Regulation of Motoneuron Firing Properties: Intrinsic and Circuit-Based Mechanisms

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REGULATION OF MOTONEURON FIRING PROPERTIES:
INTRINSIC AND CIRCUIT-BASED MECHANISMS

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

By

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SUPERVISION BY Adam Schmerge Deardorff ENTITLED Regulation of motoneuron
firing properties: Intrinsic and circuit-based mechanisms BE ACCEPTED IN PARTIAL
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ABSTRACT


Body and limb movements are controlled by regulating the activity of motor pools and their constituent motoneurons. An extensive complement of tightly regulated ion channels and second messenger systems determine active motoneuron spiking behavior, while segmental propriospinal circuits ensure the faithful execution of motor commands by providing real time sensory feedback to motoneurons and other somatosensory centers. However, current mechanistic understanding is incomplete for critical factors regulating motoneuron firing properties. Fundamental gaps in knowledge exist regarding (a) the spatial distribution and organization of specific ion channels in motoneurons, (b) the contribution of specific channels to motoneuron intrinsic properties, (c) the rules governing interactions between segmental interneuronal populations and motoneurons, and (d) patterns of motoneuron synaptic connectivity across flexor and extensor motor pools. Studies undertaken in this dissertation are aimed at filling several of these gaps in our current understanding of motoneuron behavior. Multiple factors that affect $\alpha$-MN excitability and firing are examined, including select ion channels, intrinsic membrane properties, and synaptic inputs. In addition, one series of studies was undertaken to advance understanding how some of these factors respond to peripheral nerve injury.
# TABLE OF CONTENTS

CHAPTER I: Purpose & Specific Aims ................................................................. 1

CHAPTER II. General Introduction ................................................................................... 9

CHAPTER III. General Methods...................................................................................... 22

CHAPTER IV. Expression of postsynaptic Ca\(^{2+}\)-activated K\(^+\) channels at C-bouton synapses in mammalian lumbar \(\alpha\)-motoneurons ....................................................... 36

CHAPTER V. Swimming against the tide: Investigations of the C-bouton synapse .......................................................................................... 97

CHAPTER VI. Segmental synaptic excitation of antagonist \(\alpha\)-motoneurons ................................................................. 138

CHAPTER VII. SK channel expression in mammalian lumbar \(\alpha\)-motoneurons following peripheral nerve injury .............................................. 191

CHAPTER VIII. General Conclusion............................................................................. 215

CHAPTER IX. References............................................................................................. 221

Appendix A. Commonly Used Abbreviations.............................................................. 257
LIST OF FIGURES

FIGURE 1: SK channel clusters are colocalized postsynaptically with large Kv2.1-IR clusters .............................................................. 72

FIGURE 2. SK3 immunoreactivity is present in all cat lateral lumbar spinal motoneurons ............................................................... 74

FIGURE 3. Distribution of SK3-IR cluster areas and motoneuron soma sizes .................................................................................... 76

FIGURE 4. Differential expression of SK3-IR and SK2-IR in rodent lumbar spinal α-motoneurons .......................................................... 78

FIGURE 5. Motoneuron pools innervating slow-type muscles have a higher percentage of motoneurons with SK3-IR ............................ 80

FIGURE 6. Expression of SK3 in physiologically characterized α-motoneurons ............................................................................... 82

FIGURE 7. Electrical properties in SK3 (+) and SK3 (−) tibial motoneurons. ................................................................................. 84

FIGURE 8. SK channel clusters are localized to synapses on α-motoneurons ................................................................................. 87

FIGURE 9. SK channels are localized to postsynaptic membrane sites at large C-bouton synapses on α-motoneurons ............................. 89

FIGURE 10. SK channels are clustered postsynaptically at cholinergic C-bouton synapses .............................................................. 91

FIGURE 11. SK channels do not colocalize with glutamatergic, serotonergic and inhibitory synapses ............................................... 93

FIGURE 12. SK channel clusters are colocalized postsynaptically with large Kv2.1-IR clusters ........................................................... 95

FIGURE 13. C-bouton synaptic sites contain a complex signaling ensemble ....................................................................................... 124
FIGURE 14. Postsynaptic SK channels
align with presynaptic vesicle release sites ................................................ 126

FIGURE 15. Synaptic distribution of specific ion channels
and receptors on soma and proximal dendrites of motoneurons .................... 128

FIGURE 16. The C-bouton synapse
on mammalian α-motoneurons ................................................................. 130

FIGURE 17. SK channel expression
following peripheral nerve injury ............................................................. 132

FIGURE 18. Subset of rat lumbar α-motoneurons with SK3-IR
have significantly longer AHP 1/2 decay time and increased amplitude ....... 134

FIGURE 19. Hypothesis for state dependent regulation
of motoneuron activity through the C-Bouton signaling ensemble ............... 136

FIGURE 20. Diagram depicting features of the experimental setup
for in vivo recording from anesthetized adult rats. ...................................... 166

FIGURE 21. Scatterplots of electrical and contractile properties
for MG and TAEDL α-MN and motor unit combinations ............................. 169

FIGURE 22. Quick stretch of homonymous (MG) and antagonist (TAEDL)
muscles causes the segmental synaptic excitation of MG α-MNs ................. 171

FIGURE 23. Properties of excitatory synaptic potentials evoked in MG α-MNs by
quick stretch of the homonymous (MG) and antagonist (TAEDL) muscles ..... 173

FIGURE 24. Response properties of
TAEDL proprioceptive afferents ............................................................... 175

FIGURE 25. Firing reliability index (FRI) for quick stretch and 100Hz vibration in
TA/EDL spindle and tendon organ afferents at 5g background force ......... 177

FIGURE 26. Segmental synaptic excitation of MG α-MNs by
homonymous (MG) and antagonist (TAEDL) Ia afferents .......................... 179

FIGURE 27. The functional output of TAEDL
Ia afferents is similar with quick stretch and vibration .............................. 181

FIGURE 28. Increasing MG background force
introduces synaptic inhibition ............................................................... 183
FIGURE 29. Quick stretch of antagonist (TAEDL) muscles causes the segmental synaptic excitation or inhibition of MG α-MNs following decerebration ........ 185

FIGURE 30. Quick stretch of antagonist (MG) muscles causes the segmental synaptic excitation or inhibition of TAEDL α-MNs ............... 187

FIGURE 31. A mammalian assistive reflex circuit ................................................................. 189

FIGURE 32. SK3 is expressed in more lumbar α-MNs after tibial nerve crush ................................................................. 209

FIGURE 33. Proportion of SK3(+) MG and Sol α-MNs is increased following tibial nerve injury ......................................................... 211

FIGURE 34. AHP properties and SK3 immunoreactivity in physiologically characterized tibial α-MNs following tibial nerve crush ..... 213
LIST OF TABLES

TABLE 1: Antibodies used in this study .......................................................................... 35

TABLE 2. Motor unit properties .................................................................................... 168
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Thanks for everything.
CHAPTER I: Purpose & Aims

Purpose

Body and limb movements are controlled by regulating the activity of motor pools. Current mechanistic understanding is incomplete for the factors that regulate the activity of motor pools and their constituent motoneurons. However, coordinated movement undoubtedly relies on the integration of intrinsic membrane properties with precisely timed synaptic input against a variable background of neuromodulatory drive. Studies undertaken in this dissertation are aimed at filling several gaps in our current understanding of motoneuron behavior. Multiple factors that affect α-MN excitability and firing will be examined, including selected intrinsic properties and synaptic inputs. In addition, one series of studies was undertaken to advance understanding how these factors respond to peripheral nerve injury.

Specific Aims

To address the specific aims in this dissertation, a combination of electrophysiological and anatomical methods will be employed to study the intrinsic properties, neuromodulatory mechanisms, and synaptic regulation of spinal α-motoneurons (α-MNs). Studies are designed to characterize SK channel expression in rodent lumbar α-MNs and relate expression patterns to mAHP properties (SA1) and cholinergic neuromodulatory sites (SA2). Additional investigations establish both an anatomical and theoretical
framework for the cholinergic neuromodulation of α-MN firing properties (SA3) as well as a novel proprioceptive circuit responsible for the synaptic excitation of antagonist α-MNs (SA4). Finally, SK channel expression and its role in regulating mAHP properties is investigated after peripheral nerve injury (SA5).

**Specific Aim 1: To test the hypothesis that SK3 is differentially expressed across α-MN subtypes.**

**Rationale**

AHP duration is critical in setting the firing behavior of α-MNs, so that earlier recruited α-MNs fire more slowly and later recruited α-MNs fire more rapidly (Kernell, 2006). The molecular basis for this mAHP variability has been unresolved for nearly 50 years. With the knowledge that SK channels are responsible for AHP in α-MNs (Zhang & Krnjevic, 1987), coupled with the differential kinetics of SK channel isoforms (Xia *et al.*, 1998) and the role AHP kinetics play in setting MN firing rate (Kernell, 2006), I hypothesize that SK3 is differentially expressed across α-MN subtypes (S- vs F-type).

**Methods**

*In vivo* electrophysiological approaches combined with immunohistochemistry and quantitative confocal microscopy will be used to characterize the differential expression of SK subunits in anatomically and physiologically identified α-MNs. Specific emphasis will be placed on the relationship between mAHP duration and SK3 channel expression.
**Significant Results**

These studies show that all α-MNs express SK2, while SK3 expression is markedly heterogeneous and cell-type-specific. SK3 channels, which have a longer deactivation time constant than SK2 (Xia et al., 1998), are only expressed in smaller α-MNs with longer duration/large amplitude mAHPs.

**Impact**

Results support the hypothesis that SK3 is differentially distributed across α-MN subtypes (S- vs. F-type) and, for the first time, establish a molecular mechanism for mAHP variability in α-MNs. These data give important, new insight into the differential organization of membrane properties within a motor pool.

**Specific Aim 2: To test the hypothesis that SK channels in α-MNs are postsynaptic to C-boutons.**

**Rationale**

In central neurons, SK channels are frequently localized to excitatory glutamatergic synapses on dendritic spines (Faber et al., 2005; Faber et al., 2008; Lin et al., 2008). However, there is little information about the specific subcellular distribution of SK channels in α-MNs, which lack dendritic spines. With the suggestion that C-boutons modulate α-MN AHP (Lape & Nistri, 2000; Miles et al., 2007) and the knowledge that SK channels are responsible for AHP in α-MNs (Zhang & Krnjevic, 1987), I hypothesize that SK channels in α-MNs are postsynaptic to C-boutons.
Method

Immunohistochemical approaches will be used with quantitative confocal microscopy to characterize the subcellular organization of SK channels and their relationship to specific synaptic inputs onto lumbar α-MNs.

Significant Results

There is a consistent relationship SK channel clusters in α-MNs with large, cholinergic C-type synaptic boutons on the soma and proximal dendrites of α-MNs. SK channel immunoreactivity (SK-IR) is not associated with glutamatergic, glycine/GABAergic synapses, or monoaminergic synapses.

Impact

Results support the hypothesis that SK channels in α-MNs are postsynaptic to C-boutons and provide critical insight into the synaptic regulation of mAHP properties and, consequently, α-MN firing behavior within a motor pool.

Specific Aim 3: To test the hypothesis that C-boutons express synaptic proteins uniquely organized to regulate $K^+$ currents.

Rationale

C-boutons are important modulatory loci for state dependent regulation of MN firing rate, but critical pre- and post-synaptic elements are not well characterized. With the suggestion that acetylcholine modulates α-MN somatic $K^+$ currents (Lape & Nistri, 2000;
Miles et al., 2007; Zagoraiou et al., 2009), I hypothesize that C-boutons express synaptic proteins uniquely organized to regulate $K^+$ currents.

**Method**

Immunohistochemical approaches and an extensive review of current literature will be used to characterize C-bouton circuitry and morphology. Emphasis will be placed pre-, post-, and sub-synaptic protein expression to generate a theoretical framework for the molecular mechanisms responsible for C-bouton function.

**Significant Results**

C-bouton postsynaptic sites comprise a unique structural/functional domain containing appropriate cellular machinery (a “signaling ensemble”) for the tight regulation of outward $K^+$ currents. The signaling ensemble includes clusters of SK channels, m2 receptors, connexin32, and transmitter release machinery precisely aligned across three membranous domains. $K_v2.1$ channels ‘interdigitate’ between SK and m2 clusters in the postsynaptic membrane.

**Impact**

Results support the hypothesis that C-bouton synaptic proteins are uniquely organized to regulate $K^+$ currents, and provide a theoretical framework for the molecular mechanism underlying the cholinergic modification of $\alpha$-MN firing properties via a unique structural / functional signaling ensemble.
Specific Aim 4: To test the hypothesis that reciprocal inhibition suppresses firing rate in antagonist α-MNs.

Rationale

Proprioceptive feedback is critical to the regulation of α-MN firing properties. The Ia reciprocal inhibitory system, in which Ia inhibitory interneurons (IaINs) are monosynaptically driven by Ia afferents to produce inhibitory synaptic potentials (IPSPs) in antagonist α-MNs (Hultborn et al., 1971b; Jankowska & Lindstrom, 1972; Jankowska & Roberts, 1972b), provides a motor pool specific influence on α-MNs thought to “sculpt” proper motor output from a background of diffuse neuromodulatory drive (Johnson & Heckman, 2010; Johnson et al., 2012; Johnson & Heckman, 2014). I therefore hypothesize that reciprocal inhibition suppresses firing rate in antagonist α-MNs.

Methods

In vivo electrophysiological approaches will be used to investigate reciprocal connections between muscles acting at the ankle joint in ketamine/xylazine anesthetized rats. Intracellular records of synaptic potentials from medial gastrocnemius (MG) α-MNs will be obtained during controlled muscle stretch of the antagonist tibialis anterior and extensor digitorum longus (TAEDL) muscles.

Significant Results

Quick stretch as well as 100Hz vibration of TAEDL produces a polysynaptic excitatory stretch synaptic potential (eSSP) in every MG α-MN sampled (n=34) from all rats (n=19). Intra-axonal recordings of TAEDL afferents (n = 54) shows that these physiological
stimuli are selective and specific, with quick stretch activating group Ia, II, and Ib afferents and vibration activating all Ia afferents but very few II or Ib afferents. The TAEDL→MG eSSPs, therefore, always include a contribution from Ia afferents, with additional contributions from IIs and/or Ibs.

**Impact**

Results do not confirm the hypothesis that reciprocal inhibition suppresses firing in antagonist α-MNs. Rather, these unexpected findings characterize a novel circuit mediating reciprocal excitation of antagonist α-MNs. Because this pathway is co-activated by Group Ia/Ib/II afferents, it likely operates via specific LaminaV/VI interneurons identified by Jankowska and colleagues in the cat (Jankowska & Edgley, 2010).

**Specific Aim 5: To test the hypothesis that peripheral nerve injury alters SK channel expression in lumbar α-MNs.**

**Background**

The variability of α-MN mAHP durations across motor unit subtypes (S- vs F-type) is reduced several days after peripheral nerve injury (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a), but the underlying molecular factors are unknown. Because SK channels are responsible for generating α-MN mAHP currents, and SK3 subunits are preferentially expressed in α-MNs with long duration mAHPs, I hypothesize that peripheral nerve injury alters SK channel expression in lumbar α-MNs.
Methods

In vivo electrophysiological approaches combined with immunohistochemistry and quantitative confocal microscopy will be used to determine changes in SK3 expression in both retrogradely labeled and physiologically identified α-MNs following peripheral nerve injury.

Significant Results

Following tibial nerve crush, SK3 expression is no longer correlated with long duration AHPs in injured α-MNs. Rather, SK3 is consistently expressed in a majority of α-MNs in MG and Soleus motor pools and is associated with mAHP durations that cluster tightly around the mean mAHP duration for all α-MNs analyzed. Proportions of SK3 expressing α-MNs in MG and soleus (Sol) motor pools return to approximately pre-injury levels upon successful reinnervation.

Impact

Results support the hypothesis that peripheral nerve injury alters SK channel expression in lumbar α-MNs and provide critical insight into changes in α-MN mAHP properties and, consequently, the firing behavior of α-MNs in an injured motor pool.
CHAPTER II: General Introduction

‘The thought that started me looking at the spinal motor system was a feeling that I had little likelihood of disentangling the arrangement of neurons in the cerebral cortex in a meaningful way, but I felt it was possible that ... all the higher parts of the CNS would have to be organized in a similar basic way ... even if the detail might still be capable of development.’”

-George Romanes

Neuronal architecture is organized into a complex anatomical network; a tightly woven fabric from which the self springs forth. A complete and intelligible account of neuronal wiring, were it available, would undoubtedly fail in relating neuronal operations to unified thought and purposeful action. Yet, within these threads of seemingly infinite complexity and subtlety lies our humanity, and unraveling them remains a fundamental challenge facing neuroscience. Fueled by the indelible human experience that from thought and will is born action, humankind has sought and found critical insight into neural function through its control of movement, at the interface of our internal and external world.

Theories on the neural control of movement date back to antiquity, when at the time of Hippocrates medical dogma emphasized that the brain is the principle of nerves and, through them, controls muscle (Barbara & Clarac, 2011). Since then, the neural control of movement and, in particular, the physiological and organizational properties of
motoneurons has been the exemplary system for studying and understanding neural function. During the Renaissance, Rene Descartes [1596-1650] gave the first physiological explanation of animal behavior by stressing the reciprocal coordination of antagonist muscles; though these reflex commands were still attributed to the flow of ‘animal spirits.’ A century later, again focusing on nerve-muscle interactions, Luigi Galvani [1737-1798], Alessandro Volta [1745-1827], Emil du-Bois Raymond [1818-1896], Hermann Von Helmholtz [1821-1894] and others demonstrated the electrical nature of neural tissue.

With the advent of 19th century scientific methods and the advancement of cell theory, the motoneuron was quickly elevated to and has remained the archetypal cell for studying and understanding the central nervous system. Its unambiguous identification in histological sections led to Santiago Ramon y Cajal’s [1852-1934] ‘principle of connections’ and the development of neuron doctrine. Soon after, Charles Sherrington’s [1857-1952] rigorous analysis of spinal reflexes synthesized available anatomical, physiological, and pathological data to conclude that motoneuron pools integrate descending and sensory input as the ‘final common pathway’ in activating all body and limb musculature (Burke, 2007). His conclusion emphasized that each neuron is a single cellular entity, with its own integrative capabilities, and fundamentally advanced our view of neuronal organization.

Intensive physiological investigation of the motoneuron carried into the 20th century and, by the end of its third decade, had unraveled additional threads of neural function. By
recording spike potentials directly from motor axons, Detlev Bronk [1879–1975], Edgar Adrian [1889–1977], and John Fulton [1899-1960] established the importance of motoneuron repetitive firing properties on muscle contraction, and Derek Denny-Brown [1901-1981] postulated that motoneuron firing rate is, itself, a consequence of both stimulus strength and a ‘central state of excitability’ (Denny-Brown, 1929). From these important investigations, critical concepts of frequency encoding and cellular excitability began to emerge, and with them, basic principles of the central nervous system began to take shape. In the decades since, the recording of intracellular potentials from spinal motoneurons produced a new era of neurological research.

With the direct recording of cellular potentials from spinal motoneurons, early studies of homonymous excitation (Brock et al., 1952; Coombs et al., 1955b; Eccles et al., 1957d, a; Curtis & Eccles, 1959), reciprocal inhibition (Brock et al., 1952; Bradley et al., 1953; Bradley & Eccles, 1953; Coombs et al., 1955c, d; Eccles et al., 1956; Eccles et al., 1957d; Curtis & Eccles, 1959), and recurrent inhibition (Eccles et al., 1954; Brooks et al., 1957; Curtis et al., 1957; Eccles et al., 1961) by John Eccles [1903-1997], John Coombs [1917-1993], Lawrence Brock [1923-1996], Paul Fatt [1924-2014], David Curtis [1927-], and their colleagues were critical in establishing the chemical nature of neurotransmission and the organizational features of central neural circuits (Curtis & Andersen, 2001). Moreover, these studies were critical in defining the excitatory/inhibitory nature of acetylcholine, glutamate, GABA, and glycine (Curtis & Andersen, 2001) and provided the first experimental evidence for “Dale’s Principle” of transmitter release (Eccles et al., 1954; Strata & Harvey, 1999; Alvarez & Fyffe, 2007). Wilfrid Rall
[1922-] subsequently applied cable theory to these and other data collected with Eccles to develop the first neuronal computational model (Rall, 1957, 1959, 1960, 1967; Rall et al., 1967) and, in doing so, pushed neuronal integration from the soma to the dendrites and opened new possibilities for its exploration through computational neuroscience.

Analysis of motoneuron dendrites and synaptic integration was extended with the use of intracellular dyes (Brown, 1983), which in turn provided the first structure-function studies of central neurons, as well as spike triggered averaging, which enabled a more thorough analysis of the synaptic potentials produced by individual neurons (Mendell & Henneman, 1971). More recent in vivo studies of motoneuron physiology, in particular with the use of voltage clamp technology, confirmed neuronal dendrites possess active conduction properties and demonstrated their role in variable input-output gain modulation (Schwindt & Crill, 1977; Schwindt & Crill, 1980a; Schwindt & Crill, 1980b, c; Brownstone, 2006). Altogether, these motoneuron investigations transformed the way we think about neuronal dendrites.

The foundational principles of neuroscience upon which we now stand have been indubitably tied to movement and motoneuron analysis. Now, in the 21st century, motoneurons continue to blaze the trail. Motor circuit neurobiology is at the forefront of integrating advanced electrophysiological and cellular labeling techniques with molecular-genetics and highly specific fluorescent probes to bring humankind ever closer to unraveling the governing principles of neuronal organization (Arber, 2012; Miri et al., 2013). But, motoneuron analysis provides more than just a useful model system. In his
Linacre Lecture at St. John’s College in Cambridge, Charles Sherrington said “…to move things is all mankind can do, and that for such the sole executant is muscle, whether in whispering a syllable or in felling a forest” (Eccles & Gibson, 1979). Indeed, motoneurons are the instrument of will, transforming decision into deed, aspiration into action. The final common pathway of nearly every neural circuit is the motoneuron, and neuronal wiring must, in the end, organize itself for the precise regulation of motoneuron viability and excitability. With this perspective, before we can understand purposeful thought and unified action, we must first understand motoneuron biology.

The work presented in this dissertation, therefore, seeks to incrementally advance our understanding of motoneuron biology by investigating several key intrinsic and circuit-based mechanisms (described below) that regulate motoneuron firing properties. In addition, one series of studies is undertaken to advance understanding how these factors respond to peripheral nerve injury.

**Intrinsic Mechanisms: AHP and SK channels**

Interest in α-MNs as the ‘final common pathway’ tends to shift attention toward their synaptic function. This is certainly understandable, as α-MNs have an outstanding ability to receive and integrate information from nearly 50,000 synaptic inputs. However, when considering the regulation of α-MN spiking behavior, the importance of the electrical properties that are intrinsic to the α-MN membrane – and upon which synaptic influences are exerted – cannot be understated.
Though numerous conductances regulate neuronal discharge, the importance of the medium duration afterhyperpolarization (mAHP) was recognized very early in the intracellular investigation of α-MNs (Coombs et al., 1955a; Eccles et al., 1957b; Kernell, 1965a, b, c). mAHP currents profoundly influence the repetitive discharge properties of α-MNs, including interspike interval variability, $f$-I slope, and primary-range firing rate (Kernell, 2006; Brownstone & Magown, 2013). In spinal and hypoglossal MNs, mAHP currents are generated by the opening of small conductance Ca$^{2+}$-activated K$^+$ (SK) channels (Zhang & Krnjevic, 1987; Viana et al., 1993; Lape & Nistri, 2000) activated by N- and P/Q-type Ca$^{2+}$ currents (Viana et al., 1993; Umemiya & Berger, 1994; Bayliss et al., 1995; Li & Bennett, 2007).

Early studies showed α-MN mAHP parameters (e.g. duration and amplitude) vary according to the physiological properties of the muscle fibers innervated (Eccles et al., 1957b, 1958a; Buller et al., 1960b, a; Burke, 1967). The relationships between mAHP and MN size and motor unit type are now well documented (Bakels & Kernell, 1993b; Gardiner, 1993). To achieve the minimum rate of α-MN discharge necessary for motor unit twitch summation, the mAHP in cat and rat spinal α-MNs is larger and of longer duration in S-type MNs that innervate slow twitch motor units than in F-type MNs that innervate fast twitch motor units (Eccles et al., 1957b, 1958a; Burke, 1967; Burke et al., 1982; Zengel et al., 1985; Bakels & Kernell, 1993b; Gardiner, 1993). On the other hand, cat γ-MNs display AHPs of variable duration (Kemm & Westbury, 1978). mAHP properties can be modified by cholinergic (Lape & Nistri, 2000; Miles et al., 2007) and serotonergic neuromodulatory input (Bayliss et al., 1995) as well as peripheral nerve.
injury (Kuno et al., 1974a; Gustafsson & Pinter, 1984; Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a; Prather et al., 2011). But, despite extensive characterization of mAHP properties in S- and F-type α-MNs it is not known if the observed mAHP duration and amplitude profiles reflect fundamental differences in SK channel expression, channel activation/deactivation properties, or regulation by neurotransmitters or neuromodulators.

Small conductance Ca\(^{2+}\)-activated potassium (SK) channels are widely expressed in the mammalian central nervous system (Kohler et al., 1996; Tacconi et al., 2001; Sailer et al., 2004), are activated by nanomolar concentrations of Ca\(^{2+}\) through associated calmodulin (Xia et al., 1998), and mediate the medium duration afterhyperpolarization (mAHP) of many brain and spinal cord neurons (reviewed in Faber & Sah, 2003; Fakler & Adelman, 2008; Faber, 2009). Several homologous SK channel subunits encoded by members of the KCNN gene family have been cloned and appear to be distributed in an overlapping pattern in specific neuronal and glial cell populations (Kohler et al., 1996; Ishii et al., 1997; Stocker & Pedarzani, 2000; Sailer et al., 2004; Armstrong et al., 2005).

Homomeric SK1 (K\(\text{Ca2.1}\)), SK2 (K\(\text{Ca2.2}\)) and SK3 (K\(\text{Ca2.3}\)) channels are highly sensitive to blockade by the bee venom apamin (Kohler et al., 1996; Ishii et al., 1997; Strobaek et al., 2000; Sah & Faber, 2002) and display progressively slower deactivation kinetics in the order SK1<SK2<SK3. SK channels are expressed in dendrites and dendritic spines in a variety of brain regions where they are thought to underlie a variety of physiological roles, are subject to cholinergic and adrenergic modulation, and may be involved in mechanisms of synaptic plasticity (Fakler & Adelman, 2008; Lin et al., 2008;
Power & Sah, 2008; Lujan et al., 2009). However, there is little detailed information about the expression or subcellular localization of SK subunits in spinal cord neurons, particularly in motoneurons which lack dendritic spines, in healthy adults as well as following disease or dysfunction.

Hypotheses in this dissertation will provide detailed information regarding critical organization features of SK channels and the mAHP, including (a) the expression of SK subunits across a population of α-MNs, (b) their relation to individual mAHP properties, (c) their subcellular distribution in relation to neuromodulatory or other synaptic inputs and (d) their regulation following nerve injury.

**Neuromodulatory Mechanisms: C-Boutons**

α-MN firing properties are dependent on both the amount of current reaching the soma/initial segment from widely distributed synaptic contacts as well as the cell’s own intrinsic membrane biophysics and integrative capabilities. Interestingly, α-MN membrane properties are, themselves, modifiable in a state dependent fashion by neuromodulatory activity (Heckmann et al., 2005; Miles et al., 2007; Brownstone et al., 2011). These regulatory mechanisms act via the modulation of specific ion channel conductances. Identifying these ion channels and their associated synaptic contacts has been the subject of numerous studies.

Early investigations into ‘regulated’ motoneuron properties focused on membrane bistability and self-sustained firing related to the serotonergic activation of persistent
inward Ca$^{2+}$ currents (Hultborn et al., 1975; Schwindt & Crill, 1977; Schwindt & Crill, 1980a; Schwindt & Crill, 1980b, c; Hounsgaard et al., 1988a). For decades, investigations focused on state dependent and pathologic alteration of inward current, with little attention paid to regulatory mechanisms governing outward current modulation (Brownstone et al., 1992). Recent evidence, however, points to an intraspinal, cholinergic neuromodulatory system that contributes dense presynaptic coverage, termed C-boutons, to α-MN somata and increases α-MN f-I gain via dramatic reductions in the strength of the mAHP (Miles et al., 2007; Zagoraiou et al., 2009).

C-bouton synapses are part of a recently identified neuromodulatory circuit arising from cholinergic V0-embryonic (V0C) interneurons (Miles et al., 2007; Zagoraiou et al., 2009). V0C interneurons correspond to a known population of cholinergic partition cells (Barber et al., 1984; Phelps et al., 1984; Arvidsson et al., 1997) located lateral to the central canal in Rexed's lamina X and medial lamina VII (Miles et al., 2007; Zagoraiou et al., 2009). V0C interneurons receive synaptic input from several sources, including descending serotonergic pathways, local and/or descending VGluT2 projections, inhibitory interneurons (e.g., V2b cells), lamina II/III nociceptive interneurons, and non-proprioceptive primary mechanosensors (Zagoraiou et al., 2009; Witts et al., 2014; Zampieri et al., 2014; Zhang et al., 2014). Each V0C cell sends divergent axonal projections to several α-MNs of the same or functionally equivalent motor pools and avoids α-MNs innervating antagonist muscles (Stepien et al., 2010). Numerous en passant synaptic varicosities arising from a single V0C axon contact the soma and proximal dendrites of one or more α-MNs, which in turn receive convergent input from
several V0c cells (Stepien et al., 2010). Although the precise levels of convergence / divergence are unknown, this pattern of connectivity establishes a large number of release sites from each presynaptic axon onto the α-MN, likely reflecting a high probability of transmitter release and contributing to a high safety factor for strong cholinergic neuromodulation of α-MNs (e.g., (Walmsley et al., 1998)).

The C-bouton system stands in anatomical contrast to the serotonergic system, which originates in the brainstem raphe nucleus and provides extensive synaptic input onto α-MN dendrites (Alvarez et al., 1998). It is therefore interesting to consider that while serotonin increases MN excitability by amplifying inward current (Heckmann et al., 2005; Brownstone, 2006; Li & Bennett, 2007; Heckman et al., 2008; Powers et al., 2008), acetylcholine does so by reducing outward current (Lape & Nistri, 2000; Miles et al., 2007; Zagoraiou et al., 2009). These systems likely operate synergistically to endow the CNS with remarkable control of α-MN firing properties However, questions remain regarding (a) the functional impact of C-bouton input during different behaviors, (b) the manner in which C-bouton activity is modulated to match motor demands, and (c) the mechanism of interaction between underlying acetylcholine receptors (AChRs) and K+ channels.

Hypotheses in this dissertation will shed light on the cholinergic regulation of α-MN firing properties by characterizing the subcellular organization of synaptic proteins at C-bouton synaptic sites and providing a theoretical framework for the molecular mechanisms underlying experimentally observed synaptic effects.
Synaptic Mechanisms: Proprioceptive Input

The summation of synaptic excitation and inhibition is a compulsory step in generating and regulating α-MN firing. Inhibitory and excitatory synaptic potentials from muscle proprioceptors provide motor pool specific modulation of α-MN firing properties in accordance with the biomechanical requirements of the body and limbs (Nichols et al., 1999). One important source of proprioceptive feedback is the antagonist muscle, to which α-MNs are particularly responsive (Heckman & Binder, 1991; Nichols & Koffler-Smulevitz, 1991; Nichols et al., 1999; Johnson & Heckman, 2010; Johnson et al., 2012; Johnson & Heckman, 2014).

In consideration of the antagonist regulation of α-MN firing properties, emphasis is typically placed on reciprocal inhibition between antagonist muscles. In the ‘classic’ disynaptic reciprocal inhibitory pathway, Ia afferents activated by muscle stretch (e.g. an ankle flexor) inhibit contraction of the antagonist (e.g. ankle extensor) muscle through an interposed Ia inhibitory interneuron (IaIN) (Hultborn et al., 1971b; Jankowska & Roberts, 1972b). α-MNs and their ‘corresponding’ IaINs receive parallel Ia afferent input from the same (homonymous) muscles (e.g. ankle flexors). IaINs, in turn, project glycineergic presynaptic terminals (Bradley et al., 1953; Bradley & Eccles, 1953) onto the ‘opposite’ α-MNs and IaINs, which innervate and/or receive parallel Ia afferent input from the antagonist muscle (e.g. an ankle extensor) (Hultborn et al., 1971b; Jankowska & Roberts, 1972b). Recent evidence from our laboratory demonstrates that temporally discrete recurrent IPSPs from Renshaw cells onto rodent lumbar α-MNs can cause the short term modulation of spike timing (i.e. delayed spikes and rebound spiking) with little or no
suppression of average firing rate (Obeidat et al., 2014). In contrast to Renshaw cell inputs, which are largely dendritic, glycinergic terminals from IaINs provide dense synaptic coverage to the α-MN soma and proximal dendrites (Burke et al., 1971; Fyffe, 1991b, a), which may be more effective at shunting excitatory drive and thereby suppressing α-MN firing.

Reciprocal inhibition, and its regulation by Ia input, therefore provides tightly focused inhibition between antagonist muscles (Johnson & Heckman, 2010; Johnson et al., 2012; Johnson & Heckman, 2014). However, the integration of proprioceptive feedback into ongoing motor commands is highly dynamic, and when considering antagonist motor pools, principles of reciprocal innervation are frequently overridden by central commands (Long et al., 1970; Patton & Mortensen, 1971; Llewellyn et al., 1990; Hyland & Jordan, 1997) and/or pathology (Myklebust et al., 1982; Crone et al., 1994; Xia & Rymer, 2005). During locomotion, proprioceptive input from homonymous and antagonist muscles is critical for adjusting the relative timing, phase, and amplitude of antagonist muscle activity and is particularly important when navigating an uncertain and changing environment (Pearson, 1995a; Clarac et al., 2000; Akay et al., 2014). Though in several neuronal systems, these functions depend to some degree on reversing the influence of classically described reflex pathways by modulating Ia circuitry and/or activating alternative proprioceptive pathways (Pearson, 1995a; Clarac et al., 2000).

Spinal mechanisms responsible for modulating the governing principles of reciprocal, antagonist innervation are not fully characterized but have been the subject of
investigations spanning nearly 100 years (reviewed in (Tilney & Pike, 1925; Smith, 1981; Miri et al., 2013). Though reciprocal reflex pathways can be modulated by silencing IaINs through direct or presynaptic inhibition (Pierrot-Deseilligny & Burke, 2012), questions remain regarding the degree to which spinal circuits may activate alternative reflex pathways. Interestingly, there is sparse (and indirect) evidence that short latency reciprocal excitatory circuits may coordinate activity of antagonist α-MNs (Watt et al., 1976; Jankowska et al., 1981). Early reports of Group I reflex actions demonstrated Ib afferents can evoke polysynaptic excitation of antagonist α-MNs (Laporte & Lloyd, 1952; Eccles et al., 1957c; Watt et al., 1976) via interneuronal relays under descending control (Hongo et al., 1969), with excitation typically observed from extensors onto flexors (Eccles et al., 1957c). Later studies show a considerable portion of these interneurons reside in Rexed Laminae V/VI and can be co-excited or even driven solely by Ia afferents (reviewed in Jankowska & Edgely, 2010).

Hypotheses in this dissertation will provide, for the first time in the adult rodent, a physiologic investigation of reciprocal antagonist connections by characterizing their synaptic effects, analyzing afferent contributions, and addressing fundamental questions regarding the varied influence of antagonist proprioceptors on α-MNs.
CHAPTER III: General Methods

Using a combination of in vivo electrophysiology and quantitative confocal microscopy, this dissertation will investigate electrical properties and synaptic mechanisms mammalian spinal sensorimotor circuits. General methods employed are detailed below.

Animal Use

All animal procedures conform to National Institutes of Health (NIH) guidelines and are approved by the Wright State University Institutional Animal Care and Use Committee. Detailed electrophysiological and immunohistochemical analysis are performed on adult female Sprague Dawley rats, adult female Wistar rats, adult male or female CBA/J mice, and adult female cats. Survival surgeries are performed in surgical suites located within the Laboratory Animal Resources at Wright State University.

Anatomical Techniques:

Retrograde Tracer

When required for the specific identification of distinct motor pools in the lumbar spinal cord, adult female Sprague Dawley rats undergo a single sterile survival surgery to retrogradely label the medial gastrocnemius (MG), lateral gastrocnemius (LG) and/or soleus (SOL) α-motoneurons (α-MNs) for post-hoc identification. Rats are deeply anesthetized (absent withdrawal and corneal reflex) by isoflurane inhalation (induction 4
–5%; maintenance 1–3%, both in 100% O2). Using sterile techniques, the triceps surae (MG+LG+SOL) are exposed by a midline incision through the skin and biceps femoris muscle of the left hindlimb. A total of 50 µl of 0.5% Cholera Toxin Subunit B-555 or Cholera Toxin Subunit B-488 (CTB-555 or CTB-488, Invitrogen, Carlsbad, CA, USA) is administered by a series of small injections throughout the muscle(s) of interest. The wound is subsequently irrigated and closed in layers. Animals receive 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours and Carprofen (0.25mg/250g SQ once per day for 2 days) for post-operative pain medication for 48 hours and are monitored closely by professional staff.

**Immunohistochemistry**

For basic immunostaining procedures and confocal microscopy, all animals are anaesthetized with pentobarbital (150 mg kg⁻1, I.P.) and transcardially perfused with ice-cold vascular rinse (0.01 M phosphate buffer with 0.8% NaCl, 0.025% KCl and 0.05% NaHCO₃, pH 7.4) followed by room temperature fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Lumbar spinal cord segments are quickly removed and postfixed in the same fixative for 2-12 hours at 4°C then transferred to 0.01M PBS. Transverse sections (50–75 µm thick) of L4–L5 lumbar spinal cord are cut on a cryostat or vibrating microtome, and collected in 0.01 M phosphate buffer with 0.9% NaCl (PBS, pH 7.4) for free floating immunohistochemistry. Prior to cryostat sectioning, sections are cryoprotected with 0.01M phosphate buffer with 15% sucrose at 4°C overnight, then submerged in cryoprotectant solution (500 ml 0.1 M phosphate buffer at pH 7.2, 300 g sucrose 10 g polyvinylpyrrolidone, 300 ml ethylene glycol, 200 ml double distilled H₂O)
for 5-10 min and coated in OCT (Tissue- Tek #4583).

Tissue sections are blocked with 5% normal horse serum in 0.1 M PBS with 0.1% Triton X-100 (PBS-TX) for 30-60 min, and subsequently incubated overnight at 4°C with appropriate primary antibodies (see Table 1) diluted in 0.1 M PBS-TX. All primary antibodies in this dissertation have been characterized in the past by us and other labs as to their specific labeling patterns in the spinal cord (Alvarez et al., 1998; Alvarez et al., 1999; Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Alvarez et al., 2011; Romer et al., 2014; Rotterman et al., 2014). The following day, sections are washed in PBS and immunoreactive sites are revealed with species-specific secondary antibodies raised in donkey and conjugated to FITC, Cy3, or Cy5 (Jackson Laboratories, West Grove, PA, USA), diluted 1:50-1:200 in 0.1 M PBS-Tx (pH 7.4). After a 2- to 4-h incubation in secondary antibodies the sections are thoroughly washed in PBS, mounted on gelatin-coated slides, and coverslipped with Vectashield (Vector Labs, Burlingame, CA).

Certain experiments require the use of primary antibodies raised in the same host species. These coexpression studies employ a sequential immunostaining process. The sections are incubated with the first primary antibody overnight at 4°C, and the following day are washed in PBS and incubated in species appropriate secondary antibody for 2 h at room temperature. Remaining host species IgG binding sites are subsequently blocked with an excess of unlabeled Fab fragments (1:10 in PBS-Tx for 2 h). After washing in PBS to remove unbound Fab fragments, the sections are incubated in the second primary
antibody (overnight, 4°C). The following day, these immunogenic sites are revealed with a second species appropriate secondary antibody for 2 h at room temperature.

In experiments requiring the use of a fourth fluorochrome, these immunoreactive sites are revealed with Streptavidin-405 (Invitrogen). Following primary antibody treatment, sections are incubated for 2 h at room temperature in a biotinylated species-specific secondary antibody diluted 1:100 in 0.1M PBS-TX (Jackson Laboratories, West Grove, PA, USA) followed by a second 2 h incubation in Alexa-405-conjugated Streptavidin diluted 1:50 in PBS-Tx.

**Confocal Microscopy and Quantitative Analysis**

Immunolabeled images of the lumbar spinal cord are obtained on a Fluoview FX Olympus (Center Valley, PA, USA) confocal system and Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope with 20x, 60x, and 100x dry / oil immersion objectives at 0.2-1.0 µm Z-steps and 1.0-2.5 digital zoom (NA 1.35). Molecular labels are excited with the 488-nm line of an argon laser (FITC), 568-nm line of a krypton laser (Cy-3), and 633nm of a HeNe laser (Cy-5). Image stacks are quantitatively analyzed for immunofluorescence using Fluoview software (Olympus), and immunoreactivity is considered positive for a given antigen if the fluorescence intensity is at least twofold increased over background. Background intensity is calculated per image by averaging three randomly sampled areas within the neuropil.
Experiments requiring precise measurements of ion channel cluster dimensions (maximal diameter and cluster area) and α-MN cell body diameters are measured, as previously described (Muennich & Fyffe, 2004; Romer et al., 2012), with ImagePro Plus software (Media Cybernetics, Silver Springs, MD, USA). Briefly, cluster dimensions are measured in en face single optical sections through select α-MNs, and mean cell body diameters are calculated using averaging applications based on α-MN plasma membrane labeling in a single optical section containing the nucleolus. Images are always analyzed in original, unprocessed form. For all analyses, significance was set at p<0.05 using Pairwise ANOVA and Pairwise t-Test.

Identification of α-MNs

This dissertation relies on the accurate histological identification of α-MNs. Therefore several combinations of approaches are used to ensure that α-MNs were adequately differentiated from other ventral horn neurons (γ-MNs and interneurons) during immunohistochemical analysis. Anatomically, α-MNs are readily distinguished from local interneurons by soma size, morphology, laminar location, and the expression of ChAT and NeuN as well as the presence of large cholinergic C-type synaptic contacts, which are highly specific for somatic α-MNs (Carr et al., 1998; Alvarez & Fyffe, 2007; Friese et al., 2009; Shneider et al., 2009). Moreover, the size of α-MNs has been documented for each mammalian species used here (e.g. (Burke et al., 1982; Moschovakis et al., 1991; Chen & Wolpaw, 1994; Ishihara et al., 2001)) and measurements of soma sizes in this dissertation fall within the respective published ranges. Various combinations of these strategies give consistent outcomes and enable the
accurate identification of \( \alpha \)-MNs.

**In-Vivo Electrophysiological Techniques**

Electrophysiological characterization of \( \alpha \)-MN / motor unit properties, synaptic communication between \( \alpha \)-MNs and proprioceptive afferents, and/or proprioceptive encoding, are performed in adult female wistar rats during single, terminal recording sessions lasting up to 14 hours.

**Surgical Preparation**

Rats are deeply anesthetized (absent withdrawal and corneal reflex) by isoflurane inhalation (induction: 4 –5% in 100% O\(_2\) in induction chamber; maintenance: 1–3% in 100% O\(_2\) through tracheal cannula), and vital signs are closely monitored. Respiratory rate (40–60), end-tidal CO\(_2\) (3–5%), oxygen saturation (>90%), heart rate (300-500 beats/min), and core temperature (36–38°C) are maintained by adjusting isoflurane concentration and/or radiant heat. Lactated ringers solution (0.6g/L NaCl, 0.31g/L Na-lactate, 0.03g/L KCl, 0.02 g/L CaCl\(_2\)) is given subcutaneously at a rate of 1mm/hour, per veterinarian recommendations. Because isoflurane suppresses central reflexes, rats undergoing polysynaptic circuit analysis are (a) transitioned to a ketamine-xylazine cocktail administered intraperitoneally (9:1 mg/kg/h dose); or (b) decerebrated via an intercollicular brainstem transection and mechanical aspiration of rostral brain tissue, including the entire tele- and diencephelon. These strategies permit removal of isoflurane, thereby enabling polysynaptic circuit analysis. In some cases (and in all decerebrate animals) adequate recording stability requires administration of a paralytic drug.
(pancuronium bromide 0.2 mg/kg) and mechanical ventilation of the animal. Animals remain on 100% O₂ throughout experiment.

Standard procedures are used to prepare the left hindlimb and the lumbar spinal cord for electrophysiological analysis (Seburn & Cope, 1998; Haftel et al., 2004; Haftel et al., 2005; Bullinger et al., 2011a; Bullinger et al., 2011b). A small midline incision through the skin and biceps femoris muscle of the left hindlimb exposes deep tissues of the posterior compartment, including the tibial nerve, common peroneal (CP) nerve, MG & LG/Sol muscle nerves, and the ankle extensor, triceps surae (MG+LG+Sol) muscle group. In some preparations, two additional incisions are made through the skin and biceps femoris muscle over the anterolateral compartment to expose the superficial and deep branches of the CP nerve and the tendons of the pretibial flexor tibialis anterior and extensor digitorum longus (TAEDL) muscles. One or more nerves of interest [ie. tibial, MG, and/or deep peroneal (DP) nerves] are then carefully dissected free of other tissues and suspended in continuity on a monopolar silver hook electrode for electrical stimulation. All other nerves are crushed. The leg is kept immobile by clamps inserted onto the distal femur and distal tibia.

When necessary, the MG muscle is surgically separated from the LG and Sol, freed from surrounding tissue, and its tendon of insertion (the MG portion of the fused Achilles tendon, excluding the LG, Sol, and plantaris muscle tendons) is detached from the calcaneous and tied directly to the lever of a motor system (model 309, Aurora Scientific, Aurora ON, Canada). In addition, the pretibial flexors are freed from surrounding tissue,
and their tendons of insertion are detached from the first metatarsal (TA) and the middle phalanx the second-fifth digits (EDL) and tied directly to the lever of a second motor system (model 305B-LR, Aurora Scientific). The motor system is used to stretch the MG and TAEDL with specified parameters and to record length and force at the lever (see ‘Stretch-Evoked Synaptic Potentials’ below).

With rats secured in a rigid recording frame, dorsal exposure of the lumbosacral spinal cord (L4–S1) by laminectomy and longitudinal incision of the dura mater provided access to (a) dorsal roots L4-L5, which are carefully dissected free of surrounding tissue and suspended in continuity on bipolar silver hook electrodes for intra/extracellular recording, and (b) α-MNs in the lumbar ventral horn via dorsolateral penetration of the spinal cord with microelectrodes for intra/extracellular recording. Skin flaps are used to construct pools for bathing all exposed tissues with warm mineral oil.

**α-MN and Motor Unit Properties**

Records of α-MN membrane potential, electrode current, and (when applicable) muscle force / length are collected, digitized (20 kHz), stored and analyzed with CED Power 1401 and CED Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Lumbar α-MNs are impaled by borosilicate glass microelectrodes (1.2-mm OD, 7- to 10 MΩ DC resistance, 2 M K-acetate) advanced through the spinal cord with a micromanipulator system (Transvertex Microdrive). All α-MN recordings in this dissertation are from tibial, MG, or DP motor pools, and for all analyses, significance was set at p<0.05 using Pairwise ANOVA and Pairwise t-Test.
In vivo intracellular recordings are made from tibial, MG, or DP α-MNs that are antidromically identified by MG or DP peripheral nerve stimulation. Only those α-MNs with stable membrane potential and with antidromic action potential amplitude >60 mV were deemed acceptable for further study. Failure to meet these criteria accounts for the incomplete data set obtained from some α-MNs / motor units. α-MNs were injected with current through the micropipette in order to measure their intrinsic electrical properties as previously reported in our laboratory (Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a): rheobase current (depolarizing pulses 50 ms in duration at the lowest strengths capable of initiating action potentials in 50% of trials), afterhyperpolarization (AHP) (suprathreshold pulses 0.5 ms in duration), input resistance (hyperpolarization produced by -1 and -3 nA current pulses 50 ms in duration) and axon conduction velocity (antidromic conduction delay divided by tibial nerve length). Synaptic potentials are recorded as described below (see “Stretch-Evoked Synaptic Potentials”).

Averages of motor unit twitch and endurance are made during intracellular stimulation (0.5 ms suprathreshold pulses) of the α-MN, as previously reported (Bakels & Kernell, 1993b; Gardiner, 1993). Motor unit peak force, twitch contraction time (onset of the mechanical response to peak force), twitch ½ decay time (decrease from peak force to ½-maximum value) are measured as evoked during 2Hz stimulation of the α-MN. Endurance is measured by fatigue tests in which the peak force is measured during 40Hz stimulation of the α-MN occurring in 330ms bursts repeated every second for 4 min. From these measurements a ‘fatigue index’ (the reciprocal ratio (%) between the largest
burst force and that obtained 2 min later) is calculated.

**Stretch Synaptic Potentials (SSPs)**

With passive muscle tension set to 10 g and 5 g, respectively, MG and TAEDL muscles are stretched in several paradigms, via motor systems attached separately to their distal tendons (as described above in “Surgical Preparation”). For quick stretches, muscles are lengthened by 1 mm at constant velocity (500 mm/s) in a 2 ms ramp followed immediately by a 20 ms release to resting length. For ramp-hold-release stretch, the muscles are lengthened by 1 mm at constant velocity (20 mm/s) over 50 ms rise, held at that length for 500 ms, and then released to resting length at constant velocity over 50 ms. For small amplitude, high frequency vibration, muscle lengths are oscillated +/- 40 μm from rest at a rate of 100 Hz.

The following data are recorded and averaged over 50-100 trials of each stretch paradigm.  
1) Stretch-evoked afferent volleys from MG and TAEDL muscle proprioceptors are recorded extracellularly from L4-L5 dorsal roots suspended in continuity on bipolar hook electrodes ~ 3-4 mm from the dorsal root entry zone. 2) Simultaneously, homonymous and antagonist stretch-evoked synaptic potentials are recorded intracellularly from MG and DP α-MNs. 3) Following intracellular data collection, the micropipette is moved just outside the α-MN, and the extracellular expression of the synaptic potential is measured in response to each stretch paradigm.

Records of α-MN membrane potential, electrode current, dorsal root activity, and muscle
force / length are collected, digitized (20 kHz), stored and analyzed with CED Power
1401 and CED Spike2 software (Cambridge Electronic Design, Cambridge, UK). From
these recordings, the polarity (excitatory vs inhibitory), amplitude (measured from resting
Vm to peak of the PSP), rate of rise (PSP amplitude divided by rise time), central latency
(dorsal root volley to start of the PSP), peripheral latency (start of muscle stretch to dorsal
root volley), are determined. For all analyses, significance was set at p<0.05 using
Pairwise ANOVA and Pairwise T-Test.

**Peripheral Encoding of Muscle Stretch**

Individual sensory axons are impaled by borosilicate glass microelectrodes (1.2-mm OD,
25- to 35 MΩ DC resistance, 2 M K-acetate) advanced through the dorsal root (~ 3-4 mm
from the dorsal root entry zone) with a micromanipulator system (Transvertex
Microdrive). Intraxonal recordings are made from MG or TAEDL muscle proprioceptive
afferents orthodromically identified by MG or DP peripheral nerve stimulation. Records
of action potentials and muscle force / length are collected, digitized (20 kHz), stored and
analyzed with CED Power 1401 and CED Spike2 software (Cambridge Electronic
Design, Cambridge, UK).

Afferents are classified as muscle spindle (Group Ia/II afferents) or tendon-organ (Group
Ib afferents), by whether they pause or accelerate firing, respectively, during the rising
phase of force in isometric, whole muscle twitch contractions (Matthews, 1972; Haftel et
al., 2004; Bullinger et al., 2011a; Bullinger et al., 2011b). Further classification and
characterization of proprioceptive encoding by these neurons is obtained during the
following stretch paradigms: ramp hold and release stretches (20 mm/s, 1 mm, 0.5 s hold time); triangular stretches (4 mm/s, 1 mm); quick stretches (500 mm/s, 1 mm); and small amplitude, high frequency vibration (100-330 Hz, 80µm). Muscle spindle afferents are classified as Group Ia when (a) they produce high-frequency bursts of firing at the onset of ramp hold and release or triangular stretch and (b) fire in response to each cycle of vibrations at frequencies of >100 Hz at a range of muscle length (Matthews, 1972; Bullinger et al., 2011a; Bullinger et al., 2011b). Those muscle spindle afferents exhibiting neither property designated Group II. Typical discharge thresholds as a function of passive muscle force for each afferent type were determined. For all analyses, significance was set at p<0.05 using Pairwise ANOVA or Pairwise T-Test.

**Intracellular Labeling**

In experiments requiring concurrent α-MN anatomical and physiological analyses, the micropipette solution contained 10% neurobiotin (Vector Laboratories, Burlingame, CA, USA) in Tris Buffer (0.1M Tris-OH, 1M Potassium Acetate, PH 7.6). Following physiological characterization, α-MNs are labeled by intracellular injection of neurobiotin solution. To aid neurobiotin passage into the motoneuron, positive current (3-5 nA) is delivered in 400-ms-long pulses at 2 Hz for 2–5 min. After retraction of the electrode, a minimum of 1 h waiting period is allowed for complete diffusion of neurobiotin throughout the α-MN, demonstrated by robust labeling of α-MN distal dendrites and axon collaterals, before animals are sacrificed by an intraperitoneal overdose of pentobarbital (150 mg kg⁻¹ I.P.). Animals are perfusion fixed and processed for post-hoc immunohistochemistry as described above (see “Anatomical Techniques”).
To reveal intracellular labeling, neurobiotin is visualized with 488 streptavidin (Invitrogen; diluted 1:1000 in 0.01 M PBS containing 0.1% Triton X for 2 h, room temp).

**Figure Composition**

Microscopic images are prepared by adjusting brightness and contrast in ImagePro Plus software (Media Cybernetics, Silver Springs, MD, USA) and always preserve all the information content of the images. Some images are sharpened using a high-guass filter in Image Pro. Physiological traces are prepared with Spike2 software. In some cases, traces are smoothed, DC removed, and/or filtered against 60 Hz background noise, but always preserve the information content of the original trace. Graphs are generated in Sigmaplot (v. 9.0; Systat Software, SPSS Inc, Chicago, IL) and Statistica (Statsoft). All figures are composed using CorelDraw (v. 15.0).
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CHAPTER IV: Expression of postsynaptic Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels at C-bouton synapses in mammalian lumbar α-motoneurons


This chapter, published in the Journal of Physiology, addresses SA1 and SA2 of this dissertation.

Introduction

Small-conductance Ca\textsuperscript{2+} -activated potassium (SK) channels are widely expressed in the mammalian central nervous system (Kohler et al., 1996; Tacconi et al., 2001; Sailer et al., 2004), are activated by nanomolar concentrations of Ca\textsuperscript{2+} through associated calmodulin (Xia et al., 1998), and mediate the medium-duration after-hyperpolarization (mAHP) of many brain and spinal cord neurons (Zhang & Krnjevic, 1987; Hounsgaard et al., 1988b; Viana et al., 1993; Sawczuk et al., 1997; Powers et al., 1999; Faber & Sah, 2002; Sah & Faber, 2002; Faber & Sah, 2003; Bond et al., 2004; Miles et al., 2005; Faber, 2009).

Several homologous SK channel subunits encoded by members of the KCNN gene family have been cloned and appear to be distributed in an overlapping pattern in specific neuronal and glial cell populations (Kohler et al., 1996; Ishii et al., 1997; Stocker & Pedarzani, 2000; Sailer et al., 2004; Armstrong et al., 2005). Homomeric SK1 (KCa\textsubscript{2.1}), SK2 (KCa\textsubscript{2.2}) and SK3
(K_{Ca}2.3) channels are (depending on the expression system that is used) highly sensitive to blockade by the bee venom, apamin (Kohler et al., 1996; Ishii et al., 1997; Shah & Haylett, 2000; Strobaek et al., 2000; Faber & Sah, 2002; Sah & Faber, 2002; Faber & Sah, 2003). The different channel subunits may interact to form heteromeric channel that result in increased or decreased current in comparison to homomeric channels (Benton et al., 2003; Monaghan et al., 2004; Strassmaier et al., 2005), although there is still little information about the composition of native channels. The SK channels are expressed in dendrites and dendritic spines in a variety of brain regions, where they are thought to underlie a variety of physiological roles, are subject to cholinergic and adrenergic modulation, and may be involved in mechanisms of synaptic plasticity (Faber et al., 2008; Lin et al., 2008; Power & Sah, 2008; Lujan et al., 2009); however, there is little detailed information about the expression or subcellular localization of SK subunits in spinal cord neurons.

In spinal and hypoglossal motoneurons (MNs), the involvement of SK channels in medium after-hyperpolarization (mAHP) currents is supported by observations that the mAHP is blocked by the SK channel blocker, apamin, as well as by lowering the external calcium concentration or replacing Ca^{2+} with Mn^{2+} ions (Zhang & Krnjevic, 1987; Viana et al., 1993; Lape & Nistri, 2000). Functionally, mAHP conductances contribute to the modulation of MN discharge rate and, although early studies postulated that they have a role in spike frequency adaptation, other studies indicate that mAHP conductances are non-essential for the early phase of spike frequency adaptation (Baldissera & Gustafsson, 1974; Viana et al., 1993; Sawczuk et al., 1997; Kernell, 1999; Powers et al., 1999;
Powers & Binder, 2000; Manuel et al., 2005; Meunier & Borejsza, 2005; Miles et al., 2005). There are well-documented relationships between mAHP duration and MN size and motor unit type (Burke, 1967; Bakels & Kernell, 1993b; Gardiner, 1993). In cat and rat spinal α-MNs, the mAHP is larger and of longer duration in S-type MNs that innervate slow-twitch motor units than in F-type MNs that innervate fast-twitch motor units (Eccles et al., 1957b; Burke, 1967; Burke et al., 1982; Zengel et al., 1985; Gardiner, 1993). In contrast, cat γ-MNs display AHPs of variable duration (Kemm & Westbury, 1978). Despite extensive characterization of mAHP properties in S- and F-type α-MNs, it is not known whether the observed mAHP duration and amplitude profiles reflect fundamental differences in SK channel expression, channel activation/deactivation properties, or regulation by neurotransmitters or neuromodulators. To understand more completely the physiological contributions of SK ion channels, it is essential to know the cellular and subcellular distribution of specific channel subunits in different classes of MNs.

Here, we used immunohistochemistry and in vivo electrophysiology to investigate the specific distribution and expression patterns of two apamin-sensitive SK channel subunits, SK2 and SK3, in spinal MNs. We demonstrate consistent expression of SK2 in all α-MNs and differential expression of SK3, which is expressed preferentially only in small, presumably S-type, α-MNs. The SK3-expressing α-MNs were shown by electrophysiological analysis to have significantly longer duration, larger amplitude AHPs than MNs that lack SK3. Notably, in contrast to the pattern observed in rat and mouse spinal MNs, all α-MNs in cat spinal cord express SK3 channels. The SK channel
immunoreactivity in all α-MNs, regardless of functional type or animal species, is clustered specifically at synapses on the soma or proximal dendrites and is not present in more distal dendrites. Moreover, the postsynaptic SK channels in the perisomatic region are selectively localized at large synapses formed by cholinergic C-boutons. The highly selective localization of large clusters of SK channels at specific sites in the postsynaptic membrane suggests that other molecular signaling and cellular components that are localized at C-bouton synapses, such as muscarinic m2 receptors and subsurface cisternae, may play key roles in the regulation of SK channel activity and hence of MN firing properties. Indeed, recent investigations have demonstrated that C-bouton activation of m2 receptors increases motoneuron excitability by reducing the AHP (Miles et al., 2007).

Methods
All procedures were performed according to National Institutes of Health Guidelines and approved by the Wright State University Institutional Animal Care and Use Committee. Detailed immunohistochemical analysis of SK channel expression was performed on young adult female (~250 g) Sprague–Dawley rats (n=15), adult female Wistar rats (n = 20), adult male or female CBA/J mice (~30 g; n = 12), adult female C57/B6 mice (n = 2) and adult female cats (n = 3). For basic immunostaining procedures and confocal microscopy, all animals were anaesthetized with pentobarbital (150 mg kg−1, I.P.) and transcardially perfused with vascular rinse (0.01 M phosphate buffer with 0.8% NaCl, 0.025% KCl and 0.05% NaHCO3, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). For electron microscopy, the fixative solution contained 1% glutaraldehyde (see ‘Pre-embedding immuno-electron microscopy’ below). Lumbar
spinal cord segments were then quickly removed and postfixed in the same fixative for up to 2 h. Tissue was stored in 15% sucrose at 4°C overnight. Transverse sections of L4–L5 lumbar spinal cord were cut on a cryostat at 50–75 µm thick, and then collected in 0.01 M PBS (pH 7.4). When noted, to observe SK2-immunoreactivity (IR) with antigen-retrieval procedures, tissue was incubated in 10 mM sodium citrate, pH 6.0, with 0.05% Tween 20 at 95°C for 20 min prior to immunostaining.

**Immunohistochemistry of SK channels**

Sections were rinsed with PBS-T (0.01 M PBS containing 0.1% Triton-X, pH 7.3), blocked with normal horse serum (10% in PBS-T), and then incubated in primary antibodies or cocktails of primary antibodies overnight at 4°C. All antibodies were diluted with PBS-T. The SK channel immunoreactivity was localized with polyclonal rabbit anti-SK3 (Chemicon, Temecula, CA, USA; 1:1000 dilution) or polyclonal rabbit anti-SK2 (Alomone, Jerusalem, Israel; 1:100 dilution) directed against amino acid residues 2–21 of human SK3 or against amino acids 542–559 of SK2. The specificity of the primary antibodies has been described (Khanna *et al.*, 2001; Tacconi *et al.*, 2001; Armstrong *et al.*, 2005) and was confirmed here using Western blotting and pre-absorption of the primary antibodies with the peptide antigen. Western blotting of membranes prepared from rat spinal cord tissue and probed with the anti-SK3 antibody revealed that the antibody recognizes a single ∼70 kDa molecular weight protein band, and this labeling was abolished by pre-absorption of the antibody with the peptide antigen (Alomone, Jerusalem, Israel; 1 µg peptide/µg antibody); similar specificity and abolition of staining by pre-absorption of the antibody was observed using
immunohistochemistry in all three animal species. Similar controls confirmed the specificity of anti-SK2 (which recognizes a single ~72 kDa band in Western blots).

**Dual labeling immunohistochemical studies.**

Double labeling studies were performed by combining one of the anti-SK antibodies with one of the following primary antibodies to detect specific pre- or post- synaptic proteins and their association with SK labeling: anti-synaptophysin (Calbiochem/Oncogene, Darmstadt, Germany; mouse, 1:200 dilution), anti-VACHT (vesicular acetylcholine transporter; Millipore, Billerica, MA, USA; goat, 1:5000 dilution), anti-VGLUT1 and anti-VGLUT2 (vesicular glutamate transporters; Chemicon, Temecula, CA, USA; guinea-pig, 1:5000 dilution), anti-5-HT (Dr J. Pearson Wright State University, Department of Neuroscience, Cell Biology, and Physiology; guinea-pig, 1:5,000), antigephyrin (Alexis, San Diego, CA, USA; mouse, 1:100 dilution) and anti-NeuN (neuronal nuclear protein; Chemicon, Temecula, CA, USA; mouse, 1:500 dilution). 

$\text{K}_{\text{2.1}}$ immunohistochemistry was performed using mouse anti-$\text{K}_{\text{2.1}}$ clone D4/11 at 1:1000 dilution that was developed and/or obtained from the UC Davis/NINDS/NIMH Neuromab facility, supported by NIH grant U24NS0506060 and maintained by the Department of Pharmacology, School of Medicine, University of California (Davis, CA, USA). All primary antibodies were diluted in PBS-T 0.1%, pH7.4, and incubated overnight at 4° C. The sensitivity and specificity of the primary antibodies against the various synaptic proteins and neuronal markers have been previously described by researchers in our laboratory (Alvarez et al., 1997; Alvarez et al., 1998; Alvarez et al., 1999; Deng & Fyffe, 2004; Muennich & Fyffe, 2004). Immunoreactivity was detected
with species-specific secondary antibodies conjugated to fluorescein isothiocyanate, Cy3 or DyLight 649 (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:50 in PBS-T 0.1%, pH 7.4, and incubated at room temperature for 2–4h. Given that the antibodies against SK3 and SK2 were raised in the same host species (rabbit), the coexpression studies employed a sequential immunostaining process. The sections were incubated with the first primary antibody (anti-SK3, diluted 1:1000 in 0.01 M PBS-T 0.1%), washed in PBS, and immunoreactive sites revealed with Cy3-conjugated goat anti-rabbit secondary antibody (Fab fragment, 1:50 dilution for 2 h; Jackson Immunoresearch, West Grove, PA, USA). Remaining rabbit IgG binding sites were blocked with an excess of unlabeled Fab fragments (1:10 dilution for 2 h). After washing in PBS to remove unbound Fab fragments, the sections were then incubated in the second primary antibody (anti-SK2, diluted 1:100 in 0.01M PBS-T 0.1%) and the latter immunogenic sites revealed with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:50 dilution for 30 min). Sections were mounted on gelatin-coated slides and coverslipped in Vectorshield mounting medium (Vector Laboratories, Burlingame, CA, USA).

**Confocal imaging, analysis and statistics.**

Images were obtained on a Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope with a ×10 objective at 2.0 µm Z-steps, ×20 objective at 1 µm Z-steps, and ×60 oil immersion objective at 0.5 µm steps at 1.0–2.5 digital zoom (NA 1.35). Channel cluster dimensions (maximal diameter and cluster area) and cell sizes (mean soma diameter and cross-sectional area) were measured with ImagePro Plus software (Media Cybernetics, Silver Springs, MD, USA). Immunolabeling was measured using Fluoview
software (Olympus). Immunoreactivity for SK3 was considered present if the immunoreactive intensity was at least twofold increased over background. Background intensity was calculated by averaging three randomly sampled areas within the neuropil per image analysed. Significance was set at P < 0.05 (SigmaStat; Systat Software, Port Richmond, CA, USA).

**Identification of α-MNs.**

Several approaches and combinations of approaches were used to ensure that α-MNs were adequately differentiated from other ventral horn neurons (γ-MNs and interneurons) during immunohistochemical analysis of SK channel expression. The size of MNs in lumbar spinal cord motoneuron pools has been documented for each mammalian species used here (e.g. (Burke *et al.*, 1982; Moschovakis *et al.*, 1991; Chen & Wolpaw, 1994; Ishihara *et al.*, 2001)) and our measurements of soma sizes fall within the respective published ranges. Anatomically, MNs were readily distinguished from local interneurons by soma size, morphology, laminar location and Choline Acetyltransferase (ChAT) vs. calbindin expression (Carr *et al.*, 1998; Alvarez & Fyffe, 2007). For the present study, it was particularly important to distinguish α- from γ-MNs because of the potential for overlap of the soma size distribution of γ- and small α-MNs (Moschovakis *et al.*, 1991). We used the recently reported finding that in mouse spinal cord the γ-MNs lack expression of the neuronal nuclear protein NeuN (Friese *et al.*, 2009; Shneider *et al.*, 2009); our present observations suggest this is also the case in adult mice, rats and cats. Therefore, analysis of NeuN-positive neurons in lamina IX excludes γ-MNs from the sample. Moreover, as described here in “specific association of postsynaptic SK3
channels and presynaptic C-boutons,” all of the analyzed α-MNs received synaptic inputs from large cholinergic C-boutons, which are lacking on γ-MNs (Lagerback, 1985; Johnson, 1986; Lagerback et al., 1986). Various combinations of these strategies gave consistent outcomes and enabled us to focus directly on α-MNs in this study.

**Pre-embedding immuno-electron microscopy.**

For electron microscopy, postperfusion lumbar spinal cord tissue was postfixed overnight at room temperature then stored in 0.01 M PBS (pH 7.3) at 4°C. Tissue sections 50–100µm thick were cut using a vibratome. Excess aldehydes were blocked with 1% NaBH4, and sections were rinsed thoroughly in PBS. Sections were incubated in polyclonal rabbit anti-SK3 (1:100 dilution in PBS) for 2–4 days at 4°C, and immunostaining was revealed with an ABC-peroxidase kit (Vector Laboratories, Burlingame, CA, USA) using dianinobenzidine (Sigma St. Louis, MO, USA) as the final chromogen (0.02% dianinobenzidine and 0.01% H2O2 in 0.05 M Tris buffer) with an 8–10 min reaction time. Reaction products were enhanced using silver intensification, for which sections were treated with 2.5% glutaraldehyde in 0.01 M PBS (30 min), washed three times with Tris–maleic acid buffer, rinsed with nanopure H2O, and placed in silver nitrate solution (10 min) in a dark room at 60°C. Sections were again rinsed in nanopure H2O (10 min), after which they were placed in 0.05% gold chloride solution (5 min) at room temperature and rinsed with nanopure H2O (10min). Unbound silver particles were removed by washing in 2.5% sodium thiosulfate (3 min), followed by additional washes in nanopure H2O and 0.01 M PBS. Sections were postfixed in 2% osmium tetroxide, stained en bloc with 1.0% uranyl acetate, dehydrated in a graded series of ethanols,
cleared through two 5 min washes in propylene oxide, infiltrated with 1:1 propylene oxide–Epon-Araldite, and finally flat-embedded in Epon-Araldite between two Teflon-coated coverslips. Small areas containing immunolabeled motoneurons were selected from the sections and glued onto a resin block. Ultrathin sections (60–70 nm) were obtained on a Sorvall MT 6000 ultramicrotome (Dupont Company, Newtown, CT, USA) and collected serially on copper grids coated with a thin layer of Formvar, counterstained with uranyl acetate and lead citrate (5 min), and viewed on a Philips EM201 transmission electron microscope (Philips Electron Optics, Amsterdam, Netherlands) equipped with a Gatan digital camera (Gatan Inc, Pleasanton, CA, USA). Digital images were used for synaptotological survey of the neuron surface and the determination of synapse type and distribution of immunoreactivity. Electron micrographs were produced in CorelDRAW (Corel Corp., Ottawa, Ontario, Canada) from standard electron microscopy negative plates that were scanned at 200 d.p.i.

In vivo electrophysiology

Twenty female Wistar rats were studied in single terminal recording sessions. Deep anaesthesia (absent withdrawal and corneal reflex) was induced by isoflurane (4–5% in 100% O2, by inhalation in an induction chamber) and maintained by isoflurane (1–3% in 100% O2, by inhalation through a tracheal cannula). The animal was monitored for respiratory rate (40–60 breaths min⁻¹), end-tidal CO2 (3–5%), oxygen saturation (>90%), heart rate (300–500 beats min⁻¹) and core temperature (36–38° C). These levels were variously maintained by adjusting the isoflurane concentration and radiant heat sources and by scheduled subcutaneous injection of Ringer–dextrose solution. In some cases,
adequate recording stability required intraperitoneal injection of a muscle relaxant drug (pancuronium bromide 0.2 mg kg−1).

Standard procedures were used to prepare the spinal cord and left hindlimb for electrophysiological stimulation and recording, with the animal secured in a rigid frame (Seburn & Cope, 1998; Haftel et al., 2004; Haftel et al., 2005; Bullinger et al., 2011a). The left tibial nerve was carefully dissected free of other tissues and suspended on a monopolar, silver stimulating electrode. Other hindlimb nerves were crushed, including the common peroneal nerve and sural nerves. Dorsal exposure of the lumbosacral spinal cord (L4–S1) by laminectomy and longitudinal incision of the dura mater provided access to dorsal roots L4 and L5, which were carefully dissected free of surrounding tissue and suspended in continuity on bipolar silver hook electrodes for recording, and tibial motoneurons via dorsolateral penetration of the spinal cord. Skin flaps were used to construct pools for bathing all exposed tissues with warm mineral oil.

In vivo intracellular recordings were made from antidromically identified tibial α-MNs using glass micro- electrodes (~10–25 MΩ) filled with 10% neurobiotin (Vector Laboratories, Burlingame, CA, USA) and 5% 488dextran (Invitrogen, Grand Island, NY, USA) in 0.1 M Tris-OH and 1.0 M potassium acetate. Only those MNs with stable membrane potential and with action potential amplitude >60 mV were deemed acceptable for further study. Failure to meet these criteria accounts for the incomplete data set obtained from some MNs. Motoneurons were injected with current through the micropipette in order to measure the following intrinsic electrical properties as previously
reported in our laboratory (e.g. Bullinger et al. 2011): rheobase current (depolarizing pulses, 50 ms in duration, at the lowest strengths capable of initiating action potentials from the resting membrane potential), AHP (following individual action potentials generated by suprathreshold pulses, 0.5 ms in duration), input resistance (from hyperpolarization produced by −1 and −3 nA current pulses, 50 ms in duration) and axon conduction velocity (antidromic conduction delay divided by tibial nerve length). In some animals, tibial nerve lengths and thus conduction velocity were not measured. Following physiological characterization, motoneurons were labeled by intra-cellular injection of 10% neurobiotin with 5% dextran in Tris Buffer (0.1M Tris-OH, 1M Potassium Acetate, PH 7.6). Positive current pulses (5nA), delivered as 400-ms-long pulses at 2 Hz for 2–5 min, were used to aid neurobiotin passage into the motoneuron. Records of MN membrane potential and electrode current were collected, digitized (20 kHz), stored and analysed with CED Power 1401 and CED Spike2 software (Cambridge Electronic Design, Cambridge, UK).

After data collection, animals were killed by an intra- peritoneal overdose of pentobarbital (150 mg kg−1 I.P.) and perfusion fixed as described in above methods. Spinal segments containing intracellularly labeled motoneurons were postfixed overnight. Serial transverse sections (75µm thick) were obtained on a vibrating micro- tome and were initially examined for dextran immunofluorescence using a fluorescence dissecting micro- scope. Those sections containing intracellularly labeled motoneurons were serially processed for SK3 immunoreactivity as described in “immunohistochemistry of SK channels”. To enhance intracellular labeling, neurobiotin was visualized with 488
streptavidin (Invitrogen; diluted 1:1000 in 0.01 M PBS containing 0.1% Triton X for 2 h at room temperature).

**Figure composition**

Figures were composed using CorelDRAW (version 12.0). Graphs were composed in SigmaPlot (version 9.0, Systat Software; SPSS Inc, Chicago, IL, USA). Microscope image modifications for presentation, such as adjusting contrast and brightness, were carried out in Image Pro Plus (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Some images were sharpened using a ‘high-gauss’ filter. Quantification was always carried out using original unprocessed images.

**Results**

Immunoreactivity for SK2 and SK3 (SK-IR) was observed in all laminae of the lower lumbar spinal cord, consistent with previous immunohistochemical surveys of SK channel expression (Sailer *et al.*, 2004; Mongan *et al.*, 2005). The highest levels of SK-IR were evident in the superficial dorsal horn and in the medial and lateral motoneuron pools in lamina IX of the ventral horn (Figure 1). Whilst immunoreactivity for both SK2 and SK3 was detected throughout the spinal cord grey matter, the overall intensity of SK2 immunolabeling was generally lower than that observed for SK3 and, with the fixation conditions used in our experiments, the detection of SK2-IR was more effective and consistent in mouse and rat tissue compared with cat tissue. Within a single tissue section, SK3-IR, when observed at all, could vary in intensity from modest (two to three times background) to strong (more than three times background) levels between individual
neurons; however, all SK3-positive neurons, regardless of signal intensity, were grouped
together for analysis and comparison against neurons that lacked SK3 completely. In
ventral horn neurons, SK-IR appeared to be largely surface membrane associated and was
concentrated in ‘hot spots’ that outline the perisomatic surface of MNs (soma and
proximal dendrites); punctate labeling was also distributed throughout the neuropil of the
ventral horn.

**Immunoreactivity for SK3 in motoneurons.**

In mice and rats ([Figure 1](#)) and in cats ([Figure 2](#)), SK3-IR was concentrated in disc-
shaped patches distributed in a mosaic pattern on the soma and proximal dendrites of
MNs. In proximal dendrites (up to 100 µm from the soma), SK3-IR was easily resolved
(e.g. [Figure 1C](#); see also [Figure 6A](#)), but we did not observe any SK3-IR associated with
more distal dendrites. The large, intensely labeled juxtasomatic channel cluster domains
were analyzed quantitatively using fluorescence confocal microscopy (Muennich & Fyffe,
2004). The surface areas of the SK3-IR channel clusters in mouse MNs ranged from 1.28
to 11.63 µm², with a mean of 5.02 µm² (SD ± 1.79) and mean diameter of 2.34 µm
([Figure 3](#)). The surface areas of the SK3-IR channel clusters in rat MNs ranged from
2.07 to 32.98 µm², with a mean of 9.80 µm² (SD ± 4.32) and mean diameter of 3.58 µm
([Figure 3](#)). The surface areas of the SK3-IR channel clusters in cat MNs ranged from
6.16 to 67.34 µm², with a mean of 25.91 µm² (SD ± 11.63) and mean diameter of 5.55
µm ([Figure 3](#)). Noticeably, most of the SK3-IR in MNs was concentrated in patches
greater than 1.0 µm². The lack of small clusters is not due to inability to detect or measure
smaller clusters. Our previous studies of Kv2.1 channel distribution in motoneurons
demonstrate the capability of our methods to measure smaller sized patches of immunoreactivity quantitatively (Muennich & Fyffe, 2004), and small punctae of SK-IR were observable in other spinal neurons in the present experiments. As previously noted for large $K_v2.1$ channel clusters, when individual SK3 membrane clusters are examined at high resolution they display a complex substructure that represents a non-uniform aggregation of much smaller structures (Figure 1D). For SK3-IR, in contrast to $K_v2.1$-IR at the same location, there was a tendency for immunostaining to be more concentrated around the periphery of the cluster region (see also Figure 12).

Sparsely distributed punctae of SK3-IR were observed throughout the neuropil in laminae IX and VII, in addition to immunoreactivity that could be clearly associated with individual proximal dendrites. Like the prominent juxtasomatic clusters of immunoreactivity, labeling in the neuropil was abolished in the presence of pre-incubated antigen, indicating a high degree of specificity in the observed fluorescence signal. Although these punctae could potentially represent SK expression in more distal dendrites of MNs (Li & Bennett, 2007), the cellular localization of this labeling remains undetermined, because even when distal dendrites of intracellularly labeled MNs were resolved and analyzed in detail, they lacked SK3 expression in any branches more than approximately 150–200 µm from the soma.

**Differential expression of SK3-IR in small vs. large motoneurons in rats and mice.**

In rats and mice, SK3-IR clusters were present in discrete populations of MNs (Figure 1B,F), whereas in cats the SK3-IR was observed in all presumed $\alpha$-MNs (Figure 2). In
the lateral MN pools of rat lumbar cord, 20% (254 out of 1267) of NeuN-labeled MNs expressed SK3 immunoreactivity in the surface membrane. In mice, 17% (95 out of 557) of the MNs expressed SK3.

Typically, NeuN labeling was present in the cytoplasm of the cell body and proximal dendrites as well as in neuronal nuclei, but there was a clear qualitative and quantitative difference in the level of NeuN immunostaining among cell bodies of neurons in the lateral lamina IX MN pools of both the rat and mouse spinal cord. These differences permit classification of α-MNs into two categories that reflect a direct relationship between soma size and pattern of NeuN expression. Neurons in one group, compromising the majority of α-MNs in each MN pool (Figure 1B,F), exhibit well-stained nuclei and diffuse cytoplasmic labeling that appears relatively intense and clearly extends into the proximal dendrites of the neuron. In contrast, other NeuN-positive α-MNs have well-stained nuclei but exhibit weak cytoplasmic labeling. Neurons in the latter class appear to be of smaller size than the former.

Of particular interest is the fact that the strongest levels of SK3-IR in surface membrane clusters was restricted to the α-MN population that exhibited a low level of cytoplasmic NeuN labeling (Figure 1B,C,F). Motoneurons that expressed relatively high cytoplasmic levels of NeuN generally lacked SK3-IR, although a few such neurons expressed SK3 at modest levels. In the rat, the mean soma diameter of SK3-expressing MNs (n = 156), regardless of NeuN labeling intensity, was 38.11 ± 5.39 µm (SD), which was significantly smaller (P < 0.0001) than that of MNs (n = 557) that lacked detectable SK3-
IR clusters, 42.99 ± 6.36 µm (mean ± SD; Figure 3B). A similar result was obtained when the cross-sectional areas of the cell bodies were compared. The mean cross-sectional areas for SK3-IR MNs (n = 61) was 1066.2 ± 179.2 µm², compared with 1263.6 ± 205.1 µm² for MNs (n = 183) that lacked SK3-IR (P < 0.01). Although there were other, much smaller, unidentified, but NeuN positive (and therefore unlikely to be γ-MNs), neurons interspersed among the neurons of the MN pool, these small neurons lacked SK3-IR and were presumed, based on morphology and the lack of C-bouton synaptic contacts, to be interneurons.

All rat and mouse motoneurons express SK2 channels.

Given that large-diameter MNs do not appear to express SK3, we performed dual staining of SK2 and SK3 through the use of Fab fragments to double label sections with primary antibodies from the same host, in order to determine whether the large-diameter MNs express SK2 and/or if SK2 and SK3 are coexpressed in the small MNs. The results showed that SK2 and SK3 were coexpressed in the small MNs and that SK2 was expressed in large MNs lacking SK3-IR in both rats (Figure 4) and mice. Like SK3, SK2-IR appeared to be organized in a cluster pattern over the surface membrane of the soma and proximal dendrites and appeared to colocalize with SK3 in SK3-IR MNs. The individual antibody peptide sequences were highly specific to each of the SK isoforms, and peptide pre-incubation studies for each of the two antibodies did not affect the immunoreactivity of the alternate isoform (data not shown), suggesting that there was no cross-reactivity between the antibodies. The fact that every MN was accounted for as
either SK2-IR or SK2- and SK3-IR suggests that the lack of SK3-IR in large motoneurons was not caused by antibody penetration problems or other technical issues.

*The pattern of SK3 expression in physiologically identified rat motoneurons in lateral motoneuron pools.*

It is widely accepted, on the basis of correlations between measurements of rheobase, input resistance, axonal conduction velocity and total membrane surface area, that small MNs innervate slow-twitch muscle fibers, while large MNs innervate fast-twitch muscle fibers (Henneman & Olson, 1965; Henneman *et al.*, 1965b, a; Burke, 1967; Burke *et al.*, 1982; Zengel *et al.*, 1985; Gardiner, 1993). Given the smaller size of SK3-IR cells, we injected retrograde tracer into specific hindlimb muscles to label MNs for post hoc analysis of SK3-IR. Approximately 25% (37 of 149 MNs analyzed) of MNs innervating the predominately fast-twitch medial and lateral gastrocnemius muscles (MG/LG) were SK3-IR, while 63% (84 of 133) of MNs innervating the predominately slow-twitch soleus muscle were SK3-IR (*Figure 5*).

Apamin-sensitive SK currents underlie the motoneuron AHP (Zhang & Krnjevic, 1987; Viana *et al.*, 1993; Li & Bennett, 2007), and it has been suggested that AHP ½-decay time accurately distinguishes fast- from slow-twitch motor units (Eccles *et al.*, 1957b; Zengel *et al.*, 1985; Gardiner & Kernell, 1990; Gardiner, 1993). Therefore, in a separate set of experiments, rat tibial MNs were electrophysiologically characterized, intracellularly labeled with neurobiotin, and analyzed for SK3-IR (*Figure 6*). Compared with MNs not expressing SK3, SK3-IR MNs showed a significantly longer AHP ½-decay...
time (24.2 ± 4.7 (SD; n = 8) vs. 11.0 ± 1.8 ms (SD; n = 27), Student’s unpaired t test, P < 0.001; Figure 6A) and significantly greater AHP amplitude as measured from resting membrane potential (3.1 ± 1.3 (SD; n = 8) vs. 1.5 ± 1.0 mV (SD; n = 27), Student’s unpaired t test, P = 0.001; Figure 7A). In most analyzed cells, the resting membrane potential was well documented throughout the recording period, and it was observed that there was no significant difference between individual cells (SK3-positive cells (n = 7), −53.4 ± 4.8 mV (SD); and SK3-negative cells (n = 21), −56.3 ± 8.2 mV, Student’s unpaired t test, P = 0.39). Moreover, SK3-expressing MNs had a significantly greater input resistance (3.1 ± 0.5 (SD; n = 4) vs. 1.7 ± 1.1 MΩ (SD; n = 15), Student’s unpaired t test, P = 0.035) and a significantly slower axon conduction velocity (44.2 ± 9.1 (SD; n = 7) vs. 58.6 ± 5.1 m s−1 (SD; n = 21), Student’s unpaired t test, P < 0.001; Figure 7A) than MNs not expressing SK3. No significant difference in mean rheobase was observed between cell types (SK3-positive cells (n = 9), 3.8 ± 3.3 nA (SD); and SK3-negative cells (n = 27), 8.8 ± 7.3 nA (SD), Student’s unpaired t test, P = 0.55; Figure 7A), although the observed values indicate a trend towards SK3 being present in ‘electrically smaller’ MNs.

We observed the normal tendency for AHP ½-decay time to co-vary with AHP amplitude (r = 0.61, P < 0.001; Figure 7B) and, as in previous studies (Eccles et al., 1958a; Bakels & Kernell, 1993b; Prather et al., 2011), we observed a weak but significant negative correlation between rheobase current and AHP ½-decay time (r = −0.39, P=0.028; Figure 7B) and a stronger negative correlation between axon conduction velocity and AHP ½-decay time (r = −0.85, P < 0.001; Figure 7B). In the plots shown in Figure 7, the data points representing SK3-expressing MNs cluster in regions of the overall parameter
distribution that are typically represented by MNs innervating slow-twitch motor units in rats (Bakels & Kernell, 1993b; Gardiner, 1993).

For all MNs analyzed in this study, the mean AHP amplitude ($1.9 \pm 1.2$ mV (SD; n = 35)), mean AHP $\frac{1}{2}$-decay time ($14.0 \pm 6.2$ ms (SD; n = 35)), mean input resistance ($2.0 \pm 1.2$ MΩ (SD; n = 19)) and mean conduction velocity ($55.0 \pm 8.8$ m s$^{-1}$ (SD; n = 28)) are consistent with average values previously published (Bakels & Kernell, 1993b; Seburn & Cope, 1998; Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a). In order to have an adequate sample for comparative analysis of SK3-expressing MNs, which are a minority of the total $\alpha$-MN population, we necessarily biased our sample towards small, low-rheobase cells. As a result, the mean rheobase current for all $\alpha$-MNs analyzed in this study ($7.5 \pm 6.8$ nA (SD; n = 36)) is slightly lower than previously reported by researchers our laboratory in female Wistar rats anaesthetized with isoflurane (Bullinger et al., 2011a), but still falls within ranges typical for anaesthetized rat $\alpha$-MNs (Seburn & Cope, 1998; Bichler et al., 2007a; Bichler et al., 2007b).

**Postsynaptic localization of SK3 channels.**

Double labeling with antibodies against the synaptic protein synaptophysin revealed a close apposition between the sites of presynaptic inputs (synaptophysin-labeled boutons) and postsynaptic clusters of the SK channel (Figure 8). In each mammalian species we analyzed, every postsynaptic SK3-IR cluster was closely apposed by a synaptophysin-labeled bouton, although clearly not all presynaptic boutons on the MN surface were opposed to SK3 clusters. Similar results were observed for SK2 clusters. Although
superimposed images of stacked optical sections occasionally showed a thin band of overlapping immunostaining (Figure 8), this labeling appearance was consistent with earlier observations of membrane channel labeling at synapses in MNs in which clear ultrastructural verification of apposed pre- and postsynaptic markers were obtained (Muennich & Fyffe, 2004).

Our electron microscopic analysis (Figure 9) supports the conclusion that the SK channel clusters are post-synaptically localized in the surface membrane of MNs and that there is generally very little SK protein, if any, present in the presynaptic boutons apposed to the MN soma or proximal dendrites. The postsynaptic SK clusters appeared to be apposed predominantly by the largest presynaptic boutons surrounding the soma (Figure 8). As a result of their size, these large boutons were likely to be C-boutons (Conradi, 1969a; Conradi et al., 1979a), and this hypothesis was tested by use of markers for cholinergic synapses.

**Specific association of postsynaptic SK3 channels and presynaptic C-boutons.**

Cholinergic synapses in the ventral horn were revealed by immunoreactivity against the vesicular acetylcholine transporter (VACHT), which labels distinct populations of cholinergic axon terminals, including those arising from motor axon collaterals in ventral lamina VII (that contact Renshaw cells), as well as large C-boutons that uniquely contact the soma and proximal dendrites of α-MNs in lamina IX (Alvarez et al., 1999; Hellstrom et al., 2003; Deng & Fyffe, 2004; Miles et al., 2007; Zagoraiou et al., 2009). Dual labeling of SK3 and VACHT (Figure 10) demonstrated consistent association of
motoneuron SK3-IR clusters and large VACHT-IR presynaptic C-boutons (94.9% correspondence; n = 1051 SK3-IR clusters); i.e. almost every cluster of SK-IR on the membrane surface of an SK3-expressing MN, including SK3 in proximal dendrites, was closely apposed by a cholinergic nerve terminal.

To confirm specificity and selectivity of the association of the postsynaptic SK clusters with cholinergic inputs, we also analyzed SK3-IR in conjunction with markers for glutamatergic, monoaminergic and glycine/GABAergic synapses (Figure 11). In contrast to the striking relationships between VACHT-IR boutons and SK3-IR demonstrated in Figure 10, populations of glutamatergic boutons revealed by immunostaining against two different isoforms of the vesicular glutamate transporter (VGluT1 and VGluT2; (Alvarez et al., 2004; Alvarez et al., 2011)) were not associated with SK-IR sites. Likewise, despite dense monoaminergic innervation of the ventral horn MNs (Alvarez et al. 1998), no appositions were observed between 5-HT-immunoreactive terminals and SK3 clusters. Finally, there was no overlap or relationship between SK3-immunoreactivity and immunostaining for gephyrin, a postsynaptic scaffolding protein present at inhibitory glycineergic and GABAergic synapses on MNs (Alvarez et al., 1997).

**Colocalization of SK and Kv2.1.**

We have previously demonstrated that Kv2.1 channel-IR is present in a characteristic mosaic pattern of large and small ion channel clusters in α-MNs, with the largest clusters being located postsynaptically at C-bouton synapses (Muennich & Fyffe, 2004; Wilson et al., 2004). We used double labeling with anti-SK and anti-Kv2.1 antibodies to determine
whether these respective channel properties were colocalized together at the large C-bouton synapses (Figure 12). The illustrated data focuses on SK3-IR in rat spinal cord, but equivalent results were obtained for both SK isoforms in rats and mice. There was a clear and consistent colocalization between SK-IR and Kv2.1-IR at the large cluster sites in α-MNs, with the respective SK3-IR and Kv2.1-IR signals interdigitating to ‘fill’ the space demarcated by the cluster region (Figure 12). In most cases, including those illustrated at high magnification in Figure 12, the SK3-IR tended to be located more towards the periphery of the cluster region, surrounding the Kv2.1-IR, consistent with the fact that the average size of SK3 clusters is larger than that of Kv2.1 clusters (Muennich & Fyffe, 2004). The precise colocalization between SK3-IR and Kv2.1-IR in these large cluster domains was consistent in all 341 clusters examined in 30 MNs. Note that small Kv2.1 clusters distributed widely across the surface membrane (Figure 12), independent of the large clusters, did not colocalize with SK-IR. This is consistent with the fact that Kv2.1 channel clusters are present in addition at non-cholinergic synapses on MNs and that these latter clusters are much smaller than the Kv2.1 clusters localized at C-bouton inputs (Muennich & Fyffe, 2004).

Discussion

Certain intrinsic membrane properties of spinal α-MNs vary systematically in relationship to motor unit type. It is well established that MNs of S-type motor units have larger amplitude and longer duration AHPs than do F-type MNs (Burke, 1967; Zengel et al., 1985; Bakels & Kernell, 1993b; Gardiner, 1993). In α-MNs, as in other central neurons, the AHP displays multiple phases (fast, medium and slow AHP) that can be
identified by their kinetics and pharmacological sensitivity to channel blockers (Schwindt et al., 1992; Viana et al., 1993; Sawczuk et al., 1997; Sah & Faber, 2002; Bond et al., 2004; Villalobos et al., 2004; Faber, 2009), although the slow AHP is rarely observed in MNs. There has been extensive effort to define the molecular identities, modulation and properties of the Ca$^{2+}$-activated K$^+$ channels that underlie kinetically different AHP currents in a variety of central neurons (Sah & Faber, 2002). These analyses suggest that large-conductance (BK) channels, in addition to tetraethylammonium (TEA)-sensitive voltage-gated K$^+$ channels, contribute to spike repolarization and to the fast AHP, whilst small-conductance Ca$^{2+}$-activated potassium (SK) channels, which have activation kinetics of several milliseconds and decay time constants of tens of milliseconds, contribute in large part to the mAHP (Viana et al., 1993; McLarnon, 1995; Sah & Faber, 2002). The identity of the apamin-insensitive channels that underlie slow AHPs has not yet been confirmed (Sah & Faber, 2002; Bond et al., 2004; Power & Sah, 2008). Given the major physiological role of SK channels in the generation of mAHPs, hence MN firing properties, part of the motivation for the present study was to determine whether expression of particular SK channel isoforms might be correlated with the kinetically different mAHPs produced in different functional classes of MNs.

Another impetus for the present study was the notion that compartmentalized localization of SK channels could be important for their physiological roles. In other regions of the brain, SK channels are localized at excitatory, glutamatergic synapses located on dendritic spines of hippocampal pyramidal neurons and neurons of the lateral amygdala (Faber et al., 2005; Faber et al., 2008; Lin et al., 2008). In the dendritic spines of these
neurons, SK channels, especially SK2, appear to contribute to aspects of synaptic plasticity (Faber et al., 2005; Power & Sah, 2008). These and related studies have also revealed that the activity and membrane trafficking of SK channels, as well as their calcium sensitivity, can be regulated by NMDA receptor activation and by a variety of neuromodulators, including those acting via muscarinic receptors and β-adrenoceptors, that affect calcium signaling or activation of kinases (Faber et al., 2008; Maingret et al., 2008; Faber, 2009). Given that spinal α-MNs essentially lack dendritic spines (Brown & Fyffe, 1981), it is of major interest to determine how SK channels are distributed, if at all, along spine-free dendrites, and if they are associated with excitatory synapses as they are in other neurons.

The general expression profile of SK channels in the CNS has been revealed using in situ hybridization and immunostaining against SK channel protein or myc-tagged epitopes, and the main subunit types are widely expressed in partly overlapping distributions (Stocker & Pedarzani, 2000; Tacconi et al., 2001; Sailer et al., 2004). The present study demonstrates that α-MNs in cat, rat and mouse lumbar spinal cord express specific members of the SK channel family, in accordance with observations that the mAHP in these neurons (and in hypoglossal MNs) is blocked by apamin (Zhang & Krnjevic, 1987; Hounsgaard et al., 1988b; Viana et al., 1993; Sawczuk et al., 1997; Powers et al., 1999; Miles et al., 2005). Several novel and unexpected features of SK channel expression and distribution in spinal MNs are revealed by detailed analysis of channel localization. First, we reveal in rats and mice a differential expression of specific SK subunits (SK2 vs. SK3) among differently sized α-MNs with different electrophysiological properties.
Second, SK2 and SK3 channels in the aspinous surface membrane of α-MNs are organized in a highly clustered pattern and are restricted to the soma and proximal dendrites. Third, SK channels are selectively localized at postsynaptic sites associated with cholinergic C-boutons and are thus colocalized with Kv2.1 channels and m2 muscarinic receptors (see Chapter V) at these sites. Interestingly, new evidence has indicated that state-dependent m2 receptor activation by cholinergic C-boutons increases motoneuron excitability by reducing action potential AHP (Miles et al., 2007; Zagoraiou et al., 2009); however, a mechanism of interaction between m2 receptors and SK channels has yet to be defined.

**SK2 and SK3 channels are expressed in different populations of MNs in rats and mice but not in cats.**

In this study, MNs were identified by anatomical parameters (e.g. soma size/morphology and location within lamina IX) as well as by molecular markers (e.g. VACHT and NeuN immunoreactivity) that allowed us to differentiate α-MNs from γ-MNs and interneurons (Moschovakis et al., 1991; Alvarez & Fyffe, 2007; Friese et al., 2009; Shneider et al., 2009). All MN soma measurements fell within the range of soma diameters previously described for α-MNs (Chen & Wolpaw, 1994; Ishihara et al., 2001; Bose et al., 2005). Through use of the common neuronal marker, NeuN, our analyses revealed an unexpected relationship between the level of expression of NeuN and MN soma size. Neurons that expressed high levels of NeuN in the nucleus and cytoplasm were, on average, larger than neurons that expressed low levels of NeuN in the cytoplasm. Other recent reports suggest that γ-MNs lack expression of NeuN (Friese et al., 2009; Shneider
et al., 2009) as do cerebellar Purkinje cells, mitral cells of the olfactory bulb, inferior olivary neurons, basket and stellate interneurons of the cerebellum, and retinal photoreceptors (Mullen et al., 1992; Wolf et al., 1996; Sarnat et al., 1998; Weyer & Schilling, 2003). Such observations of non-uniform expression of NeuN emphasize the need for caution when using NeuN as a presumed ubiquitous neuronal marker in spinal cord, particularly given that its level of expression may also be modulated by injury (McPhail et al., 2004; Alvarez et al., 2011).

Two α-MN populations were distinguished in rats and mice by virtue of their differential expression of SK2 vs. SK3 channel isoforms. All MNs express SK2, and a minority of MNs are distinguished by expression of SK3 plus SK2; the majority of MNs express only SK2. The SK2-IR MNs outnumber SK3-IR/SK2-IR MNs in a ratio consistent with the overall ratio of F-type to S-type motoneurons in most lumbosacral MN pools. Importantly, the expression of SK2 vs. SK3/SK2 was correlated with α-MN soma size of the respective populations. The predominant membrane SK channel in small α-MNs is SK3, with these neurons also displaying SK2-IR. Conversely, large α-MNs have prominent membrane expression of SK2 but lack SK3. Consistent with the substantial evidence that MN size varies by motor unit type in the order S < F (Burke et al., 1982; Cullheim et al., 1987), a significantly greater proportion of soleus MNs (predominantly slow-twitch) express SK3 than gastrocnemius MNs (predominantly fast-twitch), indicating that SK3 subunits are preferentially expressed in S-type rat MNs. The observation that the medial motor pools, which innervate slow-twitch postural muscles, also contain a high level of SK3-IR in mice and rats further supports MN type-specific
SK3 expression in rodent MNs. In cats, all MNs, i.e. both S- and F-types, appear to express SK3. Although the implications of this interspecies difference remain to be determined, it is of interest that the AHPs in cat MNs, even F-types, tend to be larger and longer than the AHPs observed in rat and mouse MNs.

Motoneuron electrophysiological properties in this study fell within the range of measurements previously described for rat α-MNs (Bakels & Kernell, 1993b; Seburn & Cope, 1998; Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a). Our electrophysiological analyses revealed a significantly longer duration and larger amplitude AHP in rat MNs expressing SK3. Importantly, Gardiner (1993) reports distinguishing fast from slow motoneurons in the rat medial gastrocnemius motor pool with 100% accuracy using a single parameter, namely a nominal cut-off between F- and S-types of 20 ms AHP ½-decay time (F-type AHP ½-decay < 19 ms; and S-type AHP ½-decay > 20 ms). In the present study, seven of the eight physiologically characterized, SK3-expressing MNs had AHP ½-decay times greater than 20 ms and would probably be S-type based on Gardiner’s criteria. The data of Bakels and Kernell (1993b) show several S-type MNs with AHP ½-decay shorter than 15 ms (their average was 18.3 ± 6.3 ms), indicating that all eight SK3-IR cells in our study fall within the range of AHP durations previously observed in S-type MNs. The variation among reported electrophysiological parameters from the few in vivo reports that are available underscores, as mentioned by Bakels and Kernell (1993b), that physiological properties of motor units display continuous variation in magnitude and that classification into categories, such as ‘fast’ or ‘slow,’ is still controversial (Mendell, 2005) and often made for convenience in
description, analysis and comparative study. Despite this caveat, our data appear to correlate well with the data of Gardiner (1993), and our interpretation is strengthened by further correlation with other electrophysiological properties.

While other molecular factors have been postulated to distinguish S- and F-type MNs (Forsgren et al., 1993; Piehl et al., 1993; Chakkalakal et al., 2010; Enjin et al., 2010), to date there are no indications in the literature of specific ion channels that might underlie physiological differences between S- and F-type MNs. The present results suggest that differential expression of SK isoforms contributes to the observed variability in rat motoneuron mAHP duration and, in particular, that the presence of SK3 channel subunits is associated with long-duration mAHP currents in small, presumably S-type, rat MNs. The finding that SK3-expressing MNs in this study share other known physiological properties predictive of S-type MNs, such as slower conduction velocity and a trend towards lower rheobase (differences in rheobase did not reach significance, probably due to our intentional sampling bias; see also Gardiner, 1993), strongly suggests that SK3-IR may serve as an anatomical marker for rodent S-type MNs. However, caution must be taken in interpreting motor unit classification of SK3-expressing MNs without corresponding twitch data from innervated muscle fibers, and in cases where motoneuron axons have been subjected to injury (See Aim 5).

Furthermore, the fact that all cat MNs express SK3 subunits obviously complicates any attempt to correlate systematically a specific SK channel isoform with the distinct mAHP time courses of different types of MNs across species. One speculative explanation to
reconcile the interspecies differences is to consider the ‘absolute’ mAHP duration rather than the relative differences across S- or F-type MNs in a single species. We plotted (not shown) mean values and ranges from the literature of AHP ½-decay time for cat and rat F- and S-type MNs and for mouse MNs (for which there are no type-specific data), which showed the predictable trend for cat S-type > cat F-type > rat S-type > rat F-type > mouse. However, the ranges overlap extensively, particularly between rat F-type, rat S-type and mouse data, and between rat S-type and cat F-type. Gardiner and Kernell (1990) also showed that the longest duration AHPs in rat S-type MNs are at least as long as many of the AHPs in cat F-type MNs. Our data suggest that expression of SK2 only, i.e. lack of SK3, is associated with the shortest AHPs in absolute terms, i.e. those in large F-type MNs in rat and mouse. Conversely, SK3 appears to be present in MNs with longer duration AHPs in absolute terms, namely small S-type MNs in rats and mice and all MNs in cats. Given that, in general, the relative contractile speed of muscle fibers in the three species analyzed increases in the order cat < rat < mice, it is likely that rodent F-type motoneurons lack SK3 in order for them to fire at rates necessary to match the contractile speed of their innervated muscle fibers effectively. We believe that extrapolation of this conclusion is merited, despite the absence of type specification for measured AHP values in the mouse, because the longest mouse AHPs, presumably from S-type MNs, have durations that overlap with those of rat S-type MNs. Although the molecular mechanisms that may underlie these observed differences in mAHP duration are not yet defined, this conclusion is further supported by findings that SK3 channels have slower activation and deactivation time constants than SK2 channels (Xia et al., 1998).

Moreover, SK3 expression may only be one factor underlying the observed variability in
AHP duration in rat MNs, and other factors, such as differential coupling of SK channels to Ca$^{2+}$ sources, variations in SK channel cluster sizes/density, the presence/absence of hyperpolarization-activated currents ($I_h$) and/or synaptic modulation of AHP kinetics, may need to be considered.

There is another level of uncertainty in any attempt to ascribe AHP kinetics to specific SK contributions, because we lack information on the third isoform, SK1, for which, unfortunately there is no reliable antibody available for specific detection or localization. Rat SK1, transfected and expressed alone in HEK 293 cells, was unable to produce a Ca$^{2+}$-activated K$^+$ conductance (Benton et al., 2003). However, when SK1 and SK2 were coexpressed, there was a Ca$^{2+}$-activated K$^+$ conductance that was larger in magnitude, with decreased apamin sensitivity, than when SK2 was expressed alone (Benton et al., 2003). It is now known that rat SK1 subunits can form heteromeric channels with rat SK2 but not SK3 (Benton et al., 2003; Monaghan et al., 2004; Strassmaier et al., 2005). Furthermore, SK3 can form heteromeric subunits only with SK2, suggesting SK1 may play a role in disrupting SK3 assemblies (Monaghan et al., 2004). Thus, even in rodent MNs where SK2 and SK3 exhibit differential expression patterns, SK1 could potentially be present and modify channel characteristics. Therefore, we must at present conclude that the well-documented and varying AHP properties of S- and F-type MNs cannot be ascribed simply to SK2 vs. SK3 expression but may reflect specific patterns of SK1 expression, heteromeric combinations of multiple SK isoforms, or contributions from different regulatory mechanisms or ion conductances (for example, AHP duration in cat MNs varies inversely with the magnitude of their sag current; (Gustafsson & Pinter,
Thus, complete understanding of the contribution of specific SK subunits to AHP properties awaits the following findings: (a) clear molecular evidence of the presence or absence of SK1 in MNs; (b) further determination, possibly in expression systems, of the functional properties of heteromeric SK channels; and (c) examination of other intrinsic/extrinsic mechanisms that may contribute to AHP variability.

Membrane organization of SK channels.

A novel and important finding of this study is that SK2 and SK3 channels are selectively localized at postsynaptic sites on the soma and proximal dendrites of MNs. Both confocal microscopy and ultrastructural analyses confirm that SK channel expression is enriched, particularly in large clusters immediately adjacent to cholinergic C-boutons, in a manner reminiscent of Kv2.1 channel distribution (Muennich & Fyffe, 2004).

Muscarinic m2 receptors are the dominant muscarinic acetylcholine receptor subtype in the MN plasma membrane and, like SK and Kv2.1 channels, localize directly adjacent to C-boutons (Skinner et al., 1999; Hellstrom et al., 2003; Muennich & Fyffe, 2004). Muscarinic acetylcholine receptors can be classified into five subtypes (m1 – m5), which transduce signals through distinct G-protein-coupled pathways. Recent evidence indicates that m2 receptor activation by cholinergic C-boutons during fictive locomotion increases spinal MN excitability by reducing the AHP (Miles et al., 2007; Zagoraionou et al., 2009). Similar reductions in AHP have been observed following muscarinic receptor activation of hypoglossal MNs (Lape & Nistri, 2000). The distinct subcellular localization of SK channels correlates well with these physiological data and suggests that reduced outward
SK current following cholinergic m2 receptor stimulation may mediate this reduction in AHP.

While muscarinic acetylcholine receptors can evoke cellular effects through a variety of signaling cascades (Shapiro et al., 1999; Hoshi et al., 2003; Tiran et al., 2003; Zhou et al., 2003a), m2 receptors typically couple to pertussis toxin-sensitive Gi/Go pathways which, when activated, can reduce adenylate cyclase or directly inhibit voltage-gated Ca\(^{2+}\) channels (Hille, 1994; Stewart et al., 1999; Santafe et al., 2006). Membrane delimited pertussis toxin-sensitive muscarinic blockade of N- and P/Q-type Ca\(^{2+}\) currents have been observed in a variety of cell types (Allen & Brown, 1993; Hille, 1994; Howe & Surmeier, 1995; Stewart et al., 1999; Santafe et al., 2006). Blockade is usually caused by m2-coupled, \(\beta\gamma\)-mediated, depolarizing shifts in the voltage dependence of channel activation and is transiently relieved by strong or repeated depolarization (Hille, 1994; Ikeda, 1996; Jeong & Ikeda, 1999; Shapiro et al., 1999; Herlitze & Landmesser, 2007). Interestingly, in hypoglossal and spinal MNs, conotoxin-sensitive N- and P/Q-type Ca\(^{2+}\) currents provide the calcium for SK channel activation (Viana et al., 1993; Bayliss et al., 1995; Li & Bennett, 2007); therefore, C-bouton-mediated reductions in MN AHP may occur via \(\beta\gamma\) inhibition of N- and P/Q-type Ca\(^{2+}\) currents in the vicinity of SK channels. Such a role for MN m2 receptors is intriguing in light of findings that Ca\(^{2+}\)-dependent modulation of \(K_v2.1\) channel gating increases outward K\(^+\) current and reduces neuronal firing rate (Misonou et al., 2004; Surmeier & Foehring, 2004; Mohapatra et al., 2009). It is possible that m2 receptor activation serves to regulate outward current through SK and \(K_v2.1\) channels via an intermediary effect on local [Ca\(^{2+}\)]\(_i\), and thereby provide state-dependent
modulation of MN firing rate. However, despite ample evidence for muscarine-evoked, m2 receptor-mediated reduction of neuronal N- and P/Q-type Ca\(^{2+}\) channels, Miles et al. (2007) were unable to elicit MN Ca\(^{2+}\) current modulation following bath application of muscarine in whole-cell voltage-clamp experiments. This result underscores the necessity for anatomical characterization of MN voltage-gated Ca\(^{2+}\) channels, because it is difficult to interpret pharmacological manipulation of global Ca\(^{2+}\) currents without knowledge of the subcellular localization of specific channel subtypes. It is possible that conotoxin-sensitive Ca\(^{2+}\) channels in the vicinity of MN m2 receptors represent only a minority of MN somatic Ca\(^{2+}\) sources, and their blockade may be masked in studies of global Ca\(^{2+}\) currents by other, extrasynaptic Ca\(^{2+}\) channels.

Alternatively, the direct phosphorylation of SK channels by protein kinase A and casein kinase 2 can cause channel internalization (Kohler et al., 1996; Ren et al., 2006; Faber et al., 2008; Lin et al., 2008) and reduced Ca\(^{2+}\) sensitivity (Allen et al. 2007), respectively. Although m2 receptors typically inhibit protein kinase activity, other phosphorylation pathways may be activated by m2 receptors (Zhou et al., 2003b), and it is possible that muscarinic reductions in AHP occur via direct phosphorylation of SK channels. A corollary of this is that Kv2.1 is also highly regulated by phosphorylation (Misonou et al., 2004), and the strategic localization of Kv2.1 clusters may suggest the presence of a currently undefined neuronal m2 receptor-mediated phosphorylation pathway.

It should be noted that recent electrophysiological evidence has indicated the presence of a separate population of dendritic SK channels activated by persistent inward Ca\(^{2+}\)
currents that are spatially distinct from SK channels responsible for generating the AHP (Li & Bennett, 2007). The Cav1.3 (L-type) channels mediating persistent inward Ca^{2+} currents are found in MN dendrites (Carlin et al., 2000). Subsequent studies have predicted these channels to localize in dendritic hot spots occurring at least 100 µm away from the soma (Elbasiouny et al., 2005; Bui et al., 2006). In the present study, however, dendritic SK-IR was observed only in large clusters apposed to C-bouton synapses within the initial 150–200 µm of the dendritic tree. When intracellularly labeled MN dendrites were carefully examined, we did not observe synaptic or extrasynaptic SK expression beyond the first 200 µm length of dendritic membrane. Nevertheless, we observed a significant amount of punctate SK-IR, apparently associated with dendritic profiles throughout laminae VII and IX, but could not identify the source of this labeling. It should be noted that the expression of SK1 remains undefined, but even if SK1 is present extrasynaptically in dendrites, it probably requires SK2 to construct functional heteromeric assemblies (e.g. Benton et al. (2003)). Thus, while it is possible that the dendritic SK conductances proposed by Li and Bennett (2007) represent functional homomeric SK1 channels, it is also possible that the density of extrasynaptic SK2 (or SK3) channels in the proximal and distal dendrites we have been able to analyze in detail is below the threshold for detection by our methods.

**Conclusions**

In summary, the present chapter shows the following results: (a) there is a differential expression of SK3-IR in rodent, but not cat, motoneurons; (b) rat α-MNs expressing SK3-IR are significantly smaller, have a significantly slower axon conduction velocity and
have a significantly longer AHP ½-decay time and larger AHP amplitude and than α-MNs not expressing SK3-IR; (c) rat MN pools innervating slow-twitch muscles have a higher percentage of SK3-IR α-MNs than those innervating fast-twitch muscles; and (d) SK2 and SK3 channel clusters appose cholinergic C-boutons. These data suggest that the presence of SK3 subunits may be a molecular factor differentiating between S- and F-type MNs. Furthermore, the specific clustering and synaptic localization of SK2 and SK3 together with m2 receptors and Kv2.1 channels is likely to represent a novel cellular mechanism for the state-dependent regulation of neuronal excitability.
Figure 1. **Differential expression of small-conductance calcium-activated potassium channel 3 (SK3) immunoreactivity (IR) in rat and mouse lumbar spinal motoneurons.**

Images are representative micrographs from confocal stacks exhibiting SK3-IR (red) and NeuN-IR (green) in coronal sections of spinal cord. A–E are from a rat, and F is from a mouse. A–E show successively higher magnification views (×10–×60 objectives, plus digital zoom in D and E) of the lateral lamina IX region outlined in panel A. (A) Strong SK3 immunoreactivity is observed in the superficial dorsal horn and in medial and lateral lamina IX areas of the ventral horn as indicated (12 superimposed optical sections, 1.0 µm Z-step; scale bar represents 200 µm). (B) In the lateral MN pool, arrows point to three smaller MNs expressing SK3-IR. Frequently, MNs expressing SK3-IR have less intense NeuN-IR compared with the majority of MNs (six optical sections; scale bar represents 100 µm). (C) In SK3-expressing MNs, clustered SK3-IR is distributed in a mosaic pattern on the perisomatic membrane and proximal dendrites (30 optical sections, 0.5 µm Z-step; scale bar represents 20 µm). (D) High-power micrograph of en face cluster depicted in the small square dotted box in C. Individual SK3-IR clusters display complex substructure and appear to be composed of multiple small regions of intense immunoreactivity. Image is a single optical confocal section. Scale bar represents 5 µm. (E) SK3-IR clusters on a proximal dendrite (rectangular dotted area demarcated in C; 25 optical sections (0.5 µm Z-step; scale bar represents 10 µm). (F) Similar to the expression pattern seen in B, SK3-IR in mouse MNs is exhibited in a subset of MNs in the lateral MN pools; 13 optical sections (1.0 µm Z-step; scale bar represents 50 µm).
Figure 2. **SK3 immunoreactivity is present in all cat lateral lumbar spinal motoneurons.**

In contrast to the rat and mouse (Figure 1), clusters of intense SK3-IR are observed in every MN in the pool (confocal image stack is 13 optical sections, 1.0 µm Z-step; scale bar represents 50 µm).
Figure 3. *Distribution of SK3-IR cluster areas and motoneuron soma sizes.* (A) The lines represent the distribution (calculated in increments of 2 µm²; total range 1–73 µm²) of SK3-IR cluster sizes on lumbar MNs in different species. The mean areas for cluster sizes were 5.02 µm² for mice (n = 247), 9.80 µm² for rats (n = 1497) and 25.91 µm² for cats (n = 639). (B) In rats, MNs with positive SK3-IR had a mean soma diameter of 38.11 ± 5.39 µm (SD; n = 156) whereas MNs that lack SK3-IR had a mean soma diameter of 42.994 ± 6.36 µm (SD; n = 557). The two populations are significantly different (Student’s unpaired t test, P < 0.0001).
Figure 4. Differential expression of SK3-IR and SK2-IR in rodent lumbar spinal α-motoneurons. A1, B1 & C1 are images of the same region showing three adjacent MNs (eight optical sections, 1.0 µm Z-steps; scale bar in C1 represents 20 µm) and respective SK3-IR (red), SK2-IR (green) and NeuN labeling (blue). Surface membrane regions (boxed areas in A1, B1 & C1) from each MN are shown at high magnification (scale bars represent 2.0 µm) in the three right-hand panels. Two of the neurons (see boxed areas A3 & A4) have intense (more than three times background, A3) or moderate (more than two times background, A4) clusters of SK3-IR (arrows), but SK3-IR is absent from the third MN (A2). All three neurons express SK2-IR at the same sites (arrows in B2, B3 & B4). In MNs that express both SK2 and SK3, the immunostaining is clearly colocalized at these sites (merged images in C3 & C4).
Figure 5. Motoneuron pools innervating slow-type muscles have a higher percentage of motoneurons with SK3-IR. Rat MN pools were retrogradely labeled with fluorogold from intramuscular injections into either the soleus (predominately S-type muscle) or the medial and lateral gastrocnemius (MG/LG; predominately F-type muscle). Post hoc analysis of labeled MNs classified SK3 expression as either (+) SK3-IR (more than two times background) or (–) SK3-IR (undetectable immunoreactivity). The number of MNs sampled is listed in parentheses. Approximately 63% of MNs innervating the S-type soleus muscle have (+) SK3-IR profiles and approximately 25% of MNs innervating the F-type MG/LG muscles have (+) SK3-IR. The proportion of SK3-expressing neurons in each pool is significantly different as revealed by Z-test (P < 0.001).
Figure 6. Expression of SK3 in physiologically characterized α-motoneurons. Tibial MNs were electrophysiologically characterized, labeled with neurobiotin and analyzed for SK3 expression using post hoc immunohistochemistry. (A) An SK3-expressing tibial motoneuron, with high-magnification images (insets) of clusters on the soma and proximal dendrites. The image in A is 85 optical sections (0.5 µm Z-step). Insets are four to eight optical sections (0.5 µm Z-step). (B) Intracellular physiological recordings from the same neuron. Upper traces in B show rheobase current injections (upper right) and corresponding voltage records (upper left); rheobase current in this cell, i.e. the minimal current elicting an action potential, is 3 nA. The lower trace shows the after-hyperpolarization (AHP; ½ decay time = 25.56 ms, amplitude = 2.01 mV; average of 45–60 trials; action potential amplitude is truncated to highlight AHP). (C&D) Similar image and recordings (taken at a matching resting membrane potential, ∼48 mV, to those in B) of a tibial motoneuron that does not express SK3. Image in C is 90 optical sections (0.5 µm Z-step). For the cell in C, rheobase current is 4 nA. AHP ½ decay time is 11.83 ms and AHP amplitude is 1.5 mV (average of 45–60 trials).
Figure 7. **Electrical properties in SK3 (+) and SK3 (−) tibial motoneurons.** (A) Average MN properties in SK3(+) and SK3(−) cells expressed as means ± SD. The SK3(+) cells have a significantly longer AHP ½ decay time (A1, 24.2 ± 4.7 vs. 11.0 ± 1.8 ms; Student’s unpaired t test, P < 0.001), significantly greater AHP amplitude (A2, 3.1 ± 1.3 vs. 1.5 ± 1.0 mV, Student’s unpaired t test, P = 0.001), significantly greater input resistance (A4, 3.1 ± 0.5 vs. 1.7 ± 1.1 MΩ, Student’s unpaired t test, P = 0.035) and significantly slower axonal conduction velocity (A5, 44.2 ± 9.1 vs. 58.6 ± 5.1 m s−1; Student’s unpaired t test, P < 0.001) than SK3(−) cells. Although there was no significant difference in rheobase between SK3(+) and SK3(−) cells in the present sample (A3, 3.8 ± 3.3 vs. 8.8 ± 7.3 nA, Student’s unpaired t test, P = 0.55), there was a trend towards SK3(+) cells having much lower rheobase. (B) Covariance of AHP ½ decay time with neuronal electrical properties (see also Eccles et al. 1958; Bakels & Kernell, 1993; Prather et al. 2011). Open circles indicate SK3(+) cells. Filled circles represent SK3(−) cells. All linear regression correlation coefficients (r) were significant (Pearson correlation, P < 0.05) and represent all MNs analyzed, regardless of SK3 expression. (B1) AHP ½-decay plotted vs. rheobase shows a weak but significant negative correlation (r = −0.39; P = 0.022). It is important to note that SK3(+) cells (open circles) cluster in the low-rheobase, long-AHP ½ decay time region of the graph. Another group of low-rheobase cells is apparent, which have short AHPs and lack SK3-IR; the electrical properties of these cells fit previously described distributions, confirming that the relationship between AHP and rheobase is complex. (B2) AHP amplitude plotted vs. AHP ½ decay shows a weak but positive correlation (r = 0.60; P < 0.001). (B3) Axonal conduction velocity plotted vs. AHP ½ decay shows a strong and significant negative
correlation ($r = -0.80; P < 0.001$). In this plot, SK3 (+) and SK3 (−) cells separate into distinct clusters, with SK3(+) cells being found in the slow conduction velocity and long AHP ½-decay region of the graph typical of S-type MNs. Corresponding conduction velocity and AHP measurements are unavailable for six SK3(−) and two SK3 (+) cells, including the SK3 (+) cell with the shortest AHP ½ decay.
Figure 8. SK channel clusters are localized to synapses on α-motoneurons. (A) Arrows indicate some of the close appositions of boutons labeled with the presynaptic marker synaptophysin (green) and corresponding patches of postsynaptic SK3-IR (red; rat α-MN, three optical sections at 1.0 µm Z-steps). Some synaptophysin-IR boutons (arrowheads) do not appear to have any associated SK3-IR. The SK3-IR appears to be highly associated with the largest presynaptic boutons on α-MNs. (A2) SK3-IR only. (A3) Synaptophysin-IR only. Scale bars represent 10 µm.
Figure 9. SK channels are localized to postsynaptic membrane sites at large C-bouton synapses on α-motoneurons. Ultrastructural analysis at synapses formed by presynaptic C-boutons (Pre-) shows SK3-IR labeling (arrowheads) localized to the membrane region of the postsynaptic α-MN (Post-). The synapses are characterized by the large size of the spherical synaptic vesicle-containing presynaptic boutons and by the presence of a postsynaptic subsurface cistern (arrows). (A) SK3-IR at a C-bouton contact on a proximal dendrite. In this example, the presence of cytoplasmic labeling immediately beneath the postsynaptic membrane may represent channel protein being trafficked to or from the membrane, as described for Kv2.1 channels (Muennich & Fyffe, 2004). (B) SK3-IR at a C-bouton contact on the MN soma. Scale bars represent 0.5 µm.
Figure 10. **SK channels are clustered postsynaptically at cholinergic C-bouton synapses.** Confocal images (stacks from 1 µm optical sections) of SK3-IR (red), NeuN-IR (blue) and vesicular acetylcholine transporter (VACHT-IR; green) on rat lumbar α-MNs. The areas outlined in **A1–3** (23 optical sections; scale bar represents 20 µm) are presented at higher magnification in **B1–3** (23 optical sections; scale bar represents 5 µm), respectively, to show close apposition, ‘edge on’, of large cholinergic (VACHT-IR) boutons and clusters of postsynaptic SK3-IR. **C1–3** (scale bar represents 10 µm), shown at higher magnification in **D1–3** (three optical sections; scale bar represents 2.5 µm) show ‘en face’ SK3 clusters, with one of them (boxed area in C1–3) being overlain by a cholinergic presynaptic bouton (C3 & D3).
Figure 11. SK channels do not colocalize with glutamatergic, serotonergic and inhibitory synapses. Images are representative high-power (×60 objective) rodent lumbar α-MNs. (A) Confocal image stack (21 optical sections at 1.0 µm Z-steps) shows no observable association with VGluT1-IR (green), which labels a specific population of glutamatergic synapses, and SK3-IR (red). Scale bar represents 20.0 µm. (B) Confocal image stack (32 optical sections at 1.0 µm Z-steps) shows no observable association with 5HT-IR (green), which labels serotonergic synapses, and SK3-IR (red). Scale bar represents 10.0 µm. (C) Confocal single optical section shows no observable association with VGluT2-IR (green), which labels a specific population of glutamatergic synapses, and SK3-IR (red). Scale bar represents 10.0 µm. (D) Single optical confocal section shows no observable association with gephyrin-IR (green), which labels GABAergic and glycinegic synapses, and SK3-IR (red). Scale bar represents 10.0 µm.
Figure 12. *SK channel clusters are colocalized postsynaptically with large Kv2.1-IR clusters.* Images are representative micrographs from small confocal microscopy stacks of SK3-IR (red) and Kv2.1 (green). (A) Small high-power confocal stack (×60 objective) of rat α-MN membrane, showing that SK3-IR is not present at all Kv2.1 clusters but is present at the largest Kv2.1 clusters. Confocal image stack is three optical sections (1.0 µm Z-step). Scale bar represents 10 µm. (B&C) Representative examples of *en face* SK3-IR and Kv2.1-IR clusters. Within the clusters, Kv2.1-IR tends to be more diffuse, while SK3-IR tends to be fenestrated. Confocal image stacks are two optical sections (1.0 µm Z-step). Scale bars represent 2 µm.
CHAPTER V: Swimming against the tide: Investigations of the C-bouton synapse


This chapter, published in Frontiers in Neural Circuits, addresses SA 3 of this dissertation.

Introduction
The neuromuscular system provides rapid and coordinated force generation, whereby the number and firing rate of recruited motor units are systematically adjusted to meet environmental demands (Monster & Chan, 1977; Henneman & Mendell, 1981; Clamann, 1993; Cope & Sokoloff, 1999). Indeed, the elegant simplicity with which animals navigate their environment relies on neural circuitry that is inherently modifiable, and the ability to perform a variety of motor tasks while responding quickly to unexpected perturbations and threats is essential for individual survival (Ladle et al., 2007; Miri et al., 2013). Control of α-MN repetitive firing properties is a therefore highly conserved and critical adaption of mammalian and non-mammalian species alike, and identifying the responsible spinal circuits has been of essential importance in our understanding of neuromuscular function and dysfunction (Miles & Sillar, 2011).
For more than 50 years, a particular class of synapse in the spinal cord ventral horn – the C-bouton – has generated sustained interest among α-MN anatomists and physiologists. Unambiguous identification of these conspicuously large cholinergic synaptic contacts and the characteristic postsynaptic subsurface cisternae (SSC) for which they are named has prompted numerous investigations into their distribution, source, function, and pathology. Yet despite the detailed morphologic and physiologic information generated by many neuroscientists, it is humbling to consider (a) the incrementally slow trajectory by which our understanding of this enigmatic synapse has grown and (b) that as yet there is no definitive and fully functional hypothesis regarding their distribution, their postsynaptic subcellular machinery, their contribution to motor control and behavior, and their regulation/dysregulation in health and disease.

Recently, we have learned the most elementary effect of C-boutons on α-MN f-I gain during static intracellular current injection occurs via dramatic reductions in the strength of the action potential AHP (Miles et al., 2007), which is mediated by postsynaptic small conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channels (Deardorff et al., 2013). However, the mystery of the C-bouton and its cholinergic effects on MN biophysical properties and integrative capabilities is by no means solved, as has been suggested (Frank, 2009). Using an isolated spinal cord preparation, Miles et al. (2007) demonstrate a putative role for C-boutons in ensuring appropriate levels of motor output during drug-induced fictive locomotion. But complexity arises upon behavioral assessment of adult mice with selective genetic inactivation of C-bouton synaptic inputs, which during locomotion exhibit normal flexor–extensor alternation and normal EMG amplitude. Motor deficits in
these mice primarily manifest during high-output tasks such as swimming (Zagoraiou et al., 2009). These data convincingly implicate C-boutons in the task-dependent regulation of α-MN excitability via reduction of outward K\(^+\) currents, but questions remain regarding (a) the functional impact of C-bouton input during different behaviors, (b) the manner in which C-bouton activity is modulated to match motor demands, (c) the expression of abnormal force generation as well as spasticity, rigidity, or tremor as a consequence of C-bouton dysfunction, and (d) the mechanism of interaction between underlying acetylcholine receptors (AChRs) and K\(^+\) channels.

To aid in the development of new in vivo and in vitro experimental strategies to answer these and related questions, this review details our current understanding of the cellular, synaptic, and genetic properties that underlie C-bouton function and proposes a hitherto unexplored mechanism for the cholinergic modification of α-MN excitability. It should be noted that the title of this review is intended to reflect and pay homage to the many dedicated and careful neuroscientists who have undertaken MN synaptological investigations over the years. This review will therefore also provide historical perspective on the foundational advances in our understanding of this complex and elusive, yet important, synapse. Neuroscientists have spent 50+ years at the C-bouton swimming against the tide. Significant progress has been slow and hard fought. And though we are a long way from shore, we must remember – as our murine colleagues have demonstrated – without C-boutons we cannot swim at all.
Results

*The C-bouton signaling ensemble: A contemporary view of a classic synapse.*

We are riding the crest of a wave. With the turn of the century and the application of advanced morphologic analyses, cellular neurophysiology, and selective genetic perturbations, we have built a decidedly robust picture of C-bouton form and function. C-boutons are an essential piece of an integrated control system set to regulate α-MN activity through a complex anatomical substrate: a signaling ensemble (Figures 13, 14, & 15) precisely organized for highly nuanced orchestration of somatic K+ currents.

**Precise anatomical localization and organization of signaling components: An ensemble of apposed proteins and mosaic membrane domains.**

C-type synaptic sites comprise three closely apposed membranous domains (Figure 15), spanning a breadth of <25 nm, and across which the distribution of synaptic and signaling proteins are precisely regulated. Clear and consistent immunohistochemical data demonstrate membrane clusters of α-MN Kv2.1 channels, SK2/3 channels, and m2 receptors directly apposing C-bouton presynaptic terminals (Figure 13) (Skinner *et al.*, 1999; Hellstrom *et al.*, 2003; Muennich & Fyffe, 2004; Wilson *et al.*, 2004; Deardorff *et al.*, 2013). When visualized under high resolution, these SK2/3 channel and m2 receptor clusters are composed of an intricate, non-uniform aggregation of smaller “threadlike” structures that are woven together and closely approximate/appose C-bouton pre-synaptic vesicle release sites, which are enriched with the presynaptic protein Bassoon (Figure 13 & 14). Beneath the postsynaptic membrane, in α-MN SSCs, the gap junction protein connexin32 shows a similar threadlike distribution pattern (Yamamoto *et al.*, 1990, 1991; Zampieri *et al.*, 2014) indicating that connexin32, SK channels/m2 receptors, and
transmitter release machinery are precisely aligned across the three membranous domains. 

$K_{\text{V}2.1}$ channels appear to “fill in” the remaining post-synaptic $\alpha$-MN membrane surface not occupied by SK channels or m2 receptors. The demarcated postsynaptic area, therefore, is a highly structured and mosaic domain of interdigitating clusters of $K_{\text{V}2.1}$ channels and co-localized SK2/3 channels and m2 receptors. The orderly, stacked apposition of proteins on the cisternal, postsynaptic, and presynaptic membranes as well as the spatial interdigitation of distinct channel and receptor clusters demonstrates a coordinated and specific signaling organization across all membranous domains at C-bouton synaptic sites.

**Additional signaling components.**

Additional studies have revealed, to varying levels of specificity, other signaling components that characterize the C-bouton ensemble. Certain elements, although identified within one or another membranous or cytoplasmic domain, are not well defined in regard to specific subdomain organization nor anatomic relation to other molecular components. In this category, C-bouton synaptic terminals express a range of exocytotic proteins consistent with those necessary for fast transmitter release (Hellstrom *et al.*, 1999), are highly associated with presynaptic P2X7 purinergic receptor immunoreactivity (~90% of C-boutons; Deng & Fyffe, 2004), and may also express presynaptic nicotinic acetylcholine receptors [nAChRs; (Khan *et al.*, 2003)]. In addition, the $\alpha$-MN SSC is highly enriched with S1Rs (Mavlyutov *et al.*, 2010), and with closely associated neuregulin-1 (NG1) immunoreactivity (Gallart-Palau *et al.*, 2014). Indole-N-methyl transferase (INMT), an enzyme that converts tryptamine into the S1R ligand
dimethytryptamine (DMT), is also present in close proximity to S1Rs at C-bouton postsynaptic sites (Mavlyutov et al., 2012), but the extent to which S1Rs, themselves, are diffusely distributed within the entire cisternal membrane or co-localize/interdigitate with the well-characterized connexin32 immunoreactivity is not described.

The subcellular organization of Ca\(^{2+}\) sources necessary for SK channel activation also remains poorly characterized. However, α-MN SK2/3 channels require high voltage activated (HVA) N- and P/Q-type Ca\(^{2+}\) currents to generate the mAHP (Viana et al., 1993; Umemiya & Berger, 1994; Bayliss et al., 1995; Li & Bennett, 2007), and SK channels typically couple to their Ca\(^{2+}\) source(s) by <200 nm (Fakler & Adelman, 2008; Jones & Stuart, 2013). Internally, SSCs may amplify or shape these Ca\(^{2+}\) signals via ryanodine receptors (RyRs) or connexin32, as they do in other cell types (see discussion Section “Subsurface Cisternae and the Generation of an Isolated Ca\(^{2+}\) Signal”). We, therefore, expect some proportion of HVA Ca\(^{2+}\) channels and ryanodine receptors (RyRs) to localize to the C-bouton postsynaptic membrane and/or to the associated SSC (Figure 15). In support, Wilson et al. (2004) provide evidence that P/Q-type Ca\(^{2+}\) channels are diffusely spread throughout the α-MN somatic membrane. By inference, some proportion must then appose C-boutons. The presence of N-type Ca\(^{2+}\) channels on α-MNs, however, has only been demonstrated physiologically (Carlin et al., 2000; Wilson et al., 2004).

**The crest of a wave.**

The unique aggregation of cytoplasmic and membrane bound pre- and postsynaptic proteins that constitute the C-bouton signaling ensemble provides mechanistic insight
into the cholinergic modulation of α-MN firing rate and has advanced new research at a comparatively faster pace than that of many other α-MN synaptic inputs. While uncertainties still confound our arrival at a “simple” molecular mechanism governing C-bouton synaptic function, experiments in other cell systems can help push us forward against the tide. Further exploration of this complex synapse is clearly necessitated. However, we must first review other salient features of the C-bouton system.

Discussion: Historical Review

**Moments and milestones: ultrastructure**

Pioneering EM investigations (Wyckoff & Young, 1956) provided accurate anatomical description and categorization of the structurally diverse presynaptic terminals contacting spinal α- MNs, and in general, most authors still conform to the descriptive abbreviations (S-, F-, C-, T-, and M-Boutons) introduced by Bodian (1966a, b) and Conradi (1969a). [An additional bouton type, the P bouton, makes presynaptic connections with specific excitatory boutons in contact with the MN surface and may form triadic arrangements; (Conradi, 1969a; Fyffe & Light, 1984)]. Those boutons Conradi classified as “C-type” are defined by and named for a signature 10–15 nm thick postsynaptic SSC (“C-type” for cistern): a broad, flat disc of smooth endoplasmic reticulum juxtaposed a mere 5–8 nm below the postsynaptic membrane and spanning the length of the apposing presynaptic terminal (Figure 16; Conradi, 1969a). The SSC is continuous with several lamellae of rough endoplasmic reticulum oriented in parallel with the cell membrane and frequently observed alongside free ribosomal rosettes in the subcisternal cytoplasm (Figure 16). Across a particularly narrow synaptic cleft (3–8 nm;
see discussion Davidoff & Irintchev, 1986), the C-boutons themselves contain a dense cytoplasmic matrix of glycogen particles and neurofilaments tightly packed with 25–55 nm (diameter) clear spherical/pleomorphic vesicles, abundant mitochondria, and occasionally a small number of large dense core vesicles intermingled therein (Figure 16; Bodian, 1966a, b; Conradi, 1969a; McLaughlin, 1972b; Hamos & King, 1980). Notably, several authors (Rosenbluth, 1962; Bodian, 1966a, b; Charlton & Gray, 1966; Van Harreveld & Khattab, 1967) identified these unique and prominent boutons prior to Conradi’s (1969a) classic and thorough description of their synaptic ultrastructure – which remains the gold standard for their identification.

C-boutons are among the largest of α-MN somatic and proximal dendritic synaptic inputs, ranging in size from 3–8 µm in the cat (Conradi, 1969a; McLaughlin, 1972b; Conradi et al., 1979a), 3–6 µm in the primate (Bodian, 1966a, b), 3–5 µm in the opossum (Hamos & King, 1980), 3–6 µm in the human (Pullen, 1992), and 1–8 µm in the rodent (Alvarez et al., 1999). But despite their conspicuous size, they lack quintessential active zone ultrastructure, i.e., pronounced paramembraneous densities and associated pools of readily releasable vesicles (Bodian, 1966a, b; Conradi, 1969a; McLaughlin, 1972b; Bernstein & Bernstein, 1976), prompting early speculation that vesicle release occurs across the entire synaptic interface (McLaughlin, 1972b). However, small presynaptic dense projections and local vesicle aggregations have been subsequently described (Hamos & King, 1980; Connaughton et al., 1986; Davidoff & Irintchev, 1986), and are particularly pronounced in non-osmicated tissue stained with ethanolic phosphotungstic acid [E-PTA; (Pullen, 1988a)] or uranyl acetate and lead citrate (Schroder, 1979). These
observations are commonly accepted evidence for specific synaptic vesicle release sites. Supporting this notion, C-boutons express discrete punctae of the active zone specific protein Bassoon rather than diffuse expression throughout the presynaptic membrane (Figure 13 & 14). Moreover, Bassoon immunoreactivity precisely overlies postsynaptic SK channels and m2 receptors even though traditional postsynaptic densities are not typically observed under EM. The physiologic advantage of this characteristically atypical and peculiarly subtle active zone architecture, however, is not yet fully understood, and may be further complicated by interspecies variability (see Pullen, 1988a).

C-boutons are ubiquitous and highly specific to somatic α- MNs and have been identified on α-MN somata and proximal dendrites in all mammalian species studied thus far (see Yamamoto et al., 1991 for references). Detailed analyses of γ-MNs (Lagerback, 1985; Lagerback et al., 1986; Destombes et al., 1992), autonomic MNs (Mawe et al., 1986; Leedy et al., 1988), spinal interneurons (Johnson & Sears, 1988), and Renshaw cells (Lagerback & Ronnevi, 1982; Alvarez & Fyffe, 2007) confirm these cells lack C-type synaptic inputs. C-boutons, when properly identified (see discussion Section “Moments and Milestones: Transmitter Content”), are thus a useful anatomical criterion to distinguish somatic α- MNs in the brain and spinal cord (Conradi, 1969a; Pullen, 1988b; Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Deardorff et al., 2013). Although there have been no extensive three-dimensional analyses of the total number of C-boutons per α-MN, our, and other, observations suggest on the order of 30–70 such contacts per cell (McLaughlin, 1972b; Hamos & King, 1980; Brannstrom, 1993; Brannstrom & Kellerth,
1998), and in general, there are a greater number of C-bouton synaptic contacts on large α-MNs innervating fast twitch muscle fibers, with this difference not simply due to the larger available somatic/dendritic surface area (Conradi et al., 1979a; Conradi et al., 1979b; Kellerth et al., 1979; Kellerth et al., 1983; Hellstrom et al., 2003). It should be noted, the features of C-boutons present on somatic α-MNs in ocular motor nuclei vary from those in the spinal cord and other brainstem motor nuclei. Specifically, C-boutons have been ultrastructurally identified (Tredici et al., 1976) and α-MN SSCs express connexin32 (Yamamoto et al., 1991), but no large vesicular acetylcholine transporter immunoreactivity (VAChT)-IR synaptic contacts (Hellstrom et al., 2003) nor m2 receptors are present (Vilaro et al., 1992; Hellstrom et al., 2003).

**Moments and milestones: Transmitter content.**

Correlative light-electron microscopic analysis of choline acetyltransferase immunoreactivity (ChAT)-IR confirmed C-boutons are cholinergic (Houser et al., 1983; Connaughton et al., 1986; Li et al., 1995), a suggestion first made decades prior with ultrastructural acetylcholinesterase (AChE) histochemistry (Lewis & Shute, 1966), which alone is not sufficient for cholinergic classification (Fibiger, 1982; Satoh et al., 1983; Sakamoto et al., 1985; Davidoff & Irintchev, 1986; Nagy et al., 1993). In support, VAChT is highly associated with small clear synaptic vesicles in the C-bouton presynaptic terminal (Gilmor et al., 1996) and there is a strong association throughout the brainstem and spinal cord between large ChAT-IR synaptic boutons on α-MNs and SSCs immunolabeled for connexin32 (Nagy et al., 1993). Immunoreactivity for the cholinergic markers ChAT or VAChT, combined with anatomical criteria such as bouton size and
location, therefore makes C-boutons easily identifiable in adult/neonatal histologic sections (Figure 16; (Barber et al., 1984; Phelps et al., 1984; Nagy et al., 1993; Hellstrom et al., 2003; Wilson et al., 2004; Zagoraiou et al., 2009; Alvarez et al., 2011; Deardorff et al., 2013). However, this approach should be applied with caution, as a small subset of cholinergic S-type terminals arising from recurrent α-MN axon collaterals and contacting α-MN somata may approximate C-boutons in size (Cullheim et al., 1977; Lagerback et al., 1981). Definitive confirmation of C-bouton phenotype requires ultrastructural verification of the C-bouton specific “cisternal signature” or alternatively – when systematically surveying an adequate sample of α-MNs under EM is unrealistic – light level co-localization of cholinergic makers with C-bouton specific pre- and/or postsynaptic proteins (see Section “The C-bouton Signaling Ensemble: A Contemporary View of a Classic Synapse”).

Moments and milestones: Dissecting the C-bouton circuitry.

Unlike so many α-MN synaptic inputs, for which the neurons of origin are identifiable anatomically and physiologically (Jankowska & Lindstrom, 1972; Jankowska & Roberts, 1972b, a; Brown et al., 1981; Brown, 1983; Fyffe, 1991b, a; Burke & Glenn, 1996; Bui et al., 2003), the neuronal source of C-boutons has been elusive. Early in vivo lesion studies demonstrated that C-boutons do not degenerate following dorsal root section (Conradi, 1969b; McLaughlin, 1972a; Bodian, 1975); spinal cord hemisection/transection (McLaughlin, 1972c; Bodian, 1975; Pullen & Sears, 1978, 1983), or cortical ablation (Bodian, 1975). Neither are they labeled by injection of retrograde tracers into dorsal roots (Ralston & Ralston, 1979), nor intracellular staining of Ia afferents (Brown & Fyffe,
1978c; Conradi et al., 1983; Fyffe & Light, 1984), Ib afferents (Brown & Fyffe, 1978b, 1979), group II afferents (Fyffe, 1979), hair follicle afferents (Maxwell et al., 1982), or axons innervating cutaneous mechanoreceptors (Brown et al., 1978; Brown et al., 1979; Brown et al., 1980; Brown et al., 1981; Bannatyne et al., 1984; Maxwell et al., 1984). Similarly, intracellular labeling of α-MNs showed C-boutons do not arise from α-MN axon collaterals (Lagerback et al., 1981), which is corroborated by differential protein expression in C-type synapses versus cholinergic terminals in the Renshaw cell area [see Section “The C-bouton Signaling Ensemble: A Contemporary View of a Classic Synapse;” (Hellstrom et al., 1999; Deng & Fyffe, 2004)].

Though these data collectively indicate the intraspinal derivation of C-boutons, no investigator to date has intracellularly labeled a cholinergic spinal interneuron and traced its axon to an α-MN C-type synaptic contact in vivo or in vitro; the definitive test for synaptic connectivity. Advanced molecular labeling techniques, however, have very convincingly demonstrated that C-boutons arise from cholinergic V0-embryonic (V0C) interneurons identifiable transcriptionally and phenotypically by the expression of the V0-specific homeobox protein Dbx1, the paired-like homeodomain transcription factor Pitx2, and the cholinergic proteins ChAT or VACHT (Miles et al., 2007; Zagoraiou et al., 2009). [For complete information on V0 cell ontogeny, I refer the reader to studies by Moran-Rivard et al. (2001), Pierani et al. (2001), and Lanuza et al. (2004) as well as the review by Arber (2012)]. V0C interneurons correspond to a known population of cholinergic partition cells (Barber et al., 1984; Phelps et al., 1984; Arvidsson et al., 1997) located lateral to the central canal in Rexed’s lamina X and medial lamina VII (Miles et
al., 2007; Zagoraiou et al., 2009). They can be subdivided into ipsilateral and bilateral projecting subpopulations and span several segments rostral and caudal to their innervated motor pools (Stepien et al., 2010). Cholinergic partition cells, C-type synaptic boutons, and the “signaling ensemble” appear early in postnatal development, and are well established by approximately 1 month of age (Phelps et al., 1984; Werts & Vaughn, 2001; Wilson et al., 2004).

The specific placement of V0C interneurons within segmental spinal circuitry is not fully characterized [see preliminary circuit diagrams in Zagoraiou et al. (2009) and Witts et al. (2014)]. Preliminary analysis of V0C connectivity demonstrates V0C interneurons receive synaptic input from several sources, including descending serotonergic pathways, local and/or descending VGlut2 projections, inhibitory interneurons (e.g., V2b cells), lamina II/III nociceptive interneurons, and non-proprioceptive primary mechanosensors (Zagoraiou et al., 2009; Witts et al., 2014; Zampieri et al., 2014; Zhang et al., 2014). Each V0C cell sends divergent axonal projections to several α-MNs of the same or functionally equivalent motor pools and avoids α-MNs innervating antagonist muscles (Stepien et al., 2010). Numerous en passant synaptic varicosities arising from a single V0C axon contact the soma and proximal dendrites of one or more α-MNs, which in turn receive convergent input from several V0C cells (Stepien et al., 2010). Although the precise levels of convergence/divergence are unknown, this pattern of connectivity establishes a large number of release sites from each presynaptic axon onto the α-MN, likely reflecting a high probability of transmitter release and contributing to a high safety factor for strong cholinergic neuromodulation [e.g., Walmsley et al. (1998)].
Recent work shows $\text{V0}_C$ interneurons also project numerous small synaptic contacts onto V1-derived IaINs (Siembab et al., 2010). These synapses are morphologically dissimilar to C-boutons (Siembab et al., 2010), and their postsynaptic effects are currently unknown. Still, it is intriguing to consider that $\text{V0}_C$ interneurons project to the only two neuronal types ($\alpha$-MNs and IaINs) in the ventral horn known to receive both recurrent inhibition and group Ia excitatory drive. Whether $\text{V0}_C$ interneurons, like Renshaw cells, send parallel projections to $\alpha$-MNs and their “corresponding” IaINs [i.e., those with the same Ia connections (Hultborn et al., 1971a, b; Hultborn et al., 1971d)] has yet to be elucidated. Nevertheless, these data provide further insight into segmental motor circuitry and prompt new questions into both circuit function and synaptic specificity of the $\text{V0}_C$ neuronal class.

**Moments and milestones: AHP SK, and motor unit type.**

Early in vivo use of the SK channel blocker, apamin, established that SK channels are uniquely responsible for generating $\alpha$-MN mAHP currents (Zhang & Krnjevic, 1987). In vitro investigation subsequently confirmed these findings (Viana et al., 1993; Lape & Nistri, 2000), and showed that $\alpha$-MN SK currents are reduced following m2 receptor activation at C-bouton synaptic sites (Lape & Nistri, 2000; Miles et al., 2007). Consistent with these electrophysiological data, our lab has recently shown that not only are SK channels highly enriched in the C-bouton postsynaptic membrane (Deardorff et al., 2013), but individual $\alpha$-MNs express a variable complement of SK2 and SK3 channel isoforms consistent with observed co-variability in $\alpha$-MN size and mAHP duration (Deardorff et al., 2013). In the rodent, all $\alpha$-MNs express SK2, but SK3 expression is markedly
heterogeneous and cell-type-specific (Figure 17) varying in intensity from negligible (<2× background) to modest (2 to 3× background) to strong (>3× background) between individual α-MNs in a single tissue section. SK3 channels, which have a longer deactivation time constant than SK2 (Xia et al., 1998), are only expressed (with SK2) at C-bouton postsynaptic sites in smaller α-MNs with longer duration/larger amplitude AHPs (Figure 18). Conversely, larger α-MNs with significantly shorter duration/smaller amplitude AHPs express only SK2 (with little or no SK3-IR; Figure 18).

SK3-expressing α-MNs share other physiological properties predictive of S-type MNs (i.e., slower conduction velocity, lower rheobase, and higher input resistance; Deardorff et al., 2013). SK3-IR within the signaling ensemble can therefore provide “brush stroke” differentiation of rodent α-MNs along their physiological spectrum, and is a useful tool for histologic analysis of α-MN subtypes in development and disease (Brownstone & Magown, 2013). Altogether these data strongly indicate that the relative proportion of SK2/SK3 isoforms and the channel cluster size and density regulates mAHP duration and amplitude, and the variability of these proportions accounts, in part, for the fact that mAHP properties are continuous variables across a population of α-MNs (Deardorff et al., 2013). SK channel expression may, therefore, explain the “speed match” between AHP duration of a given α-MN and the contractile speed of its innervated muscle fibers (Bakels & Kernell, 1993a, b; Gardiner, 1993). However, critical additional factors include the source and amplitude of the necessary Ca\(^{2+}\) signal, the coupling of these signals to the SK channels and, potentially, the presence/absence of hyperpolarization activated cation (Ih) currents (Gustafsson & Pinter, 1985). Nevertheless, differential SK
channel expression at the C-bouton undoubtedly contributes to α-MN input–output gain across the spectrum of α-MN subtypes by regulating mAHP properties.

**Discussion: Theoretical Framework**

*Swimming forward: A mechanism for cholinergic modulation.*

We return now to the crest of our wave. The constancy of form and the intricacy of protein expression imply a fundamental logic to C-bouton organization and engagement during motor activity. Here, we assert the signaling ensemble is built around an organizing principle (i.e., the SSC) that allows for the generation of isolated Ca$^{2+}$ signals at multiple sites on the soma. From this starting point, our intent here is to swim forward toward the synthesis of a comprehensive mechanistic hypothesis for the cholinergic modulation of α-MN firing rate. We base our rationale in the now recognized functional requirement for C-boutons in “swimming” (Zagoraiou *et al.*, 2009), in the observation that cholinergic C-bouton function is not required for regular locomotion (Zagoraiou *et al.*, 2009), and in the probable interactions of the key components of the C-bouton signaling ensemble (*Figures 13, 14 & 15*), most of which are known to generate, regulate, or be regulated by local intracellular Ca$^{2+}$.

Although C-boutons may boost recruitment gain, as proposed elsewhere (Zagoraiou *et al.*, 2009; Brownstone & Magown, 2013), we propose that the cholinergic modulation produced by C-boutons is highly task-dependent and will be maximal only during the moderate to strong physiological drive necessary for high-output motor tasks like swimming (Zagoraiou *et al.*, 2009; *Figure 19*). The mechanism we suggest accounts for
the minimal appreciable requirements and effects observed during conditions of low and/or transient drive, which are appropriate for spinal reflexes and/or low-output tasks such as walking (Zagoraiou et al., 2009; Figures 19Ai,Bi). We extend this notion further to conditions of extremely powerful physiological (or pathological) drive, during which time any effects of C-bouton activity on firing rate are negated by the molecular dynamics and kinetics of the respective m2 receptors and SK/Kv channels (Figures 19Aiv,Biv). That is, while the cumulative, combined effects of these isolated Ca$^{2+}$ signals on specific mAHP and delayed rectifier K$^+$ currents are likely to be quite significant throughout the α-MN activity spectrum, the functional impact of the C-bouton circuitry is only observed when imposed upon a restricted window of moderate to strong excitatory drive. We believe our synthesis, which is primarily based on interpretation of disparate datasets, will promote testable hypotheses. Elements of this synthetic approach are considered in the following sections.

Subsurface cisternae and the generation of an isolated Ca$^{2+}$ signal.

It is widely accepted that neuronal SSCs function as an intracellular Ca$^{2+}$ store with multiple roles in Ca$^{2+}$ homeostasis and mobilization (see Yamamoto et al., 1991 and Fuchs et al., 2014 for references). Indeed, Henkart et al. (1976) proposed that SSCs “are designed to release Ca$^{2+}$ into the cytoplasm with whatever further effects this might produce.” SSCs serve also as a physical diffusion barrier that spatially and functionally restricts this Ca$^{2+}$ signal from those originating in other cellular compartments and, during increased cellular activity, act as a Ca$^{2+}$ sink to rapidly absorb and shuttle free Ca$^{2+}$ from the cisternal microdomain (Yamamoto et al., 1990, 1991; Fuchs et al., 2014).
Ca\textsuperscript{2+} release by RyR-rich SSCs serve, in part, to activate nearby SK channels in cochlear hair cells, which share some synaptic similarities with C-boutons (Evans et al., 2000; Lioudyno et al., 2004; Grant et al., 2006), and in sympathetic ganglion cells (Akita & Kuba, 2000). RyR release of Ca\textsuperscript{2+} may also result in an increase in nearby \(K_V\textsubscript{2.1}\) channel conductances, via Ca\textsuperscript{2+}-dependent dephosphorylation pathways, as it does in hippocampal and cortical pyramidal cells (Du et al., 1998; Antonucci et al., 2001; Misonou et al., 2005). Moreover, vesicles observed budding from the cytoplasmic surface of SSCs in cochlear hair cells and \(\alpha\)-MNs are thought to be involved in removal of excess free Ca\textsuperscript{2+} from the subsynaptic cytoplasm (Yamamoto et al., 1991; Fuchs et al., 2014). In light of these factors, the SSC itself is highly indicative that the functional regulation of the C-bouton signaling ensemble (which includes SK and \(K_V\textsubscript{2.1}\) channels) occurs through precise control of an isolated Ca\textsuperscript{2+} microdomain, the mechanistic underpinnings of which are considered below.

**Involvement of the signaling ensemble with the isolated Ca\textsuperscript{2+} signal.**

The unique aggregation of cellular elements at C-bouton synaptic sites and their coordinated regulation by and/or of the isolated Ca\textsuperscript{2+} signal enables exquisite control over \(\alpha\)-MN K\textsuperscript{+} currents. Consider first the generation of the \(\alpha\)-MN AHP. Membrane bound N- and P/Q-type Ca\textsuperscript{2+} currents necessary for \(\alpha\)-MN SK channel activation (see “Additional Signaling Components”) generate this Ca\textsuperscript{2+} signal, which is isolated and shaped by the SSC. The AHP currents influence repetitive discharge properties of \(\alpha\)-MNs, in part, via reductions in the variability in the interspike interval, the slope of the \(f-I\) relation, and the maximal rate of primary-range firing (Kernell, 2006; Brownstone & Magown, 2013).
A primary effect of m2 receptor activation by C-bouton synapses is a reduction of the AHP (Lape & Nistri, 2000; Miles et al., 2007). Though their signaling pathway(s) in α-MNs are undefined, m2 receptors typically exert their effects by inhibiting N-type Ca\(^{2+}\) channels, as observed in sympathetic ganglion (Hille, 1994; Herlitze et al., 1996; Shapiro et al., 1999), cortical pyramidal (Stewart et al., 1999), neostriatal (Howe & Surmeier, 1995), and basal forebrain neurons (Allen & Brown, 1993). Ca\(^{2+}\) influx through these channels is required for activation of SK channels and dictates the number of SK channels that open. N-type channel blockade by m2 receptors is usually mediated by Gi/o protein coupled βγ subunits, which cause a depolarizing shift in the voltage dependence of channel activation (Hille, 1994; Herlitze et al., 1996; Ikeda, 1996; Jeong & Ikeda, 1999; Shapiro et al., 1999) and is negated by strong or repeated membrane depolarization (Hille, 1994).

The m2/cholinergic effect exerted by active C-boutons is quite simple and intuitive at this level: preventing N-type Ca\(^{2+}\) influx (which is largely triggered by synaptically evoked action potentials) from activating SK channels during moderate to strong physiologic drive of the MNs (Figures 19Aii,iii,Bii,iii). This would be consistent with observed reduction of the AHP and enhanced α-MN excitability when m2 receptors are, presumably, activated during swimming or other tasks requiring high motor output (e.g., Figures 19Aii,Aiii; Miles et al., 2007; Zagoraïou et al., 2009). This “upstream” mechanism of AHP modulation will have a minimal appreciable effect on individual AHPs and α-MN firing rate during low levels of physiologic drive causing transient or
“subprimary” range firing (Manuel et al., 2009; Turkin et al., 2010), due to the physiological triggering of SK channel activation by a short duration, suprathreshold stimulus (i.e., an action potential) occurring at intervals that may be longer than the duration of the mAHP itself (Figures 19Ai,Bi). This may account for observations that C-bouton function is not required for regular locomotion (Zagoraiou et al., 2009). Moreover during powerful and/or pathologic excitatory drive the m2-mediated diminution of N-type channel activity is negated (Hille, 1994), resulting in a break of the m2 generated effect and an increase in AHP size (Figures 19Aiv,Biv).

At high levels of excitatory drive we must also consider the results of modulation of other components of the signaling ensemble. Although the m2 mediated effect on mAHP is significant in a particular physiological range, the whole microdomain has an important role in setting α-MN firing rate. With this in mind, the Ca\(^{2+}\)-dependent generation of the AHP and its regulation by m2 receptor activation is one part of a coordinated series of molecular events that occur at the C-bouton, but is reliant on the complex interplay of other components in the signaling ensemble. For example, as excitatory drive increases how does the combinatorial contribution of SK and/or Kv2.1 change in the presence or absence of cholinergic input?

In the highly clustered configuration (typically) observed in hippocampal and cortical pyramidal cells, and α-MNs, K\(_v\)2.1 channels are phosphorylated and have a high activation and deactivation threshold and slow kinetics (Murakoshi et al., 1997; Misonou et al., 2004; Surmeier & Foehring, 2004; Misonou et al., 2005; Mohapatra & Trimmer,
Interestingly, some investigators have postulated that clustered Kv2.1 channels serve primarily non-conducting functions (O'Connell et al., 2010; Fox et al., 2013); for the purposes of this discussion we will consider a more traditional role for the channels in α-MNs. Importantly, upon prolonged/pathologic excitatory drive, Ca\(^{2+}\)/calcineurin dependent dephosphorylation pathways (Figures 19Aiv, Biv) rapidly decluster Kv2.1 while simultaneously lowering its activation and deactivation threshold and accelerating its kinetics (Surmeier & Foehring, 2004; Park et al., 2006; Mohapatra et al., 2009). In α-MNs, prolonged excitatory drive causes rapid Kv2.1 channel declustering (Romer et al., 2014) by a Ca\(^{2+}\)/calcineurin dependent mechanism (S. H. Romer, A. S. Deardorff, R. E. W. Fyffe, unpublished), though corresponding alterations in channel kinetics are uncharacterized.

Data from other cell types shows clustered Kv2 channels maintain steady state firing by regulating membrane potential during the interspike interval (Johnston et al., 2008; Guan et al., 2013; Liu & Bean, 2014), while declustered/dephosphorylated Kv2 channels serve to homeostatically lower firing rate (Surmeier & Foehring, 2004; Park et al., 2006; Mohapatra et al., 2009). In this way, Kv2 channels may increase or decrease cell excitability depending on the kinetics of channel activation (Liu & Bean, 2014). Brownstone et al. (2011) propose C-bouton activity during fictive locomotion (Miles et al., 2007; Zagoraiou et al., 2009) may contribute to steady state firing rates via the regulation of Kv2.1 phosphorylation and clustering. This is consistent with our hypothesis that m2-mediated inhibition of HVA Ca\(^{2+}\) current prevents the activation of Ca\(^{2+}\)/calcineurin dependent dephosphorylation pathways and thus maintains Kv2.1
clustering. However, if prolonged/pathologic excitatory drive causes large changes in intracellular Ca\textsuperscript{2+} sufficient to allow diffusion of Ca\textsuperscript{2+} from neighboring compartments, there would be rapid Kv2.1 channel declustering (Romer et al., 2014) by a Ca\textsuperscript{2+}/calcineurin dependent mechanism, negating the influence of C-boutons.

Several other components of this complex signaling ensemble likely serve to fine tune the efficacy of neuromodulation. Presynaptic nAChRs and P2X7 receptors may provide an additional regulatory mechanism for synaptic transmission, particularly if ATP is co-released with ACh as it is at other central and peripheral cholinergic synapses (Burnstock et al., 1997), and cisternal S1Rs are known to reduce the sensitivity of m2 receptors to ACh (Walker & Bourguignon, 1990; Kim et al., 2010). Altogether, we suggest the C-bouton signaling ensemble is a highly integrated system, organized around an anatomically segregated Ca\textsuperscript{2+} microdomain, for precise and nuanced regulation of cell firing. Moreover, it has a built-in fail-safe mechanism against excitotoxicity, in that this strategically organized ensemble can both be driven by, or override, the synaptic circuitry of the C-bouton.

An alternative mechanism.

Others have suggested, based on muscarine’s minimal effect on global α-MN Ca\textsuperscript{2+} currents, that m2 receptor activation results in the direct blockade of α-MN SK channels (Miles et al., 2007; Witts et al., 2014). In support of their view, the direct phosphorylation of SK channels by protein kinase A (PKA) and casein kinase 2 (CK2) can, respectively, cause channel internalization (Kohler et al., 1996; Ren et al., 2006; Fakler & Adelman, 2008; Faber, 2009) and reduced Ca\textsuperscript{2+} sensitivity (Bildl et al., 2004;
Allen et al., 2007). Moreover, neurotransmitter-initiated signaling cascades have been shown to modulate SK channel gating through CK2- or protein kinase C (PKC)-mediated phosphorylation (Maingret et al., 2008; Buchanan et al., 2010; Giessel & Sabatini, 2010). Although m2 receptors typically inhibit protein kinase activity, they can activate phosphorylation pathways in smooth muscle (Zhou et al., 2003b). Therefore it is possible the direct phosphorylation of SK channels by protein kinases could provide an alternate mechanism through which m2 receptors reduce the mAHP in α-MNs.

However, evidence that N- and P/Q-type Ca\textsuperscript{2+} channels are diffusely distributed throughout the α-MN somatic membrane (Wilson et al., 2004), and that α-MN SSCs function as Ca\textsuperscript{2+} diffusion barriers indicates that m2 receptor activation need only inhibit those α-MN Ca\textsubscript{V} channels located within or very near to the C-bouton postsynaptic membrane to exert an effect on the mAHP. In this case, m2 influence over the signaling ensemble would be masked in studies of global Ca\textsuperscript{2+} currents. The activation of CK2- or PKC-mediated phosphorylation would also be a novel finding for neuronal m2 receptors, necessitating future studies characterizing this undescribed signaling pathway. Moreover, such a mechanism would act as a binary switch, turning on and off mAHP when necessary and not requiring an elaborate signaling ensemble nor the SSC. Our hypothesis, however, of a signaling ensemble organized around fine control of a Ca\textsuperscript{2+} microsignaling domain is capable of highly nuanced and graded modulation of outward K\textsuperscript{+} current.
Discussion: C-boutons in human health and disease.

Dynamic reorganization of C-boutons and components of the postsynaptic signaling ensemble has been noted in a variety of pathologic conditions and in conditions of altered excitability (Saxena et al., 2013; Romer et al., 2014; Witts et al., 2014). The bulk of the data has thus far been obtained in animal models, and there is no consensus on whether C-bouton plasticity in these conditions is compensatory or pathologic. In part, the uncertainty results from the diversity of disease/injury models that affect C-boutons and the complexity of the signaling ensemble.

Analysis of effects on C-bouton structure in models of amyotrophic lateral sclerosis (ALS), spinal cord injury, and peripheral nerve injury demonstrate diverse and sometimes conflicting reports. In ALS, there has been interest in potential neuroprotective roles for C-boutons and this view is bolstered by studies that show an early increase in C-bouton size (Pullen & Athanasiou, 2009; Herron & Miles, 2012; Saxena et al., 2013); however, diminished C-bouton and V0C interneuronal ChAT/VACHT content (Nagao et al., 1998; Casas et al., 2013) and S1R expression (Casas et al., 2013; see Witts et al., 2014) have also been observed in similar murine models of the disease. The structural changes in animal models may also reflect a propensity for C-bouton reorganization to occur first in larger, less excitable, and more vulnerable α-MNs (Saxena et al., 2013), and the changes may be more pronounced in males (Herron & Miles, 2012). There is minimal data from autopsied human spinal cord from ALS patients, mostly from late stages of the disease, showing continued presence of C-boutons on degeneration-resistant sphincteric α-MNs (Pullen, 1992). Additionally, the duration of the mAHP in human MNs is possibly related
to disease progression (i.e., an initial shortening followed by prolongation; (Piotrkiewicz et al., 2007; Piotrkiewicz & Hausmanowa-Petrusewicz, 2011).

C-bouton organization is affected by both spinal cord and peripheral nerve injury, which generally appear to cause transient or persistent loss of and/or disconnection of C-boutons from α-MNs and changes in expression and localization of SK, HCN, and Kv2.1 channels (Kerns & Hinsman, 1973; Sumner, 1975; Alvarez et al., 2011; Romer et al., 2012; Romer et al., 2014). These specific changes may account for some, but not all, of the physiological changes that have been observed (Kuno et al., 1974a, b; Cope et al., 1986; Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a; Prather et al., 2011), including altered post-spike mAHP duration and repetitive firing properties (Kuno et al., 1974a; Gustafsson & Pinter, 1984).

The significance of C-bouton plasticity remains uncertain. After injury, the specific loss or disconnection could lead to postsynaptic receptors (m2) becoming constitutively active, analogous to observations made of the serotoninergic system (Fouad et al., 2010; Kong et al., 2010; Murray et al., 2010; Kong et al., 2011; Murray et al., 2011; Hultborn et al., 2013), but this has not been explored. Given the high vulnerability of large, F-type α-MNS in ALS, it would be interesting to determine if the graded expression of SK channel isoforms will promote new testable hypotheses regarding disease pathogenesis and C-bouton mediated compensatory adjustments (Brownstone & Magown, 2013; Deardorff et al., 2013).
Conclusion

Multiple neuromodulatory systems and a myriad of ion channels are available for the task dependent regulation of MN excitability. The serotonergic system, for example, originates in the brainstem raphe nucleus, provides extensive synaptic input onto α-MN dendrites (Alvarez et al., 1998) and is strongly linked to both behavioral and pathologic alterations of persistent inward Ca\(^{2+}\) currents (Li & Bennett, 2003; Heckmann et al., 2005; Brownstone, 2006; Li & Bennett, 2007; Heckman et al., 2008; Norton et al., 2008; Powers et al., 2008). While numerous studies have focused on inward current modulation, the state dependent regulation of α-MN outward current has only recently been investigated [see Manuel et al. (2012)]. New evidence has shown that a cholinergic modulatory system originating from spinal interneurons (V\(0_\text{C}\) interneurons), and contributing dense synaptic coverage to α-MN somata, modulates the strength of motor output via reductions in α-MN outward K\(^+\) current (Miles et al., 2007; Zagoraiou et al., 2009). It is interesting to consider that while serotonin increases MN excitability by amplifying inward current, acetylcholine does so by reducing outward current. The dynamic interplay of these two different, but rather synergistic, systems endows the CNS with remarkable control over MN output, and the interaction between the AHP and L-type Ca\(^{2+}\) currents responsible for PIC may be a critical factor in regulating α-MN firing properties (Manuel et al., 2014).

This chapter illustrates large, cholinergic presynaptic terminals, termed C-boutons (Conradi, 1969a), are important modulatory loci for state-dependent alterations in α-MN repetitive firing properties, largely mediating their effects through a unique and highly
specialized signaling ensemble organized for the state-dependent regulation of outward
K⁺ currents. To effectively manipulate signal transduction at C-bouton synaptic sites may
be critical in the development of new therapeutic interventions for a variety of
devastating neurological conditions. However, advances in patient care will first require a
complete understanding of both the transduction mechanisms, as well as which cases (if
any) C-bouton synaptic reorganization and/or alterations in α-MN mAHP (and other
intrinsic α-MN properties) contribute to disease pathology or, alternatively, maintain α-
MN viability.
Figure 13. C-bouton synaptic sites contain a complex signaling ensemble. Presynaptic Bassoon-IR and postsynaptic SK3-IR and m2-IR share a striking subsynaptic fenestrated appearance within the C-bouton. All images are small confocal stacks (3 × 1 µm Z-stacks) of en face C-boutons, indicated with VACHT-IR (blue), on rat lumbar α-motoneurons. (Top) Presynaptic active zone protein Bassoon (green) is aligned with postsynaptic ion channels SK3 and m2 receptors. (Bottom) Kv2.1-IR intercalates with SK3-IR and m2-IR, “filling in” the C-bouton postsynaptic membrane. Scale bars are 2.0 µm.
Figure 14. *Postsynaptic SK channels align with presynaptic vesicle release sites.*

**Top** and **Bottom** show *en face* view of two C-bouton synaptic sites. SK channel immunoreactivity (red) in the α-MN membrane demarcates the border of the C-bouton synapse. Vesicle release machinery in the C-bouton presynaptic terminals are enriched with the active zone scaffolding protein Bassoon (green), which precisely aligns with postsynaptic SK channels (yellow). Additional Bassoon immunoreactivity (green) is seen in other, unidentified presynaptic terminals contacting the α-MN.
Figure 15. *Synaptic distribution of specific ion channels and receptors on soma and proximal dendrites of motoneurons.* The schematic illustrates three types of motoneuron presynaptic boutons including the glycinergic/GABAergic F-type, glutamatergic S-type and cholinergic C-type with its associated postsynaptic subsurface cistern. Note the specific localization of m2 muscarinic receptors (blue) with SK channels (red) and \( K_v 2.1 \) channels (green) postsynaptic to the C-bouton. Small \( K_v 2.1 \) clusters are also found postsynaptic to some S-type synapses (see Muennich and Fyffe, 2004). The P/Q- and N-type \( Ca^{2+} \) channels \( Cav2.1/2.2 \) (light gray) are illustrated throughout the membrane, although the precise subcellular localization of this channel is currently unknown. Both connexin 32 (pink) and the sigma-1 receptor (dark gray) are specifically associated with the C-bouton subsurface cistern.
Figure 16. *The C-bouton synapse on mammalian α-motoneurons.* (A) C-bouton synapses on intracellularly labeled and reconstructed adult rat lumbar α-MN are revealed by VAChT-IR (white). Large C-boutons densely innervate the soma and proximal dendrites of α-MNs but are absent from more distal locations. Also note that C-boutons are not located on motoneuron axons (indicated by “a”). (B) C-boutons, indicated by VAChT-IR (Bi,iv, white), are presynaptic to the muscarinic m2 receptor (Bii,iv, red) and large Kv2.1 clusters (Biii,iv, green). Note that m2 receptor immunoreactivity on the α-MN soma and proximal dendrites localize exclusively to C-bouton postsynaptic sites. (Bii) Inset shows subsynaptic fenestrated distribution of m2-IR. Images are confocal stacks of 12 × 1 µm Z-stacks with nissl stain (blue) to label adult rat neuronal somata. Scale bar is 20 µm. (C) Diagrammatic representation and electron micrograph of C-bouton ultrastructure in an adult rat. (Ci) Diagram illustrates densely packed, clear spherical or pleomorphic vesicles and abundant mitochondria. Closely apposed to the postsynaptic membrane is a 10–15 nm wide subsurface cistern (SSC) that is continuous with several lamellae of underlying rough endoplasmic reticulum (rER). Free ribosomal rosettes are typically visible in the subsynaptic region. (Cii) Electron micrograph of C-bouton synapse on an α-MN soma. Arrowheads indicate a SSC extending the entire appositional length of the bouton. Note key features present in electron micrograph illustrated in diagram (Ci).
Figure 17. The potassium ion channel SK3 is part of the C-bouton signaling ensemble in a subset of α-motoneurons. Images are confocal stacks of 26×1 μm Z-stacks with nissl stain (blue) to label rat lumbar neuronal somata. Scale bar is 20 μm. (A) VACHT-IR (white) C-boutons form synapses onto all rat lumbar α-MNs on the soma and proximal dendrites. (B) SK3-IR (red) located within surface membrane of a subset of α-MNs in large distinct clusters. In rodents, SK3 channels, having slower intrinsic activation and deactivation kinetics than SK2 channels (Xia et al., 1998), are preferentially expressed in small, presumably S-type, α-MNs with long duration and large amplitude mAHP currents (Deardorff et al., 2013). (C) Large and small Kᵥ2.1-IR (green) clusters are located within the surface membrane of all α-MNs. (D&E) The large SK3-IR and Kᵥ2.1-IR clusters colocalize within the surface membrane of α-MNs and are apposed to VACHT-IR C-boutons.
Figure 18. *Subset of rat lumbar α-motoneurons with SK3-IR have significantly longer AHP \( \frac{1}{2} \) decay time and increased amplitude.* (A) Diagrammatic representation of experimental paradigms. In an adult in vivo rat preparation, tibial α-MNs, identified by antidromic activation of the tibial nerve, were penetrated with a sharp recording electrode. Neuronal electrical properties were recorded and neurons were filled with neurobiotin (green) for post hoc identification. Spinal cord tissue was harvested and processed for SK3-IR. (B–D) Neuronal electrical properties are of α-MNs depicted in micrographs below. Asterisk (*) denotes stimulus artifact. Micrographs are single optical confocal sections through the soma of intracellularly labeled α-MNs (green) processed for SK3-IR (red) and the general neuronal stain nissl (Blue). Scale bars are 20 µm. (B) SK3-IR (+) (Bii&Biii arrowheads) α-MNs have long duration and large amplitude AHP, low rheobase, and high input resistance. Micrograph insets show VACH-T-IR (White) C-bouton in apposition to an SK3-IR (+) cluster. Inset scale bar is 5 µm. (C&D) SK3-IR (−) α-MNs have short duration and small amplitude AHPs. However, even among these SK3-IR (−) cells, rheobase and input resistance show high variance along the continuum of α-MN properties. Please note the nearby SK3-IR (+) cells (Cii,Ciii&Dii,iii arrowheads).
Figure 19. Hypothesis for state dependent regulation of motoneuron activity through the C-Bouton signaling ensemble. (A) C-boutons increase motoneuron firing frequency along a widow of the α-MN activity spectrum. (Ai) With low or transient physiological drive, m2 activation is not likely to mediate an effect on AHP duration or firing rate. (Aii,iii) As excitatory drive increases, persistent m2 receptor activation inhibits local CaV channels through a Gi/Go coupled pathway, preventing both the SK channel activation and Kv2.1 dephosphorylation. Thus, outward K+ current is reduced and neuronal firing rate is increased (relative to Bii & Biii) as illustrated with spike train below. (Aiv) m2-mediated effects on CaV channels are negated by prolonged or repeated membrane depolarization (Hille, 1994) as may occur during extremely strong or pathologic excitatory drive. Here, Ca2+ influx through N-type calcium channels activates SK channels to generate AHP and to dephosphorylate Kv2.1 to increase outward K+ current and reduce firing frequency, as illustrated with spike train below. (Bi–iii) As excitatory drive increases without C-bouton activity, the N-type Ca2+ influx activates SK channels to generate AHP. Thus, the outward K+ current maintains a lower firing frequency than in corresponding images in A. Spike trains illustrated below. (Biv) As in (Aiv), during prolonged or pathologic excitatory drive, N-type Ca2+ influx results in both SK channel activation and Kv2.1 dephosphorylation, thereby increasing outward K+ current and homeostatically decreasing firing rate, illustrated with spike train below. All spike trains depicted in this figure are added for illustrative purposes only and do not represent electrophysiological recordings or computer simulations.
CHAPTER VI: Segmental synaptic excitation of antagonist α-motoneurons

This chapter, which is being prepared for publication, addresses SA 4 of this dissertation.

Introduction

Proprioceptive feedback is critical to the regulation of α-MN firing properties (Prather et al., 2001; Prather et al., 2002; Powers et al., 2008, 2012). Inhibitory and excitatory synaptic potentials from muscle proprioceptors provide motor pool specific modulation of α-MN firing in accordance with the biomechanical requirements of the body and limbs (Nichols et al., 1999). Group I and group II muscle afferents transmit encoded information on muscle length and force to α-MNs and other somatosensory centers in the central nervous system through a variety of pathways. One important pathway for the proprioceptive regulation of α-MN firing properties is the Ia reciprocal inhibitory circuit.

In the ‘classic’ disynaptic reciprocal inhibitory pathway, Ia afferents activated by muscle stretch (e.g. an ankle flexor) inhibit contraction of the antagonist (e.g. ankle extensor) muscle through an interposed Ia inhibitory interneuron (IaIN) (Hultborn et al., 1971b; Jankowska & Roberts, 1972b). α-MNs and their ‘corresponding’ IaINs receive parallel Ia afferent input from the same (homonymous) muscles (e.g. ankle flexors). IaINs, in turn,
project glycineergic presynaptic terminals (Bradley et al., 1953; Bradley & Eccles, 1953) onto the ‘opposite’ α-MNs and IaINs, which innervate and/or receive parallel Ia afferent input from the antagonist muscle (e.g. an ankle extensor) (Hultborn et al., 1971b; Jankowska & Roberts, 1972b).

Recent evidence has indicated that persistent inward Ca\(^{2+}\) currents in extensor α-MNs are particularly sensitive to reciprocal inhibition activated by flexor muscle stretch (Johnson & Heckman, 2010; Johnson et al., 2012; Johnson & Heckman, 2014). This has led to speculation that reciprocal inhibitory pathways provide tightly focused inhibition between antagonist muscles and ‘sculpt’ proper α-MN output from a background of diffuse background of excitatory neuromodulatory drive (Johnson & Heckman, 2010; Johnson et al., 2012; Johnson & Heckman, 2014). This ‘push-pull’ strategy may be particularly important in transitioning from agonist to antagonist muscle contraction, and support the out-of-phase activity of antagonist muscles observed during flexor-extensor alternation. However, critical mechanistic data necessary to support push-pull, including the manner in which α-MN firing properties are regulated by reciprocal inhibition (i.e. average frequency, spike timing, subthreshold oscillations, gain modulation, etc), are lacking.

With renewed emphasis on reciprocal inhibition, this aim sought to reexamine its capacity to regulate α-MN firing properties. Importantly, synaptic inhibition in the central nervous system can mediate a variety of postsynaptic effects. In several neuronal systems, inhibitory input can differentially modulate the average rate and timing of
repetitively firing action potentials. For example, Purkinje cell inhibition can phase lock the spike timing of postsynaptic neurons in the deep cerebellar nuclei without substantially reducing average firing rate (Person & Raman, 2012). Moreover, temporally discrete recurrent IPSPs in rodent lumbar α-MNs can cause the short term modulation of spike timing (i.e. delayed spikes and rebound spiking) with little or no suppression of average firing rate (Obeidat et al., 2014). In contrast to Renshaw cell inputs, which are largely dendritic, glycinergic terminals from IaINs provide dense synaptic coverage to the α-MN soma and proximal dendrites (Burke et al., 1971; Fyffe, 1991b, a), which may be more effective at shunting excitatory drive and thereby suppressing α-MN firing.

The goal of experiments presented in this aim was to extend the in vivo investigation of reciprocal inhibition in α-MNs. By performing electrophysiological studies on rats anesthetized with ketamine/xylazine, which is known to induce activation of persistent inward Ca^{2+} currents (Button et al., 2006; Hamm et al., 2010; Turkin et al., 2010), this aim sought to examine the effects of somatic and proximal dendritic inhibitory inputs from IaINs on medial gastrocnemius (MG) α-MN firing properties. Rather, these experiments produced the unexpected finding of activating an excitatory synaptic circuit between tibialis anterior and extensor digitorum longus (TAEDL) proprioceptive afferents and MG α-MNs. Following the physiological activation of TAEDL muscle afferents by quick stretch, polysynaptic reciprocal excitatory events were recorded in 100% of MG α-MNs (34 cells, 19 animals). These results demonstrate the presence of a powerful and predominate reciprocal excitatory pathway between ankle antagonists and are included into current models of spinal circuitry.
Methods

All animal procedures conform to National Institutes of Health (NIH) guidelines and are approved by the Wright State University Institutional Animal Care and Use Committee. Detailed electrophysiological analyses were performed on adult female Wistar rats (250-425 g; Charles River Laboratories, Wilmongton, MA) in single terminal recording sessions lasting up to 12 hours.

Terminal Experiments

Anesthesia: Deep anesthesia was induced by isoflurane (4–5% in 100% O₂, inhalation in induction chamber) and assessed by complete suppression of withdrawal and corneal reflexes. Rats were intubated, to maintain airway patency during recording sessions, and isoflurane (maintenance: 1–3% in 100% O₂) subsequently delivered by tracheal cannula. Following surgical preparation (described below), rats were transitioned to a cocktail of ketamine/xylazine (K/X; 9:1 mg/kg/h dose) administered via intraperitoneal injection. Alternatively, a small number of animals underwent bilateral carotid ligature and were acutely decerebrated via an intercollicular brainstem transection and mechanical aspiration of the tele- and diencephalon. These strategies permitted the discontinuation of isoflurane, which enabled consistent and reliable activation reflex circuitry for physiological analysis. Animals were closely monitored for respiratory rate (40–60 breaths/min), end-tidal CO₂ (3–5%), O₂ saturation (>90%), heart rate (300-500 beats/min), and core temperature (36–38°C) throughout surgical and electrophysiological recording procedures. These levels were variously maintained by adjusting anesthesia, radiant heat, and/or scheduled subcutaneous injection (1mm/hour) of Lactated ringers
solution (0.6g/L NaCl, 0.31g/L Na-lactate, 0.03g/L KCl, 0.02 g/L CaCl₂). In some cases, adequate recoding stability required intraperitoneal injection pancuronium bromide (0.2mg/kg).

*Surgical Preparation:* Standard procedures are used to prepare the left hindlimb and the lumbar spinal cord for electrophysiological analysis (Seburn & Cope, 1998; Haftel et al., 2004; Haftel et al., 2005; Bullinger et al., 2011a; Bullinger et al., 2011b). A small midline incision through the skin and biceps femoris muscle of the posterior left hindlimb exposed deep tissues of the posterior compartment, including the popliteal fossa. The MG muscle nerve was carefully dissected free of other tissues and suspended in continuity on a monopolar silver stimulating electrode. Other hindlimb nerves were crushed, including the posterior tibial nerve (just distal to the MG nerve), the sural nerve, and the nerves supplying the lateral gastrocnemius, soleus, biceps femoris, semitendonsus, and semimembranosus muscles. The MG muscle was then surgically separated from the lateral gastrocnemius and soleus muscles, freed from surrounding tissue, and its tendon of insertion (the MG portion of the fused Achilles tendon, excluding the lateral gastrocnemius, soleus, and plataris muscle tendons) was detached from the calcaneous and tied directly to the level of a motor system (model 309, Aurora Scientific, Aurora ON, Canada). A second, small incision was made through the skin and biceps femoris of the lateral hindlimb just distal to the knee to expose the common peroneal (CP) nerve and its superficial (SP) and deep (DP) branches in the lateral compartment. A plastic nerve cuff fitted with bipolar silver stimulating electrodes were secured around the DP nerve, and the SP nerve is cut distal to its branch point from the CP nerve. A third incision was
made through the skin over the lateral third of the distal hindlimb, the lateral malleolus, and the dorsum of the left foot to the digits. The pretibial flexors (TA and EDL) were carefully dissected free from surrounding tissue, and their tendons of insertion were detached from the first metatarsal (TA) and the middle phalanx of the second-fifth digits (EDL) and tied directly to the lever of a second motor system (model 305B-LR, Aurora Scientific, Aurora ON, Canada). These motor systems were used to stretch the MG and TAEDL muscles with specified parameters (described below) and to record muscle length and force at the lever. With the animal secured in a rigid recording frame, dorsal exposure of the lumbosacral spinal cord (L2-S1) by laminectomy and longitudinal incision of the dura mater provided access to (a) the L4-L5 dorsal roots, which were carefully dissected free from surrounding tissue and suspended in continuity on bipolar silver hook electrodes for intra- and/or extracellular recording, and (b) MG and or TAEDL α-MNs in the lumbar ventral horn via dorsolateral penetration of the spinal cord with microelectrodes for intra- and/or extracellular recording (Figure 20).

Data Collection
Records of α-MN and afferent membrane potential, electrode current, and muscle length and force are collected, digitized (20 kHz), stored and analyzed with CED Power 1401 and CED Spike2 software (Cambridge Electronic Design, Cambridge, UK).

α-MN and Motor Unit Properties: In-vivo intra- and extracellular recordings were made from α-MNs impaled by borosilicate glass microelectrodes (1.2-mm OD, 7- to 10 MΩ DC resistance, 2 M K-acetate) advanced through the spinal cord with a micromanipulator
system (Transvertex Microdrive). 33 MG (19 animals) and 29 TAEDL (17 animals) α-MNs were identified by antidromic action potentials elicited by electrical stimulation of the MG and DP nerves, respectively (current amplitude: 1-2.5× threshold for muscle contraction; pulse duration: 40 µs). Only those α-MNs with stable membrane potential and with action potential amplitude >60mV were selected for study. Failure to maintain these criteria accounts for the incomplete data set obtained from some α-MNs / motor units. α-MNs were injected with current through the micropipette in order to measure their intrinsic electrical properties as previously reported in our laboratory (Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a; Deardorff et al., 2013): rheobase current (depolarizing pulses, 50 ms in duration, at the lowest strengths capable of initiating action potentials from resting membrane potential), afterhyperpolarization (AHP) (following individual action potentials generated by suprathreshold pulses, 0.5 ms in duration), input resistance (from hyperpolarization produced by -1 and -3 nA current pulses, 50 ms in duration) and axon conduction delay (from antidromic action potentials). Motor unit twitch force, twitch contraction time (onset of the mechanical response to peak force), and twitch ½ decay time (decrease from peak force to ½-maximum value) were measured during intracellular stimulation (suprathreshold pulses, 0.5 ms) of the α-MN, as previously reported (Bakels & Kernell, 1993; Gardiner, 1993).

Stretch-Evoked Post Synaptic Potentials (SSPs): Resting lengths of the MG and TAEDL muscle corresponding to 90° flexion of the knee and ankle were marked with suture, and passive muscle tensions were measured at 10 g an 5 g, respectively. With passive tension maintained at these values (unless otherwise noted), MG and TAEDL muscles were
stretched in two paradigms, as previously described by our laboratory (e.g. Bullinger et al., 2011a). 1) For quick stretches, the muscles are lengthened by a 1 mm ramp at the maximum possible velocity for each motor system (MG: 250mm/s; TAEDL: 400 mm/s). In order to achieve the lowest possible temporal dispersion in activating proprioceptive afferents from the TAEDL, the faster motor was fixed to the TAEDL. This strategy allowed more precise investigation of polysynaptic circuits from TAEDL muscle proprioceptors onto MG α-MNs. Ramps were followed immediately by a slower, 20 ms release to resting length, which was found to minimize mechanical artifact in intracellular records. 2) Because small amplitude, high frequency vibration activates Ia afferents, but is below threshold for Ib and group II afferents (Dedoncker et al., 2003; Bullinger et al., 2011), 5 ms epochs of sinusoidal stretch (100 Hz, +/- 40 µm from resting length) were applied to the MG and TAEDL muscles assess the Ia contribution of all stretch-evoked synaptic potentials.

Each stretch paradigm was repeated for 75-125 trials at 0.5 ms intervals. Homonymous and antagonist stretch-evoked synaptic potentials were recorded intracellularly from MG or TAEDL α-MNs in continuity with the afferent volley recorded from L4-L5 dorsal roots (~ 2-3 mm from the dorsal root entry zone). From these recordings, the polarity (excitatory vs inhibitory), amplitude (measured from resting Vm to peak of the SSP), rate of rise (SSP amplitude / rise time), and central latency (onset of dorsal root volley to start of the SSP) are determined. Following intracellular data collection, the micropipette was moved just outside each α-MN, and the extracellular expression of the synaptic potential is recorded in response to each stretch paradigm. MG and TAEDL muscle length and
force were continuously recorded. For all analyses, significance was set at p<0.05 using Pairwise T-Test.

*Peripheral Encoding of Muscle Stretch:* Individual sensory axons are impaled by borosilicate glass microelectrodes (1.2-mm OD, 25- to 35 MΩ DC resistance, 2 M K-acetate) advanced through the dorsal root (~ 2-3 mm from the dorsal root entry zone) with a micromanipulator system (Transvertex Microdrive). 54 intraxonal recordings (4 animals) were made from TAEDL muscle proprioceptive afferents orthodromically identified by DP peripheral nerve stimulation. Afferents were classified as muscle-spindle (Group Ia/II afferents) or tendon-organ (Group Ib afferents), by whether they pause or accelerate firing, respectively, during the rising phase of force in isometric, whole muscle twitch contractions (Matthews, 1972; Haftel *et al.*, 2004; Bullinger *et al.*, 2011a; Bullinger *et al.*, 2011b). Further classification and characterization of proprioceptive encoding by these neurons is obtained during the following stretch paradigms: ramp hold and release stretches (20 mm/s, 1 mm, 0.5 s hold time); triangular stretches (4 mm/s, 1 mm); quick stretches (as described above, 400 mm/s, 1 mm); and small amplitude, high frequency vibration (as described above, 100-330 Hz, +/- 40 µm). Muscle spindle afferents are classified as Group Ia when (a) they produce high-frequency bursts of firing at the onset of ramp hold and release or triangular stretch and (b) exhibit high sensitivity to linear stretch at a range of background lengths (Matthews, 1972; Bullinger *et al.*, 2011a; Bullinger *et al.*, 2011b). Those muscle spindle afferents exhibiting *neither* property designated Group II. Typical discharge thresholds as a function of passive muscle force for each afferent type were determined.
Mechanical dispersion of the stretch stimulus: Great care was taken to minimize the mechanical transference of force generated by TAEDL muscle stretch into the MG muscle. Steps were taken prior to intracellular recording to ensure the TAEDL muscle was effectively isolated from the MG muscle in all animals included in this investigation. To reduce the dispersion of force through bony tissue, the left femur of each animal was fixed in a rigid C-clamp just proximal to the knee joint, and the medial and lateral malleoli firmly secured by a second C-clamp located at the ankle joint. To reduce soft tissue dispersion, the MG muscle of each animal was carefully dissected free from surrounding tissue, and padded with cotton kept moist with warm mineral oil. Force transference was effectively minimized with these steps, and careful analysis of force records demonstrate TAEDL stretch reduces MG force by approximately 0.2 ± 0.02 g (mean ± SE) stretch by shortening the length of the MG muscle. Finally, to verify the vector of force transfer (i.e. muscle shortening) is incapable of activating MG Ia afferents (Mathews, 1972) dorsal rootlets containing predominately MG muscle afferents were carefully examined prior to intracellular recording sessions and were not activated by the reduction of force during TAEDL quick stretch. Moreover, careful latency measurements taken during intracellular recording demonstrate that the delay between force transfer to the MG muscle and the eSSPs produced by TAEDL quick stretch is on average 0.83 to 1.77 ms faster (95% C.I., p<0.001, paired t-Test) than is required for MG stretch to produce a monosynaptic eSSP in MG α-MNs. By these criteria it was reasoned the TAEDL muscles were effectively isolated, and the eSSPs recorded in MG α-MNs following quick stretch of the TAEDL muscle could not have originated from MG Ia afferents. Rather, they are the result of a genuine reciprocal excitatory pathway between
antagonists acting at the ankle joint.

Results

Motor unit properties

Electrophysiological properties of motor units sampled in this study fell within the range of measurements previously described for the rat hindlimb (Bakels & Kernell, 1993a, b; Gardiner, 1993; Seburn & Cope, 1998; Bichler et al., 2007a; Bichler et al., 2007b; Bullinger et al., 2011a; Deardorff et al., 2013; Nardelli et al., 2013; Obeidat et al., 2014; Rotterman et al., 2014). Table 2 provides descriptive statistics for MG and TAEDL α-MN and motor units combinations included in this chapter, which extend throughout the range of slow to fast subtypes (Burke, 1967; Bakels & Kernell, 1993a, b; Gardiner, 1993). Moreover, MG and TAEDL α-MNs in this sample display the normal tendency for AHP ½ decay time to covary with rheobase and twitch contraction time, with a smaller range of twitch and AHP durations in TAEDL α-MNs (Figure 21)

Synaptic actions of homonymous and antagonist muscle stretch on extensor α-MNs

The physiologic activation of homonymous (MG) and antagonist (TAEDL) muscle afferents using the quick stretch stimulus produced marked excitatory stretch synaptic potentials (eSSPs) in all MG α-MNs (n=35) in all rats (n=20) sampled. Intracellular synaptic potentials were recorded from MG α-MNs simultaneously with extracellularly recorded afferent volleys at the dorsal root entry zone. Extracellular records of α-MN synaptic potentials were taken immediately following intracellular data collection. Typical results are displayed in Figure 22A&B, which shows α-MN intracellular, α-MN
extracellular, dorsal root records, and MG/TAEDL length and force records averaged over ~100 consecutive trials. In Figure 22, stretches were alternately applied to the MG and TAEDL muscles at 500 ms intervals. The amplitude, rate of rise, and central latency of the synaptic potentials were qualitatively and quantitatively different when evoked by homonymous (MG) muscle afferents compared to antagonist (TAEDL) muscle afferents. In Figure 23A&B, quick stretch of the MG muscle produced a 1.6 mV eSSP with a 1.4 mV/ms rate of rise and a central latency of 0.7 ms. By contrast, 500 ms later in the same MG α-MN, quick stretch of the TAEDL muscles produced a 0.8 mV eSSP with a 0.3 mV/ms rise time and a central latency of 2.8 ms (Figure 23B).

Both the size and time course of the eSSPs were measured across our sample of MG α-MNs, as these parameters regulate cell firing (Fetz & Gustafsson, 1983; Turker & Powers, 1999) and can reflect interneuronal activation (Jankowska et al., 1981). As demonstrated in Figure 23, there were significant differences in several of these parameters based on the muscle source of the afferent input. For all MG α-MNs in this study, central latency measurements for synaptic potentials obtained following quick stretch of the MG muscle ranged between 0.2 ms and 1.1 ms, averaged 0.6 ms (Figure 23C), and are within the expected range for a monosynaptic pathway recorded in vivo in the mammalian spinal cord (Eccles et al., 1956; Mendell & Henneman, 1971; Watt et al., 1976; Cope & Mendell, 1982). By contrast, central latency for eSSPs produced in MG α-MNs by quick stretch of the TAEDL muscles were significantly longer (p<0.001, Paired t-Test), ranged between 1.5 ms and 4.7 ms, and averaged 3.1 ms (Figure 23C). These values are consistent with those previously observed in vivo for a di- or tri-synaptic pathway in the
mammalian spinal cord (Eccles et al., 1956; Jankowska & Roberts, 1972b; Watt et al., 1976; Jankowska, 1992), including a synaptic delay of 0.2 – 0.7 ms (Jankowska & Roberts, 1972b; Watt et al., 1976; Cope & Mendell, 1982) for each synapse, plus \( \leq 1.0 \) ms for excitation and conduction through each interposed interneuron (Obeidat et al., 2014). In addition, eSSPs evoked in MG \( \alpha \)-MNs by TAEDL quick stretch had a significantly smaller amplitude (0.7 ± 0.1 mV vs. 1.6 ± 0.2 mV; mean ± SE; p<0.001, Paired t-Test) and a significantly slower rate of rise (0.3 ± 0.05 mV/ms vs. 1.1 ± 0.2 mV/ms; mean ± SE; p<0.001, Paired t-Test) compared to eSSPs evoked in MG \( \alpha \)-MNs by MG quick stretch. Altogether, these data indicate that the excitatory pathway between ankle antagonists revealed in these studies likely consists of 1-2 interposed interneurons.

**Synaptic contribution of afferent types**

To determine the afferent contribution to the polysynaptic eSSPs evoked by TAEDL quick stretch, the response properties of 54 primary and secondary TAEDL proprioceptive afferents were recorded in 4 animals. The axonal conduction velocities of stretch-activated TAEDL afferents ranged from 33-71 m/s and are comparable to those previously reported for adult rats (Andrew et al., 1973; Hnik & Lessler, 1973; Leslie, 1973; Lewin & McMahon, 1991; De-Doncker et al., 2003; Haftel et al., 2004; Bullinger et al., 2011b). Afferents were readily classifiable as muscle spindle afferents (\( n = 45 \)) by firing cessation during the rising phase of isometric, whole muscle twitch force (Matthews, 1972; De-Doncker et al., 2003; Haftel et al., 2004; Bullinger et al., 2011a; Bullinger et al., 2011b); whereas afferents displaying accelerated firing during isometric contraction were classified as Group Ib (Matthews, 1972; De-Doncker et al., 2003).
Spindle afferents were further differentiated as Group Ia \((n = 34)\) by high-frequency bursts of firing (i.e. initial bursting) at the onset of ramp hold stretch and high sensitivity to linear vibration at a range of passive muscle lengths (Matthews, 1972; De-Doncker et al., 2003; Haftel et al., 2004; Bullinger et al., 2011a; Bullinger et al., 2011b).

**Figure 24** shows typical discharge thresholds and firing patterns recorded in this study, with the TAEDL muscles stretched to 5 g of passive muscle force. All TAEDL afferents fired action potentials in response to TAEDL quick stretch, but 100 Hz vibration usually only activated only group Ia afferents. Interestingly, tendon organ afferents responded to quick stretch with a single action potential, while spindle afferents typically displayed high frequency doublet firing. Data for each afferent type were pooled and firing reliability indices (FRIs) were constructed to assess the percentage of stretch stimuli at 5 g background force that elicited at least 1 afferent action potential (**Figure 25**). Quick stretch FRIs were 100% for all afferent types, indicating that all afferents fired during every application of quick stretch. By contrast, only Ia afferents fired with a high reliability \((FRI = 80.7 \pm 5.5\%, \text{mean} \pm \text{S.E.})\) at 5 g background force during 100Hz vibration.

Because quick stretch does not adequately differentiate between afferent subtypes, synaptic potentials in MG \(\alpha\)-MNs in were recorded in response to 100 Hz vibration of the TAEDL muscle (**Figure 26**). These experiments allowed the direct examination of the Ia afferent contribution to eSSPs in MG \(\alpha\)-MNs evoked by TAEDL muscle stretch. **Figure 26** shows intracellular, extracellular, and dorsal root records from the same MG \(\alpha\)-MN in
response to both quick stretch and small amplitude, 100 Hz vibration of the MG (Figure 26A&B) and TAEDL (Figure 26C&D) muscles. As with quick stretch, eSSPs were evoked in MG α-MNs by high frequency vibration of the TAEDL muscles. Moreover, qualitative and quantitative differences are again observed in the eSSPs based on the muscle source of the proprioceptive afferents (i.e. homonymous vs antagonist). Notably central latency, amplitude, and rate of rise measurements of eSSPs evoked by homonymous vibration are similar to those evoked by homonymous quick stretch (Figure 25A&B). Following TAEDL vibration, however, these measurements are not only slower and smaller than those taken after MG muscle vibration, but after TAEDL quick stretch as well. For example, in the same MG α-MN, quick stretch (Figure 26A) and vibration (Figure 26B) of the MG muscle elicited an eSSPs with similar amplitude (quick stretch: 3.1 mV; vibration: 3.2 mV), central latency (quick stretch: 0.5 ms; vibration: 0.7 ms), and rate of rise (quick stretch: 2.0 mV/ms; vibration: 2.2 mV/ms). By comparison, quick stretch of the TAEDL muscle evoked a 1.3 mV eSSP that had a 0.8 0.8 mV/ms rate of rise and occurred following a 2.8 ms central latency (Figure 26C), while TAEDL vibration elicited a 0.3 mV eSSP that had a 0.1 mV/ms rate of rise and occurred following a 3.3 ms central latency (Figure 26D). The prolonged central latency, decreased amplitude, and slower rate of rise observed following antagonist vibration are consistent with a less effective activation of interposed interneurons relative to antagonist quick stretch (Jankowska et al., 1981).

Similar data were collected in 32 of the 34 MG α-MNs, in which TAEDL quick stretch and vibration elicited eSSPs. This is demonstrated in figure 27A, which shows the amplitude of polysynaptic eSSPs evoked in MG α-MNs by TAEDL vibration plotted as a
function of the eSSP amplitude evoked in the same MG α-MNs by TAEDL quick stretch. Here, the slope of linear regression line falls well below the line of identity, reflecting a limited efficacy in activating interposed interneurons with TAEDL vibration. (In the remaining 2 MG α-MNs not included in this scatterplot, TAEDL vibration did not elicit a measureable SSP.) These findings indicate the segmental synaptic excitation observed in this study can be mediated solely by TAEDL Ia afferents. However, TAEDL vibration is less effective than TAEDL quick stretch in activating the necessary interneuronal population.

There are two likely explanations that could account for decreased interneuronal activation with TAEDL vibration. First, because quick stretch reliable activates Group Ia, Ib and II afferents (Figure 25), it is possible this interneuronal population receives convergent input from all three afferent types. Second, because Ia afferents are less reliably activated (Figure 25) and exhibit greater temporal dispersion by vibration, it is possible that the diminished Ia input is less effective at bringing the interposed population to threshold, with Ib and/or group II playing no role in their synaptic activation. To test these possibilities, the synaptic output of TEADL afferents following quick stretch and 100 Hz vibration, were intracellularly recorded in TAEDL α-MNs (29 α-MNs, 17 animals). Figure 27B is a similar plot as figure 27A, this time showing the amplitude of fell eSSPs in TAEDL α-MNs evoked by TAEDL vibration and plotted as a function of the monosynaptic eSSP evoked in the same TAEDL α-MNs by TAEDL quick stretch. In this plot, the line of regression and line of identity nearly overlap, indicating the synaptic output of TAEDL afferents is similar for quick stretch and 100 Hz vibration. Altogether,
these data, provide strong evidence that the majority of antagonist evoked eSSPs in MG α-MNs include a contribution from Ia afferents, with additional contribution from IIs and/or Ibs. It is likely, therefore, that the excitatory interneuronal population interposed between TAEDL afferents and MG α-MNs in rat is co-activated by Ia/II/Ib afferents, and may operate via the Lamina V/VI interneurons demonstrated by Jankowska and colleagues in the cat.

**Change in MG force introduces an earlier inhibitory component of the SSP**

Muscle force can influence reflex amplitude (Matthews, 1968), and background MG force in this study had a strong effect on the SSP evoked by TAEDL quick stretch. Figure 28 shows data from 5 MG α-MNs in 5 animals in which background force was modulated from rest (~10 g) to approximately 30 g by adjusting the passive length of the muscle. As described above, quick stretch of the MG (Figure 28A&B, left columns) and TAEDL (Figure 28A&B, middle columns) muscles, respectively, produced mono- and polysynaptic eSSPs, in MG α-MNs with MG background force held at 10 g. Central latency and amplitude values shown in Figure 28A&B left and middle columns are included in the histograms shown in Figure 23. While recorded from these 5 MGα-MNs, background MG force was modulated to approximately 30 g by adjusting the passive length of the MG muscle. No change in eSSP properties were observed following quick stretch of the MG muscle with the MG held at 30 g (data not shown). However, figure 28A&B (right columns) shows that increased background force of the MG muscle introduced an earlier - and now inhibitory – component to the SSP. Latency and amplitude values shown in Figure 28A&B represent the *earliest* component of the
synaptic potential. Intracellular, extracellular, and dorsal root records from two of these cells are shown in Figure 28C&D. Please note traces in Figure 28Ci are also shown in Figure 22&23. These records show clear eSSPs following MG and TAEDL quick stretch, with MG background force held at 10 g (Figure 28Ci,ii&Di,ii). However, with the MG held at 30 grams, a clear inhibitory potential (an iSSP) preceded the eSSP (Figure 28Ei&ii). The range of central latencies at which these iSSPs occurred was consistent with the ‘classic’ disynaptic pathway reciprocal inhibitory pathway mediated by IaINs (Eccles et al., 1956; Jankowska & Roberts, 1972b; Watt et al., 1976; Jankowska, 1992). No change in the amplitude or latency of the excitatory component of this multiphasic SSP was observed.

Decerebration activates an excitatory or inhibitory pathway

The expression of proprioceptive reflex pathways is profoundly influenced by the activity of descending and segmental neuronal circuits (Hongo et al., 1969; Tanaka, 1974; Crone et al., 1987; Jankowska & Edgley, 2010; Geertsen et al., 2011), and the state of those neuronal circuits that may influence spinal reflex pathways are unknown for rodents under K/X anesthesia. Decerebration is an experimental preparation that has been widely used for physiologic analysis of segmental reflex circuits. Therefore, 5 animals did not receive K/X, but rather were acutely decerebrated via intercollicular brainstem transection and mechanical aspiration of the tele- and diencephalon. Quick stretch of the TAEDL muscles in 3 of these animals evoked eSSPs in all MG α-MNs sampled (n = 8). In the remaining 2 animals, quick stretch of the TAEDL muscles evoked iSSPs in all MG α-MNs sampled (n = 9). Results are displayed in Figure 29, which shows intracellular
and extracellular records for different MG α-MNs in different animals. As previously described, afferent impulses were simultaneously recorded at the dorsal root entry zone. **Figure 29A** shows a polysynaptic eSSP evoked in an MG α-MN by quick stretch of the TAEDL muscles. Central latency, amplitude, and rate of rise measurements were similar to the antagonist evoked eSSPs described above under K/X. By contrast, **Figure 29B** shows a polysynaptic iSSP evoked in an MG α-MN by quick stretch of the TAEDL muscles. Central latency values were, on average, significantly shorter for iSSPs than for eSSPs (iSSP: 2.0 ± 0.1 ms; eSSP: 3.3 ± 0.3 ms; mean ± S.E.; p=0.001, t-Test) evoked by antagonist muscle stretch (**Figure 29C**). Central latency measurements for antagonist evoked iSSPs are consistent with those mediated by Ia afferents in vivo in the mammalian spinal cord, and for MG α-MNs sampled following decerebration, central latency measurements fell within the range estimated for a di- or tri-synaptic pathway (Eccles *et al.*, 1956; Jankowska & Roberts, 1972b; Watt *et al.*, 1976; Jankowska, 1992).

**Synaptic actions of homonymous and antagonist muscle stretch on flexor α-MNs**

Proprioceptive reflex pathways were also assessed from extensor (MG) muscle afferents onto flexor (TAEDL) α-MNs using the quick stretch stimulus in 14 K/X anesthetized rats. As with experiments on MG α-MNs, intracellular synaptic potentials were recorded from TAEDL α-MNs simultaneously with extracellularly recorded afferent volleys at the dorsal root entry zone. Extracellular records of α-MN synaptic potentials were taken immediately following intracellular data collection. Typical results are displayed in **Figure 30A&B**, which shows α-MN intracellular, α-MN extracellular, and dorsal root records averaged over ~100 consecutive trials from 2 different TAEDL α-MNs.
Interestingly, the physiologic activation of antagonist (MG) muscle afferents produced either eSSPs (8 rats, 17 TAEDL α-MNs), iSSPs (1 rat, 1 TAEDL α-MN), or SSPs of mixed inhibitory and excitatory components (5 rats, 7 TAEDL α-MNs). MG quick stretch elicited a ‘simple’ eSSP in the TAEDL α-MN shown in figure 30A. Latency and amplitude measurements of the eSSP in this cell are consistent with those described above for antagonist evoked eSSPs MG α-MNs. By comparison, quick stretch of the MG muscle produced a ‘mixed’ SSP in the TAEDL α-MN shown in figure 30B. In this cell, the antagonist evoked eSSP is preceded by a clear iSSP that occurs after a central latency of 2.2 ms.

Figure 30C shows the central latencies for the earliest component of the synaptic potential recorded in all 25 TAEDL α-MNs. Central latency values were, on average, shorter for iSSPs than for eSSPs (iSSP: 2.4 ± 0.1 ms; eSSP: 3.5 ± 0.4 ms; mean ± S.E; Figure 30C) evoked by antagonist muscle stretch, and are consistent with those mediated by Ia afferents in vivo in the mammalian spinal cord (Eccles et al., 1956; Jankowska & Roberts, 1972b; Watt et al., 1976; Jankowska, 1992). Differences in latency measurements for antagonist evoked eSSP and iSSPs did not reach significance in this case (p=0.07, t-Test), most likely due to limited sample size. As expected, the physiologic activation of TAEDL muscle afferents produced marked eSSPs at latencies consistent with a monosynaptic pathway (Eccles et al., 1956; Mendell & Henneman, 1971; Watt et al., 1976; Cope & Mendell, 1982) in all TAEDL α-MNs sampled (see also Figure 27B) and are not discussed further. For all antagonist evoked SSPs recorded in TAEDL α-MNs, central latency measurements fell within the range estimated for a di- or tri-synaptic
pathway (Eccles et al., 1956; Jankowska & Roberts, 1972b; Watt et al., 1976; Jankowska, 1992). It should be noted, that quick stretch of the MG muscle produced a ‘simple’ iSSP in a single TAEDL \(\alpha\)-MN from an animal in which eSSPs were reliably recorded in MG \(\alpha\)-MNs following quick stretch of the TAEDL muscle, indicating that antagonist evoked excitatory pathways were active, but highly asymmetric in this animal.

**Discussion**

To characterize more completely the proprioceptive connections between ankle antagonists and their regulation of \(\alpha\)-MN firing properties in the adult rodent, intracellular records were obtained *in vivo* from MG \(\alpha\)-MNs during controlled stretch applied to the TAEDL muscles in ketamine/xylose anesthetized adult rats. Unexpectedly, quick stretch of the TAEDL produced antagonist eSSPs in every MG \(\alpha\)-MN (\(n=35\)) in every rat (\(n=20\)) sampled under K/X anesthesia, with no evidence of reciprocal inhibition at resting levels of background force. Central latency for antagonist eSSPs was consistent with a di-/tri-synaptic pathway and were nearly \(\frac{1}{2}\) the amplitude of homonymous eSSPs recorded in the same MG \(\alpha\)-MNs. High frequency, small amplitude vibration of the TAEDL muscle also produced a detectable antagonist eSSP in 32/34 \(\alpha\)-MNs, indicating the majority of antagonist eSSPs evoked by quick stretch are activated by Ia afferents with additional contributions from Ib and group II fibers.

A wealth of information on the ontogeny and synaptic connectivity of IaINs exist for the adult mouse (Alvarez et al., 2005; Siembab et al., 2010; Benito-Gonzalez & Alvarez, 2012; Zhang et al., 2014), providing strong indications that reciprocal inhibitory
pathways are present in the adult rodent nervous system. But, physiologic analyses of reciprocal antagonist connections, which are so well documented in the cat, are scarce in the adult rat. Data presented in this aim demonstrate that reciprocal excitatory pathways are an additional mechanism for regulating antagonist α-MN firing properties. The coordination of antagonist motor pools by muscle proprioceptors, therefore, likely occurs by premotor integration of descending and segmental systems (Hongo et al., 1969; Tanaka, 1974; Crone et al., 1987; Jankowska & Edgley, 2010; Geertsen et al., 2011), such that one or the other reciprocal excitatory or inhibitory pathways are likely to predominate, depending on the state of the spinal cord. Circuit and functional considerations of this excitatory pathway are discussed below.

**Circuit Anatomy for Reciprocal Excitation**

Renshaw cells provide parallel recurrent inhibition onto both α-MNs and their ‘corresponding’ IaINs (Hultborn et al., 1971c, a, b; Hultborn et al., 1971d; Jankowska & Lindstrom, 1972). Moreover, Renshaw cells receive inhibitory and excitatory drive from numerous segmental and descending sources (Alvarez & Fyffe, 2007; Siembab et al., 2010), which integrate local recurrent pathways into central command circuits. When Renshaw inhibition is imposed upon tonically active IaINs, the resulting disinhibition of antagonist α-MN pools causes the depolarizing ‘recurrent facilitation’ first identified by Renshaw (Renshaw, 1941; Hultborn et al., 1971d). Importantly, stretch stimuli employed in this aim show no evidence of homonymous motor unit recruitment, which would be necessary to activate Renshaw cells in phase with muscle stretch. It is, therefore, unlikely that recurrent facilitation is the underlying mechanism of antagonist evoked eSSPs
observed in this study. Rather, these eSSPs represent true synaptic excitation mediated by antagonist proprioceptive afferents.

There is sparse evidence that short-latency reciprocal excitatory pathways may link the activity of muscle proprioceptors to a small percentage of antagonist $\alpha$-MNs (Watt et al., 1976; Jankowska et al., 1981). To date, evidence detailing a potential reciprocal excitatory circuit is indirect. Early reports of Group I reflex actions demonstrated Ib afferents can evoke polysynaptic excitation of antagonist $\alpha$-MNs (Laporte & Lloyd, 1952; Eccles et al., 1957c; Watt et al., 1976) via interneuronal relays under descending control (Hongo et al., 1969), with excitation typically observed from extensors onto flexors (Eccles et al., 1957c). Later studies show a considerable portion of these interneurons reside in Rexed Laminae V/VI and can be co-excited or even driven solely by Ia afferents (reviewed in Jankowska & Edgely, 2010). Data presented in this aim provide strong evidence in support of this circuit. Intracellular and intra-axonal records show the majority of eSSPs in MG $\alpha$-MNs include a contribution from Ia afferents as well as from IIs and/or Ibs. It is likely, therefore, that the excitatory interneuronal population interposed between TAEDL afferents and MG $\alpha$-MNs in rat is co-activated by Ia/II/Ib afferents, and operates via the Lamina V/VI interneurons demonstrated by Jankowska and colleagues in the cat.

**Functional Considerations: An Assistive Reflex**

The integration of proprioceptive feedback into ongoing motor commands is highly dynamic, shapes motor output over a millisecond timescale, and provides critical support
in accordance with the biomechanical requirements of the limb (Nichols et al., 1999; Clarac et al., 2000). For example, both the intensity and sign of the stretch reflex are dynamically modulated during rhythmic motor activities (Pearson, 1995b), presumably reflecting adaptive control of circuit parameters to support limb biomechanics during locomotion (Capaday, 2002). Similarly, when considering antagonist motor pools, principles of reciprocal innervation are frequently overridden by central commands (Long et al., 1970; Patton & Mortensen, 1971; Llewellyn et al., 1990; Hyland & Jordan, 1997) and/or pathology (Myklebust et al., 1982; Crone et al., 1994; Xia & Rymer, 2005). Here, I discuss the necessity for and spinal mechanisms underlying reductions in reflex reciprocal inhibition during ongoing concentric contractions of the antagonist muscle (e.g. during stance phase of locomotion) and update current circuit diagrams to illustrate the role of reciprocal excitation in supporting such movements.

During voluntary tonic contractions stretch-evoked Ia reciprocal inhibition from the antagonist is reduced onto the $\alpha$-MNs innervating the active muscle (Tanaka, 1974; Crone et al., 1987). In this instance, IaINs coupled to the contracting muscle are activated by central drive and, in turn, silence both the antagonist MNs and the ‘opposite’ IaINs (Hultborn et al., 1976; Pierrot-Deseilligny & Burke, 2012). In locomotion, $\alpha$-MNs are similarly activated in phase with their corresponding IaINs, which in turn inhibit both their antagonist $\alpha$-MNs and their opposite IaINs (Hultborn et al., 1976; Geertsen et al., 2011; Pierrot-Deseilligny & Burke, 2012).
Figure 31 illustrates this concept. During the stance phase of locomotion, concentric contraction of the MG muscle produces a stretch-induced Ia afferent discharge from the antagonist TAEDL muscles. With ‘classic’ reflex pathways active, this Ia discharge would have two, undesirable effects: (a) monosynaptic excitation of TAEDL α-MNs, and (b) disynaptic inhibition, via TAEDL-coupled IaINs, of MG α-MNs. However, during concentric contraction of the MG muscle, MG α-MNs and their corresponding IaINs receive parallel excitatory drive from the central pattern generator (Geertsen et al., 2011; Pierrot-Deseilligny & Burke, 2012). Excitation of the MG-coupled IaINs produces inhibition of TAEDL α-MNs and inhibition of TAEDL-coupled IaINs (Hultborn et al., 1976; Geertsen et al., 2011; Pierrot-Deseilligny & Burke, 2012). Presynaptic inhibition may further reduce the efficacy of the TAEDL Ia volley onto TAEDL α-MNs and IaINs (Pierrot-Deseilligny & Burke, 2012).

The parallel activation of MG α-MNs and their corresponding IaINs, therefore, provides a relatively straightforward spinal mechanism to prevent (a) the monosynaptic excitation of TAEDL α-MNs and (b) the disynaptic inhibition of MG α-MNs by TAEDL-coupled IaINs. These intrinsic spinal circuits, however, are insufficient to direct normal locomotor flexor-extensor alternation without proprioceptive sensory feedback (Pearson, 1995a; Clarac et al., 2000; Ekeberg & Pearson, 2005; Pearson, 2008; Goulding, 2009; Akay et al., 2014), and the mechanism described above does not adequately account for the role of proprioceptive feedback in locomotor rhythm generation. Proprioceptive input during locomotion is critical for adjusting the relative timing, phase, and amplitude of flexor and extensor bursts and is particularly important when navigating an uncertain and changing
environment (Pearson, 1995a; Clarac et al., 2000; Akay et al., 2014). In several neuronal systems, these functions depend to some degree on reversing the influence of classically described reflex pathways by modulating Ia circuitry and / or activating alternative proprioceptive pathways (Pearson, 1995a; Clarac et al., 2000).

During invertebrate locomotion, as observed in the crayfish, proprioceptive feedback underlies an ‘assistive’ reflex, in which reciprocal inhibitory constraints are overridden (as in the mammal), and proprioceptors increase the excitation of active α-MNs through a polysynaptic excitatory pathway (Le Ray & Cattaert, 1997; Le Ray et al., 1997a, b; Chung et al., 2014; Bacque-Cazenave et al., 2015). While the animal is stationary, negative feedback from inhibitory ‘resistive’ reflexes stabilize against postural perturbations (Clarac et al., 2000), but once the animal is in motion, the invertebrate assistive reflex provides positive feedback to reinforce movements in coordination with central pattern generators (Pearson, 1995b; Le Ray & Cattaert, 1997; Buschges et al., 2008; Chung et al., 2014; Bacque-Cazenave et al., 2015). During locomotion reciprocal, excitatory proprioceptive feedback via the assistive reflex is necessary for proper load compensation by promoting downward torque on the limb and for triggering antagonist phase transitions, in particular, when increasing step speed (Chung et al., 2014; Bacque-Cazenave et al., 2015). With this in mind, the reciprocal excitatory pathway demonstrated in this aim may provide critical, regulatory feedback for rhythmic locomotor activity by facilitating the activity of the contracting muscle. An evolutionarily conserved assistive reflex pathway that is activated by antagonist muscle stretch accounts for the finding that
proprioceptive feedback regulates centrally generated flexor-extensor alternation during mammalian motor activities (Pearson, 1995a; Akay et al., 2014).

Figure 31 illustrates this concept. As described above, during the stance phase of locomotion, intrinsic spinal circuits reduce the undesirable effects of TAEDL Ia afferents, which would oppose ongoing MG contraction by activating TAEDL α-MNs in Lamina IX and activating TAEDL-coupled IaINs in Lamina VII. However, stretch-evoked TAEDL afferent discharge into Laminae V/VI (Brown & Fyffe, 1978a; Brown, 1983) activates interneurons receiving convergent input from group Ia/II/Ib afferents (Jankowska & Edgley, 2010). Among other synaptic effects, these interneurons mediate the reciprocal excitation of MG α-MNs observed in this aim. This would provide critical feedback to facilitate ongoing MG contraction, providing proper load compensation and resetting the central pattern generator early to accelerate the intrinsic locomotor rhythm. As a result, Figure 12 updates current circuit diagrams to demonstrate that TAEDL afferent discharge into Laminae V/VI may facilitate the ongoing MG contraction (rather than oppose it) by directly exciting MG α-MNs.

Conclusion

Despite extensive characterization of reciprocal inhibitory pathways, the prevalence of reciprocal excitation and its functional impact on mammalian motor behaviors is uncertain but have been the subject of investigations spanning nearly 100 years (reviewed in (Tilney & Pike, 1925; Smith, 1981; Miri et al., 2013). Altogether, data presented in this aim extensively characterize, for the first time in the adult rodent, a short-latency Ia
reciprocal excitatory pathway between antagonist muscles. Moreover, this pathway is present and functional in 100% of MG α-MNs. Because this pathway is co-activated by group Ia/Ib/II afferents, it likely operates via interneurons demonstrated by Jankowska and Edgley (2010) in the cat. Though additional studies need to be performed, reciprocal excitation may underlie an assistive reflex that provides proprioceptive feedback that is critical for regulating temporal parameters of flexor-extensor alternation during locomotion (Akay et al., 2014).
Figure 20. Diagram depicting features of the experimental setup for in vivo recording from anesthetized adult rats. The distal femur and distal tibia/fibula of the left hindlimb were fixed in rigid C-clamps just proximal to the knee joint and at the medial and lateral malleoli, respectively (black circles). The left MG and TAEDL muscles were dissected free from surrounding tissue. The separate attachment of MG and TAEDL tendons of insertion to force transducing servo motors enabled the application of stretch independently to extensors and flexors with minimal mechanical coupling. Intracellular records were obtained from MG and/or TAEDL α-MNs together with extracellular records from dorsal root ~1mm caudal to their entry into the spinal cord. Cuff electrodes were placed around the MG and DP nerves. All other nerves of the distal hindlimb were severed (red x).
<table>
<thead>
<tr>
<th>Muscle</th>
<th>$I_{Rh}$ (nA)</th>
<th>AHP $\frac{1}{2}$ Decay (ms)</th>
<th>AHP Amplitude (mV)</th>
<th>Twitch Contraction Time (ms)</th>
<th>Twitch $\frac{1}{2}$ Relaxation Time (ms)</th>
<th>Twitch Force (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG</td>
<td>9.8 ± 1.1</td>
<td>14.2 ± 0.8</td>
<td>2.1 ± 0.2</td>
<td>238 ± 1.3</td>
<td>25.6 ± 1.9</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(1.0-22.0)</td>
<td>(8.0-26.1)</td>
<td>(0.4-6.1)</td>
<td>(13.0-41.0)</td>
<td>(11.2-55.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
</tr>
<tr>
<td>TAEDL</td>
<td>6.9 ± 0.8</td>
<td>13.4 ± 0.8</td>
<td>1.4 ± 0.1</td>
<td>15.8 ± 0.6</td>
<td>16.3 ± 1.0</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(2.0-20.0)</td>
<td>(8.2-22.0)</td>
<td>(0.3-3.3)</td>
<td>(10.3-23.4)</td>
<td>(8.5-28.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=25</td>
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<td>n=25</td>
<td>n=22</td>
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<td>n=22</td>
</tr>
</tbody>
</table>

Mean ± S.E.; Range in italics below
Figure 21. Scatterplots of electrical and contractile properties for MG and TAEDL α-MN and motor unit combinations. For MG (A&B) and TAEDL (C&D) α-MN and motor unit combinations, α-MN AHP ½ decay time for displays the usual tendency to co-vary with rheobase (A&C) and twitch contraction time (B&D). Values reported comprise the entire range of slow to fast motor unit subtypes (Burke, 1967; Bakels & Kernell, 1993a,b; Gardiner et al., 1993), and as previously described, the range of AHP and twitch durations were smaller for TAEDL material than for MG (Bakels & Kernell, 1993a,b). Black line is line of regression.
Figure 22. *Quick stretch of homonymous (MG) and antagonist (TAEDL) muscles causes the segmental synaptic excitation of MG α-MNs.* Averaged records from approximately 100 sweeps in which quick stretch was sequentially applied, every 500 ms, to the homonymous (MG) muscle followed by the antagonist (TAEDL) muscles. Mono- and polysynaptic eSSPs were evoked in the same MG α-MN by quick stretch of homonymous MG (A) and antagonist TAEDL (B) muscles, respectively. The arrival of the afferent signal was simultaneously recorded at the dorsal root entry zone. Following the collection in intracellular data, the micropipette was retracted to just outside the MN to record the extracellular expression of the synaptic potentials.
Synaptic Potential Produced in MG MN by:

A. Homonymous (MG) Quick Stretch
B. Antagonist (TAEDL) Quick Stretch
Figure 23. Properties of excitatory synaptic potentials evoked in MG α-MNs by quick stretch of the homonymous (MG) and antagonist (TAEDL) muscles. The same records shown in Figure 20 are displayed in an expanded time scale to illustrate the differences in the quantitative properties of mono- and polysynaptic eSSPs evoked in the same MG α-MN by quick stretch of homonymous MG (A) and antagonist TAEDL (B) muscles, respectively. The central latency of the synaptic potential (measured from the arrival of the afferent volley at the dorsal root entry zone to the onset of synaptic potential in the α-MN) is ~2 ms longer following antagonist muscle stretch. In addition, the amplitude of the synaptic potential (measured from the resting membrane potential to the peak of the synaptic potential) is smaller and the rate of rise (amplitude of the synaptic potential divided by its rise time) is slower for the antagonist evoked eSSP. (C) For all MG α-MNs sampled, antagonist evoked eSSPs occurred with a mean central latency that was significantly longer (p<0.001, Paired t-Test) than corresponding homonymous evoked eSSPs. These values for central latency fell within the range estimated for a di- or trisynaptic pathway in vivo in the mammalian spinal cord (Eccles et al., 1956; Jankowska & Roberts, 1972; Watt et al., 1976; Jankowska et al., 1992). By comparison, eSSP central latency following quick stretch of the MG muscle ranged between 0.34 ms and 1.06 ms and averaged 0.6 ms, within the range previously observed for a monosynaptic pathway in vivo in the mammalian spinal cord (Eccles et al., 1956; Mendell & Henneman, 1971; Watt et al., 1976; Cope & Mendell, 1982a).
Synaptic Potential Produced in MG α-MN by:

A. Homonymous (MG)  B. Antagonist (TAEDL)
Quick Stretch       Quick Stretch

Central Latency: 0.7ms  Central Latency: 2.8ms
Amplitude: 1.6mV    Amplitude: 0.8mV
Rate of Rise: 1.4mV/ms Rate of Rise: 0.3mV/ms

C. Central Latency

- Homonymous (MG) Quick Stretch
  18 Rats, 30 Mns
  Mean ± SE: 0.6 ± 0.1ms

- Antagonist (TAEDL) Quick Stretch
  20 Rats, 35 MNs
  Mean ± SE: 3.1 ± 0.2ms
  p<0.001
Figure 24. Response properties of TAEDL proprioceptive afferents. Recordings from primary Ia muscle spindle afferents (A), group II muscle spindle afferents (B), and group 1b Golgi tendon organ (C) from TAEDL muscles. All afferent types fire in response to quick stretch, with spindle afferents frequently displaying doublet firing. Only Ia afferents display high sensitivity to high frequency, small amplitude linear vibration.
Figure 25. **Firing reliability index (FRI) for quick stretch and 100Hz vibration in TA/EDL spindle and tendon organ afferents at 5g background force.** FRI is the percentage of stretch stimuli that elicit afferent firing. Group Ia (n=34, 4 animals), Group II (n=9, 4 animals), and Group Ib (n=11, 4 animals) muscle afferents respond faithfully (FRI = 100%) to quick stretch. However, only Group Ia afferents fire with a high reliability (FRI = 80.7 ± 5.5%, mean ± SE) during each sinusoid of the 100Hz vibration.
Figure 26. **Segmental synaptic excitation of MG α-MNs by homonymous (MG) and antagonist (TAEDL) Ia afferents.** In the same MG MN, mono- and polysynaptic eSSPs evoked by quick stretch (A&C) and 100Hz vibration (B&D) of homonymous (MG) and antagonist TAEDL muscles, respectively. Intracellular records are shown with the arrival of the afferent volley at the dorsal root entry zone recorded in continuity. Extracellular field potentials show no mechanical artifact. Records are averages of ~100 sweeps. Note that the amplitude of the monosynaptic eSSP is similar following homonymous quick stretch (A) and vibration (B). However, the amplitude of the polysynaptic eSSP evoked by antagonist (TAEDL) quick stretch (C) is larger than the polysynaptic eSSP evoked by antagonist (TAEDL) vibration (D). These data, in combination with those in Figure 27, indicate antagonist quick stretch elicits an eSSP of mixed Group I/II content.
Synaptic Potential Produced in MG α-MN by:

A. Homonymous (MG) Quick Stretch

Central Latency: 0.5ms
Amplitude: 3.1mV
Rate of Rise: 2.0mV/ms

B. Homonymous (MG) 100Hz Vibration

Central Latency: 0.7ms
Amplitude: 3.2mV
Rate of Rise: 2.2mV/ms

C. Antagonist (TAEDL) Quick Stretch

Central Latency: 2.8ms
Amplitude: 1.3mV
Rate of Rise: 0.8mV/ms

D. Antagonist (TAEDL) 100Hz Vibration

Central Latency: 3.3ms
Amplitude: 0.3mV
Rate of Rise: 0.1mV/ms
Figure 27. *The functional output of TAEDL Ia afferents is similar with quick stretch and vibration.* Gray dashed line is line of identity. Black line is line of regression. (A) Quick stretch of the TAEDL muscles produce larger amplitude, polysynaptic eSSPs in MG MNs than 100Hz vibration of TAEDL muscles. Note the slope of the regression line is lower than the line of identity, as expected from intracellular records shown in Figure 25. (B) The amplitude of monosynaptic eSSPs recorded in TAEDL MNs following quick stretch or vibration of TAEDL muscles are nearly equivalent, and as expected the regression line and line of identity nearly overlap. This is consistent with intracellular records shown in Figure 25, and demonstrates the synaptic output of TAEDL afferents is similar for quick stretch and 100 Hz vibration. Therefore, the larger amplitude eSSPs evoked by TAEDL quick stretch is likely due to the convergence of Ia with group II and / or Ib afferents onto the interposed, excitatory interneurons.
A. Synaptic potential amplitude in MG MNs

B. Synaptic potential amplitude in TAEDL MNs
Figure 28. Increasing MG background force introduces synaptic inhibition. Data from 5 MG MN α-MNs in 5 animals in which background force was modulated from rest (~10 g) to approximately 30 g by adjusting the passive length of the muscle. No change in eSSP properties were observed following quick stretch of the MG muscle with the MG held at 30 g (not shown). (A&B) The central latency (A) and amplitude (B) of the earliest component of the synaptic potential evoked by MG and TAEDL quick stretch. With 10 g of force on the MG, quick stretch of the MG muscle produced an eSSP at a monosynaptic latency (purple), while quick stretch of the TAEDL produced a eSSP at a di- / tri-synaptic latency (green). With 30 of force on the MG, quick stretch of the TAEDL evoked a shorter latency (A), inhibitory (B) component of the SSP. (C,D&E) Representative intracellular, extracellular, and dorsal root records for cells represented by the green diamond and orange square. Colors correspond to those background force shown in A&B. As background force on the MG is increased from 10 g to 30 g, an earlier, inhibitory SSP is elicited with TAEDL quick stretch. Note, however, with the MG at 30 g, an excitatory pathway between antagonists is still active.
Figure 29. *Quick stretch of antagonist (TAEDL) muscles causes the segmental synaptic excitation or inhibition of MG α-MNs following decerebration.* (A&B)

Averaged intracellular, extracellular, and dorsal root records from approximately 100 sweeps in 2 different MG α-MNs from 2 different rats that had undergone acute, midcollicular decerebration, in which quick stretch was applied to the antagonist (TAEDL) muscles. Either polysynaptic eSSPs (A) or iSSPs (B) were evoked in all MG α-MNs from a given decerebrate preparation. (C) For all MG α-MNs sampled under decerebration, antagonist evoked eSSPs occurred with a mean central latency that was significantly longer (p<0.001, t-Test) than antagonist evoked iSSPs. All values for central latency fell within the range estimated for a di- or trisynaptic pathway in vivo in the mammalian spinal cord, and iSSP values are consistent with those mediated by IaINs (Eccles *et al.*, 1956; Jankowska & Roberts, 1972; Watt *et al.*, 1976; Jankowska *et al.*, 1992).
Synaptic Potential Produced in MG α-MN by:

A. Antagonist (TAEDL)
   Quick Stretch
   
   IC:
   - 0.5mV
   - -54mV
   
   EC:
   
   Aff:
   
   MG α-MN 1
   Central Latency: 3.4ms
   Amplitude: 1.1mV
   Rate of Rise: 0.4mV/ms

B. Antagonist (TAEDL)
   Quick Stretch
   
   IC:
   - 58mV
   - 0.5mV
   
   EC:
   
   Aff:
   
   MG α-MN 2
   Central Latency: 1.6ms
   Amplitude: -0.7mV
   Rate of Rise: -0.1mV/ms

C. Central Latency

   eSSP
   No. of observations
   1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 (ms)

   Antagonist (TAEDL) iSSP
   2 Rats, 9 MNs
   Mean ± SE: 2.0 ± 0.1ms

   Antagonist (TAEDL) eSSP
   3 Rats, 8 MNs
   Mean ± SE: 3.3 ± 0.3ms
   p=0.001
Figure 30. *Quick stretch of antagonist (MG) muscles causes the segmental synaptic excitation or inhibition of TAEDL α-MNs.* (A&B) Averaged intracellular, extracellular, and dorsal root records from approximately 100 sweeps in 2 different TAEDL α-MNs from 2 different rats anesthetized with K/X, in which quick stretch was applied to the antagonist (MG) muscles. Either polysynaptic eSSPs (n = 17; A), iSSPs (n = 1; not shown), or ‘mixed’ SSPs (n = 8; B), were evoked in TAEDL α-MNs following quick stretch of the MG muscle. (C) For all TAEDL α-MNs sampled, central latency measurements of antagonist evoked SSPs fell within the range estimated for a di- or trisynaptic pathway *in vivo* in the mammalian spinal cord, and iSSP latency values were consistent with those mediated by IaINs (Eccles *et al.*, 1956; Jankowska & Roberts, 1972; Watt *et al.*, 1976; Jankowska *et al.*, 1992). As expected, the physiologic activation of TAEDL muscle afferents produced marked eSSPs in TAEDL α-MNs at monosynaptic latencies (Eccles *et al.*, 1956; Henneman & Mendell, 1971; Watt *et al.*, 1976; Cope & Mendell, 1982a).
Synaptic Potential Produced in TAEDL α-MN by:

A. Antagonist (MG) Quick Stretch

- IC: 
  -62mV
- EC: 
- Aff: 

TAEDL α-MN 1
Central Latency: 3.5ms
Amplitude: 0.8mV
Rate of Rise: 0.5mV/ms

B. Antagonist (MG) Quick Stretch

- IC: 
  -61mV
- EC: 
- Aff: 

TAEDL α-MN 2
Central Latency: 2.2ms
Amplitude: -0.1mV
Rate of Rise: -0.2mV/ms

C. Central Latency

- eSSP: 14 Rats, 25 Mns
  Mean ± SE: 0.7 ± 0.1ms
- Antagonist (MG) eSSP: 8 Rats, 17 MNs
  Mean ± SE: 3.5 ± 0.4ms
- Antagonist (MG) iSSP: 6 Rats, 8 Mns
  Mean ± SE: 2.4 ± 0.1ms
**Figure 31. A mammalian assistive reflex circuit.** During locomotion, α-MNs are activated in phase with their corresponding IaINs (Geertsen et al., 2011). IaINs, in turn, inhibit antagonist the α-MNs (Geertsen et al., 2011) and the IaINs coupled to them (Hultborn et al., 1976; Pierrot-Deseilligny and Burke, 2012) In the diagram, the central pattern generator activates ankle extensor α-MNs in LIX, which cause the MG muscle to contract. The CPG simultaneously activates extensor-coupled IaINs in LVII, which inhibit flexor MNs and flexor-coupled IaINs. This prevents 1) TA muscle contraction and 2) reciprocal inhibition of extensor MNs from TA afferents. This circuit model is well-characterized, but does not address a potential role for proprioceptive afferents from the inactive, stretched muscle. Data from this chapter updates this circuit with an excitatory reflex pathway (Bold green line) relayed by interneurons residing in LV/VI and receiving convergent input from group I and group II muscle afferents (Jankowska & Edgley, 2010) provides positive feedback that reinforces the ongoing movement, as is observed during invertebrate locomotion.
CHAPTER VII: SK channel expression in mammalian lumbar $\alpha$-motoneurons following peripheral nerve injury

This chapter, which is being prepared for publication, addresses SA 5 of this dissertation.

Introduction

Peripheral nerve injury (PNI) causes major alterations in neuronal activity, excitability, and synaptic organization in the spinal cord (Cope & Clark, 1993; Cope et al., 1994; Haftel et al., 2005; Alvarez et al., 2011; Bullinger et al., 2011a; Prather et al., 2011). For example, reorganization of central circuits accounts, in part, for abnormal sensorimotor integration and stretch areflexia observed following nerve injury (Cope & Clark, 1993; Cope et al., 1994; Alvarez et al., 2011; Bullinger et al., 2011a). In addition, PNI induces well-documented alterations in $\alpha$-MN intrinsic electrical and morphologic properties (Kuno et al., 1974a, b; Gustafsson, 1979; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; MacDermid et al., 2002; MacDermid et al., 2004; Bichler et al., 2007a; Meehan et al., 2011; Romer et al., 2014).

Peripheral nerve injury and reinnervation studies depend on multiple factors (i.e. age, location of lesion, but several ‘early’ outcomes are consistently reported by a number of laboratories beginning with the early studies of Eccles and Kuno (e.g. (Eccles et al., 1958b; Kuno & Llinas, 1970a, b; Kuno et al., 1974a, b). Within several days, peripheral
nerve injury causes a reduction in α-MN rheobase, increase in input resistance, and a slowing of axonal conduction velocity (Kuno et al., 1974a, b; Gustafsson, 1979; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a). Interestingly, similar changes in rheobase are induced by α-bungarotoxin blockade of the neuromuscular junction (Nakanishi et al., 2005). In addition, there appears to be a slight prolongation in the AHP duration of MNs innervating slow twitch fibers and a lengthening of mAHP durations for MNs innervating fast twitch fibers (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a), such that the variability of α-MN mAHP durations across motor unit subtypes is reduced. Many of these membrane properties appear to return to pre-axotomy states following reinnervation of peripheral targets, particularly following crush injuries (Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a; Bullinger et al., 2011a; Prather et al., 2011).

Neuronal biophysical properties are controlled by the expression patterns and subcellular distribution of highly regulated ion channels (Magee, 2000; Lai & Jan, 2006; Cerda & Trimmer, 2010; Misonou, 2010; Duflocq et al., 2011). In the α-MN, many ion channels are non-uniformly distributed in the cell membrane, being either preferentially expressed in one or another cellular compartment or found within discrete microdomains and/or signaling ensembles (Carlin et al., 2000; Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Elbasiouny et al., 2005; Bui et al., 2006; Deardorff et al., 2013; Deardorff et al., 2014; Romer et al., 2014). The localization and density of membrane-bound ion channels within a particular subcellular domain is a critical factor regulating α-MN firing
properties, and may be dynamically modified by peripheral nerve injury (Romer et al., 2014).

Changes in mAHP conductance may be a major determinant in altered α-MN firing following nerve injury (Gustafsson, 1979; Titmus & Faber, 1990). However, molecular factors underlying injury induction alterations in mAHP properties are unknown. Because SK channels are responsible for generating α-MN mAHP currents, and SK3 subunits are preferentially expressed in α-MNs with long duration mAHPs (Deardorff et al., 2013), experiments in this aim analyze changes in SK3 channel expression at 8 days and 3 months following nerve crush.

**Methods**

All animal procedures conform to National Institutes of Health (NIH) guidelines and are approved by the Wright State University Institutional Animal Care and Use Committee. Detailed electrophysiological and immunohistochemical analysis are performed on adult female Sprague Dawley rats and adult female Wistar rats. Survival surgeries are performed in surgical suites located within the Laboratory Animal Resources at Wright State University.

**Peripheral nerve injury**

Peripheral axotomy was performed in adult female rats. Rats were deeply anesthetized (complete suppression of withdrawal and corneal reflex) with isoflurane (induction: 5% in 100% O₂; maintenance: 2-3% in 100% O₂; inhalation through nose cone) for treatment
in a single survival surgery performed under sterile conditions. A midline incision through the skin and biceps femoris in the posterior compartment of the left hindlimb expose the tibial nerve, which was dissected free from surrounding tissue within 1 cm of its bifurcation with the common peroneal nerve from the sciatic nerve. The tibial nerve was crushed between the prongs of no. 5 microsurgical forceps with firm pressure applied for 10 s, leaving the nerve intact but visibly compressed at the injury site. The wound was then irrigated and closed with suture ties. Anesthesia was discontinued, and the rat was monitored until recovering consciousness. Animals received 0.1 mL of 0.3 mg/mL buprenorphine every 12 h for post-operative pain management for 48 h and were monitored closely by professional staff. Animals were sacrificed for anatomical / in vivo physiological analysis 8 days or three months following nerve injury.

**Retrograde tracer injection**

Those animals used in retrograde tracer studies underwent an addition survival surgery, in which the medial gastrocnemius (MG) or soleus (Sol) muscles were injected with a fluorescent tracer to retrogradely label MG or Sol α-MNs for post hoc identification in the spinal cord. As described above, these surgeries were performed under sterile conditions on rats deeply anesthetized (complete suppression of withdrawal and corneal reflex) with isoflurane (induction: 5% in 100% O₂; maintenance: 2-3% in 100% O₂; inhalation through nose cone).

Rats studied 8 days after tibial nerve crush received retrograde tracer one week prior to nerve injury surgery. In these animals, a midline incision was made through the skin and
biceps femoris of the left distal hindlimb to expose the triceps surae muscles. A total of 50 µl of 0.5% Cholera Toxin Subunit B-555 (CTB-555, Invitrogen, Carlsbad, CA, USA) was administered by a series of small injections throughout the MG or Sol muscle. The wound is subsequently irrigated and closed in layers. Immediately after wound closure in the left hindlimb, a small midline incision was made through the skin and biceps femoris of the right distal hindlimb to inject a total of 50 µl of 0.5% Cholera Toxin Subunit B-488 (CTB-488, Invitrogen, Carlsbad, CA, USA) into the MG or Sol muscle of the right hindlimb. The wound was subsequently irrigated and closed in layers and anesthesia removed. Animals receive 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours for post-operative pain management for 48 hours and are monitored closely by professional staff.

Retrograde tracer in the right leg served as a contralateral control, and therefore the same muscle injected with CTB-555 in the left leg was injected with CTB-488 in the right leg.

Previous studies (Bullinger et al., 2011a) and our own observations (i.e. whole muscle twitch with nerve stimulation) demonstrate that axons sufficiently reinnervate peripheral targets within three months. Rats studied 3 months after tibial nerve crush received retrograde tracer one week prior to sacrifice. The triceps surae of the left hindlimb were exposed under deep anesthesia and sterile operating procedures, as described above, and a total of 50 µl of 0.5% Cholera Toxin Subunit B-555 (CTB-555, Invitrogen, Carlsbad, CA, USA) was administered by a series of small injections throughout the MG or Sol muscle. The wound is subsequently irrigated and closed in layers, and anesthesia was removed. Animals receive 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours for post-operative pain management for 48 hours and are monitored closely by professional staff.
**In-vivo electrophysiology**

Eight rats were studied in single terminal recording sessions. Deep anaesthesia (absent withdrawal and corneal reflex) was induced by isoflurane (4–5% in 100% O2, by inhalation in an induction chamber) and maintained by isoflurane (1–3% in 100% O2, by inhalation through a tracheal cannula). The animal was monitored for respiratory rate (40–60 breaths / min), end-tidal CO2 (3–5%), oxygen saturation (>90%), heart rate (300–500 beats / min) and core temperature (36–38°C). These levels were variously maintained by adjusting the isoflurane concentration and radiant heat sources and by scheduled subcutaneous injection of Ringer–dextrose solution. In some cases, adequate recording stability required intraperitoneal injection of a muscle relaxant drug (pancuronium bromide 0.2 mg / kg).

Standard procedures were used to prepare the spinal cord and left hindlimb for electrophysiological stimulation and recording, with the animal secured in a rigid frame (Seburn & Cope, 1998; Haftel *et al.*, 2004; Haftel *et al.*, 2005; Bullinger *et al.*, 2011a; Deardorff *et al.*, 2013). The left tibial nerve was carefully dissected free of other tissues and suspended on a monopolar, silver stimulating electrode. Other hindlimb nerves were crushed, including the common peroneal nerve and sural nerves. Dorsal exposure of the lumbosacral spinal cord (L4–S1) by laminectomy and longitudinal incision of the dura mater provided access to dorsal roots L4 and L5, which were carefully dissected free of surrounding tissue and suspended in continuity on bipolar silver hook electrodes for recording, and tibial motoneurons via dorsolateral penetration of the spinal cord. Skin flaps were used to construct pools for bathing all exposed tissues with warm mineral oil.
In vivo intracellular recordings were made from antidromically identified tibial α-MNs using glass micro-electrodes (≈10–25 MΩ) filled with 10% neurobiotin (Vector Laboratories, Burlingame, CA, USA) and 5% 488 dextran (Invitrogen, Grand Island, NY, USA) in 0.1 M Tris-OH and 1.0 M potassium acetate. Only those MNs with stable membrane potential and with action potential amplitude >60 mV were deemed acceptable for further study. Failure to meet these criteria accounts for the incomplete data set obtained from some MNs. Motoneurons were injected with current through the micropipette in order to measure the following intrinsic electrical properties as previously reported in our laboratory (e.g. (Bullinger et al., 2011a; Deardorff et al., 2013)): rheobase current (depolarizing pulses, 50 ms in duration, at the lowest strengths capable of initiating action potentials from the resting membrane potential), AHP (following individual action potentials generated by suprathreshold pulses, 0.5 ms in duration), input resistance (from hyperpolarization produced by −1 and −3 nA current pulses, 50 ms in duration) and axon conduction velocity (antidromic conduction delay divided by tibial nerve length). In some animals, tibial nerve lengths and thus conduction velocity were not measured. Following physiological characterization, motoneurons were labeled by intra-cellular injection of 10% neurobiotin with 5% dextran in Tris Buffer (0.1M Tris-OH, 1M Potassium Acetate, PH 7.6). Positive current pulses (5nA), delivered as 400-ms-long pulses at 2 Hz for 2–5 min, were used to aid neurobiotin passage into the motoneuron. Records of MN membrane potential and electrode current were collected, digitized (20 kHz), stored and analysed with CED Power 1401 and CED Spike2 software (Cambridge Electronic Design, Cambridge, UK).
After data collection, animals were killed by an intra-peritoneal overdose of pentobarbital (150 mg / kg I.P.) and perfusion fixed as described in above methods. Spinal segments containing intracellularly labeled motoneurons were postfixfixed overnight. Serial transverse sections (75µm thick) were obtained on a vibrating microtome and were initially examined for dextran immunofluorescence using a fluorescence dissecting microscope. Those sections containing intracellularly labeled motoneurons were serially processed for SK3 immunoreactivity as described below. To enhance intracellular labeling, neurobiotin was visualized with 488 streptavidin (Invitrogen; diluted 1:1000 in 0.01 M PBS containing 0.1% Triton X for 2 h at room temperature).

**Immunohistochemistry**

All animals were anesthetized with pentobarbital (150 mg/kg, i.p) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3. The spinal cords were removed, post fixed for 2 h and stored in 0.1 M phosphate buffer. Serial transverse sections (75 µm thick) obtained from the L4 and L5 spinal cord segments on a vibrating microtome immunostained free floating. Sections were rinsed with PBS-T (0.01 M PBS containing 0.1% Triton-X, pH7.3), blocked with normal horse serum (10% in PBS-T), and then incubated in primary antibodies or cocktails of primary antibodies overnight at 4°C. All antibodies were diluted with PBS-T. The SK channel immunoreactivity was localized with polyclonal rabbit anti-SK3 (Chemicon, Temecula, CA, USA; 1:1000 dilution) directed against amino acid residues 2–21 of human SK3. The specificity of the primary antibodies has been described (Khanna *et al.*, 2001; Tacconi *et al.*, 2001; Armstrong *et al.*, 2005; Deardorff *et al.*, 2013) and was confirmed
here using Western blotting and pre-absorption of the primary antibodies with the peptide antigen. Western blotting of membranes prepared from rat spinal cord tissue and probed with the anti-SK3 antibody revealed that the antibody recognizes a single ~70 kDa molecular weight protein band, and this labeling was abolished by pre-absorption of the antibody with the peptide antigen (Alomone, Jerusalem, Israel; 1 µg peptide/µg antibody). All primary antibodies were diluted in PBS-T 0.1%, pH7.4, and incubated overnight at 4°C. Immunoreactivity was detected with species-specific secondary antibodies conjugated to DyLight 649 (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:50 in PBS-T 0.1%, pH 7.4, and incubated at room temperature for 2–4h. Sections were mounted on gelatin-coated slides and coverslipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

**Confocal Imaging and Analysis**

Images were obtained on a Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope with a ×10 objective at 2.0 µm Z-steps, ×20 objective at 1 µm Z-steps, and ×60 oil immersion objective at 0.5 µm steps at 1.0–2.5 digital zoom (NA 1.35). Channel cluster dimensions (maximal diameter and cluster area) and were measured with ImagePro Plus software (Media Cybernetics, Silver Springs, MD, USA). Immunoreactivity for SK3 was considered present if the immunoreactive intensity was at least twofold increased over background. Background intensity was calculated by averaging three randomly sampled areas within the neuropil per image analysed. Physiology was analyzed with Spike2 software. Significance was set at P < 0.05 with Statistica software (Statsoft).
**Figure Composition**

Figures were composed using CorelDRAW (version 15.0). Graphs were composed in Statistica (Statsoft). Physiological traces are prepared with Spike2 software. In some cases, traces are smoothed, DC removed, and/or filtered against 60 Hz background noise, but always preserve the information content of the original trace. Microscope image modifications for presentation, such as adjusting contrast and brightness, were carried out in Image Pro Plus (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Some images were sharpened using a ‘high-gauss’ filter. Quantification was always carried out using original unprocessed images and traces.

**Results**

**SK3 expression increases but cluster sizes decrease following tibial nerve crush**

Following tibial nerve crush, the number of SK3-expressing MNs in the lumbar spinal cord increases. Figure 32 shows representative histologic images from the lumbar spinal cord 8 days after nerve crush surgery, in which ipsilateral (injured; red) and contralateral (injury-spared; green) MG MNs were retrogradely labeled. SK3 immunofluorescence is increased in all MN pools innervated by the tibial nerve following injury (Figure 32A&B). High magnification images demonstrate that a minority MG α-MNs in the contralateral spinal cord express SK3, while a majority of injured MG MNs express SK3 (Figure 32C&D); however, many of these SK3 clusters are reduced in size (Figure 32 insets). The mean surface area of SK3 channel clusters in uninjured rat lumbar α-MNs is 9.8 μm² (S.D. ± 4.3), compared to 3.3 μm² (S.D. ± 1.8; p<0.001 t-Test). Importantly, previous studies performed in our laboratory confirm that mean α-MN soma diameter is...
unaffected by the tibial nerve crush injury used in this study (Romer et al., 2014). It is therefore unlikely the observed changes in SK3 cluster area following tibial nerve injury are confounded by parallel changes in α-MN size. No lateral translocation of SK channels was observed, as occurs with α-MN Kv2.1 channels following nerve injury (Romer et al., 2014).

Quantitative analysis of retrogradely labeled cells confirms the proportion of SK3-expressing α-MNs innervating the predominately fast-twitch MG muscle increases (Figure 33A) from 18.5% (n=138 uninjured, contralateral α-MNs, 2 animals) to 83.7% (n = 147 injured α-MNs, 2 animals) 8 days following peripheral nerve crush. Moreover, the proportion of SK3-expressing α-MNs in the predominately slow twitch Sol muscle (Figure 33B) increases from 53.8% (n = 100 contralateral α-MNs, 1 animal) to 96.3% (n = 117 injured α-MNs, 1 animal). At 3 post nerve crush, after reinnervation of peripheral targets, proportions of SK3 expressing MNs in MG (20.2%; n = 190 α-MNs; 2 animals) and Sol (59.4%; n = 214 α-MNs; 2 animals) muscles approximate pre-injury levels (Figure 33A&B), consistent with observations that α-MN membrane properties return to pre-axotomy states following reinnervation of the original muscle (Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a; Bullinger et al., 2011a; Prather et al., 2011).

mAHP properties are altered following tibial nerve crush

Eight days after nerve crush, mAHP properties of tibial MNs recorded in this aim are consistent with those previously reported (Kuno et al., 1974a, b; Gustafsson & Pinter,
1984; Foehring et al., 1986b, a). Mean mAHP ½ decay time is slightly, but non-significantly, increased [control: 13.5 ± 4.7 ms (mean ± SD); injured: 15.2 ± 3.2 ms (mean ± SD); p=0.133 t-Test] while coefficient of variation is reduced (control: C.V. = 0.34; injured: C.V. = 0.21).

Subgroup analysis of intracellularly labeled tibial MNs demonstrates several differences in SK3-expressing α-MNs after nerve crush compared to the healthy control cells analyzed in specific aim 1. First, the mAHP duration of injured SK3 expressing MNs is significantly shorter [control: 24.7 ± 4.9 ms (mean ± SD); injured: 15.9 ± 1.7 (mean ± SD); p<0.01 t-Test] with reduced coefficient of variability (control: C.V. = 0.19; injured: C.V. = 0.1) (Figure 34A). Second, in a scatterplot of mAHP duration vs rheobase, SK3-expressing α-MNs in injured animals do not cluster in the region occupied by healthy SK3-expressing α-MNs (Figure 34B; see also Chapter IV). Of note, the region occupied by healthy SK3-expressing α-MNs typically represents α-MNs innervating slow twitch muscle fibers (Bakels & Kernell, 1993b; Gardiner, 1993).

Discussion

The α-MN is the archetypal cell for studying central neural function, and characterizing its responses to injury is of paramount importance for considering the effects of injury on other CNS neurons. Axotomy causes significant changes in α-MN morphology, including ‘axon-like’ growth and protein expression from distal dendritic processes (MacDermid et al., 2002; MacDermid et al., 2004; Meehan et al., 2011). Similar axonal sprouting has been observed in spinal commissural neurons following axotomy (Fenrich et al., 2007).
Nerve injury also leads to alterations in α-MN intrinsic electrical properties, including reductions in rheobase, increases in input resistance, slowing of axonal conduction velocity, and a ‘de-differentiation’ of mAHP duration (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a).

To better understand the relationship between SK channel expression and α-MN mAHP properties following peripheral nerve injury, SK3 channel immunoreactivity was analyzed in retrogradely labeled MG and Sol α-MNs 8 days and three months following peripheral nerve crush. In a separate series of experiments, intracellular records were obtained in vivo from tibial α-MNs 8 days and 3 months following tibial nerve crush. Following the collection of intracellular data, α-MNs were intracellularly labeled and spinal cords were subsequently processed for SK3 channel immunoreactivity. Results indicate that SK3 is consistently expressed in a majority of α-MNs in MG and Soleus motor pools, following crush injury to the tibial nerve, and is associated with mAHP durations that cluster tightly around the mean mAHP duration for all α-MNs analyzed. Proportions of SK3 expressing α-MNs in MG and Soleus motor pools return to approximately pre-injury levels upon successful reinnervation. These data support the hypothesis that SK3 channel expression is altered following peripheral nerve injury and provide additional insight into alterations in α-MN mAHP properties following nerve injury.
Subcellular distribution of SK3

The intrinsic electrical properties of spinal α-MNs depend on tightly regulated ion channels, and the functional roles of many of these channels are uniquely tied to their localization in specific subcellular compartments (Carlin \textit{et al.}, 2000; Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Elbsaiouny \textit{et al.}, 2005; Bui \textit{et al.}, 2006; Deardorff \textit{et al.}, 2013; Deardorff \textit{et al.}, 2014). Ion channel localization and density in the surface membrane can be dynamically modulated at a number of levels, including gene expression, posttranslational modification, protein trafficking / membrane insertion, associations with macromolecular complexes / scaffolding proteins, and interactions with neurotransmitter receptors and second messenger systems. It is likely that injury differentially affects the regulation of a number of ion channels, and recent evidence from our laboratory has shown the delayed rectifier $K_v2.1$ is dramatically redistributed in the α-MN membrane following peripheral nerve injury (Romer \textit{et al.}, 2014).

In α-MNs, SK channels are highly enriched in large clusters that are exclusively postsynaptic to C-bouton synapses. Moreover, SK channels are co-expressed with $K_v2.1$ and type 2 muscarinic acetylcholine receptors (Deardorff \textit{et al.}, 2014) in the α-MN membrane. Data presented in this aim, demonstrate that unlike $K_v2.1$, SK channels do not laterally translocate in the membrane following peripheral nerve injury. This differential response to injury may represent alternative coupling strategies to the postsynaptic domain. $K_v2.1$ clustering is highly phosphorylation dependent (Murakoshi \textit{et al.}, 1997; Misonou \textit{et al.}, 2004; Park \textit{et al.}, 2006; Zhang \textit{et al.}, 2008). Phosphorylated channels are corralled into and display lateral mobility within an actin-based cytoskeletal
fence (Tamkun et al., 2007). By contrast, SK channel clustering is independent of phosphorylation state (Fakler & Adelman, 2008). Moreover, in α-MNs, SK channel clusters are composed of an intricate, nonuniform aggregation of smaller “threadlike structures that are woven together, perfectly co-localize with m2 receptors, and align with presynaptic vesicle release sites and connexin32 on the subsurface cistern (Deardorff et al., 2014). Though the surface area of SK channel clusters significantly decreases following peripheral axotomy, this threadlike substructure remains (Figure 32C&D insets). The precise alignment of proteins across the three membranous domains at C-bouton synaptic sites, and its apparent persistence following peripheral nerve injury, strongly suggests that SK channels (and m2 receptors) are tethered to specific locations within this submembrane domain, rather than being simply corralled within an actin fence, as is Kv2.1. Future studies investigating this tethering mechanism will useful in understanding neuronal substructure and its response to injury/disease as well as trophic interactions between pre- and postsynaptic neurons (See below).

**Synaptic Stripping**

Many synaptic inputs onto α-MNs are lost following peripheral nerve injury, including a permanent loss of primary sensory afferents and a transient reduction in synaptic coverage from cholinergic C-type synapses (Blinzinger & Kreutzberg, 1968; McLaughlin, 1972a; Sumner, 1975; Brannstrom & Kellerth, 1998, 1999; Gonzalez-Forero et al., 2004; Hughes et al., 2004; Alvarez et al., 2011; Bullinger et al., 2011a; Rotterman et al., 2014). Following peripheral nerve injury, C-type synaptic contacts are displaced from the α-MN somatic membrane and the subsurface cistern is reduced in size (Sumner, 1975; Alvarez
et al., 2011). The time courses of these changes parallel the reduction in SK3 cluster size observed in this aim, strongly indicating tropism between C-boutons, SK channels, and the subsurface cistern. Interestingly, in the cochlear hair cell, trophic interactions between SK channel clusters, nicotinic acetylcholine receptors, and cholinergic presynaptic terminals have been described (Kong et al., 2008). In these cells, SK channel clusters are necessary for both the expression of nicotinic acetylcholine receptors and the formation of cholinergic presynaptic terminals (Kong et al., 2008). Better understanding of the trophic interactions between SK channels, m2 receptors, connexin32, and C-bouton presynaptic terminals will provide critical information on the organizing principles around which these unique synapses are formed and, more importantly, how the α-MN responds to injury / disease.

**SK3 channel expression and mAHP properties**

Physiological data collected in this are consistent with previously reported physiological observations following peripheral nerve injury (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a; Prather et al., 2011), including a reduced variability and non-significant increase in mean mAHP duration. The changes in SK3 expression demonstrated in this aim provide a potential molecular mechanism for these well documented alterations in mAHP duration. First, SK3 has slower deactivation kinetics than SK2 (Xia et al., 1998). Therefore, that nearly all α-MNs express SK3 after injury could account for the prolongation of mAHP properties and the increase in minimum mAHP duration observed in this and other studies [Figure 34]; (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al.,
Second, SK3 expression is no longer correlated with the very long duration mAHPs in injured MNs. Rather, SK3 is consistently expressed in a majority of α-MNs and underlies mAHP durations that cluster tightly around the mean mAHP duration for all α-MNs analyzed. This may reflect the decreased SK3 cluster sizes observed in injured MNs, though additional critical factors (i.e. differential coupling of SK channels to Ca\(^{2+}\) sources, the presence of hyperpolarization-activated currents, and/or the synaptic modulation of AHP kinetics) may also need to be considered. Nevertheless, the altered proportion of SK2:SK3 channel isoforms in a given α-MN is likely to play a role in regulating α-MN mAHP duration following nerve injury, and that the mean mAHP duration for SK3-expressing α-MNs decreases following peripheral nerve injury could account for decrease in maximum mAHP duration observed in this and other studies [Figure 34; (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986a)]. Importantly, upon successful reinnervation, mAHP properties and SK3 channel expression return to pre-injury levels (Kuno et al., 1974a, b; Gustafsson & Pinter, 1984; Foehring et al., 1986b, a; Nakanishi et al., 2005; Bichler et al., 2007a; Bullinger et al., 2011a; Prather et al., 2011).

**Conclusion**

Data in this aim show that SK3 expression is observed in nearly all MG and Sol MNs 8 days after nerve injury. However, the mean mAHP duration in tibial α-MNs is only slightly increased at this time. This paradoxical finding is explained by subgroup analysis that demonstrates SK3 expression is no longer correlated with long duration mAHPs in injured MNs. Rather, SK3 is consistently expressed in a majority of α-MNs and
underlies mAHP durations that cluster tightly around the mean mAHP duration for all α-MNs analyzed. This may reflect the decreased SK3 cluster sizes observed in injured MNs, though additional critical factors may also be involved. Nevertheless, an altered proportion of SK2:SK3 channel isoforms in a given α-MN is likely to play a role in regulating α-MN mAHP duration following nerve injury.
Figure 32. **SK3 is expressed in more lumbar α-MNs after tibial nerve crush.**

Representative histologic sections from left and right sides of the lumbar spinal cord in an adult rat 8 days after crush injury of the left tibial nerve. **(A&B)** Confocal stacks (13x2μm Z-steps) showing SK3 immunofluorescence is increased in motor pools innervated by the tibial nerve. Representative MG MN retrograde labeling is also observed. Scale bar is 200 μm. **(C&D)** High magnification (60x) of single optical sections of retrogradely labeled MG MNs. Arrows indicate SK3-IR. Note 2 of 2 injured MG MNs express SK3 compared with only 1 of 3 injury-spared MG MNs. Scale bar is 20 μm. Insets demonstrate size differences *en face* single SK3-IR clusters (2 μm scale).
Figure 33. *Proportion of SK3(+) MG and Sol α-MNs is increased following tibial nerve injury*. The percentage of MNs expressing SK3 in the MG (A) and Sol (B) muscles increases 8 days after tibial nerve crush and returns to pre-injury levels after reinnervation has occurred. Control values were measured in retrogradely labeled, injury-spared MG or Sol MNs in the contralateral spinal cord of the same rats analyzed for SK3 expression in injured MG or Sol MNs 8 days after nerve crush. (A) Control and 8 day post crush data is from 138 and 147 α-MNs, respectively, in the same two animals. 3 month post crush data is from 190 α-MNs in 2 animals. (B) Control and 8 day post crush data is from 100 and 117 α-MNs, respectively, in the same one animal. 3 month post crush data is from 214 α-MNs in 2 animals.
Figure 34. *AHP properties and SK3 immunoreactivity in physiologically characterized tibial α-MNs following tibial nerve crush.* (A) The mean mAHP ½ decay time in SK3-expressing tibial MNs is shortened (24.7 ± 4.9 ms vs 15.9 ± 1.7; mean ± SD; p<0.01, t-Test) 8 days following tibial nerve crush. Control data is from 7 SK3(+) cells in 6 animals. Injury data is from 10 SK3(+) cells in 4 animals. (B) SK3(+), injured α-MNs do not occupy the same position in a scatterplot of mAHP ½ decay tie vs rheobase as SK3(+) injury-spared MNs (see also Chapter 5).
CHAPTER VIII: General Conclusion

The neural control of movement defines our relationship with the world. For centuries, its analysis has provided insight into how cognition and behavior arise from the properties of individual neurons and their synaptic connections. To this end, the α-MN has been the exemplary cell for studying neural function. The α-MN filters discordant neuronal signals from countless sources and transforms them into cohesive and effective movement. The failure to do so, by development or disease, significantly reduces extent and/or quality of life for those afflicted. Undoubtedly, the development of new therapeutic interventions for many devastating neurological diseases will rely on both a complete understanding of the molecular mechanisms that underlie α-MN spiking behavior as well as a comprehensive characterization of α-MN synaptic inputs. Using a combination of electrophysiological and anatomical methods, this dissertation has advanced knowledge regarding several of these critical factors, including the intrinsic electrical properties of α-MNs as well as and the integration, source, and function of their synaptic inputs.

Intrinsic Properties

The pattern of SK channel expression described in this dissertation (a) gives critical insight into the molecular factors underlying α-MN mAHP variability and (b) details the precise cellular organization of membrane properties that regulate α-MN firing.
capabilities across motor unit types. These results support the hypothesis that SK3 is
differentially expressed in α-MN subtypes and provide new insight into the
organization of membrane properties across motor unit subtypes. The relative proportion
of SK2/SK3 isoforms regulates mAHP duration and amplitude, and the variability of
these proportions accounts, in part, for the fact that AHP properties are continuous
variables across a population of α-MNs. Because SK3-expressing α-MNs share other
physiological properties predictive of slow MNs (i.e., slower conduction velocity, lower
rheobase, and higher input resistance), SK channel immunoreactivity provides a new tool
for investigations requiring anatomical identification of α-MNs across their physiological
subtype. Moreover, SK channel expression may provide a window through which we can
further understand MN physiology, development, and disease (Brownstone & Magown,
2013). In particular, the pattern of expression described in this aim may be highly
relevant for studying α-MN development and diseases such as nerve injury, which causes
distinct changes in α-MN mAHP currents, and amyotrophic lateral sclerosis, in which
fast MNs are the most susceptible during disease progression (Brownstone & Magown,
2013).

This dissertation demonstrates a molecular basis for the well documented alterations in α-
MN membrane properties following peripheral nerve injury, for which mechanistic data
is surprisingly lacking. Results show that after nerve injury SK3 is consistently expressed
in a majority of α-MNs and, therefore, support the hypothesis that peripheral nerve
injury disrupts SK channel expression in lumbar α-MNs. It is important to note that SK3 expression is no longer correlated with long duration mAHPs in injured MNs. Rather, SK3 underlies mAHP durations that cluster tightly around the mean mAHP duration for all α-MNs analyzed. Caution must be exercised, however, in ascribing mAHP properties to SK3-expressing α-MNs in studies of disease without corresponding physiological data. These changes in mAHP-SK3 coupling may reflect the decreased SK3 cluster sizes observed in injured MNs, though additional critical factors (i.e. differential coupling of SK channels to Ca^{2+} sources, the presence of hyperpolarization-activated currents, and/or the synaptic modulation of AHP kinetics) may also need to be considered. Nevertheless, the altered proportion of SK2:SK3 channel isoforms in a given α-MN is likely to play a role in regulating α-MN mAHP duration following nerve injury.

Neuromodulatory Mechanisms: C-boutons

For more than 50 years, the C-type synapse has generated sustained interest among α-MN anatomists and physiologists. Using immunohistochemical labeling, this dissertation reveals SK2 and SK3 channels are organized in a highly clustered pattern, are restricted to the soma and proximal dendrites of α-MNs, and selectively localized at large synapses formed by cholinergic C-boutons. Recent investigations have demonstrated that C-type synaptic inputs increase α-MN excitability by reducing mAHP currents (Miles et al., 2007; Zagoraiou et al., 2009). The pattern of SK channel expression described in this aim, therefore, provides an anatomical basis for these physiological data and supports the hypothesis that SK channels in α-MNs localize postsynaptic to C-boutons. These data, interpreted alongside an immunohistochemical analysis of C-bouton protein expression
and an extensive review of C-bouton literature, demonstrate C-bouton synaptic sites comprise a complex signaling ensemble that is organized around a local Ca^{2+} signal to modulate outward K^{+} currents. Importantly, the features of the C-bouton signaling ensemble described in this dissertation support the hypothesis that C-bouton postsynaptic sites comprise a structural/functional domain organized for the precise regulation of somatic K^{+} currents. Moreover, the signaling ensemble shows key similarities and differences to protein ensembles in several well-characterized neuronal systems. Analysis performed in this dissertation therefore generates a comprehensive theoretical framework for C-bouton synaptic mechanisms which account for the C-bouton’s minimal effect during conditions of low and/or transient drive and extends this notion to conditions of extremely powerful physiological (or pathological) drive. This framework provides new testable hypotheses to aid in the development of new in vivo and in vitro experimental strategies to better understand C-bouton function in health and disease.

**Synaptic Mechanisms – Proprioceptive Input**

Proprioceptive feedback from the antagonist muscle is critical to regulating motor pools and their constituent α-MNs. During locomotion, proprioceptive input from antagonist muscles is critical for adjusting the relative timing, phase, and amplitude of antagonist muscle activity and is particularly important when navigating an uncertain and changing environment (Pearson, 1995a; Clarac et al., 2000; Akay et al., 2014). However, current models of spinal circuits responsible for rhythmic flexor-extensor alternation do not adequately account for the role of proprioceptive feedback from the inactive, stretched
muscle. Though experiments in this dissertation sought to examine the effects of somatic and proximal dendritic inhibitory inputs from IaINs on medial gastrocnemius (MG) α-MN firing properties, they instead produced the unexpected finding of activating an excitatory synaptic circuit between TAEDL afferents and MG α-MNs, and vice versa.

**These experiments extensively characterize, for the first time in the adult rodent, a short latency Ia reciprocal excitatory pathway between antagonist muscles.**

Furthermore, Chapter VI of this dissertation describes the necessity for and spinal mechanisms underlying reductions in reflex reciprocal inhibition during ongoing concentric contractions of the antagonist muscle (e.g. during stance phase of locomotion), and updates current circuit diagrams to account for proprioceptive feedback via reciprocal excitation. These analyses, therefore, provide critical new insight how antagonist α-MNs are coordinated in the production of purposeful movement.

**Concluding Thoughts**

Defining the elemental features of neuronal wiring poses a significant challenge. Like the work of so countless others, this dissertation begins at the end, exploiting advantages inherent in motor control systems to unravel the threads of circuit function. Here, I leave the reader with three take away messages from the work presented in this dissertation, in hopes that it will advance our understanding not just motor function, but neural function as well. 1) A differential distribution of ion channels accounts, in part, for differences in firing properties between early and late recruited α-MNs. 2) The α-MN membrane is not homogenous, but rather contains distinct microdomains that are precisely organized to regulate α-MN firing capabilities. And finally, 3) Our complete and full repertoire of
movement is regulated by a variety of segmental synaptic circuits onto the $\alpha$-MN, including excitation from proprioceptors from antagonist muscles.
CHAPTER IX: References


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### Appendix A: Commonly Used Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>IaIN</td>
<td>Ia inhibitory interneuron</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
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<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>AHP</td>
<td>afterhyperpolarization</td>
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<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<tr>
<td>CaV</td>
<td>voltage-gated calcium channel</td>
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<tr>
<td>CK2</td>
<td>casein kinase 2</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CP</td>
<td>common peroneal</td>
</tr>
<tr>
<td>DMT</td>
<td>dimethytryptamine</td>
</tr>
<tr>
<td>DP</td>
<td>deep peroneal</td>
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<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
</tr>
<tr>
<td>E-PTA</td>
<td>ethanolic phosphotungstic acid</td>
</tr>
<tr>
<td>eSSP</td>
<td>excitatory stretch synaptic potential</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated cation channel</td>
</tr>
<tr>
<td>HVA</td>
<td>high voltage-activated</td>
</tr>
<tr>
<td>Ih</td>
<td>hyperpolarization-activated cation current</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity</td>
</tr>
<tr>
<td>INMT</td>
<td>Indole-N-methyl transferase</td>
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<tr>
<td>IPSP</td>
<td>inhibitory post synaptic potential</td>
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<tr>
<td>iSSP</td>
<td>inhibitory stretch synaptic potential</td>
</tr>
<tr>
<td>Kv</td>
<td>voltage gated potassium channel</td>
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<tr>
<td>K/X</td>
<td>ketamine/xylazine</td>
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<tr>
<td>LG</td>
<td>lateral gastrocnemius</td>
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<tr>
<td>m2 receptor</td>
<td>Type 2 muscarinic acetylcholine receptor</td>
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<tr>
<td>mAHP</td>
<td>medium duration afterhyperpolarization</td>
</tr>
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<td>MG</td>
<td>medial gastrocnemius</td>
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<tr>
<td>n</td>
<td>nerve</td>
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<td>neuregulin 1</td>
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<td>nAChR</td>
<td>nicotinic acetycholine receptor</td>
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<td>PIC</td>
<td>persistent inward current</td>
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<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
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<td>protein kinase C</td>
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<tr>
<td>PNI</td>
<td>peripheral nerve injury</td>
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<tr>
<td>PPS</td>
<td>pulses per second</td>
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<td>rER</td>
<td>rough endoplasmic reticulum</td>
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<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>S1R</td>
<td>sigma 1 receptor</td>
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<tr>
<td>SCI</td>
<td>spinal cord injury</td>
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<td>small conductance calcium-activated potassium channel</td>
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<tr>
<td>Sol</td>
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<td>superficial peroneal</td>
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<tr>
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<td>subsurface cisternae</td>
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<tr>
<td>TA</td>
<td>tibialis anterior</td>
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<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
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<tr>
<td>TAEDL</td>
<td>tibialis anterior and extensor digitorum longus</td>
</tr>
<tr>
<td>VACHT</td>
<td>vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>VGluT</td>
<td>vesicular glutamate transporter</td>
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