



## Sex and hormonal variations in the development of at-level allodynia in a rat chronic spinal cord injury model

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### ABSTRACT

The development of central neuropathic pain varies among patients with spinal cord injury (SCI). The factors contributing to the development and perpetuation of segmental pain (at-level allodynia) has been the focus of ongoing experiments in our laboratory. One such factor is hormonal status. We have shown previously, using a male rat model of SCI, that a severe contusion injury is necessary for the development of allodynia in trunk regions at and just above the level of a T8 injury. In this study, we examined at-level sensitivity for SCI ovariectomized (ovx) and cycling female rats as well as for SCI males implanted with either a placebo pellet or one that slowly releases 17 $\beta$ -estradiol. The proportion of ovx SCI female rats and placebo-treated SCI males displaying pain-like behaviors to touch/pressure of at-level dermatomes up to 6 weeks post-injury (67% and 75%, respectively) was similar to our previous studies on SCI males (69%). In contrast, significantly fewer cycling SCI female rats and 17 $\beta$ -estradiol treated SCI male rats showed sensitivity to touch at-level (26% and 30%, respectively). These results implicate 17 $\beta$ -estradiol as a potential target that can readily be modulated to prevent segmental pain following SCI.

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Chronic central pain develops in the majority of spinal cord injury (SCI) patients [9,43] and is ranked highest by paraplegics as the function whose recovery would dramatically improve quality of life [2], yet relatively few studies are being done and current drug therapies and surgical interventions are inadequate [12,47]. According to the National SCI Statistical Center at the University of Alabama ([www.nscisc.uab.edu](http://www.nscisc.uab.edu) – April 2009), 259,000 people in the U.S. have SCIs. The cost to the nation is estimated at \$9.7 billion per year [3]. Classification of SCI pain by the International Association for the Study of Pain includes nociceptive pains such as musculoskeletal and visceral pain, and neuropathic pains above-level, at-level and below-level of lesion [44]. Neuropathic pains could be spontaneous or mechanically evoked by normally innocuous mechanical stimulation (allodynia) [10,22]. An understanding of the variables involved in the development and perpetuation of SCI-related pain will lead to more effective strategies for pain management.

In one longitudinal study, 81% of patients reported the presence of pain, half of whom experienced at-level neuropathic pain [43]. A high percentage of male rats with mid-thoracic spinal contusion lesions develop allodynia at-level [22,23,27]. At-level pain-like behavioral responses post-SCI may be associated with hyperactivity of remaining intact spinothalamic dorsal horn neurons [7]. After chronic SCI, these dorsal horn neurons exhibit higher background activities, altered discharge patterns, increases in responsiveness

to brush and pinch, and longer after-discharges [10,18]. A significant (62%) increase in metabotropic glutamate receptor subtype 1 immunoreactivity has been demonstrated for spinothalamic tract neurons located immediately rostral to the epicenter of a T9 contusion [36], implicating the thalamus as an important supraspinal site contributing to chronic SCI pain. Thalamic changes occur in SCI patients experiencing segmental pain [32,38] and we have shown in male rats a significant correlation between hyperexcitability of somatovisceral convergent thalamic neurons to at-level stimulation and behavioral signs of allodynia at-level following spinal contusion at T8 [23].

Although our studies on SCI pain use male rats, most animal models of SCI use females as subjects [20], despite an 80.9% prevalence of SCI among men ([www.nscisc.uab.edu](http://www.nscisc.uab.edu) – April 2009). The effect of hormonal status on the development of allodynia after SCI is not known. In the first part of this study, sex differences in the incidence of SCI-related pain in at-level dermatomes were evaluated for SCI cycling and ovariectomized (ovx) female rats and the results compared with our previous studies on male rats. In the second part, to examine the potential contribution of ovarian steroids, the incidence of pain-like behaviors evoked by touch/pressure at-level was examined in SCI male rats treated with 17- $\beta$  estradiol versus a placebo SCI control group.

A total of 56 female and 30 male Wistar rats were used and all procedures approved by the Institutional Animal Care and Use Committee at the University of Louisville in accordance with USDA regulations. Four female and two male rats were excluded from the study as they did not survive for the entire 6-week post-SCI testing

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period (these rats are not included in values provided). For the first part of the study, ovx via small lumbar incisions [19,21,37] were done on 22 of the rats 2 weeks prior to SCI surgeries (SCI on 15 of these 22 rats). The remaining 34 rats made up the cycling groups (11 intact controls and 23 SCI rats). The estrus cycle was monitored [21] and the stage noted at the time of testing for all normal cycling animals. Vaginal smear samples were taken from all ovx rats to verify those rats were non-cycling.

For the second part of the study on 30 male rats, hormones were delivered continuously throughout the recovery period with 60-day time release capsules obtained from Innovative Research of America (IRA; Sarasota, Florida – [www.innovrsrch.com](http://www.innovrsrch.com)). Pellet insertion, 1 week prior to SCI, was done with a small incision in the skin of the neck just behind the right ear (closure with one wound clip) under brief anesthesia with isoflurane. Each pellet (for 10 rats) contained 0.25 mg of 17- $\beta$  estradiol to maintain serum levels similar to the proestrus stage of the female animals' cycle [14,28]. Ovarian hormones, such as progesterone and estradiol, reach their highest level during proestrus [4,14]. The pellets are known to yield continuous serum hormone levels that are reached during proestrus [8,40]. The placebos (20 rats) are made of only the vehicle, which contains cholesterol, cellulose, lactose, phosphates and stearates.

Serum estradiol concentrations were determined with radioimmunoassays on blood samples taken during euthanasia at the end of the 6-week testing period. Blood was collected directly from the heart, allowed to clot at room temperature, centrifuged, then frozen and stored at  $-20^{\circ}\text{C}$ . Samples were subsequently shipped overnight on dry ice to the Ligand Assay and Analysis Core Laboratory at the Center for Research in Reproduction, University of Virginia Health Sciences Center ([ligandcore@virginia.edu](mailto:ligandcore@virginia.edu)). Sample labels were coded, so the assays were done blinded.

For SCI (female and male) rats, each animal was anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), injected intraperitoneally. All surgeries were performed as previously described [22,23]. A T7 laminectomy exposed the underlying T8 cord. Contusions were done using an Infinite Horizon (IH) Impactor (Precision Systems & Instrumentation, LLC, Fairfax, VA). All animals received 5 mg/kg of gentamicin (Abbott Laboratories, North Chicago, IL) once/day for five days post-surgery to control for possible bladder infections, and 2.5 mg/kg of ketoprofen (Ketofen®, Fort Dodge Laboratories, Fort Dodge, IA) once/day for two days to alleviate post-surgical pain. The urinary bladder was expressed every 8 h until reflexive voiding occurred (up to about 12 days [24]).

The dorsolateral trunk sensitivity in the T5–T7 dermatomes was tested once a week for 6 weeks as previously described [17]. Briefly, testing for pain-like behaviors (freezing and/or escape and/or grab/push probe with forepaw) began with a #5 paint brush (17 g force) stimulus. If a pain-like behavior occurred for 60% of the trials (6 of 10, 5 each being alternating between left and right sides of the trunk), the response threshold was determined using a series of 15 Semmes-Weinstein Monofilaments with forces ranging between 0.008 g and 15 g (Stoelting Co., Wood Dale, IL). If the 60% brush response criteria was not met, the rat was tested further using gentle pressure (60 g) with a pair of modified Adson tissue forceps (2.0 mm wide tips) having a load cell mounted for force measurement [17]. Note that the mean pain-like response threshold for intact rats is  $101.3 \pm 3.2 \text{ g/cm}^2$ , so all sensory testing in the SCI rats used normally innocuous levels of stimulation ( $\leq 60 \text{ g/cm}^2$ ). A 5-week mean allodynia score was calculated using our 10-point scoring system [17], which accounts for the type of pain-like behavior exhibited as well as the degree of sensitivity.

At the end of the experiment, each animal was perfused and 2 cm of cord containing the lesion at the center (based upon surface bruising and visible cord atrophy) extracted. The entire piece of cord was then sectioned transversely at 30  $\mu\text{m}$  thickness using

**Table 1**

Summary of animals with at-level allodynia.

Group	Condition	# With at-level allodynia
Male <sup>a</sup>	Intact control	0/7 (0%)
Female – ovx	Intact control	0/7 (0%)
Female – cycling	Intact control	0/11 (0%)
Male <sup>b</sup>	Complete T8 transection	0/15 (0%)
Male <sup>a</sup>	Contusion at T8	18/26 (69%)
Female – ovx	Contusion at T8	10/15 (67%)
Female – cycling	Contusion at T8	6/23 (26%) <sup>^</sup>
Male – placebo	Contusion at T8	15/20 (75%)
Male – 17 $\beta$ estradiol	Contusion at T8	3/10 (30%) <sup>^^</sup>

<sup>a</sup> Hubscher and Johnson [22,23].<sup>b</sup> Hubscher et al. [25].<sup>^</sup> Significantly different from contused males ( $\chi^2 = 3.99$ ;  $p < 0.05$ ) and ovx females ( $\chi^2 = 2.49$ ;  $p < 0.05$ ).<sup>^^</sup> Significantly different from contused placebo males ( $\chi^2 = 3.91$ ;  $p < 0.05$ ).

a cryostat, and stained with both luxol fast blue and cresyl violet (Kluver–Barrera Method [35]). All slides were viewed under a light microscope using the 4 $\times$  objective to locate the section with the largest area of damage (lesion epicenter). A SPOT RT3 camera mounted on a Nikon E400 microscope was used to capture images and SPOT version 4.6 software (Diagnostic Instruments, Inc.) for area measurements. The epicenter section image and one from a more intact portion of spinal cord (located 1 mm rostral to the epicenter) was saved for comparison and quantification for extent of white matter area sparing [17,26]. As previously described, landmarks used for white matter area measurements included medial and lateral edges of the dorsal horn, tips of the ventral horn, and surface notches where the dorsal and ventral roots enter and exit, respectively.

Behavioral testing for dorsal trunk sensitivity to low threshold mechanical stimuli revealed that only 26% (6 out of 23) of contused females with intact ovaries developed allodynia at-level, which is significantly ( $\chi^2$ ,  $p < 0.05$ ) lower than ovx SCI female rats and SCI male rats (Table 1). Those ovx female rats meeting the criteria for at-level allodynia had a mean allodynia score of  $5.1 \pm 0.4$  (out of a possible 10). The few cycling female rats that had pain-like behavioral responses to touch had a mean score of  $6.5 \pm 1.5$ , which was not significantly different. Those rats responding to brush had response thresholds to monofilament sizes ranging between 0.008 g and 1.4 g. Note that the read-out impactor forces for the cycling and ovx groups were not significantly different ( $232.1 \pm 11.2 \text{ Kdyn}$  versus  $252.4 \pm 9.6 \text{ Kdyn}$ , respectively;  $t$ -test,  $p > 0.05$ ). There were also no significant differences in the percent white matter sparing between SCI ovx and cycling groups of rats ( $11.5 \pm 2.6\%$  and  $10.4 \pm 2.8\%$ , respectively), a finding consistent with the lack of impactor force differences from the computer generated read-outs obtained at the time of injury.

For the SCI male rats, the percentage having at-level sensitivity to touch post-contusion was significantly reduced (30%;  $\chi^2$ ,  $p < 0.05$ ) for the group receiving the 17- $\beta$  estradiol treatment. For those responding to touch at-level, the mean allodynia score was  $3.9 \pm 0.5$  for the placebo group and  $6.3 \pm 1.1$  for the three estradiol treated males (approaching significance;  $p = 0.052$ ). Note that serum levels of estradiol were significantly lower ( $p < 0.001$ ) for placebos ( $8.5 \pm 1.1 \text{ pg/ml}$ ) than for the estradiol-treated ( $60.5 \pm 9.6 \text{ pg/ml}$ ) males. Although the serum levels were lower for the three allodynic estradiol-treated rats ( $48.5 \pm 10.1 \text{ pg/ml}$ ), the difference relative to the non-allodynic estradiol-treated rats was not significant ( $p > 0.05$ ).

The placebo male allodynia scores are not significantly different from ovx females ( $t$ -test;  $p = 0.083$ ). Male rats responding to brush also had similar thresholds at the low end (0.008 g and 2.0 g). Note that impactor forces for the male estradiol and placebo groups were also not different ( $221.8 \pm 2.7 \text{ Kdyn}$  versus  $224.9 \pm 2.7 \text{ Kdyn}$ ,

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