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Development of a behavioral model of TMJ pain in rats: the TMJ formalin test

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Abstract

Temporomandibular joint (TMJ) pain conditions are poorly understood. Since formalin is a noxious stimulus widely used in animal behavioral experiments for studying pain mechanisms, the aim of this study was to develop a behavioral model to study the TMJ pain conditions by characterizing the nociceptive behavioral responses induced by the injection of formalin into the TMJ region of rats. NaCl (0.9%) or different concentrations of formalin (0.5, 1.5, 2.5 or 5%) were administrated into the TMJ region. The formalin-induced behavioral responses characterized by moving the mandible, rubbing the orofacial region and flinching the head quickly were quantified for 45 min. The TMJ injection of formalin significantly increased the asymmetrical orofacial rubbing and head flinching behaviors, but not the movement of the mandible with concentrations of 1.5% and above ($P < 0.05$, Dunn's test) when compared with the NaCl (0.9%) injection. These responses were significantly reduced ($P < 0.05$, Mann–Whitney test) by the co-application of lidocaine *N*-ethyl bromide quaternary salt, QX-314 (2%), and by the administration of intraperitoneal morphine (4 mg/kg) 30 min prior to the TMJ formalin injection. This study demonstrates that the injection of formalin into the TMJ region of rats produces quantitative nociceptive behaviors constituting a novel behavioral model for TMJ pain.

Keywords: Temporomandibular joint; Formalin test; Nociception; Morphine; QX-314; Rat

1. Introduction

Although they represent an important clinical entity, temporomandibular joint (TMJ) pain conditions are poorly understood. This is due, in part, to the limited experimental models available to study these conditions. Therefore, the development of experimental models that allow the study of the mechanisms underlying these pain conditions and the efficacy of different pharmacological approaches that can be used to treat them are of great clinical relevance.

Formalin is a noxious stimulus commonly used in animal behavioral experiments. The formalin test originally described by Dubuisson and Dennis (1977) consists of a subcutaneous (s.c.) formalin injection into the rat hind paw that produces a biphasic nociceptive response which is responsive to many classes of analgesic drugs (Hunskaar and Hole, 1987; Coderre et al., 1990; Rosland et al., 1990; Taylor et al., 1995). The typical time course of the behavioral and electrophysiological responses to formalin consists of an early phase of short-lasting response, followed by a continuous prolonged response that mimics some features of post-injury pain in man (Shibata et al., 1989; Dickenson and Sullivan, 1987; Raboisson et al., 1995).

The formalin test was further adapted for assessing pain in the superficial tissues of the orofacial region by Clavelou et al. (1989). In the orofacial formalin test, formalin is subcutaneously injected into the upper lip of rats and also evokes a biphasic nociceptive response, although in this case, such a response is related to the animal's behavior of rubbing the injected area.

The orofacial formalin test is a valid and reliable model of nociception (Cadet et al., 1998; Clavelou et al., 1989, 1995; Dallel et al., 1995), but does not allow the study of the mechanisms involved in deep craniofacial pain conditions. It is important to point out that the pain response evoked by cutaneous stimuli differs from the one evoked by deep stimuli. The fact that greater sensory disturbances occur in pain conditions involving deep tissues rather than those involving cutaneous tissues has already been reported in the literature (Mense, 1986; Sessle and Hu, 1991). It has

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been suggested that orofacial deep inputs evoked by algesic substances are especially effective, versus orofacial cutaneous inputs, in inducing neuroplastic changes (Yu et al., 1993) and Fos protein expression (Zou et al., 1999) in trigeminal brain-stem nociceptive neurons. Many deep craniofacial pain conditions, such as TMJ pain conditions, are associated with manifestations of pain spread and referral (Sessle, 1995). The pathogenesis, diagnosis and treatment of these pain conditions are still controversial.

Thus, considering that the effects elicited by the injection of formalin into deep tissues such as the TMJ have not yet been explored, the aim of this study was to apply different concentrations of formalin into the TMJ region of rats to develop an experimental behavioral model of TMJ pain and verify if the model proposed is sensitive to morphine and to the hydrophilic lidocaine derivative, QX-314 (2%).

2. Methods

2.1. Animals

This study was carried out on 54 male Wistar rats (150–250 g) housed in standard clear plastic cages with soft bedding (five/cage) with free access to food and water ad libitum. They were maintained in a temperature-controlled room (23 ± 1°C) with a 12/12 h light–dark cycle with lights on at 06:00 h) for at least 1 week prior to the experiments. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

2.2. Testing procedure for TMJ pain

Testing sessions took place between 08:00 and 17:00 h in a quiet room maintained at 23°C. Each animal was first placed in a test chamber (30 × 30 × 30 cm mirrored-wood chamber with glass at the front side) for a 30 min habituation period to minimize stress (Abbott et al., 1986). Rats did not have access to food or water during the test. After the period of adaptation, the animal was removed from the test chamber and lightly anesthetized by inhalation of halothane to allow the TMJ injection.

The behavior of normal, intact rats (non-treated, $n = 5$) and of rats submitted just to needle introduction into the TMJ region (sham, $n = 5$) was evaluated during 45 min and the most frequent behaviors, such as rubbing the orofacial region and moving the mandible (animal moves the mandible in chewing-like motions), were quantified in 12 blocks of 3 min.

Rats received a 50 μl injection of different concentrations of diluted formalin or saline ($n = 10$) into the left TMJ region. Diluted formalin solutions were prepared from commercially (Sigma) available stock formalin further diluted in isotonic saline to 0.5 ($n = 7$), 1.5 ($n = 8$), 2.5 ($n = 8$) and 5% ($n = 9$). The stock formalin is an aqueous solution of 37% of formaldehyde.

In another set of experiments, the effect on the nociceptive response of blockade of peripheral inputs was studied by the co-application of 2% lidocaine *N*-ethyl bromide quaternary salt (QX-314, Research Biochemicals, Inc.) dissolved in 0.9% saline with 2.5% formalin (50 μl, $n = 6$). An additional group received an intraperitoneal injection of 4 mg/kg of morphine sulfate (Merck) also dissolved in 0.9% saline in a volume of 10 ml/kg, 30 min prior (Clavelou et al., 1989) to the injection of 50 μl of 2.5% formalin ($n = 6$) into the TMJ region. Formalin (2.5%, 50 μl) was used as a control to examine the effects of QX-314 and morphine on the formalin-induced behavioral responses.

The injections into the TMJ region were performed via a 30-gauge needle introduced into the TMJ capsule of the left TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl) previously filled with one of the different concentrations of formalin or QX-314 (2%). The volume of the TMJ injections was 50 μl.

To control for the possibility that the behaviors induced by formalin might have resulted from its effect on regions outside the immediate TMJ region, off site injections were performed. Formalin (2.5%) at the same volume previously used was injected into the left masseter muscle.

Following the TMJ injection, the rat immediately recovered from the anesthesia and was returned to the test chamber for a 45 min observation period. The most reproducible and objective behavioral responses observed were moving the mandible in a chewing-like motion, rubbing the orofacial region asymmetrically with the ipsilateral fore or hindpaw (Clavelou et al., 1989, 1995) or flinching the head in an intermittent and reflexive way characterized by high frequency shakes of the head. Flinching the head was a new behavior observed after the injection of formalin into the TMJ region.

For each block of 3 min, the behavior characterized by moving the mandible or rubbing the orofacial region was quantified by the amount of time that the animal exhibited it and the behavior characterized by flinching the head was quantified by its occurrence. The analysis of the behaviors was made by an investigator who was blind to the rat's group assignment.

After the conclusion of each experiment, Evans blue dye (0.1%, 5 mg/kg) was injected systemically in order to confirm the TMJ injection site at post-mortem, as previously described (Hass, 1992) by the visual examination of formalin-induced plasma extravasation of Evans Blue dye bound to plasma protein. This procedure also allowed confirmation that the plasma extravasation induced by the TMJ injection at the correct site was restricted to the immediate TMJ region.

The TMJ formalin nociceptive behaviors were evaluated separately first. From a theoretical perspective, the occurrence of a given behavior is proportional to the proportion of time that the behavior occupies. Thus, considering that the

flinching of head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The different TMJ formalin nociceptive behaviors were also evaluated together by their sum.

2.3. Statistical analysis

Data with homogeneity of variance were analyzed using the *t*-test or one-way analysis of variance (ANOVA) and multiple post-hoc comparisons were performed using Dunnett's test or Newman–Keuls test. Data without homogeneity of variance were analyzed using the Mann–Whitney test or the Kruskal–Wallis one-way ANOVA on ranks, and multiple post-hoc comparisons were performed using Dunn's test. The correlation between formalin concentrations and formalin-induced nociceptive behaviors was tested with Spearman's rank correlation coefficient. A probability level of less then 0.05 was considered to indicate statistical significance. Data are presented in the figures and text as means ± SEM.

3. Results

3.1. Effect of formalin injection into the TMJ region on each behavioral response

The TMJ injection of increasing concentrations of formalin significantly increased ($P < 0.05$, Dunn's test) the behavior characterized by flinching the head and rubbing the orofacial region from the concentration of 1.5% (Fig. 1), but did not significantly affect ($P > 0.05$, ANOVA) the behavior of moving the mandible compared with the saline injection (Fig. 2). In Fig. 2, it can also be seen that the behavior characterized by moving the mandible is significantly greater in the saline-treated rats compared with the non-treated rats. Taken together, these data demonstrate that

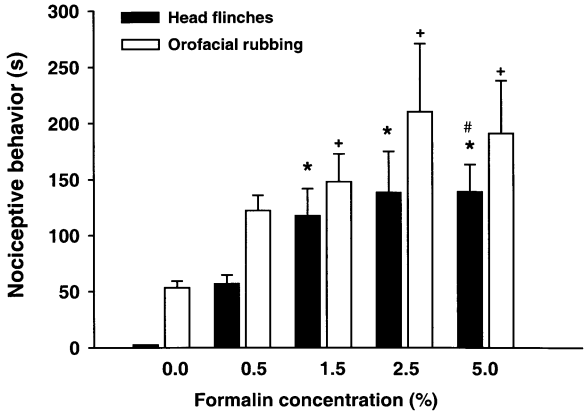

Fig. 1. Effect of increasing concentrations of TMJ formalin on the duration of the head flinches or of the orofacial rubbing behavior. Each column represents the mean. Error bars indicate the SEM. For rubbing, (+) indicates a significant difference from 0% formalin; and for head flinches, (*) and (#) indicate significant differences from 0 and 0.5% formalin, respectively ($P < 0.05$ for all comparisons by Dunn's test).

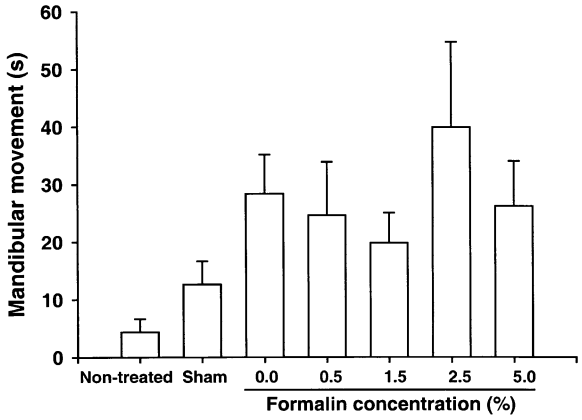

Fig. 2. Effect of increasing concentrations of TMJ formalin on the duration of mandibular movement. Each column represents the mean. Error bars indicate the SEM ($P > 0.05$, ANOVA). There was no significant difference between sham (animals submitted just to needle introduction into the TMJ region) and non-treated animals (intact animals), but there was a significant difference when animals submitted to the saline injection were compared with non-treated ones ($P < 0.05$, Newman–Keuls test).

the behavior of moving the mandible is not induced by formalin. The amplitude of the formalin-induced rubbing and flinching responses seemed to reach a maximum at a concentration of 2.5%. There was no significant difference between the formalin concentrations on the orofacial rubbing response. In contrast, the head flinching response induced by 5% formalin was significantly greater than the one induced by 0.5% formalin.

The injection of formalin into the TMJ region induced only one phase of rubbing and flinching responses as shown by the time course of the responses to the TMJ injection of 2.5% formalin (Fig. 3).

It was also observed that all animals that received the formalin injection into the TMJ region from 0.5 to 5% tumbled the head to the injected side.

In contrast, the formalin injection into the masseter muscle

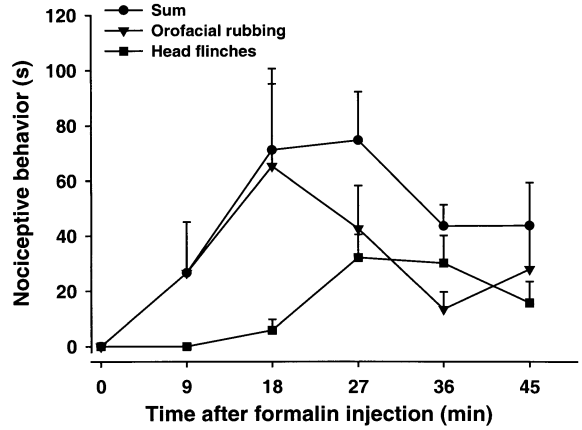

Fig. 3. Time course of the 2.5% formalin nociceptive behaviors characterized by head flinches, orofacial rubbing and by the sum of both behaviors. Each symbol represents the mean of the sum of three blocks (9 min) of the observation period. Error bars indicate the SEM.

did not induce the behaviors characterized by flinching the head or tumbling the head to the injected side in any of the animals tested nor did it exacerbate the orofacial rubbing behavior. In fact, this last behavior (72.23 ± 11.21, $n = 8$) was comparable with the rubbing behavior observed after the injection of saline into the TMJ region (53.40 ± 5.90, $n = 10$; t-test, $P = 0.14$).

3.2. Effect of the peripheral QX-314 (2%) and systemic morphine on the formalin-induced rubbing and flinching responses

The co-application with hydrophilic quaternary derived lidocaine, QX-314 (2%), significantly reduced and completely blocked the formalin-induced flinching ($P < 0.05$, Mann–Whitney test) and rubbing ($P < 0.05$, t-test; Fig. 4) responses, respectively. QX-314 is a charged quaternary lidocaine derivative that does not readily diffuse across membranes. The intraperitoneal administration of 4 mg/kg morphine (10 ml/kg) 30 min prior to the 2.5% formalin injection into the TMJ region of rats also significantly reduced the formalin-induced flinching ($P < 0.05$, Mann–Whitney test) and rubbing ($P < 0.05$, t-test; Fig. 4) responses. The behavior characterized by tumbling the head to the injected side was inhibited by both QX-314 (2%) and morphine.

3.3. Effect of formalin injection into the TMJ region on the behavioral responses evaluated together by the sum of rubbing and flinching responses

The graph shown in Fig. 3 illustrates the time course of rubbing and flinching responses induced by the injection of 2.5% formalin into the TMJ region of rats when they were evaluated separately and by their sum. It may be seen that the animals displayed the flinching and rubbing responses in an alternate manner. When the orofacial rubbing response reaches the peak (18 min post-TMJ formalin injection), the flinching response is small, however, when the lifting response reaches the peak 9 min later, the rubbing response decreases.

Table 1
Correlation coefficient (R) between formalin concentrations and flinching or rubbing responses evaluated separately and together by their sum

Behavioral response	Rubbing the orofacial region	Flinching of head	Sum (rubbing + flinching)
R	0.47*	0.76*	0.78*

* Significant correlations are indicated by asterisk.

The correlation analysis of the data demonstrated a significant correlation ($P < 0.05$) between formalin concentrations and the rubbing and flinching responses. The correlation was stronger when rubbing and flinching responses were evaluated together by their sum (Table 1; Fig. 3). When the formalin-induced rubbing and flinching responses were evaluated together, the injection of formalin into the TMJ region at the concentrations of 0.5, 1.5, 2.5 and 5% significantly increased ($P < 0.05$, Dunnett's test) the behavioral responses from the concentration of 1.5% (263.92 ± 35.47) compared with the saline administration (53.34 ± 5.93). The amplitude of these responses reached a maximum at a concentration of 2.5% and slightly decreased above that. There was a significant difference between the concentrations of 2.5 or 5% and 0.5% ($P < 0.05$, Newman–Keuls test; Fig. 5).

4. Discussion

The present study shows that the injection of formalin but not saline into the TMJ region of rats produces quantitative, and stereotyped nociceptive behaviors characterized by

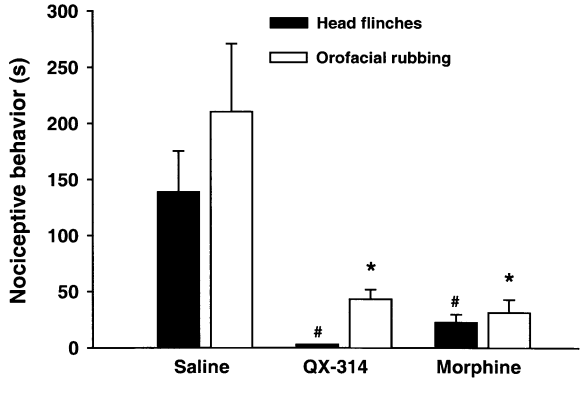

Fig. 4. Effect of local hydrophilic quaternary derived lidocaine, 2% QX-314, and of systemic morphine, 4 mg/kg, on the duration of the 2.5% formalin-induced head flinches or orofacial rubbing behavior. Each column represents the mean. Error bars indicate the SEM. Significant differences between the experimental and saline control groups are indicated by (#) for head flinches ($P < 0.001$, Mann–Whitney test) and (*) for orofacial rubbing ($P < 0.05$, t-test).

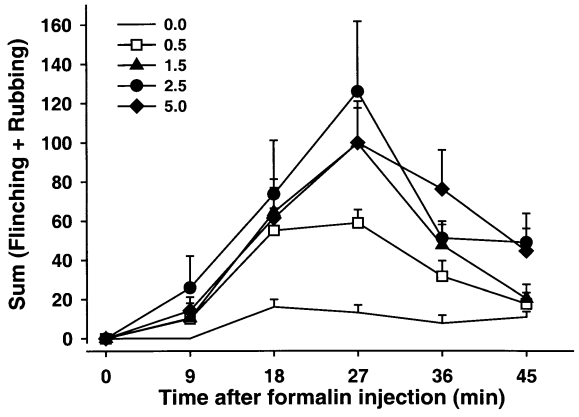

Fig. 5. Time course of the sum of flinching and rubbing behaviors after TMJ injection of increasing concentrations of formalin. Data points represent the mean of the overall nociceptive response of the sum of three blocks (9 min) of the observation period. Error bars indicate the SEM. Black symbols indicate significant difference ($P < 0.05$, Dunnett's test) compared with the 0% formalin (saline) group.

flinching the head quickly, by tumbling the head to the injected side and by rubbing the orofacial region. A similar inefficiency of saline in inducing nociceptive behaviors has already been noted in the paw (Wheeler-Aceto and Cowan, 1993) and in the upper lip formalin test (Clavelou et al., 1995).

The behaviors characterized by flinching the head and tumbling the head to the injected side were not observed in control rats (non-treated, needle introduction, saline injection and formalin injection into the masseter muscle). The act of flinching the head resembles the nociceptive behavior characterized by flinching the paw submitted to the formalin challenge. Conversely, the behavior of tumbling the head to the injected side is similar to the behavior observed in many patients suffering from intense pain in the orofacial region.

The evaluation of the behavior characterized by tumbling the head is very difficult, because the animal tumbles the head progressively along the experiment. Thus, considering that the exact moment from which the animal starts tumbling the head is very variable and subjective, we decided to evaluate it only by its incidence. The results showed that all of the animals treated with formalin tumbled the head to the injected side (data not shown).

In contrast to the behaviors described above, rubbing is a normal behavior of the animal essential for thermoregulation (Roberts et al., 1974) and for the distribution of pheromones for social signaling (Batsell et al., 1990). It consists of prolonged episodes of care and attention to the pelage in a highly organized manner (Griswold et al., 1977). In this case, the act of rubbing follows a rostrocaudal progression with stereotyped and symmetrical face rubbing sequences at the transition from one body region to the other (Gispen and Issacson, 1981). Among the multiplicity of the functions of rubbing behavior, its role as a nociceptive response is of particular importance to experimental pain research (Vos et al., 1998). In the present study, the behavior of rubbing the orofacial region was strongly exacerbated by formalin and strongly correlated with formalin concentration. The animals rubbed the orofacial region in an asymmetric manner with the forepaw and, eventually, with the hindpaw. The act of rubbing the orofacial region with the ipsilateral forepaw was frequently accompanied by similar movements of the contralateral, as previously described after the formalin injection into the perioral area (Clavelou et al., 1989).

Although the behavior of rubbing the orofacial region resembles that of washing the face, asymmetric and prolonged face rubbing is not displayed spontaneously by normal, intact rats (Vos et al., 1998).

The patterns of rubbing provoked by local irritation or by noxious stimulation have an organization different from that related to the maintenance of the pelage, thermoregulation or social signaling. In this case, the rubbing activity occurs more specifically at the painful body area (Cohen and Price, 1979; Dubuisson and Dennis, 1977; Clavelou et al., 1989, 1995; Vos et al., 1998) and it appears to be aimed at removing the cause of pain.

Another normal behavior analyzed after the formalin injection into the TMJ region was the mandible movement. In contrast to the rubbing response, the mandible movement was not exacerbated by the injection of increasing concentrations of formalin into the TMJ region since there was no significant difference in such behavior when the animals that received formalin were compared with those that received saline into the TMJ region. These data demonstrate that only rubbing and flinching responses were induced by formalin injection into the TMJ region.

However, it was observed, that mandible movement is a normal behavior slightly and significantly exacerbated by the introduction of a needle into the TMJ region and by the injection of saline, respectively. This observation indicates that such a behavior is not related to the nociceptive agent, formalin, but more likely to the volumetric stimulation of the tissue (Hu et al., 1994) although the needle introduction into the TMJ may have contributed to this.

One of the characteristics of the formalin response is its biphasic pattern, however, the injection of formalin into the TMJ region of rats demonstrated just one response phase as a consequence of the necessity of inducing anesthesia by the inhalation of halothane to allow the TMJ injections. It is important to point out that TMJ injections without any type of anesthetic induction would be practically impossible (the TMJ is a deep tissue with difficult access) and ethically unacceptable.

However, it is known that the initial response (phase 1) is generally attributed to a direct effect of formalin on the sensory receptors (Dubuisson and Dennis, 1977) and that the later response (phase 2) is related to the subsequent development of inflammation and spinal cord sensitization (Hunskaar and Hole, 1987). The later response, like the one described in the present study, better characterizes overt pain and bears more resemblance to clinical pain than that provoked by a transient stimulus. Thus, it is of greater clinical relevance.

In the present study, formalin was chosen rather than any other nociceptive agent because of its effectiveness in inducing quantifiable and well defined behaviors which were reproducible. The long-lasting nociceptive stimulus induced by formalin also allows better characterization of the onset and duration of analgesic agents (Dubuisson and Dennis, 1977). Future studies could examine the ability of other more nerve specific agents, such as mustard oil or capsaicin, in producing well defined and quantifiable nociceptive behaviors when injected into the TMJ region of rats.

The present study demonstrates that formalin-induced rubbing and flinching responses only appeared at formalin concentrations of above 0.5%. Clavelou et al. (1995), using the upper lip formalin test, demonstrated that the second phase of nociceptive rubbing activity also appeared only at formalin concentrations of above 0.5%. Similar findings for paw elevation, licking and biting the paw were obtained by Coderre et al. (1993) using the paw formalin test.

In our study, the formalin-induced rubbing and flinching

responses are related to formalin concentration and reached a maximum at 2.5%. Again, these findings are in agreement with those obtained by Clavelou et al. (1995) when using the upper lip formalin test.

The anesthetic blockade produced by the co-application of the quaternary hydrophilic lidocaine derived, QX-314 (2%), with formalin completely eliminated and significantly reduced the formalin-induced flinching and rubbing responses, respectively. Consistent with these findings, it has been demonstrated, using the paw formalin test, that QX-314 (2%) completely blocks the behavioral and cardiovascular responses induced by formalin (Taylor et al., 1995). Considering that the rubbing response is a normal behavioral response and the injection of formalin into the TMJ region produced a significant exacerbation of such a response, it is not surprising that the co-application of QX-314 completely blocked the flinching, but not the rubbing response.

Systemic morphine administration significantly reduced the formalin-induced rubbing and flinching responses at a concentration (4 mg/kg) that did not impair locomotor activity. These results may be compared with those observed in the upper lip formalin test where morphine raised the thresholds of the rubbing activity but did not abolish it (Clavelou et al., 1989). These results validate the rubbing and flinching responses as reliable pain measures.

The TMJ formalin-induced flinching and rubbing responses followed an alternate pattern, and seemed to complement each other. In fact, when animals showed a greater rubbing activity, they tended to display the flinching behavior in a frequency proportionally smaller, and vice-versa (see Fig. 3). This explains why the variability of the results between animals was greater when the rubbing and flinching behaviors were evaluated separately than by their sum.

It is noteworthy that, compared with rubbing, the head flinching behavior showed a greater correlation with the formalin concentration. This result suggests that the head flinching is a characteristic behavior of TMJ pain.

Taking into account the formalin nociceptive behavior by summing the flinching and rubbing responses allows a description of the overall changes in behavior that better reflect the pain intensity as shown by the stronger correlation between formalin concentration and the sum of such behaviors than between formalin concentration and the single behaviors. This is consistent with the idea that the combination of several behaviors provides a better measure of pain intensity than any single behavior (Abbott et al., 1995; Coderre et al., 1993; Tjolsen et al., 1992).

We conclude that the TMJ formalin nociceptive rubbing and flinching responses may be used as an index of TMJ pain. When the behaviors are evaluated separately, this permits the study of different components of the pain experience which might be modulated separately, and when evaluated together, they are extremely useful for assessing the full impact of analgesic drugs on nociception.

Acknowledgements

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