Reduced mechanical hypersensitivity by inhibition of the amygdala in experimental neuropathy: Sexually dimorphic contribution of spinal neurotransmitter receptors

Hong Wei a, Zuyue Chen a,b, Jing Lei a,c, Hao-Jun You c, Antti Pertovaara a,*  

a Department of Physiology, Faculty of Medicine, University of Helsinki, Helsinki, Finland  
b Department of Medical Imaging, School of Medicine, Shaoxing University, Shaoxing, PR China  
c Center for Translational Medicine Research on Sensory-Motor Diseases, Yan’an University, Yan’an, PR China

ARTICLE INFO

Keywords:  
Amygdala  
Descending pain modulation  
Peripheral neuropathy  
Sex-difference  
Chemical compounds:  
Atipamezole HCl (PubChem CID13649426)  
Bicuculline methiodide (PubChem CID10487871)  
Muscimol HCl (PubChem CID77108154)  
Prazosin HCl (PubChem CID68546)  
Raclopride HCl (PubMed CID57452790)  
WAY-100635 (PubChem CID5684)

ABSTRACT

Here we studied spinal neurotransmitter mechanisms involved in the reduction of mechanical hypersensitivity by inhibition of the amygdaloid central nucleus (CeA) in male and female rats with spared nerve injury (SNI) model of neuropathy. SNI induced mechanical hypersensitivity that was stronger in females. Reversible blocking of the CeA with muscimol (GABA A receptor agonist) induced a reduction of mechanical hypersensitivity that did not differ between males and females. Following spinal co-administration of atipamezole (α2-adrenoceptor antagonist), the reduction of mechanical hypersensitivity by CeA muscimol was attenuated more in males than in females. In contrast, following spinal co-administration of raclopride (dopamine D2 receptor antagonist) the reduction of hypersensitivity by CeA muscimol was equally attenuated in males and females by spinal co-administration of WAY-100635 (5-HT 1A receptor antagonist) or bicuculline (GABA A receptor antagonist). The CeA muscimol induced attenuation of ongoing pain-like behavior (conditioned place preference test) that was reversed by spinal co-administration of atipamezole in both sexes. The results support the hypothesis that CeA contributes to mechanical hypersensitivity and ongoing pain-like behavior in SNI males and females. Disinhibition of descending controls acting on spinal α 2 -adrenoceptors, 5-HT 1A , dopamine D2 and GABA A receptors provides a plausible explanation for the reduction of mechanical hypersensitivity by CeA block in SNI. The involvement of spinal dopamine D2 receptors and α 2 -adrenoceptors in the CeA muscimol-induced reduction of mechanical hypersensitivity is sexually dimorphic, unlike that of spinal α 2 -adrenoceptors in the reduction of ongoing neuropathic pain.

1. Introduction

Peripheral nerve injuries may cause long-lasting neuropathic pain conditions that are frequently associated with mechanical hypersensitivity and ongoing pain (Jensen and Finnerup, 2014). Mechanisms underlying neuropathic pain involve multiple pathophysiological changes in the processing and regulation of pain-related signals along the neuraxis from peripheral and spinal cord levels to the brain level (Bannister et al., 2020). Among brainstem structures influenced by a peripheral nerve injury is the amygdala that is important for primary emotions (LeDoux, 2000). Amygdala is also involved in processing and modulation of pain (Neugebauer, 2015). Experimental models of painful peripheral nerve injuries cause changes of amygdala neurons as shown by anatomical (Gonçalves et al., 2008), neurophysiological (Ikeda et al., 2007; Gonçalves and Dickenson, 2012), neuroimaging (Mao et al., 1993), and neuropharmacological studies (Pedersen et al., 2007; Ansah et al., 2009; Ansah et al., 2010; Bourbia et al., 2010; Wei et al., 2015; Ji et al., 2017; Sagalajev et al., 2015; Sagalajev et al., 2017). These amygdaloid changes are likely to contribute to the nerve injury-induced pain and its comorbid emotional disorders such as anxiety and
depression (Neugebauer, 2015).

The main output nucleus of the amygdala is its central nucleus (CeA) (LeDoux, 2000). Particularly laterocapsular division of the CeA plays an important role in processing of pain-related signals that arrive the CeA directly through the spinal dorsal horn (SDH) – lateral parabrachial nucleus (LPBN) pathway, or indirectly through the thalamocortical pathway, followed by relays in the lateral and basolateral amygdala (Bernard and Besson, 1990; Neugebauer, 2015). The CeA has ascending connections to the prefrontal cortex that is considered to exert a role in pain-related affective-emotional responses. The CeA may contribute to descending control of pain-related signals through multiple projections. For example, the CeA has efferent descending connections through the midbrain periaqueductal gray (PAG) – rostroventromedial medulla (RVM) pathway to the spinal cord (Fields et al., 2006; Pertovaara and Almeida, 2006). Additionally, the CeA has efferent projections to the pontine locus coeruleus (LC) (Van Bockstaele et al., 2001), the main source of descending noradrenergic pain regulation (Pertovaara, 2006), and to the LPBN (Raver et al., 2020; Hogri et al., 2022), a key relay of ascending nociceptive inputs to the CeA (Bernard and Besson, 1990). Moreover, amygdala may contribute to descending regulation of pain through an ascending-descending loop through the basolateral amygdala – prefrontal cortex pathway (Huang et al., 2019).

Descending pathways originating or relaying in the PAG and the RVM contribute to pain hypersensitivity induced by peripheral nerve injury (Pertovaara et al., 1996; Bian et al., 1998; Sung et al., 1998) or neurogenic inflammation (Pertovaara, 1998). Moreover, a number of studies have shown that the amygdala is among the structures that is involved in descending drive of pain hypersensitivity following nerve injury (Pedersen et al., 2007; Anshah et al., 2009; Anshah et al., 2010; Ji et al., 2015; Sagalajev et al., 2015; Sagalajev et al., 2018; Cooper et al., 2018; Navratilova et al., 2019; Navratilova et al., 2020). However, the roles of the various descending pathways in the amygdala-induced drive of neuropathic pain-like behavior have been only little studied.

Here we studied whether the CeA-associated neuropathic pain-like behavior involves descending controls and action on spinal neurotransmitter receptors. Therefore, we studied whether the reduction of mechanical hypersensitivity induced by reversible blocking of the CeA is reversed by various spinally administered neurotransmitter receptor antagonists in animals with experimental neuropathy. A conditioning place preference paradigm (CPP) (Sufka, 1994; King et al., 2009) was used to determine whether changes in pain hypersensitivity are accompanied by corresponding changes in ongoing pain-like behavior. Additionally, since cognitive performance has been reduced in experimental models of neuropathy (Cunha et al., 2020; Phelps et al., 2021), we determined whether blocking the CeA of nerve-injured animals improves performance in a novel object recognition (NOR) test. While this study was primarily performed in male animals, the key experiments were replicated in females to assess potential sexual dimorphism of the CeA-induced effect on neuropathic pain behavior.

2. Results
2.1. Nerve injury-induced mechanical hypersensitivity and its attenuation by amygdaloid administration of muscimol

In unoperated animals, females had significantly stronger responses to mechanical stimulation of the hind paw than males (main effect of gender: \( F_{1, 17} = 37.4, P < 0.0001 \); Fig. 1A). SNI induced a robust mechanical hypersensitivity in the operated hind limb of both males and females when compared with sham operation. This is shown by the finding that following sham operation the mean response rate to a test force of 6 g was 3 ± 8 % (± S.D.) in males and 10 ± 11 % in females, whereas following SNI the mean response rate at the test force of 6 g was increased to 50 ± 11 % in males (\( t_{20} = 8.4, P < 0.0001 \); not shown) and 80 ± 20 % in females (\( t_{21} = 8.6, P < 0.0001 \); not shown). Female SNI animals had significantly stronger mechanical hypersensitivity than male SNI animals (main effect of gender: \( F_{1, 17} = 27.7, P < 0.0001 \); not shown).

Fig. 1. Mechanical stimulation-evoked hind limb responses in male and female (red) animals before (A) and after spared nerve injury (SNI; B-G) or sham operation (Sham; H), and with co-administration of muscimol (Musc; GABA\(_A\) receptor agonist), bicuculline (Bic; GABA\(_A\) receptor antagonist) or vehicle (Veh) into the central nucleus of the amygdala (CeA; C-E, G, H) or a brain control injection site (F). A. Stimulus-response function in unoperated animals. B) Stimulus-response function in operated animals. C) Time course of the antihypersensitivity effect of CeA muscimol assessed at test stimulus force of 6 g in SNI males. D) Reduction of hypersensitivity by CeA muscimol in SNI females. E) Effect of muscimol in a control injection site, the internal capsule, in SNI males. G) Reduction of hypersensitivity by CeA muscimol, CeA bicuculline, or their co-administration in SNI males. H) Effect by CeA muscimol on mechanical sensitivity in sham-operated males.Veh: Vehicle, Mus(0.05-0.05): muscimol at the dose of 0.025 µg or 0.05 µg, Bic: bicuculline at the dose of 0.03 µg. In D–H, measurements were performed 15 min after intracerebral treatment. The symbols show the mean response rate to repetitive application of the monofilament at the defined force; increases in the response rate represent increases in mechanical sensitivity. Error bars represent S.D. (In A, \( n_{\text{female}} = 8 \) and \( n_{\text{male}} = 11 \). In B, \( n_{\text{female}} = 7 \) and \( n_{\text{male}} = 12 \). In C, D, F–H, \( n = 6 \) in all groups. In E, \( n = 7 \) in both groups). * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) (t-test with Bonferroni correction).
Administration of muscimol (a GABA<sub>A</sub> receptor agonist) at a dose of 0.025 µg or 0.05 µg into the right CeA of male SNI animals significantly attenuated mechanical hypersensitivity in the nerve-injured left hind limb (main effect of muscimol: F<sub>2,15</sub> = 28.36, P < 0.0001; Fig. 1C and D). The onset of the muscimol-induced antihypersensitivity effect was 5 min, the maximum effect was reached within 15 min, and the duration of the antihypersensitivity was <60 min (Fig. 1C). The administration of muscimol at a dose of 0.05 µg into the right CeA attenuated mechanical hypersensitivity also in female SNI animals (main effect of muscimol: F<sub>2,15</sub> = 28.36, P < 0.0001; Fig. 1E). To assess whether muscimol induced antihypersensitivity effect is due to action on the CeA or due to spread to structures adjacent to the CeA, muscimol was administered at the dose of 0.05 µg into a control injection site (the right internal capsule) in male SNI animals. In the control injection site, muscimol had no significant effect on mechanical hypersensitivity of the nerve-injured left hind limb (main effect of muscimol: F<sub>1,10</sub> = 0.55, P = 0.477; Fig. 1F). CeA co-administration of bicuculline (a GABA<sub>A</sub> receptor antagonist) at a dose of 0.03 µg prevented the antihypersensitivity effect induced by CeA administration of muscimol in male SNI animals, whereas CeA bicuculline alone had no effect on hypersensitivity (Fig. 1G). CeA administration of muscimol at doses 0.025 µg or 0.05 µg that had a significant antihypersensitivity effect in male SNI animals (as shown in Fig. 1D) had no significant effect on mechanical sensitivity in male animals with a sham nerve injury (main effect of muscimol: F<sub>2,15</sub> = 1.19, P = 0.33; Fig. 1H).

### 2.2. Spinal neurotransmitters mediating descending modulation of hypersensitivity

To study which spinal neurotransmitter receptors are involved in the attenuation of the CeA muscimol-induced reduction of mechanical hypersensitivity, various neurotransmitter antagonists were co-administered i.t. while muscimol was administered into the CeA at the dose of 0.05 µg in SNI animals. This analysis was done by assessing the drug treatment-induced changes in the response to stimulation at the force of 6 g.

CeA administration of 0.05 µg of muscimol with co-administration of various neurotransmitter antagonists had a significant main effect on hypersensitivity (F<sub>5, 73</sub> = 9.9, P < 0.0001) that varied with the gender of the animals (interaction between treatment and gender: F<sub>5, 73</sub> = 6.3, P < 0.0001; Fig. 2A). The reduction of hypersensitivity induced by CeA muscimol was significantly attenuated by i.t. co-administration of atipamezole (an α2-adrenoceptor antagonist; 5 µg) only in males, and the attenuation was significantly stronger in males than females (Fig. 2A). I.t. co-administration of prazosin (an α1-adrenoceptor antagonist; 3 µg) failed to attenuate the reduction of hypersensitivity by CeA muscimol in either males or females (Fig. 2A). I.t. co-administration of WAY-100635 (a 5-HT<sub>1A</sub> receptor antagonist; 5 µg) attenuated the reduction of hypersensitivity by CeA muscimol both in males and females, and the magnitude of the attenuation did not significantly differ between the sexes (Fig. 2A). I.t. co-administration of raclopride (a dopamine D2 receptor antagonist; 1 µg) attenuated the reduction of hypersensitivity by CeA muscimol only in females, and the attenuation was significantly stronger in females than males (Fig. 2A). I.t. co-administration of bicuculline (a GABA<sub>A</sub> receptor antagonist; 0.03 µg) attenuated the reduction of hypersensitivity by CeA muscimol both in males and females, and the magnitude of the attenuation did not vary with sex (Fig. 2A).

Currently used i.t. administered receptor antagonists alone had no significant effect on hypersensitivity induced by SNI, independent of the sex. This is shown by the finding that CeA administration of vehicle with co-administration of atipamezole (5 µg), prazosin (3 µg), WAY-100635 (5 µg), raclopride (1 µg) or bicuculline (0.03 µg) had no main effect on hypersensitivity (F<sub>5, 73</sub> = 0.95, P = 0.45), independent of the gender (interaction between treatment and gender: F<sub>5, 75</sub> = 1.3, P = 0.29; Fig. 2B).

### 2.3. Attenuation of ongoing pain-like behavior by amygdaloid administration of muscimol

CPP test was used to assess whether muscimol treatment of the CeA induces preference of the muscimol-paired chamber in SNI animals; i.e., if animal had ongoing pain that was reduced by muscimol, the animal was expected to prefer the muscimol-paired chamber. CPP test was performed predominantly in male SNI animals. In female SNI animals, CPP test was performed only in two drug treatment conditions (see details below).

CPP test results indicated that SNI animals spent significantly more time in the muscimol-paired chamber after CeA administration of muscimol, compared with CeA administration of vehicle (Fig. 2B). This is consistent with previous findings showing that CeA muscimol administration induces preference of the muscimol-paired chamber in SNI animals.
time in the chamber paired with CeA administration of muscimol at the dose of 0.05 µg than in the vehicle-paired chamber (main effect of drug treatment: \( F_{1, 28} = 24.1, P < 0.0001; \) Fig. 3A), independent of the gender (interaction between drug treatment and gender: \( F_{1, 28} = 0.2, P = 0.3; \) Fig. 3A). At a lower dose of 0.025 µg, muscimol in the CeA had no significant effect on CPP in male SNI animals (main effect of drug treatment: \( F_{1, 28} = 0.2, P = 0.3; \) Fig. 3A). Atipamezole (an \( \alpha_2 \)-adrenoceptor antagonist; 5 µg) reversed the CPP effect induced by 0.05 µg of CeA muscimol in SNI animals (main effect of drug treatment: \( F_{1, 24} = 0.1, P = 0.8; \) Fig. 3C), independent of the gender (interaction between drug treatment and gender: \( F_{1, 24} = 2.8, P = 0.1; \) Fig. 3C). Atipamezole alone (5 µg i.t.) had no significant effect on CPP in male SNI animals (Fig. 3D). For comparison, the effect of CeA muscimol (0.05 µg) on CPP was studied in a group of sham-operated males. In sham males, CeA administration of muscimol at the dose of 0.05 µg (Fig. 3E) had no significant effect on CPP.

In one group of SNI males we assessed whether the CPP effect (i.e., the association of pain attenuation with the drug-paired chamber) lasts longer than 24 h (the commonly used retention period in CPP experiments that was used in experiments shown in Fig. 3A-E). In this group, the retention period between the conditioning and the CPP assessment was prolonged to 96 h. While 0.05 µg of muscimol in the CeA of male SNI animals caused CPP when the retention period was 24 h (Fig. 3A), following prolongation of the retention time to 96 h, 0.05 µg of CeA muscimol failed to induce CPP (Fig. 3F).

### 2.4. Emotional, motor and cognitive assessments in males

Effect of amygdaloid drug treatments on anxiety-like behavior of male SNI animals was assessed using the light–dark box (LDB) test. Muscimol treatment of the CeA at doses 0.025 µg or 0.05 µg failed to have an effect on time spent in the light box \((F_{2, 19} = 1.79, P = 0.19; \) Fig. 4A). Motor effects of drug treatments were evaluated by determining ambulation distances in each drug treatment condition. Muscimol treatment of the CeA at doses 0.025 µg or 0.05 µg had no significant influence on the ambulation distance of male SNI animals \((F_{2, 19} = 1.84, P = 0.19; \) Fig. 4B).

Novel object recognition (NOR) test was used to assess whether SNI or muscimol treatment of the CeA influences cognitive performance. NOR test performed in three experimental groups (SNI males with CeA muscimol, SNI males with CeA vehicle, and Sham males with CeA vehicle) showed that there were significant main effects by the experimental group \((F_{1, 13} = 4.22, P = 0.039)\) and by the familiarity of the test object \((F_{1, 13} = 26.15, P = 0.0002; \) Fig. 4C). Additionally, interaction between the experimental group and the familiarity of the test object was significant \((F_{2, 13} = 5.83, P = 0.016). Post hoc testing indicated that the sham group spent significantly more time in observing novel objects than the vehicle- or muscimol-treated SNI group (Fig. 4C). Observation

---

**Fig. 3.** Effects of drug treatments on ongoing pain-like behavior as assessed in conditioned place-preference paradigm in male (black symbols) and female (red symbols) animals with spared nerve injury (A-D, F) or sham operation (E). A. Effect by 0.05 µg of muscimol (Mus) in the central nucleus of the amygdala (CeA). B. Effect by 0.025 µg of muscimol in the CeA. C. Effect by co-administration of 0.05 µg of muscimol in the CeA together with intrathecal (it) administration of 5 µg of atipamezole (Ati), an \( \alpha_2 \)-adrenoceptor antagonist. D. Effect by it administration of 5 µg of atipamezole. E) Effect by 0.05 µg of muscimol in the CeA. F) Effect by 0.05 µg of muscimol in the CeA following prolonged retention period. In each graph, the Y-axis shows the difference in time spent in the drug-versus vehicle-paired chamber before versus after drug-pairing sessions. Values > 0 indicate an increase in time spent in the chamber after drug-pairing. Veh: vehicle. In A-E, retention period after drug-pairing was 24 h and in F, 96 h. Error bars represent ± S.D. **\( P < 0.01 \) (t-test with Bonferroni correction).
times of familiar objects were not significant among the experimental groups, nor was the observation time of novel objects different between the vehicle- and muscimol-treated SNI groups (Fig. 4C).

2.5. Injection sites in the brain

Fig. 5A shows the injection sites in the brain.

3. Discussion

In the present study, reversible blocking of the CeA attenuated mechanical hypersensitivity and ongoing pain-like behavior in an equal fashion in SNI males and females. This is in line with previous results (see introduction for references) and with the hypothesis according to which the amygdala is involved in the maintenance of nerve injury-induced hypersensitivity and sustained pain both in males and females. Spinal pretreatment with an antagonist of the α2-adrenoceptor, 5-HT1A receptor, dopamine D2 receptor, or a GABAA receptor, unlike pretreatment with an antagonist of the α1-adrenoceptor, attenuated in a sexually dimorphic fashion the reduction of hypersensitivity induced by a reversible block of the CeA in SNI animals. Blocking spinal dopamine D2 receptors attenuated more effectively the reduction of hypersensitivity by CeA muscimol in SNI females than males, whereas following block of spinal α2-adrenoceptors the attenuation was stronger in SNI males than females. Attenuation of the CeA muscimol-induced reduction of hypersensitivity by block of spinal 5-HT1A or GABAA receptors was not significantly different between SNI males and females. Interestingly, although spinal pretreatment with an α2-adrenoceptor antagonist produced significantly stronger attenuation in the reduction of mechanical hypersensitivity by CeA muscimol in SNI males than females, the block

Fig. 4. Effects by muscimol (Mus) treatment of the central nucleus of the amygdala (CeA) on memory-related, emotional and motor behaviors in male animals with a spared nerve injury (SNIα). A. Anxiety-like behavior in the light–dark box (LDB). B) Motor activity assessed as ambulation distance. C) Memory-like behavior in novel object recognition test. Veh: vehicle, Mus: muscimol (in A and B, dose shown in µg; in C dose was 0.05 µg). In C, the Y-axis shows the change in observation time from first to second exposure; values > 0 represent prolongation of the observation time during second exposure. Graphs show mean responses and ± S.D. (n = 5–8). *P < 0.05 (t-test with Bonferroni correction).

Fig. 5. A) Injection sites in the right amygdala (black circles) and the right internal capsule (black stars). B) Time lines for experimental procedures. In A, CeA = central nucleus of the amygdala, RMg = raphe magnus. Each symbol represents 1–8 overlapping injection sites. For the stereotaxic AP coordinate, the reference is the ear bar. In B, CPP = conditioned place-preference, IT = intrathecal, LDB = light–dark box, Mus = muscimol, NOR = novel object recognition test, SNI = spared nerve injury, Veh = vehicle, wk = week.
of spinal α2-adrenoceptors reversed the CeA block-induced attenuation of ongoing pain-like behavior in a similar fashion in female and male SNI animals. Together, these findings suggest that among mechanisms contributing to the reduction of mechanical hypersensitivity by a CeA block is disinhibition of descending controls acting on spinal α2-adrenoceptors, 5-HT1A receptors, dopamine D2 receptors, and GABAergic receptors. The involvement of spinal α2-adrenoceptors and dopamine D2 receptors in the CeA block-induced control of mechanical hypersensitivity was sexually dimorphic. Independent of the sex, disinhibition of descending controls acting on spinal α2-adrenoceptors contributed to the reduction of ongoing pain-like behavior by CeA block in SNI animals.

3.1. Potential role of spinal α2-adrenoceptors in the regulation of hypersensitivity by CeA.

There is abundant evidence indicating that particularly the pontine LC, a major source of noradrenergic innervation of the spinal cord (Kwiat and Basbaum, 1992), exerts predominantly an inhibitory effect on nociception due to action on spinal α2-adrenoceptors (Pertovaara, 2006), although under some conditions LC may have a pronociceptive action (Brighswell and Taylor, 2009). Bidirectional modulation of nociception by LC can be explained by a finding that LC has different subpopulations of noradrenergic LC-spinal neurons, of which not all are promoting antinociception, but of which at least one promotes nociception (Hickey et al., 2014).

Following peripheral nerve injury, neuronal response to noxious stimulation has been increased in the LC of male animals (Viisanen and Pertovaara, 2007; Alba-Delgado et al., 2021). LC stimulation has produced spinal antinociception in nerve-injured males, although not as effectively as in controls (Viisanen and Pertovaara, 2007). Interestingly, reversible block of the LC in nerve-injured males increased hypersensitivity only during the first postoperative days, but not at later time points (≥ one week) (Llorca-Torralba et al., 2022). This recent finding indicates that sustained LC activity suppresses hypersensitivity only during the first postoperative days but not after that. This is in line with the present result showing that more than week after nerve injury, blocking the spinal α2-adrenoceptor, a pivotal mediator of the descending antinociceptive effect of noradrenergic pathways, had no effect alone in either males or females. Reduction of mechanical hypersensitivity by CeA muscimol was attenuated by spinal co-administration of an α2- but not an α1-adrenoceptor antagonist, and the attenuation was significantly stronger in SNI males than females. Together, these findings suggest that the CeA of SNI animals inhibits descending controls acting on spinal α2-adrenoceptors and thereby, the CeA has an important contribution to the maintenance of mechanical hypersensitivity in male SNI animals. In line with this interpretation, an earlier neurophysiological study showed that chemical activation of the CeA suppressed the discharge rate of LC neurons in nerve-injured but not control males (Viisanen and Pertovaara, 2007).

In an earlier behavioral study, local administration of an α2-adrenoceptor antagonist into the LC disinhibited descending noradrenergic pain inhibition in nerve-injured but not healthy male animals (Weid and Pertovaara, 2006a). This earlier finding suggests that a sustained activation of α2-adrenergic autoreceptors on cell bodies of noradrenergic LC neurons (Aghajanian and VanderMaelen, 1982) contributes to the suppression of the LC-induced descending pain inhibition following nerve injury. Findings in acute inflammation model are in line with the interpretation that activation of α2-adrenergic autoreceptors on cell bodies of noradrenergic LC neurons might play a role in the suppression of the LC-induced descending pain inhibition. Namely, while acute inflammation activates noradrenergic feedback-inhibition of pain due to action on spinal α2-adrenoceptors (Mansikka et al., 2004), a low, presumably locally acting dose of an α2-adrenoceptor agonist in the LC of males increased acute inflammation-induced pain behavior (Pertovaara et al., 1994). These earlier findings in male animals with inflammatory pain model support the proposal that α2-adrenergic autoreceptors in the LC are involved in attenuation of noradrenergic pain inhibition. It may be argued that the α2-adrenergic autoreceptor-induced inhibition of the LC might be among mechanisms contributing to the CeA-associated mechanical hypersensitivity in nerve-injured males.

3.2. Potential role of spinal 5-HT1A receptors in regulation of hypersensitivity by CeA.

RVM is a main source of descending serotonergic pathways (Kwiat and Basbaum, 1992) that modulate nociception through action on various subtypes of spinal 5-HT receptors, some of which have antinociceptive and some pronociceptive effects (Millan, 2002). It has been shown that among spinal 5-HT receptor subtypes attenuating neurophaptic hypersensitivity is the 5-HT1A receptor, whereas among subtypes facilitating neurophaptic hypersensitivity is the 5-HT3 receptor (Suzuki et al., 2002; Liu et al., 2020). The CeA may control descending serotonergic pathways through its RVM projections that have a relay in the PAG (Pertovaara and Almeida, 2006). In line with this, CeA treatments modulate neuronal activity in the RVM (Ansah et al., 2009; Palazzo et al., 2011). In the present study, blocking the spinal 5-HT1A receptor had no effect alone in nerve-injured male or female animals. Independent of the sex, however, spinal co-administration of a 5-HT1A receptor antagonist attenuated the reduction of hypersensitivity by CeA muscimol. This finding suggests that the muscimol block of the CeA in SNI males and females disinfibuted descending controls acting on spinal 5-HT1A receptors.

An earlier behavioral study showed that descending serotoninergic inhibition in neuropathic male animals could be disinhibited by RVM administration of a 5-HT1A receptor antagonist (Wei and Pertovaara, 2006b). Additionally, microinjection of a 5-HT1A receptor agonist into the RVM suppressed cortical stimulation-induced activation of descending serotonergic pathways in nerve-injured males (Sagalajev et al., 2017). These earlier findings are in line with the hypothesis that a sustained drive of 5-HT1A autoreceptors on cell bodies of serotoninergic neurons ( Rogawski and Aghajanian, 1981) suppresses serotoninergic pain controls in neuropathy (Wei and Pertovaara, 2006b). Collectively these earlier and the present findings are in line with the proposal that activation of medullary 5-HT1A autoreceptors is among downstream mechanisms contributing to mechanical hypersensitivity in peripheral neuropathy.

There are multiple 5-HT receptor subtypes. Our study using a 5-HT1A receptor antagonist does not exclude the possibility that among spinal 5-HT receptor subtypes contributing to the reduction of hypersensitivity by CeA muscimol were subtypes other than 5-HT1A. For example, among other potential 5-HT receptor subtypes contributing to the CeA muscimol-induced reduction of hypersensitivity is the spinal 5-HT7 receptor that was not studied here and that has been shown to attenuate hypersensitivity in neuropathic animals (Liu et al., 2020).

3.3. Potential role of spinal dopamine D2 receptors in regulation of hypersensitivity by CeA.

The hypothalamic A11 cell group is the main source of descending dopaminergic innervation (Hökfelt et al., 1979) that can suppress nociception in the spinal dorsal horn through action on dopamine D2 receptors (Fleetwood-Walker et al., 1988; Tamae et al., 2005; Taniguchi et al., 2011). Earlier results in male rats with spinal nerve ligation-induced model of peripheral neuropathy indicated that spinal dopamine D2 receptors contributed to heat antinociception induced by electric stimulation of the A11 cells group (Wei et al., 2009) or the motor cortex (Viisanen et al., 2012). In the present study with SNI model of neuropathy, reduction of mechanical hypersensitivity by CeA muscimol was significantly attenuated by block of spinal dopamine D2 receptors in SNI females but not in SNI males. This finding suggests a sexually dimorphic disinhibition of descending dopaminergic controls as an underlying mechanism for the attenuation.
Earlier findings indicate that spinal dopamine D5 receptors play an important role in the maintenance of chronic pain in males, whereas spinal dopamine D1 receptors contribute to chronic pain in females (Megat et al., 2018). Based on these earlier and the present findings it may be speculated that in SNI females pronociceptive spinal D1 receptors are more active than antinociceptive dopamine D2 receptors, but following CeA block the balance changes so that activation of spinal dopamine D2 receptors predominates leading to attenuation of hyper-sensitivity in females.

3.4. Potential role of spinal GABA<sub>A</sub> receptors in regulation of hypersensitivity by CeA

RVM includes a population of GABAergic neurons with anatomically verified axonal projections directly to the excitatory spinal dorsal horn neurons suggesting a role in suppression of noiception (Antal et al., 1996). In line with this anatomical finding, an in vivo patch clamp study demonstrated that the RVM has a monosynaptic GABAergic inhibitory effect on excitatory spinal dorsal horn neurons (Kato et al., 2006). In the present study, spinal co-administration of a GABA<sub>A</sub> receptor antagonist attenuated the reduction of hypersensitivity by CeA muscimol in SNI males and females. Together these findings support the hypothesis that disinhibition of descending controls acting on spinal GABA<sub>A</sub> receptors was among mechanisms attenuating the reduction of hypersensitivity by CeA muscimol in SNI males and females.

Descending noradrenergic pathways may also induce an α<sub>1</sub>-adrenoceptor-mediated activation of spinal GABAergic interneurons (Gasner et al., 2009), thereby providing an alternative explanation for the spinal GABA<sub>A</sub> receptor block-induced attenuation in the reduction of hyper-sensitivity by CeA muscimol in SNI animals. In the present study, however, drive of spinal GABAergic neurons by α<sub>1</sub>-adrenoceptors is not likely to play a significant role, since a spinally administered α<sub>1</sub>-adrenoceptor antagonist failed to influence the reduction of hypersensitivity by CeA muscimol in SNI males and females.

In contrast to the GABAergic antinociception, a recent study showed that a subpopulation of GABAergic RVM neurons has synaptic contacts with inhibitory spinal neurons and that they facilitate mechanical pain (François et al., 2017). This finding is opposite to the present results. Therefore, it does not seem likely that a descending pronociceptive projection of GABAergic RVM neurons exerted a major role in mediating the CeA block-induced effects on mechanical hypersensitivity in the present study.

3.5. Sexual dimorphism

Earlier results on sex-related differences in pain sensitivity have been variable, although a majority of studies in both humans and animals seems to suggest that pain responses are stronger in females than males (Mogil, 2020). In line with this, the present results showed that baseline responses to mechanical stimulation were stronger in female than male animals, independent whether the animals had SNI or not. In the present study, a non-selective block of the CeA induced by muscimol treatment attenuated mechanical hypersensitivity and ongoing pain-like behavior equally in female and male SNI animals. Earlier studies in non-neuropathic animals have shown that ovarian steroids in the amygdala contribute to the enhanced visceral pain sensitivity of females (Myers et al., 2011) raising a possibility that a more selective treatment of the CeA, such as block of steroid receptors, might have produced a sex-dependent modulation of hypersensitivity in SNI animals of the present study.

Previous findings in non-neuropathic animals indicate that descending pathways regulate pain in a sexually dimorphic manner. This is shown e.g. by a sex-dependent action of morphine on the anti-nociceptive PAG-RVM pathway (Loyd and Murphy, 2014). This is in line with earlier results indicating that the sex-dependent difference in the response of nociceptive spinal dorsal horn neurons disappeared following transection of the spinal cord and the consequent loss of RVM-spinal pathways (You et al., 2006). Moreover, an earlier study using neurotoxins for selective lesions of the noradrenergic or serotoninergic systems in healthy male and female rats showed that the noradrenergic system contributed to the descending heat antinociception both in males and females, whereas the serotoninergic system contributed to the descending heat antinociception in males but not in females (Lei et al., 2011). These earlier findings on sex-dependent control of heat nociception in healthy animals are only partly similar with the present results on the sex-dependent control of mechanical hypersensitivity in neuropathic animals. The differences between the earlier (Lei et al., 2011) results on heat nociception under physiological conditions and the present results on mechanical hypersensitivity under neuropathic conditions may be explained by differences in the experimental conditions, since descending control of pain-related behavior has been shown to vary from facilitation to inhibition depending on the submodality of test stimulation and pathophysiological condition (Kapulla et al., 1998; You et al., 2022). In neuropathic animals of the present study, CeA inhibition disinhibited spinal α<sub>2</sub>-adrenoceptor-mediated suppression of ongoing-like behavior in a similar fashion in males and females, whereas spinal α<sub>2</sub>-adrenoceptors contributed to the suppression of mechanical hypersensitivity by CeA block in males rather than females. The contributions of spinal 5HT<sub>2A</sub> or GAB<sub>A</sub> receptors to the suppression of mechanical hypersensitivity by CeA block did not differ between males and females. A significant contribution of spinal dopamine D2 receptors to the suppression of mechanical hypersensitivity by CeA block was observed in females rather than males. It remains to be studied whether the roles of spinal serotoninergic, dopaminergic or GABAergic receptors in the suppression of ongoing pain-like behavior by CeA block vary with sex in neuropathic animals.

3.6. Limitations and other factors to be considered.

Cognitive performance is reduced in various models of chronic neuropathic pain (Cunha et al., 2020; Phelps et al., 2021). In line with this, performance in the NOR test was reduced in SNI animals when compared with sham controls. Muscimol treatment of the CeA failed to improve performance of SNI animals in the NOR test suggesting that the CeA does not have a major role in the maintenance of cognitive deficits. In contrast, basolateral amygdala has shown to be involved in neuropathic pain-related cognitive impairment via feedforward inhibition of the prefrontal cortex (Thompson and Neugebauer, 2019). It is noteworthy that in the present study muscimol was administered unilaterally to the right CeA that has a key role in pain-related functions (Carrazquillo and Gereau, 2008; Ji and Neugebauer, 2009; Allen et al., 2021). Unilateral treatment of the CeA that was used in the present study may provide an additional or alternative explanation for not observing a CeA muscimol-induced attenuation of cognitive impairment in the NOR test as well as for the lack of a significant effect of the CeA muscimol treatment on anxiety-like behavior in the LDB test.

The currently used injection volume for muscimol administrations into the CeA was 0.5 μl that may have spread also outside of the CeA (Myers, 1966). However, muscimol injections into a control injection site, the internal capsule, failed to have any effect. Moreover, the CeA is a main source of amygdaloid outputs to the brainstem nuclei involved in descending pathways (Neugebauer, 2015). Therefore, it is likely that the amygdaloid administration of muscimol induced its effect due local action in the CeA rather than outside of it. Interestingly, CeA administration of bicuculline, a GABA<sub>A</sub> receptor antagonist, failed to influence neuropathic hypersensitivity in this or an earlier study (Pedersen et al., 2007) suggesting that endogenous release of GABA within the CeA may not explain the CeA-induced promotion of neuropathic hypersensitivity. In contrast, an earlier study showed that CeA bicuculline attenuated pain-related affective behavior (Pedersen et al., 2007), which was not studied here. Nor did we study whether CeA bicuculline might have attenuated ongoing pain-like behavior.
Concerning mechanisms within the CeA, GABAergic neurons are the main neuron type of the CeA and they form mutual inhibitory connections in the nucleus and exert inhibitory influence on various extra-amygdalar regions such as the PAG and locus coeruleus (Neugebauer, 2015). Additionally, the CeA is composed of several other types of neurons expressing various types of neurotransmitters in distinct subnuclei that form a mutually inhibitory network within the CeA. Moreover, it is known that the role of CeA neurons projecting to the PAG differs depending on the neuron types within CeA subnuclei (Li and Sheets, 2018), different classes of inhibitory neurons in the right CeA. In general, it is known that the role of CeA neurons projecting to the PAG play opposite roles in regulating mechanical hind limb sensitivity (Wilson et al., 2019), and artificial excitation of the subgroup of CeA neurons could increase or decrease pain sensitivity even under physiological conditions (Sadler et al., 2017; Sugimoto et al., 2021). Further studies are needed to pinpoint the critical sites of muscimol action on the complex intra-CeA circuitries that underlie the reduction of hypersensitivity by CeA muscimol in the present study.

It should also be noted that not only neurons but also glial cells express GABA$_A$ receptors and contribute to neuropathic pain (Malcangio, 2019). Consequently, it remains to be studied whether the reduction of mechanical hypersensitivity by CeA muscimol is at least partly due to muscimol’s effect on glia.

It has been argued that the currently used index of ongoing pain-like behavior, CPP, may actually reflect summated allodynic and hyperalgesic pains caused by the stimuli of daily life (Bennett, 2012). In the present study, block of the CeA in SNI animals induced CPP and an accompanying reduction of mechanical hypersensitivity. Both of these effects were reversed by spinally administered $\alpha_2$-adrenoceptor antagonist in males. This finding raises the possibility that in SNI males, stimulation of the hypersensitive hind paw caused by standing and walking may have exerted a role in the generation of the ongoing pain-like behavior. Accordingly, muscimol-induced CeA block may have contributed to reduced ongoing pain-like behavior by attenuating hypersensitivity rather than by a direct action on “true” ongoing pain or unpleasantness. However, reduction of hypersensitivity per se may not explain CPP in female SNI animals, since spinal pretreatment of SNI females with an $\alpha_2$-adrenoceptor antagonist reversed only CeA block-induced CPP but failed to have a significant influence on the reduction of hypersensitivity.

A change in motor activity provides a potential confounding factor when assessing pain behavior. The effect of CeA muscimol on pain-related behavior in the present study, however, may not be explained by a change in motor activity as shown by similar ambulation distances in the CeA muscimol- and CeA vehicle-treated SNI animals.

One important limitation of the present study is that all the experiments were made 1–4 weeks after nerve injury. Earlier results have shown that during this post-injury period SNI produces a strong and stable hypersensitivity to mechanical stimulation of the sural nerve area used for test stimulation (Decosterd and Woolf, 2000; Gonçalves et al., 2008; Gangadharan et al., 2022). However, clinical and experimental studies suggest that chronic pain, its co-morbidities and their underlying mechanisms may evolve with prolongation of the post-injury time (Borsook et al., 2018), which needs to be taken into account in the interpretation of the present results.

3.7. Conclusions.

Here we show in SNI model of peripheral neuropathy that among mechanisms contributing to the reduction of mechanical hypersensitivity by CeA block is disinhibition of descending controls acting in a sexually dimorphic fashion on spinal $\alpha_2$-adrenoceptors and dopamine D2 receptor, and in a sex-independent fashion on spinal 5-HT$_{1A}$ receptors and GABA$_A$ receptors. Moreover, CeA block in SNI animals contributed to reduction of ongoing pain-like behavior due disinhibition of descending controls acting in a sex-independent fashion on spinal $\alpha_2$-adrenoceptors.

4. Materials and methods

4.1. Experimental animals.

The experiments were performed in adult, male and female Hanover-Wistar rats (weight: 180–230 g; Envigo, Horst, The Netherlands). The experimental protocol was accepted by the Ethical Committee on Animal Experiments of the regional government of Southern Finland (permission # ESAVI-41116-2019). The experiments were performed according to the guidelines of European Communities Council Directive 2010/63/EU on the use of animals for scientific purposes and according to the ARRIVE guidelines (Knopp et al., 2015). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The animals were housed in polycarbonate cages with a deep layer of sawdust. Due to the brain cannulae and intrathecal catheters, only one animal was in each cage, but with visual, auditory and olfactory contact with animals of the adjacent cages. The temperature in the thermostatically controlled animal room was 24.0 ± 0.5 °C. The room was artificially illuminated from 8.30 a.m. to 8.30p.m. The home cages were environmentally enriched. The animals received commercial pelleted rat feed (CRM-P pellets, Special Diets Services, Witham, Essex, England) and tap water ad libitum.

4.2. Techniques for producing neuropathy.

The spared nerve injury (SNI) model developed by Decosterd and Woolf (2000) was used to induce peripheral neuropathy. Prior to surgery, the animal was anesthetized with sodium pentobarbital (Orion-Pharma, Espoo, Finland) administered intraperitoneally (i.p.) at the dose of 60 mg/kg. Further doses of sodium pentobarbital were given at the dose of 15–20 mg/kg as needed to keep the depth of anesthesia deep enough so that the animal did not react to noxious stimulation. SNI surgery was performed on Day 0 (D0) in the same session as installation of intrathecal (i.t.) catheter and/or brain cannula. In the SNI operation, an incision was made into the skin on the lateral surface of the left thigh, followed by a section through the biceps femoris muscle to expose the sciatic nerve and its terminal branches: the sural, common peroneal and tibial nerves. Then, the common peroneal and tibial nerves were tightly ligated with 4–0 silk, sectioned distal to the ligation and 3–4 mm of the distal nerve stump removed. The sural nerve was left intact and care was exercised not to stretch it. For comparison, sham-operated animals were studied. Sham operation was performed identically as the operation for inducing SNI, except that the common peroneal and tibial nerves were left intact. After the operations, the muscle and skin were sutured, and the rats were moved to their individual home cages for recovery. For postoperative pain treatment, animals were administered 0.01 mg/kg of buprenorphine twice daily up to the third postoperative day.

4.3. Techniques for microinjections.

For amygdaloid drug administrations, a guide cannula was installed into the central nucleus of the right amygdala (CeA) as described earlier (Sagalajev et al., 2018). The right amygdala was the target in this study, since previous rat studies indicate that the right amygdala has a more important role in pain processing than the left amygdala (Carraquillo and Gereau, 2008; Ji and Neugebauer, 2009; Allen et al., 2021), although not in all conditions (Cooper et al., 2018). Moreover, it has been reported that the descending control of nociception by the amygdala is stronger on the contralateral than ipsilateral side (Bourbia et al., 2010; Allen et al., 2021). This further supported the choice of the right amygdala for unilateral drug injections in the studied group of animals that all had a nerve injury (or sham injury) in the left hind limb.

For unilateral drug injections into the CeA or the control injection site in the internal capsule, the rats were implanted with a chronic guide cannula made of stainless steel (26 gauge; PlasticsOne, Roanoke, VA,
USA) in a standard stereotaxic frame under general anesthesia at the same time as inducing SNI. The chronic guide cannula was positioned according to the atlas of Paxinos and Watson (1997) 1.0 mm above the desired injection site. The injection target in the right amygdala was the capsule lateral of the central nucleus of amygdala (CeA): 2.56 mm posterior from the bregma, 4.2 mm lateral from the midline, and 7.8 mm ventral from the dura mater (Paxinos and Watson, 1997). A separate group of animals had a guide cannula in a control injection site (the right internal capsule: 2.1 mm posterior from the bregma, 3.6 mm lateral from the midline, and 5.0 mm ventral from the dura mater (Paxinos and Watson, 1997).

The chronic guide cannula was fixed into the skull using a dental screw and dental cement. A dummy cannula was placed into the guide cannula until the test session. When the drug was administered into the brain, it was microinjected through a 33-gauge stainless steel injection cannula (PlasticsOne) inserted through and protruding 1 mm beyond the tip of the guide cannula. The intracerebral microinjection was made using a 10 μl Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) that was connected to the injection cannula by a length of a polyethylene (PE-10) tubing (Becton Dickinson and Company, Sparks, MD, USA). The volume of intracerebral injections was 0.5 μl. The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 30 s and the injection cannula was left in place for an additional 30 s to minimize flow of the drug solution back up the injector track. During the injections, the experimenters held the animal.

For i.t. drug injections, a catheter (PE-10) was implanted into the lumbar level of the spinal cord under general anesthesia in the same operation as inducing SNI and installing the brain cannula. The procedure for the catheter installation through the lumbar route is described in detail elsewhere (Storkson et al., 1996). Following recovery from anesthesia, the correct placing of the catheter was verified by administering lidocaine (4 %, 7–10 μl followed by a 15 μl of saline for flushing) with a 50 μl Hamilton syringe. Only those rats that had no motor impairment before lidocaine injection but had a bilateral paralysis of hind limbs following i.t. administration of lidocaine were studied further. The lidocaine test was performed at least 3 days prior to the start of the drug testing sessions. For i.t. administration, the drugs were microinjected with a 50 μl Hamilton microryringe at a volume of 5–7 μl followed by a saline flush at a volume of 15 μl.

4.4. Assessment of mechanical sensitivity

Observations by clinical neurologists indicate that nerve injury most commonly produces tactile allodynia or hypersensitivity to mechanical stimulation of the skin (e.g., with brush), while heat hyperalgesia is less common (Jensen and Finnerup, 2014). Therefore, the focus of the current behavioral study was in the assessment of tactile allodynia-like hypersensitivity by determining a limb withdrawal response evoked by monofilament stimulation of the sural nerve area in the injured hind limb.

Prior to any testing, the rats were habituated to the experimental conditions by allowing them to spend 1–2 h daily in the laboratory during 2–3 days. For assessment of tactile allodynia-like hypersensitivity, the hind limb withdrawal threshold evoked by stimulation of the hind paw with monofilaments (von Frey-hairs) was determined while the rat was standing on a metal grid. At each time point, the paw ipsilateral to the spinal nerve ligation was stimulated five times with an ascending series of calibrated monofilaments (SNI animals: 1–10 g, and sham-operated animals 4–26 g; North Coast Medical, Inc., Morgan Hill, CA). At each time point, the hind paw of the operated limb was stimulated five times at each test stimulus force using an ascending series of stimulus forces. Frequency of the limb withdrawal response was used as the index of mechanical sensitivity. An increase in the withdrawal response rate was considered to represent mechanical hypersensitivity effect. When compared with the traditional or ‘up-and-down’ determination of the withdrawal threshold value, the currently used method has the advantage that it allows assessing separately drug effects on withdrawal responses evoked by stimulus forces of threshold and suprathreshold levels. To reduce redundancy in reporting, the results on the co-administration of CeA muscimol and spirally administered neurotransmitter antagonists (Fig. 2) are shown only at a stimulation force of 6 g. Among reasons for choosing the 6 g force was that an earlier study using high-speed videography, statistical modeling, and machine learning showed that to evoke limb withdrawal responses representing pain-like behavior, the test stimulus force needs to be at least 4 g in mice (Abdus-Saaboor et al., 2019). Furthermore, the force of 6 g raised no response in control animals (Fig. 1E) and a minimal response in nerve-injured animals (Fig. 1B). Thereby, the test stimulus force of 6 g allowed an optimal visualization of both increases and decreases in the response rate of nerve-injured animals. Moreover, when comparing the effects of different i.t. co-administered receptor antagonists on the reduction of hypersensitivity by CeA muscimol, the response rate to 6 g test stimulation was standardized in the following way to reduce variability: [Drug treatment induced change in response rate] = [Response rate 15 min after drug treatment] – [Response rate before drug treatment] (Fig. 2).

4.5. Assessment of ongoing pain-like behavior

Since hypersensitivity needs not be associated with ongoing pain (Kilo et al., 1994), a separate behavioral assay was used for determining ongoing pain-like behavior. For the analysis of ongoing pain-like behavior or aversiveness, CPP paradigm (Sulka, 1994; King et al., 2009) was used in a modified fashion. Earlier studies have demonstrated that i.t. clonidine produces CPP in SNI but not sham animals (King et al., 2009; Leite-Almeida et al., 2012) suggesting that the CPP paradigm allows assessing the effect of pharmacological treatments on CPP in the SNI model. Rats underwent a two-day habituation to the CPP device, in which they were placed in automated CPP boxes (Place Preference System, San Diego Instruments, Inc., San Diego, CA, USA) with access to all 3 chambers for 30 min per day during the first two days of CPP procedure (D1 and D2). The device records time spent in each chamber using a computer-controlled 4×16 array of photo beams. Among differences between the test chambers was the roughness of the floor (rough versus smooth) and the painting of the walls (black triangles versus bars on white surface). The following two days (D3 and D4) the doors between the chambers were closed and all rats received a morning injection of vehicle, after which they were placed in one of the pairing chambers for 30 min. Four hours later, all rats received drug treatment (CeA administration of muscimol or i.t. administration of atipamezole, or a combination of CeA muscimol and i.t. administration of atipamezole) and were placed in the opposite chamber for 30 min. The chamber that was paired with the drug was changed from animal to animal; i.e., the chamber with rough floor and black triangles was paired with the drug in 50 % of the animals, and with vehicle in 50 % of the animals to eliminate the possibility that properties of the chamber might cause CPP. It is also noteworthy that the plantar skin of the operated hind limb touching the floor is insensitive during the currently used observation period of 4 weeks in SNI animals (Gangadharan et al., 2022) and thereby, the environmental cues from the floor (such as roughness) are mediated by the intact paws. On the fifth CPP day (D5), 24 h following the last drug pairing, animals were placed drug-free for 30 min in the CPP boxes with access to all chambers. Furthermore, in one group we assessed whether prolongation of the retention period influences the result by allowing access to all chambers 96 h, instead of 24 h after the last drug pairing. The amount of time spent in each of the two chambers (saline- and drug-paired) before and after drug pairing session was automatically registered and used to quantify the conditioning effect by drug treatment. It was expected that if the animal had ongoing pain that was reduced by drug treatment, the animal preferred the drug-paired chamber.
4.6. Assessment of anxiety-like and locomotor behavior.

Light-dark box (LDB) test was used to assess anxiety-like behavior as described in detail in our earlier study (Chen et al., 2018). The Plexiglas LDB (30 cm × 30 cm × 30 cm) was constructed of two chambers separated by a Plexiglas board (30 cm × 30 cm × 15 cm), one of which was covered with black masking tape, the other was covered with white paper and illuminated by a cold light source of xenon lamp (100 lx). The device records automatically time spent in each chamber using a computer-controlled 4×16 array of photo beams. The box was placed in a dark room, the illumination being provided exclusively by the xenon lamp. Half of the rats were individually placed in the center of the white compartment facing the opening whereas the other half of the rats were individually placed in the center of the dark compartment. The test lasted for 5 min and the time spent in each compartment was measured. The LDB assessment was determined by calculating the percentage of the time spent in light box. A reduction in time spent in the light box was considered to represent increased anxiety-like behavior.

The same device that was used in assessment of the LDB behavior was also used in the assessment of locomotor behavior in separate groups of animals. However, when assessing locomotor behavior, all chambers of the device had equal light conditions. Computer-controlled photo beams measured the ambulation distance during a 10 min period.

4.7. Novel object recognition test

The novel-object recognition (NOR) task was used to assess cognitive performance. The test was based on protocols described previously (Moriarty et al., 2016; Chen et al., 2018) with some modifications. Testing was carried out in a circular arena with a white 85 cm diameter floor and 50 cm high wall. The arena was illuminated by four 40 W fluorescent lamps which provided a constant light level of 300 lx. A digital video camera was positioned above the arena and was used to record behavior during testing for subsequent analysis. The objects were plastic bottles (filled with water) with a base diameter of 6.5 cm and 23.5 cm height and two stacked plastic cubes with the side length of 5.7 cm and height of 11.4 cm. The objects had no apparent natural significance to the rats, and were secured to the base of the arena with adhesive plaster. Animals were habituated to the arena in the absence of objects for 20 min on the day before the test day. The test day comprised of three stages: habituation, exposure 1 and exposure 2. Rats were first introduced to the arena for a 3-min habituation period and then returned to their home cage for 7 min. During exposure 1, two identical objects (bottles) were placed in opposite quadrants of the arena, 16 cm from the perimeter. The rat was allowed to freely explore the arena and the objects for a period of 3 min, after which the animal was removed from the arena and returned to its home cage for an interval of 5 min. Prior to exposure 2, one of the bottles was replaced with the novel object (two stacked plastic cubes). The animal was again allowed to freely explore the objects for a period of 3 min in the arena and then it was returned to its home cage. The arena was cleaned with 70 % ethanol between exposures. Exploration of an object was defined as sniffing the object, rearing against the object or having the head directed towards the object within a 2 cm annulus of the object. The NOR assessment was determined by calculating the difference in the observation time between the second and the first exposure. In other words, when exposing the same object twice, the difference was “explore time 2 – explore time 1”. When exposing a novel object, the difference was “explore time 2 of the novel object – explore time of the object presented during the first exposure”. It was expected that if the animal remembers the objects presented during the first exposure, the animal spends more time exploring the novel than the familiar object during the second exposure.

4.8. Course of the study

Fig. 5 shows schematic time courses for the experiments. In general, the nerve or sham injury was performed, and the brain cannula and/or i. t. cannula was installed in one operation under general anesthesia, after which the animals were allowed to recover for one week. During the recovery, animals were habituated to the experimenters and the laboratory for 2–3 days 1–2 h daily. The CPP paradigm was tested on the second postoperative week. The CPP paradigm was performed only once and in only one drug-pairing condition for each animal. Alternatively to the CPP test, some groups of animals were tested either in the LDB or the locomotion test. The effect of drug treatments on hypersensitivity started during the second postoperative week and continued for the third postoperative week.

When assessing the effects of drug combinations on hypersensitivity, the receptor antagonists were administered 5 min before CeA administrations of muscimol that allowed having maximum effects by the currently used compounds and drug combinations at about the same time (15 min after CeA administration of muscimol). There were the following treatment groups: i) CeA muscimol, ii) CeA vehicle, iii) i.t. one of the receptor antagonists (atipamezole/prazosin/WAY-100635/raclopride/bicuculline) + CeA muscimol, iv) i.t. one of the receptor antagonists (see group iii) + CeA vehicle, v) CeA bicuculline, vi) CeA bicuculline + CeA muscimol. Each drug condition was tested on a separate day, at varying order and at an interval of at least 2 days. The number of CeA muscimol injections per one animal varied from 4 to 6. In all of treatment groups, hypersensitivity was assessed before and at various time points after the drug treatments. When comparing the effects of different i.t. administered receptor antagonists on the reduction of hypersensitivity by CeA muscimol, the response rate to 6 g stimulation was standardized in the following way: [the drug-induced change in the response rate] = [response rate 15 min after drug treatments] – [response rate before drug treatments]. In case [response change in the group treated with spinal co-administration of a receptor antagonist + CeA muscimol] > [response change in the group treated with CeA muscimol alone], then the spinally administered receptor antagonist attenuated the reduction of hypersensitivity by CeA muscimol. In other words, a more positive response change value in the co-administration group than in the CeA muscimol alone group indicates attenuation of the CeA muscimol-induced antihypersensitivity effect. The NOR test was performed during the fourth week.

Within males and females, animals were randomly selected to various experimental groups. Drug treatments were given in a blinded fashion. At the completion of the experiments, the animals were given a lethal dose of sodium pentobarbital and the brains removed for verification of the injection sites according to the atlas of Paxinos and Watson (1997).

4.9. Drugs

Muscimol (GABA<sub>A</sub> receptor agonist), bicuculline (GABA<sub>A</sub> receptor antagonist), prazosin (α<sub>1</sub>-adrenoceptor antagonist), WAY-100635 (5-HT<sub>1A</sub> receptor antagonist), and raclopride (dopamine D2 receptor antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atipamezole (α<sub>2</sub>-adrenoceptor antagonist) was purchased from Orion-Pharma (Turku, Finland). Physiological saline was used as control.

The doses of the currently used neurotransmitter receptor antagonists were chosen based on our earlier studies showing that following i.t. administration they reverse effects of agonists but have no effects alone in neuropathic animals (e.g., Viisanen et al., 2012; Wei et al., 2014).

4.10. Statistical analyses

Statistical evaluation of the data was performed using one- or two-way ANOVA, with mixed design when appropriate, followed by t-test with Bonferroni correction, or with unpaired t-test when comparing two
groups. \( P < 0.05 \) (two-tailed) was considered to represent a significant difference.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Funding

This study was financially supported by the Academy of Finland (\#315043) and the Sigrid Juselius Foundation, Helsinki, Finland.

References

Abdu-Saboor, I., Fried, N.T., Lay, M., Burdge, J., Swanson, K., Fischer, R., Jones, J., Dong, P., Cai, W., Guo, X., Tao, Y.X., Bethen, J., Ma, M., Dong, X., Ding, L., Luo, W., 2019. Development of a mouse pain scale using sub-second behavioral mapping and statistical modeling. Cell Rep. 28, 1623–1634.e4. https://doi.org/10.1016/j.celrep.2019.04.048.

Aghajanian, G.K., VanderMaelen, C.R., 1982. a2-Adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: Intracellular studies in vivo. Science. 215, 1394–1396. https://doi.org/10.1126/science.6278591.

Albo-Delgado, C., Mico, J.A., Berrocoso, E., 2021. Neuropathic pain increases spontaneous and noise-evoked activity of locus coeruleus neurons. Prog. Neurophysiopathol. Psychobiol. Psychiatry. 110, 110121. https://doi.org/10.1016/j.pnpbp.2020.110121.

Allen, H.N., Bobnar, H.J., Kolber, B.J., 2021. Left and right hemispheric lateralization of the amygdala in pain. Prog. Neurobiol. 196, 101891 https://doi.org/10.1016/j.pneurobio.2020.101891.

Anah, O.B., Gonçalves, L., Almeida, A., Pertovaara, A., 2009. Enhanced pronociception by amygdaloid group I metabotropic glutamate receptors in nerve-injured animals. Exp. Neurol. 216, 66–74. https://doi.org/10.1016/j.expneurol.2008.11.005.

Antal, M., Petkó, M., Polgár, E., Heizmann, C.W., Storm-Mathisen, J., 1996. Direct evidence of an extensive GABAergic innervation of the spinal dorsal horn by fibres descending from the rostral ventromedial medulla. Neuroscience. 79, 509–518. https://doi.org/10.1016/S0306-4522(96)00063-2.

Bannister, K., Sachau, J., Baron, R., Dickenson, A.H., 2020. Neuropathic pain: Mechanism-based therapies. Annu. Rev. Pharmacol. Toxicol. 50, 267–274. https://doi.org/10.1146/annurev-pharmtox-010818-021524.

Bennett, G.J., 2012. What is spontaneous pain and who has it? J. Pain. 13, 921–929. https://doi.org/10.1016/j.jpain.2012.05.008.

Bennett, J.F., Besson, J.M., 1990. The spinogigantocellular tract: Electrophysiological evidence for an involvement in pain processes. J. Neurophysiol. 63, 473–490. https://doi.org/10.1152/jn.1990.63.3.473.

Bian, D.i., Ossipov, M.H., Zhong, C., Malan, T.P., Porreca, F., 1998. Tactile allodynia, but not thermal hyperalgesia, of the amygdala contributes to mechanical allodynia and hyperalgesia following right-sided peripheral nerve injury. Neurosci. Lett. 284, 187–192. https://doi.org/10.1016/S0304-3940(98)01124-X.

Cooper, A.H., Brightwell, J.J., Hedden, N.S., Taylor, B.K., 2018. The left central nucleus of the amygdala contributes to mechanical allodynia and hyperalgesia following right-sided peripheral nerve injury. Neurosci. Lett. 684, 187–192. https://doi.org/10.1016/j.neulet.2018.08.013.

Cunha, A.M., Pereira-Mendes, J., Almeida, A., Guimarães, M.R., Leite-Almeida, H., 2020. Chronic pain impact on rodents’ behavioral repertoire. Neurosci. Biobehav. Rev. 119, 101–127. https://doi.org/10.1016/j.neubiorev.2020.09.025.
