Domain-selective BET inhibition attenuates transcriptional and behavioral responses to cocaine

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Abstract

Epigenetic pharmacotherapies have emerged as a promising treatment option for substance use disorder (SUD) due to their ability to reverse maladaptive transcriptional and behavioral responses to drugs of abuse. In particular, inhibitors of bromodomain and extra terminal domain (BET) reader proteins have been shown to reduce cocaine- and opioid-seeking behaviors in rodents. However, only pan-BET inhibitors, small molecules that bind to both bromodomains (BD1 and BD2) with all BET proteins, have been investigated in animal models of SUD. Given the potential side effects associated with pan-BET inhibitors, safer and more selective strategies are needed to advance BET therapeutics as a potential treatment for SUD. Here, we show that RVX-208, a clinically tested, BD2-selective BET inhibitor, dose-dependently reduced cocaine conditioned place preference in male and female mice, similar to the pan-BET inhibitor JQ1. In other behavioral experiments, RVX-208 treatment did not alter distance traveled, anxiety-like behavior, or novel object recognition memory. At the transcriptional level, RVX-208 attenuated the expression of multiple cocaine-induced genes in the nucleus accumbens in a sex-dependent manner. RVX-208 produced a distinct transcriptional response in stimulated primary neurons compared to JQ1 but had little effect on gene expression in non-stimulated neurons. Together, these data indicate that targeting domain-specific BET mechanisms may be an effective and safer strategy to reduce cocaine-induced neurobehavioral adaptations.
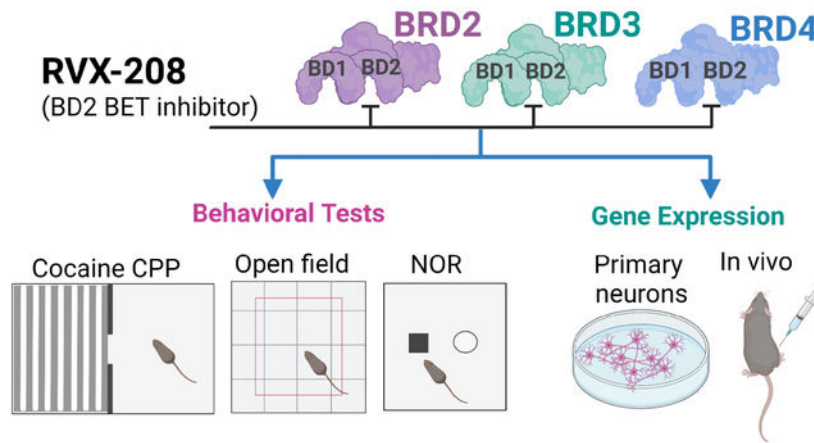
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Introduction

Repeated drug use alters histone acetylation processes in reward-related brain regions, which contributes, in part, to the molecular maladaptations involved in drug intake and relapse (Rogge and Wood, 2013). While writers (e.g., histone acetyltransferases, HATs) and erasers (e.g., histone deacetylases, HDACs) of histone acetylation have well-established roles in animal models of substance use disorder (SUD) (Kennedy et al., 2013; Malvaez et al., 2013, 2011; Rogge et al., 2013), readers of histone acetylation, called bromodomains, have also emerged as potential therapeutic targets (Egervari et al., 2017; Guo et al., 2020; Sartor et al., 2015). Specifically, pharmacological inhibition of bromodomain and extra terminal domain (BET) reader proteins (BRD2, BRD3, BRD4 and BRDT) has been shown to normalize transcriptional aberrations and behavioral symptoms in a wide range of disease models, including SUD (For review see: Singh and Sartor, 2020; Zaware and Zhou, 2017). Recognizing the growing success and usefulness of BET inhibitors, many large and small pharmaceutical companies have developed new and more selective BET therapeutics that are being tested in a variety of preclinical and clinical studies (Lu et al., 2020; Sartor, 2019).

Each BET protein contains highly conserved, tandem bromodomains (BD1 and BD2) that have been successfully targeted by small molecule inhibitors (Wu et al., 2019). First-generation BET inhibitors, also referred to as pan-BET inhibitors (e.g., JQ1, IBET151, IBET762), displayed similar affinity for BD1 and BD2 within all BET proteins and have been shown to alter disease-associated gene expression in multiple cell types by blocking BET bromodomain interactions with acetylated histones (Dawson et al., 2011; Filippakopoulos et al., 2010; Huang et al., 2009; Nicodeme et al., 2010). In preclinical SUD models, the pan-BET inhibitor, JQ1, was found to attenuate behavioral and transcriptional responses to cocaine, amphetamine, nicotine, and heroin in mice and rats (Babigian et al., 2021; Egervari et al., 2017; Guo et al., 2020; Sartor et al., 2015). However, our understanding of bromodomain-specific BET mechanisms in drug-seeking behaviors

remains limited, as JQ1 blocks both bromodomains (BD1 and BD2) within all BET proteins. Additionally, pan-BET inhibitors similar to JQ1 are known to have side effects in humans that may limit their clinical applications for SUD (Amorim et al., 2016; Berthon et al., 2016).

To reduce potential toxic liabilities associated with pan-BET inhibitors, more recent efforts have focused on developing domain-selective BET inhibitors that target specific bromodomains within BET proteins (Petretich et al., 2020). RVX-208 (also called Apabetalone and RVX000222) was one of the first small molecule BET inhibitors to display preferential binding to the BD2 domain within BET proteins (Picaud et al., 2013). In cell lines, RVX-208 only modestly affected BET-dependent gene expression and produced a unique transcriptional outcome compared to JQ1 (Picaud et al., 2013). More recent studies indicate that RVX-208 may offer therapeutic benefits in multiple disease models by blocking the transcriptional responses to inflammatory stimuli (Tsujikawa et al., 2019; Wasiak et al., 2020). In humans, RVX-208 was found to be safe and well tolerated, and it is currently being investigated in Phase II/III clinical trials for the treatment of acute coronary syndromes, atherosclerosis, and Alzheimer's disease (McNeill, 2010; Nikolic et al., 2015). Given that RVX-208 is a clinically tested, potentially safe, BD2-selective BET inhibitor, we sought to investigate its effects on cocaine-induced behavior and transcriptional activity. Here, we found that RVX-208 dose-dependently reduced cocaine conditioned place preference in male and female mice without altering locomotor activity, thigmotaxis, and novel object recognition learning. At the transcriptional level, RVX-208 reduced cocaine-induced gene expression in the nucleus accumbens (NAc) and produced a disparate transcriptional response in primary neurons compared to JQ1. Together, these data indicate that the BET BD2 plays an important role in the behavioral and transcriptional responses to cocaine and that BD2 BET inhibition has limited effects in non-stimulated neurons and other behaviors.

Materials and Methods

Drug treatments:

For *in vitro* studies, JQ1 and RVX-208 (Cayman chemicals) were dissolved in dimethyl sulfoxide (DMSO) and administered at 0.1% v/v. In *in vivo* studies, JQ1 and RVX-208 were dissolved in 10% DMSO and 5% Tween 80 (v/v) and then diluted with sterile saline. RVX-208, JQ1, and vehicle were administered by an intraperitoneal (i.p.) injection at a volume of 0.1–0.15 mL. Cocaine HCl (10 mg/kg, National Institutes of Health's National Institute on Drug Abuse Drug Supply Program) was dissolved in 0.9% sterile saline and administered by an i.p. injection.

Animals:

Male and female C57BL/6 mice (10–12 weeks old; Charles River Laboratories) were housed 4 mice per cage under a reverse 12 h/12 h light/dark cycle and had *ad libitum* access to food and water. For primary neuron experiments, a pregnant female rat (Sprague Dawley; Charles River Laboratories) carrying embryonic day 16 (E16) pups was single housed for two days with ad libitum access to food and water. Animals were housed

in a humidity- and temperature-controlled and AAALAC-accredited animal facility at the University of Connecticut. Experiments were approved by the Institutional Animal Care and Use Committee and conducted according to guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Primary neurons:

For primary cortical neuron experiments, we used similar methods described in BrainBits® primary neuron culture protocol (https://www.brainbitsllc.com/primary-neuron-plating-protocol/). Briefly, brain collection and dissection were carried out under sterile conditions. Pregnant female rats were euthanized in a carbon dioxide chamber. Embryos (E18) were removed, and brains were quickly collected and placed in cold 0.1 M PBS with 0.1% glucose wt./vol. For precise identification of the brain structures, the dissections were performed under a stereo microscope. The meninges and blood vessels were carefully removed, and the cortices were carefully separated from the brain. Frontal cortices were collected, cut into smaller pieces, and digested in papain for 10 min at 37°C (2 mg/ml papain solution in Hibernate-E minus CaCl2, BrainBits LLC). Papain solution was removed, and the tissue was washed three times with Hibernate-E solution followed by 15-minute incubation in Hibernate-E solution and DNAse (1% wt/vol.) at room temperature. Cells were mechanically dissociated with a fire-polished pasteur pipette, resuspended in NbActive4 media (BrainBits LLC), and plated at a density of ~400,000 cells on a poly-L-ornithine (100 μg/ml) and laminin (15 μg/ml) coated 12-well plates. Neurons were maintained at 5% CO_2 and 37° C for 12 days in vitro (DIV), and half the media was replaced with fresh media every three days. The use of anti-mitotic compounds to suppress glia growth is known to have toxic side effects in neurons and were not used (Meberg and Miller, 2003). In some experiments, neurons were treated with DMSO (vehicle), RVX-208 (100 nM and 500 nM) and JQ1 (100 nM and 500 nM) for 1 h, 2 h and 3 h. In other experiments, neurons received a 2 h cotreatment of AMPA (20 μM) or BDNF (30 ng/mL) with DMSO, RVX-208 (500 nM), or JQ1 (500 nM). These time points were selected to capture the early effects of BET inhibition and are based on previous studies using pan-BET inhibitors (Sartor et al., 2015; Sullivan et al., 2015). BDNF and AMPA stimulation were used because previous reports indicate that BET protein activity is regulated by glutamate and tyrosine receptor kinase B (TrKB) receptor signaling (Korb et al., 2015; Sullivan et al., 2015), and both receptor signaling pathways have been implicated in cocaine-induced neuroplasticity (Bowers et al., 2010; Li and Wolf, 2015). After treatment, neurons were collected for RNA extraction as described below.

RT-qPCR:

In *in vivo* studies, mice received an i.p. injection of vehicle or RVX-208 (50 mg/kg) 5 minutes before an i.p. injection of saline or cocaine (10 mg/kg). The nucleus accumbens (NAc) was collected and homogenized in TRIzol 2 hours after the last injection, similar to previous experiments (Sartor et al., 2015). In cell culture experiments, the media was aspirated and 1 ml of TRIzol was added to each well. RNEasy Mini Kit (Qiagen) was used for RNA extraction according to the manufacturer's instructions. RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). The genes selected for measurement were based on validated

targets of pan-BET inhibitors and/or genes involved in cocaine-induced neuroplasticity (Korb et al., 2015; Sartor et al., 2015; Sullivan et al., 2015). Using validated TaqMan primer probes for *Bdnf* (Thermo Scientific, Rn02531967_s1, Mm04230607_s1), *Gria1* (Rn06323759_m1, Mm00433752_m1), *Arc* (Rn00571208_g1, Mm01204954_g1), *Nr4a1* (Rn01533237_m1, Mm01300401_m1), *c-fos* (Rn02396759_m1, Mm00487425_m1), and *Fosb* (Mm00500401_m1), cDNA was run in triplicate and analyzed using the 2− CT method with *β-actin* (Rn00667869_m1, Mm02619580_g1) as a normalization control. Fosb levels were extremely low in non-stimulated primary neurons (RT values > 35 or undetectable), and these data were not reported.

Conditioned place preference (CPP):

The CPP apparatus (Med Associates Inc.) consisted of two compartments with distinct contextual cues on each side: black walls and grid rod-style floors in one compartment and white walls mesh style floors in the other compartment. The compartments were separated by a divider with a small door that could be opened or closed. In a pretest, drug-free acclimation session, mice were allowed to freely explore both compartments for 15 min via an opening in the partition. The time spent on each side of the CPP chamber was automatically recorded by infrared photobeam detectors on the chamber walls. Mice that exhibited a strong bias for one side of the chamber during the pretest (> 65% of time spent on one side) were excluded from further testing. For the next three consecutive days, animals were conditioned as previously described (Sartor et al., 2015; Sartor and Aston-Jones, 2012). During the 30-minute conditioning sessions, one side of the chamber was paired with an i.p. saline injection and the other side was paired with a cocaine injection (10 mg/kg, i.p.). Conditioning occurred in morning and afternoon sessions in a counterbalanced design (at least 4 h apart). Five minutes before each cocaine conditioning session, mice received an i.p. injection of vehicle, JQ1 (50 mg/kg) or RVX-208 (10, 25, or 50 mg/kg). One day after the last conditioning session, mice received a drug-free CPP test where they had free access to each side of the chamber. The preference score was calculated by subtracting the time spent on the cocaine-paired side during the posttest from the time spent on the same side during the pretest.

Open field:

The open field (Ugo Basile) consists of a 46×46 cm chamber with opaque gray walls. Baseline behavior (distance traveled and time spent in the inner and outer zone) was measured in a 30-minute, drug-free habituated test. Treatments were assigned so that baseline measurements did not differ between groups. The next day, mice were injected with vehicle or RVX-208 (50 mg/kg, i.p.) and were placed in the chamber 5 minutes post-injection. Distance traveled and time spent in the inner and outer zone were measured for 30 minutes using EthoVision tracking software (Noldus).

Novel object recognition (NOR):

On days 1–3, mice were allowed to freely explore a clean mouse home cage (without objects) for 5 min per day. On day 4, mice were exposed to two identical objects in the cage for 10 min. After the test, mice were immediately injected with vehicle or RVX-208 (50 mg/kg) and returned to the colony room. Previous studies using this approach showed that

JQ1 reduced NOR (Korb et al., 2015). The next day, mice were tested in a 10 min retention trial with one familiar object and one novel object. The objects consisted of a stack of Lego Duplo bricks and a 100 ml glass beaker. The location of the objects was counterbalanced for left and right positions in each treatment group. The objects were thoroughly cleaned with 70% ethanol after each session. Total time spent exploring and facing each object within 2.5 cm was calculated by Ethovision tracking software. Results were presented as time exploring the objects and discrimination index: [(novel object investigation time minus familiar object investigation time)/(total investigation time of both objects) * 100].

Data analysis:

Statistical analyses were performed with GraphPad Prism 7.0 software. Mean values from behavioral studies and Rq values from qRT-PCR experiments were compared between groups using one-way, two-way or three-way analysis of variance (ANOVA). When comparing multiple groups, a Bonferroni correction test was utilized. Data are expressed as means ±SEM, and the level of significance was set at $P < 0.05$.

Results

The effects of RVX-208 on gene expression in primary neurons

In *in vitro* experiments, primary neurons were treated with vehicle, RVX-208 (100 nM or 500 nM), or JQ1 (100 nM or 500 nM) and gene expression was measured after 1, 2, and 3 h of treatment (Figures 1A–E). Using a two-way ANOVA, significant treatment, time, and interaction effects were observed in *Bdnf* expression (Treatment: $F_{(4, 28)} = 22.95$, $P < 0.0001$; Time: $F_{(2, 56)} = 33.66$, $P < 0.0001$; Interaction: $F_{(8, 56)} = 6.95$, $P < 0.0001$) and *c-fos* expression (Treatment: $F_{(4, 28)} = 26.24$, $P < 0.0001$; Time: $F_{(2, 56)} = 8.57$, $P = 0.0006$; Interaction: $F_{(8, 56)} = 2.75$, $P = 0.012$) (Figure 1A and 1D). Significant treatment and interaction effects were detected in *Arc* expression, but no time effect was found (Treatment: $F_{(4, 28)} = 34.22$, $P < 0.0001$; Time: $F_{(2, 56)} = 0.92$, $P = 0.404$; Interaction: $F_{(8, 56)} = 2.61$, $P = 0.017$) (Figure 1B). A significant treatment effect was observed in *Nr4a1* expression but time and interaction effects were not detected (Treatment: $F_{(4, 28)} = 55.36$, $P < 0.0001$; Time: $F_{(2, 56)} = 0.36$, $P = 0.675$; Interaction: $F_{(8, 56)} = 1.71$, $P = 0.117$) (Figure 1C). No significant treatment or interaction effects on *Gria1* expression were found, but a time effect was observed (Treatment: $F_{(4, 28)} = 0.594$, $P = 0.670$; Time: $F_{(2, 56)} = 8.26$, $P = 0.0007$; Interaction: $F_{(8, 56)} = 1.77$, $P = 0.103$) (Figure 1E). Bonferroni *post hoc* analysis revealed that RVX-208 had minimal effect on gene expression compared to vehicle, with the only significant change occurring in *Bdnf* expression after 2 h of treatment (Figure 1A). JQ1, on the other hand, altered the expression of most genes examined compared to vehicle, including: a decrease in *Bdnf* at 1, 2 and 3 h timepoints (Figures 1A); a decrease in *Arc* at 1, 2 and 3 h timepoints (Figures 1B); a decrease in *Nr4a1* at 1, 2 and 3 h timepoints (Figures 1C); and an increase in *c-fos* at the 2 and 3 h timepoints (Figure 1D).

The effects of RVX-208 on gene expression in stimulated primary neurons

Previous experiments indicate that the BET BD2 domain is responsible for stimulus-induced gene expression in cell lines (Gilan et al., 2020). To examine if RVX-208 alters gene expression in stimulated neurons, we co-treated neurons for 2 h with vehicle, RVX-208 (500

nM), or JQ1 (500 nM) plus BDNF (Figure 2) or AMPA (Figure 3). In BDNF and AMPA stimulated neurons, all genes examined, except *Gria1*, were elevated by more than 5-fold compared to untreated neurons (baseline) (Figures 2 and 3). In BDNF simulated neurons, RVX-208 and JQ1 significantly reduced the expression of *Bdnf* (One-way ANOVA main effect: $F_{(3, 20)}$ = 44.69, P < 0.0001 followed by Bonferroni post-hoc test, P = 0.025 for BDNF vs. RVX-208 + BDNF, P = 0.022 for Vehicle + BDNF vs. RVX-208 + BDNF, P < 0.0001 for BDNF vs. JQ1 + BDNF, P < 0.0001 for Vehicle + BDNF vs. JQ1 + BDNF) (Figure 2A). *Arc* expression was reduced by RVX-208 but not by JQ1 (One-way ANOVA main effect: $F_{(3, 20)}$ = 10.65, P = 0.0002 followed by Bonferroni post-hoc test, P = 0.044 for BDNF vs. JQ1 + BDNF, P = 0.0086 for Vehicle + BDNF vs. RVX-208 + BDNF) (Figure 2B). *Nr4a1* was reduced by RVX-208 but unchanged by JQ1 (One-way ANOVA main effect: $F_{(3, 20)}$ = 8.56, P = 0.0007 followed by Bonferroni post-hoc test, P = 0.0007 for BDNF vs. RVX-208 + BDNF, P = 0.0042 for vehicle + BDNF vs. RVX-208 + BDNF) (Figure 2C), and *c-fos* was significantly elevated by JQ1 but unchanged RVX-208 in BDNF stimulated neurons (One-way ANOVA main effect: $F_{(3, 20)}$ = 111.0, P < 0.0001 followed by Bonferroni post-hoc test, P < 0.0001 for BDNF vs. JQ1 + BDNF, P < 0.0001 for vehicle + BDNF vs. JQ1 + BDNF) (Figure 2D). *Gria1* was not altered by BDNF stimulation or RVX-208 and JQ1 treatments (One-way ANOVA main effect: $F_{(3, 20)}$ = 0.923, P = 0.448) (Figure 2E).

In AMPA stimulated neurons, RVX-208 did not affect the expression of the genes measured (P values > 0.05) (Figure 3). JQ1, on the other hand, decreased *Bdnf* (One-way ANOVA main effect: $F_{(3, 20)}$ = 127.5, P < 0.0001 followed by Bonferroni post-hoc test, P < 0.0001 for AMPA vs. JQ1 + AMPA, P < 0.0001 for vehicle + AMPA vs. JQ1 + AMPA) (Figure 3A), *Arc* (One-way ANOVA main effect: $F_{(3, 20)}$ = 31.37, P < 0.0001 followed by Bonferroni post-hoc test, P < 0.0001 for AMPA vs. JQ1 + AMPA, P < 0.0001 for vehicle + AMPA vs. JQ1 + AMPA) (Figure 3B), and *Nr4a1* in AMPA stimulated neurons (One-way ANOVA main effect: $F_{(3, 20)}$ = 6.15, P = 0.004 followed by Bonferroni post-hoc test, P = 0.019 for AMPA vs. JQ1 + AMPA, P = 0.02 for vehicle + AMPA vs. JQ1 + AMPA) (Figure 3C). Expression of *c-fos* was increased by JQ1 in AMPA stimulated neurons (One-way ANOVA main effect: $F_{(3, 20)}$ = 4.32, P = 0.017 followed by Bonferroni post-hoc test, P = 0.016 for AMPA vs. JQ1 + AMPA) (Figure 3D), and expression of *Gria1* was unchanged by AMPA, RVX-208, and JQ1 (F values < 1.4, P values > 0.05) (Figure 3E).

Effects of RVX-208 on cocaine-induced gene expression in the NAc

To examine the effects of RVX-208 on cocaine-induced gene expression, male and female mice were treated with vehicle or RVX-208 (50 mg/kg) prior to an injection of cocaine (10 mg/kg) or saline (Figure 4). In the NAc, vehicle/cocaine significantly increased the expression of *Arc* in males but not females when compared to vehicle/saline (Two-way ANOVA treatment effect: $F_{(3, 56)}$ = 17.57 P < 0.0001; sex effect: $F_{(1, 56)}$ = 0.3769, P = 0.5417; interaction effect: $F_{(3, 56)}$ = 4.105, P = 0.012) (Figure 4B). *Nr4a1* was increased by cocaine in both sexes (Two-way ANOVA treatment effect: $F_{(3, 56)}$ = 19.45, P < 0.0001; sex effect: $F_{(1, 56)}$ = 4.278, P = 0.043; interaction effect: $F_{(3, 56)}$ = 0.460, P = 0.71) (Figure 4C), while *c-fos* (Two-way ANOVA treatment effect: $F_{(3, 56)}$ = 17.78, P < 0.0001, sex effect: $F_{(1, 56)}$ = 3.155, P = 0.081; interaction effect: $F_{(3, 56)}$ = 1.61, P = 0.198) (Figure

4D) and *Fosb* (Two-way ANOVA treatment effect: $F_{(3, 56)} = 27.1$, $P < 0.0001$; sex effect: $F_{(1, 56)} = 17.3$, $P = 0.0001$; interaction effect: $F_{(3, 56)} = 7.28$, $P = 0.0003$) were increased by cocaine in males but not females ((Figure 4E). Treatments had no effect on *Gria1* expression (Two-way ANOVA F values < 0.8, P values > 0.05) (Figure 4F). Although a statistically significant treatment effect was observed in *Bdnf* expression (Two-way ANOVA treatment effect: $F_{(3, 56)} = 5.622$, $P = 0.002$; sex effect: $F_{(1, 56)} = 0.9583$, $P = 0.33$; interaction effect: $F_{(3, 56)} = 1.519$, $P = 0.22$), no significant changes were observed via Bonferroni *post hoc* comparisons (P values > 0.05) (Figure 4A). When comparing vehicle/cocaine to RVX-208/cocaine, RVX-208 attenuated cocaine-induced gene expression of *Arc* in males ($P < 0.0001$), *Nr4a1* in males and females ($P = 0.04$ for males and $P = 0.0006$ for females), *c-fos* in males ($P < 0.0001$), and *Fosb* in males ($P < 0.0001$) (Figures 4B–E). When comparing sex differences in vehicle/cocaine treated mice, expression of *Fosb* was significantly higher in males compared to females ($P < 0.0001$). There were no significant changes in gene expression when comparing vehicle/saline to RVX-208/saline treated mice (P values > 0.05).

Effects of RVX-208 on cocaine conditioned place preference and other behaviors

To examine the effects of RVX-208 on cocaine-induced behavior, male and female mice were injected with vehicle, RVX-208, or JQ1 prior to each cocaine conditioning session. RVX-208 dose-dependently reduced the acquisition of cocaine conditioned place preference (CPP) compared to vehicle treated male and female mice (Two-way ANOVA treatment effect: $F_{(4, 77)} = 13.28$, $P < 0.0001$; sex effect: $F_{(1, 77)} = 4.031$, $P = 0.048$; interaction effect: $F_{(4, 77)} = 0.7318$, $P = 0.57$) (Figure 5A). In post-hoc analysis, RVX-208 significantly reduced cocaine CPP in females at 10, 25, and 50 mg/kg compared to vehicle, and in males RVX-208 significantly reduced CPP at 25 and 50 mg/kg but not 10 mg/kg. Consistent with previous results in male mice, JQ1 (50 mg/kg) attenuated acquisition of cocaine CPP compared to vehicle in male and female mice (Figure 5A). In other mice, an acute injection of RVX-208 (50 mg/kg) did not alter distance traveled in males and females, but distance traveled was higher in females vs. males, similar to previous reports (Borbélyová et al., 2019) (Two-way ANOVA treatment effect: $F_{(1, 28)} = 0.01$. $P = 0.92$; sex effect: $F_{(1, 28)} = 19.14$, $P = 0.0002$; interaction effect: $F_{(1, 28)} = 0.333$, $P = 0.57$) (Figure 5B). Time spent in the inner and outer zones in an open field was not altered by RVX-208 (Three-way ANOVA treatment effect: $F_{(1, 56)} < 0.001$, $P = 0.99$; sex effect: $F_{(1, 56)} < 0.001$, $P = 0.99$; zone effect: $F_{(1, 56)} = 666$, $P < 0.0001$) (Figure 5C). In the novel object recognition (NOR) task, time exploring the objects during acquisition was not statistically different between treatments or sexes (Two-way ANOVA F values < 0.5, P values > 0.05) (Figure 5D). During the NOR test, vehicle and RVX-208 treated mice showed an increase in time exploring the novel object (NO) compared to the familiar object (FO) (Three-way ANOVA effect on object exploration: $F_{(1, 72)} = 31.13$, $P < 0.0001$; sex effect: $F_{(1, 72)} = 5.46$, $P=0.022$; treatment effect: $F_{(1, 72)} < 0.001$, $P = 0.99$) (Figure 5E). Finally, no significant treatment effect on novel object discrimination index (DI) was observed (Two-way ANOVA F values < 0.9, P values > 0.05) (Figure 5F).

Discussion

A role for BD2-selective BET inhibition in cocaine-induced behavioral and transcriptional responses

In previous studies, we showed that BET protein activity and expression are altered by cocaine use, and BET bromodomain inhibition attenuated behavioral and transcriptional responses to cocaine (Sartor et al., 2015). Consistent with these initial findings, more recent studies have confirmed that BET inhibitors reduce other types of drug-seeking behaviors (Egervari et al., 2017; Sartor et al., 2019; Guo et al., 2020; Babigian et al., 2021). To date, however, all addiction-related BET experiments have been conducted in males using the pan-BET inhibitor JQ1, a tool compound that is not suitable for clinical testing due to its poor pharmacokinetic properties (Tanaka et al., 2016). In addition to having side effects that may restrict their clinical potential as a SUD treatment, pan-BET inhibitors similar to JQ1 block both bromodomains within all BET proteins, restricting our understanding of domain-specific BET mechanisms in drug-seeking behaviors. In the pursuit of more selective and safer therapeutic approaches for SUD, here, we report that RVX-208, a clinically tested, BD2-selective BET inhibitor, attenuated cocaine-conditioned responses in male and female mice without altering distance traveled and anxiety-like behavior in an open field. In previous studies, JQ1 reduced novel object recognition (Korb et al., 2015), an indication that pan-BET inhibitors may have undesirable effects on learning and memory. Distinct from these previous results, we found that RVX-208 did not alter novel object recognition, supporting the concept that BD2-selective BET inhibitors have fewer side effects compared to pan-BET inhibitors.

At the transcriptional level, RVX-208 attenuated cocaine-induced expression of *Arc*, *Nr4a1*, *c-fos*, and *Fosb* in the NAc, genes that play key roles in cocaine-induced neuroplasticity (Bannon et al., 2002; Carpenter et al., 2020; Cates et al., 2019; Hope, 1998; Penrod et al., 2020; Salery et al., 2017; Walker et al., 2018; Xu, 2008). The significant effects were mainly observed in males, as cocaine-induced gene expression in the NAc was generally lower in females compared to males. However, similar trends in treatment-induced gene expression were observed between sexes. Importantly, RVX-208 treatment without cocaine had no significant effect on baseline gene expression in the NAc compared to vehicle-treated mice. These results are in contrast to previous studies that showed pan-BET inhibitors (JQ1 and I-BET858) altered baseline expression of multiple neuroplasticity-associated genes in the NAc and primary neurons (Korb et al., 2015; Sartor et al., 2015; Sullivan et al., 2015). Our current results are in line with previous reports demonstrating that BET BD2 is involved in stimulus-induced gene expression, whereas the BD1 domain (a target of pan-BET inhibitors) maintains steady-state gene expression (Gilan et al., 2020).

Similar to our *in vivo* findings, RVX-208 had little effect on gene expression in non-stimulated primary neurons, but RVX-208 did reduce *Bdnf*, *Arc* and *Nr4a1* expression in BDNF, but not AMPA, stimulated neurons. JQ1, on the other hand, produced a sustained and robust change in many of the genes measured in non-stimulated and stimulated neurons. The limited effects of RVX-208 on AMPA-induced gene expression indicates that BD2-mediated transcriptional responses may differ depending on the stimulus used. Consistent with this

idea, the effects of BD2-selective inhibition on gene expression also varied depending on stimulus applied in immunoinflammatory disease models (Gilan et al., 2020). Importantly, as we only examined a few target genes in the current study, it is possible that RVX-208 alters the expression of AMPA-induced genes that were not measured.

When comparing RVX-208 to JQ1, a difference in *c-fos* and *Fosb* expression was also observed. In the current studies, JQ1 increased *c-fos* expression in stimulated and non-stimulated neurons, and in previous studies, JQ1 and I-BET858 elevated *c-fos* and *Fosb* in the NAc and primary neurons (Sartor et al., 2015; Sullivan et al., 2015). In the current studies, RVX-208 reduced *c-fos* and *Fosb* in the NAc of cocaine-treated male mice and had no effect on *c-fos* in stimulated and non-stimulated primary neurons. These data indicate that inhibition of the BD1 domain via pan-BET inhibition may be responsible for the observed elevation of *c-fos* and *Fosb*. A more recent study has confirmed that that inhibition of BD1, but not the BD2, BET domain is responsible for the induction of *c-fos* in neurons (Jones-Tabah et al., 2022). Together, these data reveal that BET BD2 regulates gene expression in stimulated neurons and brain tissue, but unlike pan-BET inhibition, BET BD2 inhibition has minimal impact on baseline expression.

The limited effects on baseline gene expression in non-stimulated cells is consistent with the improved safety profile of BD2-selective inhibitors compared to pan-BET inhibitors. For example, in multiple clinical trials, RVX-208 was shown to be well tolerated in patients without causing thrombocytopenia and gastrointestinal toxicity (Nicholls et al., 2011; Tsujikawa et al., 2019), which are dose-limiting side effects reported in humans treated with pan-BET inhibitors (Amorim et al., 2016; Berthon et al., 2016). In preclinical and clinical studies, RVX-208's primary indication has been for the treatment of cardiovascular and metabolic diseases (e.g., atherosclerosis, hypertension, hyperlipidemia) by elevating the expression of apolipoprotein AI (ApoA-I) and high-density lipoprotein (HDL) and reducing the expression of inflammation factors (Gilham et al., 2016; Tsujikawa et al., 2019). Interestingly, chronic cocaine use is also known to cause deleterious effects on the cardiovascular system, and cocaine users have an increased risk of developing coronary atherosclerosis (Bachi et al., 2017). Thus, beyond reducing drug-seeking behaviors, RVX-208 may be beneficial to the cardiovascular health of patients suffering from cocaine use disorder.

Regulation of stimulus-dependent transcription by the BET BD2 domain

Since the development of RVX-208, more selective BD2 BET inhibitors have been reported (Faivre et al., 2020; Gilan et al., 2020). For example, potent and highly selective BD1 (GSK778) and BD2 BET (GSK046) inhibitors were recently identified (Gilan et al., 2020). GSK046 displayed >300-fold selectivity for BD2 over BD1, whereas GSK778 showed 130-fold selectivity for BD1 over BD2. The BD1-selective inhibitor GSK778 exhibited similar transcriptional effects compared to pan-BET inhibitors in cancer cells, consistent with previous studies showing that BD1 plays the dominant role in maintaining established transcriptional programs (Picaud et al., 2013). However, distinct from BD1-selective and pan-BET inhibitors, the BD2-selective inhibitor GSK046 primarily affected stimulus-induced gene expression and caused limited disruptions in cell proliferation, cell viability,

and BRD4 chromatin binding. In SUD-related research, future studies examining potential differences between modestly selective (RVX-208) versus highly selective (GSK046) BD2 BET inhibitors in cocaine-induced transcriptional and behavioral responses would be of high interest and could potentially guide BET drug development efforts for cocaine use disorder.

Biochemical analysis of the BET protein, BRD4, also indicates that the BD2 domain is involved in stimulus-dependent transcriptional regulation (Chiang, 2016). For example, when the flanking regions of BD2 are dephosphorylated by protein phosphatase 2 (PP2A), the BD2 domain on BRD4 is sterically impeded, preventing it from interacting with acetylated histones and non-histone proteins (Wu et al., 2013). When these regions are phosphorylated by casein kinase 2 (CK2), protein kinase A (PKA), and potentially other kinases, BRD4 undergoes a conformational change that uncovers BD2 and promotes its interactions with other proteins. Though the upstream receptor signaling mechanisms responsible for BRD4 phosphorylation have yet to be fully explored, cocaine and BDNF stimulation were found to increase BRD4 phosphorylation levels in the NAc and primary neurons, respectively (Guo et al., 2020; Korb et al., 2015). The data reported here also suggest that upstream signaling mechanisms following BDNF and cocaine, but not AMPA, stimulation regulate transcription of multiple BET-targeted genes in a BD2-dependent manner.

Outlook of BD2-selective BET inhibitors for the treatment of cocaine use disorder.

Identifying safe and effective treatments for cocaine addiction is an urgent need as there are currently no FDA-approved medications for cocaine use disorder. Developing a brand-new drug treatment, however, takes an enormous amount of time, effort, and money due to years of preclinical and clinical testing for safety and efficacy (Morgan et al., 2011). RVX-208 has been shown to be extremely well tolerated by patients, with safety data now surpassing 2,700 patient-years (Tsujikawa et al., 2019). To date, the only dose-limiting side effect observed with RVX-208 is a reversible and transient elevation in liver enzymes (alanine aminotransferases/aspartate aminotransferases) in a small percentage of patients (Nicholls et al., 2011). With a well-established safety profile in humans, RVX-208 has the potential to make a rapid impact as an epigenetic pharmacotherapy for cocaine use disorder. However, before it can be considered a promising treatment option, RVX-208 must be tested in more sophisticated and translatable models of cocaine use disorder (e.g., short-, long-, and intermittent-access cocaine self-administration and reinstatement/behavioral economic procedures). Additional behavioral tests in female rodents are also needed, as this is the first SUD-related study to report BET inhibitor effects in female rodents. Finally, while we have identified a few target genes that potentially mediate RVX-208's effects on cocaine-seeking behavior, future transcriptomic and cell type-specific studies are necessary to fully elucidate the mechanisms by which RVX-208 regulates cocaine-induced neurobehavioral adaptations. Ongoing efforts to address these questions will determine the therapeutic potential of domain-selective BET inhibitors for the treatment of substance use disorder.

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Highlights:

- RVX-208 is a clinically tested BD2-selective BET inhibitor with limited side effects.

- RVX-208 reduced cocaine conditioned responding without affecting other behaviors.

- RVX-208 attenuated cocaine-induced gene expression in the nucleus accumbens.

- In primary neurons, RVX-208 produced a unique transcriptional profile compared to JQ1.
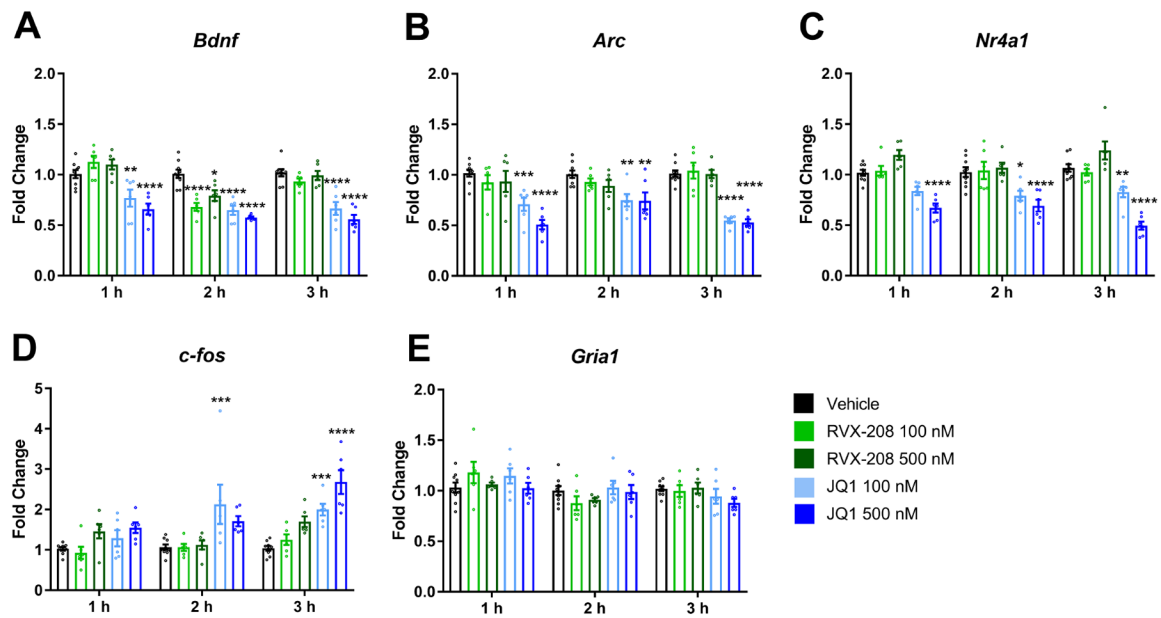
**Figure 1: RVX-208 has limited effects in non-stimulated primary neurons.**
Primary neurons were treated with vehicle (n = 9), RVX-208 (n = 6), or JQ1 (n = 6) (100 nM and 500 nM) for 1, 2, and 3 hours. Following treatment, (A) *Bdnf*, (B) *Arc*, (C) *Nr4a1*, (D) *c-fos*, and (E) *Gria1* expression levels were measured via RT-qPCR. Using Bonferroni post hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ indicate a significant difference from vehicle at the same timepoint. Data are mean ±SEM.
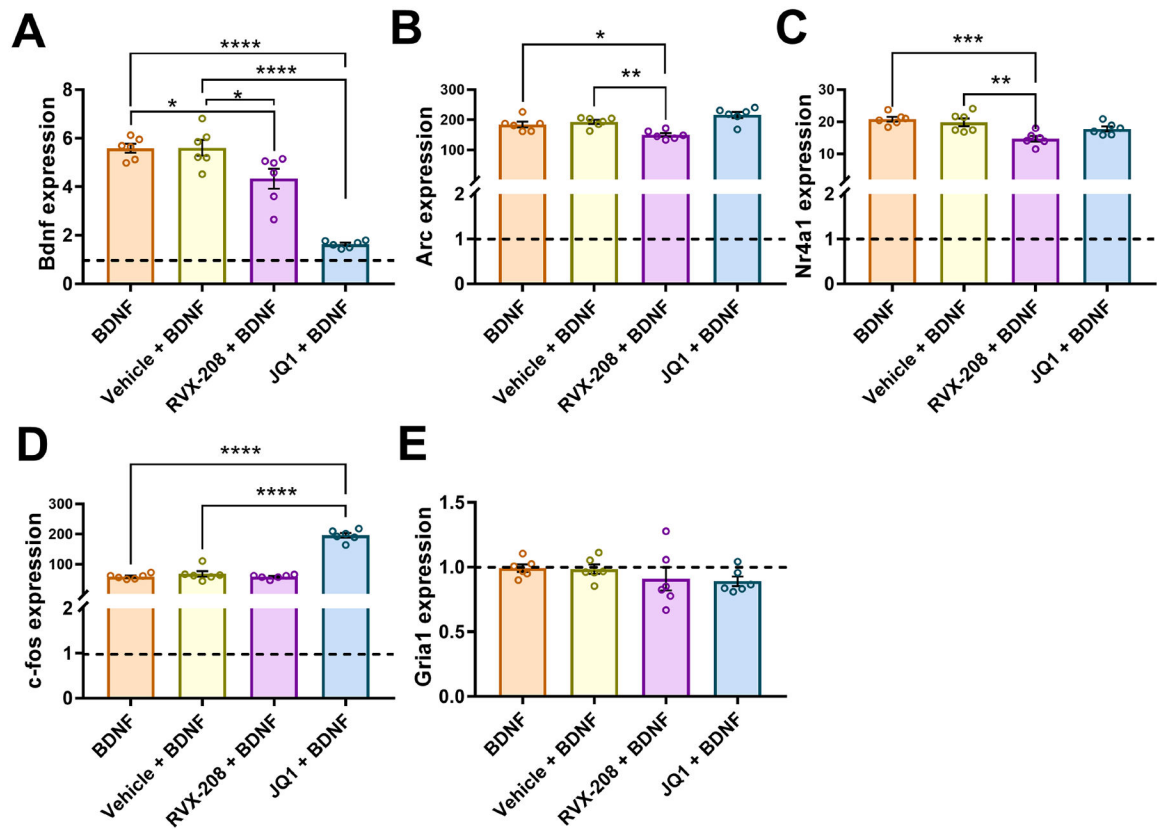
Figure 2: RVX-208 alters the expression of multiple genes in BDNF stimulated primary neurons.
Primary neurons were treated with BDNF, vehicle + BDNF, RVX-208 + BDNF or JQ1 + BDNF for 2 hours. The expression of (A) *Bdnf*, (B) *Arc*, (C) *Nr4a1*, (D) *c-fos*, and (E) *Gria1* was measured via RT-qPCR. Horizontal dotted line indicates baseline expression of untreated neurons. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ indicate a significant difference compared to BDNF or Vehicle + BDNF using Bonferroni *post hoc* test. Data are mean ±SEM, n = 6 per treatment.
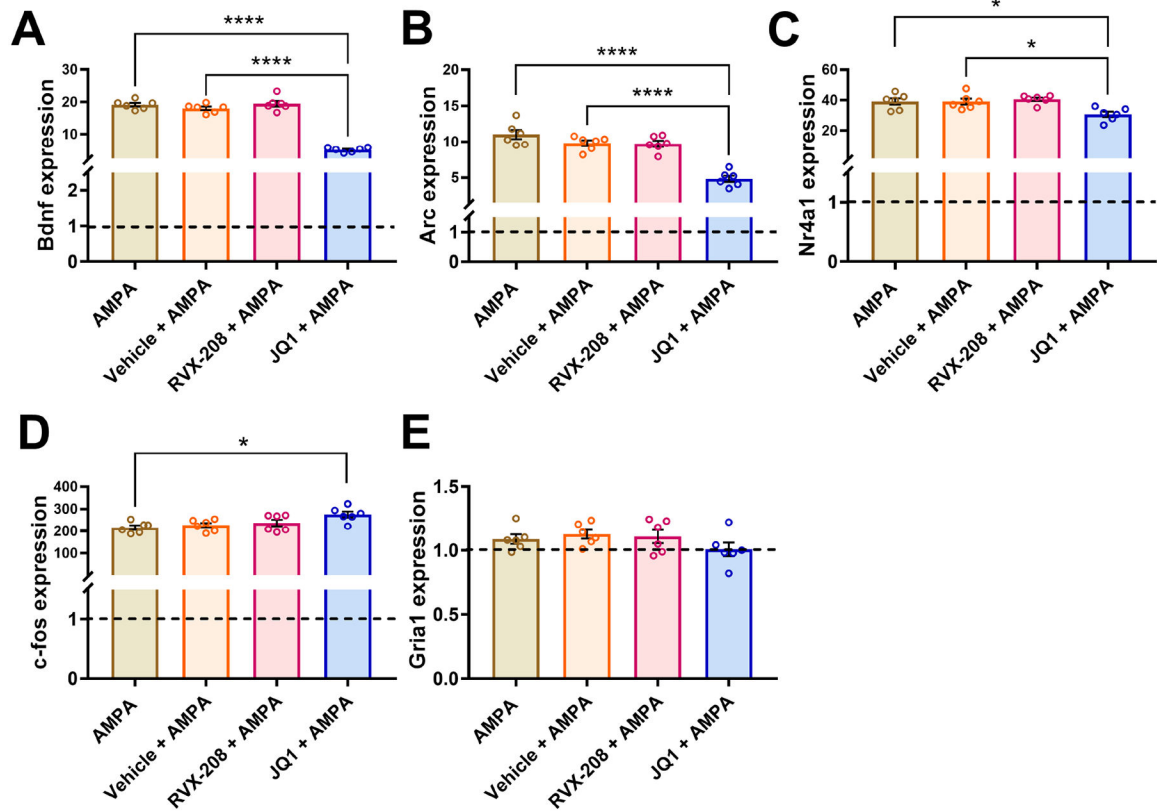
Figure 3: RVX-208 has no effect on gene expression in AMPA stimulated primary neurons. Primary neurons were treated with AMPA, vehicle + AMPA, RVX-208 + AMPA, or JQ1 + AMPA for 2 hours. The expression of (A) *Bdnf*, (B) *Arc*, (C) *Nr4a1*, (D) *c-fos*, and (E) *Gria1* was measured via RT-qPCR. Horizontal dotted line indicates baseline expression of untreated neurons. $*P < 0.05$ and $****P < 0.0001$ indicate a significant difference compared to AMPA or Vehicle + AMPA using Bonferroni *post hoc* test. Data are mean ±SEM, n = 6 per treatment.
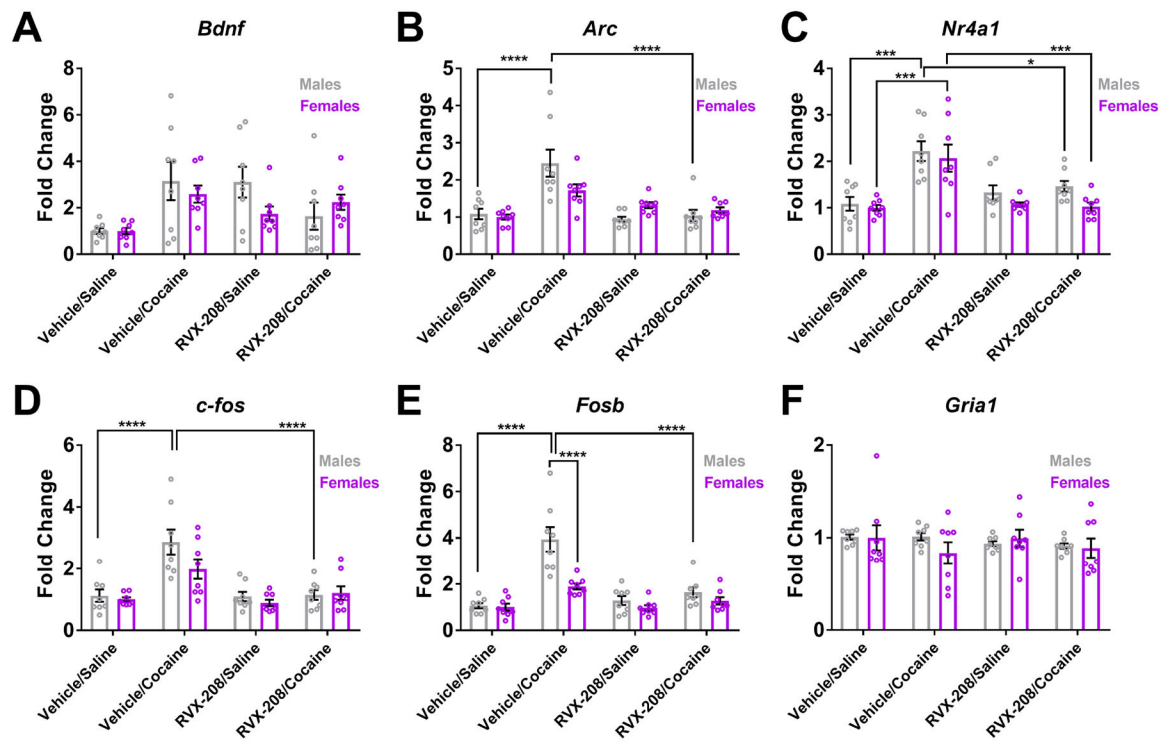
Figure 4: RVX-208 alters cocaine-induced gene expression in the NAc.
Male and female mice were treated with vehicle/saline, vehicle/cocaine, RVX-208/saline, or RVX-208/cocaine. Following treatment, (A) *Bdnf*, (B) *Arc* (C), *Nr4a1* (D), *c-fos*, (E) *Fosb*, and (F) *Gria1* expression levels were measured via RT-qPCR. $*P < 0.05$, $***P < 0.001$, and $****P < 0.0001$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean $\pm$SEM, n = 8 per treatment.
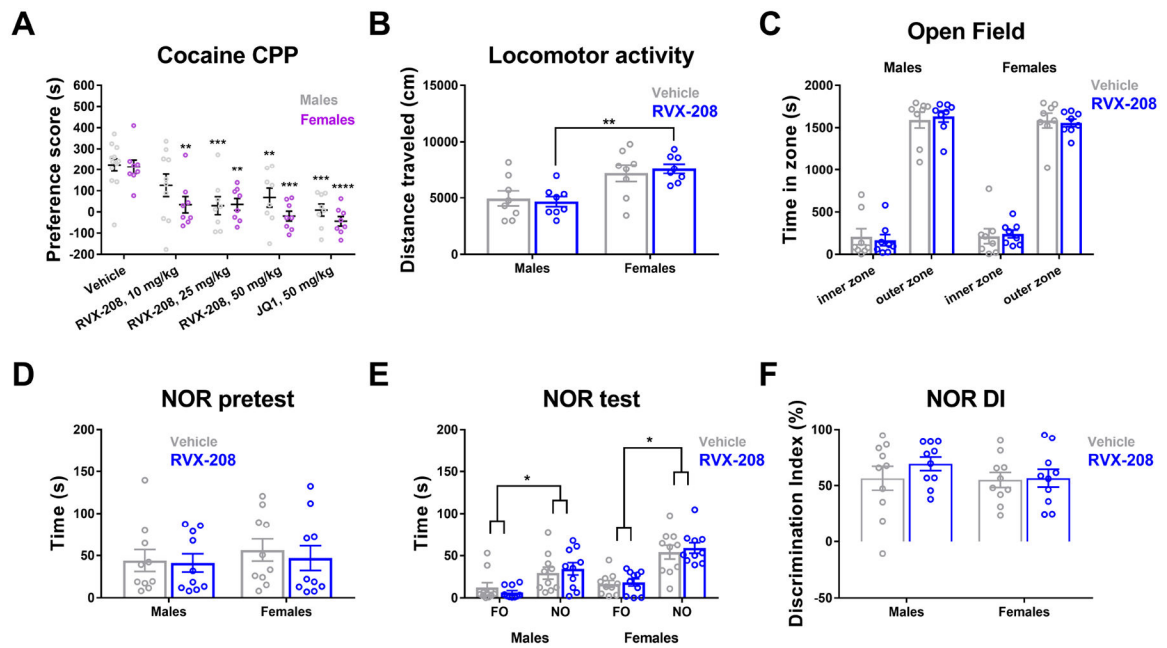
Figure 5. Effects of RVX-208 on behavior.

(A) Dose-dependent reduction of cocaine conditioned place preference (CPP) by RVX-208 compared to vehicle in male and female mice (n = 8–13). (B) The effects of RVX-208 (50 mg/kg) on locomotor activity and (C) time spent in the inner and outer zones in open field in male and female mice (n = 8). (D) Time spent exploring objects during novel object recognition (NOR) pretest in male and female mice (n = 10). (E) Time spent exploring the novel object (NO) and familiar object (FO) during the NOR test in vehicle and RVX-208 (50 mg/kg) treated male and female mice (n = 10). (F) NOR discrimination index (DI) values for vehicle and RVX-208 treated male and female mice (n = 10). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ±SEM.