Cyanidin prevents anxiety-like effects and dysregulation of cytokine systems during MDPV abstinence in rats

Saadet Inan (✉ sinan@temple.edu)
Lewis Katz School of Medicine at Temple University

Joseph J Meissler
Lewis Katz School of Medicine at Temple University

Aryan Shekarabi
Lewis Katz School of Medicine at Temple University

Jeffrey Foss
Lewis Katz School of Medicine at Temple University

Sonita Wiah
Lewis Katz School of Medicine at Temple University

Toby K Eisenstein
Lewis Katz School of Medicine at Temple University

Scott M. Rawls
Lewis Katz School of Medicine at Temple University

Short Report

Keywords: IL-17A, cytokine, glutamate, cyanidin, MDPV, psychostimulant, anxiety, GLAST

Posted Date: December 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2326678/v1

License: ☇ ️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Psychostimulant exposure and withdrawal cause neuroimmune dysregulation and anxiety that contributes to dependence and relapse. Here, we tested the hypothesis that abstinence from the synthetic cathinone MDPV (methylenedioxyprovalerone) produces anxiety-like effects and enhanced levels of mesocorticolimbic cytokines that are inhibited by cyanidin, an anti-inflammatory flavonoid and nonselective blocker of IL-17A signaling. For comparison, we tested effects on glutamate transporter systems that are also dysregulated during psychostimulant abstinence. Rats injected for 9 d with MDPV (1 mg/kg, IP) or saline were pretreated daily with cyanidin (0.5 mg/kg, IP) or saline, followed by behavioral testing on the elevated zero maze (EZM) 72 h after the last MDPV injection. MDPV abstinence caused a reduction in time spent on the open arm of the EZM that was prevented by cyanidin. Cyanidin itself did not affect locomotor activity or time spent on the open arm, or cause aversive or rewarding effects in place preference experiments. MDPV abstinence caused enhancement of cytokine levels (IL-17A, IL-1β, IL-6, TNFα, IL-10, and CCL2) in the ventral tegmental area, but not amygdala, nucleus accumbens, or prefrontal cortex, that was prevented by cyanidin. During MDPV abstinence, mRNA levels of glutamate aspartate transporter (GLAST) and glutamate transporter subtype 1 (GLT-1) in the amygdala were also elevated but normalized by cyanidin treatment. These results show that MDPV abstinence causes withdrawal-induced anxiety, and brain-region specific dysregulation of cytokine and glutamate systems, that are both prevented by cyanidin, thus identifying cyanidin for further investigation in the context of psychostimulant dependence and relapse.

1. Introduction

Among the most studied components of the neuroimmune system are cytokines, which are secreted small proteins that enable communication between cells of the immune system and contribute to neuroinflammation, neuronal activity, neuron-glia communication, neuroendocrine interactions, neurogenesis, and CNS development (Bachtell et al., 2017, 2015; Boulanger, 2009). Growing evidence suggests that crosstalk between neuroimmune and brain reward systems contributes to psychostimulant dependence and relapse. During chronic exposure to alcohol, morphine, cocaine, and methamphetamine, some proinflammatory chemokines and cytokines are elevated in brain reward areas of rodents (Trecki and Unterwald, 2009; Campbell et al., 2013; Saika et al., 2018a; Sanchez-Alavez et al., 2019; Nayak et al., 2020). Blockade of chemokine and cytokine signaling with receptor antagonists also reduces rewarding and reinforcing effects of psychostimulants and opioids in preclinical models (Kim et al., 2017; Saika et al., 2018a, b; Nayak et al., 2020; Simmons et al., 2022; Bongiovanni et al., 2022; Potula et al., 2022).

In people with psychostimulant use disorders, psychostimulant abstinence exacerbates anxiety and depression, which facilitates craving and relapse (Blanchard and Blanchard, 1999; Vorspan et al., 2015). For cocaine, anxiogenic-like behavior in rats has been consistently detected 48–72 h following cessation of chronic cocaine administration (Harris and Aston-Jones, 1993, Sarnyai et al., 1995, Basso et al., 1999, Paine et al., 2002; Philogene-Khalid et al., 2017). Similar results have been demonstrated for the ‘bath salt’ synthetic cathinone MDPV (3,4-methylenedioxyprovalerone) in rats, with anxiogenic effects
detected in the elevated plus maze (EPM) 72 h after a 10-d binge exposure (3x/d) or 72 h after a 4-d escalating binge dosing paradigm (Philogene-Khalid et al., 2017; Simmons et al., 2022). In mice, MDPV also produces anxiogenic-related behavior tested in the EPM 21 d following cessation of drug exposure (2x/d for 7 d) (Duart-Castells et al., 2019). MDPV and cocaine are both monoamine transport blockers, but MDPV displays greater selectivity and potency at dopamine transporters (DAT) and norepinephrine transporters (NET) but exerts only weak to negligible inhibition of 5-HT transporters (SERT) (Baumann et al., 2013, Simmler et al., 2013).

One cytokine that has gained recent attention for contributing to the pathogenesis of anxiety and depression is interleukin-17A (IL-17A), a member of the IL-17 family (Alves de Lima et al., 2020; Kim et al., 2021; Li et al., 2019a; Liu et al., 2012; Vieira et al., 2010; Pallavi et al., 2015). Mice exposed to stress display upregulation of IL-17, TNF-α, IL-6, and IL-1β and activation of microglia in the hippocampus, amygdala, and prefrontal cortex (PFC), and anti-IL-17 treatment rescues anxiety and depression-like behavior in the stress-exposed mice (Kim et al., 2021). IL-17 knock out (KO) mice also spend more time in the open arm of the EPM than wild type mice, suggesting that removal of IL-17 signaling results in less anxiety and vigilance (Alves De Lima et al., 2020). Furthermore, production of Th17-derived cytokines (IL-17, TNF-α) are significantly higher in cultures of peripheral blood cells taken from patients with anxiety compared to control subjects (Vieira et al., 2010).

Cyanidin is a flavonoid and a key pigment found in red berries and other related fruits. Cyanidin displays a wide range of biological functions, including anti-inflammatory, antioxidant, antiviral, and anticarcinogenic effects (Thummayot et al., 2018). In recent studies, Liu et al. (2017) used computational modeling, in vitro binding measurements, cell-based assays, and in vivo studies to show that cyanidin prevents binding of IL-17A to its reciprocal receptor IL-17 RA. Specifically, cyanidin interacts with IL-17 RA in a deep pocket at which IL-17A binds and inhibits binding of IL-17A to IL-17 RA, but not IL-17E or IL-17C to IL-17 RE, in a concentration dependent manner (Liu et al., 2017). Additionally, in HEK 293 cells expressing IL-17 RA, cyanidin concentration-dependently blocks IL-17A binding (Liu et al., 2017). Cyanidin also inhibits IL-17A-induced production of other proinflammatory cytokines and chemokines, including IL-6, IL-8, CXCL1, and CXCL2 in human cells (Liu et al., 2017). Cyanidin also inhibits IL-17A-induced skin hyperplasia in mice, indicating in vivo activity involving cytokine signaling (Liu et al., 2017).

In the present study, we determined if abstinence from chronic MDPV exposure caused anxiety-like effects on the elevated zero maze (EZM) and dysregulation of cytokine levels in brain regions related to anxiety and dependence (i.e., amygdala, ventral tegmental area (VTA), nucleus accumbens (NAC), and PFC) that are prevented by cyanidin. The EZM, like the EPM, exploits the innate aversion of rodents to exposed, well-lit spaces, presumably to avoid predation. Thus, anxiety- or exploration-related behaviors are often correlated with time spent in the open compartment of the zero-maze, with anxiogenic effects linked to decreased time spent in the open compartment and anxiolytic effects linked to increased open-compartment time. For comparison with cytokine systems, we tested effects of MDPV and cyanidin exposure on glutamate transporter systems (i.e., glutamate transporter subtype 1 (GLT-1) and glutamate aspartate transporter (GLAST)) that also dysregulated during MDPV exposure and abstinence (Hicks et
Moreover, dysregulation of glutamate transporter systems during chronic psychostimulant exposure contributes to changes in glutamate signaling that facilitates drug seeking behavior in rats (Kalivas et al., 2009, Kalivas, 2009; McFarland et al., 2003; Gipson et al., 2021; Šerý et al., 2015; Spencer and Kalivas, 2017).

2. Materials And Methods

2.1. Animals

Adult male Sprague-Dawley rats (250–300 g; Harlan Laboratories, Indianapolis, IN) were used. Animals were housed two per cage in a humidity-controlled vivarium, maintained on a 12-h light/dark cycle with lights turning on daily at 7:00AM. Rats were provided with ad libitum food and water access except during experimental testing. All procedures and animal care were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Temple University's Institutional Animal Care and Use Committee guidelines. Rats were allowed to acclimate for a week before the start of any procedures.

2.2. Compounds

(±)-MDPV was synthesized in accordance with published methods by Fox Chase Chemical Diversity Center, Doylestown, PA. Briefly, 1-Benzo[1,3]dioxol-5-yl-pentan-1-one was prepared via Friedel Crafts acylation of benzo[1,3]dioxole with pentanoyl chloride and tin (IV) chloride. Subsequent bromination of 1-benzo[1,3]dioxol-5-yl-pentan-1-one followed by displacement with pyrrolidine and salt formation provided MDPV HCl. Cyanidin chloride (A18; CAS 528-58-5) was obtained from Santa Cruz Biotechnology (Dallas, TX). Both MDPV and cyanidin were dissolved in saline. MDPV, cyanidin, and saline were administered by intraperitoneal (IP) injection in a volume of 1 ml/kg.

2.3. Effects of cyanidin on MDPV withdrawal-induced anxiety

Four groups of rats were used (N = 8/group): Saline/Saline; Saline/MDPV; Cyanidin/Saline; Cyanidin/MDPV. Rats were treated with either saline or cyanidin (0.5 mg/kg, IP) and then 30 min later they were injected with either saline or MDPV (1 mg/kg, IP) once a day for 9 days. Seventy-two hours after the last injection, withdrawal-induced anxiety was measured using the elevated zero maze (EZM). The 72-h withdrawal time point was chosen for behavioral testing because it coincides with a robust anxiogenic response (Philogene-Khalid et al., 2017). The EZM (San Diego Instruments, San Diego, CA) is a beige circular runway 4 inches wide with a 48-inch diameter outer wall and 40-inch diameter inner wall (Braun et al., 2011). The platform is elevated 30 inches above the ground and divided into equal quadrants. Two opposing quadrants are “closed” with 8-inch-high walls, separated by “open” quadrants with a half-inch-high ledge. The lighting conditions were adjusted to produce light levels of ~75 lux in the open sections and ~30 lux in the closed sections of the maze. Rats were allowed to acclimate in the testing room to the dim (~75 lux) lighting for 1 h prior to testing. Testing took place between 10:00 AM and 3:00 PM. Each rat was placed in the same orientation in the same closed section of the maze and
behavior was video recorded for 10 min. The maze was cleaned with 70% ethanol and dried between rats. Anxiety-like behavior was measured by the amount of time each rat spent in the open quadrants of the maze. An open quadrant response was scored if the head and the 2 front paws were out of the closed quadrant. Measurements were performed by an experimenter blinded to the treatments. At the end of the experiment, all rats were decapitated after brief exposure to carbon dioxide gas. Brains were quickly removed and flash-frozen in -30°C isopentane and kept at -80°C until used. Half of the brain was used for protein measurement of iL-17 and other cytokines and chemokines, and the other half of the brain was used for measurement of mRNA levels for glutamate transporters.

2.4. Effects of cyanidin on locomotor activity and conditioned place preference (CPP)

We also determined if cyanidin at 0.5 mg/kg would influence locomotion and/or induce CPP by itself. Locomotor activity was measured as previously described using a Digiscan DMicro system (Accuscan Inc., Columbus, OH) consisting of transparent plastic chambers (45 cm × 20 cm × 20 cm) set inside metal frames equipped with 16 infrared light emitters and detectors (Nayak et al., 2020). Locomotor activity was measured acutely, following one injection, as well as chronically after 9 days of once a day injections of cyanidin or saline. For acute studies, on the day of the experiment, rats were placed individually in the activity chamber and were acclimated for 60 min. Animals were then injected with either saline or cyanidin (0.5 mg/kg, IP). Locomotion was measured for 90 min. Following the acute measurements, rats were administered the drug daily for 9 days when, locomotor activity was again measured.

CPP experiments were conducted as previously described using CPP chambers (45 cm × 20 cm × 20 cm) consisting of 2 compartments separated by a removable door (Gregg et al., 2015; Hicks et al., 2018; Nayak et al., 2020). A 30-min pretest was conducted on day 1 to determine initial compartment preference. The compartment in which a rat spent less time was designated as the cyanidin-paired side. The day after the pre-test, a 4-day conditioning paradigm with morning and afternoon sessions was initiated. In the morning, rats were treated with either saline or cyanidin (0.5 mg/kg, IP) and confined to the cyanidin-paired compartment for 30 min. In the afternoon session, rats were injected with saline and placed in the opposite compartment for 30 min. On day 6, a post-test was conducted in which rats were placed into the chamber and given free access to roam both compartments for 30 min.

2.5. Measurement of chemokine and cytokine levels in brain regions

Brain region samples (nucleus accumbens (NAC), prefrontal cortex (PFC), ventral tegmental area (VTA), and amygdala were stored at -80° C in 1.5 ml microcentrifuge tubes, and were extracted for analysis by mechanical homogenization in 150 µl of 4° C PBS with Kontes® microcentrifuge pestles, followed by mixing with an equal volume of Cell Lysis Buffer 2 (R&D Systems, Minneapolis, MN) and incubation for 30 min at room temperature with gentle agitation. Samples were then centrifuged at 12,000 x g at 4° C for 10 minutes, and supernatants were transferred to new microcentrifuge tubes. Immediately after centrifugation, total protein content (mg total protein/ml) was determined using the Pierce BCA Protein
assay (ThermoFisher, Waltham, MA). Samples were stored at -80° C until assayed further. Cytokine/chemokine levels for rat IL-1β, IL-6, IL-10, IL-17A, CCL2/MCP-1, CCL5/RANTES, and TNF-α were determined by Milliplex® Luminex® multiplex assays (Millipore/Sigma, Burlington, MA). Luminex® assays were conducted using a BioRad BioPlex100 instrument. Levels of rat CXCL12/SDF-1α were determined by ELISA (Novus Biologicals, Centennial, CO). The ELISA assays were read using a FluoSTAR Omega spectrophotometer (BMC Labtech, Cary NC). Pierce BCA protein assays, Milliplex® assays, and ELISA assays were conducted following the protocols included with each kit.

2.6. Measurement of mRNA for glutamate transporters in brain regions

Gene expression of two glutamate transporters, EAAT1/GLAST and EAAT2/GLT1 were measured in the PFC, NAC, VTA, and amygdala. Experiments were conducted as described previously (Kim et al., 2017). The PFC, NAC, VTA, and amygdala were dissected from frozen slices using 1- and 2-mm round punches respectively. RNA was isolated using the Quick-RNA Miniprep kit (Zymo Research, Irvine, CA, USA), and cDNA was synthesized using RT^2 First Strand Kit (Qiagen, Germantown, MD, USA). Quantitative real-time PCR was conducted with TaqMan Fast Advanced Master Mix and the TaqMan Gene Expression Assays for glutamate transporters, EAAT1/GLAST (Rn01402419_g1), EAAT2/GLT1 (Rn00691548_m1) and the internal control gene Hbb, and the 45s rRNA (Rn03928990_g1) using the QuantStudio 3 Real-Time PCR System (Applied Biosystems). Relative gene expression was measured according to the 2^−ΔΔCT method (Nayak et al., 2020).

2.7. Statistical analysis

Two-way ANOVA followed by a Tukey’s multiple comparison test was used to analyze EZM, locomotor, chemokine and cytokine levels, and mRNA data. Student’s t-test was used to analyze CPP data. GraphPad Prism 7.02 version software was used to construct graphs and conduct statistical analyses. Data were presented as ± S.E.M. in figures, with p < 0.05 considered statistically significant in all cases.

3. Results

3.1. Cyanidin prevented MDPV withdrawal-induced anxiety

Withdrawal from MDPV induced anxiety in rats as shown by decreased time in the open arms of the EZM (Fig. 1). Chronic treatment with cyanidin prevented MDPV withdrawal-induced anxiety. Rats that received chronic cyanidin together with MDPV spent a similar amount of time in the open arms of the EZM as rats in the control groups (Fig. 1). Two-way ANOVA revealed significance for treatment ([F(1,20) = 6.516, p = 0.019], and interaction ([F(1,20) = 8.586, p = 0.008]. F values for pretreatment with cyanidin was [F(1,20) = 4.182, p = 0.054]. Tukey’s multiple analysis showed that rats treated with saline-MDPV spent significantly less time in the open arm of the maze compared to saline-saline, cyanidin-saline, and cyanidin-MDPV (p < 0.01)

3.2. Cyanidin does not affect spontaneous locomotor activity or induce CPP
Cyanidin at 0.5 mg/kg did not have any effect on locomotion. Horizontal, stereotypic and ambulatory activity were shown following acute (Fig. 2A) and chronic (Fig. 2B) administration of cyanidin. Two-way ANOVA revealed no significance for treatment and time between saline and cyanidin groups following acute or chronic injections for horizontal, stereotypic, or ambulatory activities.

Cyanidin did not induce CPP (data not shown). The time spent in the unpreferred side was similar in rats treated with either saline or cyanidin. The unpaired Student’s-t-test showed no difference between the two groups (p > 0.05).

3.3. Withdrawal from MDPV elevates IL-17A and other cytokine and chemokine levels in the VTA and cyanidin normalizes MDPV withdrawal-induced elevations in chemokines and cytokines.

Experiments were conducted to measure IL-17A levels in various brain regions, including the NAC, VTA, PFC and amygdala during withdrawal from MDPV. As IL-17A has been reported to induce the production of other proinflammatory cytokines (IL-1, IL-6, IL-8, G-CSF, GM-CSF, and TNF-α) and chemokines (CXCL1, CXCL2, CXCL5, CCL2, CCL7, CCL20) (Onishi and Gaffen, 2010; Li et al., 2019a), measurement of levels of some of these other mediators were also assessed. Figure 3 shows changes in the levels of chemokines and cytokines in the four brain regions tested.

In the VTA, IL-17A was significantly elevated in rats treated with saline-MDPV (Tukey’s analysis, (p < 0.05)). Cyanidin pretreatment decreased the IL-17A induced by MDPV, but the decrease did not reach significance. Two-way ANOVA showed no significance with treatment and pretreatment ([F(1,24) = 2.711, p > 0.05] and [F(1,24) = 0.116, p > 0.05, respectively), but a significant interaction ([F(1,24) = 9.498, p = 0.005]). In regard to the other mediators which were tested, IL-1β, IL-6, IL-10, CCL2/MCP-1, TNF-α, and CXCL12 levels were significantly increased in rats that received saline-MDPV compared to rats injected with saline-saline (Fig. 3A). Pretreatment with cyanidin blocked the increased levels of chemokines and cytokines in the VTA during withdrawal (Fig. 3A). Two-way ANOVA analysis for IL-1β revealed no significance with treatment and pretreatment ([F(1,24) = 2.028, p > 0.05] and [F(1,24) = 1.691, p > 0.05, respectively), but a significant interaction ([F(1,24) = 8.540, p < 0.01]). Tukey’s analysis showed that IL-1β significantly increased in rats treated with saline-MDPV (p < 0.01) and cyanidin pretreatment significantly lowered the level to the control level (p < 0.05). For IL-6, two-way ANOVA revealed no significance with treatment and pretreatment ([F(1,24) = 3.548, p > 0.05] and [F(1,24) = 0.403, p > 0.05, respectively), but a significant interaction ([F(1,24) = 8.019, p = 0.009]). Tukey’s analysis showed that IL-6 significantly increased in rats treated with saline-MDPV (p < 0.01) and cyanidin pretreatment significantly lowered the level to the control level (p < 0.05). For IL-10, two-way ANOVA showed no significance with treatment and pretreatment ([F(1,24) = 2.983, p > 0.05] and [F(1,24) = 0.617, p > 0.05, respectively), but a significant interaction ([F(1,24) = 8.415, p < 0.01]). Tukey’s analysis showed that IL-10 significantly increased in rats treated with saline-MDPV (p < 0.05) and cyanidin pretreatment significantly lowered the level to the control level (p < 0.05). For CCL2/MCP1, two-way ANOVA showed significance with treatment and interaction ([F(1,24) = 4.312, p < 0.05] and [F(1,24) = 9.169, p < 0.01, respectively), but no significant for pretreatment ([F(1,24) = 0.592, p > 0.05]). Tukey’s analysis showed that CCL2/MCP1 levels were
significantly increased in rats treated with saline-MDPV (p < 0.01) and pretreatment with cyanidin significantly reduced the increased levels of CCL2/MCP1 (p < 0.05). For TNF-α, two-way ANOVA revealed significance for treatment ([F(1,24) = 6.048, p < 0.05]), pretreatment ([F(1,24) = 4.323, p < 0.05]) and interaction ([F(1,24) = 15.47, p = 0.01]). Tukey’s analysis showed that TNF-α levels were significantly increased in rats treated with saline-MDPV (p < 0.01) and pretreatment with cyanidin significantly reduced the increased levels of TNF-α (p < 0.05). For CXCL12, two-way ANOVA showed significance with treatment ([F(1,24) = 4.792, p < 0.05] and pretreatment [F(1,24) = 3.992, p < 0.05, respectively), but no significant interaction ([F(1,24) = 3.267, p = 0.08]). Tukey’s analysis showed that CXCL12 levels were significantly increased in rats treated with saline-MDPV (p < 0.05) and pretreatment with cyanidin significantly reduced the increased levels of TNF-α (p < 0.05).

In the NAC, for all the chemokines and cytokines except CXCL12/SDF-1, there was a trend towards increased levels during the withdrawal in rats that received saline-MDPV, but results did not reach the statistical significance. CXCL12/SDF-1 levels were significantly increased in rats treated with saline-MDPV (p < 0.01) and cyanidin-saline compared to rats that received saline-saline (p < 0.05) (Fig. 3B, Two-way ANOVA followed by Tukey’s multiple comparison). Two-way ANOVA analysis revealed that no significance for pretreatment and treatment ([F(1,23) = 1.560, p > 0.05] and [F(1,23) = 3.889, p > 0.05], respectively) and significance for interaction ([F(1,23) = 14.54, p < 0.0001]). Cyanidin significantly lowered the levels of IL-10, CCL2/MCP-1, and CCL5 induced by MDPV treatment compared to rats treated with saline-MDPV (p < 0.05, p < 0.01, respectively) (Two-way ANOVA followed by Tukey’s multiple comparison). For IL-10, two-way ANOVA showed a significance for pretreatment ([F(1,23) = 7.631, p < 0.05]) but not for treatment ([F(1,23) = 1.016, p > 0.05]) or interaction ([F(1,23) = 1.386, p > 0.05]). For CCL2/MCP-1, two-way ANOVA showed a significance for interaction ([F(1,23) = 6.887, p < 0.05]) but not for pretreatment ([F(1,23) = 2.029, p > 0.05]) or treatment ([F(1,23) = 0.752, p > 0.05]). For CCL5, two-way ANOVA revealed significance for interaction ([F(1,23) = 6.313, p < 0.05]) and pretreatment ([F(1,23) = 5.745, p < 0.05]) but not for treatment ([F(1,23) = 0.2027, p > 0.05]).

No changes in the levels any of these mediators were observed in the PFC or amygdala (Fig. 3: Panels C and D). Thus, in the VTA, chronic MDPV treatment and withdrawal significantly induced a panel of pro-inflammatory cytokines and chemokines, and cyanidin, which inhibits IL-17A, blocked the elevation of all of the mediators tested except CCL5. IL-17 levels trended down with cyanidin treatment but did not reach significance (p = 0.0534).

3.4. MDPV withdrawal-induced increase in mRNA expression of glutamate transporters was normalized by cyanidin pretreatment in the PFC and amygdala

mRNA expression for glutamate transporters, EAAT1/GLAST and EAAT2/GLT1 were measured at four brain regions during MDPV withdrawal as well. In the amygdala, both glutamate transporter expressions were found significantly increased in rats treated with saline-MDPV and the levels were normalized in rats received cyanidin pretreatment (Fig. 4A). For EAAT1/GLAST, two-way ANOVA showed significance with interaction ([F(1,23) = 27.59, p < 0.0001], but no significance for either treatment ([F(1,23) = 3.79, p > 0.05])
or pretreatment (\(F(1,23) = 0.598, p > 0.05\)). Post-hoc analysis revealed that EAAT1/GLAST levels were significantly increased in rats received saline-MDPV (\(p < 0.001\)) and cyanidin-saline (\(p < 0.01\)) compared to saline-saline. Rats pretreated with cyanidin and then treated with MDPV EAAT1/GLAST was significantly reduced, and levels were normalized (\(p < 0.05\), Fig. 4A). For EAAT2/GLT1, two-way ANOVA showed significance with interaction (\(F(1,23) = 19.65, p < 0.001\)), but no significance for either treatment (\(F(1,23) = 0.696, p > 0.05\)) or pretreatment (\(F(1,23) = 2.93, p > 0.05\)). Post-hoc analysis revealed that EAAT2/GLT1 levels were significantly increased in rats received saline-MDPV (\(p < 0.01\)) and pretreatment with cyanidin significantly decreased EAAT2/GLT1 levels (\(p < 0.01\)) as seen in Fig. 4A.

In the PFC, EAAT1/GLAST mRNA expression was significantly increased in rats treated with saline-MDPV and the level was normalized in rats received cyanidin as pretreatment. Two-way ANOVA analysis revealed significance for treatment (\(F(1,24) = 0.01, p < 0.05\) and interaction (\(F(1,24) = 6.979, p < 0.05\)) but not for pretreatment (\(F(1,24) = 3.36, p > 0.05\)). Post-hoc analysis showed that EAAT1/GLAST levels were increased significantly in rats received saline-MDPV treatment compared to rats treated with saline-saline (\(p < 0.01\)) and rats pretreated with cyanidin and then treated with MDPV the levels of EAAT1/GLAST were significantly reduced and normalized (\(p < 0.05\)) (Fig. 4B). EAAT2/GLT mRNA expression was similar in all groups in the PFC as seen in Fig. 4B. No changes in glutamate transporters in the NAC and VTA were detected in any of the groups (Fig. 4C and D).

### 4. Discussion

The present study demonstrated that MDPV withdrawal caused anxiety-like effects accompanied by brain-region specific dysregulation of cytokine and glutamate systems. Cytokine levels (IL-17A, IL-1β, IL-6, IL-10, TNF-α, and CCL2) in the mesolimbic circuit, specifically the VTA, were enhanced during MDPV abstinence. mRNA levels of glutamate transporters (GLT-1 and GLAST) were also enhanced during MDPV abstinence but in the amygdala rather than mesolimbic substrates. Cyanidin, administered during MDPV exposure, prevented behavioral and cellular effects caused by MDPV abstinence, including normalization of withdrawal-induced (1) anxiety-like effects, (2) enhancement of cytokine levels in the VTA and (3) enhancement of glutamate transporter mRNA levels in the amygdala. Dose of cyanidin that was effective against MDPV endpoints did not elicit nonspecific behavioral effects when administered by itself. Specifically, cyanidin did not affect spontaneous locomotor activity, cause anxiolytic or anxiogenic effects, or produce aversive or rewarding effects in a standard place-conditioning paradigm.

The anxiogenic effects detected 72 h after chronic MDPV exposure concur with previous results from our laboratory, in which we detected anxiety-like effects in rats 72 h after discontinuation of a 10-d MDPV binge-type dosing paradigm or 72 h following discontinuation of a 4-d escalating dosing paradigm (Philogene-Khalid et al., 2017; Simmons et al., 2022). Anxiogenic effects during cocaine abstinence in rats are temporally similar, with anxiety-like effects detected 48-72 h following cessation of repeated cocaine exposure (Harris and Aston-Jones, 1993, Sarnyai et al., 1995, Basso et al., 1999, Paine et al., 2002; Philogene-Khalid et al., 2017). Given mechanistic similarities of MDPV and cocaine as blockers of monoamine transporters (Baumann et al., 2013, Simmler et al., 2013), comparable anxiogenic effects in
the 48 to 72 h abstinence interval is not surprising. For MDPV, however, anxiogenic effects during abstinence are not entirely the same in rats and mice and suggest the existence of species differences. Most notably, anxiogenic effects of MDPV in mice are more marked during later abstinence, with significant effects detected 21 d after cessation of MDPV exposure but not after 48 h (Duart-Castells et al., 2019).

A particularly interesting finding in our study was the brain-region specific dysregulation of cytokine signaling during MDPV abstinence, with significant changes detected almost exclusively in the VTA. MDPV-induced effects on cytokine levels in the VTA were directionally similar and broad, as we detected increases in 6 different cytokines (IL-17A, IL-1β, IL-6, TNFα, IL-10, and CCL2). None of the cytokines quantified in our assay were elevated in the PFC or amygdala, and only CXCL12 was significantly elevated in the NAC. Overall, our results are in agreement with recent evidence showing that cytokine levels (i.e., TNFα, IL-1β, IL-6 and CCL2) in multiple brain regions are elevated 24 h after discontinuation of 21 d of cathinone self-administration (Marusich et al., 2022). In the Marusich et al. (2022) study, brain cytokine levels were increased in both male and female rats, albeit with males showing a more robust enhancement, and were elicited by two mechanistically different cathinones, one being the methylenedioxy derivative α-pyrrolidinovalerophenone (α-PVP), a monoamine transporter blocker with a MDPV-like mechanism of action, and the other being 4-methylmethcathinone (4MMC), a transporter substrate that induces monoamine release (Marusich et al., 2022). Comparing effects of psychostimulants on cytokine levels across studies is challenging due to differences in experimental paradigms, dosing schedules, and time point of analysis. In fact, cytokine levels have often been quantified within 2 h of the last drug exposure when the subject is presumably still impacted by direct effects of the psychostimulant (Brown et al., 2018; Kubera et al., 2008). In our study, cytokine levels were assessed 72 h after the last MDPV exposure to better correlate neuroimmune status with established angiogenic effects, which are most robust within the 48-72 h abstinence period (Philogene-Khalid et al., 2017; Simmons et al., 2022). Thus, like the Marusich study (2022) in which cytokine levels were quantified 24 h after cathinone intake, the enhancement of cytokine levels that we detected 72 h after the last MDPV exposure should reflect effects of MDPV withdrawal rather than direct effects of MDPV.

Reasons for the VTA-specific enhancement of cytokine levels during MDPV abstinence in our study are unclear. Marusich and colleagues (2022) analyzed cytokines from multiple brain regions (amygdala, hippocampus, hypothalamus, PFC, striatum, and thalamus) and plasma but not from the VTA. Common regions of analysis between the two studies are the PFC and amygdala, where Marusich and colleagues (2022) detected significant increases in cytokine levels but we did not. Explanations for the differences between the two studies, including the lack of effect in the PFC, NAC and amygdala in our study, are the analysis of cytokines at different abstinence time points (24 versus 72 h), different routes of administration (self-administration versus non-contingent experimenter injections), and different cathinones tested (α-PVP and 4MMC versus MDPV).

Cyanidin administered during repeated MDPV exposure completely blocked development of the anxiogenic effects that normally accompany MDPV withdrawal in rats and mice (Philogene-Khalid et al.,
In control experiments conducted with MDPV-naïve rats, cyanidin did not affect spontaneous locomotor activity, cause anxiogenic or anxiolytic effects in the EZM assay, or produce rewarding or aversive in a standard place conditioning experiment. As such, the blocking effect of cyanidin on the development of MDPV withdrawal-induced anxiety-like effects could not be explained by non-specific behavioral effects such as locomotor depression or rewarding effects. A limitation of our experiments is the testing of only a single dose of cyanidin, so dose-effect data were not obtained. Interestingly, higher doses of cyanidin do reduce depression-like symptoms in mice exposed to chronic unpredictable mild stress (CUMS) (Shan et al., 2020). Further, hawthorn extract containing cyanidin along with a myriad of phenolic compounds, procyanidins, and flavonoids also has anxiolytic effects in mice (Popovic-Milenkovic et al., 2014). In a recent study, cyanidin administered at doses of 20 or 40 mg/kg for 5 d reduced the immobility time assessed by the tail suspension test (TST) and forced swim test (FST) in LPS-challenged mice, suggesting that cyanidin can reduce depression-like effects in mice with enhanced cytokine signaling (Qu et al., 2022). While it is not unusual for similar doses of the same drug (e.g. cyananidin) to produce effects in rats and mice that are not entirely congruent, it is possible that higher doses of cyanidin would have produced anxiolytic and other behavioral and neurochemical effects in our studies.

At the neuroimmune level, cyanidin administered during MDPV treatment also prevented the enhancement of cytokine levels in the VTA during MDPV abstinence. Effects of cyanidin were anatomically selective, with efficacy observed exclusively in the VTA, yet comprehensive, as cyanidin normalized MDPV withdrawal-induced elevation of 5 different cytokines (IL1-β, IL-6, IL-10, TNFα, and CCL2) with a trend toward normalization of IL-17A levels. The mechanism underlying cyanidin efficacy against MDPV withdrawal-induced anxiety is unclear, but broad-spectrum inhibition of cytokine and chemokine signaling in the VTA is plausible based on the known pharmacological profile of cyanidin and strong correlation between our behavioral and neuroimmune data. Evidence indicates that cyanidin and its derivatives, such as cyanidin-3-glucoside (C3G), display anti-inflammatory effects, including inactivation of TNFα and IL-6 signaling, inhibition of reactive oxygen species, and reduction of inflammation via PPARα-LXRα-ABCA1-dependent cholesterol efflux (Naseri et al., 2018). Furthermore, protocatechuic acid (PCA), the major human metabolite of cyanidin-glucosides, inhibits production of inflammatory cytokines (e.g., TNFα, IL-1β, IL-6) through mechanisms that may include blocking activation of nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase (ERK) (Crespo et al., 2017). In the context of our findings, one specific possibility involves TNFα, which showed a robust increase during MDPV abstinence that was completely normalized by cyanidin treatment. Among the cytokines analyzed in our study, TNFα is most closely linked to the pathogenesis of anxiety disorders. For example, circulating levels of TNFα are elevated in patients with psychiatric conditions such as anxiety, depression and cognitive decline (Bassukas et al., 2008). Preclinical studies show that genetic deletion of TNFα reduces anxiety in young mice; central TNFα blockade reduces anxiety in mice caused by experimental autoimmune encephalitis; and peripheral TNFα antagonism reduces anxiety in mice experiencing chronic pain and in mice lacking leptin receptors (Camara et al., 2013; Haji et al., 2012; Chen et al., 2013; Alshammari et al., 2020). In rats the chronic administration of etanercept, a TNFα antagonist with limited
to negligible blood-brain barrier penetrability (Boado et al., 2010), reduces anxiety- and depression-like effects in the absence of immune stimulation (i.e., under physiologically normal conditions) (Bayramgurler et al., 2013). Given that enhanced TNFα signaling facilitates anxiety-like effects, and cyanidin reduces TNFα production (Crespo et al., 2017; Naseri et al., 2018), cyanidin may have reduced MDPV withdrawal-induced anxiety in our experiments by directly or indirectly reducing TNFα levels.

In the case of an indirect mechanism involving TNFα, one possibility is upstream inhibition of IL-17A signaling by cyanidin. IL-17A induces TNFα release from macrophages and other cell types (Shen and Gaffen, 2008) and, along with TNFα, promotes a positive feedback loop of cytokine and chemokine production. In fact, IL-17A induces an array of different pro-inflammatory cytokines, and synergizes with TNFα to induce nearly all of its target genes (Shen and Gaffen, 2008; Onishi and Gaffen, 2010). Because cyanidin inhibits binding of IL-17A to IL-17 RA and blocks IL-17A-induced production of other proinflammatory cytokines and chemokines, including IL-6, IL-8, CXCL1, and CXCL2 in human cells (Liu et al., 2017), cyanidin may have prevented IL-17A-induced upregulation of TNFα and related cytokines during MDPV abstinence, leading to a reduction in anxiety-like effects. This interpretation is supported by recent data showing that genetic deletion of IL-17A reduces anxiety in mice and Th17-derived cytokines (IL-17A, TNF-α) are elevated in the plasma of patients with anxiety (Vieira et al., 2010).

For comparison to cytokines, we also studied the impacts of chronic cyanidin and MDPV exposure and abstinence (72 h) on mRNA levels of two glutamate transporters (GLAST and GLT-1) in the VTA, PFC, NAC, and amygdala. GLT-1 and GLAST gene expression were enhanced during MDPV withdrawal, but the changes were brain-region specific, and entirely distinct compared to the increases in cytokine levels. mRNA levels of both GLT-1 and GLAST were enhanced in the amygdala, a region in which no significant changes in cytokine levels were detected during MDPV withdrawal. No significant changes in mRNA levels of either glutamate transporter were detected in mesolimbic substrates (VTA and NAC), which contrasts with the marked increase in cytokine levels in the VTA that occurred during MDPV abstinence. Glutamatergic transmission in the central amygdala, and especially in the bed nucleus of the stria terminalis (BNST), is dysregulated in animal models of stress, anxiety and addiction. For example, in mice undergoing alcohol withdrawal, levels of NMDA receptors containing the NR2B subunit in the BNST are elevated (Kash et al., 2009). In addition, an increase in glutamatergic excitability in neurons projecting from the basolateral amygdala (BLA) to NAC in male mice and BLA to BNST in female mice occurs during alcohol withdrawal (Price and McCool, 2022). In mice undergoing abstinence from chronic morphine administration, anxiety-like, depressive-like, and impaired sociability behaviors are observed alongside a marked upregulation of metabotropic glutamate receptor 5 binding in the amygdala, NAC, thalamus, and hypothalamus (Zanos et al., 2016). Gene-transcript expression of GLAST and GLT-1 is increased in the cortex 10 h after repeated administration of another psychostimulant, MDMA, which could reflect either direct effects of MDMA, MDMA withdrawal, or both (Kindlundh-Högberg et al., 2008).

One possibility in our study is that abstinence from chronic MDPV exposure increased extracellular glutamate in the amygdala (Kash et al., 2009; Price and McCool, 2022; Zanos et al., 2016), which contributed to anxiety-like effects while also causing compensatory upregulation of GLT-1 and GLAST.
levels to enhance cellular glutamate uptake. Support for this interpretation is provided by a recent study that analyzed glutamate content in whole tissue homogenates obtained from the amygdala, PFC, hippocampus, thalamus, hypothalamus and striatum of rats 1 d after discontinuation of a chronic α-PVP self-administration (Marusich et al., 2019). Interestingly, in that study, the only brain region in which glutamate levels were elevated during α-PVP abstinence was the amygdala, and the effect was dependent on duration of exposure (Marusich et al., 2019). In the context of our study, the mechanism by which cyanidin normalized the increased expression of GLT-1 and GLAST during MDPV abstinence, and the relation, if any, that this effect has on the anxiogenic effects caused by MDPV withdrawal are unclear. Because cyanidin reduces agonist-induced calcium signaling in PC12 cells by inhibiting multiple pathways, including the influx of extracellular calcium and release of calcium from intracellular stores (Perveen et al., 2014), cyanidin may be capable of reducing neuronal glutamate release. If so, in the presence of cyanidin, a reduction in glutamate release may have occurred and mitigated the rise in glutamate levels in the amygdala during cathinone abstinence (Marusich et al., 2019), thereby normalizing the elevation of GLT-1 and GLAST that would normally occur to reduce the level of extracellular glutamate. Future studies will better assess how cyanidin impacts the glutamate system, and explore interactions between glutamate and cytokine systems in the context of psychostimulant dependence and addiction.

A limitation of the present study was that sex differences were not investigated. Although sex differences for established psychostimulants such as methamphetamine and cocaine are well documented (Nicolas et al., 2022; Towers and Lynch, 2021), they are less established for synthetic cathinones and perhaps less common especially regarding drug intake (Marusich et al., 2021; Fattore et al., 2020). However, in the context of cytokines, recent evidence identifies important sex differences following abstinence from cathinone self-administration, with the primary differences being more robust elevation of brain cytokines in male rats and more pronounced elevation of plasma cytokines in female rats (Marusich et al., 2022). Another limitation in our study was that a group of rats naïve to injections and experimental manipulations was not included as a comparison to vehicle-treated rats. As such, the possibility that cytokine levels were elevated by stress associated with the injection process cannot be excluded. To better discriminate effects of direct MDPV exposure from MDPV withdrawal, it will also be important in future studies to assess cytokine levels at different time points of MDPV exposure and abstinence, including from plasma, to better elucidate a role for central and peripheral cytokine mechanisms in MDPV behavioral effects.

In conclusion, abstinence from chronic exposure to a synthetic cathinone (MDPV) produced a brain-region specific enhancement of cytokines in the VTA that correlated with robust anxiogenic effects during MDPV withdrawal. Another major finding was that the flavonoid natural product cyanidin prevented the anxiety-like effects and dysregulation of cytokine signaling during MDPV abstinence. Additionally, MDPV withdrawal caused enhancement in the levels of glutamate transporters in the amygdala and cyanidin normalized increased levels of glutamate transporters. The significance of the cytokine dysregulation, especially pertaining to impacts on mesolimbic dopamine and corticolimbic glutamate systems during psychostimulant abstinence and relapse, will be investigated in future studies. Future studies will be
expanded to investigate how normalization of cytokine signaling by cyanidin during psychostimulant abstinence affects relapse to drug seeking in self-administration assays. Identifying a more specific mechanism of action for cyanidin, such as inhibition of IL-17A signaling (Liu et al., 2017), will also be important given an emerging role of IL-17A in anxiety disorders and its ability to induce downstream production of cytokines by synergizing with TNFα, which has an established role in facilitating anxiety (Vieira et al., 2010; Bayramgurler et al., 2013; Crespo et al., 2017; Naseri et al., 2018; Li et al., 2019a; Li et al., 2019b).

**Abbreviations**

BLA: basolateral amygdala; BNST: bed nucleus of the stria terminalis; C3G: cyanidin-3-glucoside; CNS: central nervous system; CPP: conditioned place preference; DAT: dopamine transporter; ERK: extracellular signal-regulated kinase; EPM: elevated plus maze; EZM: elevated zero maze; GLAST: glutamate aspartate transporter; GLT-1: glutamate transporter subtype 1; IL: interleukin; MDPV: methylenedioxypyrovalerone; NAC: nucleus accumbens; NET: norepinephrine transporters; NF-κB: nuclear factor-κB; PCA: protocatechuic acid; PFC: prefrontal cortex; SERT: 5-HT transporter; TNFα: tumor necrosis factor-alpha; VTA: ventral tegmental area

**Declarations**

**Acknowledgments**

Not applicable

**Author contributions**

SI and SMR designed the studies. SI, JJM, AS, JF, SW conducted the experiments. SI, SMR, JJM, and TKE processed, analyzed, and interpreted the results. SI, SMR, and TKE wrote the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported by the following grants from the National Institute on Drug Abuse: R01 DA045499, R01 DA039139, R01 DA051205, and P30 DA013429.

**Availability of data and materials**

All relevant data in this study are available upon reasonable request directed to the corresponding author.

**Competing interests**

The authors declare no competing interests.

**Ethics approval and consent to participate**
The animal experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Temple University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (the Guide) from the NIH.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Conflicts of Interest

None.

References

3. Bachtell RK, Jones JD, Heinzerling KG, Beardsley PM, Comer SD. Glial and neuroinflammatory targets for treating substance use disorders. Drug Alcohol Depend 2017;180:156–70


23. Gregg RA, Hicks C, Nayak SU, Tallarida CS, Nucero P, Smith GR, Reitz AB, Rawls SM. Synthetic cathinone MDPV downregulates glutamate transporter subtype I (GLT-1) and produces rewarding and locomotor-activating effects that are reduced by a GLT-1 activator. Neuropharmacology 2016;108:111–9
36. Leclercq S, Cani PD, Neyrinck AM, St´arkel P, Jamar F, Mikolajczak M, Delzenne NM, de Timary P. 2012. Role of intestinal permeability and inflammation in the biological and behavioral control of
alcohol-dependent subjects. Brain Behav Immun 2012; 26 (6), 911–918


38. Li YC, Chou YC, Chen HC, Lu CC, Chang DM. Interleukin-6 and interleukin-17 are related to depression in patients with rheumatoid arthritis. Int J Rheum Dis. 2019b; 22(6):980–985


45. Nayak SU, Cicalese S, Tallarida C, Oliver CF, Rawls SM. Chemokine CCR5 and cocaine interactions in the brain: Cocaine enhances mesolimbic CCR5 mRNA levels and produces place preference and locomotor activation that are reduced by a CCR5 antagonist. Brain Behav Immun 2020; 83:288–292


48. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 2010; 129(3):311–21


63. Šery´ O, Sultana N, Kashem MA, Pow DV, Balcar V. GLAST but not least—distribution, function, genetics and epigenetics of L-glutamate transport in brain—focus on GLAST/EAAT1. Neurochem Res 2015; 40:2461–2472


67. Simmons SJ, Oliver CF, McCloskey NS, Reitz AB, Nayak SU, Watson MN, Rawls SM. Paradoxical anxiolytic effect of the 'bath salt' synthetic cathinone MDPV during early abstinence is inhibited by a chemokine CXCR4 or CCR5 receptor antagonist. Drug Alcohol Depend 2022 27;230:109204.


Figure 1

Chronic pretreatment with cyanidin prevents MDPV withdrawal-induced anxiety in rats. Rats were treated with either saline or cyanidin (0.5 mg/kg, IP) and then 30 min later they were injected with either saline or MDPV (1 mg/kg, IP) once a day for 9 days. The elevated zero-maze (EMZ) test was performed 72 hr after the last injection. Rats that had received saline-MDPV treatment spent significantly less time in the open arm than the rats treated with saline-saline, cyanidin-saline, or cyanidin-MDPV. Chronic cyanidin treatment prevented anxiety induced by MDPV withdrawal. (Two-way ANOVA followed by Tukey's multiple test, **p<0.01, N=7-9).
Figure 2

Horizontal, ambulatory, and stereotypic activities following acute (A) and chronic (B) treatment with saline or cyanidin. Following a 30 min acclimation in the individual locomotor activity cages, rats were administered either saline or cyanidin (0.5 mg/kg, IP). Locomotion was measured for 90 min. For chronic administration, rats were injected with either saline or cyanidin (0.5 mg/kg, IP) once a day for 9 days. On day 9, rats were acclimated and then received the treatments. Locomotion was measured for 90 min. Locomotion was similar between saline and cyanidin groups following acute or chronic administration.
Chemokine and cytokine levels in the VTA, NAC, PFC, and amygdala during withdrawal from MDPV, with and without treatment with cyanidin. Chemokine and cytokine levels were measured in brains of rats treated with either saline or cyanidin and then 30 min later with saline or MDPV (1 mg/kg, IP) once a day for 9 days. Seventy-two hr after the last dose, anxiety was measured in the EZM, and then animals were sacrificed and brains were removed. No significant change in any chemokine or cytokine level was detected in the PFC, the NAC or the amygdala. In contrast, in the VTA, IL-1β, IL-6, IL-10, IL-17A, CCL2/MCP-1, TNF-α, and CXCL12 levels were significantly increased in rats which received saline-MDPV treatment. Treatment with cyanidin significantly reduced the elevated levels of all of the chemokine and cytokines tested except IL-17A, which trended down, but did not reach statistical significance (Two-way ANOVA followed by Tuckey's multiple comparison, *p<0.05, **p<0.01, n=7-9). (CXCL12/SDF-1 was below detectable levels in the PFC and amygdala, so data are not shown.)
Figure 4

Changes in mRNA expressions of EAAT1/GLAST and EAAT2/GLT1 at PFC, NAC, VTA, and amygdala during MDPV-induced withdrawal and effects of cyanidin treatment. Expressions of glutamate transporters were measured in the brain regions of PFC, NAC, VTA, and amygdala of rats treated with either saline or cyanidin and then 30 min later with saline or MDPV (1 mg/kg, IP) once a day for 9 days. Seventy two hr after the last dose, EZM were performed and brains were removed. EAAT1/GLAST expression was upregulated in PFC and both EAAT1/GLAST and EAAT2/GLT1 expressions were upregulated in amygdala during the withdrawal. Cyanidin by itself also increased EAAT1/GLAST expression at amygdala. However, pretreatment with cyanidin normalized upregulated EAAT1/GLAST and EAAT2/GLT1 in both PFC and amygdala (Two-way ANOVA followed by Tukey's multiple comparison, *p<0.05, **p<0.01, ***p<0.001, n=7-9).