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Research report

Estrogen reduces the severity of autonomic dysfunction in spinal cord-injured male mice

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Abstract

Autonomic dysreflexia is an autonomic behavioural condition that manifests after spinal cord injury (SCI) and is characterized by acute, episodic hypertension following afferent stimulation below the level of the injury. Common triggers of autonomic dysreflexia include colorectal distension (CRD), and various somatic stimuli. The development of autonomic dysreflexia is dependent, in part, upon the degree of intraspinal inflammation and the resultant spinal neuroplastic changes that occur following SCI. 17β-estradiol (E) has neuroprotective, anti-inflammatory and smooth muscle relaxant properties, and is therefore a candidate drug for the treatment and/or prevention of autonomic dysreflexia. Autonomic dysreflexia was assessed in adult male mice treated with E. We investigated whether E could be acting centrally by altering: (1) the size of the small diameter primary afferent arbor, (2) the degree of microglia/macrophage infiltration at the site of the injury, or (3) the amount of fibrous scarring present at the injury site. To determine whether E could be working through uncoupling protein-2 (UCP-2), a protein involved with inflammation and regulated by estrogen in some tissues, autonomic dysreflexia was assessed in E-treated adult male mice lacking UCP-2 (UCP-2 KO). 17β-estradiol was equipotent at reducing autonomic dysreflexia in both UCP-2 KO and WT mice following CRD but not tail pinch. We have shown that E reduces autonomic dysreflexic responses to visceral but not somatic stimulation in male mice independent of the size of the primary afferent arbour, the degree of chronic inflammation, and the presence of UCP-2.
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Keywords: α-CGRP; Estrogen; Spinal cord injury; UCP-2

1. Introduction

Traumatic spinal cord injury (SCI) occurs at an incidence ranging from approximately 10–60/1 million people annually [1]. Given that the majority of people afflicted with SCI are injured at a young age and that medical and rehabilitative therapies have improved survival time, the prevalence of SCI is large [1,52]. The general public is aware of the motor disturbances seen in people with SCI (i.e. paraplegia). Behavioural consequences of SCI not commonly thought of by non-spinal cord-injured people include impaired micturition [57], bowel function [14], sexual function [18], and autonomic control of the cardiovascular system [15,31]. Much of the present research evaluating therapies for SCI in rodents is aimed at improving sensorimotor behaviour [47,73]. However, a recent survey indicated that many spinal cord-injured people believe that improvement in bowel and bladder function, sexual function and cardiovascular control would dramatically improve their quality of life [3]. Consequently, there is a need to develop therapies for autonomic behavioural disturbances such as autonomic dysreflexia.

Autonomic dysreflexia is a potentially life-threatening condition occurring in up to 90% of people with severe SCIs at or above the level of the sixth thoracic spinal cord segment (T6) (as cited by Ref. [72]). Autonomic dysreflexia manifests as an acute, severe, uncompensated elevation in systemic arterial blood pressure in response to somatic and/or visceral stimulation below the level of the injury. A variety of hypotheses pertaining to the pathophysiology of autonomic dysreflexia have and/or are being examined [32,34,35,37,48,49,58]. Of particular interest, is the finding that intraspinal inflammation results in an increased pro-

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duction of nerve growth factor (NGF) that leads to an increase in the size of the dorsal horn primary afferent arbor which, in turn, is associated with increased severity of autonomic dysreflexia [9,12,34–36,58]. Reducing the severity of intraspinal inflammation, and presumably the production of NGF, results in the amelioration of autonomic dysreflexia [23].

17β-Estradiol, the most abundant form of estrogen in the body, has been shown to be neuroprotective and therapeutic in various models of central nervous system (CNS) disease where inflammation and immune-mediated processes predominate [6,27,30,38,42,43,55,60,63,65,66,74,76]. 17β-Estradiol exerts its neuroprotective effects, in part, by acting as an anti-inflammatory agent and an anti-oxidant [10,46,59,69,70]. The detailed mechanisms by which 17β-estradiol acts as an anti-inflammatory agent, however, are incompletely understood. There is evidence that 17β-estradiol might affect inflammatory processes by altering mitochondrial uncoupling protein-2 (UCP-2) expression in some tissues [11,56]. Uncoupling protein-2 is one of five members of the uncoupling protein family and, as its name implies, uncouples mitochondrial respiration. A consequence of uncoupling mitochondrial respiration is that oxygen-derived free radicals are reduced. Mortality rates in mice lacking UCP-2 following infection with the protozoal organism *Toxoplasma gondii* are reduced because of improved toxoplasmacidal activity of macrophages due to enhanced free radical production [5]. Further, mice over-expressing UCP-2 are resistant to brain trauma and stroke [44]. With respect to the spinal cord, UCP-2 has been located predominantly within primary afferents of the spinal cord [25]. It seems reasonable to predict that 17β-estradiol may exert, in part, some of its anti-inflammatory effects through UCP-2.

In addition to 17β-estradiol's anti-inflammatory/immunomodulatory effects, 17β-estradiol also has smooth muscle relaxant properties which may help to reduce the incidence of autonomic dysreflexia by improving poor gastrointestinal (GI) transit times and by increasing colonic compliance [17]. For example, some spinal cord-injured patients are susceptible to bowel obstruction, and subsequently autonomic dysreflexia, because of poor GI transit times and reduced colonic compliance [19,40,45].

Considering that (1) the development of autonomic dysreflexia is dependent, in part, upon the degree of intraspinal inflammation following SCI, (2) 17β-estradiol reduces inflammation, (3) 17β-estradiol has been shown to improve hind limb motor function following SCI [76], (4) 17β-estradiol has smooth muscle relaxing properties, and (5) there is evidence to suggest that slightly less women than men may be affected by autonomic dysreflexia early-on in the recovery period following SCI [13], we set out to determine whether 17β-estradiol would alter the development of autonomic dysreflexia in a mouse model of SCI. Further, we behaviourally investigated whether the potentially beneficial effects of 17β-estradiol were dependent upon UCP-2 by using a UCP-2 knock-out (UCP-2 KO) mouse. We hypothesized that the severity of autonomic dysreflexia would be less severe in animals treated with 17β-estradiol. Further, if 17β-estradiol was acting centrally (spinally), we predicted that animals would have reduced autonomic dysreflexic responses following both somatic and visceral stimulation and that the size of the CGRP-labeled primary afferent arbor, and the amount of microglia/macrophage infiltration would be less in animals treated with 17β-estradiol. Also, if 17β-estradiol had any beneficial effects, and if these effects were UCP-2 dependent, we predicted that amelioration of autonomic dysreflexia would be seen in 17β-estradiol-treated WT but not 17β-estradiol-treated UCP-2 KO animals.

2. Materials and methods

All procedures carried out in this study were approved by the University of Prince Edward Island Animal Care Committee and were conducted according to the guidelines outlined by the Canadian Council on Animal Care.

2.1. Experimental animals

Sixty-one, three- to four-month-old, B6; 129-UCP2^{tm1Lowl} (UCP-2 KO) and B6; 129 (WT) male mice were used for this study. Details regarding the development of these mice are described elsewhere [77]. B6; 129-UCP2^{tm1Lowl} mice have a targeted (knock-out) mutation of the UCP-2 gene between introns 2 and 7. All animals used were derived from C57BL/6J mice and a 129S4/SvJae ES cell line. Heterozygous animals were mated to generate WT, UCP-2 KO, and heterozygous offspring. Animals were bred and raised in the Animal Care Unit of the Atlantic Veterinary College (AVC). All animals were weaned and sexed at 1 month of age and were genotyped as previously described [77]. Mice were housed together, unless fighting occurred, with 12-h light:12-h dark photoperiod in the Animal Care Unit of the AVC. Animals were randomly assigned to a 17β-estradiol-treated ($n = 7$ WT; $n = 7$ UCP-2 KO) or placebo-treated ($n = 8$ WT; $n = 7$ UCP-2 KO) group for measuring autonomic dysreflexia, and CGRP and cd11b immunoreactivity.

Animals not used for autonomic dysreflexia measurements ($n = 32$) were randomly assigned to 17β-estradiol or placebo treatment groups for determining serum concentration of 17β-estradiol at: (1) 1 week following 17β-estradiol implantation ($n = 4$ WT; $n = 4$ UCP-2 KO); and (2) 2 weeks following either sham ($n = 6$ WT; $n = 6$ UCP-2 KO) or spinal cord transection ($n = 6$ WT; $n = 6$ UCP-2 KO) (note: 3 weeks following 17β-estradiol or placebo pellet implantation).

2.2. 17β-estradiol administration

Animals were anesthetized with 1.5% isofluorane (IsoFlo® Abbott Laboratories, Ltd., Saint-Laurent, Que., Canada) in oxygen administered nasally. One pellet containing 0.5 mg 17β-estradiol (Innovative Research of America, Sarasota, FL, USA) designed for constant rate release for 3 weeks or one placebo pellet (containing 0 mg 17β-estradiol) (Innovative Research of America, Sarasota, FL, USA) was subcutaneously implanted over the animal's right scapula. The pellet dose was chosen based on the manufacturer's recommendation so as to achieve physiological serum 17β-estradiol concentrations. Upon completion of the procedure, animals were allowed to recover and were returned to the Animal Care Unit.

2.3. 17β-estradiol concentration determination

Animals were deeply anesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Ont., Canada). A laparotomy was performed and blood was collected from the abdominal aorta. Blood was allowed to clot and serum was collected following centrifugation. Serum from each animal was stored at −80 °C. 17β-Estradiol serum concentration measurements were made using an ELISA (Calbiotech, Inc., Spring Valley, CA, USA). The lower and upper limits of detection for this test were 1 and 1000 pg/mL, respectively. When 17β-estradiol concentrations exceeded the upper limit of the ELISA, serum was diluted by 50%, the ELISA was repeated, and the concentration was determined by accounting for the dilution factor. The ELISA was performed according to the manufacturer's instructions.

2.4. Spinal cord injury

Animals were anesthetized, 1 week following pellet implantation, with 150 mg/kg ketamine HCl (Vetalar™, Vetrepharm Canada, Inc., Belleville, Ont., Canada) and 10 mg/kg xylazine (Rompun®, Bayer Inc., Toronto, Ont., Canada) administered intraperitoneally. An ocular lubricant (Tear-Gel, Novartis Pharmaceuticals (Canada) Inc., Mississauga, Ont.) was administered to prevent ocular drying. The skin at the surgical site was shaved then scrubbed three times with chlorhexidine containing soap and rinsed with isopropyl alcohol. Animals were positioned under a dissecting microscope and administered 100% oxygen nasally throughout the duration of the surgical procedure and until they recovered from anesthesia. The surgical site was draped and an incision was made over the dorsal spinous process of the second thoracic (T2) vertebra. Epaxial musculature was bluntly dissected and retracted from T2. Dorsal laminectomy was performed and the T2 spinal cord was transected with microscissors. To ensure complete transection had been performed, a 25 gauge needle, with a burred tip, was dragged along the ventral surface of the spinal canal and retracted thereby completely severing any intact ventral and ventrolateral spinal pathways. The overlying musculature was closed with 6-0 braided polyglycolic acid suture (Dexon "S", Sherwood Medical, St. Louis, MO, USA). The skin incision was closed with skin staples and the animals were allowed to recover. Post-operatively, animals were administered 0.05 mg/kg buprenorphine HCl (Temgesic, Schering–Plough Ltd., Hertfordshire, UK) subcutaneously and additional dosages were administered as required every 10–12 h. Animals were housed for 2 weeks and kept warm by placing their cages on a recirculating warm water blanket. All animals were administered 10 mg/kg enrofloxacin (Baytril™, Bayer Inc., Animal Health, Toronto, Ont., Canada) and 2.0 mL lactated ringers solution (LRS) (Baxter Corporation, Toronto, Ont., Canada) subcutaneously twice daily throughout the 14 day recovery period. All animals had their bladders partially expressed twice daily for the first 3–4 days following recovery. Because these animals were male, complete bladder expression was not possible.

2.5. Assessment of autonomic dysreflexia

Two weeks following spinal cord transection, animals were anesthetized with 1.5% isofluorane (IsoFlo®, Abbott Laboratories, Ltd., Saint-Laurent, Que., Canada) in oxygen administered nasally. The medial portion of the right hind limb was shaved and aseptically prepared as previously described. The proximal femoral artery was isolated and catheterized. Arterial catheters were constructed using PE10 polyethylene tubing (Fisher Scientific, Ottawa, Ont., Canada) stretched to an appropriate diameter to facilitate catheterization. The PE10 tubing was bonded to Tygon® tubing (0.02"ID, 0.06" OD; Cole Parmer Instrument Co., Vernon Hills, IL, USA). The catheter was sutured in place, flushed with heparinized saline and capped with a metal stopper. The catheter was subcutaneously burrowed and exteriorized over the animal's dorsum. Animals were allowed to recover for 4 h prior to evaluating autonomic dysreflexia.

Autonomic dysreflexia was assessed by a protocol similar to that previously described (Fig. 1) [28]. Blood pressure was measured using a pressure transducer (Gould P23 ID, Cleveland, OH, USA) connected to a physiograph (Gould model 2200S). Blood pressure and pulse pressure waves were recorded and displayed using Grass-Telefactor Gamma® (Warwick, RI, USA) analysis software. Animals were allowed to acclimate for at least 15 min after being connected to the pressure transducer before being tested. Acclimation was determined by a stable blood pressure recording. Blood pressure measurements were recorded following somatic (tail pinch) and visceral (colorectal distension (CRD)) stimulation as previously described [8,28]. Tail pinch was done using polypropylene forceps (Fisher Scientific Ltd.). Colorectal distension was performed by inserting a 4F Fogarty® catheter (Edwards Lifesciences™, Irvine, CA, USA) into the rectum 1 cm and injecting 0.25 mL air thereby inflating the balloon tip. Stimulus duration was 20 s and 1 min for tail pinch and CRD, respectively. The animals were allowed to recover for 15 and 30 min between two successive tail pinch and CRD tests, respectively, to permit normalization of their cardiovascular parameters. Measurements were made twice for each stimulus. Change in mean arterial blood pressure (MABP) was calculated by subtracting baseline (pre-stimulus) MABP from MABP during stimulation. The average of the two values for each test was used for statistical analysis. Responses to paired stimuli typically varied less than 15 mm Hg from each other for tail pinch and less than 10 mm Hg for CRD, which were similar to that previously reported [29]. Sham-operated animals were not included as part of the autonomic behavioural analysis as we were only interested in determining differences between 17β-estradiol-treated and placebo-treated animals. Further, autonomic dysreflexia is not observed in uninjured animals [28,33].

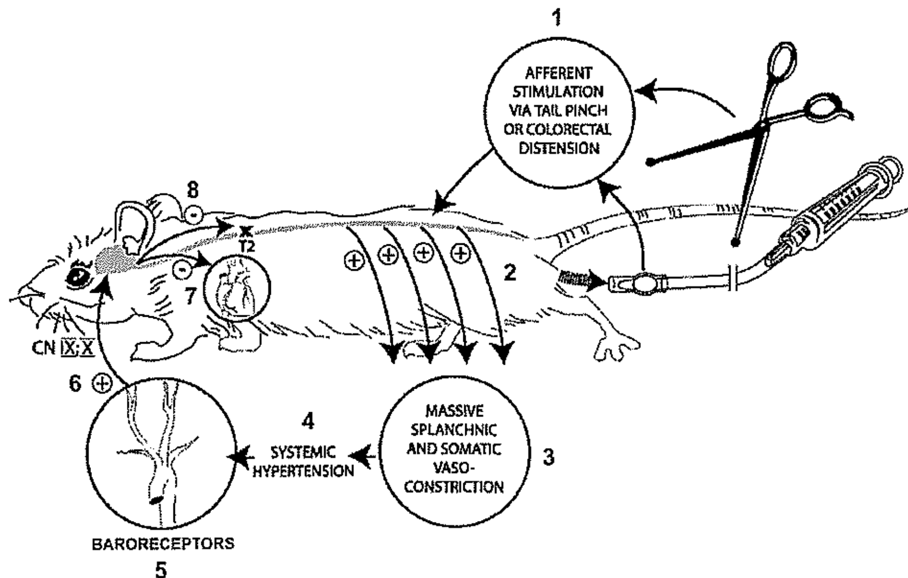

Fig. 1. Illustration depicting the neuroanatomical pathway for evoking autonomic dysreflexia: (1) afferent stimulation via either colorectal distension or tail pinch ascends to the spinal cord; (2) stimulation of sympathetic preganglionic neurons; (3) output of preganglionic sympathetic neurons results in vasoconstriction of splanchnic and vascular beds; (4) systemic hypertension occurs; (5) hypertension activates baroreceptors in the aortic arch and carotid bodies; (6) afferent input to the nucleus of the solitary tract occurs via cranial nerves 9 and 10, respectively; (7) output from the vagal nucleus results in bradycardia; (8) inhibitition of tonically active descending neurons from the rostral ventrolateral medulla via neurons in the caudal ventrolateral medulla attemps to decrease sympathetic preganglionic neuronal output without success because of the spinal cord injury.

2.6. Histology and immunohistochemistry

After autonomic dysreflexia testing, animals were deeply anesthetized with sodium pentobarbital and were transcardially perfused with 10 mL of heparinized LRS followed by 10 mL of 4% paraformaldehyde in 0.1M PBS. The spinal column was removed and post-fixed in 4% paraformaldehyde overnight. The next day the entire spinal cord was carefully removed from the spinal column and spinal cord segments C8-T4, T7-T8, T11-T12, and L2-3 were placed in 30% sucrose/0.1M PBS until they sank. Spinal cord segments were mounted in embedding media and cut at 12 μm on a cryostat and collected on cold poly-L-lysine coated slides. Spinal cord segments C8-T4 were sectioned serially along the sagittal plane. Sagittal sections were collected in an alternating fashion for separate staining with hematoxylin and eosin or immunohistochemically for cd11b. The remaining spinal cord segments were serially and transversely sectioned and were immunohistochemically stained for α-CGRP.

Hematoxylin and eosin stained sections were used to determine the completeness of the transection, to define a standardized area of interest to quantify expression of a cell surface antigen of microglia and macrophages, cd11b [64], and to confirm the extent of the fibrous tissue scar at the site of the injury.

For immunohistochemistry, slides were washed three times, for 10 min each, in 0.1M PBS pH 7.4. Slides were then washed in 0.1M PBS pH 7.4 containing 0.3% Triton-X followed by a 1 h incubation in either 10% goat or rabbit serum (Sigma–Aldrich Canada, Inc., Oakville, Ont., Canada) in 0.1M PBS pH 7.4 for α-CGRP or cd11b staining, respectively. Slides were then incubated overnight at 4 °C with 1:10,000 rabbit-anti-α-CGRP antibody (Peninsula Laboratories Inc., San Carlos, CA, USA) or 1:1000 rat-anti-mouse cd11b (M1/70) antibody (Serotec Ltd., Oxford, UK). The next day slides were washed three times for 10 min each with 0.1M PBS pH 7.4. Slides were washed once in 0.3% hydrogen peroxide in 0.1M PBS pH 7.4 for 20 min. Tissue was then incubated overnight at 4 °C with either 1:500 biotinylated-SP-goat-anti-rabbit (Cedarlane Laboratories Ltd., Hornby, Ont., Canada) or 1:500 mouse adsorbed, biotinylated rabbit-anti-rat secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) for α-CGRP and cd11b staining, respectively. The following day slides were washed three times for 10 min each with 0.1M PBS pH 7.4 and were then incubated with 1:1500 Extravidin® peroxidase (Sigma–Aldrich Canada, Inc., Oakville, Ont., Canada) for 4 h at room temperature. Sections were washed twice for 10 min each time with 0.1M PBS pH 7.4, then once for 10 min with 0.1M Na acetate buffer pH 6.0. Sections were then stained using a modified nickel-intensified diaminobenzidine (DAB) (Sigma–Aldrich) reaction using β-D-glucose (Sigma–Aldrich) and glucose oxidase (Sigma–Aldrich) to liberate H_2O_2 [61]. Sections were dehydrated in alcohol, then cleared in xylene and coverslipped.

2.7. Quantification of CGRP and cd11b expression and area of the fibrous scar

CGRP immunoreactivity was quantified to provide an estimate of the size of the small diameter primary afferent arbor as previously reported [28,29]. Because of the periodic penetration of the dorsal roots into the spinal cord, approximately every 15th section was photographed. On average, 12 sections were imaged for each spinal cord segment for each mouse. Sections were photographed at 20× magnification using a Zeiss Axioplan microscope and Axiovision software. Images were captured at 2600 × 2060 pixels. This was done throughout the extent of each of the segments for each mouse. One standard-sized oval occupying an area of 27,153 μm^2 was used to delimit the area to be quantified. The area of interest was consistent within each particular spinal cord level. These areas of interest were determined based upon the pattern of α-CGRP-afferent sprouting. Alpha-CGRP-immunoreactivity was determined by thresholding only the α-CGRP signal and ignoring any background staining and was done using the NIH ImageJ software (ImageJ Version 1.33, National Institutes of Health, USA). The total area (μm^2) of α-CGRP staining was determined by pre-calibrating the "set scale" function of the ImageJ software using a micrometer bar. Data from each segment for each mouse were imported into a spreadsheet and averaged to provide mean μm^2 α-CGRP-staining for each spinal cord segment for each mouse. All images were captured, thresholded, and quantified in a blinded fashion.

We examined the extent of the microglial/macrophage response at 2 weeks following SCI by assessing the degree of staining for the αm-integrin chain of CD11b/CD18 heterodimer found on microglia/macrophages (cd11b) at the injury site [64]. It has been shown that the degree of immunoreactivity of CD11b/CD18 is increased in activated microglia/macrophages for weeks following SCI. Quantification of cd11b immunoreactivity was carried out using a protocol similar to that previously described [2]. Briefly, hematoxylin and eosin stained sections were used to determine what sections were to be used for cd11b quantification. Three sections (separated 12 μm apart) with the central canal clearly delineated were selected. Paired sections stained for cd11b were used for cd11b quantification. Photomicrographs were taken using a Zeiss AxioPlan microscope and AxioVision software. A 2 mm scale bar was used to determine the area of the sagittal segment that was to be quantified. Briefly the scale bar was placed such that the center of the bar was at the center of the transection. The ends of the scale bar demarcated the cranial and caudal extent of the spinal cord segment to be measured, and the spinal cord was traced using the NIH ImageJ software. The total area occupied by the tracing was calculated in μm^2. Immunoreactivity for cd11b within this traced area was selected, thresholded, and measured in μm^2. Next, the perimeter of the fibrous scar (transection site) was outlined and its area was measured in μm^2. Data were collected for each of the three sections and kept separate for each mouse. The average size of the area evaluated and the average area of cd11b immunoreactivity within that area was determined. The amount of cd11b immunoreactivity was normalized for the total area of the section evaluated so comparisons could be made between groups of animals. The total fibrous tissue scar was also calculated for each animal by averaging the values obtained for each of the three sections for each mouse. All cd11b immunoreactivity and fibrous tissue scar quantifications were done in a blinded fashion.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. Change in blood pressure to either tail pinch or CRD was evaluated using two-way analysis of variance with genotype and drug treatment as the two factors. To determine the effects of spinal cord level and 17β-estradiol treatment on CGRP immunoreactivity, a two-way repeated measures analysis of variance was used with spinal cord level and drug treatment as the two factors; this was performed separately for each genotype. To determine the effects of genotype and 17β-estradiol on CGRP immunoreactivity, a two-way analysis of variance with genotype and drug treatment as the two factors for each of the spinal cord segments was used. To determine the effects of genotype and drug treatment on cd11b and fibrous scar formation two-way analysis of variance was used. All post hoc testing was performed using Student–Newman–Keuls test. All statistics were performed using SigmaStat® (SPSS Inc., Chicago, IL, USA) Version 2.03 software.

3. Results

3.1. Baseline17β-estradiol and blood pressure measurements

One week after implantation (time of SCI) mean serum 17β-estradiol measurements (± S.E.) were 1272 ± 163 and 1045 ± 119 pg/mL for WT and UCP-2 KO treated with 17β-estradiol, respectively. Three weeks after pellet implantation (2 weeks after SCI or sham surgery) mean serum 17β-estradiol measurements were still elevated in 17β-estradiol-treated animals compared to placebo-treated animals (Table 1). Baseline MABP for WT placebo, WT-17β-estradiol-treated, UCP-2 KO placebo, and UCP-2 KO 17β-estradiol-treated groups were 108 ± 3, 111 ± 6, 106 ± 5, 105 ± 3 mm Hg, respectively. There were no significant effects of 17β-estradiol treatment

Table 1
Serum concentrations (mean ± S.E.M.) of 17β-estradiol for spinal cord-transected and sham-operated WT and UCP-2 KO mice 3 weeks following pellet implantation

	WT sham (n = 3)/group	WT transected (n = 3)/group	UCP-2 KO sham (n = 3)/group	UCP-2 KO transected (n = 3)/group
Placebo	8 ± 0.8 pg/mL (20 ± 3 pmol/L)	3 ± 1 pg/mL (11 ± 3.7 pmol/L)	15 ± 8 pg/mL (56 ± 30 pmol/L)	4 ± 0.9 pg/mL (14.8 ± 3 pmol/L)
17β-estradiol	377 ± 73 pg/mL (1395 ± 270 pmol/L)	983 ± 123 pg/mL (3637 ± 455 pmol/L)	705 ± 40 pg/mL (2609 ± 148 pmol/L)	483 ± 145 pg/mL (1787 ± 537 pmol/L)

($F_{0.05(1)1,25}$ = 0.006, P = 0.94) or genotype ($F_{0.05(1)1,25}$ = 1.24, P = 0.28), nor were there any significant interactions between drug treatment and genotype ($F_{0.05(1)1,25}$ = 0.1, P = 0.78), on baseline MABP.

3.2. 17β-estradiol protects male mice from visceral, but not somatic-triggered autonomic dysreflexia

While WT and UCP-2 KO placebo-treated mice showed normal dysreflexic responses to CRD (increases in MABP of 31 ± 6 and 24 ± 5 mm Hg, respectively), WT and UCP-2 KO-17β-estradiol-treated mice had significantly reduced dysreflexic responses during CRD (MABP increases of only 9 ± 1 and 12 ± 1 mm Hg, respectively) (Figs. 2A and 3A). Dysreflexic responses to tail pinch stimulus, on the other hand, were not reduced by 17β-estradiol treatment. MABP changes in response to tail pinch for WT placebo, WT-17β-estradiol-treated, UCP-2 KO placebo, and UCP-2 KO 17β-estradiol-treated groups were 34 ± 6, 31 ± 3, 39 ± 5 mm Hg, and 32 ± 2 mm Hg, respectively (Figs. 2B and 3B).

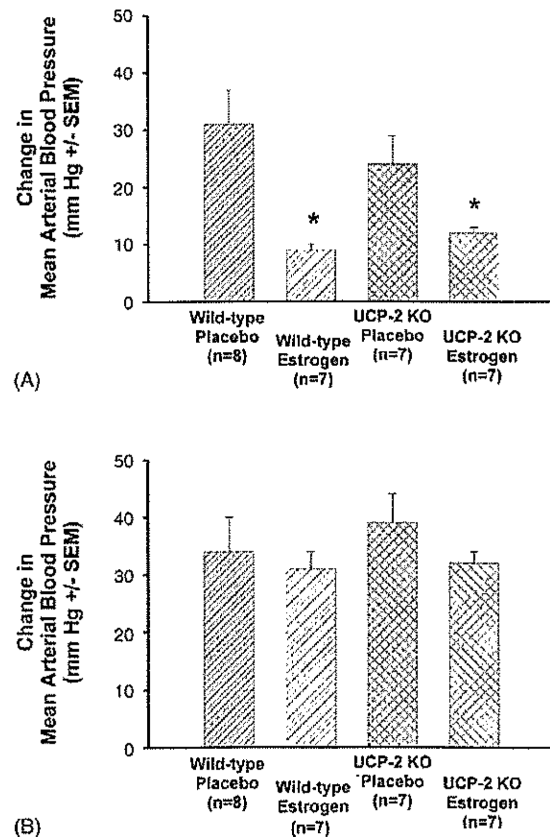

Fig. 2. Change in mean arterial blood pressure from baseline following colorectal distension for 60 s (A) and tail pinch for 20 s (B). 17β-estradiol administration dramatically reduced ($F_{0.05(1)1,25}$ = 16.2, P < 0.001) the pressor response following colorectal distension, regardless of genotype, compared to placebo-treated animals (A). Note no effect of genotype ($F_{0.05(1)1,25}$ = 0.5, P = 0.48) or 17β-estradiol administration ($F_{0.05(1)1,25}$ = 1.0, P = 0.32) following tail pinch (B) ('*' significantly different from placebo-treated groups; P < 0.001).

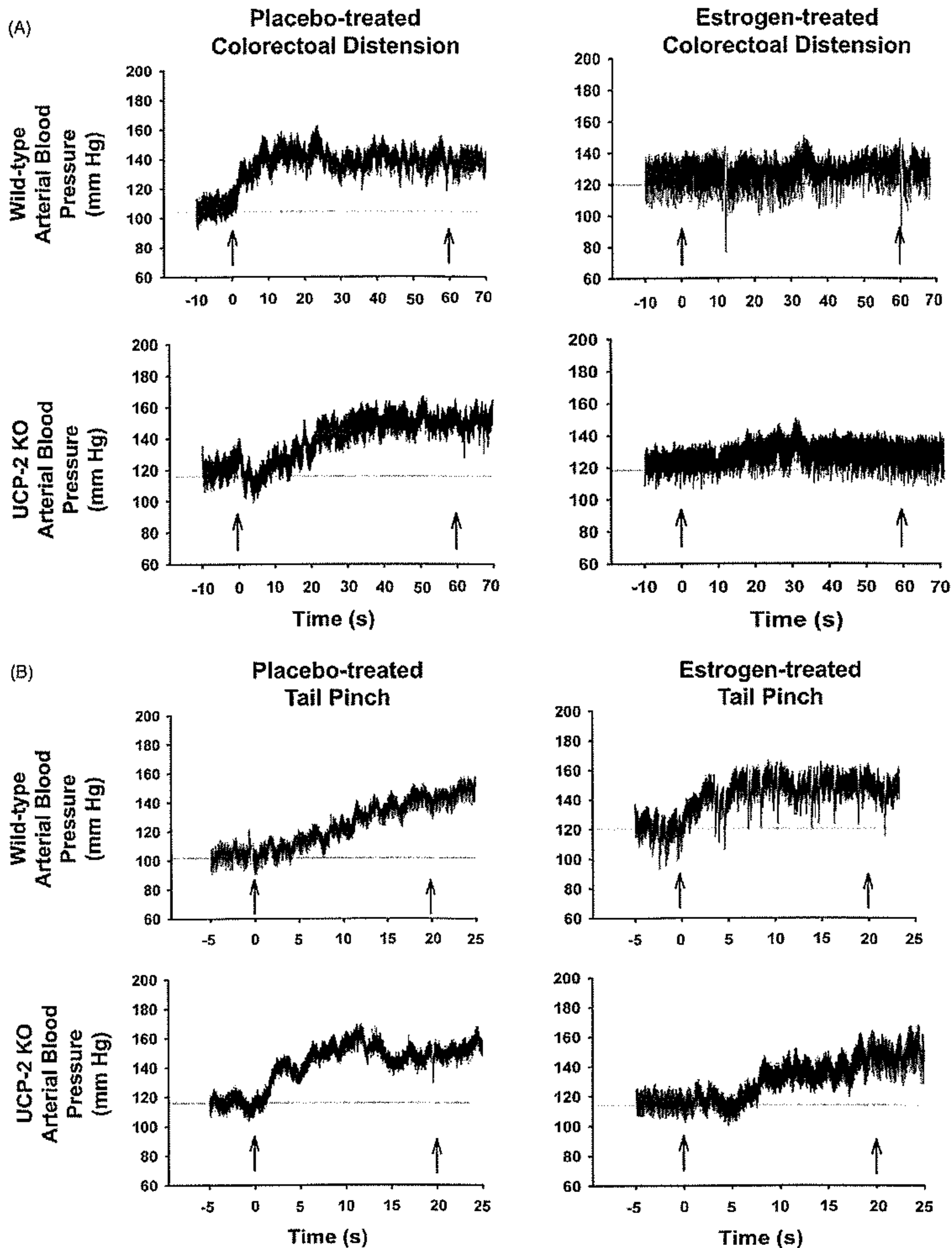

Fig. 3. Representative arterial blood pressure tracings from one animal from each group following colorectal distension (A) or tail pinch (B). Estrogen therapy lessens the dysreflexic response during colorectal distension compared to placebo-treated animals (A). Note that all four animals have similar pressure responses following tail pinch irrespective of genotype or estrogen administration (B). Periodic and rapid negative pulse-pressure spikes are indicative of cardiac arrhythmias that are not uncommonly observed during dysreflexic episodes [15] (arrows indicate beginning and end of stimulus).

3.3. Effects of 17β-estradiol on the size of the dorsal horn primary afferent arbor

The change in size of the primary afferent arbor is directly related to the severity of autonomic dysreflexia in rats and some strains of mice [8,28,29,58,71]. Furthermore, experimental treatments aimed at reducing the size of the afferent arbor are associated with a decreased severity of the autonomic dysreflexic responses [34]. Consequently, we determined whether 17β-estradiol may exert its beneficial effects by measuring the

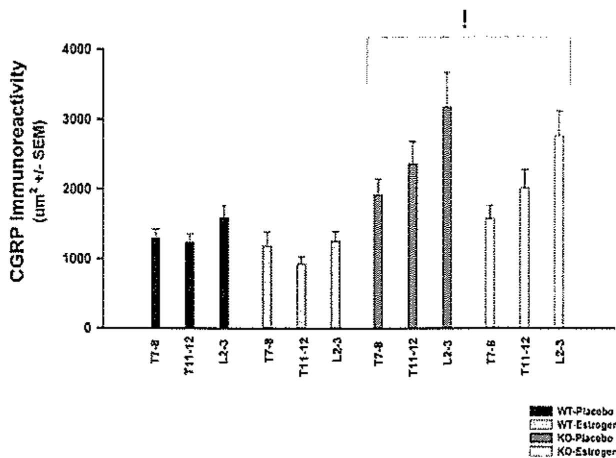

Fig. 4. Dorsal horn CGRP immunoreactivity for spinal cord segments T7-8, T11-12, L2-3. Dorsal horn CGRP immunoreactivity was greatest in the lower spinal cord segments. No effect of 17β-estradiol treatment was found for CGRP immunoreactivity. UCP-2 KO (KO) animals had significantly more dorsal horn CGRP immunoreactivity compared to wild-type (WT) animals ('!' respective spinal cord segments significantly different from corresponding WT group, $P \leq 0.01$) (WT placebo-treated $n=8$; WT estrogen-treated $n=7$; UCP2 KO placebo-treated $n=7$; UCP2 KO estrogen-treated $n=7$).

area of CGRP immunoreactivity to provide an estimate of the size of the primary afferent arbor as has been done previously [8,28,29,58,71]. In the present investigation, we were only interested in determining whether the administration of 17β-estradiol had an effect on the size of the primary afferent arbor compared to placebo-treated SCI animals, consequently the size of the primary afferent arbor was not estimated in sham-operated animals. We could not find any evidence of an effect of 17β-estradiol on the area of CGRP immunoreactivity in WT or UCP-2 KO spinal cord segments T7-8 ($F_{0.05(1)1,25} = 1.5$, $P = 0.23$), T11-12 ($F_{0.05(1)1,25} = 2.4$, $P = 0.14$), or L2-3 ($F_{0.05(1)1,25} = 1.4$, $P = 0.26$) (Figs. 4 and 5). Thus, the 17β-estradiol-induced reduction in autonomic dysreflexia observed could not be attributed to changes in the primary afferent arbor after SCI. Unexpectedly, UCP-2 KO animals had significantly more CGRP immunoreactivity compared to WT animals for comparable spinal cord segments T7-8 ($F_{0.05(1)1,25} = 7.4$, $P = 0.01$); T11-12 ($F_{0.05(1)1,25} = 26.8$, $P < 0.001$); and L2-3 ($F_{0.05(1)1,25} = 23$, $P < 0.001$) (Fig. 4).

3.4. *Effects of 17β-estradiol on the microglia/macrophage response and scar size*

To determine the severity of microglia/macrophage response and the size of the resulting intraspinal fibrous scar, following 17β-estradiol treatment, alternating serial sagittal sections throughout the lesion site were histochemically stained with hematoxylin and eosin or immunohistochemically stained for cd11b (a marker for microglia/macrophages). Hematoxylin and eosin staining, combined with intraoperative observations, revealed that spinal cord transection was complete in all of the animals examined for autonomic dysreflexia (Fig. 6C and

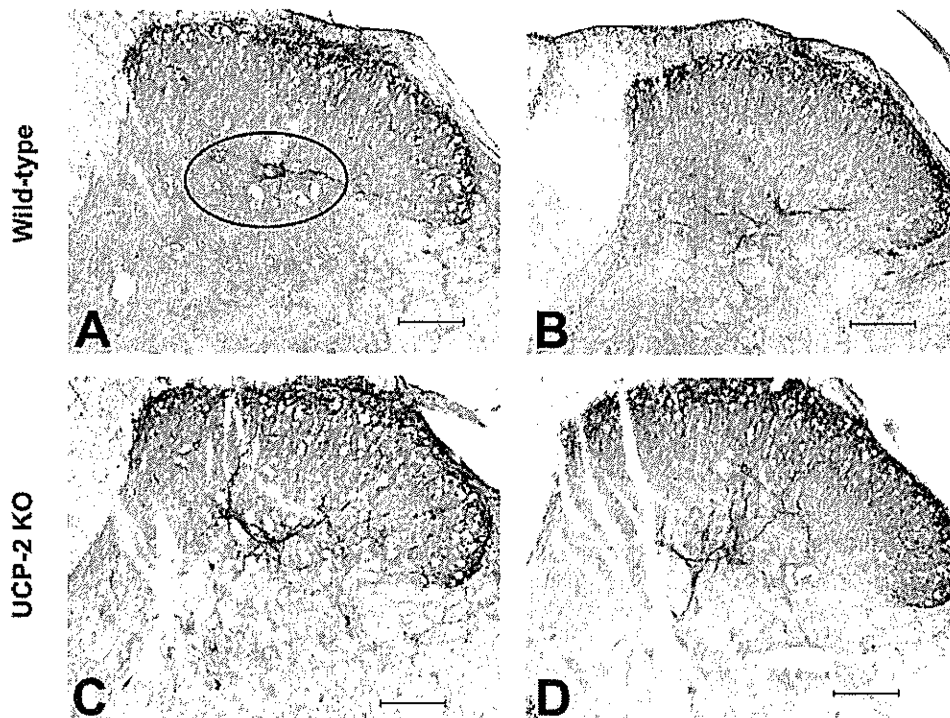

Fig. 5. Photomicrographs illustrating dorsal horn CGRP immunoreactivity for placebo (A and C) and estrogen (B and D) treated wild-type (A and B) and UCP-2 KO (C and D) treated animals. Note that UCP-2 KO (C and D) have larger CGRP arbours than wild-type (A and B) animals. 17β-estradiol did not have an effect on CGRP immunoreactivity regardless of genotype. The oval in A illustrates the region of interest. Scale bars = 100 μm.

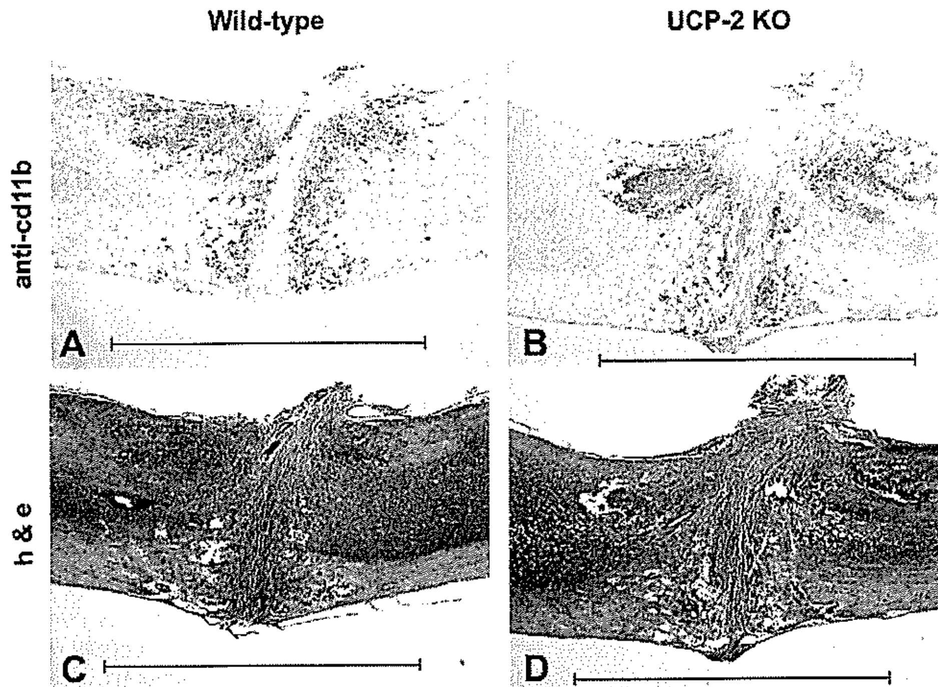

Fig. 6. Photomicrographs illustrating microglial/macrophage infiltration (using anti-cd11b immunohistochemistry) (A and B) and scar size (using hematoxylin and eosin staining) (C and D) for wild-type and UCP-2 KO animals. Note that there is no difference in the degree of cd11b immunoreactivity or the size of the scar regardless of genotype. Similarly, no differences were observed for animals treated with or without17β-estradiol (images not shown). Scale bars = 2 mm.

D). Additionally, cd11b staining combined with hematoxylin and eosin staining revealed that microglia/macrophages found along the margins of the injury were of a phagocytic phenotype as demonstrated by intracytoplasmic hemosiderin deposition and their foamy appearance. Perilesional microglia were amoeboid in appearance as opposed to the ramified (resting) appearance of microglia located distant from the site of injury. Also, cd11b immunoreactivity was much more intense for activated microglia/macrophages compared to ramified microglia in both white and grey matter. Our analysis indicates that 17β-estradiol treatment had no effect on the size of the fibrous scar or on the area of cd11b immunoreactivity that developed 2 weeks after SCI ($F_{0.05(1)1,25} = 0.7$, $P = 0.43$; Fig. 6).

4. Discussion

17β-Estradiol has been shown to be therapeutic in a variety of models of neurological disease including SCI [63,76]. Considering that 17β-estradiol is neuroprotective, anti-inflammatory, and possesses smooth muscle relaxing properties, and because the development of autonomic dysreflexia is dependent upon both central (spinal) and peripheral mechanisms, we hypothesized that 17β-estradiol would reduce the severity of autonomic dysreflexia in spinal cord-injured mice. Our results clearly show that 17β-estradiol can reduce the degree of autonomic dysreflexic behaviour that develops after SCI. Since increases in the small diameter primary afferent arbor [28,29,34,71] and inflammation-induced secondary damage [23] have been linked to the development of autonomic dysreflexia, we investigated whether 17β-estradiol might modulate plasticity or inflammation in the injured cord. Furthermore, as 17β-estradiol has been shown to signal through UCP-2, a mitochondrial protein involved in inflammation [5,26,51] and found within the spinal cord dorsal horn [25], we also evaluated whether 17β-estradiol could reduce dysreflexic behavioural responses in UCP-KO mice.

4.1. Could 17β-estradiol's anti-dysreflexic effect be due to a reduction in primary afferent plasticity after SCI?

Our study demonstrated that the size of the primary afferent arbor increases in a cranio-caudal direction, consistent with previously published work in mice and rats [28,29,35]. In rats, the size of the primary afferent arbour is related to the severity of autonomic dysreflexia [34,35]. For example, treatment of SCI rats with anti-NGF antibody reduced the size of the primary afferent arbor and the severity of autonomic dysreflexia compared to placebo-treated SCI animals [34]. Interestingly, UCP-2 KO animals have significantly more CGRP immunoreactivity in laminae III–V compared to WT animals yet the severity of autonomic dysreflexia was not different between WT and UCP-2 KO animals. We did not measure the size of the primary afferent arbor in non-spinal cord-injured mice. Consequently, we could not determine whether WT and UCP-2 KO mice exhibited sprouting of CGRP-labeled primary afferents following SCI. That is, non-SCI UCP-2 KO animals may simply have a larger primary afferent arbour compared to their WT counterparts. Our findings taken together with UCP-2 being specifically localized

to primary afferents [25] in the spinal cord indicate the need for further investigation of UCP-2 and primary afferent function and/or metabolism. It is possible that the development of autonomic dysreflexia in one or both of these two strains of mice does not depend upon the size of the CGRP-labeled primary afferent arbor. It has recently been shown, for example, that the severity of autonomic dysreflexia may not be dependent on the size of the primary afferent arbor in some strains of mice [28]. It is possible that neuroplastic changes in other classes of primary afferent neurons may account for the effect of 17β-estradiol we observed. Nevertheless, we have shown that the size of the primary afferent arbor does not differ between 17β-estradiol-treated and placebo-treated animals, regardless of their genotype. Taken together with the differences we observed for the beneficial effects of 17β-estradiol on ameliorating autonomic dysreflexia following CRD compared to tail pinch, the lack of difference in the size of the afferent arbor between 17β-estradiol-treated and non-treated mice lends further support that 17β-estradiol is likely ameliorating autonomic dysreflexia through non-central nervous system mechanisms in these strains of mice.

4.2. Could 17β-estradiol's anti-dysreflexic effect be due to a reduction in inflammation?

The development of autonomic dysreflexia is dependent, in part, upon the intraspinal inflammatory response that develops following severe traumatic SCI [23]. Consequently, we were interested in determining whether any potential benefit of 17β-estradiol on the development of autonomic dysreflexia could be related to the severity of the intraspinal inflammatory response following SCI. Further, we were interested in determining whether 17β-estradiol might have beneficial effects by acting through UCP-2. Considering that 17β-estradiol and UCP-2 have roles in modulating neuroinflammation [16,44,69], and that 17β-estradiol can influence UCP-2 expression [56], one potential way that 17β-estradiol could alter inflammation is by altering oxidative stress or oxidative capacities of microglia/macrophages by acting through UCP-2. The present study does not support 17β-estradiol's anti-inflammatory properties as a mechanism by which 17β-estradiol ameliorated autonomic dysreflexia following CRD. In fact, the serum concentrations of 17β-estradiol achieved in our mice did not substantially alter the intraspinal influx of microglia/macrophages 2 weeks following SCI. However, it should be noted that only one population of cells were examined (microglia/macrophages) at one time point (2 weeks following SCI). We chose to evaluate only microglia/macrophages because 17β-estradiol has been previously shown to have anti-inflammatory effects in vivo and in vitro by affecting this type of inflammatory cell [63,69,70]. Further, we chose to examine the intraspinal microglial/macrophage response at 2 weeks following spinal cord injury because it has been shown that this population of cells remains elevated for weeks following SCI [54,64]. In addition, it has previously been shown that anti-inflammatory therapy, with methylprednisolone acetate, reduces the influx of microglia/macrophages compared to placebo-treated animals for up to 2 months following spinal cord transection [54]. Although we did not demonstrate an effect of 17β-estradiol on the degree of microglia/macrophage infiltration, the serum concentrations of 17β-estradiol in our animals might be lower than that necessary to exert anti-inflammatory effects. This is difficult to determine, however, because serum concentrations of 17β-estradiol were not reported in three recent papers describing the beneficial effects (anti-apoptotic and anti-inflammatory) of 17β-estradiol for SCI rats [63,76]. Future studies evaluating 17β-estradiol and intraspinal inflammation following SCI need to examine multiple inflammatory cell types, similar to that previously done in studies evaluating the comparative aspects of inflammation in spinal cord-injured mice and rats [64]. In addition, planned future studies will evaluate the effect of different dosages and time-courses of adminstration of 17β-estradiol on intraspinal inflammation following SCI.

4.3. 17β-estradiol ameliorates autonomic dysreflexia following visceral, but not somatic, stimulation

Serum concentrations of 1000–1200 pg/mL of 17β-estradiol ameliorated autonomic dysreflexia following visceral but not somatic stimulation. These results suggest that 17β-estradiol likely reduced autonomic dysreflexic behavioural responses through non-central mechanisms in our mice. One plausible explanation accounting for the difference we observed between CRD and tail pinch is the smooth muscle relaxing effects that could be directly or indirectly induced by 17β-estradiol. 17β-Estradiol has recently been shown to cause rapid estrogen receptor-independent relaxation of mouse duodenum smooth muscle [17]. In addition, 17β-estradiol can indirectly cause smooth muscle relaxation by increasing circulating levels of CGRP [20,21,62,68]. Calcitonin gene-related peptide has been shown to cause smooth muscle relaxation in a variety of smooth muscle containing organs including the esophagus, colon, rectum, uterus, vasculature, and fallopian tubes [4,7,50,53,67,75]. Regardless of whether 17β-estradiol causes colorectal smooth muscle relaxation directly or indirectly, relaxation of colorectal smooth muscle would presumably result in enhanced compliance, and quiescence. For example, elevated levels of 17β-estradiol, and consequently CGRP, are thought to be important for uterine quiescence and relaxation required during pregnancy (for review see [75]). Presumably enhanced colorectal compliance and/or quiescence could result in fewer numbers of primary afferents being stimulated which could result in less stimulation of sympathetic preganglionic neurons. It has been shown that some spinal cord-injured humans have reduced colonic compliance and colonic motility thereby predisposing them to distension of the gut, and constipation [19,22,45] (for review see [39,40]). Considering that bowel dysfunction predisposes SCI patients to hemorrhoids and fecal impaction [24,41], both of which are important triggers for autonomic dysreflexia, and given what we know about estrogen's relaxing effects on the gut, future studies determining the mechanism by which estrogen reduces autonomic dysreflexia following visceral stimulation may further indicate the potential use of estrogen in SCI patients with low rectal compliance and thereby reduce the incidence or severity of dysreflexic episodes. Nevertheless, experiments to determine the mechanism by which 17β-estradiol

reduces the severity of autonomic dysreflexic behaviour are planned.

5. Conclusions

Altogether, the present work provides evidence that 17β-estradiol ameliorates the symptoms of autonomic dysreflexia following visceral but not somatic stimulation in male mice without affecting the size of the primary afferent arbor or affecting the degree of microglial/macrophage infiltration or scar size at 2 weeks following SCI. Further, we have shown that 17β-estradiol lessens the severity of autonomic dysreflexia following CRD independent of UCP-2. Taken together, these results suggest that 17β-estradiol exerts its therapeutic effect through as yet unidentified non-central (non-spinal) mechanisms. Further experiments designed to assess post-SCI administration of 17β-estradiol, combined with varying dosage regimes, are required prior to determining the clinical therapeutic utility of 17β-estradiol on ameliorating autonomic dysreflexia. In addition, it is necessary to determine the mechanism of action by which 17β-estradiol exerts this organ-specific effect.

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References

[1] Ackery A, Tator C, Krassioukov A. A global perspective on spinal cord injury epidemiology. J Neurotrauma 2004;21:1355–70.

[2] Akiyama C, Yuguchi T, Nishio M, et al. Src family kinase inhibitor PP1 reduces secondary damage after spinal cord compression in rats. J Neurotrauma 2004;21:923–31.

[3] Anderson KD. Targeting recovery: priorities of the spinal cord-injured population. J Neurotrauma 2004;21:1371–83.

[4] Anouar A, Schirar A, Germain G. Relaxant effect of the calcitonin gene-related peptide (CGRP) on the nonpregnant and pregnant rat uterus. Comparison with vascular tissue. Naunyn Schmiedebergs Arch Pharmacol 1998;357:446–53.

[5] Arsenijevic D, Onuma H, Pecqueur C, et al. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet 2000;26:435–9.

[6] Bebo Jr BF, Fyfe-Johnson A, Adlard K, Beam AG, Vandenbark AA, Offner H. Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. J Immunol 2001;166:2080–9.

[7] Boyer JC, Christen MO, Balmes JL, Bali JP. Calcitonin gene-related peptide-induced relaxation of isolated human colonic smooth muscle cells through different intracellular pathways. Biochem Pharmacol 1998;56:1097–104.

[8] Brown A, Jacob JE. Genetic approaches to autonomic dysreflexia. Prog Brain Res 2005;152:299–313.

[9] Brown A, Ricci MJ, Weaver LC. NGF message and protein distribution in the injured rat spinal cord. Exp Neurol 2004;188:115–27.

[10] Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP. Antiinflammatory effects of estrogen on microglial activation. Endocrinology 2000;141:3646–56.

[11] Bruun JM, Nielsen CB, Pedersen SB, Flyvbjerg A, Richelsen B. Estrogen reduces pro-inflammatory cytokines in rodent adipose tissue: studies in vivo and in vitro. Horm Metab Res 2003;35:142–6.

[12] Cameron AA, Smith GM, Randall DC, Brown DR, Rabchevsky AG. Genetic manipulation of intraspinal plasticity after spinal cord injury alters the severity of autonomic dysreflexia. J Neurosci 2006;26:2923–32.

[13] Chen D, Apple Jr DF, Hudson LM, Bode R. Medical complications during acute rehabilitation following spinal cord injury—current experience of the Model Systems. Arch Phys Med Rehabil 1999;80:1397–401.

[14] Chung EA, Emmanuel AV. Gastrointestinal symptoms related to autonomic dysfunction following spinal cord injury. Prog Brain Res 2006;152:317–33.

[15] Collins HL, Rodenbaugh DW, Dicarlo SE. Spinal cord injury alters cardiac electrophysiology and increases the susceptibility to ventricular arrhythmias. Prog Brain Res 2006;152:275–88.

[16] de BF, Arsenijevic D, Vallet P, et al. Resistance to cerebral ischemic injury in UCP2 knockout mice: evidence for a role of UCP2 as a regulator of mitochondrial glutathione levels. J Neurochem 2004;89:1283–92.

[17] Diaz M, Ramirez CM, Marin R, Marrero-Alonso J, Gomez T, Alonso R. Acute relaxation of mouse duodenum [correction of duodenun] by estrogens. Evidence for an estrogen receptor-independent modulation of muscle excitability. Eur J Pharmacol 2004;501:161–78.

[18] Elliott SL. Problems of sexual function after spinal cord injury. Prog Brain Res 2006;152:387–99.

[19] Fajardo NR, Pasiliao RV, Modeste-Duncan R, Creasey G, Bauman WA, Korsten MA. Decreased colonic motility in persons with chronic spinal cord injury. Am J Gastroenterol 2003;98:128–34.

[20] Gangula PR, Wimalawansa SJ, Yallampalli C. Pregnancy and sex steroid hormones enhance circulating calcitonin gene-related peptide concentrations in rats. Hum Reprod 2000;15:949–53.

[21] Gangula PR, Zhao H, Wimalawansa SJ, Supowit SC, DiPette DJ, Yallampalli C. Pregnancy and steroid hormones enhance the systemic and regional hemodynamic effects of calcitonin gene-related peptide in rats. Biol Reprod 2001;64:1776–83.

[22] Glick ME, Meshkinpour H, Haldeman S, Hoehler F, Downey N, Bradley WE. Colonic dysfunction in patients with thoracic spinal cord injury. Gastroenterology 1984;86:287–94.

[23] Gris D, Marsh DR, Oatway MA, et al. Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. J Neurosci 2004;24:4043–51.

[24] Han TR, Kim JH, Kwon BS. Chronic gastrointestinal problems and bowel dysfunction in patients with spinal cord injury. Spinal Cord 1998;36:485–90.

[25] Horvath B, Spies C, Warden CH, Diano S, Horvath TL. Uncoupling protein 2 in primary pain and temperature afferents of the spinal cord. Brain Res 2002;955:260–3.

[26] Horvath TL, Diano S, Miyamoto S, et al. Uncoupling proteins-2 and 3 influence obesity and inflammation in transgenic mice. Int J Obes Relat Metab Disord 2003;27:433–42.

[27] Ito A, Bebo Jr BF, Matejuk A, et al. Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. J Immunol 2001;167:542–52.

[28] Jacob JE, Gris P, Fehlings MG, Weaver LC, Brown A. Autonomic dysreflexia after spinal cord transection or compression in 129Sv, C57BL, and Wallerian degeneration slow mutant mice. Exp Neurol 2003;183:136–46.

[29] Jacob JE, Pniak A, Weaver LC, Brown A. Autonomic dysreflexia in a mouse model of spinal cord injury. Neuroscience 2001;108:687–93.

[30] Jansson L, Olsson T, Holmdahl R. Estrogen induces a potent suppression of experimental autoimmune encephalomyelitis and collagen-induced arthritis in mice. J Neuroimmunol 1994;53:203–7.

[31] Krassioukov A, Claydon VE. The clinical problems in cardiovascular control following spinal cord injury: an overview. Prog Brain Res 2006;152:223–9.
[32] Krassioukov AV, Johns DG, Schramm LP. Sensitivity of sympathetically correlated spinal interneurons, renal sympathetic nerve activity, and arterial pressure to somatic and visceral stimuli after chronic spinal injury. J Neurotrauma 2002;19:1521–9.
[33] Krassioukov AV, Weaver LC. Episodic hypertension due to autonomic dysreflexia in acute and chronic spinal cord-injured rats. Am J Physiol 1995;268:H2077–83.
[34] Krenz NR, Meakin SO, Krassioukov AV, Weaver LC. Neutralizing intraspinal nerve growth factor blocks autonomic dysreflexia caused by spinal cord injury. J Neurosci 1999;19:7405–14.
[35] Krenz NR, Weaver LC. Sprouting of primary afferent fibers after spinal cord transection in the rat. Neuroscience 1998;85:443–58.
[36] Krenz NR, Weaver LC. Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. J Neurochem 2000;74:730–9.
[37] Krum H, Louis WJ, Brown DJ, Howes LG. A study of the alpha-1 adrenoceptor blocker prazosin in the prophylactic management of autonomic dysreflexia in high spinal cord injury patients. Clin Auton Res 1992;2:83–8.
[38] Liao S, Chen W, Kuo J, Chen C. Association of serum estrogen level and ischemic neuroprotection in female rats. Neurosci Lett 2001;297:159–62.
[39] Lynch AC, Antony A, Dobbs BR, Frizelle FA. Bowel dysfunction following spinal cord injury. Spinal Cord 2001;39:193–203.
[40] Lynch AC, Frizelle FA. Colorectal motility and defecation after spinal cord injury in humans. Prog Brain Res 2005;152:335–43.
[41] Lynch AC, Wong C, Anthony A, Dobbs BR, Frizelle FA. Bowel dysfunction following spinal cord injury: a description of bowel function in a spinal cord-injured population and comparison with age and gender matched controls. Spinal Cord 2000;38:717–23.
[42] Matejuk A, Bakke AC, Hopke C, Dwyer J, Vandenbark AA, Offner H. Estrogen treatment induces a novel population of regulatory cells, which suppresses experimental autoimmune encephalomyelitis. J Neurosci Res 2004;77:119–26.
[43] Matejuk A, Dwyer J, Hopke C, Vandenbark AA, Offner H. 17Beta-estradiol treatment profoundly down-regulates gene expression in spinal cord tissue in mice protected from experimental autoimmune encephalomyelitis. Arch Immunol Ther Exp (Warsz) 2003;51:185–93.
[44] Mattiasson G, Shamloo M, Gido G, et al. Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. Nat Med 2003;9:1062–8.
[45] Meshkinpour H, Nowroozi F, Glick ME. Colonic compliance in patients with spinal cord injury. Arch Phys Med Rehabil 1983;64:111–2.
[46] Moosmann B, Behl C. The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. Proc Natl Acad Sci USA 1999;96:8867–72.
[47] Muir GD, Webb AA. Mini-review: assessment of behavioural recovery following spinal cord injury in rats. Eur J Neurosci 2000;12:3079–86.
[48] Naftchi NE, Lowman EW, Berard M, Sell GH, Reich T. Regulatory dysfunction of microvasculature and catecholamine metabolism in spinal cord injury. Adv Exp Med Biol 1973;37A:311–8.
[49] Naftchi NE, Lowman EW, Sell GH, Rusk HA. Peripheral circulation and catecholamine metabolism in paraplegia and quadriplegia. Arch Phys Med Rehabil 1972;53:357–61.
[50] Naghashpour M, Dahl G. Relaxation of myometrium by calcitonin gene-related peptide is independent of nitric oxide synthase activity in mouse uterus. Biol Reprod 2000;63:1421–7.
[51] Negre-Salvayre A, Hirtz C, Carrera G, et al. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB J 1997;11:809–15.
[52] O'connor PJ. Survival after spinal cord injury in Australia. Arch Phys Med Rehabil 2005;86:37–47.
[53] Ochiai T, Chijiiwa Y, Motomura Y, Yasuda O, Harada N, Nawata H. Direct inhibitory effect of adrenomedullin, calcitonin gene-related peptide, calcitonin, and amylin on cholecystokinin-induced contraction of guinea-pig isolated caecal circular smooth muscle cells. Peptides 2001;22:909–14.
[54] Oudega M, Vargas CG, Weber AB, Kleitman N, Bunge MB. Long-term effects of methylprednisolone following transection of adult rat spinal cord. Eur J Neurosci 1999;11:2453–64.
[55] Palaszynski KM, Liu H, Loo KK, Voskuhl RR. Estriol treatment ameliorates disease in males with experimental autoimmune encephalomyelitis: implications for multiple sclerosis. J Neuroimmunol 2004;149:84–9.
[56] Pedersen SB, Bruun JM, Kristensen K, Richelsen B. Regulation of UCP1, UCP2, and UCP3 mRNA expression in brown adipose tissue, white adipose tissue, and skeletal muscle in rats by estrogen. Biochem Biophys Res Commun 2001;288:191–7.
[57] Potter PJ. Disordered control of the urinary bladder after human spinal cord injury: what are the problems? Prog. Brain Res 2006;152:51–7.
[58] Rabchevsky AG. Segmental organization of spinal reflexes mediating autonomic dysreflexia after spinal cord injury. Prog Brain Res 2005;152:265–74.
[59] Ruiz-Larrea MB, Martin C, Martinez R, Navarro R, Lacort M, Miller NJ. Antioxidant activities of estrogens against aqueous and lipophilic radicals; differences between phenol and catechol estrogens. Chem Phys Lipids 2000;105:179–88.
[60] Saleh TM, Cribb AE, Connell BJ. Reduction in infarct size by local estrogen does not prevent autonomic dysfunction after stroke. Am J Physiol Regul Integr Comp Physiol 2001;281:R2088–95.
[61] Shu SY, Ju G, Fan LZ. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 1988;85:169–71.
[62] Spinetti A, Margutti A, Bertolini S, et al. Hormonal replacement therapy affects calcitonin gene-related peptide and atrial natriuretic peptide secretion in postmenopausal women. Eur J Endocrinol 1997;137:664–9.
[63] Sribnick EA, Wingrave JM, Matzelle DD, Wilford GG, Ray SK, Banik NL. Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats. J Neurosci Res 2005;82:283–93.
[64] Sroga JM, Jones TB, Kigerl KA, McGaughy VM, Popovich PG. Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. J Comp Neurol 2003;462:223–40.
[65] Toung TJ, Chen TY, Littleton-Kearney MT, Hurn PD, Murphy SJ. Effects of combined estrogen and progesterone on brain infarction in reproductively senescent female rats. J Cereb Blood Flow Metab 2004;24:1160–6.
[66] Toung TJ, Traystman RJ, Hurn PD. Estrogen-mediated neuroprotection after experimental stroke in male rats. Stroke 1998;29:1666–70.
[67] Uc A, Murray JA, Conklin JL. Effects of calcitonin gene-related peptide on opossum esophageal smooth muscle. Gastroenterology 1997;113:514–20.
[68] Valdemarsson S, Edvinsson L, Hedner P, Ekman R. Hormonal influence on calcitonin gene-related peptide in man: effects of sex difference and contraceptive pills. Scand J Clin Lab Invest 1990;50:385–8.
[69] Vegeto E, Belcredito S, Etteri S, et al. Estrogen receptor-alpha mediates the brain antiinflammatory activity of estradiol. Proc Natl Acad Sci USA 2003;100:9614–9.
[70] Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A. Regulation of the lipopolysaccharide signal transduction pathway by 17beta-estradiol in macrophage cells. J Steroid Biochem Mol Biol 2004;91:59–66.
[71] Weaver LC, Marsh DR, Gris D, Brown A, Dekaban GA. Autonomic dysreflexia after spinal cord injury: central mechanisms and strategies for prevention. Prog Brain Res 2005;152:245–63.
[72] Weaver LC, Marsh DR, Gris D, Meakin SO, Dekaban GA. Central mechanisms for autonomic dysreflexia after spinal cord injury. Prog Brain Res 2002;137:83–95.
[73] Webb AA, Muir GD. Sensorimotor behaviour following incomplete cervical spinal cord injury in the rat. Behav Brain Res 2005;165:147–59.
[74] Wen Y, Yang S, Liu R, et al. Estrogen attenuates nuclear factor-kappa B activation induced by transient cerebral ischemia. Brain Res 2004;1008:147–54.

[75] Yallampalli C, Chauhan M, Thota CS, Kondapaka S, Wimalawansa SJ. Calcitonin gene-related peptide in pregnancy and its emerging receptor heterogeneity. Trends Endocrinol Metab 2002;13:263–9.
[76] Yune TY, Kim SJ, Lee SM, et al. Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats. J Neurotrauma 2004;21:293–306.
[77] Zhang CY, Baffy G, Perret P, et al. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 2001;105:745–55.