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Psychophysical stress increases the expression of phospho-CREB, Fos protein and neurokinin-1 receptors in superficial laminae of trigeminal subnucleus caudalis in female rats

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ABSTRACT

Psychological stress and estrogen status are risk factors to develop painful temporomandibular joint disorders (TMJD); however, the neural basis for this relationship is not known. This study tested the hypothesis that repeated forced swim stress and estradiol treatment alter the phosphorylation of cAMP responsive element-binding protein (pCREB) in trigeminal subnucleus caudalis (Vc), the initial site of sensory input from the TMJ. Ovariectomized female rats were given low or high dose estradiol and subjected to repeated forced swim stress for 3 days and on day 4 an intra-TMJ injection of mustard oil or vehicle was given. Forced swim alone increased the number of pCREB-positive neurons, independent of estradiol treatment or TMJ stimulation, in superficial and deep laminae of Vc. Forced swim also increased the number of Fos-positive neurons in superficial laminae and neurokinin-1 receptor mRNA in whole dorsal Vc, independent of estradiol treatment. These results indicated that persistent psychophysical stress alone was sufficient to increase the expression of pCREB and downstream regulated genes associated with enhanced excitability in the caudal medullary dorsal horn, a brainstem region thought to be critical for TMJD pain.

Temporomandibular joint disorders (TMJD) are common conditions characterized by spontaneous pain in the temporomandibular joint (TMJ) region and muscles of mastication. Efforts to understand the neural mechanisms for persistent painful TMJD have been limited due to lack of suitable animal models. Although diverse etiologies may contribute to the development of TMJD, psychological stress and estrogen status are recognized as significant risk factors [10,14,24]. Persistent psychological stress exacerbates evoked nociceptive behavior in animal models for spinal [11], visceral [3] and TMJD pain [8]; however, most studies have used only male animals. The present study used repeated forced swim stress (FS) in female rats, an established psychophysical stress that causes persistent cutaneous and muscle hyperalgesia in male rats [22,25]. In spinal dorsal horn, phosphorylation of the transcription factor, cAMP response element-binding protein (pCREB), regulates the expression of the immediate early gene c-*fos* [13] and late response genes such as the neurokinin-1 (NK-1) receptor [23] and underlies long-term changes in synaptic activity [4]. Blockade of pCREB formation at spinal levels prevents hyperalgesia in animal models of muscle pain [9], while destruction of spinal NK-1 receptors prevents the sensitization of dorsal horn neurons and hyperalgesia to cutaneous stimuli [17]. The present study determined if repeated FS and estradiol treatment interact to alter the expression of pCREB and downstream regulated genes at the trigeminal subnucleus caudalis/cervical spinal cord (Vc/C_{1-2}) junction, a medullary dorsal horn region that processes direct input from the TMJ region in an estrogen-dependent manner [20,29] in female rats.

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and conformed to the established guidelines set by The National Institute of Health guide for the care and use of laboratory animals (Publications No. 99-158, revised 2002). Ovariectomized (OvX) female rats (225–300 g, Sprague–Dawley, Harlan, Indianapolis, IN) were injected with low (LE, 3 μg/kg) or high (HE, 30 μg/kg, sc) dose 17β-estradiol-3-benzoate (E2, Sigma, St. Louis, MO) dissolved in sesame oil for three days. The estrogen status of each rat was confirmed on the day of the experiment by the cytology of a vaginal smear sample; LE rats had mainly (>80%) small nucleated leukocytes and HE rats displayed mostly large nucleated epithelial cells. The FS protocol involved placing the rat in a plastic cylinder (30 cm × 50 cm) containing 20 cm water (25 °C) for 10 min per day for three days [22], while sham controls were placed in an empty cylinder. Immobility time, an index of behavioral stress, was measured during each swim session [21], whereas forelimb grip force, an estimate of muscle pain or weakness [16] was measured before each swim session.

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Rats received daily injections of E2, grip force measurements and a session of FS or sham swimming for 3 days. On day 4, rats were anesthetized with pentobarbital sodium (65 mg/kg, ip) and those animals assayed for pCREB were further given an intra-TMJ injection of the small fiber excitant mustard oil (20% v/v) or vehicle (mineral oil) and allowed to survive for 30 min. Animals assayed for the immediate early gene product, Fos protein, or NK-1 receptor mRNA received no TMJ injection and were killed under anesthesia. Rats processed for pCREB and Fos immunohistochemistry were perfused through the heart with heparinized saline, followed by cold fixative (4% paraformaldehyde, 0.1 M phosphate, pH 7.4), the lower brainstem was removed and post-fixed overnight at 4 °C. Transverse sections (50 μm) were cut on a vibratome, blocked in a solution of 0.1 M PBS, 0.3% Triton X-100, and 5% normal donkey serum for 60 min and incubated in primary antiserum for pCREB (Upstate, Temecula, CA; 1:3500) or Fos protein (Ab-5, Millipore; 1:15,000) for 40 h at 4 °C. After rinsing in PBS for 20 min, sections were incubated with biotinylated donkey anti-rabbit antibodies (Chemicon-Millipore, Temecula, CA, USA; 1:300, 115 min) and avidin–biotin–peroxidase complex (Vector, Burlingame, CA, USA; 60 min). pCREB- and Fos-positive neurons were visualized by incubation in a 0.025% diaminobenzidine solution containing 0.01% nickel ammonium sulfate and 0.125% cobalt chloride, activated by 0.01% hydrogen peroxide. Specific staining for pCREB and Fos was abolished by omission of primary antisera. pCREB-positive neurons were counted at 200× using a calibrated reticle of 640 μm^2 positioned over the medial superficial or deeper laminae at the trigeminal subnucleus caudalis/upper cervical cord dorsal horn (Vc/C_{1-2}) region (see Fig. 2A). We selected specific areas of the Vc/C_{1-2} region to assess the effects of forced swimming conditioning on TMJ-evoked pCREB induction, because these areas are involved in TMJ-evoked nociceptive processing [28,29]. As a control for general pCREB expression we also counted cells in ventral horn at the same Vc/C_{1-2} region. Five sections separated by 100 μm at the Vc/C_{1-2} region were counted from each treatment group. The Vc/C_{1-2} region is located approximately 4.5–6.5 mm caudal to the obex [30]. In separate rats, Fos-positive neurons at the Vc/C_{1-2} region were counted in superficial laminae (I–II), deeper laminae (III–V) using established cytological landmarks [19]. Cell counts for pCREB and Fos were compared across groups by analysis of variance (ANOVA) without prior knowledge of treatment. Individual comparisons were made by the Newman–Keuls test after ANOVA. Possible relationships between pCREB cell counts and grip force in FS and sham swim animals were assessed by Spearman's rank-order correlation. All data are presented as mean ± SEM.

NK-1 receptor mRNA was assessed by quantitative RT-PCR from fresh tissue. Under deep anesthesia the lower brainstem was removed, dorsal horn tissue dissected bilaterally and total RNA extracted (Absolutely RNA Miniprep Kit, Stratagene, La Jolla, CA). RNA was quantified by spectrophotometry, reverse transcribed (iScript cDNA synthesis Kit, Bio Rad, Hercules, CA) and quantified by a Chromo4 DNA cycler using IQ SYBR green Supermix (Bio Rad). Samples were run in triplicate 25 μL reactions using 2 μL of the 20 μL cDNA synthesis as template. Genes of interest were run against a normalizing gene (GAPDH) allowing utilization of the ΔC_T method for relative quantification of gene expression. PCR reaction parameters were: initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 58.5 °C for 30 s, and 72 °C for 45 s. A final melting curve from 55 °C to 90 °C was performed for each reaction to ensure amplicon validity. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), synthesized by Sigma Genosys (St. Louis, MO) and checked against the NCBI database to ensure gene and species specificity. Primer sets employed were: GAPDH (F: 5′ agacagccgcatcttcttgt 3′, R: 5′ cttgccgtgggtagagtcat 3′) and NK-1 receptor (F: 5′ gctgtcttgccttctggaac 3′ R: 5′ ccacacaggaagtgggaagt 3′).

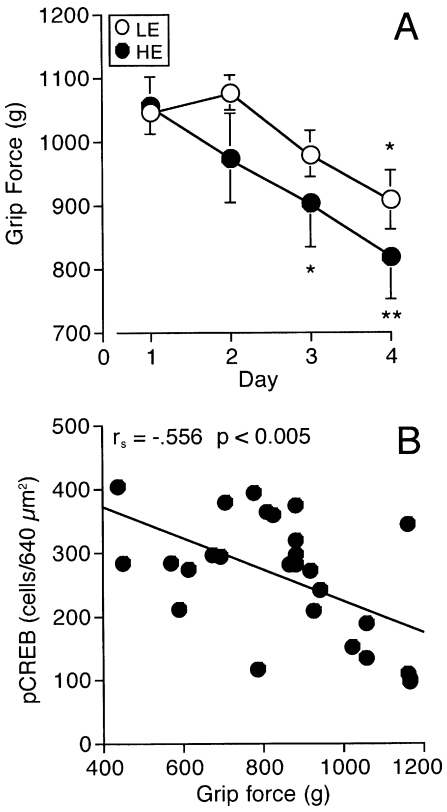

Fig. 1. Repeated FS conditioning reduces maximum forelimb grip force. (A) Grip force was reduced similarly in LE- and HE-treated rats. Sample sizes: LE = 12; HE = 14. $^*p < 0.05$, $^{**}p < 0.01$ versus day 1 value. (B) Reduction in grip force after FS was inversely correlated with the number of pCREB-positive neurons in superficial laminae. $n = 26$.

Repeated FS reduced forelimb grip force by day 4 ($F_{3,69} = 8.06$, $p < 0.001$) similarly in LE- and HE-treated rats (Fig. 1A). Sham conditioning did not affect forelimb grip force ($p > 0.05$). Although a reduction in grip force may have been due to muscle pain or weakness, we cannot exclude that other factors such as local release of agents in working muscle or central drive were involved [1,16]. Immobility times also were increased maximally by day 3 of FS conditioning in both LE and HE rats (data not shown). The number of pCREB-positive neurons in superficial laminae at the Vc/C_{1-2} region increased significantly ipsilateral to intra-TMJ injection of mustard oil in sham swim rats and bilaterally after FS alone or FS plus TMJ stimulation (Fig. 3A, $F_{7,24} = 18.6$, $p < 0.001$). As seen in the example of Fig. 2B, FS also increased the number of pCREB-positive cells in deep laminae after FS (Fig. 3B, $F_{7,24} = 6.07$, $p < 0.001$). However, TMJ stimulation in sham swim animals did not cause further increases pCREB expression in deep laminae. By contrast, estrogen status did not influence pCREB in superficial or deep laminae in any treatment group (Fig. 3). It was not likely that FS caused widespread increases in pCREB throughout the brainstem since cell counts in ventral horn at the Vc/C_{1-2} region were similar to that of sham swim rats (41 ± 2 versus 40 ± 1 pCREB cells/640 μm^2). Correlation analyses revealed a significant inverse relationship between pCREB cell counts in superficial laminae and maximum forelimb grip force on the day of the experiment (Fig. 1B, $r_s = -0.556$, $p < 0.005$). A similar analysis between pCREB cell counts in deep laminae and grip force also indicated a significant but weak relationship ($r_s = -0.423$, $p < 0.03$). To assess the effect of FS on downstream genes regulated by pCREB, we found that the number of Fos-positive neurons in superficial ($F_{3,12} = 10.85$, $p < 0.001$), but not deep laminae, was increased after FS independent of estrogen status ($F_{3,12} = 0.65$, $p > 0.05$, Fig. 4A). Similarly, NK-1 receptor expression in whole dorsal horn was