Enhanced nociception in a rabbit model of cerebral palsy

Authors
E. J. REEDICH[1,2], L. T. GENRY[1,2,3], C. F. CAVARSAN[1,2], E. MENA AVILA[1,2], M. A. SINGER[4], D. M. BOUDREAU[1,2], M. C. BRENNAN[1,2], A. M. GARRETT [1,2,3], L. DOWALIBY[1,2], M. R. DETLOFF[4*], K. A. QUINLAN[1,2*]

1 George and Anne Ryan Institute for Neuroscience
2 Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy
3 Interdepartmental Neuroscience Program
University of Rhode Island, Kingston, RI, USA
4 Department of Neurobiology and Anatomy, Marion Murray Spinal Cord Research Center, College of Medicine Drexel University, Philadelphia, PA, USA

*Corresponding Authors:

Katharina A. Quinlan
George and Anne Ryan Institute for Neuroscience
Department of Biomedical and Pharmaceutical Sciences
College of Pharmacy
University of Rhode Island
130 Flagg Rd
Kingston, RI 02881
kaquinlan@uri.edu

Megan R. Detloff
Department of Neurobiology & Anatomy
Marion Murray Spinal Cord Research Center
College of Medicine
Drexel University
2900 W. Queen Lane
Philadelphia, PA 19129
mrd64@drexel.edu

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Abstract

The most prevalent comorbidity of cerebral palsy (CP) is pain. In order to investigate the relationship between perinatal injuries that cause CP and nociception, we investigated mechanical and thermal sensitivity of New Zealand White rabbit kits after prenatal hypoxia-ischemia (HI), sham surgery without hypoxia, and after a typical, unperturbed gestation. A range of motor deficits were observed in kits born naturally after HI (40 minutes at 70-80% gestation) as previously described. We found that HI caused mechanical and thermal allodynia at postnatal day 5, which was accompanied by an expansion of peptidergic afferents (marked by expression of calcitonin gene related peptide; CGRP) in both the superficial and deep dorsal horn. Non-peptidergic afferents (marked by expression of isolectin B4; IB4) were unaltered in HI kits but overlap of the two populations (peptidergic and nonpeptidergic nociceptors) was increased by HI. Interestingly, HI-subjected rabbits exhibited allodynia, even in the absence of motor deficits. HI motor affected and unaffected kits had similar thermal sensitivity but affected kits had less mechanical sensitivity than HI unaffected kits. These findings suggest that prenatal neural injuries impact sensory and motor networks independently and that developing sensory circuits may be more vulnerable than motor circuits to perturbation by prenatal hypoxic-ischemic injury. In conclusion, pain experienced by individuals with CP could arise from developmental insults capable of causing the condition, and therapeutics that specifically target altered nociception in these individuals could be beneficial for treating and preventing chronic pain.

Introduction:

Cerebral palsy (CP) is the most prevalent motor disability in children, affecting 1:500 live births (Oskoui et al., 2013). Chronic pain is the most prevalent co-morbidity of CP (Novak et al., 2012; McDowell et al., 2017): up to 77% of children with CP experience pain (Mckinnon et al., 2018; McKinnon et al., 2020). Individuals with CP experience chronic pain at significantly higher rates than the rest of the population (Doralp and Bartlett, 2010; Badia et al., 2014; Westbom et al., 2017; García Jalón et al., 2021). Pain decreases quality of life (Findlay et al., 2016), yet the pathophysiology of pain is rarely researched in the context of CP. This is likely due to the pervasive view that pain in individuals with CP is a symptom arising from secondary effects of physical stressors, including spasticity, hip subluxation and joint contractures. Individuals with severe CP (as measured by Gross Motor Function Classification System (GMFCS) V) do experience more pain (Mckinnon et al., 2018; García Jalón et al., 2021) however, individuals across all GMFCS levels report pain (Sienko, 2018; Flanigan et al., 2020). Even individuals born very prematurely (<26 weeks gestion), who *do not* develop CP, report recurrent pain and altered thermal sensitivity as adults (Walker et al., 2018). This suggests that nociceptors may be affected by developmental insults: the same trauma that can impact developing corticospinal tracts and ultimately affect movement may also impact development of nociceptive circuitry, perhaps completely independently of motor circuits.

There is a wealth of evidence regarding the interrelatedness of sensory and motor performance (Cooper et al., 1995; Gupta et al., 2017; Matusz et al., 2018; Zarkou et al., 2020). It seems likely that somatosensory deficits are not only present in CP, but that their presence could contribute to motor dysfunction. Typically, primary afferent input onto spinal circuitry is a key mediator of functional motor performance. Proprioceptive and tactile afferent feedback informs the CNS of motor command outcomes and sculpts ongoing drive to motoneurons. The interconnection between motor and sensory systems can make it challenging to parse out their relative contribution to functional impairments. As in other CNS disorders, there is a strong correlation between the integrity of the somatosensory system (including primary afferent input) and motor performance in CP (Gupta et al., 2017; Zarkou et al., 2020). Tractography and physiology experiments in individuals with unilateral CP revealed that integrity of *somatosensory connections* has a relatively larger impact on hand function compared to the integrity of motor connections (Gupta et al., 2017), and stimulation of peripheral nerves, which provides additional sensory feedback, improved motor performance in individuals with diplegic CP (Bumin and Kayihan, 2001; Kaelin-Lang, 2008).

Physical insults during pregnancy have been linked to later development of autism, schizophrenia, and the motor impairments that are hallmarks of CP (Marriott et al., 2015), but it is not known if these prenatal events impact pain sensation. Neural development can be negatively impacted by relatively common conditions in the perinatal period, including maternal infection and inflammation, placental insufficiency, and difficult birth. These conditions can all result in varying degrees of hypoxia-ischemia (HI) and injury to the developing central nervous system. Effects of prenatal HI include white matter injury, cortical damage and motor deficits (Derrick et al., 2004; Buser et al., 2010; Drobyshevsky and Quinlan, 2017), but the effect on pain is completely unstudied.

Trauma can alter neural circuits and have long lasting effects on nociception (Costigan et al., 2009; Price and Dussor, 2014). The effect of neural injury on nociceptive primary sensory neurons is a major contributor to the development of chronic pain (Hulsebosch and Coggeshall, 1981a, 1981b; Rodin

et al., 1983; Woolf et al., 1992; Koerber et al., 1994; Ramer et al., 2012; Walters, 2012). After trauma, it is well-established that the afferent fibers of these nociceptors exhibit robust arborizations into the superficial and deep dorsal horn of the spinal cord (Krenz and Weaver, 1998; Weaver et al., 2001; Ondarza et al., 2003; Bedi et al., 2010; Detloff et al., 2014a, 2016). In addition, nociceptors increase spontaneous activity and become hyperexcitable following CNS trauma (Burchiel et al., 1985; Gracely et al., 1993; Pitcher and Henry, 2008; Bedi et al., 2010; Gold and Gebhart, 2010; Li et al., 2018; North et al., 2019). Peripheral inflammation during the neonatal period enhances collateral sprouting of primary nociceptive afferents and enhances nociception in adulthood (Ruda et al., 2000). Astrocytes and microglia are activated by neural injury and release a plethora of pro-inflammatory molecules into the spinal milieu and corresponds to the development of neuropathic pain (Zhang et al., 2007, 2016; Detloff et al., 2008; Gao et al., 2009; Hulsebosch et al., 2009; Xie et al., 2009; Chhaya et al., 2018; Ishikura et al., 2021; Lu et al., 2021). A causal link between gliosis and enhanced nociception was demonstrated in a rat model of allodynia. Intrathecal administration of fluorocitrate (a glial metabolic inhibitor) and CNI-1493 (an inhibitor of proinflammatory cytokines) fully prevented mechanical and thermal allodynia (Milligan et al., 2000).

In order to study increased pain and its potential mechanisms in the context of CP, we explored mechanical and thermal sensitivity of rabbit kits that were subjected to HI injury *in utero* and examined components of nociception at the level of the spinal cord, including the presence of reactive astrocytes and arborization of mechanosensitive and thermosensitive afferents. We characterized postnatal development of nociceptive pathways after prenatal HI insult (40 minutes at 70-80% gestation) and assessed nociceptive behavior at an age roughly corresponding to human toddlers (postnatal day [P] 5). Moreover, we used immunohistochemistry to quantify expression of molecular markers for nociceptors and activated astrocytes in spinal cord dorsal horns at P5. We found that rabbit kits exposed to prenatal HI demonstrated increased nociceptive behavior in response to cutaneous mechanical or thermal stimuli, which corresponded to aberrant distribution and density of nociceptive primary afferent fibers but almost no change in astrocyte reactivity. Interestingly, our data show that enhanced forepaw and hindpaw sensitivity occurs *independent of the severity of motor deficits*. Together, these results indicate that sensory circuits could be *more vulnerable* to perturbation by perinatal hypoxic-ischemic injury than motor circuits, and suggest that neuropathic pain could occur even without accompanying motor deficits characteristic of CP.

Materials and Methods:

Animal model: Experiments were performed in accordance with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. Approval of the University of Rhode Island's Animal Care and Use Committee was obtained for all experiments performed in this study. 84 kits from 15 different litters originating from 9 dams were used for this study; some dams provided more than one litter of rabbits for this study. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Hypoxia-ischemia surgery: Pregnant New Zealand White rabbits (bred in house or ordered from Charles River Laboratories, Inc., Wilmington MA), underwent HI procedures as previously described (Derrick et al., 2004). Briefly, at ~70-80% gestation (day 22 -26 of gestation), dams were premedicated with ketamine and xylazine (25 mg/kg and 3.75 mg/kg, respectively), anesthetized with isoflurane (1-3%; inhaled via V gel tube) and occasionally treated with buprenorphine (0.01 - 0.24 mg/kg as needed). The

left or right femoral artery was isolated. A Fogarty balloon catheter (2-4 French) was inserted and advanced to the level of the descending aorta, above the uterine arteries, and inflated for 40 minutes to cause HI in the fetuses. Sham animals underwent the same anesthesia protocols as the HI dams but without insertion and/or inflation of the catheter. After the procedures, dams recovered and later gave birth to kits.

Behavior: There is a wide range in motor phenotypes after HI, with some kits showing motor deficits while others appear unaffected. Categorization of motor deficits was performed using a modified Ashworth scale, observations/tests for activity, locomotion, posture, righting reflex, and muscle tone as previously described (Derrick et al., 2004). Based on these tests, kits were labeled as "HI motor-affected" or "HI motor-unaffected."

von Frey: Rabbit kits were tested for mechanical allodynia using von Frey filaments. Animals were allowed to acclimatize in a chamber on an elevated gridded (8 mm x 8 mm) surface. von Frey filaments (Touch Test® Sensory Evaluators; range: 1-26 g) were applied in ascending order to the plantar surface of the forepaws and hindpaws of the kit; if paw withdrawal occurred in response to application of the 1 g von Frey filament, filaments (0.6-0.008 g) were applied in descending order until no response was observed. Each filament was tested at least five consecutive times per paw to ensure the observed reaction was in response to the filament and not general movement of the animal. The force (in g) of the filament that elicited a consistent withdrawal response was recorded as the paw withdrawal threshold. If no response was elicited by application of the 26 g von Frey filament, that paw was recorded as non-responsive and given a score of 60 g. Filament forces eliciting withdrawal of left and right forepaws and hindpaws were averaged for statistical analyses.

Hargreaves: Thermal sensitivity was tested using a Hargreaves apparatus. Specifically, a model 336G Plantar/Tail Stimulator Analgesia Meter (IITC Life Sciences, Woodland Hills, CA) was used to apply a beam of radiant heat (4x6 mm area; 80% intensity; 8 s max duration) to the plantar surface of each hindpaw and forepaw. Rabbit kits were allowed to acclimatize in a chamber on a glass surface, and then each paw was tested three times with at least 30 seconds between applications. The beam of radiant heat increased temperature gradually from rest (20-30 °C) to a maximum of 83 °C at 8 s. Latency to paw withdrawal was recorded; if the animal did not withdraw its paw, that trial was recorded as a non-response and given a latency of 9 s. Withdrawal latencies of left and right forepaws and hindpaws were averaged for statistical analyses.

Immunohistochemistry: Animals were deeply anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) or sodium pentobarbital before tissue harvest at postnatal day (P)5. Transcardial perfusion was performed using chilled PBS (pH 7.4) until tissue blanching was observed, then 4% paraformaldehyde (PFA) (in PBS) was infused at a constant flow rate of approximately 5-8 mL/min (depending on rabbit kit size). Perfused spinal cords were dissected and post-fixed in 4% paraformaldehyde for ~24-72 hrs. Next, fixed spinal cords were washed with PBS and cryoprotected in 30% sucrose (in PBS) until they were observed to sink. Cryoprotected spinal cords were embedded in OCT and frozen at −80 °C until cryostat sectioning (Leica Lm 1850, Germany). Transverse lumbar spinal cord cryosections were collected in serial at 30-µm or 40-µm thickness; to avoid repeat regions of analysis, every 15th section was used for analysis.

For glial fibrillary acidic protein (GFAP) immunostaining, slides were thawed, dried in an oven at 37 °C to improve section adherence, then rehydrated in PBS. Sections were blocked in 0.2% Triton X-100 and 5%

normal horse serum (NHS) (in PBS) for 30 minutes at room temperature. Following blocking, slices were incubated in primary antibody solution containing mouse-anti GFAP antibody (diluted 1:500 in 0.1% Triton X-100 and 1% NHS in PBS; EnCor Biotechnology, cat. #MCA-5C10) overnight at room temperature. Sections were washed in PBS then incubated in secondary antibody solution consisting of Atto 488-goat anti-mouse IgG (whole molecule) (diluted 1:500 in 0.1% Triton X-100 and 1% NHS in PBS, Sigma-Aldrich, cat. # 62197) for 2 h at room temperature. Nuclei were labeled with 4',6-Diamidino-2-Phenylindole (DAPI) (Invitrogen™, cat. # D1306) and slides were mounted using Fluoromount mounting medium (SouthernBioTech). Z-stack photomicrographs of dorsal and ventral spinal cord were acquired at 10x magnification using a Nikon Eclipse Ti2 inverted confocal microscope. Maximum intensity projections were generated and stitched (if possible) using Image Composite Editor (Microsoft). Grayscale images were imported into ImageJ and blinded densitometric analysis of GFAP immunofluorescent signal (mean gray value) in regions of interest containing dorsal columns, superficial dorsal gray matter, lateral spinathalamic tract, and ventral spinothalamic tract (minus background fluorescent signal/mean gray value) was performed using Image J (NIH).

IHC for primary afferent fibers: Sections were rinsed in PBS, followed by blockage of non-specific binding of antibodies in 10% normal goat serum and 0.2% Triton-X100 in PBS for 1 hour at room temperature followed by 30 minute incubation with rabbit-to-rabbit blocking reagent (ScyTek Laboratories, Inc.). Sections were then incubated in biotinylated IB4 (1:2000, Sigma) and anti-calcitonin gene regulated peptide (CGRP; 1:1500 Peninsula Laboratories) with 0.1% Triton X-100 in PBS at room temperature for 48h. After three washes with PBS, sections were incubated in avidin conjugated with Alexafluor 594 (Invitrogen, diluted at 1:400) and goat-anti-rabbit Alexa fluor 488 (Invitrogen, diluted at 1:400) in 5% goat serum in PBS for 2 hours at room temperature. Following three washes, sections were rinsed in PBS and cover slipped with FluorSave Reagent (Calbiochem, Bedford, MA). Three images of the left and right lumbar dorsal horn were taken for each rabbit at the same magnification, lens aperture and exposure time on a Leica and captured with Slidebook software. The proportional area of IB4, CGRP and colocalized immunoreactive tissue within the cap of the dorsal horn (laminae I-III) was quantified in ImageJ as previously described (Detloff et al., 2016). The proportional area of CGRP+ fibers that expand in dorsal horn ventral to the IB4+ band was measured in the same sections.

Statistical Analyses: All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.; San Diego, CA, USA). For each dataset, normality of distributions was tested using D'Agostino and Pearson tests. For normally distributed datasets with equal variances including the Hargreaves data, a one-way ANOVA with Tukey's multiple comparisons tests were used to identify statistically significant differences between groups For datasets in which one or more distributions were non-normally distributed, including the von Frey dataset, Kruskal-Wallis tests with Dunn's multiple comparisons tests were used.

Results:

In this study, we investigated the link between prenatal HI injury and nociception in a rabbit model of CP. Rabbit kits exposed to prenatal HI and age-matched sham animals underwent testing for mechanical and thermal allodynia at P5 using von Frey and Hargreaves testing, respectively. After prenatal HI injury, neonatal rabbit kits displayed increased nociceptive behavior in response to mechanical and thermal stimuli that manifested even in the absence of motor deficits. HI kits demonstrated significantly greater sensitivity to von Frey filaments compared to sham kits, withdrawing

paws in response to application of lower forces (forepaws, *P*=0.0091; hindpaws, *P*=0.0115) (**Figure 1A**). Hindpaw von Frey scores of HI-motor-unaffected kits (8.2 g; light red symbols in Fig 1) was significantly lower than that of HI- motor-affected kits (19.4 g; dark red symbols; *P*=0.0416). Similar to the von Frey, the Hargreaves test revealed allodynia in HI kits. HI kits withdrew paws earlier than sham rabbits in response to targeted radiant heat (Figure 1B), indicating thermal allodynia (forepaws: *P*<0.0001; hindpaws: *P*=0.0012). Paw withdrawal latencies to noxious thermal stimulation were comparable between HI motor-unaffected (forepaw latency: 3.6 s; hindpaw latency: 3.0 s) and HI motor-affected kits (forepaw latency: 4.4 s; hindpaw latency: 3.8 s) (*P*=0.1637 and 0.2711, respectively). Interestingly, we found significant differences in both mechanical and thermal nociceptive behavior between control kits

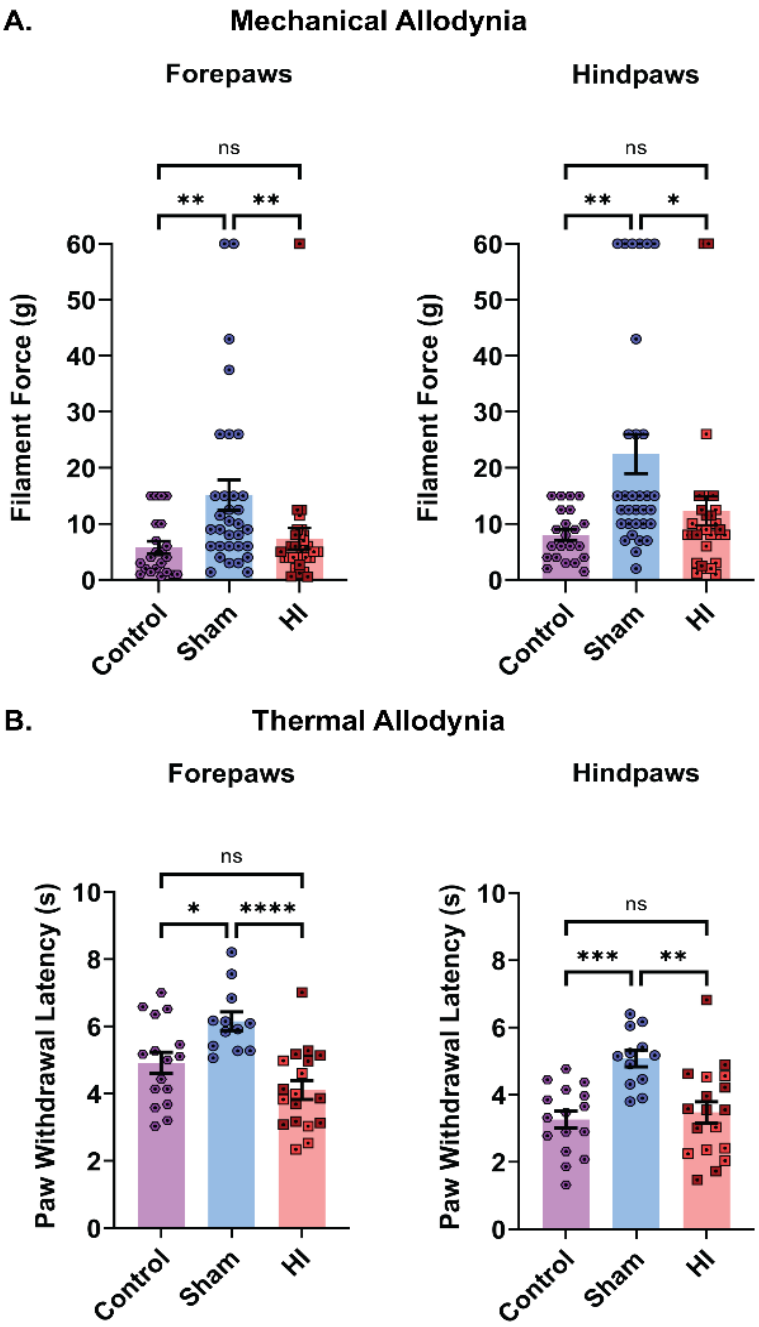

Figure 1: HI kits exhibit mechanical and thermal allodynia. **A:** Average forepaw (left) and hindpaw (right) von Frey scores at P5. Sham-operated controls (blue) have higher scores, indicating less mechanosensitivity, while HI kits (red) display greater mechanosensitivity (lower scores). Lower von Frey scores indicate mechanical allodynia in HI kits. Data are represented as mean ± SEM; N=22 control, N=32 sham, and N=30 HI rabbits (dark red, motor-affected). **P* < 0.05, ***P* <0.01, ns, not significant; Kruskal-Wallis tests with Dunn's multiple comparisons tests. **B:** Average forepaw (left) and hindpaw (right) withdrawal latencies during Hargreaves testing at P5. Shorter latencies to paw withdrawal in response to radiant heat are observed in HI kits compared to sham animals, indicative of enhanced thermal sensitivity. Data are represented as mean ± SEM; N=16 control, N=12 sham, and N=18 HI rabbits (dark red, motor-affected). **P* < 0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001, ns, not significant, one-way ANOVA with Tukey's multiple comparisons tests.

without prenatal exposure to anesthesia/sham surgery and surgical sham controls, indicating prenatal exposure to both anesthesia and HI is sufficient to alter development of nociceptors.

We hypothesized that mechanical and thermal allodynia in HI rabbits may be caused by spinal gliosis and expansion of nociceptive afferents in the spinal cord dorsal horn. To determine whether prenatal HI leads to postnatal astrogliosis in gray and white matter spinal cord regions involved in cutaneous and nociceptive sensation, immunohistochemistry was performed for glial acidic fibrillary protein (GFAP) in four lumbar spinal cord regions of interest (ROIs) containing the dorsal column, superficial dorsal gray matter, ventral spinothalamic tract, and lateral spinothalamic tract (**Figure 2**). The grey or white matter

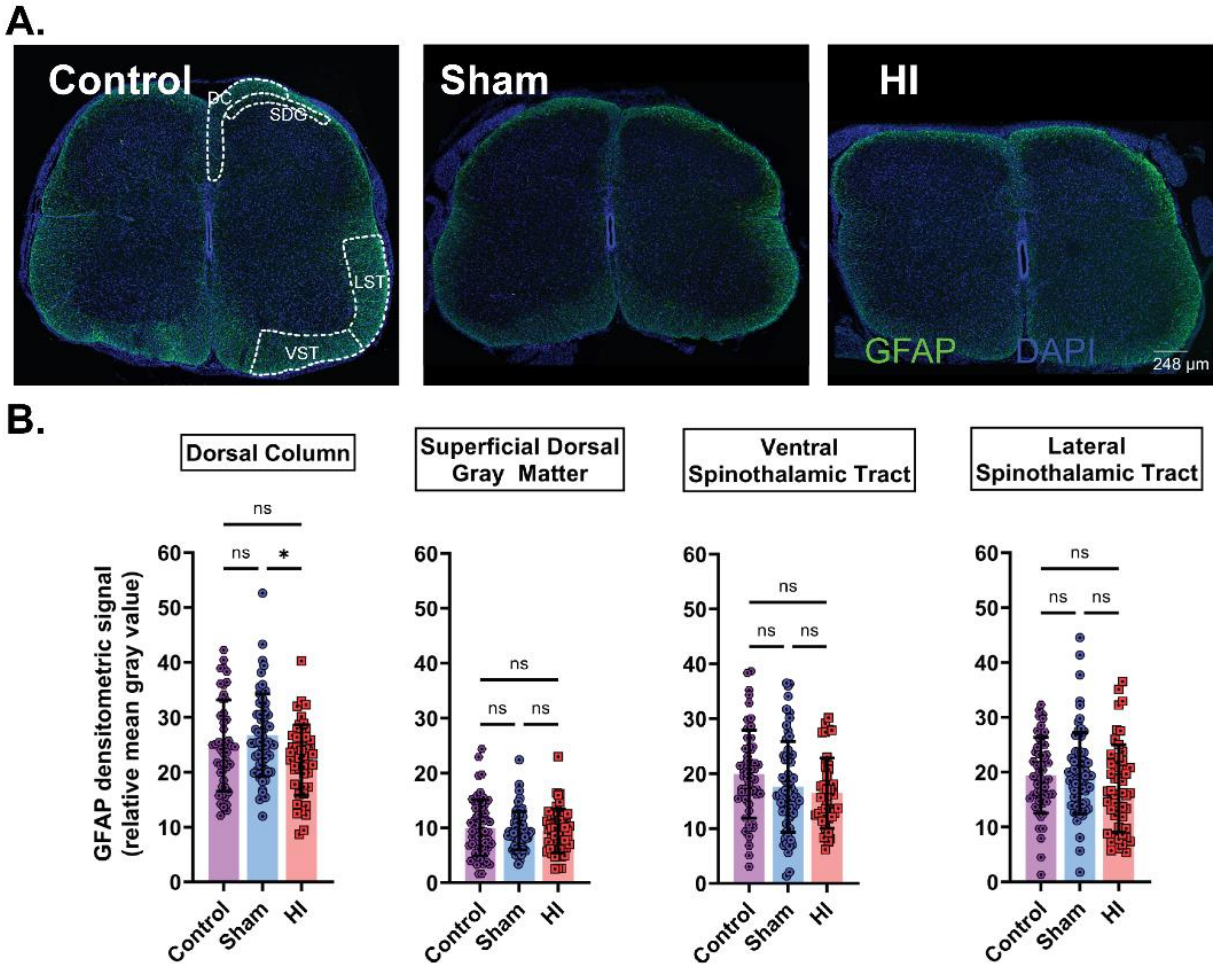

Figure 2: Astrogliosis does not contribute to enhanced nociception in HI rabbits at P5. **A**: Transverse lumbar spinal cord sections from control (left), sham (middle) and HI (right) rabbits immunostained for glial fibrillary acidic protein (GFAP; green) with nuclei labeled by DAPI (blue). Scale bar applies to all images. **B:** Quantification of GFAP densitometry in spinal regions containing tracts or cell populations of interest: dorsal column (DC), superficial dorsal gray matter (SDG), ventral spinothalamic tract (VST), and lateral spinothalamic tract (LST). GFAP expression was largely unaltered, with GFAP densitometric signal only significantly reduced in white matter containing the dorsal column (sham v. HI; P=0.0120, Kruskal-Wallis test with Dunn's multiple comparison's test). Data are represented as mean ± SD; N=48-57 control, N=60-68 sham, and N=42-51 HI sections from N=3-4 rabbits per group. *P <0.05, ns, not significant; Kruskal-Wallis tests with Dunn's multiple comparisons tests was used for statistical analysis of DC, SDG, and LST; one-way ANOVA with Tukey's multiple comparisons tests was used for statistical analysis of VST.

in our regions of interest was targeted to encompass the entire region of interest but could also contain portions of other areas. GFAP immunoreactivity was quantified by measuring its relative densitometric signal (mean gray value in ROI – background). No evidence was found to support a contribution of neuroinflammation to increased nociception at this age. The significant decrease in GFAP in the dorsal column is likely a reflection of the weakened corticospinal tract in HI animals.

To determine whether prenatal HI affects nociceptive primary afferent distribution and density within the dorsal horn of the spinal cord, lumbar spinal cord from control, sham and HI kits corresponding to the hindpaws were reacted with antibodies against CGRP and IB4 that selectively label peptidergic and non-peptidergic nociceptors, respectively. The distribution of these two nociceptor subpopulations do not overlap in the dorsal horn of control or sham kits (**Figure 3 A-A''**), however, HI led to an increased overlap in the topographic distribution of these two fiber types (Figure 3 B-B''). In addition, kits with HI exhibited increased CGRP+ fibers in the deep dorsal horn (Figure C-C''), that were not present in either control or sham groups. Quantification of the proportional area each afferent subpopulation independently revealed that CGRP fibers (Figure 3D, G) but not IB4 (Figure 3E) fibers exhibited increased density in the superficial and deep dorsal horn. Despite no increase in the density or distribution of IB4+ fibers in the dorsal horn, there was significant colocalization of green and red immunofluorescence in HI compared to Sham kits, suggesting that HI may induce plasticity or phenotype switch of these nociceptor populations.

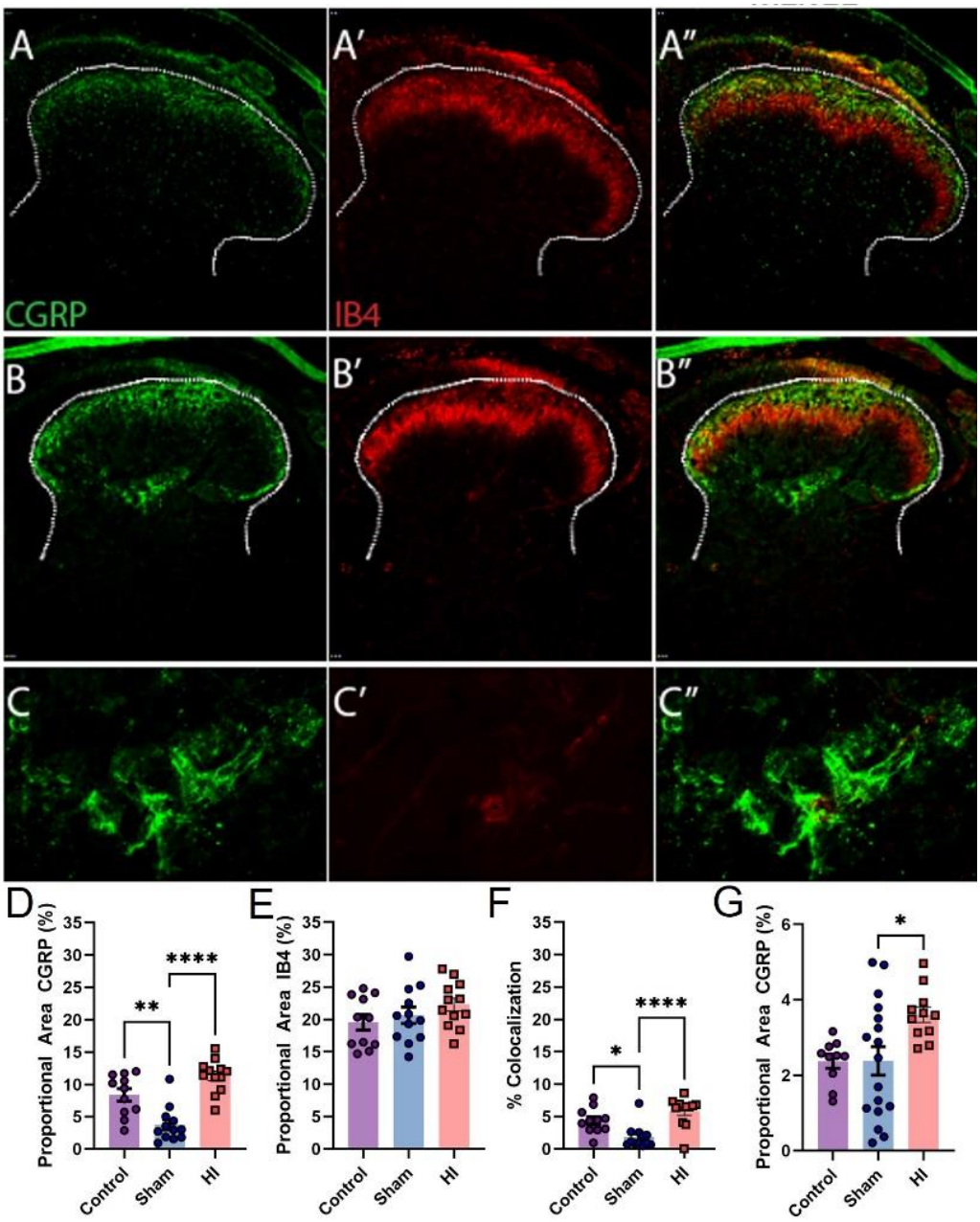

Figure 3: Increased density and overlap of nociceptive primary afferent fibers in HI kits at P5. Representative images of the lumbar dorsal horn of sham (A-A'') or HI (B-B'') kits immunolabeled for calcitonin gene-regulated peptide (CGRP; A, B, C) or isolectin B-4 (IB4; A', B', C'). Note the increased presence of CGRP+ fibers in the deep dorsal horn in the HI but not the Sham spinal cord (C-C''). The proportional area of CGRP+ tissue was significantly increased in the superficial and deep dorsal horn of HI rabbits compared to Sham (D, G), while the density and distribution of IB4+ afferents was unchanged between groups (E). The degree of overlap of these two nociceptor populations can be viewed in the merged images (A'', B'') and was significantly increased in HI kits compared to sham kits (F). Data are represented as mean ± SD; individual data points represent the average measurement from 3 separate images of left or right dorsal horns from n=4 control, n=5 sham, and n=5 HI kits. *p <0.05, **p<.01, ****p<.0001; One-way ANOVA with Tukey's post hoc tests was used for statistical analysis.

Discussion:

Experimental animal models that mimic human disease with high fidelity are more likely to drive meaningful discovery of therapeutic interventions to treat human disease. Here, we demonstrate that the rabbit HI model of CP recapitulates aberrant nociception and pain development seen in individuals with CP, in addition to motor deficits. Moreover, this is one of few studies that utilize quantitative assessments of nociception in the rabbit, despite the rabbit's extensive use in biomedical research, even in research involving osteoarthritis or intervertebral disc dislocation (Yoshioka et al., 1996; Lei et al., 2017; Mans, 2020). Our findings indicate that after prenatal HI, kits have increased sprouting of peptidergic nociceptive primary afferent fibers in the superficial and deep dorsal horn.

In individuals with CP, disruptions in somatosensory cortex connectivity (Hoon et al., 2002), and increased latency of sensory evoked potentials are present (Cahan et al., 1987; Kundi et al., 1989). Abnormality in sensory evoked potentials does not correlate with type or severity of CP, but rather the type of developmental insult (Teflioudi et al., 2011). Tactile sensitivity actually *decreases* in individuals with CP while *pain sensitivity is enhanced* (Riquelme and Montoya, 2010). This could be attributed to afferents switching after injury: in a rat model of neuropathic pain, large diameter cutaneous/somatic Aβ afferents can undergo fiber-type switching after nerve ligation and begin expressing CGRP like nociceptive fibers (Nitzan-Luques et al., 2011). This switch in identity corresponds to a period of tactile allodynia. Movement and motor unit firing patterns in neurotypically developing adults are directly affected by pain (Hodges and Tucker, 2011; Martinez-Valdes et al., 2020), perhaps through neuromodulators including serotonin (5HT) (Mesquita et al., 2020). Thus, it is unfortunate that treatment options for individuals with CP are limited to the same drugs that are available to the rest of the population even though the pathophysiology of their pain could be completely different. Nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and gabapentinoids together provide pain relief to only about 20% of suffering individuals, clearly highlighting the unmet medical need for such a common and widespread ailment (De Moulin et al., 2007, 2014; Cousins, 2012; Finnerup et al., 2015; Finnerup and Attal, 2016). Novel targets for fully alleviating pain should be explored specifically within the context of treating pain in individuals with CP or those who have survived perinatal physical trauma.

In situations where both nociception and the affective experience of pain can be altered, such as after HI, it is critical to accurately identify and classify aberrant sensory behavior. Unlike in rodent experiments, there are surprisingly few reports measuring pain or nociception in experimental rabbit models, even those modeling knee osteoarthritis or intervertebral disc degeneration. Recently, composite pain scales for rabbits in a clinical environment (Banchi et al., 2020) and the Bristol Rabbit Pain Scale (Benato et al., 2021) examine and score rabbit facial expressions and other behaviors to assess pain as the grimace scale (Keating et al., 2012; Evangelista et al., 2021). Yet, recent reports suggest that there can be a profound effect of the experimenter's presence on the animal's behavior and subsequent score on these tests (Pinho et al., 2020).

Here, we utilized methods to assess and quantify shifts in sensitivity to mechanical and thermal stimuli that are often used in rodent models of central and peripheral neuropathic pain. The stereotypical aversive paw withdrawal response to nocifensive tactile or thermal stimuli is mitigated by a segmental spinal reflex and is often inferred as a proxy for cortical perception of the stimulus. It is unclear whether these conventional methods reflect hyperreflexia of nociceptive segmental spinal cord circuits (Murphy and Zemlan, 1990; Kauppila et al., 1998; Schomburg and Steffens, 1998; Advokat, 2002) or the complicated perception of neuropathic pain. However, unlike assessments of spontaneous pain

(Rabbit grimace scale), these assessments identify the dermatomal location and relevant spinal cord or dorsal root ganglion level in which dysfunctional sensation occurs. This may be especially useful in studies where changes in primary afferent input and/or dorsal horn processing are examined.

This is the first study to show that nociceptors are altered in an animal model of CP. The increased overlap and density of nociceptive afferent fibers in the dorsal horn of HI kits may indicate intrinsic changes within the nociceptive DRG neurons. In addition to sprouting, HI could also affect nociceptor excitability. After prenatal HI, rabbit kits exhibit increased excitability of the monosynaptic H-reflex without significant anatomical changes in large diameter, myelinated primary afferent fibers (Synowiec et al., 2019). In other models of nervous system injury, chronic pain is associated with dysfunctional nociceptors that exhibit robust anatomical and electrophysiological plasticity (Dougherty et al., 2004; Bedi et al., 2010; Zhang et al., 2013; Detloff et al., 2014b, 2016; Moy et al., 2018; Shiers et al., 2020; Wangzhou et al., 2021); however, emerging evidence suggests that *nociceptive primary afferent input impedes motor output* following CNS injury (Keller et al., 2017, 2018, 2019). In a series of studies, Keller *et al*. demonstrated that nociceptor depletion reduces muscle contractures and spasticity of the hindlimbs and improves overall locomotor performance following spinal cord injury. Taken together, these findings call into question whether altered activity in proprioceptors alone could cause motor deficits in CP.

DRG neurons that extend axons into the dorsal horn are born in the first half of gestation. Dorsal horn interneurons that are involved in sensory pathways undergo growth and refinement of projections later in gestation; this process continues after birth (Olson and Luo, 2018). Since insults leading to CP occur later in gestation, like the intrauterine hypoxia surgeries performed in this study, changes in arborization of CGRP+ and IB4+ nociceptive sensory neurons in the dorsal horn without changes in their cell numbers in the DRG are consistent with the timing of injuries. Central neuropathic pain in our rabbits could also be augmented by increased in spinal serotonin after HI (Drobyshevsky et al., 2015). In a rat model of neuropathic pain, initiation of pain was blocked with 5HT2a receptor antagonists (Wang et al., 2012). These mechanisms require further investigation.

Neuroinflammation and pain are tightly intertwined (Grace et al., 2014). While astrogliosis may not contribute to enhanced nociception after HI, it does have a well-established role in other painful conditions. Astrocytes are activated by injury and release pro-inflammatory molecules into the spinal milieu. After complete spinal cord injury, increased GFAP persists for at least 3 weeks in the adult rat spinal cord (Morin-Richaud et al., 1998). However, the time course of the GFAP response to injury is different in neonates. Unlike adults, neonatal rat pups respond to spared nerve injury with an early increase in GFAP (one day after injury), along with the same elevation after 7 days that is observed in adults (Vega-Avelaira et al., 2007). In previous studies of HI rabbit kits, density of GFAP fiber length and density of GFAP+ cell bodies increased in the lumbar gray matter of hypertonic kits at P1, seven days after HI (Drobyshevsky and Quinlan, 2017; Synowiec et al., 2019). This study was performed in kits that were 4 days older (P5) and examined specific laminae / regions of interest within the gray matter. While astrogliosis is not present in the spinal regions of interest we studied at P5 (11-14 days after HI), their reactivity closer to the time of injury is likely and could be a causal link of enhanced nociception as has been demonstrated in other models (Milligan et al., 2000). Alternatively, astrocytes have been shown to exhibit different phenotypes or reactive states following similar CNS injuries that can be either destructive or pro-reparative (Liddelow and Barres, 2017; Escartin et al., 2021). Moreover, it has been

reported that ischemia can induce an "A2-like" astrocyte phenotype that upregulates thrombospondins and neurotrophic factors that promote survival and repair of neurons (Gao et al., 2005; Zador et al., 2009; Hayakawa et al., 2014). Future studies could examine more closely the time course and specific regions of elevated astrocyte activation in the rabbit after prenatal HI and its relationship to enhanced nociception.

The current study demonstrates enhanced nociceptive behavior in neonatal rabbits after prenatal HI injury, independent of the severity of their motor deficits. Identification of how prenatal physical stressors impact nociceptors could be the first step to prevent pain in those at risk, provide better clinical treatment of pain, and increase quality of life.

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Figure Legends

Figure 1: HI kits exhibit mechanical and thermal allodynia. **A:** Average forepaw (left) and hindpaw (right) von Frey scores at P5. Sham-operated controls (blue) have higher scores, indicating less mechanosensitivity, while HI kits (red) display greater mechanosensitivity (lower scores). Lower von Frey scores indicate mechanical allodynia in HI kits. Data are represented as mean ± SEM; N=22 control, N=32 sham, and N=30 HI rabbits (dark red, motor-affected). *$P < 0.05$, **$P < 0.01$, ns, not significant; Kruskal-Wallis tests with Dunn's multiple comparisons tests. **B:** Average forepaw (left) and hindpaw (right) withdrawal latencies during Hargreaves testing at P5. Shorter latencies to paw withdrawal in response to radiant heat are observed in HI kits compared to sham animals, indicative of enhanced thermal sensitivity. Data are represented as mean ± SEM; N=16 control, N=12 sham, and N=18 HI rabbits (dark red, motor-affected). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns, not significant, one-way ANOVA with Tukey's multiple comparisons tests.

Figure 2: Astrogliosis does not contribute to enhanced nociception in HI rabbits at P5. **A**: Transverse lumbar spinal cord sections from control (left), sham (middle) and HI (right) rabbits immunostained for glial fibrillary acidic protein (GFAP; green) with nuclei labeled by DAPI (blue). Scale bar applies to all images. **B:** Quantification of GFAP densitometry in spinal regions containing tracts or cell populations of interest: dorsal column (DC), superficial dorsal gray matter (SDG), ventral spinothalamic tract (VST), and lateral spinothalamic tract (LST). GFAP expression was largely unaltered, with GFAP densitometric signal only significantly reduced in white matter containing the dorsal column (sham v. HI; $P=0.0120$, Kruskal-Wallis test with Dunn's multiple comparison's test). Data are represented as mean ± SD; N=48-57 control, N=60-68 sham, and N=42-51 HI sections from N=3-4 rabbits per group. *$P < 0.05$, ns, not significant; Kruskal-Wallis tests with Dunn's multiple comparisons tests was used for statistical analysis of DC, SDG, and LST; one-way ANOVA with Tukey's multiple comparisons tests was used for statistical analysis of VST.

Figure 3: Increased density and overlap of nociceptive primary afferent fibers in HI kits at P5. Representative images of the lumbar dorsal horn of sham (A-A'') or HI (B-B'') kits immunolabeled for calcitonin gene-regulated peptide (CGRP; A, B, C) or isolectin B-4 (IB4; A', B', C'). Note the increased presence of CGRP+ fibers in the deep dorsal horn in the HI but not the Sham spinal cord (C-C''). The

proportional area of CGRP+ tissue was significantly increased in the superficial and deep dorsal horn of HI rabbits compared to Sham (D, G), while the density and distribution of IB4+ afferents was unchanged between groups (E). The degree of overlap of these two nociceptor populations can be viewed in the merged images (A'', B'') and was significantly increased in HI kits compared to sham kits (F). Data are represented as mean ± SD; individual data points represent the average measurement from 3 separate images of left or right dorsal horns from n=4 control, n=5 sham, and n=5 HI kits. *p <0.05, **p<.01, ****p<.0001; One-way ANOVA with Tukey's post hoc tests was used for statistical analysis.

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