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Article

Effects of cathinones (bath salts) on cultured primary neurons/astroglia cells and neurobehavioral functions in mice

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Abstract: This study aims to examine the cytotoxicity mechanisms of synthetic cathinone (bath salts) on rat primary cultured neurons and primary astroglial cells, and to assess their neurobehavioral effects on mice. We administered methylenedioxypyrovalerone (MDPV) to both rat primary cultured neurons and primary astroglial cells to assess cell injury. We also analyzed the effects of MDPV on these cell cultures using immunocytochemistry. We utilized western blotting to assess the breakdown of αII-spectrin and glial fibrillary acidic protein (GFAP) induced by MDPV. The western blotting experiment also included calpain and caspase inhibitors (SNJ1945 and Z-D-DCB, respectively) and pro-apoptotic and pro-necrotic agents (Staurosporine and calcium ionophore A23187, respectively). Lastly, we assessed MDPV's effects on behavioral effects using rotarod, locomotor activity, elevated plus maze, Morris water maze, forced swimming, and open field tests. MDPV caused a dose-dependent release of LDH in both cerebrocortical neuron-astroglia mixed cultures and primary astroglial cultures. MDPV also caused neurite breakages and astroglial process retraction on immunocytochemistry. Lastly, MDPV induced αII-spectrin breakdown in western blotting experiments. Co-administration of calpain and caspase inhibitors reduced the degradation of αII-spectrin and GFAP. MDPV administration also increased anxiety-like behavior and locomotor activity in the mice. Synthetic cathinones, which share structural similarities with methamphetamine, also induce significant neurotoxic effects and neurobehavioral effects on rodent models. These neurotoxic effects are likely mediated by calpain and caspase-induced apoptosis and necrosis, while astroglial death is likely only due to calpain activation. Therefore, further research may focus on pharmacological interventions targeting these pathways to mitigate the cytotoxic impact of cathinones in humans.

Keywords: Cathinones; Designer drugs; Bath salts; Neuronal injury; astroglial injury; Calpain; Caspase

1. Introduction

Cathinones are naturally occurring beta-ketone amphetamine (AMPH) analogues found in Catha edulis plant leaves. Synthetic Cathinones (SCs), often referred to as "legal highs" or "bath salts," are phenylalkylamines derivatives, classes of psychoactive

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designer drugs that act as central nervous system (CNS) stimulants by increasing synaptic neurotransmitter concentrations.[1-4] These substances are highly addictive in humans and animal models. They also result in significant morbidity and mortality as evidenced by the rise of synthetic cathinone abuse resulting in alarming emergency room incidents, police altercations, and documented toxicities including multi-organ failure and death. [5-15]

Commonly abused cathinone derivatives include methylenedioxypyrovalerone (MDPV), butylone, pyrovalerone (Fig 1), mephedrone (4-methylmethcathinone), dimethylcathinone, ethcathinone, ethylone, 3- and 4-fluoromethcathinone, mephedrone and methylone (MEPH). [16] Early clinical observations, including cerebral edema, seizures, excitatory delirium, agitation, panic attacks, psychosis, suicidal ideation, and chronic paranoia, raised concerns about the neurotoxic effects of SCs. Consequently, methedrone and MDPV were permanently classified as Schedule 1 - controlled substances under the Synthetic Drug Abuse Prevention Act in 2012. [6, 17-20]

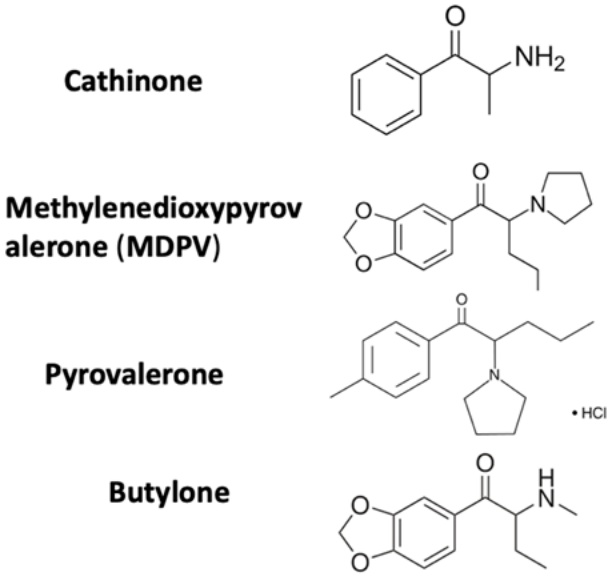

Figure 1. Structures of cathinone used in this study

SCs, being β-keto analogs of amphetamines, may be expected to have amphetamine-like effects because of their structural similarity. Psychostimulant drugs like methamphetamine (METH) and MDMA (Ecstasy) have neurotoxic properties and lead to long-lasting, destructive, astroglial and microglial activation. [7-13] METH also leads to similar psychiatric symptoms as SCs.[4, 21-23]. Given the profound acute and long-lasting brain functional and behavioral changes observed in human SC use, it is tempting to suggest that, like METH, these substances induce neurotoxic or induce sustained brain structural, functional, and psychological alterations.[24]

Several animal model studies have evaluated the mechanism of the neurotoxic effect of SCs to elucidate the mechanisms underlying these clinical manifestations. Some benchmarks used to gauge the neurotoxicity induced by amphetamines include inflammation, disruption of monoaminergic neurotransmitters, their transporters and receptors, alterations in thermoregulation, oxidative stress, and cytotoxicity.

In recent years, in-vitro studies have attempted to identify the neurotoxic potential of several SCs, [25-30] identifying oxidative stress and mitochondrial dysfunction mechanisms as significant contributors to SC-induced neurotoxicity.[28, 29, 31, 32]Moreover, the role of neuronal calcium signaling in mitochondrial dysfunction and the induction of neurotoxic cascades for SCs shown by Leong et al. extends the link to SCs disrupting neuronal Ca2+ homeostasis leading to downstream mitochondrial dysfunction in dopaminergic neuronal SH-SY5Y cells. [33] Such studies have contributed to the

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understanding of the cathinone-induced neuronal damage but the evidence about forms of toxicity, apoptosis versus necrosis and the consequent involvement of neuroinflammatory processes of different SC drugs as compared to similar structure drugs like methamphetamine is scarce where these drugs such as methamphetamine has shown adverse effects on neural cells similar to what is observed in brain injury [9, 34, 35]. Hence, the paucity of scientific research and understanding of neurotoxic mechanisms determined the two-fold aims of the study where we: first, examine the effects of various doses of MDPV, butylone, and pyrovalerone on primary cultured neurons and astroglial cells by analyzing the mechanism of cell death using a cellular marker, neuroinflammatory effects and cellular oxidative stress; and second, assess SCs' behavioral and neuromodulatory effects by administering intraperitoneal (i.p.) pyrovalerone and MDPV in mice for 7 consecutive days.

2. Results

2.1. Cathinone effects on rat primary cerebrocortical (CTX) cells

2.1.1. Lactate dehydrogenase (LDH) assay of the medium containing primary cerebrocortical (CTX) cells treated with control and MDPV

Primary cerebrocortical cells, a mixture of neuronal and astroglial cells, were cultured from the rat brain and challenged with MDPV at 20, 60, 120, 400 and 800 ◎M. The treated groups were compared with the untreated CTX cells. LDH, a cytosolic enzyme, is released into the cell culture medium as a result of primary necrosis and secondary necrosis due to apoptosis. [36-38] After 48 hr of treatment with MDPV, the CTX cells underwent a significantly increased release of LDH in a dose-dependent manner, beginning at 60 ◎M presented as the mean absorbance◎S.E.M. (Fig 2)

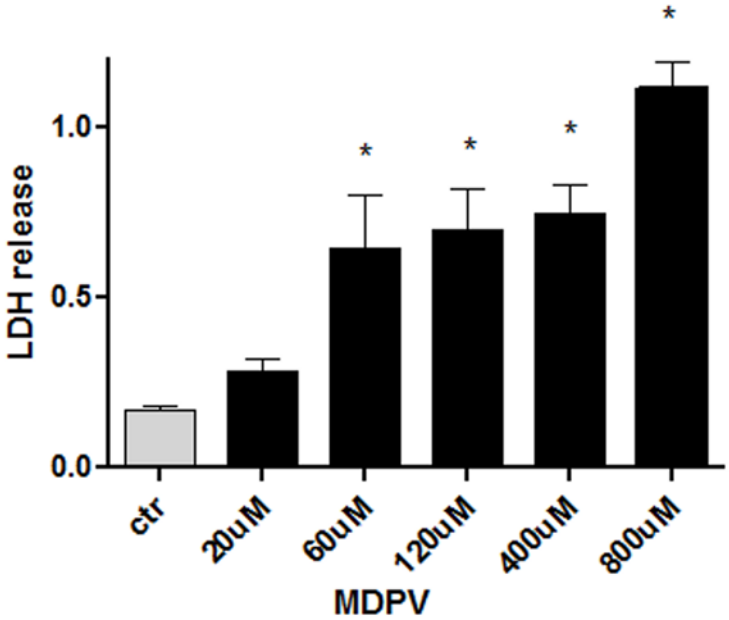

Figure 2. LDH release following MDPV administration in primary CTX mixed culture: MDPV induces dose-dependent cytotoxicity 48 h post-administration in primary cerebrocortical neuron-astroglia mixed culture (CTX) as evidenced by lactase dehydrogenase (LDH) release.

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2.1.2. Immunocytochemical changes in the CTX cell culture after drug treatment

We then assessed the structural integrity and morphological cell changes in the CTX culture using GFAP (green) to stain glia and microtubule-associated protein (MAP2) (red) to stain neurons. DAPI (blue) was used to stain the nuclei of both the neurons and astroglia. A23187 and STS were used as pro-necrosis and pro-apoptosis controls, respectively. A23187 treated CTX cells showed neuron and astroglial cell death by the near-complete disintegration of neurite and astroglial processes and cell bodies. STS, on the other hand, caused neurite and astroglial process breakage. MDPV at 400 ◎M at 24 h induced neurite breakages (yellow arrows) and dramatic astroglial process retractions (orange arrows). (Fig 3)

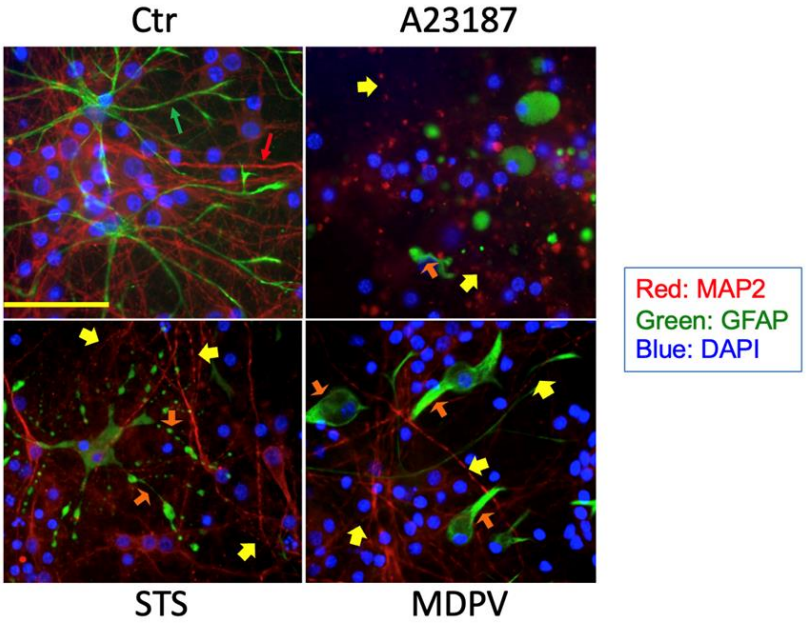

Figure 3. Immunocytochemistry demonstrating neuron and glia cytoskeletal damage in rat primary CTX mixed culture following MDPV administration. Red: MAP2, green: GFAP, blue: DAPI. Red arrow: healthy neurite. Green arrow: healthy astroglial process. 400 μM MDPV at 24 h induced neurite breakages (yellow arrows) and dramatic astroglial processes retractions (orange arrows). A23187 and STS were used as pro-necrosis and pro-apoptosis controls. A23187 caused neuron and astroglial cell death with complete disintegration of neurite and astroglial processes and cell bodies. STS conversely caused neurite and astroglial process breakage. Scale bar = 50 μm

2.1.3. Degradation of ◎II-spectrin and astroglial fibrillary acidic protein after treating CTX with MDPV

Next, we assessed MDPV toxicity in the mixed CTX culture by assessing the axonal injury markers using western blots for αII-spectrin, its breakdown products (SBDP), and astroglial fibrillary acidic protein (GFAP), and its breakdown products (GBDP). The 280kDa αII-spectrin and 50kDa GFAP parent proteins were present in both control and MDPV-treated rats. A23187 (10 μM) and STS (0.5 μM) were used as pro-necrosis and pro-apoptosis controls, respectively. They served as a positive control group and demonstrated increases in the 150, 145, and 120 kDa breakdown products of ◎II-spectrin. The total ◎II-spectrin antibody readily showed modest increases in the 150, 145 and 120 kDa SBDPs after 48 h of 400 MDPV ◎M treatment. (Fig 4a) In contrast, astroglial fibrillary acidic protein's (GFAP's) breakdown products (GBDP) were not evident from the Western blot analysis for the same 400 MDPV ◎M treatment. (Fig 4a)

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In all treatment groups, the use of the calpain inhibitor SNJ (S) attenuated increases in the calpain-mediated SBDP145, while the caspase inhibitor Z-D-DCB (Z) attenuated increases in the caspase-3 generated SBDP120.

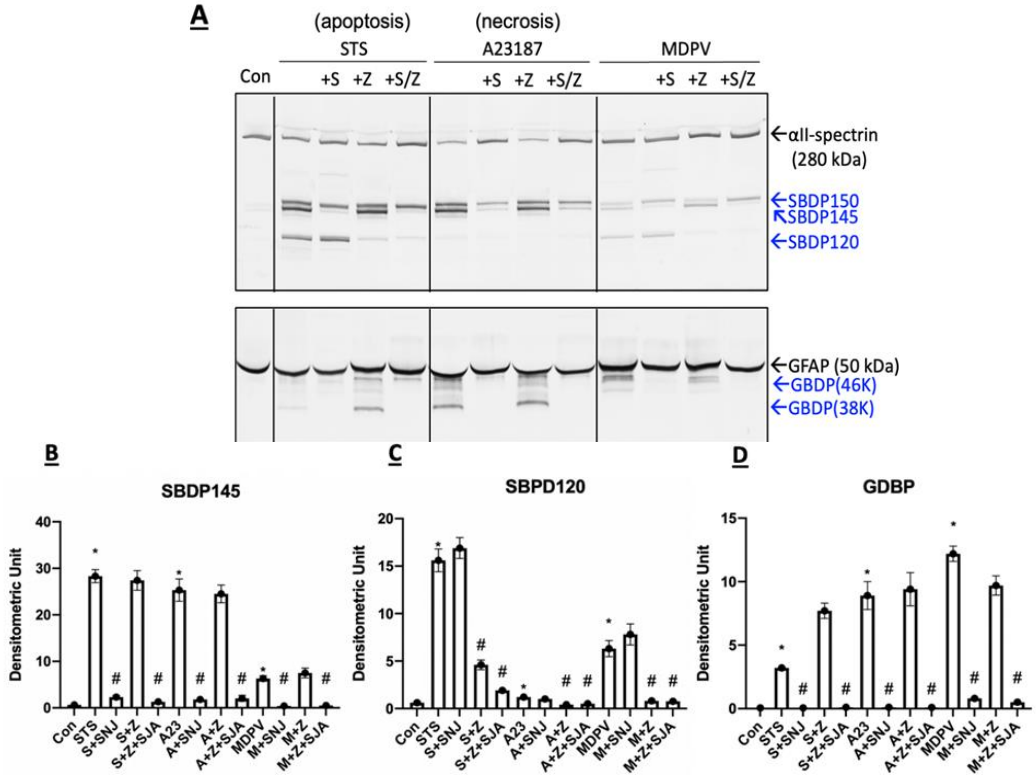

Figure 4. Western blot demonstrating MDPV-induced cytotoxicity in primary CTX cultures. Figure 4a. MDPV administration leads to neuronal injury as reflected by breakdown products of αII-spectrin (neuronal injury marker). The presence of SBDP120 is indicative of the apoptotic phenotype induced by MDPV (400 μM). For comparison, pro-apoptosis STS and pro-necrosis A23187 challenge for 48 h. The presence of calpain inhibitor SNJ1945 (S) blocks the calpain-mediated SBDP145, and GBDP46K and 38K, while caspase inhibitor Z-D-DCB (Z) blocks the caspase-3 generated SBDP120. Of note, the astroglial injury was not evident considering the lack of GFAP breakdown products (astroglial injury marker). Figures B-D represents the relative quantitative concentrations of SBDP145, SBDP120, and GDBP in the various experimental groups. # = statistically significant decrease ($p<0.05$) of the STS, A23187, or MDPV experimental groups including caspase and calpain inhibitors from those not using those inhibitors. * = statistically significant increase ($p<0.05$) of the STS, A23187, or MDPV experimental groups compared to control.

2.2. Cathinone effects on primary astroglial cells

2.2.1. Lactate dehydrogenase (LDH) assay of the medium containing primary rat astroglial cells treated with control and MDPV

We then examined the effects of cathinones on astroglial cells alone using primary rat astroglial cultures by again measuring LDH by absorbance ◎S.E.M. MDPV resulted in gliatoxicity at 24 hours as evidenced by LDH release with as low of a concentration as 125 ◎M, and more significantly with higher concentrations. (Fig. 5a) Pyrovalerone and butylone also showed dose-dependent gliatoxicity. Remarkably, 0.5 mM of pyrovalerone and 1mM of butylone both caused greater cell death than that caused by the same concentration of methamphetamine (METH) and caused nearly as much cell death as the A23187 and STS pro-necrotic and pro-apoptotic controls. (Fig. 5b)

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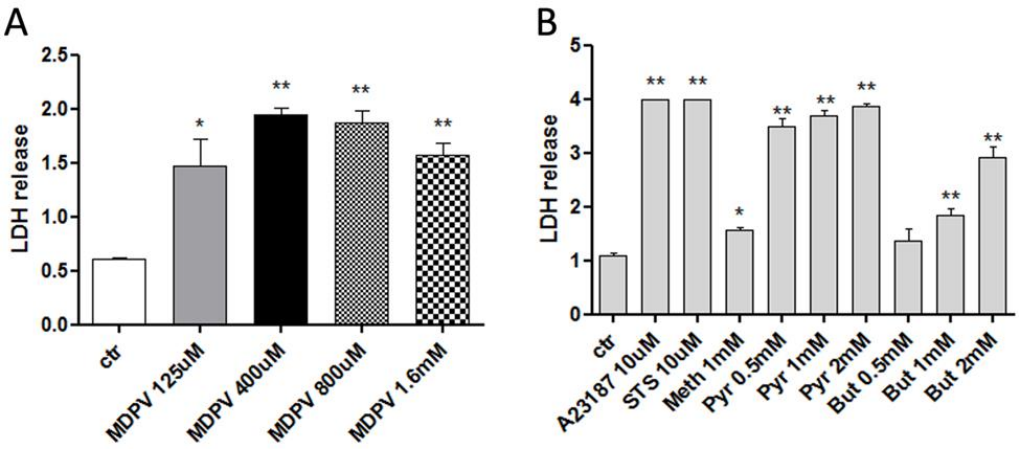

Figure 5. LDH release following MDPV administration in primary rat astroglial culture: MDPV-treated primary rat astroglial culture at 24 h show glia-toxicity as evidenced by LDH release. (A) All dosages of MDPV caused a statistically significant increase in LDH release compared to control. (B) Pyrovalerone and Butylone caused greater LDH release than methamphetamines. A23187 and STS were used as pro-necrotic and pro-apoptotic controls, respectively. Compared with control, *P<0.05, **P<0.01.

2.2.2. Immunohistochemical changes in the primary rat astroglial cells after MDPV treatment

Next, we assessed the integrity of the primary astroglial cells using immunocytochemistry. GFAP (red) was used to stain astroglia, and DAPI (blue) was used to stain their nuclei. The control group had elongated astroglial cell processes. 10 μM of the pro-necrotic control A23187 led to near-complete retraction of the astroglial processes and cell body rounding. 0.5 μM of the pro-apoptotic control STS, and 400 μM of MDPV treatment resulted in similar retractions of the astroglial processes. (Fig 6)

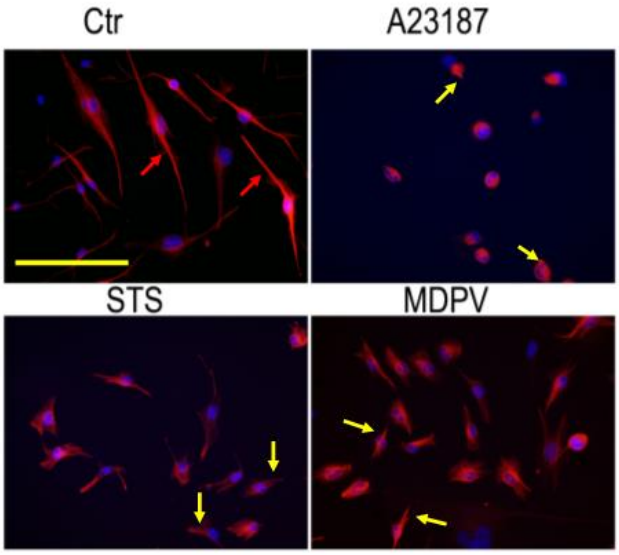

Figure 6. Immunocytochemistry demonstrating astroglial cytoskeletal damage in rat primary astroglial culture following MDPV administration Red: GFAP, blue: DAPI. MDPV treatment (400 μM) of rat astroglia culture for 24 h versus pro-necrotic A23187 (10 μM) and pro-apoptotic STS (0.5 μM) challenge. Red arrows indicate healthy astroglial processes. Shortening of astroglia processes is observed in all three challenges. Scale bar = 50 μm

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2.2.3. Degradation of ◎II-spectrin and astroglial fibrillary acidic protein after treating primary astroglial cultures with MDPV

Western-blot analyses for GFAP and its BDPs were also performed on cell lysate from the rat primary astroglial cell cultured at 48h following the MDPV drug challenge. The parent protein was observed at 50 kDa. Although not evident in the above western blot experiment on the mixed CTX culture, treatment with as little as 20 ◎M of MDPV induced the formation of BDPs. The presence of calpain inhibitor SNJ (S) blocks the MDPV-induced calpain-mediated GBDP-38K, while caspase inhibitor-D-DCB (Z) did not. These results suggest that astroglial death is due to calpain activation from MDPV. (Fig 7A) In addition, both Pyrovalerone and Butylone (0.5 or 1 mM, were used) treatment also showed increases in the calpain-mediated GFAP breakdown products of 42 K and 38K (Fig 7B)

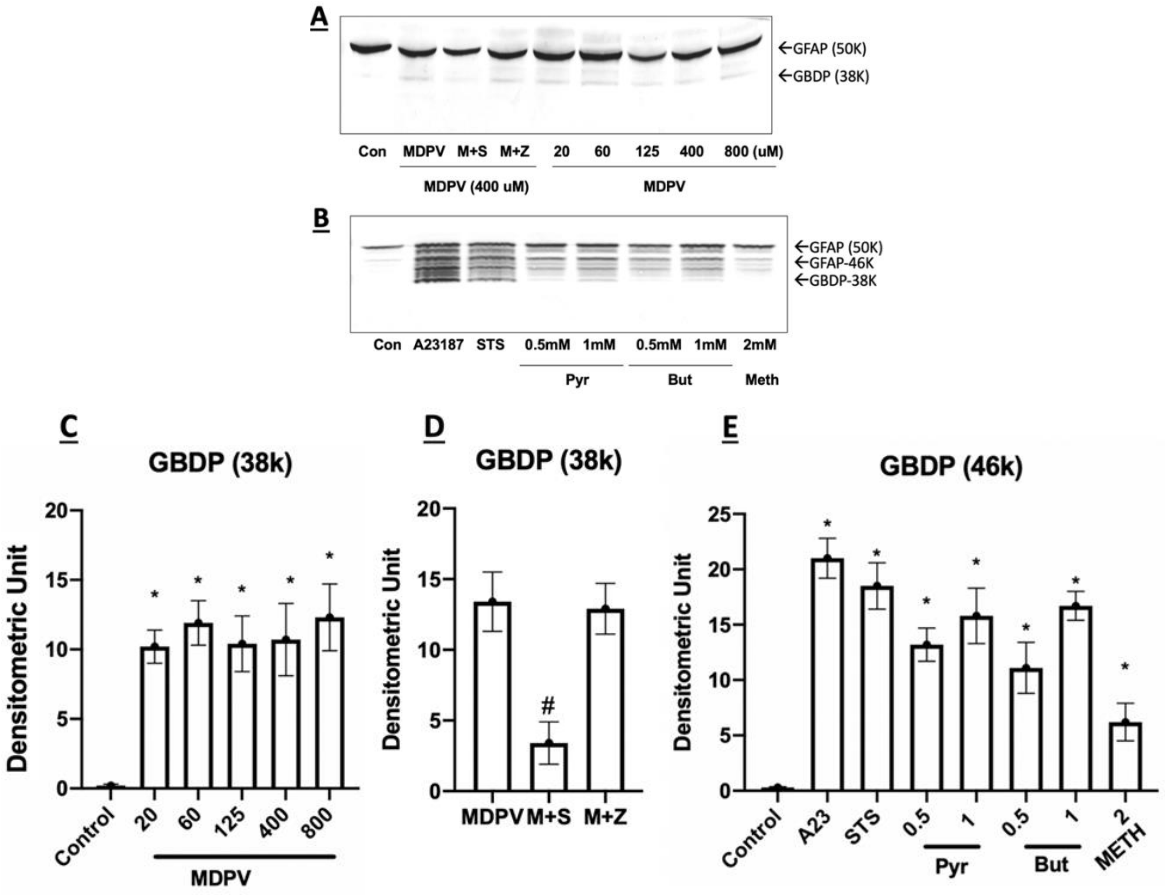

Figure 7. Western blot demonstrating MDPV-induced cytotoxicity in primary astroglial cultures. (A): Cathinones treated primary rat glial cells produce cell death as evidenced by increased GFAP-BDP formation. The presence of the 38k GFAP-BDP is indicative of astroglial death as produced by MDPV-induced cell death protease calpain activation. The presence of calpain inhibitor SNJ (S) blocks the MDPV-induced calpain-mediated GBDP-38K, while caspase inhibitor-D-DCB (Z) did not. (B) In addition, both Pyrovalerone or Butylone (0.5 or 1 mM were used) treatment also shows dose-dependent calpain-mediated 42 K and 38K GFAP-BDPs. (C-E) represent the relative quantitative concentrations of GBDP (38k and 46k) in the various experimental groups. * = statistically significant increase compared to control. # = statistically significant decrease ($p<0.05$) of GBDP (38k) with the use of calpain inhibitor CNJ.

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2.3. Neurobehavioral effects

Next, we examined the neurobehavioral effects of cathinones on mice. Mice were randomly administered saline or specific drug dosages intraperitoneally after being trained to perform various activities. Their performance was assessed at 30 minutes, 1 day, and 7 days following drug administration.

2.3.1. Effects of MDPV on rotarod test and locomotor activity in mice

The rotarod test demonstrated did not show significantly increased time spent in the rotarod with 5 mg/kg of MDPV. (Figure 7a) Therefore, motor coordination was not affected.

The locomotor activity test was then performed to assess for changes in locomotor activity and anxiety. In comparison to the rotarod test, the same concentration of MDPV produced significantly increased distance traveled at 30 mins, 1 day, and 7 days post-administration. The largest increase in locomotor activity was at 30 mins post-administration, with a tapering effect at the two later time points. (Fig 8.)

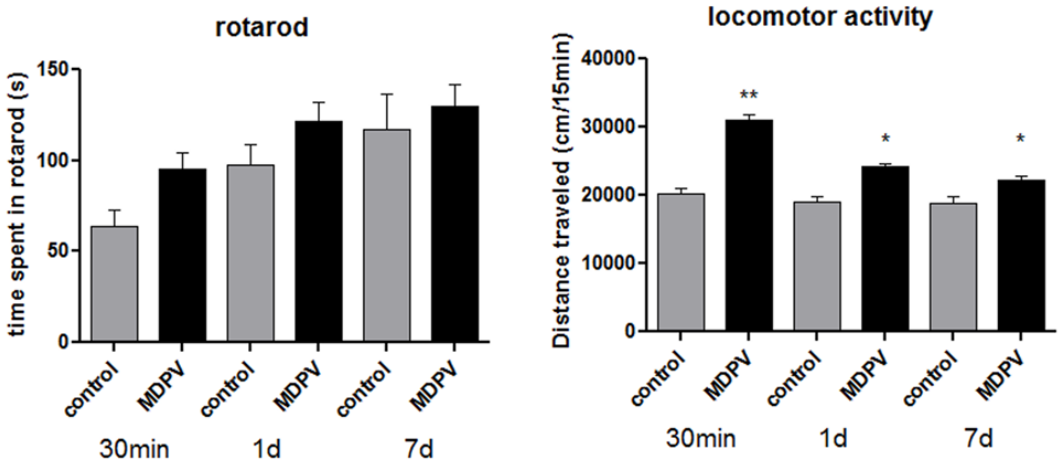

Figure 8. MDPV treatment increased locomotor activity in mice. The rotarod test showed non-significantly increased time spent in the rotarod for MDPV-treated mice compared to controls. However, the locomotor activity test showed a statistically significant increase in distance traveled compared to control at all time periods between 30 minutes and 7 days, suggesting increased locomotor activity and anxiety.

2.3.2. Effects of MDPV on the open field and elevated plus-maze test

The open-field test assesses locomotor activity and anxiety by measuring the total distance traveled and frequency of exploring the center area of the chamber in 30 mins. [39] Mice who were repetitively treated with MDPV traveled longer distances at the 1D, 3D, and 5D time points and entered the center area more frequently when compared with the saline controls. These results were statistically significant and are further reinforced by the elevated plus-maze test which suggested an increased level of anxiety-like behavior by the statistically significantly reduced open arm time in MDPV-treated rats compared to controls. (Fig 9)

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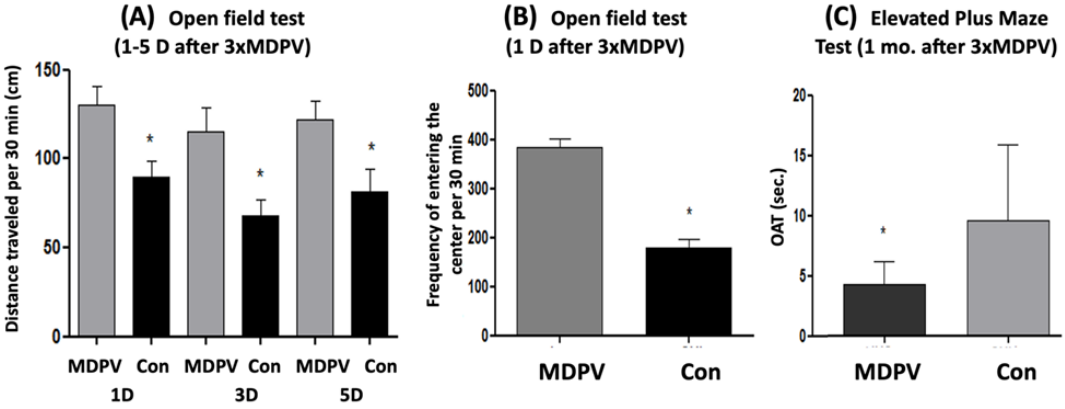

Figure 9. Acute and chronic behavioral changes induced by cathinone MDPV administration. Locomotive activity as measured in the open field test with distance traveled (A) and frequency of entering the center area (B) are significant with 1-5 repetitive 3x MDPV systemic administration (30 mg/kg each dose). In addition, by 1 month after 3x MDPV systemic administration, the mice show signs of anxiety-like behavior in elevated plus maze (EPM) tests as the MDPV groups have significantly less open arm time (OAT) than control (C).

2.3.3. Effects of MDPV on forced swimming test in mice

The forced swimming test is generally used to assess for depression, indicated by increased immobile time. While the MDPV-administered group generally showed less immobility time when compared with the saline-injected group, a statistically significant difference was only observed between one day post-MDPV administration (Fig 10). This suggests that MDPV does not induce depression-like behaviors which is consistent with previous neurobehavioral tests demonstrating increased locomotor activity.

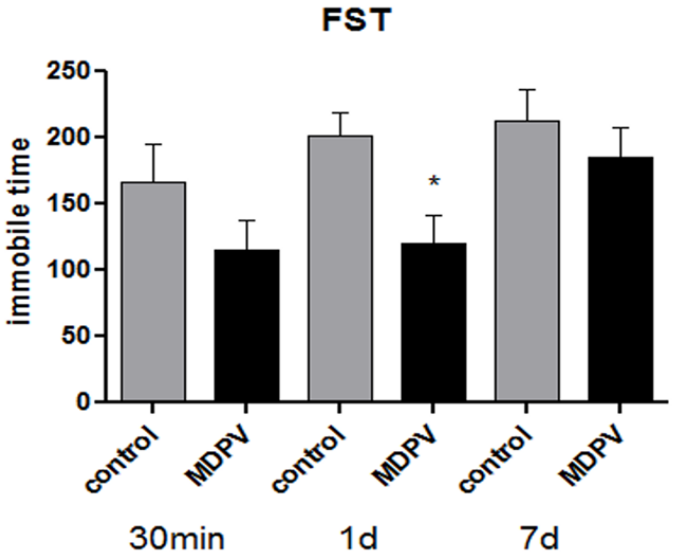

Figure 10. MDPV administration on forced swimming test in mice: MDPV caused decreased immobile time in the forced swimming test at 1d post-MDPV administration, suggesting increased locomotor activity

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2.3.4. Effects of MDPV on Morris water maze test in mice

The Morris water maze test assesses spatial learning and memory. There was no statistically significant change in distance traveled observed five days post-MDPV administration. (Fig 11)

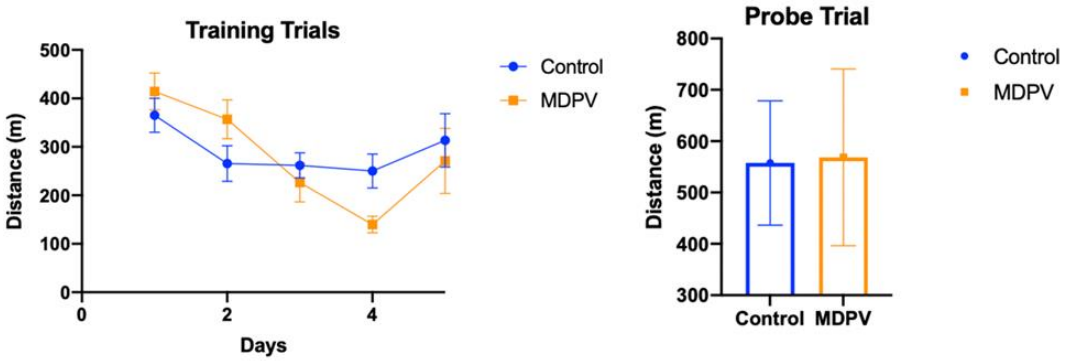

Figure 11. MDPV administration on MWM (Morris water maze) in mice: The probe trial did not reveal a difference in distance traveled between the control and MDPV-treated groups.

In summary, cathinones acutely increase locomotor activity and anxiety-like behaviors in mice. There were no effects on motor coordination, depressive-like behaviors, spatial learning, or memory.

3. Discussion

In this study, we examined the neurotoxic effects of cathinones (bath salts) on primary cerebrocortical cell cultures and primary rat astroglial cell cultures and their neuropertubative effects on mice behavior. Neurotoxic effects of bath salts have been previously reported in various studies which showed reduced cell viability, altered morphology, and apoptosis after acute exposure (24-48 h) mostly in undifferentiated and differentiated human neuroblastoma SH-SY5Y cells. [25, 40, 41] However, a limitation of these studies has been the use of cell lines instead of *in vitro* cultures or *in vivo* animal models which would translate better to human physiology. Cell lines have altered proliferation rates, trans-epithelial resistance, cell permeability, metabolic activities and transport properties compared to the normal parental tissue. Hence, primary cultures have an advantage over cell lines since they express relevant neuronal markers, showcase similar metabolic activity and cellular functions, and portray similar spatial differentiation as seen in physiological systems. [42] In our study, we utilize an in-vitro primary cell culture model that moderately overcomes the literature gap and offers a robust confirmation of the literature by observing the neurotoxic impacts of bath salts on primary cultured cells.

Consistent with the literature, our results from the effect of MDPV on primary cerebrocortical cell cultures analyzed by LDH assays, immunoblot quantifications, and immunocytochemistry indicate a dose-dependent neurotoxic effect on neuronal cells. Neurotoxic effects by MDPV and other cathinones were evident in both CTX and primary rat astroglial cell cultures. While many studies until now have attempted to contribute to the understanding of cathinone-induced neuronal damage, the evidence about the mechanism of cell damage, specifically considering apoptosis versus necrosis, is limited. In our study, we attempted to classify the neurotoxic mechanism on primary cultured cerebrocortical cells. Our findings indicate that co-administration of SNJ, a calpain inhibitor and Z-D-DCB, a caspase inhibitor, with cathinones, reduced degradation of GFAP and alpha-spectrin in the CTX cell cultures, respectively. These findings support the hypothesis that bath salts activate both the pro-apoptotic caspase system and pro-necrotic calpain system leading to neurotoxicity and neuronal death. In the case of astroglial cells, the predominant mode of injury is the activation of the calpain system evidenced by reduced degradation of GFAP when astroglial cells were co-administered SNJ with

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MDPV but no protection with Z-D-DCB. Similar results were observed when CTX cell cultures were challenged with other psychostimulant drugs such as methamphetamine. [43, 44]. The structural similarity between these drugs could be a possible reason for the activation of similar molecular mechanisms involved in neurotoxicity. These results also suggest that developing treatments for neurotoxicity prevention may be possible using these mechanisms.

In addition, we conducted a series of behavioral assessment on mice exposed to cathinones. The results from the mice experiments indicate that one of the defining effects of bath salts is on the locomotor activity of the mice. Here, MDPV-administered mice have shown significantly increased locomotor activity when compared with controls treated with saline. This effect was observed after 30 min of administration of MDPV and sustained after 1 and 7 days after the administration of MDPV. Similar consistent results are seen in other studies. [40, 45, 46] Even though the locomotor activity is increased, coordination and balance as tested by the rotarod test did not show any difference from the controls. This is inconsistent with what is observed in amphetamine exposure but this can be attributed to differences in structure, specific molecular targets, and dosage requirements to produce similar effects. [47]

In our study, there was a general trend of decreased immobility time in the forced swimming test seen in MDPV-administered mice when compared with controls. A significant difference was observed only on day 1. This is in contrast to other studies where there is a significant increase in the immobility time in mice treated with a cathinone (Mephedrone) when compared with controls treated with saline. [48, 49] This could be due to the difference in the pharmacodynamics and kinetics of these two cathinones and implies that MDPV has more stimulant effects leading to increased movements in our model animals. Another consideration could be the difference in animal strain and differences in frequency at which cathinones are administered to the animals which can lead to differences in the severity of the responses/effects.

In our study, anxiety-like behavior after the administration of MDPV was assessed using the elevated plus maze (EPM) and open-field test (OFT). In the OFT, the mice show enhanced locomotor activity with repetitive 3x MDPV systemic administration. This data is consistent with memory and neurobehavioral deficits in mice after TBI, [50-53] and in human studies where hyperactivity has been reported following pediatric TBI. [54] In addition, by 1 month after 3x MDPV systemic administration, the mice showed signs of anxiety-like behavior in elevated plus maze (EPM) tests. Previous studies have shown similar effects, showing less time present in the open arms position. [50, 52, 55, 56] These results indicate that MDPV also has anxiety-producing effects like other cathinones such as methedrone.

There is an increasing trend in the use of cathinones (bath salts) as a drug of abuse or recreational drugs due to their easy availability. Although they share a structural similarity with other psychostimulants such as methamphetamine and MDMA, there are differences in their mode of action and neurotoxic effects on the brain. Various factors such as molecular pathways, differential effects on neuronal and astroglial cells, dosage, route of administration and species could contribute to this discrepancy between cathinones and the well-established psychostimulants (methamphetamine and MDMA) and cathinones. Further characterization of these differences is crucial to evaluate the impact of the drug on human health and to characterize its various interactions with other drugs that can exacerbate neurotoxicity. One common theme among these drugs is their neurotoxic effects on the brain leading to what is observed in brain injury [43, 57-61].

Previously, our research have focused on methamphetamine and MDMA drug of abuse where we indicated that these drugs would induce a similar phenotype as observed in traumatic brain injury (TBI) where we have demonstrated that METH and

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MDMA treatment would lead to neuronal protein structural injury as shown in several studies on TBI [35, 58, 62-64]. Furthermore, applying psychoproteomics analysis on rat brain with methamphetamine highlighted the perturbed pathways involved in neuronal injury, neuroinflammation and altered cellular homeostasis which has been observed in deep proteomics studies applied on animals with TBI [24, 61-67]. Currently, we are performing similar proteomic studies on brain tissue of mice exposed to cathinones.

4. Materials and Methods

4.1. Drug supply

All drugs used in this study, namely pyrovalerone, butylone, MDPV and methamphetamine, were purchased commercially (Sigma Aldrich, St. Louis, MO).

4.2. Cell-based experiments

4.2.1. Preparation of cerebrocortical neuron-glial mixed culture (CTX) and primary rat astroglial cells

Primary cortical neurons were taken from embryonic day 18 Dawley rats and plated on Poly-L-Lysine plates (Erie Scientific, Portsmouth, NH). The cells were first dissociated using trypsin and DNase I and re-suspended in 10% plasma-derived horse serum (PDHS) in Dulbecco's modified Eagle's medium (DMEM). Then, the cells were distributed into 12-well Poly-L-lysine treated plates at the density of 1.0 × 106 cells per well and incubated for three days at 37°C in an atmosphere with 10% CO2. The cells in the 12-well plate were treated with 1 μM 4-amino-6-hydrazino-7-D-ribofuranosyl-7H-pyrrolo (2,3-D)-pyrimidine-5-carboxamide (ARC) for 2 days and then removed. Finally, the cells were grown for 10 -14 days in fresh 10% PDHS in DMEM. Primary astroglial cell cultures were obtained in the same fashion as above. However, astroglial cells were harvested from 1–3-day old pups and were not treated with ARC. All animal studies conformed to guidelines outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the University of Florida.

4.2.2. Neurotoxin challenge and pharmacological intervention

The cells (primary CTX and primary rat astroglial cultured cells), 14 -16 days post-plating, were washed in the neuro-basal medium and challenged with various neurotoxins. CTX cells were challenged with MDPV (20 ◎M to 1.6 mM) for 16 h and primary rat astroglial cells were challenged with MDPV (20 ◎M to 1.6 mM), pyrovalerone (0.5 mM to 2 mM), butylone (0.5 mM to 2 mM) and methamphetamine (1 mM). Neuronal death was assessed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) released into the medium as described previously. Cellular proteins were extracted at 16 hours, as described previously.

4.2.3. Quantification of cell injury or death

Lactate dehydrogenase (LDH) release, used as the marker for cell injury, was measured by using a commercial kit CytoToxicity 96 (Promega). The kit measures the reduction of NAD to NADH which is an indication that necrosis following apoptosis has occurred. We have previously shown the equivalence between LDH and 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltretrazolium bromide (MTT) assays. Hence, only LDH assay was used in the current experiment to measure cell injury.

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4.2.4. Cell lysate preparation

The primary CTX and primary rat astroglial cultured cells were collected and lysed by incubation in the lysis buffer consisting of 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche Biochemicals) for 90 min at 4 OC. The cell lysates were collected and centrifuged at 8000 g for 5 min at 4 OC to clear and remove insoluble debris. The supernatant was collected without disturbing the debris and stored at -80 OC till further analysis.

4.2.5. Gel electrophoresis, electrotransfer and immunoblotting analysis

The samples were added with 2x Laemmli sample buffer containing 65.8 mM Tris (pH 6.8), 0.1 mM DTT, 2% SDS, 0.01% bromophenol blue and 10% glycerol in distilled water. Using SDS-PAGE, proteins were resolved at 200 V for 60 min at room temperature, using 4-20% or 10-20% Tris-glycine gels (Invitrogen Life Technologies). The resolved proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes using the iBlot Gel transfer device for 12 minutes (Invitrogen). After the transfer of the proteins to the membrane, it was blocked in 5% non-fat dry milk in 20 mM Tris-HCl, 150 mM NaCl and 0.003% Tween-20, pH 7.5 (TBST) for 60 minutes. Then, the membrane was incubated with the primary antibody in 5% TBST overnight at 4 OC. The primary antibodies used in the current experiment were monoclonal anti-mouse αII-spectrin (Enzo Life Sciences NY, USA) and monoclonal anti-mouse GFAP (Pharmingen, MA, USA). After overnight incubation, the membrane was washed and incubated with a secondary antibody for 1 h. Immunoreactive bands were detected by developing them with 5-Bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Computer-assisted densitometric scanning (NIH ImageJ, version 1.6 software) was used for protein quantification as described previously.

4.2.6. Immunocytochemistry

Immunocytochemistry analyses were performed on both CTX and primary astroglial cultures. The following fixation for 10 minutes with 4% paraformaldehyde, the cells were washed with PBS and permeabilized with 0.1% Triton X-100 for five minutes. Staining was then performed following a 1-hour blocking step in 10% goat serum. Antibodies included polyclonal rabbit-anti-MAP 2 (Abcam ab556320) (1:200), GFAP (BD #566330) (1:200 dilution), DAPI (Proteintech Group, Chicago, IL, USA) (1:2000 dilution), Alexa 488-conjugated goat-anti-rabbit secondary antibody (Green) (Molecular Probes, Eugene, OR, USA) (1:1000), and Alexa 568-conjugated goat-anti-rabbit secondary antibody (Red) (Molecular Probes, Eugene, OR, USA) (1:1000). We captured fluorescent images using a 40 objective on the Olympus DP72 fluorescent microscope (Olympus America Inc, Center Valley, PA, USA)

4.3. Animal-based experiments

4.3.1. In vivo drug administration procedure

Mice (male C57BL/6) were randomly assigned to the saline control group and different drug groups with specific drug dosages. Before the administration of the drugs, the mice were tested and trained for various activities. The mice were randomly selected for i.p. saline or MDPV (5mg/kg) administration. The drugs were administered intraperitoneally (i.p.) on day 0 and mice were tested for various behavioral activities at 30 mins, 1 day, and 7 days following the time of drug administration.

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4.3.2. Rotarod test

An accelerating rotarod (Ugo Basile) which automatically increased rotation slowly from 4 to 40 rotations per min (rpm) over 5 min was used to test motor coordination, as previously described [23]. Each mouse was placed on the rotarod and the time duration for which the mouse stayed on the rotating rotarod was recorded. The time duration for which the mice could stay on the rotating rotarod was taken as the extent to which they could maintain motor balance and coordination.

4.3.3. Locomotor activity

A locomotor activity test was done as previously described [24]. The study was conducted using 40 Digiscan (model RXYZCM, Omnitech Electronics, Columbus, OH) locomotor activity testing chambers (40.5 X 40.5 X 30.5 cm) housed within sound-attenuating chambers in sets of two. A panel of infrared beams (16 beams) and corresponding photodetectors were located in the horizontal direction along the sides of each activity chamber. A 7.5-W incandescent light above each chamber provided dim illumination and fans provided an 80-dB ambient noise level within the chamber. Locomotor activity was expressed as the total number of beam breaks for each 10-min within the 90-min session.

The locomotor activity tests were conducted according to the procedures described in detail in our previous publications (Gregg et al. 2013, 2015). Briefly, the locomotor activity of individual rats was measured during the light cycle in activity chambers using a Digiscan DMicro System (Accuscan Inc., Columbus, OH). Two types of locomotor activity were automatically measured: (1) ambulatory activity produced by horizontal movements and (2) stereotypy resulting from recurring or focused movements.

Basal locomotor activity was measured for 30 min before rats were administered 2-PMPA or NAAG or the corresponding control condition. This time also enabled rats to acclimate to the activity chambers. Thirty minutes later, rats were injected with saline or MDPV and locomotor activity was recorded for 90 minutes. The exception to this was experiment 4A where rats did not receive an injection of saline or MDPV.

4.3.4. Elevated plus maze test

The maze experiments were conducted with a wooden elevated plus maze (EPM) with arm dimensions of 50 cm × 10 cm, closed arm sidewalls of a height of 30 cm and a central platform of 10 cm2. The maze was raised to a height of 1 m and housed in a sound-proof room. The maze was surrounded by black cloth to eliminate visual cues to the rats. All test sessions were conducted during the dark phase of a rat's light: dark cycle (1100–1500 h). The room was illuminated with red light since the sensitivity of the EPM in detecting anxiolytic properties of drugs is improved with red light illumination (Violle et al., 2009). In between explorations of the maze, the apparatus was thoroughly cleaned with a damp cloth and 70% ethanol to remove residual odor and left to dry before the next test. The test involved placing the rat in the center of the maze, facing an open arm, while monitoring its exploratory activity for 5 min. The test was recorded using a video camera from which the behaviors were scored by two observers according to the exploratory and ethological parameters described below. Each animal was tested once to avoid the phenomenon of one-trial tolerance, a condition that changes the emotional state of an animal when retested in a maze (Albrechet-Souza et al., 2007; Dawson and Tricklebank, 1995). Rats were considered to have entered an arm of the maze when they had all four paws in the arm. The number of entries into the two open arms compared to the total entries into all four arms was used to calculate the percentage of open arm entries. The percentage of the 5 min period that the rat spent in the two open arms of the maze was calculated as the open arm time.

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4.3.5. Morris water maze test

Morris water maze (MWM) test was performed as previously described [25]. This test was conducted in a round white pool 94 cm in diameter and 31 cm deep. The pool was filled to a depth of 30 cm with water made opaque with white non-toxic water-based tempura paint. The pool temperature was maintained at 24 ± 1°C by the addition of warm water. The escape platform was a 25 cm2 Plexiglas square, placed in the center of one quadrant of the pool, 15 cm from the pool's edge and submerged 1 cm beneath the water surface. The platform remained in the same position throughout the learning trials and visual cue tests and was removed from the pool during the probe test. Several distal extra-maze cues (a traffic cone, a colorful poster, and two black-and-white construction paper designs) were placed around the pool and these remained in the same position throughout the training and testing periods.

A trial began by placing the mouse on the platform for 20 s to allow orientation to extra-maze cues. After orientation, mice were gently lowered tail-first into the pool facing the wall at one of three positions, each at the center of the wall of a different quadrant, not housing the platform. After the mouse was released, the researcher retreated away from the pool to a constant position within the room, serving as an additional distal visual cue. The SMART digital tracking system (Version 2.5, Panlab, Barcelona, Spain) simultaneously began recording the trial. Maximum swim time was set to 60 s. If the mouse located the platform before 60 s had passed, it was immediately removed from the pool. If the platform was not located after 60 s of swimming, the mouse was gently guided to the platform and allowed to re-orient to the distal visual cues for an additional 20 s before being removed from the pool. After removal from the pool, mice were manually dried with a terrycloth towel and placed in a warming cage (consisting of a heating pad set to low underneath a typical shoebox cage) for at least 5 min before returning to the home cage. Mice were visually inspected to ensure thorough dryness. Mice were tested in two trials per day with an inter-trial interval of approximately 30 min. All testing was conducted at roughly the same time each day to minimize variability in performance due to the time of day.

After completion of the visual cue test, all tracks from all trials were analyzed for several behavioral parameters using SMART software (Panlab).

4.3.6. Forced swimming test

The forced swimming test (FST) was performed as described previously [26]. This test was arranged as the final test to avoid interference with other behavior tests performed on the mice. Mice were placed in a cylinder (25 cm high, 10 cm in diameter) filled with 19 cm of water. The temperature of the water was maintained at 24 ± 1 °C. Mice were allowed to swim for 6 min, the first 2 min were not taken into consideration and the immobility time was recorded for the last 4 min. The immobility and struggling time were analyzed by EthoVision XT 9.0 software (Noldus, Wageningen, Netherlands).

4.3.7. Open field test

The open-field test was performed as described previously [27]. The open-field test was performed in dim light (15 lux). The dimension of the open box apparatus used was 50 cm × 50 cm × 40 cm with the size of the center zone as 25 × 25 cm2. Mice were individually placed into the center of the open box and allowed for 5 min to explore. The activity of the mice during the exploration behaviors was tracked by video and analyzed using the EthoVision XT 9.0 software (Noldus, Wageningen, Netherlands).

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4.3.8. Statistical Analysis

Discrete variables are represented in frequencies and percentages and continuous values are represented by mean ± SEM. Group differences were evaluated by one-way or two-way analysis of variance (ANOVA) or two-tailed Student t-test using GraphPad Prism software. Statistical significance was determined by p values of <0.05.

5. Conclusions

Synthetic cathinones have neurotoxic effects as they cause increased LDH release, αII-spectrin and GFAP breakdown product formation, and immunocytochemical changes in brain cell cultures. Furthermore, they cause significant increases in agitation and anxiety in rodent models. The neurotoxic effects are likely secondary to calpain- and caspase-induced apoptosis and necrosis, respectively. Further research is needed to further investigate these mechanisms of neurotoxicity and to develop pharmaceutical interventions targeting these pathways.

Supplementary Materials: N/A

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, K.W. and F.K.; methodology, R.D.A. and A.D.; validation, A.D., T.S. and K.P.; formal analysis, T.S. and K.P.; investigation, R.D.A. and F.K.; writing—original draft preparation, K.P. and Z.Y.; writing—review and editing, M.G. and V.R.; supervision, Y.Z. and F.K.; project administration, K.W..; funding acquisition F.K.

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Institutional Review Board Statement: The animal study protocol was approved by the IACUC Study #201810118. All animal studies conformed to guidelines outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the University of Florida

Informed Consent Statement: Not applicable

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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