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NEONATAL TISSUE INJURY REDUCES THE INTRINSIC EXCITABILITY OF ADULT MOUSE SUPERFICIAL DORSAL HORN NEURONS

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Abstract—Tissue damage during the neonatal period evokes long-lasting changes in nociceptive processing within the adult spinal cord which contribute to persistent alterations in pain sensitivity. However, it remains unclear if the observed modifications in neuronal activity within the mature superficial dorsal horn (SDH) following early injury reflect shifts in the intrinsic membrane properties of these cells. Therefore, the present study was undertaken to identify the effects of neonatal surgical injury on the intrinsic excitability of both GABAergic and presumed glutamatergic neurons within lamina II of the adult SDH using *in vitro* patch clamp recordings from spinal cord slices prepared from glutamic acid decarboxylase-green fluorescent protein (Gad-GFP) mice. The results demonstrate that hindpaw surgical incision at postnatal day (P) 3 altered the passive membrane properties of both Gad-GFP and adjacent, non-GFP neurons in the mature SDH, as evidenced by decreased membrane resistance and more negative resting potentials in comparison to naïve littermate controls. This was accompanied by a reduction in the prevalence of spontaneous activity within the GABAergic population. Both Gad-GFP and non-GFP neurons displayed a significant elevation in rheobase and decreased instantaneous firing frequency after incision, suggesting that early tissue damage lowers the intrinsic membrane excitability of adult SDH neurons. Isolation of inward-rectifying K^+ (K_{ir}) currents revealed that neonatal incision significantly increased K_{ir} conductance near physiological membrane potentials in GABAergic, but not glutamatergic, lamina II neurons. Overall, these findings suggest that neonatal tissue injury causes a long-term dampening of intrinsic firing across the general population of lamina II interneurons, but the underlying ionic mechanisms may be cell-type specific.

Key words: spinal cord, development, action potential, patch clamp, lamina II, incision.

INTRODUCTION

The processing of noxious stimuli within the CNS begins in the superficial dorsal horn (SDH) of the spinal cord, where a complex network of excitatory and inhibitory interneurons integrates sensory inputs and strongly regulates the output of the spinal pain circuit by modulating the excitability of a small population of neurons which send ascending projections to the brain (Todd, 2010). Mounting evidence suggests that the level of activity within mature dorsal horn neurons is significantly influenced by sensory experience during the early postnatal period. For example, *in vivo* electrophysiological studies using extracellular recordings have demonstrated that skin wounding in the newborn rat leads to enlarged receptive fields in dorsal horn neurons at 6 weeks post-injury (Torsney and Fitzgerald, 2003). Elevated rates of spontaneous activity and exaggerated firing in response to mechanical stimulation have also been reported in the adult dorsal horn *in vivo* after peripheral inflammation during the neonatal period (Peng et al., 2003). This documented hyperexcitability following early tissue damage could be explained by long-term alterations in the balance of synaptic excitation vs. inhibition onto adult SDH neurons and/or modifications in their intrinsic membrane properties which in turn modulate their excitability in a cell-autonomous manner. While *in vivo* extracellular recordings are invaluable in measuring the responses of dorsal horn cells to natural sensory stimuli, this technique cannot distinguish between these potential underlying mechanisms.

Recent studies have focused on identifying changes in synaptic connectivity occurring within the mature SDH network following transient injuries sustained during the neonatal period. Deficits in both phasic and tonic glycinergic transmission have been observed in the adult SDH following neonatal surgical injury (Li et al., 2013a), while stronger descending inhibition to the mature dorsal horn has been reported after peripheral inflammation during early life (Zhang et al., 2010), which may be mediated by a potentiation in opioidergic tone in the CNS (Laprairie and Murphy, 2009). However, it remains unclear whether neonatal tissue damage evokes persistent alterations in the intrinsic firing properties of developing SDH neurons. It is known that

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Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; AP, action potential; C_m, membrane capacitance; E_{rev}, reversal potential; eGFP, enhanced GFP; Gad-GFP, glutamic acid decarboxylase-green fluorescent protein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IF, instantaneous firing frequency; K_{ir}, inward-rectifying K^+; R_m, membrane resistance; SDH, superficial dorsal horn; V_m, membrane potential; V_{rest}, resting membrane potential.

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the intrinsic membrane properties of SDH neurons are developmentally regulated in a cell-type specific manner (Walsh et al., 2009; Li and Baccei, 2011, 2012), and significant changes in the transcription of genes encoding voltage-dependent and voltage-independent ion channels occur during the first postnatal weeks (Blankenship et al., 2013). Given the clear importance of neuronal activity in the modulation of gene expression (Lyons and West, 2011), perturbations in sensory input resulting from injuries during this sensitive developmental period may have long-term consequences for the electrophysiological phenotype of mature SDH neurons.

Therefore, the present study was undertaken to elucidate the persistent effects of neonatal surgical injury on the intrinsic membrane excitability of both inhibitory and presumed excitatory interneurons within lamina II of the adult mouse spinal cord.

EXPERIMENTAL PROCEDURES

Ethical approval

All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee which approved this study.

Hindpaw surgical incision

At postnatal day (P)3, female glutamic acid decarboxylase-green fluorescent protein (Gad-GFP) mice (FVB-Tg(GadGFP)4570Swn; Jackson Labs, Bar Harbor, ME, USA), which express enhanced GFP (eGFP) under the control of the GAD67 promoter (Oliva et al., 2000), were anesthetized with isoflurane (2–3%) and a small incision made through the skin and underlying muscle of the plantar hindpaw as described previously (Brennan et al., 1996). The skin was immediately closed with 7-0 suture (Ethicon, Cornelia, GA, USA) and the wound fully healed in $\leqslant$2 weeks. Females were chosen based on previous work demonstrating that the long-term effects of neonatal injury on pain sensitivity are more pronounced in females (Laprairie and Murphy, 2007).

Preparation of spinal cord slices

At P49–63, Gad-GFP mice were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused with ice-cold dissection solution consisting of (in mM): 250 sucrose, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 6 $MgCl_2$, 0.5 $CaCl_2$, and 25 glucose continuously bubbled with 95% O_2/5% CO_2. The lumbar spinal cord was isolated and immersed in low-melting-point agarose (3% in above solution; Life Technologies, Carlsbad, CA, USA) and parasagittal slices (350–400 μm) were cut from the ipsilateral side using a vibrating microtome (7000smz-2; Campden Instruments, Lafayette, IN, USA). The slices were placed in a chamber filled with oxygenated dissection solution for 30 min then allowed to recover in an oxygenated artificial cerebrospinal fluid (aCSF) solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 1.0 $MgCl_2$, 2.0 $CaCl_2$, and 25 glucose for $\geqslant$1 h at room temperature.

Patch clamp recordings

After recovery, slices were transferred to a submersion-type recording chamber (~0.5 mL volume; RC-22; Warner Instruments, Hamden, CT, USA) and mounted on the stage of an upright microscope (BX51WI; Olympus, Center Valley, PA, USA) which was equipped with fluorescence to allow for the identification of GFP-expressing neurons. Slices were then perfused at room temperature with oxygenated aCSF at a rate of 3–6 ml/min.

Patch electrodes were constructed from thin-walled single-filamented borosilicate glass (1.5 mm outer diameter; World Precision Instruments, Sarasota, FL, USA) using a microelectrode puller (P-97; Sutter Instruments, Novato, CA, USA). Pipette resistances ranged from 4 to 6 MΩ and seal resistances were >1 GΩ. Patch electrodes were filled with a solution containing the following (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 MgATP, and 0.3 Na_2-guanosine-5′-triphosphate (GTP), pH 7.2 (305 mOsm).

Dorsal horn neurons were visualized with infrared-differential interference contrast and patch clamp recordings were obtained from the L4/L5 dorsal horn using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Approximately 1 min after establishment of the whole-cell configuration, the spontaneous firing patterns of dorsal horn neurons were classified at the resting membrane potential (V_{rest}). Membrane capacitance (C_m) was calculated using the built-in pClamp membrane test, while membrane resistance (R_m) was measured using the hyperpolarization produced by a −20-pA current injection from V_{rest}. To characterize the properties of evoked action potential (AP) discharge, intracellular current injections (from −20 to +150 pA in 10-pA increments; 800 ms duration) were applied from V_{rest}. Instantaneous firing frequency (IF) was calculated as 1/interspike interval (ISI), while rheobase was defined as the minimum current step (delivered in 2.5 pA increments at 50-ms duration) which evoked AP discharge.

Inward-rectifying K^+ (K_{ir}) currents were isolated as described previously (Derjean et al., 2003; Li et al., 2013b). Briefly, neurons were voltage-clamped at −55 mV in the presence of 10 μM NBQX, 25 μM AP-5, 10 μM gabazine (GBZ) and 0.5 μM strychnine to block fast synaptic transmission in the slice. Negative voltage ramps (from −55 to −155 mV) were applied at a rate of 0.2 mV/ms. $BaCl_2$ (200 μM) was bath-applied to block K_{ir} (Coetzee et al., 1999) and the Ba^{2+}-sensitive component of the current was subsequently isolated via electronic subtraction (see Fig. 4). Conductance ($g_{Ba\text{-}sensitive}$) was calculated as: $g = I/(V_m - E_{rev})$ at two different membrane potentials (V_m) that were equidistant (25 mV) from the reversal potential (E_{rev}) (Derjean et al., 2003). To estimate the degree of K_{ir} inward rectification,