The Organization of Kv2.1 Channel Proteins in the Membrane of Spinal Motoneurons: Regulation by Injury and Cellular Activity

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THE ORGANIZATION OF KV2.1 CHANNEL PROTEINS IN THE MEMBRANE OF SPINAL MOTONEURONS: REGULATION BY INJURY AND CELLULAR ACTIVITY

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

By

Shannon Hunt Romer
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M.S., Wright State University, 2005

2015
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Shannon H. Romer, ENTITLED The Organization of Kv2.1 Channel Proteins in the Membrane of Spinal Motoneurons: Regulation by Injury and Cellular Activity BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

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ABSTRACT

Romer, Shannon Hunt Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2015. The Organization of Kv2.1 Channel Proteins in the Membrane of Spinal Motoneurons: Regulation by Injury and Cellular Activity.

The intrinsic membrane properties of neurons in the central nervous system are controlled by the tight regulation of membrane-bound ion channels. Rather than existing as static entities operating only in opened or closed states in fixed locations, ion channels are dynamic molecules with the capacity to adopt multiple functional states through conformational changes and/or post-translational modification - enabling flexibility in their activity. Furthermore, the location of ion channels within certain membrane compartments and/or signaling ensembles is critical to synaptic integration and shaping of firing properties, and can also be dynamically modified by changes in neuronal activity and pathology. In mammalian motoneurons, Kv2.1 channels, which underlie delayed rectifier potassium currents, form distinct clusters, and together with other components, are assembled into a highly regulated signaling ensemble. In the ‘typical’ clustered state, these channels are phosphorylated, have slow gating kinetics and help maintain motoneuron repetitive firing. However, following pathological or prolonged excitatory drive, Kv2.1 channels are rapidly dephosphorylated by the protein phosphatase calcineurin, which has two consequences. First, Kv2.1 channels decluster and spread out in the
membrane of soma and proximal dendrites. Secondly, the channels open earlier in the time course of the action potential and stay open longer and serve to homeostatically lower firing rate. Thus, Kv2 channels have the unique capacity to both increase or decrease neuronal excitability. Characterizing the dynamic changes of Kv2.1 in motoneurons will provide insights into the homeostatic regulation of firing rate through dynamic clustering and channel kinetics and will be key to interpreting pathophysiological changes in future studies.
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DEDICATION

For Eric and Savannah

And

For William, Wanda, Ryan and Nathaniel
Chapter I. Background
Motor systems of the brain and spinal cord enable essential functions such as breathing, communicating through speech and gesture, maintaining posture and balance, as well as conducting skilled and purposeful movements. Early in the 20th century it was recognized that lower α-motoneurons (αMNs) were the final common pathway through which all motor systems converge (Sherrington, 1906) and that these MNs must generate repetitive trains of action potentials to initiate muscle contraction, thus movement (Fulton, 1926; Eccles & Hoff, 1932). It is now known that intrinsic membrane electrophysiological properties (e.g. afterhyperpolarization (AHP), rheobase, input resistance, etc) shape repetitive firing patterns of MNs (Viana et al., 1995; Meunier & Borejsza, 2005) and that these properties are framed, in large part, by membrane-bound ion channels. The elegant control of αMN repetitive firing properties is therefore both highly conserved throughout mammalian and non-mammalian species, and inherently dynamic, to provide the ability to both perform a wide variety of motor output tasks and to respond to unexpected threats and perturbations (Ladle et al., 2007; Miri et al., 2013).
Potassium Ion Channels

The fundamental components that constitute excitable cells are molecular pores in the cell membranes that form ion channels. Of all the ion channel types (i.e. \( \text{Na}^{2+} \), \( \text{Ca}^{2+} \), etc.), potassium (\( \text{K}^{+} \)) channels are the largest, most impressively diverse, and are ubiquitously expressed throughout the animal kingdom. In neurons, \( \text{K}^{+} \) currents through these channels have numerous roles, such as establishing resting membrane potentials in addition to regulation and generation of excitability, action potentials and repetitive firing properties. The diversity in \( \text{K}^{+} \) ion channel function can partially be attributed to the variability in gating kinetics of different channel types, e.g. some channels open after the membrane is depolarized others when hyperpolarized, some open quickly others slowly, some are more conductive than others, and finally some are modulated by intracellular messengers and others neurotransmitters. Well over 100 distinct pore-forming, or principal, \( \alpha \)-subunits of the \( \text{K}^{+} \) channel family have been identified, as well as auxiliary proteins (i.e \( \beta \) subunits) that can, sometimes significantly, alter channel properties (Coetzee et al., 1999). \( \text{K}^{+} \) channel subunit properties can also be regulated via post-translational modification (Misonou et al., 2004; Park et al., 2006). Despite the immense size and diversity of the \( \text{K}^{+} \) channel family, the exact physiological contribution of individual \( \alpha \)-subunits has only been identified in few cases (Wang et al., 1998; Murakoshi & Trimmer, 1999; Malin & Nerbonne, 2002; Yuan et al., 2005; Deardorff et al., 2013).

\( \text{K}^{+} \) ion channels can largely be divided into three groups based on the number of transmembrane domains (TMD); those with 2 TMDs include the inward
rectifiers, those with 4 TMDs include the leak channels, and those with 6TMD include both the Ca$^{2+}$-activated K$^+$ channels and the voltage-gated K$^+$ channels (Kv), central to this dissertation. Voltage-gated ion channels are activated or gated by changes in membrane potential in close proximity. The Kv channels comprise of at least 10 subfamilies (Kv1-9 and HCNs) with more than 70 cloned mammalian genes (Coetzee et al., 1999; Johnston et al., 2010). Two major classes of Kv currents exist in central neurons, the fast activating and inactivating A-type K$^+$ current ($I_A$) and the sustained slow activating and inactivating delayed rectifiers ($I_K$). In the 1950s the Kv channels of axons were named "delayed rectifier" because there was a delay in membrane conductance following voltage steps. This name is still used to denote 'axon-like' Kv channels, even though nearly all Kv channels have a delay from voltage trigger to conduction, and delayed rectifiers are present in neuronal dendrites and somas, as well as in the axons.

The composition of each excitable membrane consists of a unique combination of Kv channels to fulfill a specific need. The α-subunit tetramers that form functional Kv channels can also be customized by forming either homo- or heteromeric channels. Most known Kv α-subunits exist as homomeric channels, but some must coassemble with other, usually similar, subunits to form functional channels. For example, in the Kv2 subfamily, Kv2.1 can coassemble with Kv2.2 or silent α-subunits such as Kv8.1 or Kv9s (Blaine & Ribera, 1998; Kerschensteiner & Stocker, 1999; Kihira et al., 2010). Forming functional heteromeric channels can create unique channels with altered biophysical and pharmacokinetic properties,
compared to homomeric, and may be implicated in physiological and/or pathological function.
**Kv2.1**

Kv2 channels, Kv2.1 & 2.2, are atypical (see below) delayed rectifiers, and both highly conserved and expressed in many excitable cells both inside and outside the nervous system. In the central nervous system, Kv2.1 forms distinct high-density clusters on the soma and proximal dendritic membranes of neurons in the cerebral cortex, hippocampus and in spinal α-MNs (Trimmer, 1991; Murakoshi *et al.*, 1997; Du *et al.*, 1998; Antonucci *et al.*, 2001; Muennich & Fyffe, 2004; Misonou *et al.*, 2005; Mohapatra & Trimmer, 2006). In α-MNs, Kv2.1 clusters at specific postsynaptic sites, including the large cholinergic C-boutons and presumably excitatory S-type synapses in addition to apposing some glial processes, however, they do not appear to be associated with inhibitory f-type synapses (Figure 1) (Meunnich & Fyffe, 2004).

Compared to other Kv channels, Kv2.1 has the largest cytoplasmic carboxy-terminal domain consisting of 443 amino acids (Figure 2) (Frech *et al.*, 1989; Trimmer, 1991). This carboxy-terminal tail is rich in phosphorylation sites including serine, threonine, and tyrosine residues as well as 2-cAMP-dependent sites, phosphokinase A sites, and most importantly 6 calcineurin-dependent phosphorylation sites\(^1\) (Park *et al.*, 2006). Most of these calcineurin-dependent sites are within a cytoplasmic domain required for channel clustering (Scannevin *et al.*, 1996; Park *et al.*, 2006).

\(^1\) An additional calcineurin dependent phosphorylation site is located in the N-terminus cytoplasmic tail (s11) (Park et al., 2006).
In the highly clustered configuration (typically) observed in hippocampal and cortical pyramidal cells, Kv2.1 channels are phosphorylated, have high activation\(^2\) and deactivation\(^3\) thresholds and slow kinetics (Murakoshi \textit{et al.}, 1997; Misonou \textit{et al.}, 2004; Surmeier \& Foehring, 2004; Misonou \textit{et al.}, 2005; Mohapatra \& Trimmer, 2006; Misonou, 2010). These features make Kv2.1 the atypical ‘slow poke’ and generally unable to contribution to the repolarization of a single action potential, typical of other delayed rectifiers\(^4\). Interestingly, some investigators have postulated that clustered Kv2.1 channels serve primarily non-conducting functions (O’Connell \textit{et al.}, 2010; Fox \textit{et al.}, 2013). However, recent studies have reported clustered Kv2 channels can help maintain repetitive firing by regulating the membrane potential during the interspike interval to relieve Na\(^+\) channel inactivation (Johnston \textit{et al.}, 2008; Guan \textit{et al.}, 2013; Liu \& Bean, 2014).

Upon prolonged/pathologic excitatory drive, Ca\(^{2+}\)/calcineurin dependent dephosphorylation pathways rapidly decluster Kv2.1 while simultaneously causing a large hyperpolarizing (≈25 mV) shift in both the activation and deactivation thresholds (Surmeier \& Foehring, 2004; Park \textit{et al.}, 2006; Mohapatra \textit{et al.}, 2009).

\(^2\) Reported ½ max activation thresholds range from 13.1mV to 20mV (Liu \textit{et al}, 2014, Misonou \textit{et al.}, 2005, Mohapatra \textit{et al.}, 2009, \& Park \textit{et al.}, 2006).

\(^3\) Reported ½ max deactivation thresholds are -26.2 mV in recombinant HEK cells (Park \textit{et al.}, 2006) and -28.8mV in Hippocampal cultures (Mohapatra \textit{et al.}, 2009).

\(^4\) Delayed rectifier subunits in other Kv families have lower voltages for activation and faster kinetics. For example, Kv1 family delayed rectifiers can activate upon depolarization from resting membrane potentials and have a ½ max voltage for activation at ≈ -32 mV. Kv1 channels help regulate the threshold necessary for the formation of an action potential and post-synaptic potentials. Delayed rectifier channels in the Kv3 family activate at ≈ -30 mV with a ½ max voltage for activation at ≈-20 mV. Kv3 channels are only activated during action potentials to repolarize the membrane. In contrast Kv2 channels activate at approximately +15mV and do not typically contribute to the rising or falling phases of the action potential.
The culmination of increased efflux of K\textsuperscript{+} from enhanced activity of Kv2.1 provides homeostatic suppression of neuronal activity and is presumed to play a neuroprotective role in hyperexcitable conditions (Surmeier & Foehring, 2004; Misonou et al., 2005; Mohapatra et al., 2009).
Figure 1. Kv2.1 ion channels are localized to high-density clusters on the soma and proximal dendrites of lumbar motoneurons. A) Micrograph of a confocal stack (32 x 1.0 μm z-steps) of a lumbar motoneuron showing Kv2.1-IR (Red) on the soma and proximal dendrites. Scale bar is 20 μm. B) Confocal image of a small confocal stack (10 x 1.0 μm z-steps) of an en face membrane patch on a lumbar motoneuron. Please note the both the and mottled appearance and size variability in Kv2.1 clusters. Scale bar is 10 μm.
Figure 2. The Kv2.1 α-subunit. Each subunit contains six transmembrane spanning α-helices (red) labeled s1-s6. S4 contains several positively charged amino acids and is the voltage-sensing α-helix. The pore forming, P Loop, is located between S5 and S6. Both the N-Terminus and the C-terminus are located in the cytosol, where the C-terminus is especially long. On the cytoplasmic termini, the black and red dots indicate phosphorylation sites confirmed with mass spectroscopy in recombinant in vitro Kv2.1 expression systems (Park et al., 2006). The red dots indicate those residues that are specifically regulation by the enzyme calcineurin. The green dots indicate additional high probability phosphorylation sites in endogenous Kv2.1.
Kv2.1 Pharmacology

When potassium currents were first identified as underlying the repolarization phase of the action potential (Hodgkin & Huxley, 1952) the large number of protein subunits that give rise to these currents was inconceivable. How the potassium ion currents from each channel type contribute to neuronal firing is unclear, but with the discovery and development of new pharmacological agents and combinations, researchers are able to experimentally tease apart ion channel specific functions and begin to understand not only their physiological role in neurons but also their pathological role (Johnston et al., 2010).

Two widely used blocking agents, tetraethylammonium ion (TEA) (Tasaki & Hagiwara, 1957; Hagiwara & Saito, 1959; Armstrong & Binstock, 1965; Hille, 1967) and 4-aminopyridine (4-AP) (Thesleff, 1980; Glover, 1982; Baker et al., 1993), selectively and reversibly block K⁺ currents including Kv2 (Table 1). However, the pharmacological effect of these inhibitors vary among species, tissue type, cell-type, and location of binding site (internal vs external) suggesting that different Kv channel types have different affinities and sensitivity to these blockers making them unsuitable for isolating individual K⁺ currents (please refer to (Hille, 1984)).

A number of naturally occurring toxins have been identified that contain compounds with an affinity for Kv channels that have been pivotal in the further characterization of specific K⁺ currents. The best understood is α-dendrotoxin (αDTx) from the Eastern Green Mamba Snake, Dendroaspis angusticeps (Benishin et al., 1988), however, Kv2 does not appear to be sensitive to DTx. Kv2.1 is also
insensitive to other commonly used peptide inhibitors such as charybdotoxin and agitoxin (Garcia et al., 1994). Nevertheless, several peptides isolated from tarantula venom have been found to be novel inhibitors with a high affinity for Kv2 and Kv4.2 which are responsible for a rapidly inactivating A-type K+ current (Table 1). Inhibitory peptides of K+ channels generally block conductance by sterically occluding the ion channel pore. However, the isolated peptides from the tarantula venom inhibits Kv2 channels outside the pore-forming domain at the extracellular region of the S3 helix, where it modifies gating properties of the channel by shifting the activation to more depolarized voltages (Swartz & MacKinnon, 1995, 1997; Li-Smerin & Swartz, 2000). Furthermore, these toxins have multiple binding sites making them effective in the nanomolar range. These peptides include hanatoxin5, (Swartz & MacKinnon, 1995), stromatoxin6 (Escoubas et al., 2002; Johnston et al., 2008; Zhong et al., 2010), and guangxitoxin7 (Herrington et al., 2006; Herrington, 2007; Liu & Bean, 2014).

Stromatoxin-1 (STX), used in this dissertation, is a basic 34 amino acid peptide reticulated by three disulfide bridges and a molecular mass of 3.788 KDa (Escoubas et al., 2002). Through using whole-cell patch-clamp techniques, it has

---

5 Publications with HaTx have been purified from the tarantula venom and generously provided to investigators by Dr. Kenton J. Swartz. To date, attempts to manufacture the peptide using recombinant technology have failed (see Swartz & MacKinnon, 1995). Two peptides were identified, HaTx1 and 2, both found to inhibit Kv2.1 and appear to generally be used together. While the two peptides have different sequences, any any physiological or anatomical differences have not been well characterized.

6 Recombinant peptides can be generated and are effective blockers of Kv2, making STX commercially available, unlike HaTx.

7 Synthetic peptides can be generated and are effective blockers of Kv2. The successful use of these synthetic peptides in neurons was first published in 2014 (Liu et al, 2014).
been shown that STX fully inhibits both Kv2.1 and Kv2.2 at 100nM (in 100s) in a voltage-dependent manner with maximum inhibition between -30 and 0 mV (Escoubas et al., 2002). The STX effects do reverse slowly following washout, however the Kv2 current is not fully restored.

Knowledge of K⁺ channel pharmacology is dominated by in vitro studies with recombinant homomeric channel expression. In native neurons, pharmacological sensitivities can be altered by the assembly of heteromeric α subunits into functional proteins, addition of β subunits and/or additional accessory proteins. Careful characterization and interpretation must be considered when toxins are being experimentally applied. The appropriate use of toxins has contributed to exciting advances in neuroscience in the understanding of how ions move across the membrane as well as how subunit-specific currents contribute to neuronal physiology. Furthermore, toxins will play an important role in interpreting ion channel changes associated with pathology and very well could pave the way for new and targeted therapies.
### Table 1. Inhibitors of Kv2.1

<table>
<thead>
<tr>
<th>Toxin Name</th>
<th>Type</th>
<th>Kv2.1 IC\textsubscript{50}</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraethylammonium ion (TEA)</td>
<td>Ammonium Cation</td>
<td>10 mM</td>
<td>Blocks multiple K Channel Families</td>
<td>(Mathie et al., 1998)</td>
</tr>
<tr>
<td>4-aminopyridine (4-AP)</td>
<td>Organic Amine</td>
<td>0.5 mM</td>
<td>Blocks multiple K Channel Families</td>
<td>(Mathie et al., 1998)</td>
</tr>
</tbody>
</table>

#### General Voltage Gated Potassium Channel Blockers

<table>
<thead>
<tr>
<th>Toxin Name</th>
<th>Species</th>
<th>Kv2.1 IC\textsubscript{50}</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanatoxin</td>
<td>Grammostola spatulata</td>
<td>100 nM</td>
<td>Kv2.1 and Kv4.2</td>
<td>(Swartz &amp; MacKinnon, 1995)</td>
</tr>
<tr>
<td>Stromatoxin</td>
<td>Stromatopelma calceata</td>
<td>12.7 nM</td>
<td>Kv2.1,Kv2.2 and Kv4.2</td>
<td>(Escoubas et al., 2002)</td>
</tr>
<tr>
<td>Guangxitoxin</td>
<td>Plesiophriictus guangxiensis</td>
<td>5 nM</td>
<td>Kv2.1, Kv2.2, Kv4.2 and Kv4.3</td>
<td>(Herrington et al., 2006; Herrington, 2007)</td>
</tr>
</tbody>
</table>
Concluding Thoughts

Trauma and disease that affects the central nervous system can result in devastating patient outcomes with few effective therapies. Advances in patient care will require a complete understanding of the molecular events underlying the pathophysiological alterations in intrinsic membrane properties, which likely reflect cumulative dynamic modifications in multiple ion channel types that contribute to a coordinated cellular response. This project will address fundamental gaps in our knowledge of Kv2.1 ion channel dynamics in αMNs, with an emphasis on alterations resulting from activity and pathological drive, and will explore the possibility that these channels have the remarkable capacity to both increase or decrease neuronal excitability through the dynamic kinetics of channel activation and concomitant clustering properties. Characterizing these dynamics in αMNs will substantially impact the field by contributing important observations that could underlie pathophysiologic alterations. These data will lay the foundation for future studies to examine if Kv2.1 dynamics contribute to disease pathology or, alternatively, maintain α-MN viability in multiple pathological states.
Chapter II. General Methods
This dissertation is predicated on highly detailed quantification of Kv2.1 ion channel organization at high resolution (nm-μm range). To enable feasible, yet meticulous, analysis of Kv2.1 clustering dynamics, a combination of approaches and technologies (including, but not limited to, immunohistochemistry, pharmacology, confocal microscopy and electrophysiology) in both in vivo and in vitro systems are employed. The application of sophisticated methodology, together with multiple approaches, is necessary to provide unique insights into the core questions within this proposal.
Animal Use

All animal procedures were performed according to National Institutes of Health (NIH) guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University. All rats used are Sprague Dawley (Harlan Laboratories, Indianapolis, IN). Timed pregnant females were obtained from Harlan Laboratories for postnatal studies. All survival surgical procedures were performed in surgical suites located within the Laboratory Animal Resources at Wright State University.
Retrograde Labels

Adult female Sprague Dawley rats (250-350 grams) were used in a survival surgery to inject a fluorescent tracer into the medial and lateral gastrocnemius muscles to retrogradely label MNs for post-hoc identification in the spinal cord. Rats were deeply anesthetized with 5% isoflurane and maintained with 2-3% isoflurane. An incision was made using sterile technique over the left hindlimb and through biceps femoris muscle to reveal the triceps surae. A total of 40-50 µL of cholera Toxin Subunit B, CTB, (Invitrogen, Carlsbad, CA, USA; 0.5%) was injected into the medial and lateral gastrocnemius muscle in a series of multiple small injections throughout the two muscles. The wound was irrigated and closed in layers. Animals received 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours for post-operative pain medication for 48 hours and were monitored closely by professional staff. Sufficient retrograde labeling of MN somas and proximal dendrites was reached 1 week following intramuscular injection.
**Immunoblotting**

Freshly dissected L4/L5 spinal cord region was dissected from adult rats. Samples were homogenized in 0.3M sucrose in 0.1M phosphate buffer at pH 7.4 containing 10mM Hepes, 1mM EDTA, Complete Mini protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN), 10 mM NaF and 2 mM Sodium ortho-vanadate (Na$_3$VO$_4$). The homogenate was centrifuged at 3000 x g for 10 minutes at 4°C to remove the nuclei and cellular debris. The supernatant was collected and ultracentrifuged at 100,000 x g for 1 hour at 4°C. The pellet or crude membrane fraction was resuspended in 0.3M sucrose buffer at pH 7.4 containing 75mM NaCl, 10mM Tris, 20mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN), 10 mM NaF and 2 mM Sodium ortho-vanadate (Na$_3$VO$_4$). When noted the phosphatase inhibitors, NaF and Na$_3$VO$_4$ were excluded. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as a standard. Approximately 30 µg of membrane protein was loaded per lane in a 4-20% gradient SDS-polyacrylamide gel (Criterion, BioRad, Hercules, CA) and separated with electrophoresis (SDS-PAGE). Following electrophoretic transfer to nitrocellulose membrane, the membrane was probed with one of primary antibodies (see Kv2.1 antibody characterization). After primary antibody incubation, the membranes were incubated with Donkey anti-mouse AP (Promega, Madison, WI). Immunoblotting was visualized using the Western Star detection kit (Tropix, Bedford, Massachusetts) and imaged using a Fuji Bioimager 1000 (Stamford, CT).
**General Immunohistochemistry**

All animals were anesthetized with pentobarbital (150 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.3. The spinal cords were removed, post fixed in 4% paraformaldehyde for 2 hours and cryoprotected in 15% sucrose in 0.1M phosphate buffer. 50 µm transverse sections were obtained from the L4 and L5 spinal cord segments on a cryostat and immunostained free floating. Kv2.1 immunocytochemistry was performed primarily using mouse anti-Kv2.1 clone D4/11 (catalog number 75-047) at 1:1000 in PBS with 0.1% Triton X pH 7.3 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. Other Kv2.1 antibodies were that targeted different epitopes of the protein were tested to validate our antibody (see Kv2.1 antibody characterization). Additional antibodies used in this study include anti-VACHT (vesicular acetylcholine transporter; Millipore; Goat, 1:5,000), anti-VGLUT1 and anti-VGLUT2 (vesicular glutamate transporters; Chemicon; Guinea pig, 1:5,000), and anti-NeuN (neuronal nuclear protein; Chemicon; mouse, 1:500. All Immunoreactivity was detected with species specific secondary antibodies conjugated to Cy3, Alexa 488, FitC, CY5, or Alexa 647 (Jackson Immuno, West Grove, PA, USA). Nissl immunocytochemistry was performed using fluorescein-conjugated nissl (1:100, Molecular Probes, Carlsbad, CA, USA).
Identification of αMNs

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. The size of MNs in lumbar spinal cord MN pools has been documented for each mammalian species (e.g. Ishihara *et al.*, 2001; Chen and Wolpaw, 1994; Burke *et al.*, 1982; Moschovakis *et al.*, 1991) 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 CHAT vs. calbindin expression (Carr *et al.*, 1998; Alvarez & Fyffe, 2007). Moreover 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, together with retrograde labeling of specific motor pools, provided consistent outcomes and enabled us to focus directly on α-MNs in this study.
**Confocal Microscopy and Quantitative Analysis**

Immunolabeled lumbar αMN images were obtained on a Fluoview FX/FV300 Olympus (Center Valley, PA, USA) confocal system and Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope with a 60x oil immersion objective at 0.5 μm steps at 1.0-2.5 digital zoom (N.A 1.35). For *in vivo* studies, every ipsilateral injured or stimulated αMN imaged and analyzed, a contralateral αMN was also selected as an internal bilateral control. Uninjured/unstimulated MNs selected for internal control analysis were of approximate size and position within the motor pools on the contralateral side within each tissue slice. Sham surgery control animals that contained the retrograde tracers were used to verify and quantify the ipsilateral and contralateral effects observed following PNI and stimulation. For *in vitro* studies, spinal cord slices from each animal were divided among treatment groups and imaged per same parameters mentioned above. Image stacks were quantitatively analyzed for Kv2.1-IR cluster areas as previously described (Figure 3) (Muennich & Fyffe, 2004) in Image Pro Software (Media Cybernetics, Silver Springs, MD, USA). Cell body Kv2.1-IR macroclusters (diameter >1.0μm), were measured in *en face* single optical sections on MNs that innervate the medial and lateral gastrocnemius as revealed by retrograde labels. All Kv2.1-IR microcluster (diameter <1.0μm) analyses were performed by selecting regions of interest in single optical confocal sections that did not contain macroclusters. Mean cell body diameters were calculated using averaging applications in Image Pro Software based on plasma membrane labeling on single optical sections through the center of the neuron.
containing the nucleolus. For all analyses, injured MNs were compared to both contralateral internal controls as well as sham surgery control animals. Significance was set at \( p<0.05 \) using Pairwise ANOVA and Pairwise T-Test (SigmaStat; Systat Software, Port Richmond, CA, USA).
**Figure composition**

Microscope images were prepared by adjusting contrast and brightness in Image Pro Plus Software (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Figures were composed using CorelDraw (v. 12.0). Graphs were composed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA). Some images were sharpened using a “high gauss” filter in image pro. Quantification was always carried out in original unprocessed images.
Figure 3. An example of Kv2.1 cluster size quantification. The same techniques and resolution to quantify Kv2.1 cluster sizes in single optical confocal sections of ‘en face’ regions of the motoneuron surface membrane has previously been described and published from our laboratory (Muennich & Fyffe, 2004). In this dissertation, the primary focus is on larger macroclusters that have a minimum diameter of 1µm.
Chapter III. Characterization of Kv2.1 Antibodies
Multiple antibodies are commercially available against the Kv2.1 protein that specifically target different epitopes (Table 2). Following immunohistochemistry (see general immunohistochemistry methods), all 4 commercially available antibodies exhibit high-density membrane clustering in lumbar MNs (Figure 4). In lumbar spinal cord membrane preparations, separated by SDS-PAGE (see immunoblotting methods), both K89/34 (1:500; Mouse Monoclonal; Neuromab 75-014, Davis, CA) and K39/25 (1:100: Mouse Monoclonal; Neuromab 75-159, Davis, CA) clones, developed against linear epitopes, produced dominant broad bands from approximately 100 kD-120 kD representing the wide range of phosphorylation states, similar to observations made in the brain (Figure 5). The monoclonal antibody raised against a GST fusion protein to recognize a 3-D epitope, clone D4/11 (1:500; Mouse Monoclonal; Neuromab 75-047, Davis, CA), did not produce a strong band in the denaturing immunoblot (Figure 5). Finally the polyclonal antibody (1:500; Rabbit Polyclonal, Alomone APC-012, Jerusalem, Israel) produced a doublet within the 105 kD-115 kD range (Figure 5).

Previously, when isolated brain membrane preparations were incubated with alkaline phosphatase, a molecular weight shift and band narrowing was detected in immunoblots to produce a fine band at approximately 100 kDa, the predicted Kv2.1 molecular weight without post-translational modification. These results indicate that most of the post-translational modification of Kv2.1 is
phosphorylation (Misonou et al., 2004). Antibody clone K89/34 has also been used to detect activity-dependent phosphorylation shifts in Kv2.1 in the hippocampal region of the brain (Mohapatra & Trimmer, 2006; Mohapatra et al., 2009). However, in lumbar spinal membrane preparations, when incubated for 2 hours with 100U Alkaline Phosphatase (Promega, Madison, WI, USA), without phosphatase inhibitors, clone K89/34 bands appear weaker than both the controls and heat inactivated alkaline phosphatase treatment (Figure 6). The epitope bound by clone K89/34 binds contains a high probability phosphorylation site (Figure 2, high-probability site nearest the C-terminal) that is likely phosphorylated in spinal lumbar membrane preparations. Therefore, antibody clone K89/34 appears to bind the Kv2.1 protein in spinal membrane preparations in a phosphorylation state-specific manner. Tissue specific variability in Kv2.1 molecular weight is detectable using clone K89/34, possibly representing tissue-specific variability in post-translational modification (Figure 7).

Previously, Kv2.1-IR was characterized in rodent lumbar MNs with the D4/11 clone (Muennich & Fyffe, 2004). This antibody, like K89/34, produces clear immunoreactivity with low background staining, necessary for high-resolution analysis in this dissertation (See Figure 4). Because we are able to detect both the phosphorylated-regulated clustered and declustered states in immunohistological assays with clone D4/11 (please refer to Specific Aim 1 &3), indicating this clone is not biologically phosphorylation-specific, clone D4/11 was selected for all

8 Please note a rabbit polyclonal antibody was used for these studies that are not commercially available (Table 2)
immunohistochemistry and analysis in this dissertation whereas K89/34 was used for much of the immunoblot work described above.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Company</th>
<th>Host</th>
<th>Epitope Position</th>
<th>AA Sequence</th>
<th>Notes</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>K39/25</td>
<td>Neuromab</td>
<td>Mouse</td>
<td>211-229</td>
<td>LPELQSLDEFGQSTDNPQL</td>
<td>Extracellular between S1-S2 Loop</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>K89/34</td>
<td>Neuromab</td>
<td>Mouse</td>
<td>837-853</td>
<td>HMLPGGGAHGSTRDQSI</td>
<td>Cytoplasmic C-Term</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>D4/11</td>
<td>Neuromab/Upstate</td>
<td>Mouse</td>
<td>509-853</td>
<td>Produced against bacterially expressed GST fusion protein corresponding to AA 509-853</td>
<td>Cytoplasmic C-Term (Meunnich &amp; Fyffe, 2004)</td>
<td>IHC</td>
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<td></td>
<td>Alomone</td>
<td>Rabbit</td>
<td>837-853</td>
<td>HMLPGGGAHGSTRDQSI</td>
<td>Cytoplasmic C-Term</td>
<td>WB and IHC</td>
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<tr>
<td>KC</td>
<td>Commercially unavailable (Trimmer Laboratory)</td>
<td>Rabbit</td>
<td>837-853</td>
<td>HMLPGGGAHGSTRDQSI</td>
<td>Cytoplasmic C-Term Phospho-Independent</td>
<td>IHC</td>
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<td>K89/41</td>
<td>Commercially unavailable (Antonucci, 2001)</td>
<td>Mouse</td>
<td>?</td>
<td></td>
<td></td>
<td>IHC</td>
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Figure 4. Commercially available antibodies raised against different epitopes of the Kv2.1 protein all show the same ion channel clustering immunoreactivity on rat lumbar motoneurons. Arrows point to regions of the membrane where intensity profiles are shown in insets.
Figure 6. Commercially available antibodies raised against different epitopes of the Kv2.1 protein bands in immunoblots. Each antibody produces major banding in the predicted 100-120 kD range, with the exception of D4/11. Approximately 30μg of lumbar spinal membrane bound protein was loaded per well.
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<th>-Pis 37°C for 2 Hours + PIs</th>
<th>-Pis 37°C for 2 Hours + PIs</th>
<th>-Pis 37°C for 2 Hours + PIs</th>
<th>-Pis 37°C for 2 Hours + PIs</th>
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<tr>
<td>Kv2.1</td>
<td>150 kD AP IAP Cont</td>
<td>150 kD AP IAP Cont</td>
<td>150 kD AP IAP Cont</td>
<td>150 kD AP IAP Cont</td>
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<tr>
<td>B-Actin</td>
<td></td>
<td></td>
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Figure 7. Kv2.1 antibody clone K89/34 binds rat membrane preparations in a phosphorylation-specific manner. Four total experimental replicates, each from separate rats, are shown above. Approximately 30μg of lumbar spinal membrane bound protein was loaded per well. Control conditions are treated with phosphatase inhibitors (PIs). To control for the absence of phosphatase inhibitors, membrane protein was treated for 2 hours with heat inactivated alkaline phosphatase (iAP; 15 minutes at 95°C). Treatment with fully activated alkaline phosphatase (AP) for 2 hours produces a significantly lighter band compared to both control and iAP. Please note that the lowest part of the band (lower KD), which represents the fully dephosphorylated states, are most depleted. β-actin loading controls for each well are displayed below.
**Figure 8. Tissue specific variation in Kv2.1 molecular weights.** Variability in molecular weight is likely a reflection of tissue and/or cell type specific post-translational modification. Immunoblots were probed with Kv2.1 antibody clone K89/34. Approximately 30μg of lumbar spinal membrane bound protein was loaded per well.
Chapter IV. Hypothesis and Specific Aims
This dissertation will be concerned with the dynamics of Kv2.1 clustering from the perspective of altered activity levels and injury. As will be further described in the background section, the intrinsic membrane properties of neurons in the central nervous system are controlled, in part, by the tight regulation of membrane-bound ion channels. Rather than existing as static entities operating only in opened or closed states, ion channels are dynamic molecules with the capacity to adopt multiple functional states through conformational changes and/or post-translational modification - enabling flexibility in their activity. Furthermore, the location of the ion channel within certain membrane compartments, synaptic sites and/or discrete membrane clusters is critical to synaptic integration and shaping of firing properties, and can also be dynamically modified by changes in neuronal activity and pathology. Kv2.1 channels, which underlie delayed rectifier potassium currents, form distinct clusters in specific membrane compartments, in several neuronal types. In the clustered state, these channels are highly phosphorylated, have slow gating kinetics and help maintain neuronal firing rate (Guan et al., 2013; Liu & Bean, 2014). However, following pathologic or prolonged excitatory drive, Ca^{2+}-calcineurin mediated dephosphorylation leads to rapid Kv2.1 channel declustering and concomitant acceleration of gating kinetics. Declustered/dephosphorylated Kv2.1 has been described as a homeostatic mechanism to suppress neuronal firing rate (Misonou et al., 2004; Misonou et al., 2005; Misonou et al., 2008; Mohapatra et al., 2009). In spinal α-motoneurons (αMNs), Kv2.1 is also expressed in high-density clusters at specific postsynaptic sites (Muennich & Fyffe, 2004). Therefore, I hypothesize that Kv2.1 membrane-
bound ion channel clusters will dynamically reorganize following pathologic or prolonged excitatory drive in rat lumbar α-motoneurons. The 3 specific aims in this project will address fundamental gaps in our knowledge of Kv2.1 ion channel dynamics that have not been investigated in αMNs, with an emphasis on alterations in activity and pathological drive. Impact on the field will be substantial in understanding the mechanisms that underlie the modulation of MN intrinsic properties. In particular, Kv2.1 channels have been implicated in playing a neuroprotective role in hyperexcitable conditions. Characterizing the dynamic changes of Kv2.1 in αMNs will provide insights into the homeostatic regulation of firing rate through dynamic clustering and channel kinetics and will be key to interpreting pathophysiological changes in future studies.

**Specific Aim 1. Determine if in vivo peripheral nerve injury induces Kv2.1 ion channel de-clustering in lumbar αMNs.** In an *in vivo* rat preparation, I will use immunohistochemistry and quantitative confocal microscopy to analyze the dynamics of Kv2.1-IR clustering in αMNs following pathologic peripheral nerve injury. Specifically I will evaluate injury-type specific changes (crush injury vs cut), the time-course for recovery, and if there is a dependency on muscle reinnervation for complete recovery.

**Specific Aim 2. Determine if in vivo nerve stimulation disrupts Kv2.1 ion channel clustering in lumbar αMNs.** In an *in vivo* rat preparation, followed by immunohistochemistry and quantitative confocal microscopy, I will examine kinetics of any Kv2.1 membrane reorganization following tetanic nerve stimulation.
Furthermore, I will evaluate the potential contributions of antidromically stimulated MNs and orthodromically stimulated sensory input circuits on Kv2.1 clustering dynamics in αMNs.

**Specific Aim 3. Determine if prolonged excitatory drive in vitro induces Kv2.1 declustering.** In an *in vitro* spinal cord preparation, I will use pharmacology, immunohistochemistry and quantitative confocal microscopy to analyze the dynamics of clustering and underlying mechanisms. Specifically I will examine the kinetics of Kv2.1 membrane reorganization in response to prolonged excitatory drive (glutamate) and low drive (tetrodotoxin) in addition to characterizing a time-course for recovery. Furthermore, I will examine underlying mechanisms by driving cholinergic signaling (muscarine) and blocking calcineurin (cyclosporin). Finally, using patch-clamp electrophysiology, I will determine the impact on αMN activity through blocking Kv2 currents (stromatoxin).
Publications and Presentations

Much of the work done to address these specific aims have been published or presented at national and international meetings

Publications

Romer, S.H., Dominguez, K.M., Gelpi, M.W., Deardorff, A.S., Tracy, R.C., and Fyffe, R.E.W (2014). Redistribution of Kv2.1 ion channels on spinal motoneurons following peripheral nerve injury. Brain Res 1547, 1-15.\(^9\)

Deardorff, A.S., Romer, S.H., Sonner, P.M., Fyffe, R.E.W (2014). Swimming against the tide: investigations of the C-bouton synapse. Frontiers in Neural Networks 8, Article 106. Invited Review\(^{10}\)

Abstracts


\(^9\) Figure selected for journal cover artwork
\(^{10}\) Deardorff and Romer are equally contributing first authors


Chapter V. Specific Aim 1:

Redistribution of Kv2.1 ion channels on spinal motoneurons following peripheral nerve injury

Romer et al., *Brain Research* 1547, 1-15, 2014
Introduction

Injured peripheral nerves can successfully reinnervate peripheral targets, but complete functional recovery seldom occurs (Cope & Clark, 1993; Cope et al., 1994; Haftel et al., 2005; Alvarez et al., 2011; Bullinger et al., 2011; Prather et al., 2011). Reorganization of central circuits accounts, at least partially, for abnormal sensorimotor integration and stretch areflexia following peripheral nerve injury (Alvarez et al., 2011; Bullinger et al., 2011). Furthermore, injury induced changes to α-motoneuron (MN) intrinsic properties and cellular excitability are well documented (Kuno et al., 1974b, a; Gustafsson & Pinter, 1984; Foehring et al., 1986b; Nakanishi et al., 2005; Bichler et al., 2007) and may also contribute to dysfunctional or maladaptive signaling in intact spinal circuitry.

Neuronal biophysical properties are controlled in part 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; Deardorff et al., 2013). It is now recognized that certain voltage-gated ion channels are not uniformly distributed in the surface membrane of α-MNs but exhibit polarized distributions and/or are concentrated in discrete microdomains (Carlin et al., 2000; Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Elbasiouny et al., 2005; Bui et al., 2006; Deardorff et al., 2013). Ion channel localization and density in the surface membrane can be dynamically regulated at a number of levels including gene expression, protein trafficking and membrane insertion, posttranslational modifications, and interactions with macromolecular complexes or lipid rafts. Additionally, different channel subunits can interact to form heteromeric channels.
with altered channel properties (Benton et al., 2003; Monaghan et al., 2004; Strassmaier et al., 2005; Kihira et al., 2010). Moreover, channel subunits may associate with and be regulated by accessory subunits, neurotransmitter or neuromodulator receptors, scaffolding proteins and postsynaptic density proteins (e.g. PSD-95), and other signaling molecules. Yet few studies have examined channel regulation in α-MNs following peripheral nerve injury.

Delayed rectifier Kv2.1 channels regulate the excitability of a wide range of vertebrate neurons. Kv2.1 channels are typically sequestered, or ‘clustered’, via unique C-terminal phosphorylation sites into highly regulated microdomains with other ion channels, neurotransmitter receptors, and signaling molecules (Deng & Fyffe, 2004; Muennich & Fyffe, 2004; Wilson et al., 2004; Deardorff et al., 2013). Sequestration creates energetically and kinetically favorable mechanism for neurons to initiate rapid and robust responses to changes in synaptic inputs and cellular activity (Misonou et al., 2004; Misonou et al., 2005; Park et al., 2006; Misonou et al., 2008; Misonou, 2010). In α-MNs, the largest Kv2.1 clusters are located at C-bouton postsynaptic sites, where they comprise a unique cellular domain with SK2/3 channels, subsurface cisternae, and mAChR2 receptors (Muennich & Fyffe, 2004; Deardorff et al., 2013). Smaller Kv2.1 clusters are located at excitatory S-type synapses and may also be apposed to extrasynaptic neuroglia processes (Muennich & Fyffe, 2004).

Kv2.1 channel properties and membrane clustering are phosphorylation dependent (Murakoshi et al., 1997; Misonou et al., 2004; Park et al., 2006; Zhang et al., 2008). In the high-density clusters, Kv2.1 channels may be low or non-
conducting, and it is possible that the channel proteins exhibit additional non-conducting functions (O’Connell et al., 2010; Fox et al., 2013). A variety of stimuli (ex: ischemia, hypoxia, and synaptic activity) cause Ca$^{2+}$-calcineurin dependent channel dephosphorylation. (Misonou et al., 2004; Misonou et al., 2005).

Dephosphorylated Kv2.1 undergoes a large (~25 mV) hyperpolarizing shift in voltage dependence, increasing the amplitude of Kv2.1 delayed rectifier currents (Misonou et al., 2004; Mohapatra et al., 2009) and prolonging the duration of the interspike interval (Surmeier & Foehring, 2004; Mohapatra et al., 2009).

Dephosphorylated Kv2.1 channels also spatially migrate from the initially clustered state (Misonou et al., 2004). In hippocampal neurons the modulation of Kv2.1 currents by this mechanism suppresses neuronal firing frequency following prolonged depolarization and increased synaptic activity (Mohapatra et al., 2009), strongly indicating a role for Kv2.1 in homeostatic regulation of cellular excitability.

The aim of this study was to use quantitative immunohistochemical techniques to investigate the membrane organization and spatial distribution of Kv2.1 immunoreactivity (IR) in rat medial and lateral gastrocnemius α-motoneurons (MNs) following tibial nerve injury. We first tested the effects of glutamate induced excitation in in vitro spinal cord slices to demonstrate the redistribution and reduced clustering of membrane Kv2.1 channels in spinal α-motoneurons, similar to the membrane redistribution seen following increase in activity in other neurons in vitrō. We then tested two types of in vivo peripheral nerve injury in the rat including a tibial crush injury, in which peripheral reinnervation was permitted, and a tibial ligation injury, in which peripheral
reinnervation was prevented. Both *in vivo* injuries resulted in declustering of Kv2.1 suggesting that the regulation of Kv2.1 may contribute to modifications in motoneuron intrinsic excitability following peripheral nerve injury.
Experimental Procedures

All animal procedures were performed according to National Institutes of Health (NIH) guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University.
Peripheral Nerve Injuries and Retrograde Labels

Adult female Sprague Dawley rats (250-350 grams) underwent a series of two survival surgeries. In the first surgery the medial and lateral gastrocnemius muscles were injected with a fluorescent tracer to retrogradely label MNs for post-hoc identification in the spinal cord (Figure 9). Rats were deeply anesthetized with 5% isoflurane and maintained with 2-3% isoflurane. An Incision was made using sterile technique over the left hindlimb and through biceps femoris muscle to reveal the triceps surae. A total of 40-50 µL of either hydroxystilbamidine (Biotium, Inc., Hayward, CA, USA; 4%), FastBlue (Polysciences, Inc., Warrington, PA, USA; 2%) or Cholera Toxin Subunit B, CTB, (Invitrogen, Carlsbad, CA, USA; 0.5%) was injected into the medial and lateral gastrocnemius muscle in a series of multiple small injections throughout the two muscles. The wound was irrigated and closed in layers. Three days later, the incision was reopened and the tibial nerve was dissected away from surrounding tissues and exposed. The tibial nerve was either crushed, by applying pressure for 10 sec with fine jeweler’s forceps, or ligated, by using microscissors and silk suture to ligate nerve and prevent reinnervation. The wound was irrigated and closed. Surgery was not performed on the right hindlimb to provide uninjured internal controls. Animals received 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours for post-operative pain medication for 48 hours and were monitored closely by professional staff. The sham surgery control animals underwent the same surgical procedures described above except the tibial nerve was only exposed rather than injured. Nerve injured and sham control animals were analyzed at similar post-operative time points.
Figure 9. Intramuscular injections of retrograde tracer were used to identify motoneurons innervating the medial and lateral gastrocnemius (MG/LG). Only MG/LG motoneurons were selected for quantitative analysis in this study.

Yellow arrow points to a population of motoneuron somas in the lateral lamina IX in the ventral horn of spinal cord segment L5 containing cholera toxin subunit B (CTB) conjugated to 488 fluorophore (green), one of the retrograde tracers used in this study. Nissl-IR (White) was used as a general neuronal stain. Retrogradely labeled cells selected for quantitative analysis were located in appropriate position within the motoneuron pool consistent with previous reports identifying location of MG/LG motoneuron somas (Romanes, 1951). For every injured αMN a contralateral control αMN was also selected from the appropriate position within lamina IX (see methods) Scale bar is 200 μm.
**Immunohistochemistry**

All animals were anesthetized with pentobarbital (150 mg/kg, i.p) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.3. The spinal cords were removed, post fixed for 2 hours and cryoprotected in 15% sucrose in 0.1M phosphate buffer. 50 µm transverse sections were obtained from the L4 and L5 spinal cord segments on a cryostat and immunostained free floating. Kv2.1 immunocytochemistry was performed using mouse anti-Kv2.1 clone D4/11 (catalog number 75-047) at 1:1000 in PBS with 0.1% Triton X pH 7.3 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.

Immunoreactivity was detected with species specific secondary antibodies conjugated to Cy3 (Jackson Immuno, West Grove, PA, USA). Nissl immunocytochemistry was performed using fluorescein-conjugated nissl (1:100, Molecular Probes, Carlsbad, CA, USA).
**Spinal Cord Slice Preparations**

Young Sprague Dawley Rats, P14, (Charles River, Wilmington, MA, USA) were anesthetized with 65mg/ml pentobarbital and decapitated. The spinal cords were immediately dissected in <4°C sucrose modified artificial CSF (26mM NaHCO\_3, 10mM Glucose, 3mM KCl, 1.25mM NaPO\_4, 2mM MgCl\_2, and 218mM Sucrose) with bubbling 95% O\_2 and 5% CO\_2. Transverse sections, 300 μm thick, of the L4 – L5 region of the spinal cord were cut on Vibratome Series 1000 Plus (The Vibratome Co., St. Louis, MO, USA). Slices were briefly incubated in 30% PEG (Carp et al., 2008) and transferred to oxygenated aCSF (130 mM NaCl, 26mM NaHCO\_3, 10mM Glucose, 3mM KCl, 1.25mM NaPO\_4, 2mM MgCl\_2, 2mM CaCl\_2 at 300-310 mOsm) and incubated 1 hour at 37°C to stabilize. Sections were incubated in either 10 μm L-Glutamate (Sigma, St. Louis, MO) diluted in aCSF or aCSF alone for 10 minutes, longer times in glutamate increased cell death. Addition of glutamate to aCSF had minimal effect on osmotic pressure of the solution. Immediately following incubations, the slices were immersion fixed in 4% paraformaldehyde overnight. Tissue slices were sectioned further (50 μm) on cryostat and immunohistochemistry was performed as previously described (refer to immunohistochemistry methods).
Confocal Microscopy and Quantitative Analysis

Immunolabeled lumbar αMN images were obtained on a Fluoview FX Olympus (Center Valley, PA, USA) confocal system for tibial nerve crush studies and Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope for the tibial nerve ligation studies with a 60x oil immersion objective at 0.5 μm steps at 1.0-2.5 digital zoom (N.A 1.35). For every ipsilateral injured αMN imaged and analyzed, a contralateral injury spared αMN was also selected as an internal bilateral control. Uninjured MNs selected for internal control analysis were of approximate size and position within the motor pools on the contralateral side within each tissue slice. In 4 animals, the uninjured αMNs were retrogradely labeled with different color conjugated fluorophores to differentiate from the injured αMNs. No significant differences were found between these retrogradely labeled uninjured αMNs from the αMNs selected based on size and location (data not shown). Sham surgery control animals that contained the retrograde tracers were used to verify and quantify the ipsilateral and contralateral effects observed following PNI. Image stacks were quantitatively analyzed for Kv2.1-IR cluster areas as previously described (Muennich & Fyffe, 2004) in Image Pro Software (Media Cybernetics, Silver Springs, MD, USA). Cell body Kv2.1-IR macroclusters (diameter >1.0µm), were measured in en face single optical sections on MNs that innervate the medial and lateral gastrocnemius as revealed by retrograde labels (Figure 1). All Kv2.1-IR microcluster (diameter <1.0µm) analyses were performed by selecting regions of interest in single optical confocal sections that did not contain macroclusters. Mean cell body diameters were calculated using averaging applications in Image Pro.
Software based on plasma membrane labeling on single optical sections through the center of the neuron containing the nucleolus. For all analyses, injured MNs were compared to both contralateral internal controls as well as sham surgery control animals. Significance was set at p<0.05 using Pairwise ANOVA and Pairwise T-Test (SigmaStat; Systat Software, Port Richmond, CA, USA).
Dendritic Analysis and Reconstructions

Our conventional methods for measuring soma Kv2.1 cluster areas were not suitable for measuring cylindrical dendrites due to limited availability of en face dendritic membrane through which accurate 2-D measurements can be obtained. Dendritic Kv2.1 macrocluster areas and numbers were calculated by 3D reconstructions of the confocal image stacks through Imaris Software (Bitplane Scientific Software, Zurich, Switzerland). Prior to being reconstructed, the confocal images where deconvolved on Huygens Essential Software (Scientific Volume Imaging, Hilversum, Netherlands) to reduce noise and z-plane distortion. The deconvolved confocal image stacks were imported into Imaris software where the image is displayed as a 3-dimensional isometric view. Surface rendering and thresholding was used to create a 3-D solid surface of the CTB filled motoneuron and Kv2.1-IR clusters with careful attention to accurately match anatomic dimensions. A masking feature in Imaris software allowed only the Kv2.1 clusters contacting the CTB- filled motoneuron to be selected for analysis. The proximal dendrites and associated Kv2.1-IR clusters were digitally dissected from the soma for analysis. Kv2.1-IR cluster areas from proximal dendrites were exported from the software for statistical analysis. The reconstructed MNs were from 50μm thick tissue slices, an appropriate thickness for consistently analyzing dendritic lengths up to 150 μm traveling through 3-D space. Kv2.1-IR macrocluster areas located on control MN somas obtained through 3-D reconstructions were not found to be significantly different from the areas measured using our conventional techniques previously described (data not shown).
Figure composition

Microscope images were prepared by adjusting contrast and brightness in Image Pro Plus Software (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Figures were composed using CorelDraw (v. 12.0). Graphs were composed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA). Data presented in figures is mean ± SEM. Standard Deviations are listed in tables 1 and 2. Some images were sharpened using a “high gauss” filter in image pro. Quantification was always carried out in original unprocessed images.
**Results**

Redistribution of Kv2.1-IR results from increased in vitro activity in α-motoneurons.

Previous work with Kv2.1 in both primary hippocampal cells and HEK293 cultures has shown a lateral translocation of Kv2.1 from clusters to a more uniform distribution (‘declustering’) in the membrane following glutamate treatment (Murakoshi *et al.*, 1997; Misonou *et al.*, 2004; Misonou *et al.*, 2005; Mohapatra *et al.*, 2009). Here we treated lumbar spinal cord slices with 10 μM glutamate for 10 minutes to determine if Kv2.1 clusters behave similarly in αMNs. *En face* Kv2.1-IR macrocluster (diameters >1μm) areas from 20 αMNs (6 rats, 2 litters) were measured from control and glutamate-treated groups. Treatment with glutamate resulted in a significant 47% reduction in Kv2.1 macrocluster areas on lumbar αMNs in less than 10 minutes (Figure 10). The reduction of macrocluster area is consistent with the previous work in other cell types that Kv2.1-IR is redistributed following increased activity.
**Figure 10.** Kv2.1-IR cluster areas decrease in lumbar α-motoneurons following increased in vitro activity. Bath application of either artificial cerebral spinal fluid (ACSF) (panel A) or 10 μM glutamate (panel B) was applied to lumbar spinal cord slices for 10 minutes. Arrowheads point to the large Kv2.1-IR macroclusters. (A) Micrograph representation of single optical confocal section of tissue slice incubated in ACSF. Scale bar is 10 μm. (B) Micrograph representation of single optical confocal section of tissue slice incubated in artificial cerebral spinal fluid with 10 μM glutamate. Scale bar is 10 μm. (C) Quantitative analysis showing significant decrease of Kv2.1-IR macrocluster areas following glutamate treatment. Number of clusters sampled is displayed as “n” from 20 total cells of each treatment group. Data is presented as mean ± SEM.
Tibial nerve crush results in a significant decrease of both Kv2.1-IR macrocluster area and Kv2.1-IR macrocluster number.

The goal of experiments reported in this section was to determine the effects of PNI on Kv2.1-IR macroclusters in rats by implementing a tibial nerve crush injury that permits proper reinnervation of peripheral targets. Spinal MNs with axons in the tibial nerve, specifically those that innervate the medial and lateral gastrocnemius muscles (MG/LG), were visualized with retrograde tracers injected intramuscularly prior to the injury. MNs positive for the retrograde tracers were imaged with confocal microscopy and quantitatively analyzed (Figure 9). Initial observations revealed a statistically significant bilateral effect of unilateral nerve damage on Kv2.1-IR immunoreactivity in α-MNs after nerve crush or ligation (see below), although the reduction of Kv2.1 cluster sizes in contralateral MNs was always much less than on the side ipsilateral to the nerve damage. No effects were detected following sham operations. Therefore for accuracy, statistical analyses and graphs compare injury data to both sides of sham animals. However, to eliminate image variability resulting from artifacts or inconsistencies in tissue processing, confocal images included for illustrative purposes compare immunoreactivity from injured MG/LG MNs to contralateral, injury-spared MG/LG MNs within the same tissue slice (Figures 11 & 12). It should be recognized that at the time points chosen for the ‘side-by-side’ confocal images presented here (8 days post injury) there was not a significant contralateral effect; however, because there may be a contralateral effect, the illustrated data potentially underestimates the ‘absolute’ degree of change in cluster size that occurs in an injured neuron.
Kv2.1-IR macroclusters (diameters >1µm) visualized en face were selected and measured 8 days following tibial nerve crush injury (Figure 11). Kv2.1-IR macrocluster cross-sectional areas are on average reduced by about 50% on the injured αMNs (4.75 µm² ± 2.36 SD, n=937 clusters, 76 αMNs, 5 rats) compared to sham surgery control αMNs (9.29µm² ± 4.82 SD, n=543 clusters, 50 αMNs, 3 rats) and the contralateral spared αMNs (9.35µm² ± 3.76 SD, n=1123 clusters, 77 αMNs, 5 rats) (Figure 11C). The Kv2.1 cluster areas on the contralateral sides were not found to be significantly different from sham surgery control animals at this time point (see below).

In addition to the reduction in size, the crush injury also induces a significant decrease in the number of Kv2.1-IR macroclusters on the soma of medial and lateral gastrocnemius MNs, with a maximum 55% reduction in number (injured mean=24.4 ± 9.68 SD, n=44 αMNs, 5 rats) 8 days after the injury compared to sham surgery controls (sham surgery control mean=54.6± 15.91 SD, n=36 αMNs, 3 rats). There were no significant differences in macrocluster number on the contralateral side (mean=50.8 ± 13.89 SD, n=53 αMNs, 5 rats), compared to sham surgery controls (Figure 11D).

Furthermore, the linear distance between individual Kv2.1-IR macroclusters were measured in a nearest neighbor analysis, in 10 control and injured αMNs 8 days post injury. There are significant increases in distances between nearest neighbor Kv2.1 macroclusters following tibial nerve crush (Figure 11E). Altogether, these data are consistent with fewer macroclusters with reduced areas.
Kv2.1-IR macroclusters located on proximal dendrites (up to approximately 150 μm from soma) were measured through 3D reconstructions to determine if channel clusters on dendrites also were affected by PNI. Kv2.1-IR macroclusters located on the proximal dendrites are 27% reduced in area (sham surgery control 13.42 μm² ± 6.054 SD, n=299 clusters, 20 αMNs, 2 rats; injured 9.764 μm² ± 5.091 SD, n=120 clusters, 10 αMNs, 2 rats) 8 days following tibial nerve crush (Figure 12). Although dendritic Kv2.1-IR macroclusters (some of which are at least as large as clusters on the soma) are affected by PNI, they exhibit smaller on average percentage reduction of area than do macroclusters located on the soma membrane itself.
Figure 11. Kv2.1-IR macrocluster areas decrease on soma of medial and lateral gastrocnemius α-motoneurons following tibial nerve crush injury. To eliminate image variability resulting from artifacts or inconsistencies in tissue processing cells shown in panels A and B are from the same tissue section and imaged with fixed parameters. However, due to observed contralateral effects (see section 2.5) the illustrated data potentially underestimates the ‘absolute’ degree of change in cluster size that occurs in an injured neuron. (A) Micrograph of a confocal stack (32 x 1μm z-steps) of a contralateral motoneuron showing representative Kv2.1-IR clustering. Scale bar is 20 μm. (B) Micrograph of a confocal stack (32 x 1μm z-steps) of an 8 days post tibial nerve crushed motoneuron showing representative Kv2.1-IR clustering with reduced areas compared to the spared motoneuron. Scale bar is 20 μm. (C) Quantitative analysis of reduced Kv2.1-IR soma macrocluster areas on lumbar α-motoneurons 8 days following tibial nerve crush. (D) Quantitative analysis of reduced numbers of Kv2.1-IR soma macroclusters on lumbar α-motoneurons 8 days following tibial nerve crush. (E) Kv2.1 macrocluster nearest neighbor distance increases following 8 days post tibial nerve crush. In panels C, D, &E significance (p<0.05) is indicated with asterisk and determined with Pairwise T-Test and data is presented as mean ± SEM.
Figure 12. Kv2.1-IR Macrocluster areas decrease on proximal dendrites of medial and lateral gastrocnemius α-motoneurons following tibial nerve crush.

Both cells shown in these panels are from the same spinal cord tissue slice and are scaled equally. Scale bar is 30 μm. Inserts in the top right of panel A and B shows the CTB retrograde fills from gastrocnemius muscle that was used to reconstruct the cell somas and proximal dendrites. (A) Micrograph showing representative 3-D reconstruction, from confocal microscopy image stacks (57 x 0.5 μm z-steps), of a contralateral motoneuron. (B) Micrograph showing representative 3-D reconstruction, from confocal microscopy image stacks (41 x 0.5 μm z-steps), of a motoneuron 8 days following nerve crush. (C) Quantitative data from 10 control and 10 injured motoneurons showing Kv2.1-IR macrocluster areas significantly decrease on proximal dendrites following both 8 days post tibial nerve crush. Data is presented as mean ± SEM. Significance is set to p<0.05.
Tibial nerve crush results in a redistribution of Kv2.1-IR in α-motoneuron membrane.

In cultures of hippocampal cells, use of Kv2.1 surface membrane-impermeant biotinylation assays following glutamate application revealed that the amount of membrane-localized Kv2.1 did not change even though the cluster areas qualitatively appeared reduced (Misonou et al., 2004). These results indicated a lateral translocation and redistribution of Kv2.1 in the membrane following the glutamate treatment rather than Kv2.1 internalization, and we wished to determine if this is the case in αMNs after injury. Indeed, in our experiments, when the axotomized MN membrane is examined in single optical confocal sections and small stacks of en face membrane, the small distinct punctae, or microclusters, typical of Kv2.1-IR distributions in uninjured αMNs (Figure 13A&C) appear to be spread into a more diffuse field (Figure 13B&D). Our previous studies of Kv2.1 immunoreactivity demonstrates the ability of our methods to quantitatively measure very small sized patches of immunoreactivity (Muennich & Fyffe, 2004). However, following injury the diffuse Kv2.1-IR in the membrane consists largely of punctae so small that accurate area measurements are precluded. However, we were able to assess quantitatively the relative levels of immunoreactivity in areas devoid of macroclusters but containing microclusters (measurable diameters <1.0 μm) and/or even smaller punctae. Analysis of regions of interest in en face membrane were selected in 10 uninjured and 10 injured MNs 8 days post tibial nerve crush. The minimum and average intensities measured in these regions were conserved following injury, with only the maximum intensity significantly decreased (Figure
13E). However, there are no significant changes in the integrated optical density (density normalized to area sampled) of these same regions suggesting that the density of fluorescent pixels is conserved within the regions of interest (Figure 13F). Finally we evaluated the same membrane regions for the fraction of pixels that deviate more than 10% from the average intensity and found there is a significant 88% reduction in the degree of heterogeneity of the Kv2.1-IR signal after injury (Figure 13G). These results suggest that the Kv2.1-IR is less organized into microclusters, but still present in the membrane in a diffuse redistributed (or declustered) field of average intensity.
Figure 13. *Kv2.1 microcluster immunoreactivity becomes more diffuse on soma of medial and lateral gastrocnemius α-motoneurons following tibial nerve crush injury.* To eliminate image variability resulting from artifacts or inconsistencies in tissue processing cells shown in panels A and B are single optical confocal sections from the same tissue section and imaged with fixed parameters. However, due to observed contralateral effects (see *Bilateral Kv2.1-IR declustering occurs following PNI*) the illustrated data potentially underestimates the ‘absolute’ degree of change in cluster size that occurs in an injured neuron. Both panels A and B are single optical confocal sections through the center of the cell from the same tissue section and imaged with fixed parameters. Full confocal stacks of these two cells are shown in figure 11. (A1) Micrograph of single optical section of a contralateral motoneuron showing representative Kv2.1-IR clustering. (A2) Inset is zoomed region of the membrane, indicated by dotted box, with arrowheads pointing to distinct Kv2.1-IR microcluster punctae in the membrane. Scale bar is 20 μm. (B1) Micrograph of single optical section of an 8 days post tibial nerve crushed motoneuron showing representative diffuse Kv2.1 microcluster immunoreactivity compared to the spared motoneuron. (B2) Inset is zoomed region of the membrane, indicated by dotted box. Note diffuse Kv2.1 microcluster immunoreactivity in the membrane compared to distinct punctae shown by arrowheads in A2. Scale bar is 20 μm. (C&D) Micrographs of small confocal stacks (10 x 1.0 μm Z-steps) of small patches of *en face* membrane regions. The distinct microcluster punctae in the spared motoneurons (panels C) appear more diffuse 8 days after tibial nerve crush (panels D). Scale bars are 10 μm. (E) Quantitative analysis of Kv2.1-IR microcluster
intensity of lumbar α-motoneuron somas of spared and 8 day post tibial nerve crush. (F) Quantitative analysis of Kv2.1 microcluster integrated optical density of immunoreactive pixels on lumbar α-motoneuron somas of spared and 8-day post tibial nerve crush. (G) Quantitative analysis of Kv2.1-IR microcluster heterogeneity, the fraction of pixels that deviate more than 10% from the average intensity, on lumbar α-motoneuron somas of control and 8 day post crush. Data is presented as mean ± SEM. Significance (p<0.05) as indicated with asterisk was determined with t-test.
Kv2.1 macroclusters are not completely restored after peripheral reinnervation.

Properties shaping intrinsic excitability following peripheral nerve injury generally return to normal ranges upon proper reinnervation of the peripheral target (Kuno et al., 1974b; Foehring et al., 1986b, a; Bichler et al., 2007). To determine if Kv2.1 ‘reclusters’ upon reinnervation, en face Kv2.1-IR macroclusters (diameters >1µm) were selected and measured for cross sectional area at 20 minutes, 3 days, 8 days, 2 weeks, 4 weeks and 6 months following tibial nerve crush injury (Figure 14, Table 3). At the latter two of these time points it is likely that reinnervation of target muscle is underway and/or has occurred. Significant differences in Kv2.1-IR macrocluster area exist between the injured αMNs and the sham surgery control αMNs as early as 20 minutes following injury (20% reduction), progressing to a maximum 51% reduction at 3 days post-injury. Subsequently, there is a gradual increase in cluster size, eventually attaining about 87% of normal average cluster size 6 months after injury (Figure 14A).

To determine if the number of Kv2.1-IR macroclusters is restored after reinnervation, Kv2.1-IR macroclusters (diameters >1µm) were counted on MN somas at 20 minutes, 3 days, 8 days, 2 weeks, 4 weeks and 6 months following tibial nerve crush (Figure 14B). Significant differences in the number of Kv2.1-IR macroclusters exist between the injured αMNs and sham surgery control with approximately 31% reduction 3 days following nerve crush and progressing to a maximum 54% reduction at 8 days following crush and returning to about 85% of normal 6 months after injury (Figure 14B).
In a separate set of experiments, over the same time points post injury, we sought to determine the effects on Kv2.1-IR clustering when reinnervation was prevented through tibial nerve ligation (see methods). Following ligation, there is a gradual, yet ultimately significant declustering of Kv2.1-IR macrocluster areas. Significance is reached 3 days following this type of injury, when there is a 25% reduction in Kv2.1-IR macrocluster area, and a maximal 34% reduction by 4 weeks after the tibial nerve ligation when compared to sham surgery controls. Interestingly, by 6 months, the average Kv2.1-IR macrocluster areas are no longer significantly different from the sham controls (Figure 15A&B, Table 4). Similar to the tibial nerve crush injury, there is a significant decrease in the number of Kv2.1-IR macroclusters, but this too occurs over a slower time course, with a maximum 40% reduction 2 weeks after ligation (Figure 15B).

Using the same 3-D reconstruction techniques previously described for the tibial nerve crush injury, we calculated the Kv2.1 macrocluster areas on proximal dendrites of MG/LG αMNs following tibial nerve ligation. For these analyses we selected 4 weeks post ligation due to the maximum effect of ligation on somatic αMN Kv2.1-IR macroclusters. On proximal dendrites, there is a significant 23% reduction in soma Kv2.1-IR macrocluster areas (sham surgery control 13.42μm² ± 6.054 SD, n=299 clusters, 20 αMNs, 2 rats; injured 10.347μm² ± 6.388 SD, n=125 clusters, 10 αMNs, 2 rats). No significant differences of Kv2.1 macrocluster areas were ever found within the population of the control MNs that were 3-D reconstructed.
Figure 14. Time course of Kv2.1-IR declustering of gastrocnemius motoneuron somas following tibial nerve crush at key time points (20 minutes, 3 days, 8 days, 2 weeks, 4 weeks, and 6 months). Kv2.1-IR cluster sizes are still significantly smaller 6 months following injury with reinnervation. All comparisons are made to sham surgery control animals analyzed at 3 day, 8 day and 4 weeks following surgery. No significant differences were found between or within time points for the ipsilateral and contralateral sides in sham surgery control animals. Data is presented as mean ± SEM. Significance (p<0.05) was determined with pairwise ANOVA. (A) Significant decrease (indicated by single asterisk) in Kv2.1-IR areas on injured motoneurons appear as early as 20 minutes following injury and are significantly reduced at all time points following injury. There are also small, yet significant (indicated by double asterisk), decreases on the injury spared contralateral side of the rat spinal cord at 20min, 3 days, 2 weeks and 4 weeks post injury. (B) The number of Kv2.1-IR macroclusters on motoneurons somas decrease significantly (indicated with asterisk) 3 days following tibial nerve crush and are significantly reduced at all following time points.
### Table 3. Kv2.1-IR macrocluster means and sample sizes for tibial nerve crush injury

<table>
<thead>
<tr>
<th>Kv2.1-IR Mean Macrocluster Area (µm²)</th>
<th>Sham ±SD</th>
<th>20 min ±SD</th>
<th>3 Day ±SD</th>
<th>8 Day ±SD</th>
<th>2 Week ±SD</th>
<th>4 Week ±SD</th>
<th>6 Month ±SD</th>
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<tr>
<td>n=543, 50, 3</td>
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<tr>
<th>Kv2.1-IR Mean Macrocluster Area (µm²)</th>
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<td>n=504, 49, 3</td>
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±SD
A

Tibial Nerve Ligate

Kv2.1-IR Macrocluster Area (μm²)

Sham 20 m 3 D 8 D 2 W 4 W 6 M

B

Kv2.1-IR Macrocluster Number Following Tibial Nerve Ligate

Kv2.1-IR Macrocluster Number per Sense

Sham 20 m 3 D 8 D 2 W 4 W 6 M
Figure 15. Time course of Kv2.1-IR declustering of gastrocnemius motoneuron somas following tibial nerve ligation at key time points (20 minutes, 3 days, 8 days, 2 weeks, 4 weeks, and 6 months). Kv2.1-IR clusters are reduced in number but return to pre-injury size 6 months following injury without reinnervation. All comparisons are made to sham surgery control animals analyzed at 3 day, 8 day and 4 weeks following surgery. No significant differences were found between or within time points for the ipsilateral and contralateral sides in sham surgery control animals. Data is presented as mean ± SEM. Significance (p<0.05) was determined with pairwise ANOVA. (A) Kv2.1 areas are significantly reduced (indicated by single asterisk) at 3 days, 8 days, 2 weeks, and 4 weeks following injury. There are small, yet significant (indicated by double asterisk), decreases on the injury spared contralateral side of the rat spinal cord at 2 weeks and 4 weeks post injury. (B) The number of Kv2.1-IR macroclusters decrease significantly (indicated with asterisk) 3 days following tibial nerve crush and are significantly reduced at all following time points.
Table 4. Kv2.1-IR cluster means and sample sizes for tibial nerve ligation injury

<table>
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<th>Injury (ipsilateral)</th>
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<th>4 Week</th>
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<tr>
<td>Kv2.1-IR Mean Macrocluster Area (µm²) 8.19 ± 5.50 &amp; 7.77 ± 4.13 &amp; 6.51 ± 4.59 &amp; 6.18 ± 4.06 &amp; 5.66 ± 3.95 &amp; 5.43 ± 4.18 &amp; 8.07 ± 5.11</td>
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<th>8 Day</th>
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<th>4 Week</th>
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<td>Clusters, Cells, Animals sampled n=589, 58, 2 &amp; n=537, 54, 2 &amp; n=523, 61, 2 &amp; n=301, 30, 2 &amp; n=766, 72, 2 &amp; n=482, 65, 3 &amp; n=682, 74, 3</td>
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±SD
Bilateral Kv2.1-IR declustering occurs following PNI.

As mentioned previously, we found that Kv2.1-IR redistribution occurs bilaterally following PNI. Kv2.1-IR macroclusters on the αMN cell body also decrease on the uninjured contralateral side following PNI (Figure 14A). The contralateral effect in the tibial nerve crush results in a significant maximum 22% reduction at 3 days post injury compared to sham surgery controls, and there is complete recovery of cluster size by 8 days. However, this recovery appears to be transient, because a second significant reduction (16%) occurs at 28 days. While this biphasic contralateral effect is present and significant, there was, however, a greater decrease in Kv2.1-IR macrocluster area on the corresponding ipsilateral side at all time points. Like the tibial nerve crush, there is also a small yet significant contralateral effect (18% maximum reduction in Kv2.1-IR macrocluster area) on the contralateral side 28 days following ligation (Figure 15A). While not significant, there is a trend resembling the biphasic response observed in the tibial nerve crush injury, with an initial transient reduction (5%) in Kv2.1 cluster size 3 days after the ligation injury.
No Changes in Motoneuron Cell Diameter following Peripheral Nerve Injury

Axotomy can cause morphological changes in neurons such as cell shrinkage, dissolution of nissl bodies, and acentric localization of the nucleus, all referred to as chromatolysis. Axotomy, and associated chromatolysis, was used to identify central motor cell columns in the cat before the development of retrograde tracers (Romanes, 1951). However, unlike our PNIs, repeated acute injury to the peripheral nerves was necessary for generating the degree of chromatolysis necessary for identifying the motor pools. Here, we sought to determine if the changes in Kv2.1-IR cluster areas are a result of changes in MN sizes, first by determining if any correlations exist between Kv2.1 cluster sizes and MN diameter, and secondly to determine if there are detectable cell diameter changes following axotomy. In sham surgery control MNs, there was no correlation between Kv2.1-IR cluster sizes and the mean cell diameter of the MG/LG MNs (n=520 clusters, 80 cells, 2 rats) (Figure 16A). Furthermore, there were no significant changes in the mean cell diameter 8 days after the tibial nerve crush and 4 weeks after tibial nerve ligation when compared to contralateral controls (Figure 16B). It is therefore unlikely the observed changes in Kv2.1-IR macrocluster areas following axotomy are a result of changes in MN size.
Figure 16. Kv2.1-IR cluster area reduction following PNI is not a result of reduction in motoneuron size. (A) Quantitative data showing no correlation between Kv2.1 cluster sizes and soma diameters in uninjured medial and lateral gastrocnemius motoneuron somas. (B) Quantitative data showing no change in mean cell soma diameter following tibial nerve crush and tibial nerve ligate. Data is presented as mean ± SEM.
Discussion

Members of the voltage-gated potassium channel family are essential for numerous neuronal functions, and have been implicated in a wide variety of responses ranging from neuroprotection to apoptosis. Kv2.1 channels underlie delayed rectifier currents in many central neurons, including MNs where they are expressed in high-density clusters (Blaine & Ribera, 2001; Muennich & Fyffe, 2004). The striking membrane organization of Kv2.1 channels makes them a valuable model for study of channel regulation under conditions of altered activity including injury. In hippocampal cells, Kv2.1 undergoes dynamic membrane declustering following increased activity both in vivo (ischemia, hypoxia and seizure) and in vitro (glutamate application) (Misonou et al., 2004; Misonou et al., 2005). Furthermore, an increase in Kv2.1 clustering has been observed in some model systems, triggered by physiological stimuli such as repetitive firing, or removal of pathological stimuli (Misonou et al., 2004; Schulz et al., 2006; Mulholland et al., 2008; Zhang et al., 2008; Aras et al., 2009; Cerda & Trimmer, 2011). Here we show, for the first time, glutamate induced Kv2.1 cluster dispersal in lumbar spinal MNs, in vitro. This in vitro cluster dispersal occurs on a time scale (<10 minutes) similar to that previously reported for hippocampal and HEK293 cells. Moreover, we demonstrate in vivo axotomy as a novel stimulus causing significant rapid declustering of Kv2.1, as early as 20 minutes, representing the earliest accessible time point following surgical axotomy. Similar to ischemia, hypoxia, and glutamate application, axotomy induced Kv2.1 declustering is likely a product of altered activity.
Peripheral nerve injury is complex because it results in axotomy of both MNs and sensory afferents known to modulate MN function; however, given its clinical relevance, it remains a valuable model from which to extract new information on the capacity of central neurons to respond to and recover from injury. Although the results of axotomy and reinnervation studies depend on a large number of factors (e.g. lesion location, age, degree of reinnervation, etc.), several consistent outcomes have been reported by a number of laboratories (Eccles et al., 1958; Kuno & Llinas, 1970b, a; Kuno et al., 1974a, b; Kuno, 1976). Within several days following PNI there are many changes in MN intrinsic properties, such as reduction in rheobase, modulation of AHP, increase in input resistance, and slowing of axonal conduction velocity proximal to the injury (Kuno et al., 1974a; Gustafsson & Pinter, 1984; Nakanishi et al., 2005; Bichler et al., 2007). Interestingly, most membrane properties appear to return to pre-axotomy states upon reinnervation of the peripheral targets (Kuno et al., 1974b; Nakanishi et al., 2005; Bichler et al., 2007). The altered membrane properties following axotomy likely reflect the cumulative alterations to multiple channel types, including Kv2.1, that contribute to a coordinated cellular response.

In this study, we sought to determine what happens to Kv2.1-IR distribution in the absence or presence of reinnervation by the injured neurons, using a tibial nerve crush injury that permits reinnervation of the peripheral target (Fournier & Strittmatter, 2002; Nguyen et al., 2002) and a ligation injury that prevents reinnervation. As a consequence of initial axon injury in each model we expected the time course of manifestation of injury responses to be similar at least until the
time of reinnervation that occurs about 4-6 weeks after crush injury (Bichler et al., 2007; Alvarez et al., 2011). However, long before reinnervation occurs, we observed injury type-specific changes. Most surprisingly, the magnitude of effects on Kv2.1 immunoreactivity was apparently greater following nerve crush than following nerve cut and ligation. Paradoxically, after reinnervation occurs, normal Kv2.1 cluster sizes are not fully restored; however, cluster sizes do recover when reinnervation is prevented.

Other investigators have reported injury-type specific changes (Waite & Cragg, 1982; Nielsch et al., 1987; He et al., 1996; Bullinger et al., 2011; Prather et al., 2011). However, even when muscle reinnervation occurs, the initial form of injury (crush or cut) provokes different outcomes. In the cat, when muscles reinnervated by crushed nerves are stretched, MNs yield a more forceful reflex contraction despite the decrease in monosynaptic stretch evoked EPSPs (Prather et al., 2011). Contrasting results were observed in both rat and cat in which nerves were cut and the stretch reflex is absent following reinnervation (Cope & Clark, 1993; Alvarez et al., 2010; Bullinger et al., 2011). The range of pathophysiological and structural changes now observed following crush or cut injuries suggests that additional investigation into the differences between cut and crush nerve injury are warranted.

One significant structural change that occurs after peripheral nerve injury is the loss of synaptic inputs on MNs (synaptic stripping) (Blinzinger & Kreutzberg, 1968; Sumner, 1975; Brannstrom & Kellerth, 1998, 1999; Gonzalez-Forero et al., 2004; Hughes et al., 2004; Tiraihi & Rezaie, 2004; Alvarez et al., 2011). Moreover, there is a permanent loss of vesicular glutamate transporter isoform 1 (VGLUT1)
positive primary sensory afferent synapses following nerve cut and a transient reduction of synaptic coverage from cholinergic C-boutons MNs that is partially restored upon peripheral reinnervation (Sumner, 1975; Alvarez et al., 2010; Alvarez et al., 2011; Bullinger et al., 2011). Because ‘macroclusters’ of Kv2.1-IR are selectively apposed to C-boutons (Muennich & Fyffe, 2004), it is interesting that the time course of effects on these clusters mirrors the VACHT-IR stripping in nerve cut injuries. The role that synaptic stripping has in the regulation and dysregulation of Kv2.1 clusters is currently unknown, and any speculation here is complicated by the paucity of data on synaptic stripping following nerve crush injuries.
Kv2.1 and Homeostatic Plasticity

Kv2.1 has increasingly been described as a target for mechanisms of homeostatic plasticity (Cudmore & Turrigiano, 2004; Misonou et al., 2004; Surmeier & Foehring, 2004; Misonou et al., 2008; Kihira et al., 2010; Nataraj et al., 2010). Neuronal homeostatic plasticity reflects the ability of a neuron to respond to changes in activity and maintain an optimal level of output. Multiple homeostatic mechanisms have been described, invoking both pre- and post-synaptic changes (Burrone & Murthy, 2003; Murphy, 2003; Surmeier & Foehring, 2004; Schulz et al., 2006; Walmsley et al., 2006). These include ‘synaptic scaling’, reducing synaptic strength at presynaptic inputs in response to increased postsynaptic activity (Turrigiano et al., 1998). Regulation of postsynaptic machinery through either translational/transcriptional regulation or post-translational modifications may play a major role in homeostatic responses. Activation of immediate early genes (Sheng & Greenberg, 1990; Leah et al., 1991), which are known to regulate activity dependent expression of certain proteins including potassium channels (Leao et al., 2010), can occur within an hour of PNI (New et al., 1989). However, the slow time course for transcription, translation and trafficking to the membrane would not allow de novo expression or downregulation of ion channels to compensate for immediate changes in activity. Trafficking through the Golgi apparatus alone in cell lines can take up to 20 minutes (Patterson et al., 2008). Here, we saw a significant decrease in the Kv2.1 cluster area within 20 minutes following crush injury. This supports that idea that regulation of Kv2.1 in MNs is likely to be a consequence of post-translational modification, such as changes in redox state or phosphorylation.
In hippocampal pyramidal neurons and cell cultures, the lateral translocation of Kv2.1 is homeostatically regulated through calcineurin dependent dephosphorylation (Murakoshi et al., 1997; Misonou et al., 2004; Misonou et al., 2005; Park et al., 2006). This effect is mimicked by injecting phosphatases into the cell and is blocked with calcineurin inhibitors (Misonou et al., 2004; Misonou et al., 2005; Mohapatra et al., 2009). Given the speed at which declustering occurs in the spinal cord after injury (within 20 minutes), regulation through phosphorylation mechanisms appears to be a likely candidate. Regulating MN excitability and activity through modification of post-synaptic voltage-gated ion channels, such as Kv2.1, may be a more energetically favorable mechanism because regulating the firing rate can be isolated to just the cell body and proximal dendrites of the neuron rather than requiring changes in thousands of input synapses (Kihira et al., 2010).

The signal that triggers the response is not yet defined, but it is possible that the instantaneous ‘injury discharge’ in sensory and motor axons can change activity sufficiently to evoke the rapid post-translational modifications of channel state. It is also possible that the magnitude and duration of injury discharges following different types of insult may differ and be sufficient to trigger different and long-lasting regulatory changes.

Placing axotomy-induced responses of MNs in the context of homeostasis is difficult because the exact in vivo activity level of MNs, and their state dependent modifications, following PNI and the instantaneous but relatively brief injury discharge is unknown. Moreover, multiple factors, including pre- and postsynaptic changes are likely to coordinate in the injury response to increase or decrease
activity. Phosphatases regulate Kv2.1 channels in two critical ways, simultaneously altering both localization and channel activity. Modeling suggests that the disruption of Kv2.1 clusters, with all other factors constant, results in decreased excitability (Surmeier & Foehring, 2004; Mohapatra et al., 2009). However following axotomy, MNs become more excitable. One possible explanation to this paradox is that homeostatic mechanisms may not be strong enough to overcome pathophysiologically induced or sustained hyperexcitability (Misonou et al., 2004; Misonou et al., 2005; Misonou et al., 2008; O’Leary et al., 2010). It has been speculated that some ‘homeostatic’ mechanisms may instead be providing neuroprotection against neuronal damage and death (Surmeier & Foehring, 2004; Misonou et al., 2005). For example, in the brain Kv2.1 is clustered near astroglial processes expressing high levels of glutamate transporter (Misonou et al., 2008). During ischemia, the astrocytes become dysfunctional resulting in an accumulation of extracellular glutamate. The positioning of the Kv2.1 clusters permits a rapid response to the high glutamate levels thus potentially protecting the neuron from a potentially damaging excitotoxic effect (Misonou et al., 2008; Misonou, 2010). In the rat lumbar spinal cord, Kv2.1-IR clusters are selectively localized at specific synapses and at glial sites (Muennich & Fyffe, 2004) and are thus also appropriately positioned for a quick neuroprotective response to excitatory insult.

There are still unanswered questions about what happens to the phosphorylation state of the channels after the declustering of Kv2.1. The restoration of cluster areas could be explained by alterations in the ratio of kinase to phosphatase activity or because of protein turnover. However, protein turnover is
not likely to be the sole reason for reclustering. In quantum dot assays in HEK cells, new channels are regularly trafficked to the Kv2.1 clusters (O’Connell et al., 2006). This trafficking is frequent enough that within twelve minutes there is significant GFP recovery of a photobleached Kv2.1 cluster (O’Connell et al., 2006). Our data here shows that there is continued declustering of Kv2.1 clusters for at least 8 days after the injury suggesting that some other mechanism is regulating and maintaining the declustered state. Furthermore, constitutive insertion of de novo proteins to the membrane may also explain why we are never completely devoid of Kv2.1 clusters.

Kv2.1 also has a role in mediating the K+ loss in apoptosis. Increased phosphorylation of S800 in the C-terminal tail of Kv2.1 by p38 Map Kinase leads to enhanced K+ currents through SNARE mediated membrane insertion in cultured cells (Redman et al., 2007; Yao et al., 2009). This site is distinct from the identified calcineurin dependent phosphorylation sites that play a role in homeostatic plasticity (Misonou et al., 2004; Park et al., 2006; Kihira et al., 2010). Here we see evidence that Kv2.1 reversibly declusters, suggesting that the regulation of Kv2.1 after PNI is dynamic and is likely a protective, or homeostatic, response rather than an apoptotic response. This suggests that different pathological conditions can give rise to differential Kv2.1 expression and/or modification mediating multiple physiological effects.
Contralateral Effect

There are a range of well-documented pathophysiological changes in the spinal cord following unilateral peripheral nerve lesions, including effects on the contralateral side (Koltzenburg et al., 1999). Generally, as is the case with our observations here, the contralateral effects are qualitatively similar to those occurring on the ipsilateral side, but with smaller magnitudes and briefer time courses. Our comparisons of effects in control animals undergoing sham surgery as well as naïve controls, at different time points post surgery revealed that sham surgery does not trigger any changes. It is also unlikely that the contralateral effect is compensatory from the animal using the uninjured leg more, because as in injured motoneurons the decrease in cluster size is already measurable within twenty minutes, before any post-surgical locomotor activity could occur. Thus the contralateral effects must be a product of the peripheral nerve injury itself.

Evidence exists to support both multiple humoral and neuronal mechanisms contribute to a wide range of contralateral effects in rats. It is well established that inflammatory responses contribute to a variety of effects in the central nervous system within the first three days following trauma (Gaudet et al., 2011). It is unknown whether the effect on Kv2.1 expression and localization in contralateral, uninjured, motoneurons results from a systemic inflammatory response or from some other neuronal mechanism (Pachter & Eberstein, 1991; Lu & Richardson, 1993; Koltzenburg et al., 1999; Jancalek et al., 2010; Jancalek et al., 2011). The specific mechanisms underlying the biphasic contralateral response shown here are also unknown. Interestingly, studies of other biophysical properties also detect
biphasic responses with peaks at 3 and 28 day time periods following injury (Weerasuriya & Hockman, 1992; Mizisin & Weerasuriya, 2011). Nonetheless, our results further emphasize that caution should be used when using internal bilateral controls to interpret changes in spinal systems.
Conclusions

Here we show that the dynamic reorganization of Kv2.1 previously shown in other neuronal types also occurs in rat lumbar spinal MNs following increased activity. Moreover, we demonstrate and quantify novel effects on the membrane distribution of Kv2.1 ion channels on rat lumbar spinal MNs following PNI. The dynamic changes in membrane channel localization include a progressive decline in cluster size immediately after axon injury followed by gradual recovery of cluster size over the next several months. Upon motor axon reinnervation of muscle tissue, Kv2.1 clusters do not completely restore to pre-injury sizes, however in the absence of reinnervation, Kv2.1 clusters fully restore. Furthermore, unilateral peripheral nerve injury evokes parallel, but smaller, effects contralaterally in uninjured motoneurons. Altogether these results suggest that in MNs Kv2.1 declustering is likely a homeostatic response to altered activity and its regulation following axon injury is largely independent of muscle reinnervation.
Chapter VI. Specific Aim 2:

Activity-dependent redistribution of

Kv2.1 ion channels on spinal motoneurons
Introduction

Many electrophysiological and modeling studies reveal that motoneuron (MN) intrinsic properties respond to both physiologic and pathologic changes in activity (Bichler et al., 2007a; Foehring et al., 1986a; Foehring et al., 1986b; Gustafsson and Pinter, 1984; Johnson et al., 2013; Kuno et al., 1974a; Kuno et al., 1974b; Meehan et al., 2010; Prather et al., 2011; Quinlan et al., 2011; Wolpaw and Tennissen, 2001). Numerous ion channels play a role in setting intrinsic membrane properties, and modifications thereof reflect a coordinated cellular response to adjust MN activity. For example, the role of persistent inward currents in setting appropriate MN input-output gain has been extensively characterized (Johnson and Heckman, 2014; Manuel et al., 2012), and more recent studies have examined SK channels and their modulation by C-bouton synaptic contacts (Deardorff et al., 2013; Deardorff et al., 2014; Miles et al., 2007; Zagoraiou et al., 2009). Recently we have proposed the dynamic reorganization of the delayed rectifier Kv2.1, which is also clustered at C-type synaptic inputs, plays a critical role in setting appropriate MN firing following prolonged excitatory drive (Deardorff et al., 2014).

Kv2.1 undergoes activity-dependent changes in several neuronal types (Cudmore and Turrigiano, 2004; Deardorff et al., 2014; Kihira et al., 2010; Misonou et al., 2004; Misonou et al., 2008; Misonou, 2010; Mohapatra et al., 2009; Nataraj et al., 2010; Romer et al., 2014; Surmeier and Foehring, 2004). In the highly clustered configuration observed in hippocampal and cortical pyramidal cells, Kv2.1 channels are phosphorylated and have high activation and deactivation thresholds together with slow kinetics (Guan et al., 2013; Liu and Bean, 2014; Misonou et al., 2004;
Misonou et al., 2005; Misonou, 2010; Mohapatra and Trimmer, 2006; Murakoshi et al., 1997; Surmeier and Foehringer, 2004). Upon prolonged excitatory drive, Ca\(^{2+}\)/calcineurin dependent dephosphorylation pathways rapidly decluster Kv2.1 while simultaneously lowering activation and deactivation thresholds and accelerating the kinetics, thus reducing neuronal firing rate (Mohapatra et al., 2009; Park et al., 2006; Surmeier and Foehringer, 2004).

In spinal lumbar αMNs, Kv2.1 is similarly clustered into microdomains with other ion channels and neurotransmitter receptors into a highly regulated signaling ensemble on the soma and proximal dendrites (Deardorff et al., 2013; Deardorff et al., 2014; Deng and Fyffe, 2004; Mandikian et al., 2014; Muennich and Fyffe, 2004; Romer et al., 2014; Wilson et al., 2004). Following peripheral nerve injury, αMN Kv2.1 dramatically and significantly declusters within 20 minutes and slowly recovers over weeks to months suggesting this channel has the capacity to rapidly and dynamically respond to pathologic stimuli (Romer et al., 2014). Furthermore, in an in vitro spinal cord slice preparation, Kv2.1 in αMNs dynamically declusters following 10 min bath application of glutamate suggesting activity-dependent regulation (Romer et al., 2014).

Here, in an adult rat in vivo preparation, we use direct electrical stimulation of nerves to demonstrate that Kv2.1 rapid clustering dynamics (10 minutes) in αMNs are activity-dependent under physiological conditions. Moreover, we demonstrate that sensory evoked synaptic inputs also contribute to Kv2.1 clustering dynamics in αMNs. These observations are critical for not only interpreting activity-
dependent intrinsic modifications, but also lay the foundation for future studies to examine Kv2.1 dynamics in physiological and pathological states.
Experimental Procedures

Animal Use

All animal procedures were performed according to National Institutes of Health (NIH) guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University. Detailed immunohistochemical analysis of Kv2.1 channel expression was performed on adult female (250-250g) Sprague Dawley rats (n=24) following retrograde labeling of medial gastrocnemius (MG) MNs and in vivo sciatic nerve stimulations or sham control experiments. All survival and terminal surgeries were performed with rats deeply anesthetized (absent withdrawal and corneal reflex) by isoflurane inhalation (induction 4–5%; maintenance 1–3%, both in 100% O2).
Retrograde Tracer

All rats in this study underwent a single sterile survival surgery to retrogradely label MG MNs for *post-hoc* identification (Romer et al., 2014). The triceps surae were 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 (CTB, Invitrogen, Carlsbad, CA, USA) was administered throughout the MG muscle by a series of small injections. The wound was irrigated and closed in layers. Animals received 0.1 ml of 0.3 mg/ml buprenorphine every 12 hours for post-operative pain medication for 48 hours and were monitored closely by professional staff.
In Vivo Sciatic Stimulation

After an adequate time for retrograde transport of CTB (>7 days), the effects of nerve activity on MN Kv2.1 channel clustering were examined. Standard procedures were used to prepare the left hindlimb and, when necessary, the lumbar spinal cord for electrophysiological stimulation (Seburn & Cope, 1998; Haftel et al. 2004, 2005; Bullinger et al. 2011). Rats were deeply anesthetized and vital signs were closely monitored. Respiratory rate (40–60), end-tidal CO₂ (3–5%), oxygen saturation (>90%), heart rate (300-500 beats/min), and core temperature (36–38°C) were maintained by adjusting isoflurane concentration and/or radiant heat. A small midline incision was made through the skin and biceps femoris of the left hindlimb to expose the left sciatic nerve, which contains axons destined for the MG nerve in the popliteal fossa. With careful surgical dissection, the sciatic nerve was freed from surrounding tissue and placed in a bipolar cuff electrode. Experiments proceeded with one of the terminal studies described below. Optimal parameters for nerve stimulation were set with the following considerations: a) stimulus frequency (100 Hz) approximates the upper limit of MN firing rate in anesthetized rats (Bullinger et al., 2011a; Gardiner and Seburn, 1997; Haftel et al., 2005; Hamm et al., 2010; Turkin et al., 2010); and b) stimulus strength (4X MG contraction threshold) maximally activates large diameter axons, including motor axons and Group I/II afferents (Bichler et al., 2007b; Bullinger et al., 2011b).
**Experiment 1: Sciatic Nerve Stimulation.**

To study the effects of nerve activity on MN Kv2.1 channel clustering, the left sciatic nerve in three animals (n=3) was, stimulated for 10 minutes, and <5 minutes later the animals were sacrificed for histological analysis. For all analyses, Kv2.1 channel clustering in stimulated MG MNs were compared to MG MNs in three sham control animals (n=3), in which the sciatic nerve was exposed but not stimulated.
Experiment 2: Sciatic Nerve Stimulation with Tetrodoxin (TTX; n=3).

To verify the effects of nerve stimulation on MN Kv2.1 channel clustering are dependent on the centripetal conduction of action potentials, 0.5 μM TTX, a voltage-gated sodium (NaV) channel blocker, was applied to the sciatic nerve in three animals (n=3) proximal to the stimulation site to block action potential generation and propagation. The sciatic nerve was subsequently stimulated for 10 minutes, and <5 minutes later the animals were sacrificed for histological analysis. To control for any confounding effects of TTX treatment on Kv2.1 channel clustering, the sciatic nerve of an additional animal (n=1) was exposed and treated with 0.5 μM TTX for 10 minutes without nerve stimulation.
Experiment 3: Sciatic Nerve Stimulation with Recovery.

To determine if changes in Kv2.1 channel clustering that occur following nerve stimulation are reversible, the left sciatic nerve in three animals (n=3) was stimulated for 10 minutes (as in experiment 1). Animals remained under isoflurane anesthesia and two hours later were sacrificed for histological analysis.
Experiment 4: Sciatic Nerve Stimulation Following Selective Dorsal Rhizotomy (SDR).

To separate the effects of motor nerve stimulation from sensory-evoked central synaptic activity on MN Kv2.1 channel clustering, a selective dorsal rhizotomy was performed in three rats (n=3) prior to sciatic nerve stimulation. Following surgical dissection of the left hindlimb and lower back, rats were fixed prone in a rigid recording frame. Skin flaps were used to construct pools for bathing all exposed tissues with warm mineral oil. Dorsal exposure of the lumbosacral spinal cord by laminectomy and longitudinal incision of the dura mater provided access to lumbosacral dorsal roots (L1–S2), which were carefully dissected free of surrounding tissue and suspended in continuity on bipolar silver hook electrodes for recording. Dorsal roots containing sciatic nerve afferents were electrophysiologically identified and cut near their entry to the spinal cord on the left side. Any remaining dorsal roots were similarly cut. The left sciatic nerve was subsequently stimulated for 10 minutes, and <5 minutes later the animal was sacrificed for histological analysis.

Two methods were employed to control for confounding effects of SDR on Kv2.1 channel clustering. First, a pair of fine-wire silver electrodes was inserted into the MG muscle for electromyographic (EMG) recording. EMG records demonstrated no detectable MG electrical activity during SDR, indicating any spontaneous discharge of cut afferents did not bring a sufficient number of MG MNs to threshold. Second, three additional animals (n=3) underwent the same surgical procedures described above, dorsal roots were exposed and cut, but the sciatic nerve was not stimulated. These animals were subsequently sacrificed for histological analysis.
Experiment 5: Selective Dorsal Root Stimulation.

To isolate the effects of MN synaptic activation on MN Kv2.1 channel clustering, dorsal roots in three rats (n=3) were directly stimulated. As described above, rats were fixed in a rigid recording frame and dorsal roots containing sciatic nerve afferents were identified and suspended in continuity on bipolar silver hook electrodes. Dorsal roots were subsequently stimulated at 100 Hz for 10 minutes (4X threshold for a visible dorsal root volley following sciatic nerve stimulation), and <5 minutes later the animal was sacrificed for histological analysis.
**Immunohistochemistry**

Immediately following data collection and under anesthesia, rats received intraperitoneal overdose of pentobarbital (150 mg/kg ip). All rats were transcardially perfused with a vascular rinse followed with 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.3. The spinal cords were removed, post fixed for 2 hours in 4% paraformaldehyde and cryoprotected in 15% sucrose in 0.1M phosphate buffer (≈ 300 mOsm). Transverse sections (50 μm) were obtained from the L4 and L5 spinal cord segments on a cryostat and immunostained free floating. Kv2.1 immunocytochemistry was performed using mouse anti-Kv2.1 clone D4/11 (catalog number 75-047) at 1:1000 in PBS with 0.1% Triton X pH 7.3 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. Immunoreactivity was detected with species specific secondary antibodies conjugated to Alexa 488 (Jackson Immuno, West Grove, PA, USA). Nissl immunocytochemistry was performed using fluorescein-conjugated nissl (1:100, Molecular Probes, Carlsbad, CA, USA).
Confocal Microscopy and Quantitative Analysis

As previously described (Romer et al, 2014), micrographs of immunolabeled lumbar αMN images were obtained on a Olympus Fluoview 1000 (Center Valley, PA, USA) confocal microscope with a 60x oil immersion objective (N.A 1.35) at 1.0 μm z-steps. For every ipsilateral CTB-labeled MG αMN imaged and analyzed, a contralateral αMN was also selected of the same approximate size and position within the motor pools as an internal bilateral control.

Image stacks were quantitatively analyzed for Kv2.1-IR cluster areas as previously described (Muennich and Fyffe, 2004) in Image Pro Software (Media Cybernetics, Silver Springs, MD, USA). Cell body Kv2.1 immunoreactive (IR) macroclusters (diameter >1.0μm), were measured in en face single optical sections on MNs that innervate the MG, as revealed by retrograde labels (Romer et al, 2014). Significance was set at p<0.05 using Mann Whitney Rank Sum tests (SigmaStat; Systat Software, Port Richmond, CA, USA).
Figure composition

Microscope images were prepared by adjusting contrast and brightness in Image Pro Plus Software (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Figures were composed using CorelDraw (v. 12.0). Graphs were composed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA). Data presented in figures is mean ± SD. Some images were sharpened using a “high gauss” filter in image pro. Quantification was always carried out in original unprocessed images.
Results

Redistribution of lumbar motoneuron Kv2.1 is activity-dependent

Previous work with MN Kv2.1 in a rat in vitro lumbar spinal cord slice preparation has shown a lateral translocation, or declustering, of membrane Kv2.1 protein to more uniform distribution following glutamate treatment (Romer et al., 2014). These results suggest that in MNs, Kv2.1 clustering dynamics may be activity-dependent. The goal of the experiments reported in this section was to evaluate the effects of nerve activity on Kv2.1-IR clusters on medial gastrocnemius (MG) MN somas, using an in vivo preparation in adult anesthetized rats to isolate the sciatic nerve for electrophysiological stimulation. Following 10 minutes of unilateral sciatic nerve stimulation, with a stimulation frequency of 100 Hz and strength of 4X the MG contraction threshold, rats were immediately sacrificed and tissue was processed for immunohistochemistry and imaged (see experimental procedures). When en face Kv2.1-IR macroclusters (diameters >1 μm) were selected and measured on MG motoneurons, there is a significant 30% reduction in Kv2.1-IR macrocluster area (Figure 17, Table 5; control =7.37 μm² ± 5.1 SD, n=421 clusters vs. stimulated =5.15 μm² ± 3.01 SD, n=280 clusters, p<0.001, Mann-Whitney Test). When average Kv2.1 macrocluster area per MN was examined, there is a significant 35% reduction (Figure 18, Table 5, Table 5; control =7.95 μm² ± 2.4 SD, n=42 MNs vs. stimulated =5.13 μm² ± 1.3 SD, n=38 MNs, p<0.001, Mann-Whitney Test). No significant differences were found in sham stimulation control rats compared to the contralateral, unstimulated, side of the experimental rats. The reduction of macrocluster area shown here is consistent with observations made in MNs and
other neuronal types that Kv2.1-IR is declustering (Misonou et al., 2004; Misonou et al., 2005; Mohapatra et al., 2009; Romer et al., 2014).

To confirm that Kv2.1 channel dynamic clustering is a direct result of the sciatic nerve stimulation and not a confounding effect from surrounding tissue, 0.5 μM tetrodotoxin (TTX) was applied to the sciatic nerve proximal to the stimulation site to block centripetal action potential propagation. The application of both TTX alone and TTX with subsequent sciatic nerve stimulation did not affect the Kv2.1-IR macrocluster sizes on MG MN somas (Figure 17D, Table 5; TTX Control =7.45 μm² ± 3.5 SD, n=157 clusters vs. TX + Stimulation =7.75 μm² ± 3.34 SD, n= 637 clusters). These results suggest that MN Kv2.1-IR declustering is a result of the electrophysiological stimulation of the sciatic nerve, and validate that Kv2.1 clustering dynamics are activity-dependent in rat lumbar MNs.
**Figure 17. Reorganization of Kv2.1-IR clusters on rat lumbar α-motoneurons**

is activity dependent. The sciatic nerve was stimulated *in vivo* at 150 Hz and 4x tetanic threshold for 10 minutes. Motoneurons displayed in panels A and B are from the same tissue slice with fixed imaging parameters. Scale bars are 10 µm. (A) Micrograph of a confocal stack (33 x 1.0 µm z-steps) of a contralateral 'control' motoneuron showing Kv2.1-IR (red). (B) Micrograph of a confocal stack (30 x 1.0 µm z-steps) of an MG α-motoneuron following 10 min of sciatic nerve stimulation showing reduced Kv2.1-IR (red) macrocluster areas compared to the unstimulated motoneuron in panel A. (C) Quantitative analysis of reduced Kv2.1-IR soma macrocluster areas on medial gastrocnemius α-motoneurons following 10 min of sciatic nerve stimulation in all 3 rats sampled. (D) Pooled quantitative analysis of Kv2.1-IR following 0.5µM tetrodotoxin (TTX) showing no significant changes compared to the absence of TTX application. C & D N= the number of Kv2.1 macroclusters sampled. Significance (p<0.05) is indicated with asterisk and determined with Mann-Whitney and data is presented ±SD.
Kv2.1-IR Macrocluster Area Following
*In Vivo* Sciatic Stimulation

![Graph showing the average Kv2.1 macrocluster area (µm²) per motoneuron sampled for contralateral controls and sciatic nerve stimulations.](image)

- Contralateral Controls
  - (n=42)

- Sciatic Nerve Stimulations
  - (n=38)

*p< 0.001*
Figure 18. The average Kv2.1 macrocluster size per motoneuron is significantly decreased following 10 minutes of sciatic nerve stimulation.

Kv2.1 cluster sizes are highly variable in motoneurons (see Muennich et al, 2014).

Each dot represents average Kv2.1 macrocluster area (minimum diameter >1μm) for each motoneuron analyzed. Horizontal bar signifies population average and significance (p<0.05) is indicated with asterisk and determined with Mann-Whitney and data is presented ±SD.
Table 5. Kv2.1-IR macrocluster means and sample sizes following sciatic nerve stimulations

<table>
<thead>
<tr>
<th>Individual Animals Sampled</th>
<th>Mean Area ($\mu m^2$)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 Control</td>
<td>8.144 ± 5.27</td>
<td>14</td>
<td>113</td>
</tr>
<tr>
<td>#1 Stimulated</td>
<td>5.818 ± 2.82</td>
<td>14</td>
<td>139</td>
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<tr>
<td>#2 Control</td>
<td>6.860 ± 5.66</td>
<td>12</td>
<td>113</td>
</tr>
<tr>
<td>#2 Stimulated</td>
<td>5.032 ± 3.50</td>
<td>12</td>
<td>84</td>
</tr>
<tr>
<td>#3 Control</td>
<td>7.222 ± 4.54</td>
<td>16</td>
<td>195</td>
</tr>
<tr>
<td>#3 Stimulated</td>
<td>5.043 ± 2.77</td>
<td>21</td>
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</table>

<table>
<thead>
<tr>
<th>Pooled Data</th>
<th>Mean Area ($\mu m^2$)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.372 ± 5.07</td>
<td>42</td>
<td>421</td>
</tr>
<tr>
<td>Sciatic Stimulation</td>
<td>5.148 ± 3.01</td>
<td>47</td>
<td>380</td>
</tr>
<tr>
<td>TTX Control</td>
<td>7.450 ± 3.49</td>
<td>16</td>
<td>157</td>
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<tr>
<td>TTX + Stimulation</td>
<td>7.752 ± 3.34</td>
<td>36</td>
<td>637</td>
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±SD
Activity-dependent changes in Kv2.1 are reversible.

Following peripheral nerve injury, significant differences were observed in MN Kv2.1-IR macrocluster area over weeks to months (Romer et al., 2014). Here, we sought to determine if the activity-dependent impact on MN Kv2.1-IR channel clustering is reversible upon stimulus removal. For this assay, the sciatic nerve was isolated and stimulated under the same parameters previously described (see Methods: In Vivo Sciatic Stimulation). Following 10 minutes of stimulation, the rats remained anesthetized for 2 hours, then sacrificed and the lumbar spinal cord processed for immunohistochemistry subsequent imaging. No significant differences were observed in Kv2.1-IR macrocluster sizes in MG MNs 2 hours after stimulation compared to internal bilateral controls (Figure 19, Table 6; control =8.22 µm\(^2\) ± 3.0 SD, n=611 clusters vs. stimulated =8.07 µm\(^2\) ± 2.9 SD, n=583 clusters). These results suggest the impact of Kv2.1 declustering is quickly reversible (within 2 hours) upon stimulus removal and will be important in interpreting future observations of Kv2.1 clustering in pathological and non-pathological states.
Kv2.1 Macrocluster Area 2 Hours Following Sciatic Nerve Stimulation

<table>
<thead>
<tr>
<th>Rat #1</th>
<th>Rat #2</th>
<th>Rat #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral Control</td>
<td>Sciatic Nerve Stimulation</td>
<td></td>
</tr>
<tr>
<td>(n=222)</td>
<td>(n=187)</td>
<td>(n=197)</td>
</tr>
<tr>
<td>(n=252)</td>
<td>(n=192)</td>
<td>(n=144)</td>
</tr>
</tbody>
</table>

Average Kv2.1 Macrocluster Area (μm²)
Figure 19. Kv2.1-IR macroclusters on medial gastrocnemius α-motoneurons recover to control sizes by 2 hours following sciatic nerve stimulation in all 3 rats sampled. Absence of significance was determined with Mann-Whitney T-test and data is presented ±SD. N=number of Kv2.1 macroclusters sampled
Table 6. Kv2.1-IR macrocluster means and sample sizes 2 hours following sciatic nerve stimulations

<table>
<thead>
<tr>
<th>Individual Animals Sampled</th>
<th>Mean Area (µm²)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 Control</td>
<td>7.384 ± 2.79</td>
<td>11</td>
<td>222</td>
</tr>
<tr>
<td>#1 Stimulated</td>
<td>7.250 ± 3.11</td>
<td>14</td>
<td>187</td>
</tr>
<tr>
<td>#2 Control</td>
<td>8.404 ± 2.88</td>
<td>21</td>
<td>197</td>
</tr>
<tr>
<td>#2 Stimulated</td>
<td>8.288 ± 2.53</td>
<td>21</td>
<td>252</td>
</tr>
<tr>
<td>#3 Control</td>
<td>8.994 ± 3.06</td>
<td>15</td>
<td>192</td>
</tr>
<tr>
<td>#3 Stimulated</td>
<td>8.750 ± 3.13</td>
<td>15</td>
<td>144</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pooled Data</th>
<th>Mean Area (µm²)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.22 ± 2.98</td>
<td>47</td>
<td>611</td>
</tr>
<tr>
<td>Stimulation</td>
<td>8.07 ± 2.94</td>
<td>50</td>
<td>583</td>
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</tbody>
</table>

±SD
Motor axon stimulation and sensory-evoked synaptic activation impacts Kv2.1-IR channel clustering on motoneuron somas.

During sciatic nerve stimulation, not only are the MN axons antidromically driven, but sensory circuitry is also driven that can evoke central synaptic activity onto MNs. A selective dorsal rhizotomy was performed to isolate the effects of motor axon stimulation from the sensory-evoked synaptic activity on MN Kv2.1-IR clustering dynamics. With motor axon volley isolated and the sciatic nerve stimulated with fixed parameters (see Experimental Procedures: In Vivo Sciatic Stimulation) there was still a significant ≈ 28% reduction in Kv2.1 macrocluster areas on MG MNs compared to internal bilateral controls (Figure 20A, Table 7; control =10.60 µm² ± 4.0 SD, n=450 clusters vs. antidromic stimulation =7.66 µm² ± 3.25 SD, n=560 clusters, p<0.001, Mann-Whitney Test).

However, cutting of dorsal roots results in an injury discharge (Eschenfelder et al., 2000; Liu et al., 2000; Sun et al., 2005). To control for this discharge, the impact of dorsal rhizotomy on Kv2.1 cluster sizes was carefully analyzed in addition to measuring any motor output within the MG muscle with EMG recordings during the rhizotomy period. Following dorsal rhizotomy, we report an approximately 11% significant reduction in Kv2.1-IR macrocluster area (Figure 20A, Table 7; control =9.45 µm² ± 3.2 SD, n=453 clusters vs. dorsal rhizotomy =8.43 µm² ± 3.54 SD, n=578 clusters, p<0.001, Mann-Whitney Test). These results suggest that the rhizotomy itself has an impact on Kv2.1 clustering. However, in MG EMG studies of all 6 rats analyzed, the sensory injury discharge was not sufficient to bring MNs to threshold and cause motor discharges in the muscle (Figure 20B).
Alternatively, to determine if sensory-evoked synaptic input could impact Kv2.1-IR channel clustering on MG MNs, all dorsal roots containing sciatic nerve afferents were isolated and stimulated at 100 Hz to selectively drive sensory input circuitry. In all rats tested, 10 minutes of dorsal root stimulation caused a significant 19% reduction of Kv2.1-IR macrocluster areas on MN somas (Figure 21, Table 8; control =8.06 µm² ± 3.1 SD, n=558 clusters vs. dorsal stimulation =6.51 µm² ± 2.8 SD, n=724 clusters, p<0.001, Mann-Whitney Test).

These results suggest that both motor nerve activity and sensory-evoked synaptic activity can induce Kv2.1-IR channel declustering on MNs. Although intrinsic stimulation of the motor axons themselves contribute to Kv2.1 channel declustering on the soma and proximal dendrites, a portion of this declustering is likely caused by the selective dorsal rhizotomy.
A  Kv2.1 Macrocluster Area Following Dorsal Rhizotomy with Sciatic Nerve Stimulation

- Contralateral Controls
- DR + Sciatic Stims
- DR Only

B  Medial Gastrocnemius EMG During Dorsal Rhizotomy

MGN Stim  DR Cut Period  MGN Stim
Figure 20. Kv2.1-IR macroclusters on α-motoneurons significantly reduce when motor axons are stimulated. (A) Quantitative analysis of reduced Kv2.1-IR soma macrocluster areas on lumbar α-motoneurons following sciatic nerve stimulation with dorsal rhizotomy in all 3 rats sampled. The impact of dorsal rhizotomy on Kv2.1-IR macrocluster areas on lumbar α-motoneurons was quantified in 3 rats. N= the number of Kv2.1 macroclusters sampled. Significance (p<0.05) is indicated with asterisk and determined with Mann-Whitney T-test and data is presented ±SD. (B) Electromyography (EMG) of medial gastrocnemius (MG) muscle indicates that the injury discharge during dorsal root cuts did not cause motor output activity in the muscle. The MG nerve (MGN) itself was stimulated before and after the dorsal roots were cut to confirm EMG recordings.
Table 7. Kv2.1-IR cluster means and sample sizes following dorsal rhizotomy (DR) and sciatic nerve stimulation

<table>
<thead>
<tr>
<th>Individual Animals Sampled</th>
<th>Mean Area ($\mu m^2$)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
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</thead>
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<tr>
<td>#1 Control</td>
<td>10.49 ± 4.75</td>
<td>18</td>
<td>169</td>
</tr>
<tr>
<td>#1 DR + Stim</td>
<td>7.01 ± 3.05</td>
<td>20</td>
<td>202</td>
</tr>
<tr>
<td>#2 Control</td>
<td>11.30 ± 3.81</td>
<td>10</td>
<td>108</td>
</tr>
<tr>
<td>#2 DR + Stim</td>
<td>7.35 ± 3.40</td>
<td>10</td>
<td>149</td>
</tr>
<tr>
<td>#3 Control</td>
<td>10.26 ± 3.28</td>
<td>13</td>
<td>173</td>
</tr>
<tr>
<td>#3 DR + Stim</td>
<td>8.51 ± 3.28</td>
<td>14</td>
<td>209</td>
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<table>
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<th>Clusters Sampled</th>
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<td>#1 Control</td>
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<tr>
<td>#1 DR</td>
<td>9.36 ± 4.15</td>
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<td>144</td>
</tr>
<tr>
<td>#2 Control</td>
<td>9.30 ± 3.49</td>
<td>13</td>
<td>151</td>
</tr>
<tr>
<td>#2 DR</td>
<td>8.10 ± 3.85</td>
<td>13</td>
<td>171</td>
</tr>
<tr>
<td>#3 Control</td>
<td>9.37 ± 3.30</td>
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<td>156</td>
</tr>
<tr>
<td>#3 DR</td>
<td>8.14 ± 2.81</td>
<td>13</td>
<td>263</td>
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<table>
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<tr>
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<th>Clusters Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR + Stim</td>
<td>10.60 ± 4.02</td>
<td>41</td>
<td>450</td>
</tr>
<tr>
<td>DR Control</td>
<td>7.66 ± 3.25</td>
<td>44</td>
<td>560</td>
</tr>
<tr>
<td>DR</td>
<td>9.45 ± 3.75</td>
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<td>453</td>
</tr>
<tr>
<td></td>
<td>8.43 ± 3.54</td>
<td>40</td>
<td>578</td>
</tr>
</tbody>
</table>

±SD
Kv2.1 Macrococluster Area Following Dorsal Root Stimulation

- **Rat #1**
  - Contralateral Control: (n=231)
  - Dorsal Root Stimulation: (n=285)
  - p < 0.001

- **Rat #2**
  - Contralateral Control: (n=64)
  - Dorsal Root Stimulation: (n=134)
  - p = 0.003

- **Rat #3**
  - Contralateral Control: (n=263)
  - Dorsal Root Stimulation: (n=305)
  - p < 0.001
Figure 21. Kv2.1-IR macroclusters on α-motoneurons significantly reduce when sensory afferent circuitry is driven through dorsal root stimulations. Quantitative analysis of Kv2.1-IR on medial gastrocnemius α-motoneuron somas in all three animals samples demonstrates significant decrease in area. N= the number of Kv2.1 macroclusters sampled. Significance (p<0.05) is indicated with asterisk and determined with Mann-Whitney T-test and data is presented ±SD.
Table 8. Kv2.1-IR macrocluster means and sample sizes following dorsal root stimulation

<table>
<thead>
<tr>
<th>Individual Animals Sampled</th>
<th>Mean Area (µm²)</th>
<th>Cells Sampled</th>
<th>Clusters Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 Control</td>
<td>8.63 ± 2.81</td>
<td>11</td>
<td>231</td>
</tr>
<tr>
<td>#1 Stimulated</td>
<td>6.97 ± 2.98</td>
<td>14</td>
<td>285</td>
</tr>
<tr>
<td>#2 Control</td>
<td>8.10 ± 3.52</td>
<td>21</td>
<td>64</td>
</tr>
<tr>
<td>#2 Stimulated</td>
<td>6.55 ± 2.88</td>
<td>21</td>
<td>134</td>
</tr>
<tr>
<td>#3 Control</td>
<td>7.55 ± 3.23</td>
<td>15</td>
<td>263</td>
</tr>
<tr>
<td>#3 Stimulated</td>
<td>6.08 ± 2.62</td>
<td>15</td>
<td>305</td>
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</table>

<table>
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<th>Clusters Sampled</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7.46 ± 3.14</td>
<td>36</td>
<td>558</td>
</tr>
<tr>
<td>Stimulation</td>
<td>5.97 ± 2.84</td>
<td>39</td>
<td>724</td>
</tr>
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</table>

±SD
Discussion

The striking membrane organization of Kv2.1 channels in rodent lumbar MNs, and along with their strategic positioning at specific synapses within signaling ensembles (Deardorff et al., 2013; Deardorff et al., 2014; Muennich and Fyffe, 2004), make them a valuable model for the study of channel regulation under conditions of altered activity. We have previously shown that both in vitro application of glutamate and in vivo axotomy induce rapid Kv2.1 cluster dispersal in lumbar spinal MNs, suggesting clustering dynamics may be activity-dependent (Romer et al., 2014). Here, we use a sophisticated in vivo preparation that provides the advantage of direct control of electrophysiological nerve stimulation in intact and physiological conditions to evaluate activity dependent changes in MN Kv2.1 channels. Following increases in activity, Kv2.1 ion channels rapidly decluster in the MN soma membrane. Moreover, MN Kv2.1 channels are sensitive to motor axon activity, sensory-evoked synaptic activity, and even dorsal rhizotomy that provides an injury discharge below firing threshold in MNs (Figure 22).

These observations of activity-dependent Kv2.1 channel localization, further bolsters the argument that Kv2.1 channels are likely a mechanism for homeostatic plasticity (Cudmore and Turrigiano, 2004; Deardorff et al., 2014; Kihira et al., 2010; Misonou et al., 2004; Misonou et al., 2008; Misonou, 2010; Mohapatra et al., 2009; Nataraj et al., 2010; Romer et al., 2014; Surmeier and Foehring, 2004). Homeostatic mechanisms, in particular, contribute to stabilizing the activity of neurons and neuronal circuits to maintain optimal firing levels (Burrone and Murthy, 2003; Ganguly and Poo, 2013; Katz and Shatz, 1996; Marder and Goaillard, 2006; Murphy,
Because neuronal firing arises from an intimate choreography of synaptic currents and intrinsic membrane properties, homeostatic mechanisms exist that specifically target each of these facets (Marder and Goaillard, 2006; Turrigiano, 2011). A complex amalgamate of these mechanisms are utilized in a neuronal and tissue specific manner to optimize and adapt to different demands (see Turrigiano, 2011). Specifically, intrinsic homeostatic mechanisms have been shown to directly influence ion channels, coordinated by intracellular calcium concentrations, and can lead to rapid dramatic changes in neuronal firing patterns (Marder and Goaillard, 2006; O’Leary et al., 2014; Turrigiano et al., 1994).

While the impact of Kv2.1 channel clustering on MN firing rate has yet to be elucidated, many electrophysiological and modeling studies reveal that MN intrinsic properties do, in fact, respond to alterations in activity in both pathologic and non-pathologic states (Bichler et al., 2007a; Foehring et al., 1986a; Foehring et al., 1986b; Gustafsson and Pinter, 1984; Johnson et al., 2013; Kuno et al., 1974a; Kuno et al., 1974b; Meehan et al., 2010; Prather et al., 2011; Quinlan et al., 2011; Wolpaw and Tennissen, 2001). However, only a few motoneuron studies have characterized the ion channel alterations that could underlie these effects, and of these studies, most examined transcriptional expression changes over days to weeks (Alvarez and Fyffe, 2007; Anneser et al., 1999; Anneser et al., 2000; Woodrow et al., 2013). The rapid (10 minutes) activity-dependent alterations in MN Kv2.1 channel clustering dynamics shown here is not likely a product of transcriptional regulation but
consistent with observations in hippocampal and cortical pyramidal neurons that calcium-dependent dephosphorylation pathways post-translationally regulate Kv2.1 clustering (Mohapatra et al., 2009; Park et al., 2006). The rapid response of MN Kv2.1 channels is likewise consistent with observations that intrinsic homeostatic mechanisms can respond more rapidly than synaptic mechanisms (Karmarkar and Buonomano, 2006).

Kv2.1 cluster sizes rapidly return to their original sizes within 2 hours following the cessation of nerve stimulation. Whether these clusters are physically ‘reclustering’ or protein turn-over rates are restoring original cluster sizes is unknown. However, that the cluster sizes rapidly restore upon stimulus removal is further support that Kv2.1 channels are homeostatically driven to respond to alterations in activity. These findings are especially important when interpreting long-term pathological changes in Kv2.1 channel clustering. For instance, following peripheral nerve injury, MN Kv2.1-IR macrocluster areas were reduced over weeks to months (Romer et al., 2014), suggesting the continued existence of some pathological stimulus.
**Figure 22. Summary of spinal motoneuron Kv2.1 ion cluster responses to various in vivo stimuli.** Kv2.1 ion channels significantly reduce by 30% following sciatic nerve stimulation (Stim), an effect inhibited by the application of tetrodotoxin proximal to the stimulation site (TTX+STIM) that results in an insignificant 4% increase in cluster size. Channel cluster sizes are restored to the original sizes, with a insignificant 2% reduction, 2 hours following the sciatic nerve stimulation (2 Hr Post Stim). A significant 28% reduction in cluster size was produced when the effect of the sciatic nerve stimulation was isolated to just antidromic activation of motor axons (AntiD Stim) through dorsal rhizotomy. However, the dorsal rhizotomy (Dorsal Rhiz.) itself, without nerve stimulation, also induced a significant 11% reduction cluster areas. Finally, Sensory evoked synaptic activity, through dorsal root stimulations (D. Root Stim), caused a significant 19% reduction of cluster areas.
The role of Kv2.1 at the C-bouton.

The largest Kv2.1 clusters are localized within a signaling ensemble at C-bouton synapses (Deardorff et al., 2014), important cholinergic loci for state-dependent modifications of MN firing rate (Miles et al., 2007; Zagoraiou et al., 2009). Our hypothesis on the modulatory effects of cholinergic signaling in αMNs is centered on a highly-regulated system surrounding a Ca\(^{2+}\) microdomain for the precise and nuanced regulation of cell firing (Deardorff et al, 2014). Specifically, activation of the muscarinic m2 receptors, concentrated at C-boutons, likely inhibit Ca\(^{2+}\) currents preventing activation of Ca\(^{2+}\)/calcineurin dependent pathways, thus maintaining Kv2.1 clustering. However, if prolonged/pathologic excitatory drive causes large changes in intracellular Ca\(^{2+}\) sufficient to allow diffusion of Ca\(^{2+}\) from neighboring compartments, there would be rapid Kv2.1 channel declustering, to homeostatically suppress repetitive firing (Deardorff et al., 2014; Romer et al., 2014). While the impact of Kv2.1 channel clustering on MN physiology is still undefined, our observations here, that Kv2.1 synaptic reorganization at C-boutons following prolonged excitatory drive do, indeed, offer support to their potential role at the C-bouton.
A remarkable feature of the central nervous system is its capacity to respond and adapt to a variety of environmental demands through diverse activity-dependent mechanisms. Through the novel use of a fully intact, *in vivo* rat preparation, we show through electrophysiological nerve stimulation that Kv2.1 channel clustering dynamics in lumbar MNs are, indeed, activity-dependent. Moreover, we demonstrated that this effect is reversible (within 2 hours) upon stimulus removal. Finally, we demonstrated that MN Kv2.1 channels respond to not only intrinsic activity, but also sensory evoked synaptic activity. Altogether evidence of dynamic activity-dependent clustering in MNs further supports the global theory that Kv2.1 channels are a mechanism for intrinsic homeostatic regulation of neuronal firing properties.
Chapter VII. Specific Aim 3:

The physiological role of Kv2.1 ion channels in spinal motoneurons
Introduction

Mammalian neurons express a rich diversity of membrane bound voltage-gated potassium channels (Kv) that have an array of properties that coordinate to shape and/or modify firing properties (Coetzee et al., 1999; Hille, 2001; Johnston et al., 2010; Liu & Bean, 2014). Kv2 subunits, in particular, are widely expressed throughout the mammalian brain and spinal cord (Trimmer, 1991; Scannevin et al., 1996; Du et al., 1998; Antonucci et al., 2001; Muennich & Fyffe, 2004; Trimmer & Rhodes, 2004; Guan et al., 2007; Johnston et al., 2008; Sarmiere et al., 2008; King et al., 2014), where they produce delayed rectifier currents ($I_{K}$) that activate slower than nearly all other Kv channel subunit types (Misonou et al., 2005; Park et al., 2006; Mohapatra et al., 2009; Liu & Bean, 2014). In hippocampal and cortical pyramidal cells, knockdown of Kv2 suggests that the channels are minimally active during single action potentials (Du et al., 2000; Guan et al., 2013), whereas they significantly contribute to the repolarization of the action potential in sympathetic neurons that contain much broader action potentials (Malin & Nerbonne, 2002). Interestingly, in all these neuronal types, the loss of Kv2 disrupts repetitive firing properties (Du et al., 2000; Malin & Nerbonne, 2002; Guan et al., 2013; Liu & Bean, 2014), an effect also observed in computer modeling of neurons within the medial nucleus of the trapezoid body (Johnston et al., 2008).

In the brain, Kv2.1 channels have generated profound interest. Stimuli such as ischemia, seizure and neuromodulator activity trigger calcineurin-dependent dephosphorylation pathways that rapidly decluster the channels while simultaneously lowering activation and deactivation thresholds and accelerating the
kinetics of channel behavior - thereby reducing neuronal firing rate (Misonou et al., 2004; Surmeier & Foehring, 2004; Misonou et al., 2005; Mohapatra & Trimmer, 2006; Park et al., 2006). Thus, Kv2.1 is increasingly been described as a mechanism for intrinsic homeostatic plasticity that may even provide protection against neuronal damage and death (Cudmore & Turrigiano, 2004; Surmeier & Foehring, 2004; Misonou et al., 2005; Kihira et al., 2010; Misonou, 2010; Nataraj et al., 2010; Romer et al., 2014).

In rat spinal lumbar motoneurons (MNs), Kv2.1 channels form distinct clusters in highly regulated signaling ensembles on the soma and proximal dendrites (Muennich & Fyffe, 2004; Deardorff et al., 2013; Deardorff et al., 2014; Romer et al., 2014). Immediately following peripheral nerve injury, αMN Kv2.1 channels reorganize by and rapidly declustering (Romer et al., 2014), an effect also observed following direct stimulation of peripheral nerves (see Specific Aim 2), suggesting Kv2.1 channels on αMNs have the capacity to dynamically respond to pathological and/or prolonged excitatory stimuli.

Despite their relative abundance on MNs, the physiological role of the Kv2.1 ion channels in mammalian MNs is unknown. To explore the impact of alterations in Kv2.1 clustering in rat lumbar αMNs, we use an in vitro spinal cord system that permits the application of multiple pharmacological agents, including the tarantula toxin stromatoxin (STX) to block Kv2 channel conductances (Escoubas et al., 2002). In particular, using whole cell patch clamp electrophysiology we focus on the role of Kv2.1 in repetitive firing properties of MNs. The results obtained from each experimental question addressed in this report generate an important foundation
for interpreting molecular underpinnings in both physiological and pathological states.
Experimental Procedures

All animal procedures were performed according to National Institutes of Health (NIH) guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University.
**Spinal Cord Slice Preparations**

Young Sprague Dawley Rats, P8- P14, (Charles River, Wilmington, MA, USA) were anesthetized with 65mg/ml pentobarbital, submerged up to their necks in ice until loss of withdrawal reflexes then decapitated. The spinal cords were immediately dissected in <4°C sucrose modified artificial cerebral spinal fluid (aCSF) (in mM: 26 NaHCO3, 10 Glucose, 3 KCl, 1.25 NaPO4, 2 MgCl2, and 218 Sucrose) with bubbling 95% O2 and 5% CO2. Transverse sections, 300 µm thick, of the L4 – L5 region of the spinal cord were cut on Vibratome Series 1000 Plus (The Vibratome Co., St. Louis, MO, USA). Slices were briefly incubated in 30% PEG (Carp et al., 2008) and transferred to oxygenated aCSF (in mM: 130 NaCl, 26 NaHCO3, 10 Glucose, 3 KCl, 1.25 NaPO4, 2 MgCl2, 2 CaCl2 at 300-310 mOsm) and incubated 1 hour at 37°C to stabilize. For pharmacological studies, sections were incubated in either 10 µM L-Glutamate (Sigma, St. Louis, MO), 10 µM Muscarine (Sigma, St. Louis, MO), 5 & 10µM Cyclosporine A (Sigma, St. Louis, MO), 500nM Tetrodotoxin (Alomone, Jerusalem Israel) and 100nM Stromatoxin (Alomone, Jerusalem Israel). Addition of pharmacological agents to aCSF had minimal effect on osmotic pressure of the solution. Control tissue from each rat was incubated and time-matched in aCSF without pharmacological treatment. Immediately following incubations, the slices were immersion fixed in 4% paraformaldehyde for 20 minutes. Tissue slices were sectioned further (50 µm) on cryostat and immunohistochemistry was performed as previously described (refer to immunohistochemistry general methods).
Lumbar spinal cord slices were transferred to a submersion style chamber for recording and perfused (approximately 3 ml/min) with oxygenated aCSF at room temperature. Thin-walled (1.5 mm o.d., 1.12 mm i.d.) borosilicate glass (TW150F-3, World Precision Instruments, Inc., Sarasota, FL, USA) was used to pull patch pipettes (3-5 MΩ) on a horizontal Flaming/Brown micropipette puller (P-80, Sutter Instruments, Novato, CA, USA). Patch pipettes were filled with an internal solution (in mM: 122.5 potassium D-gluconate, 17.5 KCl, 9 NaCl, 1 MgCl2, 10 HEPES, 0.2 EGTA, 3 Mg-ATP, and 0.3 Na-GTP; pH 7.2-7.3). On the day of the experiments, 0.08% Alexa Fluor 488 Hydrazide (Invitrogen, Carlsbad, CA, USA) was added to the internal solution. MNs were visualized for whole-cell recordings using differential interference contrast microscopy (25x2; water immersion; Olympus FV1000 Fluoview confocal microscope, Olympus, Center Valley, PA, USA). Whole-cell patch-clamp recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data was digitized at 16-bit resolution, 10 kHz (Digidata 1440A, Molecular Devices), and all protocols used an output gain of 2 and a Bessel filter of 10 kHz. To evoke individual action potentials, neurons were subjected to 5ms depolarizing current pulses at their resting membrane potential. Several properties were recorded from these action potentials including rheobase, action potential threshold, peak amplitude, half-width, rise-time, decay-time, AHP amplitude measured from baseline and half decay-time. Repetitive firing properties and corresponding frequency-current plots were evoked by injecting 500 ms square
pulses from 0-1000 pA in 50 pA steps. Input resistance was calculated by injecting 500 ms hyperpolarizing currents (-100 and -500 pA). Long square pulses (45s) were used to analyze spike frequency adaptation. All recordings were analyzed using Clampfit 10.3 (Molecular Devices). Before a MN was used experimentally, several conditions must exist, specifically, a GΩ seal must have formed with the pipet before breaking the membrane, the series resistance must be <25 MΩ with minimal change throughout the duration of the recording session, the neuron must fire an action potential with an amplitude greater than 70 mV and exhibit repetitive firing properties with a stable resting membrane potential < -55 mV.
**Immunohistochemistry**

Immediately following data collection and/or incubation times, spinal cord slices were immersion fixed for 45 minutes in 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.3. The spinal cord slices were removed and cryoprotected in 15% sucrose in 0.1M phosphate buffer (≈ 300 mOsm). Transverse sections (50 μm) on a cryostat and immunostained free floating. Kv2.1 immunocytochemistry was performed using mouse anti-Kv2.1 clone D4/11 (catalog number 75-047) at 1:1000 in PBS with 0.1% Triton X pH 7.3. The antibodies were developed by and 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. Immunoreactivity was detected with species-specific secondary antibodies conjugated to Alexa 488 or Cy3 (Jackson Immuno, West Grove, PA, USA). Nissl immunocytochemistry was performed using fluorescein-conjugated nissl (1:100, Molecular Probes, Carlsbad, CA, USA).
Confocal Microscopy and Quantitative Analysis

As previously described (Romer et al, 2014), micrographs of immunolabeled lumbar αMN images were obtained on a Olympus Fluoview FV1000 (Center Valley, PA, USA) confocal microscope with a 60x oil immersion objective (N.A 1.35) at 1.0 μm z-steps. All control slices from the same rats were imaged and analyzed with the same imaging parameters as experimental slices.

Image stacks were quantitatively analyzed for Kv2.1-IR cluster areas as previously described (Muennich & Fyffe, 2004) in Image Pro Software (Media Cybernetics, Silver Springs, MD, USA). Cell body Kv2.1 immunoreactive (IR) macroclusters (diameter >1.0μm), were measured in en face single optical sections on MNs that innervate the MG, as revealed by retrograde labels (Romer et al, 2014). Significance was determined using SigmaStat Software (Port Richmond, CA, USA) was set at p<0.05.
**Figure composition**

Microscope images were prepared by adjusting contrast and brightness in Image Pro Plus Software (Media Cybernetics, Bethesda, MD, USA) and always preserved all the information content of the images. Figures were composed using CorelDraw (v. 12.0). Graphs were composed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA). Data presented in figures is mean ± SD. Some images were sharpened using a “high gauss” filter in image pro. Quantification was always carried out in original unprocessed images.
Results

Motoneuron Kv2.1 channel clustering is dynamic and calcineurin dependent.

Previously we have demonstrated in lumbar MNs in in vitro rat spinal cord slices that Kv2.1 channels decluster, exhibiting a reduction in the en face surface area of large Kv2.1 macroclusters (diameters >1μm) following bath application of glutamate (Figure 23) (Romer et al., 2014). These observations are consistent with studies in both primary hippocampal and HEK293 cultures where glutamate treatment and subsequent increase of intracellular Ca$^{2+}$ also induces Kv2.1 channel declustering (Murakoshi et al., 1997; Misonou et al., 2004; Misonou et al., 2005; Mohapatra et al., 2009).

The protein phosphatase calcineurin mediates intracellular signaling by coupling Ca$^{2+}$ signals to many cellular responses (Klee et al., 1998; Aramburu et al., 2000; Rusnak & Mertz, 2000) and has been previously demonstrated to mediate Kv2.1 ion channel clustering (Murakoshi et al., 1997; Misonou et al., 2004; Misonou et al., 2005; Mohapatra & Trimmer, 2006; Mohapatra et al., 2009). To determine whether Kv2.1 declustering in αMNs is a calcineurin mediated event, cyclosporine A, an FDA-approved inhibitor of calcineurin activity, was added to artificial cerebral spinal fluid (ACSF) and bath applied to lumbar spinal cord slices for 10 minutes with and without 10.0 μM glutamate treatments. Use of cyclosporine concentrations at or above 15 μM increased the osmotic pressure of the bath and was not used in this study. Application of 10 μM Cyclosporine co-incubated with glutamate fully inhibited glutamate-induced Kv2.1 ion channel declustering (Figure 24; control=...
6.9μm ±3.0 SD, n=202 clusters, 32 MNs; glutamate= 4.8 μm ±4.1 SD, n=225 clusters, 30 MNs; 10 μM Cyclosporine= 6.6μm ±3.9 SD, n=150 clusters, 40 MNs; Glutamate +5 μM Cyclosporine= 5.5 μm ±3.2 SD, n=103 clusters, 34 MNs; Glutamate +10 μM Cyclosporine= 6.7μm ±3.3 SD, n=179 clusters, 43 MNs; 9 rats from 3 litters). These results suggest that, in rat lumbar MNs, Kv2.1 declustering dynamics are calcineurin-dependent.

In addition to dephosphorylation in high activity conditions, Kv2.1 has also been shown to be hyper-phosphorylated when activity was suppressed for 1 hour, but the impact on clustering was not evaluated (Misonou et al., 2006). To determine the impact of low activity on Kv2.1 clusters in αMNs, in vitro lumbar spinal cord slices from 3 rats each from separate litters were treated with 500 nM Tetrodotoxin (TTX), to block all neuronal activity, for 1 hour. Compared to ACSF controls, after 1 hour in low activity conditions, Kv2.1-IR ion channel macroclusters are approximately 20% larger (control, 34 MNs, 212 clusters, mean area 5.696 μm² ± 3.3 SD vs. TTX treated, 19 MNs, 196 clusters, mean area 6.835 μm² ± 3.9 SD) (Figure 25). These data suggest that in addition to declustering in high activity conditions, the Kv2.1 channels in MNs can be induced to form clusters of increased size in low activity conditions.
**Figure 23. Kv2.1-IR ion channels decluster in lumbar motoneurons following glutamate application.** Bath application of either artificial cerebral spinal fluid (ACSF) (A1) or 10 µM glutamate (B1) was applied to lumbar spinal cords slices for 10 minutes. Micrograph of single optical confocal sections through the center of lumbar MNs with membrane bound Kv2.1-IR (Red) and neuronal nissl staining (Green). Scale bars are 10 µm. A2 & B2 Intensity heat maps of the Kv2.1-IR in confocal micrographs above. Please notice in the ACSF treatment (A2) that pixels are localized to larger high-density clusters. Following glutamate treatment (B2) pixels are less organized indicated by smaller, less dense clusters indicating ion channel dispersal.
ANOVA (Dunn’s Method) p<0.001

Kv2.1-IR Macrocluster area (μm²)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kv2.1-IR Macrocluster area</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSF</td>
<td>(202)</td>
</tr>
<tr>
<td>10 μM Glut</td>
<td>(225)</td>
</tr>
<tr>
<td>10 μM Cyclo</td>
<td>(150)</td>
</tr>
<tr>
<td>5 μM Cyclo</td>
<td>* (103)</td>
</tr>
<tr>
<td>10 μM Cyclo</td>
<td>(179)</td>
</tr>
<tr>
<td>10 μM Glutamate Treatment</td>
<td></td>
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</table>
Figure 24. Kv2.1-IR ion channel dispersal is regulated by calcineurin. Bath application of either artificial cerebral spinal fluid (ACSF), 10 μM glutamate (glut) and 5 or 10 μM cyclosporine (cyclo), an inhibitor of calcineurin activity, was applied to lumbar spinal cords slices for 10 minutes. The significant decrease, indicated by asterisk, of Kv2.1 channel declustering following glutamate treatment, was partially inhibited with 5 μM cyclosporine treatment, and fully inhibited by 10 μM cyclosporine treatment. Error bars represent ±SEM.
Figure 25. Kv2.1-IR ion channel clustering increases in low activity conditions.

Bath application of either artificial cerebral spinal fluid (ACSF) or 500 nM tetrodotoxin (TTX; Red), to suppress neuronal activity, was applied to lumbar spinal cords slices for 1.0 hour. (A) En face Kv2.1-IR ion channel macrocluster sizes are significantly larger following TTX treatment (ACSF Mean= 5.97 µm; TTX Mean= 6.51 µm) and n=number of macroclusters sampled. (B) Average Kv2.1 ion channel macrocluster sizes per motoneuron are significantly larger following TTX treatment and n= number of motoneurons sampled. Horizontal bars represent mean values (ACSF Mean= 6.5 µm; TTX Mean= 8.8 µm). Significance is indicated by asterisk and determined with Mann-Whitney Rank Sum. Error bars ± SEM.
Motoneuron Kv2.1 channels do not disperse upon cholinergic modulation.

Modulation of muscarinic receptors in cultured hippocampal pyramidal cells that contain predominately the m1 receptor subtypes (Levey et al., 1995) causes rapid Kv2.1 declustering (Mohapatra & Trimmer, 2006). However, in lumbar αMNs, the largest Kv2.1 clusters colocalize with m2 receptors into a signaling ensemble apposed by the enigmatic cholinergic C-bouton presynaptic terminals (Hellstrom et al., 2003; Muennich & Fyffe, 2004; Wilson et al., 2004; Deardorff et al., 2013; Deardorff et al., 2014) (Figure 26). While the signaling pathway of m2 receptors is undefined in MNs, in other cell types their activation leads to an inhibition of N-type voltage-gated Ca\(^{2+}\) channels (Allen & Brown, 1993; Hille, 1994; Howe & Surmeier, 1995; Herlitze et al., 1996; Shapiro et al., 1999; Stewart et al., 1999). Thus, the net effect of m2 receptor activation would be lowering of internal Ca\(^{2+}\) - in direct contrast to the effects of m1 receptor activation, that typically raises internal Ca\(^{2+}\) (Gallo et al., 1993).

To determine if Kv2.1 clustering is sensitive to cholinergic modulation in αMNs, 10 μM muscarine, a muscarinic receptor agonist, was bath applied with and without 500 nM TTX, to block confounding results from intra-spinal cholinergic circuits that could evoke synaptic activity onto MNs, for 10 minutes in 6 total rats from 2 separate litters. Bath application of muscarine without TTX did cause a
significant declustering of Kv.21 when compared to ACSF controls (Figure 26; ACSF, 108 clusters, 7.668 μm² ± 3.5 SD vs. muscarine, 135 clusters, 4.434 μm² ± 4.4 SD). However, when muscarine was applied with TTX to isolate the muscarinic effect on the MN receptors, there was no significant Kv2.1 ion channel dispersal (Figure 26; TTX Controls, 391 clusters, 7.009 μm² ± 5.9 SD vs. muscarine +TTX, 368 clusters, 6.537 μm² ± 5.4 SD). These results suggest that m2 receptor activation on motoneurons does not induce Kv2.1 cluster dispersal. The small, yet insignificant, effect that was noted could be due to the delay in TTX inhibition relative to muscarinic activation. Because Kv2.1 ion channel declustering mechanisms are related, through calcineurin, to increases in internal Ca²⁺, these observations are consistent with m2 channel functions defined in other neurons and supports the hypothesis that cholinergic modulation through C-bouton activity maintains Kv2.1 clustering in MNs (please see Deardorff et al., 2014).
Figure 26. The muscarinic type 2 (m2) acetylcholine receptor is colocalized with large Kv2.1-IR clusters within a signaling ensemble located at C-boutons. Images are confocal stacks of 12 x 1 μm Z-stacks with nissl immunoreactivity (blue) to label neuronal somas. Scale bar is 20 μm. (A) m2-IR (red) located within surface membrane of α-motoneurons in large distinct clusters. (B) Kv2.1-IR (green) located within the surface membrane of all α-motoneurons in a distinct mosaic pattern of large and small clusters. (C) The largest Kv2.1-IR (green) clusters colocalize with m2-IR (red) clusters. Panels D, E, and F are zoomed images of specific clusters indicated by white boxes in panels A-C. (G) Kv2.1 ion channels do not decluster upon motoneuron cholinergic modulation. Bath applied 10 μM muscarine for 10 minutes causes Kv2.1 declustering in MNs, however, when synaptic evoked activity is blocked with tetrodotoxin (TTX), muscarine application does not disperse Kv2.1 channel clusters to a significant extent. Significance, indicated by asterisk, was calculation via ANOVA, Dunn’s method, p<0.05 and n= the number of Kv2.1 macroclusters sampled.
Motoneuron Kv2 channels regulate repetitive firing properties.

The goal of the experiments reported in this section was to determine the effects of Kv2 currents on MN firing through using the tarantula toxin stromatoxin (STX). First, however, to confirm that STX application would not directly alter Kv2.1 ion channel clustering, 100nm STX in ACSF was bath applied to postnatal rat lumbar spinal cord slices in 3 rats from separate litters for 10 minutes. When compared to ACSF controls from the same rats, no significant differences in Kv2.1 ion channel clustering on lumbar MNs were found (Figure 27; ACSF, 40 MNs, 316 clusters, mean area 6.490 μm² ± 4.6 SD vs. STX treated, 42 MNs, 367 clusters, mean area 5.845 μm² ± 3.5 SD).

The clustering properties of Kv2.1 are linked to the gating kinetics of the channel and can impact repetitive firing properties in other neurons (Johnston et al., 2008; Mohapatra et al., 2009; Guan et al., 2013; Liu & Bean, 2014). To determine the contribution that Kv2.1 clustering has on αMN activity, STX was used to block Kv2 currents in whole-cell current clamp electrophysiological recordings of visually identified MNs using DIC optics in in vitro rat lumbar spinal cord slices.

There are no significant differences in the measured parameters of single MN action potentials when Kv2 channels are blocked (Figure 28, Table 9), with the exception of a modest but significant 23% decrease in the after-hyperpolarization (AHP) amplitude (Table 9; Before STX= 7.72 mV ± 4.7SD vs. After STX= 5.95 mV ±4.9SD). Furthermore, we found no significant differences in the resting membrane potential (RMP, Table 9), input resistance (R_{in}, Table 9), and rheobase current (I_{min})
in all 16 MNs measured. This is consistent with observations in cortical pyramidal
cells, where because Kv2 channels have slow activation kinetics and depolarized
activation ranges they do not influence the parameters of individual isolated action
potentials (Guan et al., 2007; Guan et al., 2013),

Although Kv2 currents have minimal involvement in both single action
potential and passive properties in MNs the effect of blocking Kv2 channels becomes
more dramatic during repetitive firing evoked by longer current pulses (Figure 29).
After STX application, lumbar MNs had an average 41% significant reduction in
firing frequency (Wilcoxon Signed Rank, P<0.001) (Figure 29), and in longer spike
trains there was both an increase in failures to fire and increased inability to
maintain repetitive firing (Figure 30). Furthermore, the slope of firing frequency vs.
injected current (f-I curve) was significantly less steep (lower gain) following STX
application indicating that the overall excitability is reduced (Figure 29B&C; before
STX 70.0 Hz/nA ± 35 SD vs. after STX 58.1 Hz/nA ± 35 SD, p<0.05 pairwise T-Test).
While all the MNs sampled had a reduction in firing frequency, some were more
impacted by STX application that others (Figure 29D). To facilitate comparisons
between cells, the change in firing frequency was plotted against the threshold for
repetitive firing to illustrate a significant correlation (Figure 29E), suggesting that
Kv2 properties may also exist on a continuum similar to many MN properties.

Although Kv2 does not affect the current required to evoke a single action
potential, it does impact the threshold for which MNs fire repetitively. In every MN
sampled, more current was required (500ms square pulses) to evoke repetitive
firing following STX application (Figure 31; Before STX 225 pA ± 190 SD vs. After
STX 314 pA ± 206 SD, n=16 motoneurons, p<0.001 via Paired T-Test). These observations further support the argument that Kv2 currents regulate MN excitability.

To confirm that the STX effect on repetitive firing is in fact, a result of the toxin itself and not some other confounding effect arising from the health of the cell, a 10 minute ACSF washout was performed in 6 MNs and examined for partial recovery. In all MNs sampled, both the firing frequency (following STX ≈62% reduced vs. washout ≈ 46% reduced; P=0.002, paired t-test) and threshold for repetitive firing (Before mean = 224 pA ± 290 SD; STX mean= 329.17 pA ± 300 SD vs. washout mean = 245 pA ± 299 SD, P=0.011, paired t-test) partially, yet significantly, restored following STX washout (Figure 32).
**Figure 27. Stromatoxin application does not cause Kv2.1 ion channel dispersal on lumbar motoneurons.** Bath application of either artificial cerebral spinal fluid (ACSF) or 100 nM stromatoxin was applied to lumbar spinal cords slices for 10 min. (A) No significant differences found when individual Kv2.1 clusters were compared from 3 rats from different litters. Mean values for ACSF = 6.5 μm² and STX = 5.8 μm² and n= the number of clusters measured. Error bars equal ±SD. (B) No significant differences found when average Kv2.1 cluster size per motoneuron was compared from 3 rats from different litters. Horizontal bars represent mean values, where ACSF = 6.7 μm² and STX = 6.2 μm² and n= number of motoneurons sampled. The Mann-Whitney rank sum test was used to determine lack of significance (p =0.653).
Figure 28. Stromatoxin application does not alter single action potential properties in rat lumbar motoneurons. Single action potentials were evoked by brief, 10 ms, depolarizing current injections. Voltage traces shown are from the same motoneuron and recorded before (black) and after (red) 10min bath application of 100 nM stromatoxin in lumbar spinal cord slices.
Table 9. Individual Action Potential Properties

<table>
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<tr>
<th></th>
<th>$R_n$ (MΩ)</th>
<th>$I_{\text{min}}$ (pF)</th>
<th>RMP (mV)</th>
<th>$V_m$ (mV)</th>
<th>$A_{\text{amp}}$ (mV)</th>
<th>$A_{\text{area}}$ (mV.ms)</th>
<th>$A_{\text{spike}}$ (ms)</th>
<th>$dV/dT$ up (V.s⁻¹)</th>
<th>$dV/dT$ down (V.s⁻¹)</th>
<th>$A_{\text{H}}$ (mV)</th>
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<tr>
<td>CTL</td>
<td>107.46</td>
<td>630.56</td>
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<td>84.88</td>
<td>166.43</td>
<td>1.81</td>
<td>126.62</td>
<td>-68.82</td>
<td>7.72</td>
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<td></td>
<td>±59.1</td>
<td>±467</td>
<td>±5.53</td>
<td>±9.94</td>
<td>±18.8</td>
<td>±31.7</td>
<td>±0.50</td>
<td>±57.49</td>
<td>±36.19</td>
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<td>STX</td>
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<td>-61.41</td>
<td>-35.16</td>
<td>88.18</td>
<td>208.56</td>
<td>1.97</td>
<td>113.68</td>
<td>-56.78</td>
<td>5.95</td>
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<td>±548</td>
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<td>±16.3</td>
<td>±22.9</td>
<td>±63.6</td>
<td>±0.58</td>
<td>±40.57</td>
<td>±28.12</td>
<td>±4.9</td>
</tr>
</tbody>
</table>

±SD, n=16
Figure 29. Stromatoxin sensitive currents maintain firing rate and excitability in rat lumbar motoneurons. All motoneuron electrophysiological recordings were made before and after 10min bath application of 100 nM stromatoxin (STX) in lumbar spinal cord slices. (A) Voltage trace is response to 500 pA current injection for 500 ms before and after STX application. (B) Plot of current injected ($I$) vs firing frequency ($f$) of cell shown in A. Please note the faster firing and steeper $f$-$I$ slope (gain) before STX application. (C) In all 16 cells evaluated, there is a significant decrease in $f$-$I$ slope (gain) following STX application. Significance was determined with pairwise T-test. (D) Normalized to the control frequency, every cell sampled had a reduction in firing rate following STX treatment. Data displayed for each motoneuron is at 3x firing threshold, n=14 (E) Significant relationship (Spearman rank order correlation value = 0.660, P = 0.009) between percent change in normalized firing frequency, at 3x firing threshold, and repetitive firing threshold.
Figure 30. Stromatoxin-sensitive currents help maintain repetitive firing properties. Voltage traces are 300pA square pulses for 45s from the same motoneuron before and after bath application of 100nM stromatoxin. Not only do motoneurons fire slower, there is also an increase failure to fire and the inability to maintain repetitive firing.
Figure 31. Stromatoxin sensitive currents lower the threshold for repetitive firing in rat lumbar motoneurons. In all 16 motoneurons recorded, there was a significant (P<0.001, paired T-test) increase in the threshold for repetitive firing in a 500ms square pulse after STX-sensitive currents were blocked. The red line represents mean values.
Figure 32. Firing frequency and the threshold for repetitive firing partially restore following stromatoxin washout in rat lumbar motoneurons. The effects of stromatoxin (STX) are partially reversible upon 10-minute washout with ACSF. (A) Representative voltage trace from a single motoneuron. Repetitive firing was evoked in a 500ms depolarizing square pulse with fixed parameters before, immediately following (red) and after 10 min washout. (B) The firing frequency increased following STX washout in all 6 motoneurons tested. (C) The threshold for repetitive partially restored following STX washout in all 6 motoneurons tested.
Motoneuron Kv2 channels regulate interspike interval.

Because of their slow kinetics, Kv2 channels do not alter single action potential properties in postnatal rat lumbar MNs (see above). However, following STX application, the action potentials in spike trains become significantly broader (Figure 33A; before STX 68.8 mV*ms ± 16.5 SD vs. after STX 79.8 mV*ms ± 19.2 SD, n=6 MNs, P<0.001 Wilcoxon Signed Rank Test), where both the rising maximum slopes (Figure 33B) and decaying maximum slopes (Figure 33C) are significantly reduced (n=6, Pairwise ANOVA). That the maximum $dV/dt$ action potential upstroke is highly correlated with Na$^+$ channel activation and that both the action potential threshold and spike rise rate can covary with the preceding interspike interval (ISI) (Henze & Buzsaki, 2001), we and others argue that Kv2 channels exert effects on repetitive firing by regulating the membrane potential of the ISI, thus relieving Na$^+$ channel inactivation (Johnston et al., 2008; Guan et al., 2013). The ISI occurs during steepest part of the inactivation range for the Na$^+$ current, thus small changes in interspike voltage will have a large effect on Na$^+$ channel availability. Here we show that, during repetitive firing, the ISI is depolarized following STX application (Figure 34) and that this effect is significant (Figure 35; before STX -47.3 mV ± 4.5 SD vs. after STX -34.1 mV ±5.5 SD, n=16, P=0.004 Wilcoxon Signed Rank Test).
Figure 33. Stromatoxin-sensitive currents maintain narrower action potentials in spike trains of rat lumbar motoneurons. (A) Bath application of 100nM stromatoxin (STX) creates a broader action potential (Pairwise ANOVA, P=0.05). (B) The maximum rate of rise ($dV/dt$) decreased with spike number during firing and this effect was significantly enhanced following STX treatment (Pairwise ANOVA, P<0.001). (C) The maximum rate of decay ($dV/dt$) decreased with spike number during firing and this effect was significantly enhanced following STX treatment (Pairwise ANOVA, P<0.001).
Before STX (30 Hz)

-64 mV

400 pA

After STX (24 Hz)

-64 mV

400 pA

Merge

-64 mV
Figure 34. Stromatoxin-sensitive currents maintain a more hyperpolarized interspike interval. Voltage traces are from same motoneuron before and after the treatment with 100 nM stromatoxin. In the merged traces, please note that the black traces are more hyperpolarized in the interspike interval.
Interspike Interval Membrane Polarization

Before STX: Mean -47.3mV
After STX: Mean -35.2mV

* P=0.004
Figure 35. Stromatoxin-sensitive currents maintain hyperpolarized interspike interval. In spike trains the interspike interval minimum becomes significantly depolarized (paired T-Test, p=0.004, n=16), following 10 min bath application of stromatoxin (STX).
Motoneuron Kv2 channels homeostatically suppress firing following increased activity.

Previous studies have indicated that the Kv2.1 channel clustering properties and kinetics are intimately associated through phosphorylation states and following glutamate-induced dephosphorylation, there is a large (~25mV) hyperpolarizing shift in both the activation and deactivation of Kv2.1 channels (Murakoshi et al., 1997; Misonou et al., 2004; Misonou et al., 2005; Park et al., 2006; Misonou et al., 2008; Mohapatra et al., 2009). These activity-dependent alterations would enable more channels to open during the AP upstroke and more current flowing after the spike and suppresses repetitive firing (Mohapatra et al., 2009) and, when simulated in computer models, generates smaller action potential amplitudes and a more profound afterhyperpolarization (Surmeier & Foehring, 2004).

In all the rat lumbar MNs sampled here, repetitive firing properties are dramatically affected following bath application of 10 μM glutamate for 10 minutes, applied to induce Ca\(^{2+}\)/Calcineurin-dependent declustering (Figure 36). Specifically, glutamate treatment significantly suppresses repetitive firing by approximately 60% with a maximum effect occurring 10-15 minutes following treatment (Figure 37). This provides an experimental window to test what the contribution of Kv2 channels are on the reduction in firing rate caused by the glutamate treatment. Moreover, following glutamate treatment, MNs also had an increase failure to fire (Figure 36) and in some cases, MNs lost the ability to repetitive fire (Figure 38). When STX was applied to block Kv2 channels during the peak glutamate effect window, the single action potential was not altered (Table 10), but repetitive firing
properties were remarkably and significantly restored (Figure 38 and 39) suggesting Kv2 is mediating the suppression in repetitive firing. As predicted with models, here we show for the first time in any neurons, that activity-induced alterations in Kv channels both decrease spike amplitude in repetitive firing trains and increase the afterhyperpolarization to slow firing (Figure 40).
Figure 36. Glutamate treatment suppresses repetitive firing properties in rat lumbar motoneurons. Both voltage traces are from the same motoneuron before (top) and after (bottom) 10-minute bath application of 10 µM glutamate, recorded with fixed parameters. Please note that following glutamate treatment there is an increase failure to fire and the inability to maintain repetitive firing.
Normalized Firing Frequency

Time following 10min 10μm Glutamate Application
Fig 37. Glutamate treatment reduces firing frequency in rat lumbar motoneurons. Following 10-minute bath application of 10 µM glutamate, repetitive firing properties were recorded every 5 minutes with 500 ms square pulses at current injections 2x repetitive firing threshold in a total of 3 motoneurons. Approximately 10-15 minutes following glutamate treatment there is a maximum, significant (Pairwise ANOVA, P<0.05), as indicated with asterisk, reduction in firing. Error bars represent ±SD.
Figure 38. Kv2 ion channels underlie glutamate specific reduction in repetitive firing. Voltage traces are recorded from the same motoneuron, evoked by a 45 second 400 pA square pulse. In some neurons, 10-minute bath application of 10 µM glutamate fully inhibits repetitive firing (middle trace). Following 10 minute bath application of 100 nM stromatoxin (STX), repetitive firing properties were restored (red trace). Please note that the glutamate + STX trace was recorded during the window of maximum glutamate effect on repetitive firing.
Table 10. Single Action Potential Properties After Glutamate and STX Treatments

<table>
<thead>
<tr>
<th></th>
<th>$C_m$ (pF)</th>
<th>$R_{in}$ (MΩ)</th>
<th>$I_{\text{min}}$ (pF)</th>
<th>RMP (mV)</th>
<th>$V_{th}$ (mV)</th>
<th>AP$_{\text{amp}}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>88.67±16.8</td>
<td>163.69±156</td>
<td>691.67±269</td>
<td>-66.34±2.0</td>
<td>-38.94±7.7</td>
<td>99.03±8.7</td>
</tr>
<tr>
<td>STX+Glut</td>
<td>80.33±6.7</td>
<td>156.01±104</td>
<td>679.17±232</td>
<td>-65.99±2.1</td>
<td>-35.66±12.2</td>
<td>87.62±10.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AP$_{\text{area}}$ (mV*ms)</th>
<th>AP$_{\text{HfT}}$ (ms)</th>
<th>$dV/dT$ up (V*s$^{-1}$)</th>
<th>$dV/dT$ down (V*s$^{-1}$)</th>
<th>AHP$_{\text{amp}}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>161.89±16.5</td>
<td>1.47±0.2</td>
<td>176.77±53.5</td>
<td>-69.10±12.7</td>
<td>3.28±1.9</td>
</tr>
<tr>
<td>STX+Glut</td>
<td>187.15±22.9</td>
<td>1.79±0.2</td>
<td>111.54±28.5</td>
<td>-52.86±10.8</td>
<td>2.06±2.3</td>
</tr>
</tbody>
</table>

±SD, n=9
Fig 39. Stromatoxin-sensitive currents underlie the reduction of repetitive 
fire rates following glutamate treatment. In all 9 motoneurons sampled, 10- 
minute bath application of 10 µM glutamate significantly reduced firing rate (P 
=0.004; Wicoxon Signed Rank Test). Graph above shows normalized decrease 
compared to firing rate before pharmacological manipulation. When 100nm 
Stromatoxin (STX) was applied for 10-minutes (during the window of maximum 
glutamate effect), repetitive firing rate is restored and no longer significantly 
different from the control recording before pharmacological manipulation.
Before Treatments (20 Hz)

Glutamate (14 Hz)

Glutamate + STX (24 Hz)

Merge

30 mV

50 ms
**Fig 40. Kv2 currents reduce repetitive firing rates and underlie shorter action potentials and large after-hyperpolarizations following glutamate treatment.**

Voltage traces are recorded from the same motoneuron, evoked by a 500ms 400 pA square pulse. Please note, when compared to control (Black), the glutamate trace (Blue) has shorter action potentials and larger AHPs, an effect reversed upon Kv2 blockade with 100nM stromatoxin (STX; red). The glutamate + STX trace was recorded during the window of maximum glutamate effect on repetitive firing.
Discussion

Mammalian neurons express multiple types of membrane bound voltage-gated potassium channels (Kv) (Coetzee et al., 1999; Hille, 2001; Johnston et al., 2010; Liu & Bean, 2014). Over 40 genes have been identified that give rise to multiple families of Kv channel subunits and knowledge of these individual subunits comes largely from studies in recombinant homomeric expression systems in cell cultures (see Johnston et al., 2010). In native environments, each excitable membrane consists of a unique combination of Kv subunits to meet the demands of that neuron. These subunit compositions can be further customized by the formation of heteromeric channels, protein-protein interactions, and both post-translational and transcriptional modifications. Knowledge of how these channel subunits are behaving in their native and dynamic environments may be critical to the development of new therapies for a variety of devastating neurological pathologies.
Kv2.1 membrane clustering is dynamic in mammalian motoneurons.

Here we show that Kv2.1 channel clusters are dynamic in lumbar MNs, not only in their calcineurin-dependent channel declustering following excitatory drive, but also, and for the first time, we show that Kv2.1 channel clusters have the capacity to significantly increase in size in low-activity conditions. What is more remarkable, is that these cellular changes occur rapidly, within minutes to hours, supporting the notion that these channels can respond to sudden increases and/or decreases in synaptic input to homeostatically shape MN output (see below). Rapid alterations in intracellular Ca^{2+} are intimately linked to Kv2.1 channel clustering dynamics in mammalian MNs through calcineurin. Thus Kv2 can be added to the increasing list of K^+ channels that mediate changes in K^+ conductance largely through posttranslational modifications, driven by intracellular signaling events (see Misonou, 2010).

Unlike observations in the brain, cholinergic modulation did not cause significant declustering of Kv2.1 in αMNs (Mohapatra & Trimmer, 2006), likely because of differential expression of muscarinic receptor subtypes. Although uncharacterized in the MN, the m2 receptor subtype expressed in MNs, typically couples to pertussis toxin (PTX)-sensitive Gi/Go pathways, which can reduce adenylate cyclase (via G-protein α subunits) and/or inhibit conotoxin (CTX)-sensitive voltage gated N-type Ca^{2+} channels (via G-protein βγ subunits). The net effect of m2 receptor activation would be lowering of internal Ca^{2+} and Kv2.1
declustering mechanisms are related, through calcineurin, to increases in internal 
Ca$^{2+}$.

This leads to the question as to why is Kv2.1 localized at the C-bouton? Our 
hypothesis on the modulatory effects of cholinergic signaling in αMNs is centered on 
a highly integrated system organized around a Ca$^{2+}$ microdomain for precise and 
nuanced regulation of cell firing (Deardorff et al, 2014). Specifically, m2-mediated 
inhibition of Ca$^{2+}$ currents 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$^{2+}$ 
sufficient to allow diffusion of Ca$^{2+}$ from neighboring compartments, there would be 
rapid Kv2.1 channel declustering (Romer et al., 2014), thus, Kv2.1 is a built-in fail-
safe at the C-bouton against excitotoxicity.
Stromatoxin-Application and Limitations

A challenge in studying the function of Kv2 channels has been primarily due to the lack of a highly selective blocker. Several peptides isolated from tarantula venom have been found to be novel inhibitors with a high affinity for Kv2 and Kv4.2/3 including the gating-modifier stromatoxin (STX) (Escoubas et al., 2002; Johnston et al., 2008; Zhong et al., 2010). As monitored using whole-cell patch-clamp techniques, bath applied STX fully inhibits both Kv2.1 and Kv2.2 at 100nM (in 100s) in a voltage-dependent manner with maximum inhibition between -30 and 0 mV (Escoubas et al., 2002). Although Kv2.2 ion channel mRNA is expressed in Xenopus MNs (Burger & Ribera, 1996; Blaine & Ribera, 2001), in rat MNs, only Kv2.1 mRNA has been detected (Alessandri-Haber et al., 2002). Thus we conclude that the STX-sensitive current in rat lumbar MNs is largely from the Kv2.1 subunit. It should also be noted that STX effects on Kv2 reverse slowly following 10 min washout, however the Kv2 current is not fully restored.

Because STX also inhibits the A-type currents from Kv4.2 and Kv4.3, its use experimentally is often precluded. However in rat lumbar MNs, Kv4.2 and Kv4.3 are only transiently embryonically expressed, with channel expression undetected after the first postnatal week (Huang et al., 2006). Here, we show that STX-sensitive currents do not alter the single action potential, which offers further support that confounding STX-sensitive A-type current is minimal. Thus STX a suitable candidate for studying Kv2 channels in postnatal and adult rats.
Despite the fact that only a fraction of Kv2 channels turn on in a single action potential, their slow deactivation allows the activity to accumulate, and this effect is more dramatic at higher frequencies, where Kv2 currents are also most likely to contribute to spike frequency adaptation (Du et al., 2000; Johnston et al., 2008; Mohapatra et al., 2009; Guan et al., 2013). In this study, we can see evidence of this accumulation over the first several ISIs in our spike trains (Figures 34 & 40), but there are experimental limitations with the use of stromatoxin-1 (STX) that prevent us from testing higher frequencies of firing. STX modifies gating properties of the channel by shifting the activation to more depolarized voltages, functionally blocking conductance. Thus, STX inhibits in a voltage-dependent manner with maximum inhibition between -30 and 0 mV (Escoubas et al., 2002). While STX provides the opportunity of paired measurements in each MN, the significant effects on MN repetitive firing, reported here, and are likely further enhanced during high frequency firing rates requiring higher evoked current injections - beyond the effective range for STX inhibition.
Physiological role of Kv2 in mammalian motoneurons

How Kv2 ion channels influence neuronal excitability is of particular interest because of accumulating evidence that these channels are exceptionally dynamic and targets of a remarkable range of influences that can alter their functions (Murakoshi et al., 1997; Misonou et al., 2004; Misonou et al., 2005; O’Connell et al., 2006; Redman et al., 2007; Mohapatra et al., 2009; O’Connell et al., 2010; Plant et al., 2011; Steinert et al., 2011; Fox et al., 2013). In some cultured neurons, high-density clusters of Kv2 channels are largely low or nonconducting, and it is possible that the channel proteins have additional ‘nonconducting’ functions (O’Connell et al., 2010; Fox et al., 2013). However, the data presented here together with other reports (Du et al., 2000; Malin & Nerbonne, 2002; Guan et al., 2007; Johnston et al., 2008; Mohapatra et al., 2009; Guan et al., 2013; Liu & Bean, 2014) suggests that the electrical function of Kv2 channels in native neuronal membranes is substantial enough to strongly regulate repetitive firing properties.

The high threshold, slow activation range of Kv2 currents prevents them from contributing to the single action potentials in neurons with half-widths under approximately 2.0 ms (Du et al., 2000; Blaine & Ribera, 2001; Malin & Nerbonne, 2002; Johnston et al., 2008; Guan et al., 2013; Liu & Bean, 2014). Here, we show in lumbar MNs, that Kv2 channels also do not affect the depolarization and repolarization properties in a single action potential. Furthermore, Kv2 channels do not affect passive membrane properties such as rheobase, input resistance, membrane capacitance and resting membrane potential. These observations are
consistent with other findings and suggest Kv2 subunits are ‘high-threshold’ channels in MNs and do not behave as traditional delayed rectifiers ($I_k$). It is likely that other $I_k$ subunits in Kv1 and Kv3 families, with traditionally lower activation thresholds and faster activation ranges, contribute action potential properties in MNs (Takahashi, 1990; Viana et al., 1993; McLarnon, 1995; Lape & Nistri, 1999; Alessandri-Haber et al., 2002).

The most prominent finding is that in mammalian MNs, Kv2 channels maintain excitability, evident with increased $f$-$I$ slope (gain) and decreased threshold for repetitive firing, and increased repetitive firing frequency. Maintaining repetitive firing properties of MNs, especially in higher frequency ranges, is necessary and required to generate maximum muscle force (Monster & Chan, 1977; Thomas et al., 1991; McNulty et al., 2000). The hypothesis that the slow kinetics of Kv2 channels function to maintain repetitive firing through relieving Na⁺ channel inactivation are consistent with our observations, that Kv2 currents significantly hyperpolarized interspike intervals and underlie increased maximum slope upstroke in the subsequent spike, a reflection of increased Na⁺ channel availability. Because STX application does not alter the single isolated action potential, the results presented herein are not likely a confounding affect of STX acting directly upon Na⁺ channels.
Homeostatic role of Kv2 in mammalian motoneurons

As previously discussed (above and Romer et al., 2014), Kv2.1 ion channels rapidly decluster in lumbar MNs following glutamate treatment. This same treatment in hippocampal neurons leads to pronounced changes in voltage-dependent gating properties of Kv2.1 channels, specifically activation parameters (Control $G_{1/2} = 16 \text{ mV}$; Glutamate $G_{1/2} = -8.6\text{ mV}$) and inactivation parameters (Control $V_i = -28.8 \text{ mV}$; Glutamate $V_i = -54.7\text{ mV}$) hyperpolarize (Mohapatra et al., 2009). Thus, the channels will open earlier in the action potential cycle, with some even opening during the spike upstroke, and remain open longer after the spike, hence suppressing the firing rate. Here, we show that 10-minute glutamate application does decrease repetitive firing in lumbar MNs, and that this effect is Kv2 specific. When modeled in spike trains of pyramidal neurons, the alterations Kv2.1 channel kinetics caused a modest shortening of the spike amplitudes together with a prominent increase in the ISI (Surmeier & Foehring, 2004). Here, we show evidence for the first time in any native neuron, that activity-dependent changes of Kv2.1 kinetics do, in fact, cause shorter spikes and increases in the interspike intervals of spike trains. The net effect of slowing down the firing rate in high activity conditions to restore an optimal range of firing is the ultimate goal of homeostatic plasticity.
Table 11. The impact of the ion channel Kv2.1 on firing properties in mammalian lumbar motoneurons

<table>
<thead>
<tr>
<th></th>
<th>‘Typical’ Physiological State</th>
<th>Activity-Dependent Physiological State</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Input Resistance</strong></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Rheobase</strong></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Voltage of Threshold</strong></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Single Action Potential Amplitude</strong></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Single Action Potential Area</strong></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Afterhyperpolarization</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Repetitive Firing Rate</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Repetitive Firing Threshold</strong></td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Repetitive Firing Spike Amplitude</strong></td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Repetitive Firing Interspike Interval</strong></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table indicates positive (+), negative (-) or neutral (+/-) effects
Conclusions

The clustering properties of Kv2.1 are linked to the channel gating kinetics of the channel and can impact repetitive firing properties in other neurons (Johnston et al., 2008; Mohapatra et al., 2009; Guan et al., 2013; Liu & Bean, 2014). Here, we demonstrate that the clustering properties in MNs are profoundly dynamic and respond to both high and low activity conditions. Furthermore, we show that the enzyme calcineurin regulates Kv2.1 ion channel declustering. To determine the contribution that Kv2.1 clustering has on αMN activity, the Kv2 inhibitor Stromatoxin (Escoubas et al., 2002; Johnston et al., 2008; Zhong et al., 2010) was used to block Kv2 currents in whole-cell current clamp electrophysiological recordings. We report that in rat lumbar MNs, Kv2 currents contribute to maintenance of MN repetitive firing properties and membrane excitability (Table 11). Specifically, Kv2 currents may relieve Na⁺ channel inactivation, permitting shorter interspike intervals and higher repetitive firing rates. Conversely, in the presence of prolonged 10μM glutamate treatment, increased outward current through Kv2.1 channels may play a role in homeostatic regulation by limiting cell firing. These results are consistent with the notion that differential modulation of Kv2.1 channel kinetics allows these channels to act in a variable way across a spectrum of MN activity states.
Chapter VIII:

Swimming against the tide:

Investigations of the C-bouton synapse

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¹Equally Contributing Authors

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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 and Chan, 1977; Henneman and Mendell, 1981; Clamann, 1993; Cope and 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-motoneuron (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 and 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 afterhyperpolarization (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 and colleagues (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 can’t swim at all.
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 41 & 42) precisely organized for highly nuanced orchestration of somatic K⁺ currents.
<table>
<thead>
<tr>
<th></th>
<th>Presynaptic</th>
<th>Postsynaptic</th>
<th>Ensemble</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A1</strong></td>
<td>VACt-IR</td>
<td>Bassoon-IR</td>
<td>SK3-IR</td>
</tr>
<tr>
<td></td>
<td><img src="image1" alt="Image" /></td>
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</tr>
<tr>
<td><strong>A2</strong></td>
<td>VACt-IR</td>
<td>Bassoon-IR</td>
<td>m2-IR</td>
</tr>
<tr>
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<td><img src="image7" alt="Image" /></td>
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</tr>
<tr>
<td><strong>B1</strong></td>
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<td>Kv2.1-IR</td>
<td>SK3-IR</td>
</tr>
<tr>
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<td><img src="image13" alt="Image" /></td>
</tr>
<tr>
<td><strong>B2</strong></td>
<td>VACt-IR</td>
<td>Kv2.1-IR</td>
<td>m2-IR</td>
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<tr>
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<td><img src="image18" alt="Image" /></td>
</tr>
</tbody>
</table>
**Figure 41. 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 x 1μm Z-stacks) of *en face* C-boutons, indicated with VACHT-IR (Blue), on rat lumbar α-motoneurons. (A) Presynaptic active zone protein bassoon (green) is aligned with post-synaptic ion channels SK3 (Ai, red) and type 2 muscarinic acetylcholine receptors (Aii, red). (B) Kv2.1-IR (green) intercalates with SK3-IR (Bi, red) and m2-IR (Bii), ‘filling in’ the C-bouton postsynaptic membrane. Scale bars are 2.0 μm.
Figure 42. Synaptic distribution of specific ion channels and receptors on soma and proximal dendrites of motoneurons. The schematic illustrates 3 types of motoneuron presynaptic boutons including the Glycinergic/GABAergic F-type, Glutamatergic S-type and Cholinergic C-type with its associated post-synaptic subsurface cistern. Note the specific localization of m2 muscarinic receptors (blue) with SK channels (red) and Kv2.1 channels (green) postsynaptic to the C-bouton. Small Kv2.1 clusters are also found postsynaptic to some S-type synapses (see Muennich et al, 2004). The N-type Ca\(^{2+}\) channel Cav2.2 (light grey) is 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 grey) are specifically associated with the C-bouton subsurface cistern.
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 42), spanning a breadth of <25nm, 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 type 2 muscarinic acetylcholine (m2) receptors directly apposing C-bouton presynaptic terminals (Skinner et al., 1999; Hellstrom et al., 2003; Muennich and Fyffe, 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 bassoon (A.S. Deardorff, S.H. Romer, R.E.W. Fyffe unpublished observations, see Figure 41). Beneath the post-synaptic 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. Kv2.1 channels appear to ‘fill in’ the remaining post-synaptic α-MN membrane surface not occupied by SK channels or m2 receptors. The demarcated post-synaptic area, therefore, is a highly structured and mosaic domain of interdigitating clusters of Kv2.1 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 and Fyffe, 2004)), and may also express presynaptic nicotinic acetylcholine receptors (nAChRs; (Khan et al., 2003)). In addition, the α-MN SSC is highly enriched with sigma-1 receptors (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
AHP (Viana et al., 1993; Umemiya and Berger, 1994; Bayliss et al., 1995; Li and Bennett, 2007), and SK channels typically couple to their Ca\textsuperscript{2+} source(s) by <200nm (Fakler and Adelman, 2008; Jones and Stuart, 2013). Internally, SSCs may amplify or shape these Ca\textsuperscript{2+} signals via ryanodine receptors (RyRs) or connexin32, as they do in other cell types (see discussion). We, therefore, expect some proportion of HVA Ca\textsuperscript{2+} channels and RyRs to localize to the C-bouton postsynaptic membrane and/or to the associated SSC (Figure 42). In support, Wilson and colleagues (2004) provide evidence that P/Q-type Ca\textsuperscript{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\textsuperscript{2+} channels on α-MNs, however, has only been demonstrated physiologically (Carlin et al., 2000; Wilson et al., 2004).
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.
Moments and Milestones: Ultrastructure

Pioneering EM investigations (Wyckoff and 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 motoneuron surface and may form triadic arrangements (Conradi, 1969a;Fyffe and Light, 1984)]. Those boutons Conradi classified as “C-type” are defined by and named for a signature 10-15nm thick postsynaptic SSC (‘C’ for cistern): a broad, flat disc of smooth endoplasmic reticulum juxtaposed a mere 5-8nm below the postsynaptic membrane and spanning the length of the apposing presynaptic terminal (Figure 43) (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 43). Across a particularly narrow synaptic cleft (3-8nm; (Davidoff and Irintchev, 1986)), the C-boutons themselves contain a dense cytoplasmic matrix of glycogen particles and neurofilaments tightly packed with 25-55nm (diameter) clear spherical / pleomorphic vesicles, abundant mitochondria, and occasionally a small number of large dense core vesicles intermingled therein (Figure 43) (Bodian, 1966a;b;Conradi, 1969a;McLaughlin, 1972b;Hamos and King, 1980). Notably, several authors (Rosenbluth, 1962;Bodian, 1966a;b;Charlton and Gray, 1966;Van
Harreveld and 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 3-8µm in the cat (Conradi, 1969a; McLaughlin, 1972b; Conradi et al., 1979a), 3-6µm in the primate (Bodian, 1966a;b), 3-5µm in the opossum (Hamos and King, 1980), 3-6µm in the human (Pullen, 1992), and 1-8µm 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 and 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 and King, 1980; Connaughton et al., 1986; Davidoff and Irintchev, 1986), and are particularly pronounced in non-osmicated tissue stained with E-PTA (Pullen, 1988) 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 (A.S. Deardorff, S.H. Romer, R.E.W. Fyffe unpublished observations, see Figure 41). Moreover, bassoon immunoreactivity precisely overlies postsynaptic SK channels.
and m2 receptors, but traditional post-synaptic 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 et al., 1988).

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 and Sears, 1988), and Renshaw cells (Lagerback and Ronnevi, 1982; Alvarez and Fyffe, 2007) confirm these cells lack C-type synaptic inputs. C-boutons, when properly identified (see discussion Section), are thus a useful anatomical criterion to distinguish somatic α-MNs in the brain and spinal cord (Conradi, 1969a; Pullen, 1988; Deng and Fyffe, 2004; Muennich and 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 and King, 1980; Brannstrom, 1993; Brannstrom and 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 VACHT-IR synaptic contacts (Hellstrom et al., 2003) nor m2 receptors are present (Vilaro et al., 1992; Hellstrom et al., 2003).
**Figure 43. The C-bouton synapse on mammalian α-motoneurons.** (A) C-bouton synapses on intracellularity 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 & Biv, white), are presynaptic to the muscarinic type 2 (m2) acetylcholine receptor (Bii & Biv, red) and large Kv2.1 clusters (Biii & Biv, 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 x 1μm Z-stacks with nissl immunoreactivity (blue) to label adult rat neuronal somas. 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).
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 and Lindstrom, 1972; Jankowska and Roberts, 1972b; a; Brown et al., 1981; Brown, 1983; Fyffe, 1991b; a; Burke and 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 and Sears, 1978; 1983), or cortical ablation (Bodian, 1975). Neither are they labeled by injection of retrograde tracers into dorsal roots (Ralston and Ralston, 1979), nor intracellular staining of Ia afferents (Brown and Fyffe, 1978; Conradi et al., 1983; Fyffe and Light, 1984), Ib afferents (Brown and Fyffe, 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., 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 2; Deng & Fyffe, 2004; Hellstrom et al., 1999).

Though these data collectively indicate the intraspinal derivation of C-boutons, to date, no investigator 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, we 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; Wiggs and 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 a 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 V0c interneurons also project numerous small synaptic contacts onto V1-derived Ia inhibitory interneurons (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 V0c interneurons project to the only two neuronal types (α-MNS and IaINs) in the ventral horn known to receive both recurrent inhibition and group Ia excitatory drive. Whether, like Renshaw cells, V0c interneurons send parallel projections to α-MNs and the ‘corresponding’ IaINs (i.e. those with the same Ia connections; (Hultborn et al., 1971a;b; Hultborn et al., 1971c) 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 V0<sub>C</sub> 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 a-MN AHP currents (Zhang and Krnjevic, 1987). *In vitro* investigation subsequently confirmed these findings (Viana et al., 1993; Lape and Nistri, 2000), and showed that a-MN SK currents are reduced following m2 receptor activation at C-bouton synaptic sites (Lape and 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 that individual a-MNs express a variable complement of SK2 and SK3 channel isoforms consistent with observed co-variability in a-MN size and AHP duration (Deardorff et al., 2013). In the rodent, SK3 expression is markedly heterogeneous and cell-type-specific (Figure 44), and varies in intensity from negligible (<2x background) to modest (2-3x background) to strong (>3x background) between individual α-MNs in a single tissue section. Notably, 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 45). Conversely, larger α-MNs with significantly shorter duration/smaller amplitude AHPs express only SK2 (with little or no SK3-IR) (Figure 45).

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 and Magown, 2013). Altogether these data strongly indicate that the relative proportion of SK2/SK3 isoforms and the channel cluster size and density regulates AHP 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 (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 and Kernell, 1993; 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 \(I_h\) currents (Gustafsson and 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 AHP properties.
Figure 44. 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 x 1μm Z-stacks with nissl stain (blue) to label rat lumbar neuronal somas. 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.
In Vivo Intracellular Recording / Ionophoretic Neurobiotin Injection

Tibial Nerve Stimulation

Bi

Cii

Di

Bii

SK3-IR
Nissl

Ci

Di

Biii

Neurobiotin
SK3-IR
Nissl

Ci

Di

VACHT-IR

VACHT-IR

VACHT-IR

Peak = 3.4mV

Peak = 1.5mV

Peak = 1.7mV

4pA

4pA

10pA

I_0 = 2nA

I_0 = 3nA

I_0 = 11nA

R_0 = 2.3MO

R_0 = 2.2MO

R_0 = 0.8MO

% Decay = 27.7ms

% Decay = 11.2ms

% Decay = 14.7ms

Peak Amplitude

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Figure 45. Subset of rat lumbar α-motoneurons with SK3-IR have significantly longer AHP $1/2$ decay time and increased amplitude. Data shown is review of previous study reported by Deardorff et al. (2013). (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. Micrographs are single optical confocal sections through the soma of neurobiotin intracellular filled 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) MNs have long duration and large amplitude AHP, low rheobase, and high input resistance. Micrograph insets show VACHT-IR (White) C-bouton in apposition to an SK3-IR (+) cluster. Inset scale bar is 5 μm. (C&D) Along the continuum of αMN properties, SK3-IR (-) have short duration and small amplitude AHPs. However, even among these SK3-IR (-) cells, rheobase and input resistance show high variance. Please note the nearby cells with SK3-IR (C&Dii & C&Diii arrowheads).
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 \(\alpha\)-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, most of which are known to generate, regulate, or be regulated by local intracellular Ca\(^{2+}\) (Figure 42).

Although C-boutons may boost recruitment gain, as proposed elsewhere (Zagoraiou et al., 2009; Brownstone and 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 46). 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; Figure 46, Ai & 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 (Figure 46, Aiv & Biv). That is, while the cumulative, combined effects of these isolated Ca\(^{2+}\) signals on specific AHP and delayed rectifier K\(^{+}\) currents are likely to be quite significant throughout the \(\alpha\)-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.
**Figure 46. 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 & Aiii) 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- Biii) 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.
Subsurface cisternae (SSCs) 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\(^{2+}\) 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 and Kuba, 2000). RyR release of Ca\(^{2+}\) may also result in an increase in nearby Kv2.1 channel conductances, via Ca\(^{2+}\)-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 α-MNs are thought to be involved in removal of excess free Ca\(^{2+}\) 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 Kv2.1 channels) occurs through precise control of an isolated $\text{Ca}^{2+}$

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 α-MN K\textsuperscript{+} currents. Consider first the generation of the α-MN AHP. Membrane bound N- and P/Q-type Ca\textsuperscript{2+} currents necessary for α-MN SK channel activation generate this Ca\textsuperscript{2+} signal, which is isolated and shaped by the SSC. The AHP currents influence repetitive discharge properties of α-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 and Magown, 2013).

A primary effect of m2 receptor activation by C-bouton synapses is a reduction of the AHP (Lape and 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\textsuperscript{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 and Surmeier, 1995), and basal forebrain neurons (Allen and Brown, 1993). Ca\textsuperscript{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 is usually mediated by $G_{i/o}$ protein coupled βγ subunits, which cause a depolarizing shift in the voltage dependence of channel activation (Hille,
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 motoneurons (Figure 46Aii/iii & Bii/iii). This would be consistent with observed reduction of the AHP and enhanced \(\alpha\)-MN excitability when m2 receptors are, presumably, activated during swimming or other tasks requiring high motor output (e.g. Figure 46Biii) (Miles et al., 2007; Zagoraiou et al., 2009). This ‘upstream’ mechanism of AHP modulation will have a minimal appreciable effect on individual AHPs and \(\alpha\)-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 AHP itself (Figure 46Ai & 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 (Figure 46Aiv & 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 AHP 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, Kv2.1 channels are phosphorylated and have a high activation and deactivation threshold and slow kinetics (Murakoshi et al., 1997; Misonou et al., 2004; Surmeier and Foehring, 2004; Misonou et al., 2005; Mohapatra and Trimmer, 2006; Misonou, 2010). 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 αMNs. Importantly, upon prolonged/pathologic excitatory drive, Ca\(^{2+}\)/calcineurin dependent dephosphorylation pathways (Figure 46Aiv & Biv) rapidly decluster Kv2.1 while simultaneously lowering its activation and deactivation threshold and accelerating its kinetics (Surmeier and 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.
(Romer SH, Deardorff, AS, Fyffe REW, unpublished observations), 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 and Bean, 2014), while declustered/dephosphorylated Kv2 channels serve to homeostatically lower firing rate (Surmeier and 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 and 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\(^{2+}\) sufficient to allow diffusion of Ca\(^{2+}\) from neighboring compartments, there would be rapid Kv2.1 channel declustering (Romer et al., 2014) by a Ca\(^{2+}\)/calcineurin dependent mechanism (Romer SH, Deardorff, AS, Fyffe REW, unpublished observations), 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 P2X\(_7\) 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 and 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$^{2+}$ 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\(^{2+}\) 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 and Adelman, 2008; Faber, 2009) and reduced Ca\(^{2+}\) 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 and Sabatini, 2010). Although m2 receptors typically inhibit protein kinase activity, they can activate phosphorylation pathways in smooth muscle (Zhou et al., 2003). Therefore it is possible the direct phosphorylation of SK channels by protein kinases could provide an alternate mechanism through which m2 receptors reduce the AHP in α-MNs.

However, evidence that N- and P/Q-type Ca\(^{2+}\) channels are diffusely distributed throughout the α-MN somatic membrane (Wilson et al., 2004), and that α-MN SSCs function as Ca\(^{2+}\) diffusion barriers indicates that m2 receptor activation need only need inhibit those α-MN Ca\(_V\) channels located within or very near to the C-bouton post-synaptic membrane to exert an effect on the AHP. 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 heretofore undescribed signaling pathway. Moreover, such a mechanism would act as a binary switch, turning on and off AHP 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+} micro-signaling domain is capable of highly nuanced and graded modulation of outward K\textsuperscript{+} current.
**C-boutons in Human Health & 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 and Athanasiou, 2009; Herron and 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. 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). 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...
and Miles, 2012). The duration of the AHP in human MNs is possibly related to disease progression (i.e. an initial shortening followed by prolongation (Piotrkiewicz and 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 and 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., 2011; Prather et al., 2011), including altered post-spike AHP duration and repetitive firing properties (Kuno et al., 1974a; Gustafsson and 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 serotonergic 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 and 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 and Bennett, 2003; Heckmann et al., 2005; Brownstone, 2006; Li and 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 (V0c 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 motoneuron 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 accordingly, recent data suggests an interaction between the AHP and L-type Ca\(^{2+}\) currents responsible for PIC regulates α-MN firing properties (Manuel et al., 2014).

Here, we illustrate large, cholinergic presynaptic terminals, termed C-boutons...
(Conradi, 1969a), are important modulatory loci for state-dependent alterations in 
MN repetitive firing, 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 $\alpha$-MN AHP (and other intrinsic $\alpha$-MN properties) contribute to 
disease pathology or, alternatively, maintain $\alpha$-MN viability.
Chapter IX:

Conclusions
The results presented in this dissertation were predicated on highly detailed analysis of Kv2.1 ion channel localization dynamics in lumbar motoneurons at high resolution through a combination of approaches, technologies and sophisticated methodology, all necessary to answer the core questions. The three specific aims within this dissertation were designed to address fundamental gaps in knowledge of Kv2.1 ion channel organization in mammalian lumbar motoneurons and support the central hypothesis that **Kv2.1 membrane-bound ion channel clusters will dynamically reorganize following pathologic or prolonged excitatory drive.**

Specific aim 1 was designed to address the pathological portion of the hypothesis, by investigating Kv2.1 channel dynamics following *in vivo* peripheral nerve injury. Specific aim 2 was designed to address the direct impact of prolonged excitatory drive on Kv2.1 channel dynamics via *in vivo* nerve stimulations. Finally, Kv2.1 clustering dynamics undergo a variety of neuromodulatory influences and the impact of Kv2.1 regulation on motoneuron physiology were investigated in *in vitro* assays in specific aim 3.

Within this dissertation, evidence is provided to support the dynamic role of Kv2.1 in lumbar motoneurons. Specifically, Kv2.1 ion channels are clustered into highly regulated signaling ensembles on the soma and proximal dendrites where they have high activation and deactivation thresholds and slow kinetics (specific aim 3). Clustered Kv2.1 channels maintain steady state firing by regulating membrane potential during the interspike intervals (specific aim 3). Upon prolonged pathological (specific aim 1) or excitatory drive (specific aims 2 &3),
Kv2.1 channels are rapidly dephosphorylated by the calmodulin-dependent protein phosphatase calcineurin (specific aim 3). Dephosphorylating Kv2.1 channels has two consequences. First, Kv2.1 channels decluster and spread out in the soma and proximal dendrites (specific aims 1 & 2). Channel dispersal may have implications for its regulation by nearby calcium stores and local signaling ensembles (see dissertation discussion), and it also serves to move channels away from signaling components that are presumed to maintain channel phosphorylation. Secondly, the channels now open earlier in the action potential and stay open for a longer duration and serve to homeostatically lower firing rate (specific aim 3). Thus, Kv2 channels have the capacity to both increase or decrease neuronal excitability (see Figure 46).

To date, no cures exist for motor neuron diseases, and the development of new and novel therapies will undoubtedly rely on a complete understanding of underlying pathophysiological alterations in neuronal membrane properties. Whether or not Kv2.1 dynamics contribute to disease pathology or maintain MN viability is currently unknown. However, the results presented in this dissertation will provide a foundation for examining and interpreting Kv2.1 channel dynamics in multiple motor neuron disease states.
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