SPINAL CORD INJURY INDUCES CHANGES IN ION CHANNELS OF RETICULOSPINAL NEURONS IN LARVAL LAMPREY

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ABSTRACT

After spinal cord injury (SCI) the axons of RS neurons are damaged and animals become paralyzed. Adult higher vertebrates, such as mammals, are incapable to regenerate their axons (Schwab and Bartholdi, 1996). In embryonic stage before myelination begins, higher vertebrates can regenerate axons and motor functions can be recovered. In contrast to adult higher vertebrates, adult lower vertebrates, such as lamprey, fish and certain amphibians, can regenerate there axons through SCI site and locomotion can be recovered completely (McClellan, 1998).

After SCI in larval lamprey, RS neurons regenerate their axons (Davis and McClellan, 1994a,b), restore synaptic contacts and most of the original locomotor functions (McClellan, 1998). It was previously demonstrated that calcium influx in RS neurons in culture results in inhibition of neurite outgrowth (Ryan et al., 2007). In addition, RT-PCR data indicated there was a significant down-regulation of calcium and SKKCa channels in RS neurons (McClellan et al., 2008). Thus, there is a hypothesis that injured RS neurons may undergo a number of changes in their electrical properties to maintain relatively low intracellular calcium concentrations and to promote axonal regeneration (Ryan et al., 2007; McClellan et al., 2008).

In the present study we used various pharmacological agents to determine types of ion channels present in uninjured as well as axotomized RS neurons. Also, the effect of the pharmacological on different components of AHP as well as firing properties of the RS neurons was analyzed.

For uninjured RS neurons, blocking calcium channels with various calcium channel blockers significantly reduced or eliminated the sAHP and produced changes in firing patterns in response to applied depolarizing current pulses that mimicked some of the effects of SCI. Furthermore, blocking SKKCa channels with apamin significantly reduced the sAHP compared to control values. However, blocking calcium and SKKCa channels in injured RS neurons did not have an apparent effect on sAHP and firing properties of the neurons. Moreover, computer modeling indicated that the action potential, AHP and firing patterns of axotomized RS neurons could be mimicked by substantially reducing HVA calcium channels and SKKCa channel conductances compared to those in models of uninjured neurons. The model can closely mimic the behavior (firing properties and proportions in the components of AHP) of real neurons before and after SCI. This work provides additional information for understanding changes in ion channel expression in RS neurons that might be important to promote axonal regeneration in higher vertebrates, including humans.

INTRODUCTION

Organization of Locomotor Systems

In vertebrates locomotion is produced by a neural network that has three components (Stein, 1978). 1) Command system is located in the brain and activates spinal central pattern generators (CPG). 2) Pattern generators are located along the spinal cord and generate oscillatory activity. 3) Coordinating system connects oscillators and coordinates locomotion for different body parts. Reticulospinal neurons (RS) receive inputs from locomotor centers in the brain and send axons to spinal cord to initiate locomotion.

Spinal Cord Injury in Higher and Lower Vertebrates

After spinal cord injury (SCI) the axons of RS neurons are damaged and animals become paralyzed. Adult higher vertebrates, such as birds and mammals, are incapable to effectively regenerate their axons in the CNS (Schwab and Bartholdi, 1996). In embryonic stage before myelination begins, higher vertebrates can regenerate CNS axons following SCI and motor functions can be recovered. In contrast to adult higher vertebrates, adult lower vertebrates, such as lamprey, fish and certain amphibians, can regenerate there axons through SCI site and locomotion can be recovered completely (McClellan, 1998).

After SCI in larval lamprey, which is a lower vertebrate, RS neurons regrow their axons and completely restore motor functions (Rovainen, 1976; McClellan, 1992, 1994). However, the RS neurons do not restore the original neural connections in the spinal cord

(Rovainen, 1976; Davis and McClellan, 1994a,b). The majority of RS neurons send axons to all levels of the spinal cord. After the axonal regeneration most of the RS neurons make contacts just below the SCI site (Davis and McClellan, 1994a,b).

Effects of Spinal Cord Injury on Neuronal Properties and Ion Channel Expression

Spinal cord injury (i.e. axotomy) causes a number of morphological, electrophysiological and molecular changes to injured neurons. However, the changes vary between types of neurons as well as organism.

For sympathetic neurons of frogs there is a decrease in both calcium and calcium-activated potassium currents resulting in reduction of the slow afterhyperpolarization (sAHP) (Jassar et al., 1993). In sensory neurons in rat DRG (Baccei and Kocsis et al., 2000; Kim et al., 2001) calcium channels are reduced. Axotomized rat DRG neurons display a decrease in currents for slow inactivating (persistent) sodium currents (pNa), and molecular data suggests a downregulation of these channels (Dib-Hajj et al., 1998, Sleeper et al., 2000). Injured rat DRG neurons also have decreased expression of certain potassium channel subtypes, while other subtypes are unchanged (Ishikawa et al., 1999; Yang et al., 2004).

Effects of Axotomy on Lamprey Neurons

SCI causes some morphological, electrophysiological and molecular changes to lamprey neurons. After SCI in dorsal cells of lamprey spinal cord spike width, action potential amplitude and conduction velocity increase (Yin et al., 1981). In sensory spinal neurons there was a decrease in cell diameter and increase in nuclear diameter when SCI

was <30 mm from cell body (Yin et al 1981). When SCI was performed <500 μm from the cell body of RS neurons dendritic sprouting occurred. On the other hand, distal SCI ~15 mm from the cell body elicits mostly axonal sprouting (Hall and Cohen, 1988).

After spinal cord transection in larval lamprey, RS neurons regenerate their axons for progressively greater distances below the lesion with increasing recovery times (Davis and McClellan, 1994a,b), and there is a gradual recovery of locomotor functions (McClellan, 1998). In addition, behavioral recovery appears to be largely due to axonal regeneration of preexisting RS neurons and not addition of new neurons (Zhang et al., 2002). Moreover, isolated from larval lamprey RS neurons in culture extend neuritis, and manipulations that induce calcium influx through voltage-gated and/or ligand gated channels induce retraction or inhibit neurite outgrowth (Ryan et al., 2007). In addition, at relatively short recovery times (1-3 wks) following axotomy of RS neurons and during the time when these neurons are regenerating their axons, a component of the action potential, that is dependent on calcium influx via HVA calcium channels and activation of calcium-activated potassium channels (SKKCa), was significantly reduced or abolished (McClellan et al., 2008), and RT-PCR data indicated there was a significant down-regulation of these calcium and SKKCa channels (McClellan et al., 2008). At long recovery times (12-16 weeks) electrophysiological properties as well as the level of mRNA returned to normal (McClellan et al., 2008). The above changes at the properties of neurons after axotomy might be important for subsequent regeneration of RS neurons or might be just a response to axotomy.

Specific Background and Rationale for the Present Study

In the present study we used various pharmacological agents to determine types of ion channels present in uninjured as well as axotomized RS neurons. Also, the effect of the pharmacological on different components of AHP as well as firing properties of the RS neurons was analyzed.

Blocking calcium and/or SKKCa channels in uninjured neurons changed firing pattern of the neurons to be more like in injured cells. Moreover, blocking calcium and SKKCa channels significantly reduced sAHP in uninjured cells, but did not have a significant effect on sAHP as well as firing properties of axotomized neurons (1-3 weeks of recovery). In addition, computer modeling of RS neurons and affects of injury was performed for the project. The model shows a correlation with the real data and can closely mimic the behavior (firing properties and proportions in the components of AHP) of real neurons before and after axotomy.

METHODS

Animal Care

Larval sea lamprey (*Petromyzon marinus*) were maintained in 10 gallon aquaria at 23–25°C and used for all neurophysiological experiments. The procedures in the present study have been approved by the Animal Care and Use Committee at the University of Missouri (protocol reference No. 1506-1).

Spinal Cord Hemi-Transections

Lampreys (83-130 mm, n = 22 animals) were anesthetized in tricaine methanosulfonate (MS222, ~100 mg/200 ml; Sigma Chemical Co., St. Louis, MO). Hemi-transections (HTs) were made on the right side of the spinal cord at 10% body length (BL, normalized distance from the head) using iridectomy scissors and fine forceps (Fig. 2A, HT). In the lamprey, descending brain neurons (Fig. 1) can have either ipsilateral or contralateral descending axons, but a large fraction of reticulospinal (RS) neurons have ipsilateral descending axons (Shaw et al., 2001). In contrast, all Müller cells, which are large, identified RS neurons (Fig. 1), on the right (left) side of the brain project descending axons in the right (left) side of the spinal cord (Rovainen, 1979). Thus, after spinal cord hemi-transection, all Müller cells on the right side of the brain were injured and ~80% of the neurons on the left side of the brain were uninjured because of a non-perfect hemi-transection (see Results and McClellan et al., 2008). Thus, we were able to compare the properties of RS neurons with uninjured axons on the left side of the brain with axotomized axons on the right side of the brain. Some animals

had long recovery times (~4 months, 10 animals), while other animals had short recovery times (1-3 weeks, 12 animals).

Dissection and Experimental Set Up

For normal animals or following recovery for animals with spinal cord HTs, brains and spinal cords, up to ~30-40% BL, were removed as previously described (Rouse et al., 1998) and transferred to a recording chamber containing cooled lamprey Ringer's solution (6-9^{o}C, pH 7.4) (McClellan, 1990). A suction electrode (SC2) was used for all preparations with hemi-transections and for ~90% of control preparations (all preparations before MK90). This spinal cord electrode was used to record orthodromic responses to test whether RS neurons were injured or not.

Intracellular Recording

Intracellular microelectrodes were pulled using 1.2 mm fiber filled glass tubing, filled with 5 M KAc (45-70 MΩ), and inserted into a microelectrode holder containing 3 M KCl. The holder was inserted into a probe of an intracellular amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA). To examine the effects of pharmacological blockers, the recording were performed from uninjured and/or axotomized identified Müller cells in the MRRN (B1, B2, B3, B4), MRN (M1, M2, M3) and ARRN (I1, I2). Only neurons, that had membrane resting potential (V_{rest}) less negative than -65 mV, were analyzed (Tables 1-2).

The intracellular recordings were performed in the following modes: (a) In "Bridge mode" the neurons were stimulated with 0.1-10 ms, +10 nA current pulses to

elicit action potentials and to test for orthodromic responses in the spinal cord electrode; (b) In the discontinuous current clamp mode (DCC, 5-6 kHz) a series of 200 ms depolarizing and hyperpolarizing current pulses (≤ 1 nA) were applied to determine membrane resistance ($R_m = \Delta V_m/\Delta I_m$; Table1); (c) In the DCC mode a series of 2s current pulses (1-10 nA) were applied to determine firing properties of the cells; and (d) in the Bridge mode a series of short (0.1-10 ms, +10 nA) current pulses was applied with 10 and 20 Hz frequency to examine possible summation of certain components of the afterpotential (sAHP, see below).

Electrophysiological Properties

The following electrophysiological parameters were measured: (1) Resting membrane potential (V_{rest}) was measured as the baseline when the membrane potential was stable and constant. (2) Action potential amplitude (V_{ap}) was measured as the difference in voltage between V_{rest} and the peak of the action potential. (3) The amplitudes of the fast (fAHP) and slow (sAHP) afterhyperpolarization were determined as the difference in voltage between V_{rest} and the most hyperpolarized point of these components of the AHP. (4) The amplitude of the afterdepolarising potential (ADP) was defined as the difference in voltage between V_{rest} and the most depolarized point after the fAHP. Since the action potential, fAHP, ADP and sAHP occur in rapid succession, their peak amplitudes depend on each other, and sometimes fAHP and ADP were not measurable (McClellan et al., 2008). Thus, if the peak of the fAHP was above and/or the

peak of the ADP was below V_{rest}, these parameters were not measured and not included in the analysis. (5) Membrane resistance (R_m) was calculated as evoked membrane hyperpolarization divided by relatively small applied negative current (typically 0.1 – 0.5 nA) for 200 ms pulses applied in the DCC mode ($Rm = (\Delta V_m/\Delta I_m)$.

Pharmacological Manipulations

To examine the pharmacology of the components of the action potential (AP) and firing properties of uninjured RS neurons, one or more of the following pharmacological agents was added to the bath: (a) 400 μM $NiCl_2$ and 200 μM $CdCl_2$ to block calcium influx through virtually all calcium channels; (b) 2 mM $CoCl_2$ to block calcium channels; (c) 2 μM ω-conotoxin MVIIC (Tocris Bioscience, Ellisville, MO) to block high-voltage-activated (HVA) N- and P/Q-type calcium channels (Bussieres and El Manira, 1999); (d) 2 μM ω-conotoxin GVIA (Tocris Bioscience, Ellisville, MO) to block HVA N-type calcium channels; (e) 5 mM nifedipine (Sigma) to block L-type calcium channels; and (f) 20 μM apamin (Sigma) to block calcium-activated potassium channels (SKKCa). Typically, these drugs were made up in stock solutions at 100X concentration, and an appropriate volume of the stock solution was added to the bath. All the recordings were performed at least 15 minutes after a drug was added to the bath. The effect of some of the above blockers was also examined on axotomized RS neurons. All of the above blockers, besides nifedipine, were made up as a 100X solution in water and were added to the side of the recording chamber. Nifedipine was made up as a 1000X solution in 95% ETOH.

To examine the effect of axotomy on "calcium action potentials", the following drugs were added to the bath: 3 μM tetrodotoxin (TTX) to block voltage-gated sodium channels; and 10 mM tetraethylammonium (TEA) and 5 mM aminopyridine (4-AP) to block several voltage-gated potassium channels. In the DCC mode, a series of increasing amplitude 400 ms depolarizing current pulses was applied to elicit calcium action potentials. All the recordings were performed at least 60 minutes after the drugs were added to the bath. In many of the experiments, 10 mM kynurenic acid was added to reduce the synaptic noise in the preparations. Similar results were obtained with and without kynurenic acid, suggesting that measured activity of the neurons is due to the neuronal properties and not due to effects of modulatory substances.

Statistics

For experiments from animals without spinal HTs, the parameters for the electrophysiological properties of uninjured RS neurons were compared before and after adding various pharmacological agents either by paired t-test, or by Wilcoxon's signed rank test. Statistical significance was assumed when $p \leq 0.05$.

Computer Modeling

Uninjured RS neurons (Fig. 3 and 4) were modeled in SNNAP (www.snnap.uth.tmc.edu) using parameter sets similar to those used for adult lamprey spinal neurons in Ekeberg et al., 1991 for voltage-gated sodium and potassium channels (see Table 3). High voltage-activated HVA calcium and SKKCa channels were added from McClellan et al., 2008 and exact conductances were set up empirically so that the

model would produce AHP and firing patterns similar to real neurons. Moreover, a "slow" and a "fast" calcium ion pools were added to the model with parameters k1=10 and k2=3 for the fast pool and k1=1, k2=5 for the slow pool. These coefficients were set up empirically to mimic behavior real RS neurons. In the real neurons the ADP is not blocked by calcium channel blockers (see Fig. 3A-3D), and therefore persistent sodium channels (pNa) were added the model because they are the best candidates for generating the ADP (Table 3). Low voltage-activated (LVA) calcium channels, which have been shown to be present in spinal neurons (El-Manira et al., 1994), were not included in the model because pharmacological blocking of HVA calcium channels virtually eliminates the sAHP, which is due, in part, to calcium influx during depolarization. To mimic the changes in properties of real RS neurons at short recovery times following axotomy (Fig. 2D), HVA calcium and Ca-activated K channels (SKKCa) were reduced by 90% to produce a relatively small sAHP (Fig. 9B2), and slow-inactivating (persistent) sodium channels (pNa) were substantially reduced to produce no ADP (Fig. 9C2, 9D2 and Table 3).

FIGURE 1: DESCENDING BRAIN NEURONS IN LARVAL LAMPREY

(top) Diagram of the lamprey brain (left) and upper spinal cord (right) showing (upper) labeled descending brain neurons resulting from application of HRP to the spinal cord at 20% body length (BL, normalized distance from the head), and (lower) outlines around cell groups containing descending brain neurons (Swain et al., 1993; Davis and McClellan, 1994b). Note that non-reticular nuclei have been omitted for simplicity. Abbreviations: mesencephalic reticular nucleus (MRN), and anterior (ARRN), middle (MRRN), and posterior (PRRN) rhombencephalic retricular nuclei. (bottom) Enlargements of reticular nuclei showing large, identified reticulospinal (RS) neurons: M1-M3 (MRN), I1-I4 (ARRN), and (B1-B5, Mau (MRRN). (modified from Zhang et al., 2002)

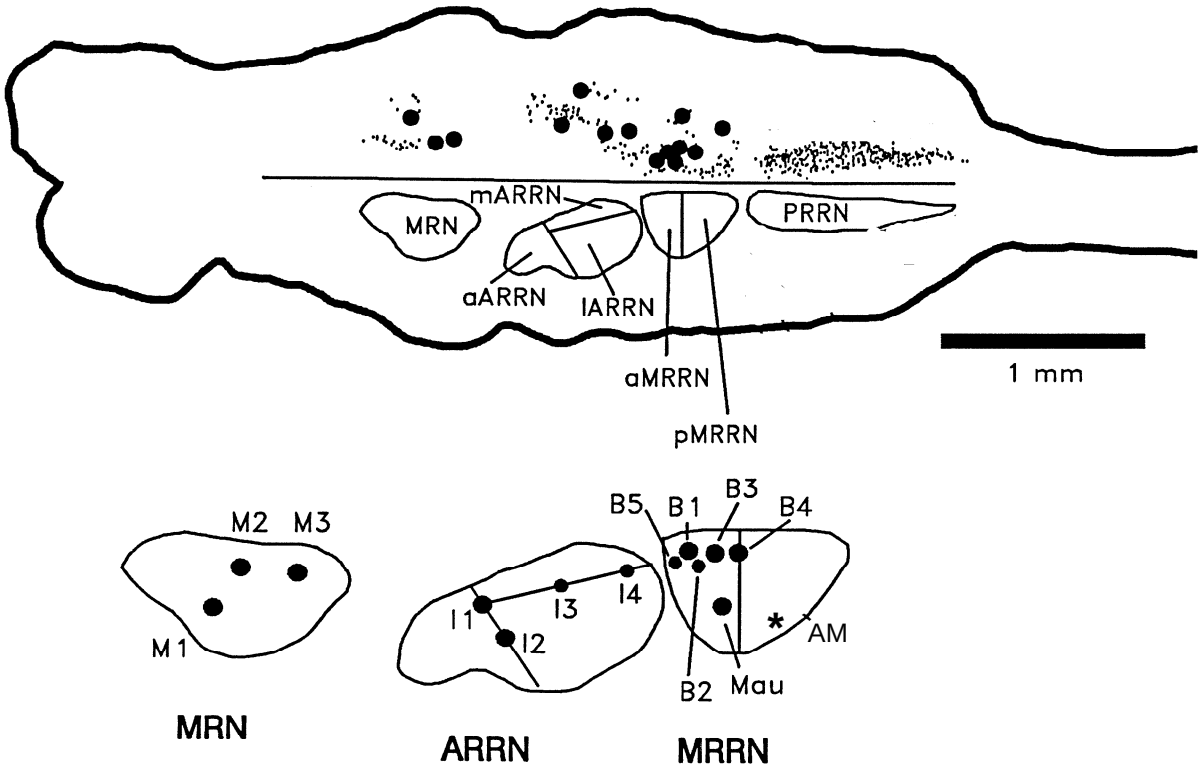
mARRN
MRN
PRRN
aARRN
lARRN
aMRRN
pMRRN
1 mm
M2 M3
M1
MRN
B5 B1 B3 B4
I1 I3 I4
I2
AM
B2 Mau
ARRN
MRRN

Figure 1

FIGURE 2: PROPERTIES OF UNINJURED AND AXOTOMIZED IDENTIFIED RS NEURONS 2 WEEKS AFTER A SPINAL CORD HEMI-TRANSECTION (**A**) Isolated brain/spinal cord preparation, showing the brain (left) and rostral spinal cord, right spinal cord hemi-transection (HT) at 10% BL, intracellular recording micropipette (IC), suction electrode on the caudal spinal cord just below the hemi-transection was used for all preparations with hemi-transections and for ~90% of control preparations (all preps before MK90) (SC2). (**B,C**) Membrane potential (V), current (I), and instantaneous firing frequency (F) of (**B1,C1**) uninjured RS neurons and (**B2,C2**) the same contralateral axotomized neurons in a single preparation in response to depolarizing current pulses: (B) "M2"; (C) "M3". Note "delayed excitation" (DE, arrows) in both uninjured and axotomized "M2" following a hyperpolarizing current pre-pulse. (**D**) Uninjured (black trace) and axotomized (red trace) "M2" Müller cells in the same preparation. The resting membrane potential and action potential amplitude were similar in value in uninjured and axotomized RS neurons. The fAHP (arrow) was significantly larger in axotomized RS neurons ($p \leq 0.05$, Sign test). The ADP and sAHP were present in uninjured RS neurons but sAHP was absent or significantly reduced in axotomized neurons ($p \leq 0.05$, Sign test). (B,C) Vertical scale bar = 100 mV/14 nA/40 Hz; horizontal = 2 s. (D) Vertical/horizontal 2 mV/100 ms. (data from McClellan, 2003; McClellan et al., 2002, 2005).

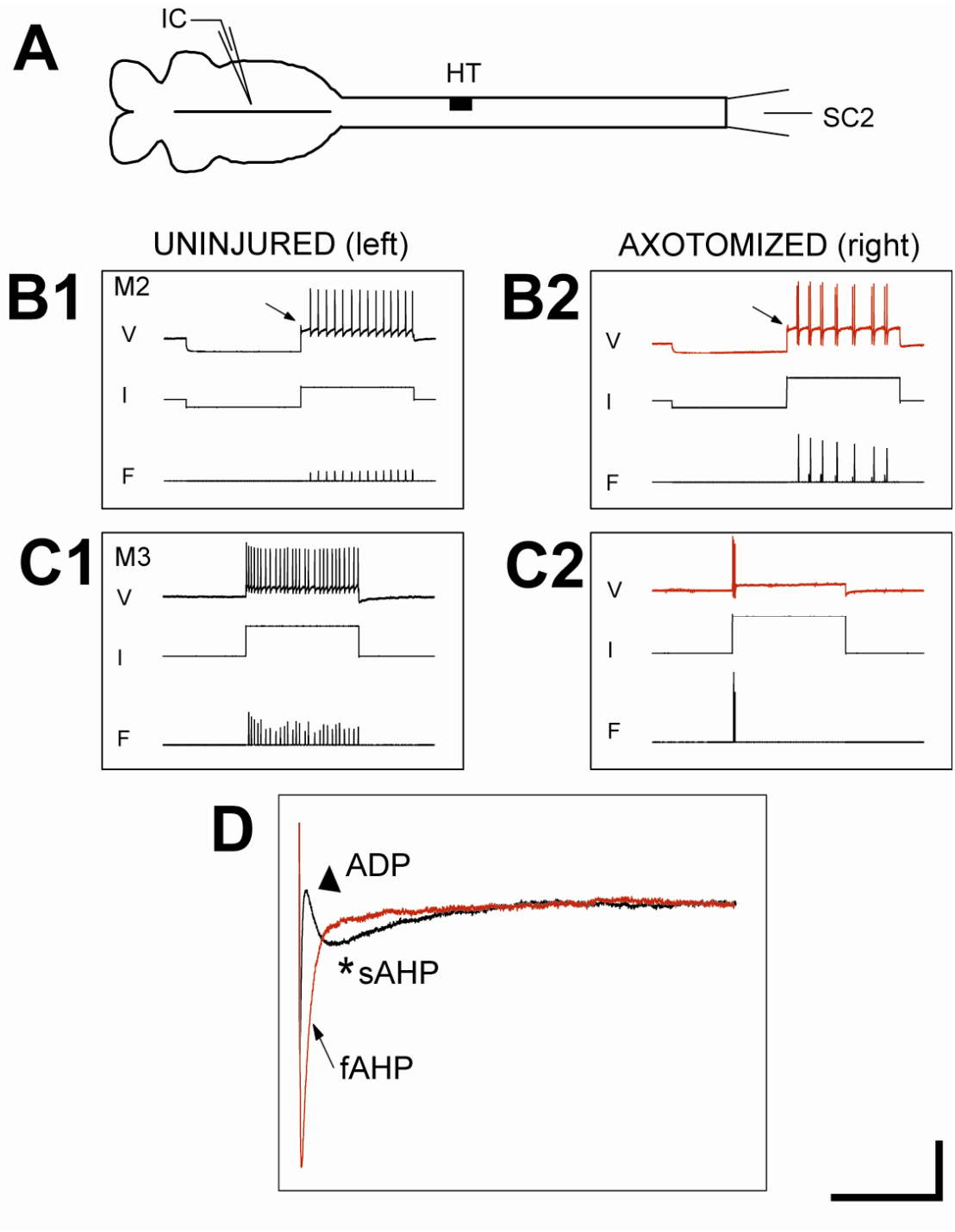
A
IC
HT
SC2
UNINJURED (left)
AXOTOMIZED (right)
B1
M2
V
I
F
B2
V
I
F
C1
M3
V
I
F
C2
V
I
F
D
ADP
*sAHP
fAHP

Figure 2

RESULTS

Pharmacology of sAHP in Uninjured RS Neurons

To identify ion channels contributing to sAHP, the pharmacology of this component was examined in the RS neurons of normal animals that did not receive spinal cord hemi-transections (HTs). Blocking calcium channels with 2 mM cobalt in the bath significantly reduced the sAHP to 8.8±13.2% of control values ($p \leq 0.0001$, paired t test; n=24 neurons) (Fig. 3A). In addition, blocking calcium channels with 400 μM nickel and 200 μM cadmium also significantly reduced the sAHP to 1.1±3.3% of control values ($p \leq 0.01$, Wilcoxon's signed rank test; n=9) (Fig. 3B).

Applying 2 μM ω-conotoxin MVIIC to the bath to block high-voltage-activated (HVA) calcium channels (N- and P/Q-type) also significantly reduced the sAHP to 2.5±5.8% of control values (n=12 neurons; $p \leq 0.001$, Wilcoxon's signed rank test) (Fig. 3C). In addition, application of the specific N-type calcium channel blocker 2 μM ω-conotoxin GVIA also significantly reduced sAHP to 5.6±9.7% of control values (n=11 neurons; $p \leq 0.001$, Wilcoxon's signed rank test) (Fig. 3D). However, the L-type channel blocker 5 mM nifedipine did not have much of an effect on the sAHP (n=11 neurons; $p > 0.05$, unpaired t test) (Fig. 3E). Moreover, blocking SKKCa channels with 20 μM apamin also significantly reduced the sAHP to 8.1±14.1% of control values (n=11; $p \leq 0.0001$, one-sample t test) (Fig. 3F). Finally, application of a series of short repetitive depolarizing pulses (+10 nA, f=10/20 Hz, Fig. 7A1,A2) to uninjured neurons after addition of calcium channels blockers Ni and Cd, cobalt or conotoxins with the above concentrations (n = 21) did not appear to have a significant effect on sAHP. That makes it unlikely that a part of sAHP is due to sodium activated potassium channels. The same

is applicable to axotomized (n=7) RS neurons (Fig. 7B1, 7B2). Although changes in V_{rest} can alter the amplitude of the sAHP and V_{rest} was significantly different before as compared to after addition of 2 mM cobalt ($p \leq 0.002$, paired t-test) (Table 1), this factor did not contribute substantially in the present study because in ~90% of the experiments after addition of the cobalt, nickel and cadmium, conotoxins or apamin, sAHP was close to zero no matter whether V_{rest} increased or decreased as compared to the controls. Thus, in larval lamprey the sAHP of RS neurons is primarily due to calcium influx through N-type calcium channels and activation of SKKCa channels.

Firing Properties of Uninjured RS Neurons Before and After Addition the Blockers

Prior to application of channel blockers, uninjured RS neurons fired a smooth train of action potentials in response to applied depolarizing current pulses (Fig. 4A1 – D1). Addition 400 μM nickel / 200 μM cadmium (n=7 neurons) or 2 mM cobalt (n=16 neurons), both of which are general calcium channel blockers, resulted in changes in firing patterns from a smooth train of action potentials to a single short burst of action potentials (Fig. 4A2, 4B2) in 21 out of 23 cells. When the applied current was increased, a relatively short burst of action potentials was still elicited that did not continue for the entire time of depolarization. Also, application of 2 μM ω-conotoxin MVIIC (n=10 neurons) or 2 μM ω-conotoxin GVIA (n=9 neurons) changed the firing of the uninjured RS neurons from a smooth train of action potentials in ~80% of neurons (~20% were erratic or short bursts) (Fig. 4C1, 4D1) to short bursts (Fig. 4C2, 4D2) or erratic firing in ~70% of the neurons (~30% exhibited smooth firing). With an increase of the applied current, the bursting and erratic firing increased in frequency. Thus, pharmacological

blockade of calcium channels in uninjured RS neurons results in injury-like firing patterns, and these results also supports the hypothesis for a down-regulation of calcium channels in axotomized RS neurons after spinal cord injury in larval lamprey (McClellan et al., 2008).

Pharmacology of the ADP

Interestingly, none of the above calcium channel blockers appeared to have a significant effect on the ADP in uninjured RS neurons (Table 1), suggesting that this component is due to persistent sodium channels (pNa). First, in one experiment after adding 10 mM riluzole, a potential blocking agent for persistent sodium channels (Zhong et al., 2007), the amplitude of the ADP did not change (n=1 neuron) (Fig. 5A). In this experiment, recordings were started ~20 minutes after the drug was added to the bath. In the other 5 experiments, recordings were started one hour after the drug was added to the bath and no recording were made from the neurons before riluzole was added to the bath. ADP in these experiments appears to be reduced (n=5 neurons) (Fig. 5B). However, riluzole might affect the duration of AP and that could mask the ADP. The duration of APs in these cells needs to be measured. Moreover, additional experiments, perhaps with longer exposure to riluzole, will be needed to prove the hypothesis that ADP in lamprey RS neurons is due to persistent sodium channels.

Pharmacology of sAHP in Axotomized RS Neurons

To test the hypothesis that calcium and SKKCa channels are down-regulated in RS neurons after spinal cord injury (McClellan et al., 2008), the calcium channel blockers

400 μM nickel and 200 μM cadmium as well as SKKCa channel blocker apamin were applied to axotomized RS neurons. Thirty minutes later the firing properties of axotomized neurons did not change significantly (Fig. 6A1, 6A2), presumably because calcium and/or SKKCa channels were already down-regulated (McClellan et al., 2008). In addition, there were no apparent changes in the shape of the AHP (Fig. 6A3), and these results also supports the above hypothesis (McClellan et al., 2008).

Computer Modeling

The properties of RS neurons were modeled using SNNAP software and parameters similar to those used to model spinal neurons in adult lamprey (Ekeberg et al., 1991). The model for uninjured RS neurons (Table 3) displayed an increase in the frequency of repetitive firing in response to increases in injected current and spike frequency adaptation (Fig. 8A1, 8A3), similar to properties of real uninjured RS neurons (Rouse et al., 1998). In addition, the model generated single action potentials (Fig. 8A2) as well as components of the AHP (Fig. 8A4; fAHP, ADP, sAHP) that were similar to those of real uninjured RS neurons (McClellan et al., 2006, 2008; Kovalenko et al., 2007).

To mimic the effect of axotomy in lamprey RS neurons, the conductance values for HVA calcium, SKKCa, and/or persistent sodium channels were reduced by 90% (Table 3) (Fig. 9B1,B2). Similar to effect of calcium channel blockers on a real uninjured RS neurons (Fig. 4B2 and Fig. 3A-3D) this model cell displayed a short burst in the very beginning of a long depolarizing pulse (Fig. 9B1) as well as components of AHP (9B2) after a 90% reduction in Ca and SKKCa channels. Also, the model was

successful to show firing properties (Fig. 9C1) and AHP (Fig. 9C2) similar to a real axotomized RS neuron (Fig. 2C2, 2D) after a 90% reduction in calcium, SKKCa and pNa conductances. In the model the reduction only pNa channels by 90% as compares to the model of a normal cell resulted in no change in firing (9D1). However, the shape of AHP (9D2) became like in injured neurons (2D). This result suggests that injury may also affect pNa channels.

In the present model of axotomized neurons that displayed a short, single burst of action potentials, a hyperpolarizing current pulse resulted in rebound action potentials (Fig. 9C1), a property that is similar to that displayed by real axotomized RS neurons (McClellan et al., 2002). Presumably, a brief hyperpolarizing pulse removes inactivation of sodium channels so that when the membrane is returned to a depolarized level, a few action potentials can be generated. When a shorter hyperpolarizing current pulse is applied no resonance occurred suggesting that the model is unable to reproduce all properties of RS neurons.

By changing the parameters in the model for sodium channel activation (B and C in Table 3) from 40 and -40 to 45 and -45, 49 and -49 to 45 and -45 respectively, as well as reducing the conductance for calcium channels by 80%, the model will display a series of short repetitive bursts in response to maintained depolarization and membrane potential oscillations (Fig. 8E1), which is one of the firing types for axotomized RS neurons (Fig. 2B2) (McClellan et al., 2006, 2008). Thus, the model can mimic many of the biophysical properties of real uninjured and axotomized RS neurons.

Calcium Action Potentials

Right spinal cord hemi-transections were made at 10% BL, and at 2 week recovery times, recording were made from right and left RS neurons to examine calcium action potentials (n = 8 neurons; 4 animals). In addition to that calcium action potential were examined in 2 normal animals (n=6 neurons). Voltage gated sodium channels were blocked with 3 μM tetrodotoxin (TTX), and several fast voltage gated potassium channels were blocked with 10 mM TEA and 5 mM 4-AP. For both right (n=8 neurons) and left (n=6) RS neurons, depolarization with 400 ms current pulses resulted in relatively slow depolarizing potentials, followed in some cases by slight hyperpolarization (Fig. 10A,10B). Nickel and cadmium were applied to 12 out of 14 neurons and the calcium action potentials were completely blocked in 11 out of 12 neurons. Thus, it confirms that the action potentials are calcium action potentials. In these experiments, spinal cord electrodes (Fig. 2A) were not used to confirm the presence or absence of a descending axon. However, from previous studies it is known that after a right spinal cord hemi-transection, virtually all RS neurons on the right side of the brain are injured and ~80% of RS neurons on the left side are uninjured (McClellan et al., 2008). Thus, it is reasonable to compare and analyze calcium action potentials from right and left RS neurons as injured versus uninjured neurons, respectively.

In uninjured RS neurons during calcium action potentials, activation of HVA calcium and SKKCa channels have opposing effects on the membrane potential, and it is possible that this results in calcium action potentials that are smaller and shorter than in axotomized neurons. Thus, in axotomized neurons, the net effect of a partial reduction in

HVA calcium and SKKCa conductance might be to generate calcium action potentials that are larger and longer than in uninjured neurons (Fig. 10B). This hypothesis could be tested on uninjured neurons by application of apamin, which blocks SKKCa channels, to determine if the calcium action potentials become longer and larger in amplitude.

FIGURE 3: PHARMACOLOGY OF THE AHP OF UNINJURED RS NEURONS **(A)** AHP in a left B1 neuron before (control) and after application of 2 mM $CoCl_2$ to the bath to block HVA and LVA calcium channels. Note that blocking calcium channels abolished the sAHP but not the fAHP or ADP (n = 17 neurons). (**B**) AHP in a right "B3" neuron before and after application of 200 μM Cd/400 μM Ni to the bath to block HVA and LVA calcium channels. Again, blocking calcium channels abolished the sAHP but not the fAHP or ADP (n = 10 neurons). (**C**) AHP for a left "B2" RS neuron before and after application of 2 μM ω-conotoxin MVIIC to block N- and P/Q-type calcium channels (n = 12 neurons). (**D**) AHP for a left "I1" RS neuron before and after application of 2 μM ω-conotoxin GVIA to block N-type calcium channels (n = 11). (**E**) AHP in a left "B1" neuron before and after application 5 mM of nifedipine to block calcium channels (n = 9 neurons), and after addition of 2 μM ω-conotoxin GVIA to also block N-type calcium channels. (**F**) AHP in a left "B1" RS neuron before and after application of 20 μM apamin, to block Ca-activated K channels (n = 11). (A) Vertical/horizontal scale bars = 7.5 mV/200 ms. (B,C,D,E,F) Vertical/horizontal scale bars = 5 mV/200 ms.

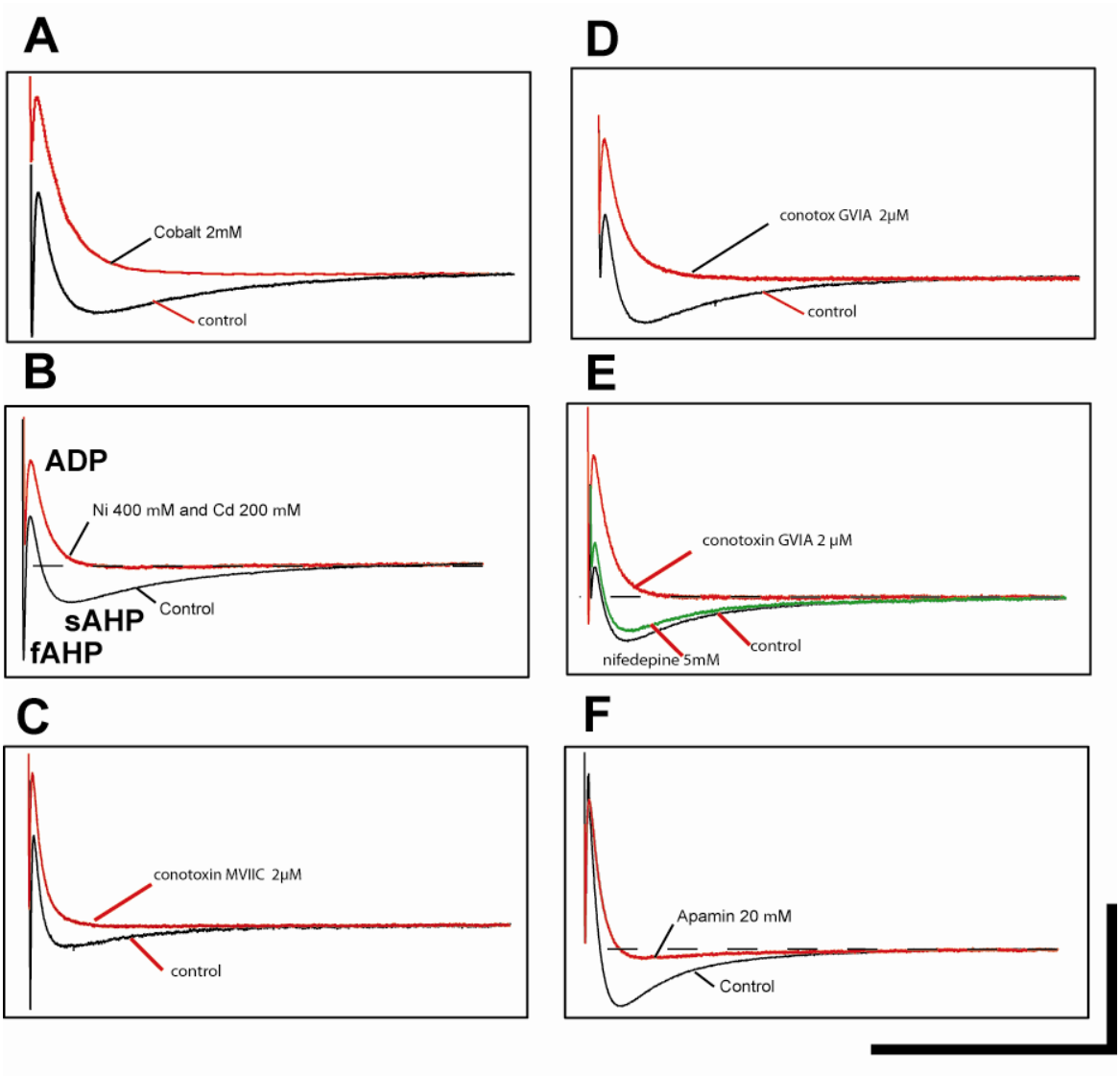
A
Cobalt 2mM
control
D
conotox GVIA 2μM
control
B
ADP
Ni 400 mM and Cd 200 mM
Control
sAHP
fAHP
E
conotoxin GVIA 2 μM
control
nifedepine 5mM
C
conotoxin MVIIC 2μM
control
F
Apamin 20 mM
Control

Figure 3

FIGURE 4: EFFECTS OF PHARMACOLOGICAL AGENTS ON FIRING PROPERTIES OF UNINJURED RS NEURONS (A) Firing of a "B3" cell in response to a depolarizing current pulse (I) before (A1) and after (A2) adding 400 μM nickel and 200 μM cadmium. (B) Firing of a "B1" cell before (B1) and after (B2) adding 2 mM cobalt (same cell as in Fig. 3A). (C) Left "B2" cell before (C1) and after (C2) adding 2 μM ω-conotoxin MVIIC (same cell as in Fig. 3C). (D) Left "I1" cell before (D1) and after (D2) adding 2 μM ω-conotoxin GVIA (same cell as in Fig. 3D). (E) Left "B1" cell before (E1) and after (E2) adding 20 μM apamin (same cell as in Fig. 3E). Vertical = 75 mV/15 nA; Horizontal bar = 2 s.

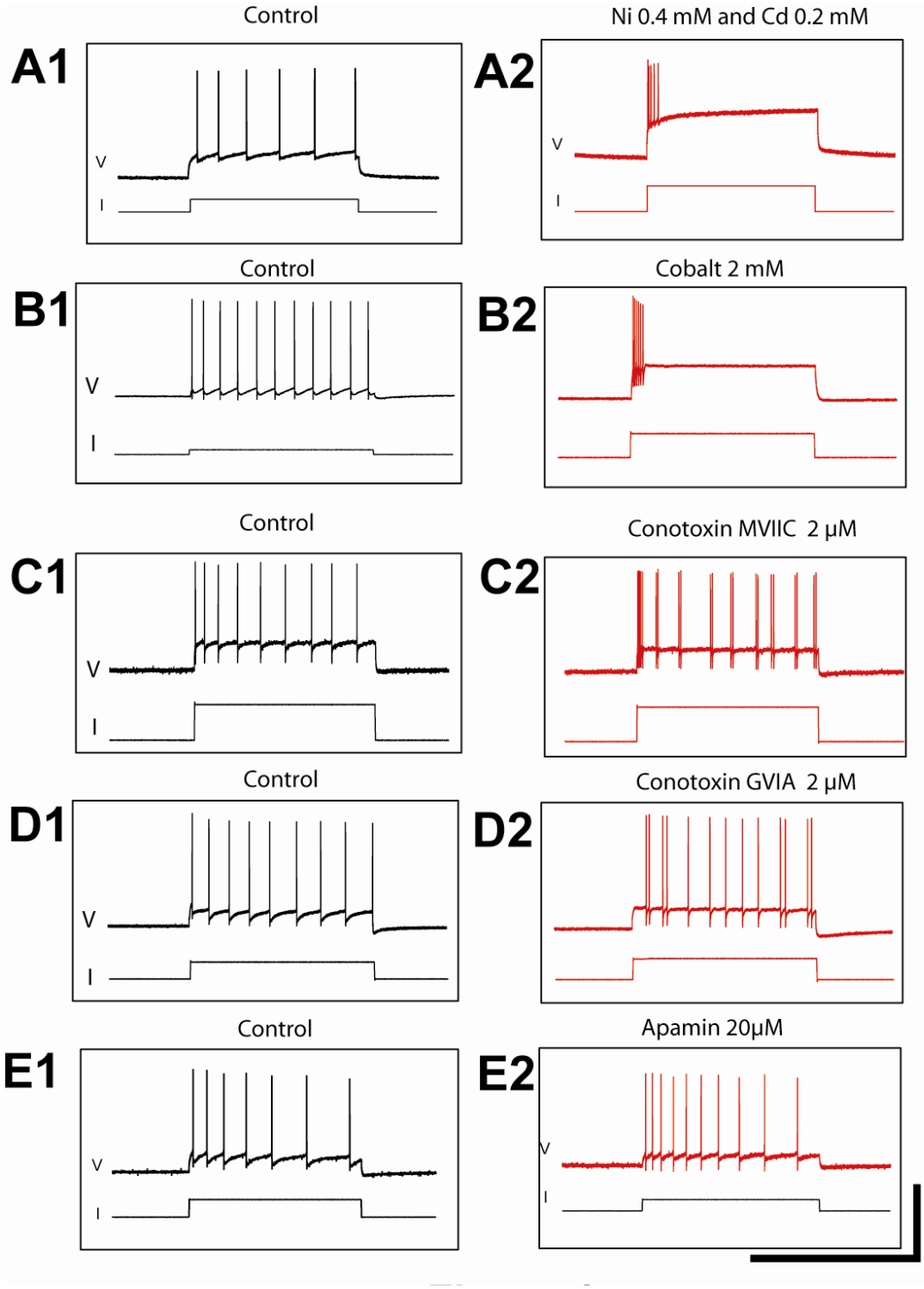
Control
Ni 0.4 mM and Cd 0.2 mM
A1
A2
V
I
Control
Cobalt 2 mM
B1
B2
V
I
Control
Conotoxin MVIIC 2 µM
C1
C2
V
I
Control
Conotoxin GVIA 2 µM
D1
D2
V
I
Control
Apamin 20µM
E1
E2
V
I

Figure 4

FIGURE 5: EFFECT OF RILUZOLE ON ADP IN UNINJURED RS NEURONS
(A) ADP in a "I1" right cell before (control) and ~20 minutes after addition 10 mM riluzole (n=1 neuron). (B) ADP in a B cell ~60 minutes after adding 10 mM riluzole (n=5 neurons).

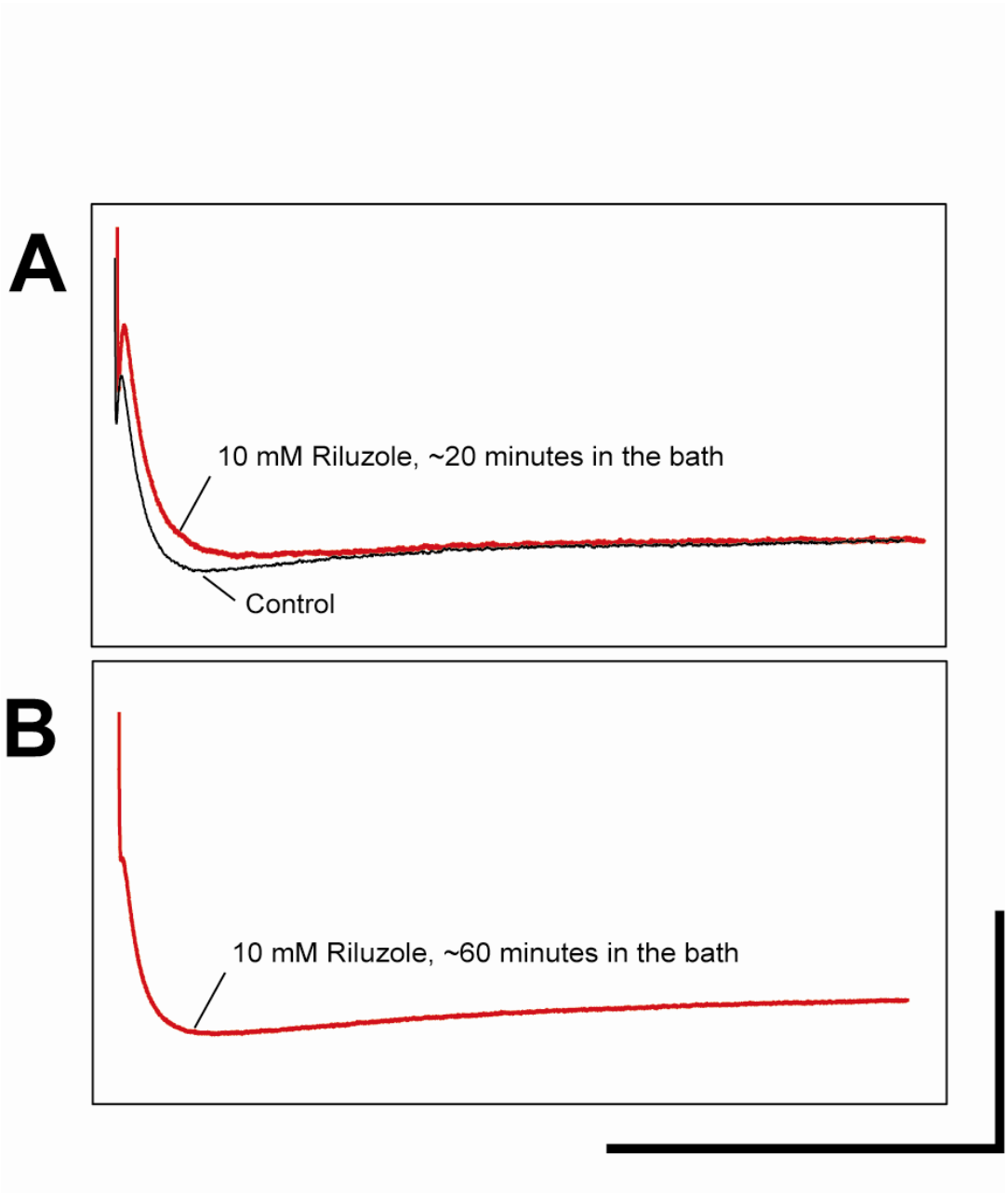
A
10 mM Riluzole, ~20 minutes in the bath
Control
B
10 mM Riluzole, ~60 minutes in the bath

Figure 5

FIGURE 6: PHARMACOLOGY OF AXOTOMIZED RS NEURONS (A1,A2) Axotomized right "B3" cell displayed similar firing in response to depolarizing current pulses (I) before blockers (A1) and after addition of 20 μM apamin, 400 μM $NiCl_2$, and 200 μM $CdCl_2$ in the bath (A2). (A3) The AHP before blockers was reduced slightly with the above blockers in the bath. Vertical/horizontal scale bars = 100 mV, 20 nA/2 s (A1,A2); 5 mV/120 ms (A3).

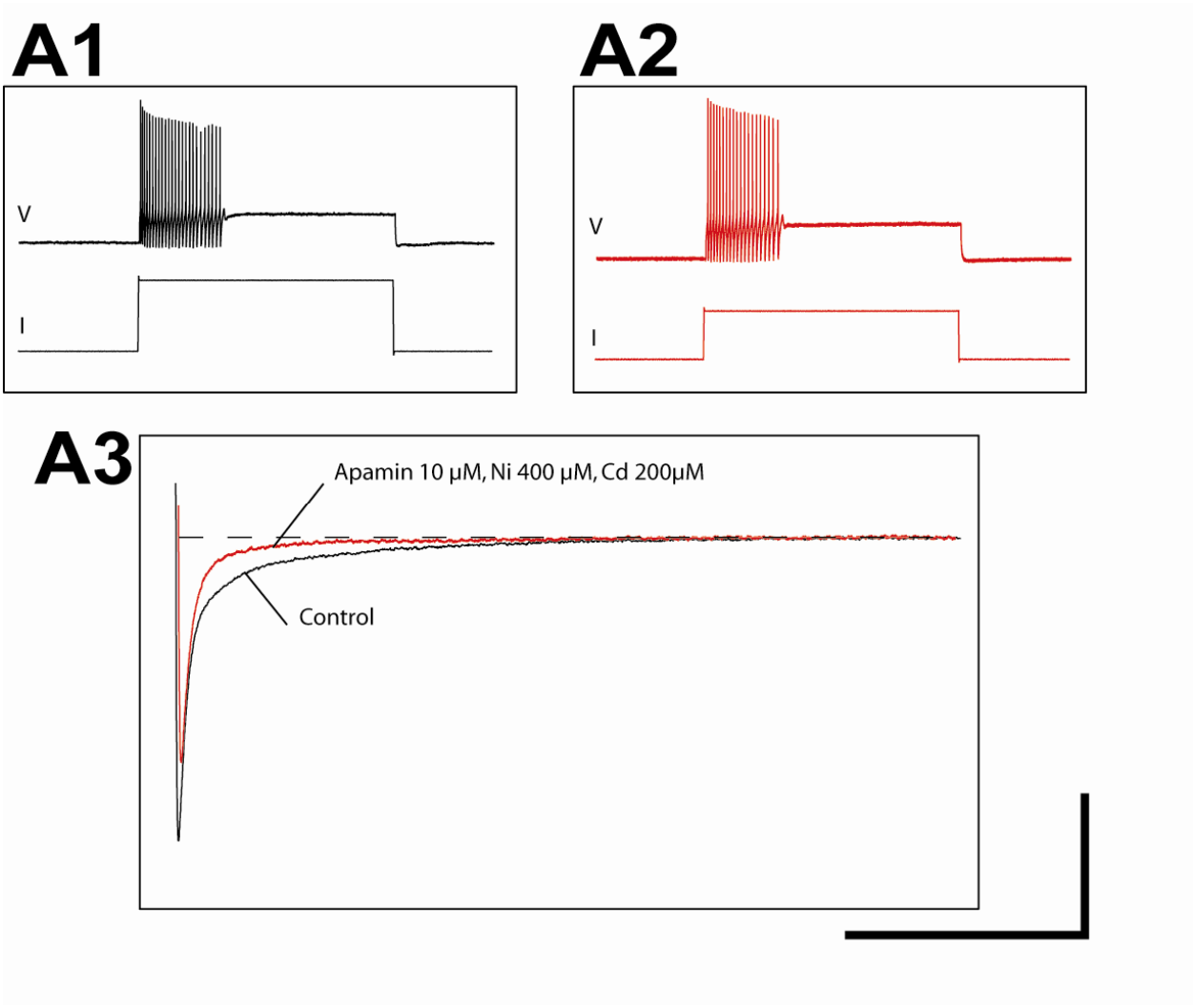

Figure 6

FIGURE 7: EFFECT OF APPLICATION OF A SERIES OF 10/20 Hz SHORT PULSES ON sAHP IN RS NEURONS (A1,A2) Response of an uninjured right "B1" cell to a series of 10 Hz (A1) and 20 Hz (A2) pulses after application of 400 μM Ni and 200 μM Cd. (B1,B2) Response of an axotomized right "B3" cell to a series of 10 Hz (B1) and 20 Hz (B2) pulses after application of 400 μM Ni and 200 μM Cd and apamin 20 μM. There was little summation of the AHP during evoked high-frequency action potential bursting at 10 Hz (A1,B1) and 20 Hz (A2.B2). Vertical/horizontal scale bars = 5 mV/1 s.

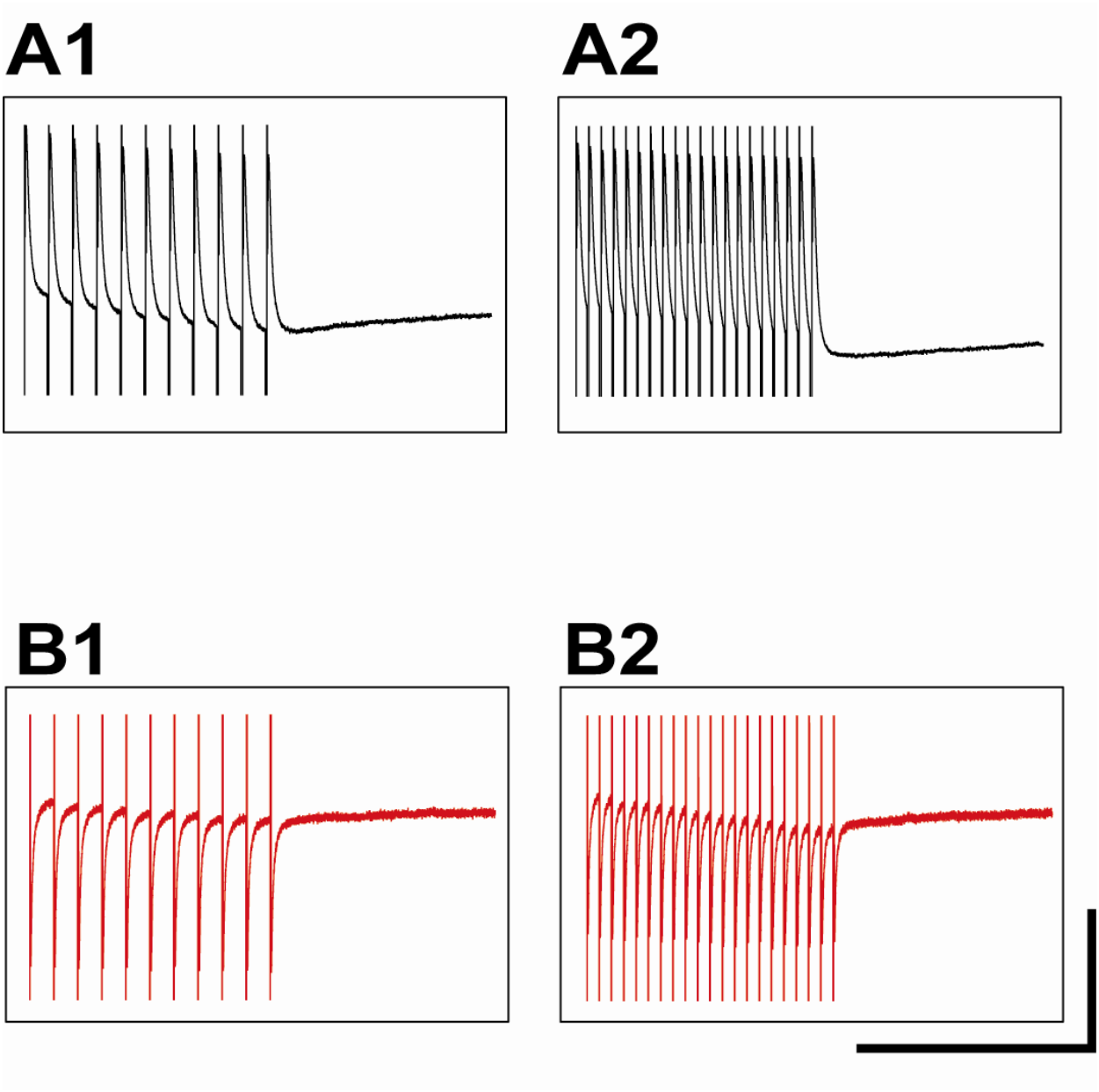

Figure 7

FIGURE 8: COMPUTER MODELING OF UNINJURED RS NEURONS (A) Properties of a computer model for an uninjured RS neuron (see Table 1) showing (A1) continuous, smooth firing pattern in response to a (A1) small and (A3) larger depolarizing current pulse. (A2) Single action potential and (A4) AHP. The afterpolarization had three components: fAHP due to fast K+ channels; ADP due to slow-inactivating sodium channels (pNa); and sAHP due to calcium influx and activation of SKKCa channels. Vertical/horizontal scale bars = (A1, A3) 100 mV, 2 nA/1 s; (A4) 7 /200 ms; (A2) 100 mV/20 ms.

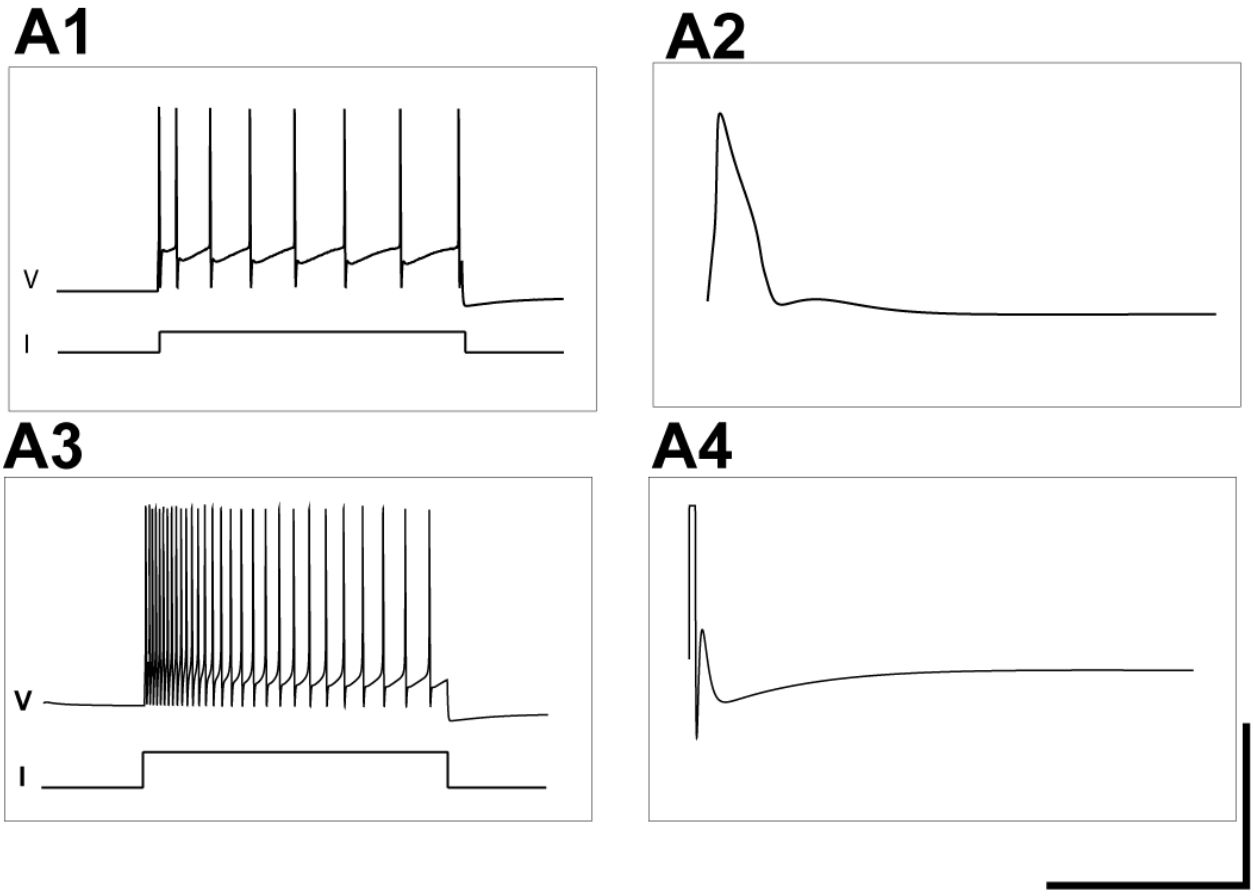

Figure 8

FIGURE 9: COMPUTER MODELING OF AXOTOMIZED RS NEURONS (B) Properties of a model of an axotomized RS neuron (see Table 1) with a 90% reduction in the conductance of HVA-Ca and SKKCa channels showing (B1) brief burst in response to a depolarizing current pulse. (B2) fAHP and ADP but absence of sAHP. (C) Properties of a model axotomized RS neuron with a 90% reduction in the conductance of HVA-Ca, SKKCa, and pNa channels showing (C1) brief initial burst in response to a depolarizing current pulse, and an additional action potential in response to a brief hyperpolarizing pulse resulting from removal of inactivation of sodium channels. (C2) Afterpolarization showing only the fAHP. (D1) Firing of an RS neuron with reduced by 90% conductance of pNa channels as compared to the normal neuron from Figure 8A3. (D2) AHP of the neuron at D1. (E1) A model of a neuron, firing a series of short bursts as compared to a real neuron from Fig. 2B2. (E2) AHP of the neuron from E1. Vertical/horizontal scale bars = (B1, C1, D1, E1) 100 mV, 2 nA/1 s; (B2, C2, D2, E2) 7 mV/200 ms.

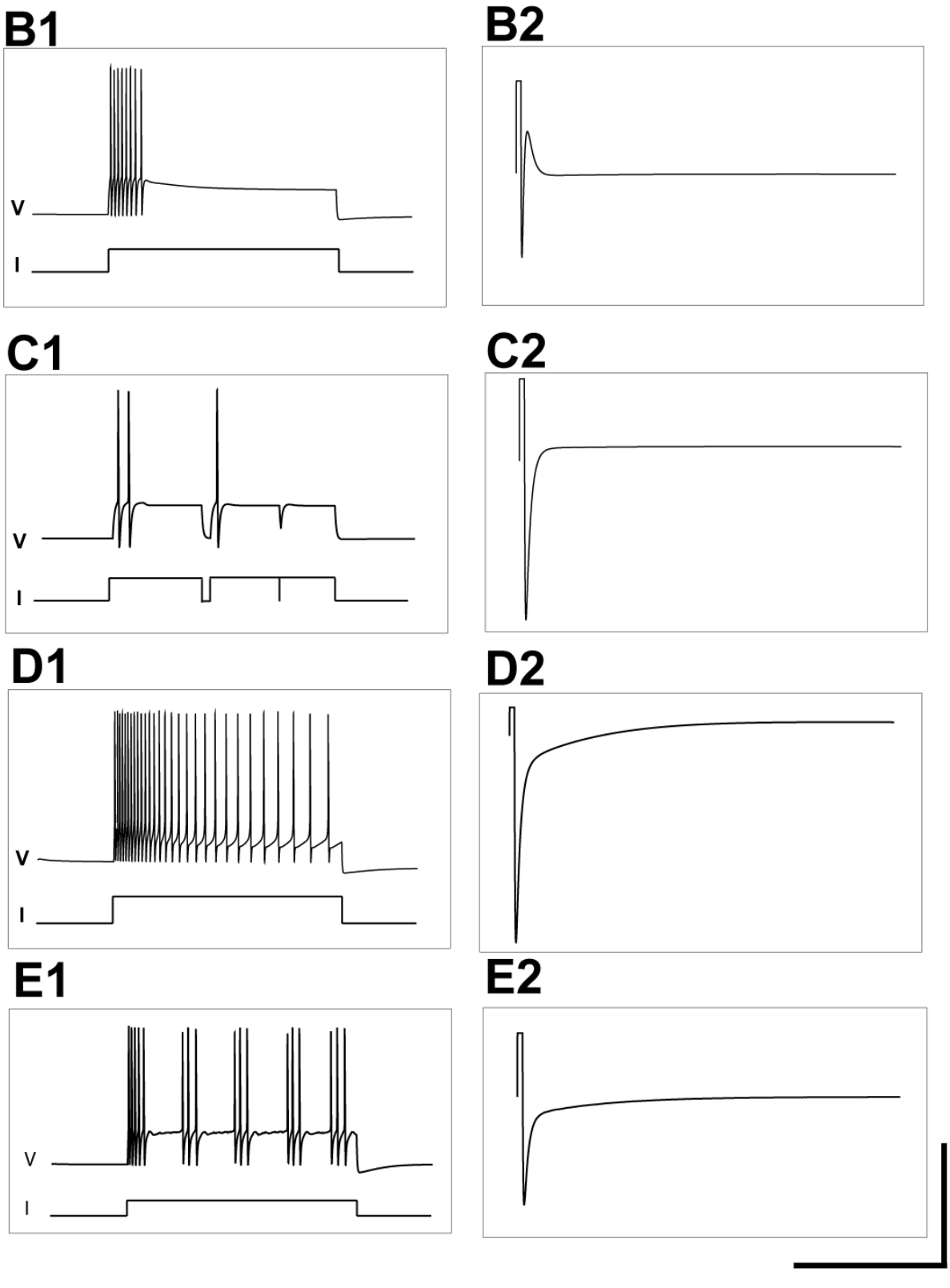

Figure 9

FIGURE 10: CALCIUM ACTION POTENTIALS IN AXOTOMIZED RS NEURONS The bath contained 3 μM TTX to block voltage-gated sodium channels, as well as 30 mM TEA, and 5 mM 4-AP to block several voltage-gated potassium channels (see Methods). (A) Calcium action potentials in a left "B3" cell (~80% chance of being uninjured; see McClellan et al., 2008) evoked in response to a series of increasing depolarizing current pulses. (B) Calcium action potentials evoked in response to a series of increasing depolarizing current pulses in a right axotomized "B3" cell in the same preparation as in (A). Vertical bar = 50 mV/10 nA; Horizontal bar = 200 ms.

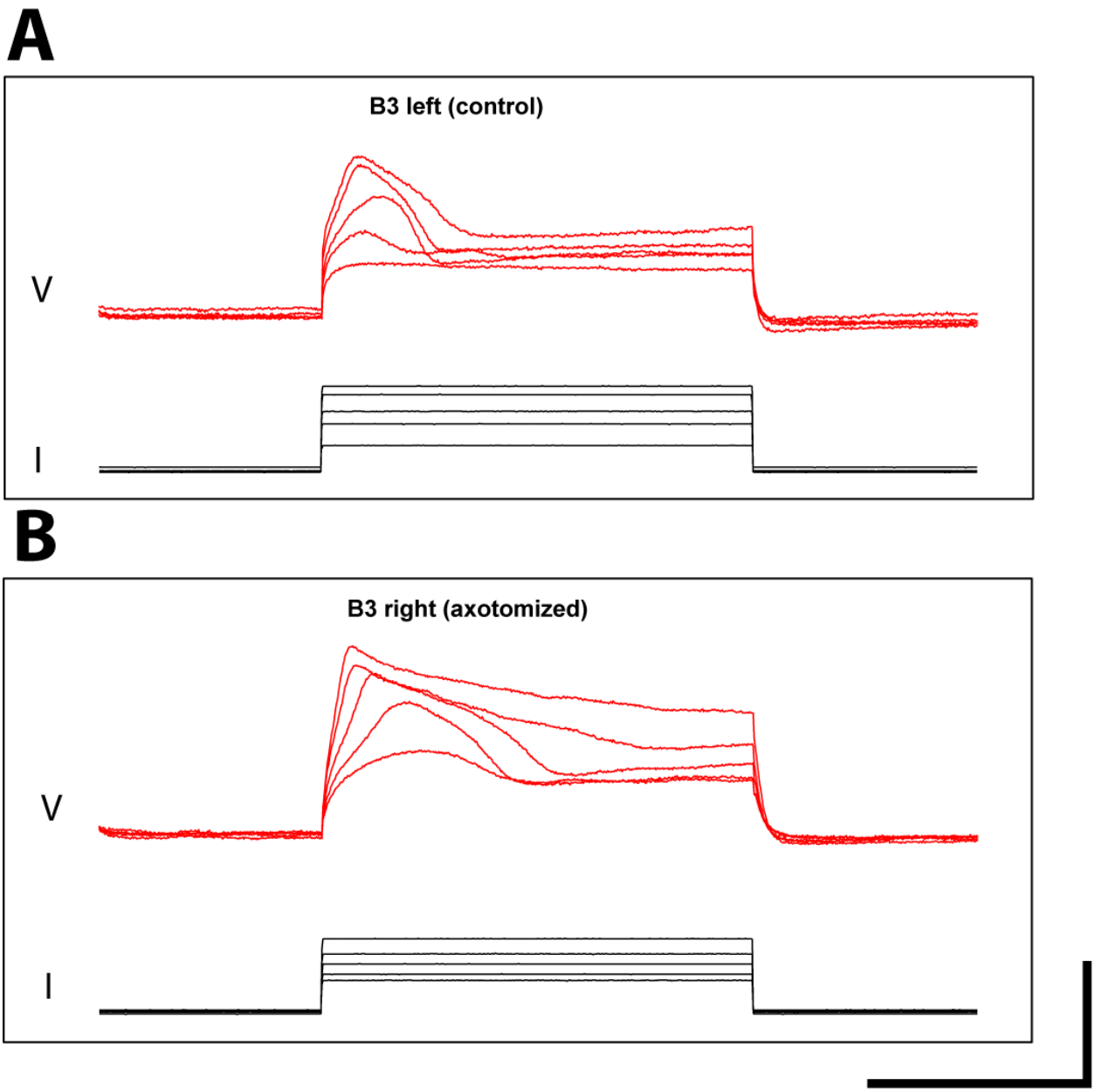

Figure 10

TABLE 1

Electrophysiological properties of Müller cells in normal animals before and after addition of the blockers

	Vrest	Vap	fAHP	ADP	sAHP	Rm
Control (n = 17)	-71.62± 5.08[a]	96.77 ± 8.24	-4.55±2.18	2.50±1.96	-2.06±1.07	7.55±3.63
cobalt (n = 17)	-67.05± .47***	88.44±9.64	-3.6±0	7.15±2.47***	-0.18 ±0.25***	7.21±3.65

	Vrest	Vap	fAHP	ADP	sAHP	Rm
Control (n = 10)	-71.65±3.89	98.3±8.08	-4.56±2.46	3.23±2.30	-2.08±1.73	9.14±4.39
Ni and Cd (n = 10)	-73.55±6.10	104±11.19	-1.32±1.79***	3.64±3.50	-0.26 ±0.78***	8.56±5.84

	Vrest	Vap	fAHP	ADP	sAHP	Rm
Control (n = 11)	-71.41± 4.93	100.72±9.09	-4.07±3.45	3.44±2.75	-1.88±0.91	5.40±1.27
Conotoxin GVIA (n = 11)	-71.95± 4.38	103.45±7.32	-4.66±2.79	3.49±2.56	-0.06 ±0.10***	4.32±0.88***

	Vrest	Vap	fAHP	ADP	sAHP	Rm
Control (n = 12)	-72.27 ± 2.86	103±8.12	-1.36±1.20	4.45±1.69	-0.91±0.43	7.8±4.90
Conotoxin MVIIC (n = 12)	-76.5±4.77	103.2±7.28	-1.72±1.18	5.86±2.94	-0.025 ±0.04***	6.52±3.37

	Vrest	Vap	fAHP	ADP	sAHP	Rin
Control (n = 11)	72.77±2.28	101.54±6.95	-1.88±0.72	3.64±1.94	-1.03±0.49	3.74±1.43
Apamin (n = 11)	71.13±3.18	98.45±5.57	-2.28±3.48	4.14±1.35	-0.07±0.11***	2.74±0.71***

a - Values are means ± S.D. Rm is in MΩ, the rest of the parameters are in mV.
*** - $p<0.002$. Significantly different than control recordings (paired t-test).

TABLE 2

Electrophysiological properties of Müller cells in hemi-transected animals after 4 months of recovery.

	Vrest	Vap	fAHP	ADP	sAHP
Control (n = 21)	-69.35±6.32	99.80±10.18	-3.32±2.99	2.28±1.55	-1.93±1.42

a - Values are means ± S.D (mV).

TABLE 3

PARAMETERS OF THE MODEL

Uninjured / Injured (w/o sAHP) / Injured (w/o ADP and sAHP)

Cell Area and Capacitance		
Dia =	45	μm
Area =	$6.36*10^{-5}$	cm^2
C_m =	$6.36*10^{-5}$	μF

Membrane Time Constant		
τ =	6.36	ms
Passive Leakage Channels		
E_L =	-70	mV
G_L =	0.01	μS

Potassium (K+)			
E_k =	-90	mV	
G_k =	0.18	μS	
	Activation:	α	β
	A =	0.02	-0.005
	B =	31	28
	C =	-31	-28
	D=	0.8	-0.4

HVA Calcium (Ca++)			
E_{Ca} =	150	mV	
G_{Ca} =	0.03 / 0.003 / 0.003	μS	
	Activation:	α	β
	A =	0.08	-0.001
	B =	-10	-10
	C =	10	10
	D =	11	-0.5

Sodium (Na+)			
E_{Na} =	50	mV	
G_{Na} =	1	μS	
	Activation:	α	β
	A =	0.2	-0.06
	B =	40	49
	C =	-40	-49
	D =	1	-20
	Inactivation:	α	β
	A =	-0.08	0.4
	B =	40	-36
	C =	-40	2
	D=	-1	

LVA Calcium (Ca++)			
E_{Ca} =	150	mV	
G_{Ca} =	0	μS	
	Activation:	α	β
	A =	0.02	-0.05
	B =	35	61
	C =	-35	-61
	D =	4.5	4.5
	Inactivation:	α	β
	A =	-0.0001	0.03
	B =	63	-61
	C =	-63	4.8
	D=	-7.8	

Sodium Activated Potassium			
	slow	fast	
E =	-90	-90	mV
G_k =	0	0	μS
k1 =	1	10	
k2 =	5	3	

Calcium Activated Potassium			
	slow	fast	
E =	-90	-90	mV
G_k =	0.01 / 0.001 / 0.001	0.01 / 0.001 / 0.001	μS
k1 =	1	10	
k2 =	5	3	

Slow-Inactivated Sodium (Na+)			
E_{Na} =	50	mV	
G_{Na} =	0.0055 / 0.0055 / 0.0005	μS	
	Activation:	α	β
	A =	0.091	-0.01
	B =	38	38
	C =	-38	-38
	D =	5	-5
	Inactivation:	α	β
	A =	-0.003	0.5
	B =	48	-37
	C =	-48	2.4
	D=	-2.5	

DISCUSSION

Ion Channels Contributing to sAHP in Uninjured vs Axotomized Lamprey RS Neurons

In the present study various pharmacological agents were used to identify ion channels contributing to sAHP in uninjured RS neurons. General calcium channel blockers such as, nickel/cadmium or cobalt significantly reduced or eliminated the sAHP. More specific calcium channel blockers such as ω-conotoxin MVIIC (N and P/Q type) and ω-conotoxin GVIA (N-type) also significantly reduced or eliminated the sAHP. However, the L-type channel blocker nifedipine did not have much of an effect on the sAHP. Moreover, blocking SKKCa channels with apamin also significantly reduced or eliminated the sAHP. Thus, in larval lamprey the sAHP of RS neurons is primarily due to calcium influx through N-type calcium channels and activation of SKKCa channels.

In contrast to uninjured cells, axotomized RS neurons display a significantly reduced sAHP (McClellan et al., 2008). It can be explained by the hypothesis that calcium and SKKCa channels are down-regulated in RS neurons after spinal cord injury (McClellan et al., 2008). To test that the calcium channel blockers nickel/cadmium as well as SKKCa channel blocker apamin were applied to axotomized RS neurons. Thirty minutes later the firing properties of axotomized neurons did not change significantly, presumably because calcium and/or SKKCa channels were already down-regulated (McClellan et al., 2008). In addition, there were no apparent changes in the shape of other components of the AHP, and these results also support the above hypothesis.

Effect of Pharmacological Agents on Firing Properties of Uninjured RS Neurons

Prior to application of channel blockers, uninjured RS neurons fired a smooth train of action potentials in response to applied depolarizing current. Nickel/cadmium or cobalt, both of which are general calcium channel blockers, resulted in changes in firing patterns from a smooth train of action potentials to a single short burst of action potentials in ~90% of the neurons. When the applied current was increased, a relatively short burst of action potentials was still elicited that did not continue for the entire time of depolarization, which is similar to injured RS neurons (McClellan et al., 2008). Also, application of ω-conotoxin MVIIC or ω-conotoxin GVIA changed the firing of the uninjured RS neurons from a smooth train of action potentials in ~80% of neurons (~20% were erratic or short bursts) to short bursts or erratic firing in ~70% of the neurons (~30% exhibited smooth firing). With an increase of the applied current, the bursting and erratic firing increased in frequency. Thus, pharmacological blockade of calcium channels in uninjured RS neurons results in injury-like firing patterns, and these results also supports the hypothesis for a down-regulation of calcium channels in axotomized RS neurons after spinal cord injury in larval lamprey (McClellan et al., 2008). Interestingly, blocking SKKCa channels in uninjured RS neurons with 20 μM apamin increased the frequency of firing but did not have as profound effect on firing pattern in ~ 40% of neurons (Fig 4E1, 4E2). These results suggest that a reduction in the sAHP is not the only factor that alters the firing patterns of axotomized RS neurons.

Riluzole as a Potential Blocker of the ADP

Interestingly, none of the above calcium channel blockers appeared to block the ADP in uninjured RS neurons, suggesting that this component is, probably, due to persistent sodium channels. To test that riluzole, a potential blocking agent for persistent sodium channels (Zhong et al., 2007), was applied to uninjured RS neurons. ADP in these cells appears to be reduced (n=5 neurons) (Fig. 5B). However, riluzole might affect the duration of AP and mask the ADP. The duration of APs in these cells needs to be measured. Moreover, additional experiments, perhaps with longer exposure to riluzole, will be needed to prove the hypothesis that ADP in lamprey RS neurons is due to persistent sodium channels.

Computer Modeling

The properties of RS neurons were modeled using SNNAP software and parameters similar to those used to model spinal neurons in adult lamprey (Ekeberg et al., 1991). The model for uninjured RS neurons displayed an increase in the frequency of repetitive firing in response to increases in injected current and spike frequency adaptation, similar to properties of real uninjured RS neurons (Rouse et al., 1998). In addition, the model generated single action potentials as well as components of the AHP that were similar to those of real uninjured RS neurons (McClellan et al., 2006, 2008; Kovalenko et al., 2007).

To mimic the effect of axotomy in lamprey RS neurons, the conductance values for HVA calcium, SKKCa, and/or persistent sodium channels were reduced by 90%.

Similar effect of axotomy can be obtained by 70 or 80% reduction in the above conductances.

Similar to effect of calcium channel blockers on a real uninjured RS neurons (Fig. 4B2 and Fig. 3A-3D) this model cell displayed a short burst in the very beginning of a long depolarizing pulse (Fig. 9B1) as well as components of AHP (Fig. 9B2) after a 90% reduction in Ca and SKKCa channels. Also, the model was successful to show firing properties (Fig. 9C1) and AHP (Fig. 9C2) similar to a real axotomized RS neuron (Fig. 2C2,2D) after a 90% reduction in calcium, SKKCa and pNa conductances. In the model the reduction only pNa channels by 90% as compares to the model of a normal cell resulted in no change in firing (Fig. 9D1). However, the shape of AHP (Fig. 9D2) became like in injured neurons (Fig. 2D). This result suggests that injury may also affect pNa channels.

Comparison to other vertebrate studies

Axotomy has variable effect on neurons in other model organisms. There are organisms that display similar response to axotomy as shown in the present study. For example, in sympathetic neurons of frogs there calcium and calcium-activated potassium currents decreased after axotomy resulting in reduction sAHP (Jassar et al., 1993). Moreover, sensory neurons in rat DRG (Baccei and Kocsis et al., 2000; Kim et al., 2001) exhibit a reduction in calcium channels after axotomy. Injured rat DRG neurons also display a decrease in currents for slow inactivating (persistent) sodium currents (pNa), and molecular data suggests a downregulation of these channels (Dib-Hajj et al., 1998, Sleeper et al., 2000). Injured rat DRG neurons also have decreased expression of certain

potassium channel subtypes, while other subtypes are unchanged (Ishikawa et al., 1999; Yang et al., 2004).

Conclusion

In lamprey sAHP depends on calcium influx through N-type calcium channels and activation of SKKCa channels. Application of calcium and SKKCa channel blockers did not have an apparent effect on components of AHP and firing properties of injured RS neurons, as compared to uninjured ones. Application of the above blockers to uninjured neurons resulted in injury-like effect on firing of the neurons. These and other findings suggest that SCI results in calcium and/or SKKCa channels downregulation, which might be important for subsequent axonal regeneration.

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