NEUROSCIENCE
RESEARCH ARTICLE
S. Darvish-Ghane et al. / Neuroscience 498 (2022) 249–259

Inflammatory Pain Alters Dopaminergic Modulation of Excitatory Synapses in the Anterior Cingulate Cortex of Mice

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Abstract—Pain modulation of dopamine-producing nuclei is known to contribute to the affective component of chronic pain. However, pain modulation of pain-related cortical regions receiving dopaminergic inputs is understudied. The present study demonstrates that mice with chronic inflammatory injury of the hind paws develop persistent mechanical hypersensitivity and transient anxiety. Peripheral inflammation induced by injection of complete Freund's Adjuvant (CFA) induced potentiation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAR) currents with a presynaptic component in layer II/III of the ACC. After four days of inflammatory pain, the dopamine-mediated inhibition of AMPAR currents was significantly reduced in the ACC. Furthermore, dopamine enhanced presynaptic modulation of excitatory transmission, but only in mice with inflammatory pain. High-performance liquid chromatography (HPLC) analysis of dopamine tissue concentration revealed that dopamine neurotransmitter concentration in the ACC was reduced three days following CFA. Our results demonstrate that inflammatory pain induces activity-dependent changes in excitatory synaptic transmission and alters dopaminergic homeostasis in the ACC.

Key words: ACC, Dopamine, CFA, Inflammation, Pain, AMPAR, Glutamate, Synaptic potentiation.

INTRODUCTION

There is evidence for the involvement of central dopaminergic circuits in pain modulation (Navratilova et al., 2012; Elman and Borsook, 2016; Mitsi and Zachariou, 2016; Serafini, et al., 2020). Altered dopamine receptor binding and dopamine metabolism have been observed in patients with burning mouth syndrome (Hagelberg et al., 2003b), atypical facial pain (Hagelberg et al., 2003a), fibromyalgia (Wood et al., 2007), and chronic back pain (Martikainen et al., 2015). Accordingly, chronic pain conditions may cause homeostatic changes in central dopaminergic pathways. Animal studies demonstrate that pain modulates midbrain dopaminergic neurons and may reduce dopamine output (Huang et al., 2019a, b; Markovic et al., 2021; Yang et al., 2021). However, the effect of inflammatory injury-induced pain on dopamine function in cortical regions associated with pain and learning remains mostly unexplored.

The anterior cingulate cortex (ACC) is intimately involved in the sensory and affective components of pain (Rainville et al., 1999; Zhuo, 2016). In humans, ACC neurons are activated by noxious stimuli (Apkarian et al., 2005), while rodent experiments have demonstrated synaptic potentiation of ACC neurons in mice with inflammatory or nerve injury-induced pain (Zhuo, 2008; Koga et al., 2015; Bliss et al., 2016). This suggests that pain may be stored as memories in the glutamatergic synapses of ACC neurons (Zhuo, 2008; Koga et al., 2015; Bliss et al., 2016). In this regard, the ACC has a functional role in the top-down descending facilitation of target areas (Zhuo, 2008; Beier et al., 2015; Chen et al., 2018; Smith et al., 2021), including direct facilitatory projections to dopaminergic neurons within the ventral tegmental area (VTA) (Beier et al., 2015).

As part of the mesocorticolimbic dopaminergic pathways, activation of the ACC induces burst firing of VTA neurons (Gariano and Groves, 1988), and VTA activation inhibits nociceptive responses in the ACC triggered by noxious mechanical stimuli (Sogabe et al., 2013). In

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Abbreviations: ACC, Anterior Cingulate Cortex; ACSF, Artificial cerebrospinal fluid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic receptors; CFA, Complete Freund's adjuvant; CPP, Conditioned place preference; D1R, D1 receptor; D2DR, D2 receptor; DA, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; EPSCs, excitatory postsynaptic currents; EPM, Elevated plus maze; GABA, gamma aminobutyric acid; $GABA_A$, gamma aminobutyric acid subtype a receptor; HPLC, high-performance liquid chromatography; IPSC, inhibitory postsynaptic current; LTP, Long-term Potentiation; OFT, Open field test; mPFC, Medial prefrontal cortex; NMDAR, N-Methyl-d-aspartic acid or N-Methyl-d-aspartate receptor; PPR, Paired pulse ratio; VTA, Ventral tegmental area.

https://doi.org/10.1016/j.neuroscience.2022.07.010
0306-4522/

addition, direct microinjection of dopamine into the ACC produces antinociceptive effects in neuropathic mice (Lopez-Avila et al., 2004), and dopamine receptor activation in ACC slices inhibits α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) currents that are associated with pain processing (Bliss et al., 2016; Darvish-Ghane et al., 2016; Darvish-Ghane et al., 2020). More recent studies have demonstrated that endogenous dopamine release mediated by optogenetic stimulation of VTA projections to the prelimbic cortex is also antinociceptive (Huang et al., 2020), and activation of D1 receptors in the ACC alleviates the sensory and affective components of neuropathic pain in rodents (Lançon et al., 2021). Since the ACC plays an integral role in pain processing, we sought to investigate the effect of chronic inflammatory pain on local modulation of AMPAR transmission by dopamine in the ACC of mice.

In the present study, we used an electrophysiological approach to measure AMPAR transmission and dopaminergic modulation of AMPAR excitatory postsynaptic currents (EPSC) in the ACC of mice injected with complete Freund's adjuvant (CFA), an inflammatory pain stimulus. CFA-treated mice displayed persistent mechanical hypersensitivity and anxiety-like behavior three days following injection as measured by the elevated plus-maze (EPM) and open field test. The anxiety phenotype was not present in mice injected with CFA and tested four days later. Input-output responses of ACC pyramidal neurons showed that AMPAR currents were potentiated three days post-CFA injection compared with naïve mice; however, no difference was observed between three- and four-days post-CFA when compared directly. Furthermore, dopamine-mediated inhibition of AMPAR postsynaptic currents was reduced four-days post-CFA when compared with three-days post-CFA and naïve mice. Finally, HPLC analysis showed decreased dopamine concentrations, but not its metabolite, DOPAC in the ACC of mice three days post-CFA treatment. Our results demonstrate that inflammatory injury induces an activity-dependent decrease in dopaminergic function in the ACC of mice.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME); aged 6–8 weeks for behavior and 4–6 weeks for electrophysiology experiments. All mice were housed in groups of four upon arrival and allowed one week to habituate before experiments started. The housing facility was temperature-controlled (20 ± 1 °C) with a 12:12 h normal light: dark cycle in mouse rooms. All cages contained a cotton nesting square and crinkled paper bedding. Mice were provided with unrestricted access to food (Harlan Teklad 8604) and water. All procedures were carried out according to the Canadian Council on Animal Care (CCAC) guidelines and approved by the University of Toronto's Animal Care Committee.

CFA model of inflammatory pain

Complete Freund's adjuvant (CFA; 20 μl) was injected intraplantar into both hind paws using a 100-μl microsyringe with a 30-gauge needle as we have previously done (Darvish-Ghane et al., 2020). Bilateral hind paw injections were used because this allowed us to record from both hemispheres for electrophysiology studies. In addition, the ACC is bilaterally activated in unilateral injury models of neuropathic (Zhao et al., 2018) and inflammatory pain (Cao et al., 2009). Thus, bilateral hind paw injections allowed us to treat each hemisphere equally and removed ipsilateral/contralateral specific effects as this was not the main goal of the present paper.

Behavioral study design

Mice were randomized to condition using within-cage randomization, and all behavioral experiments were performed by an experimenter blinded to CFA condition. Given that the open field and elevated plus maze tests cannot be repeated on the same mouse, each test environment was novel and independent mice were used for each timepoint of the behavioral experiments.

Mechanical threshold testing

Mice were placed on a metal mesh platform within small (9 × 5 × 5 cm high) testing cubicles made of Plexiglas and allowed to habituate for 1 h before testing. We used the plantar anesthesiometer (Ugo Basile, Gemonia, Italy) to assess mechanical sensitivity by measuring paw withdrawal responses. This device slowly increases pressure until the mouse withdraws its hind paw and the force (g) is displayed.

Elevated plus maze (EPM)

The EPM is a commonly used test of anxiety behavior in mice. In our experiments, the EPM was performed for 5 min with mice initially placed in the center of the maze. Mice were video tracked, and the time spent in the open arm was measured visually as we have previously done (Ramzan et al., 2020). Light levels were recorded to be 150 lux and 130 lux in the open and closed arms, respectively (Light Meter; LuxMaster 11010067).

Open field test (OFT)

The OFT was performed by placing each mouse in the corner of a large Plexiglas box (40 cm × 40 cm × 40 cm) in the center of a normally lit (400 lux) room and videotaping their behavior for 10 min. The time spent in the center (25 % of the total surface) and total distance were analyzed using Noldus Ethovision.

Tissue preparation for electrophysiology

Brain slices were prepared as described previously (Darvish-Ghane et al., 2016; Yamanaka et al., 2016). Mice were exposed to 5 % isoflurane, and brains were

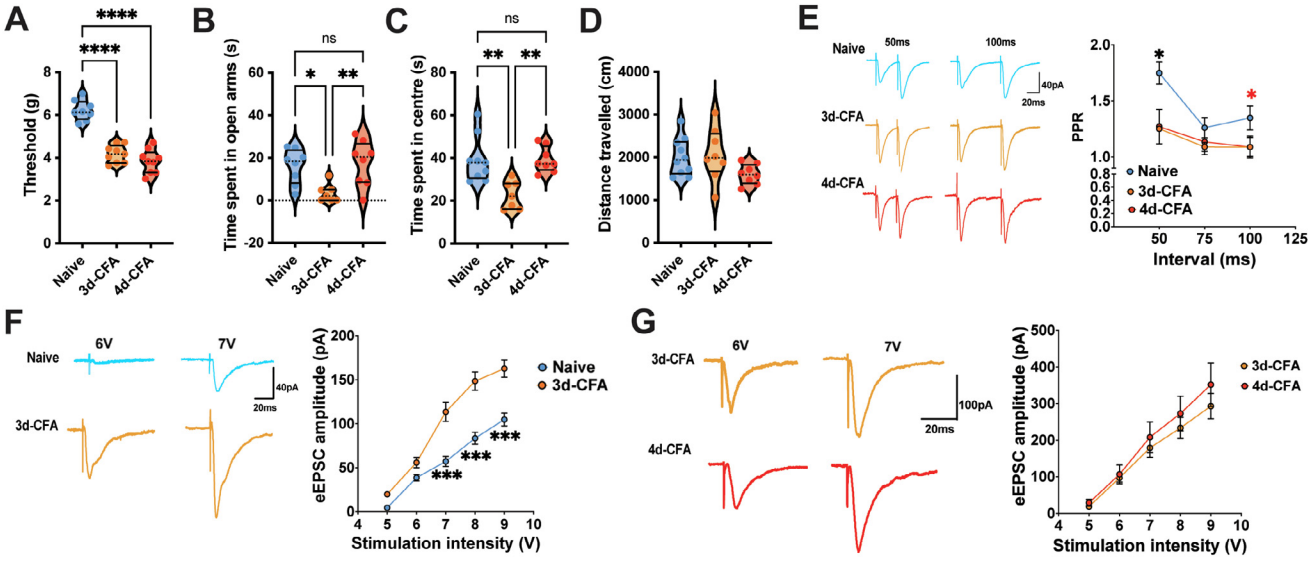


Fig. 1. Behavioral changes and ACC excitatory synaptic potentiation in mice with CFA-induced inflammatory pain. (**A**) CFA-induced mechanical sensitivity in mice three days (3d-CFA) and four days (4d-CFA) following hind paw injection (one-way ANOVA, $F_{2,23} = 59.86$, $p < 0.001$). (**B**) Mice spend significantly less time in the open arms of the elevated plus maze in the 3d-CFA group than naïve and 4d-CFA (one-way ANOVA, $F_{2,20} = 6.4$, $p < 0.01$). (**C**) Mice spend significantly less time in the center of the open field in the 3d-CFA group than naïve and 4d-CFA (one-way ANOVA, $F_{2,21} = 9.93$, $p < 0.001$). (**D**) No significant difference between the groups for total distance traveled in the open field (one-way ANOVA, $F_{2,23} = 2.6$, $p = 0.1$). Violin plots in A–D show distribution and individual data points with solid black lines indicating the upper and lower quartiles, while dashed lines represent the median. (**E**) **Left:** Representative paired pulse ratio (PPR) traces at intervals of 50 ms and 100 ms. **Right:** The PPR of naïve mice (n = 6) was enhanced compared with 3d-CFA (n = 6) and 4d-CFA (n = 8) mice at the 50 ms interval and 4d-CFA mice at the 100 ms interval (two-way repeated measures (RM) ANOVA, main effect of CFA: $F_{2,17} = 3.37$, $p = 0.055$; main effect of time interval (RM): $F_{2,25} = 15{,}27$, $p < 0.001$; CFA × interval interaction: $F_{4,34} = 2.16$, $p = 0.09$). (**F**) **Left:** Representative evoked traces from 6- and 7-volt stimulation recorded from naïve (n = 5) and 3d-CFA (n = 5) mice. Input-output analysis of eEPSCs from naïve and 3d-CFA mice showed enhanced responses in 3d-CFA mice at 7-, 8-, and 9-volt stimulation (two-way RM ANOVA, effect of CFA: $F_{1,8} = 63.34$, $p < 0.0001$; effect of voltage (RM): $F_{2,17} = 119.8$, $p < 0.0001$; CFA × voltage interaction: $F_{4,32} = 6.3$, $p < 0.001$). (**G**) **Left:** Representative evoked from 6- and 7-volt stimulation recorded from 3d-CFA and 4d-CFA mice. **Right:** No difference in input–output responses of eEPSCs from 3d-CFA (n = 6) and 4d-CFA (n = 7) (two-way RM ANOVA, effect of CFA: $F_{1,11} = 0.57$, $p = 0.46$; effect of voltage (RM): $F_{2,17} = 77.73$, $p < 0.0001$; CFA × voltage interaction: $F_{4,44} = 0.59$, $p = 0.67$). Stars (*) represent posthoc comparisons using Tukey's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). In panel (E) the red star represents the comparison between naïve and 4d-CFA. For electrophysiology experiments *n* represents the number of neurons recorded from three to four mice.

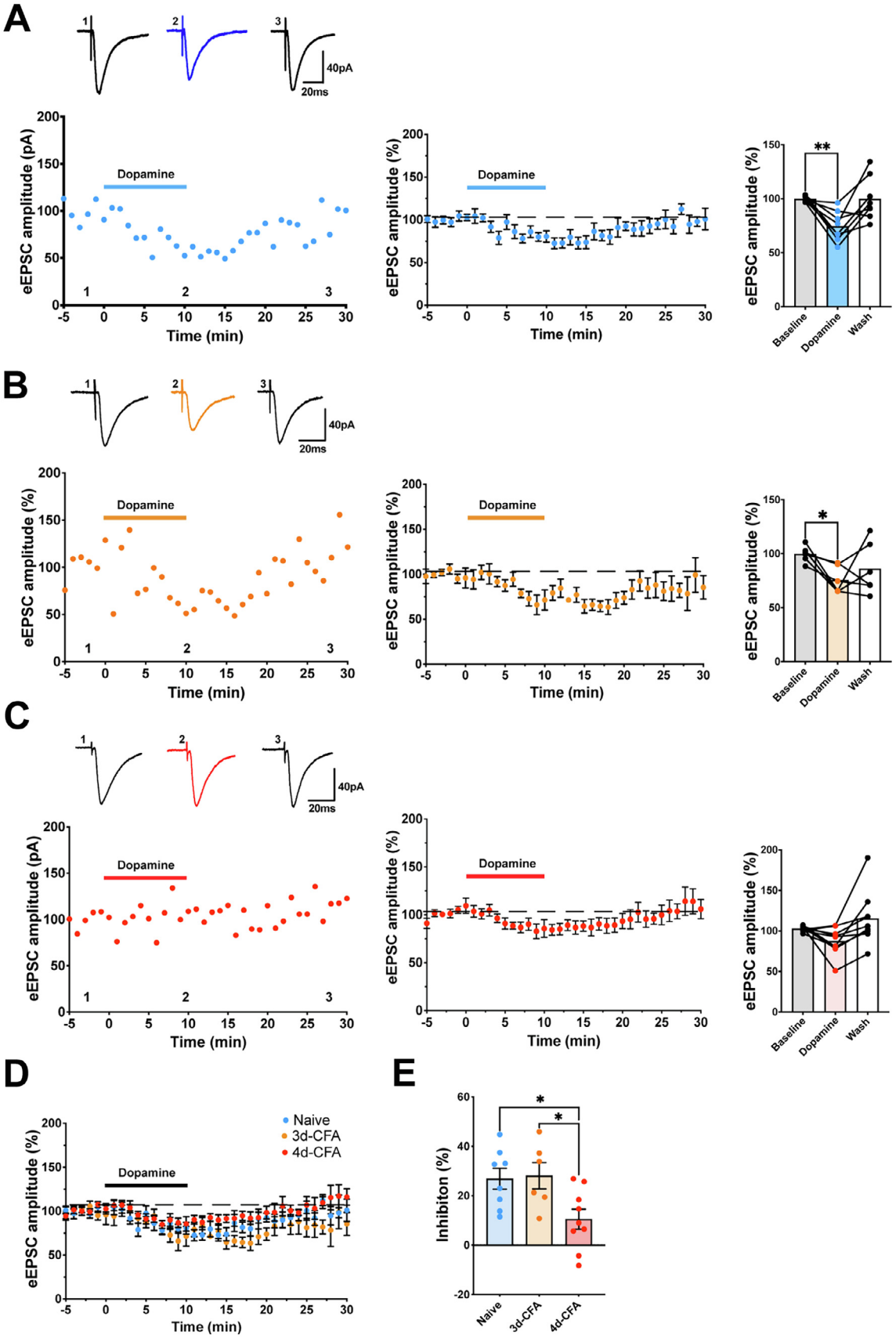
quickly removed following decapitation. Coronal ACC brain slices (300 μm) were prepared with a VT1200S tissue slicer (Leica, Concord, ON) in cold (4 °C) artificial cerebrospinal fluid (aCSF; aerated with 95 % O_2; 5 % CO_2): 124 mM NaCl, 4.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 10 mM glucose. Slices recovered for at least 60 min undisturbed in a submerged holding chamber (25 °C) before recording.

Whole-cell patch-clamp recording

Whole-cell voltage-clamp recordings from layer II/III pyramidal neurons of the ACC cg1 region were performed at room temperature. Slices were placed in a submerged recording chamber where they were continuously perfused with aCSF at a rate of 2 ml per min. Glass pipettes (4–6 MΩ) were made with a horizontal puller (P1000; Sutter, Novato, CA) and filled with an internal recording solution: 145 mM K-gluconate, 5 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM Na_3-GTP (adjusted to pH 7.2 with KOH). Neurons were visualized using a 40X objective on a Zeiss Axioskop FS upright microscope and voltage-clamped at − 60 mV. Recordings were performed using a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz, and digitized at 10 kHz with Clamplex (version 10.6; Molecular Devices). A tungsten bipolar stimulating electrode (Microprobes, Gaithersburg, MD) was placed in deep layers of the ACC to evoke EPSCs. AMPAR-mediated EPSCs were isolated by adding picrotoxin (100 μM) to the aCSF to block $GABA_A$ (γ-aminobutyric acid type A)-receptor-mediated inhibitory synaptic currents. For paired-pulse facilitation recordings, evoked responses were spaced 50 ms apart and performed every 30 s. Stable baseline recordings were obtained for 5 min, followed by perfusion of pharmacological agents. Only recordings with series resistance below 25 MΩ and input/access resistance that varied less than 15 % were included in the analyses.

Pharmacological agents used for electrophysiology

The drugs used in the electrophysiology experiments include dopamine hydrochloride, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and picrotoxin. All drugs were purchased from Sigma Aldrich, CA.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Wash
Naive
3d-CFA
4d-CFA
Inhibiton (%)

High-performance liquid chromatography analysis

Mice were humanely euthanized by decapitation following exposure to 5 % isoflurane. Brains were rapidly removed, frozen on liquid nitrogen, and stored at − 80 °C until processed. The ACC and striatum were microdissected with each sample suspended in aCSF (30 μl) and subjected to sonication. Sonicated brain samples (2 μl) were analyzed for protein levels using the BioRad protein assay (BioRad, Hercules, CA, United States). The neurochemical stabilizers, perchloric acid (1 μl of 0.2 N solution), and ascorbic acid (1.0 M) were added to all samples. Samples were subsequently sonicated and centrifuged (10,000 rpm) for 10 min at 4 °C. Supernatants were collected and stored at −80 °C.

HPLC analysis of dopamine and DOPAC were performed on the supernatant using a BAS 460 MICROBROPE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) as previously described (Chatterjee and Gerlai, 2009). A Uniget c-18 reverse-phase microprobe column was used as the stationary phase (BASi, Cat no. 8949). The mobile phase consisted of buffer (0.1 M monochloroacetic acid, 0.5 mM Na-EDTA, 0.15 g/L Na-octylsulfonate and 10 nM sodium chloride, pH 3.1). Standard dopamine and DOPAC were used to measure and identify chromatograph peaks. Neurochemicals were detected using 10 μl of samples, and peaks of dopamine and DOPAC were compared with standard peaks for concentration analysis.

Data and statistical analysis

All statistical analysis was performed using GraphPad Prism 9. Behavioral and HPLC data were analyzed using a one-way analysis of variance (ANOVA), using Tukey's post-hoc tests corrected for multiple comparisons. Electrophysiological data were collected and analyzed using pClamp 9.2 software (Molecular Devices, San Jose, Ca). A one-way ANOVA with repeated measures was used for electrophysiology experiments where a washout phase after drug application was measured. Dunnett's case comparison was used for post-hoc analysis with baseline recording as the comparison. For baseline analysis, the time between − 4 min and + 1 min was used for analysis as stable baseline responses are expected to be present + 1 min following drug application. We used paired t-test comparisons to determine whether baseline and drug effects were significantly different in the absence of the washout phase. For t-test analysis of unpaired groups, Welch's correction was used for unequal sample size between groups. Statistical outliers were defined via Studentized residuals of more than three and excluded before analyses were run. Based on this criterion, *two outliers* were removed from the EPM data set, while *two outliers* were removed from the time spent in the center of the open field data set. For the HPLC data, a total of *five* samples across all groups and brain regions were classified as outliers. *p < 0.05 was considered statistically significant.

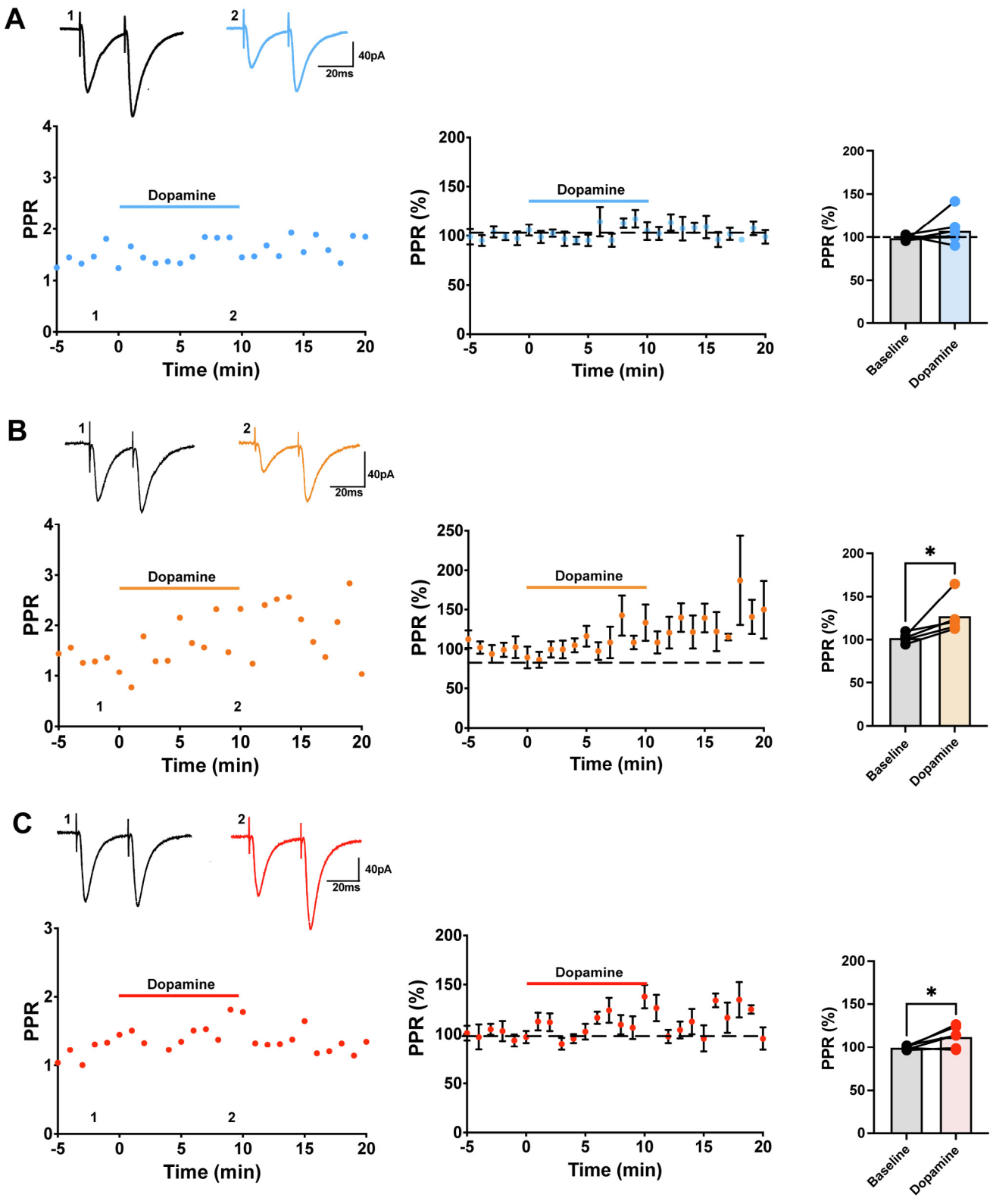
RESULTS

CFA induces behavioral changes and potentiates ACC glutamatergic currents

Bilateral CFA injections have been used in studies that measure spontaneous pain responses (Wang et al., 2017) and the effect of pain on voluntary behavior (Cobos et al., 2012; Sheahan et al., 2017). In addition, other models of inflammatory pain have been performed bilaterally including ankle zymosan, which we have previously used (Sorge et al., 2014). Thus, mice were injected with CFA in both hind paws, which we validated by testing mechanical sensory thresholds and behavior on the elevated plus maze (EPM) as a measure of anxiety. CFA induced a significant decrease in paw withdrawal thresholds three- and four-days post-CFA (3d-CFA and 4d-CFA, respectively) relative to the naïve group (Fig. 1 (**A**)). In addition, 3d-CFA mice displayed a significant decrease in time spent in the open arms of the EPM relative to naïve mice; however, this anxiety-like phenotype was not present in 4d-CFA mice (Fig. 1(**B**)). Furthermore, mice were tested using the open field test (OFT) as another proxy of anxiety and to measure locomotion. CFA treatment significantly reduced the time spent in the center of the OFT in the 3d-CFA group (Fig. 1(**C**)). Total distance traveled was not different between the groups (Fig. 1(**D**)). These results indicate that inflammation induces mechanical sensitivity and an anxiety phenotype that is present three, but not four days following CFA.

Fig. 2. Altered synaptic transmission and dopaminergic modulation of EPSCs in the ACC of mice with inflammatory pain. (**A**) **Left:** Representative trace (top) and time course (bottom) of eEPSCs from an ACC neuron recorded from a naïve mouse before (1), during (2), and following the washout (3) of dopamine. **Middle:** Normalized data showing the time course of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μM) reversibly inhibits eEPSCs (one-way repeated measures ANOVA, $F_{2,12} = 9.79$, $p < 0.01$). (**B**) **Left:** Representative trace (top) and time course (bottom) of eEPSCs from an ACC neuron recorded from a 3d-CFA mouse before (1), during (2), and following the washout (3) of dopamine. **Middle:** Normalized data showing the time course of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μM) reversibly inhibits eEPSCs (one-way repeated measures ANOVA, $F_{2,12} = 3.42$, $p < 0.05$). (**C**) **Left:** Representative trace (top) and time course (bottom) of eEPSCs from an ACC neuron recorded from a 4d-CFA mouse before (1), during (2), and following the washout (3) of dopamine. **Middle:** Normalized data showing the time course of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μM) marginally inhibits eEPSCs (one-way repeated measures ANOVA, $F_{2,12} = 3.13$, $p = 0.07$). (**D**) Comparison of eEPSCs before, during, and following dopamine application in naïve, 3d-CFA, and 4d-CFA mic. (**E**) Percentage of dopamine inhibition is significantly less in 4d-CFA compared with naïve and 3d-CFA mice (one-way ANOVA, $F_{2,20} = 5.16$, $p = 0.02$). Negative inhibition indicates a potentiated response. Stars (*) represent posthoc comparisons using Tukey's multiple comparisons test (*$p < 0.05$, **$p < 0.01$). Bar and symbols represent mean ± SEM.

Next, we used whole-cell patch-clamp recordings of ACC pyramidal neurons in layers II/III to determine whether CFA altered synaptic transmission in ACC neurons. First, we measured the paired-pulse ratio (PPR) of evoked excitatory postsynaptic currents (eEPSC) as a measure of glutamate presynaptic release probability (Zhao et al., 2006; Koga et al., 2015) from naïve and CFA-injected mice. Consistent with previ-



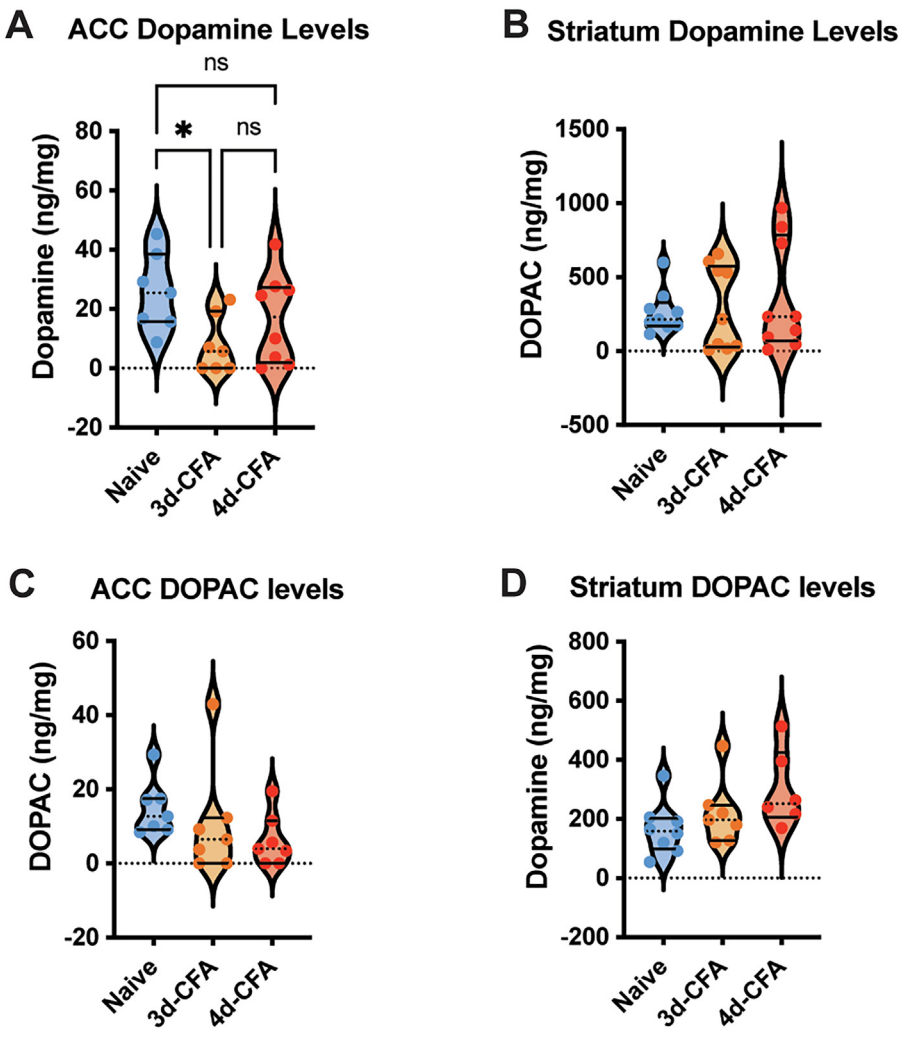


Fig. 4. Dopamine concentration in the ACC and striatum of mice with inflammatory pain. (**A**) In comparison to naïve mice, there is a significant decrease in the dopamine concentration (ng/mg) three days post-CFA (3d-CFA) injection in the ACC (one-way ANOVA, $F_{2,19} = 3.28$, $p < 0.05$). (**B**) No significant change in dopamine concentration in the striatum of CFA-treated mice (one-way ANOVA, $F_{2,24} = 0.30$, $p = 0.74$). (**C**) No change in DOPAC levels in the ACC of CFA-treated mice (one-way ANOVA, $F_{2,18} = 1.17$, $p = 0.33$). (**D**) No change in DOPAC levels in the striatum in CFA-treated mice (one-way ANOVA, $F_{2,18} = 2.62$, $p = 0.1$). Violin plots in A–D show distribution and individual data points with solid black lines indicating the upper and lower quartiles, while dashed lines represent the median. *$p < 0.05$, compared to naive mice.

ous reports (Zhao et al., 2006; Koga et al., 2015), ACC neurons had a significantly lower PPR in CFA-injected mice than naïve mice at all three inter-stimulus intervals (Fig. 1(**E**), indicating potentiation of presynaptic glutamate release in mice with inflammatory pain. However, there was no difference between 3d-CFA and 4d-CFA slices. Next, input–output responses of layer II-III pyramidal neurons in the ACC demonstrated enhanced AMPAR responses in the 3d-CFA group compared with naïve mice, as measured by plotting the stimulation intensity (input) vs the eEPSC amplitude (output) (Fig. 1(**F**)). Albeit, directly comparing 3d-CFA and 4d-CFA indicated no differences between these groups on input–output responses (Fig. 1(**G**)).

Dopamine-mediated inhibition of eEPSCs is reduced four days following CFA injection

Since several recent studies have shown disruption in dopamine in frontal cortical circuits following nerve injury (Huang et al., 2020; Liu et al., 2020; Lançon et al., 2021), we next sought to determine whether dopamine-mediated inhibition of AMPAR transmission was altered in CFA injected mice. After obtaining five min of stable baseline recording, dopamine (50 μM) was applied for 10 min and washed out by applying fresh aCSF (Fig. 2(**A and D**)). Application of dopamine significantly inhibited eEPSCs in ACC slices prepared from naïve and 3d-CFA mice, with responses returning to baseline following

Fig. 3. Paired pulse responses are enhanced by dopamine application in CFA-treated (**A**) **Left:** Representative trace (top) and time course (bottom) for paired pulse ratio (PPR) of eEPSCs from an ACC neuron recorded from a naïve mouse before (1) and during (2) dopamine application. **Middle:** Normalized data showing the time course for PPR of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μM) does not change PPR in the ACC of naïve mice (paired t-test, $t_6 = 1,27$, $p = 0.25$, $n = 7$). (**B**) **Left:** Representative trace (top) and time course (bottom) for paired pulse ratio (PPR) of eEPSCs from an ACC neuron recorded from a 3d-CFA mouse before (1) and during (2) dopamine application. **Middle:** Normalized data showing the time course for PPR of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μM) enhances the PPR in the ACC of 3d-CFA mice (paired t-test, $t_4 = 2.78$, $p < 0.05$, $n = 5$). (**C**) **Left:** Representative trace (top) and time course (bottom) for paired-pulse ratio (PPR) of eEPSCs from an ACC neuron recorded from a 4d-CFA mouse before (1) and during (2) dopamine application. **Middle:** Normalized data showing the time course for PPR of eEPSC responses throughout the recording. **Right:** Summary data showing that application of dopamine (50 μμM) enhances the PPR in the ACC of 3d-CFA mice (paired t-test, $t_5 = 2.84$, $p < 0.05$, $n = 6$). *$p < 0.05$. Bar and symbols represent mean ± SEM.

dopamine washout (Fig. 2(**A, B, D**)). However, in mice from the 4d-CFA group, dopamine-induced marginal inhibition that was not significantly different than baseline and the washout phase (Fig. 2(**C and D**)). Dopamine-mediated inhibition of AMPAR eEPSCs in the 4d-CFA group was significantly lower than the inhibition of eEPSCs in naïve mice (Fig. 2(**E**)).

Dopamine modulates presynaptic transmission in mice with inflammation

Previous work suggests that dopamine-mediated inhibition of eEPSCs during basal transmission in naïve mice is mediated by postsynaptic G-proteins in the ACC (Darvish-Ghane et al., 2016; Darvish-Ghane et al., 2020). However, the role of dopamine-mediated presynaptic modulation of glutamatergic transmission in the ACC is unknown. Thus, we next tested whether dopamine modulates presynaptic transmission in naïve and CFA-treated mice. Dopamine application did not affect PPR in naïve mice (Fig. 3(**A**)). However, dopamine significantly increased PPR in 3d-CFA and 4d-CFA mice (Fig. 3(**B, C**)). These results demonstrate that inflammation activates presynaptic dopaminergic mechanisms to modulate glutamatergic transmission in the ACC.

Inflammatory pain induces time-dependent changes in the ACC dopamine concentration

Activation of the ACC has been shown to enhance VTA activity (Gariano and Groves, 1988), and subpopulations of VTA dopaminergic neurons receive direct glutamatergic synapses from the ACC (Beier et al., 2015). Since CFA treatment increased ACC neuronal output, and activated a presynaptic dopaminergic mechanism to modulate glutamatergic transmission in the ACC, we next examined if CFA treatment altered endogenous dopamine concentration in the ACC. We used high-performance liquid chromatography (HPLC) to measure concentrations of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC), the metabolite of dopamine in the ACC and striatum (Chatterjee and Gerlai, 2009; Jensen et al., 2017). Since cortical dopamine concentrations may be low, we included the striatum, a brain region with high dopaminergic activity, as an internal control for HPLC neurotransmitter peak detection. Our results showed significantly lower dopamine concentration in the ACC of 3d-CFA mice (Fig. 4(**A**)). In the ACC, dopamine concentration was no different in 4d-CFA mice compared with the naïve group (Fig. 4 (**A**)). In the striatum, we did not observe a significant change in dopamine concentrations in CFA-treated mice (Fig. 4(**B**)). Analysis of DOPAC, a dopamine metabolite revealed no significant changes across both brain regions (Fig. 4(**C, D**)). Hence, in the ACC there is a significant decrease in dopamine concentration in 3d-CFA mice, which coincides with the behavioral anxiety phenotype of these mice.

DISCUSSION

In the present study, we investigated the effect of CFA-induced inflammatory pain on ACC dopaminergic function. CFA treatment induced a robust and persistent mechanical sensitivity accompanied by anxiety. CFA-induced anxiety-like behavior was observed in the EPM three, but not four days following CFA injection, while mechanical sensitivity was present on days three and four. Patch-clamp experiments demonstrated enhanced presynaptic glutamate release and potentiated AMPAR currents from brain slices of mice with CFA. In naïve mice, dopamine application reversibly inhibited AMPAR currents; however, four days following CFA treatment, dopamine-mediated inhibition of AMPAR currents was significantly reduced. Moreover, dopamine inhibition of AMPAR currents in CFA-treated mice may operate through a presynaptic event based on our PPR analysis showing that bath application of dopamine enhances presynaptic glutamate release in the ACC. Further, dopamine concentration in the ACC was decreased three days following CFA injection. Collectively these results demonstrate that peripheral inflammation induces activity-dependent changes in the ACC that reduce the inhibitory effect of dopamine in these neurons and may act to reduce overall dopaminergic function.

In the ACC, there are spatial differences between mechanisms of glutamatergic potentiation and function on pain- and anxiety-related behavior. Presynaptic LTP of incoming glutamatergic synapses onto ACC neurons are activated in anxiety states (Koga et al., 2015; Zhuo, 2016), and postsynaptic NMDAR-dependent potentiation is a characteristic of mechanical sensitivity related to injury (Li et al., 2010; Chen et al., 2014a, b; Zhuo, 2016). In the 3d-CFA group, an anxiety phenotype was observed that was not apparent in the 4d-CFA group; however, both groups showed equal sensitivity to mechanical stimuli. Moreover, our electrophysiology studies revealed a comparable decrease in PPR in 3d-CFA and 4d-CFA slices that was similarly increased by dopamine application. Inhibition of postsynaptic AMPARs by dopamine was reduced in 4d-CFA slices when compared with 3d-CFA and naïve slices; however, this was not associated with a behavioral phenotype. Further experiments examining the correlation between dopamine concentrations in the ACC, sensory thresholds, and the level of anxiety in individual mice will be necessary to understand whether altered dopaminergic transmission in the ACC is a critical modulator of pain and pain-related anxiety.

We and others (Koga et al., 2015) have demonstrated enhanced glutamatergic release probability in the ACC following inflammatory pain, indicating excessive glutamatergic transmission in the ACC. Thus, enhanced glutamatergic transmission and dopamine signaling may work together to desensitize dopamine receptors in the ACC following inflammatory pain. The persistent activation of D2DRs has been shown to desensitize dopamine receptors in the mPFC (Bates et al., 1991), while D2DR-

mediated inhibition of evoked IPSCs in the mPFC was abolished in a mouse model of cocaine addiction (Kroener and Lavin, 2010). These results suggest that persistent dopaminergic activity may alter dopamine receptor functionality in the mPFC, including the ACC. Moreover, our HPLC analysis revealed reduced dopamine concentrations in the ACC of 3d-CFA mice, indicating that CFA may induce a dopamine deficit that corresponds with the anxiety phenotype of these mice. Although, it is conceivable that reduced ACC dopamine in the 3d-CFA group may trigger long-lasting disruption of ACC dopaminergic signaling which could manifest as less dopamine-mediated inhibition of AMPAR transmission in the 4d-CFA group. However, our studied failed to uncover a behavioral phenotype linked to the reduced dopaminergic inhibition of EPSCs in the 4d-CFA group.

Pain can inhibit subpopulations of VTA dopaminergic neurons (Huang et al., 2020; Markovic et al., 2021; Yang et al., 2021), with specific subpopulations of medial VTA dopaminergic neurons receiving direct projections from the ACC (Beier et al., 2015), which are known to be activated by aversive stimuli (de Jong et al., 2019). Since CFA treatment activates the ACC, CFA may upregulate VTA dopaminergic activity of neurons receiving excitatory projections from the ACC (Beier et al., 2015). Additionally, inflammatory pain enhances the phasic firing of VTA dopaminergic neurons in response to reward delivery based on VTA dopaminergic calcium transients (Markovic et al., 2021). In this regard, ACC projections onto subpopulations of VTA dopaminergic neurons may regulate the phasic firing of dopamine neurons and reward-seeking behavior during chronic pain states. In line with this, enhanced ACC activity is observed in both humans and rats following pain relief as a rewarding signal (Becerra et al., 2013). Reduced dopamine concentration in the ACC may indicate a deficit of dopamine signaling, which corresponds with CFA-induced negative affect. This downregulation may be caused by a dysregulation of homeostatic pathways such as VTA signaling to decrease ACC dopaminergic activity that has been observed in rodent chronic pain models (Huang et al., 2019a, b; Markovic et al., 2021; Yang et al., 2021). However, our study did not examine altered dopamine concentration in the VTA, but rather the striatum and in the 3d-CFA group, striatal dopamine levels did not change.

In conclusion, our results indicate that CFA treatment causes ACC peripheral mechanical hypersensitivity, transient anxiety, and plasticity of ACC glutamatergic and dopaminergic transmission. In the ACC and PFC, dopaminergic transmission is analgesic in chronic pain conditions (Lopez-Avila et al., 2004; Huang et al., 2020), and our results indicate that persistent sensory input alters ACC dopaminergic function. There is evidence for crosstalk between dopaminergic and opioidergic signalling in the ACC as injections of morphine into the ACC induce conditioned place preference (CPP) and dopamine release in the nucleus accumbens (Navratilova et al., 2015), while D1R-mediated dopaminergic signaling in the ACC induces CPP and pain relief (Lançon et al., 2021). Thus, our study adds to the growing literature of dopamine in corticolimbic circuits that modulate sensory deficits and negative affect. Future studies are required to investigate the connectivity between the ACC and dopaminergic transmission to pain and anxiety during inflammatory states. Ultimately, uncovering the mechanism that alters cortical dopaminergic transmission and how this contributes to the development of sensory and affective pain perception is critical for novel non-opioid drug development and understanding the broader utility of dopaminergic drugs that could be used as adjuncts to current pain therapies.

AUTHOR CONTRIBUTIONS

SDG performed electrophysiological and HPLC experiments. BL performed behavioral experiments. AF and DC provided training for HPLC and analysis. SDG and LJM analyzed the data. SDG and LJM designed the experiments and wrote the manuscript. LJM supervised the overall project. All authors reviewed, edited, and approved the final manuscript.

ACKNOWLEDGEMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC; RGPIN-2016-06284), Canadian Institutes of Health Research (CIHR PJT-166171), Canadian Foundation for Innovation, and the Canada Research Chairs program.

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(Received 19 April 2022, Accepted 12 July 2022)
(Available online 18 July 2022)