Published in final edited form as:

Pain. 2013 July ; 154(7): 1129–1139. doi:10.1016/j.pain.2013.03.030.

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.

Keywords

Spinal cord; Superficial dorsal horn; Neonatal; Patch clamp; Synapse; Glycine; Development; Incision; Nociception

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

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Conflict of interest statement

The authors declare that they have no conflicts of interest with any of the work presented in this manuscript.

[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.

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 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 NaHCO₃, 1.0 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, and 25 glucose continuously bubbled with 95% O₂/5% CO₂. 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 NaHCO₃, 1.0 NaH₂PO₄, 1.0 MgCl₂, 2.0 CaCl₂, and 25 glucose for 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 M Ω and seal resistances were >1 G Ω . 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 CaCl₂, 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 μ M strychnine while glycine receptor (GlyR)-mediated mIPSCs were recorded in the presence of 10 μ M gabazine. Where indicated, a measurement of overall glycinergic “synaptic drive” onto a given lamina II neuron was calculated as: GlyR mIPSC frequency $\times$ GlyR mIPSC amplitude and expressed in units of Hz $\times$ pA.

In some experiments, monosynaptic GlyR-mediated IPSCs were electrically evoked via focal stimulation (0–20 μ A, 100 μ s 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 μ M NBQX and 25 μ M AP-5 to block glutamatergic transmission and 10 μ M gabazine to block GABA_AR activation. The threshold to evoke an IPSC was defined as the current intensity that evoked a measurable IPSC in $\geq$ 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 $\times$ 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 μ M) and gabazine (GBZ; 10 μ M) 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 mPSC detection was set at twice the mean amplitude of the background noise. The first 50 events in a given neuron were also used to construct cumulative probability distributions of interevent intervals and amplitudes in the Naïve and Incision groups, and between-group comparisons were then made using the Kolmogorov-Smirnov test (MiniAnalysis). Evoked IPSCs were analyzed using Clampfit (Molecular Devices) software. Nonparametric statistical tests (Mann-Whitney test for 2 groups; Prism 5.0 software; Graph-Pad Software, La Jolla, CA, USA) were used in cases in which the distribution of data failed the D'Agostino & Pearson normality test (Prism) or when the number of observations was insufficient ($n < 24$) to definitively conclude that data were distributed in a Gaussian manner. “n” refers to the number of neurons sampled in a given group unless specified otherwise. Data are expressed as means $\pm$ SEM.

2.5. Quantitative polymerase chain reaction (qPCR)

Adult Gad-GFP mice (P49–63) were deeply anesthetized with sodium pentobarbital (60 mg/kg; intraperitoneal) and perfused with ice-cold dissection solution as described above. The lumbar (L4–L5) spinal cord was removed, placed in cold oxygenated dissection solution, and hemisected. The SDH was localized via the band of translucence commonly used to identify the substantia gelatinosa and dissected free with a scalpel, although we cannot completely exclude the possibility that some samples contained tissue from deeper laminae. Any remaining dorsal roots or residual dura mater was then removed and as much white matter was cut away as possible before the tissue was rapidly frozen and stored at -80°C until use.

One microgram of RNA per sample was reverse transcribed with the iScript cDNA kit (Bio-Rad Life Science, Hercules, CA, USA) containing a mixture of random hexamer and oligo(dT) primers according to manufacturer's instructions, under the following thermal cycler conditions: 5 minutes at 25°C , 40 minutes at 45°C , 5 minutes at 85°C , and held at 4°C . Each 20- μL reaction was aliquoted to minimize freeze-thaw cycles of the resultant cDNA and stored at -20°C until use.

Primers were designed with the National Center for Biotechnology Information/Primer-BLAST (Basic Local Alignment Search Tool) tool and BLASTed against the mouse Refseq_mRNA database to test for specificity. Whenever possible, primer pairs were designed to contain at least one sequence that crossed an exon–exon boundary to minimize amplification of genomic DNA. The specificity of all primers was confirmed using quantitative polymerase chain reaction (qPCR) melting point analysis (MxPro, Agilent Technologies, Santa Clara, CA, USA) and gel electrophoresis. The following primers were used: *gephyrin* Forward (F): CATGGCAAATCATGGGCAGCCC, Reverse (R): TGCTGCACCTGGACTGGACTTTT; *1 integrin* (F): GCGGACGCTGCGAAAAGATGA, (R): ACCACCCACAATTTGGCCCT GC; *3 integrin* (F): TGTTCCGGCCATGGGCAATGTAAC, (R): CTTCAT GGAGCGTTCCCCGGTT; *Gira1* (F): TGCACCCCCATAACTCGTGGAC, (R): CCTCTTTGGAAGCAGCAAGGCTGAA; *Gira2* (F): TGGAAAACATCCCTCGCAGACCC, (R): TGTTCACTCGGTAGTCCATGGTGGT; *Gira3* (F): TTTCTGGTCCCCCATGCCTTGGA, (R): TGGCCACCAAACCTCAGTAACAGTGC; *Gira4* (F): GGTCTGCTCAGTGTGGCTTTG, (R): TCGGGCCTTCTCGGCTTCAA; *Girb* (F): TACCTCTGGGCATCTTCT CCGTGC, (R): TCGGGCCTTCTCGGCTTCAA. Primers for several candidate reference genes were designed and tested for expression stability using GeNorm [53] and BestKeeper software [37] under the experimental conditions employed in this study, resulting in the choice of hypoxanthine-guanine phosphoribosyltransferase (HPRT) (F: TCACTGCTTTCCGGAGCGGTA, R: ATCATCGCTAATCACGACGCT GGG) and β -tubulin (F:TCAGTAAACCGTAGCCATGAGGGAA, R: GGACATACTTGCCACCTGTGGCTT) as reference genes.

Each sample was run in sets of 3 technical replicates, using 12.5 ng of cDNA, 0.75 μ L of 10 μ M target-specific primer working solution, 15 μ L of prepared SYBR Green qPCR Master Mix (Roche, Indianapolis, IN, USA), and nucleic-acid free H₂O to a final volume of 25 μ L per reaction. qPCR was performed on a Stratagene MxPro 3500 thermal cycler (Agilent Technologies, Santa Clara, CA, USA) with an initial 95°C Taq activation step for 10 minutes, followed by 40 cycles of the following: 95°C denaturing step for 15 seconds, 59°C annealing step for 30 seconds, and 72°C extension step for 30 seconds, with a fluorescence value recording step (30 seconds) obtained at 76°C after every cycle. Following cycling, samples were evaluated for correct product by melting point analysis and considered contamination-free if no-template controls were negative (MxPro, Agilent Technologies).

Raw fluorescence data were normalized to an internal reference dye (ROX) using MxPro software and imported into LinReg software (Heart Failure Research Center, Amsterdam, The Netherlands) to obtain threshold cycles (C_t), N_0 values, and average plate efficiencies generated from sample amplification kinetics [44]. Replicate outliers were defined as having either a difference of 3 C_t values from the sample mean or efficiency outside of 5% of the plate mean, and were excluded from further analysis. Data were analyzed according to the “gene expression’s C_t difference” formula described previously [46], which incorporates a correction for efficiency in its calculation of fold change in expression. Briefly, the C_t for the target gene in the Incision group was normalized to a reference gene index (the geometric mean of HPRT and β -tubulin expression) from the same sample, then normalized to the same measure in the Naive group. Significant effects of neonatal incision on the expression of a given target were determined using the Mann-Whitney test. A value of $P < 0.05$ was considered significant. “n” refers to the number of animals in a given group. Data are expressed as means $\pm$ SEM.

3. Results

3.1. Surgical injury during the neonatal period decreases miniature inhibitory postsynaptic currents (mIPSCs) in the adult superficial dorsal horn (SDH)

To investigate whether early tissue damage evokes persistent, cell type-dependent alterations in synaptic transmission within mature spinal nociceptive circuits, unilateral hind paw surgical incision [6] was administered at postnatal day (P)3 in Gad-GFP mice, which selectively express enhanced GFP (eGFP) in GABAergic neurons [35]. Naïve littermate-matched controls (handled in an identical manner including exposure to anesthesia) were used for all experiments. At P49-63, in vitro whole-cell patch clamp recordings were obtained from Gad-GFP and adjacent, non-GFP neurons within lamina II of spinal cord slices prepared from the ipsilateral side. It is known that eGFP labels >60% of the GABAergic neurons in the adult SDH of these mice [11]. Given previous estimates that GABAergic cells comprise ~30% of lamina II neurons in the rodent [38,51], along with the observation that glycinergic neurons predominantly represent a subset of the GABAergic population [21,51], one can estimate that the vast majority (>85%) of non-GFP cells will correspond to glutamatergic neurons. mEPSCs (Fig. 1A) and mIPSCs (Fig. 1B) were recorded from the same SDH neurons.

As illustrated in Fig. 1E and F, P3 incision evoked a significant reduction in mIPSC frequency in both GABAergic ($n = 38$; $P = 0.0007$; Mann-Whitney test; Fig. 1E, left) and presumed glutamatergic neurons ($n = 36$; $P = 0.018$; Fig. 1F, left) within the adult SDH compared to naïve littermate controls ($n = 35$), with no accompanying changes in mIPSC amplitude (Fig. 1E and F, right). In contrast, the frequency of mEPSCs onto both Gad-GFP (Fig. 1C, left) and non-GFP lamina II neurons (Fig. 1D, left) during adulthood was unaltered by surgical injury during the neonatal period, although P3 incision did cause a significant increase in mEPSC amplitude within the mature Gad-GFP population (Fig. 1C, right; $P = 0.040$; Mann-Whitney test).

Importantly, hind paw incision at P17 failed to significantly influence either miniature excitatory (Fig. 2A and B) or inhibitory (Fig. 2C and D) transmission within the adult SDH network. These results indicate that tissue injury during the neonatal period leads to a prolonged, widespread deficit in inhibitory synaptic efficacy within spinal nociceptive circuits.

3.2. Early tissue damage selectively compromises glycinergic signaling within the adult SDH

The above alterations in inhibitory transmission after neonatal injury could reflect a decrease in signaling at GABA_ARs and/or GlyRs within the mature dorsal horn. To distinguish between these possibilities, GlyR- or GABA_AR-mediated mIPSCs were pharmacologically isolated (see Methods) in adult Gad-GFP and non-GFP neurons within lamina II following P3 surgical incision. Within the GABAergic population of SDH neurons, neonatal injury significantly reduced the frequency of GlyR-mediated mIPSCs compared to naïve littermate controls (Naïve: 0.27 ± 0.04 Hz, $n = 51$; P3 Incision: 0.13 ± 0.02 Hz, $n = 52$; $P = 0.007$; Mann-Whitney test; Fig. 3A, left), although mIPSC amplitude was increased ($P = 0.048$; Fig. 3A, right). Cumulative probability distributions also illustrated a significant increase in the GlyR mIPSC interevent intervals after incision ($P < 0.0001$; Kolmogorov-Smirnov test; Fig. 3E) along with a significant elevation in amplitudes ($P < 0.0001$; Kolmogorov-Smirnov test; data not shown). Notably, the net effect of P3 incision was to reduce the strength of glycinergic synaptic drive (ie, mIPSC frequency multiplied by mIPSC amplitude) onto these neurons (Naïve: 3.36 ± 0.49 Hz*pA, $n = 51$; P3 Incision: 1.93 ± 0.28 Hz*pA, $n = 52$, $P = 0.029$; Mann-Whitney test, data not shown). Meanwhile, the properties of miniature

GABAergic transmission onto mature Gad-GFP neurons was unaffected by surgical injury at P3 (Fig. 3B).

A similar effect of neonatal tissue damage was observed in the non-GFP population of adult SDH neurons, as P3 incision significantly reduced the mean frequency of GlyR-mediated mIPSCs (Naïve: 0.25 ± 0.05 Hz, $n = 36$; P3 Incision: 0.15 ± 0.03 Hz, $n = 45$; $P = 0.036$; Mann-Whitney test; Fig. 3C, left). This was accompanied by a significant rightward shift in the cumulative probability curve for interevent intervals ($P = 0.036$; Kolmogorov-Smirnov test; Fig. 3F). Meanwhile, no significant effect on the frequency ($n = 32$ – 34 in each group; $P = 0.542$; Mann-Whitney; Fig. 3D, left) or amplitude ($P = 0.859$; Mann-Whitney; Fig. 3D, right) of GABA_AR-mediated mIPSCs was observed.

To determine if early injury evokes a global alteration in glycinergic function in mature spinal nociceptive circuits, GlyR-mediated mIPSCs were recorded in adult lamina II neurons located in the lumbar spinal cord contralateral to the site of surgical incision. P3 injury failed to modulate the frequency (Naïve: 0.24 ± 0.08 Hz; $n = 20$; Incision: 0.21 ± 0.05 Hz, $n = 20$; $P = 0.695$; Mann-Whitney test) or amplitude (Naïve: 16.6 ± 2.5 pA; Incision: 16.3 ± 2.1 pA; $P = 0.563$) of GlyR mIPSCs on the contralateral side. Collectively, the results demonstrate that early tissue damage evokes selective, long-term changes in glycinergic tone within mature spinal nociceptive circuits.

To evaluate the degree to which neonatal incision also decreased evoked glycinergic signaling within the adult SDH, we recorded monosynaptic GlyR-mediated IPSCs in adult Gad-GFP or non-GFP lamina II neurons in response to minimal electrical stimulation near the cell of interest (Fig. 4A and B). Similar results were observed for the Gad-GFP and non-GFP populations, therefore, the data were pooled for analysis. P3 surgical injury did not affect the threshold to evoke an IPSC within the adult SDH (Naïve: 9.25 ± 0.50 μ A, $n = 21$; Incision: 9.37 ± 0.56 μ A, $n = 26$; $P = 0.882$; unpaired *t*-test). Surprisingly, P3 incision failed to significantly modulate evoked GlyR-mediated currents in the adult SDH, as we observed no differences in the mean amplitude ($P = 0.566$; Mann-Whitney test; Fig. 4D) or coefficient of variation ($P = 0.369$; unpaired *t*-test; Fig. 4E) of the IPSCs between the neonatally incised and naïve groups. In addition, early injury did not influence the response of mature glycinergic synapses to paired-pulse stimulation ($P = 0.554$; Mann-Whitney; Fig. 4C and F).

3.3. Cell type-specific alterations in tonic glycinergic inhibition within the mature SDH after neonatal incision

It is now appreciated that tonic GlyR- and GABA_AR-mediated conductances carry significantly more negative charge across the membrane compared to spontaneous, phasic IPSCs [13]. They can also strongly regulate neuronal excitability within the SDH of Gad-GFP mice, as evidenced by reduced spike thresholds and elevated firing frequencies in the presence of strychnine and bicuculline [50]. To evaluate the effect of early tissue damage on the expression of tonic GABAergic and glycinergic currents in mature SDH neurons, STR (0.5 μ M) and GBZ (10 μ M) were sequentially bath-applied in the presence of TTX and the resultant shifts in holding current were measured (Fig. 5A; see Methods). As illustrated in Fig. 5B, neonatal surgical injury significantly reduced the density of tonic GlyR-mediated current (I_{Gly}) in mature non-GFP SDH neurons ($n = 12$ – 13 in each group; $P = 0.005$; Mann-Whitney test; Fig. 5B, left) without altering the density of tonic GABA_AR current ($P = 0.619$; Fig. 5B, right) in the same cells. Interestingly, no such change in the density of tonic I_{Gly} was observed within the GABAergic population of adult SDH neurons following P3 incision ($n = 12$ – 13 in each group; $P = 0.497$; Mann-Whitney test; Fig. 5C, left). The level of tonic GABA_AR-mediated current (I_{GABA}) in the Gad-GFP group was similarly unaffected by early injury ($P = 0.620$; Fig. 5C, right).

3.4. Neonatal injury does not alter the transcription of genes involved in glycinergic signaling within adult spinal nociceptive circuits

The above results suggest that neonatal tissue damage can lead to a persistent reduction in both phasic and tonic inhibition within the mature SDH, but the effect is restricted to glycinergic transmission. These selective alterations in glycinergic transmission following early injury could potentially reflect changes in: 1) the postsynaptic expression of GlyR subunits within the mature SDH; 2) the levels of the anchoring protein gephyrin, which is required for the appropriate targeting of GlyRs to postsynaptic sites in developing spinal neurons [27]; and/or 3) the relative postsynaptic expression of $\alpha 1$ vs. $\alpha 3$ integrins, which exert opposite actions on gephyrin stabilization and the number of GlyRs that are “trapped” at inhibitory synapses [8]. However, quantitative PCR analysis of the adult SDH (see Methods) revealed no significant differences in the mRNA levels of the GlyR $\alpha 1$ subunit ($P = 0.394$; Mann-Whitney test; Fig. 6A), GlyR $\alpha 2$ ($p = 0.937$; Fig. 6B), GlyR $\alpha 3$ ($P = 1.0$; Fig. 6C), or the β subunit of the GlyR ($P = 0.485$; Fig. 6D) between the naïve ($n = 6$) and neonatally incised groups ($n = 6$). In addition, we observed no effect of P3 incision on the transcriptional expression of gephyrin ($P = 0.937$; Fig. 6E), $\alpha 1$ integrin ($P = 0.818$; Fig. 6F), or $\alpha 3$ integrin ($P = 1.0$; data not shown).

4. Discussion

These findings provide the first evidence that transient tissue damage during early life produces long-term alterations in inhibitory synaptic function within adult spinal nociceptive circuits. The data indicate that neonatal injury can compromise both phasic and tonic inhibitory signaling within the mature SDH, but the effects appear selective for glycinergic circuits as miniature GABA_AR-mediated transmission remains unaffected.

4.1. Neonatal tissue injury decreases glycinergic tone within adult spinal nociceptive circuits

The results demonstrate that tissue damage during the first, but not third, postnatal week modulates synaptic inhibition onto both GABAergic and presumed glutamatergic neurons within lamina II of the adult spinal cord (Figs. 1 and 2). Since neonatal surgical incision fails to alter inhibitory signaling within the SDH of these mice at 1–2 days postinjury [29], modifications within dorsal horn inhibitory circuits appear to occur with a delayed time course following early tissue damage. While GABA and glycine are transported into individual synaptic vesicles by the same vesicular inhibitory amino acid transporter [2,58] and co-released within the spinal cord [22], the persistent deficits in inhibitory transmission after neonatal injury were restricted to GlyR-mediated signaling (Fig. 3A–C, 5). This could reflect differential modulation of GABA and glycine release by second messenger pathways, as adenylate cyclase activity selectively modulates glycinergic IPSCs within lamina II via presynaptic mechanisms [9].

Neonatal tissue damage could also influence the developmental reorganization of inhibitory neurotransmitter receptors within the postsynaptic membrane [26]. Newborn lamina II neurons lack GlyR-mediated mIPSCs and yet invariably respond to exogenous glycine application [3], suggesting that functional GlyRs are expressed within the membrane but not appropriately targeted to the synapse. Therefore, early tissue damage could interfere with the process by which GlyRs are targeted to synaptic locations during development. Although our results (Fig. 6) suggest that neonatal injury does not persistently alter the transcription of key genes previously implicated in GlyR trafficking [12], this does not exclude the possibility that posttranscriptional changes in these proteins contribute to the prolonged alterations in inhibitory signaling within the SDH. Future experiments examining the relationship between GlyT2-immunoreactive presynaptic terminals and α subunits of the

GlyR [39] would help to determine if the postsynaptic localization of GlyRs within the mature SDH is disrupted by neonatal incision.

4.2. Early incision reduces miniature glycinergic currents within the adult SDH

It is currently unclear why neonatal tissue injury decreases miniature glycinergic signaling (Fig. 3) while sparing GlyR-mediated IPSCs evoked by focal stimulation within the SDH (Fig. 4), given that inhibitory synaptic input onto an individual lamina II neuron predominantly originates from regions immediately surrounding the cell [23,24]. One possibility is that neonatal tissue damage selectively decreases spontaneous glycinergic signaling in the adult SDH. Indeed, prior evidence suggests that distinct mechanisms regulate miniature and evoked neurotransmitter release within the spinal cord [4,32].

Alternatively, the selective effect on mIPSCs could be explained if early injury reduces the overall number of glycinergic inputs to a given lamina II neuron without altering the strength of individual glycinergic connections that have successfully developed onto that cell. Such a decrease in glycinergic innervation could reflect a loss of input from nearby lamina II interneurons [45], interneurons located in deeper laminae of the L4/L5 dorsal horn where a greater number of glycinergic neurons reside [51,61], cells located in more distant segments of the spinal cord [17,33], or glycinergic neurons contributing to descending inhibitory pathways from the brain stem [25].

However, if glycinergic neurons predominantly represent a subset of the GABAergic population [21,51], such a loss of innervation would also be predicted to decrease GABA_AR-mediated signaling, which was not observed in our experiments. Nonetheless, a selective impairment of glycinergic synaptic function after early injury could result from alterations in the number of dorsal horn neurons expressing the neuronal glycine transporter GlyT2. While GlyT1 is primarily localized to glia in the central nervous system [1], GlyT2 is responsible for transporting glycine into the presynaptic terminal [2] and is known to be required for glycinergic inhibition [16]. The complete loss of GlyT2 expression in a subset of developing dorsal horn neurons after neonatal tissue damage could effectively lower the number of glycinergic neurons within the mature spinal nociceptive circuit without affecting the prevalence of GABAergic inputs within the region. Alternatively, there is evidence for the existence of axons in the dorsal horn that express GlyT2 but not GAD [31,61], suggesting the possibility that the observed reduction in glycinergic signaling (Fig. 3) reflects persistent, injury-evoked alterations in the function of a subpopulation of glycinergic dorsal horn neurons that do not synthesize GABA.

4.3. Do persistent alterations in the organization of the SDH synaptic network contribute to the long-term changes in pain sensitivity after neonatal tissue damage?

Early tissue damage in the rodent evokes 2 prolonged shifts in nociceptive processing that are temporally and spatially distinct: 1) a global reduction in baseline pain sensitivity that has a delayed (~4 weeks) onset [28,42]; and 2) an exacerbated hyperalgesia following re-injury of the paw, which is site-specific and can be evoked immediately after recovery from the initial neonatal injury [10,42,57]. Both effects last into adulthood and are observed only if the original insult occurs during a critical period of development corresponding to the first postnatal week [42,57]. Importantly, recent human studies suggest that neonatal tissue damage can lead to higher pain thresholds in response to acute noxious stimuli but enhanced perceptual sensitization during prolonged nociceptive input [20,56,59].

The difference in onset times between the global hypoalgesia and the increased sensitivity to repeat injury following neonatal tissue damage suggests that they are mediated by distinct mechanisms. The delayed appearance of the global hypoalgesia coincides with the gradual

maturation of descending modulatory pathways from the rodent brainstem [18], and stronger descending inhibition from the adult rostroventral medulla has been reported following neonatal hind paw inflammation [62]. Meanwhile, recent work indicates that the persistent enhancement in hyperalgesia following subsequent injuries does not arise from alterations at the injury site, but instead from changes within the spinal dorsal horn [5], which are triggered by primary afferent input [57]. This suggests that neonatal tissue damage causes a localized “priming” of adult spinal nociceptive circuits, which may be masked under baseline conditions by enhanced descending inhibition but becomes dominant during prolonged nociceptive stimulation such as that resulting from a repeat injury.

In this context, the decreased efficacy of glycinergic signaling within the mature dorsal horn after neonatal tissue damage (Figs. 1, 3 and 5) could compromise feed-forward and/or feedback inhibition of SDH neurons during repetitive noxious stimulation, thus facilitating action potential discharge in these cells. Although neonatal injury reduced phasic glycinergic inhibition onto both GABAergic and presumed excitatory interneurons within lamina II (Figs. 1 and 3), glutamatergic neurons represent the majority (~70%) of the lamina II population [51] and dominate sensory processing within this lamina [45]. As a result, the above changes in phasic glycinergic transmission within lamina II are predicted to enhance the overall excitability of the SDH network. In addition, the injury-evoked decrease in tonic glycinergic current occurred selectively in the non-GFP population during adulthood (Fig. 5), although it remains to be determined if the change in tonic GlyR signaling significantly enhances neuronal excitability within this population of mature SDH neurons.

If the above modification of glycinergic circuits results in elevated firing within the SDH, this could amplify the activation of spinal microglia [19], which play a key role in the priming of adult nociceptive reflexes by neonatal injury [5]. While it remains to be determined whether the reduced glycinergic tone is linked to an exaggerated neuroimmune response to subsequent injury during adulthood, it is interesting that the persistent deficits in glycinergic function and the long-term behavioral effects [42,57] may share the same critical period, as surgical incision during the third week failed to alter synaptic inhibition within the adult SDH (Fig. 2). However, it should be noted that future experiments characterizing the effects of injury at additional time points will be required before one can clearly define the critical period for long-term plasticity within developing spinal glycinergic circuits.

4.4. Conclusion

The present results suggest that synaptic integration within nociceptive networks of the adult spinal cord is highly dependent upon sensory experience during early postnatal development. Additional investigations into the mechanisms underlying these persistent alterations in synaptic function may yield insight into novel strategies to prevent long-term changes in pain sensitivity following neonatal tissue damage.

Acknowledgments

This work was supported by the US National Institutes of Health grants NS060858 and NS072202 (M.L.B.).

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Fig. 1.

Neonatal surgical incision selectively reduces miniature inhibitory neurotransmission within the adult mouse superficial dorsal horn (SDH). (A) Example of miniature excitatory postsynaptic currents (mEPSCs) at a holding potential of -70 mV. (B) Miniature inhibitory postsynaptic currents (mIPSCs) isolated at a holding potential of 0 mV in the same lamina II neuron. (C and D) Hind paw surgical incision at postnatal day 3 failed to alter mEPSC frequency in either GABAergic ($n = 34-35$ in each group; $P = 0.214$; Mann-Whitney test; C, left) or presumed glutamatergic (non-GFP) neurons ($n = 35-37$; $P = 0.660$; D, left) within lamina II of the adult SDH, although mEPSC amplitude in the Gad-GFP population was increased by neonatal incision ($*P = 0.040$; Mann-Whitney, C, right). (E and F) P3 injury significantly decreased mIPSC frequency in both mature Gad-GFP ($***P = 0.0007$; Mann-Whitney; E, left) and adjacent non-GFP SDH neurons ($*P = 0.018$; F, left) with no

accompanying changes in mIPSC amplitude ($P = 0.408$ for Gad-GFP and $P = 0.997$ for non-GFP; E and F, right).

Fig. 2.

Surgical injury during the third postnatal week fails to evoke long-term changes in synaptic signaling within mature spinal nociceptive circuits. (A) Hind paw incision at postnatal day 17 failed to modulate the frequency ($P = 0.602$; Mann-Whitney; left) or amplitude ($P = 0.962$; right) of miniature excitatory postsynaptic currents (mEPSCs) in adult Gad-GFP neurons ($n = 16-17$ in each group). (B) Similarly, mEPSC frequency ($P = 0.895$; left) and amplitude ($P = 0.985$; right) were similar in non-GFP superficial dorsal horn (SDH) cells from the Naïve ($n = 16$) and Incision ($n = 16$) groups. (C and D) P17 incision did not significantly alter miniature inhibitory postsynaptic current frequency in GABAergic ($P = 0.611$; C, left) or presumed glutamatergic ($P = 0.763$; D, left) neurons of the adult SDH, with mIPSC amplitudes in these populations also unchanged by early tissue damage (C and D, right).

Fig. 3.

Selective reduction in GlyR signaling within the adult SDH after early surgical injury. (A) Within the Gad-GFP population of adult lamina II neurons, surgical incision at P3 significantly decreased the frequency of GlyR-mediated miniature inhibitory postsynaptic currents (mIPSCs) compared to naïve littermates (** $P = 0.007$; Mann-Whitney; left) while enhancing mIPSC amplitude in these cells (* $P = 0.048$; right). (B) Meanwhile, the frequency ($P = 0.118$; left) and amplitude ($P = 0.807$; right) of GABA_AR-mediated mIPSCs within this population of mature SDH cells were similar in the naïve ($n = 43$) and neonatally-incised ($n = 35$) groups. (C and D) Similar effects of P3 injury were observed in adult non-GFP neurons, as GlyR mIPSC frequency was reduced (* $P = 0.036$; Mann-Whitney; C, left) in the

absence of significant alterations in GABA_AR-mediated transmission (D). (E and F) Cumulative probability functions demonstrating a significant rightward shift in the distribution of GlyR-mediated mIPSC inter-event intervals in Gad-GFP (E; $P < 0.0001$; Kolmogorov-Smirnov test) and non-GFP (F; $P = 0.036$) lamina II neurons after neonatal hind paw incision.

Fig. 4.

Neonatal tissue damage does not modify the properties of inhibitory postsynaptic currents (IPSCs) evoked by local stimulation within the mature superficial dorsal horn (SDH). (A) Example of monosynaptic GlyR-mediated IPSCs evoked by focal stimulation (0–20 μ A) within lamina II from a holding potential of 0 mV. (B) Plot of evoked IPSC amplitude as a function of stimulus intensity, with the step-like increase in IPSC amplitude consistent with the activation of a small number of presynaptic inputs to the SDH neuron. (C) Examples of GlyR-mediated IPSCs evoked by paired stimulation at an interstimulus interval of 100 ms. Trace represents average of 10 trials. (D–F) P3 hind paw incision failed to significantly influence the mean amplitude ($P = 0.566$; Mann-Whitney test; D), coefficient of variation ($P = 0.369$; unpaired t -test; E) or paired-pulse ratio ($P = 0.554$; Mann-Whitney; F) of evoked GlyR-mediated IPSCs in the adult SDH compared to naïve littermate controls. Data reflect pooled results from Gad-GFP and non-GFP cells.

Fig. 5.

Early injury decreases tonic glycinergic input to adult superficial dorsal horn (SDH) neurons in a cell type-selective manner. (A) Top: Example of protocol involving the sequential bath application of tetrodotoxin (TTX; point #1), the GlyR antagonist strychnine (STR; point #2) and the GABA_AR antagonist gabazine (GBZ; point #3) in an adult lamina II neuron from a holding potential of 0 mV. The amplitudes of some miniature inhibitory postsynaptic currents (mIPSCs) have been truncated for illustrative purposes; Bottom: Currents shown with expanded time base corresponding to points #1–3 in the above trace. (B) P3 surgical incision significantly reduced the density of tonic glycinergic current (I_{Gly}) in mature, non-GFP SDH neurons (** $P = 0.005$; Mann-Whitney test; left) without altering the level of tonic

GABA_AR responses (I_{GABA}) in this population (right). (C) In contrast, P3 tissue damage failed to influence either the density of tonic I_{Gly} (left) or I_{GABA} (right) within the Gad-GFP group of adult SDH neurons.

Fig. 6.

Neonatal surgical injury does not evoke long-term changes in the transcription of genes implicated in GlyR signaling within spinal nociceptive networks. (A–F) Quantitative polymerase chain reaction (PCR) analysis suggests that neonatal hind paw incision does not influence the mRNA levels (normalized to hypoxanthine-guanine phosphoribosyltransferase [HPRT] and α -tubulin expression) of GlyR 1 (A), GlyR 2 (B), GlyR 3 (C), the α subunit of the GlyR (D), gephyrin (E), or $\beta 1$ integrin (F) within the adult mouse superficial dorsal horn.