Drug and Alcohol Dependence 197 (2019) 22–27

Contents lists available at ScienceDirect

Drug and Alcohol Dependence

journal homepage: www.elsevier.com/locate/drugalcdep

Short communication

Synthetic cathinone MDPV enhances reward function through purinergic P2X7 receptor-dependent pathway and increases P2X7 gene expression in nucleus accumbens

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ARTICLE INFO

Keywords:
Purine
P2X7
Psychostimulant
MDPV
Cathinone
ICSS
ATP
Accumbens

ABSTRACT

Background and purpose: Purinergic P2X7 receptors are present on neurons, astrocytes and microglia and activated by extracellular ATP. Since P2X7 receptor activation releases endogenous substrates (*e.g.,* pro-inflammatory cytokines, dopamine, and glutamate) that facilitate psychostimulant reward and reinforcement, we investigated the hypothesis that the synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV) produces rewarding effects that are dependent on active P2X7 receptors.

Methods: Reward function was measured in male mice using intracranial self-stimulation (ICSS). MDPV (0.1, 0.3, 0.5 mg/kg, SC) and a selective P2X7 antagonist (A438079) (5, 10, 50 mg/kg, IP) were tested alone and in combination. In separate mice, gene and protein expression of P2X7 and mitochondrial adenosine triphosphate (ATP) synthase (an enzyme that catalyzes synthesis of ATP, an endogenous ligand for P2X7 receptors) in the nucleus accumbens (NAcc) were quantified following MDPV exposure (0.1, 0.5, 5 mg/kg, SC).

Key results: MDPV (0.5 mg/kg, SC) facilitated ICSS as quantified by a significant reduction in brain reward threshold. A438079 (5, 10, 50 mg/kg, IP) did not affect ICSS by itself; however, for combined administration, A438079 (10 mg/kg, IP) inhibited facilitation of ICSS by MDPV (0.5 mg/kg, SC). At the cellular level, MDPV exposure increased gene and protein expression of P2X7 and ATP synthase in the NAcc.

Conclusion and implication: We provide evidence that a psychostimulant drug produces reward enhancement that is influenced by P2X7 receptor activity and enhances P2X7 receptor expression in the brain reward circuit.

1. Introduction

Synthetic cathinones share pharmacological features with established psychostimulants but differ structurally from amphetamines by possessing a ketone oxygen atom (C═O) on the β position of the side chain (Glennon, 2014). MDPV (3,4-methylenedioxypyrovalerone) is a first-generation synthetic cathinone that has provided a template for development of new-generation analogs, including α-pyrrolidinovalerophenone (α-PVP) and α-pyrrolidinopropiophenone (α-PPP) (Marusich et al., 2014). Acute MDPV induces more robust ambulation and stereotypy than cocaine or methamphetamine (Marusich et al., 2014), while repeated MDPV exposure produces locomotor sensitization (Berquist et al., 2016; Kohler et al., 2018). In abuse liability studies, MDPV produces more robust rewarding effects in the conditioned place preference (CPP) paradigm than amphetamine and is self-administered at higher rates than cocaine or methamphetamine (Aarde et al., 2013; Schindler et al., 2016). Notably, MDPV potently facilitates intracranial self-stimulation (ICSS) in rats (Bonano et al., 2014; Watterson and Olive, 2017).

Neuroinflammation contributes to psychiatric and neurological diseases (*e.g.,* depressive disorders, mania, obsessive-compulsive disorder (OCD) and neuropathic pain) (Dao-Ung et al., 2015). Cytokines, such as IL-1β, IL-6 and TNF-α, and reactive oxygen species are released from microglia following P2X7 receptor activation (He et al., 2017; Fernandes et al., 2016), suggesting a link between the pro-inflammatory actions of P2X7 and CNS diseases. Neuroinflammation is

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https://doi.org/10.1016/j.drugalcdep.2018.12.022
Received 21 July 2018; Received in revised form 14 November 2018; Accepted 7 December 2018
Available online 31 January 2019
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also associated with psychostimulant addiction [*e.g.,* cocaine-induced activation of cytokine and chemokine systems contributes to pathological alterations in the brain and cocaine-induced behavioral effects (Bachtell et al., 2017)], but a role for P2X7 receptors in psychostimulant reward has not been shown in preclinical models of abuse liability. In fact, the most relevant behavioral evidence is that P2X7 antagonism or genetic deletion reduces hyperactivity induced by amphetamines (Gubert et al., 2016; Bhattacharya et al., 2013; Csölle et al., 2013). Evidence also indicates that purines activate monoamine neurons and, in the nucleus accumbens (NAcc), enhance dopamine release that contributes to facilitation of psychostimulant-induced reward (Krügel et al., 1999, 2001; Kittner et al., 2001). Thus, we first tested the hypothesis that MDPV produces a reduction in brain reward threshold, as measured by intracranial self-stimulation (ICSS), that depends on P2X7 receptor activity. For behavioral experimentation we selected the competitive and reversible P2X7 antagonist A438079 (*e.g.,* IC_{50} = 100 and 300 nm at rat and human P2X7 receptors, respectively), which is largely devoid of activity at other P2 receptors ($IC_{50} \gg 10\ \mu M$), shows little or no activity at non-purinergic receptors and ion channels, is brain-penetrable, and displays antinociceptive activity in models of neuropathic pain following peripheral administration (Nelson et al., 2006; Donnelly-Roberts and Jarvis, 2007). At the cellular level, we determined effects of MDPV exposure on P2X7 and ATP synthase gene and protein expression in the NAcc of mice.

2. Materials and methods

2.1. Animals and chemicals

Adult male C57Bl/6 mice (Jackson Laboratories; Bar Harbor, ME) were group-housed (2–5/cage), kept in a humidity- and temperature-controlled vivarium, and maintained under a 12-h light/dark cycle on a regular chow diet. Studies were conducted in accordance with the Institutional Animal Care and Use Committee at Temple University. (±)-MDPV was synthesized according to published methods (Doylestown, PA, USA) (Gregg et al., 2015). A438079 hydrochloride was purchased from Tocris Biosciences (St. Louis, MO, USA). Drugs were dissolved in 0.9% saline and administered in a volume of 1 ml/kg subcutaneously (SC) (MDPV) or injected intraperitoneally (IP) (A438079).

2.2. Intracranial self-stimulation (ICSS)

Mice were stereotaxically implanted with a monopolar stimulating electrode (Plastics One; Roanoke, VA) targeting the medial forebrain bundle (from bregma: AP: −1.9, ML: +0.8, DV: −4.8 mm). Mice were trained to respond to electrical stimulation using computer-controlled operant chambers (Med Associates; St. Albans, VT) in daily sessions at the lowest current that sustained responding at a rate of one response per s ± 10%. This current was maintained through testing in which mice responded for stimulation frequencies (158–34 Hz) in descending order (each frequency for 50 s) across a 15 min trial (herein termed "Pass"). Experimental sessions proceeded when reward thresholds, computed using a least-square line of best fit analysis to determine the frequency at which operant responding is no longer supported, reached stable performance criteria (± 10% for 3 consecutive days).

Experimental sessions proceeded using a within-subjects, counterbalanced, Latin-Square design. Each experimental session began with three Passes. For all experimental sessions, within-session baselines were established as mean data (reward thresholds and maximal response rates) from Passes 2 and 3. For the MDPV dose-response sessions, mice were injected with MDPV (0.0, 0.1, 0.3, 0.5 mg/kg, SC), and data were averaged across Passes 5 and 6. In other sessions, A438079 (0, 5, 10, 50 mg/kg, IP) was injected and data were averaged across Passes 6 and 7. Similarly, for combinatorial studies, A438079 was injected after Pass 3, MDPV (0.5 mg/kg, SC) after Pass 4, and data were obtained across Passes 6 and 7. In this manner, A438079 was allowed 30 min for maximal onset of action, and MDPV was allowed 15 min. Reward Threshold was quantified as: ([calculated threshold post − drug/calculated threshold baseline] * 100). Maximal Response Rate was measured as the greatest number of operant responses performed within 1 min intervals during each Pass (baseline and post-drug) and quantified as described for Reward Threshold

2.3. Gene expression (quantitative RT-PCR) of P2X7 and ATP synthase

Mice (separate group) were injected with MDPV (0, 0.1, 0.5, 5 mg/kg, SC), once daily for 4 consecutive days. RNA was extracted from NAcc, 120 min following the last MDPV injection, and converted into cDNA, as described (Kim et al., 2017). TaqManTM PCR primer/probe sets for detection of P2X7, ATP synthase and 18S genes were purchased from Thermo Fisher. Analyses were executed using the ΔΔCt method utilizing the StepOnePlus real-time PCR system (Thermo Fisher) by normalization to housekeeping ribosomal 18S gene and fold-change calculated from the difference between experimental condition and untreated control (n = 7–8 mice). To eliminate biased data, two people performed analysis in blinded fashion.

2.4. Protein expression of P2X7 and ATP synthase

Mice (separate group) were injected with MDPV (0, 0.1, 0.5, 5 mg/kg, SC), once daily for 4 consecutive days. Protein was extracted from NAcc, 120 min following the last MDPV injection, with RIPA buffer (Thermo Scientific™) following the manufacturer's instructions. Primary antibodies were purchased for the following proteins: P2X7 (Alomone Labs, Jerusalem, Israel) and ATP5F1 (ProSci Inc., Poway, CA), actin (Santa Cruz Biotechnology, Dallas, TX). Western blots were performed as previously described (Rom et al., 2013, 2015).

2.5. Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc analysis. Statistical significance was $p < 0.05$.

3. Results

3.1. A438079 normalizes MDPV-elicited changes in reward thresholds during ICSS (Fig. 1)

For experiments testing MDPV alone (Fig. 1A,B), one-way ANOVA indicated an effect of MDPV dose on reward thresholds [$F(3, 17) = 9.30$, $p < 0.01$] but not on maximal response rates [$F(3, 17) = 1.972$, $p > 0.05$]. The percentage of baseline threshold in mice treated with MDPV (0.5 mg/kg) was lower than saline-injected mice ($p < 0.01$; Fig. 1A). Thus, in subsequent experiments, we utilized 0.5 mg/kg MDPV to facilitate brain reward function. No effects of A438709 (5, 10, 50 mg/kg; Fig. 1E,F) on reward threshold [$F(3, 30) = 0.26$, $p > 0.05$] or maximal response rates [$F(3, 30) = 0.06$, $p > 0.05$] were observed. An effect was, however, detected when testing efficacy of A438079 against MDPV-elicited changes in reward thresholds [$F(3, 31) = 3.31$, $p < 0.01$] (Fig. 1C,D), whereas no effect was found when examining maximal response rates [$F(3, 30) = 0.06$, $p > 0.05$]. Post-hoc analysis indicated that a dose of 10 mg/kg A438079 normalized MDPV-elicited reductions in reward threshold ($p < 0.05$)

3.2. MDPV enhances P2X7 and ATP synthase gene and protein expression in NAcc (Fig. 2)

For P2X7 mRNA levels (Fig. 2A), an effect of MDPV dose on gene expression [$F(3, 29) = 6.08$, $p < 0.01$] was observed. A dose of MDPV (0.5 mg/kg) that lowered brain stimulation reward threshold also

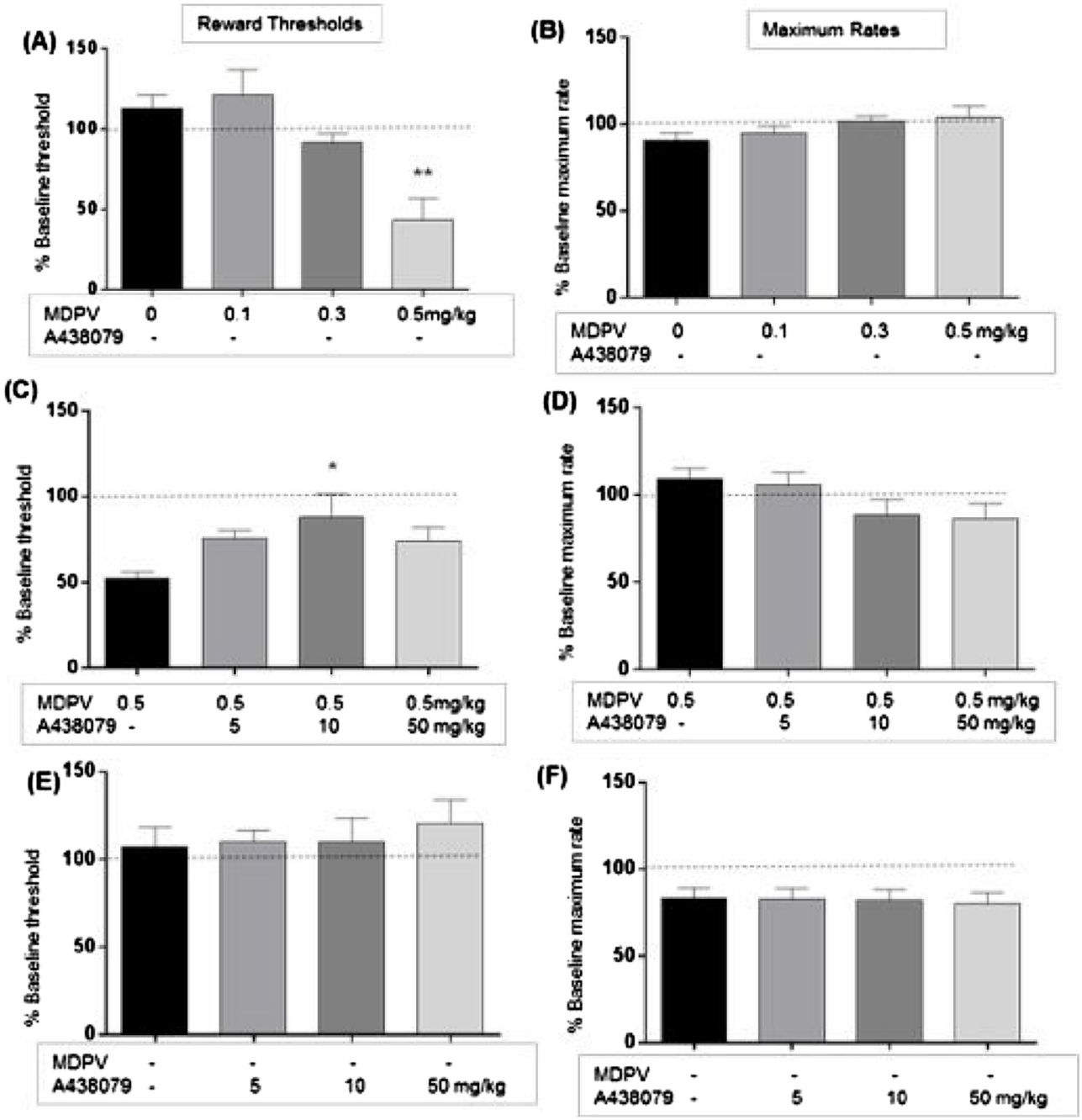

Fig. 1. P2X7 antagonist A438079 inhibits reduction in brain reward threshold produced by MDPV. Percentage change in baseline reward threshold + *S.E.M.* shown in (A, C, E) and maximum response rate shown in (B, D, F). **(A,B)** MDPV (0.5 mg/kg) reduces brain reward threshold without affecting maximal response rate. N = 4–7. $^{**}p < 0.01$ compared to vehicle-injected control subjects. **(C,D)** A438079 (0, 5, 10, 50 mg/kg) inhibits reduction in brain reward threshold produced by MDPV (0.5 mg/kg). N = 8–9. $^{*}p < 0.05$ *versus* 0.5 mg/kg MDPV control (*i.e.* leftmost column). **(E,F)** A438079 by itself (0, 5, 10, 50 mg/kg) does not affect ICSS. N = 7.

enhanced P2X7 mRNA levels relative to saline-treated mice ($p < 0.001$). For Western blot analysis (Fig. 2B), an effect of MDPV dose on P2X7 protein expression [$F(2, 14) = 11.66$, $p < 0.01$] was observed, with post-hoc analysis revealing that 5 mg/kg of MDPV increased P2X7 protein expression relative to saline ($p < 0.001$).

For ATP synthase (ATP5F1) mRNA levels (Fig. 2C), an effect of MDPV dose on gene expression was detected [$F(3, 30) = 6.13$, $p < 0.01$]. Post-hoc analysis revealed that mice treated with 5 mg/kg of MDPV displayed greater ATP synthase gene expression than saline-treated controls ($p < 0.01$). An effect of MDPV dose on ATP synthase protein expression was also detected [$F(2, 11) = 12.49$, $p < 0.01$] (Fig. 2D), with 5 mg/kg MDPV increasing protein levels relative to saline ($p < 0.01$).

4. Discussion

MDPV reduced brain reward thresholds in intracranially self-stimulating mice without affecting maximal response rates, thereby suggesting an increase in reward sensitivity rather than enhanced motor activity. Mechanistically, MDPV is similar to cocaine but with enhanced potency at DAT and NET (50- and 10-fold more potent, respectively) and negligible effects on SERT (Baumann et al., 2013; Simmler et al., 2013). Because selective DAT inhibitors, but not NET or SERT inhibitors, facilitate ICSS (Negus and Miller, 2014), the most

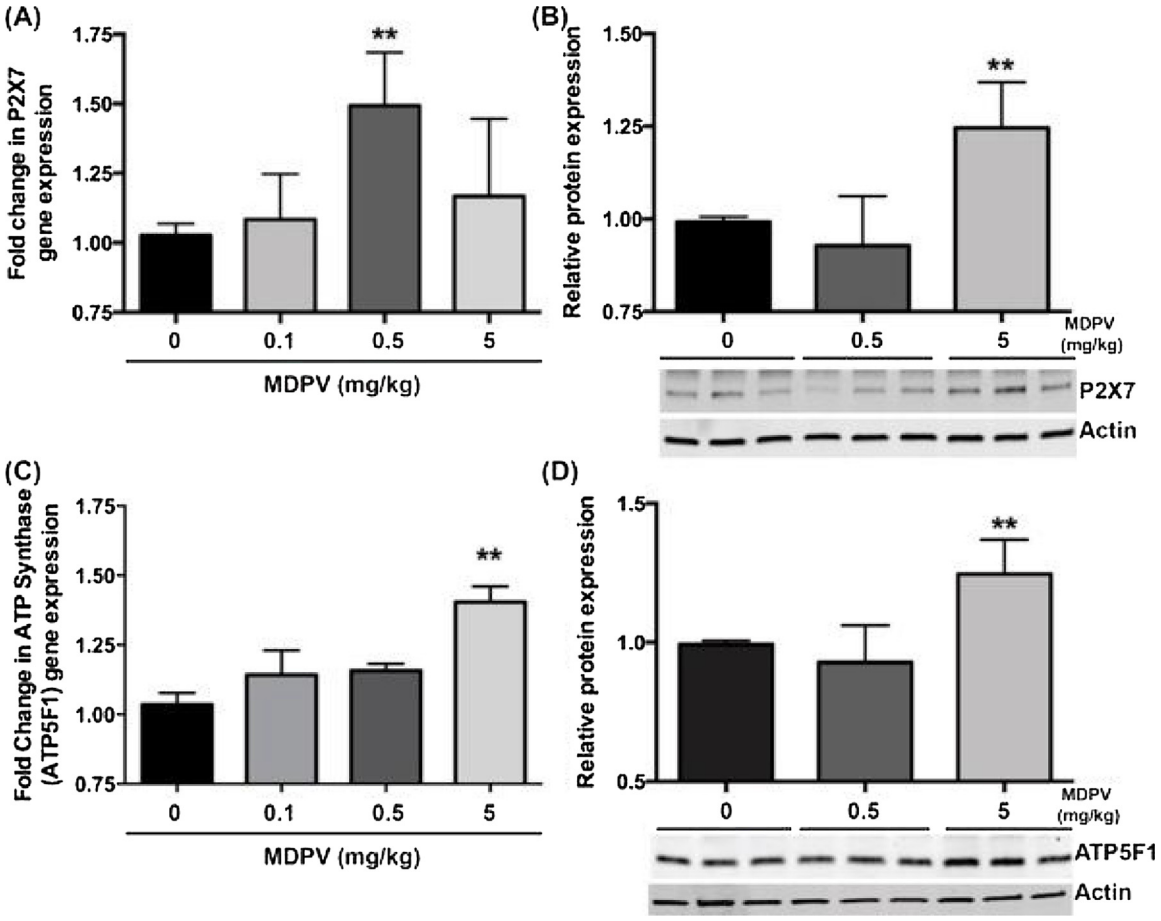

Fig. 2. MDPV increases P2X7 and ATP synthase gene and protein expression in NAcc. Data were obtained from mice treated with MDPV (0. 0.1, 0.5, 5 mg/kg) for 4 days (brains extracted 120 min after last MDPV injection). **(A,C)** qRT-PCR quantification of mRNA levels in NAcc tissue. Data presented as fold-change in gene expression + S.E.M. N = 7–8. **(B,D)** Representative images of Western Blot for P2X7, ATP synthase (ATP5F) and actin proteins, and quantitative image densitometry in NAcc tissue. Data are presented as fold-change in protein expression (normalized to actin) + S.E.M. N = 4–6. $^{**}p < 0.01$ *versus* saline control (MDPV 0 mg/kg).

parsimonious explanation for reduction of brain reward thresholds by MDPV is inhibition of DAT.

The P2X7 receptor antagonist A438079 inhibited MDPV-induced facilitation of ICSS without altering brain reward function itself. A438079 is selective for P2X7 with good bioavailability, brain penetration and preclinical efficacy (Nelson et al., 2006). Accordingly, P2X7 antagonism is the most probable explanation for the efficacy of A438079, which, in turn, suggests that rewarding effects of MDPV require active P2X7 receptors. This interpretation is supported by our gene expression experiments, as P2X7 mRNA levels in the NAcc were enhanced by a dose of MDPV that also facilitated ICSS. P2X7 protein levels in the NAcc were also increased by MDPV, albeit only at the highest dose. These directionally similar effects of MDPV suggest a correlation between P2X7 gene and protein expression in the NAcc. Although the P2X7 gene is inducible, the turnover rate may not be matched to P2X7 protein production, which might be regulated by factors such as post-transcriptional modification and small non-coding RNAs (Khan et al., 2013), and will be investigated in future studies.

The relevance of the dose-effect differences of MDPV on P2X7 gene and protein expression to behavioral outcomes is not entirely clear. P2X7 mRNA, but not protein, was increased by a dose of MDPV that produced facilitation of ICSS that was P2X7-receptor dependent. However, increased P2X7 message in the NAcc could conceivably lead to enhanced protein expression in regions extraneous to the NAcc, such as the ventral tegmental area (VTA) and ventral pallidum, which are innervated by medium spiny projection neurons originating in the NAcc (Francis and Lobo, 2017). Because the P2X7 antagonist (A438079) was injected systemically in our studies, the observed efficacy against MDPV-induced facilitation of ICSS could be due to antagonism of P2X7 receptors intrinsic and extrinsic to the NAcc. Future studies, through local administration of antagonists and analysis of gene and protein expression in brain regions outside the NAcc, will better define neural circuits underlying the MDPV-P2X7 interaction. It should also be mentioned that facilitation of ICSS has been shown by other groups at doses as high as 3.2 mg/kg, which is closer to the range of doses in which we detected an effect on P2X7 protein (Bonano et al., 2014; Watterson et al., 2014).

mRNA and protein levels of mitochondrial ATP synthase, an enzyme that catalyzes synthesis of ATP, an endogenous ligand for P2X7, was also enhanced by MDPV. Because increased ATP synthase gene and protein expression occurred only after exposure to a dose of MDPV that was 10-fold higher than that required to facilitate ICSS, the relevance of changes in ATP synthase message to MDPV-induced reward is unclear. ATP itself also has low affinity for P2X7 and, under homeostatic conditions, is unlikely to reach a high enough concentration to activate P2X7 receptors. However, MDPV can produce sustained elevation of extracellular monoamines, locomotor activity and body temperature that could increase extracellular ATP to a level sufficient for P2X7 activation, while simultaneously causing enhancement of neuronal ATP utilization and depletion of ATP stores that could eventually lead to enhanced ATP synthase gene and protein expression. Methamphetamine, in fact, rapidly increases local cerebral glucose utilization in multiple brain regions but induces striatal ATP loss 1.5 h after discontinuation of repeated exposure (Chan et al., 1994). Thus, an

increase in extracellular ATP, not measured in the present experiments, may have occurred with the lower dose of MDPV (0.5 mg/kg) and contributed to the facilitation of ICSS whereas increased ATP synthase expression was only apparent at higher MDPV doses.

Neurochemical mechanisms by which enhanced P2X7 activity facilitated MDPV reward may be dopaminergic-related. ICSS induces dopamine release in the NAcc, and is further enhanced by drugs that increase extracellular dopamine in NAcc but blocked by drugs that reduce dopamine or block dopamine receptors, indicating that mesolimbic dopamine neurons are important mediators of ICSS, particularly when the stimulating electrode is located in the medial forebrain bundle or ventral tegmental area (Negus and Miller, 2014). Endogenous purines appear to support dopamine release in the NAcc by influencing physiology of afferents in the VTA (Krügel et al., 1999, 2001; Kittner et al., 2001). Application of ATP or the P2X/Y receptor agonist 2-methylthio ATP into rat striatum or NAcc, respectively, increases extracellular dopamine (Krügel et al., 1999; Zhang et al., 1995). The latter agonist also enhances explorative locomotion (Kittner et al., 1999). Thus, in our study, the P2X7 block by A438079 may have reduced the MDPV-induced enhancement of extracellular dopamine that underlies facilitation of ICSS, leading to inhibition of MDPV-induced reward. A P2X7 block might not only prevent direct facilitative effects of ATP on extracellular dopamine but also lead to a predominance of adenosine over ATP effects under conditions of MDPV exposure, resulting in greater activation of adenosine A_1 and A_{2A} receptors that inhibit drug-induced neuronal activation and reward (Krügel, 2016).

Both psychostimulant abuse and P2X7 activation induce neuroinflammation, which in turn contributes to psychostimulant dependence. Thus, another possibility is that MDPV and related psychostimulants (*e.g.,* cocaine, methamphetamine), through downstream P2X7 upregulation, release pro-inflammatory cytokines (*e.g.,* IL-1, IL-6 and TNFα) that contribute to abuse liability (Krügel, 2016; Bachtell et al., 2017). Still another possibility, as shown with MDMA (Rubio-Araiz et al., 2014), is that MDPV altered BBB permeability through a P2X7R-mediated event, leading to increased brain concentrations of MDPV that enhanced rewarding efficacy.

In summary, we showed that a psychostimulant dysregulates the P2X7 system in the brain reward circuit and produces rewarding effects through a P2X7 receptor-dependent pathway. Future studies will study P2X7-MDPV interactions in additional abuse liability models, such as operant self-administration and conditioned place preference, assess the efficacy of P2X7 receptor antagonism against the rewarding effects of related psychostimulants (*e.g.,* cocaine, methamphetamine), and uncover downstream mechanisms underlying the P2X7-dependent inhibition of psychostimulant-induced reward enhancement.

Role of funding source

The present work was supported by National Institutes of Health and National Institute on Drug Abuse through grants R01 DA039139, R01DA045499, R01DA031064, P30 DA013429, and T32 DA007237.

Contributors

Taylor Gentile, Steven Simmons, Mia Watson and Shu Su conducted ICSS experiments. Slava Rom and Chris Tallarida conducted gene and protein expression experiments to analyze P2X7 and ATP synthase expression. Allen Reitz synthesized MDPV. Scott Rawls and Raghava Potula designed experiments and contributed to data analysis, interpretation, and manuscript preparation. Scott Rawls prepared and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

No conflict declared.

Acknowledgement

None.

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