

# Atypical dopamine efflux caused by 3,4-methylenedioxypyrovalerone (MDPV) via the human dopamine transporter



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## ABSTRACT

Synthetic cathinones are similar in chemical structure to amphetamines, and their behavioral effects are associated with enhanced dopaminergic signaling. The past ten years of research on the common constituent of bath salts, MDPV (the synthetic cathinone 3,4-methylenedioxypyrovalerone), has aided the understanding of how synthetic cathinones act at the dopamine (DA) transporter (DAT). Several groups have described the ability of MDPV to block the DAT with high-affinity. In this study, we demonstrate for the first time a new mode of action of MDPV, namely its ability to promote DAT-mediated DA efflux. Using single cell amperometric assays, we determined that low concentrations of MDPV (1 nM) can cause reverse transport of DA via DAT. Notably, administration of MDPV leads to hyperlocomotion in *Drosophila melanogaster*. These data describe further how MDPV acts at the DAT, possibly paving the way for novel treatment strategies for individuals who abuse bath salts.

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## 1. Introduction

The neurotransmitter dopamine (DA) mediates behaviors relating to reward, motivation, attention, and cognition (Iversen and Iversen, 2007; Bjorklund and Dunnett, 2007). Important to dopamine neurotransmission is the dopamine transporter (DAT). DAT is a presynaptic membrane protein responsible for the reuptake and recycling of DA following vesicular release (Giros and Caron, 1993). Dysfunctions in DAT can lead to dopamine-associated neuropsychiatric disorders including ADHD, autism spectrum disorders, schizophrenia, and bipolar disorder

(Gowrishankar et al., 2014). DAT is also the target of commonly abused psychostimulants and controlled substances, namely cocaine and amphetamine (AMPH). Cocaine acts as a high-affinity antagonist of the transporter and blocks DA uptake, whereas AMPH acts as a substrate of the transporter and, through a series of intracellular mechanisms, causes DAT to reverse transport or “efflux” DA into the extracellular space (Schmitt and Reith, 2010). The actions of cocaine and AMPH on the DAT are well-known to play a role in their rewarding properties and abuse potential. Thus, determining the effects of psychostimulants on DAT function is important for understanding the neural and molecular mechanisms underlying psychostimulant drug action.

In recent years, the abuse of synthetic cathinones or “bath salts” has become a major world-wide health concern (German et al., 2014). These substances are synthetic derivatives of the naturally-occurring stimulant, cathinone, found in the flowering plant *Catha*

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*edulis* (Brenneisen et al., 1990). The psychoactive effects of synthetic cathinones vary from the cocaine-like stimulant effects seen with 3,4-methylenedioxypropylvalerone (MDPV) (Baumann et al., 2013) to the MDMA-like empathogenic effects of methylone (3,4-methylenedioxymethcathinone) (Liechti, 2015). Among a number of identified biological sites, cathinones are known to target proteins that modulate dopamine neurotransmission, increasing dopaminergic signaling and associated behaviors (Glennon et al., 1987; Nguyen et al., 2016; King et al., 2015; Novellas et al., 2015; Kehr et al., 2011; Bonano et al., 2014), including drug-seeking (Watterson et al., 2013; Lisek et al., 2012). When consumed in small doses, cathinones can lead to euphoria, alertness, increased libido, and elevated blood pressure. When consumed at higher doses, tremors, seizures, paranoia, violent behavior, psychoses, tachycardia (Borek and Holstege, 2012), delusions/hallucinations (Penders and Gestring, 2011), and death (Wyman et al., 2013) can occur. A recent report released by the Substance Abuse and Mental Health Services Administration (SAMHSA) showed that nearly 23,000 emergency room visits in 2011 were a result of cathinone abuse (SAMHSA 2013 Bath Salts Report [https://www.dea.gov/druginfo/drug\\_data\\_sheets/Bath\\_Salts.pdf](https://www.dea.gov/druginfo/drug_data_sheets/Bath_Salts.pdf)). Due to the high risk associated with the use and the abuse potential of these compounds, the Drug Enforcement Administration (DEA) designated mephedrone (4-methylmethcathinone), methylone and MDPV as Schedule 1 substances under the Controlled Substances Act [http://www.who.int/medicines/areas/quality\\_safety/4\\_13\\_Review.pdf](http://www.who.int/medicines/areas/quality_safety/4_13_Review.pdf) (DEA Drug Fact Sheet on Synthetic Cathinones). Nonetheless, illegal manufacturers continue to circumvent this ban by synthesizing “designer” substances with novel chemical structures but which produce similar psychostimulant effects (Marusich et al., 2014). These compounds are readily available and sold with fraudulent labels such as “plant food”, “research chemicals”, or “bath salts” at gas stations, tobacco stores, and over the Internet with a warning that the contents are not intended for human consumption. Their continued production and availability make it nearly impossible to control the exponentially rising sales and consumption of synthetic cathinones.

Despite increased data regarding the use and abuse of cathinones (Zawilska and Wojcieszak, 2013), little is known about their mechanism of action. To address this issue, several research groups have begun to study the chemistry, pharmacology, and behavioral effects of various synthetic cathinones. Of these, MDPV is most commonly implicated in high-risk use (Borek and Holstege, 2012; Wyman et al., 2013; Marusich et al., 2014; Coppola and Mondola, 2012a; Ross et al., 2012; Wright et al., 2013; Murray et al., 2012; Kriikku et al., 2011). First synthesized in 1969, MDPV gained popularity much later in 2010 (2014 World Health Organization Critical Review Report on MDPV). As a highly lipophilic analogue of the synthetic cathinone pyrovalerone (Coppola and Mondola, 2012b), MDPV readily crosses the blood-brain barrier. Importantly, MDPV, when administered to animals exhibits striatal distribution, a brain region enriched in DA projections (Novellas et al., 2015). MDPV also shows high abuse potential in animal behavioral tasks (King et al., 2015; Novellas et al., 2015; Kehr et al., 2011; Watterson et al., 2014).

Early research on MDPV demonstrated that this drug acts similarly to cocaine (a known DAT blocker), but with a 10- to 50-fold higher potency (Baumann et al., 2013; Cameron et al., 2013). However, increasing data suggests that there may be more to the action of MDPV. Work from Baumann et al. showed that after intravenous administration of MDPV, DA levels remain elevated for far longer than after cocaine administration (Baumann et al., 2013). In addition, MDPV administration results in long lasting cross-sensitization in mice, similar to the effects of methamphetamine (Watterson et al., 2013). These results suggest that MDPV, in

addition to acting as a DAT blocker, may also display other modes of action. To examine further the molecular actions of MDPV on the DAT, we performed amperometric studies. Specifically, to obtain greater temporal resolution, we studied MDPV action on human DAT (hDAT) by employing single cell amperometry. This assay has been previously used to discriminate AMPH versus cocaine actions in a single cell and these results have been reproduced in different model systems (Cartier et al., 2015). Further, we assessed MDPV-induced behaviors in *Drosophila melanogaster*, specifically focusing on known DAT-associated behaviors. *Drosophila* is a powerful genetic model for studying behaviors that are associated with DA as well as promoted by psychostimulants (Cartier et al., 2015; Hamilton et al., 2014; Hamilton et al., 2013), as several genes that regulate DA transport, synthesis, and signaling are conserved between flies and humans (Yamamoto and Seto, 2014).

## 2. Methods

### 2.1. Drugs

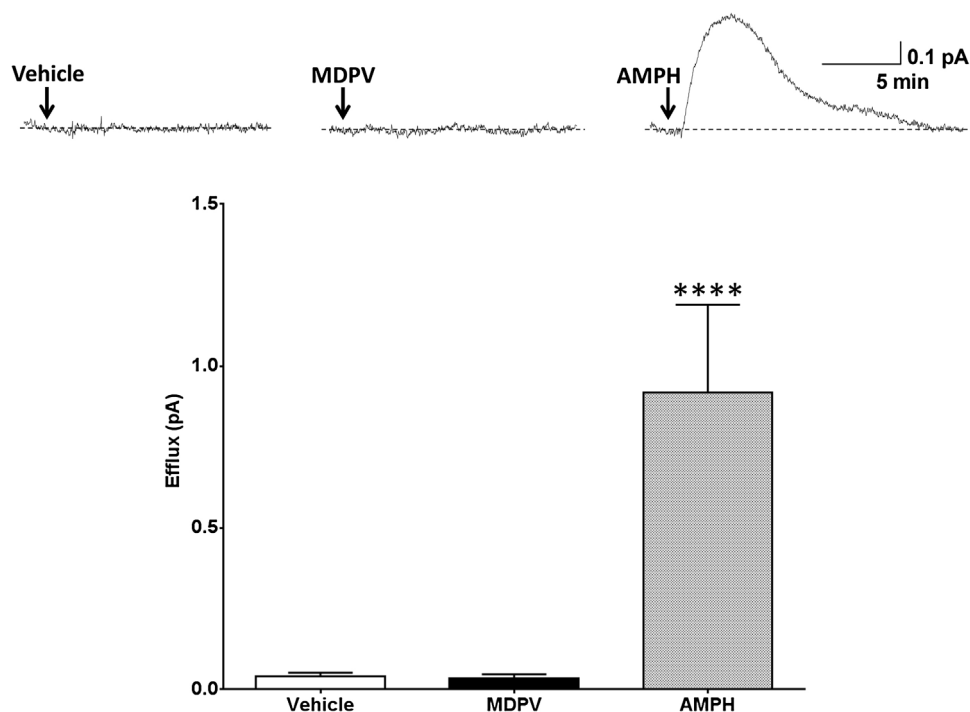
(±)-3,4-Methylenedioxypropylvalerone HCl (MDPV), was synthesized in racemic form in our laboratories. Chemical and structural analysis included nuclear magnetic resonance spectroscopy, gas- and liquid- chromatography/mass spectrometry, thin layer chromatography, and melting point determination. All data were consistent with the expected structures. All other drugs used in this study including their salt and enantiomeric forms were as follows and purchased from Sigma-Aldrich (St. Louis, MO): Dopamine, D-amphetamine hemisulphate salt and cocaine hydrochloride.

### 2.2. Amperometry

Chinese hamster ovary (CHO) cells stably expressing hDAT (here defined as hDAT cells) were plated at a density of ~20,000 per 35-mm culture dish. To preload cells with DA, dishes were washed with KRH assay buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM HEPES, 1.1 mM MgSO<sub>4</sub>, 2.2 mM CaCl<sub>2</sub>, pH 7.4) supplemented with 10 mM dextrose, 100 μM pargyline, 1 mM tropolone, and 100 μM ascorbic acid, and incubated with 1 μM DA in KRH assay buffer for 20 min at 37 °C. To record DA efflux, a carbon fiber electrode (ProCFE; fiber diameter of 5 μm; obtained from Dagan Corporation) juxtaposed to the plasma membrane and held at +700 mV (a potential greater than the oxidation potential of DA) was used to measure DA flux through oxidation reactions. Amperometric currents in response to the addition of 1 nM MDPV were recorded using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) with a low-pass Bessel filter set at 1 kHz; traces were digitally filtered offline at 1 Hz using Clampex9 software (Molecular Devices, Union City, CA). DA efflux was quantified as the peak value of the amperometric current.

### 2.3. *Drosophila melanogaster* behavior

To measure the locomotor response to MDPV we used the TriKinetics *Drosophila* Activity Monitoring (DAM) system as described in earlier studies (Pfeiffenberger et al., 2010; Andretic et al., 2005). Wild-type Oregon-R male flies were entrained for seven days in 12:12 h light:dark (LD) cycles at 25 °C on standard cornmeal-molasses medium. On day two, flies were transferred individually to activity tubes and acclimated for a period of five days. On day seven, flies were transferred into identical activity tubes containing 20 μM MDPV or vehicle (water) in standard medium. Flies were continuously monitored for movement using activity monitors (DAM5, Trikinetics). Activity was measured as the number of times a fly crossed the infrared beam (beam crosses)



**Fig. 1.** MDPV (1 nM) does not elicit an amperometric current in the absence of hDAT cells. Top: Representative amperometric traces recorded from hDAT cells in response to application of vehicle to (Vehicle), application of 1 nM MDPV to bath in the absence of hDAT cells (MDPV), or application of 10  $\mu$ M AMPH to hDAT cells (AMPH). Bottom: Quantitation of the peak amperometric current amplitude measured after vehicle or drug treatment (\*\*\*\* =  $p < 0.0001$  by One-way ANOVA with Dunnett's multiple comparisons post-hoc analysis;  $n = 5-6$ ).

per 30 min. Activity data was recorded for six hours after drug administration. Change in activity in response to drug treatment was reported as beam crosses normalized to average beam crosses 30 min prior to drug administration.

### 3. Results

#### 3.1. 1 nM MDPV alone does not produce an amperometric signal

As a first control experiment, we demonstrated that hDAT cells pre-loaded with DA (see Methods Section) did not release DA upon application of vehicle (Fig. 1, Vehicle). Next, we tested whether MDPV alone reacts at the carbon fiber electrode. At concentrations as low as 1 nM, MDPV did not elicit any amperometric current when applied to a bath chamber in the absence of hDAT cells (Fig. 1, MDPV). Finally, to demonstrate that we can record DA efflux with our amperometric electrode, we show that bath application of 10  $\mu$ M AMPH causes a robust DA efflux in the presence of hDAT cells (Fig. 1, AMPH). We have previously shown that this AMPH-induced DA efflux is mediated by the hDAT and is cocaine-sensitive (Mazei-Robison et al., 2008). These control experiments were conducted to ensure that amperometry is a suitable technique to elucidate the actions of MDPV in terms of DA efflux and that MDPV at a concentration of 1 nM does not produce a non-specific amperometric signal.

#### 3.2. New mode of action of MDPV

24 h after plating, hDAT cells were preloaded with DA (see Methods). Amperometric measurements were taken from individual hDAT cells after application of a low concentration of MDPV (1 nM) or cocaine (10  $\mu$ M). As expected, 10  $\mu$ M cocaine did not cause DA efflux as reflected by a lack of an upward deflection of the amperometric trace (Fig. 2, Cocaine). These data are in agreement

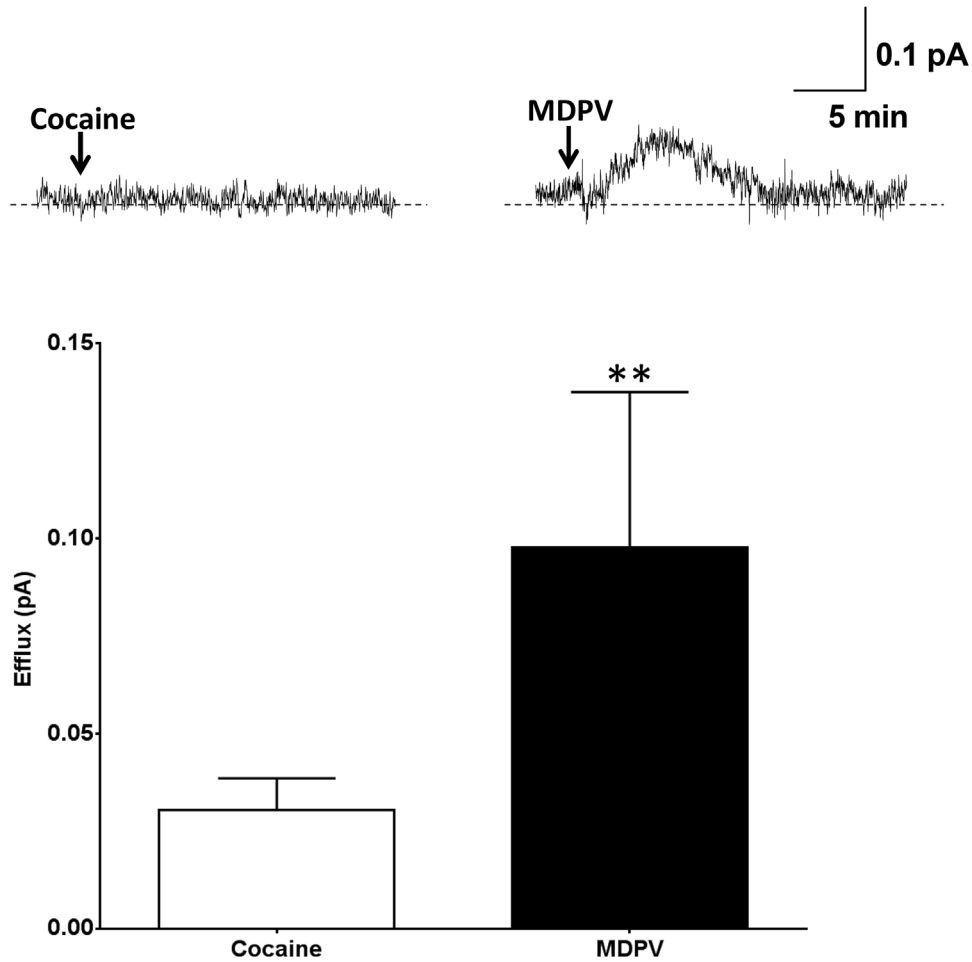
with previously published studies (Hamilton et al., 2013; Mazei-Robison et al., 2008) and also establishes that there is no anomalous dopamine efflux or “leak” associated with the transporter in this *in vitro* system, as previously shown (Mazei-Robison et al., 2008). Surprisingly, and in contrast to the effects of cocaine, amperometric traces recorded in response to 1 nM MDPV show a clear upward deflection of the amperometric current. This upward deflection reflects DAT-mediated DA efflux (Fig. 2, MDPV). In hDAT cells, the peak amperometric responses for MDPV were smaller than those recorded for AMPH (positive controls; compare Fig. 1 (AMPH) to Fig. 2 (MDPV)).

#### 3.3. MDPV causes hyperlocomotion in flies

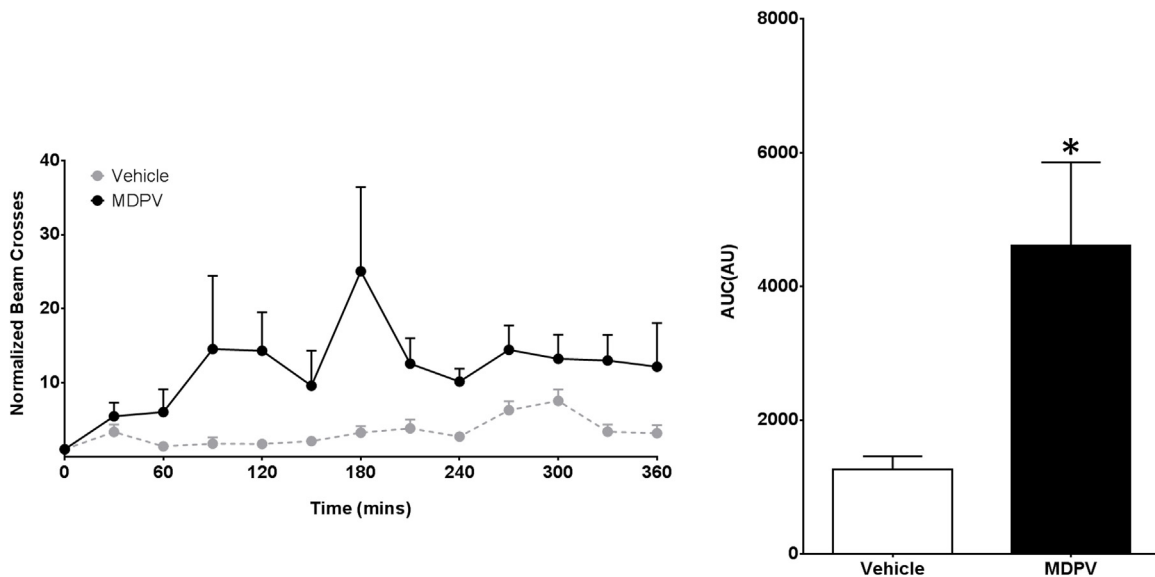
Building on our *in vitro* findings, we examined MDPV's role in modifying DA-associated behaviors in *Drosophila melanogaster*. Wildtype flies were placed in locomotion chambers and acclimated for a period of five days. 20  $\mu$ M MDPV or vehicle was administered orally *via* voluntary consumption. Locomotion was quantified as average beam crosses per 30 min normalized to pre-treatment conditions. Flies administered MDPV ( $n = 16$ ) show an elevated rate of locomotion compared to those administered vehicle ( $n = 15$ ) (Fig. 3, left). Cumulative beam breaks over a period of 6 hours show a greater than two-fold increase in locomotion in flies administered MDPV compared to vehicle (Fig. 3, right).

### 4. Discussion

DA homeostasis in the central nervous system is essential to regulating important brain functions, including reward. Synthetic cathinones disrupt normal dopaminergic neurotransmission and thus affect DA-associated behaviors. These drugs elicit behaviors indicative of enhanced dopaminergic signaling. The past ten years of research on MDPV and other synthetic cathinones demonstrate



**Fig. 2.** MDPV, but not cocaine, induces reverse transport of DA via hDAT. Top: Representative amperometric traces recorded in response to 10  $\mu$ M cocaine or 1 nM MDPV in hDAT cells. Bottom: Quantitation of DA efflux measured as peak amplitude of the amperometric current after drug or vehicle treatment (\*\* =  $p < 0.01$  by Student's  $t$ -test;  $n = 5-8$ ).



**Fig. 3.** MDPV induces hyperlocomotion in flies. Left: Locomotion was measured by average beam crosses following 20  $\mu$ M MDPV ( $n = 16$ ) or vehicle ( $n = 15$ ) administration. Beam crosses were normalized to pre-treatment conditions for each fly. Right: Cumulative beam breaks were quantified for up to six hours post drug or vehicle administration. Flies exposed to MDPV displayed an increase in cumulative beam breaks compared to vehicle-controls (\* =  $p < 0.05$  by Student's  $t$ -test;  $n = 15-16$ ).

that the rewarding properties of synthetic cathinones are derived, in part, from their actions on monoamine transporters (Eshleman et al., 2013; Kolanos et al., 2013). Understanding how MDPV disrupts normal DA neurotransmission *via* its actions on the DAT is essential to the development of novel treatment options that can restore normal DA homeostasis in individuals who abuse MDPV and other synthetic cathinones. In this study, we aimed at revealing new modes of action by which MDPV causes an elevation in extracellular DA levels, and the behavioral consequences of its actions on DAT.

Using single cell amperometry we reveal that low concentrations of MDPV (1 nM) cause reverse transport of DA *via* DAT. Amperometry is a well-established paradigm that has been used by our group and others in several studies to determine different aspects of monoamine release mediated by catecholamine transporters (Cartier et al., 2015; Hamilton et al., 2014; Hamilton et al., 2013; Hamilton et al., 2015). We first established that this assay is suitable for studying reverse transport of DA mediated by MDPV. We conducted an initial characterization of MDPV to demonstrate that at low concentrations, MDPV does not interact with the carbon fiber electrode to produce an artificial signal. Next, we demonstrated that in hDAT cells, MDPV (1 nM) causes reverse transport of DA mediated by hDAT. To note is that higher concentrations of MDPV, such as 100 nM, produced a non-specific amperometric signal (*i.e.* an amperometric current is recorded with MDPV in the absence of hDAT cells). These data describe for the first time a novel mode of action of MDPV at the DAT. Interestingly and importantly, previous work with hDAT has shown that higher concentrations of MDPV can block DAT function (20–30 nM) (Baumann et al., 2013). Taken together, these data suggest that MDPV might have multiple modes of action that are concentration-dependent, where at low concentrations MDPV works to cause DAT reverse transport, and at high concentrations MDPV primarily causes DAT blockade.

*Drosophila melanogaster* has been used as a model organism to study the behavioral consequences of newly discovered molecular mechanisms of AMPH and cocaine (Pizzo et al., 2012; Freyberg et al., 2016). In *Drosophila*, locomotion requires functional DA neurotransmission. Therefore, to understand the significance of the actions of MDPV *in vivo* in terms of changes in extracellular DA levels, we used flies as a behavioral model. Here, we show that MDPV administration leads to hyperlocomotion in *Drosophila melanogaster*. These data point to *Drosophila melanogaster* as a suitable animal model to further characterize *in vivo* the multiple actions of MDPV at the hDAT. This increase in locomotor activity has been previously documented to be associated with an increase in extracellular DA promoted by DAT blockers (*e.g.* cocaine) as well as DA effluxers (*e.g.* AMPH). In this study, we did not determine whether the increase in *Drosophila* locomotor activity is driven by the ability of MDPV to block the hDAT, to cause DA efflux, or both. In the near future, we aim to explore these different possibilities by generating flies that are insensitive to the ability of MDPV to cause DA efflux as we have already done for AMPH (Cartier et al., 2015; Hamilton et al., 2014).

Further studying the different mode of actions of MDPV and other synthetic cathinone drugs as well as their specific behavioral consequences will not only pave the way toward better treatment strategies for those who abuse them, but also lead to better recognition and prediction of the dangers posed by novel designer cathinones that are emerging in the market today.

### Conflict of interest

The authors declare no conflict of interest.

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