Neuroscience Letters 553 (2013) 110–114

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Sex-independent suppression of experimental inflammatory pain by minocycline in two mouse strains

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HIGHLIGHTS

- Minocycline suppresses inflammatory pain irrespective of mouse sex and strain.
- Lack of motor coordination does not account for suppression of nociceptive response.
- These results may be translated to minocycline's analgesic actions in men and women.

ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 9 August 2013
Accepted 13 August 2013

Keywords:
Sex
Gender
Mice
Tetracyclines
Nociception
Formalin test

ABSTRACT

The research on sex differences in nociception and antinociception as well as sex and gender differences in pain and analgesia is a maturing field. There is a vast literature showing experimental and clinical pain suppressive effects induced by minocycline, especially in inflammatory pain. However, as far as we know, possible qualitative or quantitative sex differences in those effects remained to be examined. By employing the formalin test, which has two phases of experimental pain behavior that models nociceptive pain (i.e., first phase) and inflammatory pain (i.e., second phase), we initially evaluated the effect induced by minocycline in female or male C57BL/6 mice. The treatment reduced the second phase of licking behavior in both females and males, and the effects were quantitatively similar in both sexes. Likewise, the same sex-independent effect was observed in Swiss mice, suggesting a genotype-unspecific sex-independent effect. While minocycline is already being tested in clinical trials, this appears to be the first preclinical investigation of sex differences in the experimental pain suppressive effects induced by this widely studied drug. The independence of sex in the antinociceptive effect induced by minocycline may be hopefully translated to gender-independent analgesic effects, which would be surely promising in a therapeutic paradigm.

1. Introduction

Data from many studies show that pain/nociception and analgesia/antinociception, both in humans and experimental animals, exhibit great interindividual variability and the factors that may contribute to such differences have been intensely investigated recently [18]. Furthermore, data from large epidemiological studies clearly show that pain is more prevalent in women than in men [21]. Nevertheless, there is a clear male-orientated bias in experimental subject choice in the field of pain and others; and the reasons for this bias are believed to be the inertia of pain researchers and their over-concern about estrous cycle-related variability [18]. The inclusion of females is mandatory in clinical but not in preclinical studies. For this reason, the awareness of this matter has been increasing and the investigation of sex and gender differences in nociception, pain and inflammation and their inhibition has been maturing [6,12]. Currently and more frequently, sex is used to refer to a biologically based dichotomous variable, whereas gender refers to a socially based phenomenon, considered to range from exclusively feminine to exclusively masculine. Therefore, in human studies, group differences are likely to be attributable to either sex or gender. On the other hand, only the term "sex" applies to preclinical data and will be used throughout this manuscript [12].

Clinical trials have been conducted to test the clinical utility of repositioning minocycline as an analgesic or antirheumatic drug [11,20,30]. Indeed, this second-generation tetracycline exhibits anti-inflammatory effects unrelated to its antibacterial one [1], and anti-inflammatory effects help explain either its antinociceptive [2]

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http://dx.doi.org/10.1016/j.neulet.2013.08.026

or neuroprotective actions [16,27]. Minocycline is neuroprotective after experimental stroke, a pathological condition characterized by massive inflammatory response [29]. A study suggested that the neuroprotective effect in the middle cerebral artery occlusion model is dependent on sex in C57BL/6 mice [19], whereas another study suggested that neuroprotective effects in a thromboembolic stroke model are independent of sex in the same mouse strain [13]. Therefore, we wondered whether there might be qualitative or quantitative sex differences in the effect induced by minocycline on inflammatory pain.

There is a very high number of studies showing suppression of experimental pain by tetracycline derivatives in rodents, especially inflammatory pain [2]. However, as far as we know, just in a few studies the authors clearly informed the use of female animals only [3,24,32], and therefore a "head-to-head" comparison between the effects induced by minocycline in females and males remained to be performed. Then, we designed a straightforward study to compare the effect induced by minocycline in female and male mice using the formalin test, which models both nociceptive (i.e., first phase of licking behavior) and inflammatory (i.e., the second phase) pain [14,31]. To evaluate whether a sex difference is a genotype specific or unspecific effect, we evaluated the activity of minocycline in two mouse strains.

2. Materials and methods

2.1. Animals

Seven to nine week-old C57BL/6 or Swiss mice of both sexes were used. Efforts were made to minimize both animal distress and the number of animals used. The animals had free access to food and water and were maintained in a room with a 12 h light–dark cycle. The experiments were performed at room with temperature controlled between 26 and 28 °C, which ranges within the thermoneutral zone for mice [10] and it is suitable for carrying out the temperature-sensitive formalin test [31]. All experiments were performed according to the ethical guidelines for the investigation of experimental pain in conscious animals [33]. Experimenters were blinded to treatments. Each experiment was performed in a separate group of mice.

2.2. Drugs

Minocycline hydrochloride (Galena, Campinas, Brazil), formaldehyde 37% (m/v) (Sigma–Aldrich, St Louis, MO, USA) and phenobarbital (Aventis Pharma, São Paulo, Brazil) were used. Solutions and suspensions were prepared in isotonic saline immediately before the intraperitoneal (i.p.) injections. The volume injected was 5 ml/kg.

2.3. Effect induced by minocycline on the nociceptive response induced by formalin in mice

For three consecutive days before the experiment, the animals were habituated for approximately 30 min to the testing apparatuses to minimize stress-induced antinociception on the testing day. Each mouse was placed under a transparent glass funnel (18 cm diameter, 15 cm high). Formalin (2.5%, v/v, 20 μl) was injected subcutaneously (s.c.) into the dorsum of the right hind paw of mice 1 h after the i.p. administration of minocycline (12.5, 25, 50 or 100 mg/kg) or saline. This minocycline dose range was chosen on the basis of previous studies performed by our research group and others [1,3–5,25]. The amount of time the animal intermittently licked the injected paw was counted manually by using stopwatches between 0 and 5 min (first phase) and 15 and 30 min (second phase) after the injection of formalin.

2.4. Evaluation of the motor coordination of mice in the rota-rod

The motor coordination of the animals was evaluated in a rota-rod apparatus. This test is essential when studying experimental pain to examine the possibility of reduced display of nociceptive behavior due to lack of motor coordination that may result from central nervous system depression or muscle relaxation [9,15].

The animals were trained on the apparatus for three days before the experiment. On the testing day, the animals were placed on the rotating rod (12 r.p.m.) and the latency to fall was measured. The cut-off time was 120 s. After confirming that all animals were sufficiently trained to stay on the rotating rod for at least 120 s, they were treated with minocycline (100 mg/kg, i.p.) or phenobarbital (40 mg/kg; i.p., positive control) and 1 h later they were again tested on the apparatus. Phenobarbital, a central nervous system depressant, was used as a positive control because of its well-known ability to impair the performance of rodents in the rotarod test [1,4,7].

2.5. Data analysis

The pain behavior data were analyzed by two-way ANOVA, taking sex and drug treatment as main factors, followed by Bonferroni post hoc test for multiple comparisons. Calculations of half-maximal inhibitory doses (ID_{50}) were performed by using the software JFlashCalc (M.H. Ossipov, University of Arizona, AZ, USA). As rota-rod data did not display normal distribution (analyzed by Kolmogorov–Smirnov test), Kruskal–Wallis test, followed by Dunn's multiple comparison test, was applied. Values of $p < 0.05$ were considered to show significant differences between means or medians. The software Prism® 5 (GraphPad Software Inc., San Diego, CA, USA) was used for such analyses. For ease of reading, the basic statistical values are shown in the text, whereas the more extensive statistical information can be found in the figure captions.

3. Results

The s.c. injection of formalin (2.5%, 20 μl) in mice induced a biphasic nociceptive response characterized mainly by licking the injected paw. Fig. 1A and 1B show that the first phase of licking behavior was unaffected by minocycline treatment in C57BL/6 mice, whereas the second phase was attenuated by this tetracycline derivative, as revealed by two-way ANOVA. However, the minocycline's antinociceptive activity was not affected by sex. Reinforcing the sex-independent effect induced by minocycline in this experimental setting, minocycline inhibited the second phase of licking behavior in either female or age-matched male mice of a different mouse strain (i.e., Swiss mice; Fig. 2B), whereas the first phase was unaffected (Fig. 2A). In male C57BL/6 and female Swiss mice, the half-maximal inhibitory doses (ID_{50}) were 89 mg/kg (95% confidence interval: 20–386 mg/kg) and 61 mg/kg (95% confidence interval: 24–155 mg/kg), respectively. By extrapolation, the ID_{50} values in female C57BL/6 and male Swiss mice were 123 and 140 mg/kg, respectively; however, these estimated numbers should be treated cautiously because the highest dose used in the present study was 100 mg/kg (i.p.). We have not tried higher doses because of the risk of toxicity – 100 mg/kg is already a high dose. Indeed, as far we know, there is no study on experimental pain that used a dose of minocycline higher than 100 mg/kg (i.p.).

Importantly, lack of motor coordination seems not to account for reduced display of experimental pain behavior, as the highest dose of minocycline (100 mg/kg) did not affect the performance of mice in the rota-rod test (Table 1). On the other hand, phenobarbital, used