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Cortex glial cells activation, associated with lowered mechanical thresholds and motor dysfunction, persists into adulthood after neonatal pain

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Abstract

We investigated if changes in glial activity in cortical areas that process nociceptive stimuli persisted in adult rats after neonatal injury. Neonatal pain was induced by repetitive needle prickling on the right paw, twice per day for 15 days starting at birth. Wistar rats received either neonatal pain or tactile stimulation and were tested behaviorally for mechanical withdrawal thresholds of the paws and gait alterations, after 15 (P15) or 180 (P180) days of life. Brains from rats on P15 and P180 were immunostained for glial markers (GFAP, MCP-1, OX-42) and the following cortical areas were analyzed for immunoreactivity density: prefrontal, anterior insular, anterior cingulate, somatosensory and motor cortices. Withdrawal thresholds of the stimulated paw remained decreased on P180 after neonatal pain when compared to controls. Neonatal pain animals showed increased density for both GFAP and MCP-1 staining, but not for OX-42, in all investigated cortical areas on both experimental times (P15 and P180). Painful stimuli in the neonatal period produced pain behaviors immediately after injury that persisted in adult life, and was accompanied by increase in the glial markers density in cortical areas that process and interpret pain. Thus, long-lasting changes in cortical glial activity could be, at least in part, responsible for the persistent hyperalgesia in adult rats that suffered from neonatal pain.

Keywords

Neonatal pain; Glial cells; Cortex; Development; GFAP

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Conflict of interest statement

There is no conflict of interest.

1. Introduction

Painful procedures are routinely performed during neonatal intensive care on a daily basis (Anand, 2000; Grunau, 2013). Over the last decade, increased awareness about pain in neonates resulted in reduced tissue-damaging procedures and increased analgesic usage prior to painful procedures (Johnston et al., 2011). Despite these positive changes, the management of pain in newborns still depends on the hospital's protocols and is not always adequate (Johnston et al., 2011).

Experimental studies are important for a better understanding of the changes resulting from pain in the neonate. In rats, painful procedures, such as needle pricking, incisions, or nerve injury cause local tissue damage and decrease pain thresholds (hyperalgesia) in neonates (Anand et al., 1999; Walker et al., 2009; Pertin et al., 2012; Knaepen et al., 2013). It is well documented that injury in the neonatal period results in enhanced and persistent nociceptive sensitivity in the adult animals (Knaepen et al., 2012) and is accompanied by changes in neural processing that occur centrally (Hathway et al., 2012; Vega-Avelaira et al., 2007); however the mechanisms for this persistent changes is yet to be explained.

There is substantial evidence that both microglia and astrocytes in the spinal cord are activated in a variety of pain models (Gwak and Hulsebosch, 2009). For instance, nerve injury in young rats is known to cause acute changes in the glial cells of the spinal cord (Vallejo et al., 2010). Also, electrical stimulation of C fibers, which are activated by noxious stimuli, can lead to sensitization of neurons in the central nervous system (Wu et al., 2012). Nevertheless, further studies are necessary to clarify whether there is a relation between glial cell activation and hyperalgesia in the neonatal period and if this activation is a persistent phenomenon that may lead to hyperalgesia in adults.

Nociceptive stimuli are not only processed within the spinal cord, but must engage cortical sites for perception of pain. Cortical sites involved in nociceptive processing include the somatosensory cortices (primary and secondary) which mediate the sensation of pain, the anterior insular and anterior cingulate cortices that are related to the emotional component of pain, the prefrontal cortex which is involved in complex cognitive behaviors related to pain, and the motor cortices involved with movement responses to pain (Peltz et al., 2011). It is well known that astrocytes participate in the formation of neuronal synapses (Eroglu and Barres, 2010) and their control (Dallérac et al., 2013) and release inflammatory mediators that cause neuronal loss (Deng et al., 2013). The microglia, in neurodegenerative disorders are recognized as neurotoxic cells (Eggen et al., 2013) and when activated, can cause tissue remodeling, synaptic plasticity and neurogenesis (Eggen et al., 2013). Little is known about the role of glial cells in the cortical sites involved in nociceptive processing. We hypothesized that increased astrocytes and microglial activation in the cortex occurs in the neonates submitted to painful stimuli and that this increase persists throughout the adult life. Therefore, we tested if (A) hypersensitivity develops, (B) motor function is altered, and (C) glial activity in the cortex is enhanced after early-life insult, and if these changes persist into adulthood.

2. Material and methods

2.1. Animals

All experiments were approved by the Institutional Ethics Committee for Animal Research (CETEA—Comitê de Ética em Experimentação Animal, protocol number 025/2009) from the School of Medicine of Ribeirão Preto, University of São Paulo, and were carried out according to the guidelines of the International Association for the Study of Pain and National Institutes of Health.

Animals were provided by the Animal Care Facility at the University of São Paulo, and they were kept in a carefully regulated environment maintained at 21 to 23 °C, 40 to 70% relative air humidity and 12/12 h light/dark cycle, and received tap water and normal rat chow ad libitum. Mature Wistar females cohabitated with mature Wistar males for 21 days. After birth, females were single housed until their litter was weaned on 22th day. On the day of birth, P0, each litter was randomly assigned into 8 different groups ($N = 10$ each), as shown on Table 1: Control male or female neonates followed for 15 days; Pain male or female neonates followed for 15 days; Control male or female adults followed for 180 days; Pain male or female adults followed for 180 days. Pain groups received noxious stimuli that consisted of a 30-ga needle insertion rapidly into the plantar foot pad and the lateral surface of the right paw, twice per day for 15 days starting at birth. Control groups received tactile stimuli with a cotton-swab into the plantar and the lateral surface of the right paw for twice per day for 15 days starting at birth. All animals were separated from their mothers during the stimulation. The protocol for repetitive tactile and nociceptive stimulation of the rats was adapted from Anand et al. (1999).

2.2. Behavioral testing

In preparation for testing, mothers were removed from the maternity cages and transferred to holding cages. The pups were carried to the testing laboratory, identified individually with a felt-tip marker, and weighed. Before the behavioral tests were applied, animals were acclimated to the testing room for 20 min in transparent acrylic cubicles ($24.6 \times 7.5 \times 7.5$ cm) on an elevated mesh platform for the paw withdrawal threshold test. During this period of time the animals explored the new environment and got comfortable with the mesh floor. Fifteen days after birth, animals were weighed and submitted to the behavioral functional analysis as described above. A separate group of animals was submitted to the same procedures 180 days after birth.

For the paw withdrawal threshold test, a mechanical stimulus with von Frey filaments was applied to the lateral portion of the plantar surface of the hind paw, as previously described (Chaplan et al., 1994). To test withdrawal thresholds to mechanical stimuli von Frey filaments with different bending forces (0.05, 0.2, 2.0, 4.0, 10.0 and 300.0g) were progressively applied perpendicularly to the plantar aspect of the hind paw. The observer started the test with the filament of smaller value. In the absence of a paw withdrawal response, the application of filaments occurred in ascending order. Each filament had one trial that consisted of two consecutive applications of the filament. The lowest bending force

at which the rat withdrew its paw from one of the two applications was recorded as the paw withdrawal threshold.

For the calibrated forceps test, the animals were acclimated in a glove for 5 min (no longer, since restraining is stressful for the animals) and they were caressed prior to the test. The animal remained in the glove, the investigator extended one hind limb, and the plantar area of the paw was compressed with the forceps tips. The device (developed and provided by Bonther®—Equipments for teaching and research, Ribeirão Preto, SP, Brazil) consists of a pair of large blunt forceps (15 cm long; flat contact area; 7 mm × 1.5 mm with smooth edges) equipped with 2 strain gauges connected to a modified electronic dynamometer, as described by Luis-Delgado et al. (2006). The contact area of the forceps was approximately 30 mm². The tips of the forceps were placed around the hind paw, and care was taken to apply the same tip length on the hind paw for each trial. The force applied was then incremented by hand at a speed of approximately 200g every 3 s until the paw withdrawal or animal vocalization. An analogical display unit on the dynamometer allows the experimenter to train for this device and to check mechanical force level over time during the test. The maximum force applied on the paw was automatically recorded and displayed by the dynamometer. Compression was stopped when the animal withdrew the limb forcefully or when it vocalized. The maximum compression force applied at withdrawal was recorded as the baseline compression threshold in millinewtons for the plantar area of the corresponding limb.

2.3. Gait analysis

Walking track test was performed on an enclosed acrylic apparatus (43 × 8.7 × 5.5 cm) ending in a darkened cage, similar to that described by Varejão et al. (2001). The walking apparatus was illuminated on both sides with fluorescent lamps of 120 W each and gait was recorded with digital video cameras (DVD 203, SONY), positioned under and in front of the apparatus. The videos were analyzed off line with Adobe Premium software assistance. All rats are first allowed two or three conditioning trials, during which they often stop to explore the corridor, thereafter they walk steadily to the darkened cage.

The paw print, digitized with Adobe Premium software, was measured with Image J software (NIH). Four different parameters were evaluated as follows: toe spread (TS), the distance between first and fifth toes; intermediate toe spread (IS), the distance between the second and fourth toes; print length (PL), the distance between the third toe and the hind pad; and the orthogonal distance from the third toe of one paw to the contralateral hind pad, designated TOF. All measurements are taken from the experimental (E) and normal (N) sides. Several prints of each foot are obtained on each track. The formula used to measure the tibial functional index (TFI) was described previously (Varejão et al., 2001) and is a measure of a step distance. The score varies between 0 and –100%. An IFT of 0 is normal. An IFT of –100 indicates total impairment of the gait.

$$IFT = 125 \times \left(\frac{NPL - EPL}{NPL} \right) - 43.8 \times \left(\frac{ETS - NTS + 252}{NTS} \right) \times \left(\frac{EIS - NIS}{NIT} \right)$$

2.4. Brain analysis

Either on the 15th day after the last stimulation or when the rats reached 6 months of age (180 days), the animals were anesthetized with 25% urethane (1 ml/100 g). Transcardial perfusion through the left ventricle was performed using PBS 0.05 M, followed by 4% phosphate-buffered paraformaldehyde. The brain was then removed, kept in paraformaldehyde for 4 h, transferred to 30% sucrose solution for 48 h, frozen on dry-ice and stored at -80°C until sectioned. Coronal sections (20 μm) of the brain were cut at a cryostat and placed on slides in serial order. Coronal sections were cut to include regions of the prefrontal, anterior insular, anterior cingulate, somatosensory (S1—hindlimb area and S2) and motor (M1 and M2) cortices bilaterally.

For immunohistochemistry, 48 brains were chosen (24 of each treatment group, 12 from 15 days old animals and 12 from 180 days old animals, 6 from males and 6 from females). In order to ensure that the areas of interest were analyzed, Nissl staining was performed. The examination for microglia was performed after OX-42 labeling. The OX-42 is a microglia/macrophage marker of the cells that express neurotrophin-3 (Elkabes et al., 1996). The sections were first blocked with 3% normal goat serum for 30 min, followed by Avidin-Biotin Block (15 min each). Afterwards, they were incubated overnight with mouse anti-rat CD11b IgG (AbDSerotec, Raleigh, NC, USA—1:2500, Cat. no. MCA275G). On the second day, the sections were incubated in biotinylated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h, followed by incubation in Strep-568 (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h, and then NeuroTrace fluorescent Nissl (Invitrogen, Carlsbad, CA, USA—1:1000) for 20 min. Slides were coverslipped using Vectashield (Vector Laboratories, Burlingame, CA, USA).

A second set of slides were double-labeled with anti-GFAP (Glial Fibrillary Acidic Protein) and anti-MCP-1 (Monocyte Chemotactic Protein-1) to visualize astrocytes. GFAP is one of a family of intermediate filament proteins essential for the process of reactive astrogliosis (Sofroniev and Vinters, 2010) and MCP-1 is a neuroprotective chemokine (Madrigal et al., 2009). The slides were first blocked with 3% normal goat serum for 30 min, followed by Avidin-Biotin Block (15 min each). Then, the sections were incubated overnight with a monoclonal anti-mouse anti-GFAP (Millipore, Billerica, MA, USA—1:5000, Cat. no. MAB360). On the second day, the sections were incubated with biotinylated goat antimouse IgG (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h followed by Strep-568 (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h. Slides were then incubated overnight in rabbit anti-rat MCP-1 (Millipore, Billerica, MA, USA—1:500, Cat. no. 1834P). Afterwards, the sections were incubated with biotinylated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h followed by Strep-488 (Invitrogen, Carlsbad, CA, USA—1:1000) for 1 h. Slides were covered using Vectashield (Vector Laboratories, Burlingame, CA, USA). All sections were stained simultaneously to avoid differences between days of testing. All images were taken with the same settings for each stain and each section.

The stained sections were examined by a single investigator, who was blind to group identity, with a fluorescence microscope (Olympus BX-51—Japan) and images taken for off-line analysis. All images for one stain were taken with the same setting on the microscope for comparison. The digital images were submitted to density readings with

Image J software as previously described (Hoeger-Bement and Sluka, 2003). Five sections per area of brain were averaged for each animal for each staining, and the number of pixels occupied by immunoreactive cells was measured. Specifically, each tissue section was first converted to eight-bit gray scale, inverted, and then each tissue section was calibrated independently using the “uncalibrated OD” function with pixel values ranging from 0 to 255. The density values represent pixels per area.

2.5. Statistical analysis

All data were analyzed with SPSS statistical software. All values were expressed as mean $\pm$ SEM. Data were analyzed by Repeated Measures ANOVA (sex, time after injury, and side) followed by Tukey's post hoc multiple comparison tests. Differences were considered significant if $p < 0.05$.

3. Results

Since there was no significant difference between genders (p values close to 1.00 except for the analyses of GFAP in the anterior cingulate cortex which showed $p < 0.05$), the present study combined data from male and female rats, resulting in four groups: (1) Control-Neonate group with 15 days of life ($N = 20$, 10 female and 10 male); (2) Pain-Neonate group with 15 days of life ($N = 20$, 10 female and 10 male); (3) Control-Adult group with 180 days of life ($N = 20$, 10 female and 10 male); (4) Pain-Adult group with 180 days of life ($N = 20$, 10 female and 10 male).

3.1. Behavioral and gait analysis

On day 15, paw withdrawal thresholds (von Frey test) did not differ between neonates exposed to repeated noxious stimuli compared to those exposed to repeated innocuous stimuli ($p = 0.647$). However, 6 month old adult rats (day 180) that received repeated noxious stimuli during the neonatal period showed a significant decrease in paw withdrawal thresholds ipsilaterally when compared to controls exposed to repeated innocuous stimuli ($p < 0.001$) (Fig. 1A). Control groups showed significant differences in paw withdrawal thresholds between ages on both paws ($p < 0.001$); neonates being significantly lower than adults. This difference was not observed for the painful group ($p = 1.000$) which showed decrease in paw withdrawal thresholds on P180 for the paw that received noxious stimuli.

In contrast, there was no difference in the calibrated forceps test between control and experimental groups during either neonatal (day 15) or adult (day 180) periods. Similar to paw withdrawals thresholds, the adult control group showed significantly higher thresholds than the neonatal control group (Fig. 1B).

There was an alteration in the gait (Fig. 1C) in neonates ($p < 0.001$) and adults ($p < 0.001$) that received repeated noxious stimulation when compared to controls. The animals that received noxious stimulation, both neonates and adults, avoided stepping on the stimulated (right) paw and took longer to walk the entire runway.

3.2. Brain immunochemistry

Positive astrocytes, marked with both GFAP and MCP-1, were found throughout the brain, in control exposed to repeated innocuous stimuli and pain groups exposed to repeated noxious stimuli, for both ages (neonates and adults). Similar results were obtained with the microglia cells which were marked with OX-42.

For the limbic system areas studied, (pre-frontal, anterior cingulate and anterior insula), we observed bilateral immunostaining for both astrocytes and microglia markers. Increased staining density was observed bilaterally for GFAP and MCP-1 on day 15 ($p < 0.001$) and day 180 ($p = 0.01$) in the groups that received repeated noxious stimulation compared to controls that received repeated innocuous stimuli (Figs. 2A and B and 3). The prefrontal cortex, anterior insular cortex and anterior cingulate cortex showed no differences for side (ipsilateral and contralateral) or timing after stimulation (neonate and adult) indicating that effects were bilateral, acute and remained increased in adults. The prefrontal and anterior insular cortices showed no gender differences. However, the anterior cingulate showed a significant difference for GFAP staining, where females in the Pain-Neonate group presented higher values than males on the ipsilateral (0.21 ± 0.02 female; 0.15 ± 0.02 male, $p = 0.035$) and contralateral sides (0.18 ± 0.02 female; 0.10 ± 0.01 male, $p = 0.001$). No significant differences were observed with the microglial marker OX-42 in the prefrontal, insular and anterior cingulate cortices.

For the primary somatosensory cortex of the hind limb (S1HL) and the secondary somatosensory cortex (S2), both groups, neonate and adult exposed to repeated noxious stimuli showed significant increases in staining density for GFAP and MCP-1 when compared controls exposed to repeated innocuous stimuli ($p < 0.001$) (Figs. 4 and 5). No differences for side (ipsilateral and contralateral) or gender were found. The analysis of OX-42 staining showed that neither S1HL nor S2 were altered in the group that received repeated noxious stimulation compared to the controls that received repeated innocuous stimuli.

The motor areas, represented by primary (M1) and secondary (M2) motor cortex presented a significant increase in GFAP and MCP-1 staining density in the group that received repeated noxious stimuli when compared to controls that received repeated innocuous stimuli ($p < 0.001$ for both) (neonate and adult) (Figs. 6 and 7). No significant differences were observed between sides or sex for GFAP and MCP-1 staining in the motor cortices. OX-42 staining showed no significant differences in these areas.

4. Discussion

The current study showed, for the first time, increased activity in glial cells in specific cortical sites after neonatal noxious stimuli. In particular we showed enhanced astrocyte activity in somatosensory, motor, prefrontal, insular and anterior cingulated cortices that lasted throughout adulthood after neonatal noxious stimulation. We also added to the literature that repeated noxious stimulation in the neonate alters gait patterns that persist into adulthood, and confirm prior data showing enhanced nociceptive sensitivity to paw withdrawal threshold test. The absence of differences in nociceptive sensitivity 15 days after

insult could be explained by the immaturity of the somatosensory system at this age (Baccei and Fitzgerald, 2005). Thus, our results suggest that activation of cortical glial cells in this critical maturation period could underlie the motor and sensory changes observed in the adult.

Several brain areas are activated by noxious stimuli and are involved in the perception of pain. In adult rodents, nerve injury increases activation of the sensory, motor, prefrontal, anterior cingulate, and insular cortices, measured by ERK, IL-1 β , AMPA, GFAP or c-fos immunoreactivity (Takeda et al., 2009; Wiech et al., 2010). On the other hand, lesions of the somatosensory cortex, cingulate or prefrontal cortex in adults reduce nociceptive behaviors (Kuo et al., 2009; Uhelski et al., 2012). Despite the data showing the importance of cortical processing in pain, only one prior study (Anand et al., 1999) examined cortical sites after repeated needle stick (7 days), showing reduced c-fos immunoreactivity to the hot-plate test on P65, suggesting reduced responses to noxious stimulation in the somatosensory cortex. This is in direct contrast to the current study which shows enhanced glial activity in the neonatal cortex of multiple nociceptive sites. Nevertheless, one must take into consideration that c-fos immunoreactivity could be due to neuronal activation and its reduction could suggest remodeling with neuronal loss. Recently, it was suggested that chronic pain could be a result of dysregulation of glial functions in the central nervous system (Ji et al., 2013), possibly related to upregulation of glial markers, such as GFAP. If this is the case, that agrees with our finding of astrocyte activation in the limbic system, somatosensory and motor cortical areas, indicating that repeated exposure to noxious stimuli in the neonatal period alters nociceptive processing in a multidimensional way, not only at the time of injury but also persists into adulthood. Interestingly, the OX-42 density was very low and not different between the experimental groups, suggesting that the involvement of the microglia in this process is not important and that the alterations found are not inflammatory or immunemediated.

Our results are in agreement to prior studies showing that during development sensory experiences play a critical role in the refinement of cortical connections. Specifically, early noxious experiences affect hippocampal neurogenesis (Leslie et al., 2011), with increased expression of a neurogenesis marker and a marker for immature neurons observed 14 days after injection of Freund's adjuvant (CFA) on day P8. Previous investigators also showed that the brainstem and spinal cord are key sites in nociceptive circuit development (Hathway et al., 2012). In normal adult rats high intensity stimulation of the rostral ventral medulla (RVM) in the brainstem inhibits activation of the nociceptive reflex (Hathway et al., 2012), while the same stimulation in neonates facilitates this reflex. Blockade of opioid receptors between days 21 and 28 prevents the development of this inhibition in adults while in animals where opioid receptors were blocked between days 14 to 21 or 28 to 33, the inhibition develops normally (Hathway et al., 2012). In summary, pain in the neonatal period alters different areas of the brain including the brainstem, hippocampus, frontoparietal, sensory and motor cortices. Our studies extend these finding and show glial cell activation in multiple sites, as well as the involvement of sites not previously observed after neonatal injury.

Numerous studies have examined the consequences of early exposure to noxious stimuli in the neonate using different models such repetitive heel stick (Anand et al., 1999), nerve and skin injury (Li et al., 2009), and injection of inflammatory agents (Leslie et al., 2011; Uhelski et al., 2012;). In general these studies show that pain in the neonatal period is capable of altering nociceptive behavioral output in adults with decreases in thermal and mechanical withdrawal thresholds (Luis-Delgado et al., 2006) and increase in the susceptibility of developing stress and anxiety (Anand et al., 1999).

It has become clear that after nerve injury or inflammation there is activation of glial cells in the spinal cord and blockade of glial cells reduces pain behaviors (Gwak and Hulsebosch, 2009). Interestingly, at the spinal level there are also changes in microglia after neonatal nerve injury; however this is time-dependent. Newborn animals with injury show minimal or no immunostaining of microglia in the spinal cord at the time of injury or when they are adults (Vega-Avelaira et al., 2007); however animals that received neonatal injury and are re-injured when they are adults show increased immunostaining for microglial markers in the dorsal horn (Vega-Avelaira et al., 2012). In contrast, astrocytic activation in the dorsal horn occurs in direct response to injury in the early neonatal period directly after injury (Vega-Avelaira et al., 2012). This is in agreement with the current study that shows no microglia activation in injured newborns and increased activation of the astrocytes in multiple cortical sites after neonatal injury that persists into adulthood.

Previous studies (Anand et al., 1999) showed that injury in the neonatal period can change locomotor activity in the open field test, immediately after injury. However, the current study is the first to show that pain in the neonatal period there are alterations in gait patterns and these alterations remain in adulthood. Altman and Sudarshan (1975) showed that locomotion in laboratory rats develops during the first three weeks after birth. Rats are able to walk and run on the second week with a steady level of ambulation similar to that of an adult rat suggesting maturation of sensory and motor systems. In critical periods of development, early noxious stimuli can alter spinal nociceptive processes changing activity in dorsal horn circuits that can alter the nociceptive behavior for life (Anand et al., 1999). These long-lasting alterations in dorsal horn circuits could explain the alteration in the motor function evidenced in the present study. Furthermore, unknown connections between different cortical areas could underlie the sensory and motor dysfunction that persists into adulthood. Although, some studies observed differences between sexes after neonatal injury (Knaepen et al., 2012) we were unable to detect such differences, in agreement with results from a previous study (Beggs et al., 2012).

The number of painful stimuli in neonatal intensive care units is high and effective analgesic measures to prevent pain are often inadequate (Johnston et al., 2011). As significant alterations in nociceptive sites throughout the nervous system occur and persist into adulthood after neonatal injury, and adults with neonatal injury are more sensitive to noxious stimuli, it is important to establish a pain management policy for the neonates that includes efforts to reduce the number of painful procedures. The current study show that, in rats, neonatal injury (repetitive needle pricking) results in long-lasting alterations in responses to noxious stimuli, alterations in gait, and is associated with widespread increases in activation of astrocytes across multiple cortical sites. It still remains unknown what

triggers glial activation following neonatal noxious stimuli. Thus, further studies are needed to understand the role of cortical glia in nociceptive processing and particularly in continued nociceptive sensitivity in the adult after neonatal injury.

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Fig. 1. von Frey test (A), calibrated forceps (B) and gait analysis (C) average values from Control neonatal group (P15), Pain neonatal group (P15), Control adult group (P180) and Pain adult group (P180). Comparison was made between paws in the same group (pain or controls, 15 days or 180 days) and between experimental times (15 days $\times$ 180 days) for the von Frey and calibrated forceps tests. The gait analysis comparison was performed between experimental groups (pain $\times$ control) in the same experimental time and between experimental

times. ⁺ indicates difference compared to Pain neonatal group ($p < 0.05$). ^{*} indicates difference compared to Pain-Adult group ($p < 0.05$).

Fig. 2.

Average density values of GFAP (A), MCP-1 (B), and OX-42 (C) immunostaining in areas of the limbic system (prefrontal, anterior cingulate and anterior insular cortices) (left column), somatosensory cortex (somatosensory of hindlimb region—S1HL and secondary somatosensory—S2) (middle column) and motor cortex (primary motor—M1 and secondary motor—M2) (right column). Note that the pattern of GFAP and MCP-1 staining is similar in all experimental groups while the OX-42 density is very small and does not follow a specific pattern. There is a significant increase in the activation of glial cells (astrocytes) but not of the microglia in neonates and adults after painful stimulation. # indicates difference compared to Pain neonatal group ($p < 0.05$). * indicates difference compared to Pain-Adult group ($p < 0.05$).

Fig. 3. Representative coronal cryo-sections immunostained for GFAP (red), MCP-1 (green) and merge (GFAP + MCP-1) in the limbic system: prefrontal (A and B), anterior cingulate (C and D) and anterior insula (E and F). Note that pain (B, D and F) groups are densely marked, compared to their respective controls (A, C and E). Bar = 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Representative coronal cryo-sections immunostained for GFAP (red), MCP-1 (green) and merge (GFAP + MCP-1) in somatosensory cortex: primary somatosensory of hindlimb region (S1HI—A and B) and secondary somatosensory (S2—C and D). Note that pain groups, acute (B) and chronic (D) are densely marked, compared to their respective controls (A and C). Bar = 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Representative coronal cryo-sections immunostained for GFAP (red), MCP-1 (green) and merge (GFAP + MCP-1) in motor cortex: primary motor region (M1—A and C) and secondary motor region (M2—B and D). Note that pain groups, acute (B) and chronic (D) are densely marked, compared to their respective controls (A and C). Bar = 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Experimental groups according to gender, treatment and evaluation time. Pain groups received noxious stimuli (needle pricking twice a day for 15 days after birth) while control groups received tactile stimuli (with a cotton-swab, twice a day for 15 days after birth). Independent groups were evaluated 15 days and 180 days after birth.

Gender (N = 40)	Treatment (N = 20)	Evaluation time (days) (N = 10)
Male	Pain	15
		180
	Control	15
		180
Female	Pain	15
		180
	Control	15
		180

N = number of animals per group.