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Ibutilast attenuates expression of behavioral sensitization to cocaine in male and female rats

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Abstract

There are no FDA-approved pharmacotherapies for cocaine use disorder, indicating a need to identify novel reagents with therapeutic potential. Ibutilast is an anti-inflammatory glial attenuator and non-selective phosphodiesterase inhibitor currently undergoing clinical evaluations for methamphetamine, opiate, and alcohol abuse disorders. We previously showed that twice daily (b.i.d.) ibutilast reduces the development of methamphetamine sensitization in male mice. However, nothing is known about the ability of ibutilast to modulate the expression of sensitization that occurs after drug re-exposure during abstinence, effects on cocaine-mediated behaviors, or potentially sexually dimorphic effects. Male and female rats were administered cocaine for 7 days and expression of sensitization was assessed by cocaine challenge after 21 days abstinence. On test days, 15 mg/kg i.p. cocaine was evaluated, whereas 30 mg/kg was administered on intervening days. Lower test doses avoid competition of non-motor behaviors with locomotion. In all measures where sensitization was expressed, ibutilast (7.5 and 10 mg/kg, i.p., b.i.d. for 3 days and once on test day) reversed this behavior to levels seen after acute exposure, but not below. There were some intriguing sexually dimorphic effects that were not a function of estrous cycle. Specifically, distance travelled in the center of the test arena and rearing only sensitized in male rats, and ibutilast reversed these behaviors to levels seen after acute cocaine exposure. In females, center distance travelled was reduced below acute cocaine levels by 7.5 mg/kg ibutilast. Increased distance travelled in the center versus periphery is thought to model

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6. Disclosures

The authors have no competing or conflicting interests to declare.

anxiolytic-like behavior due to increased predation risk. Taken together, these data suggest that the clinical evaluation of ibudilast could be extended to cocaine use disorder.

Graphical Abstract

Keywords

cocaine; sensitization; sex; Ibudilast; AV411; MN-166

1. Introduction

Cocaine abuse extracts large socioeconomic resources, and the United Nations estimates that 17 million individuals use cocaine worldwide (Crime, 2015). Upon initial use, cocaine produces profound subjective well-being and intensification of almost all normal pleasures (Gawin, 1991). With repeated use, a compulsive desire for the drug often develops that can supplant most other thoughts and activities (Gawin, 1991). Cocaine use disorder commonly results in both acute and long-term physical (e.g., cardiovascular and gastrointestinal disorders) and psychiatric (e.g., depression, psychosis, anxiety, and impulsivity) problems, and is a significant contributor to morbidity and mortality (Shorter and Kosten, 2011).

A number of receptor-mediated (e.g., dopamine, serotonin, GABA, and glutamate) and non-receptor mediated (e.g., disulfiram and vaccines) approaches have been evaluated for their capacity to reduce cocaine abuse or prevent relapse (Bowers et al., 2010; Fischer et al., 2015). Overall, these treatments have not shown wide efficacy (Fischer et al., 2015), and there are currently no FDA-approved pharmacotherapies for cocaine use disorder. Consequently, there is a need to identify new therapeutic entities, perhaps by examining reagents that counteract multiple cocaine effects.

Ibudilast (aka AV411, MN-166, and 3-isobutyl-2-isopropyl-pyrazolo-[1,5-a]pyridine) is a blood-brain barrier permeant, non-selective glial modulator, phosphodiesterase inhibitor, and anti-inflammatory agent (Kishi et al., 2001; Gibson et al., 2006) that has shown some promise for methamphetamine use disorder. Pre-clinical ibudilast studies show reduced development of methamphetamine sensitization in male mice (Snider et al., 2012) in addition to reduced methamphetamine self-administration (Snider et al., 2013) and reinstatement of methamphetamine-seeking behavior in male rats (Beardsley et al., 2010). Consequently, ibudilast is currently undergoing clinical evaluation for methamphetamine dependence (NCT01860807).

Ibutilast has also shown efficacy in reducing ethanol consumption (Bell et al., 2015) and morphine withdrawal signs while promoting opioid analgesia (Hutchinson et al., 2009) in preclinical models. Moreover, ibutilast reduced self-reports of methamphetamine reward-related subjective effects (Worley et al., 2016), but intriguingly, nothing is known about the ability of ibutilast to modulate cocaine-induced behaviors or sexually dimorphic effects in the context of any abused drug. Because ibutilast can modulate some neurobiological effects implicated in cocaine abuse including cAMP accumulation, glial activation, and neuroinflammation, we sought to determine if ibutilast alters the expression of behavioral sensitization to cocaine and if this response differs between male and female rats. Sensitization is defined as increased motoric responses following repeated, intermittent drug exposure that can occur in at least two phases called development (or induction) and expression. The expression of sensitization is observed following drug challenge during abstinence (Post and Rose, 1976; Kalivas et al., 1993; Vanderschuren and Kalivas, 2000). Our focus was on the expression of sensitization because it is thought to model enduring psychostimulant-induced plasticity occurring within circuitry that parse motivationally relevant stimuli (Kalivas et al., 1993; Vanderschuren and Kalivas, 2000). Accordingly, sensitization is thought to model some enduring behaviors observed in human drug addicts (Segal and Schuckit, 1983). Thus, reagents that reduce the expression of sensitization may have at least some capacity to reduce drug-seeking behavior in addicts. Here, we show that ibutilast was effective at reducing expression of cocaine sensitization in both sexes, although effects on anxiety-like behavior were sexually dimorphic.

2. Materials and Methods

2.1 Subjects

Male and Female, adult Long Evans rats (11 wks upon arrival, ENVIGO, Indianapolis, IN) were allowed 1 wk to acclimate to the temperature-controlled (22°C), Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited facility prior to experimentation. Rats were singly housed and kept on a 12 h light/dark cycle (lights on 0700 h) with *ad libitum* access to food and water. All testing was conducted by the same investigators during the light phase. All procedures adhered to the 2011 Guide for the Care and Use of Laboratory Animals, ARRIVE guidelines (Kilkenny et al., 2010), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2 Reagents

All reagents were freshly prepared. Cocaine HCl (NIDA Drug Supply Program, RTI, NC) was dissolved in sterile saline. Ibutilast (AvaChem, San Antonio, Tx) was dissolved in 35% PEG 200 (Electron Microscopy Sciences, Hatfield, PA), 10% Cremophore EL (Sigma-Aldrich, St. Louis, MO), and 55% sterile saline with gentle heating and cooling.

2.3 Apparatus

The test arenas (Omnitech Electronics, Columbus, OH) were Plexiglas® cubes measuring 40.6 × 40.6 × 40.6 cm that were illuminated to 16 lux via diffused white LEDs placed above the center of the test arena. Arenas were housed within sound attenuating chambers. A series

of 16 photobeam arrays (8 on each horizontal axis) surrounded the periphery and 2 sets of 8 photobeam arrays were located 10 cm above the arena floor. Motoric activity was detected by photobeam breaks and recorded by a computer.

2.4 Treatment Regimen

Rats were treated, as we have described previously (Bowers and Kalivas, 2003; Bowers et al., 2004). **Figure 1** illustrates the procedure. Briefly, rats were habituated to the locomotor chamber for 1 h, injected with saline (1 ml/kg, i.p.) and returned to the locomotor chamber for 1 h. Distance travelled was recorded, and this procedure was repeated daily until total distance travelled in the 1 h test phase was less than 5,000 cm, which generally required 3 days. After satisfying habituation criteria, rats experienced their first test session (Day 1), in which they were habituated to the chamber (1 h), injected with cocaine (15 mg/kg, i.p.), and returned to the locomotor chamber for a 1 h test. During the subsequent 6 consecutive days, rats received cocaine injections (30 mg/kg, i.p.) in the home cage. Next, rats underwent 3 wks of cocaine abstinence, and assigned to treatment groups balanced by total distance travelled on Day 7. Ibudilast (0, 7.5, or 10 mg/kg, i.p., b.i.d., 7 h apart) was given during the last 3 days of abstinence (Days 26 – 28) in the home cage. The following day (Day 29), ibudilast (or vehicle) was injected, and rats were placed into the locomotor chamber for 1 h. This regime was shown to reduce the development of methamphetamine sensitization in male mice and methamphetamine self-administration and reinstatement in male rats (Hutchinson et al., 2009; Beardsley et al., 2010; Snider et al., 2012; Snider et al., 2013; Bell et al., 2015). Next, rats were injected with cocaine (15 mg/kg, i.p.), and motor responses recorded for 1 h (see **Figure 1**). An ibudilast-free habituation period was not used on the final test day to avoid over exposure to the chamber which can impact motor responding, and 3 wks abstinence was studied because behavioral sensitization is often absent during early abstinence due to homeostatic events (Vanderschuren and Kalivas, 2000).

2.5 Estrous Cycle

The estrous stage was determined by the phenotype of vaginal cells, as described previously (Goldman et al., 2007; McLean et al., 2012). Briefly, vaginal cells were collected by smearing the vaginal canal opening (100 μ l sterile ddH₂O), and the smear was immediately examined under light microscopy at 10x to determine cell morphology present. **Table 1** enumerates estrous cycle phases during the second week of abstinence as well as after the test session.

2.6 Statistical Analysis

Locomotion was analyzed by measuring total distance traveled as well as distance travelled in the center and periphery of the open field. Distance travelled was quantified via breaking of adjacent photobeams. The central region consisted of an 20.32 $\times$ 20.32 cm area located in the center of the open field. The periphery was defined as a 10.16 $\times$ 40.64 cm area (or 40.64 $\times$ 10.16 cm) that surrounded the central region. Rearing was quantified by the breaking of elevated photobeams, and stereotyped behavior was estimated via quantification of repeated breaking of the same photobeam. Data were analyzed (Statview, Berkeley, CA) by a 1- or 2-

way ANOVA or repeated measures ANOVA, as appropriate. Scheffé post-hoc tests were used to further characterize the results, and a Bonferroni correction was applied to multi-comparison time course data. Significance was set at $p < 0.05$. Expression of sensitization was considered significant if motoric behavior was different in vehicle-treated rats when comparing the first cocaine exposure (Day 1) to the cocaine challenge on test day (Day 29).

3. Results

3.1 Total Distance Travelled

Figure 2 illustrates total distance travelled. Expression of sensitization was evident in both female (**Figure 2A**) and male (**Figure 2A'**) vehicle-treated rats. A two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons using sex and day as factors indicated a main effect of treatment day, but no effect of sex or interaction [Sex: $F(1,28) = 0.18$, $p = 0.67$; Day: $F(1,28) = 12.54$, $p < 0.001$; Sex $\times$ Day: $F(1,28) = 6 \times 10^{-5}$, $p < 0.001$]. A one-way repeated measures ANOVA of these data within each sex indicated a main effect of day in both females ($p < 0.05$) and males ($p < 0.05$). Thus, both sexes expressed sensitized total distance travelled. The effect of ibudilast is shown in subsequent panels. A two-way ANOVA over the 1 h test period, with sex and ibudilast dose as factors, revealed that there was a main effect of dose [$F(2,42) = 12.81$, $p < 0.001$], but no effect of sex [$F(1,42) = 0.36$, $p = 0.55$] and no interaction [Dose $\times$ Sex: $F(2,42) = 0.006$, $p = 0.99$]. Thus, ibudilast appeared to reverse the expression of sensitized total distance travelled in both sexes. Given the above, the effect of ibudilast was analyzed within sex, and cocaine day 1 data are included in subsequent summary graphs for comparison. In females, a one-way ANOVA over the 1 h test period with ibudilast dose as the factor indicated that ibudilast reversed sensitized total distance travelled, and post-hoc analysis revealed that this effect was dose-related (**Figure 2B**). However, a two-way ANOVA showed that ibudilast did not reduce total distance travelled below that induced by acute cocaine ($p = 0.97$). A one-way repeated measures ANOVA failed to detect an effect of ibudilast alone during the 60 min prior to cocaine challenge, but some of this may be due to a floor effect (**Figure 2B'**). In males, a similar effect was seen (**Figure 2C**). Specifically, ibudilast dose-relatedly reversed sensitized total distance travelled to levels seen after the first cocaine exposure, but not below ($p = 0.21$). In contrast to females, ibudilast alone had a greater initial effect, but no ibudilast effect was seen for the remaining 45 min prior to cocaine challenge (**Figure 2C'**). Again, this could be partially attributed to a floor effect. Lastly, estrous phase on test day (**Table 1**) did not effect either the response of female rats to ibudilast pretreatment [Phase: $F(3,14) = 0.95$, $p = 0.95$] or to cocaine challenge [Phase: $F(3,14) = 0.68$, $p = 0.58$].

3.2 Center Distance Travelled

Because time spent in the center versus periphery of the test arena can be indicative of anxiolytic-like behavior, total distance travelled was further analyzed as distance travelled in the center and periphery. **Figure 3** illustrates distance travelled in the center of the test arena. In contrast to total distance travelled expression of sensitization was not seen in females (**Figure 3A**), but it was evident in male (**Figure 3A'**) vehicle-treated rats. Specifically, a two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons using sex and day as factors indicated a main effect of treatment day, but no effect of sex or

interaction [Sex: $F(1,28) = 0.55$, $p = 0.47$; day: $F(1,28) = 4.0$, $p < 0.05$; Sex $\times$ Day: $F(1,28) = 2.76$, $p = 0.11$]. A one-way repeated measures ANOVA of these data within each sex showed no main effect of day in females ($p = 0.78$). However, a main effect of day was detected in males ($p < 0.05$). Thus, only vehicle-treated males expressed cocaine-sensitized center distance travelled. The effect of ibudilast is shown in subsequent panels. In further contrast to total distance travelled, a two-way ANOVA over the 1 h test period, with sex and ibudilast dose as factors, revealed a main effect of both dose [$F(2,42) = 8.85$, $p < 0.001$] and sex [$F(1,42) = 4.48$, $p < 0.05$], but no interaction [Dose $\times$ Sex: $F(2,42) = 0.25$, $p = 0.78$]. Subsequently, the effect of ibudilast was analyzed within sex, and cocaine day 1 data are included in subsequent summary graphs for comparison. In females, a one-way ANOVA over the 1 h test period with ibudilast dose as the factor indicated a main effect of dose, and post-hoc analysis revealed that this effect was dose-related (**Figure 3B**). Moreover, even through sensitized center distance travelled was not expressed by females, a 2-way ANOVA followed by a Scheffé post-hoc indicated that the 7.5 mg/kg dose reduced center distance travelled below that seen after acute cocaine ($p < 0.05$). Thus, although center distance travelled did not sensitize in females, ibudilast reduced cocaine-stimulated locomotion below vehicle-treated rats on test day within a narrow dose range. A one-way repeated measures ANOVA failed to detect an effect of ibudilast alone during the 55 min prior to cocaine challenge, but some of this may be due to a floor effect (**Figure 3B'**). In contrast to females, ibudilast reversed the expression of sensitized distance travelled in the center in males. However, this reduction was not below levels seen after the initial cocaine exposure ($p = 0.15$; **Figure 3C**). In males, ibudilast alone had a greater initial effect than in females, but no ibudilast effect was seen for the remaining 45 min prior to cocaine challenge (**Figure 3C'**). However, this could be partially attributed to a floor effect. Lastly, estrous phase on test day (**Table 1**) did not effect either the response of female rats to ibudilast pretreatment [Phase: $F(3,14) = 0.43$, $p = 0.73$] or the cocaine challenge [Phase: $F(3,14) = 0.62$, $p = 0.62$].

3.3 Periphery Distance Travelled

Figure 4 illustrates periphery distance traveled. Similar to total distance travelled, expression of sensitization was seen in both female (**Figure 4A**) and male (**Figure 4A'**) vehicle-treated rats. Specifically, a two-way repeated measures ANOVA (with Bonferroni correction for multiple comparisons using sex and day as factors) indicated a main effect of treatment day, but no effect of sex or interaction [Sex: $F(1,28) = 0.05$, $p = 0.83$; Day: $F(1,28) = 12.81$, $p < 0.01$; Sex $\times$ Day: $F(1,28) = 0.49$, $p = 0.49$]. A one-way repeated measures ANOVA of these data within each sex showed a main effect of day in both females ($p < 0.01$) and males ($p < 0.05$). Thus, both females and males expressed sensitized distance travelled in the periphery of the test arena. The effect of ibudilast is shown in subsequent panels. In further contrast to total distance travelled, a two-way ANOVA over the 1 h test period, with sex and ibudilast dose as factors, revealed a main effect of dose [$F(2,42) = 11.45$, $p < 0.001$], but neither a main effect of sex [$F(1,42) = 0.01$, $p = 0.96$] nor an interaction [Dose $\times$ Sex: $F(2,42) = 0.06$, $p = 0.94$]. To further describe the data, the effect of ibudilast was analyzed within sex, and cocaine day 1 data are included in subsequent summary graphs for comparison. In females, a one-way ANOVA over the 1 h test period with ibudilast dose as the factor indicated a main effect of dose, and post-hoc analysis revealed that this effect was dose-related (**Figure 4B**). Thus, ibudilast reversed the

expression of sensitized distance travelled in the periphery by females. However, a 2-way ANOVA indicated that this reduction was not below levels seen after the initial cocaine exposure ($p = 0.08$). Moreover, a one-way repeated measures ANOVA failed to detect an effect of ibudilast alone during the 60 min prior to cocaine challenge (**Figure 4B'**). Similar to females, ibudilast reversed the expression of sensitized distance travelled in the periphery in males. However, this reduction was not below levels seen after the initial cocaine exposure ($p = 0.19$; **Figure 4C**). In males, ibudilast alone had a greater initial effect than in females, but no ibudilast effect was seen for the remaining 50 min prior to cocaine challenge (**Figure 4C'**). However, this could be partially attributed to a floor effect. Lastly, estrous phase on test day (**Table 1**) did not effect either the response of female rats to ibudilast pretreatment [Phase: $F(3,14) = 0.14$, $p = 0.94$] or the cocaine challenge [Phase: $F(3,14) = 0.26$, $p = 0.85$].

3.4 Rearing

Figure 5 illustrates rearing behavior. Similar to center distance travelled, expression of sensitization was only seen in vehicle-treated male rats (females: **Figure 5A**; males: **Figure 5A'**). A two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons using sex and day as factors to analyze sensitized rearing indicated a main effect of treatment day and sex, but no interaction [Sex: $F(1,28) = 13.58$, $p < 0.01$; Day: $F(1,28) = 6.42$, $p < 0.05$; Sex $\times$ Day: $F(1,28) = 1.82$, $p = 0.19$]. A one-way repeated measures ANOVA of these data within each sex failed to find a main effect of day in females ($p = 0.24$), but a main effect was seen in males ($p < 0.05$). Thus, only males expressed sensitized rearing throughout the 1 h test session. The effect of ibudilast is shown in subsequent panels. A two-way ANOVA over the 1 h test period, using sex and ibudilast dose as factors, revealed a main effect of Dose [$F(2,42) = 7.82$, $p < 0.01$] and sex [$F(1,42) = 16.60$, $p < 0.001$], but no interaction [Dose $\times$ Sex: $F(2,42) = 1.46$, $p = 0.24$]. To further describe the data, the effect of ibudilast was analyzed within sex, and cocaine day 1 data are included in subsequent summary graphs for comparison. A one-way ANOVA over the 1 h test period with ibudilast dose as the factor indicated a main effect of dose (**Figure 5B**). However, a 2-way ANOVA indicated that this reduction was not below levels seen after the initial cocaine exposure ($p = 0.61$). Moreover, a one-way repeated measures ANOVA indicated that ibudilast pretreatment had no effect in females beyond the initial 10 min. Thus, no effect was observed for the 50 min prior to cocaine challenge (**Figure 5B'**). In contrast to females, ibudilast reversed the expression of sensitized rearing in males. However, this reduction was not below levels seen after the initial cocaine exposure ($p = 0.06$). (**Figure 5C**). In males, ibudilast alone had a greater initial effect than in females, but no ibudilast effect was seen for the remaining 45 min prior to cocaine challenge (**Figure 5C'**). However, this could be partially attributed to a floor effect. Lastly, the estrous cycle phase on test day (**Table 1**) had no effect on rearing after either ibudilast alone [Phase: $F(3,14) = 0.73$, $p = 0.73$] or following cocaine challenge [Phase: $F(3,14) = 0.62$, $p = 0.61$].

3.5 Stereotypy

Figure 6 illustrates stereotyped behavior. A two-way repeated measures ANOVA with Bonferroni correction for multiple comparisons using sex and day as factors to analyze

sensitized stereotypy indicated no main effect of treatment day or sex [Sex: $F(1,28) = 1.44$, $p = 0.24$; Day: $F(1,28) = 2.71$, $p = 0.11$]. Moreover, a one-way repeated measures ANOVA of these data within each sex failed to find a main effect in either (**Figure 6A**) females ($p = 0.25$) or (**Figure 6A'**) males ($p = 0.61$). Thus, cocaine sensitized stereotypy was not observed in vehicle-treated rats, which is consistent with other reports using this regimen (Bowers et al., 2004). Lastly, no ibudilast effect was seen in either females (**Figure 6B, 6B'**) or males (**Figure 6C, 6C'**) following cocaine challenge.

4. Discussion

We studied the effect of ibudilast on component behaviors that comprise the expression of behavioral sensitization. The following dependent measures were examined: total distance travelled, distance travelled in the center of the test area, distance travelled in the periphery, rearing, and overall stereotypy. In brief, we found that ibudilast dose-relatedly reversed sensitized motor behavior in all measures where sensitization was expressed. There were some interesting sexually dimorphic effects. Only males expressed sensitized distance travelled in the center of the test area in addition to sensitized rearing; ibudilast reversed this sensitization in males. These data suggest ibudilast may reverse heightened risk-taking or anxiolytic-like behavior in males to levels seen after acute cocaine. Moreover, our data suggests that ibudilast may reduce risk-taking or anxiolytic-like behavior in females below that seen after acute cocaine. Some of these effects, especially in males, may arise via mild motor suppression.

Sensitization is predominantly studied by total distance travelled. This measure includes both horizontal movements that occur parallel to the axes as well as diagonal movements. The present results show that both female and male rats expressed sensitized total distance travelled. Moreover, ibudilast dose-relatedly reversed this sensitization to levels seen after acute cocaine exposure, but not below. While ibudilast alone can reduce initial locomotion in both sexes, with males being especially sensitive, no effect was observed for at least 45 min prior to cocaine challenge. Thus, because there was no effect of ibudilast-alone when examining responses for the full hour pre-treatment period, it is unlikely that ibudilast produced its effects on cocaine sensitization via gross locomotor suppression.

An important aspect of this study was the examination of sensitization component behaviors to understand how ibudilast may affect individual cocaine-mediated phenotypes such as anxiety-like behavior. Thus, it is intriguing that sensitization of center distance travelled was only expressed in male rats and that ibudilast reversed this sensitization to the levels seen after acute cocaine exposure. Although center distance travelled did not sensitize in females, 7.5 mg/kg ibudilast reduced center distance travelled in females below levels seen after acute cocaine. Rodents commonly exhibit a high preference for locomotion near walls when exploring an open field, presumably to avoid predation. This behavioral preference is known as thigmotaxis. Because thigmotaxis can be increased by stressful stimuli and decreased by clinically effective anxiolytic reagents, distance travelled in the center is thought to model anxiolytic-like behavior (Treit and Fundytus, 1988). Thus, in our study, it appears that male rats became disinhibited in their normal thigmotactic behaviors upon cocaine challenge, or the proclivity to travel in the center was unmasked by cocaine challenge. Regardless, this

response was reversed by ibudilast. The specificity of this effect is partially supported by the fact that both sexes expressed sensitization of distance travelled in the periphery. If substantiated by further evaluation in future studies (e.g., elevated plus maze, light-dark shuttle box, and electrified grid crossing), these data suggest that ibudilast could reduce risk-taking behavior seen during cocaine abstinence (Bornovalova et al., 2005).

The second component behaviors studied were non-locomotor, and sexually dimorphic effects were also observed. Only males expressed sensitized rearing throughout the 1 h test session, which was reversed by ibudilast to levels seen with acute cocaine, but not below. While sensitized rearing was not expressed by females across the 1 h test, ibudilast reduced rearing in females at the 7.5 mg/kg dose. These effects are similar to our observations with center distance travelled. In contrast to the other behaviors studied, we failed to detect an expression of stereotypes, as estimated here via repeated horizontal photobeam breaks. Moreover, there was no ibudilast effect on overall stereotyped behavior seen for either sex. Stereotypies often develop at high psychostimulant doses and can compete with ambulatory movements and goal-directed behaviors. Because stereotypy can be suppressed in order to complete a goal, our failure to observe stereotypy may be an artifact of the design where cocaine was administered in the home cage on days 2 – 7. In other words, rats can acquire the ability to suppress stereotypy when competing goals (i.e., food) are present (Wolgin, 2000).

4.1 Sex differences

In general, females are considered to be more sensitive to cocaine effects, but there are interactions with genetic background. For example, cocaine-associated cues induced more craving in female addicts than in males (Robbins et al., 1999), and female humans tend to consume more cocaine than males (Kosten et al., 1993). Similarly, stressed female Long Evans rats consume more cocaine than stressed male rats (Holly et al., 2012). Drug-naïve female rats exhibit greater locomotor activity in the open field (Beatty, 1979), and greater sensitization to repeated cocaine administration was found in female Wistar (van Haaren and Meyer, 1991), Roman High Avoidance (Haney et al., 1994), and Fischer rats (Chin et al., 2001). In contrast, only modest sex differences were observed in the development of cocaine sensitization in the Wistar Kyoto Hyperactive, Spontaneous Hypertensive, and Wistar Kyoto rats (Caillhol and Mormède, 1999). In Sprague-Dawley rats, females respond greater to an acute cocaine injection (Walker et al., 2001), but both sexes are equivalently sensitive to the discriminative stimulus effects of cocaine (Craft and Stratmann, 1996) despite no difference in brain or blood cocaine levels following intraperitoneal injection (Bowman et al., 1999). Moreover, both sexes of Long Evans rats developed a conditioned place preference to cocaine equivalently, but reinstatement of an extinguished place preference was higher in females (Bobzean et al., 2010). Thus, while we were surprised that the female Long Evans rats used in this study did not show a more robust sensitization over males, other studies have found modest-to-no differences in cocaine effects between sexes, and genetic background appears to be an important factor. Long Evans rats were used here to facilitate comparison with the methamphetamine ibudilast literature (Beardsley et al., 2010; Snider et al., 2013). Intriguingly, when analyzing center distance travelled or rearing, females were more sensitive to ibudilast after cocaine challenge whereas males were sensitive to both

initial ibudilast effects as well as to the combined effect of cocaine challenge. That male rats exhibited greater anxiety-like behavior is also interesting given that human males report significantly higher anxiety ratings when treated with d-amphetamine (Gabbay, 2003) and cocaine dependent men have significantly higher corticoadrenal response to stress than cocaine dependent women (Fox et al., 2006). However, women are more likely to report stress and anxiety prior to drug relapse than men (Back et al., 2005). The emergence of sexually dimorphic cocaine effects likely emerge from ovarian hormones since female Fischer rats in estrus were more sensitive to cocaine (Quiñones-Jenab et al., 1999), and ovariectomized females with pustule estradiol supplementation sensitized to cocaine greater than intact males or non-supplemented ovariectomized females (Hu and Becker, 2003). Moreover, cocaine-dependent women with high progesterone exhibit lower cue-induced anxiety and blood pressure compared to women with low progesterone (Sinha et al., 2007), and anxiety levels negatively correlate with estradiol levels in female rats (Mora et al., 1996; Díaz-Véliz et al., 1997). Thus, an important aspect of this study was the inclusion of rats at multiple phases of the estrous cycle (**Table 1**); estrous phase was not found to contribute to any ibudilast effect reported. However, there were not enough subjects in any given phase to attempt a correlation. Thus, the genesis of the sexually dimorphic effects observed here are not clear at present, and further study is required.

4.2 Brief neurobiology of psychostimulant sensitization

Repeated, intermittent exposure of rats to psychostimulants produces a progressive and enduring increase in psychomotor effects (Stewart and Badiani, 1993; Vanderschuren and Kalivas, 2000). Sensitization is studied in two phases: development (or induction) and expression. Development can occur after repeated, intermittent drug exposure, whereas expression occurs after drug re-exposure during protracted abstinence. While the precise neural substrates of sensitization have not been fully elucidated, sensitization appears to depend upon the interplay of dopaminergic, glutamatergic, and GABAergic neurotransmission within the mesocorticolimbic circuit. How this circuit is engaged and modulated by repeated drug exposure depends on the mechanism of action of each drug. For cocaine, the primary mechanism is blockade of monoamine (dopamine, serotonin, and norepinephrine) re-uptake transporters located predominantly on presynaptic terminals of the mesoaccumbens and mesostriatal projections. Due to long-term hypersensitivity of mesolimbic dopaminergic pathways that are involved in goal-directed behavior and incentive salience (Salamone, 1994), cocaine sensitization is thought to model critical synaptic processes underlying substance use disorder. Moreover, sensitization is sensitive to the context in which the drug is administered (Torres and Rivier, 1992; Wise and Leeb, 1993), which akin to other drug-associated behaviors, indicates the involvement of associative learning processes. Thus, sensitization has been identified as one of the key elements supporting the acquisition and maintenance of compulsive drug-seeking behavior (Pierre and Vezina, 1997; De Vries et al., 1998; De Vries et al., 1999; Deroche et al., 1999; Vanderschuren and Kalivas, 2000). Cocaine also increases extracellular glutamate in the ventral tegmental area, nucleus accumbens and prefrontal cortex in a dopamine-dependent manner (Pierce and Kalivas, 1997; Wolf, 1998; Vanderschuren and Kalivas, 2000). The excitatory ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) is strongly implicated in the development of sensitization since NMDA channel blockers, competitive

antagonists, or glycine site partial agonists block the development of behavioral and neurochemical sensitization (Pierce and Kalivas, 1997; Wolf, 1998; Vanderschuren and Kalivas, 2000). The expression of cocaine sensitization is closely associated with increased activity of descending corticofugal glutamatergic efferents into the nucleus accumbens core and ventral tegmental area as well as with altered transmission through both ionotropic and metabotropic glutamate receptors (Pierce and Kalivas, 1997; Wolf, 1998; Vanderschuren and Kalivas, 2000).

4.3 Ibudilast mechanism of action

Ibudilast is an anti-inflammatory with three main mechanisms of action. First, ibudilast is an anti-inflammatory agent that appears to act via glial attenuation (Suzumura et al., 1999; Mizuno et al., 2004). Glial cells comprise the majority of cells in the brain (Sherwood et al., 2006), and a sensitizing cocaine administration regime has been shown to impact glia in a number of ways. For example, repeated, intermittent cocaine administration increased forebrain astroglia number and reactivity in a region-specific manner (Bowers and Kalivas, 2003), whereas oligodendrocyte number and myelination are reduced (Beardsley and Hauser, 2014). The effect of cocaine on microglia is less clear; cocaine increased the number of activated microglia in the rat cerebellum (Lopez-Pedrajas et al., 2015) and post-mortem human brain (Little et al., 2009), but increased microglia activation was not seen in a human imaging study of cocaine abusers (Narendran et al., 2014). While it is difficult to pharmacologically isolate glial subtypes or glia from neurons, chemogenetic approaches have shown important roles of astroglia in relapse-like behavior for cocaine (Scofield et al., 2015) and alcohol (Bull et al., 2014).

Psychostimulants including cocaine can activate innate immune responses (Clark et al., 2013), which is hypothesized to be a necessary step in addiction etiology (Crews et al., 2011; Clark et al., 2013). Unlike neurons and oligodendrocytes, microglia and to some extent astroglia mediate innate CNS immune responses. Some of these responses to cocaine are related to interferon- γ production (Kubera et al., 2004). Ibudilast attenuates astroglia and microglia immune-related activation by suppressing lipopolysaccharide and interferon- γ mediated production of several pro-inflammatory products including tumor necrosis- α , nitric oxide, and the interleukins IL-1 β and IL-6 (Suzumura et al., 1999; Kawanokuchi et al., 2004; Mizuno et al., 2004). Moreover, ibudilast also increases nerve growth factor, glial derived nerve growth factor (GDNF), and the anti-inflammatory cytokine IL-10 (Suzumura et al., 1999; Kawanokuchi et al., 2004; Mizuno et al., 2004). Microinjection of GDNF into the ventral tegmental area inhibits cocaine-mediated upregulation of the rate limiting enzyme in dopamine synthesis tyrosine hydroxylase and blocked the positive conditioning effects of cocaine (Messer et al., 2000). Thus, ibudilast could reverse some cocaine-induced plasticity via suppressed interferon- γ production and GDNF upregulation.

Akin to ibudilast, other glial-mediated anti-inflammatory drugs block cocaine effects as well. For example, minocycline hydrochloride significantly attenuates microglial activation (Sriram et al., 2006), and minocycline blocks the development of cocaine sensitization in male mice (Chen et al., 2009). Taken together, these data suggest that reagents that attenuate

glial activity or that act as anti-inflammatory agents reduce some cocaine effects that are thought to model the addicted phenotype.

Second, ibudilast preferentially inhibits specific phosphodiesterase (PDE) isoforms including PDE3A, PDE4, PDE10, and PDE11 (Gibson et al., 2006). PDEs breakdown cyclic nucleotides such as cAMP, and both PDE4 and PDE10 are expressed in brain. Thus, inhibition of PDE4 and PDE10 would be expected to facilitate brain cAMP accumulation. Cocaine is well known to indirectly activate and upregulate signaling through the cAMP pathway, and this is hypothesized to be a homeostatic mechanism to decrease drug action that can lead to tolerance, dependence, and increased drug self-administration (Nestler, 2015). Thus, PDE inhibitors that prevent cAMP hydrolysis would be expected to promote some drug-associated behaviors. In contrast, we found that ibudilast reduced expression of cocaine sensitization in female and male rats. Similarly, the PDE4 inhibitors rolipram and Ro 20-1724 blocked the acquisition of a cocaine conditioned place preference (Zhong et al., 2012), and cocaine self-administration was blocked by the PDE inhibitor zaprinast. Rolipram also reduced methamphetamine-mediated motor activation (Iyo et al., 1995; Mori et al., 2000). However, PDE inhibitors do more than increase cAMP accumulation (Deschatrettes et al., 2013). For example, the non-specific PDE inhibitor isobutylmethylxanthine reduced cocaine sensitization, but these effects are likely explained by inhibition of adenosine production rather than cAMP accumulation (Schroeder et al., 2012).

Third, ibudilast has been shown to protect against high levels of glutamate *in vitro* (Tominaga et al., 1996). While modulation of glutamatergic neurotransmission is not a primary mechanism of ibudilast action, this effect may be significant given that the expression of cocaine sensitization is associated with increased nucleus accumbens glutamate levels following cocaine challenge during abstinence (Pierce et al., 1996). Taken together, it is not yet clear which of ibudilast's many actions are responsible for the reduction in drug-stimulated behaviors seen here and in other studies (Hutchinson et al., 2009; Beardsley et al., 2010; Snider et al., 2012; Snider et al., 2013; Bell et al., 2015; Worley et al., 2016). Thus, now that a role for ibudilast in reducing cocaine-mediated behavior has been established by the present work, further study is needed to precisely delineate the underlying mechanism(s).

4.4 Conclusion

Earlier work showed that ibudilast reduced methamphetamine self-administration (Snider et al., 2013) as well as reinstatement of drug- and cue-primed methamphetamine-seeking behavior in male rats (Beardsley et al., 2010). Ibudilast was also found to block the development of methamphetamine sensitization in male mice (Snider et al., 2012). More recently, ibudilast was shown to reduce the reward-related subjective effects of methamphetamine (Worley et al., 2016). These data are supported by work of Becker and colleagues, which found that ibudilast reduced ethanol self-administration in non-dependent male rats as well as in dependent male mice (Bell et al., 2015). Moreover, ibudilast attenuated morphine effects including activation of microglia, astroglia, and withdrawal signs, while promoting opioid-mediated analgesia effects (Hutchinson et al., 2009). Thus, a

growing body of literature indicates that ibudilast may reduce drug use. Accordingly, ibudilast is undergoing clinical evaluation for methamphetamine (NCT01860807) and opioid (NCT01740414) and alcohol (NCT02025998) use disorder. Because interventions that alter sensitization provide the impetus to study more complex behaviors such as self-administration and relapse-like behavior, the present study indicates the need for further investigation of the ability of ibudilast to ameliorate cocaine use disorder.

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Highlights

- Ibutilast reversed expression of cocaine sensitization in female and male rats - 77/85 characters
- Total distance and peripheral distance travelled were reversed in both sexes - 76/85 characters
- Sexually dimorphic effects were observed for center distance travelled - 71/85 characters
- No estrous cycle effect was observed - 35/85 characters

A. Experimental Timeline

B. Timecourse of Test Day

Figure 1. Experimental timeline

Habitation and experimental sessions consisted of 2 phases: a 1 h habitation phase and a 1 h post-cocaine (or saline) injection test period. Only saline was injected on habitation sessions. (A) After 3 days habituation, rats were given 7 daily cocaine injections (closed arrow) and then placed into 3 wks abstinence. Ibudilast was injected twice per day during the last 3 abstinence days (open arrow). (B) On test day (Day 29), rats were pre-treated with ibudilast or vehicle (open arrow) 1 h prior to cocaine challenge (closed arrow). Behavior was recorded during habituation, day 1, and day 29. Hab: habituation; h: hour.

Figure 2. Total distance travelled: Ibudilast reversed the expression of sensitization by both male and female rats

The expression of sensitized total distance travelled was detected via a one-way repeated measures ANOVA in both (A) female vehicle-treated rats [Day: $F(1,14) = 6.42$, $p < 0.05$; Day $\times$ Time: $F(11,154) = 3.19$, $p < 0.001$] as well as (A') males [Day: $F(1,14) = 6.13$, $p < 0.05$; Day $\times$ Time: $F(11,154) = 1.92$, $p < 0.05$]. (B) Ibudilast (open bars) dose-relatedly reversed sensitized total distance travelled in females [Dose: $F(2,21) = 7.52$, $p < 0.001$]. Locomotion on cocaine treatment day 1 (closed bars) is included for comparison. Thus, ibudilast reversed the expression of sensitized total distance travelled, but it did not reduce it below levels seen after acute cocaine exposure. (B') Time course data illustrate that a strong

trend for an initial ibudilast effect dissipated prior to cocaine challenge [Dose: $F(2,21) = 0.88$, $p = 0.43$]. (C) In males, ibudilast dose-relatedly reversed sensitized total distance travelled [Dose: $F(2,21) = 5.55$, $p < 0.01$]. (C') Ibudilast alone decreased total distance travelled for the first 15 min in males, but no effect was seen for the subsequent 45 min prior to cocaine challenge [Dose: $F(2,21) = 1.56$, $p = 0.23$]. Data represent mean $\pm$ SEM, * $p < 0.05$; $n = 8$ per treatment.

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Figure 3. Center distance travelled: Ibudilast reversed the expression of sensitization by males, and reduced distance travelled by female rats

(A) The expression of sensitized center distance travelled was not detected in vehicle-treated females via a one-way repeated measures ANOVA [Day: $F(1,14) = 0.08$, $p = 0.78$]. (A') However, sensitized center distance travelled was detected in vehicle-treated males [Day: $F(1,14) = 5.19$, $p < 0.05$; Day $\times$ Time: $F(11,154) = 1.67$, $p < 0.05$]. (B) Ibudilast (open bars) dose-relatedly reduced distance travelled in the center by females [Dose: $F(2,21) = 6.50$, $p < 0.001$]. Locomotion on cocaine treatment day 1 (closed bars) is included for comparison, and the 7.5 mg/kg dose significantly reduced center distance travelled below levels seen after acute cocaine [Dose: $F(2,42) = 3.34$, $p < 0.05$]. Thus, the 7.5 mg/kg ibudilast dose

reduced center distance travelled despite no expression of sensitization. **(B')** The ibudilast effect observed during the first 5 min after treatment was not apparent during the 55 min prior to cocaine challenge [Dose: $F(2,21) = 2.43$, $p = 0.11$]. **(C)** In males, ibudilast dose-relatedly reversed sensitized total distance travelled [Dose: $F(2,21) = 3.9$, $p < 0.05$]. **(C')** Ibudilast alone decreased total distance travelled for the first 15 min in males, but no effect was seen for the subsequent 45 min prior to cocaine challenge [Dose: $F(2,21) = 2.28$, $p = 0.13$]. Data represent mean $\pm$ SEM, * $p < 0.05$; $n = 8$ per treatment.

Figure 4. Periphery distance travelled: Ibudilast reversed the expression of sensitization by both female and male rats

(A) The expression of sensitized periphery distance travelled was detected in female vehicle-treated rats via a one-way repeated measures ANOVA [Day: $F(1,14) = 9.85$, $p < 0.01$; Day x Time: $F(11,154) = 2.93$, $p < 0.01$]. (A') Moreover, sensitized periphery distance travelled was also detected in vehicle-treated males [Day: $F(1,14) = 3.87$, $p < 0.05$; Day x Time: $F(11,154) = 1.91$, $p < 0.05$]. (B) Ibudilast (open bars) dose-relatedly reversed sensitized distance travelled in the periphery by females [Dose: $F(2,21) = 6.88$, $p < 0.01$]. Locomotion on cocaine treatment day 1 (closed bars) is included for comparison, but there was only a strong trend for reduction beneath these levels ($p = 0.08$). Thus, ibudilast reversed sensitized

periphery distance travelled in females. However, ibudilast did not reduce this activity below that produced by acute cocaine. **(B')** No effect of ibudilast pre-treatment was seen in females [Dose: $F(2,21) = 0.39$, $p = 0.68$]. **(C)** In males, ibudilast dose-relatedly reversed sensitized total distance travelled [Dose: $F(2,21) = 4.72$, $p < 0.05$]. **(C')** Ibudilast alone decreased total distance travelled for the first 10 min in males, but no effect was seen for the subsequent 50 min prior to cocaine challenge [Dose: $F(2,21) = 2.11$, $p = 0.15$]. Data represent mean $\pm$ SEM, * $p < 0.05$; $n = 8$ per treatment.

Figure 5. Rearing behavior: Ibudilast reduced the expression of sensitization by males and reduced rearing by female rats

(A) Sensitized rearing was not detected in female vehicle-treated rats via a one-way repeated measures ANOVA [Day: $F(1,14) = 1.53$, $p = 0.24$; Day $\times$ Time: $F(11,154) = 3.22$, $p < 0.001$]. (A') However, sensitized rearing was detected in vehicle-treated males [Day: $F(1,14) = 4.90$, $p < 0.05$; Day $\times$ Time: $F(11,154) = 1.64$, $p = 0.09$]. (B) Ibudilast (open bars) dose-relatedly reduced rearing by females [Dose: $F(2,21) = 3.76$, $p < 0.05$]. Rearing on cocaine treatment day 1 (closed bars) is included for comparison, but rearing did not sensitize in females. Moreover, the 7.5 mg/kg ibudilast dose reduced rearing in females below acute cocaine levels when analyzed by a Student's t-Test ($p < 0.05$), but not when all doses are

considered together by ANOVA ($p = 0.61$). **(B')** No effect of ibudilast pre-treatment was seen in females for the 50 min preceding cocaine challenge [Dose: $F(2,21) = 1.53$, $p = 0.24$]. **(C)** In males, ibudilast dose-relatedly reversed sensitized rearing [Dose: $F(2,21) = 5.04$, $p < 0.05$]. **(C')** Ibudilast alone decreased total distance travelled for the first 15 min in males, but no effect was seen for the subsequent 45 min prior to cocaine challenge [Dose: $F(2,21) = 2.44$, $p = 0.11$]. Data represent mean $\pm$ SEM, * $p < 0.05$; $n = 8$ per treatment.

Figure 6. Stereotyped behavior: Ibudilast did not effect the expression of sensitization in either male or female rats

A one-way repeated measures ANOVA failed to detect expression of sensitized stereotyped behavior in either (A) females [Day: $F(1,14) = 1.42$, $p = 0.25$] or (A') males [Day: $F(1,14) = 0.27$, $p = 0.61$]. No effect of ibudilast (open bars) on stereotypy was observed in either (B, B') females [Dose: $F(2,21) = 0.91$, $p = 0.42$] or (C, C') males [Dose: $F(2,21) = 1.21$, $p = 0.32$] following cocaine challenge. Data represent mean $\pm$ SEM; $n = 8$ per treatment.

Table 1

Estrous cycle.

Rat	Ibutilast	Abstinence day					
		7	8	9	10	11	29
1	Vehicle	Estrus	Estrus	Metestrus	Diesterus	Proestrus	Diesterus
2	Vehicle	Diesterus	Estrus	Estrus	Proestrus	Proestrus	Diesterus
3	Vehicle	Metestrus	Proestrus	Estrus	Metestrus	Proestrus	Proestrus
4	Vehicle	Estrus	Diesterus	Proestrus	Estrus	Metestrus	Proestrus
5	Vehicle	Metestrus	Estrus	Metestrus	Diesterus	Proestrus	Proestrus
6	Vehicle	Diesterus	Estrus	Diesterus	Proestrus	Metestrus	Estrus
7	Vehicle	Proestrus	Proestrus	Estrus	Proestrus	Metestrus	Proestrus
8	Vehicle	Estrus	Metestrus	Estrus	Proestrus	Diesterus	Estrus
9	7.5 mg/kg	Metestrus	Proestrus	Estrus	Proestrus	Proestrus	Diesterus
10	7.5 mg/kg	Diesterus	Estrus	Diesterus	Metestrus	Proestrus	Metestrus
11	7.5 mg/kg	Proestrus	Proestrus	Estrus	Metestrus	Proestrus	Proestrus
12	7.5 mg/kg	Metestrus	Diesterus	Proestrus	Estrus	Metestrus	Estrus
13	7.5 mg/kg	Diesterus	Proestrus	Metestrus	Diesterus	Proestrus	Metestrus
14	7.5 mg/kg	Proestrus	Estrus	Metestrus	Estrus	Proestrus	Proestrus
15	7.5 mg/kg	Estrus	Metestrus	Diesterus	Metestrus	Diesterus	Diesterus
16	7.5 mg/kg	Metestrus	Proestrus	Diesterus	Estrus	Proestrus	Metestrus
17	10 mg/kg	Diesterus	Proestrus	Proestrus	Estrus	Metestrus	Estrus
18	10 mg/kg	Metestrus	Proestrus	Metestrus	Diesterus	Proestrus	Diesterus
19	10 mg/kg	Proestrus	Estrus	Diesterus	Proestrus	Estrus	Proestrus
20	10 mg/kg	Metestrus	Diesterus	Estrus	Metestrus	Proestrus	Proestrus
21	10 mg/kg	Proestrus	Estrus	Diesterus	Estrus	Proestrus	Proestrus
22	10 mg/kg	Diesterus	Estrus	Estrus	Proestrus	Proestrus	Proestrus
23	10 mg/kg	Estrus	Diesterus	Proestrus	Estrus	Estrus	Diesterus
24	10 mg/kg	Estrus	Metestrus	Proestrus	Estrus	Proestrus	Diesterus

Visual inspection of vaginal smears under 10× magnification was used to estimate estrous phase (Goldman et al., 2007; McLean et al., 2012).