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Small Conductance Calcium-activated Potassium (SK) Channels in Mammalian Spinal Motoneurons

Zhihui Deng

Wright State University

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SMALL CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM (SK) CHANNELS IN MAMMALIAN SPINAL MOTONEURONS

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

By

ZHIHUI DENG
M.S., Tianjin Medical University, 1998
B.S., Shandong Medical University, 1993

2009
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Zhihui Deng ENTITLED Small conductance calcium-activated potassium (SK) channels in mammalian spinal motoneurons BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

Robert E.W. Fyffe, Ph.D.
Dissertation Director

Gerald M. Alter, Ph.D.
Director, BMS Ph.D. Program

Joseph F. Thomas, Jr., Ph.D.
Dean, School of Graduate Studies

Committee on Final Examination:

Robert E.W. Fyffe, Ph.D.

Kathrin Engisch, Ph.D.

Paula Bubulya, Ph.D.

Timothy C. Cope, Ph.D.

David R. Cool, Ph.D.
ABSTRACT

Deng, Zhihui, Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2009. Small conductance calcium-activated potassium (SK) channels in mammalian spinal motoneurons.

Three homologous small conductance calcium-activated potassium (SK) channel subunits (SK1, SK2 & SK3) are expressed in distinct and overlapping patterns in mammalian central nervous system. SK channels likely mediate the medium afterhyperpolarization (mAHP), which plays an essential role in regulating neuron repetitive firing frequency. In spinal motoneurons (MNs) the mAHP duration is shorter on average in fast (F-type) MNs than that in slow (S-type) MNs. To better understand the molecular basis for mAHP, we determined the expression and sub-cellular distribution of SK channels in normal, axonally-injured, and developing spinal MNs in vivo using immunohistochemistry and quantitative confocal imaging techniques.

SK2 and SK3 channels are clustered on the surface membrane of MN soma and proximal dendrites. SK clusters are post-synaptically localized at synapses associated with cholinergic C-terminals. In complementary pattern, SK2-immunoreactive (-IR) and SK3-IR clusters are expressed in different subpopulations of rat and mouse spinal α-MNs; on average, SK3-IR MNs are smaller than SK2-IR MNs. Comparison of SK3 expression in rat soleus versus gastrocnemius MNs, together with intracellular electrophysiological data suggests that SK3-IR MNs are S-type whereas SK2-IR MNs are F-type. Moreover a subpopulation of motor axon terminals innervating slow muscle fibers expresses SK3 channels.
In postnatal developing mouse MNs, differential expression of SK2 and SK3 channels becomes apparent around the same time that muscle fiber differentiation occurs (around P9). The SK channel clustering develops in concert with the establishment and maturation of pre-synaptic cholinergic C-terminals, corresponding to the maturation of motor function.

Injury to the motor axon results in a decreased AHP duration in S-type MNs but an increased AHP duration or no change in F-type MNs. Here, we characterized the effects on SK3 channel clustering in rat spinal MNs following nerve crush. SK3 clusters appear unaltered until the 3rd day after axotomy. By the 8th day post-injury, the average sizes of SK3 clusters are much smaller than in the normal control MNs. In contrast, co-localized Kv2.1 clusters start to fragment and become reduced in size within hours following injury, suggesting differential regulation and dynamics of discrete channel populations at these synapses.
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DEDICATION

To my husband Shihong who encourages and supports me, brings me love and happiness, and shares every moment of joy and sorrow with me…

To our dear baby boy Joshua who brings us lots of joy and happiness…

To my parents who give me incredible inspiration and unconditional love and support throughout all these years…

To my brother Yuwen and my parents-in-law, who have been tremendous supportive during my difficult times and helped me go through them…
CHAPTER I  Background and Significance

I SK channels

i) What are SK channels?
    Potassium (K\(^+\)) channels are the most diverse ion channel family which is composed of functional distinct subfamilies including voltage-gated K\(^+\) (Kv) channels, calcium (Ca\(^{++}\))-activated K\(^+\) (Kca) channels, and others. In response to different stimuli, K\(^+\) channels mediate outward K\(^+\) currents with different kinetics. Unlike Kv channels which are driven solely by the changes of membrane potential, Kca channels are activated by a rise of cytosolic Ca\(^{++}\) concentration.

    According to their electrophysiological, pharmacological, and molecular structural properties, Kca channels may be divided into three subfamilies: BK (large conductance Kca), IK (intermediate conductance Kca) and SK (small conductance Kca) channels. In contrast to the large BK single-channel conductance ~250 pS, cloned SK channels exhibit unitary conductance ~10 pS [Vergara et al, 1998].

ii) Cloned and native SK channels
    Three homologous SK subunits (SK1, SK2 & SK3) have been cloned. Their sequences code proteins of 561 (hSK1), 580 (rSK2) and 553 (rSK3) amino acids respectively. The trans-membrane segments of three isoforms are highly conserved, but the sequences and length of N- and C- termini are divergent. Similar as the members of
Kv channels, SK channels express tetrameric structure of four α pore forming subunits; each α subunit consist of six trans-membrane segments (S1~S6) with N- and C-termini inside the cell. The pore forming domain is located between S5 and S6 [Kohler et al., 1996]. The following cartoon demonstrates the structure of SK α subunit.

![Figure 1-1 Structure of SK channel α subunit](image)

At least three isoforms of SK2 subunit (SK2-std, SK2-short and SK2-long) were identified in mouse brain [Strassmaier et al., 2005; Murthy et al., 2008]. When transfected into COS cells, the homomeric SK2-std channels were distributed uniformly on cell surface whereas the homomeric SK2-long channels clustered into punctae [Strassmaier et al., 2005]. The elevated expression of SK2-short was suggested to be related to aging and Alzheimer’s disease [Murthy et al., 2008].

Direct phosphorylation of K⁺ channels is known as a post-translational modification mechanism underlying the modulation of channel kinetics, K⁺ current amplitude, or channel density in plasma membrane [Jonas & Kaczmarek 1996]. For example, Kv2.1 channels, which are believed to mediate delayed rectifier in mammalian central neurons, have up to 60 potential phosphorylation sites [Park et al., 2006]. Kv2.1 channels are constitutively highly phosphorylated and a graded dephosphorylation can be triggered by neuron activity or injury. The Kv2.1 dephosphorylation results in an
alteration of channel expression and biophysical properties [Misonou et al., 2004, 2005; Mohapatra & Trimmer 2006; Park et al., 2006]. (see section II for more details)

Similarly, multiple potential phosphorylation sites that may be targeted by protein kinases were shown in primary sequences of cloned SK channels [Kohler et al., 1996]. The direct phosphorylation of several serine residues within C-terminus of SK2 channel by AMP-dependent protein kinase (PKA) led to a reduction of the cell surface expression of these channels in transfected COS cells [Ren et al., 2006]. Recent studies showed the plasticity of native SK channels in hippocampal CA1 cells and amygdale pyramidal neurons that long term potentiation (LTP) induced a protein kinase A (PKA) dependent internalization of postsynaptic SK2 channels [Lin et al., 2008; Faber et al., 2008]. In contrast to the internalization/ recycling of SK channels following phosphorylation, Kv2.1 distribution in cell surface membrane is reorganized that Kv2.1 clusters disperse when dephosphorylated [Misonou et al., 2004, 2005; Mohapatra & Trimmer 2006].

So far, cloned and native SK channels are the only known target of bee venom apamin, which makes it an important tool for the study of these channels. Three cloned SK channels express different sensitivity to apamin: SK2 and SK3 channels show higher affinity to apamin than SK1 channels [Kohler et al., 1996; Hirschberg et al., 1998].

iii) Ca\(^{++}\) sensitivity of SK channels

Calcium ions function as ubiquitous intracellular second messengers. Transmembrane transporters, intracellular Ca\(^{++}\) store system, and many Ca\(^{++}\) binding proteins in the cytoplasm maintain intracellular free Ca\(^{++}\) ion concentration at very low level (10-100nM) in eukaryotic cells, which makes Ca\(^{++}\) suitable as an intracellular messenger for rapid cellular responses. SK channels are highly Ca\(^{++}\) sensitive in a
voltage-independent manner. Their gating with the application of Ca\(^{++}\) is rapid, occurring within a few milliseconds. All three cloned SK channels exhibit similar dose-response relationship: Ca\(^{++}\) concentration for half-maximal activation (K\(_{0.5}\)) is about 0.3-0.7 \(\mu\)M [Kohler et al., 1996; Hirschberg et al., 1998; Xia et al., 1998].

The Ca\(^{++}\) sources activating SK channels in neurons include distinct subtypes (including L-type, N-type, P/Q-type) of voltage-dependent Ca\(^{++}\) channels (VDCC), intracellular Ca\(^{++}\) stores and Ca\(^{++}\) permeable ionotropic neurotransmitter receptors [Li & Bennett 2007; Marrion & Tavalin 1998; Stocker 2004; Bowden et al., 2001]. SK channels are activated by increased intracellular Ca\(^{++}\) during an action potential and their activity contributes to medium duration afterhyperpolarization (mAHP), thus they play an essential role in neuron firing properties [Zhang & Krnjevic 1987; Sah & Faber 2002; Faber & Sah 2003].

iv) Calmodulin constitutively bound with SK

SK channel \(\alpha\) subunits are constitutively associated with calmodulin (CaM). SK channel protein does not directly bind to Ca\(^{++}\) when cytosolic Ca\(^{++}\) concentration increases; instead, calmodulin works as Ca\(^{++}\) sensor by binding to Ca\(^{++}\) [Xia et al., 1998; Maylie et al., 2004; Stocker 2004]. SK channel activation by Ca\(^{++}\) binding to CaM results in channel opening whereas channel deactivation is the reverse process by dissociation of Ca\(^{++}\) from CaM [Bildl et al., 2004; Pedarzani et al., 2001; Xia et al., 1998].

CaM binds to SK \(\alpha\) subunit through CaM binding domain (CaMBD) and this constitutive interaction between SK and CaM is very stable. Crystallographic studies showed a dimeric complex: two CaMBDs are arranged in an antiparallel configuration, and two CaMs are woven around CaMBDs symmetrically at each end. Thus, each CaM
molecule contacts both subunits of the CaMBD dimer. Therefore the tetrameric SK channels function as dimers-of-dimers. Upon Ca\(^{++}\) binding to CaM, a large conformational rearrangement of CaM occurs, which induces the conformational changes of associated transmembrane domains of the channel protein. As a result, the ionic conductance pore selective for K\(^{+}\) is triggered to open leading to hyperpolarization of membrane potential [Maylie et al., 2004; Schumacher et al., 2001].

The calcium independent interaction between SK \(\alpha\) subunit and CaM is also suggested to be necessary for the cell surface expression of the channels. When this constitutive interaction was interrupted by site mutations (SK2:64/67), SK2 channels fail to target cell surface membrane in transfected COS cells [Lee et al., 2003; Maylie et al., 2004].

**v) Modulation of SK channel Ca\(^{++}\) sensitivity**

In addition to CaM, the cytoplasmic domains of SK channel protein also interact with protein kinase CK2 and protein phosphatase 2A (PP2A) forming a multi-protein complex [Bildl et al., 2004; Allen 2007]. CaM is a substrate for CK2 [Marin 1999], and CK2 was demonstrated to be able to efficiently phosphorylate CaM associated with SK CaMBD, but SK CaMBD was not a substrate for CK2. The phosphorylation of CaM by CK2 may reduce the Ca\(^{++}\) sensitivity of SK channels [Bildl et al., 2004; Allen 2007]. The shift of Ca\(^{++}\) sensitivity was suggested to result mainly from the accelerated deactivation kinetics of SK channels, which reflects the stability of interaction between Ca\(^{++}\) and CaM [Bildl et al., 2004; Schumacher et al., 2001; Wissmann et al., 2002]. The following cartoon demonstrates the microdomain multi-protein organization of SK channel in cytoplasmic membrane (not in proportion).
Recent evidence showed this mechanism in native neurons. Neurotransmitters including noradrenaline were found to increase neuronal excitability by reducing SK2 channel calcium sensitivity gating in dorsal root ganglion cells, and this effect is through CK2-dependent phosphorylation of SK2-bound CAM [Maingret et al., 2008].

In addition, some drugs (eg. 1-EBIO, CyPPA & NS4591) may increase Ca$^{++}$ sensitivity of SK channels, which brings a clue for the mechanism underlying the positive regulation of native SK channels [Hougaard et al., 2007].

**vi) Heteromeric co-assembly of different SK subunits in expression system**

The interaction between co-expressed different SK subunits was studied and shown to cause alteration in protein trafficking, cell surface membrane targeting, and channel pharmacological properties. The expression of rSK1 gene alone in mammalian cell lines does not form functional channels. When rSK1 and rSK2 were co-expressed, the SK channel current magnitude was larger, and apamin sensitivity was reduced compared with the cells expressing rSK2 only [Benton et al., 2003]. The rSK3 can form functional heteromeric channels with rSK2 but not with hSK1 subunit, since hSK1 may interrupt functional SK3 assemblies on cell surface [Monaghan et al., 2004].
These *in vitro* studies suggested the possibility of heteromeric assembly of SK channels *in vivo*. However, *in vivo* studies showed controversial results that some suggested SK channels exclusively form homomeric channels [Sailer et al., 2002] whereas others suggested the co-assembly of different SK subunits in mouse brain [Strassmaier et al., 2005].

**vii) SK channels in mammalian neurons**

As mentioned before, SK channels mediate medium AHP following action potential and thus determine neuron firing properties (detailed discussion in next section). In addition, SK channels were also shown involved in dendritic excitability, synaptic integration and neurotransmission in mammalian neurons.

Persistent inward currents (PICs) amplify synaptic inputs and govern the dendritic excitability in motoneurons. PICs are voltage-activated and facilitated by neuromodulatory inputs of monoamines including serotonin and other neurotransmitters such as glutamate and acetylcholine [Svirskis & Hounsgaard 1998]. Plateau potential, a latent firing property of motoneurons, is PICs dependent. A persistent calcium current (Ca\(^{++}\) PIC) and a persistent sodium current (Na\(^{+}\) PIC) in dendritic tree plays a major role in generating PICs. Low voltage-activated L-type Ca\(^{++}\) channels contribute to Ca\(^{++}\) PIC, but the channel mediating Na\(^{+}\) PIC has not been identified [Heckman et al., 2003; Li & Bennett 2003; Schwindt & Crill 1980]. Recent evidences showed that in some central neurons including spinal motoneurons, dendritic SK channels may be activated by dendritic L-type Ca\(^{++}\) channels. The activation of these SK channels terminates Ca\(^{++}\) PIC and thus participates in dendritic excitability. Therefore, these dendritic SK channels are spatially and functionally different from the SK channels mediating mAHP which are
believed to locate around soma and be activated by N, P-type Ca\textsuperscript{++} channels [Li & Bennett 2007; Bond et al., 2005; Cai et al., 2004].

Long-term potentiation (LTP) in different brain areas such as hippocampus and lateral amygdala is believed to underlie learning and memory. LTP requires the activation of ionotropic NMDA receptors. Recent studies showed that SK channels were co-localized and tightly coupled to NMDA receptors in hippocampus CA1 neurons and lateral amygdala pyramidal neurons. The activation of these SK channels by Ca\textsuperscript{++} entry via NMDA receptors reduced the amplitude of Ca\textsuperscript{++} transient and thus forms a negative feedback loop to depress synaptic potential [Ngo-Anh et al., 2005; Faber et al., 2005]. The plasticity of these SK channels by internalization from the postsynaptic membrane was mediated by protein kinase A (PKA) and suggested to contribute to LTP [Lin et al., 2008; Faber et al., 2008].

In addition, SK3 channels were found to be expressed in cholinergic motor axon terminals at rat neuromuscular junctions (NMJs) [Roncarati et al., 2001] and glutamatergic presynaptic terminals of cultured mouse hippocampal neurons [Obermair et al., 2002]. These results suggest that SK3 may involve in the regulation of excitatory synaptic neurotransmission.

SK channel knock-out transgenic mouse lines were constructed, and the animals lacking any one of the SK genes (SK1, SK2, or SK3) were viable. Whole cell patch clamp recording showed that only SK2 channels are necessary for the mAHP in hippocampal CA1 neurons [Bond et al., 2004]. Unfortunately no available information shows the properties of motoneurons in SK knock-out mice.
Three SK channels (SK1, SK2 & SK3) have been shown to be expressed in distinct and overlapping patterns in mammalian central nervous system [Stocker et al., 2000, Sailer et al., 2004]. However, the expression of SK channels in many neurons including motoneurons is still unknown, though electrophysiological evidences suggested the existence and the functions of these channels in motoneurons [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. This dissertation will address this issue aiming to illustrate the molecular basis for the recorded AHP and dendritic SK currents and to shed light on the mechanisms underlying motoneurons excitability and firing properties (see aim I).
II Spinal motoneurons and motor units

i) An introduction to spinal motoneurons

Spinal motoneurons, described as “final common pathway” by C. S. Sherrington, transmit signals from central nervous system to their target skeletal muscles. There are two major types of motoneuron in mammalian spinal cord: larger $\alpha$-MN and smaller $\gamma$-MN. $\alpha$-MNs innervate extrafusal muscle fibers throughout the skeletal muscles whereas $\gamma$-MNs innervate intrafusal muscle fibers within muscle spindles. A third type of motoneuron, $\beta$-MN, innervates both extrafusal and intrafusal muscle fibers. All motoneurons discussed in this study are $\alpha$-MNs, which contribute to voluntary skeletal muscle contraction and muscle tone.

Motoneuron pool is defined as the population of motoneurons innervating a particular muscle. The topographic distribution of sciatic motoneuron pools have been identified in cat and rat lumbar spinal cord. The sciatic motoneurons form a continuous cell column in the dorsolateral quadrant of ventral horn, within which the motoneurons of each sciatic branches occupy spatially distinct and overlapping sub-compartments [Burke 1981; Swett et al 1986].

ii) Control of motoneuron excitability

Motoneuron excitability and firing properties are essential in motor control. Motoneuron input-output function describes the relationship between synaptic inputs (or injected currents) onto motoneurons and resulting firing of motoneurons. The transformation of synaptic inputs to the initiation of action potential depends on: i) motoneuron type (S-type or F-type) and its membrane properties; ii) location of synaptic
contacts; iii) effects of neuromodulators on synaptic transmission and repetitive firing properties [Rekling et al., 2000].

On the one hand, different types of synaptic inputs (excitatory versus inhibitory) onto motoneurons regulate their excitability and firing behavior; on the other hand, the intrinsic properties of motoneuron including ionic channels shape their response to synaptic inputs.

A) Synaptic input onto motoneurons

In the past 30 years Robert Fyffe and colleagues have been using both light and electron microscopy to study synapses in spinal motoneurons and inhibitory interneuron Renshaw cells. They demonstrated the quantity and distribution of Ia afferent boutons [Brown & Fyffe 1981; Fyffe & Light 1984] and inhibitory glycine/GABAergic terminals from Renshaw cells and other inhibitory interneurons [Fyffe 1991; Alvarez et al., 1996, 1997] contacting motoneurons. They also quantitatively analyzed the descending serotonergic synapses upon motoneurones [Alvarez et al., 1998].

Each mammalian α-MN receives tens of thousand synaptic contacts of various origins, and the majority of synaptic inputs are located on dendrites which constitutes ~97% of total membrane surface area of the motoneuron [Brannstrom 1993; Cullheim et al., 1987].

Synaptic inputs on motoneurons can be divided into two categories which influence their excitability and firing properties in different ways. 1) The synapses acting via ionotropic receptors, such as glutamate and glycine/GABA, cause depolarizing (excitatory) or hyperpolarizing (inhibitory) postsynaptic potential. 2) The synapses
working through metabotropic receptors, such as amines and peptides, change motoneuron intrinsic input/output properties [Kernell 1999; Rekling et al 2000].

According to ultrastructural studies, there are distinct types of presynaptic boutons (S, F, C and others) contacting motoneurons [Conradi et al., 1979; Kellerth et al., 1979; Lagerback et al., 1986; Johnson, 1986]. S-type boutons, which contain spherical synaptic vesicles, distribute more frequently on distal dendrites than on proximal dendrites. Descending serotonergic boutons and most glutamatergic boutons including large Ia primary afferent terminals are S-type [Conradi et al., 1983; Fyffe & Light 1984; Ornung et al., 1998]. F-type boutons, which contain pleomorphic or flattened synaptic vesicles, are the most numerous type of synapse on motoneuron somatic membrane and are assumed to be inhibitory terminals such as glycinergic and GABAergic terminals arising from inhibitory interneurons [Fyffe 1991; Ornung et al., 1996]. C-terminals, large cholinergic boutons exhibiting postsynaptic sub-surface cisternae, arise from a group of interneurons located close to the central canal of spinal cord [Maxwell et al., 2003]. Small S-type and F-type boutons contact both α-MNs and γ-MNs whereas large C-type and Ia primary afferent boutons exclusively target α-MNs [Lagerback, 1985; Lagerback et al., 1986; Johnson, 1986; Ichiyama et al., 2006].

B) Ionic channels in motoneuron

Like all other central neurons, motoneuron has its unique combination of ionic channels which matches its specific physiological role. Motoneurons express diverse ionic channels in their surface membrane including Na⁺, K⁺ and Ca²⁺ channels.

(1) Na⁺ channels
Voltage-gated Na\(^+\) channels play an essential role in neuron excitability. Several Na\(^+\) currents were found in motoneurons but the Na\(^+\) channels underlying these currents are not well defined yet. Fast inactivating TTX sensitive Na\(^+\) current is responsible for action potentials initiation and propagation in all motoneurons [Rekling et al., 2000]. A TTX-sensitive persistent Na\(^+\) current contributes to persistent inward current (PIC), which produces a prolonged depolarization, amplifies excitatory synaptic inputs, and is turned off by inhibitory inputs to motoneurons [Heckman et al., 2003; Li & Bennett 2003]. Recent studies suggested that the Na\(^+\) PIC plays an essential role in action potential initiation, determining firing frequency and sustaining repetitive firing in motoneurons [Lee & Heckman 2001; Kuo et al., 2006; Harvey et al., 2006; Li & Bennett 2007]. Several subtypes of voltage-gated Na\(^+\) channels including Na\(_{\text{v}}\)1.5, Na\(_{\text{v}}\)1.8 and Na\(_{\text{v}}\)1.9 are activated at more hyperpolarized potential (lower than -60mV) and show slower inactivation kinetics than fast inactivating Na\(^+\) channels [Diss et al., 2004]. However, the Na\(^+\) channel subtypes mediating PIC remain unclear [Kiss 2008]. In addition, there exists a TTX insensitive Na\(^+\) current in motoneurons [Rioult-Pedotti 1997]. Since motoneurons are not ideal for voltage-clamp techniques, the biophysical properties of Na\(^+\) channels in motoneurons are difficult to study. One type of TTX-sensitive Na\(^+\) channel, with a conductance of 14.0 pS, was found in the soma of neonatal rat spinal motoneurones [Safronov & Vogel 1995].

(2) K\(^+\) channels

There are many K\(^+\) currents contributing to the integrated function of motoneurons. K\(^+\) currents are responsible for shaping action potential and determining the sub-threshold membrane behavior and firing properties of motoneurons. Moreover,
neuromodulators target these K⁺ currents and regulate motoneuron excitability. Several K⁺ currents in motoneurons such as delayed rectifier and Ca²⁺ dependent K⁺ currents have been studied extensively.

Delayed rectifier (Ik), which is activated by depolarization, contributes to the falling phase of action potential and fast AHP. Kv2.1 channels (Shab) are abundant in spinal motoneurons and might be the major contributor to the delayed rectifier in motoneurons. Kv2.1 was shown to form small irregularly shaped and large disc-like clusters in the surface membrane of soma and proximal dendrites in α-MNs. Prominent disc-like Kv2.1 clusters were invariably apposed to presynaptic cholinergic C-terminals and co-localized with postsynaptic muscarinic (m2) receptors [Muennich & Fyffe 2004].

Recent studies illustrated the dynamic regulation of native and cloned Kv2.1 channels in hippocampal neurons and HEK cells induced by neuron activity, glutamate or muscarine stimulation, ischemia and CO2 application. Kv2.1 channels are constitutively maintained highly phosphorylated. All the above events cause a graded dephosphorylation of Kv2.1 and lead to a dispersing of Kv2.1 clusters in cell surface membrane and a hyperpolarizing shift of channel activation. This effect was calcineurin (PP2B) dependent and resulted from a calcium influx or release from intracellular calcium store [Misonou et al., 2004, 2005; Mohapatra & Trimmer 2006; Park et al., 2006]. Similar Kv2.1 de-clustering effect was observed in spinal motoneuorns induced by sciatic stimulation, glutamate or muscarine application [unpublished results from Fyffe lab]. If the motoneuron injury may induce similar modulation of Kv2.1 clusters will be examined in aim V.
Ca++-activated K+ channels are gated by increased intracellular Ca++, which play an essential role in determining firing properties of motoneurons and serve to terminate long bursts of action potentials. Two types Ca++-activated K+ channels, large conductance BK channels and small conductance SK channels are expressed in motoneurons. SK channels were shown to mediate medium AHP in motoneurons [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. As discussed before, electrophysiological evidences suggested that there exist dendritic SK channels in motoneurons which were spatially and functionally different from the SK channels mediating mAHP [Li & Bennett 2007; Bond et al., 2005; Cai et al., 2004]. The expression and sub-cellular distribution of SK channels will be studied in this dissertation. Moreover, how motoneuron injury may cause the regulation of SK expression in cell surface membrane will also be examined in aim V.

(3) Ca++ channels

Voltage-dependent Ca++ conductances in motoneurons contribute to afterdepolarization (ADP) and afterhyperpolarization (AHP) and thus play a role in determining the duration of action potential. In addition, voltage-dependent persistent inward calcium currents (Ca++ PIC), which are facilitated by monoamines, play a major role in enhancing excitatory synaptic inputs and sustaining repetitive firing [Heckman et al., 2003]. Ca++ PIC may also be promoted by other neurotransmitters such as glutamate and acetylcholine [Svirskis & Hounsgaard 1998].

Voltage-dependent Ca++ channels (VDCCs) may be classified into low voltage-activated (LVA) and high voltage-activated (HVA) channels; HVA channels may be
subdivided into L-type, P/Q-type, N-type and others by pharmacological study [Lacinova 2005].

Ca\textsubscript{v}1.3 (or \(\alpha 1D\)), a subtype of L-type Ca\textsuperscript{++} channels, is activated by relatively hyperpolarized membrane potential compared with other HVA VDCCs and has been shown to contribute to Ca\textsuperscript{++} PIC in spinal motoneurons [Carlin et al., 2000; Alaburda et al., 2002; Simon et al., 2003; Elbasiouny et al., 2006]. Electrophysiological studies demonstrated a dendritic origin of Ca\textsuperscript{++} PIC [Bennett et al., 1998; Carlin et al., 2000; Hultborn et al., 2003]. Computational models of cat spinal motoneuron suggested that Ca\textsubscript{v}1.3 channels mediating Ca\textsuperscript{++} PIC are localized in the dendritic tree around hundreds of micrometers from soma [Elbasiouny et al., 2005; Bui et al., 2006]; in addition, these channels form discrete hot spots instead of uniform density distribution [Bui et al., 2006;]. Immunohistochemical studies also showed the dendritic location of Ca\textsubscript{v}1.3 channels in spinal motoneurons of cats, rats, mice and turtles but their results are of variation. Ca\textsubscript{v}1.3 channels were shown to distribute mainly in the soma and proximal dendrites of rat motoneurons whereas in the dendritic tree of mouse motoneurons [Westenbroek et al., 1998; Carlin et al., 2000]. In turtle motoneurons, Ca\textsubscript{v}1.3 channels form clusters in the cell membrane of soma, proximal and distal dendrites. Ca\textsubscript{v}1.3 clusters were always located at synaptic sites [Simon et al., 2003]. At the ultrastructural level, Ca\textsubscript{v}1.3 were found in the soma and dendrites of cat motoneuron, associated with both intracellular organelles and plasma membrane [Zhang et al., 2008]. Dendritic SK channels activated by Ca\textsuperscript{++} PIC were suggested to be close (within 100nm) to Ca\textsubscript{v}1.3 channels in motoneuron dendritic tree [Li & Bennett 2007].
N-type and P/Q-type Ca\textsuperscript{++} channels were suggested to activate SK currents mediating mAHP during motoneuron firing [Bayliss et al., 1995; Li & Bennett 2007]. Since action potentials are initiated in axon initial segment and hillock [Oomura & Maeno 1963], and AHP is initiated within millisecond after initial spike, N-, P/Q-type channels and SK channels account for mAHP are assumed to localize close to motoneuron cell body. The cellular distribution of SK channels in motoneuron will be investigated in this dissertation.

**iii) Motor unit types**

Motor unit, a single motoneuron and all its innervated muscle fibers, is the functional unit of motor activity. Motor units may be classified into fast (F-type) and slow (S-type) types according to their twitch properties including twitch half-relaxation time (RT1/2) and twitch time-to-peak tension (TPT). Slow motor units have longer RT1/2 than fast units; however, there is some overlapping between F and S units in TPT. Slow motor units are fatigue resistant and their contraction tends to produce smaller tension than that of fast units. Fast motor units may further be subdivided into fast twitch fast fatiguing (FF), fast twitch with intermediate fatigue resistance (FI), and fast twitch fatigue resistance (FR). Histochemical properties of muscle fibers, such as myofibrillar ATPase, mitochondrial oxidative enzymes, and myosin heavy chain isoforms, can also be used to distinguish muscle unit types [Burke 1981; Gardiner 1993; Staron et al 1999].

Most mammalian muscles are heterogeneous because they contain diverse histochemical types of muscle fibers, so the motoneuron pools innervating these muscles are also heterogeneous. As an exceptional example, cat soleus is histochemically homogeneous slow muscle. Rat gastrocnemius (fast) and soleus (slow) muscles will be
used in this study, both of which are shown heterogeneous. Staron and colleagues studied muscle fiber composition of hindlimb muscles of adult rats by identifying myosin heavy chain (MHC) isoforms of muscle fibers. Over 80% soleus muscle fibers (81.9±7.4%) were type I (slow type). They separated gastrocnemius muscle into pure deep-red (GDR) and superficial-white (GSW) portions. Over 99% GSW fibers were type II (fast type) whereas over 65% GDR fibers were fast type. In gastrocnemius muscle, slow fibers were the smallest; in contrast, slow fibers were the largest within soleus muscle [Staron et al 1999].

Though there is no anatomical characteristic that may clearly distinguish motoneurons innervating different types of motor units, on average, fast motoneurons are larger than slow motoneurons. During movements distinct types of motor units in different muscles are recruited in an orderly fashion. Within heterogeneous muscles, motor units are recruited sequentially related to motor unit types: S → FR → FF, which means from low to high force output, high to low resistance to fatigue, and from small to large motoneurons referred to as “size principle” [Burke 1981]. “Size principle” was proposed by Elwood Henneman about 50 years ago that the orderly recruitment of motoneurons within a pool is based on neuron sizes, with the smaller neurons activated first and de-activated last. There is still disagreement on the mechanisms underlying this orderly recruitment including active presynaptic inputs onto motoneurons and motoneuron intrinsic properties [Mendell 2005].

Motoneurons of different type motor units may also be distinguished by their electrophysiological properties. Generally, slow motoneurons have slower axonal conduction velocity, larger and longer AHP, lower rheobase, higher input resistance and greater overshoot of action potential than fast motoneurons. However, these differences
are quantitative rather than qualitative, and there is always overlapping of all these properties between motoneuron types [Burke 1981; Zengel et al., 1985; Gardiner 1993].

The differential AHP expressed by distinct types of motoneurons is of interest in this dissertation. Better understanding how distinct types of motoneurons express SK channels will provide insight into the molecular basis for differential AHP in motoneurons (aim II).
III Afterhyperpolarization (AHP) in motoneuron

An action potential is comprised of several distinct components including a fast initial spike, afterdepolarization (ADP), and afterhyperpolarization (AHP). The currents that contribute to AHP display three distinct kinetic components: fast (IfAHP), medium (ImAHP) and slow (IsAHP).

Figure 1-3 Action potential elicited from neonatal rat hypoglossal MN [Lape & Nistri 2000]

Fast AHP results primarily from the activation of voltage-gated K\(^+\) channels and large conductance Ca\(^{++}\)-activated K\(^+\) (BK) channels. It contributes to the falling phase of action potential in spinal motoneurons but not in hypoglossal motoneurons [McLarnon 1995; Viana et al., 1993]. Medium AHP is mediated by small conductance Ca\(^{2+}\)-activated potassium (SK) channels. Slow AHP is commonly seen following a train of spikes. It is activated by a rise of Ca\(^{++}\) but none of the known SK channels or BK channels underlies IsAHP. So far the ionic channels underlying IsAHP is not identified yet [Sah and Faber, 2002; Bond et al., 2004].
Medium AHP in motoneurons

Medium AHP in motoneurons is independent of membrane potential, and may be blocked by bee venom apamin indicating it is mediated by SK channels [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. Medium AHP does not contribute to the repolarization of action potential, but it plays an important role in the repetitive firing of neurons. Medium AHP is activated rapidly, and its decay reflects the reduction of intracellular Ca\(^{++}\) levels back to normal, so its time course is Ca\(^{++}\) dependent. The greater the calcium entry, the larger the peak amplitude is, and the slower the current decays [Sah 1992 & 1996; Faber & Sah, 2003]. Close co-localized voltage-gated Ca\(^{++}\) channels, Ca\(^{++}\) permeable neurotransmitter receptors, and Ca\(^{++}\) release from intracellular stores are proposed to be responsible for the activation of SK channels in neurons [Marrion & Tavalin 1998; Bowden et al., 2001; Stocker 2004; Bond et al., 2005; Li & Bennett 2007].

The AHP conductance recruited by single action potential in cat lumbar motoneurons was wide spread (0.3-1.4 μS; 0.7±0.3 μS) [Manuel et al., 2005]. During the repetitive firing of motoneurons, the mAHP conductance increases progressively following successive spikes until saturation. An earlier study of cat spinal motoneurons showed that the maximal AHP conductance (1.78 - 3.46 μS) due to the summation was 1.8-2.7 times larger than the conductance recruited by a single action potential [Baldissera et al., 1978]. The cytoplasmic calcium is accumulated during repetitive firing which leads to AHP conductance summation. The deactivation kinetics of SK channels also contributes to the AHP summation because the complete AHP decay is not allowed between spikes during high frequency firing resulting in accumulated SK activation until saturation [Teagarden et al., 2008].
The maximal AHP conductance and the kinetics of SK channels determines the gain (the slope of F-I curve) of motoneurons. Unlike many other central neurons, Motoneurons express a limited range of firing rate that their steady state firing rate is confined at 10-50 Hz [Meunier 2005; Manuel et al., 2005]. Motoneurons innervating slow twitch motor units have lower firing frequency than those innervating fast twitch motor units. The matching between motoneurons firing rate and the contractile properties of target muscle fibers is mostly attributable to the medium AHP [Kernell 1999].

Spike frequency adaptation (SFA) is a prominent property of many neurons, including motoneurons. After the onset of firing, there are several phases (initial, early, late) of SFA during which firing rate drops. The mechanism underlying SFA is not very clear, but medium AHP was assumed to play a role in SFA [Baldissera et al., 1974; Kernell 1999]. Some studies suggested that mAHP is responsible for the initial SFA in rat hypoglossal motoneurons [Powers et al., 1999] but is not necessary for the SFA in mouse lumbar spinal motoneurons [Miles et al., 2005].

The duration of the mAHP varies with spinal motoneuron types, and it is closely correlated with the contraction time of its innervated muscle fibers. On average, slow (S-type) motoneurons express larger and longer mAHP than fast (F-type) motoneurons [Gardinar 1993; Zengel et al., 1985]. The medium AHP conductance in slow motoneurons was shown similar as that in fast motoneurons. Since slow motoneurons have lower input conductance, it is explained that why they express higher AHP amplitude than fast motoneurons [Manuel et al., 2005]. However, why slow motoneurons have longer AHP decay time than fast motoneurons remains unknown. In this
dissertation, how different types of motoneurons express SK channels will be studied
aiming to shed light on mechanism underlying the differential AHP in motoneurons.

ii) Modulation of motoneuron mAHP

As mentioned before, motoneurons receive thousands of synaptic inputs, which
may be divided into two categories: the synapses causing depolarizing (excitatory) or
hyperpolarizing (inhibitory) postsynaptic potential via ionotropic receptors, and the
synapses modulating motoneuron intrinsic input/output properties through metabotropic
receptors. One of these neuromodulating effects is the regulation of the amplitude and
duration of AHP which leads to the alteration of motoneuron firing properties. Serotonin
and acetylcholine have been shown to act as neuromodulators regulating motoneuron
AHP [Kernell 1999; Rekling et al 2000].

A) Serotonergic synapses

Serotonin (5-HT) is a potent neuromodulator in mammalian central nervous
system. Spinal motoneurons receive direct descending serotonergic innervation from
Raphe premotor neurons in brain stem. Fyffe and colleagues showed that each cat lumbar
spinal motoneuron receives about 1,500 (1,570 ± 487) 5-HT-immunoreactive bouton
contacts with the vast majority apposed to dendrites rather than soma [Alvarez et al.,
1998].

Serotonin may increase motoneuron excitability by inhibiting mAHP [Van
Dongen et al 1986; Bayliss et al., 1995; Grunnet et al., 2004], hyperpolarizing the
threshold for action potential [Fedirchuk & Dai 2004], facilitating Ca^{++} PIC [Perrier &
Hounsgaard 2003] and Na^{+} PIC [Harvey et al., 2006]. Pharmacological studies showed
that 5-HT_{2} receptors mediated the facilitation of PIC and plateau potentials in
motoneurons [Perrier & Hounsgaard 2003; Harvey et al., 2006; Perrier & Cotel 2008]. The mechanism underlying the mAHP inhibition of serotonin in motoneurons is uncertain though 5-HT1A receptors were suggested to mediate this modulatory effect [Bayliss et al., 1995; Grunnet et al., 2004].

As a G protein coupled receptor, the activation of 5-HT1A receptor was shown to inhibit Ca\(^{++}\) influx via N- and P-type channels in rat hypoglossal motoneurons. This effect of serotonin might be responsible for the reduction of mAHP mediated by SK channels in hypoglossal motoneurons [Bayliss et al., 1995]. Similarly, the activation of 5-HT1A receptors was suggested to reduce mAHP in turtle and lamprey spinal motoneurons [Perrier & Cotel 2008; Grunnet et al., 2004; Hill et al., 2003]. In rat spinal motoneurons N- and P-type Ca\(^{++}\) channels were also suggested to mediate calcium currents activating SK channels underlying mAHP [Li & Bennett 2007]; however, serotonin was shown to have no appreciable effect on high voltage activated Ca\(^{++}\) currents including N- and P-type channels [Berger & Takahashi 1990]. Future study is needed on the mechanism underlying serotonin-induced AHP modulation in mammalian spinal motoneurons.

**B) Cholinergic C-terminals**

Spinal motoneurons receive synaptic input from large cholinergic C-terminals over their soma and proximal dendritic membrane. C-terminals arise from a subpopulation of cholinergic interneurons located close to the central canal [Maxwell et al, 2003]. These cholinergic synapses are characterized of sub-surface cisternae, which are continuous with rough endoplasmic reticulum. Postsynaptic muscarinic m2 receptors were shown to appose to large cholinergic C-terminals in motoneurons. [Hellstrom et al,
In addition to C-terminals, motoneurons also receive cholinergic synaptic inputs from nearby homonymous motoneurons [Cullheim et al., 1977].

Voltage gated Kv2.1 channels were observed to localize postsynaptic to C-terminals and co-localize with M2 receptors in spinal motoneuorns [Muennich & Fyffe 2004]. Cholinergic stimulation in cultured hippocampal neurons induced a dispersing of Kv2.1 clusters in cell surface membrane and a hyperpolarizing shift of channel activation via the dephosphorylation of Kv2.1 channels. This effect was calcineurin dependent and resulted from a calcium release from sub-surface cisternae via muscarinic receptors [Mohapatra & Trimmer 2006]. Similar de-clustering effects were observed in vitro neonatal spinal motoneuorns by muscarine application [Katie’s SfN poster 2007; unpublished results from Fyffe lab].

The activation of M2 receptor may lead to a reduction of AHP and thus increase the input-output gain (slope of f-I curve) of motoneurons [Brownstone 2006; Lape et al., 2000]. The mechanism underlying the decreased AHP by M2 receptors activation in motoneurons is not clear. If the calcium sequestration/release is involved in this modulation needs to be clarified in future study.

As discussed before, direct phosphorylation of K+ channels is a post-translational modification mechanism underlying the modulation of channel kinetics, K+ current amplitude, or channel density in plasma membrane [Jonas & Kaczmarek 1996]. SK channels have multiple potential phosphorylation sites and the direct phosphorylation of cloned and native SK2 channel by PKA was shown to cause an internalization of these channels from cell surface membrane [Kohler et al., 1996; Ren et al., 2006; Lin et al., 2008; Faber et al., 2008]. At the same time, constitutively bound calmodulin is a substrate
for CK2 and its phosphorylation may reduce SK channel Ca\(^{++}\) sensitivity [Bildl et al., 2004; Allen 2007]. Neurotransmitters such as noradrenaline was found to inhibit SK2 channels by a CK2 dependent calmodulin phosphorylation resulting in the reduction of calcium sensitivity of channel gating in dorsal root ganglion cells [Maingret et al., 2008]. Whether these mechanisms are involved in the SK channel modulation by acetylcholine and/or serotonin needs to be clarified in future study.

In order to explore the mechanism underlying the effects of these neuromodulators on spinal motoneurons, the synaptic localization of SK channels in motoneuron plasma membrane will be examined to detect if they are spatially associated with any specific types of presynaptic terminals. The expected results may shed light on the signaling pathway of neuromodulation in motoneurons (see aim III).
**IV Postnatal development of motoneurons**

During the early postnatal period, the motor function matures with both growth and reorganization in mammalian central nervous system. The mature motor skill depends on the complicated networks of neurons and the complex intrinsic properties of individual motoneurons. Functioning as the final common pathway, proper postnatal maturation of both intrinsic properties and synaptic connectivity of spinal motoneurons is essential for the development of motor output control.

At birth motoneurons have higher excitability, express abundant spontaneous activity, and receive both appropriate and inappropriate synaptic contacts. With increasing age during postnatal development, motoneuron excitability decreases with increased cell sizes, membrane properties change, and the synaptological organization onto motoneuron matures [Carrascal et al., 2005; Wilson et al., 2004; Vinay et al., 2000; Ulfhake & Cullheim 1988a; Conradi & Ronnevi 1977].

**i) Postnatal anatomical development of motoneurons**

Ulfhake and colleagues studied the anatomical development of cat hind limb motoneurons after birth. The postnatal increase of cell size varied among motoneurons, resulting in a wider cell size range in adult cats than that in kittens. On average, motoneuron soma area doubled and total dendritic membrane area increased 400% postnatally [Ulfhake & Cullheim 1988a].

More than half of the terminal dendritic branches of newborn kitten motoneurons possessed growth cones, filopodia, and/or fusiform processes [Ulfhake & Cullheim 1988b]. During postnatal development, the dendritic branching structure of motoneuron...
remodeled and the spatial distribution of dendritic branches changed; however, there was no net change in the number of dendrites or in the degree of dendritic branching [Ulfhake et al 1989].

**ii) Postnatal maturation of synaptic inputs on motoneurons**

Vinay and colleagues showed that neonatal rat motoneurons received both appropriate and inappropriate connections from the periphery, and supraspinal control was weak and acted mainly through polysynaptic connections. During the 1st postnatal week, inappropriate sensomotor contacts disappeared, and supraspinal control increases gradually because of myelin formation of descending axons. They suggested that the spontaneous activity of motoneurons and primary afferent terminals may play a role in the refinement of sensomotor connections and neuron maturation [Vinay et al., 2000].

As discussed before, spinal motoneurons receive synaptic input of large cholinergic C-terminals, which arise from a subpopulation of cholinergic interneurons located close to the central canal. Wilson and colleagues demonstrated that these presynaptic C-terminals develop on the membrane of soma and proximal dendrites of mouse spinal motoneurons after birth, which contributes to postnatal motor function maturation. Postsynaptic potassium channel subunit Kv2.1 occurred during early postnatal period in concert and colocalized with the maturation of presynaptic C-terminals [Wilson et al, 2004].

Conradi and Ronnevi studied the synaptic inputs onto initial axon segment (IS) of cat spinal $\alpha$-MNs. During the first postnatal week, the IS received the boutons of different ultrastructural types, and these boutons disappeared during the second postnatal week [Conradi & Ronnevi 1977].
iii) **Postnatal differentiation of motor units**

The maturation of motoneuron properties is associated with the differentiation of muscle fibers. The duration of motoneuron AHP, which limits the maximal firing frequency of motoneurons, is positively correlated to the contraction time of their innervated muscle fibers.

All skeletal muscles show slow contraction in new born kitten, and the contraction speed of gastrocnemius muscle becomes progressively faster during postnatal development whereas soleus muscle shows little changes. In contrast, the AHP duration in soleus motoneurons becomes progressively longer with age whereas that in gastrocnemius motoneurons remains unchanged [Huizar 1975]. Postnatal changes in the contractile properties of skeletal muscles were thus shown to be independent of the duration changes of AHP in their innervating motoneurons. Since AHP is an important determinant for firing frequency, it is not clear whether postnatal muscle differentiation is independent of the firing pattern maturation of its innervating motoneurons.

The average size of adult soleus motoneurons is smaller than that of gastrocnemius motoneurons. During early postnatal period (around P10), the difference of the soma sizes between kitten soleus and gastrocnemius motoneurons was found to be significant. The differentiation of motoneurons into γ-MNs and α-MNs was suggested to occur earlier than the motoneuron differentiation into slow (tonic) and fast (phasic) types, so it was supposed to occur before P10 in kitten [Sato 1977]. Moreover, motoneurons in different motoneuron pools were shown to mature differentially [Vinay et al., 2000]. Similar as the motoneuron differentiation in kitten, rat muscle fibers differentiate into different types around P7-P12 [Ishihara & Taguchi 1991; Picquet et al., 1997].
iv) Postnatal development of intrinsic motoneuron membrane properties

Compared with adult rat motoneurons, neonatal rat motoneurons have higher input resistance, longer duration of action potential, lower maximal firing frequency, higher gain (slope of f-I curve), and no clear threshold for repetitive firing [Fulton1986; Carrascal et al., 2005]. As for kitten spinal motoneurons, with increasing age, the resting membrane potential changes toward more hyperpolarized; the EPSPs evoked by dorsal root stimulation in motoneurons decreases; action potential amplitude increases but duration decreases [Kerllerth 1971].

The development of AHP in neonatal motoneurons differs between different species. The AHP of neonatal rat spinal motoneurons is longer than that in adult motoneurons; in contrast, the AHP of kitten spinal motoneurons is shorter than that in adult cat motoneurons [Carrascal et al., 2005; Fulton 1986; Huizar 1975].

Gao and colleagues studied the ionic currents underlying the action potential in embryonic and postnatal rat spinal motoneurons and suggested a large increase of the density of existing voltage-gated ion channels rather than expressing new channel types. Among these channels, Ca\(^{++}\) dependent K\(^{+}\) current (Ikca) was shown a significant postnatal increase resulting the shorter action potential duration and the AHP generation [Gao & Ziskind-Conhaim 1998]. Delayed rectifier K\(^{+}\) currents were developmentally regulated and shown to play an essential role in the maturation of action potential from long duration Ca\(^{++}\) dependent to a brief Na\(^{+}\) dependent in Xenopus spinal neurons including motoneurons [Ribera 1999]. Kv2 channels were suggested to function as delayed rectifier channels in vertebrate neurons [Blaine & Ribera 2001].
The high voltage-activated (HVA) Ca\(^{++}\) currents including L-type, which mediate PIC and are assumed to be responsible for the activation of Ca\(^{++}\) dependent K\(^{+}\) current (Ikca), increase during perinatal period in rat motoneurons [Vinay et al., 2000]. Similarly, in mouse motoneurons, L-type Ca\(^{++}\) channels were found to develop both physiologically and anatomically during early postnatal period (before P18), which parallels the motor function development [Jiang 1999].

The development of the ionic currents underlying motoneuron membrane properties is very important for the maturation of motor function. Since mAHP is an important determinant for motoneuron firing properties and SK channels mediate mAHP, studying the development of SK channels in motoneurons at different postnatal stage will shed light on the postnatal maturation of motoneuron properties. This question will be explored in aim IV.
**V Responses of motoneurons to axonal injury**

Peripheral nerves are subject to different types of injury which may lead to the dysfunction of motor and sensory system. In order to find effective solution for functional recovery following nerve injury, the responses of motoneurons to axotomy and the factors that determine motoneuron survival and axon regeneration have been studied broadly. Following sections contain some important results showing our current understanding of the overall effects of axotomy on motoneurons.

**i) Motoneuron survival after axotomy**

Two key factors determine motoneuron survival following axotomy: the age and the location of injury. Motoneuron survival becomes less target dependent during postnatal development [Pollin et al., 1991]. The vulnerability of rat motoneurons to nerve injury was shown to decline rapidly during the first week (P7) after birth. The critical period when axonal regenerative capability started was around two weeks (P14) [Chan et al., 2002]. The location of injury is another determinant factor for motoneuron survival; proximal axon injury causes higher motoneuron death rate than distal axon injury [Dai et al., 2000].

A recent study showed the electrophysiological properties of axotomized rat neonatal motoneuron ones that the electrical properties were profoundly altered shortly after axotomy. There was a marked increase in motoneurone excitability associated with a shift in the motoneurone firing pattern from a predominantly phasic pattern to a tonic pattern [Mentis et al., 2007].

The mechanism underlying the different fate of adult and neonatal motoneurons following axotomy has been explored. The neonatal motoneuron death is likely due to the
deprivation of the critical target-derived trophic factors for motoneuron survival; moreover, the responses of neurotrophin factor receptors such as glial cell line-derived neurotrophic factor (GDNF) receptor GFRalpha1 in motoneurons were also suggested to be crucial for motoneuron fate after axonal injury [Honma et al., 2002]. Axotomy causes an increased intracellular Ca\(^{++}\) concentration; up-regulated Ca\(^{++}\) buffering proteins such as Calbindin and Parvalbumin in axotomized motoneurons were shown to facilitate the survival of injured motoneurons [Krebs et al., 1997; Dassesse et al., 1998; Dekkers et al., 2004].

ii) Synapse stripping

Another predominant sign after axotomy is the loss of synaptic inputs onto motoneurons [Gonzalez-Forero et al., 2004; Tiraihi et al., 2004]. Ultrastructural studies showed that excitatory (S type) terminals were eliminated more than inhibitory (F type) terminals after motoneuron axonal injury, and this preferential glutamatergic inputs elimination was suggested to help diminishing excitotoxicity of axotomy [Linda et al., 2000].

A transient increase of F type boutons on motoneurons at 3-week and 6-week after axotomy was observed in a cat study; however, at 12-week the number of boutons decreased to half of normal level [Brannstrom et al., 1998]. Two years after re-innervation, synaptic covering recovered, but the distribution of S type and F type terminals was different compared with normal control [Brannstrom et al., 1999].

A number of studies also showed the modulation of postsynaptic receptors in motoneurons after axotomy. AMPA receptor subunits are differentially regulated in axotomized motoneurons that GluR1 subunits were increased whereas GluR2/3 and
GluR4 subunits were decreased [Garcia Del Cano et al., 2000; Alveraz et al., 2000]. Metabotropic glutamate receptor 1a was downregulated after axotomy [Alveraz et al., 1997]. Abundance of glycine receptor GlyR subunits and gephyrin decrease sharply in axotomized facial motoneurons [Eleore et al., 2005].

iii) Morphological changes of motoneurons after axotomy

The survival rate of newborn motoneurons is much lower than that of adult motoneurons following axonal injury. There are noticeable morphological changes in neonatal rat spinal motoneurons within 24 hours post axotomy. The changes include soma shrinking, the thickness of dendrites decreasing, and the length of dendritic branches decreasing [Peyghambari et al., 2005; Mentis et al., 2007]. At birth, the entire somato-dendritic surface of rat spinal motoneurons is covered with fine filopodial growth-associated processes, which are eliminated during the first postnatal week. Following neonatal axotomy, the postnatal elimination of growth associated processes was halted and axonal-like processes sprouted from soma and proximal dendrites in some axotomized motoneurons. These results indicate that synaptic interaction with target muscle during early postnatal period is essential for the maturation of motoneurons [Dekkers et al., 1998].

Several studies focused on the responses of adult cat neck motoneurons to axotomy. In contrast to other motoneurons, 11-17 weeks after injury, the dendrite area increased, the total number of dendritic branches increased, and growth cone-like lamellipodia and filopodia formed [Rose & Odlozinski, 1998]. 8-12 weeks post injury axon-like structures formed from distal motoneuron dendrites [Rose et al., 2001], and 20-35 weeks post injury some of these axon-like structures acquired synaptic connections.
Permanent transection of motor nerve may cause a gradual elimination of whole axon collateral system in injured spinal motoneurons [Havton et al., 1990].

Morphological changes were also studied at ultrastructural level which aims to understand what really occur in degenerating and regenerating motoneurons following axotomy. The reported results include cytoskeleton enlargement, chromatolysis, and the changes of organelles including Nissl bodies, Golgi bodies, and mitochondria [McIlwain et al., 2005; Guntinas-Lichius et al., 1996; Johnson et al., 1989].

iv) Electrophysiological properties of axotomized adult motoneurons

Besides the alteration of synaptic inputs onto axotomized motoneuron, the intrinsic membrane properties of motoneurons were also changed after axonal injury, resulting in altered neuron excitability and firing properties.

Neuronal excitability may be indirectly measured by rheobase, the threshold current of a long depolarizing current pulse for eliciting an action potential. Following axotomy the adult motoneurons showed a decreased rheobase in cats and rats [Kuno et al., 1974; Gustafsson 1979; Foehring et al., 1986; Nakanishi et al., 2005; Bichler et al., 2007]. This change was suggested to result from increased input resistance due to the elevated specific membrane resistivity, decreased cell size and altered dendritic geometry of injured motoneurons [Gustafsson & Pinter 1984; Foehring et al., 1986]. The modulation of ionic (K⁺, Na⁺, Ca++) conductance in cell surface membrane at sub-threshold or threshold membrane potential level might also contribute to the reduction in rheobase [Titmus & Faber 1990].
Axotomy resulted in a differential modulation of AHP duration in cat motoneurons that slow (S-type) motoneurons decrease AHP duration whereas fast (F-type) motoneurons slightly increase AHP duration. The differences in this electrophysiological property between fast and slow motoneurons became less prominent in axotomized animals compared with normal control, resulting in an axotomy-induced de-differentiation of spinal α-MNs [Kuno et al., 1974; Foehring et al., 1986]. The duration of AHP in rat motoneurons innervating fast (gastrocnemius) muscles was also observed to increase following axotomy [Gardiner & Seburn 1997]. The slopes of the frequency-current (f-I) curves for first interspike intervals and steady-state firing rates were steeper in cat axotomized motoneurons than those in normal control [Gustafsson 1979]. A significant reduction of AHP conductance was demonstrated in cat axotomized motoneurons and suggested to be major determinant for their altered AHP and firing pattern, though several other factors may also play a role in motoneuron property changes [Gustafsson 1979; Titmus & Faber 1990].

In addition, axotomized motoneurons also showed decreased axonal conduction velocity and increased amplitude of overshoot of action potential compared to normal control [Kuno et al., 1974; Foehring et al., 1986; Gardiner et al., 1997].

The mechanism underlying axotomy-induced electrical properties changes in motoneurons has been studied but many issues are still unresolved. Pinter and colleagues used botulinum toxin to block the release of acetylcholine at the neuromuscular junctions (NMJs). Their results showed that the existence of neurotransmission at a small number of motor terminals was sufficient to prevent axotomy-like changes in motoneuron electrical properties, but the complete elimination of neurotransmission at all motor
terminals resulted in axotomy-like changes [Pinter et al., 1991]. Later, Cope and colleagues showed that inhibition of acetylcholine release from motor terminals using botulinum toxin only resulted in minor changes in motoneuron properties; in contrast, the blockade of acetylcholine receptors at endplates using α–bungarotoxin induces motoneuron properties changes equivalent to axotomy. Their results suggested a retrograde signaling mechanism that maintains normal motoneuron excitability which depended on the activation of acetylcholine receptors at NMJs [Nakanishi et al., 2005].

Decreased AHP conductance in axotomized motoneurons was observed in electrophysiological study and was suggested to play a major role in their altered AHP and firing pattern [Gustafsson 1979; Titmus & Faber 1990]. This electrical membrane properties change might result from the altered expression and/or the gating properties of ionic channels mediating AHP in motoneurons. Since SK channels mediate medium AHP, studying SK expression and organization in axotomized motoneurons will shed light on the mechanisms underlying changed AHP and firing properties in motoneurons induced by axonal injury (see aim V).
CHAPTER II Hypothesis and Specific Aims

Spinal alpha-motoneurons (α-MNs), also known as “final common pathway”, transmit signals from central nervous system to their target skeletal muscles and thus control voluntary muscle contraction and muscle tone. Motoneuron excitability and firing properties are essential in motor control, and their underlying mechanisms have been studied extensively. Medium afterhyperpolarization (mAHP) and dendritic persistent inward currents (PICs) are two important properties controlling motoneuron excitability and firing behavior.

Medium AHP, which occurs following an action potential, determines the gain (the slope of F-I curve) of motoneurons and thus plays an essential role in their repetitive firing [Meunier 2005; Manuel et al., 2005]. The duration of the mAHP varies with motoneuron types; on average, slow (S-type) motoneurons express larger and longer mAHP than fast (F-type) motoneurons [Burke 1981; Zengel et al., 1985; Gardiner 1993]. The amplitude and duration of AHP in motoneurons is subjected to the modulating effect of neuromodulators such as serotonin and acetylcholine [Kernell 1999; Rekling et al 2000]. SK channels are suggested to mediate ionic currents underlying mAHP in motoneurons [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005].

Persistent inward currents (PICs) amplify synaptic inputs and play an essential role in controlling motoneuron excitability. PICs are enhanced by monoamines including serotonin and other neurotransmitters such as glutamate and acetylcholine [Svirskis & Hounsgaard 1998]. A calcium current (Ca++ PIC) mediated by L-type Ca++ channels and
a sodium current \((Na^+ \text{PIC})\) are the major components of PICs [Heckman et al., 2003; Li & Bennett 2003; Schwindt & Crill 1980]. Recent physiological observation suggested that there exist SK channels in dendrites which may be activated by dendritic \(Ca^{++}\) PIC in motoneurons. The activation of these SK channels decreases PICs and thus plays a role in the control of motoneuron excitability [Li & Bennett 2007]. Therefore, there exist two spatially and functionally distinct subpopulations of SK channels in same motoneurons.

Though there are numerous electrophysiological evidences for the existence of SK channels in motoneurons, so far the understanding of SK channel expression and distribution in motoneurons is very poor. The molecular basis for the recorded AHP and dendritic SK currents need to be defined. This dissertation will address these issues.

Development and injury are two important fields in neuroscience; many properties of developing and injured motoneurons have been characterized. Motor function matures during early postnatal period and the development of AHP after birth contributes to the maturation of motoneuron firing properties [Carrascal et al., 2005; Fulton 1986; Huizar 1975]. However, the mechanism underlying the maturation of AHP in developing motoneurons is to be clarified. Peripheral nerves are subject to different types of injury and axotomy results in a differential modulation of AHP duration in motoneurons. Slow motoneurons were shown to reduce AHP whereas fast motoneurons increase AHP or do not change after axonal injury [Kuno et al., 1974; Gustafsson et al., 1984]. The mechanism underlying this differential modulation of AHP in motoneurons is unknown. This dissertation will also explore these questions.

I hypothesize that SK channels are expressed differentially in distinct types of motoneurons which is responsible for their differential AHP. I also hypothesize that SK
channel maturation after birth underlies the postnatal development of AHP in
motoneurons. Finally, I hypothesize that the regulation of SK channel expression induced
by axotomy is responsible for the AHP modulation following injury.

The following aims are proposed in order to test my hypotheses:

I. Characterize the expression, distribution and membrane organization of SK channels
   in spinal motoneurons;

II. Determine the differential expression of SK channels in distinct types of spinal
    motoneurons;

III. Address the synaptic localization of SK channel clusters spinal motoneurons;

IV. Reveal the postnatal maturation of SK channel clusters in spinal motoneurons;

V. Investigate the modulation of SK channel expression by axonal injury in spinal
    motoneurons;

Aim I will characterize the expression of different SK channel isoforms in rat, mouse and cat lumbar spinal cord, in vivo. One transgenic mouse model (Hb9-eGFP) will be employed for the identification of α-motoneurons. Immunohistochemistry and confocal microscopy will be performed and the antibodies against SK2 and SK3 channels will be used. This aim will be continued in aim III which is focused on the synaptic localization of SK channels. The results in this aim will lay the foundation for the next four aims in this dissertation.

In aim II, the expression of SK channels in distinct types (slow & fast) of spinal motoneurons will be determined. This study will use rats (Sprague-Dawley or Wistar). Fluorogold retrograde tracing will be employed to indentify specific motoneuron pools in spinal cord. In addition, in vivo intracellular recording of single motoneurons will be
performed for the physiological identification of motoneuron type followed by immunohistochemical staining for detecting SK expression. The expected results will test our hypothesis if SK channels are expressed differentially in distinct types of motoneurons.

Aim III will address the synaptic localization of SK channels in the surface membrane of spinal motoneurons. To identify different subsets of synapses in motoneurons, antibodies against several pre-synaptic and post-synaptic markers will be used. The results in this aim will provide us insight into the mechanism of AHP and its modulation in motoneurons.

In aim IV, mouse pups of different age will be used to follow the postnatal development of SK channels in motoneuron membrane. The expected results of the postnatal maturation of SK2 and SK3 channel clusters in the surface membrane of motoneurons will shed light on the mechanism of postnatal AHP development in mototneurons.

One peripheral nerve crush model using Wistar rats will be employed in aim V in order to study the modulation of SK channels induced by axonal injury in spinal motoneurons. At different time points after the nerve crush animals will be sacrificed for the immunohistochemical study. The results are expected to explain the electrophysiological observation of the changes of AHP in injured motoneurons.
CHAPTER III Materials & methods

Tissue preparation

Sprague-Dawley rats, Wistar rats, CBA mice, or Hb9-eGFP mice of both sexes were euthanized with an overdose of pentobarbital, Nembutal, or euthe nal, and perfused transcardially with vascular rinse phosphate buffer (0.01M phosphate buffer with 0.8% NaCl, 0.025% KCl and 0.05% NaHCO₃, pH 7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Spinal cords and gastrocnemius/soleus muscles were quickly removed and post-fixed in the same fixative for 2 ~ 4 hours at room temperature. Tissue was stored in 15% sucrose at 4°C until sectioning on a freezing/sliding microtome or cryostat. Transverse sections of lumbar spinal cord (usually L4 or L5 segments) and neuromuscular junction rich regions of the gastrocnemius/soleus muscles were cut at 20 μm to 50 μm thickness and collected in PBS (0.01M phosphate buffered saline; pH 7.3).

For electron microscopy, Sprague-Dawley rats were perfused transcardially with 3% paraformaldehyde and 1% gluteraldehyde in 0.1M phosphate buffer (pH 7.4). Lumbar spinal cord tissue was post-fixed in the same fixative overnight at room temperature. Then tissue was stored in 0.01M PBS (pH 7.3).

All animal procedures were performed according to the Laboratory Animal Use Committee at Wright State University.
**Immunohistochemistry / immunofluorescence**

Transverse spinal cord or muscle sections were obtained using freezing/sliding microtome or Cryostate microtome. Sections were cut at 30~60µm thickness for floating procedure whereas at 10~20µm thickness for on-slide staining.

Two sets of antibodies are used: a primary antibody directed against the protein of interest, and a secondary dye-coupled antibody recognizing the primary antibody. The primary antibodies may be the same ones as those used in Western blotting.

In dual or triple immunolabeling, sections were rinsed with PBS (0.01M phosphate buffered saline; pH 7.3), blocked with normal horse/goat serum (10% in PBS) and then incubated in primary antibody mixtures diluted in PBS-T (0.01M PBS with 0.1% Triton-X-100, pH 7.3). Sections were incubated in primary antibodies overnight at 4oC, and immunoreactivity was detected with Fluorescein (FITC), Cy3-, or Cy5- conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) directed against the appropriate primary antibody species. Sections were mounted and cover-slipped under Vectashield mounting medium (Vector Laboratories).

When the respective antibodies were raised in same host species (rabbit), dual immunolabeling of SK3 and SK2 (or SK and CGRP) employed a sequential process. After the incubation of the first (anti-SK3) primary antibody, the immunoreactivity was detected with FITC-conjugated goat anti-rabbit secondary antibody (Fab fragment). Then all available binding sites were blocked by excess Fab fragment followed by the incubation in the second (anti-SK2) primary antibody. Finally Cy3-conjugated goat anti-rabbit secondary antibody was used to detect these sites.
**Electron microscopy pre-embedding immunoassay**

Ricardo Zerda helped with this study. Lumbar spinal cord tissue was sectioned at 50~100μm thickness using Vibratome. Sections were treated with 1% NaBH4 in PBS for 30mins to reduce glutaraldehyde and increase the permeability and affinity of primary antibody; then sections were rinsed with PBS thoroughly.

Sections were incubated with the polyclonal rabbit anti-SK3 (1:100; Chemicon) in PBS for 3 days at 4ºC on shaker. Immunoreactive site were then revealed using avidin-biotin complex (ABC kits from Vector Laboratories) followed by peroxidase protocols using DAB as substrate (0.02% diaminobenzidine and 0.01% H2O2 in 0.05M Tris Buffer) with reaction time of 10 minutes.

Next, German Silver Intensification was performed to enhance the reaction products. Sections were treated with 2.5% glutaraldehyde in H2O for 30mins followed by tris maleic acid rinse for 3 times and nanopure H2O rinse for 10mins. Then sections were incubated in Silver Nitrate solution for 10mins at 60 ºC in dark room followed by nanopure H2O rinse for 10mins and 0.05% Gold Chloride solution for 5mins to stabilize silver staining. Another nanopure H2O rinse for 10mins and 2.5% Sodium Thiosulfate wash for 3mins to dissolve unbound silver were followed by 10mins nanopure H2O rinse and 0.01M PBS.

Then, sections were post-fixed using 0.25% Osmium Tetroxide with 1% potassium ferricyanide for 20mins at 4ºC with light protection followed by a graded series of dehydration with ethanol and then 2 x 5mins propylene oxide.

Finally, section were incubated in 1:1 propylene oxide / Epon-Araldite overnight followed by flat-embedding in Epon-Araldite using coated coverslips and
drying for 2 days in 60 °C oven. Motoneuron areas in lamina IX with immuno-labeling were selected for ultrathin sectioning (60-70nm) using Sorvall MT 6000 ultramicrotome. Serial sections were collected onto grids, contrasted with 2% Uranyl Acetate and Lead Citrate for 5mins each, and viewed using Philips EM201 transmission electron microscopy.

**Western blotting**

Western blot can detect the protein of interest in a protein mixture and give information about the molecular weight of the protein. This method is dependent on a highly specific antibody directed against the target protein. In this dissertation western blotting was used to test the specificity of polyclonal antibodies rabbit anti-SK2 and anti-SK3.

Isolated lumbar spinal cords of Sprague-Dawley rats were disrupted by sonication in a buffered solution containing protease inhibitors (on ice). The isolation solution contained 178mM Na+, 1.5mM Mg2+, 153mM Cl-, 50mM HEPES, 10mM EDTA, 10% glycerol, 1% Triton X-100, and 1.0mM 4-(2-aminoethyl) benzenesulfonyl fluoride, as well as 1.54M aprotinin, 23.5M leupeptin, and 14.6M pepstatin A. In order to obtain membrane protein samples, spinal cord tissue samples were centrifuged at 6,000 g for 10 min at 4°C and then the resulting supernatant was centrifuged at 100,000 g for 60 min at 4°C. Protein content was determined using the Bradford method.

Protein samples were electrophoresed using 9% acrylamide SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 10% non-fat dry milk in TBS-T (TBS with 0.1% Tween 20), followed by
incubation with primary antibody (anti-SK2 or anti-SK3; rabbit 1:1,000; Alomone) for 2 hours and then with horseradish peroxidase-conjugated secondary antibody. Membranes were developed (90 s) with LumiGLO (Cell Signaling Technology, Beverly, MA) and then detected using Fuji film.

**Electrophysiological intracellular recording of MNs**

This *in vivo* electrophysiological study was performed by Katie Bullinger in Dr. Tim Cope’s lab. Adult female Wistar rats were anesthetised with isoflurane (4-5% in 100% O2, inhalation in induction chamber) and maintained by isoflurane (1-3% in 100% O2, through tracheal cannula). Respiratory rate, end tidal pCO2, Heart rate, O2 saturation and core body temperature were monitored. The lumbar spinal cord was exposed dorsally by laminectomy. Spinal MG motoneurons were identified by antidromic stimulation of MG muscle nerves placed on a stimulating hook electrode. Motoneurons were impaled with microelectrodes and the intrinsic membrane properties were measured, including Rheobase, Input resistance and AHP ½ decay time. To determine rheobase, depolarizing current pulses (50 ms) were delivered intracellularly in 1 nA steps until the motoneuron fired. To determine AHP, depolarizing current pulses (0.5 ms) greater than rheobase were given intracellularly to elicit single action potentials. The AHP ½ decay time is defined as the time for the membrane potential to decay from the peak of the hyperpolarization to the one-half of that level. To measure input resistance, intracellular injection of hyperpolarized current pulses (50 ms) was used and the resulting membrane potential
change was recorded. After the intracellular recording the motoneurons were filled with neurobiotin and the animals were transcardially perfused for immunoassays.

**Fluorogold retrograde tracing**

Lori Goss helped with the surgeries and fluorogold injection. Fluorogold retrograde tracing was used to identify specific spinal motoneuron pools. Survival surgeries were performed on rats anesthetized with isoflurane. An incision was made on the left hindlimb to expose the gastrocnemius or soleus muscles, which were then injected in multiple sites with 4% fluorogold. 3 days later animals were transcardially perfused for immunohistochemical assays described as above.

**Peripheral nerve crush**

Lori Goss helped with the surgeries and fluorogold injection. Two survival surgeries were performed using aseptic procedures on rats anesthetized by isoflurane. A skin incision was made on the posterior aspect of the left hindlimb to expose muscles or nerves, and then closed by staples. In the first surgery gastrocnemius and soleus muscles were injected in multiple sites with fluorogold, to retrogradely label motoneurons. Three days later in the second surgery the tibial nerve was crushed with fine jewlers forceps. 0, 1, 3 or 8 days after nerve damage, the animals were transcardially perfused and the lumbar spinal cords were harvested for immunoassays.
Analysis of en face ion channel clusters using ImagePro Plus 5.1

For the measurement of SK and Kv2.1 cluster size, only the ‘en face’ ion channel clusters were analyzed, which was ensured by the selection of single optical section and the creation of an area of interest (AOI) containing ‘en face’ clusters. A threshold of 25%-33% of maximal fluorescence intensity was used to distinguish immuno-labeled clusters from the background. The manually measured smallest clusters in confocal images are 0.05μm^2 or 0.222μm in diameter which corresponds to about 4 pixels (pixel size 0.111μm in diameter), so they are clearly different from background noise. See figure 3-1 for the details.

Statistical analysis

The statistical analysis of motoneuron average sizes was conducted using t-test to compare SK3-IR motoneurons with SK2-IR motoneurons or Non SK3-IR motoneurons. The statistical significance level was set at p<0.05.

Two-factor ANOVA was conducted with factors TIME and SIDE for Kv2.1 and SK3 clusters analysis after nerve crush. Wright State University Statistical Consulting Center helped with this analysis.
Figure 3-1 Quantitative analysis of Kv2.1-IR clusters from confocal image.

ImagePro Plus 5.1 was used for the analysis. A: One motoneuron labeled with NeuN (green) expresses Kv2.1 clusters (red) in the surface membrane of soma and proximal dendrites. This is a superimposed image of multiple optical sections taken on confocal microscopy. B: One single optical section from the motoneuron in A shows Kv2.1-IR clusters in the surface membrane of motoneuron. C: Create an area of interest (AOI) to restrict measurement of *en face* Kv2.1 clusters only. D: Select the range of fluorescence intensity from 0 to 4095 for display and set a threshold 25% of maximal intensity (1024 ~ 4095) for cluster measurement. E: Then the program selects all clusters from the background that contain fluorescence intensity greater than the threshold. The out-of-focus halo is excluded. F: All the selected clusters are numbered and their areas and diameters are measured.
CHAPTER IV Aim I. The expression, distribution and membrane organization of SK channels in spinal motoneurons

Introduction

Compared with many other central neurons, the gain (the slope of frequency-current curve) in motoneurons is low which matches the contractile properties of their target muscle fibers. Spinal motoneurons innervating slow twitch motor units express lower firing rate than those innervating fast motor units. The medium afterhyperpolarization (mAHP) following action potential plays an important role in limiting steady state firing frequency of motoneurons [Kernell, 1999; Manuel et al., 2005; Meunier 2005]. The duration of the mAHP varies with motoneuron types; on average, slow motoneurons express larger and longer mAHP than fast motoneurons. Differential mAHP in spinal α-MNs innervating slow and fast twitch motor units have been well characterized in cat and rat [Burke 1981; Zengel et al., 1985; Gardiner, 1993]. However, the molecular basis underlying the recorded currents remains unknown.

Based on previous results of other researchers, SK channels are believed to mediate the ionic currents underlying mAHP in motoneurons [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. Thus, understanding the expression, distribution, and membrane

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organization of SK channels will bring insight into the mechanisms underlying the excitability and firing properties of motoneurons. The work in this dissertation addresses this issue.

Three SK channels (SK1, SK2 & SK3) have been cloned [Kohler et al., 1996] and shown to be expressed in distinct and overlapping patterns in mammalian central nervous system [Stocker et al., 2000, Sailer et al., 2004]. However, the expression of SK channels in many neurons is still unknown. So far the structural and functional properties of SK channels in vivo motoneurons are not clear yet. What types of SK channels are expressed in motoneurons needs to be clarified; furthermore, the cellular distribution and membrane organization of these channels is also desirable for understanding the mAHP properties in motoneurons. On one hand, the spatial distribution of SK channels over somatic and dendritic membrane of motoneurons governs their mAHP characteristics. On the other hand, how SK channels are organized in the surface membrane, i.e., forming clusters or diffusively distributed, is important in determining SK channels Ca$^{++}$ gating properties and thus influencing the mAHP properties during motoneuron discharge.

Recent electrophysiological observation suggested that there also exist SK channels in dendrites which were spatially and functionally different from those mediating mAHP in motoneurons. These dendritic SK channels could be activated by persistent inward calcium current (Ca$^{++}$ PIC) mediated by L-type Ca$^{++}$ channels in motoneurons [Li & Bennett 2007]. This will also be examined in this immunohistochemical study.

My hypothesis is that motoneurons express SK channels and the subcellular distribution and the membrane organization of SK channels may explain the
electrophysiological recording of mAHP currents in motoneurons. In this aim, the immunoreactivity against SK2 and SK3 channels will be studied in rat, mouse and cat spinal cord using immunohistochemistry and confocal microscopy. Motoneurons innervating lower limbs that located in the ventral horn (Rexed’s lamina IX) of lumbar spinal cord (L4-5 in rats & mice; L6-7 in cats) are the focus of my study. Neuronal marker NeuN will be used to reveal all the neurons in lamina IX. Microtubule associated protein 2 (MAP2) will be employed to label motoneuron dendrites in the same area. Immunoreactivity against choline acetyltransferase (CHAT) and calcitonin gene-related peptide (CGRP) will be used to identify motoneurons in lamina IX [Barber et al., 1984; Réthelyi et al., 1989].

The homeodomain protein HB9 is expressed selectively by motoneurons in developing vertebrates and consolidates the identity of postmitotic motoneurons. In Hb9-eGFP transgenic mice motoneurons are supposed to be identifiable by the expression of enhanced green fluorescent protein (eGFP) [Arber et al., 1999; Wichterle et al., 2002]. During the postnatal development of these transgenic mice, the GFP intensity in spinal motoneurons is decreasing, and the GFP is only observed in spinal α-MNs rather than γ-MNs in adult animals [Alvarez unpublished results]. In this study adult Hb9-eGFP transgenic mice will be used to identify spinal α-MNs.

Since other investigators observed the expression of SK3 in some excitatory presynatic terminals including motor axon terminals innervating muscles [Roncarati et al., 2001; Obermair et al., 2002], the presence of SK channels in motor axon terminals in spinal cord and neuromuscular junctions (NMJs) will be tested in this study. Renshaw cells receive excitatory motor axon collaterals and mediate recurrent inhibition of
homonymous and synergistic α-MNs [Baldissera et al., 1981]. Most Renshaw cells are located in the ventral portion of Rexed’s lamina VII in spinal cord [Thomas and Wilson 1965], and they may be identified by calbindin D28K immunoreactivity [Carr et al., 1998]. The Renshaw cell area (ventral lamina VII) contains a high density of small to medium sized (2.26 +/- 0.94 microm in diameter) cholinergic motor axon terminals contacting Renshaw cell dendrites [Fyffe 1991; Alvarez et al., 1999]. The SK expression in these intra-spinal motor axon terminals will be examined as well as that in motor axon terminals at NMJs in muscles.

The results in this aim will lay the foundation for the next four aims in this dissertation.

**Methods & materials**

The specificity of polyclonal antibodies rabbit anti-SK2 and anti-SK3 were tested by pre-adsorption test using immunohistochemistry (rats, mice, & cats) and Western blotting (rats). The peptide antigen used are SK2 (amino acid residues 542-559, from a putative intracellular C-terminal part of SK2; Alomone); and SK3 (amino acid residues 2-21, from a putative intracellular N-terminal part of SK3; Alomone & Chemicon). The protocols for immunohistochemistry and western blotting have been described (see Chapter III).

Adult Spray-Dawley rats, CBA mice, Hb9-eGFP transgenic mice and cats were used for this *in situ* study. Lumbar spinal cords and soleus/gastrocnemius muscles were processed for analysis. Tissue preparation has been described in Chapter III. The immunohistochemical procedures performed in this aim are listed in Table 1.
The primary antibodies used to label SK channels include anti-SK2 (Alomone; rabbit 1:100) and anti-SK3 (Alomone, rabbit 1:100; or Chemicon, rabbit 1:1,000). The neuronal markers used are anti-NeuN (neuronal nuclei; Chemicon; mouse 1:400), anti-calbindinD28K (Swant; mouse 1:1,000), and Map2 (microtubule associated protein 2; Upstate; mouse 1:200). Anti-CHAT (Chemicon; Goat 1:500) or anti-CGRP (Peninsula; rabbit 1:100) were used to identify motoneurons and anti-VACHT (vesicular acetylcholine transportor; BD Pharmigen; Goat 1:5,000) to label cholinergic motor axon terminals.

Immunoreactivity of primary antibodies was detected with Cy3-, Fluorescein (FITC), or Cy5- conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). After immunostaining, the sections are examined with confocal microscopy (Olympus Fluoview) and subsequent analysis is performed using ImagePro Plus and CorelDraw software.

**Results**

1. The specificity of SK antibodies

The specificity of SK2 and SK3 antibodies were confirmed using western blotting. Western blots of the membranes prepared from rat spinal cord showed that the anti-SK3 recognizes a major protein band of ~70 kDa molecular weight. The pre-incubation using the peptide antigen (amino acid residues 2-21 of intracellular sequence of human SK3; Alomone) abolished the staining of membranes. Similarly as SK3, anti-SK2 recognizes a major protein band of ~72 kDa molecular weight. The pre-incubation of the peptide antigen (amino acid residues 542-559, from a putative intracellular C-terminal part of
SK2; Alomone) abolished the staining of membranes (figure 4-1). No cross-reactivity between SK2 and SK3 was observed.

The pre-adsorption control test also confirmed the specificity of the SK antibodies (see below).

2. Distribution of SK immunoreactivity in spinal cord

Transverse sections of rat and mouse spinal cord (L4 ~ L5) with Nissl staining are shown in figure 4-2, in which motoneuron area (lamina IX) and Renshaw cell area (ventral lamina VII) are illustrated. The SK3-like immunoreactivity (SK3-IR) was observed in whole gray matter of rat lumbar spinal cord, with the highest intensity in superficial dorsal horn, motoneuron area (lamina IX) and Renshaw cell area (ventral lamina VII) (figure 4-2). The distribution of SK3-IR in mouse spinal cord is similar to that in rat tissue (figure 4-6). This result is consistent with previous studies from other researchers (Sailer et al., 2004; Mongan et al., 2005). All SK3-like immunoreactivity was abolished with peptide antigen pre-absorption (hSK3 amino acid 2-21; Alomone).

Higher magnification confocal images show that, in lamina IX motoneuron area, SK3-IR forms large disk-shaped clusters in the surface membrane of soma and proximal dendrites of a subpopulation of neurons (figure 4-3). The en face SK3 clusters in single optical sections showed aggregates of smaller punctae with discrete bright spots within each cluster (figure 6-8). There was also relatively small amount SK3 immunoreactivity within cytoplasm. The SK3-IR neurons were confirmed to be motoneurons using dual labeling of choline acetyltransferase (CHAT) or calcitonin gene-related peptide (CGRP) (figure 4-4). Approximately, each SK3-IR motoneurons express about 50 SK3 clusters totally and half of which are located on cell body. Double labeling with dendritic marker
Map2 showed that there was no detectable SK3 immunoreactivity in the intermediate and distal dendrites of motoneurons (figure 4-5).

In the parallel study using cat spinal cords, SK3-IR also forms large clusters in the surface membrane of soma and proximal dendrites of motoneurons in lamina IX. However, unlike the differential expression of SK3 in motoneurons of rats and mice, SK3-IR clusters were seen in almost all presumed α-motoneurons in lamina IX of cat lumbar spinal cord (figure 4-6).

In ventral lamina VII Renshaw cell area and lamina IX, SK3 immunoreactivity also appears in punctate fashion and seems to be expressed by presynaptic terminals (see below).

Unfortunately anti-SK2 did not work with rat or cat tissue, so SK2-IR was analyzed only in mouse spinal cord. The SK2-IR was observed in whole gray matter of mouse spinal cord. Higher magnification images show that SK2-IR forms clusters over the surface membrane of soma and proximal dendrites of neurons in lamina IX motoneuron area, similar as SK3-IR in the same area. However, the SK2-IR clusters seem to be expressed by different subpopulation of motoneurons (see below).

3. Differential expression of SK channels in rat and mouse motoneurons

Using dual labeling of SK3 with neuronal marker NeuN, we found that in lamina IX only about 23% (73/316) of presumed α-motoneurons expressed SK-IR clusters. The intensity of NeuN-IR in motoneurons is not uniform that some neurons exhibit higher level of cytoplasmic staining than others. Noticeably, SK3-IR motoneurons expressed less NeuN intensity than those lacking SK3-IR (figure 4-3).
More interestingly, on average, the soma size of motoneurons expressing SK3-IR clusters was smaller than that of neurons lacking SK3-IR clusters. The difference between the mean soma diameters of rat SK3-IR motoneurons (36.7 ± 3.4 μm; n=61) and that of motoneurons without SK3-IR clusters (40.2 ± 3.5 μm; n=183) was of statistical significance (P<0.001) (figure 4-3). The cross-sectional areas of motoneuron cell bodies also showed similar results (SK3-IR MNs: 1066.2 ± 179.2 μm², n=61; non-SK3 MNs: 1263.6 ± 205.1 μm², n=183; p<0.01).

The SK3-IR motoneurons are presumed to be α-MNs rather than γ-MNs according to the size range of rat hindlimb α-MNs reported by other investigators [Chen & Wolpaw, 1994; Ishihara et al., 2001; Bose et al., 2005; Ichiyama et al., 2006]. Moreover, all SK3-IR motoneurons receive synaptic inputs of large cholinergic C-terminals (see aim III for details), which do not target γ-MNs [Lagerback, 1985; Lagerback et al., 1986; Johnson, 1986; Ichiyama et al., 2006]. In addition, Hb9-eGFP transgenic mice were used to confirm SK3-IR motoneurons are α-MNs (figure 4-8).

Since larger α-MNs do not express SK3, if they express SK2 is an important question to be answered. SK2 immunoreactivity in mouse spinal cord also appeared clusters over the surface membrane of α-MN soma and proximal dendrites in lamina IX. Dual labeling of SK2 and SK3 showed that SK2-IR and SK3-IR clusters are expressed, in complementary pattern, in different subpopulations of spinal α-MNs (figure 4-7). No co-expression of two SK isoforms in any motoneurons was observed. Similar as the results in rat, SK3-IR motoneurons exhibit comparatively weaker NeuN staining than SK2-IR motoneurons. As expected, the mean soma diameter of SK2-IR motoneurons (29.7 ± 3.2
um; n=87) was larger than that of SK3-IR motoneurons (27.3 ± 3.6 um; n=72), and the difference was of statistical significance (p<0.001) (figure 4-7).

4. Expression of SK3 in presynaptic motor axon terminals

SK3 was reported to target some excitatory presynaptic terminals by other investigators [Roncarati et al., 2001; Obermair et al., 2002], so if SK channels are trafficked to motor axon terminals is examined in this study. In ventral lamina VII cholinergic motor axon recurrent collaterals contacting Renshaw cells were revealed by VAChT immunoreactivity [Fyffe, 1991; Alvarez et al., 1999]. Numerous SK3-IR punctae were observed in this Renshaw cell area and almost all of them were colocalized with VAChT (98.7%; n=163). However, only a small portion of VAChT-IR terminals exhibited SK3-IR in this area (figure 4-9), which showed that a minority of motor terminals express SK3. Dual labeling of SK3 with calbinfinD28K revealed that some SK3-IR punctae were in apposition to the dendrites of Renshaw cells (figure 4-9), which indicated that those SK3-IR punctae are motor axon terminals. Similarly, a small portion of motor axon terminals at neuromuscular junctions also exhibited SK3-IR (figure 4-10). In contrast, SK2 was not observed in any presynaptic motor axon terminals.

Discussion

Using immunohistochemistry, it was shown that spinal motoneurons express SK channels, which is consistent with the electrophysiological observation that the mAHP in motoneurons may be blocked by apamin [Zhang and Krnjevic, 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. SK channels form large clusters in the surface membrane of motoneurons soma and proximal dendrites. The high magnification images showed that the SK clusters were
composed of aggregates of smaller punctae with discrete bright spots within each clusters. This characteristic immunoreactivity of SK clusters was similar as that of Kv2.1 macro-clusters reported by our lab [Muennich & Fyffe 2004]. The small amount SK immunoreactivity within cytoplasm is presumed to represent the intracellular compartments involved in channel production and trafficking as well as channel internalization and recycling. Interestingly, in rat and mouse SK2 and SK3 channels are expressed differentially in distinct subpopulations of spinal motoneurons. Since there is a significant difference of sizes between SK2-IR motoneurons and SK3-IR motoneurons, whether the differential expression of SK channels is related to motoneuron types will be examined in aim II.

It was expected that the findings in cats would resemble the results from rats and mice. However, we didn’t see the differential expression of SK3 in cat motoneurons. This different result in cats raises the question: if it represents the real difference in the SK channel expression or if it is due to the methodology and experiment condition. The epitope of the antibody is amino acid 2-21 of human SK3. The sequence of the N-terminus of the channel gene containing this epitope is shown to be well preserved across different species including rat, mouse, pig and human (from the company data sheets), though the cat SK3 channel is not cloned. The specificity of SK3 is confirmed in rat using western blot. The antigen pre-adsorption control was also performed in cats and all the SK3 staining was blocked by SK3 peptide. So our results suggested a different SK expression in spinal motoneurons among different species.

Recent electrophysiological evidences showed that there exist dendritic SK channels located close to L-type Ca^{++} channels mediating Ca^{++} PIC [Li & Bennett 2007,
Bond et al, 2005]. However, in this study, no SK3-like or SK2-like immunoreactivity was observed in the intermediate and distal dendrites of motoneurons revealed by MAP2 immunoreactivity besides the large SK clusters in proximal dendrites. Since the computational models of motoneuron suggested that Ca\textsubscript{v}1.3 channels mediating Ca\textsuperscript{++} PIC are localized in the dendritic tree around hundreds of micrometers from soma [Elbasiouny et al., 2005; Bui et al., 2006], the SK clusters in proximal dendrites can not account for the dendritic SK currents found in Li’s study.

The failure of identifying dendritic SK channels may result from: (i) The limitation of the current technical and experiment conditions. The ion channel density might be too low for the current technique to detect; (ii) SK1 channels mediate the recorded currents in Li’s eletrophysiological finding, which was not characterized in this immunohistochemical study due to the lack of available good antibody against SK1 channels.

A minority of presynaptic motor axon terminals was found to display SK3 immunoreactivity, which is partially consistent with previous report from Roncarati and colleagues who observed that mature motor axon terminals at NMJs in hind limb muscles expressed SK3 [Roncarati et al., 2001]. The mechanism underlying the differential expression of SK3 in motor terminals is not clear; whether it is related to motor unit types will be studied in aim II. Since the muscle fiber types were not addressed in Roncarati’s study, one possible explanation is that they investigated one specific type (eg. S-type) muscle fibers by chance whereas the motor axon terminals innervating other type (F-type) muscle fibers were lacking SK3 and not involved in their study.
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¹ Chemicon; USA  
² Alomone; Jerusalem  
³ BD Pharmingen; San Diego, CA  
⁴ Swant; Bellinzona, Switzerland  
⁵ Jackson ImmunoResearch Laboratories; West Grove, PA  
⁶ Peninsula; Belmont, California
The specificity of SK2 and SK3 antibodies was tested using western blotting. Membrane proteins extracted from rat lumbar spinal cord were separated by electrophoresis using 9% acrylamide SDS-PAGE gel. Ponceau stain was used to demonstrate separated proteins, which were then transferred to the membranes. Western blots of the membranes showed that the anti-SK3 (Alomone; rabbit 1:1000) recognizes a major protein band of ~70 kDa molecular weight (A), and anti-SK2 (Alomone; rabbit 1:1000) recognizes a major protein band of ~72 kDa molecular weight (B). There is a faint band of ~60 kDa revealed in both blots, which may result from non-specific binding of primary or secondary antibody. Another possible explanation for this extra band is that SK channel proteins were fragmented during the experiment and the faint protein band represents a portion of SK proteins.
A: Semi-transverse section of rat lumbar spinal cord with Nissl staining. Approximate lamina boundaries in the ventral horn are marked with dashed lines including motoneuron area in Lamina IX and Renshaw cell area in ventral Lamina VII.

B: Confocal image of SK3 immunofluorescence in rat lumbar spinal cord. SK3-like immunoreactivity (SK3-IR) was present in all laminae of rat lumbar spinal cord. The highest levels of SK3 labeling intensity are in the superficial dorsal horn and in lamina IX. Within lamina IX motoneuron area and lamina VII, SK3-IR appears to be expressed in both pre- and post-synaptic neuronal structures. SK3-IR is prominent in a subpopulation of presumed motoneuron (boxed area). The higher magnification image of the boxed area shows the SK3-IR form clusters in the surface membrane of motoneuron soma and proximal dendrites.
A LIX LVIII Renshaw cell area

Sk3

B 200 μm

Sk3
A: Dual immunofluorescence for NeuN and SK3 in lamina IX motoneuron area of rat lumbar spinal cord. Motoneuron cell bodies and proximal dendrites are revealed by NeuN immunoreactivity (green). A small portion of motoneurons (arrows) express SK3-IR clusters (red) on their surface membrane while other motoneurons do not exhibit SK3 immunoreactivity. SK3-IR motoneurons are comparably smaller and have less NeuN intensity than others.

B: Quantitative analysis of SK3-IR motoneuron sizes versus those lacking SK3-IR in rat lumbar spinal cord. Motoneurons with average diameter greater than 30µm were analyzed. The distribution of the soma average diameter of SK3-IR motoneurons is represented by red bars whereas blue bars show motoneurons lacking SK3 immunoreactivity. The mean soma diameter of SK3-IR motoneurons (36.7 ± 3.4 µm; n=61) was significantly smaller than that of motoneurons without SK3-IR clusters (40.2 ± 3.5 µm; n=183), P<0.001.
A

Sk3 NeuN

B

Motoneuron soma average diameter (μm)
Percentage of MNs observed
SK3-IR
Non-SK3-IR
In lamina IX motoneuron area of rat lumbar spinal cord, SK3-IR neurons are confirmed to be motoneurons by their immunoreactivity against CGRP (calcitonin gene-related peptide) and CHAT (choline acetyltransferase).

A: Dual immunofluorescence for CGRP (green) and SK3 (red) in motoneurons. Four motoneurons are revealed by CGRP immunoreactivity, one of which expresses SK3-IR clusters in the surface membrane of its soma and proximal dendrites (arrow).

B: Dual immunofluorescence for CHAT (green) and SK3 (red) in motoneurons. Three motoneurons exhibit CHAT immunoreactivity, one of which expresses SK3-IR clusters in the surface membrane of its soma and proximal dendrites (arrow).
Figure 4-5 Dual immunofluorescence for SK3 and Map2 in rat lumbar spinal motoneurons.

Motoneuron dendrites are revealed by immuoreactivity against Map2 (microtubule associated protein 2) (green). There are 6 motoneuron cell bodies surrounded by neuropil in this confocal image and three of them express SK3-IR clusters in their soma and proximal dendrites. There is no detectable SK3 immunoreactivity in the intermediate and distal dendrites of motoneurons labeled with Map2.
Figure 4-6 SK3 immunofluorescence in mouse and cat lumbar spinal motoneurons.

A. Confocal image of SK3 immunofluorescence in mouse lumbar spinal motoneurons. In lamina IX a group of neurons are revealed by NeuN immunoreactivity (green). Among these presumed motoneurons one comparably smaller motoneuron (arrow) express SK3-IR clusters in its soma and proximal dendrites while other larger ones lack SK3 immunoreactivity.

B. Confocal image of SK3 immunofluorescence in cat lumbar spinal motoneurons. In lamina IX motoneuron area a group of neurons are revealed by NeuN immunoreactivity (green). Different from rat and mouse, almost all the presumed cat motoneurons express SK3-IR clusters in their soma and proximal dendrites.
A

Mouse

B

Cat

Sk3
NeuN

Sk3
NeuN
Figure 4-7 Differential expression of SK2 and SK3 channels in different subpopulations of mouse spinal motoneurons.

A: Dual immunofluorescence for SK2 and SK3 in mouse spinal motoneurons. Two motoneurons (asterisks) are revealed in this confocal image that the larger one exclusively expresses SK2-IR clusters (green) whereas the smaller one SK3-IR clusters (red) in the surface membrane of their soma and proximal dendrites.

B: Quantitative analysis of SK3-IR motoneuron sizes versus SK2-IR motoneurons in mouse spinal cord. The diagram shows the distribution of soma average diameter of SK3-IR motoneurons (red bars) versus SK2-IR motoneurons (blue bars). On average, SK2-IR motoneurons are larger than SK3-IR motoneurons. The mean soma diameter of SK2-IR motoneurons (29.7 ± 3.2 μm; n=87) was significantly greater than that of motoneurons with SK3-IR clusters (27.3 ± 3.6 μm; n=72), P<0.001.
A

Sk2
Sk3

Motoneuron soma average diameter (μm)
Percentage of MNs observed
SK2-IR
SK3-IR

B

Percentage of MNs observed

Motoneuron soma average diameter (μm)
Hb9 is a transcription factor exclusively expressed by developing motoneurons. In adult Hb9-eGFP transgenic mouse, only spinal $\alpha$-motoneurons express green fluorescence protein (GFP) rather than $\gamma$-motoneurons. Using adult Hb9-eGFP mouse, SK2-IR and SK3-IR motoneurons are confirmed to be $\alpha$-motoneurons.

A. SK2 immunofluorescence in spinal alpha-motoneurons of Hb9-eGFP mouse. This is a superimposed confocal image. A couple of $\alpha$-motoneurons (asterisks) revealed by GFP (green) express SK2-IR clusters (red) in the surface membrane of their soma and proximal dendrites.

B. SK3 immunofluorescence in spinal alpha-motoneurons of Hb9-eGFP mouse. There are several $\alpha$-motoneurons revealed by GFP (green) in this confocal image. Two smaller ones (asterisks) express SK3-IR clusters (red) in their soma and proximal dendrites whereas the largest one (arrow) lacks SK3 immunoreactivity.
Figure 4-9 Expression of SK3 in a subpopulation of intra-spinal motor axon terminals.

A. Dual immunofluorescence for VACHT and SK3 in Renshaw cell area of rat lumbar spinal cord. Cholinergic motor axon terminals in ventral laminal VII Renshaw cell area are revealed by VACHT immunoreactivity (green). A minority of VACHT-IR motor terminals express SK3 (red) resulting in yellowish color that indicates the co-localization of the two staining whereas other terminals lack SK3 immunoreactivity. Conversely, almost all SK3-IR punctae exhibit VACHT immunoreactivity. The intensity of the VACHT and SK3 staining on a motor axon terminal was measured indicated by the white line in the image. The boxed diagram shows the measurement of the fluorescence intensity that the peaks of the two labeling overlap indicating co-localization.

B. Dual immunofluorescence for CalbindinD28K and SK3 in Renshaw cell area of rat lumbar spinal cord. Several Renshaw cells (asterisks) are revealed by CalbindinD28K immunoreactivity (green). Renshaw cell dendrites are contacted by some SK3-IR punctae (red), indicating these SK3-IR punctae are motor axon terminals.
A  VACHT
SK3

B  Calbindin
SK3

*
Similar as intra-spinal motor axon terminals, SK3 channels are expressed in a subpopulation of motor axon terminals at neuromuscular junctions (NMJs). Two NMJs in rat gastrocnemius muscle are revealed by presynaptic VACHT immunoreactivity (green). One presynaptic motor axon terminal (A1) expresses SK3 (red, A2) while the other one (B1) lacks SK3 immunoreactivity (B2).
A1

B1

A2

B2

10.0um VACHT

10.0um SK3
CHAPTER V Aim II. The expression of SK channels in distinct types of rat spinal motoneurons

Introduction

The amplitude and duration of the mAHP varies with motoneurons innervating different types of motor units. On average, slow (S-type) motoneurons express larger and longer mAHP than fast (F-type) motoneurons. Since SK channels are proposed to mediate mAHP, investigating the expression and membrane organization of SK channels in distinct types of motoneurons will be of great importance for our understanding of differential AHP expressed by spinal motoneurons.

Though there is no anatomical characteristic that may clearly distinguish different types of motoneurons, generally, fast motoneurons are larger than slow motoneurons [Burke 1981]. Based on the results in aim I, SK2 and SK3 channels are expressed, in complementary pattern, by different subpopulations of rat and mouse spinal motoneurons. On average, SK3-IR motoneurons are smaller than SK2-IR motoneurons. In order to explain the differential AHP expressed by distinct types of motoneurons, it is important to determine if the differential expression of SK channels in motoneurons is related to the motor unit types. My hypothesis is that slow motoneurons express SK3 channels whereas
fast motoneurons express SK2 channels. To determine the expression of SK channels in
distinct types of motoneurons is the focus of this study.

As discussed before, most mammalian muscles are heterogeneous containing diverse
histochemical types of muscle fibers. For example, in the rat, soleus muscle is slow and
gastrocnemius muscle is fast; however, the muscle fibers of both muscles are not
histochemically pure. Muscle fiber type composition studies of myosin heavy chain
(MHC) isoforms showed that the majority (>80%) of rat soleus muscle fibers are slow
type expressing MHCI whereas rat gastrocnemius muscle fibers are primarily (> 80%)
fast type expressing MHCII [Staron et al 1999]. In this study, SK3 expression in rat
soleus motoneurons and gastrocnemius motoneurons will be examined respectively.
Fluorogold retrograde tracing will be used to identify specific motoneuron pools in
lumbar spinal cord. The expected results will shed light on the SK expression in
motoneurons innervating distinct types of motor units.

Another important technique, electro-physiological recording, will be used here
to identify motoneuron types. Intracellular recording is a powerful technique that enables
the electrophysiological membrane properties of single motoneurons to be determined.
Generally, slow motoneurons have slower axonal conduction velocity, larger and longer
AHP, higher input resistance and greater overshoot of action potential than fast
motoneurons; however, there is always overlapping of all these properties between
motoneuron types [Burke 1981; Zengel et al., 1985; Gardiner 1993]. In this study
membrane properties including input resistance, rheobase, the amplitude and duration of
AHP will be recorded for the identification of motoneuron types. Intracellular recording
followed by intracellular filling of neurobiotin and immunocytochemistry will be performed to explore the SK expression in recorded motoneurons.

Aim I also showed that a small portion of presynaptic motor axon terminals in rat spinal cord and neuromuscular junctions displayed SK3-IR immunoreactivity. This result is partially consistent with the results from other investigators who did not examine muscle fiber types in their study [Roncarati et al., 2001]. According to our results it is of interest to understand what type of muscle fibers SK3-IR terminals innervate and whether it is related to motor unit types. Neuromuscular junctions in rat soleus (slow) muscle and gastrocnemius (fast) muscles will be examined separately. The expected results will provide us insight on the differential expression of SK3 in presynaptic motor axon terminals.

**Methods & materials**

Adult Spray-Dawley and wistar rats were used for the in situ study of the SK3 expression in soleus motoneurons and gastrocnemius motoneurons. Retrograde labeling of spinal motoneurons by fluorogold injection into muscles (soleus or gastrocnemius) was performed for the identification of specific motoneuron pools in lumbar spinal cord. The surgery and fluorogold injection was performed by Lori Goss. Three days after fluorogold injection lumbar spinal cords were harvested for immunohistochemical assays. Protocols for fluorogold injection are described in Chapter III.

Intracellular recording of in vivo motoneurons was performed in Dr. Tim Cope’s lab in collaboration with Katie Bullinger. Adult Wistar rats were anesthetised with
isoflurane. Spinal motoneurons identified by antidromic stimulation of medial gastrocnemius (MG) muscle nerve were intracellularly recorded and filled with neurobiotin after the measurement of membrane properties including input resistance, rheobase, and AHP amplitude and duration. Animals were then transcardially perfused and spinal cords containing recorded and neurobiotin-filled motoneurons were processed for immunohistochemical analysis. Neurobiotin was revealed by Alexa Fluor 405 conjugated Streptavidin (Vector Laboratories; 1:50), then the sections were incubated in primary antibodies followed by fluorescence conjugated secondary antibodies.

For the study of SK3 expression in motor axon terminals in muscle, neuromuscular junctions (NMJs) in soleus and gastrocnemius muscles of adult Spray-Dawley rats were analyzed. NMJs were revealed by immunoreactivity against α-bungarotoxin which targets postsynaptic acetylcholine receptors in muscle fibers. Anti-Myosin type I (Sigma; mouse 1:500) was used to identify slow type muscle fibers. At the same time, SK3 immunoreactivity was examined.

Immunoreactivity of primary antibodies was detected with Cy3-, Fluorescein (FITC), or Cy5- conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All immunohistochemical procedures performed in this aim are listed in Table 2. The immunostaining results were examined with confocal microscopy (Olympus Fluoview).
Results

1. SK3 expression in rat soleus versus gastrocnemius motoneurons

Soleus motoneurons and gastrocnemius (MG/LG) motoneurons in rat lumbar spinal cords were identified by retrogradely labeled fluorogold which was revealed using epi-fluorescence microscope with UV filter (figure 5-1). Since both α-MNs and γ-MNs were labeled with fluorogold, and only α-MNs are of interest in this study, all presumed γ-MNs were discarded whose soma average diameters were less than 25 um [Chen & Wolpaw, 1994; Ishihara et al., 2001; Bose et al., 2005; Ichiyama et al., 2006]. A total of 133 soleus α-MNs were observed and (63%) 84 of them expressed SK3-IR clusters; in contrast, 37 out of 149 (25%) MG/LG α-MNs exhibited SK3-IR clusters. The SK3 immunoreactivity was then arbitrarily divided into 2 subgroups: strong and weak. Among the 84 SK3-IR soleus motoneurons, 50 (60%) expressed strong SK3 intensity whereas 34 (40%) weak. As for the 37 SK3-IR MG/LG motoneurons, 25 (68%) exhibited strong SK3 immunoreactivity and 12 (32%) weak (figure 5-1).

2. SK3 expression in intracellularly recorded motoneurons

In vivo electrophysiological recordings were performed in Dr. Tim Cope’s lab in collaboration with Katie Bullinger. 10 medium gastrocnemius (MG) motoneurons were recorded followed by immunohistochemical analysis of SK3 expression. Shannon Romer was of great help with the immunostaining and imaging on confocal microscopy.

Motoneuron types were classified by their AHP ½ decay time (presumptive slow MN > 20ms; presumptive fast MN < 20ms) according to the previous results of rat gastrocnemius motoneurons [Gardiner 1993].
The electric membrane properties (rheobase & AHP ½ decay time) and SK3 immunoreactivity of the 10 recorded motoneurons are listed in Table 3, which are ranked by AHP ½ decay time. Among the 10 recorded motoneurons, one presumptive slow motoneuron (rheobase 1.3nA, AHP 1/2 decay time 25.0ms) expressed SK3 clusters over its surface membrane of soma and proximal dendrites (figure 5-2). Eight presumable fast motoneurons did not exhibit SK3 immunoreactivity, one of which was shown in figure 5-2. These results support the hypothesis that slow motoneurons rather than fast motoneurons express SK3 channels.

However, one presumable fast motoneuron with rheobase 12nA and AHP ½ decay time 15.7ms exhibited SK3 clusters. This result challenges the above hypothesis.

3. SK3 expression in rat fast versus slow motor axon terminals
Since a subpopulation of motor axon terminals was found to express SK3 channels in aim I, the SK3 expression in motor axon terminals at neuromuscular junctions (NMJs) was studied here. NMJs in rat soleus and MG/LG muscles were examined using immunohistochemistry. The NMJs were labeled by α-bungarotoxin, which targets postsynaptic nicotinergic acetylcholine receptors. Slow muscle fibers were identified with myosin type I and fast muscle fibers were lack of myosin I. All observed NMJs were randomly selected from muscle sections. The majority of NMJs innervating slow type muscle fibers exhibited presynaptic expression of SK3 (figure 5-3). In contrast, SK3 expression was seldom observed in NMJs innervating fast type muscle fibers. This finding of SK3 in slow rather than fast motor axon terminals suggests a differential expression of SK channels in distinct types of spinal motoneurons.
**Discussion**

1. **Soleus motoneurons versus gastrocnemius motoneurons**

   In this study two different sets of experiments were performed in order to test if the differential SK3-IR cluster expression in rat motoneurons is related to motor unit types. The first one detected the SK3 expression in rat soleus versus MG/LG motoneurons. As a result, the majority (63%) of soleus motoneurons expressed SK3-IR clusters; in contrast, only a minority (25%) of gastrocnemius motoneurons expressed SK3.

   The exact proportion of each motor unit type in both motoneuron pools are not clear, but the proportion of each muscle fiber type in both muscles were studied that more than 80% of rat soleus muscle fibers are slow whereas more than 80% rat gastrocnemius muscle fibers are fast [Staron et al 1999]. Electrophysiological study also showed the consistent results of the proportion of each motor unit type (slow type 16.7% whereas fast type 83.3%) in rat MG [Kanda & Hashizume 1992]. Based on these previous reports, our results indirectly suggest that slow motoneurons rather than fast motoneurons tend to express SK3 channels, though our numbers do not perfectly match the proportion of each muscle fiber types.

   One important thing needs to be mentioned here is the technical consideration. The fluorogold retrograde tracing technique is not 100% reliable that the possibility of injected fluorogold leaking into adjacent muscles may affect the final results due to the juxtaposition of soleus and gastrocnemius muscles.
2. Intracellular recording from fast motoneurons versus slow motoneurons

The physiological properties of gastrocnemius motoneurons in cat and rat indicate that slow motoneurons express higher input resistance, lower rheobase and longer AHP ½ decay time than fast motoneurons [Zengel et al., 1985; Gardiner 1993]. However, there is always overlapping of all these properties between distinct motoneuron types, so none of these properties is perfect predictor for classifying motor units. Another thing needs to be considered is the variation of these electrophysiological parameters, for example, rheobase is of physiological variation that it varies during recording. Compared to rheobase, the measurement of AHP is relatively more stable.

Zengel and colleagues studied cat medial gastrocnemius motoneuron properties and suggested that rheobase was the best individual predictor for classifying motor units as fast twitch fast fatiguing (FF), fast twitch with intermediate fatigue resistance (FI), fast twitch fatigue resistance (FR) or slow twitch fatigue resistant (S). The AHP ½ decay time was the most accurate predictor in discriminating between slow and fast motoneurons [Zengel et al., 1985]. Unfortunately there is not as much information of motoneuron properties available for rat as for cat. Gardiner studied rat gastronemius motoneurons and found that slow motoneurons always had AHP ½ decay time longer than 20ms whereas fast motoneurons never had the value greater than 19ms [Gardiner 1993]. The cutoff point of AHP ½ decay time (20ms) between slow and fast units set by Gardiner was used in this study for classifying motoneurons. The absolute definition of motoneurons can only be obtained by determining the mechanical twitching properties or myosin types of their innervated muscle fibers, which was not available in this study.
So far, only one recorded motoneuron was typed as slow by its long AHP ½ decay time (25.0ms) and low rheobase (1.3nA). This presumptive “slow” motoneuron expressed SK3 clusters in its soma and proximal dendrites as expected. In contrast, eight out of the nine (89%) presumable “fast” motoneurons did not express SK3 channels. All these expected results supported our proposed hypothesis.

Unexpectedly, one presumable fast motoneuron (AHP ½ decay time 15.7ms) expressed SK3 clusters. As a fact, the classification of motoneuron types based on their membrane properties is not 100% reliable, and there always exists uncertainty when using any motoneuron physiological properties as predictors. The SK3-IR “fast” motoneuron had AHP ½ decay time 15.7ms, which is close to the cutoff point (20ms), so there is a possibility that it is a slow rather than fast motoneuron.

With current data, it is difficult to draw a conclusion due to the following reasons:

1) The size of our sample (n=10) is too small; only one presumptive slow motoneuron was recorded. More recorded motoneurons in future study, especially slow type, will bring more information for a conclusion;

2) There is always overlapping of motoneuron membrane properties innervating different types of motor units, so using the cutoff point of 20ms AHP ½ decay time to discriminate slow and fast motor units is not 100% reliable and the mistyping is inevitable;

3) The limitation of current techniques: there may be mistakes when using immunohistochemistry to identify recorded neurons because of the possible leaking of neurobiotin into adjacent neurons.
Though it is too early to conclude that SK3 expression is expressed by slow rather than fast motoneurons, the present results are promising. There was a clear bias of SK3 expression in: i) retrogradely labeled soleus versus gastronemius motoneurons; ii) physiologically identified fast versus slow recorded motoneurons. But in each case, the identity of “fast” and “slow” is not absolute. Future study is needed for a conclusion.

3. Motor axon terminals of slow motoneurons versus fast motoneurons

Our results show that a subpopulation of motor axon terminals in ventral horn spinal cord and neuromuscular junctions express SK3 channels. This is consistent with the previous report [Roncarati et al., 2001]. We suggested that motor axon terminals of slow motoneurons rather than those of fast motoneurons express SK3 channels. This result suggests a differential expression of SK channels in nerve terminals by motoneurons innervating distinct types of motor units. However, it is unknown if what is expressed in axon terminals such as SK3 channel is also expressed in motoneuron cell body.

4. Mechanism underlying the differential mAHP in motoneurons

Though more data are required for a conclusion, our results in this aim suggested a differential expression of SK channels in rat spinal motoneurons that slow type is more likely to express SK3 whereas fast type SK2. Whether this may explain the differential AHP expressed by distinct types of motoneurons is a question that needs to be answered. The mAHP conductance in slow motoneurons was shown similar as that in fast motoneurons [Manuel et al., 2005]. The lower input conductance of slow motoneurons may explain their higher AHP amplitude; however, why slow motoneurons have longer
AHP duration is unknown. Why SK3 in slow motoneurons mediates longer AHP than SK2 does in fast motoneurons needs to be explored in future study.

In order to confirm that SK3 is responsible for the mAHP in slow motoneuron and SK2 for fast ones, the deletion of SK3 or SK2 genes would be the ideal model. As discussed before, SK channel knock-out transgenic mouse lines have been constructed [Bond et al., 2004]. However, few studies, if any, have been performed in mouse for the electrophysiological recording of motoneurons. Unfortunately, no knock-out rat is available at this time.

As discussed in Background, the biophysical properties of three cloned SK isoforms (SK1, SK2, & SK3) in expression system are not different from each other, including their unitary conductance and kinetics of calcium activation. However, the environment of *in vivo* motoneurons is different from the isolated cells in expression system, which lack many regulatory proteins and scaffolding proteins in native motoneurons. If any other proteins interact with SK channel clusters and contribute to the different kinetics of SK3 and SK2 channels in rat and mouse motoneurons needs to be examined in future study.

Another possibility is that the clustering of SK channels results in different Ca\(^{++}\) sensitivity and kinetics of SK3 channels from that of SK2 channels. As discussed in Background, the phosphorylation state of calmodulin constitutively bound to SK2 or SK3 channels may be regulated by CK2 / PP2A leading to altered channel Ca\(^{++}\) sensitivity [Bildl et al., 2004; Allen 2007]. When SK channels aggregate into clusters in motoneuron surface membrane, the phosphorylation state of calmodulin might be changed and this change is not at the same level for SK2 and SK3 channels leading to different Ca\(^{++}\)
sensitivity and channel kinetics. The exact mechanism of differential AHP in 

motoneurons needs to be clarified in future study.

Since the differential expression of SK channels was not observed in cat, the 
mechanism underlying the differential AHP in cat motoneurons might be different from 
that in rodents.
Table 2  Tissue, fixation, sectioning and antibodies for immunofluorescence in Aim II

| Dual Immunofluorescence for SK3 and NeuN | Tissue | Fixation | Sections | Primary Abs | Secondary Abs
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat spinal cord after Fluorogold injection</td>
<td>4% paraformaldehyde with 2 hour post-fix</td>
<td>40 µm floating sections</td>
<td>NeuN (mouse 1:400)(^1)</td>
<td>FITC anti-mouse IgG 1:200 Cy3 anti-rabbit IgG 1:300</td>
<td></td>
</tr>
<tr>
<td><strong>Triple immunofluorescence for SK3, VAChT, and Neurobiotin</strong></td>
<td>Rat spinal cord after intracellular recording and neurobiotin filling of MNs</td>
<td>4% paraformaldehyde with 2 hour post-fix</td>
<td>40 µm floating sections</td>
<td>SK3 (rabbit 1:100)(^2) VAChT (goat 1:5,000)(^2)</td>
<td>Alexa Fluor 405 Streptavidin 1:50 Cy3 anti-rabbit IgG 1:300 Cy5 anti-goat IgG 1:50</td>
</tr>
<tr>
<td><strong>Triple immunofluorescence for SK3, kv2.1, and Neurobiotin</strong></td>
<td>Rat spinal cord after intracellular recording and neurobiotin filling of MNs</td>
<td>4% paraformaldehyde with 2 hour post-fix</td>
<td>40 µm floating sections</td>
<td>SK3 (rabbit 1:100)(^2) Kv2.1 (mouse 1:1,000)(^4)</td>
<td>Alexa Fluor 405 Streptavidin 1:50 Cy3 anti-rabbit IgG 1:300 FITC anti-mouse IgG 1:200</td>
</tr>
<tr>
<td><strong>Triple immunofluorescence for α-bungarotoxin, SK3, and myosin type I</strong></td>
<td>Rat soleus/gastrocnemius muscles</td>
<td>4% paraformaldehyde with 4 hour post-fix</td>
<td>20 µm on-slide sections</td>
<td>SK3 (rabbit 1:100)(^2) Myosin I (mouse 1:500)(^5) α-bungarotoxin-Alexa 488 (1:200)(^7)</td>
<td>Cy3 anti-mouse IgG 1:300 Cy5 anti-rabbit IgG 1:50</td>
</tr>
</tbody>
</table>

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1 Chemicon; USA  
2 Alomone; Jerusalem  
3 BD Pharmingen; San Diego, CA  
4 Neuromab; Davis, California  
5 Sigma-Aldrich; St. Louis, MO  
6 Jackson ImmunoResearch Laboratories; West Grove, PA  
7 Molecular Probes; Carlsbad, California  
8 Vector Laboratories; Burlingame, CA
Table 3 The membrane properties of 10 recorded MNs

<table>
<thead>
<tr>
<th>Recorded MN</th>
<th>Rheobase (nA)</th>
<th>AHP 1/2 decay (ms)</th>
<th>AHP amplitude (mV)</th>
<th>SK3-IR</th>
<th>Putative identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>8.5</td>
<td>1.17</td>
<td>no</td>
<td>Fast</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>9.1</td>
<td>0.64</td>
<td>no</td>
<td>Fast</td>
</tr>
<tr>
<td>3</td>
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<td>9.6</td>
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<td>5</td>
<td>9</td>
<td>10.2</td>
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<td>6</td>
<td>28</td>
<td>11.6</td>
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<tr>
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<td>12</td>
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<tr>
<td>9</td>
<td>3.7</td>
<td>18.9</td>
<td>3.75</td>
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</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>25.0</td>
<td>3.99</td>
<td>Yes</td>
<td>Slow</td>
</tr>
</tbody>
</table>
Figure 5-1 SK3 expression in motoneurons innervating slow versus fast muscles.

A. Gastrocnemius motoneurons retrogradely labeled with fluorogold. 3 motoneurons (asterisks) are revealed by fluorogold staining under UV light after the fluorogold injection into gastocnemius muscles. Only the brightest presumed alpha-motoneurons were selected for this study. Motoneurons with average diameter less than 25 um were discarded.

B. SK3 expression in soleus motoneurons versus gastrocnemius motoneurons. A total of 133 soleus motoneurons and 149 gastrocnemius motoneurons were analyzed for the SK3 expression analysis. A majority (63%) of soleus motoneurons express SK3 whereas a minority (25%) of gastrocnemius motoneurons do so. The SK3 immunoreactivity is arbitrarily divided into strong and weak, and 60% SK3-IR soleus motoneurons and 68% SK3-IR MG/LG motoneurons express strong SK3 intensity.
Figure 5.2 Intracellular recording of rat spinal motoneurons.

A. Triple immunofluorescence for Neurobiotin, VACHT and SK3 in a recorded ‘slow’ motoneuron. This recorded motoneuron was classified as a slow motoneuron due to its low rheobase (1.3nA) and long AHP ½ decay time (25.0ms).

B. The measurement of rheobase and AHP for the recorded motoneuron shown in A. (i) Action potential elicited by intracellular current injection to determine AHP ½ decay time. The AHP ½ decay time was defined as the time for the membrane potential to decay from the peak of the hyperpolarization to the one-half of that level. (ii) To determine rheobase, depolarizing current pulses (50 ms) were delivered intracellularly in 1 nA steps until the motoneuron fired.

C. Triple immunofluorescence for Neurobiotin, Kv2.1 and SK3 in a recorded ‘fast’ motoneuron. This recorded motoneuron was classified as a fast motoneuron due to its higher rheobase (10nA) and shorter AHP ½ decay time (15.5ms).

D. The measurement of rheobase and AHP for the recorded motoneuron shown in C. (i) Action potential elicited by intracellular current injection to determine AHP ½ decay time. (ii) To determine rheobase, depolarizing current pulses (50 ms) were delivered intracellularly in 1 nA steps until the motoneuron fired.
Figure 5-3 SK3 immunofluorescence at NMJs innervating slow muscle fibers.

A. Transverse section of rat soleus muscle fibers with triple immunofluorescence for α-bungarotoxin, Myosin type I and SK3. Slow muscle fibers are identified with immunoreactivity against myosin I (red). Two NMJs are revealed by immunostaining of α-bungarotoxin (green). The immunofluorescence of one NMJ (arrow) is demonstrated in higher magnification images shown in B.

B. Triple immunofluorescence for α-bungarotoxin, Myosin type I and SK3 in the NMJ shown in A. The NMJ labeled with α-bungarotoxin (green, B1) innervates slow muscle fiber stained with Myosin I (red, B2) and expresses SK3 (white, B3). B4 is the merging image of the triple immunolabeling.
Myosin type I

B1  a-bungarotoxin
B2  Myosin type I
B3  SK3
B4

Soleus
50 μm

A

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CHAPTER VI Aim III The synaptic localization of SK channel clusters in spinal motoneurons

Introduction

Each mammalian α-motoneuron receives thousands of synaptic contacts of various origins, some of which modulate motoneuron intrinsic input/output properties by working through metabotropic receptors. One of these modulating effects is the regulation of the amplitude and duration of AHP that leads to the alteration of motoneuron firing properties [Kernell 1999; Rekling et al 2000].

Medium AHP in motoneuron was found to be reduced by serotonin and muscarine/acetylcholine, and the activation of 5-HT1A receptors and M2 receptor were suggested to mediate their modulatory effect respectively [Van Dongen et al 1986; Bayliss et al., 1995; Grunnet et al., 2004; Brownstone 2006; Lape et al., 2000]. However, the exact signaling pathway of these neuromodulation is unknown.

Medium AHP in motoneurons may be blocked by apamin indicating it is mediated by SK channels [Zhang et al., 1987; Hounsgaard et al., 1988; Viana et al., 1993; Powers et al., 1999; Sawczuk et al., 1997; Miles et al., 2005]. Based on the results of aim I, SK2 and SK3 channels form clusters over the surface membrane of soma and proximal dendrites of motoneurons. If this characteristic organization contributes to the neuromodulatory effects of neurotransmitters is an important question to be answered.
My hypothesis is that SK clusters in motoneuron surface membrane are located at synaptic sites in association with one specific type of presynaptic terminals, for example, cholinergic C-terminals, because of the large size of SK clusters. This aim will focus on examining the synaptic localization of SK clusters in motoneurons. The expected results will provide insight into the mechanism underlying the neuromodulation in motoneurons.

In order to identify different subsets of synapses, several pre-synaptic or post-synaptic markers will be used to reveal cholinergic, serotonergic, glutamatergic, and glycine/GABAergic synapses in motoneurons. Antibody against vesicular acetylcholine transporter (VACChT) reveals presynaptic cholinergic bountons including large C-terminals contacting α-motoneurons in lamina IX and motor axon collaterals contacting Renshaw cells in lamina VII [Alvarez et al., 1999; Deng & Fyffe 2004]. Antibody against 5-HT labels descending serotonergic inputs from Raphe neurons in brainstem [Alvarez et al., 1998]. Antibodies against vesicular glutamate transporters (VGLUT1 & 2) reveal glutamatergic boutons that VGLUT1 labels primary afferent terminals and VGLUT2 descending and segmental glutamate inputs onto motoneurons [Alvarez et al., 2004]. Gephyrin is the clustering protein for glycine receptors and antibody against gephyrin post-synaptically reveals glycine/GABAergic synapses in motoneurons and interneurons [Alvarez et al., 1997].

Voltage gated potassium Kv2.1 channel, the contributor to the delayed rectifier, was abundant and distributed in clusters in the surface membrane of soma and proximal dendrites of α-motoneurons. Large Kv2.1 clusters were apposed to presynaptic
cholinergic C-terminals and colocalized with postsynaptic muscarinic (m2) receptors [Muennich & Fyffe 2004]. Kv2.1 will also be tested in this study.

**Methods & materials**

Dual labeling of SK (SK2 or SK3) channels combined with one of synaptic markers was performed for this immunohistochemical study.

The antibodies against pre-synaptic markers include: 1) anti-Synaptophysin (Calbiochem/Oncogene; mouse 1:200) un-specifically labels presynaptic terminals; 2) anti-VACHT (vesicular acetylcholine transportor; BD Pharmigen; Goat 1:5,000) labels cholinergic presynaptic terminals; 3) anti- 5-HT (Dr. Pearson; Guinea Pig 1:5,000) labels serotonergic presynaptic terminals; 4) anti-VGLUT1 (Chemicon; Guinea pig 1:5,000), and anti-VGLUT2 (Chemicon; Guinea pig 1:5,000) label glutamatergic presynaptic terminals.

The antibody against gephyrin (Alexis; mouse 1:100) post-synaptically labels inhibitory glycine/GABAergic synapses. The antibody against Kv2.1 (Upstate Biotechnology; mouse 1:250) labels voltage-gated Kv2.1 channels.

All immunohistochemical procedures performed in this aim are listed in Table 4. The resulting immunostaining was visualized and imaged using confocal microscopy. The SK3 cluster sizes were analyzed using ImagePro sofeware, and only the *en face* clusters in individual optical sections were selected for this analysis (detailed method in Chapter III).
The synaptic localization of SK was confirmed at ultra-structural level using electron microscopy. Pre-embedding immunostaining of SK3 channel (Chemicon; rabbit 1:100) was performed on vibratome sections of rat lumbar spinal cord (see Chapter III for details). The resulting labeling was visualized on Philips EM201 transmission electron microscopy. Ricardo Zerda helped with the EM study.

**Results**

1. **Postsynaptic location of SK channel clusters**
   Double labeling of presynaptic protein synaptophysin with SK3 revealed a close correspondence between presynaptic boutons and SK3-IR clusters in the soma and proximal dendrites of rat spinal motoneurons. Each SK3-IR cluster was apposed to (not colocalized with) a presynaptic terminal revealed by synaptophysin, though some presynaptic boutons were not apposed to any SK3-IR clusters on the surface membrane of motoneurons. Using mouse spinal cord similar results were observed for SK2 channels. This finding suggests a postsynaptic location of SK channel clusters in the surface membrane of motoneurons. Moreover, the pre-synaptic terminals apposing SK-IR clusters are the largest ones compared with those not associating any SK channels (figure 6-1). The co-localization of SK clusters with Kv2.1 channels also confirm the postsynaptic location of SK clusters in motoneurons (see below).

2. **Specific localization of SK clusters at cholinergic synapses**
   In order to identify what type of synapses SK clusters are localized at, several pre- and post-synaptic markers were used to label different subsets of synapses on
motoneurons. Cholinergic C-terminals contacting the soma and proximal dendrites of α-motoneurons were revealed by the immunoreactivity against VACHT, which are the largest boutons among various synaptic inputs onto motoneurons [Alvarez et al., 1999; Deng & Fyffe 2004]. Double labeling of SK3 with VACHT showed that the majority (94.9%; n=1051) of SK3-IR clusters in rat motoneurons are associated with VACHT-IR presynaptic C-terminals. The immunoreactivity against VACHT and SK3 was closely apposed to rather than overlapping with each other, which is consistent with the results of synaptophysin indicating a postsynaptic rather than presynaptic localization of SK3-IR clusters (figure 6-2). Similar results were observed for SK2-IR clusters in mouse spinal motoneurons (figure 6-4).

The electron microscopy results confirmed the postsynaptic localization of SK3 channels at cholinergic synapses in rat spinal motoneurons. The immunoreactivity against SK3 targeted the postsynaptic membrane apposed to presynaptic C-terminals. There was some SK3 immunoreactivity within cytoplasm, which may demonstrate SK3 protein trafficking to and recycling from motoneuron surface membrane (figure 6-3).

Then serotonergic, glutamatergic and glycine/GABAergic synapses were also examined in order to test if postsynaptic SK3 clusters are particularly associated with cholinergic C-terminals. Dual labeling of SK3 with 5-HT showed no close apposition of SK3-IR clusters with any serotonergic bountons (figure 6-5C). Two subsets of glutamatergic boutons were revealed by two isoforms of vesicular glutamate transporter VGLUT1 and VGLUT2; as a result, none of SK3-IR clusters in motoneurons was found to associate with any presynaptic glutamatergic terminals (figure 6-5AB). Inhibitory glycine/GABAergic synapses were labeled by immunoreactivity against gephyrin, and no
overlapping of post-synaptic gephyrin immunoreactivity and SK3-IR clusters was observed (figure 6-5D).

3. Co-localization of SK channels and Kv2.1 channels

Voltage gated Kv2.1 channel, the contributor to the delayed rectifier, forms clusters in the surface membrane of soma and proximal dendrites of α-motoneurons. Large Kv2.1 clusters are postsynaptic and apposed to pre-synaptic cholinergic C-terminals and co-localized with muscarinic (m2) receptors. The post-synaptic location of large Kv2.1 clusters was confirmed at ultra-structural level using electron microscopy [Muennich & Fyffe 2004]. Since SK-IR clusters were found to be post-synaptically localized at cholinergic synapses, they were expected to co-localize with kv2.1 channels. Then double labeling of SK3 combined with Kv2.1 was performed using rat spinal cords and the results showed the perfect overlapping of the immunoreactivity against the two ion channels in motoneurons (figure 6-6). This result confirms the post-synaptic location of SK channel clusters in motoneurons.

It needs to be mentioned that all motoneuorns of both large and small sizes expressed kv2.1-IR clusters whereas only a subpopulation of motoneurons exhibited SK3-IR clusters. In SK3-IR motoneurons, only large kv2.1-IR clusters co-localize with SK3-IR clusters, and small kv2.1-IR clusters do not contain SK3 immunoreactivity (figure 6-6). Totally 341 SK3-IR clusters in 30 motoneurons were examined and all of them were co-localized with Kv2.1. Similar results were observed for SK2-IR clusters in mouse spinal motoneurons (figure 6-7).

The sizes of en face SK3-IR clusters in rat motoneurons were then quantitatively analyzed. The range of the maximal diameters of SK3-IR clusters was from 1.29 μm to
7.14 μm (mean ± SD 3.51 ± 0.88; n=245); and the cluster areas were from 1.03 μm² to
15.62 μm² (mean ± SD 6.75 ± 2.68; n=245). This result of SK3-IR cluster sizes is
comparable to that of large Kv2.1 clusters in rat motoneurons [Muennich & Fyffe 2004]
(figure 6-8).

**Discussion**

In this immunohistochemical study SK2-IR and SK3-IR clusters in motoneurons
were shown to be specifically localized post-synaptically at cholinergic synapses
associated with C-terminals, which was confirmed at ultra-structural level using electron
microscopy. The co-localization of SK clusters with Kv2.1 channels at these synaptic
sites are also good evidences for the post-synaptic location of SK clusters. None of the
observed SK3-IR or SK2-IR clusters in motoneurons was localized at serotonergic,
glutamatergic, or glycine/GABAergic synapses. No evidence of presynaptic localization
of SK immunoreactivity was found at any synapses in motoneurons.

M2 muscarinic acetylcholine receptors (mAChR) were shown to enrich beneath C-
terminals in the postsynaptic membrane and suggested to be the major muscarinic
acetylcholine receptors in spinal motoneurons [Rekling et al., 2000; Hellström et al., 2003].
Voltage gated Kv2.1 channels were found to be co-localized with M2 receptors at these
sites [Muennich & Fyffe 2004]. Recent studies showed the dynamic regulation of Kv2.1
localization in cell surface membrane and channel biophysical properties plays an
important role in the control of neuron excitability [Misonou et al., 2004, 2005;
Mohapatra & Trimmer 2006]. In this study SK channels were observed to be localized in

- 109 -
the same postsynaptic domains, which may bring a new clue of the modulatory effect of acetylcholine/ muscarine on motoneurons.

The cholinergic synapses in motoneurons formed by large C-terminals are characterized of sub-surface cisternae, which are endoplasmic reticulum-derived organelles rich in ryanodine receptors, the intracellular calcium release channels [Antonucci 2001]. The activation of postsynaptic M2 receptors leads to a reduction of mAHP and increases the input-output gain (slope of f-I curve) in motoneurons [Brownstone 2006; Lape et al., 2000]. One possible explanation is the activation of m2 receptors results in the alteration of calcium sequestration into and/or release from subsynaptic calcium store, which reduces the free Ca++ ion concentration at those microdomains and thus decreases SK channel activation resulting in AHP reduction.

However, several evidences suggested the activation of M2 receptors leads to an increased calcium release from SCC [Zhou et al., 2003]. The cholinergic modulation of Kv2.1 clusters in cultured hippocampal neurons was shown to result from calcium release from subsurface cistern via muscarinic receptors causing a dispersing of Kv2.1 clusters in cell surface membrane [Mohapatra & Trimmer 2006]. Similar Kv2.1 de-clustering effects were also observed in neonatal spinal motoneurons by muscarine application [Katie’s SfN poster 2007; unpublished results from Fyffe lab]. Since the co-localization of Kv2.1 and SK clusters at cholinergic synapses, if the calcium sequestration/release is involved in the acetylcholine/ muscarine modulation of SK channel / AHP in motoneurons needs to be clarified in future study.

In rat motor axon terminals the activation of M2 receptors was suggested to inhibit neurotransmission by decreasing protein kinase A (PKA) activity, and P/Q-type
calcium channels were involved in this muscarine regulatory effect [Santafe et al., 2006]. However, other researchers suggested a membrane delimited pathway of (N-type and P/Q-type) Ca²⁺ channel modulation by G protein-coupled neurotransmitter receptors including M2 receptors. G-protein βγ dimmers can directly bind to and inhibit these Ca²⁺ channels by shifting their gating toward depolarizing membrane potential [Herlitze et al., 1996; Ikeda 1996; Shapiro et al., 1999]. N-type and P/Q-type Ca²⁺ channels were suggested to activate SK channels mediating mAHP during motoneuron firing [Bayliss et al., 1995; Li & Bennett 2007]. The activation of postsynaptic M2 receptors at C-terminals sites in motoneuron could lead to a reduction of Ca²⁺ influx through N-type and P/Q-type channels resulting in a decreased mAHP via SK channels. However, the sub-cellular localization of these Ca²⁺ channels in motoneurons is not defined yet. At the other hand, this fast modulation by G-protein βγ dimmers binding to ion channels might also occur to SK channels at the same sites.

SK channels have multiple potential phosphorylation sites and the direct phosphorylation of cloned and native SK2 channel by protein kinase A (PKA) causes an internalization of these channels from cell surface membrane [Kohler et al., 1996; Ren et al., 2006; Lin et al., 2008; Faber et al., 2008]. Though PKA activity was found to be inhibited by M2 activation [Santafe et al., 2006], other phosphorylation pathways were also observed to be initiated by the activation of M2 receptors [Zhou et al., 2003], so there exist a possibility that direct phosphorylation of SK channels is involved in muscarinic/ acetylcholine regulation in motoneurons.

Constitutively bound calmodulin is a substrate for CK2 and its phosphorylation may reduce SK channel Ca²⁺ sensitivity [Bildl et al., 2004; Allen 2007].
Neurotransmitters such as noradrenaline was found to inhibit SK2 channels by a CK2 dependent reduction of calcium sensitivity of channel gating in dorsal root ganglion cells [Maingret et al., 2008]. Muscarine/acetylcholine might decrease the calcium gating of SK channels in the same way through calmodulin phosphorylation by CK2.

As a conclusion, our results of SK localization at C-terminal synapses indicate an important role of postsynaptic M2 muscarinic receptors in the AHP modulation of motoneurons. However, the detailed molecular signaling pathway of acetylcholine/muscarine regulation of motoneuron excitability and firing properties is yet to be fully defined in future study.

So far the expression of SK channels in normal adult spinal motoneurons has been studied. Postnatal maturation of motoneuron functions and their changes induced by axonal injury are two important areas in neuroscience. How motoneuron firing properties develop after birth and what changes occur after the axon is injured are fundamental questions need to be clarified. Since SK channels mediate mAHP, investigating SK channels in neonatal motoneurons and injured motoneurons will help answer these questions. Aim IV and aim V will focus on SK channels in developing and injured motoneurons respectively.
Table 4  Tissue, fixation, sectioning and antibodies for immunofluoresence in Aim III

<table>
<thead>
<tr>
<th>Dual immunofluorescence for SK3 and Synaptophysin</th>
<th>Tissue</th>
<th>Fixation</th>
<th>Sections</th>
<th>Primary Abs</th>
<th>Secondary Abs</th>
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<tbody>
<tr>
<td>Rat spinal cord</td>
<td>4% paraformaldehyde with 0 hour post-fix</td>
<td>20 µm on-slice sections</td>
<td>SK3 (rabbit 1:100)¹</td>
<td>FITC anti-mouse IgM 1:200</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Synaptophysin (mouse 1:200)²</td>
<td>Cy3 anti-rabbit IgG 1:300</td>
<td></td>
</tr>
<tr>
<td>Dual immunofluorescence for SK3 and VChT</td>
<td>Rat spinal cord</td>
<td>4% paraformaldehyde with 0 hour post-fix</td>
<td>40 µm floating sections</td>
<td>SK3 (rabbit 1:100)¹</td>
<td>FITC anti-goat IgG 1:200</td>
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<td></td>
<td>VChT (goat 1:5,000)³</td>
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<td>Dual immunofluorescence for SK3 and VGLUT1</td>
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<td>VGLUT2 (Guinea Pig 1:5,000)⁴</td>
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<td></td>
<td></td>
<td>Kv2.1 (mouse 1:200)⁷</td>
<td>Cy3 anti-rabbit IgG 1:300</td>
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</table>

¹ Alomone; Jerusalem
² Calbiochem/Oncogene; Cambridge, MA
³ BD Pharmingen; San Diego, CA
⁴ Chemicon; USA
⁵ Dr. J. Pearson; Wright State University, Dayton, OH
⁶ Alexis; San Diego, CA
⁷ Upstate; Lake Placid, NY
⁸ Jackson ImmunoResearch Laboratories; West Grove, PA
Figure 6-1 Synaptic localization of SK3-IR clusters in rat spinal motoneurons.

A. One spinal motoneuron (asterisk) in rat spinal cord is surrounded by presynaptic boutons labeled with synaptophysin (green).
B. The same motoneuron expresses SK3-IR clusters (red) in the surface membrane of its soma and proximal dendrites.
C. Merging image of the immunofluorescence for synaptophysin and SK3 shows a close correspondence between presynaptic boutons and postsynaptic SK3-IR clusters. Each postsynaptic SK3-IR cluster is closely apposed by a synaptophysin-IR presynaptic terminal although some presynaptic boutons on the motoneuron are not associated with any SK3-IR clusters.
Synaptophysin

SK3

*  *  *

merging

A

B

C

merging

10.0μm

10.0μm
Figure 6-2 Postsynaptic SK3-IR clusters in rat spinal motoneurons are specifically located at cholinergic synapses apposed to presynaptic C-terminals.

A. One motoneuron cell body is surrounded by presynaptic cholinergic C-terminals labeled with immunofluorescence for VACht (green).
B. The same motoneuron expresses SK3-IR clusters (red) in its surface membrane.
C. The merging image of the immunofluorescence for VACht and SK3 shows the close apposition of the two immunolabeling indicating that SK-IR clusters are postsynaptically associated with presynaptic C-terminals.
D. The analysis of VACht and SK3 staining intensity across one SK3-IR cluster/C-terminal indicated by the white line in C. The measurement of the fluorescence intensity shows the two labeling closely appose to each other.
Figure 6-3 At ultra-structural level, SK3-IR is restricted to the postsynaptic membrane at cholinergic synapses in spinal motoneurons.

A. Electron microscopy image of one cholinergic synapses on motoneuron proximal dendrite (M) formed by presynaptic large C-terminal (asterisk), which contains spherical synaptic vesicles. This type of synapse is characterized of sub-surface cistern. Distinct membrane labeling (arrows) of SK3 is postsynaptic and associated with subsurface cisternae. Some cytoplasmic labeling is visible in the motoneuron close to the synaptic membrane (within 0.5μm).

B. Another C-terminal (asterisk) forming synapse on motoneuron soma (M) contains spherical synaptic vesicles and expresses characteristic subsurface cistern. Distinct membrane labeling of SK3 is clearly postsynaptic (arrow).

C. One more C-terminal (asterisk) synapse on motoneuron is postsynaptically labeled with SK3 (arrows). There is some cytoplasmic labeling around the synaptic site.

Notably, it appears that the immunolabeled synaptic sites illustrated in these electronmicrographs are closely associated with one or more mitochondria. The significance of the presence of mitochondria so close to the region of SK3-IR is unclear, but it is known that mitochondria may serve as an intracellular calcium store, same as the characteristic sub-surface cistern at these synapses.
Figure 6-4 Postsynaptic SK2-IR clusters are located at cholinergic synapses in mouse spinal motoneurons.

Same as SK3 channels, SK2-IR clusters are also associated with presynaptic cholinergic C-terminals in the surface membrane of spinal motoneuron soma and proximal dendrites.

A. One motoneuron cell body is surrounded by presynaptic cholinergic C-terminals labeled with immunofluorescence for VACHT (green).

B. The same motoneuron expresses SK2-IR clusters (red) in its surface membrane.

C. The merging image of the immunofluorescence for VACHT and SK2 shows the close apposition of the two immunolabeling indicating that SK2-IR clusters are postsynaptically associated with presynaptic C-terminals.

D. The analysis of VACHT and SK2 staining intensity across one SK2-IR cluster/C-terminal indicated by the white line in C. The measurement of the fluorescence intensity shows the two labeling closely appose to each other.
Figure 6-5 SK3-IR clusters are not associated with any glutamatergic, serotonergic or inhibitory (glycinergic/ GABAergic) synapses in rat spinal motoneurons.

A. Dual immunofluorescence for VGLUT1 and SK3 in rat spinal motoneurons. Two motoneuorns (asterisks) expressing SK3-IR clusters (red) are surrounded by glutamatergic presynaptic boutons labeled with VGLUT1 (green). No SK3-IR clusters are associated with any VGLUT1-IR terminals.

B. Dual immunofluorescence for VGLUT2 and SK3 in rat spinal motoneurons. One motoneuron (asterisk) exhibits SK3-IR clusters (red) in the surface membrane of its soma and receives some glutamatergic presynaptic inputs stained with VGLUT2 (green). There is no close apposition of any SK3-IR clusters with VGLUT2-IR terminals. This is a superimposed confocal image and the yellowish spots are not the co-localization of the two immunolabeling in single optical sections.

C. Dual immunofluorescence for 5-HT and SK3 in rat spinal motoneurons. One motoneuron (asterisk) expresses SK3-IR clusters (red) in the surface membrane of its soma and proximal dendrites. Descending serotonergic boutons are labeled with 5-HT (green). No close apposition of SK3-IR clusters with 5-HT-IR terminals is observed. This is a superimposed confocal image and the yellowish spot is not the co-localization of the two immunostaining in single optical sections.

D. Dual immunofluorescence for Gephyrin and SK3 in rat spinal motoneurons. One motoneuron (asterisk) expresses SK3-IR clusters (red) in the surface membrane of its soma and proximal dendrites. Inhibitory glycinergic synapses are labeled with postsynaptic marker Gephyrin (green). There is no overlapping of SK3 with Gephyrin in the surface membrane of the motoneuron. Since this is a superimposed confocal image, a couple of SK3-IR clusters are out of register.
Figure 6-6 Postsynaptic SK3-IR clusters are co-localized with Kv2.1 clusters in spinal motoneurons.

A. Superimposed confocal image of Kv2.1 immunostaining shows three rat spinal motoneurons express Kv2.1 clusters (green) in the surface membrane of their soma and proximal dendrites.

B. The same confocal image with SK3 staining shows that only the smaller motoneuron (astrisk) express SK3-IR clusters (red) whereas two larger ones lack SK3 immunoreactivity.

C. The merging image for Kv2.1 and SK3 immunofluorescence indicates that all SK3-IR clusters are co-localized with Kv2.1-IR clusters showing yellowish color.

D. Single optical section of the SK3-IR motoneuron shown in A-C. The fluorescence intensity of VChT and SK3 staining across one cluster indicated by the white line was quantitatively analyzed (E).

E. The analysis of the fluorescence intensity across the cluster shown by the white line in D indicates the two immunolabeling overlap (co-localize) with each other.
Figure 6-7 Postsynaptic SK2-IR clusters are co-localized with Kv2.1 channel clusters in spinal motoneurons.

Same as SK3-IR clusters, SK2-IR clusters in mouse spinal motoneurons are co-localized with Kv2.1-IR clusters.

A. Confocal image of Kv2.1 immunofluorescence shows that two mouse spinal motoneurons (asterisks) express Kv2.1 clusters (green) in the surface membrane of their soma and proximal dendrites.

B. The same confocal image with SK2 immunostaining shows that the two motoneurons also express SK2-IR clusters (red).

C. The merging image for Kv2.1 and SK2 immunofluorescence indicates that all SK2-IR clusters are co-localized with Kv2.1-IR clusters showing yellowish color.
**A**

* * *

**B**

* * *

---

**C**

* * *

**SK2**

**Kv2.1**

Merging

---

**20.0µm**
Figure 6-8 Quantitative analysis of SK3-IR cluster size in rat spinal motoneurons.

A. Dual immunofluorescence for NeuN and SK3 in the surface membrane of motoneuron. This single optical section of confocal image shows *en face* SK3-IR clusters (red) in the surface membrane of motoneuron cell body (green). The SK3 clusters are aggregates of smaller punctae with discrete bright spots within clusters.

B. The quantitative analysis of SK3 cluster sizes using ImagePro software. The range of cluster area is from 1.03 μm$^2$ to 15.62 μm$^2$ (mean ± SD 6.75 ± 2.68, n=245). This result is comparable to that of large Kv2.1 clusters in rat motoneuron according to the previous results from our lab.

C. Distribution of Kv2.1 cluster size in rat spinal motoneurons. This diagram is from Muennich & Fyffe 2004.
Distribution of SK3 cluster sizes in rat spinal MNs

~ from Muennich & Fyffe 2004 ~
CHAPTER VII Aim IV  Postnatal maturation of SK channels in spinal motoneurons

Introduction

Rat spinal motoeneurons are produced on embryonic E11 to E14 day; about half of generated motoneurons die before birth around E22. The motor behavior is immature at birth and develops rapidly during the first 2 weeks [Vinay et al., 2000]. From birth to P4 or P5, neonates start to be able to lift heads; from then until P12 or P13, pups are able to lift their trunks and walk; from then until P16, they developed adult-like locomotion, and complex motor acts [Geisler et al., 1993]. Similarly, at around P10 mice start to be able to bear weight and walk with their abdomens suspended, which is considered to be motor functionally mature [Jiang 1999].

The ionic currents underlying the motoneuron intrinsic membrane properties are very important for motor function. Understanding how the ion channels mediating these currents develop after birth will shed light on the mechanism underlying the postnatal maturation of motoneuron properties. Medium AHP is an important determinant for motoneuron firing properties and SK channels are believed to mediate mAHP, so studying the development of SK channels in motoneurons will provide us insight into the maturation of spinal motor control.

Based on the results in aim I and III, SK2 and SK3 channels form clusters at cholinergic synapses over the surface membrane of soma and proximal dendrites in
motoneurons, where SK clusters are co-localized with voltage gated Kv2.1 channels and M2 receptors. The mechanism of this specific synaptic organization is unknown, but it is supposed to play an important role in determining the intrinsic membrane properties of motoneurons. This aim will focus on the postnatal development of SK channel clusters at cholinergic synapses, which is expected to shed light on the mechanism underlying the maturation of motoneuron intrinsic properties.

The synaptic inputs onto spinal motoneurons also mature during early postnatal period. There are inappropriate monosynaptic contacts from primary afferent fibers on rat spinal motoneurons at birth and around seven days (P7) the majority synaptic connections are appropriate [Vinay 2000]. Similarly, cholinergic synapses formed by C-terminals on spinal motoneurons show a postnatal development which corresponds to the maturation of pup’s motor function [Wilson 2004; Ronnevi 1979]. If the formation of SK clusters parallels the development of the cholinergic C-type synapses in motoneurons will be investigated in this aim. At the same time, Kv2.1 channels will also be examined at distinct postnatal ages. The expected results will provide valuable information for understanding the mechanism underlying postnatal maturation of motoneuron firing properties.

**Methods & materials**

Neonatal and adult CBA mice of both sexes will be used for this *in vivo* study. Lumbar spinal cords of animals at distinct postnatal ages will be harvested for the immunohistochemical analysis. The day of birth is considered P0, and the animals will
be grouped in one of the following age categories: P1-2, P5-6, P8-10, P13-14 and adult (> 6 weeks). The protocols for tissue preparation and immunohistochemistry are described in Chapter III.

The primary antibodies against ionic channels will be used including anti-SK2 (Alomone; rabbit 1:100), anti-SK3 (Alomone; rabbit 1:100), and anti-Kv2.1 (Upstate Biotechnology; mouse 1:250). In addition, neuronal marker anti-NeuN (neuronal nuclei; Chemicon; mouse 1:400) and presynaptic marker anti-VAChT (vesicular acetylcholine transportor; BD Pharmigen; Goat 1:5,000) will be employed in this study. All these antibodies have been used in previous aims. All immunohistochemical procedures performed in this aim are listed in Table 5. The resulting immunostaining will be examined and imaged on confocal microscopy.

**Results**

The development of SK channels was investigated by studying the immunoreactivity against SK2 and SK3 at distinct postnatal ages. At the same time, the immunoreactivity against VACht and Kv2.1 were also examined in order to illustrate the maturation of pre-synaptic cholinergic C-terminals and post-synaptic Kv2.1 channels in motoneurons respectively.

**1. Postnatal development of SK channels**

The immunoreactivity against SK2 in motoneurons was evident shortly after birth (P1-2) showing discrete punctae over the surface membrane of soma and proximal dendrites of presumed α-motoneurons in lamina IX which were revealed with NeuN
immunoreactivity. Some NeuN-IR presumed α-motoneurons were lack of SK2-IR clusters. At that time the intensity of NeuN immunoreactivity in motoneuorns did not show much difference. Then the number and the size of SK2 clusters in motoneurons increased whereas the size of motoenurons also increased. Gradually immunoreactivity against NeuN in presumed α-motoneurons started to show differences. At P8-10 SK2-IR started to demonstrate differential expression among motoneurons that some motoneurons with weaker NeuN intensity did not exhibit SK2-IR clusters. At P13-14 the SK2 immunoreactivity in motoneurons resembled adult pattern (figure 7-1).

In contrast, no obvious SK3 immunoreactivity is visible after birth though a few SK3-IR clusters were occasionally observed in some motoneurons from P2 to P6, and these clusters were of very low intensity of immunoreactivity. At around P8 the immunoreactivity against SK3 increased and started to focus on a subpopulation of motoneurons with comparatively weaker NeuN immunoreactivity. It was around that time motoneurons began to show differential NeuN intensity. A small portion of presumed α-motoneuron exhibited SK3-IR clusters in the surface membrane of their soma and proximal dendrites whereas others were lacking of SK3. At P10 it was evident that SK3-IR motoneurons were smaller and expressed less NeuN intensity than those lacking SK3. At P13-14 SK3 immunoreactivity looked mature and resembled adult motoneurons (figure 7-2).

2. Postnatal development of VACHT-IR C-type synapses in motoneurons

VACHT-labeled cholinergic presynaptic terminals showed small punctea on motoneurons and in the neuropil surrounding motoneurons at birth. At P1-2, SK2-IR clusters in motoneurons were associated with some VACHT-IR bontons; however, the
sizes of these cholinergic terminals were so tiny compared with SK2 clusters that no
close apposition was observed. Then the size and the number of VACHT-IR boutons
contacting motoneurons gradually increased. The close apposition of SK2-IR and SK3-IR
clusters to VACHT-IR cholinergic presynaptic terminals was demonstrated by around P6
and the mismatching was seldom observed at this time. At P13-14 the VACHT-IR C-
terminals showed adult-like distribution and organization and their association with SK
clusters looked mature (figure 7-3; figure 7-4). Our results were consistent with previous
report in mice and rats [Wilson et al., 2004; Wets & Vaughn 2001].

3. Postnatal development of Kv2.1 channels in motoneurons

At birth the immunoreactivity against Kv2.1 channels was shown a
continuous labeling of the surface membrane of motoneuron soma and proximal
dendrites. Then Kv2.1 channels started to form clusters, and at P5-6 Kv2.1-IR clusters
were evident in the surface membrane, and some of the Kv2.1 clusters were co-localized
with SK2-IR clusters. At P8-10 the continuous labeling of membrane disappeared and
there appeared both large and small Kv2.1-IR clusters instead. At that time all observed
SK2-IR and SK3-IR clusters were co-localized with Kv2.1. By P13-14 the
immunoreactivity against Kv2.1 and their association with VACHT-IR C-terminals and
SK channels appeared mature [Muennich & Fyffe 2004] (figure 7-5; figure 7-6).

Discussion

This study showed a different time course of SK2 versus SK3 expression after
birth. SK2 channels were expressed and showed clusters in cytoplasm membrane at birth
whereas SK3 channel expression was not evident. At P8 SK3 expression became evident and showed clusters in cytoplasm membrane. In contrast, voltage gated Kv2.1 channels were expressed at birth but the sub-cellular organization in cell surface membrane was not mature. At P8-10 distribution and organization of Kv2.1 matured showing macro- and micro- clusters in cytoplasm membrane instead of continuous expression. Though the two different types of K+ channels are co-localized at same sites in motoneurons, they show differential development after birth before clustering at cholinergic synapses.

Differential expression of SK channels appeared to become mature-like at around P8-10 according to the immunohistochemical results in this study. This is about the same time that muscle fiber differentiation occurs in rat. The studies of rat hindlimb muscle fibers showed differentiating into slow contracting and fast contracting types at around P7-P12 [Ishihara & Taguchi 1991; Picquet et al., 1997]. Our result suggests a concurrent development of motoneurons and their target muscles that SK channel maturation in motoneurons is in concert with muscle fiber differentiation. Motoneurons that will differentiate into distinct types may start to show differential mAHP at this age. However, whether motoneuron properties guide the differentiation of its’ target muscle fibers or vice versa needs to be clarified in future study. Our result also supports the hypothesis that the differential expression of SK channels in motoneurons is related to motor unit types.

This study examined the postnatal development of both pre- and post-synaptic components at cholinergic synapses in motoneurons. The development of post-synaptic ionic (Kv2.1 & SK) channels occurred in concert with pre-synaptic cholinergic C-terminals contacting motoneurons. At around P10 all three synaptic components showed
close apposition and co-localization. At the same time the pups start to be able to bear weight and walk with their abdomens suspended, which is considered to be motor functionally mature [Jiang 1999]. The time course of the postnatal development of the components at cholinergic synapses corresponds to the maturation of motor function.
Table 5  Tissue, fixation, sectioning and antibodies for immunofluorescence in Aim IV

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<th>Tissue</th>
<th>Fixation</th>
<th>Sections</th>
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<th>Secondary Abs</th>
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<td>20 µm on-slice sections</td>
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¹ Chemicon; USA
² Alomone; Jerusalem or Millipore/Chemicon/Upstate; Temecula, CA
³ NeuroMab; Davis, CA
⁴ BD Pharmingen; San Diego, CA
⁵ Jackson ImmunoResearch Laboratories; West Grove, PA
Figure 7-1 Expression of SK2 channels in postnatal developing mouse motoneurons.

A. Dual immunofluorescence for SK2 and NeuN in spinal motoneurons of P2 mouse. In lamina IX several motoneurons are revealed by NeuN immunoreactivity. Each NeuN-IR motoneuron expresses a few SK2-IR clusters in the surface membrane of soma and promixal dendrites.

B. Dual immunofluorescence for SK2 and NeuN in spinal motoneurons of P5 mouse. The sizes of motoneurons and the number of SK2-IR clusters on each motoneuron increases.

C. Dual immunofluorescence for SK2 and NeuN in spinal motoneurons of P8 mouse. The range of motoneuron sizes increases at this age, so does the NeuN intensity. Most motoneurons express SK2-IR clusters whereas one motoneuron (asterisk) with weaker NeuN staining lacks SK2 immunoreactivity.

D. Dual immunofluorescence for SK2 and NeuN in spinal motoneurons of P10 mouse. The SK2 and NeuN staining is more mature-like that larger motoneurons with brighter NeuN staining express SK2-IR clusters whereas smaller motoneurons (asterisks) with weaker NeuN labeling lack SK2 immunoreactivity.
A. Dual immunofluorescence for SK3 and NeuN in spinal motoneurons of P2 mouse. In lamina IX several motoneurons are revealed by NeuN immunoreactivity. No obvious SK3 immunoreactivity is visible at this age though a few SK3-IR clusters were occasionally observed in motoneurons.

B. Dual immunofluorescence for SK3 and NeuN in spinal motoneurons of P5 mouse. The sizes of motoneurons increases but there is still little SK3 immunoreactivity.

C. Dual immunofluorescence for SK3 and NeuN in spinal motoneurons of P9 mouse. NeuN intensity in motoneurons varies at this age and one motoneuron (asterisk) with weaker NeuN staining expresses SK3-IR clusters while other brighter motoneurons lack SK3 immunoreactivity.

D. Dual immunofluorescence for SK3 and NeuN in spinal motoneurons of P13 mouse. The SK3 and NeuN staining is more mature-like that smaller motoneuron (asterisks) with weaker NeuN intensity express SK3-IR clusters whereas larger motoneurons with brighter NeuN staining lack SK3 immunoreactivity.
Figure 7-3 Postnatal development of presynaptic cholinergic C-terminals and postsynaptic SK2 channels.

A1-3: Dual immunofluorescence for SK2 and VAChT in P2 mouse spinal cord. One motoneuron expresses a few SK2-IR clusters (red) in its surface membrane (A1). Some small VAChT-IR punctae (green) contact this motoneuron (A2). The merging image (A3) shows the mismatching of the two labeling though each SK2-IR cluster is associated with some small VAChT-IR punctae.

B1-3: Dual immunofluorescence for SK2 and VAChT in P5 mouse spinal cord. One motoneuron expresses SK2-IR clusters (red) and receives some VAChT-IR boutons (green). Since the presynaptic boutons are larger compared to P2, the two labeling start to match each other.

C1-3: Dual immunofluorescence for SK2 and VAChT in P8 mouse spinal cord. The size of both postsynaptic SK2-IR clusters (C1) and presynaptic VAChT-IR terminals (C2) are larger, and the two labeling start to show close apposition.

D1-3: Dual immunofluorescence for SK2 and VAChT in P13 mouse spinal cord. The number and size of the presynaptic VAChT-IR boutons (D2) and postsynaptic SK2-IR clusters (D1) are mature-like and the two labeling are closely apposed to each other (D3).
Figure 7-4 Postnatal development of presynaptic cholinergic C-terminals and postsynaptic SK3 channels.

A1-3: Dual immunofluorescence for SK3 and VAChT in P1 mouse spinal cord. One motoneuron expresses few SK3-IR clusters with weak immunoreactivity at this age (A1). Some small VAChT-IR punctae (green) contact this motoneuron (A2).

B1-3: Dual immunofluorescence for SK3 and VAChT in P8 mouse spinal cord. One motoneuron expresses some visible SK3-IR clusters (red) and receives larger presynaptic VAChT-IR boutons (green). The merging image shows the good matching of the two immunolabeling (B3).

C1-3: Dual immunofluorescence for SK3 and VAChT in P10 mouse spinal cord. The size and the number of both postsynaptic SK3-IR clusters (red) in motoneuron and presynaptic VAChT-IR boutons (green) increase, and the two labeling start to show close apposition (C3).

D1-3: Dual immunofluorescence for SK3 and VAChT in P13 mouse spinal cord. The presynaptic VAChT-IR boutons (D2) and postsynaptic SK2-IR clusters (D1) in motoneuron are mature-like and the two labeling are closely apposed to each other (D3).
Figure 7-5 Postnatal development of Kv2.1 channels and SK2 channels in motoneurons.

A1-3: Dual immunofluorescence for SK2 and Kv2.1 in one spinal motoneuron of P2 mouse. The motoneuron expresses a few SK2-IR clusters (A1). Different from SK2, the immunoreactivity against Kv2.1 channels shows a continuous labeling of the surface membrane of motoneuron soma and proximal dendrites (A2). A3 is the merging image of the two immunostaining.

B1-3: Dual immunofluorescence for SK2 and Kv2.1 in one spinal motoneuron of P5 mouse. At this age Kv2.1 channels start to form clusters (B2), and the merging image shows some co-localization of the two channel clusters (B3).

C1-3: Dual immunofluorescence for SK2 and Kv2.1 in one spinal motoneuron of P8 mouse. The continuous labeling of Kv2.1 in cell surface membrane disappears; instead, there appear both large and small Kv2.1-IR clusters (C2). The merging image indicates the co-localization of SK2-IR and Kv2.1-IR clusters (C3).

D1-3: Dual immunofluorescence for SK2 and Kv2.1 in one spinal motoneuron of P13 mouse. At this age both types of ion channels look mature-like and all SK2-IR clusters are co-localized with Kv2.1 clusters (D3).
Figure 7-6 Postnatal development of Kv2.1 channels and SK3 channels in motoneurons.

A1-3: Dual immunofluorescence for SK3 and Kv2.1 in two spinal motoneurons of P1 mouse. The motoneurons express continuous Kv2.1 immunoreactivity in their surface membrane (A2). There is little SK3 immunoreactivity at this age (A1).

B1-3: Dual immunofluorescence for SK3 and Kv2.1 in one spinal motoneuron of P6 mouse. SK3 immunoreactivity (B1) is still weak and Kv2.1 channels start to form clusters (B2).

C1-3: Dual immunofluorescence for SK3 and Kv2.1 in one spinal motoneuron of P8 mouse. The motoneuron expresses visible SK3-IR clusters in its surface membrane (C1). Kv2.1 immunoreactivity shows both large and small clusters in the surface membrane of motoneuron soma and proximal dendrites.

D1-3: Dual immunofluorescence for SK3 and Kv2.1 in one spinal motoneuron of P13 mouse. Both types of ion channel clusters are mature-like and all SK3-IR clusters are co-localized with Kv2.1 clusters (D3).
CHAPTER VIII Aim V  Modulation of SK channel expression in spinal motoneurons following axonal injury

Introduction

Motoneurons serve to transmit neuronal commands from central nervous system to skeleton muscles and thus control voluntary movement. Motoneuron axonal injury resulting from peripheral nerve traumas interrupts this pathway and leads to the dysfunction of motor control and muscle atrophy. Understanding the responses of motoneurons to injury and the key factors determining neuron survival and axon regeneration is of great importance for designing pharmaceutical and surgical intervention for recovery. The long-term goal of this study is to define the structural and functional properties underlying the excitability of motoneurons and the changes induced by axonal injury, which will provide insight to help establish effective treatment of traumatic nerve injuries.

The expression, distribution or physiological properties of intrinsic ionic channels in motoneurons are subject to different types of modulation under certain circumstances such as increased neuronal activity and ischemia [Misonou et al., 2006; Misonou et al., 2005]. The modulation of specific ion channels underlying intrinsic currents may lead to the increased excitability and changed firing pattern of motoneurons after axotomy.

Axotomy results in a differential modulation of AHP duration in cat motoneurons that slow (S-type) motoneurons decrease AHP duration whereas fast (F-type)
motoneurons slightly increase AHP duration, leading to a de-differentiation of spinal α-MNs [Kuno et al., 1974; Gustafsson 1979; Gustafsson & Pinter 1984; Foehring et al., 1986]. The duration of AHP in rat motoneurons innervating fast (gastrocnemius) muscles was also observed to increase following axotomy [Gardiner & Seburn 1997]. The gain (slope of the frequency-current curve) was higher in cat axotomized motoneurons than those in normal control [Gustafsson 1979]. A significant reduction of AHP conductance was suggested to play a major role in the altered AHP and discharge pattern in axotomized motoneurons [Gustafsson 1979; Titmus & Faber 1990]. However, the mechanism underlying this modulation is unknown.

The time course of the AHP changes following axon injury has also been studied. The decreased AHP duration in cat axotomized soleus motoneurons was observed within 10-20 days after injury; in contrast, the AHP duration in cat MG motoneurons started to show increase about 4 weeks after axotomy [Kuno et al., 1974]. Similar results in rats were reported. The AHP ½ decay time in rat tibial motoneurons and MG motoneurons did not show changes 3 day and 5 day after nerve crush respectively [Bichler et al., 2007; Nakanishi et al., 2005]. 4 weeks after axotomy the AHP ½ decay time in rat gastrocnemius motoneurons was found to increase [Gardiner & Seburn 1997].

Since SK channels mediate ionic currents underlying medium AHP, axotomy-induced reduction of AHP conductance in motoneurons may result from the down-regulated gene expression, reorganized cell surface localization, and/or altered calcium gating of SK channels. Based on the results in aim I, SK3 channels might be expressed exclusively by slow motoneurons in rats and mice. The reduced AHP in slow motoneurons following axon injury may result from the decreased SK3 channel density.
in cell surface membrane, and/or decreased Ca\textsuperscript{++} sensitivity of SK3 channels. This study will test the hypothesis that the axonal injury induced mAHP reduction in slow motoneurons results from the decreased SK3 expression in cell surface membrane.

The results in previous aims show that SK3 clusters co-localize with Kv2.1 channels in somatic and proximal dendritic membrane of spinal motoneurons. Kv2.1 clusters were found to disperse following neuron activity and ischemic injury [Misonou et al., 2006; Misonou et al., 2005]. How Kv2.1 clusters in spinal motoneurons are modulated after axonal injury will also be tested in this aim. The expected results will shed light on the mechanism underlying the modulation of ionic channels in motoneurons by axonal injury.

**Methods & materials**

8 Adult wistar rats were used for this in vivo study. Lori Goss helped with the surgeries and fluorogold injection. Two survival surgeries were performed in order to make the axotomy model. In the first surgery, fluorogold was injected into left side soleus muscles to retrogradely label soleus motoneurons in lumbar spinal cord. Three days later a second surgery will be performed that left side tibial nerve was isolated and crushed for 5 seconds using forceps. The detailed procedure is described in Chapter III.

At each time points (0, 1, 3, 8 days) following nerve crush 2 animals were transcardially perfused with fixative and the spinal cords were harvested for immunoassay. Anti-SK3 (Alomone; rabbit 1:100) and anti-Kv2.1 (Upstate Biotechnology; mouse 1:250) were used for this *in situ* immunohistochemical study. The resulting
immuno-staining was visualized and imaged on confocal microscopy. The en face SK3 and Kv2.1 clusters were analyzed using ImagePro software. The motoneurons in the right side of the same spinal cord sections were selected for internal normal control. The method of en face clusters analysis is described in Chapter III.

**Results**

We observed a differential regulation of postsynaptic SK clusters and Kv2.1 clusters over the surface membrane of motoneuron soma and proximal dendrites. The presynaptic cholinergic C-terminals did not show apparent stripping/retracting from injured motoneurons.

1. **The modulation of SK3 channel expression in motoneurons after nerve crush**

   The SK3 immunoreactivity in rat lumbar spinal motoneurons at different time points (0, 1, 3, and 8 days) following nerve crush was visualized and imaged using confocal microscopy. From the day of injury till 3 days following axotomy the SK3 clusters in the surface membrane of motoneuron soma and proximal dendrites did not show much difference compared with normal control. Till the 8th day after injury the SK3 clusters were still evident but the sizes were strikingly decreased compared with the normal control. The smaller SK3 clusters did not seem resulting from cluster dissociation because the immunoreactivity did not show any dispersion/diffusion and the number of clusters in each motoneuron was not increased compared with the normal control (figure 8-1).
Motoneurons from both injury (left) side and control (right) side were sampled for this study. The *en face* clusters were analyzed using ImagePro. The mean sizes (µm²) of SK3 clusters at day 0 (mean ± SD 7.2 ± 3.8, n=67), day 1 (mean ± SD 6.5 ± 2.8, n=29), and day 3 (mean ± SD 7.2 ± 3.6, n=76) following injury were not significant different from the control side. The mean cluster sizes (µm²) at day 8 (mean ± SD 3.3 ± 2.1, n=71) after injury were statistically significantly smaller than control side. The sizes of control side SK3 clusters in all 8 animals are not statistically different from each other. See table 6 and figure 8-1 for details.

2. The alteration of Kv2.1 channel expression in motoneurons after nerve crush

In contrast to the comparatively slow modulation of SK3 after injury, Kv2.1 clusters started to show changes hours after nerve crush in the same day of injury. The Kv2.1 clusters showed dispersing and started to distribute diffusely instead of aggregating in the surface membrane of motoneurons. The *en face* cluster areas were analyzed using ImagePro. However the mean cluster area (µm²) at day 0 (mean ± SD 0.27 ± 0.70, n=407) was not statistically different from the control side. From day 1 to day 8 following injury the dispersing of Kv2.1 clusters continued and the average area of Kv2.1 clusters decreased gradually. At day 1, 3 and 8 the Kv2.1 cluster areas (µm²) were (mean ± SD 0.24 ± 0.57, n=482), (mean ± SD 0.25 ± 0.57, n=355), and (mean ± SD 0.17 ± 0.33, n=691) respectively, and they were statistically different from the control side (p<0.05). The sizes of control side Kv2.1 clusters in all 8 animals are not statistically different from each other. See table 7 and figure 8-2 for details.
**Discussion**

This study showed the modulation of Kv2.1 and SK3 channel expression after nerve crush with different time courses and in different ways in spite of the co-localization of the two types of ion channels in motoneurons. Kv2.1 clusters in cell surface membrane started to break up within hours following injury, and the dispersing continues in the first week. At the 8th day the immuoreactivity of Kv2.1 was diffuse and all large clusters disappeared. In contrast, SK3 clusters did not show any change till the 3rd day after axotomy. At the 8th day the average sizes of SK3 clusters were much smaller than the normal control, but the immunoreactivity against SK3 did not show any dispersion/diffusion, suggesting SK3 clusters did not dissociate as Kv2.1 did.

1. **Regulation of Kv2.1 clusters following axotomy**

Recent studies of in vivo or cultured hippocampal neurons showed the dynamic regulation of Kv2.1 localization in cell surface membrane and channel biophysical properties, which plays an important role in the control of neuron excitability. Kv2.1 channels are constitutively maintained highly phosphorylated. Various factors such as neuronal activity, glutamate or muscarine stimulation, and ischemia may induce a graded Kv2.1 dephosphorylation resulting in a dispersion of Kv2.1 clusters in cell surface membrane and a hyperpolarizing shift of channel activation within minutes. These fast modulatory effects involve calcium influx or release from intracellular calcium store and the activation of calcineurin (PP2B) [Misonou et al., 2004, 2005; Mohapatra & Trimmer 2006]. Similar effects were observed in vitro or in vivo spinal motoneurons that Kv2.1 clusters in surface membrane break up after sciatic nerve stimulation and glutamate or muscarine application [unpublished results from Fyffe lab].
Using FRAP and quantum dot tracking techniques O’Connell and colleagues found that Kv2.1 channel is mobile in cell surface membrane but restrained within a dynamic microdomain, perimeter fence, rather than tethering to a static scaffolding protein. Cytoskeletal protein actin may play a role in cluster maintenance and localization [O’Connell et al., 2006]. Kv2.1 channel proteins within clusters may interact with each other by C terminus in a phosphorylation dependent way. The de-phosphorylation could interrupt this interaction leading to cluster dissociation and altered channel voltage-dependent gating properties [Mohapatra & Trimmer 2006].

In this study we observed that axotomy caused Kv2.1 cluster dispersion in motoneurons similar as the effect led by neuron activity, glutamate or acetylcholine/muscarine stimulation, and ischemia. However, the dynamic modulation of Kv2.1 clusters induced by axotomy expressed a slower and longer time course starting from hours and lasting for more than one week following injury. The Kv2.1 channel de-clustering may result from the channel protein de-phosphorylation, and the channel gating properties may be altered at the same time as discussed before. The hyperpolarizing shift of Kv2.1 channels may result in a decrease of neuron excitability and thus provide a protective mechanism from hyper-excitability after injury.

2. Regulation of SK clusters following axotomy

Compared to the faster regulation of Kv2.1 clusters, SK3 clusters did not show changes within 3 days after injury. At the 8th day the sizes of SK3 clusters were much smaller than the normal control and it did not seem to result from the cluster dissociation as Kv2.1 clusters did. The more stable SK clusters than Kv2.1 clusters suggest different mechanisms for channel clustering and regulation at the same microdomains in
motoneuron surface membrane. In contrast to the mobile Kv2.1 proteins within clusters, SK channels may be tethered to scaffolding proteins such as PDZ domain proteins which cluster and organize postsynaptic neurotransmitter receptors and ion channels [Kim & Sheng 2004; O'Connell et al., 2006]. As a result, SK channels do not show lateral mobility as Kv2.1 when they are regulated by injury or any other factors. The smaller SK clusters at 8\textsuperscript{th} day may result from the internalization of channel proteins, but the exact molecular mechanism needs to be clarified in future study.

Our results are in consistent with the electrophysiological observation of no AHP changes within 5 days after axotomy in rat motoneurons [Bichler et al., 2007; Nakanishi et al., 2005]. 4 weeks after axotomy the AHP $\frac{1}{2}$ decay time was shown to change in rat gastrocnemius motoneurons [Gardiner & Seburn 1997]. The immunohistochemical results in this study suggest that AHP conductance in axotomized motoneurons may be decreased as early as 8 days. However, so far there is no available electrophysiological data to confirm this anatomic observation.

Tetrodotoxin (TTX) is a potent neurotoxin that blocks action potentials in nerves by binding to the pores of voltage gated Na\textsuperscript{+} channels in surface membrane. The duration of AHP in cat soleus motoneurons was found to decrease within 8 days after TTX block of soleus nerve, which mimics the transection of soleus nerve [Czeh et al., 1978]. However, the study in rats showed different results that TTX block did not induce axotomy like changes in motoneuron properties [Gardiner & Seburn 1997].
Table 6 Regulation of SK3 cluster sizes in rat spinal motoneurons after axonal injury

<table>
<thead>
<tr>
<th></th>
<th>Control side</th>
<th></th>
<th>Injury side</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD (μm²)</td>
<td>Number of clusters</td>
<td>Mean ± SD(μm²)</td>
<td>Number of clusters</td>
</tr>
<tr>
<td>Day 0</td>
<td>7.1 ± 3.1</td>
<td>n=71</td>
<td>7.2 ± 3.8</td>
<td>n=67</td>
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<tr>
<td>Day 1</td>
<td>6.7 ± 2.3</td>
<td>n=65</td>
<td>6.5 ± 2.8</td>
<td>n=29</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.1 ± 2.7</td>
<td>n=75</td>
<td>7.2 ± 3.6</td>
<td>n=76</td>
</tr>
<tr>
<td>Day 8</td>
<td>7.0 ± 2.7</td>
<td>n=53</td>
<td>3.3 ± 2.1*</td>
<td>n=71</td>
</tr>
</tbody>
</table>

*Significantly different from control side, P<0.001.
Table 7  Regulation of Kv2.1 cluster sizes in rat spinal motoneurons after axonal injury

<table>
<thead>
<tr>
<th></th>
<th>Control side</th>
<th>Injury side</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD (μm²)</td>
<td>Number of clusters</td>
</tr>
<tr>
<td>Day 0</td>
<td>0.32 ± 0.85</td>
<td>n=335</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.32 ± 0.66</td>
<td>n=444</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.39 ± 0.83</td>
<td>n=440</td>
</tr>
<tr>
<td>Day 8</td>
<td>0.33 ± 0.94</td>
<td>n=906</td>
</tr>
</tbody>
</table>

*Significantly different from control side, P<0.05;
** Significantly different from control side, P<0.01;
*** Significantly different from control side, P<0.001.
A. Confocal images of SK3 immunofluorescence in injured motoneurons. Four motoneurons from 4 different animals identified as axonally injured with their fluorogold staining. The left side tibial nerves were crushed in 4 animals, and the spinal cords of the 4 animals were harvested at different time points (0, 1, 3 or 8 days after injury). At Day 0, 1, and 3, the SK3-IR clusters in the surface membrane of motoneuron soma and proximal dendrites do not show much difference compared with control side. At day 8, SK3 clusters are still evident but the sizes are strikingly decreased compared with control side. B. Quantitative analysis of SK3-IR cluster sizes in injured motoneurons. Motoneurons from both injury (left) side and control (right) side were sampled for this study. The en face clusters were analyzed using ImagePro. The mean sizes (μm²) of SK3 clusters at day 0 (mean ± SD 7.2 ± 3.8, n=67), day 1 (mean ± SD 6.5 ± 2.8, n=29), and day 3 (mean ± SD 7.2 ± 3.6, n=76) following injury were not significant different from the control side. The mean cluster sizes at day 8 (mean ± SD 3.3 ± 2.1, n=71) after injury were statistically significantly smaller than control side.
A

Day 0

Day 1

Day 3

Day 8

10 μm

10.5 μm

B

SK3 cluster area (μm²)

Control side

Injury side

Day 0

Day 1

Day 3

Day 8

0

2

4

6

8

Control side

Injury side

Control side

Injury side

*
Figure 8-2 Kv2.1 channels in axonally injured motoneurons.

A. Confocal images of Kv2.1 immunofluorescence in injured motoneurons. Four motoneurons from 4 different animals identified as axonally injured with their fluorogold staining. The left side tibial nerves were crushed in the 4 animals, and the spinal cords of the 4 animals were harvested at different time points (0, 1, 3 or 8 days after injury). The immunofluorescence of Kv2.1 in the 4 motoneurons is shown here. Kv2.1 clusters started to break up hours after nerve crush in the same day of injury. From day 1 to day 8 following injury the dispersing of Kv2.1 clusters continued and the average area of Kv2.1 clusters decreased gradually.

B. Quantitative analysis of Kv2.1-IR cluster sizes in injured motoneurons. The *en face* cluster areas were analyzed using ImagePro. The mean cluster area (μm$^2$) at day 0 (mean ± SD 0.27 ± 0.70, n=407) was not statistically different from the control side. At day 1, 3 and 8 the Kv2.1 cluster areas were statistically different from the control side (p<0.05).
To better understand the molecular basis for mAHP currents and their regulation, we used immunohistochemistry and quantitative confocal imaging techniques to determine the expression and sub-cellular distribution of SK channels in normal, developing, and axonally-injured spinal motoneurons.

Several major findings emerged from my studies.

- Rat and mouse spinal motoneurons differentially express specific SK channel isoforms. Analysis of motoneuron size, intrinsic electrophysiological properties, and target muscle profiles, as well as SK-labeling of neuromuscular junctions on type-identified skeletal muscle fibers, supported my hypothesis that slow motoneurons express SK3 whereas fast motoneurons express SK2. This differential expression pattern may be partly responsible for the different AHP properties in these neurons, although much future study is needed for an explanation of how SK2 and SK3 mediate differential mAHP in distinct types of motoneurons in rodents. Interestingly, I did not observe differential SK expression in cat motoneurons, suggesting that different mechanisms underly differential AHP properties in cat motoneurons.

- I determined that SK2 and SK3 channels form clusters over the surface membrane of the motoneuron soma and proximal dendrites. The SK clusters are postsynaptically localized at cholinergic synapses associated with presynaptic C-terminals and co-localized with Kv2.1 channels and M2 receptors. This observation provides clues for a potential mechanism underlying acetylcholine regulation of motoneuron firing properties; however, the detailed molecular
signaling pathway for acetylcholine/ muscarine modulation needs to be clarified in future study.

- Differential expression of SK2 & SK3 channels in motoneurons becomes apparent around the same time that muscle fiber differentiation occurs. Postnatal development of the key components (Kv2.1, SK, and C-terminal) at cholinergic synapses in motoneurons corresponds to the maturation of motor function.

- Axonal injury induces the modulation of SK3 and Kv2.1 clusters in rat motoneurons with different time courses and in different ways. SK3 expression is dramatically decreased 8 days after injury, which might explain the AHP reduction in slow motoneurons.
The following cartoons demonstrate the SK2 and SK3 channels in normal, developing and axonally injured rat and mouse spinal motoneurons.
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