Research report
Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies

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1. Introduction

Stress can have bilateral effects on pain-related phenomena. First, acute stress can produce analgesia in animals and humans (stress-induced analgesia; see [4] for review). On the other hand, stress has also been reported to produce hyperalgesia (increased sensitivity to painful stimuli) or allodynia (pain triggered by innocuous stimuli). It appears that the repetitive nature of the stress seems to favor the induction of hyperalgesia/allodynia in rodents. Hence, repeated exposure to loud sound, cold environment, restraint, swim stress or sleep deprivation potentiates pain perception in rats. However, acute stress produced by non-noxious stimuli (hand-holding, vibration, novel environment, etc.) can also trigger hyperalgesia (see [24] for review).

In humans too, exposure to chronic stress may increase pain sensitivity and reduce pain threshold [11]. In laboratory studies, Caceres and Burns [11] reported that human volunteers exposed to stress before a cold pressor test, had lower pain tolerance and a decrease pain threshold. At the clinical level, stress has a major impact on painful pathologies; for example, patients suffering from chronic back pain, from neuropathic, cancer-induced or arthritic pains, are known to report higher levels of suffering during stressful episodes [12,16,48]. There are also painful pathologies where stress is often a precipitating factor: the chronic shoulder/neck pain syndrome [32], the complex regional pain syndrome [21] and fibromyalgia (FM) [45]. FM is a common chronic pain syndrome that affects about 2–4% of the population, and is characterized by widespread muscle pain and tender points, according to the classification criteria adopted by the American College of Rheumatology in 1990 [47]. FM patients display qualitative as well as quantitative abnormalities in pain perception, which are reflected by an allodynia and/or an hyperalgesia [39,40].

Numerous pre-clinical models have been developed to reproduce various pain modalities (acute, neuropathic, inflammatory, surgery- and cancer-related) that are encountered in the clinic. There are also a multitude of pre-clinical models of stress, whether acute or chronic. However, animal studies assessing the interrelationship between stress and hyperalgesia/allodynia are rather scant, despite the close relationship between stress and pain observed in the clinic.

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Here, we evaluated, in rats, the impact of long-term intermittent stress (restraint in a cylinder, 1 h/day Monday to Thursday, during 5 weeks) on nociception, using behavioural pain tests involving either noxious or non-noxious mechanical (Randall–Selitto, von Frey filament) or thermal (tail immersion test, paw-acetone) stimuli, in addition to a noxious chemical stimulus (formalin test). The period of stress was limited to 5 weeks, due to the difficulty of fitting rats into cylinders beyond that. (weight gain). The aim was to determine which model(s) would be the most appropriate to demonstrate hyperalgesia and/or allodynia following such a stress.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Lyon, France) weighing 160–180 g upon arrival were quarantined for 4–5 days. They were housed 5 to a cage in polycarbonate type III cages (internal dimensions 42.6 cm × 27.8 cm × 15.4 cm, L × W × H) in an environmentally controlled room (temperature 21 ± 1°C, relative humidity 55 ± 5%) under a 12-h light-dark cycle (lights on at 07:00 AM) with food (pellets A04, SAFE, Epinay sur Orge, France) and tap water freely available. Rats were brought into the experimental room 1 week before starting the experiment; thereafter, they were individually housed in plastic hanging cages with a grid floor (30.5 cm × 10.8 cm × 18.4 cm, L × W × H) where they had free access to water and food. Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility, in strict compliance with all applicable French regulations, and with the local Ethical Committee guidelines for animal research.

2.2. Protocols

2.2.1. Chronic restraint stress procedure

Rats were first daily habituated over 4 days (i.e., Monday to Thursday) to handling and to the test apparatus used for the “von Frey” and acetone allosthenia models (see below). They were then, on the 5th day, subjected to nociceptive tests (see below for detailed descriptions) for determination of basal scores, each rat being subjected to at most two tests (chosen at random for a given rat). The chronic restraint stress procedure was started on the following Monday: rats were restrained 1 h daily (Monday to Thursday) in the morning, by placement into 25 cm × 6 cm (L × D) Plexiglas tubes, whose length was adjusted with a piston, so that the animal was unable to move. On Fridays, rats were subjected to the two nociceptive tests to which they had been allocated before. This cycle was repeated for 5 consecutive weeks. In addition, on the Monday of the 6th week, rats randomly selected were subjected to a formalin test (this test could only be implemented once, see below for explanation). Rats from the control group were handled like those of the stress group, except that they were not submitted to the restraint procedure, the rats being kept in their home cages instead.

2.2.2. von Frey mechanical allodynia

The protocol was adapted from the one described by Tai and Bennett [44]. Each rat was placed in a 30 cm × 24 cm (H × D) Plexiglas cylinder resting on a fine mesh floor (1.3 cm × 1.3 cm apertures) with mesh itself resting on a metal frame positioned underneath the floor at a 45° angle to allow an unobstructed view of the paws. Each cylinder was filled with water maintained at the temperature of body temperature (37°C). Rats were allowed to adapt to the testing environment for at least 10 min. Filaments (Semmes–Weinstein monofilaments, Stooling, IL, USA; forces: 0.6, 1.4, 2.4, 6, 8, 10, and 15 g) were applied in an ascending order of forces to the plantar surface of each hindpaw from below the mesh floor. For each filament, the test was repeated five times consecutively to each paw, with an interval of 1 min between each stimulus. The threshold was determined as the lowest force that evoked a withdrawal response to five out of ten stimuli.

2.2.3. Paw pressure test

Mechanical hyperalgesia was tested as previously described by Randall and Selitto [35]. Nociceptive threshold, expressed in grams (g), was measured with a Ugo Basile algosimeter (Apexlec; tip diameter of the probe: 1 mm; weight: 30 g) before applying an increasing pressure to the left hindpaw until a squeak (vocalization threshold) was obtained. The measure was taken only once, and the cut-off weight was set at 750 g. 

2.2.4. Tail immersion test

Cold hyperalgesia or allodynia (4 or 15°C water temperature, respectively) or hot allodynia or hyperalgesia (42 or 48°C) were assessed using the tail immersion test [2]. The tail was gently held and the tail (5 cm distal part) was immersed in a rectangular Plexiglas container filled with water maintained at the right temperature by a circulation pump and an electrical resistance, or the addition of ice cubes. The duration of tail immersion was manually recorded (0.1 s precision), with a cut-off time of 20 s.

2.2.5. Acetone-induced cold allodynia

The same apparatus (Plexiglas cylinder resting on a mesh floor) as the one described above for the von Frey test was used. Rats were allowed to adapt to the testing environment for at least 10 min. Then, a drop of 5(50) μl of acetone was applied with a glass syringe fitted with a blunt needle at the centre of the plantar face of a hindpaw. Acetone was applied alternately twice to each hindpaw, with 5 min between each successive application.

2.2.6. Formalin test

Considering the relatively persistent nature of tissue damage produced by a plantar injection of formalin, this test could only be carried once, at the end of chronic stress period, after all other tests had been carried out. Methodological details are described in Bardin et al. [6]: rats were placed in a clear plastic chamber (31 cm × 22 cm × 28 cm, L × W × H), resting on a metal rack with a mirror placed underneath the floor at a 45° angle to allow an unobstructed view of the paws. Each animal was first placed in the chamber for a 15 min habituation period. Thereafter, it received a 50 μl s.c. injection of diluted formalin (2.5%, v/v); formaldehyde in distilled water) into the plantar surface of the right hindpaw. Following the injection, rats were returned to the observation chamber, and the number of paw licking and paw flinching were recorded for 60 min (12 blocks of 5 min). The number of both behaviours was cumulated to give a global pain score for each block of 5 min.

2.3. Statistical analysis

Data are expressed as the mean ± SEM, and were analysed (SigmaStat 3.5, Systat Software, Inc., Point Richmond, CA, USA) using a mixed design two-way analysis of variance (ANOVA) for repeated measures, with the time (week of testing, or block of 5 min for the formalin test) as the within-subjects factor, and the condition (stress/no stress) as the between-subjects factor. Post hoc comparisons were carried out, where appropriate, using the Dunnett’s method for multiple comparisons, at each time point, between non-stressed and stressed animals. In addition, for the formalin test, data across time (in min) were collated into an ‘early’ (0–5 min) and a ‘late’ (15–20 min) phase, and these cumulated data were analysed by a Student’s unpaired t-test to compare non-stressed and stressed animals for each phase.

3. Results

3.1. General considerations on this stress protocol

Chronic restraint stress produced a significant decrease in body weight gain [F(1,1148) = 10.31, P < 0.001; F(25,1148) = 86.22, P < 0.001 and F(25,1148) = 2.84, P < 0.001 for the condition, time, and for the interaction condition × time, respectively] (Fig. 2A). The withinsubjects factor. Post hoc comparisons were carried out, where appropriate, using the Dunnett’s method for multiple comparisons, at each time point, between non-stressed and stressed animals. In addition, for the formalin test, data across time (in min) were collated into an “early” (0–5 min) and a “late” (15–20 min) phase, and these cumulated data were analysed by a Student’s unpaired t-test to compare non-stressed and stressed animals for each phase.

3.2. Chronic restraint stress produces mechanical allodynia but not hyperalgesia

Chronic restraint stress induced significant mechanical allodynia in the von Frey test [F(1,70) = 8.65, P = 0.01; F(5,70) = 11.91, P = 0.001 and F(5,70) = 3.33, P < 0.001 for the condition, time and for the interaction condition × time, respectively] (Fig. 2A). The withdrawal thresholds were significantly lower from the end of the first week of stress, with a maximum diminution of 60.5% at the end of the period of stress (week 5).
Chronic restraint stress had no significant impact on thermal hyperalgesia induced either by a noxious cold (4 °C) or hot (48 °C) stimulus [all F’s < 4.06, all P’s ≥ 0.05] (Fig. 3A and D).

3.4. Chronic restraint stress produces cold allodynia in the acetone drop test

Rats subjected to the stress regimen presented a significant score increase in responses to application of acetone to the hindpaws: \[ F(1,35) = 34.25, P < 0.001; F(5,35) = 2.83, P < 0.05 \text{ and } F(5,35) = 1.93, P > 0.05 \] for the condition, time and for the interaction condition × time, respectively (Fig. 4). Except for week 2, all other scores of stressed rats were above those of the non-stressed controls.

3.5. Chronic restraint stress enhances hyperalgesia in the formalin model

In non-stressed rats, formalin injection in the hindpaw induced a well-characterized biphasic pattern of nociceptive behaviours (Fig. 5A). The affected paw was shaken and/or licked, during the 0–5 min and 15–60 min post-injection periods, which correspond to the so-called early and late phases of formalin-induced nociceptive behaviours. In chronic restraint stress rats, pain scores were significantly higher \[ F(1,15) = 6.29, P = 0.05; F(11,55) = 11.59, P = 0.001 \text{ and } F(11,55) = 1.93, P = 0.05 \] for the condition, time and for the interaction condition × time, respectively. Statistical analysis of cumulated data for both phases confirmed that scores were significantly increased (Student’s unpaired \( t \)-test, \( t = -2.50, \text{df} = 24, P < 0.05 \text{ and } t = -2.09, \text{df} = 103, P < 0.05 \) for the early and late phases, respectively: Fig. 5B) by stress in the early (+160% versus non-stressed rats) and late (+75%) phases.

4. Discussion

This study is the first, to our knowledge, to assess the impact of protracted (5 weeks) application of a daily 1 h restraint stress in a cylinder in a wide number of models (eight) of thermal, mechanical and chemical hyperalgesia, as well as thermal and mechanical allodynia. Such a stress regimen gave rise to marked hyperalgesia in the formalin paw test, as well as cold (acetone drop test), hot (tail immersion test) thermal, and mechanical (von Frey) allodynia. In contrast, this type of stress elicited neither mechanical hyperalgesia in the Randall–Sellito paw pressure test, nor cold thermal allodynia/hyperalgesia or hot hyperalgesia in tail immersion tests.
4.1. Chronic restraint stress produces mechanical alldynia but not hyperalgesia

Chronic restraint produced mechanical alldynia, as revealed by the von Frey test, starting from week 1, with a maximum plateau effect from week 2 to 5 (over 50% decrease as compared to baseline value). An overall similar effect (circa 40% reduction) was observed by Nishiyori and Ueda [33], who used an intermittent cold stress to produce mechanical alldynia (von Frey test) in mice, or after repeated forced swim stress, using a muscular grip strength method in rats [42]. In contrast, the present stress regimen had no effect on mechanical hyperalgesia (i.e. no change in baseline nociceptive threshold), as determined in the paw pressure (Randall–Selitto) test. In line with our data, Khasar et al. [26] also reported that unpredictable sound stress did not cause mechanical hyperalgesia, using the same pressure test. In contrast, it has been reported that rats develop mechanical hyperalgesia following exposure to repeated exposure to cold environment (4 °C for 30 min every 1 h for 1 day), with the Randall–Selitto method [37]. It thus appears that the nature of the stress has a substantial impact on the development of mechanical hyperalgesia, at least as assessed by the paw pressure method.

4.2. Chronic stress has a moderate effect in only one model of tail immersion thermal alldynia/hyperalgesia, but produces cold alldynia in the acetone test

On the whole, the impact of chronic restraint stress on the tail immersion test was either nil (4, 15 and 48 °C water temperature) or of modest magnitude for hot (42 °C) alldynia. It thus appears that such a stress regimen has very limited effects on thermal-induced
alldynia/hyperalgesia measured by the tail immersion procedure. In particular, negative results for the 15 °C variant of the protocol, aimed at detecting cold allodynia, are in sharp contrast with those obtained in the model of cold allodynia with the acetone drop on the paw. In this latter model, chronic restraint stress induced a gradual development of cold hypersensitivity, with a maximal effect at week 4. The most obvious discriminant element between the two protocols is anatomical: tail versus hindpaw. Other factors, such as the kinetics of the cold stimulus (acetone cools the skin by a rather gradual evaporative mechanism, whereas the water bath method provokes an abrupt cooling), or the extent of the surface involved (much greater in the case of the tail), could also play a part. Complementary experiments using alternative methods, such as the cold plate (with a fixed temperature or a ramping increment/decrement protocol), in which all four paws are in contact with cold, should help in clarifying the importance of anatomical factors.

Our results on thermal hyperalgesia contrast with those of Nishiyori and Ueda [33], who found that mice subjected to an intermittent cold stress where more hyperalgesic than their control congeners to a hot stimulus applied to the paw. Sub-chronic swim stress also triggered thermal hyperalgesia in the hot plate test in rats [34,41,42]. Our results also diverge from studies [13,14,18,23] using rats subjected to a restraint stress, and tested in a tail flick procedure. Of note, these latter studies used a light source to trigger hyperalgesia, instead of hot water. This protocolar difference might play a primary role in the outcome of stress on this type of nociception: it might be that the more localized nociceptive stimulus produced by a light source presents higher sensitivity to the impact of stress. Alternatively, in our protocol, rats were gently held and the tail was immersed in water, whereas in the studies of Da Silva Torres et al. [13,14], they were wrapped in a towel and their tail placed under the light beam. It is possible that wrapping produced an additional restraint-type of stress that might have facilitated/triggered hyperalgesia. Finally, rats from the study by Imbe et al. [23] were subjected to 6 h of restraint per day, and those from the Brazilian team to 1 h per day but for 40 days (i.e. 8 weeks) [13,14,18]. This greater cumulated period of stress may have contributed to an enhanced hyperalgesia.

4.3. Chronic restraint stress potentiates chemical hyperalgesia

In rats subjected to chronic restraint stress, pain scores were significantly increased during the early and late phases of the formalin test, suggesting that pain in these rats was more pronounced compared to that in non-stressed rats. Using a similar stress protocol (1 h of restraint in a cylinder, for 40 days), Gameiro et al. [19] also reported enhanced hyperalgesia following injection of formalin into the temporomandibular joint. Various other stress modalities have also been shown to augment chemical-induced nociception: for instance, sub-chronic swim stress and prenatal stress increase nociceptive behaviours in the formalin test [10,34,43]. Carageenan-induced muscle nociception was also increased after repeated forced swim stress [41]. Repeated sound stress enhanced bradykinin-induced inflammatory pain in rats, measured with the Randall–Selitto method [25]. Lastly, multiple exposures to a cold stressor potentiates acetic acid and phenylquinone-induced writhing reflex in mice [27].

It thus appears that various forms of stress share in common the ability to potentiate chemically induced inflammatory pain. It would be interesting to see if the impact of stress generalizes to other types of inflammatory pain, such as that produced by thermal (UV skin exposure) or mechanical (surgery/injury) insults.

4.4. Possible applications of the present model to the study of human painful pathologies with a strong stress component

Stress has a major impact on and/or has a causal effect in numerous pathologies, most notably those in which pain is an essential or associated component. For example, stress amplifies nociception in irritable bowel syndrome [7], and higher psychological distress results in higher levels of pain in cancer patients [49]. Some studies also indicate that stress negatively impacts headaches and/or abdominal pain in children [1,8]. FM patients appear to be particularly sensitive to the negative effects of stress [15,45]. Accordingly, 22–49% of post-traumatic stress disorder subjects meet criteria for FM [3,5]. Compared to healthy controls, FM patients have significantly lower thresholds for non-painful [28] and painful [29] cold stimuli. They also show allodynia to warmth stimulation and heat-induced hyperalgesia [29], enhanced sensitivity to pressure [20], as well as hypersensitivity to intramuscular hypertonic saline [22,38], that produces a mild form of inflammatory pain [36]. Interestingly, allodynia and hyperalgesia in these patients is widespread, and not restricted to the so-called deep muscular “tender points”, as both can be observed by mechanical/thermal stimuli applied to remote parts of the body as well [29]. As a consequence, a rodent model
where stress provokes enhanced inflammatory pain response and widespread temperature and/or pressure hyperalgesia/allodynia, could offer an interesting investigational tool to probe putative physiological mechanisms underlying stress-associated chronic pain disorders.

Furthermore, substantial co-morbidity exists between chronic pain pathways. Indeed, FM patients often also suffer from irritable bowel syndrome (IBS), a form of visceral pain [46]. Consequently, experiments aimed at assessing the impact of such a chronic stress on models related to IBS (such as rectal chemical irritation with butyrate or mechanical distension: [9,31]) would be warranted. Also, considering the high prevalence of co-morbid symptoms in chronic pain pathways such as FM, including impaired memory and insomnia (see Table 1 [30]), future studies addressing the impact of prolonged stress in rodents on sleep/wake patterns (electroencephalographic recordings), and on models of cognition/mememory, would also prove valuable. However, such an approach constitutes a major undertaking, and is clearly out of the scope of the present study that focuses on pain models.

Finally, such a model could prove useful in dissecting out mechanisms of action of clinically active compounds used in the management of chronic pain disorders, as well as in searching for novel, improved compounds. There are already reports that compounds clinically effective in FM patients attenuate hyperalgesia/allodynia produced by sustained stress. For instance, the noradrenaline-serotonin reuptake inhibitor milnacipran, and the voltage-gated calcium channel alpha-2-delta subunit ligand, gabapentin have shown beneficial effects against stress-induced hyperalgesia/allodynia in rodents [33,41].

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References

[8] Butkevich IP, Vershinina EA. Maternal stress differently alters nociceptive impaired memory and insomnia (see Table 1 [30]), future studies warranting. Also, considering the high prevalence of co-morbid hyperalgesia/allodynia in rodents [33,41].


