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Rodent Estrous Cycle Response to Incomplete Spinal Cord Injury, Surgical Interventions, and Locomotor Training

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Abstract

Estrous cycle disruption after spinal cord injury (SCI) in female rats is a common phenomenon. It remains unknown, however, if the aberrant estrous cycle is a result of an injury to the spinal cord itself or due to the general stress associated with surgical interventions. We addressed this issue by determining estrous cyclicity in female rats after a spinal cord hemisection (HX), implantation of EMG wires into selected hindlimb muscles, and/or injections of tracer dyes into the spinal cord. Since it is known that aerobic exercise can enhance the recovery of locomotor function in rodents with an incomplete SCI, we also determined if locomotor training positively impacts the disrupted estrous cycle after a HX. Estrous cycle assessments were made during a 5-8 week period in 27 female rats before and after HX, EMG, and/or dye injection surgeries and in HX rats that recovered spontaneously or underwent locomotor training. Our results show that estrous cyclicity was disrupted (cycle length >5 days) in approximately 76%, 46%, and 50% of the rats after HX, EMG, and dye injection surgeries, respectively. The cyclicity, however, was disrupted for a longer period after HX than after EMG or dye injection surgeries. Furthermore, estrous cycle mean length was shorter in the trained than non-trained HX group. These results suggest that estrous cycle disruption after a major SCI is a consequence of both the direct injury to the spinal cord and to the associated stress. Moreover, moderate aerobic exercise initiated early after a spinal cord HX returns the duration of the estrous cycle towards normal.

Keywords

spinal cord injury; surgical intervention; EMG surgery; estrous cycle; rodents

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Introduction

The majority of women report amenorrhea or dysmenorrhea after a spinal cord injury (SCI), irrespective of the severity and level of the lesion (Huang, Wang, Lai, Chang, & Lien, 1996; Reame, 1992). In the rodent model of SCI, the estrous cycle (menstrual cycle equivalent in rodents) is disrupted for six to ten days after both a transection (Gelderd & Peppler, 1979) or a contusion (Hubscher, Armstrong, & Johnson, 2006) injury of the spinal cord. Irregularities in the estrous cycle are largely associated with imbalance of plasma prolactin, estradiol, and progesterone concentrations (Samantaray, Matzelle, Ray, & Banik, 2010) which in turn impact reproductive functions such as sexual receptivity and ovulation (Mahesh & Brann, 1998). Disturbance in the rodent estrous cycle is characterized by an undue persistence of a specific stage or a random sequence of the otherwise orderly progression of the estrous cycle stages, i.e., pro-estrus, estrus, diestrus, or metestrus (Goldman, Cooper, & Murr, 2007). Estrous cycle stages are reliably quantified by obtaining vaginal smear cytology in female rodents and identifying the morphological changes in the vaginal epithelium as cells desquamate (Goldman, Cooper *et al.*, 2007; Hubscher, Brooks, & Johnson, 2005). Characterizing the estrous cycle by cell counts is a rather sensitive measure (Goldman, Cooper *et al.*, 2007), but is subject to change by several environmental stressors such as chronic restraint and immobilization, varied temperature and light, electrical shock, and noise (Gonzalez, Rodriguez Echandia, Cabrera, & Foscolo, 1994). Since an experimentally induced injury is a rather invasive procedure capable of inducing a stress response (Hager, Hagman, Wikstrom, & Strommer, 2004), it remains unknown if the aberrant estrous cycle after an experimental SCI (Hubscher *et al.*, 2006) is a result of a direct impact of the injury on the spinal cord or due to the presence of general stress associated with a surgical intervention. We assessed estrous cyclicity in female rodents after a variety of surgical interventions, i.e., implantation of EMG wires into selected hindlimb muscles, hemisection of the spinal cord, and injections of tracer dyes into the spinal cord to address this issue.

In part, reproductive hormonal changes in SCI males and females are a consequence of the neurochemical responses to stress after the injury (Rutberg, Friden, & Karlsson, 2008). Stress-related enhanced activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, which is the key neural drive controlling reproductive functions, leads to the release of glucocorticoids into the general circulation (Campeau *et al.*, 2010). As such, it is safe to presume that exercise might potentially negate some inhibitory effects of stress and perhaps reverse the estrous cycle disruption commonly observed after an injury. For example, non-injured rats that are given the opportunity to exercise while stressed with inescapable shock have lower corticosterone levels than shocked animals that are unable to exercise (Starzec, Berger, & Hesse, 1983). Importantly, voluntary exercise regimens attenuate several stress-related responses in rodents (Greenwood & Fleshner, 2008), including HPA axis activation under some acute and repeated stress conditions (Campeau *et al.*, 2010). Given that rehabilitation interventions that include exercise regimens play a critical role in the recovery of locomotor function of rodents with an incomplete SCI (Heng & de Leon, 2009; Thota, Carlson, & Jung, 2001), in the present work we determine the impact of locomotor training on the estrous cycle of female rodents after a hemisection injury to the spinal cord (HX). We hypothesized that locomotor training would attenuate the effects of HX on the estrous cycle.

Methods

Animals

Twenty-seven adult female Sprague Dawley rats (200-250 g body weight) underwent a chronological succession of surgeries, i.e., implantation of EMG recording electrodes in selected hindlimb muscles, spinal cord HX at a mid-thoracic level, and tracer dye injections in the spinal cord, each separated by three to four weeks. Longitudinal estrous cycle period

assessments were determined throughout the study in 15/27 animals that underwent EMG surgeries, 27/27 animals that underwent both EMG and HX surgeries and 23/27 animals that underwent all three surgeries. Normal estrous cycle data for all animals were obtained for at least three estrous cycles (~12 days) prior to each surgery (B00-B10). Data then were analyzed during the first ten days (P00-P10) and the subsequent ten days (P11-P21) after each surgery. Animals were eliminated from the study when there was an aberrant estrous cycle length prior to any surgery (>5 days). Accordingly, estrous data are reported from 13/15, 25/27 and 20/23 animals that underwent EMG, EMG+HX, and EMG+HX+dye injection surgeries, respectively. After the HX surgery, animals were assigned randomly to either a locomotor trained (N=16) or a non-trained (N=9) group.

All surgeries were performed under aseptic conditions with the animals deeply anesthetized with isoflurane gas (1.0 to 2.5% via facemask as needed) and on a water-circulating heating pad maintained at 37°C to prevent hypothermia. All incisions were closed in layers using 4.0 Dexon for the muscle and fascia and 4.0 Vicryl for the skin, respectively. After surgery the rats were placed in an incubator maintained at 37°C until fully recovered. Animals were administered analgesics (Buprenorphine (Buprenex), 0.05 mg/kg s.c.) and antibiotics (enrofloxacin (Baytril) 0.5 mg/kg s.c.) once or twice per day for 3-4 days as needed and housed in a room maintained at 26±1°C and 40% humidity and on a 12:12 h light:dark cycle with access to food and water *ad libitum*. All experimental procedures were approved by the University of California Los Angeles Chancellor's Animal Research Committee and complied with the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

EMG implantation procedures—The details of the EMG electrode implantation procedures have been described previously (Roy, Hutchison, Pierotti, Hodgson, & Edgerton, 1991). Both forelimb (biceps brachii, triceps brachii) and hindlimb (vastus lateralis, semitendinosus, medial gastrocnemius, and tibialis anterior) muscles were implanted bilaterally with EMG recording electrodes.

Spinal cord hemisection procedures—A dorsal midline skin incision was made from ~T6 to T10 and the paravertebral muscles and fascia from ~T7 to T9 were reflected laterally to expose the vertebrae. To expose the spinal cord, a partial laminectomy was performed via removal of the spinous processes and a portion of the lateral bodies of the T7 and T8 vertebrae. The exposed dura was incised longitudinally at the midline of the spinal cord with microscissors and then cut laterally to at both ends to expose one-half of the spinal cord. A 30-gauge needle then was used to form a small dorsal-ventral tunnel through the entire extent of the spinal cord and microscissors were used to transect the one-half of the spinal cord. Post-surgery, the bladders of all animals were expressed manually three times daily for the first three days and twice daily thereafter.

Tracer dye injection procedures—The general procedures for exposing the spinal cord were the same as described above. Partial laminectomies were performed at the T11-T12 vertebrae to expose the spinal cord: tracer dyes were injected into the L1 segment of the spinal cord for retrograde labeling using a 10 µl Hamilton syringe and stereotaxic equipment. Results of these experiments will be published elsewhere.

Locomotor training procedures—An upper body harness was used to position a subset of HX rats over a treadmill belt and to partially support their body weight for bipedal locomotion (N=8). Another subset of HX rats (N=8) was trained to step quadrupedally on a moving treadmill belt without any body weight support. For both training regimens, all rats

were trained 7 days/week, 20 min/session for 17 days starting at 4 days after HX. The speed of the treadmill belt was increased progressively from 6 to 13.5 to 21 cm/s.

Vaginal smear procedures—Vaginal smears from all animals were taken throughout the entire study starting for at least three estrous cycles (minimum of 12 days) prior to the EMG surgery. All animals underwent three surgeries with at least 3-4 weeks allowed between each surgery. Animals with an aberrant estrous cycle prior to any of the surgeries (>5 days) were eliminated from the study. All animals were handled for a couple of min prior to the lavage. Each rat was restrained gently via the “cone shape towel method” (Marcondes, Bianchi, & Tanno, 2002). Sterile saline solution (~0.10-0.15 ml) was heated to 40°C and inserted into the vagina using a 5-3/8” sterilized Fisher pipette. The pipette (~5 mm length) was inserted carefully to keep the animals at ease. Drops containing vaginal smears were mounted on glass slides.

Determination of the estrous cycle phase—Two technicians classified the raw (unstained) vaginal smears by their cytology under a light microscope at 5X and 10X magnifications. Estrous cycle phases of each animal were analyzed independently by three testers. Four distinct stages have been identified in a normal rat estrous cycle which is typically 4 to 5 days (Goldman, Murr, & Cooper, 2007; Hubscher *et al.*, 2005). Each stage (pro-estrus, estrus, metestrus, and diestrus) of the cycle has a unique proportion of 3 types of cells: epithelial, cuboidal, and leukocytes (Marcondes *et al.*, 2002). The pro-estrus phase is characterized by clusters of nucleated, epithelial cells (Figure. 1a, A2); the estrus phase by numerous cuboidal cells as well as needle-like cornified cells in aggregates (Figure. 1a, B2); the metestrus phase, which is used to describe the early part of the first day of diestrus, has a mix of leukocytes, epithelial, and cuboidal cells in approximately the same proportions (Figure. 1a, C2); and the diestrus phase exhibits the same mix of cells as the metestrus phase but shows a greater relative percentage of leukocytes (Figure. 1a, D2). A normal rodent estrous cycle lasts for 4 to 5 days and consists of 1 day of pro-estrus, 1 or 2 days of estrus, 6-12 hours of metestrus, and 2 days of diestrus. Extended (>3 to 4 days of estrus or >4 to 5 days of metestrus/diestrus) and irregular cycles (random alternations between the pro-estrus, estrus, metestrus, and diestrus phases) also exist (Goldman, Murr *et al.*, 2007) and were chosen as criteria in our study to identify an aberrant estrous cycle. Accordingly, animals with estrous cycle lengths of more than 5d were considered as having an aberrant cycle length.

Each phase of the estrous cycle was identified according to the relative percentages of leukocyte, epithelial, and cornified cells observed in the smears (Goldman, Murr *et al.*, 2007). Pro-estrus was defined as having more than 70% epithelial cells. Estrus was defined as having more than 70% cornified cells or having 50% epithelial and 50% cornified cells since this is characteristic of late estrus (Hubscher *et al.*, 2005). Metestrus was characterized as having nearly the same percentages of all three types of cells. Diestrus was defined in a similar manner as metestrus, but with a much higher relative percentage of leukocytes. Due to the difficulty of discriminating between the metestrus phase (which is technically the transitional period during the early part of the first day of diestrus and with the occasional chance of missing the metestrus phase because of its short period) and diestrus phases, we merged the data for these two phases in our analyses. Estrous cycle length was calculated as the number of days from the onset of the estrus phase of one estrous cycle to the onset of the estrus phase of the next cycle.

Statistical analyses—All data are reported as mean $\pm$ SEM. For all surgical interventions (EMG, HX, and dye injection surgeries), the differences in estrous cycle lengths at B00-B10, P00-P10, and P11-P21 were determined using one-way repeated measures ANOVA. Comparisons in estrous cycle lengths between animals immediately after the HX, EMG, and

dye injection surgeries (P00-P10) were measured using a between subject one-way ANOVA. When the overall main effects were significant, adjusted (p level/number of groups, 0.05/3) Bonferroni post-hoc tests were used to identify significant differences between individual time points and surgery groups. Differences in the estrous cycle length between the trained and non-trained HX sub-groups were determined using two-sample Student's t -tests. Differences were considered statistically significant at $p < 0.05$.

Results

Impact of the HX injury

Prior to HX, the vaginal smears of most animals (25/27) demonstrated an orderly progression of one of the four cell types that represent distinct estrous cycle stages (Figure 1a). The average length of an estrous cycle before HX was approximately 4 days. The progression of a representative animal through the estrous cycle before and after HX is shown in Figure 1b. Quantitatively, the average estrous cycle length of 19/25 rats (76%) increased to ~9 days ($p < 0.05$, Figure 1c) during the first 10 days post-HX. Most of the rats (17/25) showed a prolonged metestrus/diestrus phase. As reported in other studies (Hubscher *et al.*, 2006), the transient prolonged cycle periods reverted to normal cyclicity in all animals between 11 to 21 days post-HX. The long cycle periods began within the first two days post-injury for 16/19 animals, and at seven to nine days post-injury for the remaining 3 animals.

Impact of locomotor training interventions

Although most rats showed prolonged estrous cycle periods post-HX, the mean length of the estrous cycle periods was shorter in the trained than non-trained sub-group ($p < 0.05$, Figure 1d).

Impact of EMG and dye injection surgeries

Six of 13 rats after EMG surgeries (~46%) and 10/20 animals after dye injection surgeries (50%) showed prolonged cycle periods starting from the day of surgery (Figure 2a and b). The prolonged cycle periods were due to either an increase in the duration of the estrus or diestrus phases of the cycle. The duration of the estrous cycle phases was shorter after the EMG and dye injection surgeries than after the HX surgery (Figure 2a). Note that Figure 2a represents the average estrous cycle period from all rats including those showing a 4-day normal estrous cycle length after the respective surgery.

Discussion

One of the factors for the estrous cycle dysfunction after a HX in rodents is a consequence of direct injury to the spinal cord. Multi-synaptic neuronal connections between the brain, spinal cord, and ovary/testes are well established in the neuronal control of reproductive organs (Gerendai, Kocsis, & Halasz, 2002). Hubscher *et al.* (Hubscher *et al.*, 2006) reported that the recovery to a normal rodent estrous cycle after a spinal cord contusion injury was, in part, a function of the amount of spared ventromedial white matter. Although the precise mechanisms that deregulate the estrous cycle rhythm after SCI in rats remain unclear, it is well known that alterations in vaginal epithelium are controlled by cyclic changes in ovarian steroids throughout the 4-5 day estrous cycle (Axelson, 1987; Westwood, 2008). Specifically, absence of cornified vaginal epithelial cells or persistence of the diestrus phase is generally considered to be a good indication of a failure of follicular development due to its association with low serum luteinizing hormone (LH) (Huang *et al.*, 1996) and elevated levels of prolactin (hyperprolactinemia) (Rutberg *et al.*, 2008). LH and prolactin are key hormonal regulators of follicular development and are greatly altered after SCI. This

hormonal dysregulation ultimately manifests as amenorrhea. An imbalance in the central neurotransmitter system, such as a decrease in central dopaminergic tone (Huang *et al.*, 1996; Huang, Wang, & Lien, 1995) and GABAergic tone (Gwak & Hulsebosch, 2011), after a SCI is thought to alter the endocrine interactions among the hypothalamic, pituitary, and ovarian components of the reproductive axis that collectively disrupt the ovarian estrous cyclicity (Huang *et al.*, 1996). A decrease in dopamine and GABA activity decreases the overall gonadotrophin releasing hormone (GnRH) activity that is ultimately responsible for the release of gonadotrophins (LH and follicle stimulating hormone (FSH)) from the anterior pituitary. Additionally, central neurotransmitters directly trigger hyperprolactinemia that alters the role of estrogen in follicular development.

We observed an alteration of the estrous cycle in female rodents after muscle electrode implant surgeries (that do not involve exposure of the spinal cord) and dye injections into the spinal cord (that involve the exposure, but minimal invasion, of the spinal cord). This implies that the general stress associated with surgery can affect the neuroendocrine control of the reproductive axis and lead to amenorrhea. In fact, elevated levels of glucocorticoids and hyperglycemia are common consequences of activation of the HPA axis secondary to surgical interventions (Hager *et al.*, 2004; Hager *et al.*, 2009). Desborough (Desborough, 2000) suggests that surgery is one of the most potent activators of adrenocorticotrophin and cortisol release. While elevated levels of cortisol maintain normal body homeostasis after surgery via anti-inflammatory and catabolic effects, corticotrophic releasing hormone release produces a dose-dependent decrease in GnRH from the mediobasal hypothalamus (Gambacciani, Yen, & Rasmussen, 1986), which in turn can result in stress amenorrhea (Genazzani *et al.*, 1991). Additionally, the neuroendocrine portion of the HPA axis can be activated by cytokines or endotoxins released by immunoinflammatory challenges secondary to surgery (Xiao & Ferin, 1997). Unlike a SCI (Huang *et al.*, 1996), however, a non-SCI surgical intervention has a rather variable impact on the levels of FSH and LH (Desborough, 2000). Therefore, it appears that while central neurotransmitters play a predominant role in inducing amenorrhea after SCI, stress associated with surgery alone can disrupt normal functioning of the HPA axis. One could also argue that the differences observed across manipulations depend in some fashion on the order in which they were performed. The widely varying estrous cycle lengths observed after the surgeries, however, do not support this notion. For example, there is a clear mean difference in the length of the cycle delay after the EMG and HX surgeries (~9d vs. ~6d). In addition, the percentage of animals that showed a delayed estrous cycle was lower after a non-SCI (50%) than a SCI (76%) surgical intervention. These findings are consistent with there being both central neurotransmitter and stress hormonal factors that alter the reproductive axis after a SCI. This interpretation is further supported by the observations that stress receptors (β 2-adrenergic) become hyper-responsive to catecholamines after a SCI (Lucin, Sanders, & Popovich, 2009).

Unlike intense exercise regimens that disrupt reproductive function (Axelson, 1987; Chatterton, Hrycyk, & Hickson, 1995), the training protocol used in our study positively impacted the estrous cycle. Specifically, rats trained to step on a treadmill after HX (20 min of training at 13.5 to 21 cm/s without inclination) regained a normal estrous cycle length earlier (~4 days) than those that were not trained. Aerobic exercise regimens have been shown to reduce susceptibility to several stress-related responses in rodents, including diminishing HPA axis activation (Greenwood, Strong, Dorey, & Fleshner, 2007) and up-regulating brain neurotrophins (Adlard & Cotman, 2004; Gomez-Pinilla, Ying, Roy, Molteni, & Edgerton, 2002). During treadmill running, noradrenergic and dopaminergic activity levels are significantly increased in the hypothalamus (Kitaoka *et al.*, 2010). Neurotransmitters released from the hypothalamus eventually serve as a permissive factor in the release of GnRH into the anterior pituitary, which in turn retains adequate serum

concentrations of circulating reproductive hormones. Collectively, these exercise-associated mechanisms likely ameliorated the impact of HX in the trained animals.

In conclusion, the present results clearly demonstrate that surgical interventions can transiently impact the estrous cycle of female rats. Furthermore, a severe injury to the spinal cord, i.e., HX, has a larger impact on the rodent estrous cycle than less invasive surgeries, i.e., EMG electrode implantation of hindlimb muscles or dye injections into the spinal cord. The results also indicate that moderate aerobic exercise initiated early after a HX positively impacts the estrous cycle. These data provide unique insight into the effects of surgical interventions on acute reproductive or hormonal imbalances in female rats that may influence the outcome measures of a study.

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Figure 1.

(a) Microphotographs demonstrating the phases of a 4-5 day normal estrous cycle in a female rat (top panels 5X, bottom panels 10X magnification). Arrows in the bottom panels are directed towards specific cell types. A1, A2: Pro-estrus Phase (PE; nucleated epithelial cells in clusters); B1, B2: Estrus Phase (ES; cornified cuboidal cells); C1, C2: Metestrus Phase (ME; mixture of all three cell types in approximately equal amounts); D1, D2: Diestrus Phase (DE; predominantly leukocytes). (b) Graphical representation of estrous cycle phases in a hemisected (HX) rat throughout the entire study. Note the normal estrous cycles before HX (at B16, B10, B4), the prolonged cycles immediately after HX (P2, P8), and the normal cycles later after HX (P14, P20). Note that data from the ME phase has been grouped with the DE phase (see text for details). B, indicates days before HX; P, indicates days post-HX. (c) Average ($\pm$ SEM) length of an estrous cycle before (Pre-HX) and after (Post-HX) injury in rats that showed a prolonged cycle (>5 d) after HX ($N = 19$). Cycle length is significantly (*) delayed up to an average of 9 days after HX and returns to normal thereafter. (d) Histograms showing the average ($\pm$ SEM) estrous cycle length for trained vs. non-trained HX rats that showed longer estrous lengths (>5 d) after HX. *, significantly

longer than the trained group at $p < 0.05$. Note that data presented in Figure 1c (for Post-HX) represent the average of the data presented in Figure 1d.

Figure 2.

(a) Average ($\pm$ SEM) estrous cycle length before, 10 days after, and 21 days after HX, EMG electrode implantation, or dye injection surgery. Note that all tested rats are included in this analysis irrespective of estrous cycle length post-surgery. *, significantly longer than at B0-B10; †significantly shorter than at P0-P10; and ‡, significantly shorter estrous cycle length than HX at P0-P10 at $p < 0.05$. (b) Mean estrous cycle lengths before and after each surgery are shown for individual animals that were either trained (filled circles) or non-trained (open triangles) after the HX surgery.