Blockade of the kinin B₁ receptor counteracts the depressive-like behaviour and mechanical allodynia in ovariectomised mice

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Menopause is related to a decline in ovarian oestrogen production, affecting the perception of the somatosensory stimuli, changing the immune-inflammatory systems, and triggering depressive symptoms. It has been demonstrated that the inhibition of the kinin B₁ and B₂ receptors (B₁R and B₂R) prevented the depressive-like behaviour and the mechanical allodynia that was induced by immune-inflammatory mediators in mice. However, there is no evidence regarding the role of the kinin receptors in the depressive-like and nociceptive behaviour in female mice that were subjected to bilateral ovariectomy (OVX). This study has shown that the OVX mice developed time-related mechanical allodynia, together with an increased immobility time as indicative of depression. Both of these changes were reduced by the genetic deletion of B₁R, or by the pharmacological blockade of the selective kinin B₁R antagonist R-715 (acute, i.p.). The genetic deletion or the pharmacological inhibition of B₂R (HOE 140, i.p.) did not prevent the OVX-elicited behavioural changes. The data has suggested a particular modulation of kinin B₁R in the nociceptive and depressive-like behaviour in the OVX mice. The selective inhibition of the B₁R receptor may be a new pharmacological target for treating pain and depression symptoms in women during the perimenopause/menopause period.

1. Introduction

In women, the ageing process is linked to the onset of the perimenopause/menopause period, which occurs approximately at the age of 45–55. It is related to a progressive decrease in the circulating levels of the gonadal hormones [1–3]. These hormonal changes include an increase in the serum levels of the follicle-stimulating hormone (FSH), which is associated with diminished oestradiol and inhibin B [3]. This oestrogen decrease is associated with several physical and psychological symptoms [2, 4–6]. Some of these symptoms especially occur during the perimenopause period. Some such examples are vasomotor alterations, sleep disturbances, depression, sexual dysfunctions, cognitive impairment, and joint pain. A range of these alterations can accompany women for the rest of their lives [7]. The most effective therapeutic option to treat menopausal symptoms remains hormone replacement therapy (HRT). All the same, HRT increases some risks, such as post-menopausal breast cancer, thrombosis, an increase of cardiovascular disease, and stroke [3, 8, 9].

Besides the importance of oestrogen receptors (ERs) in mediating the sexual growth and differentiation in women, these receptors are widespread in the central nervous system (CNS) [10]. The activation of these ERs in the CNS regulates mood, alertness, cerebral blood flow, and neurotransmitter activity [2]. Previous clinical evidence has shown that one of the most prevalent symptoms during perimenopause/menopause...
is chronic pain [3,11,12]. Furthermore, the ERs regulate the immune systems and the inflammatory mediators [13,14].

Major depressive (MD) disorder affects more than 350 million people globally, and the prevalence is higher in women than in men [15,16]. Notably, the data from clinical studies has indicated that perimenopause is a critical period for the development of MD signs [4]. In treating MD symptoms, the currently available antidepressant therapy shows mixed results in its effectiveness. In addition, many patients discontinue the therapy because of the variable side effects of the antidepressants [17]. Preclinical studies have shown that OVX rats or mice developed depressive-like behaviour and nociception changes, both of which were counteracted by an oestradiol treatment [18–20]. Despite that, these HRT adverse effects cannot be disregarded [8]. As a result, to treat menopause-associated MD and pain symptoms, the development of new approaches is essential.

Over the past few years, kinin receptors have been associated with a series of pathophysiological conditions [21–24]. The biological effects of kinins are mediated via the activation of two metabotropic receptors, namely, B₁R and B₂R. While B₁R is constitutively expressed in several tissues and is preferably activated by bradykinin (BK), the B₂R receptor is weakly expressed under physiological conditions but is upregulated in traumatic and immune-inflammatory conditions [25,26]. The activation of B₁R is modulated by the kinin metabolites and it contributes to chronic allodynia and hyperalgesia, which are associated with the production of the inflammatory cytokines [27,28]. The activation of B₂R also has been implicated in some central nervous system (CNS) conditions, such as Alzheimer’s disease, epilepsy, and bipolar disorder [29–31]. Furthermore, our previous work has shown that B₂R participated in the depressive-like behaviour that was induced by the systemic administration of E. coli lipopolysaccharide (LPS) in mice [22]. Such an effect seemed to be related to an increase in the TNF levels and the microglia activation in the brains of the mice [22].

When considering the impact of an ovarian oestrogen depletion on MD, pain, and the immune-inflammatory system [4,14], this study has hypothesised that the kinin receptors may participate in the OVX-induced behavioural changes in rodents [20]. The researchers have also postulated that kinin receptor inhibition might attenuate the beneficial changes in OVX mice. This current study has assessed the effects of the pharmacological and genetic inhibition of B₁R and B₂R on mechanical nociception and the depressive-like behaviour in OVX female mice.

2. Materials and methods

2.1. Animals

The experiments were conducted when using four different adult female mice (8 weeks of age, at 25–30 g), namely, Swiss and the C57BL6 wild type (WT), together with B₁ and B₂ receptor knockout mice (B₁-/- and B₂-/-, UNIFESP-EPM). The animals were housed under standard conditions of light, temperature, and humidity (12 h light-dark cycle, 22 ± 1 °C, under 60–80 % humidity), with food and water provided ad libitum. The Swiss and the C57BL6 mice were obtained from the Central Animal Facility at the Universidade Federal de Pelotas (UFPEL, Brazil). The B₁ and B₂ receptor knockout mice were obtained from the Animal Facility at the Department of Biophysics, Universidade Federal de São Paulo (UNIFESP-EPM, São Paulo, Brazil). All of the procedures were conducted in accordance with the Brazilian Council for Animal Experimentation (COBEA) guidelines, which comply with international laws and policies for the investigation of experimental pain in conscious animals. The protocols were approved by the local Ethical Committee (protocol number CEUA-PUCRS 12/00,290). Every effort was made to minimise the animal’s suffering and with an aim to reduce the number of animals used. The experiments were conducted between 08:00–17:00 (during the light phase of the 24-h cycle), with randomisation of the experimental groups.

2.2. Ovariectomy surgical procedure

The surgical procedure was conducted as previously described in the literature [18,19], with minor modifications. Briefly, the animals were anaesthetised by an intraperitoneal (i.p.) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). After the onset of anaesthesia, the lumbar dorsal was shaved. The exposed skin was prepared for aseptic surgery by a povidone-iodine (10 %) scrub, followed by a sterile saline wipe. The ovary was resected bilaterally at 8 weeks of age, with a one to two cm incision in the skin in the midline on the lumbar vertebral line. The ovary was pulled through a small opening in the musculature. A ligature was placed around the exposed ovary and an initial segment of the fallopian tube was removed (OVX group). The skin and muscle incision were then sutured (4–0 non-absorbable). The Sham group underwent the same procedure as the OVX group but without the resection of the ovaries and the initial segment of the fallopian tube. The behavioural tests were initiated after a period of recovery of 7 days.

2.3. Measurement of the uterine weight

Uterine atrophy is indicative of the success of ovariectomy surgical model [18]. After the battery of behavioural tests, all of the animals of both the OVX and Sham groups were euthanised by a deep inhalation of isoflurane. The uterus of each animal was resected quickly without the periovary fat and was immediately weighted. The values were expressed in mg.

2.4. Mechanical allodynia

The measurement of the mechanical paw withdrawal threshold (PWT) was carried out when using the up-down paradigm, as described in the literature [32,33], with minor modifications. The mice were individually acclimatised for one hour in elevated clear plexiglass boxes, with a wire mesh floor to allow for access to the right hind paw plantar surface. Von Frey filaments of increasing stiffness (0.02–10 g) were applied to the right hind paw plantar surface of the animals, with a pressure that was high enough to bend the filament. The tests were initiated with a 0.4 g filament. The absence of a paw lifting after 5 s led to the use of the next filament with an increased weight, whereas a paw lifting indicated a positive response, which led to the use of the next weaker filament. This paradigm continued for a total of 6 measurements, including the one before the first paw-lifting response had been made, or until four consecutive positives (assigned a score of 0.030) or four successive negatives (assigned a score of 6.76) responses occurred. The mechanical paw withdrawal threshold response was then calculated as described previously [34], using the following formula:

\[\text{Threshold (mg)} = \log \text{of the last filament used} - (\text{K.mean})\]

where K was the constant based on the Dixon Table, and mean referred to the mean difference (in log units) between the stimuli (for the mice, 0.44). The paw withdrawal threshold was expressed in grams (g) and was evaluated before (baseline) and 7, 14, 21, and 28 days after the surgical procedure. A significant decrease in the paw withdrawal threshold when compared to the baseline values was considered as mechanical allodynia.

2.5. Tail suspension test

According to the methodology as was described initially [35], the study used the tail-suspension test (TST) to assess the depressive-like behaviour. At different time-points, following the ovariectomy surgical procedure (7, 14, 21, and 28 days), the animals were suspended 50 cm above the floor by using adhesive tape that was placed approximately 1 cm from the tip of the tail. The mice were exposed to the TST, 30 min after the PWT test. The time during which mice remained immobile was
quantified in seconds for 6 min [22].

2.6. Open-field locomotor activity

The locomotor activity was assessed in the open-field arena at 7, 14, 21, and 28 days, after the ovariectomy surgical procedure. The experiments were conducted 30 min after the TST in a sound-attenuated room, under low-intensity light. The mice were individually placed in the centre of an acrylic box (40 × 60 × 50 cm), with the floor divided into nine squares. The number of squares that were crossed with the four paws was registered for 6 min [22].

2.7. Pharmacological treatments

To assess the involvement of the B₁ and B₂ receptors on the behavioural changes that were induced by the ovariectomy surgery, the animals were pre-treated with the selective B₁ receptor antagonist R-715 (0.5 mg/kg; i.p.) and the selective B₂ receptor antagonist Hoe-140 (50 nmol/kg; i.p.). The positive and negative control groups were treated with pregabalin (30 mg/kg; i.p.), or saline (0.9 %; i.p.), respectively. All of the treatments were administered 30 min before the behaviour PWT test. The doses and the treatment schedules were determined based on the previous literature, or on the pilot experiments [22,28,36].

2.8. Drugs and reagents

The following drugs and reagents were used: the B₁ receptor antagonist R-715 was kindly provided by Dr Fernand Gobeil (Department of Pharmacology, University of Sherbrooke, QC, Canada) and the B₂R antagonist Hoe-140 was of the commercial source (Bachem, USA; #4,043,056). Ketamine (Cristália), Xylazine (Syntec), 10 % povidone-iodine, and pregabalin (Lyrica®) were obtained from Pfizer (UK). All of the drugs were diluted in sterile saline immediately before the injection (NaCl 0.9 %) solutions.

2.9. Statistical analysis

The results are presented as the mean ± SEM. The statistical analyses were performed by One-Way or Two-Way Analysis of Variance (ANOVA), followed by Bonferroni’s post hoc test, or by using the unpaired Student t test. P-values less than 0.05 (p < 0.05) were considered significant. All of the tests were performed when using GraphPad Prism Software version 8.0 (San Diego, USA).

3. Results

3.1. The ovariectomised mice showed mechanical allodynia and depressive-like behaviour

Initially, the researchers characterised the behavioural changes that were induced by the menopause surgical model (Fig. 1A). The ovariectomised Swiss mice (OVX group) showed mechanical allodynia, which was characterised by a significant reduction in the right hind PWT (OVX = F (1, 20) = 42.72, p = 0.0001, N = 11 by group; at 7, 14, and 21 days, respectively; Fig. 1B). On the 28th day after the surgical procedure, there were no significant differences between the groups (OVX x Sham, P = 0.49; Fig. 1B). The same group of mice demonstrated a significant increase in immobility time in the TST (OVX = F (1, 40) = 31.20, p =
3.2. The B$_2$ receptor antagonist R-715 attenuated the mechanical nociceptive and depressive-like behaviour in the ovariectomised mice

At the next step, the study assayed the effects of an effective dose of pregabalin and the B$_2$ receptor antagonist peptide for kinin R-715 in mechanical allodynia (von Frey filament test) and the immobility time in the TST (Fig. 2A). The results showed that the ovariectomised Swiss mice displayed a significant decrease in the PWT, when measured at 21 days, following the surgical procedure (OVX = F (1, 42) = 29.43, p = 0.0001, N = 13; Fig. 2B). The acute treatment with pregabalin (30 mg/kg; i.p.; 30 min. before the behaviour test), or with the B$_2$R antagonist R-715 (0.5 mg/kg, i.p.; 30 min. before the behaviour test), significantly inhibited the mechanical allodynia that was induced by the ovariectomy procedure (interaction = F (3, 42) = 12.51, p = 0.0001, N = 7–13; Fig. 2B). Only the treatment with R-715 significantly reduced the TST immobility time (F (3, 41) = 26.84, p = 0.0001, N = 7–13; Fig. 2C) in the ovariectomised female mice. Both of these pharmacological treatments were not able to reverse the decrement of uterus weight in the ovariectomised mice (p = 0.99, N = 7–13; Fig. 2D).

3.3. The B$_2$ receptor knockout mouse was protected from the behavioural changes elicited by the ovariectomy

To confirm the role of the B$_2$ receptor for kinin in the mechanical allodynia and depressive-like behaviour that was induced by the ovariectomy, the B$_2$ receptor knockout female mouse (KO-B) was used (Fig. 3A). The results showed that the ovariectomised C57BL/6 mice (WT) displayed a significant decrease in the PWT value when measured at 21 days, following the surgical procedure (OVX = F (1, 42) = 14.02, p = 0.0005, N = 10; Fig. 3B). Both the KO-B mice that were submitted to the ovariectomy procedure (OVX-KO-B) and the OVX-WT mice that were treated with R-715 (0.5 mg/kg; i.p.; 30 min. before the behaviour test) significantly exhibited an inhibition of the mechanical allodynia induced by the ovariectomy procedure (interaction = F (4, 42) = 10.19, p = 0.0001, N = 8–10; Fig. 3B). Relevantly, both the OVX-KO-B group and the OVX-WT group that were treated with R-715 had a significant decrease of the immobility time in the TST (F (4, 39) = 9.0, p = 0.0001, N = 8–10; Fig. 3C). Either the pharmacological treatment with the B$_2$ receptor antagonist, or the gene deletion, were unable to reverse the decrease of uterus weight in the ovariectomised mice (p < 0.05; N = 8–10, Fig. 3D).

3.4. The pharmacological inhibition or gene deletion of the B$_2$ receptor failed to protect against the behavioural changes that were induced by the ovariectomy

The present study also investigated the participation of the kinin B$_2$ receptor in the behavioural changes as elicited by the ovariectomy surgery (Fig. 4A). The B$_2$ receptor knockout mouse (KO-B2) and the selective B$_2$ receptor antagonist Hoe-140 (50 nmol/kg; i.p., 30 min before the behaviour test) were used. Similarly, the OVX-WT group and the KO-B2 female mice that were submitted to ovariectomy (OVX-KO-B2 group) developed mechanical allodynia when measured at 21 days, following the surgical procedure (OVX = F (1, 33) = 129.2, p = 0.0001, N = 6–9; Fig. 4B). The OVX-KO-B2 group, and the OVX-WT group that were treated with Hoe-140, did not prevent the mechanical allodynia that was induced by the ovariectomy surgery (interaction; F (4, 33) = 17.84, p = 0.99; N = 6–9; Fig. 4B). Furthermore, both the OVX-KO-B2 group and the OVX-WT group that were treated with Hoe-140 did not present a reduction of the immobility time in the TST (F (4, 32) = 18.30, p = 0.99, N = 6–9; Fig. 4C). Either the pharmacological treatment with the B$_2$ receptor antagonist or the gene deletion were unable to reverse the decrement of uterus weight in the ovariectomised mice (p < 0.05, N = 6–9; Fig. 4D).

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**Fig. 2.** Evaluation of the acute treatment with the B$_1$ antagonist in the ovariectomised Swiss mice. (A) Timeline of the experimental approach. The effects of ovariectomy surgery at 21 days. (B) Mechanical threshold, as analysed by using the von Frey test, (C) Immobility time in the tail suspension test, and (D) Uterus weight (mg). The effects of the treatment with pregabalin (30 mg/kg), or R-715 (0.5 mg/kg); both by i.p., injected 30 min before behavioural tests. Each column represents the mean ± SEM of 7–13 animals per group. *P < 0.05 shows a significant difference from the Sham Swiss group; #P < 0.05 shows a significant difference from the OVX Swiss group; One or Two-Way (repeated-measure) ANOVA (time x OVX + treatment), followed by Bonferroni’s post hoc test. PWT = paw withdrawal threshold; TST = tail suspension test; OVX = ovariectomy.
4. Discussion

The present study has described the participation of the bradykinin receptors on the depressive-like behaviour and mechanical allodynia that were triggered by a preclinical model of menopause in mice. Both the pharmacological inhibition and the genetic deletion of the B1 receptor mitigated the ovariectomy-induced behavioural changes. Neither the pharmacological or the genetic inhibition of the B2 receptor for kinin attenuated the bilateral ovariectomy-induced mechanical hypernociception and the increase in immobility time. The data has indicated
a specificity for the B2 receptor subtype for bradykinin in the OVX-induced mechanical allodynia and depressant-like behaviour. This specificity has suggested a potential pharmacological target to treat pain and the symptoms of major depression in women during the menopause period.

During the perimenopause/menopause period, an increase in depression and pain symptoms is associated with a decrease of ovarian hormones [37]. In preclinical studies, the decline of ovarian hormones by the bilateral ablation of ovaries has induced mechanical allodynia and thermal hyperalgesia, with an increase of immobility time in the FST or TST [18–20,38]. Corroborating previous data, it was observed that the bilateral ovariectomy significantly decreased the uterine weight, being an indirect indicator of oestrogen decrease [18,20]. Uterine atrophy was associated with mechanical allodynia at 7, 14, and 21 days after surgery, with a significant increase in immobility time in the TST at 14, 21, and 28 days. The locomotor activity had no significant changes when compared with the Sham group, indicating that an increase of immobility time in the TST was not due to motor impairment. This study has, therefore, assumed that a depletion of the ovarian hormone levels facilitated the development of mechanical allodynia and the acute coping behaviour (helplessness state) in the female mice.

Clinical and preclinical studies have demonstrated the relationship between a decrease in the ovarian hormones and an increase in the pro-inflammatory markers when associated with molecular mechanisms of pain perception and the symptoms of major depression [39–41]. Examples of such markers are interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) [39,42]. Certain researchers have postulated that the pro-inflammatory cytokinies, by activating the B1 kinin receptor, elicit mechanical nociception and the acute coping behaviour in mice [22,43]. Such mechanisms are associated with an increased expression of B1R in the peripheral (e.g. terminal nerves) and central (e.g. hippocampus and cortex) nervous system structures, both of which are related to allodynia and major depression [22,26,43–45]. This evidence is similar to the results from the present study, wherein pre-gabalin (positive control) and the B2R antagonist R-715 effectively inhibited mechanical allodynia in the OVX mice. Notably, only R-715 was able to decrease the immobility time in the TST. On the other hand, for the ovariectomised mice, the selective B2R antagonist HOE-140 failed to prevent an increase of mechanical nociception or immobility time. Additionally, the B1R knockout mice were unresponsive to the OVX-induced mechanical allodynia and the increased immobility time, while the B2R knockout mice were responsive. Thus, in the ovariectomised mice, this decrease in the ovarian hormones may increase the B1R expression/activity and modulate the nociception and depressive-like behaviour.

In humans and rodents, post-ovariectomy oestrogen reduction is associated with an increase of the systemic and central immune-inflammatory mediators [41]. In the ovariectomised mice, the 17 beta-oestradiol treatment significantly inhibited the LPS-induced increase in the microglia activation [46]. In a previous study by the same researchers, the systemic LPS injection induced an increase in the immobility time in the TST, and significantly increased the TNF levels in the whole brain, serum, and cerebrospinal fluid (CSF). These increases preceded an acute elevation of the B1R mRNA expression in the hippocampus and cortex [22]. The acute systemic treatment with the B1R antagonists decreased the microglia activation in the hippocampus. In addition, the LPS-treated TNF P55 knockout mice displayed no depressive-like behaviour or an increase in the B1R expression [22]. Together, this data has suggested that 17 beta-oestradiol and the selective B2R antagonist (R-715) may be acting via similar mechanisms to protect against the behavioural changes and the molecular alterations, as modulated by the increasing TNF levels. Accordingly, clinical and preclinical data suggest that a decrease of ovarian hormones induces: (I) an alteration of the angiotensin-converting enzyme (ACE) activity, (II) an increase in oxidative stress, and (III) an increase of the pro-inflammatory cytokinies [47–49], which leads to an increased production of kinin, receptor activation, and/or upregulation [41,50].

In a clinical study, hormone replacement therapy (HRT), as administered in hypertensive postmenopausal women, decreased the serum levels of ACE activity and increased the plasma levels of angiotensin II (ANG II) and bradykinin [47]. The cultured primary hypothalamic neurons of the mice that were treated with ANG II showed a significant increase in the B1R expression, an increase of oxidative stress, and the proinflammatory cytokinies, which were prevented by a pre-treatment with the B2R antagonist (R-715) [51]. Hence, HRT may facilitate the kinin-kallikrein system and the B2R activity.

A reduction of the oestrogen levels has been associated with an increased reactive oxygen species (ROS) [52–54]. In preclinical studies, an increase of ROS induced the upregulation of B1R in the brain of a rat model with insulin resistance [49]. This increase of B1R activity led to downstream pathways, which involved an increase of nitric oxide (NO), glutamate, and substance P (SP) [49]. The ovariectomised mice demonstrated an increase of nitric oxide (NO) in the hippocampus. An acute treatment with the non-selective NO synthase inhibitor (L-NAME) significantly decreased the immobility time of the OVX mice in the FST [19]. Consequently, these decreasing ovarian hormones may increase the B1R activity via oxidative stress and the pro-inflammatory cytokinies [19,49].

When comprehended together, this data has suggested that the antagonists of the kinin receptors could be a new pharmacological approach to treat pain and major depression symptoms during the menopause period. Having said that, it is not clear how the ovarian hormones modulate the B2R expression or activity. Further investigation is needed to address the direct role of the ovarian hormones on the kinin-kallikrein system when related to major depression.

5. Conclusion

In conclusion, this study has shown the specificity of B2R on the mechanical nociception and depressive-like behaviour in female mice when submitted to ovariectomy. To the best of the authors’ knowledge, this is the first evidence that the pharmacological or genetic inhibition of B2R has prevented the behavioural changes that were induced by the surgical menopause model in female mice. The inhibition of B1R could be a new pharmacological target to treat pain and major depression during the perimenopause/menopause period.

Author contributions

ISM designed the study, performed the experiments, collected, analysed, and interpreted the data, as well as writing the manuscript draft. VMA and PO performed the experiments, collected, and analysed the data. MMC designed the study, analysed and interpreted the data, and wrote the manuscript.

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Author statement

Izaque de Sousa Maciel designed the study, performed the experiments, collected, analysed, and interpreted the data, as well as writing the manuscript draft. Vanessa Machado Azevedo and Patricia Oliboni performed the experiments, collected, and analysed the data. Maria Martha Campos designed the study, analysed and interpreted the data,
and wrote the manuscript. All the authors read and approved the final version to be published in the Behavioural Brain Research.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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