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THE PUERPERIUM ALTERS SPINAL CORD PLASTICITY FOLLOWING PERIPHERAL NERVE INJURY

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Abstract—Tissue and nerve damage can result in chronic pain. Yet, chronic pain after cesarean delivery is remarkably rare in women and hypersensitivity from peripheral nerve injury in rats resolves rapidly if the injury occurs in the puerperium. Little is known regarding the mechanisms of this protection except for a reliance on central nervous system oxytocin signaling. Here we show that the density of inhibitory noradrenergic fibers in the spinal cord is greater when nerve injury is performed in rats during the puerperium, whereas the expression of the excitatory regulators dynorphin A and neuregulin-1 in the spinal cord is reduced. The puerperium did not alter spinal cord microgial and astrocyte activation. Astrocyte activation, as measured by glial fibrillary acidic protein (GFAP) expression, was not evident in female rats with injury, regardless of delivery status suggesting sex differences in spinal astrocyte activation after injury. These results suggest a change in the descending inhibitory/facilitating balance on spinal nociception neurotransmission during the puerperium, as mechanisms for its protective effect against injury-induced hypersensitivity.

Key words: nerve injury, puerperium, spinal cord plasticity.

INTRODUCTION

There is a strikingly low incidence of chronic pain from childbirth even though it can be accompanied by considerable tissue and potential nerve damage (Eisenach et al., 2013). This low incidence is unexpected, given the enhanced excitability, enhanced TRPV1 expression, and sprouting of sensory afferents innervating the lower uterine segment and cervix at the end of pregnancy (Tong et al., 2006). In addition, labor is associated with an inflammation-like response (Tornblom et al., 2005) in these tissues, which results in their increased compliance to allow passage of the fetus. This inflammation-like response also sensitizes peripheral afferents. Despite these changes in afferent sensitization peripheral nerve injury-induced hypersensitivity, as a model of neuropathic pain, resolves rapidly in rats when surgery is performed surrounding the time of delivery (Gutierrez et al., 2013). This protection appears to be an effect of the post-delivery period rather than pregnancy *per se* and is not dependable on circulating progesterone and estrogen which decline rapidly following delivery and involves oxytocin signaling in the central nervous system. The primary goal of the current study is to better understand the mechanisms by which the puerperium hastens the resolution of hypersensitivity after peripheral nerve injury.

Peripheral nerve injury alters both primary afferent and second-order spinal cord neurons, resulting in sensitization and abnormal responses to peripheral stimuli. Glia in the spinal cord also reacts to peripheral nerve injury, with an initial wave of activation of microglia, followed by a more sustained activation of astrocytes resulting in release of pronociceptive cytokines and neuronal sensitization (Zhang and De Koninck, 2006; Gao et al., 2009). The increased immunoreactivity to the ionized calcium-binding adaptor molecule (IBA1) and the glial fibrillary acidic proteins (GFAP) are good indicators of microglia and astrocyte activation, respectively and were used in the current study to determine the effect of the puerperium on spinal cord glial activation following peripheral nerve injury.

Spinal cord neurons are also modulated by descending pathways from the pons and the medulla, as proposed in the original description of the gate control theory of pain (Melzack and Wall, 1965). The balance between descending inhibition and facilitation has been proposed to be disrupted after peripheral nerve injury, leading to spinal sensitization, increased ascending nociceptive signaling, and consequently pain. A key inhibitory pathway, noradrenergic fibers descending from the pons, sprout after peripheral nerve injury in mice and rats (Ma and Eisenach, 2003; Hayashida et al., 2008) and presence of this system may play an inhibitory role in glial activation in the spinal cord following peripheral nerve injury (Hayashida et al., 2012). On the other hand, a key facilitatory pathway from the nucleus raphe magnus releases serotonin into the spinal cord, resulting in the increased synthesis of dynorphin (Kondo et al., 1993; Hentall et al., 2006). This endogenous opioid peptide paradoxically drives injury-induced hypersensitivity (Wang et al., 2001; Gardell

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Abbreviations: DβH, anti-dopamine β hydroxylase; EIA, enzyme immune-assay; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IBA1, ionized calcium-binding adaptor molecule; NRG1, neuregulin-1; PBS, phosphate-buffered saline; PP, postpartum; SNL, spinal nerve ligation.

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et al., 2002) by actions on *N*-methyl-D-aspartate receptors (Laughlin et al., 1997). Another aim of the current study was to determine the effect of the puerperium on noradrenergic fiber density, using immunostaining for dopamine β-hydroxylase (DβH) and dynorphin content in the spinal cord after nerve injury.

Finally, most previous work on the mechanisms of neuropathic pain or hypersensitivity has been performed in male animals, despite a predominance of many common chronic pain conditions in women. We therefore asked whether the glial and neuronal plasticity after nerve injury, classically defined in male rats, applied to female rats. As part of this effort, we focused on the role of the puerperium on neuregulin-1 (NRG1), which was shown to be important in maintaining persistent pain among female rats (Lacroix-Fralish et al., 2008).

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley rats (250–350 g) from Harlan Industries (Indianapolis, IN, USA), housed under a 12-h light–dark cycle with food and water *ad libitum*, were used. All experiments were approved by Animal Care and Use Committee at the Wake Forest University, School of Medicine (Winston Salem, NC, USA).

Tissues from a total of 108 (98 virgin females and 10 males) age-matched Sprague–Dawley rats (age = 16–17 weeks, weigh = 250–350 g) were used in this study. The behavioral data from 28/108 animals were reported previously (Gutierrez et al., 2013) and the tissue collected from those animals was used in the immunocytochemistry analysis ($n = 7$ in each group). Withdrawal thresholds were not determined in the remaining 80 animals (10 males (non-breeders) and 70 females). The tissue collected from these 80 animals was used in Western blots ($n = 7$ in each group of females and $n = 5$ in each group of males) and enzyme immune-assay ($n = 7$ in each group of postpartum and $n = 4$ in each group of virgin females).

Surgical procedures

Within 24 h after the delivery of the last pup and when virgin females and males rats were 16–17 weeks old (to match the age of postpartum rats at the time of delivery), spinal nerve ligation (SNL) surgery was performed as previously described (Hayashida et al., 2012). Animals were anesthetized with 2% isoflurane in oxygen and SNL or sham surgery was performed. For SNL, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were tightly ligated using 5.0 silk sutures. Sham surgery consisted of exposure of the L5 and L6 spinal nerves, but no ligation. After surgery, animals were housed with their pups (postpartum) or pair housed (males and virgin females) in plastic cages in a climate-controlled room under a 12 h–12 h light–dark cycle, with free access to food and water.

Tissue preparation for Immunocytochemistry

Seventeen days following SNL or sham surgery, rats were anesthetized with sodium pentobarbital (i.p; 100 mg/kg), the thorax was opened, and fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) was perfused through the left ventricle with a peristaltic pump (20 ml/min) for 15 min. The spinal cord L4–L6 segments were then identified based on anatomy (lumbar enlargement and identification of the L4–L6 spinal nerves), removed and immersed in fixative (post-fixation) for 3 h at 4 °C. After post-fixation a needle was inserted in the ventral dorsal horn, ipsilateral to SNL surgery and the spinal cord immersed in 30% sucrose at 4 °C for cryoprotection until sectioned. Transverse sections (40 μm) were cut on a cryostat. At least three experiments were performed per marker (IBA1, GFAP and DβH). Sections from 2 to 3 animals per group from the L5–L6 segments were processed simultaneously in each experiment. Antibodies for IBA-1, GFAP and DβH were used to examine glia, astrocytes and noradrenergic fibers (Zhang and De Koninck, 2006; Hayashida et al., 2008). To analyze these aspects, the sections were incubated over night at 4 °C with the primary antibody rabbit anti-IBA1 (1:1000, #019-19741, Wako Chemicals, Richmond, VA, USA); rabbit anti-GFAP (1:5000, #RZ0334, Dako, Carpinteria, CA, USA) or mouse monoclonal anti-dopamine β hydroxylase (DβH, 1:500, #MAB308, Chemicon International Inc., Temecula, CA, USA) followed by the corresponding biotinylated donkey anti-rabbit or anti-mouse IgG (1:500, Vector Laboratories, Burlingame, CA, USA) in 1.5% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), 0.1% Triton X-100 in 0.01 M phosphate-buffered saline (PBS). Then the sections were reacted using Elite Vectastain ABC kit (Vector Laboratories, AC, USA).The Elite Vectastain ABC kit was used to link the antigen antibody complex to horseradish peroxidase (HRP; ABC Elite, Vector), which was then visualized with 3,3-diaminobenzidinetetrahydrochloride (DAB) histochemistry. Finally, the sections were washed thoroughly in PBS, mounted on plus-slides, air-dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX.

Image analysis

Coded numbered slides with the sections were examined with brightfield illumination on a Nikon E600 epifluorescence microscope and images were captured with a CCD digital camera attached to the microscope using a 10× objective. Images of ipsilateral L5/6 dorsal horn were captured, corresponding to projection sites of denervated regions of the spinal cord. Quantification for each image was performed calculating the average from three to five randomly selected spinal cord sections per rat. The images were quantified using Image J (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011). In order to conduct the regional analysis of the spinal cord, a macro was defined using a digital template to contain laminae I to IV of the dorsal horn. The number of pixels occupied by immunoreactive processes with intensity above a fixed threshold (used for the analysis of all sections) and within the defined area was counted automatically. The average was then calculated per animal and per group. The experimenter performing image analysis was blinded to group.

Tissue preparation for enzyme immune-assay (EIA) and Western blots

Animals were euthanized 17 days after SNL or sham surgery by deep halothane anesthesia followed by decapitation. The spinal cord was removed, the segment containing in the lumbar enlargement dissected, cut (coronal plane) and ipsi and contralateral dorsal quarters were rapidly dissected on ice, then frozen in dry ice-cooled 2-methylbutane and stored at −80 °C.

Preparation of spinal cord extracts for dynorphin A EIA

Dynorphin A content of spinal cord was determined as previously described with minor modifications (Parra et al., 2002). The dissected segments of the spinal cord were sonicated on ice in