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Examination of the Role of Neuroimmune Signaling in Drug Addiction

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Examination of the Role of Neuroimmune Signaling in Drug Addiction

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Abstract

Cocaine and oxycodone addictions are chronic, relapsing disorders characterized by maladaptive patterns of drug seeking that create significant impairment for the user. While the homeostatic functions of neuroimmune cells such as microglia and astrocytes have been well characterized, little work has examined the structural and functional alterations of these neuroglial cells following repeated drug intake. The first experiment of this study used male and female Sprague-Dawley rats to examine sex differences in microglial cells following repeated cocaine intake. The second experiment used a rodent model of oxycodone self-administration to test the ability of ibudilast, a non-selective anti-inflammatory drug, to reduce drug seeking behavior following 14 days of forced abstinence. The present study found no apparent difference in markers of microglial activity between males and females following cocaine intake. Further, it was demonstrated that administration of ibudilast attenuates incubation of oxycodone craving following 14 days of forced abstinence. This was accompanied by a reduction in markers of astrocyte, but not microglial, activity in the ventral tegmental area and nucleus accumbens. These results are consistent with the hypothesis that drug-induced neuroinflammation contributes to heightened oxycodone craving and seeking following a prolonged period of abstinence.
Introduction

Opioid drugs, such as oxycodone, remifentanil, and morphine, possess strong analgesic properties that render them effective for pain management in clinical settings. However, the high abuse potential for such drugs and absence of non-addictive alternatives necessitates an understanding of their neural actions. Drug addiction is characterized by a viscous cycle of drug use, withdrawal, and intensified craving, ultimately leading to repeated use of the drug and persistent drug craving. It has been estimated that over 115 people each day die from opioid overdoses in the United States (Hedegaard et al., 2017). This imposes an economic burden of $78.5 billion dollars each year that accounts for costs of healthcare, addiction treatment, and legal fees (Florence et al., 2016). With a lack of effective treatment for chronic pain, 21-29% of the chronic pain population has been calculated to misuse prescription opioids (Richards, 2018). Elucidating the mechanisms of opioid reward and relapse within the brain may contribute to future development of abuse-deterrent opioid formulas.

Reward processing is carried out by the mesolimbic dopamine system, a circuit composed of dopaminergic projections from the ventral tegmental area (VTA) to regions of the ventral striatum, including the nucleus accumbens (Venniro, Caprioli, & Shaham, 2016). Initial links between positive emotions associated with a drug and related environmental or experiential cues are largely dependent on projections both from the VTA to the amygdala—a structure that promotes reward learning—and projections from the VTA to the nucleus accumbens, thought to be involved in the transformation of impulses to action (Kalivas & McFarland, 2003; Mahler & Aston-Jones, 2012). Glutamatergic projections from the prefrontal cortex (PFC) to the nucleus accumbens help regulate such impulsive actions, aligning immediate motivational cues with situationally appropriate behaviors and long-term goals of an organism.
Essential for communication in the mesolimbic pathway is dopamine, a neurotransmitter seen to be involved in modulation of value-based decision making (Steinberg et al., 2013; Willuhn et al., 2010). Recurrence of rewarding experiences—such as repeated oxycodone or cocaine use—facilitates pathological learning, where even the prospect of drug taking triggers the VTA to release large amounts of dopamine throughout mesolimbic pathway (Mameli et al., 2009; Simmons, Wheeler, & Mazei-Robison, 2019). Dysregulation of the prefrontal cortex following repeated drug use may compound impulsive decision making by allowing for disinhibition of GABAergic neurons in the nucleus accumbens (McFarland & Kalivas, 2001). Consequently, the motivational effects of drug-related stimuli may be enhanced to promote drug-seeking behavior.

Opiates, such as oxycodone, fentanyl, and morphine, are thought to confer analgesic effects by binding µ-opioid receptors (MORs) within the CNS (Terrie, 2011). MORs are G\textsubscript{i}-coupled receptors that diminish activity in central pain pathways through inhibition of adenylyl cyclase to dampen transmission of pain signals. The high abuse liability of oxycodone comes from its stimulatory effects in the mesolimbic dopamine system. After crossing the blood-brain barrier (BBB), oxycodone can bind G\textsubscript{i}-coupled receptors on GABAergic interneurons that monitor VTA dopamine neuron activity. Such inhibition of regulatory GABAergic interneurons allows the VTA to release large amounts of dopamine into the nucleus accumbens, signaling immediate prospects of reward (Simmons, Wheeler, & Mazei-Robison, 2019).

In contrast to oxycodone, the reinforcing effects of cocaine are classically thought to come from its inhibition of the dopamine transporter (DAT), a membrane-spanning protein that helps shuttle dopamine out of the synapse and into the cytosol to terminate its signal. Blocking DAT on VTA terminals results in an accumulation of dopamine in the nucleus accumbens, leading to
the acutely reinforcing effects of cocaine. Despite the known role of DAT in facilitating the acutely rewarding effects of cocaine, it has been seen that DAT knockout mice continue to show elevated dopamine concentrations following cocaine intake (Carboni et al., 2001). This suggests additional mechanisms by which cocaine increases dopamine concentrations within the nucleus accumbens.

Previous studies have provided evidence for a role of neuroimmune signaling in the development and maintenance of substance use disorders (SUDs), demonstrating that drugs of abuse, such as ethanol, opioids, cocaine, and methamphetamine, produce an acute inflammatory response within the central nervous system (CNS) largely through microglial cells (Hutchinson & Watkins, 2014; Hutchinson et al., 2010; Kohno et al., 2019). Microglia are resident macrophage cells of the CNS, acting as the primary form of immune defense by scavenging their environment for cell damage, debris, or pathogens. Drugs of abuse are recognized as foreign by the innate immune system, as they are non-endogenous substances not expected to be present within the CNS (Bachtell et al., 2015). Upon drug detection, microglia launch an inflammatory response through toll-like receptor 4 (TLR4), a pattern recognition receptor capable of detecting small-molecule xenobiotics that cross the BBB. TLR4 induces an intracellular signaling cascade that leads to the activation of nuclear factor kappa B (NFκB), a transcription factor largely responsible for the production of proinflammatory cytokines. Evidence that TLR4 inhibitors, such as (+)-naltrexone, attenuate opiate- or cocaine-seeking in rodent models self-administration has implicated microglia-mediated TLR4 signaling in drug addiction (Bachtell et al., 2015; Northcutt et al., 2015). Further studies have illustrated that microglia form positive feedback loops, where repeated activation leads to a “primed” state (Dilger & Johnson, 2008; Watkins &

Chronic neuroinflammation is characterized by a persistent increase in proinflammatory cytokine expression within the CNS (Beardsley & Hauser, 2014). Although not fully elucidated, it has been demonstrated that chronic neuroinflammation may play a role in heightened relapse vulnerability by dysregulating dopamine release from the VTA, thereby altering the rewarding properties of a drug (Northcutt et al., 2015; Taylor et al., 2015). Studies that have attenuated drug-induced inflammatory responses by blocking the actions of proinflammatory cytokines at their receptors or disrupting cleavage of proinflammatory cytokines to their active form have successfully reduced drug-seeking behaviors following cocaine self-administration (Brown et al., 2018). Further experiments using the anti-inflammatory agent ibudilast have illustrated diminished methamphetamine, opioid, and cocaine seeking behavior using similar rodent models of self-administration (Kohno et al., 2019). Together, these studies provide evidence for a prominent role of the innate immune system in the etiology of substance use disorders.

The possibility that astrocytes may be involved in the maintenance of substance use disorders has gained wider recognition, in part due to their prominent role in glutamate homeostasis. Astrocytes are immunocompetent glial cells of the CNS that help modulate synaptic activity and plasticity, in addition to participating in proinflammatory signaling (Dong & Benveniste, 2000; Eroglu & Barres, 2010). Previous studies have demonstrated that elevated microglial activity following TLR4 signaling can subsequently lead to astrocyte reactivity, amplifying proinflammatory cytokine signaling and increasing glutamate release (Bal-Price & Brown, 2001; Tanga, Nutile-McMenemy, & DeLeo, 2005; Watkins, Hutchinson, Milligan, & Maier, 2007). These neuroexcitatory effects may potentiate plasticity in the mesolimbic
dopamine system to heighten the rewarding effects of cocaine and opiates, or promote a shift from phasic to burst firing by VTA dopamine neurons (Northcutt et al., 2015). Astrocyte-mediated shifts in excitatory signaling within the mesolimbic dopamine system warrant further studies on the role of astrocytes in drug-seeking behavior.

While previous work has demonstrated the contribution of TLR4 signaling to the acutely reinforcing effects of cocaine (Northcutt et al., 2015), the necessity of TLR4 within the context of drug-primed cocaine reinstatement, and the role of IL-1β circulation in drug-seeking behavior (Brown et al., 2018), few studies have examined the connections between behavioral models of reinstatement and observable glial alterations. Despite the known role of astrocytes in promoting inflammatory responses, little data on astrocyte alterations following oxycodone intake exists. Because projections from the VTA to the nucleus accumbens are known to be essential components of reward modulation, both areas were targeted as possible regions of high glial activity following cocaine or oxycodone intake. To that end, the present study aimed to elucidate the effects of cocaine and oxycodone on astrocytes and microglia signaling in the nucleus accumbens and VTA.

**Materials and Methods**

**Animals**

Male and female Sprague-Dawley rats (ENVIGO, Indianapolis, IN) were housed individually in the animal vivarium at the University of Colorado Boulder. All animals were maintained on a 12h light/12h dark cycle, and all experiments were conducted during the light cycle. The animal vivarium was maintained at a constant temperature (21°C). Animals had *ad libitum* access to food and water during the 7-day habituation period. Experiments were
performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

**Drugs**

Oxycodone was obtained from B&B Pharmaceuticals (Englewood, CO) and diluted to a concentration of 1.5 mg/mL with sterile saline (0.9% NaCl). Cocaine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO) and diluted with sterile saline (0.9% NaCl) to a concentration of 5 mg/mL. Ibudilast was obtained from SelleckChem and reconstituted to 3.75 mg/mL in poly(ethylene glycol) (PEG). PEG was obtained from Arcos Organics (New Jersey, USA).

**Surgery**

Rats were allowed to habituate to their vivarium conditions for 7 days prior to surgery. Rats were anesthetized with isoflurane and implanted with indwelling intravenous catheters (Norfolk Medical Technologies Skokie, IL). Catheters were inserted into the right jugular vein, and attached cannulas were passed subcutaneously to the back. Following surgery, rats were injected with Baytril, (5 mg/kg) and Ketofen (5 mg/kg) to manage postsurgical infection and inflammation. Rats were given a 7-day recovery time before testing, where catheters were flushed daily with heparinized saline containing 0.1 mL Gentamicin (100 mg/mL). Surgical procedures adhered to guidelines for aseptic technique.

**Experiment 1: Sex differences in microglia following repeated cocaine intake**

**Repeated Experimenter-delivered Cocaine Administration**

A separate cohort of male and female Sprague-Dawley rats was used to measure differences in microglial morphology following cocaine intake between sexes. Rats were divided
into two treatment groups (saline or cocaine) and given i.p injections of either saline or cocaine (20 mg/kg) once a day for 7 days. Animals were sacrificed for tissue collection two hours after their last injection.

Experiment 2: Effects of ibudilast on astrocytes and microglia following oxycodone self-administration

Food Training & Oxycodone Self-Administration

Self-administration procedures were conducted in operant conditioning chambers (Med Associates, Fairfax, VT) consisting of two retractable levers, stimulus cue lights above each lever, and a house light that was illuminated throughout each session. To facilitate lever responding, rats were food restricted for 2 days prior and subsequently trained to self-administer banana-flavored sucrose pellets (Bio-Serv, Flemington, NJ) on a fixed ratio 1 (FR1) schedule of reinforcement, where each press on the active lever resulted in delivery of a sucrose pellet.

Oxycodone self-administration took place over twelve 6-hour sessions, where presses on the active lever resulted in a 5-s oxycodone infusion (75 µg/kg/infusion). Presses on the active lever were coupled with presentation of a cue light directly above the lever. Each infusion was followed by a 20-s time-out period during which responding was recorded, but did not result in an additional drug infusion.

Forced Abstinence & Cue Testing

Following oxycodone self-administration, animals underwent a 14-day period of forced abstinence where they were divided into two groups and given i.p injections of either 35% PEG (2.5 mL/kg) or the anti-inflammatory agent, ibudilast (7.5 mg/kg). Animals were weighed daily during abstinence to monitor potential effects of ibudilast on appetite.
On the fourteenth day of abstinence, animals were injected with 35% PEG or ibudilast, then placed in operant conditioning chambers for a 2-hour cue-seeking test. Cue seeking was measured by presses on the active, formerly drug-paired lever. Each press on the active lever resulted in the presentation of the previously drug-associated cue light. Immediately following the cue test, animals were sacrificed for tissue extraction.

**Tissue Collection**

All rats were euthanized with sodium pentobarbital (65 mg/kg, i.p) and transcardially perfused with approximately 250 mL of chilled saline (0.9% NaCl), followed by 250 mL of 4% paraformaldehyde (PFA). Tissue for Experiment 1 was harvested 2 hrs following the final saline or cocaine injection. In Experiment 2, tissue was harvested immediately following the 2 hr cue seeking test. Upon extraction, brains were post-fixed in 4% PFA for 4 hours, 20% sucrose diluted in 1x phosphate-buffered saline (1x PBS) for 24 hours, then 30% sucrose in 1x PBS (pH=7.4). Following a 48-hour immersion in 30% sucrose, all brains were flash-frozen in 2-methylbutane at -20°C for approximately 2 minutes, then stored at -80°C.

**Immunohistochemistry**

Tissue was sectioned into 30 µM sections on a cryostat at approximately -17°C. Sections were stored in free-floating wells containing 1x PBS and 0.01% sodium azide for tissue preservation. Rat brain sections were washed in PBS buffer with 0.2% Triton X-100 (Sigma Aldrich, USA) for 30 minutes at room temperature and blocked for 4 hours at room temperature in 0.2% PBS-Triton (PBS-T) with 10% normal goat serum (NGS). Tissue sections were incubated for 48 hours at 4°C in 0.2% PBS-T and 3% NGS, with a purified primary antibody rabbit polyclonal anti-Iba1 (Wako, LKF6437) at a dilution of 1:1000, or anti-GFAP at a dilution of 1:2000 (Abcam, AB7260). Tissue was washed 3 times for 10 min/wash, and incubated in
0.2% PBS-T with the secondary antibody Alexa Fluor™ 488 goat anti-rabbit IgG (Life Technologies Corporation, Eugene, Oregon, 2 mg/mL) diluted at 1:500 for 2 hours at room temperature. Tissue sections were mounted in PBS, cover slipped in Vectashield® (Maravai LifeSciences), and stored at 4°C. Fluorescence was detected with AxioVision 4.6. Channel sensitivity was optimized and maintained for each set of stained sections. Mosaic images of the ventral tegmental area, nucleus accumbens, and caudate nucleus were taken with the Zeiss AxioCam MRc5 and stitched together in AxioVision 4.6. Slice location and identification of brain regions were determined by comparison to images in The Rat Brain Atlas (Paxinos and Watson, 1998).

Data Analysis

Microglia and astrocyte quantity and size were analyzed in ImageJ®. A customized ImageJ® protocol for cell quantification and morphological analyses was used to perform quantitative measures of astrocytes and microglia (Courtesy of Russel Ravenel). Astrocytes were analyzed at 20x magnification, where fluorescent signals greater than 40 square pixels were automatically counted as cells using ImageJ®. In order to obtain a more representative picture of microglial expression, separate images of microglia from the nucleus accumbens were stitched together in a 3x3 array to create a mosaic image of the entire region. Mosaic images were made with images of microglia at 20x magnification. Microglia in the ventral tegmental area were similarly stitched into mosaic images. To account for the smaller size of microglia in mosaic images, ImageJ® was programmed to count fluorescent signals greater than 10 square pixel units as cells. All statistical analyses were performed in Prism 7 (GraphPad Software Inc., San Diego, CA).
A 2x2 ANOVA was used to analyze the effects of ibudilast on active and inactive lever responses following 14 days of forced abstinence. A 2x2 mixed design ANOVA was used to analyze possible effects of ibudilast on weight gain over the 14-day abstinence period. Microglia and astrocyte quantity, size, and density in the nucleus accumbens were analyzed using separate unpaired t-tests. Microglia and astrocyte quantity and size in the VTA were analyzed with an unpaired t-test. Results were normalized to the parameter area used to obtain cell quantity and size in ImageJ®.

**Results**

*Cocaine increases microglial quantity in the VTA in a sex-independent manner*

Images of the VTA following cocaine administration were qualitatively assessed in order to examine differences in microglial expression between sexes (Figure 1). Immunohistochemical images of the VTA were visually assessed to examine sex differences in microglial size and quantity following repeated cocaine administration. Reactive microglial subtypes were visually determined using representative images from a previous study of sex differences in microglia activity (Doyle et al., 2017). Microglia were characterized as ramified if microglia displayed long, thin projections branching off the soma. Amoeboid microglia were characterized by large, round somas with few projections. Intermediate microglia were identified by short, thick processes emanating from the soma.

Although no quantitative data was gathered due to the small sample size, there appeared to be an increase in total number of microglial cells in animals who received cocaine as compared to saline. No visual differences in microglial quantity in the VTA were evident between males and females. There did not appear to be a difference in microglia morphology
between sexes, where the majority of microglia in animals receiving either saline or cocaine appear to be ramified, with a small number of intermediate microglia containing thicker processes more characteristic of reactive microglia.

**Ibudilast attenuated active lever pressing following prolonged abstinence from oxycodone**

In order to examine the anti-inflammatory effects of ibudilast on oxycodone seeking, animals self-administered oxycodone followed by a 2-week period without access to the drug. Animals learned to acquire oxycodone over 12 days of self-administration, as demonstrated by increased responding on the drug-paired lever over multiple sessions (Figure 2A). Animal weights increased over 14 days of forced abstinence (Figure 2B). There was no significant effect of ibudilast on animal weight trends over the 14 day abstinence period (F(1, 10)=1.013, ns). There was a main effect of treatment, where ibudilast administration significantly reduced responding on the formerly drug-paired lever as compared to animals who received saline (F(1, 20)=5.428, p<0.05; Figure 2C). Additionally, there was a main effect of lever, where animals pressed significantly more on the active lever as compared to the inactive lever, (F(1, 20)=9.681 (p<0.01), suggesting that they were able to discriminate between the two. There was no significant interaction between ibudilast administration and lever (F(1, 20)=2.094). These results suggest that ibudilast may attenuate incubation of opiate craving.

**Ibudilast had no effect on microglial quantity or size in the VTA**

The relative presence and size of microglia in the VTA and nucleus accumbens following ibudilast administration was analyzed due to their involvement in inflammatory responses in the CNS. There was no significant effect of ibudilast on microglial quantity in the nucleus accumbens (t(8)=0.323, ns; Figure 3A). Ibudilast treatment appeared to reduce microglial quantity in the VTA (Figure 3B), however, this trend was not statistically significant (t(8)=1.727,
ns). No significant effects of ibudilast were observed on microglial size in the nucleus accumbens ($t(8)=0.439$, ns; Figure 3C). Similarly, ibudilast showed no significant effects on average cell size in the VTA ($t(8)=1.042$, ns; Figure 3D). Overall, these results suggest that ibudilast treatment was ineffective in altering the number or morphology of microglia in the nucleus accumbens or VTA following oxycodone intake.

**Ibudilast reduced total number and size of astrocytes in the nucleus accumbens and VTA**

While ibudilast is thought to exert anti-inflammatory effects on microglia, it may additionally impact other immunocompetent cells, such as astrocytes. The present study therefore examined whether the quantity and size of astrocytes was altered following ibudilast treatment. There was a significant effect of ibudilast on astrocyte quantity in the nucleus accumbens, where treatment of ibudilast reduced the observed number of astrocytes ($t(9)=3.745$, $p<0.05$; Figure 4A). Further, ibudilast treatment reduced the number of astrocytes in the VTA ($t(9)=5.064$, $p<0.05$; Figure 4B). Average astrocyte size was significantly diminished in the nucleus accumbens following ibudilast treatment ($t(9)=2.519$, $p<0.05$; Figure 4C). Similarly, ibudilast had a significant effect on astrocyte size in the VTA, where ibudilast administration reduced astrocyte size ($t(9)=3.149$, $p<0.05$; Figure 4D). Taken together, these results suggest that ibudilast may reduce markers of astrocyte activity in the nucleus accumbens and VTA following oxycodone self-administration. When combined with behavioral results, this data may point to a role of astrocyte activity in incubation of oxycodone craving.

**Discussion**

A defining feature of drug addiction is the high rate of relapse during periods of abstinence, often precipitated by exposure to a previously abused drug, drug-associated cues, stress, or protracted withdrawal symptoms. The regularity with which drugs of abuse are used or
prescribed within US society warrant a need to understand the neural adaptations taking place both during drug use, and in the weeks following cessation of drug use. The present study sought to examine how drugs of abuse impact non-neuronal glial cells in the mesolimbic system. In particular, we evaluated sex differences in microglial activity following repeated cocaine intake. We further aimed to examine the effects of ibudilast on cue-seeking behavior following prolonged abstinence from oxycodone. In concert with behavioral results, the effects of ibudilast on microglia and astrocyte activity in the nucleus accumbens and VTA were also evaluated.

Preliminary results showed no sex differences in microglial activity following cocaine intake. While there appeared to be a modest difference in microglial quantity between animals who received saline as compared to cocaine, no conclusions can be definitively drawn due to the small sample size used for comparisons. Studies of this nature would benefit from further investigation, as microglia in this study did not show any drug-induced morphological alterations characteristic of heightened activity, as has been described in previous studies (Kohno et al., 2019; Wang et al., 2012). Intraperitoneal cocaine administration, such as was used in this study, is subject to hepatic first-pass elimination, diffusion into tissues or lymph, and breakdown by various tissue enzymes (Intraperitoneal Drug Administration, 2013). Future studies may examine sex differences in microglial activity with more potent routes of administration, such as intravenous infusion or vapor inhalation, in order observe sufficient activation of the innate immune system.

The current study demonstrated an ability of ibudilast to reduce incubation of oxycodone craving following prolonged abstinence without altering broader motivated behavior, as indicated by continued food consumption and weight gain throughout the abstinence period. Weight gain was not an effect of ibudilast treatment, as animals who received ibudilast showed
similar increases in weight over the 14-day abstinence period compared with the vehicle-treated 35% PEG group. Treatment with ibudilast reduced astrocyte activity following extended oxycodone intake but interestingly, had little influence on microglia. Taken together, the reduction of astrocyte activity and active lever pressing following ibudilast administration suggests a role of astrocytes in heightened oxycodone craving following cessation of drug use.

Although previously thought to be a selective microglial inhibitor, ibudilast has more recently been seen to block glial proinflammatory responses at large (Hutchinson et al., 2009; Lacagnina, Rivera, & Bilbo, 2017; Snider et al., 2012). Results of this study provide further evidence of the non-selective effects of ibudilast on glial cells more broadly, however, the specific mechanisms by which ibudilast influences different cell types remains unknown.

Ibudilast is a TLR4 antagonist, a non-selective phosphodiesterase (PDE) inhibitor, a glial cell modulator, an inhibitor of macrophage migration inhibitory factor, and a glial cell line-derived neurotrophic factor (GDNF) inducer (Snider et al., 2012). It has further been seen to reduce glial activity by suppressing release of proinflammatory cytokines such as TNF-alpha, IL-6, IL-1β, and nitric oxide (Mizuno et al., 2004). The effects of ibudilast on astrocytes in this study could be due to any combination of these mechanisms. One possibility is that increased levels of cAMP following ibudilast-induced PDE inhibition reduces TNF-alpha synthesis from astrocytes (Shames et al., 2001), which would in turn reduce downstream glial activation. In support of this idea, previous studies have demonstrated that AV1013—an ibudilast analogue with minimal PDE inhibition—has less potent anti-inflammatory effects relative to ibudilast (Snider et al., 2012).

It has been proposed that astrocytes may act as a secondary form of innate immune defense in response to cell damage or xenobiotics (Lacagnina, Rivera, & Bilbo, 2017), becoming
activated by microglia either through direct contact or through soluble factors released by microglia. Such astrocyte activation leads to a second cascade of proinflammatory cytokine release, and can heighten proinflammatory cytokine release by microglia. In light of the finding that ibudilast reduces astrocyte size and quantity in the nucleus accumbens and VTA while having little impact on microglia, it may be hypothesized that reactive astrocytes—as a secondary form of immune defense—are the first to be downregulated when the immediate prospect of a threat is absent.

Astrocytes have been seen to express a damage-associated molecular pattern (DAMP) sensor protein known as nucleotide-binding leucine-rich repeat caspase recruitment domain-containing 4 (NLRC4) in greater numbers than microglia, which strongly expresses NLR family pyrin domain containing 3 (NLRP3) (Freeman et al., 2017). Although these molecular sensors both allow for proteolytic activation and increased circulation of proinflammatory cytokines, they respond to different environmental threats, and show different specificities for different ligands (Freeman et al., 2017). This affords the possibility that ibudilast may be attenuating NLRC4 to a greater extent than NLRP3, which would help to explain the significant reduction of astrocyte activity as compared to microglial activity in this study. Future experiments may seek to examine the ability of different anti-inflammatory agents to reduce the expression of proinflammatory cytokine release for clarification of the mechanisms by which this may occur.

A major impediment to the development of pharmacological interventions for treatment of substance use disorders is the ability to reduce drug craving while leaving adaptive motivated behavior intact. The present study examined oxycodone seeking with the knowledge that heightened craving following cessation of opioid drugs can impede attempts to maintain sobriety. In addition to acute drug cravings, drug-associated cues, environmental stressors, and
exposure to previously abused drugs are all sufficient to trigger a relapse, suggesting that activation of G-protein coupled MORs facilitates long-lasting alterations in synaptic plasticity that maintain context-dependent memories of one’s environment surrounding the time of MOR activation.

The data presented in this study indicate that astrocytes may play a significant role in incubation of oxycodone craving during periods of prolonged abstinence. Furthermore, it was demonstrated that ibudilast attenuates astrocyte activity to a greater extent than microglial activity following oxycodone intake, providing evidence for a non-specific anti-inflammatory effect of ibudilast. The contribution of glial-induced neuroinflammation to the acutely rewarding effects of opioids and cocaine may help guide the development of future pharmacotherapies that can reduce vulnerability to drug addiction.
Figure 1. Qualitative sex differences in microglial alterations following cocaine intake. There were no apparent differences in microglial quantity or morphology between males and females following cocaine intake. Microglial morphology did not appear to change following cocaine administration.
**Figure 2.** Oxycodone self-administration, withdrawal weights, and cue testing. (A) Animals showed an increase in oxycodone self-administration over 12 days. Each self-administration session lasted 6 hours. (B) Animal weights increased over 14 days of forced abstinence, with no significant effect of treatment on weight. (C) Ibudilast treatment significantly reduced active lever presses following 14 days of forced abstinence.
Figure 3. Microglial quantity and size in the nucleus accumbens and VTA. (A) Microglial cell quantity was not significantly reduced in the nucleus accumbens following ibudilast treatment. (B) A trend towards reduction in microglial quantity in the VTA following ibudilast treatment was observed, but was not statistically significant. (C, D) There were no significant differences in microglial size in either the nucleus accumbens or VTA following ibudilast treatment.
Figure 4. Astrocyte quantity and size in the VTA and nucleus accumbens. (A, B) Treatment with ibudilast reduced the observed number of astrocytes in both the nucleus accumbens and VTA. (C) Average astrocyte size was significantly different in the nucleus accumbens between animals who received ibudilast or a vehicle. (D) Average astrocyte size was significantly different in the VTA following treatment with ibudilast.
Figure 5. Comparative analysis of astrocyte and microglia quantity in the nucleus accumbens and VTA following 14 days of ibudilast administration. Top images show astrocytes in the nucleus accumbens in animals that received a vehicle (right) or ibudilast (left) following oxycodone self-administration (20x magnification). Bottom images show microglial cells in the VTA of animals who received a vehicle (right) or ibudilast (left) following oxycodone self-administration (20x magnification, mosaic image).
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