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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 cingulated, 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.

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.

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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.

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 cingulated 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 Experimentacão 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 × 7.5 × 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 be 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 mm^2. 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