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Deficits in glycinergic inhibition within adult spinal nociceptive circuits after neonatal tissue damage

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ABSTRACT

Tissue injury during a critical period of early postnatal development can alter pain sensitivity throughout life. However, the degree to which neonatal tissue damage exerts prolonged effects on synaptic signaling within adult spinal nociceptive circuits remains unknown. Here we provide evidence that a transient surgical injury of the hind paw during the neonatal period compromises inhibitory transmission within the adult mouse superficial dorsal horn (SDH), while the same incision occurring during the third week of life failed to evoke these long-term modifications of the SDH synaptic network. The decrease in phasic inhibitory signaling after early tissue damage reflected a selective reduction in glycine receptor (GlyR)-mediated input onto both GABAergic and presumed glutamatergic neurons within lamina II of the adult SDH. Meanwhile, neonatal incision significantly decreased the density of tonic GlyR-mediated current only in the presumed glutamatergic population during adulthood. These persistent changes in synaptic function following early injury occurred in the absence of significant alterations in the transcription of genes known to be important for glycinergic transmission. These findings suggest that aberrant sensory input during early life has permanent consequences for the functional organization of nociceptive synaptic circuits within the adult spinal cord.

1. Introduction

It has been estimated that ~10% of all children will require a stay in a neonatal intensive care unit due to prematurity or other underlying medical conditions. Unfortunately, tissue damage represents an inevitable consequence of their medical treatment, with many infants experiencing >10 invasive procedures per day of hospitalization [7,48]. These repeated interventions result in pain hypersensitivity that can last for weeks in the presence of the injury [14,15,49].

Considerable evidence now suggests that such tissue damage during early life can also evoke more persistent alterations in pain sensitivity in both humans [20,47,56] and rodents [10,30,42]. It is now clear that these long-term changes in pain behaviors are observed only if the injury occurs during a critical period of development, corresponding to the first postnatal week in the rodent [28,42,57]. However, less is known about the cellular and molecular mechanisms that underlie these prolonged shifts in nociceptive processing following neonatal tissue damage. A better understanding of how early injury modifies the functional organization of central nociceptive circuits could provide insight into the emerging link between pediatric and adult chronic pain conditions [54,55].

The persistent effects of neonatal tissue injury may result, at least in part, from alterations at the level of the spinal cord, as peripheral inflammation in the neonate leads to significant changes in gene expression within the adult dorsal horn [43]. In addition, recent work suggests that the ability of early tissue damage to "prime" adult pain responses following repeat injury results from changes in the excitability of the dorsal horn network [5]. Indeed, in vivo electrophysiological studies have shown that mature dorsal horn neurons exhibit enlarged receptive fields and elevated spontaneous firing following injury during the neonatal period [36,52]. However, it remains unclear whether the above changes in spinal nociceptive processing reflect prolonged alterations in synaptic signaling within the superficial dorsal horn (SDH) network.

The present findings indicate that neonatal tissue damage leads to selective deficits in phasic and tonic glycinergic signaling within spinal nociceptive circuits long after the injury has healed. These alterations may contribute to the complex, long-term changes in pain sensitivity that are observed following noxious sensory experience during early life.

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2. Materials and methods

All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.1. Hind paw surgical incision

Female Glutamic acid decarboxylase-Green Fluorescent Protein (Gad-GFP) mice [FVB-Tg(GadGFP)4570Swn, postnatal days 3 or 17; Jackson Laboratory, Bar Harbor, ME, USA], which express enhanced GFP under the control of the GAD67 promoter [35], were anesthetized with isoflurane (2%-3%) and a small incision made through the skin and underlying muscle of the plantar hind paw as described previously [6]. The skin was immediately closed with 5-0 (at P17) or 7-0 (at P3) suture (Ethicon, Cornelia, GA, USA) and the wound fully healed in $\leqslant$2 weeks. Female mice were chosen based on previous work demonstrating that the long-term effects of neonatal injury on pain sensitivity are more pronounced in females [28], along with our prior studies showing that the acute effects of neonatal hind paw incision on synaptic signaling occurred selectively in female Gad-GFP mice [29]. However, it should be noted that the stage of estrous cycle at the time of electrophysiological recording was not accounted for.

2.2. 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 Vibroslice tissue slicer (HA-752; Campden Instruments, Lafayette, IN, USA). The slices were placed in a chamber filled with oxygenated dissection solution for 30 minutes 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 hour at room temperature.

2.3. Patch clamp recordings

After recovery, slices were transferred to a submersion-type recording chamber (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. Voltage-clamp recordings used an intracellular solution containing the following (in mM): 130 Cs-gluconate, 10 CsCl, 10 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid, 11 ethylene glycol tetraacetic acid, 1.0 $CaCl_2$, and 2.0 MgATP, pH 7.2 (300-305 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). Miniature postsynaptic currents (mPSCs) were isolated via the bath application of 500 nM tetrodotoxin (TTX), and miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of −70 mV. Under these conditions, mEPSCs are abolished by bath application of the selective AMPAR antagonist NBQX. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded at a holding potential of 0 mV, thus minimizing the contribution of *N*-methyl-D-aspartate and AMPA/kainate receptor-mediated events [60]. $GABA_AR$-mediated mIPSCs were isolated via the bath application of 0.5 μM strychnine while glycine receptor (GlyR)-mediated mIPSCs were recorded in the presence of 10 μM gabazine. Where indicated, a measurement of overall glycinergic "synaptic drive" onto a given lamina II neuron was calculated as: GlyR mIPSC frequency × GlyR mIPSC amplitude and expressed in units of Hz*pA.

In some experiments, monosynaptic GlyR-mediated IPSCs were electrically evoked via focal stimulation (0-20 μA, 100 μs duration) delivered via a second patch electrode that was connected to a constant-current stimulator (Master-8, Jerusalem, Israel) and placed directly on the soma of a nearby Gad-GFP lamina II neuron under visual control, although it should be noted that it is impossible to conclusively identify the source of the glycine released. GlyR IPSCs were pharmacologically isolated in the presence of 10 μM NBQX and 25 μM AP-5 to block glutamatergic transmission and 10 μM gabazine to block $GABA_AR$ activation. The threshold to evoke an IPSC was defined as the current intensity that evoked a measurable IPSC in $\geqslant$50% of the trials. To determine whether surgical incision altered the probability of glycine release in the dorsal horn, pairs of identical stimuli (at 1.2× threshold at a frequency of 0.10 Hz) were delivered at an interstimulus interval of 100 ms, and the paired-pulse ratio (PPR) was calculated as: PPR = Mean IPSC2/Mean IPSC1. The coefficient of variation of the evoked IPSC amplitudes (30 trials) was defined as SD/mean.

To evaluate the effect of early injury on the expression of tonic GABAergic (I_{GABA}) and glycinergic (I_{Gly}) currents, neurons were voltage-clamped at 0 mV and perfused with TTX, after which strychnine (STR; 0.5 μM) and gabazine (GBZ; 10 μM) were sequentially added to the perfusate. To quantify the mean tonic current, 2 all-points histograms were constructed for each drug condition (TTX, TTX+STR, and TTX+STR+GBZ) using 10-second epochs taken at 2-minute intervals. Each histogram was fitted with a Gaussian distribution, and the mean of the Gaussian fits used to measure the average holding current for a given drug condition [34]. Tonic I_{Gly} was then calculated as the difference in holding current between the TTX and TTX+STR conditions, while tonic I_{GABA} is measured as the difference in holding current between the TTX+STR and TTX+STR+GBZ conditions. The densities of tonic I_{Gly} and I_{GABA} were then calculated by normalizing current amplitude to cell capacitance. However, it should be noted that since tonic $GABA_AR$ currents can be strongly influenced by membrane potential independently of the driving force on anions [40,41], the levels of tonic $GABA_AR$ (and GlyR) conductance measured in our experiments may not necessarily be indicative of those present at membrane potentials close to the resting potential.

Membrane voltages were adjusted for liquid junction potentials calculated using JPCalc software (P. Barry, University of New South Wales, Sydney, Australia; modified for Molecular Devices) unless otherwise specified. Currents were filtered at 4-6 kHz through a −3-dB, 4-pole low-pass Bessel filter, digitally sampled at 20 kHz, and stored on a personal computer (ICT, Cincinnati, OH, USA) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.0 software; Molecular Devices).

2.4. Data analysis and statistics

mPSCs occurring during a 3-minute recording period (up to 300 events) were analyzed via visual inspection using MiniAnalysis (version 6.0.3; Synaptosoft, Decatur, GA, USA). The threshold for