Neurotoxicology 78 (2020) 36–46

Contents lists available at ScienceDirect

Neurotoxicology

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

Full Length Article

Potential of the zebrafish model for the forensic toxicology screening of NPS: A comparative study of the effects of APINAC and methiopropamine on the behavior of zebrafish larvae and mice

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ARTICLE INFO

Keywords:
APINAC
Methiopropamine
Novel Psychoactive Substances
Mice
Zebrafish larvae

ABSTRACT

The increased diffusion of the so-called novel psychoactive substances (NPS) and their continuous change in structure andconceivably activity has led to the need of a rapid screening method to detect their biological effects as early as possible after their appearance in the market. This problem is very felt in forensic pathology and toxicology, so the preclinical study is fundamental in the approach to clinical and autopsy cases of difficult interpretation intoxication. Zebrafish is a high-throughput suitable model to rapidly hypothesize potential aversive or beneficial effects of novel molecules. In the present study, we measured and compared the behavioral responses to two novel neuroactive drugs, namely APINAC, a new cannabimimetic drug, and methiopropamine (MPA), a methamphetamine-like compound, on zebrafish larvae (ZL) and adult mice. By using an innovative statistical approach (general additive models), it was found that the spontaneous locomotor activity was impaired by the two drugs in both species: the disruption extent varied in a dose-dependent and time-dependent manner. Sensorimotor function was also altered: i) the visual object response was reduced in mice treated with APINAC, whereas it was not after exposure to MPA; ii) the visual placing responses were reduced after treatment with both NPS in mice. Furthermore, the visual motor response detected in ZL showed a reduction after treatment with APINAC during light-dark and dark-light transition. The same pattern was found in the MPA exposed groups only at the dark-light transition, while at the transition from light to dark, the individuals showed an

Abbreviations: 5F-ADBINACA, (1-(5-fluoro-pentyl)1H-indole-3-carboxylic acid-(1-carbamoyl-2-methyl-propyl)-amide); 5F-AKB48, N-(1-adamantyl)-1-(5-fluoropentyl)-1H-indazole-3- carboxamide; Δ9-THC, Δ9-tetrahydrocannabinol; AB-FUBINACA, N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide; AKB48, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide; APINAC, 1-adamantyl 1-pentylindazole-3-carboxylate; dpf, days post fertilization; GAM, generalized additive model; JWH-018, 1-pentyl-3-(1-naphthoyl)indole; JWH-073, 1-butyl-3-(1-naphthoyl)indole; JWH-250, 1-pentyl-3-(2-methoxyphenylacetyl)-indole; MPA, N-methyl-1-thiophen-2-ylpropan-2-amine, methiopropamine; NPS, Novel Psychoactive Substances; VMR, Visual Motor Response; STS-135, N-(Adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide; WIN 55,212-2, (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

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https://doi.org/10.1016/j.neuro.2020.02.003
Received 4 October 2019; Received in revised form 7 February 2020; Accepted 8 February 2020
Available online 09 February 2020

increased response. In conclusion, the present study highlighted the impairment of spontaneous motor and sensorimotor behavior induced by MPA and APINAC administration in both species, thus confirming the usefulness of ZL as a model for a rapid behavioural-based drug screening.

1. Introduction

The recent appearance in the international market of the so-called Novel Psychoactive Substances (NPS), newly synthesized molecules or relatively old compounds, which for different reasons never ended into commerce as therapeutic drugs, is posing new challenges to regulating bodies as well as to clinical and forensic toxicologists. In fact, in order to escape the legislations against the production, commerce and abuse of narcotics, their availability in the market changes in type and structure with high rate, thus overtaking in speed the legislative procedures to be adopted for their ban. The interest of forensic and clinical toxicologists for these new compounds is not limited to their inherent toxicity, but includes the psychoactive consequences of their use which have direct impact on the public health and safety. If the new analytical approaches largely based on high-resolution mass spectrometry, high performance chromatography, nuclear magnetic resonance, infrared spectrometry may greatly shorten the time needed for the identification and characterization of these new molecules, little improvements have been reported on the side of the study of drug activity and particularly in the early understanding of the behavioral effects, a crucial characteristic for a suspected psychoactive compound.

Rodents are a traditional, universally accepted model to study the behavioral alterations caused by psychoactive drugs, and, more in detail, mouse has widely been used to study the pharmaco-toxicological effects of NPS, because it exhibits a wide range of well recognizable behaviors and allows the evaluation of various physiological and neurological parameters (Vigolo et al., 2015; Ossato et al., 2015; Canazza et al., 2016; Ossato et al., 2017; Marti et al., 2019). Moreover, mice have distinct motor behaviors like spatial preference and stereotyped movements and show complex social interactions. However, this approach is limited by costs, time and complexity, as well as by ethical issues arising from the on-purpose testing of non-therapeutically compounds in mammals expectedly inducing discomfort and suffering.

In the last decade, however, zebrafish has increasingly been reported as a new model in the neuropsychopharmacology area as a complementary approach to mammals (Khan et al., 2017). Zebrafish may offer important benefits, such as the rapid and external development of hundreds of embryos per time (Kimmel et al., 1995), the small size, the low cost, and the high level of homology with mammals (Kalueff et al., 2014). In particular, zebrafish larvae (ZL) are widely used for drug screening: at 4 days post fertilization (dpf) because all neuronal cell groups, and their projections, are present, and at 5 dpf it is possible to study the locomotor activity as the fish display functional anatomical and sensory-motor systems with a full ability to swim (Kalueff et al., 2014; Schmidt et al., 2013; Peterson and Fishman, 2011; Drapeau et al., 2002). Differently from mice, ZF are mostly active during the day time (Di Rosa et al., 2015) and react to changes in ambient illumination with a series of stereotyped motor responses, called the visual motor response (VMR), which are used to characterize neurobehavioral responses to xenobiotics and drugs (Burgess and Granato, 2007; Irons et al., 2013). It has been well established that known psychostimulant drugs, such as D-amphetamine and methamphetamine, induce in zebrafish locomotor impairments similar to rodents, characterized by a biphasic dose-dependent response (Irons et al., 2010; Mi et al., 2016). Furthermore, similarly to rodent models, ZL have been used to understand behavioral responses to cannabis use (Luchtenburg et al., 2019).

Psychotic disorders are the most common risks associated to two categories of NPS, such as structural analogues of methamphetamine and synthetic cannabinoids. In particular, for the present study, methiopropamine (MPA; N-methyl-1 (thiophen-2-yl) propan-2-amine), and APINAC (AKB-57, ACBL(N)-018, adamantan-l-yl l-pentyl-l*H*-indazole-3-carboxylate) were chosen as test compounds. MPA is a powerful and addictive analogue of methamphetamine, in which the benzene ring has been bioisostericaly exchanged with a thiophene ring. It was synthesized in 1942 and first documented use as recreational drug dates back to 2011 in Finland (New drugs in Europe, 2012), but it was reported as commercially available on-line us “legal highs” already in 2010 (Welter et al., 2013). Many reports have indicated that MPA acts similarly to stimulant drugs; it blocks preferentially the reuptake of dopamine and norepinephrine, with a minor activity on serotonin reuptake (Iversen et al., 2013). The main recreational effects of the MPA are alertness, euphoria with subsequent behavioral disturbances like anxiety, fear, aggression, psychosis and the sympathomimetic toxicity (sweating, fever, palpitations, dilated pupils, and chest tightness) (Kamijo et al., 2014; Anne et al., 2015). Psychosis is the highest risk related to the use of such drug, as more than 36 % of MPA addicts suffer from psychosis (Akindipe et al., 2014).

APINAC, is a new cannabimimetic drug detected for the first time in herbal mixture in Australian (Queensland Health Forensic and Scientific Service) and South Korean drug markets (Lee et al., 2016), and eventually confiscated in Russia as pure compound at the end of September 2015. APINAC is structurally similar to the second-generation drug AKB-48, the only difference being the replacement of the NH group with oxygen (Canazza et al., 2016). There is no data regarding toxicity caused by this drug, but on the basis of its structure that is similar to the synthetic cannabinoids (as the presence of a tetramethycyclopropyl ketone indazoles group), the typical effects could be hallucinations, excitement, drowsiness, vomiting and tachycardia. Recently, a pharmacodynamics study *in vivo*, showed that APINAC shares the same metabolic pathway with PB-22 (Savchuk et al., 2017).

The preclinical study of MPA and APINAC in animal models and in ZL is also very useful in forensics to evaluate the possible effects of these drugs and to perform toxicological investigations in cases of NPS intoxication.

The main aim of the present study was to test if zebrafish could become a rapid, simple and high-throughput behavioral model for a first screening of NPS. For this scope, using two different NPS (MPA and APINAC), we carried out a detailed dose-response study comparing the responses to drug exposure in adult mice and zebrafish larvae (ZL). Zebrafish exhibit about 200 complex behaviors and the majority of these responses are conserved in mammals including rodents (Kalueff et al., 2013; Mathur and Guo, 2010) For instance, the visual and acoustic startle response are defensive reactions to adverse stimuli in many vertebrates, including zebrafish (Kalueff et al., 2014). Among the behavioural tests available we choice to investigate in both species the drugs effect on the spontaneous locomotor activity and on the visual responses, such as visual object response and visual placing response in mice and visual motor response in ZL. Moreover, in order to develop a reliable tool, also the statistical method of data treatment was revisited moving from univariate to multivariate methods of data treatment. In fact, univariate statistical tests usually used to assess behavioral activities have been criticized (Wood, 2006), as they do not consider the time dependence of measures and repeated-measures. In particular, ANOVA requires that data variance satisfying sphericity assumption, which is hardly satisfied in behavioral researches (Liu et al., 2015). To address these issues, multivariate approaches using linear models have been recently proposed (Liu et al., 2015), but here we propose a further improvement using generalized additive models (GAM). GAM is a semi-parametric extension of generalized linear model, which assumes that link functions are additive and

components are smooth. The main characteristic of GAM is that it does not force the relationships between response and predictor variables to be linear or monotonic, which are rare in biological phenomena, but is able to fit non-linear trends (Wood, 2006).

2. Materials and methods

2.1. Ethics statement

All husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian animal protection standards (D.lgs. 26/2014). General license for zebrafish maintenance and breeding for the University of Ferrara was approved by the Italian Ministry of Health (auth. num. 18/2017). Experimental protocols performed in the present study were approved by the Ethics Committee of the University of Ferrara and by Italian Ministry of Health (auth. num. 335/2016-PR and 801/2017-PR). Moreover, adequate measures were taken to reduce the number of animals used and their pain and discomfort according to the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Animal husbandry

2.2.1. Mouse

Male ICR (CD-1®) mice, 25–30 g (ENVIGO Harlan Italy, Italy), were group-housed (8–10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a 12:12 light-dark (LD) cycle (lights on 6:30 am; lights off 6:30 pm), temperature of 20 − 22 °C, humidity of 45–55 % and were provided ad libitum access to food (Diet 4RF25 GLP, Milan, Italy) and water.

2.2.2. Zebrafish

Wild-type adult zebrafish were raised according to standard methods (Nüsslein-Volhard and Dahm, 2002) at constant temperature of 28 C° and 12:12 LD cycle (lights on 6:30 AM; lights off 6:30 PM). Reproductive zebrafish were fed 3 and 8 h after lights on with *Artemia* nauplii and dry food, respectively (TetraMin, Tetra GmbH, D). Sexually mature zebrafish pairs were transferred into breeding cage (Techniplast, Varese, I) during the late afternoon. Natural spawning took place the next morning, approximately 2 h after lights on. Embryos were immediately collected and raised in E3 medium (E3 solution composition: 5 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl2; 0.33 mM MgSO4; 1 % of methylene blue) in the same environmental conditions of light and temperature of adults.

2.3. Drug preparation and doses determination

MPA and APINAC were purchased from LGC Standards (LGC Standards, Milan, Italy).

2.3.1. Mouse

Drugs were initially dissolved in absolute ethanol (final concentration was 2 %) and Tween 80 (2 %) and brought to the final volume with saline (0.9 % NaCl). The solution made of ethanol, Tween 80 and saline was also used as vehicle. Drugs were administered by intraperitoneal injection at a volume of 4 µl/g. In this trial, as already described in a previous study (Ossato et al., 2015; Ossato et al., 2016), we used a wide range of doses for APINAC (0.01 − 6 mg/kg) and MPA (0.01 − 100 mg/kg) for the purpose of better evaluating the behavioral effects of the two compounds on spontaneous locomotion and visual responses.

2.3.2. Zebrafish

ZL (N = 18–36/dose) were exposed to a wide range of doses (0.001–10 µM) of APINAC and MPA. We performed 6 tests, each test was conducted with a different group of larvae obtained larvae from different parents. Reproductions occurred between zebrafish haphazardly selected from various maintenance tanks. The drugs were initially dissolved in absolute ethanol (final concentration was 2 %) and Tween 80 (2 %) and brought to the final concentrations with saline solution (0.9 % NaCl). The reference vehicle used is the one with the maximum concentration of ethanol and tween used for the preparation of the highest dose tested (10 µM). The drug concentrations were selected based on previous reports (Burgess and Granato, 2007; Wood, 2006). ZL at 5 dpf were arrayed in a 96-well plate (1 larvae/well, each well filled with 600 µl of E3 medium without methylene blue). The drug treatments were delivered at 6 dpf at 11 a.m. The timing of drug administration was chosen to exclude interferences with the increase of locomotor activity induced by lights on (Di Rosa et al., 2015). The starvation during the assay was reported not altering growth and survival of larvae (Hernandez et al., 2018).

2.4. Behavioral tests

2.4.1. Mouse

For the overall study 228 mice were used. In the analysis of spontaneous locomotion in the open field test for MPA experiments for each treatment (vehicle or 6 different MPA doses, 0.01, 0.1, 1, 10, 30 and 100 mg/kg) were used 10 mice (total mice used: 70), while for APINAC experiments for each treatment (APINAC experiment: vehicle or 4 different APINAC doses, 0.01, 0.1, 1 and 6 mg/kg) were used 10 mice (total mice used: 50).

In the visual object and visual placing responses test for MPA experiments for each treatment (MPA experiment: vehicle or 6 different MPA doses, 0.01, 0.1, 1, 10, 30 and 100 mg/kg) were used 8 mice (total mice used: 56), while for APINAC experiments for each treatment (APINAC experiment: vehicle or 5 different APINAC doses, 0.01, 0.1, 1, 3 and 6 mg/kg) were used 8 mice (total mice used: 48). Since 4 mice died after acute administration of MPA at 100 mg/kg, they were excluded from the behavioral tests and were replaced with new 4 animals in order to maintain the correct statistical numerosity in the experimental groups.

Spontaneous locomotor activity was measured using the ANY-maze video tracking system (Ugo Basile, application version 4.99 g Beta). In open field test MPA and APINAC were given intraperitoneally (i.p.) in mice and each animal was singularly located in the open field box. After 5 min of habituation in the open field box, the recording section started. The mouse was placed in a square plastic cage (60 × 60 cm) located in a sound- and light-attenuated room and motor activity was monitored for 240 min (Ossato et al., 2015). Four mice placed in separate boxes were monitored simultaneously in each experiment. The parameter measured was the distance travelled (meters; m). Variation of the distance travelled was analyzed every 15 min for a maximum of 240 min. To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5 %) ethanol solution and washed with water between animal trials. All experiments were performed between 9:00 AM to 1:00 PM.

Visual response was verified by two behavioural tests, which evaluated the ability of the mouse to capture visual information when the animal is stationary (the visual object response) or when it is moving (the visual placing response). In the sensorimotor tests (visual object and visual placing tests) MPA and APINAC were given i.p. in mice and animal sensorimotor tests started 10 min after drug administration. As previously reported (Ossato et al., 2015; Canazza et al., 2016; Ossato et al., 2016; Canazza et al., 2017) these visual sensorimotor tests were conducted consecutively in the same animals and started 10 min (visual object test) and 15 min (visual placing test) after drug administration. Visual object response test was used to evaluate the ability of the mouse to see an object approaching from the front or from the side, then inducing the animal to shift or turn the head or retreat it (Ossato et al., 2015). For the frontal visual response, a white horizontal bar was moved frontally to the mouse head and the manoeuvre was repeated 3 times. For the lateral visual response, a small dentist mirror was moved

into the mouse's field of view in a horizontal arc, until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally and was repeated 3 times. The score assigned was a value of 1 if there was a reflection in the mouse movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240, and 300 min post injection. Visual Placing response test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al., 2015). The downward movement of the mouse is videotaped by a camera. The analysis frame by frame allows to evaluate the beginning of the reaction of the mouse while it is close to the floor. When the mouse starts the reaction, an electronic ruler evaluates the perpendicular distance in millimeters between the eyes of the mice to the floor. The mice untreated control perceives the floor and it prepares to contact at a distance of about 27 ± 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 35, 70, 125, 185, 245, and 305 min post injection.

2.4.2. Zebrafish

ZL locomotor activity were recorded for 3 days from 5 to 7 dpf (Fig. S1). The first day of recording at 5 dpf was exclusively used to verify the expected behaviour (i.e. the diurnal activity and the visual motor response) in ZL from different clutches. After drug administration at 6 dpf the locomotor activity was recorded for the following 42 h. This experimental protocol allows us to detect: i) the acute dose response (activities shown the first 4 h following the drug delivery); ii) the long-term effect, even on the daily rhythm of locomotor activity; and iii) the VMR after 12 h of dark- or light-adaptation.

The spontaneous activity was videorecorded at 25 frames per second (fps) under constant infrared illumination using a high-throughput system for videotracking (DanioVision, Noldus Information Technology, The Netherlands). The larvae plate was placed into the DanioVision observation chamber where temperature was maintained at 28 °C by means of a thermostatic unit (Noldus Information Technology, The Netherlands). A 12:12 LD cycles routines were set up (lights on at 6:00 am, lights off 6:00 pm). As light source an array of white LED strips was used and irradiances were set at 0.17 W/m^2. The ZL locomotor activity was detected by an automated online image analysis consisting of a dynamic subtraction algorithm (Ethovision XT 11, Noldus Information Technology, The Netherlands). To minimize the effect of missing samples a threshold of 0.2 mm/frame was set up. Locomotor activity of each larva was calculated as the total distance moved during a 6 min time window. The effects of APINAC and MPA on VMR of zebrafish were assessed by analysis of the spontaneous activity in the first 30 min after the first light-dark and dark-light transitions.

2.4.3. Survival assay

Mice used for the behavioral tests were also evaluated for viability in order to identify mortality caused by the substances tested. Immediately after the recording routine, the mice from each treatment groups were moved into their husbandry cage and they have free access to food and water". Survival was monitored during one week after treatments.

ZL were evaluated for viability in order to identify any increased mortality provoked by the drug administration. Immediately after the recording routine, the larvae from each treatment groups were moved into Petri dishes filled with embryo medium in the incubator at a 12:12LD and 28 °C of constant temperature. Larvae were daily fed with powered food (GEMMA Micro 75, Skretting Italy). The survival rates from each group were daily detected until 11 dpf, 5 days after the drug treatment.

2.4.4. Statistical analysis

Liu and colleagues (Liu et al., 2015) pointed out that activity data from zebrafish experiment are affected by multiple factors, even in laboratory trials. Hence, we used a multivariate approach: GAMs are an ideal tool for such analysis as they are flexible in modelling the shape of non-linear relationships. Non-parametric smoothing functions are used on sections of the data and the response curves are connected at their end-points to generate an overall smooth curve (Wood, 2006). In addition to the non-parametric smoothing functions, parametric variables may also be included, as in a conventional generalized model. The distance travelled by mice and ZL was modelled as the response variable by fitting separate GAMs, implemented by means of the package "mgcv" (version 1.7) (Wood, 2006) in R (version 3.5.0; R Core Team 2018). In order to evaluate the effect of the drugs on ZL behavior, we constructed two models for each NPS: the first one considering the data recorded in the first 4 h after the drug delivery, the second one analyzing all dataset (48 h of recording). In so doing, we were able to compare the mice and zebrafish responses (4 h model) as well as, for zebrafish only, to estimate the effects of drugs in a longer period (48 model).

The predictor variables considered were dose, minutes after the assumption of active substance, and light (on/off, for zebrafish only). Moreover, in order to assess the effects of different doses in the fluctuating patterns of distance travelled throughout the trial, we inserted the interaction term minute * dose. Effects of continuous predictor variables were initially modelled as natural cubic spline functions and the optimal roughness of the smoothing terms was determined by minimizing the generalized cross-validation value. When the effective degrees of freedom (edf) of a predictor variable were 1 and the graphical inspection confirmed a linear relationship with the response variable, we refitted the model omitting the smoothing function. We confirmed the global goodness-of-fit (i.e., homoscedasticity, normality of errors and independence) of the best models by visual inspection of residuals (Zuur, 2009).

The effect of APINAC and MPA on VMR of zebrafish was assessed by means of separate ANOVA, considering the travelled distance as response variable and dose as explanatory variable. The Tuckey multiple comparison was used as post-hoc test to compare the effects of different doses.

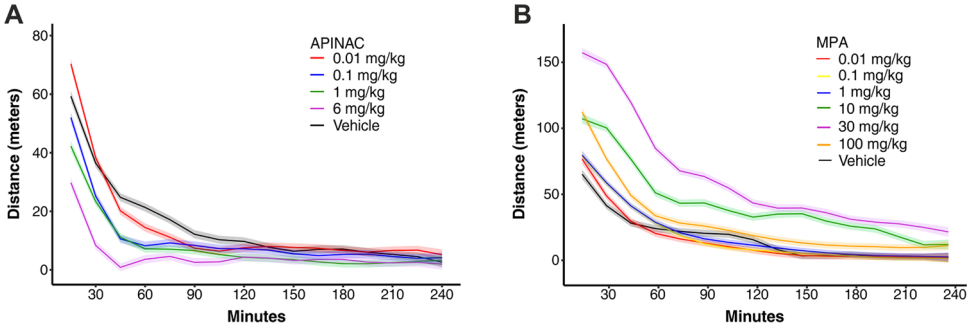

Fig. 1. Mice locomotor activity. Values (mean ± standard error) of distance travelled by mice predicted by GAM. The figure shows effect of the systemic administration of APINAC (0.01–6 mg/kg i.p.; panel A) and MPA (0.01 − 100 mg/kg; panel B). Data are expressed as meters travelled. Estimated mean values and standard errors are represented by the continuous line and colour-shaded areas, respectively.

3. Results

3.1. Locomotor activity in mice

In mice, the systemic administration of APINAC (0.01–6 mg/kg i.p.) reduced in a dose-dependent manner the total distance travelled (Fig. 1A; Table 1S), but, paradoxically, at the lowest dose (0.01 mg/kg) APINAC showed a transient facilitation of spontaneous locomotion. Such facilitation appeared only in the first 15 min, decreasing subsequently and so, overall, the behavioral patterns of this group were not statistically different to the controls. Conversely, the highest dose 6 mg/kg induced a greater and prolonged inhibition of locomotion in mice (~50 % reduction of the total distance respect to the vehicle-treated mice). Systemic administration of MPA (0.01–100 mg/kg i.p.) in mice produced a biphasic dose-response pattern of the spontaneous locomotor activity (Fig. 1B; Table 2S). In particular, the doses of 0.01 and 0.1 mg/kg did not significantly affect mice mobility, whereas the dose of 1 mg/kg slightly increased the total distance travelled in the first hour after the drug administration only. Similarly, the doses of 10 mg/kg and, mainly, 30 mg/kg exerted a potent stimulatory effect on spontaneous locomotion in mice. The highest dose of MPA (100 mg/kg i.p.) partially facilitated the total distance travelled causing a facilitation of locomotor activity, similar or lower to that induced by MPA at 10 mg/kg.

3.2. Locomotor activity in zebrafish

All ZL tested at 5 dpf displayed a daily rhythm of locomotor activity, the typical diurnal pattern with the higher activity during the light phase. The administration of APINAC (0.001–10 μM) to ZL reduced in a dose-dependent manner the total distance travelled. The lower doses (0.001, 0.01, 0.1, 1 μM) caused an inhibitory effect on locomotion during the acute phase (Fig. 2A), while in the long term exposure (Fig. 3A), the curve trend follows the vehicle treated pattern, indeed no difference in the distance travelled is detected within this groups (Table 3S). (Fig. 3A). Conversely, the highest dose (10 μM) induced a reduction of mobility immediately after the treatment (Fig. 2A) that persists until the second day after treatment (Fig. 3A). The analysis involving the whole dataset (i.e., including the ZL activities in the 42 h after the administration) showed that ZL mobility was affected by light, dose and interaction term light*minutes after APINAC administration (Table 4S): in addition to the findings of the 4 h model, this model showed that animals were more active during light hours, as expected in this diurnal species. Instead, the highest dose impaired the capability to synchronized locomotor activity to the active phase of the day showing no difference in the distance travelled between the light and the dark phase (Fig. 3A).

The drug solutions of MPA (0.001-0.1 μM) alter the total distance travelled in a dose-dependent manner in the zebrafish model during the acute treatment (first 4 h; Table 5S; Fig. 2B). At the lowest doses (0.001 and 0.01 μM) a facilitation of spontaneous locomotion is detected lasting for 4 h. Conversely, a significant reduction in the distance travelled was observed at the highest drug concentration (10 μM). The highest dose lead to a reduction of more than 50 % of the total distance travelled during the 30 min after administration, that remained significantly lower for all the acute phase.

The results of the model fitting all data showed that the lowest doses (0.001-0.01 μM) did not affect locomotion during the first dark periods (Table 6S; Fig. 3B). The travelled distances covered by zebrafish treated with 0.001 μM were longer in respect to the control in the second light period. No significant effect is reported for 0.01 μM dosage in respect to the control in the second light period, whereas a significant reduction in the distance travelled is reported by the greater dosage (1 μM).

3.3. Visual object and visual placing in mice

Visual object response was not affected by the administration of the vehicle (Fig. 4 A and B). The systemic administration of APINAC (0.01 – 6 mg/kg i.p.) induced in a dose-dependent manner, a decrease of the visual object response of mice (Fig. 4A). In particular, the higher doses tested (3 and 6 mg/kg i.p.) rapidly and fully inhibited the visual object response at 10 min after the drug injection and effects persisted until the end of the test. (Fig. 4A; significant effect of treatment ($F_{5.42}$ = 25.32, $p < 0.0001$). Differently, systemic administration of MPA (0.01 – 100 mg/kg i.p.) did not affect the visual object response in mice over the course of the 5 h analysis (Fig. 4B; ($F_{6.49}$ = 1.144, p = 0.3513).

Visual placing response was not affected by the administration of the vehicle (Fig. 4C and D). The systemic administration of APINAC (0.001 – 6 mg/kg i.p.) decreased in a dose-dependent manner the visual placing response of mice. In particular, at the higher doses (3 and 6 mg/kg) the visual response of mice was inhibited to about 80 % after 10 min from drug injection and the inhibitory effect persisted up to 5 h (Fig. 4C; $F_{(5,42)}$ = 17.63, $p < 0.0001$). The systemic administration of MPA (0.01 – 100 mg/kg i.p.) reduced in a dose-dependent manner the visual placing response in mice and the effect persisted up to 5 h at the higher doses (30 and 100 mg/kg i.p.; Fig. 4D; $F_{(6,49)}$ = 15.21, $p < 0.0001$).

3.4. Visual motor response in zebrafish

We elicited the VMR at the dark to light and light to dark transitions during the second day of recording. The increase in locomotor activity during the first 30 min after transitions were significantly altered in

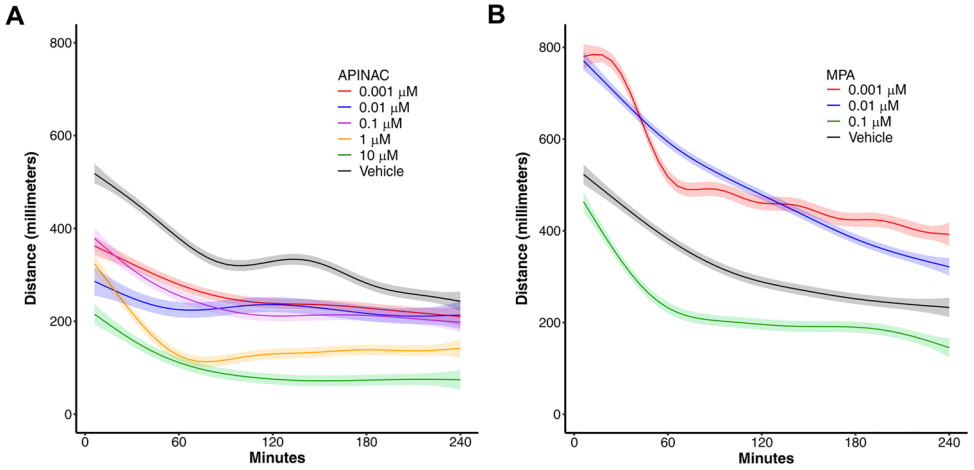

Fig. 2. Zebrafish locomotor activity (acute phase). Values (mean ± standard error) of distance travelled by larvae of zebrafish during the first 4 h after the treatment predicted by GAM. The figure shows effect of the systemic administration of APINAC (0.001–1 μM.; panel A) and MPA (0.001-0.1μM; panel B). Data are expressed as millimeters travelled. Estimated mean values and standard errors are represented by the continuous line and colour-shaded areas, respectively.

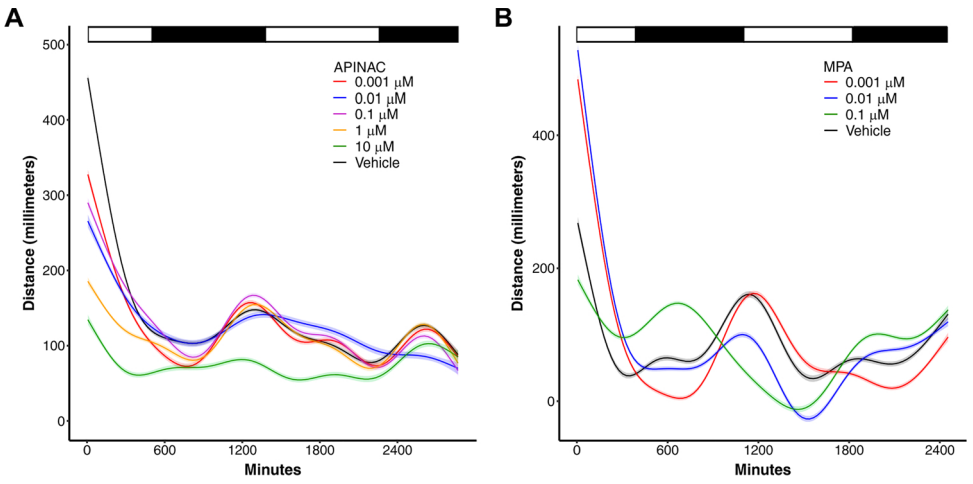

Fig. 3. Zebrafish locomotor activity (long term). Values (mean ± standard error) of distance travelled by larvae of zebrafish during the 48 h after the treatment predicted by GAM. The figure shows effect of the systemic administration of APINAC (0.001–1 µM.; panel A) and MPA (0.001-0.1µM; panel B). Data are expressed as millimeters travelled. Estimated mean values and standard errors are represented by the continuous line and colour-shaded areas, respectively.

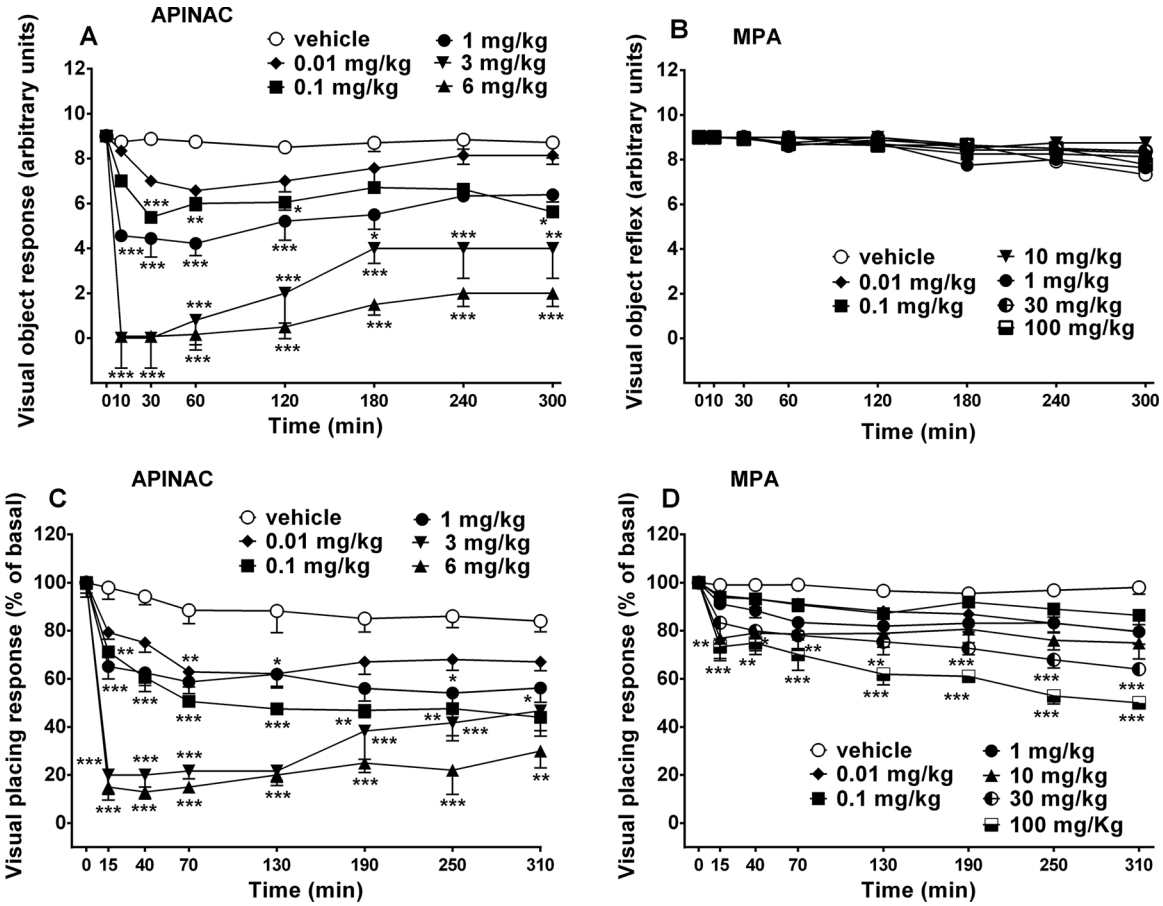

Fig. 4. Visual placing and object response in mice. The effect of the systemic administration of APINAC (0.01 − 6 mg/kg i.p.) and MPA (0.01 − 100 mg/kg i.p.) on the visual object response (panel A and B) and visual placing response (panel C and D) in mice. Data are expressed (see "Materials and methods") as arbitrary units (panel A and B) or percentage of basal response (panel C and D) and represent the mean ± SEM of 8 animals for each treatment. Statistical analysis was performed by a two-way ANOVA followed by the Tukey's test for multiple comparisons for the dose response curve at different times. *p < 0.05, **p < 0.01, ***p < 0.001 versus the vehicle.

zebrafish treated with APINAC (Fig. 5A–B; Lights on: $F_{(5,773)} = 2.86$, $p = 0.014$; Lights off: $F_{(5,772)} = 8.83$, $p < 0.001$). Interestingly, the highest APINAC dose (10 µM) significantly reduced the response of ZL to the lights on respect to controls (−45 %). A major effect of APINAC has been found at the lights off; different doses dampened the dark photokinesis (Fig. 5B). All MPA doses induced a significant increase of the VMR respect to controls at the lights on (Fig. 6A–B; $F_{(35,260)} = 95.37$, $p < 0.001$). Differently, at the lights off only ZL treated with 0,001 µM MPA showed a significant reduction of activity (Fig. 6A; $p = 0.01$, Tukey post-hoc test).

3.5. Survival

3.5.1. Mice

The injection of APINAC (0.01 − 6 mg/kg i.p.) didn't cause mortality in mice while the administration of MPA at the doses of 10, 30 and 100

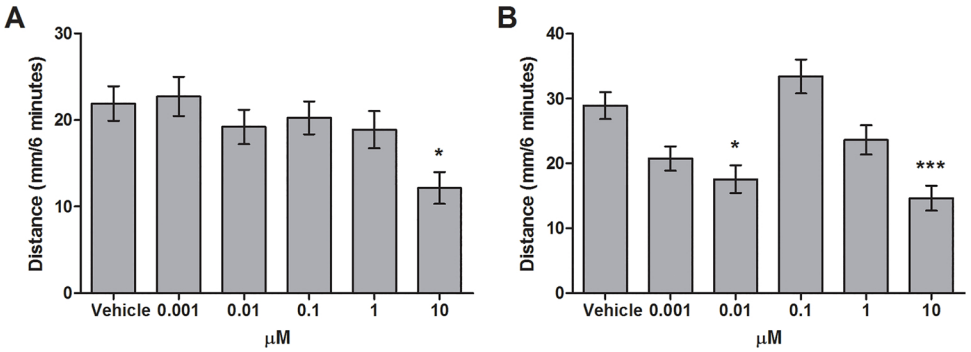

Fig. 5. Visual motor response. Effect of the systemic administration of APINAC (0.001–1 μM) on spontaneous activity of larvae of zebrafish in the first 30 min of the first light-dark (panel A) and dark-light transitions (panel B). Data are expressed mean ± SEM. *p < 0.05, ***p < 0.001 versus the vehicle.

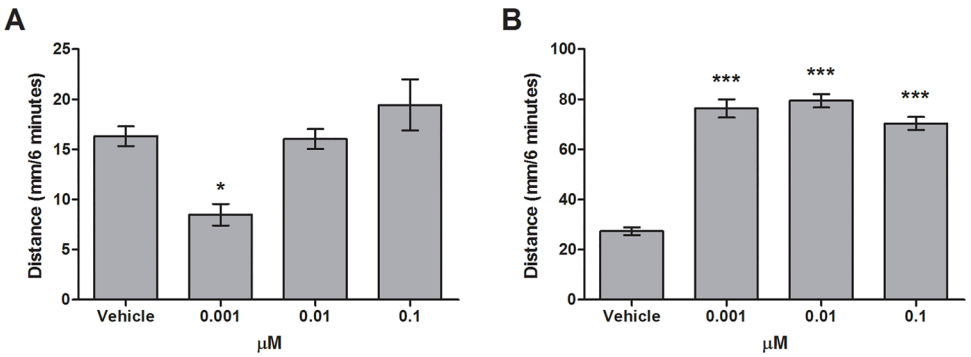

Fig. 6. Visual motor response. Effect of the systemic administration of MPA (0.001-0.1 μM) on spontaneous activity of larvae of zebrafish in the first 30 min of the first light-dark (panel A) and dark-light transitions (panel B). Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001 versus the vehicle.

mg/kg induced death in 30 %, 30 % and 40 % of the groups of mice, respectively. To be noted that the injection of the 10 and 30 mg/kg did not cause death immediately in mice, neither during the 5 h of the manipulation but mice were found dead the next day in cage. Differently the group of mice treated with dose of 100 mg/kg died on the same day of the treatment: 2 mice died after 10 min of injection, one died during the second hour of the test and the last one died on the end of the fifth hour of the treatment.

3.5.2. Zebrafish

After locomotor recording the mortality of larvae at different concentration was recorded. In the APINAC treatments larvae mortality were absent until 9 dpf. Subsequently a mortality > 50 % were found in the higher doses (1 and 10 μM). MPA treatments did not induce a relevant mortality in the ZL. No morphological abnormality was observed in all larvae survived.

4. Discussion

In the present study, we reported for the first time the comparison of the behavioral motor and visual sensorimotor responses induced by the two novel psychoactive substances (NPS), the synthetic cannabinoid APINAC and the stimulant MPA on two different animal models: mice and ZL.

Both NPS induced comparable behavioral responses in the two species. In the mouse model, APINAC at the lowest dose tested (0.01 mg/Kg) induced a transient facilitation of the motor activity, while caused a potent inhibition of spontaneous locomotion at the other doses tested (0.1 − 6 mg/kg). These results are in agreement with most preclinical studies on cannabinoids proving that cannabinoid receptor agonists, in time and dose-dependent manner, modulated, with a biphasic curve, spontaneous locomotion in rodents, with a facilitation or an inhibition at low and high doses, respectively. This biphasic effect has been displayed by the endocannabinoid anandamide (Sulcova et al., 1998), Δ^9-tetrahydrocannabinol (Δ^9-THC) (Ossato et al., 2015) along with the synthetic compounds WIN 55,212-2 (Kari et al., 2007), JWH-018 (Ossato et al., 2015; Ossato et al., 2017), AKB48, 5F-AKB48 (Canazza et al., 2016), 5 F-ADBINACA, AB-FUBINACA, and STS-135 (Canazza et al., 2017), suggesting that it is typical of the cannabinoid system and not of the single molecules (Rodriguez de Fonseca et al., 1998). Motor impairment and akinesia are some of the main behavioral effects observed after systemic administration of cannabinoid receptor agonists (Rodriguez de Fonseca et al., 1998) and they are mainly caused by stimulation of cerebellar and basal ganglia CB_1 receptors (Sanudo-Pena et al., 1999). Synthetic cannabinoids-induced motor stimulation can be related to their ability to facilitate dopamine (DA) release in the nucleus accumbens (NAcc) shell possibly by facilitating the meso-accumbal dopaminergic pathway through a CB_1 receptor-dependent mechanism (Ossato et al., 2017).

In ZL the administration of APINAC reduced in a dose-dependent manner the total distance travelled during the first 4 h after the treatment. These observations are consistent with previous investigations on the effect of Δ^9-THC (Achenbach et al., 2018). Interestingly, both Δ^9-THC and cannabidiol (CBD) have an inhibitory effect also in adult zebrafish that showed a reduced swimming speed and distance travelled following exposure to these compounds (Kalueff et al., 2014; Stewart and Kalueff, 2014; Jensen et al., 2018). These effects have been considered an indication of anxiolytic behavior. However, a recent investigation showed that cannabinoid treatment in zebrafish embryo altered synaptic activity at neuromuscular junctions, reduced heart rates and locomotor responses to sound (Ahmed et al., 2018). This may indicate that the reduction in activity may not be purely due to an anxiolytic response.

The biphasic effect on the locomotor activity (stimulation at low concentrations and suppression at high concentrations) that was found in mice after APINAC treatment has also been reported in zebrafish

after CBD and Δ^9-THC treatments (Akhtar, 2013; Carty et al., 2018). Differently, in the present study APINAC did not induce the biphasic effect. This dissimilarity could be related to the concentration of APINAC used or to the differences between the experimental protocols (i.e. time of day or stage of the administration, strain used, light intensity).

We also find that the stimulant MPA produced a biphasic dose-response pattern of the spontaneous locomotor activity in mice. Similarly to other psychostimulants such as cocaine, amphetamine and methamphetamine, MPA may stimulate spontaneous locomotion in mice by facilitating the DA release in the NAcc (Ossato et al., 2017; Iversen et al., 2013; Koob, 2009). The reduction of the stimulatory effect caused by MPA at the highest dose (100 mg/kg) could be related to the appearance of animal stereotypies as also reported for other psychostimulants (Ossato et al., 2017). These stereotypes could be caused by an excessive stimulation of pre- and post-synaptic dopaminergic receptors with a consequent dysregulation of the glutamatergic transmission which leads to an excessive activation of NMDA and mGlu5 receptors (Shoblock et al., 2004; Hashimoto et al., 2007). The description of the MPA effect, produced in mice, can help the clinical diagnosis of intoxication of unknown origins and address the toxicologist in the search for substances of abuse.

ZL are also sensitive to psychostimulants such as d-amphetamine. The acute administration of methamphetamine induces avoidant behavior and increases swim speed in the open field and mirror stimulation tasks (Mi et al., 2016). The cholinergic system may also play a role in modulating the rewarding effects of various psychoactive drugs, and genetic impairment of AChE reduced the amphetamine-induced conditioned place preference in adult zebrafish (Ninkovic et al., 2006). Many neuroactive substances produce a biphasic dose–response pattern on spontaneous locomotor behavior, where lower doses increase activity and higher doses decrease activity (Irons et al., 2010). This peculiar pattern has been found also after MPA administration. In the first hours after treatment, at the lowest doses (0.001 and 0.01 μM), a stimulation of locomotion activity was present, whereas a significant reduction in the distance travelled was consequence of the highest dosage (0.1 μM).

Our study showed that the highest dose of APINAC (10 μM) and MPA (0.1 μM) affect the daily rhythm of activity in ZL. The behavior protocol applied in the murine model in our investigation is not designed to consider the circadian rhythms of mice. However, it has been reported that cannabinoids and d-amphetamine influence of treatment on the circadian clock and attenuate the ability of the circadian clock to synchronize to LD cycle in mice (Acuna-Goycolea et al., 2010; Wongchitrat et al., 2013; Jones et al., 2014). These findings could reveal a similar action of cannabinoids and psychostimulant on affecting the circadian system of zebrafish and rodent models. Further circadian investigations in zebrafish are crucial to understand whether this species could be useful to study the alteration of the circadian behavior induced by drugs.

The statistical analyses applied in this study has been implemented by means of GAM, which takes into account the time dependency among response variables and reveals other dependency structures of continuous responses data. This multivariate model does not force the relationships between response and predictor variables to be linear or monotonic. In so doing, we have been able to describe and statistically discriminate the effects of different concentrations of NPS on mice and ZL activities. This approach proved useful in the analysis of ZL long-term data (48 h) giving us the opportunity to point out the alteration of the LD cycle by higher concentrations of MPA.

This statistical framework is recently used in wildlife to assess drug effects on movement rate after capture events by chemical immobilization (Brivio et al., 2015) or to understand the role of ecological variables on activity rhythms in large herbivore (Brivio et al., 2017; Grignolio et al., 2018). Our results suggest to apply this framework of analysis in other experiments on locomotor activities of mice or ZL with a similar structure of the data.

We have also demonstrated that APINAC (0.01 − 6 mg/kg) induces an inhibition of the visual response in mice in a dose-dependent manner while they are moving (visual placing response) and also when they are stationary (visual object response). This action profile on visual sensorimotor responses is analogous to that observed after administration of other synthetic cannabinoids, such as JWH-018 (Ossato et al., 2015), JWH-073, JWH-250 (Ossato et al., 2016), 5F-ADBINACA, ABFUBINACA, STS-135 (Canazza et al., 2017) and in particular to the adamantine derived indazole groups, such as the AKB48 and 5F-AKB48 (Canazza et al., 2016). Although it was not identified a specific anatomical substrate responsible for the cannabinoid-induced visual impairment in rodents, some studies have shown that CB_1 receptors are critically involved in the modulation of visual cortical plasticity in mice and that Δ^9-THC inhibits the visual processes in rat by impairing the thalamocortical transmission (Dasilva et al., 2012). Moreover, a recent study has shown that visual information in mice is elaborated in a subpopulation of neurons selectively localized in the dorsomedial striatum (Reig and Silberberg, 2014), a brain area of the basal ganglia in which CB_1 receptors are expressed (Tsou et al., 1998; Marsicano and Lutz, 1999). Even though in our study we were not able to understand which brain areas and neural mechanisms are responsible for the reduced visual response of the mouse, it is possible to hypothesize that APINAC, similarly to other synthetic cannabinoids, could reduce visual function through the stimulation of CB_1 receptors expressed in thalamocortical-striatal visual circuitry (Tsou et al., 1998; Marsicano and Lutz, 1999).We also evaluated the effect of MPA in visual placing and object responses in mice. It is interesting to note that although MPA does not alter the visual response in the visual object test, it reduces the sensorimotor response in the visual placing test. This different response is due to the fact that the two behavioral tests are performed under different experimental conditions that require different behavioral responses of the mouse. In fact, in the visual object test the animal is not engaged in coordinating the movements for the correct landing but it is still and only engaged to receive the visual stimulus caused by the object that is approaching, while in the visual placing test the mouse must correctly integrate visual, tactile (from vibrissae stimulation) and vestibular information to prepare the correct extension movement of the muscles of the neck and forelegs for correct contact with the ground (Lambert et al., 2016). In particular, vestibule-spinal pathways play a major role in the control of posture and movement by stabilizing body posture in the face of gravity and movement by activating scapula and forelimb muscles (Clarac et al., 1998; Tosolini and Morris, 2012; Tosolini et al., 2013). In combination with proprioceptive information, vestibular inputs to spinal motoneurons stabilize the head relative to the trunk and engage limb and trunk musculature to produce postural adjustments during active and passive body motion (Angelaki and Cullen, 2008; Cullen, 2012). Therefore, in the visual placing test different circuits for motor and postural control come into play, as well as those closely related to the visual perception. This suggests that MPA inhibits the visual placing response in mice not for the reduction in the perception of visual stimuli, but possibly for an alteration of vestibular signals, since MPA by releasing NE could change the vestibulo-ocular reflex and the optokinetic response in mice through the β- and α2-receptor activation (Wakita et al., 2017). Moreover, MPA could also affect vestibular response in rodents by enhancing the DA transmission in the auditory/vestibular system (Eybalin et al., 1993; Karadaghy et al., 1997; Darrow et al., 2006; Inoue et al., 2006; Maison et al., 2012; Drescher et al., 2010).

The study about the visual response in mice has important implications in the forensic field, because the use of MPA and APINAC, as well as the other synthetic cannabinoids, is misunderstood in patients or in autopsy, since very often the assumptions are not recognized, as associated with others substances. Consequently, mice responses to MPA and APINAC are very important and useful to verify a clinical suspect of intoxication and a correct forensic toxicological analysis.

To investigate the sensorimotor function and to characterize neurobehavioral responses in ZL the analysis of the VMR has been extensively used. In physiological conditions the transition from light to dark, or *vice versa*, induces drastic propulsive and turning movement for 10 − 15 min, following which activity falls to the baseline (Burgess and Granato, 2007). The sensory and motor mechanisms that drives the VMR is not fully understood. It depends on the integrity of brain function, nervous system development, and visual pathways and it is attributed to the increased stress/anxiety level determined by lighting transition (Burgess and Granato, 2007). VMR is mediated by retinal and extraretinal photoreception and activated by reticulospinal neurons (Fernandes et al., 2012). Furthermore, a fundamental role to set basic behavioral states and to drive a coordinated response to environmental changes has been attributed to the Orthopedia-dependent DA system (Ryu et al., 2007). Both NPS tested altered the VMR at lighting transitions. It is interesting to note that the effect of MPA is diametrically opposed at the lights on respect to the lights off. Previous investigation showed the central role of deep brain photoreceptors of the preoptic area expressing melanopsin (*opn4a*) in the "dark photokinesis", the VMR induced by lights off (Fernandes et al., 2012). In the zebrafish brain some *opn4a* expressing neurons are part of DA cell clusters that project to the hindbrain and the spinal cord and are involved in the modulation of behavioural responses (Tay et al., 2011). We could hypothesize that different effects of the MPA depend on a different action of the MPA during the day on these DA cell clusters.

Finally, we have investigated also the rate of death induced by NPS. In ZL, MPA treatments did not induce a relevant mortality, whereas the higher APINAC dose induced a marked mortality 5 days after treatment. In mice only MPA at 30 and 100 mg/kg concentration induced fatality with a percentage of 30 % and 40 %, respectively. This rate of dead mice is very high showing the important vulnerability of this substance. The causes of death were not deeply investigated since our study was limited to behavioral analysis. Based on our previous study we showed that high doses of MPA induced a significant increase in the heart rate associated with a persistent vasoconstriction that could lead to myocardial ischemia (Foti et al., 2019). The cellular mechanism by which this drug could damage the heart and induce lesions is not yet discovered and further studies will be undertaken. Interestingly, the higher ZL survivor after MPA exposure could depend to a different mechanism of action at cardiovascular level. In ZL previous investigations showed lethal neurotoxicity and hepatotoxicity induced by different substances that could be associated to the mortality after APINAC treatments (Cornet et al., 2017).

In conclusion, overall, the present findings demonstrate that the effect of the NPS on the motor and sensorimotor behavior in ZL and mice is roughly similar. The acute treatment with APINAC and MPA on zebrafish impairs in a dose-dependent manner the locomotor activity and the VMR. The same effects have been observed on the spontaneous locomotion and on the visual object and placing responses in mice. Our results confirm the *bona fide* of the high-throughput behavior-based screening of drugs in zebrafish (Kalueff et al., 2014; Peterson and Fishman, 2011). These screening do not require a deep knowledge of physiological and molecular mechanisms because they use behavioral responses as outputs. Since different compounds could have different mechanisms of action, a catalogue of behavioural responses could help to predict the mechanisms of action. Hence, a continuous and intense behavioural phenotyping of new compounds will increase the predictive power of the zebrafish high-throughput behavior-based screening to classify new drugs and compounds and identify their mechanisms of action. Moreover, it positively supports the 3Rs principles because a prediction will permit to reduce the number of animals required for a test, to refine the evaluation of the drug toxicity or mortality.

Author contributions

CB, MM, EM: conceived and designed the experiments.
EM, SB, MT, RA: performed the experiments.
SG, EM, SB, MT: analyzed the data.
CB, MM, AF, SS, SA, PF: contributed reagents, materials, and analysis tools.
EM, CB, MM, FT, MN: wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research has been funded by the Anti-Drug Policies Department, the Presidency of the Council of Ministers, Italy (project: "*Effects of NPS: development of a multicentre research for the information enhancement of the Early Warning System"* to M. Marti), local funds from the University of Ferrara (FAR2016, FAR2017, FAR2019 and FIR2018 to M. Marti and C. Bertolucci), and FIRB 2012 from the Italian Ministry of Education, University and Research (Grant no. RBFR12LDOW to F. De-Giorgio).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2020.02.003.

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