Effects of Sex and Stress on Trigeminal Neuropathic Pain-Like Behavior in Rats

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Aims: To investigate the effects and interactions of sex and stress (provoked by chronic restraint [RS]) on pain-like behavior in a rat model of trigeminal neuropathic pain. **Methods:** The effects of sex and RS (carried out for 14 days as a model for stress) on somatosensory measures (reaction to pinprick, von Frey threshold) in a rat model of trigeminal neuropathic pain were examined. The study design was 2 × 4, with surgery (pain) and sham surgery (no pain) interacting with male restrained (RS) and unrestrained (nRS) rats and female RS and nRS rats. A total of 64 Sprague Dawley rats (32 males and 32 females) were used. Half of the animals in each sex group underwent RS, and the remaining half were left unstressed. Following the RS period, trigeminal neuropathic pain was induced by unilateral infraorbital nerve chronic constriction injury (IOCCI). Half of the animals in the RS group and half in the nRS group (both males and females) were exposed to IOCCI, and the remaining halves to sham surgery. Elevated plus maze (EPM) assessment and plasma interferon gamma (IFN-γ) levels were used to measure the effects of RS. Analysis of variance (ANOVA) was used to assess the effects of stress, sex, and their interactions on plasma IFN-γ levels, changes in body weight, EPM parameters, tactile allodynia, and mechanohyperalgesia. Pairwise comparisons were performed by using Tukey post hoc test corrected for multiple comparisons. **Results:** Both male and female RS rats showed significantly altered exploratory behavior (as measured by EPM) and had significantly lower plasma IFN-γ levels than nRS rats. Rats exposed to RS gained weight significantly slower than the nRS rats, irrespective of sex. Following RS but before surgery, RS rats showed significant bilateral reductions in von Frey thresholds and significantly increased pinprick response difference scores compared to nRS rats, irrespective of sex. From 17 days postsurgery, RS-IOCCI rats showed significantly reduced von Frey thresholds and significantly increased pinprick response difference scores compared to nRS-IOCCI rats, and the von Frey thresholds were significantly lower in females than in males. RS-sham females—but not RS-sham males—developed persistently reduced thresholds and increased pinprick response difference scores. **Conclusion:** RS produced an increased bilateral sensitivity to stimuli applied to the vibrissal pad following infraorbital nerve injury, irrespective of sex. This observed sensitivity subsequently persisted in RS-sham female rats but not in RS-sham male rats. Stress induced a significant but moderate increase in pain-like behavior in female rats compared to male rats. RS had no significant sex effects on IFN-γ levels, EPM parameters, or body weight gain. This suggests that stress may have a selective effect on pain-like behavior in both sexes, but the possible mechanisms are unclear. *J Oral Facial Pain Headache 2017;31:381–397. doi: 10.11607/ofph.1807*

Keywords: *elevated plus maze, interferon γ, nerve injury, neuropathic pain, trigeminal*

Differential effects of sex on experimental pain in animals have been extensively reported.[1–4] These effects are likely multifactorial and include hormonal, genetic, and environmental factors.[2,5–8] Largely, behavioral differences are related to female subject hyper-responsiveness, although in some rodent strains no differences have been shown.[9–11]

Studies of experimental pain in humans replicate these findings, showing greater pain sensitivity among females compared to males for most pain modalities.[12–15] However, sociocultural factors and gender

role stereotypes in humans likely play a prominent role. Additionally, stress is viewed as an important modulator of pain responsiveness,[16] and when anxiety levels and gender role stereotypes are controlled for, sex differences in experimental pain in humans may be eliminated.[17] Notwithstanding, a female predilection is apparent in many chronic pain syndromes, including migraine, fibromyalgia, and temporomandibular disorders (TMD).[18–20]

Although sex differences have been examined mostly with acute nociception experimental models, an increasing number of animal studies have used models of neuropathic pain.[21–27] Generally, the mouse has been the focus of attention for genetic pain studies; however, behavioral assessment in the mouse is more complicated than in the rat, particularly when the trigeminal nerve is examined. There are therefore a number of studies examining strain and sex effects in rat models of neuropathic pain.[6,7,9,11,27–36] However, the majority of these studies have focused on spinal nerve neuropathic pain models. There are only a few studies on the interacting effects of sex and stress on nociception, particularly in acute pain models.[37–39] Moreover, no reports on the interacting effects of sex and stress on trigeminal neuropathic pain have been reported, and there are only a few on spinal neuropathic pain.[21]

Both animal and human studies indicate a significant interaction between sex and chronic stress in establishing increased pain or risk for pain. Many of the clinical syndromes preferentially occurring in females are frequently comorbid with psychosocial issues, which are considered to play an important role in establishing chronic pain.[40,41] Similarly, in preclinical studies, chronic stress also has been shown to lead to visceral and peripheral hyperalgesia,[42–47] with sex-dependent differences.[39] Therefore, the aim of this study was to investigate the effects and interactions of sex and stress (provoked by chronic restraint [RS]) on pain-like behavior in a rat model of trigeminal neuropathic pain. It was hypothesized that:

- Chronic stress would induce an increase in pain-like behavior in naïve male and female rats, and that the effect of chronic stress on behavioral measures of pain would be more pronounced in female rats.
- Infraorbital nerve chronic constriction injury (IOCCI) would induce pain-like behavior in all rats, but pain-like behavior measures would be more severe in female rats than in male rats.
- In rats with IOCCI, stress would exacerbate pain-like behavior, and the pain-like behavior would be more pronounced in female rats.

The effects of sex and stress on behavioral measures of trigeminal neuropathic pain induced with a controlled nerve injury were examined by using somatosensory testing during 14 days of restraint (stress induction). The primary outcomes were behavioral measures of pain- and stress-like behavior and a biochemical stress marker.

Materials and Methods

All experimental procedures and protocols were approved by Rutgers University Institutional Animal Care and Use Committee (IACUC) protocol No. 14043.

Animals

Healthy adult male and female Sprague Dawley rats (225 to 250 g on arrival) were purchased from Charles River Laboratories. All animals were housed in rat polycarbonate and poly-sulfone, solid bottom, open shoebox cages with Beta Chip bedding. Same-sex animals were housed two per cage in a temperature- and humidity-controlled facility with ad libitum access to a standard rodent chow and reverse osmosis water. The facility was under veterinary supervision and maintained a 12-hour light/dark schedule (lights on at 7:00 am and off at 7:00 pm). Animals were allowed to acclimatize for 72 hours after arrival at the animal facility. All rats were habituated for 14 days prior to any behavioral testing.

Study Design

An overview of the experimental timeline is shown in Fig 1. RS was the model used to induce stress, and IOCCI was used to induce neuropathic pain.

In brief, baseline sensory testing and interferon gamma (IFN-γ) measurements were performed in all rats on experimental day 15 following the habituation period. Following baseline sensory assessment, half of the rats in each sex group (16 males and 16 females) were exposed to RS for a period of 14 days (experimental days 19 to 33) for 2 hours a day.[48,49] Following 7 and 14 days of restraint (experimental days 26 and 33, respectively), behavioral testing was performed, and IFN-γ levels were measured. These measures were all on naïve rats that had not yet undergone any type of surgical intervention.

On day 36, neuropathic pain was induced by IOCCI surgery. Half of the animals in the RS group (8 males and 8 females) underwent IOCCI, and the other half (8 males and 8 females) underwent sham surgery. Similarly, half of the animals that were not exposed to restraint (nRS group) underwent either IOCCI (8 males and 8 females) or sham (8 males and 8 females) surgery. This was a 2 × 4 design, with surgery (pain) and sham surgery (no pain) interacting with male RS and nRS rats and female RS and nRS rats. Following IOCCI, pain-like behavior was

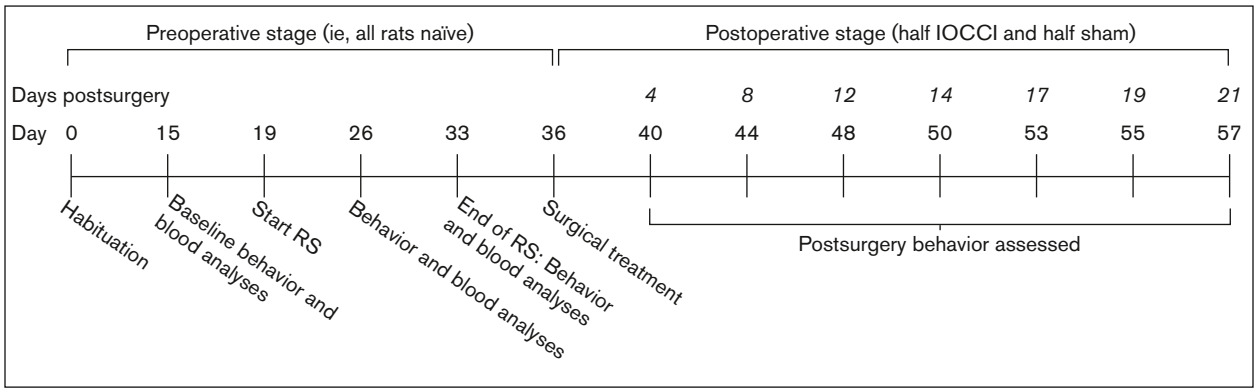

Fig 1 Experimental timeline. Following 14 days of habituation, baseline (BL) behavioral data and blood samples were obtained (experimental day 15). On day 19, chronic restraint (RS) was initiated in half of the rats and lasted for 14 days, ending on day 33. In the middle and at the end of the RS time period, further behavioral data and blood samples were obtained. Surgical treatment (infraorbital nerve chronic constriction injury [IOCCI] or sham surgery) was performed on day 36. Further behavioral data were collected on postoperative days 4, 8, 12, 14, 17, 19, and 21 (experimental days 40, 44, 48, 50, 53, 55, and 57, respectively).

assessed at postoperative days 4, 8, 12, 14, 17, 19, and 21 (experimental days 40, 44, 48, 50, 53, 55, and 57, respectively) (Fig 1).

Sample Size

Based on previously published data,[27] a power of 0.8, and an alpha of 0.05, the sample size was estimated at eight animals per group. Therefore, a total of 64 rats (32 males and 32 females) were used in the experiment, split into four experimental groups made up of RS and nRS rats interacting with sham and IOCCI rats. Sample size was calculated using JMP Pro software version 11.2.1 (SAS Institute).

Experimental Procedures

Animals in both sex groups were randomly assigned to the RS and nRS groups. Following the 14 days of RS, half of the animals in both the RS and nRS groups were randomly assigned to the IOCCI and sham groups.

Chronic Restraint

To induce chronic stress, animals were restrained in the laboratory for a period of 2 hours once daily for 14 consecutive days between 9:00 am and 12:00 pm.[48,49] Restraint was carried out by placing the male and female rats (in separate batches) into a Perspex restraining enclosure comprised of four compartments, each 6 cm wide × 7 cm high × 18 cm long.[49] The partitions between animals were opaque. During restraint, animals had no access to food or water.

Surgical Procedures

Anesthesia. For surgical procedures, rats were anesthetized with ketamine (50 mg/kg) and xylazine (7.5 mg/kg) solution administered intraperitoneally (ip).

IOCCI Surgery. Chronic constriction injury of the left infraorbital nerve was performed in male (n = 16) and female (n = 16) rats as previously described.[50] The infraorbital nerve is a major branch of the maxillary division of the trigeminal nerve in rats and is composed of sensory fibers.[50–52] IOCCI is a validated[51,52] model of trigeminal neuropathic pain, displaying spontaneous and evoked pain-like behavior.[53,54] In brief, rats were anesthetized, and an incision about 1 cm long was made along the gingivobuccal sulcus. The incision began just proximal to the first molar. Approximately 0.5 cm of the left side of the infraorbital nerve was freed from adherent tissue, and two chromic gut ligatures were loosely tied around it (2 mm apart). The incision was closed at three points with the use of 4-0 silk sutures.

Animals exposed to sham surgery (16 males and 16 females) were used as controls. In sham surgery, animals were anesthetized and an incision about 1 cm long was made along the gingivobuccal sulcus, as described above. The left infraorbital nerve was exposed but not manipulated. The incision was closed at three points with 4-0 silk sutures. To minimize variability, all surgical procedures were performed by a single investigator (J.K.).

Assessment of Stress

Elevated Plus Maze. The elevated plus maze (EPM) is a frequently used and validated behavioral test for measuring anxiety-like behavior in rodents.[55,56] The maze is a plus-shaped platform with two opposite open arms (50 × 10 cm) and two opposite closed arms (50 × 10 cm) extending out from a central platform (10 × 10 cm) (Stoelting). The closed arms have walls 40 cm high, and the maze is elevated 40 cm above the floor. During the test procedure, each rat was placed in the center of the maze facing one of the open arms, away from the investigator. Animals were allowed to explore the open or closed arms of the maze for 5 minutes. Each test was recorded with

a video camera placed above the maze, and the behavioral parameters were calculated by an automated activity-monitoring system (AnyMaze, Stoelting). Additionally, the investigator (O.K.) handling the animals for the EPM was blinded to the experimental treatment (RS/nRS).

Data automatically captured in the EPM included the amount of time spent by the rats in the closed arms or in the open arms as a percentage of the total time they were observed (stressed/anxious rodents tend to spend more time in the closed arms[57]), as well as the frequency of open-arm and closed-arm entries (entry into an arm was defined as all four paws entering the arm).

Both male and female naïve animals were tested in the EPM after 14 days of RS. This was the only phase of the experiment in which there were surgically naïve rats; ie, EMP testing occurred prior to the surgical interventions. Males and females were tested on separate days, and the maze was cleaned with 70% ethanol after each test.

IFN-γ Measurements. Stress has been shown to induce consistent suppression of IFN-γ production.[58–60] This cytokine has been shown to respond robustly to chronic stress,[58–60] and therefore plasma IFN-γ concentrations were used in this study to reflect stress levels in the animals. Blood was drawn from the lateral vein of the tail and collected in EDTA-Vacutainer tubes (0.25 mL). This method does not require surgery or anesthesia, is simple and minimally invasive, and, most importantly, allows for multiple blood draws from the same animal. Blood was drawn at baseline prior to any manipulations, then 24 hours after days 7 and 14 of RS. Blood draws were between 9:00 am and 11:30 am and were completed rapidly (< 2 minutes). Blood was immediately placed on ice and centrifuged at 1,500× for 15 minutes at 4°C. Plasma was stored at −80°C until processing.

IFN-γ levels were quantified by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (ELISA # ERIFNGALPHA, Rat IFN-γ ELISA Kit, Thermo Scientific Pierce). A sandwich-type ELISA was performed: 50 microliters (μL) of standards or samples were pipetted into a clear 96-well microtiter plate coated with an antibody specific for IFN-γ, followed by the addition of a biotinylated second antibody. The plate was then incubated for 30 minutes, during which time the IFN-γ antigen bound simultaneously to the immobilized antibody on one site (capture) and to the solution-phase biotinylated antibody on a second site. Following removal of excess second antibody, streptavidin-peroxidase enzyme—which binds to the biotinylated antibody—was added to complete the four-member sandwich. After 30 minutes, the incubation plate was washed again to remove all unbound enzyme, and a substrate solution was added to produce color. The intensity of the colored product is directly proportional to the concentration in the IFN-γ present in the original specimen and was detected in a microtiter plate reader EL×800 (BioTek, Winooski, VT) at 450 nm (wavelength). The concentration of IFN-γ in the sample was calculated by using Gen5 software available with the EL×800 plate reader (BioTek, Winooski, VT).

Assessment of Pain-Like Behavior

Testing was conducted from 9:00 am to 3:00 pm with a 30-minute pretest habituation period in a darkened room with the test area illuminated by a 40-W light bulb placed 1 m above the center of the test area. The animals were placed individually in perforated testing cages (4-cm wide × 21-cm long × 8-cm high). The animals were allowed to habituate to the testing cage and reaching movements for 10 minutes prior to testing. To minimize variability and bias, the same investigator (O.A.K.), who was blinded to the experimental groups but not to the sexes, performed all tests. Males and females were tested on separate days, and rats from the experimental groups were tested randomly. Assessment of female estrus cycle was not performed, as it has been shown to generate a negligible source of variation.[1]

Tactile allodynia was measured by applying bilateral mechanical stimulation within the infraorbital nerve territory in the area of the vibrissal pad. Each stimulus filament was applied three consecutive times (~1-second intervals) to the same area of the vibrissal pad at each examination. Stimuli were applied in ascending order of intensity with a graded series of 15 von Frey filaments (EXACTA Precision & Performance monofilaments, Stoelting). The filaments produce a bending force ranging from 0.008 to 15.0 g. The tactile withdrawal threshold was considered as the lowest force that produced a brisk head withdrawal, touching, or scratching the facial region on mechanical stimulation.[51,61–63] A decrease in the withdrawal threshold was considered to indicate tactile allodynia.

Mechanohyperalgesia was tested by using a modified pinprick test.[51,64] The rat's reaction to a stimulus applied with a blunted acupuncture needle (0.2-mm diameter) was assessed. The needle was applied once within the infraorbital nerve territory of the left and right vibrissal pads at each examination without puncturing the skin. The response to stimulation was scored as: 0 = no response; 1 = non-aversive response (such as a face swipe ipsilateral to the stimulated area without pulling the head away); 2 = mild aversive response (ie, the rat turned the head away or pulled it away briskly when the stimulus was applied, sometimes accompanied by a single face swipe ipsilateral to the stimulated area); 3 = strong aversive response (ie, escape/attack

Table 1 Summary Data for Baseline and Period of Chronic Restraint (RS)

	Female		Male	
	RS (n = 16)	nRS (n = 16)	RS (n = 16)	nRS (n = 16)
BL				
Body weight (g)	274.21 ± 6.0	265.71 ± 4.7	362.53 ± 4.0	346.97 ± 5.4
Log (IFN-γ % of BL)	1.63 ± 0.2	1.30 ± 0.2	1.52 ± 0.3	1.40 ± 0.2
Allodynia (% change from BL)	0 ± 0.01	0 ± 0.02	0 ± 0.02	0 ± 0.02
Hyperalgesia scores (difference from BL)	0 ± 0.1	0 ± 0.1	0 ± 0.1	0 ± 0.1
7 d RS				
Body weight	272.17 ± 4.6	275.13 ± 4.8	384.13 ± 5.3	380.08 ± 6.7
Log IFN-γ	1.23 ± 0.1	1.78 ± 0.1	1.35 ± 0.2	1.88 ± 0.1
Allodynia	–12.5 ± 2	–1.7 ± 3.8	–17.4 ± 1.1	–10.1 ± 2.2
Hyperalgesia scores	0.4 ± 0.2	–0.1 ± 0.2	1 ± 0.2	0.3 ± 0.2
14 d RS				
Body weight	273.61 ± 4.3	275.63 ± 4.9	402.96 ± 6.7	404.36 ± 7.5
Log IFN-γ	0.44 ± 0.2	1.71 ± 0.1	1 ± 0.2	1.68 ± 0.1
Allodynia	–17.5 ± 1.7	–9.2 ± 2.4	–19.3 ± 1.6	–9.5 ± 2.3
Hyperalgesia scores	0.9 ± 0.1	0 ± 0	0.7 ± 0.2	0.2 ± 0.1
Closed arm time (s)	175.60 ± 10.1	129.39 ± 9.1	208.09 ± 10.2	161.04 ± 10.2
Open arm time (s)	63.31 ± 9.2	95.04 ± 10.7	32.84 ± 6.3	59.60 ± 10.8
Closed arm entries (n)	16.89 ± 0.7	11.67 ± 0.8	18.72 ± 1.5	13.57 ± 0.9
Open arm entries (n)	10.76 ± 0.8	14.86 ± 1.1	6.91 ± 0.9	11.41 ± 1.1

Ipsilateral side data reported. Raw data reported as mean ± standard error of the mean (SEM). BL = baseline.

response, the animal attacks the stimulus object, making biting and grabbing movements); 4 = prolonged aversive behavior (ie, strong aversive behavior as described in the score of 3 accompanied by uninterrupted facial grooming of at least three face-wash strokes ipsilateral to the stimulated area).[51,61–64]

Data Analyses

Statistical analyses were performed by using JMP software version 11.2.1 (SAS) with two-tailed alpha set at ≤ .05 for all analyses. Data were evaluated for normal distribution prior to analyses (goodness of fit test). Results are reported as mean ± standard error of the mean (SEM) unless otherwise specified. Analysis of variance (ANOVA) was used to assess the effects of stress, sex, and their interactions at specific time points of interest on log-plasma IFN-γ levels, changes in body weight, percent time spent in the closed and open arms of the EPM, number of entries to the closed and open arms, tactile allodynia, and mechanohyperalgesia. When suitable, post hoc comparisons were performed by using Tukey post hoc test with correction for multiple comparisons.

Results

Summary data for each experimental group are presented in Table 1.

IFN-γ Measurements

To correct for baseline differences in IFN-γ levels, the data were analyzed as the IFN-γ level of the individual subject relative to the group baseline mean. Additionally, since plasma IFN-γ levels were not normally distributed, a logarithmic transformation was used to normalize the data. Day 14 of RS was the critical endpoint for stress; therefore, IFN-γ levels for days 7 and 14 are reported, but the statistical analysis for day 14 only is presented.

Following 14 days of RS, there was a significant effect of stress—but not sex—on the plasma IFN-γ levels measured in male and female rats (stress F = 27.6, $P < .0001$; sex F = 2.0, $P = .17$). Stressed animals showed significantly lower plasma IFN-γ levels than nonstressed animals in both sexes (males $P = .01$; females $P = .0002$) (Fig 2, Table 1).

Changes in Body Weight

Figure 3 shows the percent increase from baseline in body weight of RS and nRS rats during the 14 days of RS prior to instigating neuropathic pain. Significant differences were observed between the experimental groups after 7 (stress F = 70.9, $P < .0001$; sex F = 107.12, $P < .0001$; stress*sex interaction F = 5.6, $P = .02$) and 14 (stress F = 44.1, $P < .0001$; sex F = 205.8, $P < .0001$; stress*sex interaction F = 10.3, $P = .002$) days of RS. Specifically, after 7 days, percent weight gain in RS males (4.48% ± 0.44%) and RS females (0.7% ± 0.46%) was significantly lower than in nRS males (9.6% ± 0.53%, Tukey $P < .0001$) and nRS females (3.578% ± 0.45%, Tukey $P < .02$), respectively. After 14 days, percent weight gain in RS males (9.4% ± 0.8%) and RS females (1.27% ± 0.61%) was significantly lower than in nRS males (16.6% ± 0.85%, Tukey $P < .0001$) and nRS females (3.77% ± 0.61%, Tukey $P < .0001$), respectively.

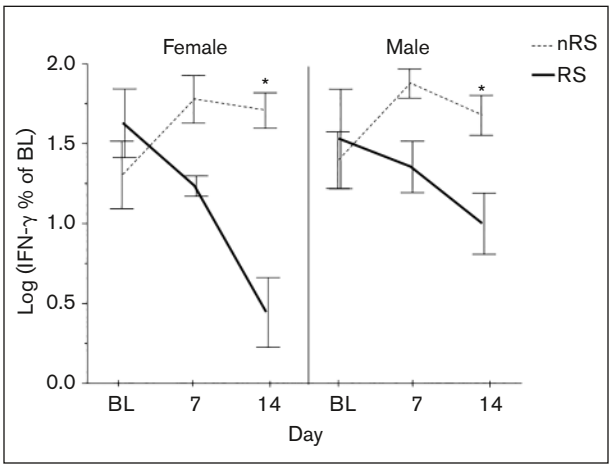

Fig 2 Influence of chronic restraint (RS) on interferon-γ (INF-γ) plasma levels. RS significantly decreased INF-γ plasma levels in both male and female rats. To correct for baseline (BL) differences in IFN-γ levels, the data were analyzed as the individual level relative to the baseline mean, which was made equal to 100%. Plasma IFN-γ levels were not normally distributed; therefore, a logarithmic transformation was used to normalize the data. Each value therefore represents the mean log (IFN-γ % of BL) ± standard error of the mean (SEM). **P* < .05.

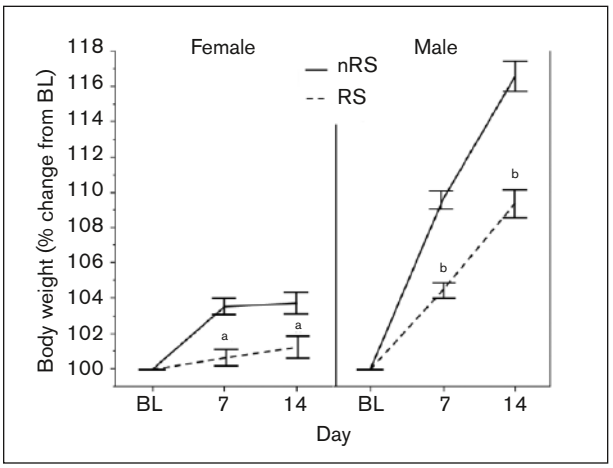

Fig 3 Body weight over the chronic restraint (RS) time period. Mean ± standard error of the mean (SEM) baseline body weight at the beginning of the experiments was 354.52 ± 3.5 g for males and 269.96 ± 3.8 g for females. After 14 days of RS, percent body weight gain in RS male (9.4% ± 0.8%) and female (1.27% ± 0.61%) rats was significantly lower than in nonrestrained (nRS) males (16.6% ± 0.85%) and females (*P* < .0001), with a significant sex*stress interaction. [a]*P* < .05 for RS females compared to nRS females. [b]*P* < .05 for RS males compared to nRS males.

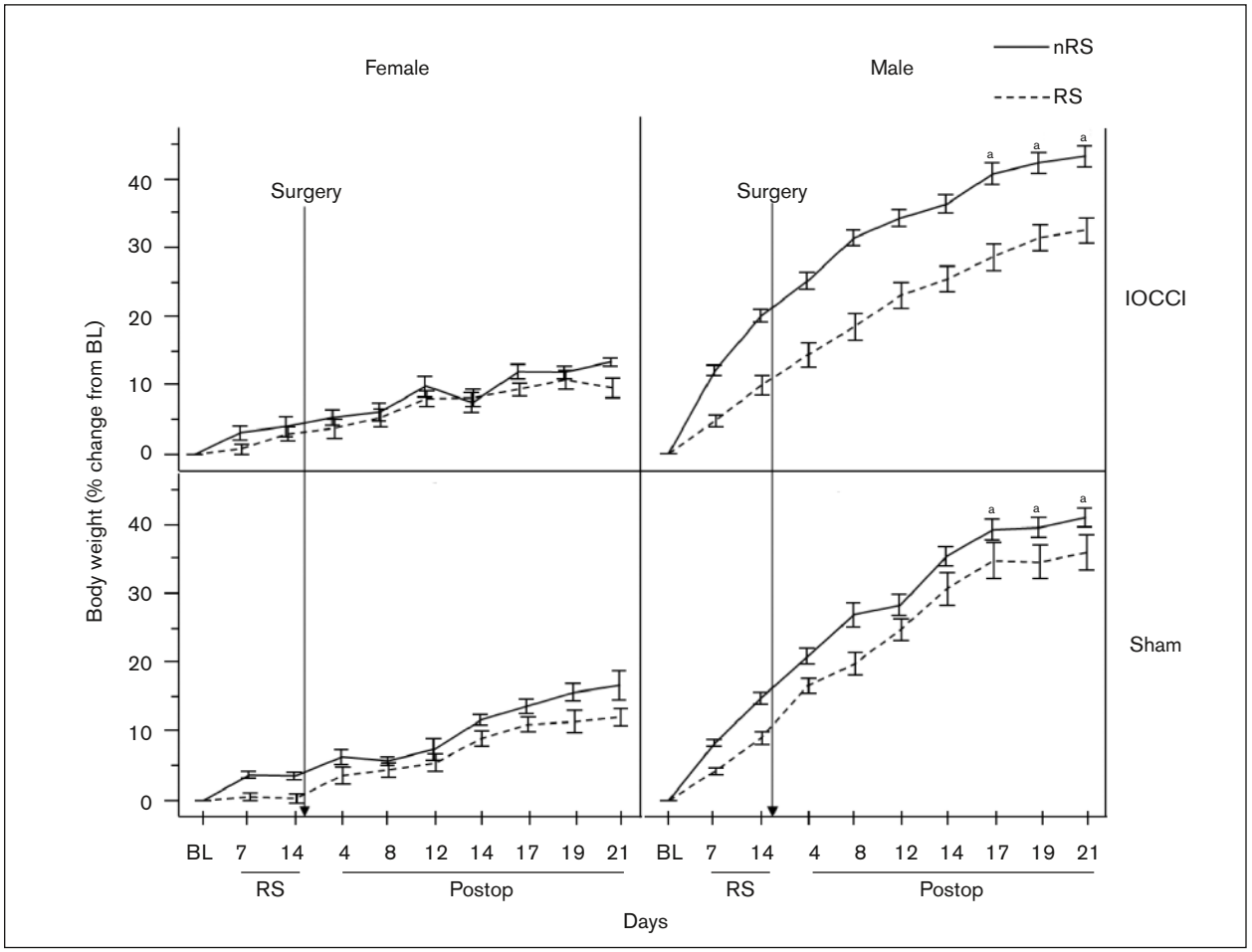

Fig 4 Changes in mean body weight over entire experimental period. Significant differences were observed between the experimental groups for stress and sex (but not surgical intervention [pain]) at days 17, 19, and 21 postsurgery. A significant stress*sex interaction was observed at days 17 and 19, but not 21. **P* < .05 for RS vs nRS males.

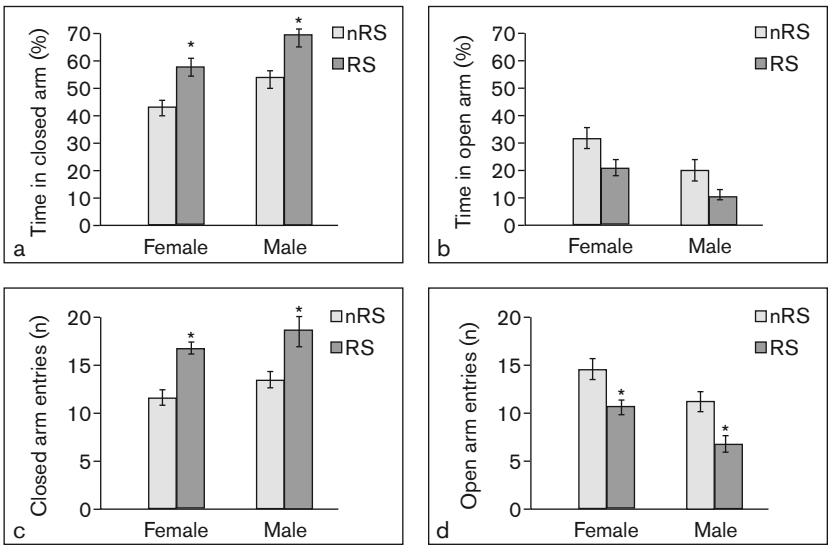

Fig 5 Influence of chronic restraint (RS) on elevated plus maze (EPM) parameters. In both female and male rats, chronic stress induced by RS **(a)** significantly increased percent time spent in the closed arms, **(b)** decreased percent time spent in the open arms, **(c)** significantly increased number of closed arm entries, and **(d)** significantly decreased number of open arm entries in both female and male rats. *$P < .05$. Data are reported as mean ± SEM.

Figure 4 shows changes in weight throughout the experiment, including the stages after neuropathic pain was induced. Significant differences were observed between RS and nRS rats at postoperative days 17 (stress F = 22.7, $P < .0001$; pain F = 3.3, $P = .07$; sex F = 449.2, $P < .0001$; stress*sex interaction F = 6.1, $P = .02$), 19 (stress F = 21.1, $P < .0001$; pain F = 1.3, $P = .3$; sex F = 449.2, $P < .0001$; stress*sex interaction F = 2.1, $P = .03$), and 21 (stress F = 24, $P < .0001$; pain F = 2.2, $P = .15$; sex F = 413.4, $P < .0001$).

Stress-Like Behavior

Elevated Plus Maze (EPM). Following 14 days of RS, there was a significant difference in the percentage of time spent in the closed arms of the EPM among the different experimental groups (stress F = 22, $P < .0001$; sex F = 10.4, $P = .002$; stress*sex interaction F = .002, $P = 1$). RS males (69.4% ± 3.4%) and RS females (58.5% ± 3.4%) spent significantly more time in the closed arms than nRS males (53.7% ± 3.4%, Tukey $P = .009$) and nRS females (43.1% ± 3.1%, Tukey $P = .006$), respectively. Taken together, this implies that the effect of restraint on the percentage of time spent in closed arms was similar in both sexes (RS male vs RS female Tukey $P = .7$). No significant differences were observed in percent time spent in the open arms between the RS and nRS animals in males or females ($P > .05$), and no significant differences were noted between RS males and RS females in percent time spent in closed or open arms (Tukey $P > .05$) (Figs 4a and 4b).

RS males and females made significantly more entries into the closed arms (18.7 ± 1.5 and 16.9 ± 0.7, respectively) than nRS males (13.6 ± 0.9, Tukey $P = .003$) and females (11.7 ± .8, Tukey $P = .04$), respectively. No significant differences were noted between RS males and females in the number of entries into the closed arms (Tukey $P > .05$). Both RS males and females made significantly fewer entries into the open arms (7 ± 1 and 10.8 ± 0.8, respectively) than the nRS males (11.4 ± 1.1, Tukey $P = .01$) and females (14.9 ± 1.1, Tukey $P = .02$), respectively. RS males made significantly fewer entries into the open arms than RS females (Tukey $P = .04$) (Fig 5).

Pain-Like Behavior

Analysis of behavioral measurements (tactile allodynia and mechanohyperalgesia) according to the subsequent experimental groups (sex, stress, surgery) revealed significant differences (tactile allodynia F = 5.01, df = 7, $P < .0001$; mechanohyperalgesia F = 4.72, df = 7, $P = .0001$) at baseline (Table 1). These were attributable to the random allocation of rats to the surgical and stress groups in both sexes. Therefore, to correct for this effect, the data were analyzed as percent change from baseline for tactile allodynia and difference scores from baseline for mechanohyperalgesia, which were calculated by subtracting the score assigned to the pinprick stimulus response at baseline from the pinprick score at the time point of interest (Table 2).

In the early period following IOCCI or sham surgery (postoperative days 4 to 14), animals that underwent IOCCI showed an ipsilateral increase in thresholds (hypoesthesia) and decrease in difference scores (hypoalgesia), most likely due to surgical trauma. This effect has been observed in previous experiments. From postoperative day 17, there was an increase in ipsilateral sensitivity to pinprick and von Frey filaments, which became robust on days 19 and 21. The contralateral side demonstrated parallel but milder somatosensory changes, and there was no period of hypoesthesia preceding the mild pain-like behavior.

Table 2 Summary Data for Each Experimental Group Following Surgical Treatment

	Female			
	RS		nRS	
	IOCCI (n = 8)	Sham (n = 8)	IOCCI (n = 8)	Sham (n = 8)
4 d postop				
Body weight (g)	271.38 ± 4.5	289.69 ± 6.0	264.5 ± 3.2	290.25 ± 6.4
Allodynia (% change from BL)	30.7 ± 7.2	−18.8 ± 2	32.5 ± 8.7	−12.1 ± 3.8
Hyperalgesia scores (difference from BL)	−0.75 ± 0.16	0.63 ± 0.2	−0.63 ± 0.46	−0.31 ± 0.2
8 d postop				
Body weight	275.5 ± 4.6	292.13 ± 6.2	266.5 ± 3.5	288.4 ± 6.8
Allodynia	18.0 ± 5.5	−20.3 ± 2.1	20.3 ± 8.4	−17.8 ± 4.0
Hyperalgesia scores	−0.25 ± 0.25	0.56 ± 0.26	−0.38 ± 0.26	0 ± 0
12 d postop				
Body weight	282.63 ± 3.8	294.81 ± 6.3	275.88 ± 3.8	293.13 ± 6.6
Allodynia	−14.5 ± 8.2	−19.5 ± 2.2	2.2 ± 7.8	−15.7 ± 3.0
Hyperalgesia scores	0.5 ± 0.19	0.63 ± 0.15	−0.13 ± 0.35	−0.13 ± 0.15
14 d postop				
Body weight	283 ± 4.6	279.75 ± 2.8	270 ± 4	282 ± 3.3
Allodynia	−32.8 ± 5.0	−26.7 ± 0	−23.0 ± 7.9	−12.5 ± 2.4
Hyperalgesia scores	1 ± 0.27	1 ± 0	0.63 ± 0.38	0.38 ± 0.18
17 d postop				
Body weight	286.2 ± 3.6	285.0 ± 2.6	281.7 ± 2.8	287.0 ± 3.7
Allodynia	−44.7 ± 1.4	−26.7 ± 0	−20.5 ± 6.5	−11.1 ± 2.1
Hyperalgesia scores	2 ± 0.27	1.5 ± 0.19	0.25 ± 0.25	0.25 ± 0.25
19 d postop				
Body weight	289.9 ± 5.1	286.0 ± 1.9	281.1 ± 3.4	292.0 ± 3.4
Allodynia	−44.7 ± 1.4	−26.7 ± 0	−41.7 ± 0	−13.8 ± 0
Hyperalgesia scores	2 ± 0	1.5 ± 0.19	1.5 ± 0.19	0.38 ± 0.18
21 d postop				
Body weight	286.9 ± 5.4	287.7 ± 2.9	286.5 ± 2.9	299.0 ± 9.3
Allodynia	−45.2 ± 1.3	−26.7 ± 0	−41.7 ± 0	−15 ± 0.6
Hyperalgesia scores	2 ± 0	1.13 ± 0.23	1.88 ± 0.23	0.38 ± 0.18

Ipsilateral side data are reported as mean ± standard error of the mean (SEM). BL = baseline; IOCCI = infraorbital chronic constriction injury; RS = chronic restraint; nRS = unrestrained.

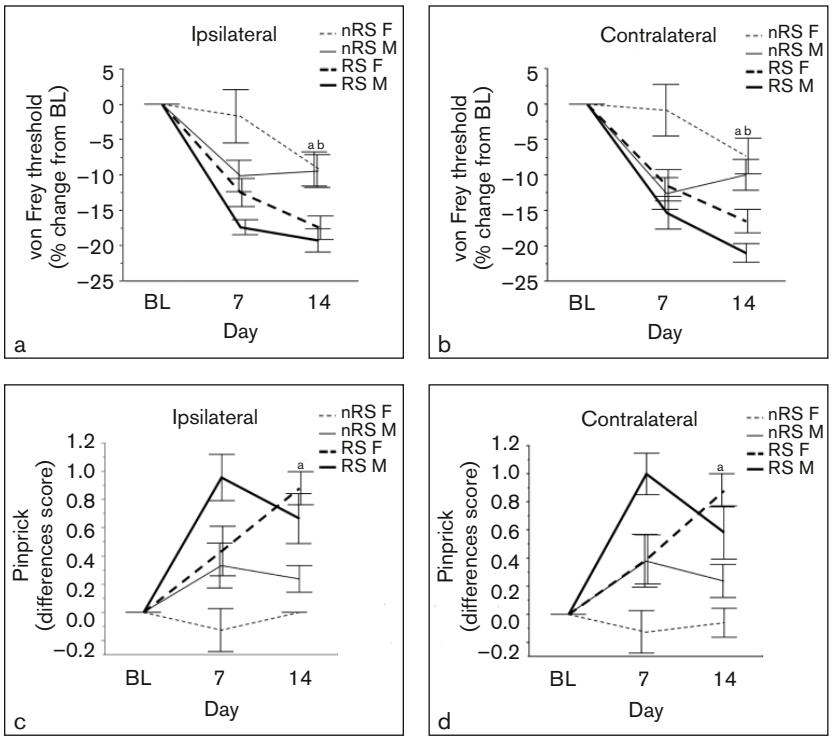

Fig 6 Pain-like behavior during chronic restraint (RS). **(a)** RS rats showed a significant reduction compared to nRS rats in both males and females in ipsilateral side thresholds (tactile allodynia) (stress $P < .0001$; sex $P = .6$) and in **(b)** contralateral side thresholds (stress $P < .0001$; sex $P = .04$). **(c)** RS rats compared to nRS rats in both males and females showed significantly increased pinprick response difference scores (mechanohyperalgesia) **(c)** on the ipsilateral side (stress $P < .0001$; sex $P = .93$) and **(d)** on the contralateral side (stress $P < .0001$; sex $P = .99$). [a]$P < .05$ for RS females compared to nRS females. [b]$P < .05$ for RS males compared to nRS males. Data are reported as mean ± SEM.

Tactile Alloydnia. *Effects of RS* As this stage was prior to surgery, the statistical analyses were performed on rats according to stress and sex categories only, and the sensory changes over the RS period and prior to surgery are shown first in Fig 6.

Male			
RS		nRS	
IOCCI (n = 8)	Sham (n = 8)	IOCCI (n = 8)	Sham (n = 8)
436.83 ± 14	423.67 ± 8.4	397.57 ± 9.5	436.41 ± 10.3
13.1 ± 8.4	−18.9 ± 1.9	59.4 ± 9.1	−14.9 ± 2.8
0 ± 0	0.63 ± 0.22	−1 ± 0	0 ± 0
447.38 ± 12.2	436.25 ± 10.3	417.14 ± 10.5	456.93 ± 11.2
0.07 ± 5.8	−19.0 ± 1.8	60.5 ± 8.3	−9.2 ± 3.8
−0.57 ± 0.2	0.44 ± 0.22	−0.83 ± 0.31	−0.31 ± 0.12
464.38 ± 11.8	454.5 ± 11.0	426.71 ± 10.9	463.41 ± 11.8
−10.2 ± 2.7	−15.5 ± 2	22.2 ± 5.3	−12 ± 3.4
0.38 ± 0.32	0 ± 0.2	−1 ± 0	0 ± 0
473.13 ± 11.7	499 ± 12.4	433.11 ± 11.2	495.69 ± 15.0
−14.6 ± 0.9	−12.9 ± 3.3	19.8 ± 3.8	−10.23 ± 3.2
0.75 ± 0.41	−0.25 ± 0.25	0 ± 0.44	−0.5 ± 0.19
485.7 ± 11.7	514.1 ± 13.4	447.1 ± 11.7	509.9 ± 15.3
−19.5 ± 1.9	−12.9 ± 3.3	−0.3 ± 0.1	−8.2 ± 3.3
2 ± 0	0 ± 0.38	1.43 ± 0.2	0 ± 0
496.4 ± 13.0	513.4 ± 12.6	452.3 ± 12.3	512.2 ± 17.2
−25.9 ± 5.1	−13.8 ± 0	−12.2 ± 2.8	−7.7 ± 4.4
2 ± 0	−0.25 ± 0.25	1.43 ± 0.2	−0.5 ± 0.19
500.7 ± 12.9	518.6 ± 12.9	455.7 ± 12.7	517.4 ± 17.1
29.0 ± 5.3	−4.5 ± 4.1	−24.1 ± 5.5	−3.0 ± 4
2.29 ± 0.18	0.13 ± 0.3	1.29 ± 0.18	−0.5 ± 0.19

Subsequently, the data are presented in Fig 7 according to surgery, sex, and stress groupings to demonstrate the complete timeline of the animals within each group.

After 14 days of RS, significant differences in tactile detection thresholds were observed between the experimental groups on both the ipsilateral (stress F = 19.2, $P < .0001$; sex F = 0.26, $P = .6$) and contralateral (stress F = 26.0, $P < .0001$; sex F = 4.4, $P = .04$) sides. Significant differences in response to pinprick stimulation were also observed between experimental groups on both the ipsilateral (stress F = 25.0, $P < .0001$; sex F = 0.01, $P = .93$) and contralateral (stress effect F = 18.5, $P < .0001$; sex effect F = 0.0, $P = .99$) sides.

Pairwise analyses revealed that RS females had significantly lower tactile detection thresholds (ipsilateral: −17.5% ± 1.7%; contralateral: −16.5% ± 8.1%) than the nRS females (ipsilateral: −9.2% ± 2.4%, $P = .03$; contralateral: −6.4% ± 12.7%, $P = .004$), with a similar effect observed in males (ipsilateral RS male: −19.3% ± 1.6%, nRS male: −9.5% ± 2.3%, $P = .006$; contralateral RS male: −21.0% ± 6.29%, nRS male: −10.3% ± 11.3%, $P = .002$). No significant differences were noted between RS females and males or between nRS females and males on either the ipsilateral ($P = .9$ for both) or contralateral (RS females vs RS males: $P = .4$; nRS females vs nRS males: $P = .5$) sides.

Effects of Surgery. 17 Days Postsurgery. At 17 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 47, $P < .0001$; pain F = 7.9, $P = .007$; sex F = 44.5, $P < .0001$; with a significant stress*pain interaction F = 6.1, $P = .02$). Pairwise comparisons revealed that RS-IOCCI males had significantly lower thresholds (−19.5% ± 1.9%) than nRS-IOCCI males (−0.3% ± 0.2%, $P = .007$) and that RS-IOCCI females had significantly lower thresholds (−44.7% ± 1.4%) than nRS-IOCCI females (−20.5% ± 6.5%, $P < .0001$). RS-IOCCI females also had significant decreases in percent thresholds compared to RS-IOCCI males ($P < .0001$), and nRS-IOCCI females had significant decreases in percent thresholds compared to nRS-IOCCI males ($P < .0001$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in ipsilateral thresholds between RS-sham males (−13% ± 3.3%) and nRS-sham males (−8.2% ± 3.3%, $P = .9$); however, RS-sham females had significantly lower thresholds (−26.7% ± 0%) than nRS-sham females (−11.1% ± 2.1%, $P = .03$). There were no significant differences between nRS-sham males and females ($P = .9$) or between RS-sham males and females ($P = .07$).

A significant overall difference for the contralateral side was also noted between the experimental groups at 17 days postsurgery (stress F = 23, $P < .0001$; pain F = 9.6, $P = .003$; sex F = 15.8, $P = .0002$; with a significant stress*sex interaction F = 9.2, $P = .004$) (Fig 7). Pairwise comparisons revealed that RS-IOCCI females had significantly lower thresholds (−41.1% ± 11.4%) than nRS-IOCCI females (−19.5% ± 12.0%, $P = .0007$). No significant difference in thresholds was observed between RS-IOCCI and nRS-IOCCI males ($P = 1$). RS-IOCCI females also had significant decreases in percent thresholds (−41.1% ± 11.4%) compared to RS-IOCCI males (−17.2% ± 4.4%; $P = .0002$). No significant

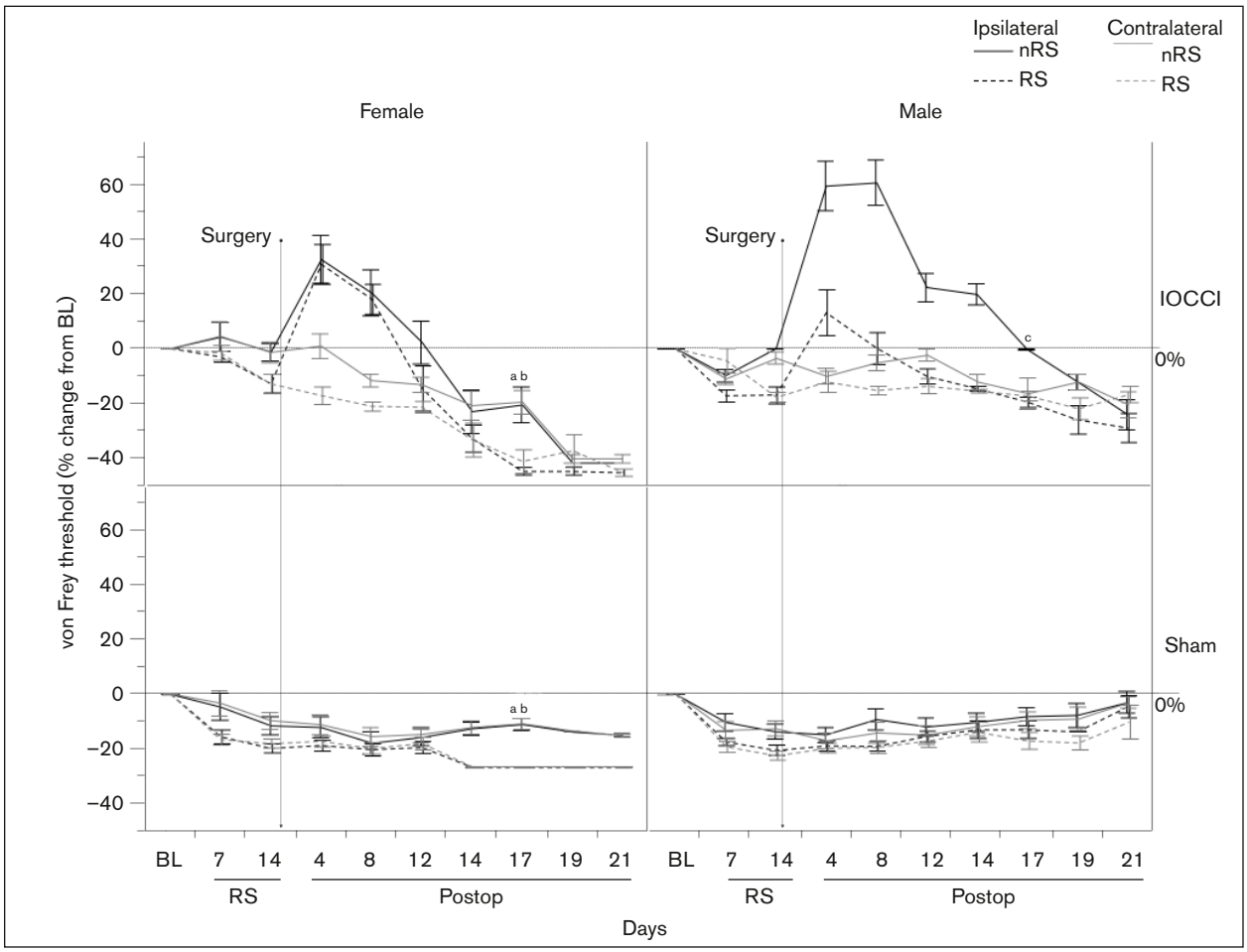

Fig 7 Percent change in ipsilateral and contralateral von Frey withdrawal thresholds over the experimental period. An early increase in thresholds (hypoesthesia) from postoperative days 4 to 12 was noted in the groups that underwent IOCCI, probably due to surgical trauma. From postoperative day 17, there was a significant decrease in thresholds to von Frey hair stimulation (allodynia) in the IOCCI groups, which became robust on days 19 and 21. RS-IOCCI rats showed significantly reduced thresholds compared to nRS-IOCCI rats, and this was more prominent in female than in male rats (with significant main effects for sex, stress, and pain at 17, 19, and 21 days, and significant stress*pain interactions at days 17 and 19). RS-sham female rats developed persistently reduced thresholds lasting from postoperative day 14 to day 21 compared to nRS-sham females, an effect not observed in male rats. There were parallel but milder changes observed on the contralateral side, with significant differences observed at postoperative day 17 between RS-IOCCI and nRS-IOCCI females ($P < .05$), but with no increased detection thresholds immediately after surgery. [a]$P < .05$ for RS females compared to nRS females on the ipsilateral side. [b]$P < .05$ for RS females compared to nRS females on the contralateral side. [c]$P < .05$ for RS males compared to nRS males. Data are reported as mean ± SEM.

differences were found between nRS-IOCCI females and males ($P = 1$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in contralateral thresholds between RS-sham males (–17% ± 8.7%) and nRS-sham males (–9.7% ± 8.9%, $P = .7$). RS-sham females had significantly lower thresholds (–26.7% ± 0%) than nRS-sham females (–11.1% ± 6%, $P = .03$). There were no significant differences between nRS-sham males and females ($P = 1$) or between RS-sham males and females ($P = .5$) at this time point.

19 Days Postsurgery. At 19 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 15.5, $P = .0003$; pain F = 47.4, $P < .0001$; sex F = 55, $P < .0001$; with a significant stress*pain interaction F = 10.4, $P = .002$). Pairwise comparisons showed no significant difference in thresholds between RS-IOCCI males (–25.9% ± 5.1%) and nRS-IOCCI males (–12% ± 2%, $P = .06$) or between RS-IOCCI females (–44.7% ± 1.4%) and nRS-IOCCI females (–41.7% ± 0%, $P = .9$). RS-IOCCI females also had significant decreases in percent thresholds compared to RS-IOCCI males ($P = .001$). Similarly, nRS-IOCCI females had significant decreases in percent thresholds compared to nRS-IOCCI males ($P < .0001$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in ipsilateral thresholds between RS-sham males (–13.8% ± 0%) and nRS-sham males (–7.7% ± 4.4%, $P = .9$) or between RS-sham females (–26.7% ± 0%) and nRS-sham females (–13.8% ± 0%, $P = .13$). There were no significant differences between nRS-sham males and females ($P = .9$) or between RS-sham males and females ($P = .17$) at this time point.

A significant overall difference between the experimental groups was also noted on the contralateral side at 19 days postsurgery (stress F = 7.4, $P = .009$; pain F = 17.6, $P = .0001$; sex F = 29.5, $P < .0001$; with a significant sex*pain interaction F = 8.3, $P = .006$). Pairwise comparisons showed no significant difference in tactile percent thresholds between RS-IOCCI males (–21.9% ± 11.4%) and nRS-IOCCI males (–12.2% ± 6.9%, $P = .53$) or between RS-IOCCI females (–37.2% ± 15.3%) and nRS-IOCCI females (–40.1% ± 3.8%, $P = 1$). Significantly decreased percent thresholds were also observed in nRS-IOCCI females compared to males ($P = .0001$). There was no significant difference in tactile percent threshold between RS-IOCCI females and males ($P = .05$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in thresholds between RS-sham males (–18% ± 5.7%) and nRS-sham males (–9.2% ± 12.1%, $P = .7$) or between RS-sham females (–26.7% ± 0%) and nRS-sham females (–13.8% ± 0%, $P = .3$). There were no significant differences between nRS-sham males and females ($P = 1$) or between RS-sham males and females ($P = .8$) at this time point.

21 Days Postsurgery. At 21 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 4.4, $P = .04$; pain F = 77, $P < .0001$; sex F = 42.9, $P < .0001$; with no significant interactions). Pairwise comparisons showed no significant difference in thresholds between RS-IOCCI males (–29.1 ± 5.3%) and nRS-IOCCI males (–24.1 ± 5.5%, $P = .06$) or between RS-IOCCI females (–45.2 ± 1.3%) and nRS-IOCCI females (–41.7 ± 0%, $P = .9$). RS-IOCCI females had significant decreases in percent thresholds compared to males ($P = .04$). Similarly, nRS-IOCCI females had significant decreases in percent thresholds compared to males ($P = .04$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in ipsilateral thresholds between RS-sham males (–4.5% ± 4.1%) and nRS-sham males (–3% ± 4%, $P = 1$) or between RS-sham females (–26.7% ± 0%) and nRS-sham females (–15% ± 0.6%, $P = .4$). There were no significant differences between nRS-sham males and females ($P = .3$). RS-sham females showed a significant decrease in percent threshold compared to RS-sham males ($P = .003$).

A significant overall difference between the experimental groups was also noted on the contralateral side (stress F = 4.4, $P = .04$; pain F = 50.9, $P < .0001$; sex F = 66.8, $P < .0001$; with a significant sex*pain interaction F = 4.4, $P = .04$). Pairwise comparisons showed no significant difference in thresholds between RS-IOCCI males (–16.7% ± 8.33%) and nRS-IOCCI males (–20.5% ± 12.6%, $P = 1$) or between RS-IOCCI females (–45.2% ± 3.8%) and nRS-IOCCI females (–40.1% ± 3.8%, $P = 1$). RS-IOCCI females had significant decreases in percent thresholds compared to RS-IOCCI males ($P < .0001$). Similarly, nRS-IOCCI females had significant decreases in percent thresholds compared to nRS-IOCCI males ($P = .005$).

In the sham-operated rats, pairwise comparisons revealed no significant differences in contralateral thresholds between RS-sham males (–10.4% ± 17.1%) and nRS-sham males (–3.2% ± 6%, $P = .7$) or between RS-sham females (–26.7% ± 0%) and nRS-sham females (–15.4 ± 1.5%, $P = .2$). There were no significant differences between nRS-sham males and females ($P = .3$). RS-sham females showed a significant decrease in percent threshold compared to RS-sham males ($P = .03$).

Mechanohyperalgesia. *Effects of RS*. Since this stage was prior to surgery (ie, all rats were naïve), statistical analyses were performed with only stress and sex categories as the independent variables (Fig 6). After 14 days of RS (prior to surgery), significant differences in response to pinprick stimulation were observed between the experimental groups on both the ipsilateral (stress F = 25, $P < .0001$; sex F = .01, $P = .9$) and contralateral (stress F = 18.5, $P < .0001$; sex F = 0.0, $P = .99$) sides.

Pairwise analyses revealed that RS females had significantly higher difference scores (0.9 ± 0.1) than the nRS females (0 ± 0, $P = .0002$) for both the ipsilateral and contralateral sides (RS females = 0.9 ± 0.5, nRS females = –0.0 ± .4, $P = .0004$). No significant differences were seen in pinprick response scores in males on either the ipsilateral (RS male = 0.7 ± 0.2, nRS male = 0.2 ± 0.1, $P = .07$) or contralateral (RS male = 0.6 ± 0.9, nRS male = 0.2 ± 0.5, $P = .3$) sides. No significant differences were noted between RS females and RS males or between nRS females and nRS males ($P = .9$ for both).

Figure 8 shows the complete timeline of the animals within each group according to surgery, sex, and stress groupings.

17 Days Postsurgery. At 17 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 27.1, $P < .0001$; pain F = 32.8, $P < .0001$; sex

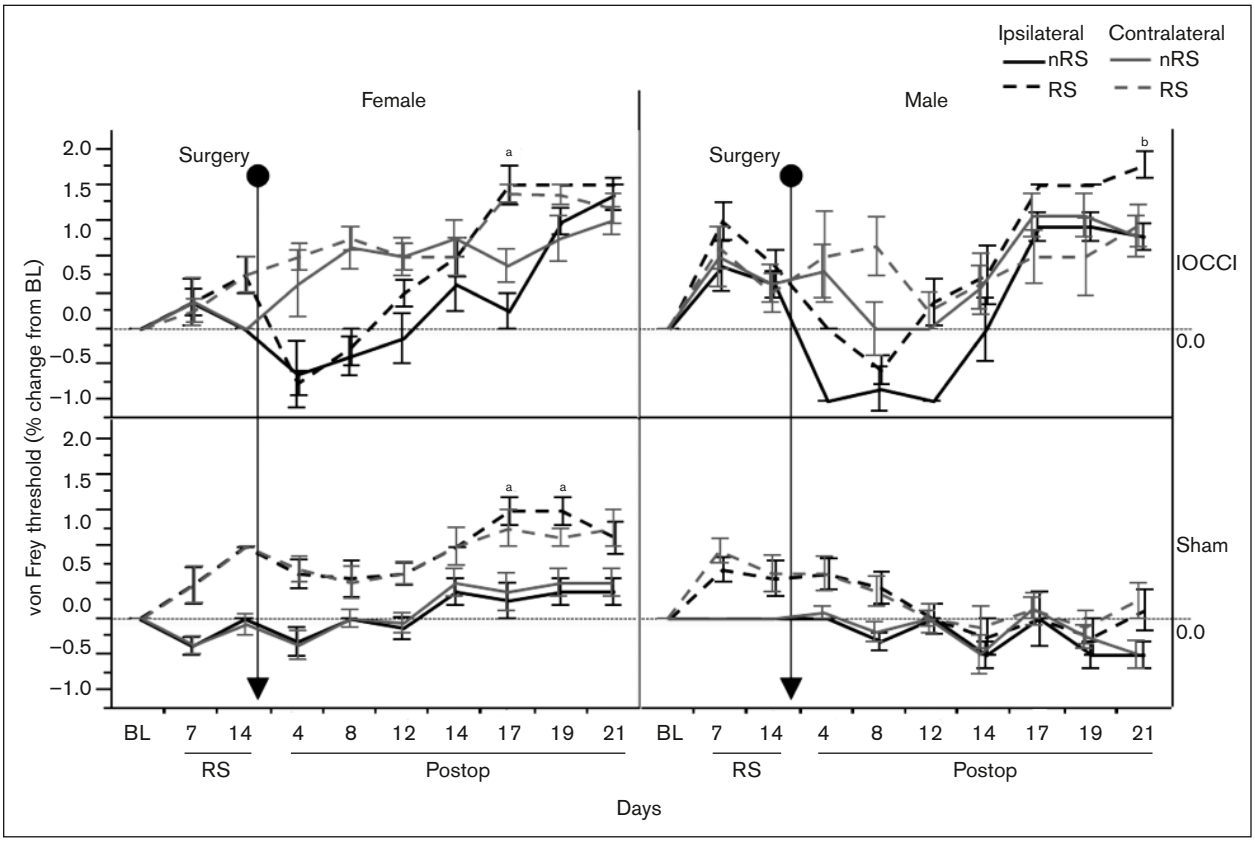

Fig 8 Difference scores for response to pinprick stimulation over the experimental period. An early decrease in difference scores (hypoalgesia) was noted in the groups that underwent IOCCI from postoperative days 4 to 14, probably due to surgical trauma. From postoperative day 17, there was a significant increase in difference scores (hyperalgesia) in the IOCCI groups, which became robust on postsurgical days 19 and 21. RS-IOCCI rats showed significantly increased difference scores compared to nRS-IOCCI rats, with no apparent sex differences (significant main effects for stress and pain at day 17; for sex, stress, and pain at 19 and 21 days; significant pain*sex interaction at days 17, 19, and 21; and stress*sex interaction at day 17). RS-sham female rats developed persistently increased difference scores lasting from postoperative days 4 to 21 compared to nRS sham females. This was not observed in male rats. There were parallel but milder changes ($P > .05$) observed on the contralateral side, but with no contralateral reduced scores immediately after surgery. [a]$P < .05$ for RS females compared to nRS females. [b]$P < .05$ for RS males compared to nRS males. Data are reported as mean ± SEM.

F = 0.7, $P = .4$; with significant stress*sex F = 12.5, $P = .001$ and pain*sex F = 18.2, $P < .0001$ interactions). Pairwise comparisons revealed no statistical significance in the difference scores between RS-IOCCI males (2 ± 0) and nRS-IOCCI males (1.4 ± 0.2, $P = .8$), but RS-IOCCI females (2 ± 0.3) had significantly higher scores than nRS-IOCCI females (0.3 ± 0.3, $P < .0001$). There were no differences between RS-IOCCI females and males ($P = 1$), but nRS-IOCCI females had significantly lower difference scores compared to nRS-IOCCI males ($P = .02$).

In the sham-operated rats, no significant differences in response to ipsilateral pinprick stimulation were found between RS males (0 ± 0.4) and nRS males (0 ± 0, $P = 1$). RS-sham females showed a significant increase in scores (1.5 ± 0.2) compared to nRS-sham females (0.3 ± 0.3, $P = .01$) and to RS-sham males ($P = .001$), but there were no significant differences between nRS-sham males and females ($P = 1$).

A significant overall difference was noted between the experimental groups at 17 days on the contralateral side (stress F = .5, $P = .07$; pain F = 25.1, $P < .0001$; sex F = 5, $P = .03$). Pairwise comparisons revealed no statistically significant effects of stress on the difference scores of the contralateral side in IOCCI animals within each sex group or between sexes ($P > .05$).

In the sham-operated rats, no significant differences in response to contralateral pinprick stimulation were found between RS and nRS animals within each sex group ($P > .05$). RS-sham females had significantly higher response scores to pinprick stimulation than RS-sham males ($P = .03$).

19 Days Postsurgery. At 19 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 23, $P < .0001$; pain F = 129.4, $P < .0001$; sex F = 27.9, $P < .0001$; with a significant pain*sex interaction F = 25.1, $P < .0001$). Pairwise compari-

sons revealed no statistical significance between the difference scores in RS-IOCCI males (2 ± 0) and nRS-IOCCI males (1.4 ± 0.2, P = .4) or between RS-IOCCI females (2 ± 0) and nRS-IOCCI females (1.5 ± 0.2, P = .5). There were no differences between RS-IOCCI females and males (P = 1) or between nRS-IOCCI females and males (P = 1).

In the sham-operated rats, no significant differences in response to ipsilateral pinprick stimulation were observed between RS-sham males (–0.25 ± 0.25) and nRS-sham males (–0.5 ± 0.2, P = 1). RS-sham females showed a significant increase in scores (1.5 ± 0.2) compared to nRS-sham females (0.4 ± 0.2, P = .001), and RS-sham females showed a significant increase compared to RS-sham males (P < .0001). Also, nRS-sham males had a significantly lower difference score in response to pinprick stimulation than nRS-sham females (P = .02).

A significant overall difference between the experimental groups was also noted on the contralateral side (stress F = 1.1, P = .3; pain F = 33.6, P < .0001; sex F = 11, P = .002).

IOCCI and sham animals demonstrated no statistical significance in the difference scores between RS and nRS animals within each sex group (P > .05). Also, in the IOCCI animals, no statistically significant differences in response to pinprick stimulation on the contralateral side were observed between sexes in either the RS or nRS group (P > .05).

In the sham-operated animals, RS females had significantly higher responses to pinprick stimulation on the contralateral side compared to RS males (P = .03).

21 Days Postsurgery. At 21 days postsurgery, a significant overall difference between the experimental groups was noted on the ipsilateral side (stress F = 5.8, P = .0001; pain F = 111.2, P < .0001; sex F = 13.2, P = .0006; with a significant pain*sex interaction F = 6.9, P = .02). Pairwise comparisons revealed that RS-IOCCI males had a significantly higher difference score (2.3 ± 0.2) than nRS-IOCCI males (1.3 ± 0.2, P = .04), but there was no significant difference between RS-IOCCI females (2 ± 0) and nRS-IOCCI females (1.9 ± 0.2, P = 1). No significant differences were found between RS-IOCCI females and males (P = 1) or between nRS-IOCCI females and males (P = .5).

In the sham-operated rats, no significant differences in response to ipsilateral pinprick stimulation were observed between RS-sham males (0.1 ± 0.3) and nRS-sham males (–0.5 ± 0.2, P = 1) or between RS-sham females (1.13 ± 0.2) and nRS-sham females (0.4 ± 0.2, P = .2). RS-sham females had a higher difference score than RS-sham males (P = .02), but there was no significant difference between nRS-sham males and females (P = .07).

At 21 days postsurgery, a significant overall difference between the experimental groups was noted on the contralateral side (stress F = 7.4, P = .009; pain F = 43.1, P < .0001; sex F = 13.5, P = .0006; with a significant pain*sex interaction F = 5.4, P = .02). IOCCI and sham-operated animals demonstrated no statistical significance between the difference scores in RS and nRS animals within each sex group (P > .05). No statistically significant differences in response to pinprick stimulation on the contralateral side were observed between RS males and females or between nRS males and females in either IOCCI or sham animals (P > .05).

Discussion

Overall, the present study has demonstrated important sex differences in rats in their responses to stress and trigeminal nerve injury and how these factors interact differently between sexes.

The results of this study negate part of the first hypothesis: Although chronic stress did induce an increase in pain-like behavior in naïve male and female rats, pain-like behavior was not more pronounced in females at the end of the restraint period. In RS animals, there was a drop of 17% to 19% in von Frey monofilament detection thresholds compared to baseline. Interestingly, this is comparable to what has previously been observed in the trigeminal neuritis model.[64] In this model, Complete Freund's Adjuvant (CFA) was applied around the infraorbital nerve, and there was a drop of about 22% in von Frey detection thresholds following 5 days of exposure. Similarly, following 14 days of restraint, pinprick response difference scores in RS animals in the present study were between 0.7 and 0.9, which is comparable to the effects seen in neuritis (difference of approximately 1).[64] This is a striking result, as it indicates stress is able to induce pain-like behavior that equals the effects of an inflammatory insult.

Both RS male and female rats showed a significant decrease in plasma IFN-γ levels compared to nRS rats, which reflects the effects of stress on the immune system. Stress leads to a decrease in innate IFN-γ production.[60] The mechanisms responsible for the suppression of the innate IFN-γ synthesis include release of stress hormones and glucocorticoid receptor activation.[60,65] Laboratory routines—such as cage change or movement, transport, handling, exposure to novel environment, and blood collection—have been shown to cause mild animal stress,[66] but no significant changes in IFN-γ levels were observed in the nRS rats in the present study. Furthermore, evidence from the EPM data supports the view that RS results in significant behavioral changes in both sexes, with three of the four measures showing statistical significance and pointing toward stress-like behavior in the RS rats. It is not clear why the fourth

parameter (percent time spent in the open arms) was not significantly different between the RS and nRS animals. Although sex has been shown to have an effect on EPM results,[67] the EPM data at 14 days of restraint show no sex differences in rats.

Body weight change in rodents is a validated and generally accepted indicator/parameter of stress.[68] RS has been well documented to reduce weight gain.[69,70] Changes in body weight indicated that the RS animals were gaining less weight than the nRS animals,[71] and the statistical analyses indicate that the effect of stress on weight was more pronounced in female than in male rats. However, taking into account the normal patterns of weight gain in male and female rats,[72] it appears that there were no significant differences between sexes in the effects of restraint on weight gain.

The second hypothesis received support from the present findings. IOCCI is a behavioral animal model of trigeminal neuropathic pain based on the original work performed on painful sciatic neuropathy.[53] In a similar fashion, chronic constriction of the infraorbital nerve results in somatosensory changes in the territory of the injured nerve consistent with a neuropathic pain-like condition.[50,51,73–75] However, these previous studies were carried out in male rats and did not examine the effects of chronic stress. IOCCI is characterized by an early period of hyporesponsiveness to mechanical stimulation (most likely due to nerve manipulation in the tight surgical area), followed by hyper-responsiveness to mechanical stimulation applied to the affected area beginning 1 to 2 weeks postsurgery.[51,73,74,76,77] The present findings are therefore consistent with those reported previously,[51,73,76,77] but expand the data to include female rats and the effects of RS. Other models of trigeminal neuropathic pain have been described, including partial infraorbital nerve transection,[78] insult to the trigeminal ganglion by mechanical compression or injection of the mitogen lysophosphatidic acid,[79,80] and inflammation of the infraorbital nerve.[64] All these models induce pain-like behavior in rodents with different temporal and somatosensory profiles than with IOCCI.

Ipsilateral tactile allodynia was more severe in females at 17, 19, and 21 days postsurgery. IOCCI females had significantly lower von Frey thresholds compared to IOCCI males in both the RS and nRS subgroups. The observed effects of sex in the development of hypersensitivity following IOCCI are comparable to those of a previous study on sex differences in rats undergoing IOCCI.[27] Previous data also indicate that female rats are more sensitive to chemical, heat, and electrical stimuli than male rats.[81] The mechanisms of the observed sex differences in the development of hypersensitivity are unclear, and it has been suggested that testosterone may reduce the severity of nerve injury pain by inhibiting the associated inflammatory response.[82] A significant protective effect of testosterone on pain behavior has been demonstrated in sciatic nerve chronic constriction injury.[33] Ipsilateral pinprick testing showed no significant sex differences at days 17 and 19 postsurgery. There were also no significant differences between the sexes in RS- or nRS-IOCCI rats.

Importantly, sex differences in pain behavior have not been consistent across all strains of rats and mice.[10,11] Also, site differences have been reported, with one study examining neuropathic pain showing significant sex differences in the face but not in the leg.[27]

The final hypothesis was in part supported by the present findings. The combined effects of sex and stress on somatosensory changes following IOCCI have not been reported previously. The present findings indicate that stress does exacerbate pain-like behavioral measures, particularly in the early stages of hypersensitivity. This finding is similar to previously published evidence that stress exacerbates neuropathic pain in mice[83]; however, there was little evidence that this was more pronounced in females than in males. This finding is consistent with the behavioral data at the immediate postrestraint time point, which showed an effect of stress, but not sex, on pain-like behavior. To the best of the authors' knowledge, this is the first study to report the effects of stress and sex on somatosensory changes in rats following IOCCI, and the data suggest that in the presence of stress, the effects of sex on pain-like behavior are diminished.

In the context of hypersensitivity measures, interesting findings were observed in the sham groups. RS-sham females showed persistent hypersensitivity throughout the experimental period in both the pinprick and von Frey assessments. RS-sham males demonstrated some hypersensitivity early on associated with exposure to RS, but these effects waned over the experimental time course. At later time points (17, 19, and 21 days postsurgery), RS-sham and nRS-sham males showed no differences in pinprick response scores or von Frey assessments. These data suggest a more persistent effect of stress on stimulus sensitivity in the female rats, particularly in the sham groups. No increase in hypersensitivity in the affected area was observed in the sham animals not exposed to RS; therefore, the observed increase in hypersensitivity to stimulation in the affected region in RS-sham groups is most likely caused by increased levels of stress and anxiety, not by specific somatosensory changes in the affected area. In the presence of neuropathic pain, stress-induced, sex-specific, hyper-responsive effects seem to be masked. The severity of ongoing pain may be the

parameter that masks this effect. In support of this view, the level of hyper-responsiveness of sham rats was only about 50% to 65% of that observed in the IOCCI rats, depending on whether pinprick or von Frey responses were examined.

As has been previously observed with IOCCI,[84] the contralateral side demonstrated parallel and statistically significant but milder changes in somatosensory measures compared to the ipsilateral side. Interestingly, this phenomenon has been previously reported in trigeminal neuropathic pain models[52,78] and has also been shown in humans with painful traumatic trigeminal neuropathy.[85]

Study Limitations

Although a power calculation and strong 2 × 4 experimental design were used, the number of animals per group was low (n = 8), and this may be a limiting factor contributing to the variability observed in the data set and potentially to lack of significance of some data comparisons. No assessment of female estrus cycle was performed; however, evidence suggests that this is a negligible source of variation.[1,14] The scoring of behavior was done by only one researcher, and although that researcher was blinded to the experimental conditions, a second observer would be desirable.

Seven measurements were made over a period of 21 days, or, on average, measurements were made every 3 days. This type of repeated testing may cause behavioral changes in the experimental animal. There is always a trade-off to be made when testing nociceptive sensitivity.[86] Too few dependent measure determinations and the risk of inaccurate data are high, while too many may alter nociceptive sensitivity. Repeated measurements may lead to a phenomenon known as behavioral tolerance, which can induce changes in latencies or withdrawal thresholds.[87] These are the limitations of pain-related behavioral testing in animals. The use of high-intensity noxious stimuli can minimize this problem, as shown by the remarkable consistency of the suprathreshold tail flick test data,[88] and therefore pinprick response measurements may also be a more consistent test. However, the von Frey assay to measure withdrawal to a non-noxious stimulus was also used, and this may be more affected by repeated testing.

Previous data[51] have shown that following IOCCI, pain-like behavior develops at around 14 to 17 days postsurgery and becomes robust after about 20 days. For the specific aims of this experiment and to minimize animal suffering, the end of the experiment was set at 21 days. However, the differences in pain-like behavior between the sexes may have changed over time. Therefore, a study continuing for a prolonged period of time following IOCCI is needed to elucidate the longer-term effects of sex on pain-like behavior.

Conclusions

An increased sensitivity to stimuli applied to the trigeminal nerve area of the vibrissal pad was observed in rats following RS, irrespective of sex. This sensitivity subsequently persisted in RS-sham females, but not in RS-sham males. Following IOCCI, female RS rats developed significantly greater tactile allodynia, but not greater mechanohyperalgesia, than male RS rats. Although these findings indicate that stress may induce a significant increase in pain-like behavior in female compared to male rats, these results need to be balanced with the finding that RS had no significant sex effects on IFN-γ levels, EPM parameters, or percent body weight gain. This suggests that stress may have a selective effect on pain-like behavior in the two sexes, but this mechanism is unclear. Alternatively, based on the small sample size, an argument could be made that RS had approximately equal effects across all experimental parameters. Nevertheless, these results encourage further investigations of the interactions between stress and sex in trigeminal pain states.[1] Studying the interaction with stress adds a new dimension to enhancing understanding of the modulation of responses to nerve injury in complex life situations in both sexes.

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