Central amygdala inflammation drives pain hypersensitivity and attenuates morphine analgesia in experimental autoimmune encephalomyelitis

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Abstract

Chronic pain is a highly prevalent symptom associated with the autoimmune disorder multiple sclerosis (MS). The central nucleus of the amygdala plays a critical role in pain processing and modulation. Neuropathic pain alters nociceptive signaling in the central amygdala, contributing to pain chronicity and opioid tolerance. Here, we demonstrate that activated microglia within the central amygdala disrupt nociceptive sensory processing and contribute to pain hypersensitivity in experimental autoimmune encephalomyelitis (EAE), the most frequently used animal model of MS. Male and female mice with EAE exhibited differences in microglial morphology in the central amygdala, which were associated with heat hyperalgesia, impaired morphine reward, and reduced morphine antinociception in females. Animals with EAE displayed a lack of morphine-evoked activity in cells expressing somatostatin within the central amygdala, which drive antinociception. Induction of focal microglial activation in naive mice via injection of lipopolysaccharide into the central amygdala produced a loss of morphine analgesia in females, similar to as observed in EAE animals. Our data indicate that activated microglia within the central amygdala may contribute to the sexually dimorphic effects of morphine and may drive neuronal adaptations that lead to pain hypersensitivity in EAE. Our results provide a possible mechanism underlying the decreased efficacy of opioid analgesics in the management of MS-related pain, identifying microglial activation as a potential therapeutic target for pain symptoms in this patient population.

Keywords: Chronic pain, Central amygdala, Opioids, Microglia, Reward, Sex differences, Multiple sclerosis, Central inflammatory pain

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease that is characterized by chronic inflammation and demyelinating lesions within the central nervous system, ultimately leading to neurodegeneration.19,67 Chronic pain is a highly prevalent and debilitating symptom associated with MS, affecting between 50% to 80% of patients over the course of their disease.26,59 Unfortunately, MS-related pain responds poorly to classical analgesics, contributing to overall disease burden and reduced quality of life. Despite the high incidence of chronic pain in MS, the underlying mechanisms remain poorly understood.

Microglia, the resident macrophages of the central nervous system, are key players in the initiation and maintenance of chronic pain. Elevated microglial reactivity and proliferation are consistently observed in animal models of acute, inflammatory, and neuropathic pain.8,22,58,75,76,87–89 In experimental autoimmune encephalomyelitis (EAE), an animal model commonly used to study MS, changes in glial cell reactivity within the spinal cord correlate with the development of cold and tactile allodynia.16,27,58 Although much of the evidence about glial contribution to chronic pain emanates from studies in the spinal cord, more recent reports have found widespread pain-induced microglial activation throughout the neural axis in both animal models and human patients, particularly in brain regions associated with pain and affect.6,70,78,79 No previous research has examined how microglia within affective circuitry contribute to sensory and affective disturbances associated with MS-related pain.

The amygdala is a small nucleus within the limbic brain that is well-known for its role in integrating emotional and sensory information.44,82 The central nucleus of the amygdala, in particular, has emerged as a neural substrate critical to pain processing and regulation that serves as the major output nucleus for amygdala-driven pain-related functions.35,55,56,80,85 Chronic pain is associated with alterations in amygdala neurocircuitry and anatomy, suggesting that these maladaptive changes are an important contributor to pathological pain states.20,34,35,39,80 By contrast, however, earlier studies demonstrated that the central amygdala is an important locus for analgesia and opioid-dependent pain modulation.31,49,50,54 Recent research has further shown that the central amygdala functions to maintain a balance of pronociceptive and antinociceptive signaling, contributing to the overall nociceptive tone of the animal.85
Despite the wealth of data implicating the amygdala as a critical node for pain modulation and endogenous analgesia, there are few studies examining how neuroinflammation influences amygdala function in the context of chronic pain. Here, we describe a novel mechanism by which activated microglia in the central amygdala disrupt the balance of inhibitory and excitatory pain pathways that gate nociceptive information processing in the EAE mouse model of MS. The current study identifies central amygdala inflammation as a driving factor of pain chronicity and reduced opioid-mediated analgesia in EAE.

2. Materials and methods

2.1. Subjects and experimental testing

All animal experiments and procedures were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with approval from the University of Alberta Health Sciences Animal Care and Use Committee. Male and female C57BL/6 mice aged 10 to 12 weeks old were received from Charles River Canada Laboratory in QC, Canada. Animals were housed in wire-top cages (4-5 per cage) in a temperature-controlled environment with food and water available ad libitum. Mice were kept on a 12-hour light/dark cycle and all experimental procedures were performed during light cycles only.

2.2. Experimental autoimmune encephalomyelitis induction and assessment

Male and female C57BL/6 mice were randomly assigned to vehicle control or experimental (EAE) groups. To induce EAE, animals were immunized by subcutaneous injection of 50 μg/100 μL of myelin oligodendrocyte glycoprotein peptide 35 to 55 (MOG35-55; Stanford University Peptide Synthesis Facility) emulsified in Complete Freund’s Adjuvant (CFA; 1.5 mg/mL; Sigma-Aldrich, ON, Canada) in the hind flanks. Control mice received a subcutaneous injection of CFA (1.5 mg/mL) and were used as vehicle control for EAE induction. All mice received an intraperitoneal injection of pertussis toxin (300 ng; List Biological Laboratories, Cedarlane, ON, Canada), administered at the time of induction and 48 hours later to facilitate an immune response. Throughout the experiment, mice were monitored daily and the clinical signs of EAE were classified using the following criteria: grade 0, normal mouse; grade 1, flaccid or paralyzed tail (disease onset); grade 2, mild hind limb weakness with quick righting reflex; grade 3, severe hind limb weakness with slow righting reflex; and grade 4, hind limb paralysis in one hind limb or both. All behavioural testing was performed before the onset of clinical motor symptoms (grade 1), when animals did not exhibit any hind limb weakness, paralysis, or righting reflex impairment. Animals that progressed beyond clinical grade 1 were excluded from behavioural experiments.

2.3. Thermal tail withdrawal assay

Mice were habituated to the testing room before the experiment. On the test day, mice were gently restrained, and approximately 2.5 cm of the tail was immersed in 49°C water. The time between tail submersion and withdrawal response was recorded. A cut-off latency of 15 seconds was used to prevent the possibility of tissue damage. Three baseline measurements were recorded and later averaged. Morphine analgesic potency was determined using cumulative dose–response curves. Immediately after the last baseline measurement, animals were injected with an initial dose of 0.1 mg/kg morphine, followed with successiveescalating doses (3, 10, 30 mg/kg, i.p.). Tail withdrawal latencies were measured once every 15 minutes after each injection. Mice were returned to their home cages between testing. Data are expressed as the raw tail withdrawal latencies (seconds), as well as the percent maximum possible effect (%MPE), calculated as: ([test latency – baseline latency]/[cut-off latency – baseline latency]) × 100.

2.4. Conditioned place preference

Morphine-conditioned place preference (CPP) was conducted using a 2-chamber, counter-balanced, and unbiased apparatus. The 2 conditioning chambers (24 × 24.5 × 26 cm) were distinguishable by visual cues and separated by a wall with a guillotine door. To control for inherent bias to either chamber, mice were placed in the CPP apparatus and allowed free access to both chambers for 25 minutes. The time spent in each chamber was recorded using an infrared charge coupled-device camera attached to a computer running behavioural tracking software (Noldus EthoVision, Leesburg, VA). The drug-paired chamber was then assigned such that any inherent bias for one chamber was balanced among treatment groups. Conditioning sessions consisted of mice receiving 2 trials of morphine sulfate (0.5 or 10 mg/kg, i.p.) and 2 trials of saline vehicle (0.9% NaCl, i.p.) on alternating days over 2 days. Animals were confined to the chambers for 25 minutes after injection and immediately returned to their home cages. On the final testing day, animals were allowed free access to both chambers in a drug-free state, and the time spent in the morphine-paired chamber compared with the vehicle-paired chamber was assessed over 25 minutes. Male and female vehicle control and EAE mice were tested on days 5 to 8 post-disease induction. The amount of raw time (seconds) spent in the vehicle-paired chamber and morphine-paired chamber is presented, as well as the difference score. Difference scores were calculated as the time spent in the drug-paired chamber minus the time spent in the vehicle-paired chamber. A positive difference score is indicative of a CPP. Locomotor activity was acquired during testing and was used to ensure that locomotor function was retained for the duration of the experiment.

2.5. Intracranial lipopolysaccharide injections

Naïve male and female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with isoflurane and mounted on a stereotaxic frame. Saline vehicle (0.9% NaCl; 100 nL per side) or lipopolysaccharide (LPS; 1 μg dissolved in 100 nL saline per side; Sigma-Aldrich) was bilaterally injected into the central amygdala (coordinates from bregma: anterior-posterior (AP) −1.06, medial-lateral (ML) ± 2.25, dorsal-ventral (DV) −4.50) using a sterile glass micropipette over a period of 1 minute per injection. Animals were allowed to recover for 72 hours after surgery, at which point thermal pain hypersensitivity and morphine antinociception were assessed.

2.6. Immunohistochemistry

On the day of clinical signs of disease presentation (grade 1), animals were euthanized using an intraperitoneal injection of Euthasol (sodium pentobarbital, 0.1 mL of 340 mg/mL). For immunohistochemical experiments, mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Frozen brains and spinal cord were sectioned on a cryostat at 25 μm and mounted onto glass slides. Sections were incubated with primary antibodies overnight at room temperature. The following primary antibodies and concentrations were used: rat anti-cluster of differentiation 4 (CD4; 1:200; Bio-Rad Laboratories, ON, Canada), rabbit anti-ionized calcium-binding adapter molecule (Iba-1; 1:500; Wako, Richmond, VA), rabbit anti-cFOS (1:3000; Cell Signaling, Danvers, MA), mouse anti-protein kinase C delta
(PKCs; 1:500; BD Biosciences, San Jose, CA), and rat anti-somatostatin (SOM; 1:250; MilliporeSigma, ON, Canada). The next day, secondary immunolabeling was performed with donkey anti-rabbit Alexa Fluor 488 (1:200; Thermo Fisher Scientific, Carlsbad, CA), donkey anti-rat Alexa Fluor 594 (1:200; Thermo Fisher Scientific), or donkey anti-mouse Alexa Fluor 647 (1:250; Thermo Fisher Scientific). Tissue sections were counterstained with ProLong Gold mounting medium containing fluorescent 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) to visualize cellular nuclei.

2.7. Image acquisition and analysis

All image acquisition and analyses were performed blind to experimental condition and sex. For each given immunohistochemical stain, only sections with identifiable nuclei were analyzed. Three to 4 separate sections per region per animal were analyzed and averaged to give a single value per animal. For analysis of Iba-1, CD4, cFOS, SOM, and PKCα staining, images were acquired on a Zeiss AxioImager microscope equipped with a 20X objective lens (Carl Zeiss, Oberkochen, Germany). To examine Iba-1+ microglial morphology, Z-stack images were captured on a Zeiss AxioObserver inverted confocal microscope equipped with a 63X objective lens (LSM 710; Carl Zeiss). Representative images were acquired on a Zeiss AxioObserver inverted confocal microscope using either a 20X, 40X, or 63X objective lens. Iba-1 staining intensity (optical density) in the lumbar spinal cord was quantified with ImageJ Software by measuring the area fraction of stain occupied within the region of interest. To objectively differentiate Iba-1-positive cells from diffuse staining, a staining intensity threshold that selectively identified concentrated staining surrounding the cell nuclei was applied to all images. Iba-1-positive microglial activation in the amygdala, nucleus accumbens, and primary somatosensory cortex was assessed based on morphological criteria, including cell body size and cell branching.70,86 Microglial morphology was analyzed with MetaXpress High-Content Image Acquisition and Analysis Software. Approximately 20 cells per section were measured. The number of CD4-positive, cFOS-positive, SOM-positive, and PKCα-positive cells were quantified using the ImageJ Software Analyze Particles function with constant cell identification parameters across all images. Only clearly labeled and in-focus cells were counted in the entire optical region. For analysis of cFOS colabelling with SOM-positive and PKCα-positive cells, colabelled cells were identified using Zen Pro Imaging Software (Zeiss) and then manually counted. Quantifications of cell types were randomly sampled throughout the central amygdala at approximate coordinates relative to bregma: AP –0.9 to –1.2, ML ± 2.3 to 2.5, and DV –4.5 to –4.9.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 8.3.1. Data were tested for normality and parametric or nonparametric statistics were used accordingly for all experiments. Group means were compared using unpaired Student t test or Mann–Whitney test. For comparisons using data sets with more than 2 groups or conditions, a 2-way or 3-way analysis of variance (ANOVA) followed by an appropriate post hoc test was conducted. Intersex comparisons were performed for all experiments using either a 2-way or 3-way ANOVA. The data were presented with the sexes pooled if the main effect of sex was not statistically significant. Either Tukey or Sidak post hoc analysis was used to correct for multiple comparisons. All data were expressed as mean ± standard error of the mean. Significance threshold was set at P < 0.05 in all experiments.

3. Results

3.1. Presymptomatic experimental autoimmune encephalomyelitis is associated with spinal cord T-cell infiltration, microgliosis, and thermal hyperalgesia

In humans, patients often present with pain as an initial sign of MS or as their only symptom before the development of other symptoms.51 Here, we found that before the onset of clinical motor symptoms (presymptomatic phase), EAE mice displayed significantly lower thermal tail withdrawal thresholds than vehicle-treated controls (Fig. 1B, F_disease (1,56) = 10.35, P = 0.0022, F_time (1,56) = 8.826, P = 0.0044, and F_interaction (1,56) = 5.558, P = 0.0219). Analysis with a 3-way ANOVA revealed no significant effect of sex (supplementary Fig. 1, F_sex (1,52) = 1.836, P = 0.2066, available at http://links.lww.com/PAIN/B364); therefore, males and females were pooled for these analyses. This presymptomatic timepoint was also associated with cellular signs of disease, including T-cell infiltration and microgliosis in the lumbar spinal cord (Fig. 1C–E). The number of lumbar spinal cord CD4+ cells (Fig. 1D, t = 2.650, P = 0.0243) and Iba-1+ immunoreactivity (Fig. 1E, t = 2.277, P = 0.0488) were significantly increased in EAE mice compared with controls. These results indicate that EAE is associated with changes in pain hypersensitivity, as well as microglial and immune cell reactivity within the spinal cord that precede deficits in locomotor function.

3.2. Experimental autoimmune encephalomyelitis impairs morphine analgesia in females

We next sought to assess the analgesic potency of systemic morphine in EAE. The analgesic effects of morphine on EAE-induced pain hypersensitivities of the hind paw could not be assessed because of drug-induced hyperlocomotion; therefore, morphine antinociception was measured by the tail withdrawal assay. Escalating doses of morphine (0.1-30 mg/kg, i.p) produced dose-related elevations in tail withdrawal thresholds (analgesia) that were not significantly different between male EAE and control animals (Fig. 2B, F_disease (1,40) = 3.333, P = 0.0754, F_dose (4,40) = 155.1, P < 0.0001, and F_interaction (4,40) = 0.7390, P = 0.5710). In female mice, morphine produced dose-dependent increases in withdrawal thresholds in controls, whereas this analgesic effect was significantly reduced in the EAE group (Fig. 2B, F_disease (1,40) = 29.29, P < 0.0001, F_dose (4,40) = 177.5, P < 0.0001, and F_interaction (4,40) = 9.261, P < 0.0001). This loss of morphine antinociception was most evident at higher doses of morphine (10 mg/kg; P = 0.0048; 30 mg/kg; P < 0.0001). A significant sex difference in the antinociceptive potency of morphine was also noted, such that morphine analgesia was markedly reduced in EAE females compared with males (F_sex (1,31) = 74.3, P = 0.0001). These comparisons remained when data were expressed as the %MPE induced by 30 mg/kg morphine (Fig. 2C, F_disease (1,16) = 13.21, P = 0.0022, F_sex (1,16) = 31.63, P < 0.0001, and F_interaction (1,16) = 11.48, P = 0.0037). These data suggest that morphine-mediated antinociception is sexually dimorphic in EAE.

3.3. Experimental autoimmune encephalomyelitis impairs morphine reward in both males and females

We have previously shown that chronic pain causes disruptions within affective and reward circuitry, resulting in impaired reward behaviour.78 To probe the function of the reward system in EAE, we used morphine-CPP testing. No significant effect of sex was detected for the raw time spent in the vehicle-paired and
Figure 1. Experimental autoimmune encephalomyelitis as a model of MS-related pain. (A) Schematic of typical EAE disease course and progression. (B) Baseline thermal tail withdrawal thresholds compared between control and EAE animals before and after disease induction (day 18 post-induction; 2-way ANOVA, N = 9-20 per group). (C) Representative immunomicrographs (20X) of Iba-1+ (microglia, green) and CD4+ (CD4+ T cells, red) cells in the spinal cord from control and EAE animals. (D) The number of infiltrating CD4+ cells and (E) Iba-1+ cells in the lumbar spinal cord was significantly increased in the presymptomatic phase of EAE compared with controls (Student t test, N = 5-7 per group). Scale bar = 100 µm. Data presented as mean ± SEM. ANOVA, analysis of variance; AU, arbitrary units; CTL, control; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; SEM, standard error of the mean.
Figure 2. Experimental autoimmune encephalomyelitis impairs morphine analgesia in females and morphine reward in both sexes. (A) Timeline and drug regimen of the behavioral experiments. Animals were induced with EAE, followed by 4 days of morphine-conditioned place preference testing (days 5–8 post-induction), before being subjected to the tail withdrawal assay (day 8 post-induction). Testing day is shown above the long solid black line and the conditioned place preference phase (blue) or experiment below. (B) Morphine (0.1, 3, 10, and 30 mg/kg) dose–response curve was measured using thermal tail withdrawal and compared between male and female control and EAE animals. Morphine antinociception was significantly reduced in female mice with EAE (2-way ANOVA, **P < 0.0048, ****P < 0.0001, N = 5 per group). (C) Transformed data (%MPE) of tail withdrawal thresholds from animals treated with 30 mg/kg morphine (2-way ANOVA). (D) No significant differences between males and females were detected so the sexes are presented pooled (see supplementary Fig. 2, available at http://links.lww.com/PAIN/B364). Conditioned place preference to 10 mg/kg (i.p.) morphine in control and EAE animals. Left: systemic morphine induced a significant place preference selectively in control animals (2-way ANOVA, N = 8-9 per group). Right: the difference score calculated as the time spent in the morphine-paired chamber minus the time spent in the vehicle-paired chamber, indicating that only control mice showed a morphine place preference (Student t test). (E) Conditioned place preference to 0.5 mg/kg (i.p.) morphine in control and EAE animals. Left: low-dose morphine failed to elicit a place preference in control animals and produced a place aversion in EAE animals (2-way ANOVA, N = 9-10 per group). Right: the difference score for mice with EAE was significantly lower than that of the control group, indicating a place aversion (Student t test). (F) Locomotor behaviour was not affected in EAE mice at this stage of disease. Mice with EAE showed no impairments in basal locomotor activity or in morphine-induced (5 mg/kg) hyperlocomotion when compared with controls (Student t test, N = 9-10 per group). Data presented as mean ± SEM. ANOVA, analysis of variance; CTL, control; EAE, experimental autoimmune encephalomyelitis; %MPE, percent maximum possible effect; SEM, standard error of the mean.
morphine-paired chambers (supplementary Fig. 2A, \(F_{\text{sex}} (1,24) = 0.04466, P = 0.8343\), available at http://links.lww.com/PAIN/B364) or for CPP difference scores (supplementary Fig. 2B, \(F_{\text{sex}} (1,13) = 0.3873, P = 0.5445\), available at http://links.lww.com/PAIN/B364) when mice were conditioned to 10 mg/kg morphine. In a similar manner, no main effect of sex was found for the raw time spent in either chamber (supplementary Fig. 2C, \(F_{\text{sex}} (1,30) = 0.08526, P = 0.7723\), available at http://links.lww.com/PAIN/B364) for CPP difference scores (0.5 mg/kg: supplementary Fig. 2D, \(F_{\text{sex}} (1,15) = 0.00246, P = 0.9611\), available at http://links.lww.com/PAIN/B364) when animals were conditioned to 0.5 mg/kg morphine. Therefore, males and females were pooled for the following analyses. It may be noted that the sample size is too small to draw definitive conclusions about the absence of sex differences. Analysis with 2-way ANOVA indicated a significant effect of drug-pairing when animals were administered 10 mg/kg morphine (Fig. 2D, \(F_{\text{drug-pairing}} (1,30) = 31.68, P < 0.0001\) and \(F_{\text{disease}} (1,30) = 0.02354, P = 0.8791\)) and a significant interaction between drug-pairing and disease (\(F_{\text{interaction}} (1,30) = 16.70, P = 0.0003\)). As expected, control animals displayed robust place preference for the morphine-paired compared with the vehicle-paired chamber (\(P < 0.0001\)). By contrast, morphine did not induce a significant place preference in EAE mice (\(P = 0.8800\)). The difference in time spent in the drug-paired chamber over the unpaired chamber for control mice was significantly greater in comparison with that of EAE mice (Fig. 2D, \(t = 2.942, P = 0.0101\)). This confirms that morphine produces place preference only in control animals.

A significant effect of drug-pairing (Fig. 2E, \(F_{\text{drug-pairing}} (1,34) = 7.405, P = 0.0102\), \(F_{\text{disease}} (1,34) = 0.009937, P = 0.9210\) and an interaction between drug-pairing and disease (\(F_{\text{interaction}} (1,34) = 4.991, P = 0.0322\)) were found when animals were injected with a lower dose of morphine (0.5 mg/kg). No significant place preference was observed in the control group when animals were conditioned to this low dose of morphine (\(P = 0.9997\)), whereas EAE animals showed a preference for the vehicle-paired chamber (\(P = 0.0060\)). This dose of morphine produced an unanticipated place aversion in mice with EAE, as indicated by a significant negative difference score (Fig. 2F, \(t = 2.124, P = 0.0486\)). To confirm that mice with EAE had no gross locomotor deficits that would interfere with the ability to develop or exhibit a place preference, locomotor activity was measured. No significant deficits in gross locomotor function (Fig. 2F, \(t = 1.075, P = 0.2993\)) or in morphine-induced hyperlocomotion (\(t = 0.5431, P = 0.5937\)) were observed in these animals at this stage of disease.

4.3. Experimental autoimmune encephalomyelitis induces microglial activation in the amygdala in males and females

Mounting evidence suggests that inflammation and changes in microglial reactivity can influence pain sensitivity in a variety of injury models. Although reactive microgliosis and inflammation at the spinal level are established characteristics of the EAE model, few studies have examined the expression of Iba-1 immunolabeling, we found that EAE animals displayed significant microglial activation selectively in the amygdala at this stage of disease (Fig. 3A–D). No significant effect of sex for microglial cell body size (supplementary Fig. 3A, \(F_{\text{sex}} (1,24) = 2.156, P = 0.1550\), available at http://links.lww.com/PAIN/B364) or branching (supplementary Fig. 3A, \(F_{\text{sex}} (1,24) = 1.774, P = 0.1954\), available at http://links.lww.com/PAIN/B364) was detected in the amygdala. As such, males and females were pooled for these analyses. Amygdala microglia in EAE mice exhibited a stereotypical activated phenotype, including an enlarged cell body (Fig. 3B, \(t = 3.221, P = 0.0034\)) and reduced cell branching compared with controls (Fig. 3B, \(t = 3.535, P = 0.0025\)). We have previously shown that these morphological changes correlate with the activation state of microglia. Experimental autoimmune encephalomyelitis microglia in the somatosensory cortex showed no change in cell body size (Fig. 3C, \(t = 1.277, P = 0.2117\)) but a significant reduction in branching (Fig. 3C, \(t = 3.052, P = 0.0049\)). Experimental autoimmune encephalomyelitis did not induce significant changes in microglial morphology in the nucleus accumbens (Fig. 3D, cell body size: \(t = 1.136, P = 0.2653\); cell branching: \(t = 1.404, P = 0.1756\)).

3.5. Noxious signaling in the central amygdala is disrupted in experimental autoimmune encephalomyelitis

The central amygdala receives direct noxious projections from the spinal cord and parabrachial nucleus via the spino-parabrachio–amygdaloid pathway, playing a critical role in nociceptive processing. Recent work indicates that the central amygdala contains 2 populations of GABAergic neurons (PKCδ+ and SOM+) that regulate pain perception in an opposing manner. PKCδ+ neurons are activated by nociceptive stimuli and increase pain behaviours, whereas activation of SOM+ neurons drives antinociception. This study also found that noxious activity within the central amygdala is altered in chronic neuropathic pain. Here, we investigated how central amygdala activity is altered in EAE. Analysis of double immunostaining for cFOS and SOM with 2-way ANOVA demonstrated no significant effect of sex for total cFOS+ (supplementary Fig. 4A, \(F_{\text{sex}} (1,22) = 0.5839, P = 0.4529\), available at http://links.lww.com/PAIN/B364) or total SOM+ cell counts in the central amygdala (supplementary Fig. 4B, \(F_{\text{sex}} (1,22) = 0.3144, P = 0.5807\), available at http://links.lww.com/PAIN/B364). Three-way ANOVA indicated no significant effect of sex for cFOS colabelling with SOM+ (supplementary Fig. 4C, \(F_{\text{sex}} (1,22) = 0.5988, P = 0.4439\), available at http://links.lww.com/PAIN/B364). Similarly, analysis of double immunolabeling for cFOS and PKCa revealed no significant effect of sex for total cFOS+ (supplementary Fig. 5A, \(F_{\text{sex}} (1,22) = 0.009806, P = 0.9220\), available at http://links.lww.com/PAIN/B364), total PKCα+ (supplementary Fig. 5B, \(F_{\text{sex}} (1,22) = 0.02739, P = 0.8700\), available at http://links.lww.com/PAIN/B364), or colabelled cFOS+/PKCα+ cells (supplementary Fig. 5C, \(F_{\text{sex}} (1,38) = 1.560, P = 0.2192\), available at http://links.lww.com/PAIN/B364). The sexes were therefore pooled for the following analyses. On day of disease onset, EAE animals displayed an increased number of cFOS+ cells in the central amygdala compared with controls (Fig. 4B, \(t = 2.210, P = 0.0369\)). To determine the neuronal identity of the FOS+ cells in the EAE tissue, we colabelled for cFOS alongside markers for SOM and PKCδ. We found no significant difference in the total number of SOM+ (Fig. 4C, \(t = 0.3505, P = 0.7290\)) or PKCa+ neurons (Fig. 4C, \(t = 0.1145, P = 0.9098\)) between vehicle control and EAE mice. Systemic morphine (5 mg/kg) evoked an increase in the activity of antinociceptive central amygdala (Cas)-SOM+ cells in control mice but failed to elicit this response in mice with EAE (Fig. 4D, \(F_{\text{disease}} (1,41) = 7.632, P = 0.0085\), \(F_{\text{drug}} (1,41) = 33.43, P < 0.0001\), and \(F_{\text{interaction}} (1,41) = 14.63, P = 0.0004\)). Co-localization of cFOS with PKCa was not found to be significantly different between control and EAE animals (Fig. 5D, \(F_{\text{disease}} (1,42) = 1.341, P = 0.2534\), and \(F_{\text{interaction}} (1,42) = 0.3201, P = 0.5757\))
Morphine treatment had no significant effect on cFOS-PKC colocalization in either control or EAE mice (Fig. 5D, $F_{\text{drug}}(1,42) = 2.501$, $P = 0.1213$). Taken together, these results indicate that a lack of responsivity within the antinociceptive neurons in the central amygdala may be driving impaired morphine efficacy in EAE.

### 3.6. Activated microglia in the central amygdala attenuate morphine analgesia in naïve female mice

Given the robust microglial activation observed specifically within the amygdala (Fig. 3), we next asked whether activated microglia contribute to the altered central amygdala nociceptive processing in EAE. Saline vehicle or LPS was injected directly into the central amygdala of EAE-naïve male and female mice to focally activate microglia (Fig. 6A). We confirmed that intra-CeA LPS injection induced significant microglial activation, as evidenced by increased cell body size ($F_{\text{injection}}(1,27) = 165.6$, $P < 0.0001$, $F_{\text{sex}}(1,27) = 2.775$, $P = 0.1073$, and $F_{\text{interaction}}(1,27) = 2.277$, $P = 0.1430$) of Iba-1 immunopositive cells surrounding the injection site. Focal LPS injection did not induce microglial activation in surrounding areas, such as the secondary sensory cortex, indicating that LPS injection did not lead to diffuse inflammation throughout the brain (Fig. 6C, $F_{\text{injection}}(1,26) = 3.260$, $P = 0.0826$, $F_{\text{sex}}(1,26) = 0.4877$, $P = 0.4912$, and $F_{\text{interaction}}(1,26) = 0.1284$, $P = 0.7229$).

Morphine produced dose-dependent increases in tail withdrawal thresholds that were not significantly different between saline-treated and LPS-treated males (Fig. 6D, $F_{\text{injection}}(1,65) = 1.179$, $P = 0.2815$, $F_{\text{drug dose}}(4,65) = 143.0$, $P < 0.0001$, and $F_{\text{interaction}}(4,65) = 0.8252$, $P = 0.5139$). Strikingly, injection of LPS into the central amygdala interfered with morphine antinociception in females. Females treated with intra-CeA LPS displayed significantly lower tail withdrawal latencies in response to morphine than saline-treated controls (Fig. 6D, $F_{\text{injection}}(1,65) = 7.591$, $P = 0.0076$, $F_{\text{drug dose}}(4,65) = 145.3$, $P < 0.0001$, and $F_{\text{interaction}}(4,65) = 3.003$, $P = 0.0345$). Analysis with a 3-way ANOVA revealed a significant effect of sex ($F_{\text{sex}}(1,26) = 9.578$, $P = 0.0047$) and a significant interaction between sex and treatment ($F_{\text{interaction}}(1,26) = 5.177$, $P = 0.0314$). Thus, injection of LPS into the central amygdala induced a loss of morphine analgesia in female but not male mice. Saline-treated animals did not exhibit any changes in morphine antinociception, suggesting that the observed reduction in morphine potency was due to the presence of microglial activation in the central amygdala. These trends remained when data were transformed to %MPE induced by 30 mg/kg morphine (Fig. 6E, $F_{\text{sex}}(1,25) = 3.947$, $P = 0.0580$, $F_{\text{injection}}(1,25) = 3.017$, $P = 0.0947$, and $F_{\text{interaction}}(1,25) = 6.889$, $P = 0.0146$). These findings demonstrate that microglial activation within the central amygdala contributes to mu-opioid sensitivity in a sex-dependent manner.

### 4. Discussion

The central amygdala is an important brain region involved in the bidirectional modulation of pain.80-83 Here, we show that markers of cellular central amygdala activity after nociceptive and...
analgesic stimuli are altered in mice with EAE in a sex-dependent manner. We also identified activated microglia within the central amygdala as a key mediator in this dysfunction.

Activity within affective and motivational brain regions influence the experience of pain and analgesia, and disruptions to this circuitry contribute to pain chronicity. Activity within affective and motivational brain regions influence the experience of pain and analgesia, and disruptions to this circuitry contribute to pain chronicity.29,78 Chronic pain conditions, including MS, are highly comorbid with affective and mood disorders, suggesting that disturbances in affective circuitry are indeed prevalent within this patient population.24,43 Several studies have reported depression and anxiety-like symptoms in the EAE model,1,66 and there is evidence indicating that amygdala function is affected early on in the MS disease course. For instance, patients with relapsing–remitting MS display reduced amygdala volume and loss of functional connectivity with other cortical regions.62 These changes have been correlated with cognitive deficits and mood disorders associated with this disease.65 However, how alterations in amygdala function contribute to pain hypersensitivity and analgesia in EAE remained unexplored.

In this study, we found that morphine (10 mg/kg) produced a CPP in vehicle control animals but failed to elicit a place preference in EAE mice (Fig. 2). These results indicate disrupted reward processing in the EAE model and are consistent with previous reports showing that opioids, including morphine, are less rewarding in animals with chronic pain.52,57,60,61,64 In contrast to our findings, however, other studies have noted that low-dose and high-dose opioids remain reinforcing in neuropathic pain models, likely through their analgesic effects.13,78 We found that administration of low-dose morphine (0.5 mg/kg) did not produce a place preference in controls, whereas EAE animals displayed an unexpected aversion to the morphine-paired chamber. These observations indicate changes in dose–response sensitivity to morphine reward and suggest that EAE animals retained the capacity for associative learning, however, function contribute to pain hypersensitivity and analgesia in EAE remained unexplored.

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cognitive performance was not directly measured. Additional studies are warranted to assess how EAE-induced pain influences associative learning in the context of drug reward. Opioid analgesics frequently provide inadequate relief for MS and neuropathic pain patients, except at high doses that might enhance the risk for adverse side effects. Animal models of chronic pain show reduced opioid analgesia compared with controls.3,17,42,47,48,90 In line with previous work, we show that morphine-induced analgesia was significantly impaired in female EAE animals (Fig. 2). We found robust microglial activation in the central amygdala that correlated with heat hypersensitivity and loss of morphine efficacy in female mice. Focal activation of microglia in the central amygdala induced by LPS was associated with a loss of opioid-mediated analgesia in a similar manner to EAE animals (Fig. 5). Although we confirmed that microglial activation was limited to the region surrounding the injection site, we acknowledge that LPS-induced inflammation likely extended throughout the amygdala and was not limited to the central amygdala. This effect was only observed in females, again reminiscent of the EAE condition. These results indicate a greater role for microglia in modulating central amygdala nociceptive processing. In particular, our findings suggest that inflammation within the central nucleus of the amygdala may impair nociceptive signaling specifically in females. Further research is necessary to investigate causal relations between microglial activation and nociceptive signaling within the central amygdala.

Our findings within the central amygdala complement a vast literature indicating that microglia throughout the central nervous system contribute to morphine anti-analgesia and pain hyper-sensitivity. Microglial activation induced by neuropathic pain, persistent inflammatory pain, or intraperitoneally administered LPS attenuates the analgesic efficacy of acute morphine.25,41,68,83 This effect is prevented by blocking microglial activation with systemic or intrathecal glial inhibitors.25,28,38,41,68,84 It is clear that the physiological mechanisms that underlie opioid analgesia and pain are inextricably linked.
Although much of this previous work has been limited to spinal loci, we indicate a role for supraspinal microglial cell activity as well. The converging evidence of this study and previous research support the notion that modulation of microglial and neuro-immune activation are potential therapeutic targets for pain reduction and enhanced morphine analgesia.

Recent work indicates that SOM-expressing neurons in the central amygdala contribute to an endogenous analgesic tone and display suppressed excitability during nerve injury–induced chronic pain.85 Chemogenetic activation of CeA-SOM1 cells results in robust analgesia that reverses nerve injury–induced hypersensitivity.85 The role of these neurons in modulating exogenous opioid-induced analgesia had not yet been established. In this study, systemic morphine evoked an increase in the number of SOM1 central amygdala neurons that colocalized with cFOS and was associated with significant analgesia in vehicle control animals. Compared with controls, SOM+ cells in EAE were less responsive to systemic morphine at disease onset, a time point which correlates with peak pain hypersensitivity58 and lower morphine analgesia (Fig. 2). The consistent inhibition of CeA-SOM+ neurons in both central inflammatory (Fig. 4) and neuropathic85 pain states strongly points to their physiological relevance in the modulation of analgesic signals emerging from the central amygdala. The distribution of SOM and PKCδ expression varies across the anterior–posterior axis of the central amygdala.2,53 Our imaging analyses were primarily focused within the central capsular division of the central amygdala, where SOM expression is relatively low. The combined results from our immunohistochemical analyses of SOM+ and PKCδ+ central amygdala neurons suggest that the observed changes in the SOM cell population may be contributing to alterations in morphine analgesia. Additional investigations are required to determine the functional relevance of these changes on a behavioural level. Taken together, our findings suggest that reduced activity of SOM-expressing neurons within the central amygdala may influence pain hypersensitivity and opioid analgesia in EAE. We observed an increase in CeA-cFOS expression at the onset of EAE that was not localized to SOM- or PKCδ-positive cells (Fig. 4). Although nonoverlapping neurons expressing SOM and PKCδ make up most of the central amygdala cell population,32,36,37,59 these data suggest that additional neuronal populations are likely recruited in central inflammatory pain.
Here, we show that morphine-induced analgesia was significantly impaired in EAE animals in a sexually dimorphic manner. In EAE female, but not male mice, morphine analgesia was dramatically reduced compared with controls (Fig. 2). These results are consistent with previous reports that indicate sex differences in opioid analgesia. Male rodents generally display greater analgesic responses to systemically administered mu-opioid agonists than females. Enhanced sensitivity to morphine analgesia exhibited by male animals has been documented with several pain assays, including those assessing thermal and visceral nociception. Our results build on this previous literature and demonstrate that females exhibit an even greater reduction in opioid analgesic efficacy in chronic pain. Although both males and females displayed a similar impairment in opioid-induced CeA-SOM+ neuronal activity, only females exhibited a reduction in morphine analgesia. It is therefore likely that this sexual dimorphism in opioid-induced analgesia reflects differences in signaling pathways downstream to the amygdala. The amygdala is involved in mediating descending pain modulation through projections to the periaqueductal gray (PAG), which communicates with the rostral ventromedial medulla (RVM) to project to the spinal cord to inhibit pain signals. The physiological organization and the functional activation of the PAG-RVM pathway are sexually distinct. Female rats have significantly more PAG-RVM output neurons than males, whereas this circuit is preferentially engaged in males during persistent inflammatory pain states. Systemic morphine activates the descending modulatory pathway to a greater degree in male than female rats. Recent work indicates that sex differences in microglial reactivity within the PAG contribute to the sexually dimorphic effects of morphine. Collectively, these studies suggest that descending projections from the central amygdala may provide an anatomical basis and central mechanism for the observed sex differences in morphine antinociception in EAE.

Previous research has described differences in the microglial regulation of pain between the sexes, such that spinal microglia contribute to pain hypersensitivity particularly in males. However, this male-dependent microglial effect on pain is confined to the spinal cord. Intrathecal LPS injection elicits pain only in male mice, whereas administration to the brain and hind paw produces equivalent mechanical allodynia in males and females. Notably, LPS induces a similar degree of spinal microgliosis in both sexes. These results suggest that differences in spinal microglial function and signaling, not in the degree of activation or proliferation, contribute to the sexually dimorphic role of microglia in pain. This aligns with our current study, which describes similar levels of microglial activation in the amygdala between male and female EAE mice (Fig. 3). A clear limitation of the current investigation is that only changes in microglial morphology were assessed. Additional research that examines microglial cell function and signaling is needed to dissect out the sexually distinct contributions of microglial activation to EAE-induced pain.

In conclusion, our present data indicate that activated microglia within the central amygdala contribute to the sexually dimorphic effects of morphine and may drive neuronal adaptations that lead to pain hypersensitivity in EAE. Novel treatment approaches for pain in MS must, therefore, take the effects of sex on pain regulation and treatment outcomes into consideration. Results from this study provide mechanistic insight into why opioids are less effective at treating chronic pain and suggest that inhibiting microglial activation may be a viable target to improve analgesic efficacy in this patient population.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found at http://links.lww.com/PAIN/B364.

Supplemental video content
A video abstract associated with this article can be found at http://links.lww.com/PAIN/B365.

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