Enhanced nociceptor sprouting in a rabbit model of cerebral palsy

Authors

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Abstract

Spastic cerebral palsy (CP) is a movement disorder marked by hypertonia and hyperreflexia, and the most prevalent comorbidity is pain. Since spinal nociceptive afferents contribute to both the sensation of painful stimuli as well as reflex circuits involved in movement, we investigated the relationship between prenatal hypoxia-ischemia (HI) injury which can cause CP, and possible changes in spinal nociceptive circuitry. To do this, we examined nociceptive afferents and mechanical and thermal sensitivity of New Zealand White rabbit kits after prenatal HI or a sham surgical procedure. As described previously, a range of motor deficits similar to spastic CP was observed in kits born naturally after HI (40 minutes at ~70-80% gestation). We found that HI caused an expansion of peptidergic afferents (marked by expression of calcitonin gene-related peptide; CGRP) in both the superficial and deep dorsal horn at postnatal day (P)5. Non-peptidergic nociceptive afferent arborization (labeled by isolectin B4; IB4) was unaltered in HI kits but overlap of the two populations (peptidergic and non-peptidergic nociceptors) was increased by HI. Density of glial fibrillary acidic protein (GFAP) was unchanged within spinal white matter regions important in nociceptive transmission at P5. We found that mechanical and thermal allodynia was present in HI kits even in the absence of motor deficits. These findings suggest that prenatal HI injury impacts spinal sensory pathways in addition to the more well-established disruptions to descending motor circuits. In conclusion, changes to spinal nociceptive circuitry could disrupt spinal reflexes and contribute to pain experienced by individuals with CP.

Key words: cerebral palsy, hypoxia-ischemia, sensation, nociception, rabbit, primary afferent

Abbreviations: Serotonin (5HT); calcitonin gene-related peptide (CGRP); central nervous system (CNS); cerebral palsy (CP); Diamidino-2-Phenylindole (DAPI); dorsal column (DC); dorsal root ganglion (DRG); glial fibrillary acidic protein (GFAP); Gross Motor Function Classification System (GMFCS); hypoxia-ischemia (HI); isolectin B4 (IB4); immunohistochemistry (IHC); lateral spinothalamic tract (LST); normal horse serum (NHS); nonsteroidal anti-inflammatory drugs (NSAIDs); postnatal day (P); phosphate buffered saline (PBS); paraformaldehyde (PFA); regions of interest (ROIs); superficial dorsal gray matter (SDGM); ventral spinothalamic tract (VST)

Introduction:

Cerebral palsy (CP) is the most common motor disability in children, affecting 1:500 live births (Oskoui et al., 2013). Chronic pain is the most prevalent co-morbidity of CP (McDowell et al., 2017; Novak et al., 2012): 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 (Badia et al., 2014; Doralp and Bartlett, 2010; García Jalón et al., 2021; Westbom et al., 2017). 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 (García Jalón et al., 2021; Mckinnon et al., 2018); however, CP-affected individuals across all GMFCS levels report pain (Flanigan et al., 2020; Sienko, 2018). 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). Thus, we hypothesized that the same trauma that can impact developing corticospinal tracts in CP could impact development of nociceptive circuitry in the spinal cord.

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). Somatosensory deficits are present in CP, and 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 central nervous system (CNS) of motor command outcomes and sculpts ongoing drive to motoneurons. In particular, the role of primary nociceptive afferent fibers in motor function is critically overlooked—especially after CNS damage. Importantly, there are a few exceptions, including the restriction of motor unit activation in response to excessive stretch, detection of dangerous pH and other metabolite levels during muscle fatigue, and the exercise pressor reflex and motor unit modulation during exercise-induced fatigue (Laurin et al., 2015; Zając et al., 2015). Cutaneous nociceptors (mechanosensitive C, thermosensitive C and Aδ neurons) are small, pseudounipolar neurons in the dorsal root ganglion (DRG) that are analogous to Type III (Aδ) and Type IV (C) primary afferent fibers from the muscle, though their axons terminate in different dorsal horn laminae (Kandel et al., 2013; Lallemend and Ernfors, 2012; Laurin et al., 2015). Importantly, nociceptive and pain information can be relayed from muscle and joints via the nociceptive primary afferent reflex arcs, modulating motor output by generating a withdrawal reflex (Kandel et al., 2013; Kimura et al., 2004). 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., 2017), but it is not

known if these prenatal events impact nociceptive afferents. 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 CNS. Effects of prenatal HI include white matter injury, cortical damage and motor deficits (Buser et al., 2010; Derrick et al., 2004; Drobyshevsky and Quinlan, 2017; Rocha-Ferreira and Hristova, 2016), but the effect on nociceptors is completely unstudied. Notably, cell death in the deep dorsal horn (where synaptic targets of nociceptors are located) is present after HI in rabbits and in cases of infant death after hypoxia (Clancy et al., 1989; Drobyshevsky and Quinlan, 2017; Sladky and Rorke, 1986). Dorsal root ganglia (DRG) neurons undergo perinatal growth and refinement of projections during the period of susceptibility to injuries causing CP (Marmigère and Ernfors, 2007; Olson and Luo, 2018), though afferent plasticity is retained in adulthood (Jiang et al., 2016; Tan et al., 2012). Changes to nociceptive DRG neurons are entirely unexplored in the context of CP.

In this study, we have chosen to focus on nociceptors at the spinal level because they are the neuronal link between sensation of stimuli in the periphery (that is perceived as painful by higher brain centers) and hyperreflexia that is the hallmark of spastic CP. Nociceptors impact movement by modulating spinal reflex circuits via primary afferent depolarization (PAD)(Black, 2019; Lucas-Osma et al., 2019). PAD profoundly alters reflex responses (Hari et al., 2021; Hochman et al., 2010; Rudomin and Schmidt, 1999) and could directly contribute to the hyperreflexia and reflex irradiation that is observed in people with CP (Achache et al., 2010; Leonard et al., 1991; Leonard and Hirschfeld, 1995). Importantly, monosynaptic stretch reflexes can be potently suppressed via nociceptor inhibition (Black, 2019; Lucas-Osma et al., 2019). Thus, not only could expanded distribution of nociceptive afferents contribute to the perception of pain by higher brain centers, but also to hyperreflexia and disruptions in motor coordination that are the hallmarks of CP.

Thus, we examined nociceptors and altered nociception at the level of the spinal cord in rabbit kits that were subjected to HI injury *in utero* (40 minutes at ~70-80% gestation) using both immunohistochemistry to quantify molecular markers for nociceptors and *in vivo* behavioral testing at an age roughly corresponding to human toddlers (postnatal day [P] 5). We found that rabbit kits exposed to prenatal HI demonstrated increased nociceptive behavior in response to cutaneous mechanical and thermal stimuli. This increased nociceptive response corresponded to aberrant distribution and density of nociceptive primary afferent fibers but not astrogliosis at this age. Interestingly, our data show that enhanced forepaw and hindpaw sensitivity occurs independent of motor deficits produced by this model. Together, these results indicate that sensory circuits are vulnerable to perturbation by perinatal HI injury, and suggest that neuropathic pain could accompany motor deficits characteristic of CP.

Materials and Methods:

Animal model: Experiments were performed in accordance with the ARRIVE guidelines and the United States National Institutes of Health's Guide for Care and Use of Laboratory Animals. Approval of the University of Rhode Island's Institutional Animal Care and Use Committee was obtained for all experiments performed in this study. 62 kits of both sexes from 11 different litters originating from 6 dams were used for this study; some dams provided more than one litter of rabbits. The inherent

144 variability in behavioral data warranted large sample sizes. All efforts were made to minimize animal
145 suffering and to reduce the number of animals used.

146 **Hypoxia-ischemia surgery:** Pregnant New Zealand White rabbits (bred in house or ordered from Charles
147 River Laboratories, Inc., Wilmington MA), underwent HI procedures as previously described (Derrick et
148 al., 2004). Briefly, at ~70-80% gestation (day 22 -26 of gestation), dams were premedicated with
149 ketamine and xylazine (25 mg/kg and 3.75 mg/kg, respectively), anesthetized with isoflurane (1-3%;
150 inhaled via V gel tube) and occasionally treated with buprenorphine (0.01 - 0.24 mg/kg, as needed). The
151 left or right femoral artery was isolated. A Fogarty balloon catheter (2-4 French) was inserted and
152 advanced to the level of the descending aorta, above the uterine arteries, and inflated for 40 minutes to
153 cause HI in the fetuses. Dams subjected to sham surgical procedures underwent the same anesthesia
154 protocols as the HI dams but without insertion and/or inflation of the catheter.

155 After the procedures, dams recovered and later gave birth to kits. Neonatal kits of both sexes were
156 characterized for motor deficits, nociceptive behavior, and used for anatomical investigations as
157 described below. Since we examined behavior in neonatal animals far from sexual maturity (NZW
158 rabbits typically reach sexual maturity at ~6 months of age), and whose external genitalia were not yet
159 visible, we grouped together male and female kits.

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

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

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

186 **Immunohistochemistry (IHC):** Kits were deeply anesthetized by intraperitoneal injection of
187 ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) or sodium pentobarbital before tissue
188 harvest at P5. Transcardial perfusion was performed using chilled 0.01 M phosphate buffered saline
189 (PBS; pH 7.4) until tissue blanching was observed, then 4% paraformaldehyde (PFA) (in PBS) was infused
190 at a constant flow rate of approximately 5-8 mL/min (depending on rabbit kit size). Perfused spinal cords
191 were dissected and post-fixed in 4% paraformaldehyde for ~24-72 hrs. Next, fixed spinal cords were
192 washed with PBS and cryoprotected in 30% sucrose (in PBS) until sinking was observed. Cryoprotected
193 spinal cords were embedded in optimal cutting temperature (OCT) compound and frozen at −80 °C until
194 cryostat sectioning (Leica Lm 1850, Germany). Transverse lumbar spinal cord cryosections were
195 collected in serial at 30-µm or 40-µm thickness; to avoid repeat regions of analysis, every 15th section
196 was used for analysis.

197 *IHC for glial fibrillary acidic protein (GFAP)*: Slides were thawed, dried in an oven at 37 °C to improve
198 section adherence, then rehydrated in PBS. Sections were blocked in 0.2% Triton X-100 and 5% normal
199 horse serum (NHS) in PBS for 30 minutes at room temperature. Following blocking, slices were
200 incubated in primary antibody solution containing mouse-anti GFAP antibody (diluted 1:500 in 0.1%
201 Triton X-100 and 1% NHS in PBS; EnCor Biotechnology, cat. #MCA-5C10) overnight at room temperature.
202 Sections were washed in PBS and then were incubated in secondary antibody solution consisting of Atto
203 488-goat anti-mouse IgG (whole molecule) (diluted 1:500 in 0.1% Triton X-100 and 1% NHS in PBS,
204 Sigma-Aldrich, cat. # 62197) for 2 h at room temperature. Nuclei were labeled with 4',6-Diamidino-2-
205 Phenylindole (DAPI) (Invitrogen™, cat. # D1306) and slides were mounted using Fluoromount mounting
206 medium (SouthernBioTech). Z-stack photomicrographs of dorsal and ventral lower lumbar spinal cord
207 were acquired at 10x magnification using a Nikon Eclipse Ti2 inverted confocal microscope. Maximum
208 intensity projections of the green channel were generated and stitched (if possible) using Image
209 Composite Editor (Microsoft). Grayscale images were imported into ImageJ and blinded densitometric
210 analysis of GFAP immunofluorescent signal (mean gray value) in regions of interest containing dorsal
211 columns, superficial dorsal gray matter, lateral spinothalamic tract, and ventral spinothalamic tract
212 (divided by background fluorescent signal/mean gray value) was performed using Image J (NIH). The
213 grey or white matter in our regions of interest was targeted to encompass the entire region of interest
214 but could also contain portions of other areas.

215 *IHC for primary afferent fibers*: Sections were rinsed in PBS, blocked in 10% normal goat serum and 0.2%
216 Triton-X100 in PBS for 1 hour at room temperature followed by a 30-minute incubation with rabbit-to-
217 rabbit blocking reagent (ScyTek Laboratories, Inc.). Sections were then incubated with a solution of
218 biotinylated IB4 (1:2000, Sigma) and anti-calcitonin gene-regulated peptide (CGRP; 1:1500 Peninsula
219 Laboratories) in 0.1% Triton X-100 in PBS at room temperature for 48 h. After three washes with PBS,
220 sections were incubated in avidin-conjugated with Alexa Fluor 594 (Invitrogen, diluted at 1:400) and
221 goat-anti-rabbit Alexa Fluor 488 (Invitrogen, diluted at 1:400) in 5% goat serum in PBS for 2 hours at
222 room temperature. Following three washes, sections were rinsed in PBS and cover slipped with
223 FluorSave Reagent (Calbiochem, Bedford, MA). Three images of the left and right lumbar dorsal horn
224 were taken for each rabbit at the same magnification, lens aperture and exposure time on a Leica
225 DM5500B epifluorescent microscope and captured with Slidebook software. Images were averaged to
226 calculate values per animal. The proportional area of IB4, CGRP and colocalized immunoreactive tissue
227 within the cap of the dorsal horn (laminae I-III) was quantified in ImageJ as previously described (Detloff

228 et al., 2016). The proportional area of CGRP+ fibers that expand in dorsal horn ventral to the IB4+ band
229 was measured in the same sections.

230 **Statistical Analyses:** All statistical analyses were performed using GraphPad Prism (GraphPad Software
231 Inc.; San Diego, CA, USA). For each dataset, normality of distributions was tested using D'Agostino and
232 Pearson tests. For normally distributed datasets with equal variances, unpaired t-tests were used to
233 identify statistically significant differences between groups. For datasets in which one or more
234 distributions was non-normally distributed, Mann-Whitney tests were used.

235 **Results:**

236 In this study, we investigated the link between prenatal HI injury and nociception in a rabbit model
237 of CP. Rabbit kits exposed to prenatal HI and age-matched sham animals underwent testing for
238 mechanical and thermal allodynia at P5 using von Frey and Hargreaves' testing, respectively. After
239 prenatal HI injury, neonatal rabbit kits displayed increased nociceptive behavior in response to
240 mechanical and thermal stimuli that manifested even in the absence of motor deficits. HI kits
241 demonstrated significantly greater sensitivity to von Frey filaments applied to the paws compared to
242 sham kits, withdrawing paws in response to application of lower forces (forepaws, P=0.0014; hindpaws,
243 P=0.0043) (**Figure 1A**). Hindpaw von Frey scores of motor-unaffected HI kits (mean: 8.2 g; light red
244 symbols in Figure 1A) were significantly lower than that of motor-affected HI kits (mean: 19.4 g; dark red
245 symbols; P=0.0416). There was a similar but non-significant trend in forelimb paw withdrawal thresholds
246 between motor-unaffected (mean: 5.6 g; light red symbols in Figure 1A) and motor-affected HI kits
247 (mean: 10.5 g; dark red symbols; P=0.9068).

248 Similar to von Frey, the Hargreaves' test revealed allodynia in HI kits. HI kits had reduced paw
249 withdrawal latencies compared to sham rabbits in response to targeted radiant heat (**Figure 1B**),
250 indicating increased thermal sensitivity (forepaws: P<0.0001; hindpaws: P=0.0011). Unlike with
251 mechanical stimulation, paw withdrawal latencies to noxious thermal stimulation were comparable
252 between motor-unaffected HI (forepaw latency: 3.6 s; hindpaw latency: 3.0 s) and motor-affected HI kits
253 (forepaw latency: 4.4 s; hindpaw latency: 3.8 s) (P=0.1637 and 0.2711, respectively).

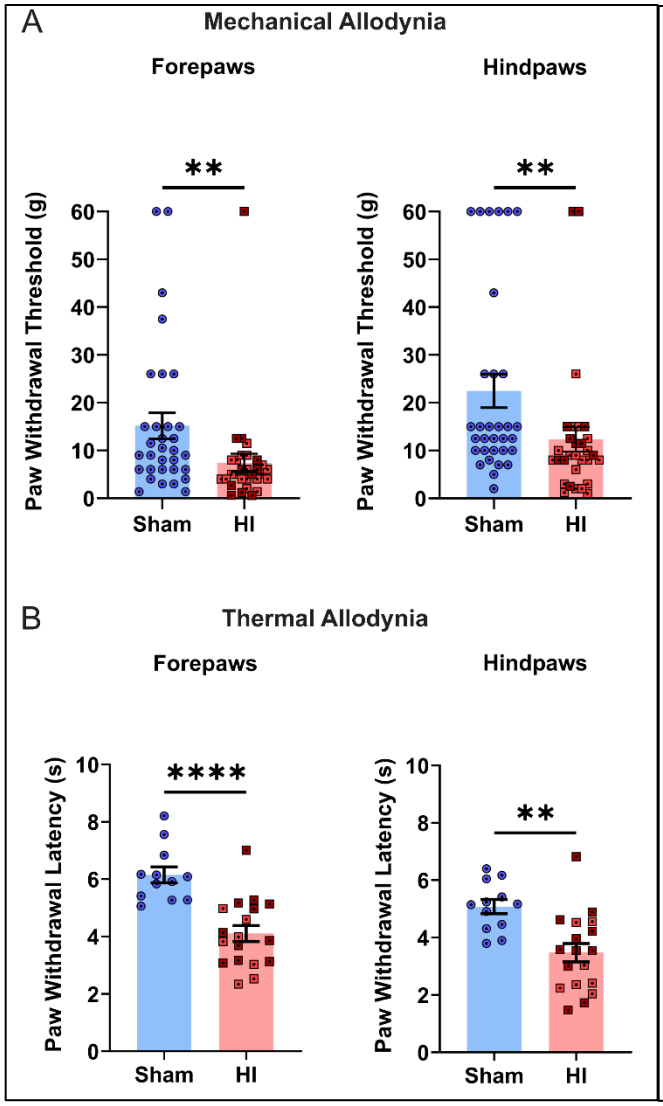

Figure 1: HI kits exhibit mechanical and thermal allodynia. **A:** Average forepaw (left) and hindpaw (right) von Frey scores at P5. Sham-operated controls (blue) have higher scores, indicating less mechanosensitivity, while HI kits (red) display greater mechanosensitivity (lower scores). Lower von Frey scores indicate mechanical allodynia in HI kits. Data are represented as mean ± SEM; N=32 sham and N=30 HI rabbits (dark red, motor-affected). ***P* <0.01; Mann-Whitney 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=12 sham and N=18 HI rabbits (dark red, motor-affected). ***P* <0.01, ****P* <0.0001; unpaired t-tests.

We hypothesized that mechanical and thermal allodynia in HI rabbit kits may be caused by spinal astrogliosis and/or expansion of nociceptive afferents in the spinal cord dorsal horn. To determine whether prenatal HI leads to long-lasting postnatal astrogliosis in gray and white matter spinal cord regions involved in cutaneous sensation or nociception, the degree of glial fibrillary acidic protein (GFAP) expression in four lumbar spinal cord regions of interest (ROIs) was quantified. These ROIs included tracts/laminae involved in cutaneous sensation or nociceptive neurotransmission: the dorsal column, superficial dorsal gray matter, ventral spinothalamic tract, and lateral spinothalamic tract. Densitometric analysis revealed no significant differences in GFAP labeling in these ROIs at P5 (**Figure 2B**). Baseline GFAP immunoreactivity in the white matter of neonatal rabbits found here is consistent with the role of astrocytes in myelination during early postnatal development (Landry et al., 1990; Liedtke et al., 1996; Nash et al., 2011). No evidence was found to support a contribution of neuroinflammation to increased nociception at this age.

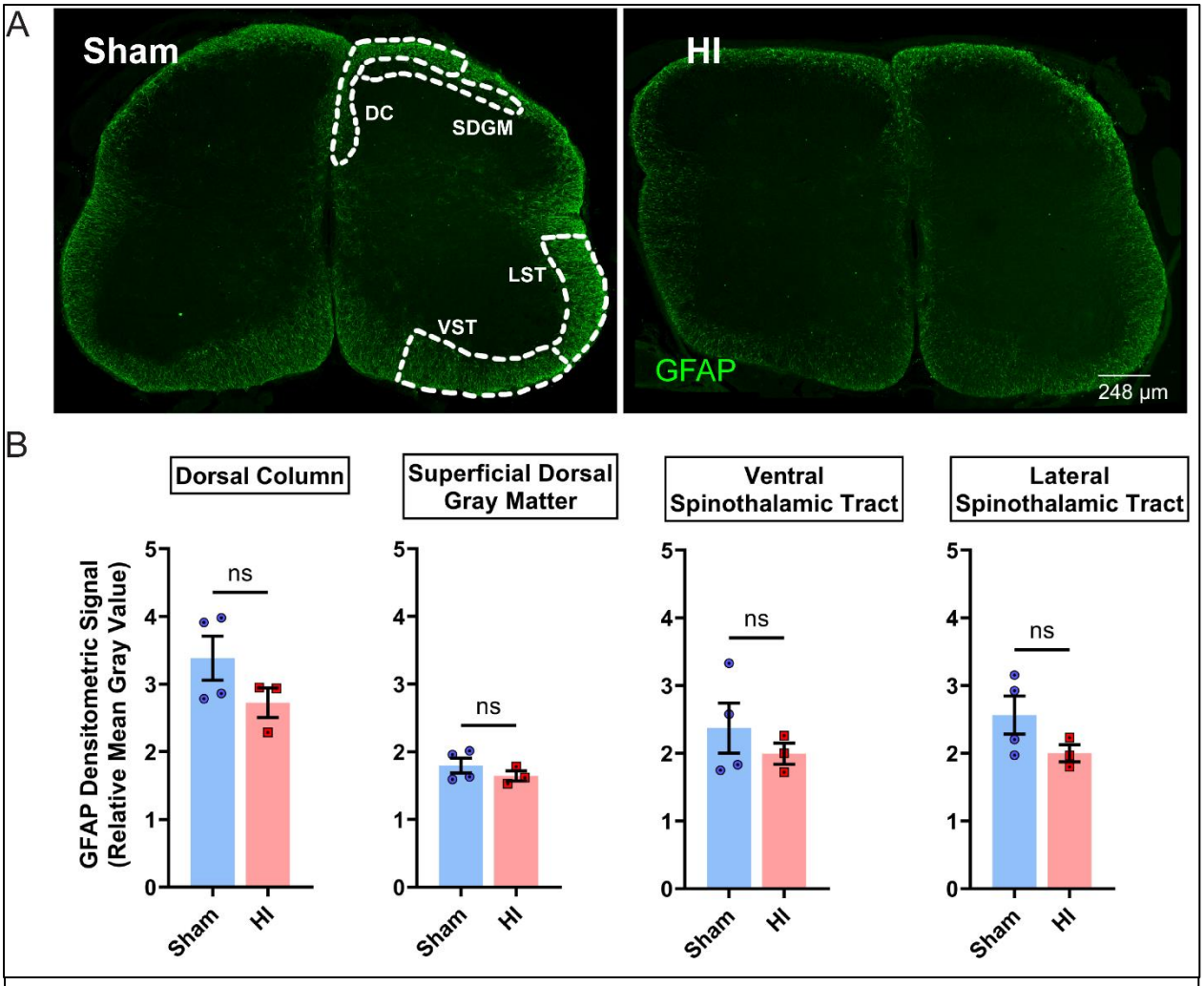

Figure 2: Astrogliosis is not present in HI rabbits at P5. **A**: Transverse lumbar spinal cord sections from sham (left) and HI (right) rabbits immunostained for GFAP (green). Scale bar applies to both images. **B:** Quantification of GFAP densitometry in spinal regions containing tracts/laminae of interest: dorsal column (DC), superficial dorsal gray matter (SDGM), ventral spinothalamic tract (VST), and lateral spinothalamic tract (LST). GFAP expression was unchanged in all ROIs in HI spinal cords (ns, not significant; unpaired t-tests). Data are represented as mean ± SEM; N=3-4 rabbits per group.

To determine whether prenatal HI affects nociceptive primary afferent distribution and density within the dorsal horn of the spinal cord, lumbar spinal cords (which include hindpaw dermatomes) from sham and HI kits were labeled for CGRP and IB4 to identify peptidergic and non-peptidergic nociceptors, respectively. The distributions of these two nociceptor subpopulations had little overlap in the dorsal horn of 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, HI kits exhibited increased CGRP-positive fibers in the deep dorsal horn (Figure C-C'') that were not present in sham kits. Quantification of the proportional area of 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-positive fibers in the dorsal horn,

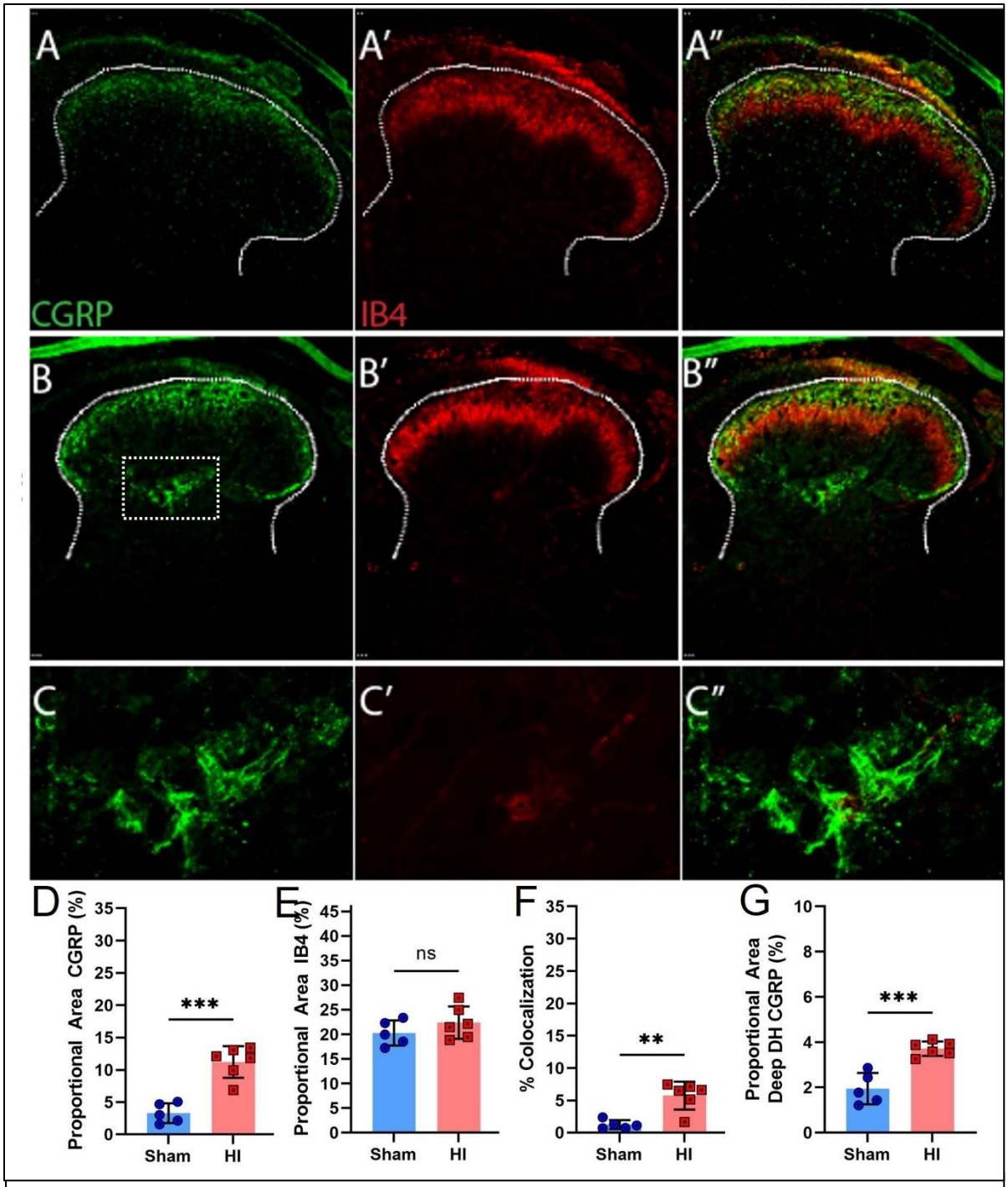

Figure 3: Increased density and overlap of nociceptive primary afferent fibers in HI kits at P5. Representative images of the lumbar dorsal horn of sham (A-A'') and HI (B-B'') kits immunolabeled for calcitonin gene-regulated peptide (CGRP; A, B, C) or labeled by isolectin B-4 (IB4; A', B', C'). Note the increased presence of CGRP-positive fibers in the deep dorsal horn in the HI but not the sham spinal cord (C-C''). The proportional area of CGRP-positive 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-positive 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 ± SEM; individual data points represent each animal (n=5 sham and n=6 HI kits) from averages of 3 separate images of left and right dorsal horns per animal. ***P*<0.01, ****P*<0.001; unpaired t-tests were used for statistical analysis.

279 there was significant colocalization of CGRP and IB4 labeling in the dorsal horn of HI kits compared to
280 sham kits, suggesting that HI may induce plasticity or phenotype switching of these nociceptor
281 populations.

282

283 **Conclusions:**

284 Experimental animal models that mimic human disease with high fidelity are more likely to drive
285 meaningful discovery of therapeutic interventions to treat such diseases. Here, we use a preclinical
286 model that displays prominent motor deficits to show that nociceptive afferents are also disrupted, in
287 addition to the previously characterized damage to the cortex, thinning of the corticospinal tract, loss of
288 spinal motoneurons and altered motoneuron excitability (Buser et al., 2010; Drobyshevsky et al., 2007;
289 Drobyshevsky and Quinlan, 2017; Steele et al., 2020). Importantly, both rabbits after HI and human
290 infants after asphyxiation show cell death in the dorsal horn (Clancy et al., 1989; Drobyshevsky and
291 Quinlan, 2017; Sladky and Rorke, 1986). Moreover, this is one of few studies that utilize quantitative
292 assessments of nociception in the rabbit, despite the rabbit's extensive use in biomedical research (Lei
293 et al., 2017; Mans, 2020; Yoshioka et al., 1996). Our findings indicate that after prenatal HI, sprouting of
294 peptidergic nociceptive primary afferent fibers in the superficial and deep dorsal horn is present along
295 with behavior that is suggestive of allodynia.

296 This is the first study to show that nociceptors are altered in an animal model of CP. The
297 increased overlap and density of nociceptive afferent fibers in the dorsal horn of HI kits may indicate
298 intrinsic changes within nociceptive DRG neurons. Despite significantly lower von Frey scores in HI kits
299 indicating increased mechanosensitivity, non-peptidergic IB4-positive mechanical nociceptors, which are
300 activated by noxious mechanical stimuli (Marmigère and Ernfors, 2007), were not found to have
301 expanded afferent arborizations in the dorsal horn. It could be that these IB4-positive nociceptors are
302 hyperexcitable, and that altered activity, not anatomy, contributes to mechanical allodynia in HI kits. In
303 other models of nervous system injury, chronic pain is associated with dysfunctional nociceptors that
304 exhibit robust anatomical and electrophysiological plasticity (Bedi et al., 2010; Detloff et al., 2016, 2014;
305 Dougherty et al., 2004; Moy et al., 2018; Zhang et al., 2013); further, emerging evidence suggests that
306 nociceptive primary afferent input impedes motor output following CNS injury (Keller et al., 2017, 2019,
307 2018). In a series of studies, Keller *et al*. demonstrated that nociceptor depletion reduces muscle
308 contractures and spasticity of the hindlimbs and improves overall locomotor performance following
309 spinal cord injury. Nociceptor silencing via activation of inhibitory $5HT_{1D}$ receptors has also been found
310 to be helpful in reducing clonus and spasms after spinal cord injury (Lucas-Osma et al., 2019). Taken
311 together, these findings indicate that altered activity in nociceptors contributes to motor deficits in
312 cases of CNS injury.

313 In adults, physical trauma is known to alter neural circuits and have long lasting effects on
314 nociception (Costigan et al., 2009; Price and Dussor, 2014). The effect of neural injury on nociceptive
315 primary sensory neurons is a major contributor to the development of chronic pain (Hulsebosch and
316 Coggeshall, 1981a, 1981b; Koerber et al., 1994; Ramer et al., 2012; Rodin et al., 1983; Walters, 2012;
317 Woolf et al., 1992). After trauma, it is well-established that the afferent fibers of these nociceptors
318 exhibit robust arborizations into the superficial and deep dorsal horn of the spinal cord (Bedi et al.,
319 2010; Detloff et al., 2016, 2014; Krenz and Weaver, 1998; Ondarza et al., 2003; Weaver et al., 2001). In

320 addition, nociceptors increase spontaneous activity and become hyperexcitable following CNS trauma
321 (Bedi et al., 2010; Burchiel et al., 1985; Gold and Gebhart, 2010; Gracely et al., 1993; Li et al., 2018;
322 North et al., 2019; Pitcher and Henry, 2008). In neonates, effects of neural injury on nociception are less
323 studied. Peripheral inflammation in neonatal rats enhances collateral sprouting of primary nociceptive
324 afferents and enhances nociception in adulthood (Ruda et al., 2000). Astrocytes and microglia are
325 activated by neural injury and release a plethora of pro-inflammatory molecules into the spinal milieu,
326 and their activation corresponds to the development of neuropathic pain (Chhaya et al., 2018; Detloff et
327 al., 2008; Gao et al., 2009; Hulsebosch et al., 2009; Ishikura et al., 2021; Lu et al., 2021; Xie et al., 2009;
328 Zhang et al., 2016, 2007).

329 Neuroinflammation and pain are tightly intertwined (Grace et al., 2014) and astrogliosis has a
330 well-established role in other painful conditions. After complete spinal cord injury, increased GFAP
331 persists for at least 3 weeks in the adult rat spinal cord (Choi et al., 2021; Ducza et al., 2021; Morin-
332 Richaud et al., 1998; Nesic et al., 2006; Xu et al., 2021). However, the time course of the GFAP response
333 to injury is different in neonates. Unlike adults, neonatal rat pups respond to spared nerve injury with an
334 early increase in GFAP (one day after injury), along with the same elevation after 7 days that is observed
335 in adults (Vega-Avelaira et al., 2007). Density of GFAP fiber length and density of GFAP+ cell bodies are
336 increased in the lumbar gray matter of motor-affected (hypertonic) HI rabbit kits at P1, seven days after
337 HI (Drobyshevsky and Quinlan, 2017; Synowiec et al., 2019). The current study was performed in HI kits
338 that were 4 days older (P5) and examined specific laminae/tracts involved in cutaneous sensation or
339 nociception: (1) the dorsal column, the ascending pathway for cutaneous sensation; (2) the superficial
340 dorsal gray matter, containing second-order projection neurons relaying nociception; (3) the ventral
341 spinothalamic tract, carrying crude touch and pressure information; and (4) the lateral spinothalamic
342 tract, the ascending pathway for nociception. While astrogliosis is not present in the spinal regions of
343 interest we studied at P5 (11-14 days after HI), their reactivity closer to the time of injury could be a
344 causal link of enhanced nociception as has been demonstrated in other models (Milligan et al., 2000).
345 Astrocytes have been shown to exhibit different phenotypes or reactive states following similar CNS
346 injuries that can be either destructive or pro-reparative (Escartin et al., 2021; Liddelow and Barres,
347 2017). Moreover, it has been reported that ischemia can induce an “A2-like” astrocyte phenotype that
348 upregulates thrombospondins and neurotrophic factors that promote survival and repair of neurons
349 (Gao et al., 2005; Hayakawa et al., 2014; Zador et al., 2009). Future studies could examine more closely
350 the time course and specific regions of elevated astrocyte activation in the rabbit after prenatal HI and
351 its relationship to enhanced nociception observed here at P5.

352 The stereotypical aversive paw withdrawal response to nocifensive tactile or thermal stimuli is
353 mitigated by a segmental spinal reflex and is often inferred as a proxy for cortical perception of the
354 stimulus. It is unclear whether the results of the von Frey and Hargreaves tests performed here reflect
355 hyperreflexia of nociceptive segmental spinal cord circuits (Advokat, 2002; Kauppila et al., 1998; Murphy
356 and Zemlan, 1990; Schomburg and Steffens, 1998) or the complicated perception of neuropathic pain. It
357 is likely these stimuli were offensive to the rabbits because paw withdrawal was typically accompanied
358 by paw licking and/or a rapid jump away from the stimuli. Unlike in rodent experiments, there are
359 surprisingly few reports measuring pain or nociception in experimental rabbit models, even those
360 modeling knee osteoarthritis or intervertebral disc dislocation (Lei et al., 2017; Mans, 2020; Yoshioka et
361 al., 1996). Composite pain scales for rabbits in a clinical environment (Banchi et al., 2020) and the Bristol
362 Rabbit Pain Scale (Benato et al., 2021) examine and score rabbit facial expressions and other behaviors
363 to assess pain, such as the grimace scale (Evangelista et al., 2021; Keating et al., 2012). Yet, recent

364 reports suggest that the experimenter's presence can have a profound effect on the animal's behavior
365 and subsequent score on these tests (Pinho et al., 2020). In the present study, unlike assessments of
366 spontaneous pain using the rabbit grimace scale, these assessments identify the dermatomal location
367 and relevant spinal cord or dorsal root ganglion level in which dysfunctional sensation occurs. This is
368 especially useful in examining changes in primary afferent input and/or dorsal horn processing in
369 specific spinal segments. Also, since our study examined an early postnatal age (P5) when the neonatal
370 rabbits' eyes are not yet open, the tests we performed were more appropriate to assess nociceptive
371 behavior than using the grimace scale, place-preference or aversion tests. However, future work to
372 assess pain perception and an associated fear response at later postnatal ages and could include these
373 assessments to further characterize the perception of pain and translate these findings clinically.
374
375 Individuals with CP have disruptions in somatosensation in addition to motor dysfunction.
376 Disruptions in somatosensory cortex connectivity (Hoon et al., 2002) and increased latency of sensory
377 evoked potentials (Cahan et al., 1987; Kundi et al., 1989) have been well documented in individuals with
378 CP. Innocuous tactile sensitivity actually decreases in individuals with CP while pain sensitivity is
379 enhanced (Riquelme and Montoya, 2010). The mechanism of this is not clear, but it is possible that this
380 is due to afferent switching after injury: in a rat model of neuropathic pain, large diameter
381 cutaneous/somatic Aβ afferents can undergo fiber-type switching after nerve ligation and begin
382 expressing CGRP, like nociceptive fibers (Nitzan-Luques et al., 2011). This switch in identity corresponds
383 to a period of tactile allodynia. Another possibility is that the impaired CST creates a permissive
384 environment for afferent sprouting. When competition between CST and afferent input to the spinal
385 cord is lost after transection of the pyramidal tracts in adult rats, afferent sprouting into the deep dorsal
386 horn is observed (Jiang et al., 2016; Tan et al., 2012). Afferent sprouting after CST transection is
387 accompanied by loss of rate dependent depression of the H reflex, a hallmark of CP (Tan et al., 2012).
388 The common thread in both of these mechanisms is the remarkable plasticity of afferents, even in
389 adulthood. This raises the possibility that aberrant nociceptive sprouting arising during perinatal
390 development could be corrected later in life.
391
392 Movement and motor unit firing patterns in neurotypical adults are directly affected by pain
393 (Hodges and Tucker, 2011; Martinez-Valdes et al., 2020), perhaps through neuromodulators including
394 serotonin (5HT) (Mesquita et al., 2020). In our study, we found that HI kits were more sensitive to
395 mechanical and thermal stimuli than sham control kits. When comparing HI kits that were motor-
396 affected and those that were motor-unaffected, motor-affected HI kits appeared less sensitive to
397 mechanical stimuli and their response to thermal stimuli trended in the same direction. However, we
398 attribute this to the greater difficulty these kits had in making movements to respond to stimuli due to
399 their hypertonia. No kits in this study were completely incapable of movement and unable to react
400 physically to the stimuli, but their hypertonia could have impeded their reaction. Further investigation
401 into differences between motor-affected and motor-unaffected HI kits warrants further study.
402
403 Each individual's pain perception includes complex psychosocial components that were not the
404 focus of this study. In situations where both nociception and the affective experience of pain can be
405 altered, such as after HI, it is critical to accurately identify and classify aberrant sensory behavior. Here,
406 we utilized methods to assess and quantify shifts in sensitivity to mechanical and thermal stimuli that
407 are sensitive, reliable, and validated in models of central and peripheral neuropathic pain across species.
408 We found that prenatal HI heightened nociception at the level of spinal nociceptors and reflexive
409 responses to mechanical and thermal stimuli. It could be that CP-causative perinatal injuries contribute
410 to other aspects of the pain experience in individuals with CP, including changes to mood and cognition,
411 in addition to altered nociception. Thus, treatment options for chronic pain in individuals with CP should

be explored with greater specificity, considering that the pathophysiology of their pain could be distinct from that of chronic pain derived from other etiologies. Novel targets for fully alleviating CP-induced chronic pain should be explored specifically within the context of treating individuals with CP or those who have survived perinatal physical trauma.

The current study demonstrates enhanced nociceptive behavior and nociceptor sprouting in neonatal rabbits after prenatal HI injury. Identification of how prenatal physical stressors impact nociceptors could be the first step to reducing hyperreflexia and preventing aberrant nociception and ultimately chronic pain in those at risk.

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Author contributions

EJ Reedich contributed to data curation, formal analysis, investigation, methodology, supervision, validation and writing, editing and reviewing the manuscript. LT Genry contributed to data curation, formal analysis, investigation, methodology, supervision, validation, and reviewing and editing the manuscript. MA Singer contributed to data curation, formal analysis, investigation, methodology, and validation. CF Cavarsan contributed to formal analysis, investigation, methodology, and validation. E Mena Avila contributed to data curation, formal analysis, investigation, methodology, validation, and reviewing the manuscript. DM Boudreau contributed to data curation, formal analysis, investigation, methodology, validation, and reviewing the manuscript. MC Brennan contributed to data curation, formal analysis, investigation, methodology, validation, and reviewing the manuscript. AM Garrett contributed to data curation, formal analysis, investigation, methodology, validation, and reviewing the manuscript. L Dowaliby contributed to investigation, methodology and supervision of this project. MR Detloff contributed to contributed to conceptualization, data curation, funding acquisition, project administration, resources, supervision, and writing, editing and review of the manuscript. KA Quinlan contributed to conceptualization, data curation, funding acquisition, project administration, resources, supervision, and writing, editing and review of the manuscript.

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