Neurotoxicology and Teratology 73 (2019) 15–21

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

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

When fish take a bath: Psychopharmacological characterization of the effects of a synthetic cathinone bath salt 'flakka' on adult zebrafish

Tatiana O. Kolesnikova[a,1], Sergey L. Khatsko[a,1], Oleg S. Eltsov[a], Vadim A. Shevyrin[a], Allan V. Kalueff[b,c,d,e,f,g,h,i,*]

[a] Ural Federal University, Ekaterinburg 620002, Russia
[b] School of Pharmacy, Southwest University, Chongqing 400700, China
[c] Institute of Translational Biomedicine (ITBM), St. Petersburg State University, St. Petersburg 199034, Russia
[d] Granov Russian Research Center of Radiology and Surgical Technologies, Ministry of Healthcare of Russian Federation, St. Petersburg 197758, Russia
[e] ZENEREI Research Center, Slidell, LA 70458, USA
[f] The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA 70458, USA
[g] Vivarium, Ural Federal University, Ekaterinburg 620002, Russia
[h] Institute of Experimental Medicine, Almazov National Medical Research Centre, Ministry of Healthcare of Russian Federation, St. Petersburg 197341, Russia
[i] Scientific Research Institute of Physiology and Basic Medicine, Novosibirsk 630017, Russia

ARTICLE INFO

Keywords:
Zebrafish
Drugs of abuse
α-PVP
Anxiety behavior
Sedation
Synthetic cathinone

ABSTRACT

Alpha-pyrrolidinopentiophenone (α-PVP) is a synthetic cathinone which exerts robust mental and physiological effects clinically, as well as causes aberrant stereotypic behaviors and altered locomotion in rodents. Given the rich spectrum of pharmacological activity of α-PVP in rodents and humans, as well as its high abuse potential, further studies are needed to better understand the pharmacology and toxicology of this drug. The zebrafish (*Danio rerio*) is a relatively novel model organism in neuropharmacology and toxicology research. Here, we characterize behavioral effects of α-PVP in adult zebrafish following its acute (1, 5, 25 and 50 mg/L for 20 min) and chronic (1, 5 and 10 mg/L for 7 days) treatments. Overall, acute exposure to α-PVP evoked psychostimulant (but not anxiolytic-like) effects in zebrafish novel tank test, with characteristic stereotypic 'side-to-side' bottom swimming at 5, 25 and 50 mg/L. The high-performance liquid chromatography/high-resolution mass spectrometry (HPLC/HRMS) analyses of zebrafish brains showed detectable levels of α-PVP following its acute administration, likely underlying the observed behavioral effects. Although acute 2-day discontinuation of chronic 7-day α-PVP at 1, 5 and 10 mg/L produced no effects, hypolocomotion occurred after a 7-day chronic treatment and repeated withdrawal, resembling rodent effects of some chronic psychostimulants. Collectively, these findings support zebrafish sensitivity to α-PVP and show some parallels with its effects in mammals and humans. This study also suggests that aquatic models based on zebrafish can help further examine the CNS effects evoked by α-PVP and screen for related synthetic new psychoactive drugs.

1. Introduction

Alpha-pyrrolidinovalerophenone (α-PVP) is a pyrrolidine *N*-substituted synthetic cathinone (Katselou et al., 2016; Wood et al., 2016; Zawilska and Andrzejczak, 2015). A potent psychotropic drug, α-PVP belongs to a large family of *β*-keto-amphetamine compounds derived from cathinone (Wood et al., 2016; Zawilska and Andrzejczak, 2015), which is the primary psychoactive alkaloid of khat (*Catha edulis*) (Sykutera et al., 2015). Developed in the 1960s as a CNS stimulant, α-PVP has recently reached drug market as an abused substance (Katselou et al., 2016; Kolesnikova et al., 2019), sold under street names "flakka", "bath salts" or "gravel" (Kaizaki et al., 2014; Sellors et al., 2014; Sykutera et al., 2015). The drug is currently a Schedule 1 agent in USA, and is strictly controlled globally (Kolesnikova et al., 2019).

Poorly understood, the psychopharmacological profile of α-PVP includes potent inhibition of the dopamine (DAT) and norepinephrine (NET) transporters, with no effects on the serotonin (SERT) transporter (Kolanos et al., 2013; Liechti, 2015; Marusich et al., 2014; Tyrkko et al., 2016). The IC_{50} of this drug ranges between 12.8 and 205 nM for DAT, 4.2–20 nM for NET, and > 10,000–30,000 nM for SERT in rats

* Corresponding author at: School of Pharmacy, Southwest University, Chongqing 400700, China.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).
[1] These authors have equally contributed to this study.

https://doi.org/10.1016/j.ntt.2019.02.001
Received 27 August 2018; Received in revised form 16 February 2019; Accepted 19 February 2019
Available online 20 February 2019
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(Marusich et al., 2014) and humans (Kolanos et al., 2013; Liechti, 2015), respectively. A-PVP is more potent than cocaine and amphetamine as a DAT/NET blocker (Liechti, 2015), suggesting its likely high abuse potential. Thus, given the rich spectrum of pharmacological activity and strong behavioral/physiological effects of α-PVP in rodents and humans, further studies are needed to understand the psychopharmacology of this and related compounds in various model organisms.

The zebrafish (*Danio rerio*) is a novel, rapidly accepted model organism in psychopharmacological research (Bhandari, 2016; Kalueff et al., 2014; Stewart et al., 2015). Due to its cost-efficiency, easy housing, rapid reproduction, sensitivity and high physiological and genetic homology to humans, zebrafish are a useful tool for studying complex brain disorders (Kalueff et al., 2014; Maximino et al., 2013; Neelkantan et al., 2013; Sackerman et al., 2010; Wong et al., 2010). Zebrafish have well-developed and conserved glutamatergic, opioidergic, monoaminergic and other neurotransmitter systems (Stewart et al., 2011a,b,c), making them a useful model to study CNS drugs (Kalueff et al., 2016). Zebrafish are also highly sensitive to all major classes of neurotropic drugs (Kalueff et al., 2014), including various drugs of abuse (Kyzar et al., 2012; Mi et al., 2016; Neelkantan et al., 2013; Stewart et al., 2011a), and their toxicity (McGrath and Li, 2008; Stewart et al., 2011a). Further characterizing the effects of α-PVP in-vivo, the present study aimed to examine its acute and chronic behavioral effects in adult zebrafish.

2. Methods

2.1. Animals and housing

A total of 540 mature (~5–7 months old) male and female wild type short-fin zebrafish (~50:50 male:female ratio) were obtained from a local commercial distributor and housed in groups of 15 fish per 20-L tank, filled with filtered system water maintained at 25 °C. Illumination (950–960 lx) was provided by 36 ceiling-mounted 18-Wt fluorescent light tubes with a 10/14 light/dark cycle according to the standards of zebrafish care (Westerfield, 2000). All fish used in this study were experimentally naive and fed twice daily with Tetramin-Pro (Terta GMBH, Osnabruck, Germany). Animal experiments were approved by the Institutional IACUC, and fully adhered to National and International guidelines and regulations.

2.2. Behavioral testing and pharmacological manipulations

Behavioral testing was performed between 11.00 and 17.00 h using tanks with water adjusted to the holding room temperature, to assess zebrafish behavior in the novel tank test. Prior to testing, fish were pre-exposed in a 0.5-L plastic beaker for 20 min to either drug-treated or drug-free vehicle, 0.1% solution of dimethyl sulfoxide (DMSO, Tathimpharmpreparaty Inc., Kazan, Russia). This concentration of DMSO is known to be devoid of own behavioral effects in zebrafish, as consistently reported in the literature (e.g., Cachat et al., 2013; Goldsmith, 2004; Kolesnikova et al., 2017; Kyzar and Kalueff, 2016), and is commonly used in zebrafish drug studies. In Experiment 1, the fish were randomly divided into 5 groups: drug-free control as well as 1, 5, 25 and 50 mg/L α-PVP-treated fish (n = 12–16 per group). In Experiment 2, behavioral effects of chronic 1-week α-PVP treatment (n = 20 fish per group) in controls vs. 1, 5 and 10 mg/L were assessed. We also analyzed zebrafish behavior 2 days following the acute withdrawal of the respective α-PVP concentrations (Experiment 3), and after a 1-week repeated (5 h/day) withdrawal of these α-PVP concentrations (n = 20 fish per group, Experiment 2 Part 2).

A-PVP for this study was obtained from the Russian Federal Drug Control Service (FDCS, Ekaterinburg, Russia), as part of a 2009 joint pilot collaborative research project with Ural Federal University to screen a battery of new psychoactive substances. The chemical structure and identity of the drug were confirmed by nuclear magnetic resonance (NMR) and high-performance liquid chromatography/high-resolution mass spectrometry (HPLC/HRMS), fully corresponding to those previously reported for α-PVP in the literature (Casale and Hays, 2012; Tyrkkö et al., 2013). The concentrations used in the present study were chosen based on our pilot experiments with the drug. The standard 20-min pre-treatment time for Experiment 1, and 1-week chronic treatment protocol, were chosen here based on our pilot studies α-PVP and on our prior experience with screening various CNS drugs in zebrafish models (Riehl et al., 2011). The two-day acute withdrawal model for Experiment 2 was selected based on a previously published similar zebrafish withdrawal protocol (Cachat et al., 2010). The repeated withdrawal protocol for Experiment 3 was chosen here based on similar zebrafish models of repeated drug withdrawal reported previously (Cachat et al., 2010).

For behavioral testing, fish were individually exposed to the novel tank test, a simple, standardized and sensitive method to assess zebrafish anxiety and locomotion (Levin et al., 2007; Stewart et al., 2011a). The tank consisted of a 1.5-L rectangle tank (15 cm height × 20 cm length x 5 cm width; Aquatic Habitats, Apopka, FL, USA) filled with water filled up as full as possible and divided into two equal virtual horizontal portions by a line marking the outside walls. Trials were recorded by camera for further analyses. Zebrafish behavior was then processed by a highly trained observer blinded to the treatments, using the RealTimer software (Open Science, Krasnogorsk, Russia) to manually score different behavioral endpoints (intra-rater reliability > 0.85), including top entries frequency, top duration (s), and the latency to enter the top (s), the number and duration (s) of freezing bouts and total erratic movements. Additionally, characteristic drug-evoked stereotypic side-to-side (wall-to-wall) lateral swimming ~1-1.5 cm from the bottom (Fig. 1 inset) was assessed here. Because this phenotype includes continuous episodes of stereotypic activity, it was impossible to separate them into individual bouts to reliably quantify their frequency and duration. Thus, since this behavior did not occur in control fish, we quantified the number of animals presenting this phenotype (Table 1). Freezing was defined as a total absence of movement (except for the gills and eyes) for > 2 s (Goldsmith, 2004). Endpoints recorded automatically by Noldus Ethovision XT10 software (Noldus IT, Wageningen, Netherlands) included maximal and mean velocity (cm/s) as well as distance travelled (cm).

Zebrafish locomotor traces were also generated using Noldus Ethovision XT10 software and saved as image files to be independently rated (on a consensus basis, ranked from 1 to n) by three highly trained observers blinded to the treatments (Cachat et al., 2011; Green et al., 2012). This visual assessment was based on general similarity of generated 2D traces (to each other) in terms of spatial distribution of activity (top/bottom), overall amount of locomotion (high/low), and pattern of observed activity (typical/aberrant) within each group. Generally characterized by a tight clustering of the independent raters' scores, this approach enables a rapid selection of the median trace, to be used as representative of the group for the visual illustration reflecting global spatiotemporal patterns of zebrafish swimming (Cachat et al., 2011; Green et al., 2012).

2.3. High-performance liquid chromatography/high-resolution mass spectrometry (HPLC/HRMS) analysis of α-PVP

To examine whether behavioral effects of α-PVP are associated with detectable brain levels of the drug, a separate study on 225 zebrafish performed HPLC/HRMS analysis of zebrafish brain α-PVP levels following acute 20-min systemic treatment with 1 and 10 mg/L. A total 15 brain samples (n = 15) were used per group, each sample representing 5 pooled zebrafish brains (total 75 brains per concentration). Immediately after exposure, zebrafish were euthanized in ice water, decapitated, and their brains quickly extracted on ice, pooled 5 brains per sample, placed into 2-mL plastic centrifuge tubes with 0.3 mL of

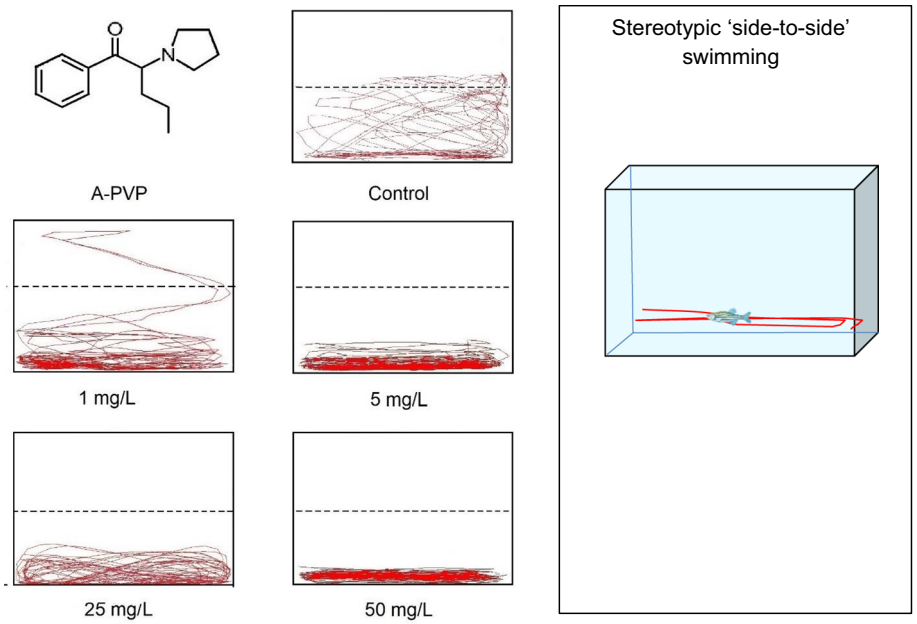

Fig. 1. The chemical structure of α-PVP and typical locomotor traces following its acute 20-min exposure at 1, 5, 25 and 50 mg/L in adult zebrafish tested in the 5-min novel tank test (Experiment 1). Representative traces shown here were selected (on a consensus basis) as median tracks based on ranking from 1 to n by three highly-trained raters blinded to the treatments. Inset: characteristic stereotypic 'side-to-side' swimming (also see Table 1).

Table 1
Effects of acute 20-min α-PVP exposure on stereotypic 'side-to-side' swimming in adult zebrafish (Experiment 1, see Fig. 1 inset for details) assessed by RealTimer software. NS – no significance ($p > 0.05$, Chi-square test).

Groups	The number of fish showing phenotype	Chi-square test data with Yates correction
Control (n = 15)	0	NS
1 mg/L (n = 15)	0	NS
5 mg/L (n = 15)	9	10.16, p = 0.0014
25 mg/L (n = 16)	6	5.21, p = 0.023
50 mg/L (n = 12)	9	13.67. p = 0.0002

0.25-M formic acid in water added to each tube, and shaken for 15 min. The extracts were then centrifuged using MicroCL 17 centrifuge (Thermo Scientific, USA) at 13000 rot/min for 10 min, removing supernatant for analyses using the Agilent 1290 Infinity II HPLC system connected with a tandem quadrupole time-of-flight (QTOF) accurate mass detector (Agilent 6545 Q-TOF LC/MS; Agilent Technologies, USA). Chromatographic separation was achieved on a Zorbax Eclipse XDB-Phenyl 2.1 mm × 50 mm × 5.0 µm column (960967-912, Agilent Technologies), with the column thermostat temperature at 35 °C. The mobile phase was prepared from 0.2% aqueous formic acid with 10 mM ammonium formate (component A) and 0.2% formic acid in acetonitrile (component B). The gradient program was as follows: 0 min 0% B, constant at 0% B to 3 min, linear to 60% B at 5 min, constant at 60% B to 7 min, back to 0% B and equilibration for 5 min.

The flow rate was 0.25 mL/min. QTOF instrument was operated with an electrospray ion source (Dual AJS ESI) in positive ion mode. Nitrogen at 300 °C, flow rate 8 L/min was used as a drying gas. Sheath gas temperature was set at 400 °C, sheath gas flow rate 12 L/min. The nebulizer pressure was 40 psi, the capillary voltage 3000 V and the fragmentor voltage 75 V. In MS/MS mode the quadrupole was adjusted to isolate precursor ion with bandwidth $\Delta m/z = 1.3$ (mass-to-charge ratio). Collision induced dissociation (CID) spectra of the precursor ion were recorded with collision energy 25 eV, using nitrogen (99.9%) as a collision gas. Spectra were recorded in the mass ranges 100–1700 Da in MS mode and 50–500 Da in MS/MS mode. The *α*-PVP identification was based on exact mass and CID spectrum generated in the MS/MS mode for protonated molecule of *α*-PVP. The chromatographic peaks for *α*-PVP showed the retention time of 5.41 min in all drug-treated fish brains at both concentrations (1 and 10 mg/L) used. The signal peaks for *α*-PVP in the brain were consistent with the systemic concentrations of the drug given (Fig. 3), and their CID spectra fully corresponded to those previously reported for α-PVP in the literature (Casale and Hays, 2012; Tyrkkö et al., 2013). For the purpose of this study, HPLC/HRMS results were expressed as categorical data (0 – absence, 1 – presence of α-PVP peaks) appropriate for statistical analyses.

2.4. Statistical analyses

Data were analyzed using the Kruskal-Wallis test followed by post-hoc non-parametric Mann-Whitney *U* test with Bonferroni correction, and expressed as mean ± SEM. Additionally, the experimental fish groups were also analyzed for patterns of aberrant (e.g., stereotypic) swimming. To characterize this aspect in detail, we noted the number of zebrafish displaying stereotypic 'side-to-side' swimming from one wall of the tank to another (0 – normal swimming, 1 – stereotypic 'side-to-side' swimming, Fig. 1 inset). The Chi-square test with Yates correction was then applied to these categorical data, to compare the occurrence of this behavior in control and experimental groups. The Chi-square testing was also applied to categorical HPLC/HRMS data. All behavioral analyses planned for this study were included in the Results without omission. All animals tested were included in analyses, without removing outliers. Due to damaged video files during recording, 3 fish (out of 73 tested) were excluded from analyses in Experiment 1. Due to software detection problems, two other fish (out of total 80 tested) were excluded from part 2 of Experiment 2. The intra-rater reliability was assessed by Spearman correlation. Statistical significance was set at $p < 0.05$ in all tests.

3. Results

Overall, acute α-PVP in Experiment 1 did not alter anxiety-sensitive novel tank endpoints (the duration, frequency and latency of top entries, as well as freezing frequency and duration) at all concentrations tested, but induced fewer erratic movements at 25 mg/L (Table 2). However, α-PVP significantly reduced mean, frequency and duration of regular mobility state ($H_{(4,70)} = 17.64$, 26.39 and 26.39, p = 0.00001–0.0015, respectively) and low mobility/immobility ($H_{(4,70)} = 24.72$, 24.91 and 23.11, p = 0.0001, respectively) at 25 and 50 mg/L, without affecting distance travelled in any concentration. The later profile is generally consistent with a psychostimulant effect of the

Table 2
Behavioral effects of acute 20-min exposure to α-PVP in adult zebrafish tested in the 5-min novel tank test (Experiment 1, n = 12–16 per group). There were no significant Kruskal-Wallis (KW) data (NS), except for total erratic movements (*p < 0.05 vs. control, *U* test); see text for other endpoints assessed in this Experiment.

Endpoints	Control	1 mg/L	5 mg/L	25 mg/L	50 mg/L	KW statistics, p
Assessed by RealTimer software						$H_{(4,73)}$
Top entries frequency	4.1 ± 2.5	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	8.76, p = 0.07, NS
Top duration, s	15 ± 8	0.8 ± 0.8	3.2 ± 1.9	8 ± 7	0.3 ± 0.2	7.40, p = 0.12, NS
Top latency, s	226 ± 25	272 ± 20	257 ± 26	267 ± 22	296 ± 3	6.97, p = 0.14, NS
Freezing frequency	1.1 ± 0.2	1.5 ± 0.4	1.0 ± 0.3	1.5 ± 0.5	0.7 ± 0.3	4.49, p = 0.34, NS
Freezing duration, s	126 ± 27	96 ± 28	119 ± 27	195 ± 31	127 ± 41	6.20, p = 0.18, NS
Erratic movements	0.88 ± 0.52	1.33 ± 0.51	1.20 ± 0.66	0.0 ± 0.0*	0.0 ± 0.0	18.36, p = 0.001
Assessed by Noldus Ethovision XT10 software[a]						$H_{(4,70)}$
Distance travelled, m	6.2 ± 1.2	5.7 ± 1.1	4.9 ± 0.9	3.5 ± 1.1	5.0 ± 1.5	4.18, p = 0.38, NS
Mean velocity, cm/s	2.1 ± 0.4	1.9 ± 0.4	1.6 ± 0.3	1.2 ± 0.4	1.7 ± 0.5	4.18, p = 0.38, NS
Angular velocity, deg/s	0.0 ± 0.0	2.8 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.12, p = 0.28, NS

[a] Three animals out of total 73 tested were undetectable as 'objects' during video-tracking, and their video-files were excluded from analyses.

drug as it promotes more rapid movements in zebrafish. In line with this notion, *α*-PVP also lowered acceleration minimum at 25 and 50 mg/L ($H_{(4,70)}$ = 27.48, p = 0.00001) and evoked characteristic stereotypic 'side-to-side' swimming (Table 1, Fig. 1) not typically seen in control fish.

Analyses of chronic effects of α-PVP in Experiment 2 reveal fewer total erratic movements ($H_{(3,80)}$ = 9.62, p = 0.02) and longer freezing duration ($H_{(3,80)}$ = 29.56, p = 0.00001) at all concentrations tested, suggesting a hypolocomotor profile of this drug given chronically. Freezing frequency was significantly lower at 10 mg/L of α-PVP ($H_{(3,80)}$ = 8.25, p = 0.041), while its duration was higher in all experimental groups vs. control ($H_{(3,80)}$ = 29.56, p = 0.00001, Fig. 2). Although top entries frequency and duration remained unaltered in all concentrations tested (Table 3), chronic α-PVP decreased distance travelled and maximal velocity ($H_{(3,80)}$ = 46.03–46.07, p = 0.00001) at 1, 5 and 10 mg/L, reduced mean, frequency and duration of mobility states ($H_{(3,80)}$ = 19.46, 39.57 and 39.69, p = 0.00001–0.0002) in all concentrations, and increased mean, frequency and duration of immobility state ($H_{(3,80)}$ = 37.72, 38.79 and 28.39, p = 0.00001, respectively). Consistent with putative hypolocomotor profile of chronical α-PVP, Part 2 of Experiment 2 showed that 1, 5 and 10 mg/L of this drug do not alter major novel tank endpoints following a 2-day withdrawal period (Table 4), except for evoking higher frequency and duration of high mobility ($H_{(3,78)}$ =10.94-10.95, p = 0.012) at 10 mg/L (p < 0.05, *U* test vs. control for both endpoints). No stereotypic 'wall-to-wall' swimming was observed in Experiment 2 (data not shown).

In the repeated withdrawal study (Experiment 3), α-PVP increased total freezing duration ($H_{(3,\ 80)}$ = 44.25, p = 0.00001) in all three concentrations, and freezing frequency at 1 mg/L ($H_{(3,80)}$ = 8.23, p = 0.0415; p < 0.05, U test vs. control). The number of erratic movements ($H_{(3,80)}$ = 26.61, p = 0.00001) declined in all experimental groups vs. controls, whereas the latency, frequency and duration of top entries were unaffected (Table 5). In contrast, all concentrations lowered distance travelled ($H_{(3,80)}$ = 46.03–46.06, p = 0.00001) and mean, frequency and duration of mobility state, increased mean, frequency and duration of immobility state ($H_{(3,80)}$ = 19.46, 39.57 and 39.69, p = 0.00001–0.0002 for mobility state, and $H_{(3,80)}$ = 37.72, 38.79 and 28.39, p = 0.00001 for immobility state). Minimum and maximum acceleration ($H_{(3,80)}$ = 24.49 and 24.41, p = 0.00001) measures were also lower in the 5- and 10-mg/L groups vs. controls (both p < 0.00001, U test), consistent with a hypolocomotor profile of chronic α-PVP noted earlier. Similar to chronic treament in Experiment 2, no stereotypic 'wall-to-wall' swimming was observed in Experiment 3 (data not shown).

Finally, HPLC/HRMS analyses of *α*-PVP in zebrafish brains in Experiment 4 revealed chromatographic peaks with the retention time of 5.41 min in all drug-treated fish brains at both acute concentrations (1 and 10 mg/L) used. Predictably, no *α*-PVP signal was detected in all brains of untreated control fish (Fig. 3). The Chi-square test with Yates correction applied to these categorical data revealed statistically significant differences between control and both drug-treated groups (n = 15 samples per group, each sample representing 5 pooled zebrafish brains; Chi-square = 9.11, p = 0.0025 vs. controls). Confirming chemical identity of the drug in zebrafish brains, the observed CID spectrum fully corresponded to those previously reported for α-PVP in the literature (Casale and Hays, 2012; Tyrkkö et al., 2013).

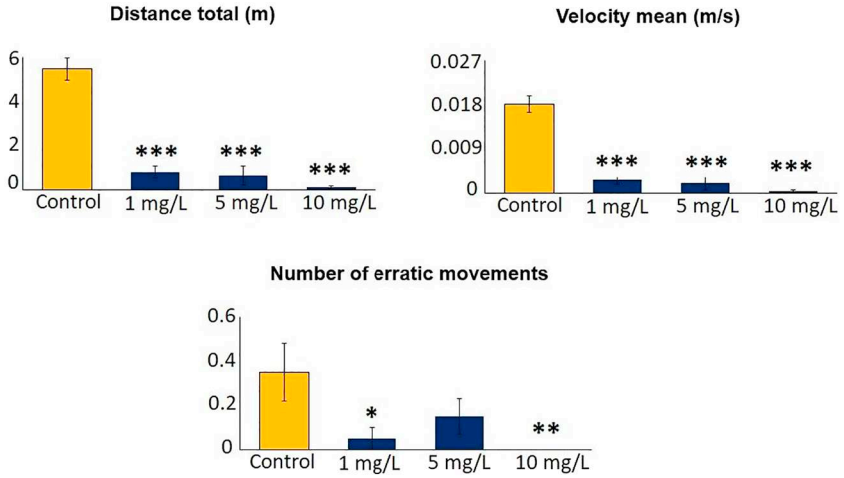

Fig. 2. Summary of significant behavioral effects of chronic (7-day) exposure to 1, 5 and 10 mg/L α-PVP in adult zebrafish tested in the 5-min novel tank test (Experiment 2). Distance travelled and velocity were assessed by Noldus Ethovision XT10 software, the number of erratic movements were assessed by RealTimer software. *p < 0.05, **p < 0.01, ***p < 0.001, U test with Bonferroni correction vs. control for significant Kruskal-Wallis data (n = 20 per group).

Table 3
Selected behavioral effects of chronic (7-days) α-PVP in adult zebrafish tested in the 5-min novel tank test (Experiment 2). There were no significant Kruskal-Wallis (KW) data (NS; n = 20 per group), see text and Fig. 2 for other endpoints assessed in this experiment.

Endpoints	Control	1 mg/L	5 mg/L	10 mg/L	KW $H_{(3,80)}$ statistics, p
Assessed by RealTimer software					
Top entries frequency	0.25 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.08, p = 0.11, NS
Top duration, s	1.16 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.08, p = 0.11, NS
Top latency, s	294 ± 4.3	300 ± 0	300 ± 0	300 ± 0	6.08, p = 0.11, NS
Assessed by Noldus Ethovision XT10 software					
Angular velocity, deg/s	3.0 ± 2.1	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	3.83, p = 0.28, NS

4. Discussion

A-PVP is a relatively new, powerful, addictive and potentially toxic psychoactive substance, currently tightly controlled in the US and other countries worldwide (Kolesnikova et al., 2019). In humans, α-PVP causes robust acute mental (agitation, aggression, hallucinations, delirium, reduced consciousness, confusion, anxiety and/or psychosis) and physiological effects (hypertension, tachycardia, mydriasis, fever, diaphoresis, miosis, seizures and/or hypokalemia), as well as insomnia, headache and lethality (Beck et al., 2016; Hohmann et al., 2014; Katselou et al., 2016; Liechti, 2015; Namera et al., 2013; O'Connor et al., 2015; Saito et al., 2013; Sellors et al., 2014; Sykutera et al., 2015; Wright and Harris, 2016). In rodents, α-PVP activates locomotion, exploration and circular ambulation, also causing flat body posture, other atypical behaviors (e.g., stereotyped head circling and weaving), Straub tail and piloerection (Kaizaki et al., 2014; Marusich et al., 2014; Marusich et al., 2016). Hyperactivity induced by α-PVP is dopamine D_1/D_2 receptor-dependent, and can be reduced by antagonists of these receptors (Kaizaki et al., 2014; Marusich et al., 2014). Compared to methamphetamine, α-PVP evokes higher locomotion with a lesser rise of extracellular dopamine (Kaizaki et al., 2014). A-PVP also exerts overt rewarding effects (e.g., in animal models of intracranial (Watterson and Olive, 2014) or intravenous self-administration (Aarde et al., 2015)) and substitutes cocaine in rodent and monkey drug discrimination tasks (Gatch et al., 2015a; Gatch et al., 2015b; Smith et al., 2016).

Complex neurobehavioral effects evoked by α-PVP in rodents and humans show that the CNS profile of this drug remains poorly understood, therefore necessitating additional models (e.g., simpler vertebrate organisms) to evaluate these responses. The rationale for this approach is that simpler organisms may provide more clear-cut drug responses to a CNS drug in question, likely representing evolutionarily conserved, 'core' mechanisms shared by different taxa. The present study is the first report characterizing neurobehavioral effects of α-PVP in zebrafish.

Overall, acute treatment with α-PVP evoked psychostimulant effects on adult zebrafish (without affecting anxiety-like behaviors) at 5, 25 and 50 mg/L. We also observed a characteristic stereotypic 'side-to-side' bottom swimming pattern at these three concentrations, which was not typical for normal, usually more flexible swimming of control fish (Fig. 1, Table 1). In general, our results in zebrafish are consistent with the data obtained in rodents, where 3 and 10 mg/kg of α-PVP increase locomotor activity in mice after both vapor and injection administration (Marusich et al., 2016). The 10 and 30 mg/kg concentrations also induce atypical rodent behaviors, such as jumping or rearing while facing away from the wall, ataxia, flattened body posture or retropulsion (Marusich et al., 2014).

In the present study, we observed reduced zebrafish activity following a 7-day chronic treatment with α-PVP, suggesting that prolonged intake of this drug inhibits animal locomotor activity, in contrast to acute treatment, which promotes mobility, agitation and stereotypic swimming (Fig. 1, Table 1). Such profile is not unusual, since similar effects of decreased locomotor activity were observed in rodents subjected to chronic administration of amphetamine (Cryan and Markou, 2003). Interestingly, there were no signs of withdrawal-like behavior 2 days after α-PVP discontinuation (1, 5 and 10 mg/L), likely because a longer (or, alternatively, shorter) acute withdrawal period may be needed to affect zebrafish. To further examine the possibility of α-PVP withdrawal syndrome in zebrafish, we applied the repeated withdrawal protocol, noting a general hypolocomotion in zebrafish at 1, 5 and 10 mg/L (similar to chronic exposure) without

Table 4
Summary of behavioral effects of 2-day withdrawal from chronic α-PVP (7 days) in adult zebrafish tested in the 5-min novel tank test (Experiment 2, Part 2). There were no significant (NS) Kruskal-Wallis (KW) data, except for top latency and duration, which yielded no significant differences vs. control by U test (n = 19–20 per group)[a].

Endpoints	Control	1 mg/L	5 mg/L	10 mg/L	KW $H_{(3,78)}$ statistics, p
Assessed by RealTimer software					
Top entries frequency	0.25 ± 0.14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.93, p = 0.03[b]
Number of erratic movements	0.15 ± 0.11	0.06 ± 0.06	0.20 ± 0.12	0.0 ± 0.0	3.42, p = 0.33[b]
Top duration, s	0.87 ± 0.57	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.93, p = 0.03[b]
Freezing duration, s	195 ± 19	201 ± 17	146 ± 21	181 ± 19	4.59, p = 0.20, NS
Freezing frequency	2.10 ± 0.31	2.67 ± 0.33	2.15 ± 0.33	2.95 ± 0.49	3.05, p = 0.38, NS
Top latency, s	285 ± 9	300 ± 0	300 ± 0	300 ± 0	8.93, p = 0.03[b]
Assessed by Noldus Ethovision XT10 software					
Distance travelled, m	3.23 ± 0.69	2.83 ± 0.42	4.39 ± 0.60	3.72 ± 0.51	5.67, p = 0.13, NS
Mean velocity, cm/s	1.08 ± 0.23	0.94 ± 0.14	1.46 ± 0.20	1.24 ± 0.17	5.67, p = 0.13, NS
Mobility state mean	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	1.21, p = 0.75, NS
Mobility state frequency	103 ± 29	89 ± 13	112 ± 14	118 ± 19	4.72, p = 0.19, NS
Mobility state total duration, s	3.41 ± 0.98	2.96 ± 0.42	3.76 ± 0.45	3.96 ± 0.63	4.81, p = 0.19, NS
Immobility state mean	10.1 ± 3.7	5.6 ± 1.0	10.6 ± 7.4	6.0 ± 2.1	5.03, p = 0.17, NS
Immobility frequency	104 ± 29	91 ± 13	114 ± 14	121 ± 19	4.99, p = 0.17, NS
Immobility total duration, s	296 ± 1	297 ± 0.5	296 ± 0.5	296 ± 0.6	4.96, p = 0.17, NS

[a] Video files for 2 fish (of the total 80 fish tested) were damaged during the recording, could not be analyzed using video-tracking software kits, and were not included in analyses reported here.

[b] These significant KW data yielded no significant differences from control by post-hoc *U* test.

Table 5
Selected behavioral effects of repeated withdrawal from chronic (7 days) α-PVP in adult zebrafish tested in the 5-min novel tank test (Experiment 3). There were no significant Kruskal-Wallis (KW) data (NS, n = 20 per group); see text for other endpoints assessed in this experiment.

Endpoints	Control	1 mg/L	5 mg/L	10 mg/L	KW $H_{(3,\ 80)}$ statistics, p
Assessed by RealTimer software					
Top entries frequency	0.05 ± 0.05	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.00	3.00, p = 0.39, NS
Top duration, s	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.00	3.00, p = 0.39, NS
Top latency, s	299 ± 0.2	300 ± 0	300 ± 0	300 ± 0.0	3.00, p = 0.39, NS
Assessed by Noldus Ethovision XT10 software					
Angular velocity, deg/s	3.0 ± 2.1	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	3.83, p = 0.28, NS

anxiety-like behaviors. Thus, regardless of the duration of exposure to α-PVP, its long-term effects on zebrafish were the overall behavioral hypoactivity. In line with this, stereotypic 'wall-to-wall' swimming (observed for hyperlocomotor action of acute α-PVP exposure) was not seen following a chronic treatment.

Notably, the present study clearly has several limitations. For example, the sex effects on behavioral outcomes, including CNS drug responses, are an important factor in drug screening in various model organisms, including zebrafish (Lopez Patino et al., 2008; Volgin et al., 2019). Although this aspect has not been addressed here, future follow-up studies may be needed to examine whether male and female zebrafish display different neurobehavioral effects of α-PVP. Likewise, other factors contributing to intraspecies variability, including individual- ('individuality'), population- and strain-specific differences in zebrafish CNS responses (Volgin et al., 2019), merit further scrutiny in relation to this drug. While the main focus of this study was behavioral characterization of acute and chronic effects of α-PVP in zebrafish, we also recognize that it is critical to explore potential molecular (especially monoaminergic) mechanisms underlying such effects, as well as their genomic, proteomic and epigenetic signatures in zebrafish and other model organisms. Analyses of putative neurodevelopmental effects of α-PVP in zebrafish, as well as its delayed effects (e.g., on behavior, neuroprotection and CNS aging) represent another line of research to be considered.

In Experiment 4, HPLC/HRMS analyses of zebrafish brains detected *α*-PVP in all brain samples of drug-treated, but not in untreated, fish (Fig. 3). As shown in this figure, the signal peaks for *α*-PVP in the brain directly depended on the systemic concentration (small for 1 mg/L, large peaks for 10 mg/L) of *α*-PVP given. Taken together, these findings confirm that *α*-PVP quickly penetrates the blood-brain barrier of zebrafish following its acute 20-min systemic exposure, likely contributing to neurobehavioral effects of the drug observed here (Figs. 1–2).

In summary, the growing societal impact of α-PVP and its abuse globally (Zawilska and Andrzejczak, 2015) call for further in-depth neurobehavioral analyses of this agent in both clinical and preclinical studies (Kolesnikova et al., 2019). In the present study, acute treatment of α-PVP provoked stimulant-like effect (without anxiolytic-like action) in adult zebrafish at 5, 25 and 50 mg/L. We also observed a stereotypic 'side-to-side' swimming at 5, 25 and 50 mg/L (Table 1, Fig. 1) – an atypical fish phenotype that may be relevant behavioral hyperactivity and stereotypy commonly observed in humans under the acute influence of α-PVP. In contrast to hyperlocomotor effects of acute α-PVP, hypolocomotion in this study followed a 7-day chronic exposure or repeated withdrawal. Collectively, this supports zebrafish sensitivity to acute and chronic α-PVP, and suggests that aquatic models based on these fish can be a sensitive tool to further examine the CNS effects evoked by α-PVP and related drugs of abuse. Our present findings also support the growing value of this non-mammalian aquatic model species to study neurotropic and toxic properties of synthetic cathinones and related small molecules in-vivo.

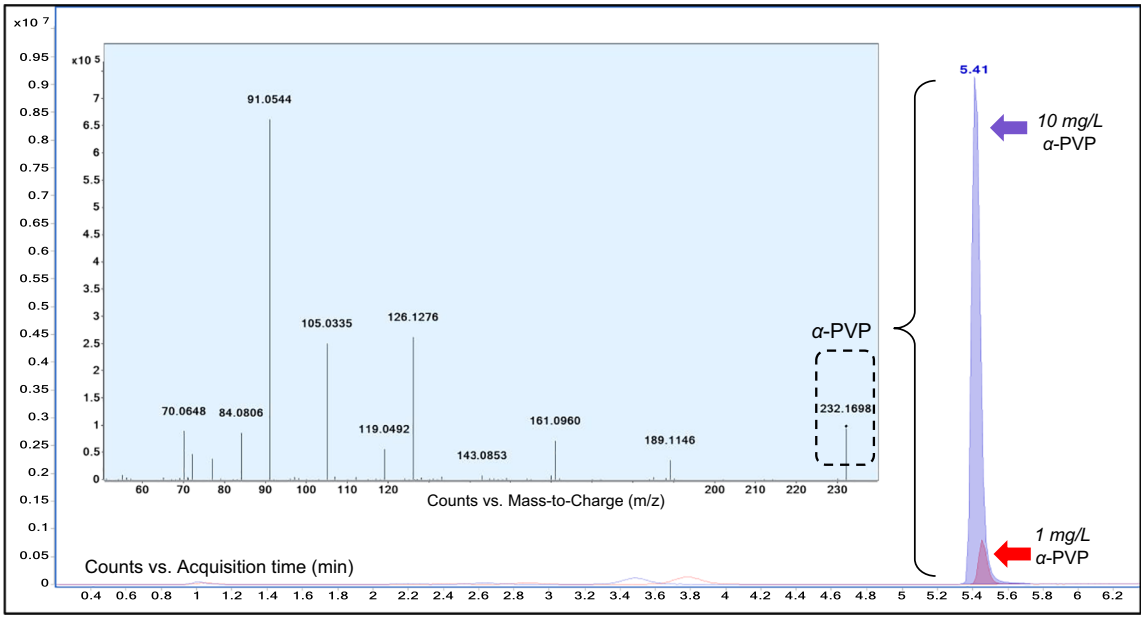

Fig. 3. Extracted ion chromatograms of protonated molecule of *α*-PVP (mass-to-charge ratio *m/z* = 232.1698, dotted line) in zebrafish brains (Experiment 4; n = 15 samples per group, 5 brains per sample, a total 75 brains per concentration). Note that systemic concentrations of *α*-PVP (1 and 10 mg/L) used for zebrafish treatment correspond to the respective small and large peaks (arrows) detected in brain samples. Inset illustrates the detected collision induced dissociation (CID) spectrum fully corresponding to the CID spectrum previously reported for of *α*-PVP in the literature (Tyrkkö et al., 2013). Collectively, this confirms that systemically administered *α*-PVP reaches zebrafish brain following acute 20-min treatment, underlying the observed behavioral effects reported here (Figs. 1–2).

Conflict of interest statement

The authors declare no conflict of interest.

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