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The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ

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Abstract

It has been reported that stress can alter nociception from superficial tissues, such as skin and subcutaneous region. However, the influence of stress on an experimental deep nociception model is not understood. In this study, the temporomandibular joint (TMJ) formalin test was used to evaluate the effects of acute and chronic restraint stress on nociceptive responses in rats. Animals were initially submitted to one session of acute restraint stress (1 h) or exposed to chronic stress (40 days—1 h/day). Then, animals were killed immediately to collect blood for hormonal determinations by radioimmunoassay, or submitted to the TMJ formalin test to evaluate nociception. Rats submitted to acute restraint presented a performance similar to unstressed controls in the TMJ formalin test, whereas chronically stressed rats showed an increase in nociceptive responses. After 40 days of restraint, morphine was injected i.p. (1, 5 mg/kg or saline). The stressed rats displayed decreased morphine effects on nociception compared to unstressed controls. These findings suggest that repeated stress can produce hyperalgesia, which is, at least in part, due to alterations in the activity of opioid systems. This model may help elucidate the underlying neural mechanisms that mediate the effects of repeated stress on orofacial pain.

Keywords: Stress; Hyperalgesia; Formalin test; Temporomandibular joint; Nociception

1. Introduction

Different effects upon the nociceptive response have been observed with exposure to acute and chronic stress in rats (Vidal and Jacob, 1982; Watkins et al., 1982; Bodnar, 1986; Kavaliers and Innes, 1992; Quintero et al., 2000). Acute exposure to a variety of stressors produces an immediate analgesia in several pain tests (Lewis et al., 1980; Urca et al., 1985; Terman et al., 1986; Vaccarino and Kastin, 2001). Some studies, although, have reported that under some experimental conditions both acute and chronic stress can elicit hyperalgesia instead of analgesia (Satoh et al., 1992; Quintero et al., 2000, 2003; Imbe et al., 2004). Repeated exposure to a cold environment (4 °C for 30 min every hour for 1 day) induces 3-day long mechanical hyperalgesia (Satoh et al., 1992). One hour restraint a day for 40 days produces thermal hyperalgesia, which persists for at least 28 days after suspension of the chronic treatment (Torres et al., 2003a). Finally, repeated non-noxious swim stress (10–20 min a day for 3 days) elicits a delayed (after 24–48 h) and long-lasting (8–9 days) thermal and chemical cutaneous hyperalgesia (Quintero et al., 2000). Mechanisms regulating stress-induced changes in nociception include alterations in: endogenous opioid (Lewis et al., 1980; Przewlocki et al., 1987; Amit and Galina, 1988; Yamada and Nabeshima, 1995), serotoninergic (Quintero et al., 2000), adenosinergic (Torres et al., 2003b) and noradrenergic systems (Watkins and Mayer, 1982), as well as the hypothalamic–pituitary–adrenal (HPA) axis (Bodnar et al., 1979).

Although the precise mechanisms involved in the development of hyperalgesia observed after repeated stress are not well known, there are strong evidences that they could be related, at least in part, to alterations in the central or peripheral opioid activity (Gamaro et al., 1998; Torres et al.,

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2001a). The absence of novelty-induced antinociception, which has been attributed to opioid activation (Netto et al., 1987; Siegfried et al., 1987), in chronic stressed animals supports this theory. Therefore, one of the aims of the present work is to verify the effect of chronic restraint stress on morphine-induced antinociception, as measured by the TMJ formalin test.

The formalin test has been used to evaluate the effect of stressful stimuli in numerous experimental animal models, such as swim stress in mice (Carmody and Cooper, 1987; Vaccarino et al., 1992) and the exposure to a cat odour in rats (Lester and Fanselow, 1985). Our understandings of the influence of stress on nociception are largely based on experimental models of nociception in animals (Le Bars et al., 2001). Most of these models of nociception measure the output responses induced by superficial stimuli, for example tail-flick (Gamaro et al., 1998), hot-plate (King et al., 2003) and formalin injected in the paw (Aloisi et al., 1998). It is important to point out that deep pain conditions differ from the one evoked by superficial stimuli. There are different sensory disturbances in pain conditions involving deep tissues rather than cutaneous tissues (Sessle and Hu, 1990). Many deep craniofacial pain conditions, such as TMJ pain, are associated with manifestations of pain spread and referral (Sessle, 2002). Indeed, TMJ inflammation results in more robust changes in central nervous system when compared to perioral inflammation (Iwata et al., 1999). It is, nevertheless, poorly understood due in part to the limited options of experimental models available for the investigation of this condition.

Thus, considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of orofacial deep pain (Roveroni et al., 2001), the aim of this study was to evaluate the effects of acute and chronic restraint stress on the nociceptive responses induced by TMJ formalin test.

2. Methods

2.1. Animals

Male Wistar rats (weighing 200–230 g at the beginning of experiment) obtained from Centro Multi-disciplinar de Bioterismo-Cemib, Unicamp, Campinas, Brazil were used in this study. The rats were housed in groups of five and maintained in a temperature-controlled room (23 ± 1 °C) with a 12/12 light–dark cycle (lights on at 7:00 AM) and food and water were available ad libitum. Rats were adapted to the testing apparatus and handled prior to behavioral testing. Procedures were performed between 08:00 and 15:00 h. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

2.2. Stress exposure

The animals were stressed by restraint 1 h daily, 5 days per week for 40 days in the chronic model (Ely et al., 1997). In the acute model, there was a single exposure (Gamaro et al., 1998). Restraint was carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight) for 1 h. The area of the tube could be adjusted individually to each rat with a mobile inside wall and the tube was held firmly in place with Velcro straps. There was a 1 cm hole in the far end for breathing. The control group was not submitted to restraint. The immobilization procedure was carried out in a separate quiet room between 10:00 and 12:00 h.

2.3. Hormonal assays

Plasma corticosterone and ACTH levels were determined by radioimmunoassay (RIA) after plasma extraction using ethanol or silic acid (Castro et al., 1995), respectively. The rats were decapitated immediately after the last stress session and the whole blood was collected. The time interval between the stress procedure and manipulations until sacrifice was strictly maintained similar (30 s) among the different groups (acute restraint group $n=8$; chronic restraint group $n=8$; acute control group $n=8$; chronic control group $n=8$).

2.4. Testing procedure for TMJ pain

The design of this study follows that used by Roveroni et al. (2001). Testing sessions took place between 08:00 and 15:00 h in a quiet room maintained at 23 ± 1 °C. Immediately after the period of stress procedures, each animal was lightly anesthetized by inhalation of halothane to allow the TMJ injection.

Rats received a 50-μl injection of formalin diluted in saline (1.5%) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl) previously filled with formalin 1.5%.

Following the TMJ injection, the rat was placed in the test chamber (30 × 30 × 30 cm mirrored-wood chamber with glass at the front side) and nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time—seconds) and flinching the head (number of head flinches) were quantified for 30 min (10 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni et al., 2001; Gameiro et al., 2003). An investigator, who was blind to the rat's group assignment, made the analysis of the behaviors.

At the end of each experiment, Evans blue dye (0.1%, 5 mg/kg) was injected systemically (via penile vein) in order to confirm the TMJ injection site at postmortem, as previously described (Haas et al., 1992) by the visual examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

2.5. Drug treatments

In order to evaluate the role of endogenous opioids in nociceptive changes induced by stress, one opioid antagonist (naloxone) and one agonist (morphine) were used. In experiment 1, naloxone 10 mg/kg (Vissers et al., 2004) was administered i.p. immediately after the acute restraint stress (1 h) and before the TMJ formalin test. In experiment 2, the animals were submitted to chronic stress as described above. After 40 days of treatment (control group was left undisturbed in their home cage), the rats were injected i.p. with morphine 1.0 mg/kg (Torres et al., 2003a), 5.0 mg/kg (D'Amato et al., 1999) or saline ($n=6$/group) 30 min before the administration of formalin 1.5% into the TMJ. Morphine sulfate was dissolved in 0.9% saline and administered i.p. immediately after the last stress session in a volume of 1.0 ml/kg.

2.6. Statistical analysis

Statistical analysis of plasma corticosterone and ACTH data was made using the Student's *t*-test. Data were previously transformed to square-root or log, as indicated by the program SAS (version 8.2 for windows). The sum of rubbing and flinching responses exhibited by each animal was computed. The comparison between two groups was made by Student's *t*-test. The comparison of more than two groups (morphine effect analysis) was made by two-way analysis of variance (ANOVA). All values are given as mean±standard error of the mean (SEM). A level of 5% was taken as evidence of statistical significance. Data were analyzed using SAS (version 8.2 for windows) by Institute Inc., Cary, NC, USA—licensed to Universidade Estadual de Campinas.

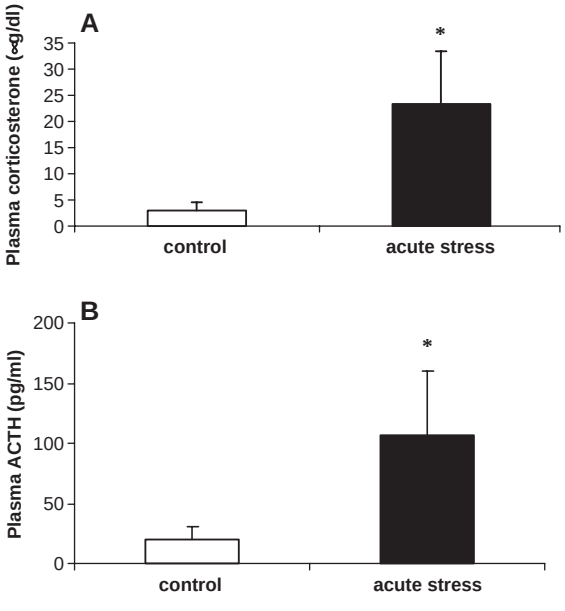

Fig. 1. (A) Plasma corticosterone level after a single restraint session (1 h). Each data point represents mean±SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student's *t*-test. (*) Indicates significant difference compared with the control rats at $p<0.0001$. (B) Plasma ACTH level after a single restraint session (1 h). Each data point represents mean±SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student's *t*-test. (*) Indicates significant difference compared with the control rats at $p=0.0011$.

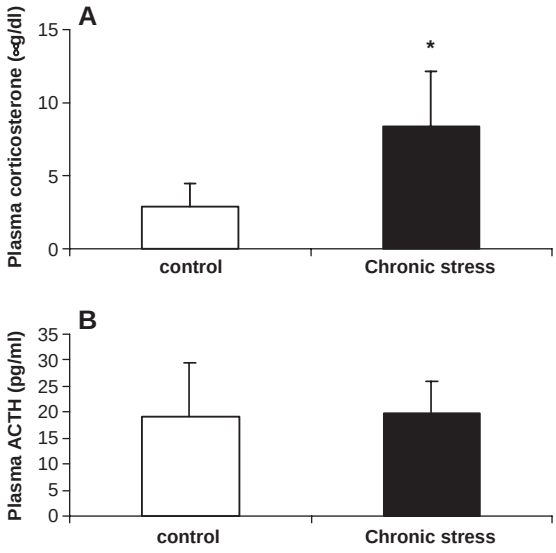

Fig. 2. (A) Plasma corticosterone level after the last session of chronic stress (8-week). Each data point represents mean±SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student's *t*-test. (*) Indicates significant difference compared with the control rats at $p=0.0261$. (B) Plasma ACTH level after the last session of chronic stress (8-week). Each data point represents mean±SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student's *t*-test. There was no statistical difference between control and stressed groups ($p=0.4134$).

3. Results

3.1. Effects of stress procedures on plasma corticosterone and ACTH levels

This experiment was carried out to define the efficacy of restraint in inducing stress-like hormonal modifications in the acute and chronic groups. There was a significant increase in plasma corticosterone ($p<0.0001$, *t*-test, Fig. 1A) and ACTH levels ($p=0.0011$, *t*-test, Fig. 1B) after a single restraint session for 1. The chronically stressed rats showed higher levels of corticosterone than control animals ($p=0.0261$, *t*-test, Fig. 2A). However, there was no difference in plasma ACTH

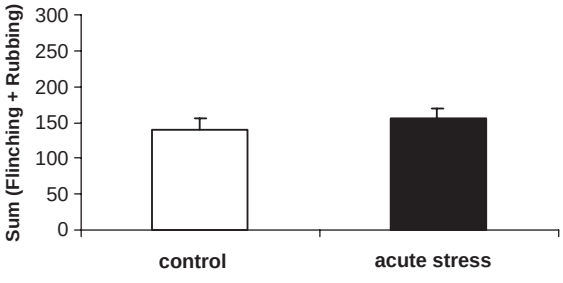

Fig. 3. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 μl, 1.5%) previously submitted to 1 h of restraint ($n=6$) or left undisturbed in their home cage ($n=6$). Each column represents the mean. Error bars indicate the SEM. No significant differences were found in nociceptive responses for control vs. stressed group ($p=0.125$, *t*-test).

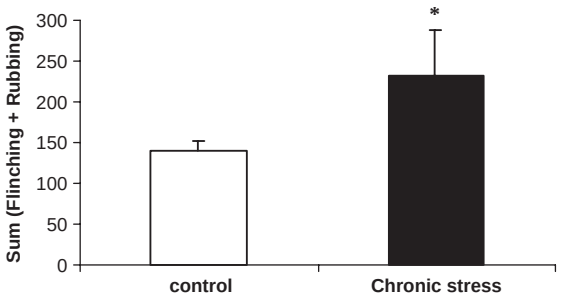

Fig. 4. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 μl, 1.5%) previously submitted to chronic stress ($n=6$) or left undisturbed in their home cage ($n=6$). Each column represents the mean. Error bars indicate the SEM. (*) Significant difference between the control and stressed group ($p<0.05$, t-test).

levels between chronically stressed vs. control rats ($p=0.4134$, t-test, Fig. 2B).

3.2. Effect of acute stress on nociceptive behavioral responses

The exposure to a single restraint session for 1 h did not affect the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats (Fig. 3). There was no statistical difference ($p=0.125$) between the control group (non-stressed) and the stressed group.

3.3. Effect of chronic stress on nociceptive behavioral responses

Results are shown in Fig. 4. Immediately after the last restraint session (1 h/40 days), the chronically stressed animals were hyperalgesic. A statistically significant increase in the nociceptive behavioral responses was observed in the stressed group when compared with the control group ($p<0.05$, t-test).

3.4. Effect of chronic restraint stress on rubbing spontaneous behaviors

We also evaluated the spontaneous rubbing in order to exclude the possibility of an increased motor behavior induced by the chronic stress procedure. The chronic stressed rats exhibited a similar behavior than those of the control group

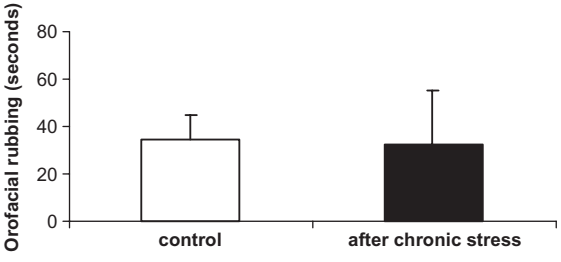

Fig. 5. Duration of the orofacial rubbing behavior in rats previously submitted to chronic stress ($n=6$) or left undisturbed in their home cage ($n=6$). Each column represents the mean. Error bars indicate the SEM. There was no statistical difference between control and stressed groups ($p=0.7488$, Mann–Whitney test).

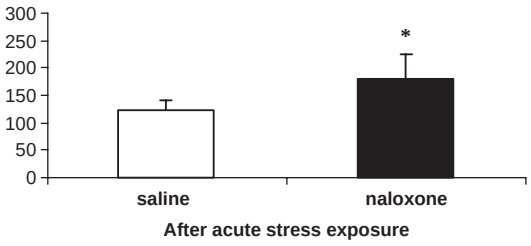

Fig. 6. Effects of naloxone or saline on formalin-treated animals (50 μl, 1.5%) previously submitted to acute restraint stress ($n=6$/group). Each column represents the mean. Error bars indicate the SEM. (*) Indicates significant difference compared with the saline group ($p=0.0011$, t-test).

(non-stressed) when saline was administered in the rat's TMJ ($p=0.7488$, Mann–Whitney test, Fig. 5).

3.5. Effect of naloxone on nociception in rats submitted to acute restraint stress

After 1 h of immobilization, the injection of naloxone evoked an increase in nociceptive behaviors (180.69±45.29), when compared with saline (123.14±16.53). The increase in the sum of nociceptive behaviors (flinching+rubbing) was statistically significant ($p=0.0489$, t-test, Fig. 6).

3.6. Effect of morphine on nociception in repeatedly stressed and control rats

Results referring to the analgesic effect of morphine are shown in Fig. 7. ANOVA revealed significant interaction between stress and morphine ($p=0.003$). Post hoc tests (Tukey)

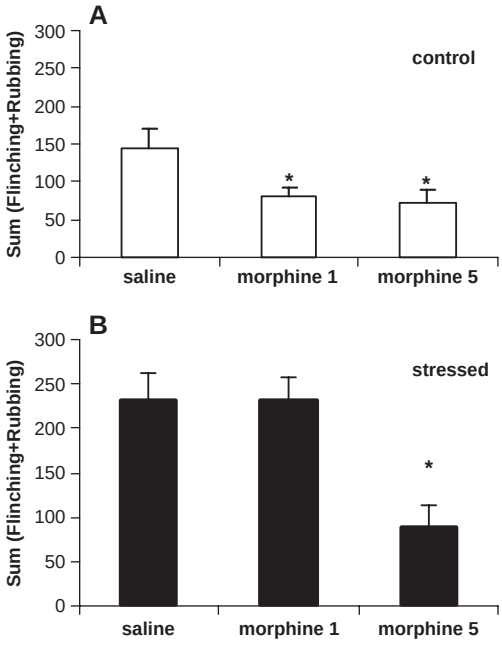

Fig. 7. Sum of nociceptive responses to morphine (1 or 5 mg/kg, i.p.) or saline after 40 days chronic restraint stress. *Panel A:* control groups ($n=6$/group); *Panel B:* stressed groups ($n=6$/group). Each column represents the mean. Error bars indicate the SEM. (*) Significant difference compared to saline group ($p<0.05$, ANOVA+Tukey).

revealed that morphine administration produced a significant reduction of nociceptive behavioral responses in the control group (non-stressed). Morphine 1 mg/kg reduced the nociceptive responses 30 min after the administration ($p<0.05$), and morphine 5 mg/kg also had this effect ($p<0.05$). In the stressed group, morphine had an effect only at the dose of 5 mg/kg ($p<0.05$) when compared to the saline group.

4. Discussion

A variety of environmental and/or stressful stimuli have been shown to elicit analgesia, a phenomenon often referred to as stress-induced analgesia (SIA) (Amir and Amit, 1978; Watkins et al., 1982; Furuta et al., 2003). In the present study, a single exposure (1 h) to restraint stress did not reduce the nociceptive behavioral responses evoked by nociceptive chemical stimulation (formalin 1.5%) of the rat's TMJ. The ability of the procedure to induce stress was confirmed by higher corticosterone and ACTH levels in restraint rats than those of control rats. One effect of acute stress exposure is a reduction of reflex responses that include tail or hinpaw withdrawal and licking in rats (Bodnar et al., 1980; Lewis et al., 1980; Gamaro et al., 1998). Although most of these responses involve a spinal–brain stem–spinal loop and appear to be purposeful, they do not depend upon cortical processing of nociceptive signals that result in pain perception (Mauderli et al., 2000; Vierck et al., 2002). King et al. (2003) showed that acute stress diminishes reflex responses to nociceptive input while enhancing operant responding to the same stimuli (nociceptive thermal stimuli), suggesting that stress-induced hyporeflexia can coexist with stress-induced hyperalgesia. According to these findings, we speculate that a single restraint session did not induce an analgesic effect on rats submitted to the TMJ formalin test, which evokes nociceptive responses that have an organization different from those related to innate reflexes, for example tail-flick response that can be modulated directly at spinal levels (King et al., 2003). Moreover, the absence of stress-induced analgesia in our model may be related to the different site of formalin injection. As described in the Introduction, nociceptive response evoked by cutaneous stimuli differs from the one evoked by deep stimuli. The discrepancy between nociception models in their susceptibility to modulation by stress is evident not only in the present results, but also in the partial and transient analgesic effects found in other studies employing the formalin test (Amir and Amit, 1979; Fuchs and Melzack, 1996; Aloisi et al., 1998).

The increase in nociceptive behavioral responses produced by chronic restraint stress has important implications in relation to other studies that have reported a hyperalgesic effect after exposure to a variety of stressors (Satoh et al., 1992; Quintero et al., 2000; Torres et al., 2003a,b). The present study confirmed the previously reported results for nociceptive responses, using an experimental model for the study of nociception from deep tissue injury: the TMJ pain. Although an extensive literature has reported the relationship between stress and chronic facial pain (Grzesiak, 1991; Vanderas, 1994; Korszun, 2002), little is known about the physiopathology of neural mechanisms that mediate the effects of repeated stress on pain sensitivity and affective states. The development of experimental models such as the present one may provide further information about the mechanisms involved in these painful conditions and may be used to test the efficacy of drugs. In the current study, we were able to induce an increase in nociceptive behaviors following a repeated restraint stress procedure. In agreement with our results, previous studies have also found that chronic stress can elicit hyperalgesia rather than hypoalgesia (Lewis et al., 1980; Quintero et al., 2000; Torres et al., 2003a,b). Previous works have suggested that, when animals are repeatedly submitted to the same stressor, some behavioral and physiological consequences of stress exposure are reduced (habituation). For example, ACTH or corticosterone levels are reduced after repeated exposure to the same stressor (Marti and Armario, 1998; Torres et al., 2001b), although negative results have been reported (Dal-Zotto et al., 2000). In our model, corticosterone and ACTH levels were reduced after the end of stress session in 8-week restraint rats. However, the ability of the procedure to induce stress was confirmed by higher corticosterone levels in 8-week restraint rats than those of control rats. We also evaluate the spontaneous rubbing in order to exclude the possibility of an increased motor behavior induced by the chronic stress procedure. The chronic stressed rats exhibited a similar behavior than those of the control group (non-stressed) when saline was administered in the rat's TMJ. This result suggests the increase of flinching and rubbing behaviors is a hyperalgesic effect induced by chronic stress. The mechanism through which repeated stress produces hyperalgesia is not clear; in fact, more than one mechanism could be involved. Satoh et al. (1992) suggested that mechanical hyperalgesia induced by prolonged cold stress involves peptide-containing primary afferents (substance-P and calcitonin-gene-related peptide). Quintero et al. (2000) showed that the increased thermal and chemical nociception observed after sub-chronic swimming stress might be mediated by changes in the activity of the central serotoninergic system. Torres et al. (2003b) suggested that repeated restraint stress could induce an adaptative response in chronically stressed rats, which can lead to a desensitization of adenosine receptors. In other study, Torres et al. (2003a) also showed that chronically stressed rats displayed decreased morphine effects on nociception.

In the last experiment, we tested control and repeatedly restrained rats injected with morphine (1 and 5 mg/kg) in the TMJ formalin test. Our results demonstrate that repeatedly stressed rats display decreased morphine effects on nociception compared to non-stressed controls. Although it has been described that morphine induces analgesia in a dose-related manner, in the present work there was no observation of any difference between the two doses of morphine administrated in the control group (non-stressed). This discrepancy may be due to the different nociception assay used. We know that nociceptive transmission and modulation are different even

when distinct superficial nociceptive assays are used (Fang and Proudfit, 1998). The stressed group needed an increased dose to show the classic analgesic effect of morphine. This change in sensitivity to morphine may be a result of alterations in treatment-induced peptide release, i.e., persistent activation of opiate peptide receptors by endogenous opioids released during restraint stress could lead to receptor down-regulation, but it is possible that interactions with other released neurotransmitter could induce these effects, for example, serotonin, glutamate, adenosine and other opioid receptor systems have also been involved (Fitzgerald et al., 1996; Torres et al., 2003b). The tolerance of response to morphine observed in the present study agrees with the hypothesis suggested by previous studies that chronic restraint stress could modify the activity of opioid systems (for review, see Drolet et al., 2001). Changes in the analgesic effect of morphine observed in stressed rats might be due to alterations in central or peripheral opioid receptors, both in their affinity or number, or these changes might be due to alterations in other neuro-transmitter or hormonal systems able to interact with these receptors. Omiya et al. (2000) showed that hypofunction of the supraspinal mu-opioid receptor may explain the hyperalgesic effect of repeated cold stress loading in mice. Since morphine exerts its antinociceptive effects primarily through mu-opiate receptor subtype, the altered responses observed in animals submitted to TMJ formalin test after chronic stress might be due to changes at the level of these receptors. Future studies should evaluate the activity of the opioid receptors in this model. We suggest the influence of endogenous opioids released during chronic stress on the development of tolerance to morphine antinociceptive effects. This conclusion was based in the fact that restraint stress can release endogenous opioids, as was observed by the effect of naloxone on the augment of nociceptive responses in rats submitted to acute stress. In this case, it was expected that acute stress would reduce formalin-induced nociception, a finding not observed in our study. We believe that, in our model, the effects of endogenous opioids were counter-balanced by the enhancement in pain perception evoked by stress-induced anxiety. Studies have shown that hyperalgesia is elicited by some experimental conditions (Cornwall and Donderi, 1988; Al Absi and Rokke, 1991; Meaguer et al., 1998). In our laboratory, we have demonstrated that a single exposure to restraint stress (1 h) induced a high level of anxiety in the elevated-plus-maze (data not shown). This factor could also be a determinant in the absence of stress-induced analgesia. Continued research concerning the mechanisms of stress-induced hyperalgesia may be relevant to the study of the etiology of chronic pain disorders, like the temporomandibular disorder.

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