaard et al., 1984). This characterization of the motoneuron input-output relationship shows that the same excitatory input can produce different firing rates, depending on the presence of modulators.

2.3.2 Block of SK channels -> reduction of the mAHP and facilitation of PICs on dendrites

SK channels, or small conductance calcium-activated potassium channels, are present on most neurons, including motoneurons, and are responsible for modulating the medium-duration postspike afterhyperpolarization (mAHP) of action potentials (McLarnon, 1995). Unlike the L-Type Ca^{2+} channels, SK channels are not activated by voltage, but instead open when the
Figure 2.2 (A) Diagram of the “hysteresis” of the input-output relationship in the presence of modulators (adapted from Hounsgaard et al., 1988). Graph on the left shows a triangular current ramp colour coded for orange on the upslope of current and purple for the downslope of current. Graph on right shows the corresponding changes in motoneuron firing rate. (B) The effects of SK channel suppression adapted from Berger et al., 1992; Bayliss et al., 1995. Trace on the left shows the diminished mAHP after the application of 5-HT. Graph on the right shows the resulting changes in motoneuron firing rate.
intracellular Ca$^{++}$ concentration rises. As listed in Table 2.1, 5-HT, NA and DA have been found to block the actions of SK channels on motoneurons (Fung and Barnes, 1987; Berger et al., 1992; Bayliss et al., 1995; Han et al., 2007). The block of SK channels diminishes the magnitude of the mAHP, and as a result the motoneuron fires at a higher frequency (Figure 2.2B). However, in a physiological setting this effect is dependent on whether a source of 5-HT, NA or DA exists near the soma and axon initial segment, where action potentials are initiated. Li and Bennett (2007) have investigated the importance of SK current on the dendrites of motoneurons in providing a negative feedback for PICs. They showed that SK channels provide a hyperpolarizing outward K$^+$ current in response to the large influx of Ca$^{++}$ carried by PICs. In this case, the application of 5-HT or DA may enhance the PIC by blocking the hyperpolarizing SK current on dendrites.

2.3.3 Block of TASK-1 Channels -> dendrite depolarization and less leak

Tandem of P domain weak inward related acid sensitive K+ channels 1 (TASK-1) channels are non-voltage gated channels that provide an outward K$^+$ current at rest (Binder et al., 2011). Hence, TASK-1 channels contribute to making the dendrite “leaky” to current by decreasing the effective membrane resistance. It has been shown that both 5-HT and NA are capable of blocking the TASK-1 channels on motoneurons (Elliott and Wallis, 1992; Larkman and Kelly, 1992; Lindsay and Feldman, 1993; Parkis et al., 1995; Hsiao et al., 1997; Talley et al., 2000; Sirois et al., 2002). There are two consequences. First, blockage of TASK-1 channels causes a depolarization that can exceed 20mV in magnitude. This is because block of an outward K$^+$ current shifts the Vm away from the reversal potential for K$. Secondly, blockage of TASK-1 channels reduces the amount of current that leaks through the membrane and thus increases the effective membrane resistance. A decrease in the effective membrane resistance means
that the current generated on dendrites can travel a further distance (see equation 3 and 4). Both of these effects together may lead to an increase in the excitability of the motoneuron.

**2.3.4 Facilitation of HCN channel mediated current -> dendrite depolarization and more leak**

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels pass a positive inward current in response to hyperpolarization. HCN channels oppose both excitatory and inhibitory influences on the membrane potential: they are deactivated when the membrane is depolarized, and they provide positive current when the membrane is hyperpolarized (Accili et al., 2002). The current provided by HCN channels is facilitated in the presence of 5-HT (Figure 2.1B) (Takahashi and Berger, 1990; Larkman and Kelly, 1992; Hsiao et al., 1997). There are two consequences. First, the facilitation of HCN current causes a membrane depolarization due to an increase in positive inward current. Taken together with data on the TASK-1 current, the membrane depolarization caused by adding 5-HT is likely a combination of both the TASK-1 channels and the HCN channels. Secondly, unlike the effects 5-HT has on TASK channels, the facilitation of HCN current increases the amount of leak through the membrane.

**2.3.5 Facilitation of fast Na⁺ current -> lower threshold for action potential generation**

The characteristics of the fast Na⁺ current responsible for the generation of action potentials in the initial segment were described in detail in section 1.2. Another change to the motoneuron input-output relationship can be made by hyperpolarizing the voltage threshold needed to activate these channels (Dai et al., 2002; Fedirchuk and Dai, 2004; Gilmore and Fedirchuk, 2004). Both 5-HT and NA have been shown to shift the voltage activation range of the fast activating Na⁺ current. However, the modulation of these channels is highly dependent on whether or not a source of either modulator exists near the soma and initial segment.
2.3.6 Block of \( I_{Ka} \) - decrease first spike latency

\( I_{Ka} \) is a slowly inactivating K\(^+\) current known to be present on several neuronal cell types and important in determining the interval between consecutive action potentials (Cai et al., 2007). DA was shown to selectively block the \( I_{Ka} \) in motoneurons and this resulted in a decreased first spike latency, which is the time between the onset of current step and first spike (Han et al., 2007). This results in an increase in motoneuron firing frequency. There is evidence for the presence of Kv4.2 and Kv4.3 channels (responsible for \( I_{Ka} \)) in motoneurons (Alessandri-Haber et al., 2002). However, based on the limited data available it is unclear whether these effects are direct on motoneurons, or indirect through an intermediary pathway.

2.4 Why are the locations of modulatory synapses on motoneurons important?

This chapter described what is currently known about the effects of modulators on motoneurons. These effects have been determined with both the understanding of the biochemical pathways leading to receptor activation, and the resulting electrophysiological changes to the input-output relationship. However, what has not been considered in much detail as of yet are the locations of modulatory synapses on the motoneurons. Why would these locations be of such crucial importance to our understanding of these mechanisms? Consider for example the TASK-1 channels described in section 2.3.3. There is convincing evidence to show that TASK-1 channels are blocked by both 5-HT and NA. For hypothetical purposes consider that TASK-1 channels are located on motoneuron dendrites, soma and initial segment. If 5-HT synapses were only present on the motoneuron soma and initial segment, TASK-1 block in these areas could cause a membrane depolarization and mediate cell firing. This means that any synaptic input that reaches the soma is amplified, because TASK-1 channels influence the motoneuron output. Now consider another scenario: 5-HT synapses are only on medially located dendrites. In this case the block of TASK-1 channels by 5-HT is not to directly change the
motoneuron firing rate, but to reduce the leak in medial motoneuron dendrites, thereby allowing greater magnitudes of current in those dendrites to reach the soma. In this case, the block of TASK-1 would affect only medial synaptic inputs. This example demonstrates how the location of modulatory synapses can demonstrate an important cellular mechanism for selectively amplifying synaptic inputs.

Consider further that 5-HT, NA and DA have overlapping signaling cascades as well as effectors. Given that the actions of modulatory synapses are local, knowledge of the proximity of synapses from these systems can provide evidence into how they may interact in a physiological setting. Continuing the previous example, if both 5-HT and NA synapses are present only on medial dendrites, the co-activation of these systems allows for an interaction that may provide a more substantial amplification of current on medial dendrites than activation of either system alone. Conversely, if 5-HT synapses are located medially and NA synapses laterally, then co-activation of these systems may instead be a way of selectively amplifying inputs from either medial or lateral dendrites. These interpretations can only be made if the locations of modulatory synapses are known.
Chapter 3. Introduction Part III: What is currently known about the distributions of synapses on motoneurons, and what questions remain?

3.1 The known distributions of ionotropic synapses, ion channels, and modulatory synapses on motoneurons

There are two ways that synapses and ion channels can be arranged on the dendritic trees of motoneurons. The first possibility is that all synapses and voltage dependent channels are arranged in exactly the same way; in this case there would be no reason to suspect these locations are important for any aspect of motoneuron control. The second possibility is that different types of synapses and channels are confined to specific regions of the motoneuron, presumably placed to meet the demand of specific motor tasks. This section outlines the mounting evidence that makes the second possibility much more likely.

3.1.1 Distributions of ionotropic synapses

Given that a large percentage of total surface area in motoneurons is on the dendritic tree, it is not surprising that most ionotropic synapses are located on the dendrites of motoneurons. Of particular interest however is evidence supporting that the locations of synapses on motoneurons are not random, but instead organized in a specific manor depending on the source of the synapses. Some ionotropic synapses show a preference for proximal dendrites: inhibitory synapses projecting from Renshaw cells are located on only the proximal first third of motoneuron dendrites (Fyffe, 1991). On the other hand, some synapses are arranged with respect to motoneuron orientation: synapses from ipsilateral vestibulospinal axons have the highest density on motoneuron dendrites that project rostrally and caudally (Rose et al., 1995). Other synapses show a preference for specific regions of the dendritic tree: contralateral vestibulospinal synapses are found to be biased to medial dendrites (Grande et al.,
Finally, some synapses are not biased to any specific region: synapses from la afferents are distributed proportional to the available surface area (Brown and Fyffe, 1981; Burke and Glenn, 1996). These examples demonstrate that ionotropic synapses from different sources have different distributions on the dendritic tree.

### 3.1.2 Distributions of voltage dependent channels

As mentioned in section 1.5, of the 11 types of ion channels that are thought to exist on motoneurons, the locations of very few are known. However, it is known that KV2.1 channels are located on the soma, proximal and distal dendrites of motoneurons, with a propensity to aggregate near cholinergic synaptic sites (Muennich and Fyffe, 2004). Similarly, HCN-1 channels are located on the soma, proximal and distal dendrites and distributed proportional to surface area (Zhao et al., 2010). In contrast, the locations of L-type Ca$^{++}$ channels are more restricted; immunohistological evidence suggests that L-Type Ca$^{++}$ channels exist in 50µm-200µm zones, located 350 µm to 1250 µm from the soma. (Ballou et al., 2006; Zhang et al., 2006). The immunohistochemical evidence of L-Type Ca$^{++}$ channel hotspots is also supported by computational models, using the geometry of the dendritic tree and matching existing electrophysiological data (Elbsaiony et al., 2005; Bui et al., 2006; Grande et al., 2007; Shapiro and Lee, 2007). These studies found that the hotspots were approximately 100µm in length and at further distances away from the soma in large cell compared to small cells. Much more work will be required to fully characterize the distributions of ion channels on motoneurons. However, existing data show that ion channels have distinctive arrangements on the dendritic tree.
3.1.3 The distributions of neuromodulatory synapses

In section 2.2, eight different types of transmitters were listed as modulators of motoneurons. Of these, the distributions of only ACH, TRH, 5-HT and NA synapses have been investigated in depth on motoneurons. Like ionotropic synapses and ion channels, different types of modulatory synapses seem to innervate specific regions on motoneurons. For example, ACH terminals from local interneurons form synapses at the soma and proximal dendrites of motoneurons (Muennich and Fyffe, 2004; Wilson et al., 2004). On the other hand, TRH synapses on phrenic motoneurons are more widespread and exist on the soma, proximal and distal dendrites with a relatively low density (Murphy et al., 1995). The distributions of 5-HT synapses were first reported on hindlimb motoneurons in 1998 (Alvarez et al., 1998). This study found that 5-HT synapses were present on the soma, proximal and distal dendrites, and were distributed in proportion to the available surface area.

In a recent study conducted in our lab, the distributions of 5-HT and NA were simultaneously investigated on five neck extensor motoneurons (Montague, 2008). In contrast to the results presented by Alvarez et al (1998), there were very few 5-HT contacts found on the soma. In addition, the densities of both 5-HT and NA contacts on small diameter dendrites (<2 μm) were five to ten times higher than the densities of 5-HT synapses on larger diameter dendrites. NA synapses were also found to have a bias to dendrites dorsal of the soma. At a local level, pairs of like contacts (ie 5-HT and 5-HT or NA and NA) were closer together than predicted by a generated uniform distribution. On the other hand, unlike contacts (5-HT and NA) were further apart than would be predicted. The large discrepancy in the distribution of 5-HT synapses in the hindlimb compared to the distribution seen in neck extensor motoneurons suggests that the spatial arrangement of 5-HT synapses may be dependent on the type of motoneuron.
3.2 Statement of the problem

The distributions of modulatory synapses on motoneurons are not all the same. These distributions vary depending on the source of the synapses or the type of motoneuron being innervated. This suggests that the distribution of modulatory synapses may be crucial to the specific actions of modulators, or the specific tasks of motoneurons. The long term goal of this project is to answer the following question: what is the functional reason modulatory synapses have these distributions? This thesis asks two specific questions:

3.2.1 (I) Are 5-HT and NA synapses distributed differently on flexor versus extensor motoneurons?

One of the most basic motor tasks is the ability to use muscles for extension and flexion to execute a motor task. Recently, two lines of evidence have shown that flexor motoneurons have different intrinsic properties than extensor motoneurons. A study of plateau potentials in feline hindlimb motoneurons found that of 20 flexor motoneurons examined, none were capable of eliciting plateau potentials (Hounsgaard et al., 1988). More recently, a group studying hindlimb flexor motoneurons in neonatal rats reported that of 20 flexor motoneurons examined, none produced self sustained firing (Cotel et al., 2009). As mentioned in section 2.3.1, plateau potentials and self sustained firing are a result of PICs, which are only activated in the presence of 5-HT and NA. We propose four mechanisms that could explain this data. (i) 5-HT and NA synapses may be absent on flexor motoneurons, or present with a lower density. (ii) Alternatively, 5-HT and NA synapses may be present on flexor motoneurons with a different distribution. For example, if all 5-HT and NA synapses on flexor motoneurons exist on the soma, then L-type Ca\(^{2+}\) channels on the dendrites could not be facilitated to sustain the activation of PICs. (iii) The density of the voltage dependent Ca\(^{2+}\) and Na\(^{+}\) channels responsible for PICs may be lower on flexor motoneurons. (iv) Finally, flexor motoneurons may have a higher level of
inhibitory background activity that overcomes the ability of PICs to be self-sustaining. To decide which of these mechanisms are most likely, we have investigated the distribution of 5-HT and NA synapses on neck flexor motoneurons. There is anatomical and electrophysiological evidence to suggest that the ventrally located recti capitis anterior (RCA) muscle is primarily responsible for flexion movements of the head (Crouch, 1969; Selbie et al., 1993). Conversely, the dorsally located splenius muscles are activated unilaterally to yaw the head, and bilaterally to keep the extended upwards (Crouch, 1969; Wickland et al., 1991; Pettorossi et al., 1993). A study on the distributions of 5-HT and NA on RCA motoneurons provides an ideal comparison to previous 5-HT and NA distributions collected from splenius motoneurons (Montague, 2008).

### 3.2.1 (II) Are there DA synapses on motoneurons, and if so how are they distributed?

Although the distributions of only four different types of modulatory synapses have been investigated to date, there are some differences in distributions depending on the type of modulator. One hypothesis is that these differences in distribution facilitate the functional roles of each modulator. For example cholinergic terminals from local interneurons modulate motoneuron excitability by decreasing the AHP of action potentials (Miles et al., 2007). It is logical then that cholinergic terminals are restricted to the cell body and initial segment where action potentials are generated (Muennich and Fyffe, 2004; Wilson et al., 2004). Although there is mounting electrophysiological and immunohistological evidence that DA exist on motoneurons (see section 2.2.1, 2.3.2 and 2.3.6), there have been no investigations of the specific distributions of DA contacts on motoneurons. Electrophysiological evidence suggests that DA can change the output of motoneurons: DA facilitates SK current and shortens the AHP and DA blocks $I_{KA}$ which results in a decreased first spike latency on rat motoneurons (Han et al., 2007). Since these effects change the kinetics of action potential generation, the prediction is that DA synapses would be located on the soma and initial segment. To test this hypothesis, we
have also investigated the distribution of DA synapses on RCA motoneurons. If DA is restricted to these regions it will provide another example of a modulator that strictly adjusts motoneuron output.

3.3 Statement of the goal

The goal of this project is to describe the distributions of 5-HT, NA and DA synapses on neck flexor motoneurons in the adult feline.
Chapter 4. Methods

4.1 Animal Preparation

Experiments were performed on 5 adult female felines weighing 3.1-4.6 kg. All experimental protocols were approved by the Queen’s University Animal Care Committee. Animals were premedicated with glycopyrrolate (0.01 mg/kg; Sandoz), hydromorphone (0.075 mg/kg; Purdue Pharma Inc.), ketamine (5.0 mg/kg; Bioniche) and medetomidine (0.03 mg/kg; Pfizer) to induce anesthesia. Deep anesthesia was maintained with doses of sodium pentobarbital (2.5-5 mg/kg; CEVA Sante Animale) administered intravenously upon sudden increase of heart rate. This protocol maintained the heart rate between 150-160 beats per minute. Heart rate and oxygen saturation was continuously monitored with a digital monitor (Nonin Inc.). Rectal temperature was monitored using a YSI tele-thermometer system and maintained at 35 °C -37 °C with a heating blanket. Normosol-R (10 ml/kg/hr; Hospira) was administered intravenously to maintain fluid level.

Rectus Capitus Anterior (RCA) are deep ventral neck muscles located below the trachea (Crouch, 1969). Surgery to access the RCA nerves was completed as described by Kitamura and Richmond (1994). To access the nerves, the animal was placed in dorsal recumbency and a midline incision was made on the ventral surface of the neck. The superficial musculature and trachea were gently retracted to locate C1, C2 and C3 RCA nerves. Isolated nerves were then positioned on bi-polar stimulating electrodes. Musculature and trachea were returned to original locations. Animals were then placed in a ventral recumbency and a laminectomy extending from C1 to C4 was performed. Animals were subsequently secured in a stereotaxic frame (Transvertex, AB). The nerves supplying the neck muscles trapezius, biventer cervicis, complexus, and splenius were isolated. All isolated nerves were then positioned on bi-polar
stimulating electrodes. The animals were paralyzed with gallamine triethiodode (3 mg/kg/hour; Sigma) and artificially respired to maintain end tidal CO₂ between 3.7-4.5%. A bilateral pneumothorax was performed to reduce respirator-related movements.

### 4.2 Motoneuron Identification and Intracellular Injections

Intracellular recordings were obtained with glass micropipettes (ranging from 1.5-1.9 μm in diameter) broken to create sharp tips. The pipettes were filled with 12% Neurobiotin (Vector Laboratories) in 0.5 M KCl and 0.10 M Trizma buffer (pH 8.2). Pipette resistance varied from 5-15 MΩ. To prevent leakage of positively charged neurobiotin while searching for motoneurons, a negative current of 5 nA was used. RCA motoneurons from C1 to C3 were antidromically identified by stimulating the C1, C2 or C3 nerves that supply the RCA muscle.

RCA motoneurons were stained by passing positive current pulses (450 ms at 2 Hz) of 6-10 nA for 2-3 min (Figure 4.1A). Total charge delivered varied from 21 na•min to 25 na•min. In order to minimize overlap of dendritic trees from adjacent cells, each motoneuron was separated by at least 3 mm. In a preliminary experiment, stained cells closely resembled trapezius motoneurons in both ventral horn location and morphological characteristics (Vanner and Rose, 1984). It was concluded that these cells were antidromically activated due to current spread from stimulating electrodes in the RCA nerve to the nearby spinal accessory nerve. As such, we took special care to limit current spread and incorrect cell identification in subsequent experiments using two measures: (1) Fluid buildup in the neck was drained to limit any current spread to the spinal accessory nerve. (2) After processing, the cell must have an axon that exits the motoneuron pool ventrally as opposed to trapezius motoneurons which have axons that exit the motoneuron pool dorsally (Vanner and Rose, 1984). As a final general criterion for all selected cells, the motoneuron must be well stained with neurobiotin.
Figure 4.1 Figures A, B and C illustrate the three step methodology involved in motoneuron reconstruction and contact placement. Image in C is a 63x oil immersion confocal image of motoneuron dendrites and boutons. 5HT, NA and DA boutons are visualized in magenta, yellow/orange and green, respectively. Examples of contacts are indicated with stylized arrows.
4.3 Perfusion and Histology

Prior to perfusion, heparin (5ml) was intravenously administered and followed by a lethal dose of sodium pentobarbital. Animals were then perfused with saline (1000 ml), followed by 2 liters of fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The upper cervical cord was removed and refrigerated overnight in fixative. After 15-20 hours, the tissue was removed from fixative and placed in a 15% sucrose buffer for 3 days. Subsequently, 50μm horizontal sections of the spinal cord were cut using a freezing microtome (Leitz Wetzlar Germany; Model 44011). Sections were then placed into 30% ethylene glycol, 30% sucrose in phosphate buffer, and stored at 4 °C for a minimum of two weeks prior to further processing.

In order to visualize NA, 5-HT and DA axon terminals as well as the neurobiotin filled cell, a quadruple labeling approach was taken (Figure 4.1B). Sections were taken out of at 4°C storage and placed into buffer (0.02 M potassium phosphate buffer solution (KPBS)). To inhibit autoflouresence, sections were incubated in 1% sodium borohydride in 0.02 M KPBS for 20 minutes. To expose the antigenic sites in tissue, sections were incubated in 10 mM sodium citrate buffer (pH 8.3-8.5) at 80 °C for 30 minutes (Jiao et al., 1999). In order to block non-specific labeling, sections were then incubated overnight in 5% donkey serum. To stain for NA boutons, sections were incubated overnight in rabbit anti-dopamine beta hydroxylase (1:1000; Biomol DZ1020) in 0.02M KPBS and 3% donkey serum. Sections were then incubated with CY3 conjugated donkey anti-rabbit IgG (1:300; Jackson 711-165-152) for 2 hours at room temperature. To stain for NA and DA boutons, sections were incubated in sheep anti-tyrosine hydroxylase (1:300; Abcam AB1542) for 36 hours at room temperature in 0.02M KPBS and 3% donkey serum with sodium azide. Sections were then incubated with ALEXA488 conjugated donkey anti-sheep (1:300; Invitrogen A11015) for 2 hours at room temperature. To stain 5-HT boutons sections were incubated in rat anti-5-HT (1:1000; Invitrogen SZ1011) for 36 hours at
room temperature in 0.02M KPBS and 3% donkey serum with sodium azide. Sections were then incubated with AMCA donkey anti-rat (1:200; Jackson 712-155-150) for 2 hours at room temperature. Finally, to visualize filled cells, sections were incubated with CY5 conjugated streptavidin (1:100; Jackson 016-170-084) for 3 hours at room temperature.

4.4 Identification of 5-HT, NA, DA and 5-HTTH boutons

Three biochemical markers were used to identify boutons: 5-HT, DβH, TH. In the case of 5-HT, boutons were directly identified based on axonal swellings that were positive for the transmitter 5-HT. Examples of 5-HT boutons can be seen in Figure 4.1C as magenta swellings. A small subset of 5-HT positive boutons were also positive for TH. The existence of 5-HTTH positive boutons has been observed previously (Vanhatalo et al., 1995). These varicosities were counted independently of 5-HT-only boutons, however for the purposes of comparison to previous studies on 5-HT distributions, 5-HTTH boutons were treated as additional 5-HT boutons. NA and DA varicosities were identified indirectly, based on the enzymes required to synthesize the transmitter. The biosynthetic pathway of DA and NA is shown below: (Nestler et al., 2001).

\[
\text{Tyrosine} \xrightarrow{\text{hydroxylase}} \text{Dihydroxyphenylalanine} \xrightarrow{\text{\textit{l}-Aromatic amino acid decarboxylase}} \text{Dopamine} \xrightarrow{\text{Dopamine-\textit{\beta} hydroxylase}} \text{Noradrenalin}
\]

To synthesize NA, boutons must possess both TH and DβH enzymes. Hence, boutons positive for both DβH and TH were classified as NA boutons. These swellings appeared as orange or red (see Figure 4.1C), presumably because each NA bouton had different relative concentrations of TH and DβH enzymes. TH is required to produce DA, but DβH is not. Hence, any TH positive swellings that were negative for DβH were classified as DA boutons. These swellings appeared
as bright green (Figure 4.1C). This method of classifying DA boutons has been used in past studies (Bjorklund and Hokfelt, 1984; Allard et al., 2010).

### 4.5 Specificity of Antibodies

The rabbit anti- DβH (Biomol DZ1020) antibody has been used by several previous groups to visualize NA boutons in both cat spinal cord (Jankowska and Gladden, 1999; Maxwell et al., 2000; Hammar and Maxwell, 2002; Hammar et al., 2004). However these previous studies did not describe controls for specificity in the cat spinal cord, and to our knowledge there are no other detailed descriptions of DβH in the spinal cord.

The anti sheep anti-TH (Abcam AB1542) antibody has been used in by three previous studies to visualize TH in the rat spinal cord (Kalous et al., 2007, 2009; Ramer, 2008). Ramer et al (2008) used double labeling with another polyclonal rabbit anti-TH (AB152), which labeled the same structures as the sheep anti-tyrosine hydroxylase (Abcam AB1542). According to equation 5 in section 4.4, all NA terminals require the colocalization of both DβH and TH enzymes. As confirmation of specific labeling for both DβH and TH antibodies, 100% of the boutons positive for DβH were also positive for TH. In addition, DA boutons near the central canal (lamina X) and dorsal horn (lamina I-IV) were particularly dense, which matches previous descriptions of DA immunoreactivity in the cat spinal cord (Holstege et al., 1996).

The rat anti-5-HT (Invitrogen SZ1011) has been used in past studies in the feline to visualize the distribution of 5-HT terminals on the dendrites of lamina VII interneurons (Maxwell et al., 2000), γ-motoneurons (Gladden et al., 2000) spinocerebellar neurons (Hammar and Maxwell, 2002) spinal commissural interneuons (Hammar et al., 2004), and dorsal horn interneurons (Dougherty et al., 2005). The pattern of labeling seen in this study (dense collections of swellings, usually 1 um in diameter, separated by thin processes) was identical to
previous studies using different antibodies to visualize 5-HT in the cat lumbar spinal cord (Arvidsson et al., 1990; Alvarez et al., 1998). The immunoreactivity of this antibody are completely blocked with the serotonin-formaldehyde condensation product 6-hydroxy-1,2,3,4-tetrahydro-β-carboline according to the manufacturers information.

4.6 Imaging

All RCA motoneurons were reconstructed with the aid of an Olympus BX60 microscope and Neurolucida (V9.03; Microbrightfield Inc) neuron tracing software. The bandpass and emission filters used (Chroma Technology Corp., VT, USA) were selected to isolate fluorescence signals from CY5 (650abs, 670ems), CY3 (550abs, 570ems) ALEXA488 (462abs, 519ems) and AMCA (350abs, 450ems). Double labeling of axon processes and boutons under two different filters was found. To ensure that these observations were not a result of “bleedthrough” of fluorescence from one flourochrome that could be seen under both filters we ran several controls. As a first control, only DβH was labeled and visualized using a CY3 fluorescent marker in test tissue. Subsequently, this test tissue was examined under the CY5, CY3, ALEXA488 and AMCA filters. Only the CY3 filter showed any signal, indicating that there is no bleedthrough. This control was repeated for CY5 labeled dendrites, ALEXA488 labeled axons and AMCA labeled axons, respectively. There was no evidence of bleedthrough in any of these cases, leading us to conclude that bleedthrough was negligible in our preparation.

4.7 Reconstruction

Four image stacks of the same region containing dendrites were taken using the CY5, CY3, ALEXA488 and AMCA filters. These image stacks were then merged and could be mapped to any given colour and toggled on and off using Neurolucida image adjustment. This allowed for simultaneous view of the dendrites as well as 5-HT, NA and DA boutons. 1-18 regions were
captured using this process for a given 50μm section in order to acquire all dendrites in that section. The location of each region was saved by Neurolucida such that dendrites extended across multiple regions. After pictures were taken, dendrites were traced by placing points along the dendritic tree. Each point has an associated diameter that corresponds to the diameter of the dendrite at that location. This process was repeated 30-36 times for each of the 50μm sections that contained dendrites for a given cell until the entire cell was reconstructed from the soma to the distal tips of dendrites.

4.8 Contact Identification

After the dendrites in a section were traced and prior to merging with dendrites in the adjacent section, contacts from 5-HT, NA and DA boutons were placed on the dendritic tree. All contacts were subject to the same criteria for inclusion: i) the bouton must be a round elliptical swelling whose diameter was twice that of the adjacent collateral shaft, ii) there was no discernable gap between the swelling and the dendrite, and iii) both the bouton and the dendrite at the site of the putative contact were in the same focal plane (see Figure 4.1C for examples of contacts). These criteria have been used in several previous studies and nearly 9 out of every 10 appositions have been shown to correspond to synaptic contacts at the electron microscopic level (Fyffe, 1991; Alvarez et al., 1998; Carr et al., 1999; Lübke et al., 2000; Grande et al., 2005). The locations of 5-HT, NA and DA contacts were added to the reconstruction of the dendritic tree with the aid of Neurolucida (V9.03; Microbrightfield Inc).

4.9 Defining Dendritic Sub-trees

For some types of analysis, the dendritic tree was divided into sub-trees. Each sub-tree consisted of all of the branches emerging from a primary dendrite. However, identification of primary dendrites can be unclear due to the rapid tapering of the dendrite at the junction with
the soma and the emergence of early dendrite branches. Thus what constitutes the soma and what belongs to primary dendrites can often be ambiguous. We have adapted a strategy used by previous groups to define primary dendrites (Ulfhake and Kellerth, 1981; Rose et al., 1985). If the process emerging from the soma has a branch point 30 µm or less from the edge of the soma, then the two emerging processes are considered to be separate primary dendrites. The edge of the soma is defined as the boundary of an ellipse that can be traced over the shape of the soma.
Chapter 5. Results

5.1 5-HT, NA and DA boutons are present in flexor and extensor motoneuron nuclei

The numbers of 5-HT, NA and DA boutons in the regions containing splenius motoneuron cell bodies and RCA cell bodies were systematically compared (Figure 5.1A). These regions were defined based on previous descriptions (Keirstead and Rose, 1983; Kitamura and Richmond, 1994). The relative abundance of 5-HT, NA and DA boutons in RCA versus splenius motoneuron nuclei is 11:8:1 and 19:11:1, respectively (Figure 5.1B). Absolute counts of boutons indicate that the numbers of NA boutons are comparable in RCA and splenius motoneuron regions, as are the numbers of DA boutons. However, the number of 5-HT boutons in splenius motoneuron regions is 1/3 greater than in RCA motoneuron regions.

5.2 RCA motoneurons have a unique morphology

Five RCA motoneurons from three different experiments were reconstructed: SNDA32, SNDA42, SNDA43, SNDA51 and SNDA52 (Figure 5.2). RCA motoneurons have large arching dendrite sub-trees which project into the white matter, cross the midline and terminate in the contralateral ventral horn. Every RCA motoneuron had at least one of these sub-trees. Table 5.1 lists the morphological characteristics of RCA motoneurons. The number of primary dendrites ranged from 9-11 and total dendritic surface area ranged from 353,171μm² to 678,436 μm², which are comparable to previous studies of feline motoneurons (Ulfhake and Kellerth, 1981, 1983; Rose et al., 1985; Cullheim et al., 1987; Grande et al., 2005). All RCA motoneuron cell bodies were located in the medial 1/3 of the ventral horn of the spinal cord in segments ranging from C1 to C3, which satisfies the previous description of RCA motoneuron cell body locations (Kitamura and Richmond, 1994).
Figure 5.1 (A) Picture of DβH in the C2 spinal cord ventral horn with the regions containing RCA and splenius motoneuron cell bodies outlined in red. (B) The numbers of swellings in two splenius motoneuron regions and two RCA motoneuron regions with dimensions of 250 μm x 250 μm x 50 μm.
Figure 5.2 The reconstructed dendritic trees of RCA motoneurons from C1 (SNDA43) (A), C2 (SNDA52) (B), and C3 (SNDA32) (C) spinal segments. Cells are shown from a horizontal view (left) and transverse view (right). Ventral horn outline and midline are shown in gray.
<table>
<thead>
<tr>
<th>Cell</th>
<th>Spinal segment</th>
<th>Number of primary dendrites</th>
<th>Total dendritic length (µm)</th>
<th>Total dendritic surface area (µm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>C3</td>
<td>9</td>
<td>92,287</td>
<td>678,436</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>C1</td>
<td>12</td>
<td>77,913</td>
<td>588,138</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>C1</td>
<td>13</td>
<td>70,589</td>
<td>572,378</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>C2</td>
<td>13</td>
<td>62,516</td>
<td>353,171</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>C2</td>
<td>11</td>
<td>66,718</td>
<td>517,482</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>11.6</strong></td>
<td><strong>74,005</strong></td>
<td></td>
<td><strong>541,921</strong></td>
</tr>
</tbody>
</table>

**Table 5.1** Morphological characteristics of RCA motoneurons.
5.3 5-HT, NA and DA contact the dendrites of RCA motoneurons, but the density of DA contacts is low compared to 5-HT and NA contacts

The distribution of 5-HT, NA and DA contacts are shown on SNDA52 in Figure 5.3. There is a relatively high abundance of both 5-HT and NA contacts on the dendritic tree, without any obvious bias to any particular region on the tree. DA contacts are less dense on the dendritic compared to 5-HT or NA contacts. The numbers and densities of contacts on the dendritic trees of all five cells are listed in Table 5.2A and Table 5.2B. The average number of 5-HT contacts over all five cells was 793, with an average density of 1.40 contacts/1000 μm². The average number of NA contacts was 897, with a average density of 1.65 contacts/1000 μm². There were far fewer DA contacts on the dendritic tree with an average of 63 contacts over five cells and an average density of 0.11 contacts/1000 μm². There were also several examples of 5-HTTH contacts, however the average density of these contacts was low at about 0.04 contacts/1000 μm².

Only one NA contact was located directly on the cell body of SNDA52 (Figure 5.3B). This near absence of somatic innervation was typical of the distribution of all cells. The high concentration of neurobiotin that is present in the soma after an intracellular fill can result in the spread of fluorescence when using an epi-flourescent microscope. This fluorescence spread makes it difficult to define the edge of the somatic membrane and hence it is difficult to confidently identify somatic contacts. It is possible that the absence of labeled contacts on the cell body could have been due to fluorescence spread. To rule out this possibility, high resolution confocal image stacks (63X oil immersion, 0.4 μm steps) were taken of the soma for all five cells. In total, three somatic NA contacts were identified across all five cells and no 5-HT or DA contacts were identified. In addition, no contacts were found to contact the axon hillock or initial segment.
Figure 5.3 The distribution of 5HT, NA and DA contacts on SNDA52. Two distally located dendrites (A and C), and the soma (B) are enlarged.
Table 5.2A – Summary of 5-HT and NA contacts on each motoneuron, reported as the absolute number and density with respect to dendritic surface area. 5-HT contact numbers includes contacts that were classified as having both 5-HT and TH present in axon terminals.

<table>
<thead>
<tr>
<th>Cell</th>
<th>5HT contacts</th>
<th>NA contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Number</td>
<td>Contacts/1000 μm²</td>
</tr>
<tr>
<td>SNDA3-2</td>
<td>1430</td>
<td>2.11</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>815</td>
<td>1.39</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>696</td>
<td>1.21</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>381</td>
<td>1.07</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>643</td>
<td>1.24</td>
</tr>
<tr>
<td>Average</td>
<td>793</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Table 5.2B – Summary of DA and 5-HTTH contacts on each motoneuron, reported as the absolute number and density with respect to dendritic surface area.

<table>
<thead>
<tr>
<th>Cell</th>
<th>DA Contacts</th>
<th>5HTTH Contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Number</td>
<td>Contacts/1000 μm²</td>
</tr>
<tr>
<td>SNDA3-2</td>
<td>132</td>
<td>0.19</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>47</td>
<td>0.08</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>58</td>
<td>0.10</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>39</td>
<td>0.11</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>41</td>
<td>0.08</td>
</tr>
<tr>
<td>Average</td>
<td>63.4</td>
<td>0.11</td>
</tr>
</tbody>
</table>
5.4 5-HT, NA and DA have a bias to distal and small diameter dendrites

Figure 5.4 shows the relationship between distance from the cell body and the density of contacts. Distances were grouped into 250μm bins and plotted with respect to normalized density. There is a significant positive relationship (P<0.05 Spearman rank order correlation) between distance from the cell body and the average normalized density of 5-HT, NA and DA contacts, respectively. The average normalized density of contacts in each distance bin for all cells is summarized in Table 5.3. The average density of contacts in the furthest distance bin (1500-1750 μm) compared to the closest distance bin (0-250 μm) is greater by a factor of 1.6, 2.0 and 3.4 for 5-HT, NA and DA contacts, respectively.

Dendrites decrease their diameter as they extend distally away from the soma (Rose et al., 1985; Cullheim et al., 1987). Given that 5-HT, NA and DA contacts tend to be biased to distal dendrites, it is reasonable to expect that there is also a bias to dendrites with smaller diameters.

Figure 5.5 shows the relationship between dendrite diameter and the density of contacts. Diameters were grouped into six bins and plotted with respect to normalized density. There is a significant relationship (P<0.05 Spearman rank order correlation) between dendrite diameter and the average normalized density of 5-HT, NA and DA contacts. The bias to small diameter dendrites is more pronounced than the bias to distal dendrites. The average normalized density of contacts in each diameter bin for all cells is summarized in Table 5.4. The average density of contacts in the smallest diameter bin (<1 μm) compared to the largest diameter bin (>7.9 μm) is greater by a factor of 5.7, 16.4, and 6.2 for 5-HT, NA and DA contacts, respectively.
**Figure 5.4** Bias to distal dendrites for 5HT, NA and DA contacts. The normalized density of contacts per distance bin for all five cells is shown with the average superimposed for 5-HT (A), NA (B) and DA (C) respectively. Normalized density is calculated by dividing absolute density of contacts in each bin by the average density of contacts for each cell.
<table>
<thead>
<tr>
<th>Distance from soma (μm)</th>
<th>Average normalized 5-HT Density</th>
<th>Average normalized NA Density</th>
<th>Average normalized DA Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-250</td>
<td>0.93</td>
<td>0.75</td>
<td>0.41</td>
</tr>
<tr>
<td>250-500</td>
<td>0.82</td>
<td>0.95</td>
<td>0.87</td>
</tr>
<tr>
<td>500-700</td>
<td>0.83</td>
<td>1.06</td>
<td>1.00</td>
</tr>
<tr>
<td>700-1000</td>
<td>1.02</td>
<td>1.11</td>
<td>1.21</td>
</tr>
<tr>
<td>1000-1250</td>
<td>1.38</td>
<td>1.31</td>
<td>1.27</td>
</tr>
<tr>
<td>1250-1500</td>
<td>1.48</td>
<td>1.14</td>
<td>2.02</td>
</tr>
<tr>
<td>1500-1750</td>
<td>1.50</td>
<td>1.47</td>
<td>1.39</td>
</tr>
</tbody>
</table>

**Table 5.3** Table listing the average normalized density in each distance bin across all five cells. Normalized density is calculated by dividing absolute density of contacts (contacts/1000 μm²) in each bin by the average density of contacts for each cell.
Figure 5.5 Bias to small diameter dendrites for 5HT, NA and DA contacts. The normalized density of contacts per diameter bin for all five cells is shown with the average superimposed for 5-HT (A), NA (B) and DA (C). Note that data points for some cells in some bins are not shown. These data points were excluded because they represented <1% of the dendritic surface area or <1% of the total contacts.

** Indicates Relationship between diameter bin and normalized density is significant (p<0.05) according to a spearman rank order correlation test.
<table>
<thead>
<tr>
<th>Diameter bin (µm)</th>
<th>Average normalized 5-HT Density</th>
<th>Average normalized NA Density</th>
<th>Average normalized DA Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;7.9</td>
<td>0.50</td>
<td>0.16</td>
<td>0.37</td>
</tr>
<tr>
<td>6-7.9</td>
<td>0.76</td>
<td>0.54</td>
<td>0.39</td>
</tr>
<tr>
<td>4-5.9</td>
<td>0.82</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>2-3.9</td>
<td>0.80</td>
<td>0.85</td>
<td>0.92</td>
</tr>
<tr>
<td>1-1.9</td>
<td>1.40</td>
<td>1.37</td>
<td>1.36</td>
</tr>
<tr>
<td>&lt;1</td>
<td>2.83</td>
<td>2.63</td>
<td>2.30</td>
</tr>
</tbody>
</table>

**Table 5.4** Table listing the average normalized density in each diameter bin across all five cells. Normalized density is calculated by dividing absolute density of contacts in each bin by the average density of contacts for each cell.
5.5 Reference distributions of contacts can be used to statistically prove biases

To determine whether the observed distributions of 5-HT, NA and DA contacts are statistically different than one would expect if the same number of contacts were uniformly distributed, we developed a series of tests customized to each dendritic tree. This technique is illustrated in Figure 5.6. In this example, the goal is to determine if 643 5-HT contacts on SNDA52 are distributed differently than one would expect if the contacts were distributed uniformly. To do this, the dendritic tree is stripped of all contacts and then assigned over 20,000 contacts which are uniformly distributed on the tree (one contact every 28 μm²). From this population of over 20,000 contacts, 643 are randomly selected to create a reference distribution (RD) of contacts. This process is repeated 1000 times to produce 1000 different RDs. Because each RD is drawn from a population of contacts that are distributed based on surface area alone, the RDs are representative of uniform distributions.

1000 RDs can be compared to the observed 5-HT distribution. For example, if the average coordinates of the observed 643 5-HT contacts are more dorsal than the average coordinates of 643 contacts on all 1000 RDs, we can say that the observed location of 5-HT contacts is more dorsal than would be expected if the distribution were uniform. More specifically, there is less than a 1/1000 chance that the observed 5-HT distribution is representative of a uniform distribution. If the observed distribution is more dorsal than a maximum of 25 of the RDs then the observed distribution is significantly biased with a P value of less than 0.05 according to the equation below.

\[
\frac{25}{1000} = 0.025 \quad 0.025 \times 2 \text{ (for a two tailed test)} = 0.05 \quad (6)
\]

A second test that can be used to measure significance between the observed distributions and RDs is a non-parametric alternative to the t-test for independent samples known as the
Figure 5.6 The statistical technique used to generate and use reference distributions representative of uniform distributions. See text for details.
Wilcoxon Signed-Rank Test. The Wilcoxon Signed-Rank test is used when comparing the observed result to the RDs for all five cells. If observed data from all five cells are consistently biased in one direction compared to RDs, the bias is significant (P<0.05).

5.5.1 5-HT and NA contacts have higher densities on small diameter dendrites than would be predicted if the distribution were uniform

Figure 5.7 compares the observed density of 5-HT, NA and DA contacts in each diameter bin to 1000 RDs for a representative cell (SNDA52). For 5-HT and NA contacts, the observed density in the two smallest diameter bins (<2.0 μm) are significantly higher than predicted by the 1000 RDs (P<0.05 for 4/4 comparisons). In the remaining four large diameter bins (>2.0 μm), with the exception of the largest diameter bin for 5-HT contacts, the density of 5-HT and NA contacts was significantly lower than predicted by 1000 RDs (P<0.05 for 7/8 comparisons). In contrast the density of DA contacts in each diameter bin was not different than predicted by the RDs. Hence DA contacts are not statistically more biased to smaller diameter dendrites than would be predicted based on RDs representative of uniform distributions for SNDA52.

Table 5.5A, 5.5B and 5.5C summarize the differences between the observed density of contacts in each diameter bin, and the density of contacts for the median RD in each diameter bin across all five cells. Consistent with the representative cell shown in Figure 5.6, the observed densities of 5-HT and NA contacts on small diameter dendrites (<2.0 μm) were always greater than predicted by the median RD (5-HT: P<0.05 for 7/8 comparisons; NA: P<0.05 for 8/8 comparisons). Conversely, with the exception of 5-HT contacts on SNDA43 in the 6-7.9 μm diameter bin, 5-HT and NA contacts on large diameter dendrites (>2.0 μm) were always less
Figure 5.7 Bias to small diameter dendrites for 5HT, NA and DA contacts. The observed absolute density in each diameter bin is compared to 1000 RDs represented as box plots on SNDA 5-2 for 5-HT (A), NA (B) and DA (C).
Table 5.5A – Difference between the observed 5-HT synapse density in each diameter bin and the median predicted 5-HT contact density of 1000 RDs in each diameter bin. A positive value indicates that the density of 5HT in a given bin was greater than would be expected and a negative value indicates that the density of 5HT was less than expected.

<table>
<thead>
<tr>
<th>Cell</th>
<th>&gt;7.9 μm</th>
<th>6-7.9 μm</th>
<th>4-5.9 μm</th>
<th>2-339 μm</th>
<th>1-1.9 μm</th>
<th>&lt;1 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-1.21*</td>
<td>-0.58*</td>
<td>-0.91*</td>
<td>-0.23*</td>
<td>0.97*</td>
<td>3.44*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-0.91*</td>
<td>-0.65*</td>
<td>-0.17</td>
<td>-0.34*</td>
<td>0.59*</td>
<td>3.90</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-0.19</td>
<td>0.27</td>
<td>-0.07</td>
<td>-0.32*</td>
<td>0.75*</td>
<td>-0.36</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>0.00</td>
<td>1.94</td>
<td>-0.15</td>
<td>-0.27*</td>
<td>0.12</td>
<td>0.78</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-0.66</td>
<td>-0.45*</td>
<td>-0.17*</td>
<td>-0.16*</td>
<td>0.32*</td>
<td>3.19</td>
</tr>
<tr>
<td>Average</td>
<td>-0.74</td>
<td>-0.35</td>
<td>-0.29**</td>
<td>-0.26**</td>
<td>0.55**</td>
<td>2.47</td>
</tr>
</tbody>
</table>

*Indicates that the density was significantly greater or lesser than expected using a generated uniform distribution for 1000 trials (p<0.05). Values in red were excluded because the surface area of the dendrites found in that bin was <1% of the total surface area of the dendritic tree.

Table 5.5B – Difference between the observed NA synapse density in each diameter bin and the median predicted NA contact density of 1000 RDs in each diameter bin. A positive value indicates that the density of NA in a given bin was greater than would be expected and a negative value indicates that the density of NA was less than expected.

<table>
<thead>
<tr>
<th>Cell</th>
<th>&gt;7.9 μm</th>
<th>6-7.9 μm</th>
<th>4-5.9 μm</th>
<th>2-339 μm</th>
<th>1-1.9 μm</th>
<th>&lt;1 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-1.46*</td>
<td>-0.58*</td>
<td>-0.42*</td>
<td>-0.28*</td>
<td>0.78*</td>
<td>3.44*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-1.30*</td>
<td>-0.98*</td>
<td>-0.51*</td>
<td>-0.23*</td>
<td>0.74*</td>
<td>5.32</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-1.22*</td>
<td>-0.65*</td>
<td>-0.22</td>
<td>-0.08</td>
<td>0.45*</td>
<td>5.08</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>0.00</td>
<td>0.49</td>
<td>-0.21</td>
<td>-0.27*</td>
<td>0.19*</td>
<td>1.37</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-1.00*</td>
<td>-0.45*</td>
<td>-0.31*</td>
<td>-0.23*</td>
<td>0.72*</td>
<td>2.68</td>
</tr>
<tr>
<td>Average</td>
<td>-1.25</td>
<td>-0.67</td>
<td>-0.33**</td>
<td>-0.22**</td>
<td>0.58**</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Table 5.5C – Difference between the observed DA synapse density in each diameter bin and the median predicted DA contact density of 1000 RDs in each diameter bin. A positive value indicates that the density of DA in a given bin was greater than would be expected and a negative value indicates that the density of DA was less than expected.

<table>
<thead>
<tr>
<th>Cell</th>
<th>&gt;7.9 μm</th>
<th>6-7.9 μm</th>
<th>4-5.9 μm</th>
<th>2-339 μm</th>
<th>1-1.9 μm</th>
<th>&lt;1 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-0.05</td>
<td>-0.03</td>
<td>-0.02</td>
<td>0.02</td>
<td>-0.02</td>
<td>0.37</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>0.00</td>
<td>-0.07</td>
<td>-0.02</td>
<td>-0.03*</td>
<td>0.06*</td>
<td>0.71</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>0.00</td>
<td>-0.05</td>
<td>-0.02</td>
<td>-0.03*</td>
<td>0.10*</td>
<td>0.00</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>0.00</td>
<td>0.00</td>
<td>-0.06</td>
<td>0.01</td>
<td>-0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-0.08</td>
<td>-0.04</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Average</td>
<td>-0.03</td>
<td>-0.05</td>
<td>-0.02</td>
<td>-0.01</td>
<td>0.03</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Indicates that all 5 cells were biased one way and significant according to a Wilcoxon Signed-Rank Test (P<0.05)
than predicted by the median RD (5-HT: P<0.05 for 12/18 comparisons; NA: P<0.05 for 15/18 comparisons). There is no consistent evidence that DA is distributed non-uniformly with respect to diameter.

5.5.2 NA contacts are more biased to lateral dendrites and DA contacts are biased to dorsal dendrites than expected if the distribution were uniform

The center of mass (COM) analysis has been used in previous studies to test if the observed distributions of contacts are biased to dorsal-ventral or medial-lateral, or rostral-caudal regions on the dendritic tree (Grande et al., 2005). The COM of a given distribution of contacts is the average X, Y and Z coordinates of all the contacts. The COM of contacts from the observed distribution can be compared to the COMs of 1000 RDs. This process is shown for a representative cell (SNDA42) in Figure 5.8. In this example, the COMs of the observed 5-HT and NA distributions are located more laterally than the COMs of 1000 RDs. The COM of the observed DA contacts is located more dorsally than the COMs of 1000 RDs.

Table 5.6A, Table 5.6B and Table 5.6C summarize the differences between the COMs of the observed distributions and the average COMs of 1000 RDs for 5-HT, NA and DA contacts, respectively. A value of zero indicates that there was no COM difference between the observed distribution of contacts and the average COMs of 1000 RDs. The COMs of 5-HT contacts across all cells was heterogeneous; three cells are significantly biased more medially (P<0.05 compared to 1000 RDs) and two are significantly biased more laterally (P<0.05 compared to 1000 RDs) than would be expected if the distribution were uniform. On the other hand, the COMs of NA contacts for all five cells are more lateral than the average COMs of 1000 RDs (P<0.05 for 4/5 comparisons). In addition, the COMs of DA contacts for all five cells are more dorsal than the average COMs of 1000 RDs (P<0.05 for 3/5 comparisons). Since the COMs of NA contacts are
Figure 5.8 The COM analysis from SNDA42 for 5-HT (A), NA (B) and DA (C) contacts, respectively. The observed COMs are illustrated with coloured stars, and the COMs from 1000 RDs are illustrated as black dots (cyan stars mark the average of all RD COMs). Note that the X and Y axis in C is expanded compared to A and B.
Table 5.6A – Differences in μm between the observed COM of 5-HT contacts and the average COM of 1000 RDs.

<table>
<thead>
<tr>
<th>Cell</th>
<th>x-axis (+medial)</th>
<th>y-axis (+rostral)</th>
<th>z-axis (+dorsal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-84*</td>
<td>28*</td>
<td>-49*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-86*</td>
<td>21</td>
<td>-14</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-43*</td>
<td>5</td>
<td>34*</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>127*</td>
<td>6.8</td>
<td>28</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>35*</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Average</td>
<td>-10</td>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

*Indicates that the location of the observed COM was significantly different than the average COM of 1000 RDs.

Table 5.6B – Differences in μm between the observed COM of NA contacts and the average COM of 1000 RDs.

<table>
<thead>
<tr>
<th>Cell</th>
<th>x-axis (+medial)</th>
<th>y-axis (+rostral)</th>
<th>z-axis (+dorsal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-24*</td>
<td>-10</td>
<td>20*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-54*</td>
<td>-14</td>
<td>-15</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-2</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>-34*</td>
<td>-23</td>
<td>56*</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-45*</td>
<td>-44*</td>
<td>13</td>
</tr>
<tr>
<td>Average</td>
<td>-32**</td>
<td>-18</td>
<td>19</td>
</tr>
</tbody>
</table>

**Indicates that all 5 cells were biased one way and significant according to a Wilcoxon Signed-Rank Test (P<0.05)

Table 5.6C – Differences in μm between the observed COM of DA contacts and the average COM of 1000 RDs.

*Indicates that the location of the observed COM was significantly different than the average COM of 1000 RDs.

<table>
<thead>
<tr>
<th>Cell</th>
<th>x-axis (+medial)</th>
<th>y-axis (+rostral)</th>
<th>z-axis (+dorsal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>15</td>
<td>61</td>
<td>38</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-56</td>
<td>196*</td>
<td>158*</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>59</td>
<td>-170*</td>
<td>134*</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>116</td>
<td>-9</td>
<td>121*</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>91</td>
<td>-75</td>
<td>107</td>
</tr>
<tr>
<td>Average</td>
<td>45</td>
<td>1</td>
<td>112**</td>
</tr>
</tbody>
</table>
more lateral, and the COMs of DA contacts are more dorsal than expected compared to 1000 RDs in all five cells, these observations are significant (P<0.05 Wilcoxon Signed Rank Test). The bias of DA contacts to dorsally located dendrites was particularly strong; in the cell with the largest bias (SNDA42), the average density of DA contacts on dendrites dorsal of the soma was 3.4 times greater than the average density of DA contacts on dendrites ventral of the soma. The bias of NA contacts to lateral dendrites was more modest; in the cell with the largest bias (SNDA42), the average density of NA contacts on dendrites lateral of the soma was 1.5 times greater than the average density of NA contacts on dendrites medial of the soma.

5.5.3 Dendritic sub-trees are dominated by either 5-HT contacts, NA contacts, or neither

Each sub-tree emerging from the soma has both 5-HT and NA contacts. However, it is unknown how the relative densities of 5-HT and NA contacts vary between different sub-trees. We compared the observed number of 5-HT and NA contacts on each sub-tree to what would be expected if the distribution were uniform. For each cell, two separate RDs were created: one to match the total number of 5-HT contacts, and one to match the total number of NA contacts. This process was repeated 1000 times to create 1000 RDs for 5-HT and NA contacts. The ratios of 5-HT to NA contacts (number of 5-HT contacts/number of NA contacts) on each sub-tree was then compared between 1000 RDs and the observed distributions of 5-HT and NA contacts. This analysis is shown for a representative cell (SNDA32) in Figure 5.9. Sub-trees 5 and 9 had significantly more 5-HT contacts compared to NA contacts (P<0.05 compared to 1000 RDs) than would be expected if the distribution were uniform. We have termed these trees as being “dominant” for either 5-HT contacts. Figure 5.9B illustrates the sub-trees on SNDA32 that are dominant for either 5-HT or NA contacts. The two NA dominant sub-trees are located medially and the two 5-HT dominant sub-trees are located laterally. This division of medial sub-trees
Figure 5.9 (A) The ratio of 5-HT contacts to NA contacts on each sub-tree from the observed distribution (red diamonds), and 1000 RDs represented as boxplots for SNDA32. (B) Illustration of the trees dominant for either 5-HT or NA on SNDA32.
and lateral sub-trees dominant for either 5-HT and NA was seen across four cells, with the exception of SNDA42 which did not have any sub-trees dominant for 5-HT. SNDA32 and SNDA43 had medial dominant NA sub-trees and lateral 5-HT dominant sub-trees, while SNDA51 and SNDA52 had the opposite arrangement. The total number of sub-trees dominant for either 5-HT or NA across all five cells are listed in Table 5.7. For each cell there were an average of 1.2 sub-trees dominant for 5-HT, 2.0 sub-trees dominant for NA, and 8.4 sub-trees not significantly biased to either.

5.5.4 Like contacts are separated by less volume and unlike contacts are separated by more volume than would be expected based on uniform and diameter weighted distributions

Figure 5.3 A and C exemplify the local arrangement of 5-HT and NA contacts on the dendritic tree of RCA motoneurons. In general, 5-HT contacts are closer to other 5-HT contacts, and NA contacts are closer to other NA contacts. The “nearest neighbor” analysis has been used in previous studies to assess the proximity between neighboring contacts and provide a quantitative description of this observation for the entire dendritic tree (Montague, 2008). The purpose of this analysis is to ask the following question: Is there a relationship between the locations of adjacent contacts? For example, if there is one 5-HT contact on the dendritic tree, is there typically another 5-HT contact in close proximity, or another NA contact in close proximity? The nearest neighbor algorithm uses each contact on the dendritic tree as a reference point. It then finds the next closest contact on the dendritic tree in either direction and records the cytoplasmic volume between the reference point and this contact. This value represents the least volume between neighboring contacts (VBNC). This process is repeated for every contact on the dendritic tree to produce a complete set of VBNCs. A set of VBNCs can be calculated between like contacts (5-HT:5-HT or NA:NA), and unlike contacts (5-HT:NA or NA:5-HT).
Table 5.7 – Summary of the sub-tree analysis in all five cells. Columns indicate the number of sub-trees dominated by either 5-HT contacts, NA contacts or neither compared to 1000 RDs.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Number of sub-trees dominated by 5-HT contacts</th>
<th>Number of sub-trees dominated by NA contacts</th>
<th>Number of trees not dominant for 5-HT or NA</th>
<th>Total number of trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Average</td>
<td>1.2</td>
<td>2.0</td>
<td>8.4</td>
<td>11.6</td>
</tr>
</tbody>
</table>
To assess if the VBNCs are higher or lower than expected if the location of contacts were independent of one another, we compared the VBNCs from the observed distributions of 5-HT and NA contacts to the VBNCs of 50 RDs. Importantly, because RDs are randomly drawn from a population of contacts that are distributed based on surface area, the locations of contacts in RDs are independent of one another. The VBNCs from a given cell can be plotted in a cumulative histogram (Figure 5.10). For both the 5-HT:5-HT and NA:NA pairings (Figure 5.10A and C), the red cumulative histogram representing the observed VBNCs is to the left of the gray cumulative histograms representing the VBNCs of 50 RDs. This indicates that there were more instances of lower VBNC’s than predicted by 50 RDs. On the other hand for the 5-HT:NA pairing (Figure 5.10E), the red cumulative histogram representing the observed VBNCs is to the right of the gray cumulative histograms representing the VBNCs from 50 RDs. This indicates that there were more instances of higher VBNCs than predicted by a 50 RDs.

To acquire a value which represents the set of VBNCs in a cumulative histogram, we calculated the area over the curve for each cumulative histogram. The area over each curve was divided by 100, and this represents the average VBNC based on the area over the curve. We have termed this the “geometric mean” VBNC because it is the average VBNC as calculated from the curve of the cumulative histogram. Figure 5.10B, D and F compare the geometric mean VBNCs from the observed distribution of 5-HT and NA contacts to the average geometric mean VBNCs of 50 RDs. 5-HT:5-HT and NA:NA pairings produce a lower geometric mean VBNC, and the 5-HT:NA pairing produces a higher geometric mean VBNC than the average geometric mean VBNC of 50 RDs. Table 5.8A summarizes the differences between the geometric mean VBNCs produced from the observed distribution of 5-HT and NA contacts and the average geometric mean VBNC of 50 RDs for all five cells. The geometric mean VBNCs of 5-HT:5-HT and NA:NA pairings were significantly lower (P<0.05 for 5/5 comparisons) than the average geometric
Figure 5.10 Cumulative histograms of the volumes between nearest contacts (VBNCs) of the observed distribution versus 50 RDs for the pairings of 5-HT:5-HT (A), NA:NA (C) and NA:5-HT (E) on SNDA43. Note that the VBNCs for the NA:5-HT pairing were calculated using two RDs to account for both the NA and 5-HT distributions. Pictured on the right are the geometric mean VBNCs of the observed distribution versus the average geometric mean VBNCs of 50 RDs for the pairings of 5-HT:5-HT (B), NA:NA (D) and NA:5-HT (F).
*Indicates that the geometric mean VBNC was significantly greater or lesser than expected using 50 uniform RDs (p<0.05)

**Table 5.8A** – Differences in $\mu m^3$ between the observed geometric mean VBNC and the average geometric mean VBNCs of 50 RDs representative of uniform distributions. A positive value indicates that the volume between contacts was greater than expected and a negative value indicates that the volume between contacts was less than expected.

<table>
<thead>
<tr>
<th>Cell</th>
<th>5HT to 5HT</th>
<th>NA to NA</th>
<th>5HT to NA</th>
<th>NA to 5HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNDA3-2</td>
<td>-49*</td>
<td>-41*</td>
<td>19*</td>
<td>71*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-89*</td>
<td>-49*</td>
<td>32*</td>
<td>85*</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-86*</td>
<td>-58*</td>
<td>115*</td>
<td>111*</td>
</tr>
<tr>
<td>SNDA5-1</td>
<td>-59*</td>
<td>-35*</td>
<td>42*</td>
<td>125*</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-119*</td>
<td>-72*</td>
<td>114*</td>
<td>156*</td>
</tr>
<tr>
<td>Average</td>
<td>-80**</td>
<td>-51**</td>
<td>64**</td>
<td>110**</td>
</tr>
</tbody>
</table>

*Indicates that the geometric mean VBNC was significantly greater or lesser than expected using 50 diameter weighted RDs (p<0.05)

**Table 5.8B** – Differences in $\mu m^3$ between the observed geometric mean VBNC and the average geometric mean VBNCs of 50 RDs that match the bias of contacts to small diameter dendrites. A positive value indicates that the volume between contacts was greater than expected and a negative value indicates that the volume between contacts was less than expected.

<table>
<thead>
<tr>
<th>Cell</th>
<th>5HT to 5HT</th>
<th>NA to NA</th>
<th>5HT to NA</th>
<th>NA to 5HT</th>
</tr>
</thead>
<tbody>
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<td>-32*</td>
<td>30*</td>
<td>78*</td>
</tr>
<tr>
<td>SNDA4-2</td>
<td>-78*</td>
<td>-36*</td>
<td>30*</td>
<td>108*</td>
</tr>
<tr>
<td>SNDA4-3</td>
<td>-81*</td>
<td>-42*</td>
<td>91*</td>
<td>124*</td>
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<td>130*</td>
</tr>
<tr>
<td>SNDA5-2</td>
<td>-104*</td>
<td>-49*</td>
<td>114*</td>
<td>184*</td>
</tr>
<tr>
<td>Average</td>
<td>-72**</td>
<td>-38**</td>
<td>62**</td>
<td>125**</td>
</tr>
</tbody>
</table>

*Indicates that all 5 cells were biased one way and significant according to a Wilcoxon Signed-Rank Test (P<0.05)
mean VBNCs for 50 RDs in each cell. The geometric mean VBNCs of 5-HT:NA and NA:5-HT pairings were significantly higher (P<0.05 for 5/5 comparisons) than the average geometric mean VBNCs for 50 RDs in each cell. Since 5-HT:5-HT and NA:NA pairs were always separated by less volume and 5-HT:NA and NA:5-HT pairs were separated by more volume than predicted by 50 RDs in all five cells, these observations are significant (P<0.05 Wilcoxon Signed Rank Test).

5-HT and NA contacts have a significant bias to small diameter dendrites. It is possible that a high density of contacts on small diameter dendrites accounts for the tendency for there to be lower volumes between neighboring 5-HT contacts and lower volumes between neighboring NA contacts than predicted. To test whether or not the bias to small diameter dendrites accounts for this observation, we created “diameter weighted” RDs. Diameter weighed RDs are drawn a uniform distribution of over 20,000 contacts, however the number of contacts within each diameter bin are chosen to match the observed distribution. For example, if the cell has 643 5-HT contacts and 128 of these contacts are in the diameter bin 1-1.9 μm, then 643 contacts are chosen, and 128 contacts are designated to dendrites with a 1-1.9 μm diameter. Hence, the diameter weighted RD mimics the bias of contacts to small diameter dendrites seen in the observed data. Table 5.8B summarizes the differences between the geometric mean VBNCs from the observed distribution of 5-HT and NA contacts and the average geometric mean VBNC of 50 diameter weighted RDs for all five cells. The observed geometric mean VBNCs of 5-HT:5-HT and NA:NA pairings were significantly lower (P<0.05 for 5/5 comparisons) than the average geometric mean VBNCs for 50 diameter weighted RDs in each cell. Hence the bias to small diameter dendrites does not account for the tendency of like contacts to be separated by a small volume. However, the differences between the geometric mean VBNCs of the observed distributions and 50 diameter weighted RDs are less than the corresponding differences between the geometric mean VBNCs of the observed distributions.
and 50 non-diameter weighted RDs. Hence, the VBNC’s from the diameter weighted RDs more closely resemble the observed data.
Chapter 6. Discussion

6.1 Summary of Findings

The primary goal of this study was to investigate the distributions of 5-HT, NA and DA synapses on RCA motoneurons. The dendritic trees of RCA motoneurons were unlike other neck motoneurons due to a distinctive collection of dendrites that projected medially and crossed into the contralateral spinal cord. 5-HT, NA and DA contacts were primarily located on the dendrites of RCA motoneurons, with the exception of a small number of NA contacts found on the soma. This suggests that 5-HT, NA and DA systems are primarily responsible for modulating the strength of synaptic input to RCA motoneurons, rather than acting directly on mechanisms that control their output (e.g. Na⁺ channels on the soma and/or initial segment). 5-HT and NA were found to have a bias to distal and small diameter dendrites. This arrangement suggests that these modulators may facilitate the transmission of current to the soma from distal excitatory inputs on smaller dendrites. NA contacts were more common on the lateral half of the dendritic tree and DA contacts were more frequent on the dorsal half of the dendritic tree. The source of these regional biases is unknown, but they may be linked to the systematic differences in the density of NA and DA varicosities in different regions of the ventral horn and adjacent white matter. Based on the relative density of 5-HT and NA contacts, the sub-trees on RCA motoneurons formed 3 groups: Dominant for 5-HT contacts, dominant for NA contacts or no dominant for neither. Hence the ionotropic inputs on a given sub-tree may be more strongly influenced by either the 5-HT system, the NA system or evenly influenced by both systems than predicted by a uniform distribution. Finally, like contacts (5-HT/5-HT and NA/NA) were separated by less volume than expected and unlike contacts (5-HT/NA) were separated by more volume than expected based on the assumption that the location of each contact is not related to the location of its neighboring contacts. Pairing of synapses that release the same modulator
may lead to the coactivation of the same G-coupled protein receptors in close proximity. The resulting rise in the concentration of intracellular messenger (such as PKC) may be higher than if two G-coupled receptors were activated in isolation.

6.2 Limitations

6.2.1 The number of cells examined was small

The number of motoneurons examined (five) is typical of these types of studies (Rose et al., 1995; Murphy et al., 1995; Alvarez et al., 1998; Grande et al., 2005). One of the greatest challenges with a small sample size is the danger of the samples being non-representative of the population. Kitimura and Richmond (1994) showed that RCA motoneurons are located in spinal segments C1, C2, C3, and C4. To obtain a representative sample from this collection, 2 cells from C1, 2 cells from C2, and one cell from C3 were selected. In addition, the cells were collected from three different animals so as not to bias the chosen cells to only one animal. Finally, RCA motoneuron surface areas ranged from 353,171 µm² to 678,436 µm², which when compared to reconstructions of other motoneurons suggests that this is a typical range of motoneuron sizes (Ulfhake and Kellerth, 1981, 1983; Rose et al., 1985; Grande et al., 2005).

6.2.2 It is likely that a small percentage of contacts are not synapses

As stated in section 4.8, strict criteria were used to identify contacts based on previous studies, in which nearly 9 out of every 10 appositions have been shown to correspond to synaptic contacts at the electron microscopic level (Fyffe, 1991; Alvarez et al., 1998; Carr et al., 1999; Lübke et al., 2000; Grande et al., 2005). Importantly, this confirmation rate is independent of the type and location of contacts. Based on these observations, this discussion assumes that the contacts identified in the experiments are representative of the distribution of synapses. However, it must be made clear that not all identified contacts are likely synapses. In fact, based
on estimations from the previously mentioned sources, 10% of identified contacts likely do not correspond to the locations of synapses. All swellings which form appositions with motoneuron dendrite are counted, and this suggests that the total number of functioning synapses are overestimated. There are two reasons why this source of variability would not invalidate any conclusions. First, the total number of misidentified contacts is small, most likely representing only 10% of the contacts. Second, contacts that do not correspond to functioning synapses are not added in such a way that they are biased to a specific region, specific distance from the cell body, specific dendrite diameter, specific sub-tree, or specific distance from neighboring contacts. Thus the only error is a 10% over-estimate of contact density unrelated to the distribution of contacts.

6.2.3 Volume transmission

There have been several morphological and functional observations suggesting the existence of an alternate form of neuronal cell signaling other than point-to-point synaptic transmission which has been termed extra-synaptic transmission or volume transmission (Agnati et al., 1995; Zoli et al., 1999). Synaptic transmission classically involves the release of transmitter from a synapse which travels a very short distance to bind to post-synaptic receptors that are associated with that synapse. Volume transmission is the release of transmitter from a synapse which spreads over a large region, affecting many receptors. There is evidence for volume transmission of 5-HT, NA (Beaudet and Descaries, 1978), and DA (Law-Tho et al., 1994; Bast et al., 2002) in cortex. This mode of conduction would allow dendrites a far distance from the 5-HT, NA and DA varicosities to be affected by transmitter release. This would preclude selective amplification of specific regions on the dendritic tree by these modulators.
To our knowledge, there are no reports of volume transmission for these transmitters in the ventral horn of the spinal cord. One of the morphological features of volume transmission is the presence of boutons that are not associated with any post-synaptic membrane specializations when analyzed with electron microscopy. There is ultrastructural evidence that appositions between 5-HT and NA varicosities and dendrites in the ventral horn are associated with post-synaptic membrane thickenings (Ulfhake et al., 1987; Maxwell and Foster, 1991; Rajaofetra et al., 1992; Alvarez et al., 1998; Hammar and Maxwell, 2002). Electron microscopic examination of DA appositions in the rat spinal cord indicate that DA transmission in the ventral horn is mostly point-to-point (Ridet et al., 1992). In addition to ultrastructural evidence, 5-HT, NA and DA synapses on dendrites are distributed non-uniformly and are highly organized to specific regions on the dendritic tree. For example, the density of 5-HT and NA contacts is strongly biased to small diameter dendrites and very low or absent at the soma. The density of DA contacts is much higher dorsal of the cell body. These observations are not consistent with a broad release of neuromodulator that can diffuse to any region of the motoneuron. However to gain a complete understanding of the mode of transmission of 5-HT, NA and DA in the ventral horn of the spinal cord, descriptions of the co-localization of synapses and receptors are needed, similar to studies performed in cortex for other modulators (eg Yamasaki et al., 2010).

6.3 Similarities and differences between the distribution of 5-HT and NA contacts on RCA (flexor) motoneurons and splenius (extensor) motoneurons

6.3.1 5-HT and NA modulate the input of neck motoneurons: not the output

Similar to the observations based on RCA motoneurons, 5-HT and NA contacts were widely distributed on the dendritic trees of splenius motoneurons. In addition, similar to RCA motoneurons, there were very few contacts located on the cell bodies of splenius motoneurons.
This consistency between neck flexor and extensor motoneurons suggests that 5-HT and NA likely work to modulate the input, rather than the output of these cells. This is consistent with the literature in that many effects of 5-HT and NA are dendritic (see section 2.3). However there are examples of 5-HT and NA affecting channels at the soma and initial segment. For example, 5-HT blocks SK channels which reduces the action potential AHP in hypoglossal motoneurons (Berger et al., 1992; Bayliss et al., 1995). A similar decrease in AHP has been reported through the actions of NA on hindlimb motoneurons (Fung and Barnes, 1987). In addition, several groups have reported that 5-HT and NA can hyperpolarize the threshold of action potential for action potential generation by facilitation fast activating Na⁺ current (Dai et al., 2002; Fedirchuk and Dai, 2004; Gilmore and Fedirchuk, 2004). The observation that there are an average of 52 5-HT contacts are present on the cell bodies of lumbar motoneurons (Alvarez et al., 1998) suggests that these 5-HT contacts are ideally positioned and in large enough numbers to regulate the threshold of action potentials and therefore directly control the output of lumbar motoneurons. By this same logic, the paucity of somatic 5-HT contacts or NA contacts on RCA and splenius motoneurons suggests that this type of modulation is absent in neck motoneurons.

6.3.2 The bias of 5-HT and NA contacts to distal, small diameter, dendrites may be a fundamental feature of neck motoneurons

Another consistent pattern between RCA and splenius motoneurons is a bias to distal and small diameter dendrites. We propose two possible consequences of this observation. According to equations 3 and 4 in section 1.4, the magnitude of current attenuation in a passive cable is proportional to the diameter of the cylinder; current in a small diameter cylinder attenuates more with distance, than a large diameter cylinder. In compartmental models which included the full reconstructions of neck motoneurons, most current attenuation occurred along distal and small diameter dendrites (Bui et al., 2003). Hence, 5-HT and NA contacts are biased to
the dendrites in which current attenuates most en route to the soma. 5-HT and NA are both capable of facilitating inward Na\(^+\) and Ca\(^+\) currents on dendrites which could help compensate for the current attenuation on small diameter dendrites (see section 2.3.1). Li and Bennett (2007) have shown that 5-HT and NA may also potentiate PICs on motoneurons by blocking SK channels on dendrites, and this could also augment the current on small diameter dendrites (see section 2.3.2). Finally, 5-HT and NA could block TASK-1 channels and decrease the leakiness of the membrane in these regions (see section 2.3.3).

5-HT and NA contacts may also be biased to small diameter dendrites to increase the efficacy of the G-protein coupled signaling pathway. As illustrated in Figure 2.1, the metabotropic receptors activated by modulators produce a rise in the intracellular concentration of active second messenger. Given that small diameter dendrites have less intracellular volume than large diameter dendrites, the concentration of active second messenger would be greater in smaller diameter dendrites after transmitter binding. A higher concentration of second messenger could allow for a greater proportion of nearby ion channels to be phosphorylated. Both of these consequences support the hypothesis that the bias of 5-HT and NA contacts to small diameter dendrites may be crucial to the function of these modulators.

### 6.3.3 There is a high density of NA contacts lateral of RCA and dorsal of splenius motoneurons

The COM analysis in section 5.5.2 shows convincing evidence that NA contacts on RCA motoneurons are located more laterally than would be expected based on a uniform distribution. On the other hand, the COM of NA contacts on splenius motoneurons indicated that they were located more dorsally than expected. To reconcile this data, it is instructive to observe the locations of RCA and splenius nuclei (see Figure 5.1A). The ventral horn region lateral to RCA motoneuron nuclei and dorsal to splenius motoneuron nuclei is in common. It is
possible that this region in the center of the ventral horn has a higher density of NA boutons than the surrounding regions, and consequently NA boutons will form more contacts on motoneuron dendrites. Equally possible is that this region has the same density of NA boutons as surrounding regions, but there are more contacts specific to motoneuron dendrites in this region. A systematic study of NA bouton density in several regions of the ventral horn is required to fully make this distinction.

Since 5-HT contacts were not significantly biased to the central region of the ventral horn in splenius or RCA motoneurons, this suggests that dendrites entering this region may be preferentially modulated by the NA system. However, as is discussed in section 6.4, preferential modulation of dendrites from one modulator over another may be also related to the division of dendritic sub-trees.

6.3.4 The absence of plateau potentials in flexor motoneurons may be due to a low density of 5-HT and NA contacts on flexor motoneurons

Section 3.2.1 describes four possible mechanisms that explain why plateau potentials are absent on flexor motoneurons. We proposed that flexor motoneurons (i) receive a weaker monoaminergic innervation, (ii) have a somatic monoaminergic innervation that would avoid the locations of L-type Ca\(^{++}\) channels on dendrites, (iii) have a lower density or absence of effector channels, or (iv) have a higher level of inhibitory activity compared to extensor motoneurons. Given that 5-HT and NA contacts are widely distributed on the dendritic trees of both flexor and extensor motoneurons, the second mechanism seems unlikely. On the other hand, our data speak directly to the first mechanism. The average overall density of 5-HT contacts on splenius motoneurons is 2.3 times higher than on RCA motoneurons. Similarly, the average density of NA contacts on splenius motoneurons is 1.4 times greater than on RCA
motoneurons. When all five cells were compared between RCA and splenius motoneurons, these differences were significant (Mann-Whitney test of independent samples P<0.05). This observation suggests that the density of 5-HT and NA synapses on motoneurons may be a crucial factor in determining their input-output properties. More specifically, the density of 5-HT and NA contacts may be tailored to meet the demands of different motoneurons.

Lee and Heckman (1998) provide evidence for two populations of lumbar motoneurons that they term as fully bistable and partially bistable. Fully bistable motoneurons had an input-output relationship similar to the previous description (see Figure 2.2A right panel). On the upslope of the current ramp there is an acceleration in firing rate (ii), and on the downslope of the current ramp the motoneurons continue to fire at a higher frequency than on the upslope (iii). They also continue to fire in the absence of injected current (iv) for > 3 seconds. As explained in section 2.3.1, this is due to the activation of PICs on the dendrites, which provide a self-sustaining positive inward current to create plateau potentials. In contrast, partially bistable motoneurons showed an acceleration in firing rate (ii) on the upslope of the current ramp, however on the downslope of the current ramp, the cells did not continue to fire at a higher frequency than on the upslope. In addition, these cells did not continue to fire for >3 seconds in the absence of injected current. This clockwise hysteresis of the input-output relationship was caused by the inability of partially bistable cells to maintain PICs to create plateau potentials. We propose that the density of 5-HT and NA synapses may determine whether a motoneuron is fully bistable or partially bistable. Fully bistable cells may receive a high density of 5-HT and NA synapses like splenius motoneurons, and exhibit both firing rate acceleration and sustained plateau potentials, causing sustained firing. On the other hand, partially bistable cells may receive a low density of 5-HT and NA contacts like RCA motoneurons, and can provide enough current from PICs only to create an acceleration in firing rate, without sustained plateau
potentials capable of causing sustained firing. Importantly, both fully bistable and partially bistable cells both exhibit a firing rate acceleration caused by PICs. Hence both populations would be able to produce PICs needed to amplify current generated by excitatory synapses en route to the soma, which is a crucial function given the high degree of current attenuation in motoneurons (see section 1.4).

The biomechanical tasks of the splenius muscle as an extensor and the RCA muscle as a flexor supports the hypothesis that their corresponding motoneurons are fully bistable and partially bistable, respectively. Selbie et al (1993) suggest that RCA muscle fibers have a phasic rather than a postural role, involved in periodic activation for the downward flexion of the head. On the other hand the bilateral activation of splenius muscles hold the head upright, which is a postural task requiring long term contraction (Crouch, 1969). Lee and Heckman (1998) postulate that the functional role of partially bistable motoneurons is to accommodate muscles requiring brief, high forces, while the fully bistable cells may be more suited to accommodate long duration low force tasks required for posture. Curiously, Cotel et al (2009) did not observe firing rate acceleration in either flexor or extensor motoneurons in the neo-natal rat. This may be due to the fact that the animals were still in a developmental stage, or that the PICs were already activated before the cell was impaled.

6.3.5 The absence of plateau potentials in flexor motoneurons may additionally be due to a larger volume between pairs of like contacts

An equally plausible mechanism to explain the absence of plateau potentials in flexor motoneurons comes from an unexpected result. Pairs of 5-HT contacts and pairs of NA contacts were separated by less volume than predicted by 50 RDs representative of uniform distributions and 50 RDs created to match the bias to small diameter dendrites. However the volume
between like pairs of contacts on RCA motoneurons were separated by a larger volume than like pairs on splenius motoneurons. The geometric mean VBNC for pairs of 5-HT contacts on splenius motoneurons was 3.0 times less than the geometric mean VBNC for pairs of 5-HT contacts on RCA motoneurons. Similarly the geometric mean VBNC for pairs of NA contacts on splenius motoneurons was 1.8 times less than the geometric mean VBNC for pairs of NA contacts on RCA motoneurons. When all five cells were compared between RCA and splenius motoneurons, these differences were significant (Mann-Whitney test of independent samples P<0.05). Hence, the absence of plateau potentials in flexor motoneurons may also be due to the fact that 5-HT and NA nearest neighbor pairs of synapses have a greater volume of separation compared to extensor motoneurons. Continuing the hypotheses in section 6.3.2, the efficacy of modulator response may depend on factors which influence the concentration of active intracellular second messenger. Having modulators on small diameter dendrites with a low intracellular volume is a way to increase this concentration. Another way to increase this concentration may be to place two modulatory synapses in close proximity, separated by a small volume. A basic feature of G-coupled protein systems is that the activation of nearby enzymes to catalyze the formation of second messengers amplifies the original signal by creating many second messengers (Purves et al., 2008). Having two modulatory synapses in close proximity may create more active second messengers in a multiplicative fashion. For example, if the release of 5-HT or NA at one synapse led to the activation of 100 second messengers, release of 5-HT or NA from 2 nearby synapses may activate 100 x 100 =10000 active second messengers. In contrast, the release of 5-HT or NA from two synapses that are widely separated would only activate 200 second messengers. This effect would depend largely on the how far second messengers diffuse in an intracellular volume. Studies of the intracellular diffusion properties of IP3 (Santamaria et al., 2006), ERK1/2 (Wiegert et al., 2007), RAS (Harvey et al., 2008), Ca\(^+\) (Jia et al., 2010), RhoA
and Cdc42 (Murakoshi et al., 2011) show that these messengers have a very limited diffusion in dendrites (in the range of 5-10 μm). Harvey and Svoboda (2007) supplement this data with the observation that long term potentiation of channels caused by the uncaging of glutamate spreads roughly 10 μm in dendrites. Given that the diffusion of second messengers is limited in dendrites, the specific volume between synapses may be a crucial feature of a non-linear amplification of second messengers.

Overall, our data suggests that the absence of plateau potentials on flexor motoneurons may be due to a combination of lower density of 5-HT and NA contacts on flexor motoneurons, and a larger volume between nearest neighbor pairs of like contacts. However this does not exclude the third and fourth possible mechanisms that could contribute to the differences in plateau potentials between flexor and extensor motoneurons. The density of voltage dependent and independent ion channels that contribute to the plateau potentials may be low or absent on flexor motoneurons. Alternatively, flexor motoneurons may have a higher level of inhibitory background activity that overcomes the ability of PICs to be self-sustaining. The observations in this study do not address either of these possible mechanisms. Further work defining the distributions of dendritic ion channels and inhibitory synapses would be instructive.

6.4 The integration that each sub-tree performs will be different depending on whether the 5-HT system or NA system is activated

Motoneurons typically have 5-15 large primary dendrites that emerge from the soma which proceed to branch and together form the complete dendritic tree (Ulfhake and Kellerth, 1983; Rose et al., 1985; Cullheim et al., 1987; Ulfhake and Cullheim, 1988; Rose and Neuber-Hess, 1991). Each sub-tree is by definition separate from every other sub-tree because the soma acts as an electrical sink and thus the voltage and current in one sub-tree does not affect other
sub-trees (Rall, 1977). Hence each sub-tree is an integrative unit that will depend primarily on the composition of synaptic inputs on that sub-tree. Our data showed that the ratio of 5-HT contacts to NA contacts on each sub-tree is highly variable. Some sub-trees were dominant for 5-HT contacts, while other trees were dominant for NA contacts. In addition, some sub-trees showed no dominance for one or the other. Consequently, independent activation of NA axons from the locus coeruleus and 5-HT axons from the raphe nucleus would have differential effects on different sub-trees. For example, the synaptic inputs on a sub-tree that is dominant for 5-HT synapses will be more strongly influenced by activation of the 5-HT system than the NA system (than would be predicted by a uniform distribution). It is unknown whether the distribution of ionotropic synapses or ion channels are also confined to specific sub-trees. Future studies of these topics would help decide whether the separation of sub-trees is fundamental to the integration of inputs from different sources on motoneurons.

6.5 DA modulates the inputs to neck motoneurons: not intrinsic properties that directly control the output

As predicted by the low number of DA boutons in the RCA motoneuron pool, the density of DA contacts on the dendritic tree of RCA motoneurons was roughly an order of magnitude less than the density of 5-HT or NA contacts. Like the distributions of 5-HT and NA, DA contacts were restricted to the dendrites and there were no DA contacts on the soma or initial segment of motoneurons. This data is in contrast to the effects of DA seen in literature. As shown in sections 2.3.2 and 2.3.6, electrophysiological evidence from lumbar motoneurons suggests that DA can change the output of motoneurons: DA facilitates SK current and shortens the AHP and DA blocks I_{KA} which results in a decreased first spike latency on rat motoneurons (Han et al., 2007). This suggests that the DA system may be operating in fundamentally different way on neck motoneurons. The low density of DA on motoneurons also suggests that the actions of this
system are confined to local regions on the dendritic tree. The mechanism by which a very small number of DA synapses on the dendritic tree can affect the input-output properties of motoneurons remains unknown.

DA contacts were also found to be located more dorsally than would be expected based on a uniform distribution. Holstege et al (1996) and our observations indicate that DA boutons near the central canal (lamina X) and are particularly dense. Lamina X is located dorsal to the RCA nuclei. Hence the bias of contacts to more dorsally located dendrites may reflect this increase in bouton density near the central canal. Although the bias of DA contacts to dorsal dendrites was particularly strong, there was not a complete absence of DA contacts on ventral dendrites. This suggests that the influence of this system on dorsal dendrites may be stronger on dorsal dendrites, but not absent on ventral dendrites.

6.6 Concluding Statements

The results of this study emphasize that not all motoneurons have the same intrinsic properties. Previous experiments have shown that there may be differences in the firing properties of flexor and extensor motoneurons. We postulate that the reasons for these observations may stem from differences in the density and pairing characteristics of 5-HT and NA synapses. In contrast, we have also proved that there are some similarities in 5-HT and NA synapse distribution across neck flexor and extensor motoneurons that may be crucial to the tasks of these modulators. 5-HT and NA systems are likely involved in modulating the input, but not the output of neck motoneurons. In addition, the bias of 5-HT and NA synapses to small diameter dendrites may be fundamental to how 5-HT and NA regulate the input-output properties of neck motoneurons.
This study also explores the concept of how multiple modulators interact on motoneurons. The bias of NA synapses laterally and DA synapses dorsally suggest that dendrites in these regions are more strongly influenced by these systems. There are also several examples of sub-trees on RCA motoneurons that are dominant for either 5-HT synapses, NA synapses or neither. This suggests that the preferential modulation of different sub-trees from either 5-HT or NA systems is also possible.

The distributions of 5-HT, NA and DA all suggest that they modulate the input rather than the output of motoneurons and this is inconsistent with electrophysiological data from lumbar motoneurons. Electrophysiological studies in neck motoneurons could be of value as a comparison. In addition, the observations in this study strongly speak to the value of interaction between synapses as a means of controlling intrinsic properties. Future studies on the diffusion properties of PKA and PKC second messengers and the synergistic effects of multiple modulator systems on motoneurons would be particularly instructive.
References


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