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Review Article

Preclinical Studies of Pediatric Pain: Functional Modulation of Spinal Pain Circuitry by Early Life Tissue Injury

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ABSTRACT

Aim of review: Incomplete knowledge regarding the biological basis of pediatric pain has prompted the need for more research into how tissue injury during early life evokes hyperalgesia in infants, and the degree to which neonatal insults can alter nociceptive processing throughout development. This paper mainly reviews preclinical studies investigating the short- and long-term modifications of spinal nociceptive circuitry evoked by neonatal tissue damage. A better understanding of the plasticity resulting from neonatal injury may ultimately guide new clinical strategies to relieve pain in infants and children.

Methods: We conducted a review of recent clinical and preclinical publications relating to pediatric pain, with a focus on neuronal networks in the superficial dorsal horn (SDH) of the spinal cord that are directly innervated by primary sensory inputs and serve as a main site for the integration and transmission of nociceptive signals within the central nervous system (CNS).

Recent findings: Aberrant peripheral input following neonatal tissue damage produces a selective and transient enhancement in excitatory synaptic transmission in the SDH during early postnatal development. In addition, deficits in glycinergic inhibition were observed that persisted into adulthood, suggesting prolonged alterations in synaptic function within spinal pain circuits in response to neonatal tissue injury. As a result of a disrupted balance of inhibition vs. excitation onto mature lamina I projection neurons ascending to the parabrachial nucleus, neonatal tissue damage also produced a greater signaling "gain" in this population, as evidenced by more action potential discharge in response to primary afferent stimulation. Furthermore, tissue injury during the early life created a more permissive environment for spike timing-dependent long-term potentiation (t-LTP) to occur at sensory synapses onto mature projection neurons, thus allowing the repetitive activation of more weakly correlated sensory inputs and postsynaptic activity to produce LTP. This provides a novel potential mechanism that ascending nociceptive signals could be amplified within the spinal cord.

Summary: The development of spinal pain circuits is subject to modifications by aberrant peripheral inputs that accompany tissue insults. Tissue injury during early life can lead to profound alterations in synaptic function in the SDH, such as an increased gain of projection neurons and facilitated LTP at sensory synapses onto these output neurons of the spinal pain circuit. Therefore, more effort is needed to further illustrate the consequences of neonatal tissue injury for the maturation of nociceptive pathways, with the ultimate goal of providing more age-appropriate clinical strategies to minimize the long-term impact of these injuries on the developing CNS. (Funded by the National Institutes of Health.)

It is estimated that 14.9 million babies were born preterm (< 37 completed weeks of gestation) in 2010, corresponding to 11.1% of all live births worldwide, which often necessitates admittance to the neonatal intensive care unit (NICU) (1). Infants in the NICU are exposed to a high number of painful procedures such as heel lance, suctioning, venepuncture and insertion of peripheral venous catheters (2), which are performed frequently and often with inadequate pain management (2). The consequences of exposure to procedural pain in neonates have been associated with adverse neurodevelopment and altered pain responses (3), poorer cognitive and motor scores (4), and impairments of growth (5). For instance, following neonatal circumcision without anesthetics, a stronger pain response to vaccination was observed many months later (6). Persistent changes in pain sensitivity have been reported in school-aged children with experience in the NICU (7). Additional clinical studies demonstrated that initial surgery in neonates led to increased analgesics requirement during a subsequent surgery in the same dermatome, suggesting the potential for changes in spinal and supraspinal nociceptive networks following neonatal surgery (8).

Similar results were obtained from preclinical studies, as re-inflammation of the same hindpaw during adulthood in rats with neonatal injury led to excessive hyperalgesia (9). Moreover, the incision in the hindpaw during the first week of life, but not older ages, enhanced pain responses to repeat incision two weeks later, pointing to a critical period in early life during which noxious stimulation can prime future pain processing (10). Further studies demonstrate that repeated stimulation in the neonatal period can alter the mechanical sensitivity of adult spinal sensory neurons (11), which serve as the first relay center of nociceptive signals within the central nervous system (CNS).

Overview of the Spinal Superficial Dorsal Horn

Although nociceptive circuits in the CNS are functional at the time of birth, considerable structural and functional refinement in these pathways occurs postnatally which can be strongly influenced by the pattern of sensory input. A disruption of the normal peripheral input by tissue insults during a critical period of life can provoke profound alterations in the function of spinal pain circuitry. In this review, we focus upon the impact of early life tissue injury on spinal nociceptive processing as revealed by recent electrophysiological analysis of synaptic signaling within the spinal superficial dorsal horn (SDH).

The SDH contains the first synaptic relay of primary afferent fibers from skin, muscle and viscera, thereby serving as the initial processing center of peripheral nociceptive signals. Due to the relative lack of myelination, the SDH is very distinct from other regions of gray matter in the spinal cord. The earliest description of its gelatinous appearance by Italian anatomist Luigi Rolando in the 1820s led to the naming of this zone as Substantia Gelatinosa (12). In 1952, Rexed divided the entire gray matter of the spinal cord into ten laminae according to their cytoarchitecture, with the SDH consisting of lamina I (the marginal zone) and lamina II (the substantia gelatinosa) (13).

The majority of neurons in the SDH have axons that remain within the spinal cord, and are therefore defined as interneurons or propriospinal neurons. It is believed that all of these neurons give rise to locally arborizing axons to make synaptic connections between neurons located in different segments or laminae in the spinal cord. The inhibitory neurons compose around 30% of the total neuronal population in the SDH, releasing γ-aminobutyric acid (GABA) and/or glycine as a neurotransmitter upon the arrival of presynaptic impulses (14, 15). The vast majority of the remaining SDH neurons are excitatory interneurons, which account for 60%-70% of the overall population in the region and release glutamate to activate postsynaptic AMPA/kainate and NMDA receptors. The neurons that have axons ascending to the thalamus, periaqueductal grey matter (PAG), lateral parabrachial nucleus (PB) and other medullary nuclei are defined as projection neurons, which are concentrated in the marginal area (comprising ~5% of the total population in lamina I) as well as the deep dorsal horn. They serve as the major output neurons to convey the processed and integrated nociceptive signals to their direct targets in the brain, such as PAG and PB. The ascending pain signals are eventually transmitted to multi-

ple cortical regions including, but not limited to, primary somatosensory cortex, which is critically involved in the perception of noxious stimulus intensity (16, 17).

Besides receiving input from the peripheral nociceptors, the SDH also receives descending modulation from the brain, which includes the facilitatory and inhibitory pathways from the rostral ventromedial medulla (RVM) (18-20). Prolonged noxious stimulation can cause pronounced changes in the activity of nociceptive modulatory neurons in the RVM (21).

Synaptic function within the SDH undergoes substantial postnatal refinement, and is therefore highly susceptible to modulation by aberrant sensory input arising from tissue damage during the neonatal period. These injury-evoked changes in synaptic transmission serve as the main focus of the present review.

Modulation of Synaptic Function by Neonatal Injury: SDH Interneurons

The SDH consists of complex networks of excitatory and inhibitory interneurons and the proper balance of activity in these populations is crucial for normal nociception. Indeed, pharmacological blockade of receptors for GABA and glycine, or chemogenetic silencing of inhibitory neurons in the spinal cord, has been shown to produce allodynia, hyperalgesia and spontaneous pain (22, 23). Meanwhile, excitatory interneurons are indispensable for acute, inflammatory and neuropathic pain states. Selective ablation of excitatory interneurons in SDH, via the deletion of testicular orphan nuclear receptor 4 (TR4), is associated with the loss of nerve injury-induced mechanical hypersensitivity (24) and loss of somatostatin-positive glutamatergic neurons lead to deficits in noxious mechanical sensation (25). These findings (and others) highlight the importance of better understanding how tissue injury during the neonatal period alters synaptic signaling onto these populations of interneurons in the SDH.

Acute Changes in Synaptic Transmission Within the Developing SDH After Early Life Injury

Neonatal tissue damage enhanced excitatory synaptic function within the developing SDH in an age- and activity-dependent manner. Subcutaneous injection of carrageenan (CARR) (26), or hindaw surgical injury, in rats at postnatal day 3 (P3) selectively increased the frequency (but not amplitude) of miniature excitatory postsynaptic currents (mEPSCs) at 1-3 days after tissue damage, without altering the frequency or amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in the same neurons (26, 27). The potentiation of glutamatergic signaling was transient, as the difference in mEPSC frequency induced by CARR had resolved by P10-11. In addition, the alterations in synaptic function seen after tissue injury are age-dependent, since these same injuries administered during the second or third postnatal week failed to significantly alter excitatory or inhibitory synaptic efficacy in the SDH (26, 27). Further studies demonstrated that this selective modulation of the excitatory synaptic drive after neonatal tissue damage is activity-dependent, as blocking the primary afferent input from the time of injury via the delivery of bupivacaine hydroxide or tetrodotoxin to the sciatic nerve prevented the facilitation of mEPSC frequency (27). It should be noted that the SDH exhibits extensive heterogeneity in terms of neuronal subpopulations, and these initial studies did not identify the functional subtype of interneurons that were affected by the neonatal tissue damage. However, in the GAD GFP mouse line, enhanced green fluorescence protein (eGFP) is expressed under the GAD1 promoter in ~80% of GABAergic inhibitory neurons in neonatal SDH (28) and glycinergic cells predominantly represent a subset of this GABAergic population (29), therefore indicating that the vast majority (>80%) of the non-GFP neurons corresponds to excitatory interneurons in the SDH. Patch clamp recordings using GAD GFP mice illustrated a widespread facilitation of mEPSC frequency in both inhibitory and presumed excitatory interneurons at 1-2 days after hindpaw incision occurring at P3 (30).

Higher mPSC frequency is generally attributed to a higher number of neurotransmitter release sites (n) and/or higher probability of transmitter release (Pr). To further explore the mechanisms underlying the increased frequency of mEPSCs after early injury, focal stimulation of

the dorsal root was used to minimally activate afferent fibers and isolate monosynaptic ESPCs mediated by low threshold and high threshold afferents. The paired-pulse ratio (PPR), calculated using the amplitude of EPSCs evoked by paired-pulse stimulation, is thought to be inversely related to Pr (31, 32). Meanwhile, the coefficient of variation (CV) inversely depends on both Pr and the number of transmitter release sites (n) (33). We found that, following neonatal surgical injury, the EPSCs mediated by high threshold afferent fibers exhibited larger amplitudes with a significant reduction in CV, while the PPR remained unchanged. Collectively, these findings suggest that the increase of mEPSC frequency is likely mediated by an elevation in the number of synapses made by high threshold nociceptive afferents within the developing SDH. Interestingly, the extensive sprouting of nociceptive afferent fibers has been reported to occur within the neonatal rat dorsal horn following peripheral inflammation during the early postnatal period (34, 35). In addition, the elimination of the $TRPV^{1+}$ afferent fibers by capsaicin treatment in newborn rats at birth abolished the increase of mEPSC frequency following early hindpaw incision, pointing to an important contribution of $TRPV^{1+}$ fibers to the increased mEPSC frequency (30).

Neonatal tissue damage evokes a robust increase in nerve growth factor (NGF) expression within the skin (36) and elevated NGF levels in the periphery result in the expansion of developing primary afferent inputs to the SDH (37). Peripheral administration of NGF in the neonatal rats mimicked the effects of hindpaw incision on SDH glutamatergic synapses, which were prevented by blocking trkA receptors. Additionally, hindpaw incision in neonatal rats pretreated with NGF didn't exhibit additive facilitation of glutamatergic function (30). Taken together, these results indicate that NGF and early tissue damage regulate excitatory synaptic function within the SDH via similar downstream mechanisms.

Persistent Alterations in Synaptic Functioning within the Adult SDH After Neonatal Injury

Behavioral studies have shown that early tissue damage evokes a global reduction in baseline pain sensitivity that displays a delayed onset at ~ 4 weeks of age (38, 9). In contrast, re-injury at the same site led to an exacerbated hyperalgesia, which could be evoked immediately upon recovery from the initial neonatal injury (39, 9, 10), suggesting that neonatal tissue damage causes a localized "priming" within the spinal nociceptive circuit. These two temporally and spatially distinct effects evoked by neonatal tissue injury can last into adulthood (40, 9). However, it remained unknown how neonatal tissue injury affects synaptic function within the mature SDH.

Interestingly, recent studies illustrated that hindpaw incision during early life compromises inhibitory synaptic transmission within the adult mouse SDH (41), as evidenced by a selective reduction in the frequency of glycine receptor (GlyR) - mediated IPSCs onto both GABAergic and presumed glutamatergic neurons within lamina II of the adult SDH. Along with the reduction of phasic inhibitory signaling, neonatal incision significantly decreased the density of tonic GlyR-mediated current in the presumed glutamatergic population during adulthood. In contrast to the spontaneous IPSCs mediated by glycine receptors, the GlyR-mediated IPSC evoked by focal stimulation of the surrounding SDH was not influenced by the neonatal injury. It is currently unclear why neonatal tissue injury decreases miniature glycinergic signaling while sparing GlyR-mediated IPSCs evoked by focal stimulation within the SDH. It might be due to the different mechanisms regulating spontaneous and evoked neurotransmitter release within the spinal cord (42, 43). Although inhibitory interneurons in the spinal cord co-release GABA and glycine as neurotransmitters, the selective reduction in the frequency of GlyR-mediated IPSCs may suggest that subpopulations of dorsal horn neurons synthesizing glycine only were persistently affected by the neonatal injury. Indeed, there are axons in the dorsal horn expressing GlyT2 but not glutamic acid decarboxylase (GAD) (44, 45), supporting the notion that some inhibitory neurons in the SDH do not synthesize GABA.

The above long-term changes in glycinergic transmission following neonatal hindpaw incision are predicted to increase the overall excitability of the mature SDH network. Similar disinhibition was also observed in the adult lumbosacral spinal cord following neonatal bladder in-

flammation (46). It seems that the reduced inhibition is inconsistent with the behavioral global hypoalgesia following the initial neonatal tissue injury. However, it should be noted that the delayed hypoalgesia coincides with the gradual maturation of descending modulatory pathways from the rodent brainstem (47). Stronger descending inhibition from the adult RVM has been reported following neonatal hindpaw inflammation (48) or surgical injury (49), and increased endogenous opioid tone has been proposed as an underlying mechanism for the observed hypoalgesia (40). Therefore, the predicted increase of excitation resulting from reduced glycinergic transmission within the mature SDH may be masked by enhanced descending inhibition.

It is noteworthy that the priming of nociception can occur at different ages and be mediated by different mechanisms. In the hyperalgesic priming model, in which a previous inflammatory stimulus triggers a long-lasting increase in responsiveness to pro-algesic mediators, female rats were found to be primed by TNFα injection at earlier ages (< postnatal 3 weeks old), while the males were primed by TNFα administration at later ages (> postnatal 4 weeks old) (50). The hyperalgesic priming was also reported in adult mice in which the priming might be through altered GABAergic signalings within the spinal circuits (51). In the mice primed by interleukin 6 (IL-6), GABAAR agonists or its positive allosteric modulators failed to alleviate the prostaglandin E2 (PGE2)-evoked hyperalgesia. In contrast, GABAAR antagonists significantly attenuated the mechanical hypersensitivity. The priming is at least partially modulated by the descending dopaminergic system (18), as evidenced by the activation of dorsal horn neurons that express PAX2 following the spinal application of a D1/5 receptor agonist.

Functional Impact of Early Injury on Ascending Projection Neurons in the Mature SDH

Given the role of lamina I projection neurons as the major outlet of the SDH, and their clear importance for the generation of neuropathic and inflammatory pain (52, 53), it is essential to elucidate the influence of neonatal tissue injury on this specific population during adulthood. Since up to 85% of lamina I projection neurons target the parabrachial nucleus (PB), and 90% of the projection neurons ascending to periaqueductal gray (PAG) have collaterals entering the PB (54), the functional studies of projection neurons following neonatal tissue injury were mainly conducted on the subpopulation of neurons projecting to the PB.

Altered Balance of Excitation vs. Inhibition onto Spinal Projection Neurons Following Neonatal Injury

In vivo studies showed that adult dorsal horn neurons exhibit greater excitability when preceded by neonatal tissue damage (55, 56), which could be mediated in part by the prolonged deficits in glycinergic inhibition within lamina II following early surgical injury (41). This would predict that neonatal injury increases the overall excitability, and therefore output, of adult spinal nociceptive circuits. Since only approximately 5% of lamina I neurons convey noxious sensory information to the brain, the general sampling of unidentified neurons in the SDH cells (mainly in lamina II) is unlikely to include this important population. If the changes described above ultimately fail to significantly alter synaptic integration and/or membrane excitability in the ascending projection neurons, it is uncertain how the neonatal injury can influence pain sensitivity during adulthood.

Following dye injection into lateral PB, the ascending neurons targeting PB were retrogradely labeled in lamina I of the spinal cord, which enabled patch clamp recordings from this specific population (57). Compared to naïve mice, hindpaw incision at P3 significantly enhanced sensory drive to adult projection neurons, as evidenced by a significantly greater peak amplitude (and area under the curve) of monosynaptic primary afferent-evoked EPSCs (58). Concomitantly, the pattern of direct sensory input to the mature projection neurons is persistently altered, as demonstrated by a higher prevalence of projection neurons receiving direct input from low threshold afferents in mice that were incised as neonates, compared to the naïve littermate controls. Perhaps more importantly, ear-

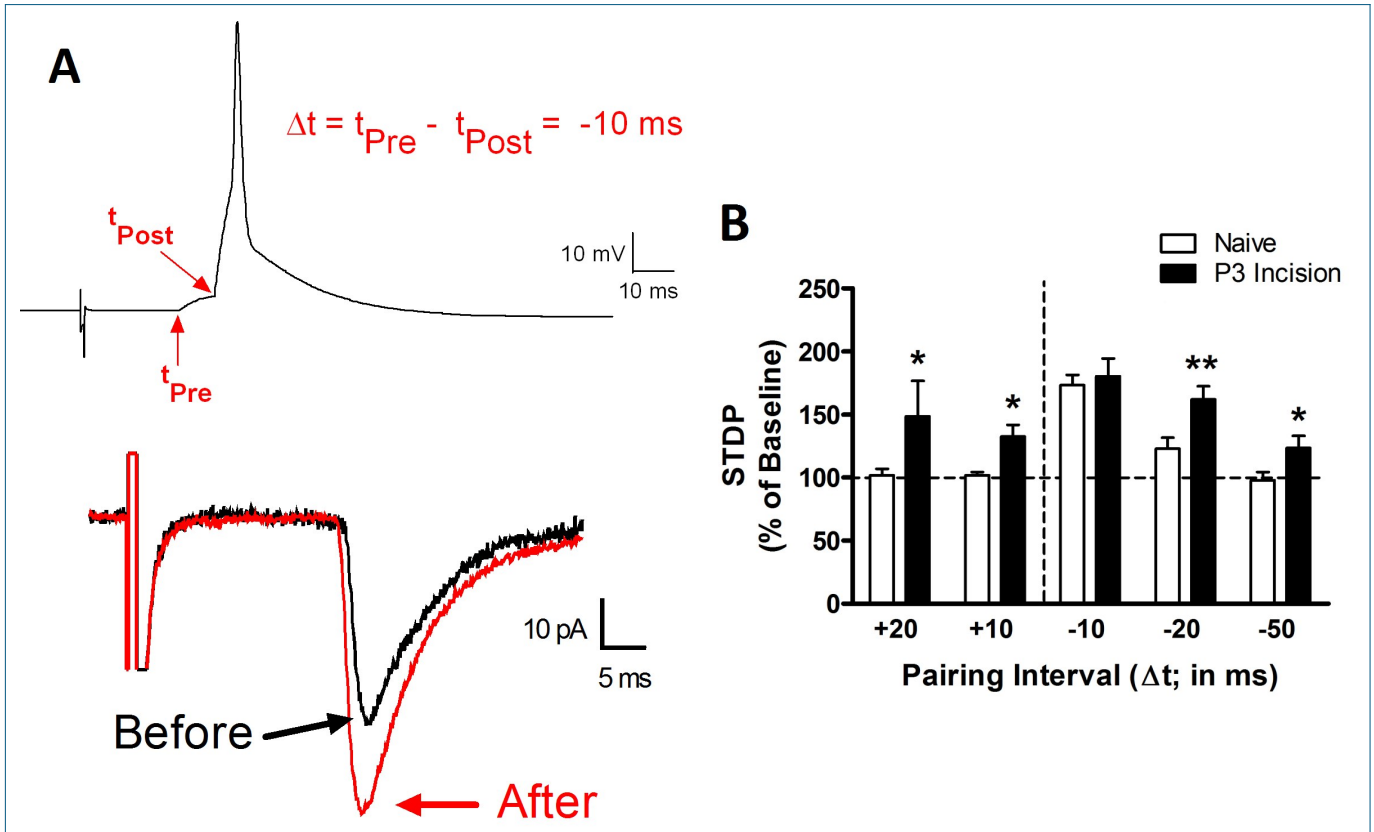

Figure 1. Representative t-LTP and its Facilitation by Neonatal Tissue Injury.
Panel A illustrates an example of t-LTP induced by pairing protocol in a mature projection neuron targeting the parabrachial nucleus. In this particular cell, the administration of the pairing protocol (30 times at 0.2 Hz) in which the presynaptic input preceded the postsynaptic spike by 10 ms (Δt = -10 ms, top) significantly potentiated EPSC amplitude (bottom). Panel B shows the broadened timing window for t-LTP at afferent synapses onto adult projection neurons following neonatal tissue damage. Pairing protocols with longer intervals (Δt =-20 and -50 ms) or in the reversed order (Δt =10 and 20 ms) led to significantly stronger t-LTP following neonatal tissue injury compared to naïve littermate controls. (Figures are modified from Li and Baccei, J Neurosci, 2016).

ly tissue damage compromised feedforward inhibition (FFI) of projection neurons during the adulthood, which has been proposed to gate mechanical allodynia (59). Upon high-threshold electrical stimulation of the dorsal root, polysynaptic IPSCs were evoked in projection neurons and GABAA receptor- and glycine receptor-mediated components of the IPSC were dissected by perfusion of the selective antagonists gabazine and strychnine, respectively. Hindpaw incision at P3 significantly decreased the efficacy of primary afferent-evoked glycinergic input, which is consistent with the persistent deficit in phasic glycinergic transmission observed in the mature SDH following neonatal surgery. Surprisingly, a reduction in the strength of GABAAR-mediated FFI onto adult projection neurons was also observed. The reduced inhibition onto adult projection neurons is unlikely mediated by diminished innervation of projection neurons by GABAergic interneurons since the immunochemistry failed to show a significant difference in the number of appositions between $VGAT^+$ boutons and spino-PB neurons between the neonatally incised and naïve littermates (58). However, the significant decrease in the intrinsic excitability of adult SDH neurons after neonatal injury can lead to reduced activa-

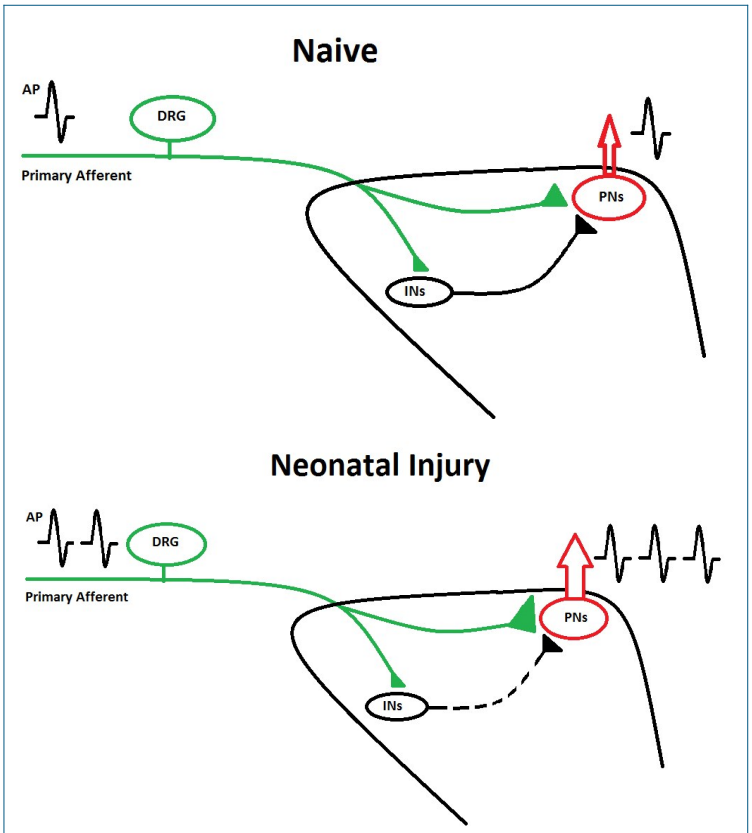

Figure 2. Schematic Diagram of the Synaptic Integration Within the Mature SDH After Early Life Injury.
Under naïve conditions, nociceptive inputs mediated by primary afferents result in normal output discharge in the projection neurons targeting supraspinal pain centers. Following neonatal tissue injury, however, multiple factors including enhanced peripheral inputs, diminished inhibition and facilitated LTP onto projection neurons lead to the excessive amplification of nociceptive signals, as evidenced by the increased action potential discharge from this population.
AP, action potential; DRG, dorsal root ganglion; INs, inhibitory neurons; PNs, projection neurons.
Dashed line, reduced inhibitory synaptic transmission.

tion in response to primary afferent stimulation, which may contribute to the reduced FFI despite the apparent lack of structural changes (60). As a result, the neonatal injury permanently increased the gain of nociceptive output from the mature projection neurons, as manifested by a greater level of action potential discharge evoked by dorsal root stimulation.

Facilitated Spike Timing-Dependent Long-Term Potentiation at Sensory Synapses onto Adult Projection Neurons Following Neonatal Injury

Long-term potentiation (LTP) has been proposed as a mechanism of central sensitization by which the excitation in ascending nociceptive pathways can be amplified within the spinal cord (61, 62). It is well established from studies in adolescent rodents that the repetitive stimulation of nociceptive afferents evokes LTP at their synapses onto spinal projection neurons (63, 64). Interestingly, the high-frequency stimulation (HFS, 3 trains at 100 Hz, 1 s) of primary afferents evoked a significantly greater potentiation in the amplitude of monosynaptic primary afferent-evoked EPSCs in adult mice when preceded by hindpaw incision at P3 compared to naïve littermates (65).

Besides the enhanced magnitude of LTP, neonatal tissue injury could prime the nociceptive circuits within the dorsal horn by increasing the likelihood that spike timing-dependent LTP (t-LTP) occurs, in which coincident presynaptic input and postsynaptic firing within a narrow time window cause synaptic potentiation. Indeed, the properties of t-LTP at excitatory synapses in the CNS have been extensively studied (66, 67). In naïve adult mice, the administration of a pairing protocol (30 trials at 0.2 Hz) in which the presynaptic primary afferent input preceded the postsynaptic action potential discharge by a short interval led to marked potentiation of monosynaptic EPSC amplitude in lamina I projection neurons (as evidenced in Figure 1A), while reversal of the stimulus order failed to affect EPSC amplitude (65). The t-LTP involves alterations in the presynaptic function, as the pairing protocol significantly reduced the PPR of EPSCs evoked by afferent stimulation (65). Moreover, the t-LTP depends on intracellular Ca^{2+} elevation and NMDAR activation in the postsynaptic neurons, as evidenced by the fact that intracellular BAPTA or bath perfusion of the NMDAR antagonist AP5 completely abolished t-LTP (65).

Importantly, neonatal tissue damage resulted in profound alterations in the temporal rules governing t-LTP in adult lamina I projection neurons (65). First, early tissue injury widened the timing window of pairing protocols that induced t-LTP, and even paired stimuli presented in the

reverse order (which normally fails to alter synaptic efficacy) led to LTP in mice with P3 hindpaw incision (as summarized in Figure 1B). Second, some of the projection neurons from the neonatally-incised mice exhibited robust potentiation in EPSC amplitude in the presence of AP5, suggesting a reduced dependence on NMDAR activation. Meanwhile, the neonatal incision unmasked a novel contribution of Ca^{2+}-permeable AMPARs to t-LTP in projection neurons, as a higher fraction of the EPSC amplitude was depressed by the selective antagonist IEM1460 in the projection neurons from adult mice receiving P3 incision compared with naive littermate controls. These findings suggest that neonatal tissue injury creates a more permissive environment for the occurrence of LTP at peripheral afferent synapses onto the major output neurons of the spinal nociceptive network, which allows for a higher likelihood for a certain nociceptive input to be strengthened within the spinal cord after repetitive activation.

In summary, transient neonatal injury persistently primes sensory synapses onto ascending projection neurons by multiple mechanisms including, but not limited to, strengthened sensory inputs and reduced inhibition within SDH, a stronger magnitude of frequency-dependent LTP and a higher prevalence of t-LTP onto ascending projection neurons. These long-term changes are predicted to amplify ascending nociceptive signals (Figure 2) and thereby contribute to the increased pain severity following repetitive tissue injury.

Conclusion

Collectively, the above studies suggest that neonatal tissue injury provokes a persistent shift in the balance of synaptic excitation vs. inhibition within the spinal nociceptive network, resulting in a greater gain in the output of nociceptive signals from projection neurons to the brain. Moreover, neonatal injury also creates a more permissive environment for spike timing-dependent LTP to occur in projection neurons in response to the activity which is well correlated with incoming sensory input. These results highlight the importance of further studies into the mechanistic basis for the pediatric pain, in order to eventually develop more age-appropriate interventions to minimize the long-term consequences of early life pain exposure on the maturation of spinal nociceptive pathways, which is particularly relevant for infants requiring hospitalization (68). In addition, the persistent alterations observed in mature projection neurons following neonatal injury also emphasize the potential utility of targeting this neuronal population via therapeutic intervention as a means to control the strength of ascending nociceptive transmission to the brain.

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