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ORIGINAL INVESTIGATION

Abuse potential of methylenedioxymethamphetamine (MDMA) and its derivatives in zebrafish: role of serotonin 5HT2-type receptors

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Abstract
Rationale The synthetic phenethylamines are recreational drugs known to produce psychostimulant effects. However, their abuse potential has not been widely studied.
Objectives Here, we investigated the rewarding and the hallucinatory effects of 2,5-dimetoxy-4-bromo-amphetamine hydrobromide (DOB) and *para*-methoxyamphetamine (PMA) in comparison with the classical 3,4-methylene dioxymethamphetamine (MDMA). In addition, the role of serotonin 5-HT_2-like receptor on the abovementioned effects was evaluated.
Methods Zebrafish were intramuscularly (i.m.) treated with a wide range of doses of DOB (0.1–20 mg/kg), PMA (0.0005–2 mg/kg), or MDMA (0.5–160 mg/kg). Animals were submitted to a conditioned place preference (CPP) task, to investigation of the rewarding properties, and to the evaluation of hallucinatory behavior in terms of appearance of a trance-like behavior. The serotonin 5-HT_2 subtype receptor antagonist ritanserin (0.025–2.5 mg/kg) in association with the maximal effective dose of MDMA, DOB, and PMA was given i.m., and the effect on CPP or hallucinatory behavior was evaluated.
Results MDMA and its derivatives exhibited CPP in a biphasic fashion, being PMA the most potent. This effect was accompanied, for DOB (2 mg/kg) and PMA (0.1 mg/kg), by a trance-like hallucinatory behavior. MDMA at a high dose as 160 mg/kg did not induce any hallucinatory behavior. Ritanserin significantly blocked the rewarding and hallucinatory effects suggesting the involvement of serotonin $5HT_2$ subtype receptor.
Conclusion Collectively, these findings demonstrate for the first time that the rewarding properties of DOB and PMA are accompanied by hallucinatory behavior through a serotonergic system and reinforce zebrafish as an emerging experimental model for screening new hallucinogens.

Keywords Phenethylamines · Conditioned place preference · Trance-like · PMA · DOB · Hallucinogens

Introduction

Phenethylamines refer to a class of substances with documented psychoactive and stimulant effects and include amphetamine, methamphetamine, and 3,4-methylenedioxyme thamphetamine (MDMA) also known as ecstasy, all of which are now under control (Hill and Thomas 2011). Then, a new generation of phenethylamines that are synthetic analogues of mescaline such as 2C-B and 2,5-dimetoxy-4-bromo-amphetamine hydrobromide (DOB) was found to exceed that of many naturally occurring hallucinogens (Monte et al. 1997), a class of drugs which affect sensory, motor, affective, and cognitive responses in both humans and animals (Nichols 2004, 2016; Liechti 2015) associated with significant toxicity

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(Balíková 2005). To overcome national or international laws, other phenethylamines have been placed on the market during the last years (UNODC 2015). Among them, *para*-methoxy methamphetamine (PMMA) and *para*-methoxyamphetamine (PMA) have been frequently found in tablets that carry a similar logo to "ecstasy" (European Monitoring Centre for Drugs and Drug Addiction 2003). There is concern about the growing usage of synthetic drugs which can often be sold openly through websites on the internet (Lawn et al. 2014) especially for their limited knowledge of pharmacological effects. Recently, there has been a remarkable resurgence of interest in hallucinogenic drugs, focusing on the mechanisms of their action in various species, as well as side effects and potential clinical applications (Neelkantan et al. 2013).

Even if hallucinogen drugs have a relatively low addicting potential, they are frequently abused even if no successful attempts are reported to demonstrate self-administration of classical hallucinogens in animals (Nichols 2004). MDMA (0.2–3.2 mg/kg/injection) has been found to be self-administered in baboons (Lamb and Griffiths 1987) and produce conditioned place preference (CPP) in rats (García-Pardo et al. 2015). No data are available about PMA apart from a very old paper (Winter 1984) in which this drug produced only limited response on the amphetamine appropriate lever in rats while DOB on the LSD appropriate lever (Benneyworth et al. 2005).

Recent evidence indicates that novel animal models are emerging as useful tool to screen addicting drugs. Among them, zebrafish showing high physiological and genetic homology to humans (Cheng et al. 2011) appears a sensitive and promising model for the investigation of hallucinogen-evoked states. In a recent review, elegantly written by Kalueff's group (Neelkantan et al. 2013), the effect of MDMA and different hallucinogens, including LSD, mescaline, PCP, MK-80, ketamine, ibogaine, salvinorin A, atropine, and scopolamine in adult zebrafish, has been reported. However, there are no currently available data on the rewarding effects of MDMA derivatives thus representing an important area of extensive investigation.

CPP paradigm serves as a measure of the rewarding properties of a given substance. CPP is a commonly used technique to evaluate the motivational effects of compounds associated with a positive or negative reward, which capitalizes on the basic principles of Pavlovian conditioning. If the animal develops an increase in preference for a drug-paired environment, it is inferred that the drug has positive-reinforcing properties. Similar to many rodent behavioral paradigms, CPP has been extensively adopted in zebrafish (Collier et al. 2014).

On this basis, the purpose of the current study aimed to evaluate the classical MDMA and its two derivatives, PMA and DOB, on CPP, a classical technique previously set up in our laboratory using a hallucinogenic drug such as salvinorin A (Braida et al. 2007). Furthermore, the study was accomplished by the evaluation of a hallucinatory behavior in terms of trance-like behavior and number of crossed lines.

As serotonin 5-HT_{2A} receptor agonists have typically been referred to "hallucinogens" and some of them (LSD, mescaline) tested also in zebrafish (Neelkantan et al. 2013), we investigated the role of serotonin $5HT_2$-like receptors, previously identified in zebrafish (Schneider et al. 2012), on the reward and the appearance of trance-like behavior .

Methods

Animals and housing

Adult wild-type short fin zebrafish (*Danio rerio*) (0.4–1 g) of heterogeneous genetic background and aged between 6 and 12 months were obtained by a local aquarium supply store (Aquarium Center, Milan, Italy). In all of the experiments, the sex ratio was 50–50 %. Males and females were identified according to Streisinger (2000). The animals were kept at approximately 28.5 °C on a 14/10-h light/dark cycle (lights on at 0.8:00 h). In order to minimize stress, the experiments were began 1 month after their arrival in the lab. Furthermore, the fish were habituated to the apparatus for 1 h a day in the week preceding the beginning of the experiments. During the experiments, the observer, who was blind to the treatment allocation, sat 2 m away from the tank. Behavioral testing took place during the light phase between 9.00 and 14.00 h. Tank water consisted of deionized H_2O and sea salts (0.6 g/10 l of water; Instant Ocean, Aquarium Systems, Sarrebourg, France). The home tanks with groups of approximately 30 adult fish were maintained with constant filtration and aeration. Fish were fed daily with brine shrimp and flake fish food (tropical fish food, Consorzio G5, Italy). All the fish were drug naive, and each fish was used only once. Ten fish per group were used. All experiments were conducted in strict accordance with the European Community Council Directive No. 86/609/EEC and the subsequent Italian Law on the Protection of animals used for experimental and other scientific reasons. All efforts were made to minimize the number of animals used and their discomfort.

CPP

The fish were tested in a two-chamber tank (10 × 20 × 15 cm) as previously described (Braida et al. 2007). The tank was divided into two halves (10 × 10 cm) containing distinct visual cues (three black polka dots) with a perforated wall that allowed complete, albeit somewhat impeded, movement. On the first day, after a previous introduction to the apparatus, the fish were tested for baseline preference by calculating the percent of time spent on a given side during a 15-min trial (pre-conditioning phase: PRE). Six hours later, the fish were

given intramuscular (i.m.) injections with the different drugs and then restricted to the least preferred side for 30 min. Twenty four hours after, fish receiving vehicle were confined in the opposite compartment for 30 min. Drug-texture pairings were always counterbalanced. On the third day (post-conditioning phase: POST), the fish were free to access to two sides for 15 min, and the time spent in each compartment was recorded. The change in preference, obtained by subtracting the baseline value from the final value in the drug-paired compartment, reflected rewarding or aversive properties. Data were expressed as Δ% versus PRE.

Hallucinatory behavior

A simplified method to the one described previously (Braida et al. 2007) to evaluate the appearance of hallucinatory behavior through the fish observation was used. Hallucinatory behavior consisted of the appearance of a typical behavior in terms of "trance-like" effect (horizontal motionless position on bottom tank maintained for 2–3 min at a time at the peak of the narcosis and broken by a very brief change of position by means of a slight stimulus). The number of fish showing such behavior was counted. Immediately after treatment, each subject was placed in the transparent tank (20 × 10 × 15 cm) as previously described containing home tank water filled at a level of 12 cm. During the evaluation of hallucinatory behavior, in order to better quantify the swimming activity, the number of crossed lines, made by each fish, was measured in the same tank previously divided into ten equal-sized 2 × 10 cm rectangles marked with permanent marker. Using a time-sampling procedure, swimming activity was monitored by counting the number of lines crossed in a 30-s observation period, every min for a total of 5 observation bins, over 5 min, according to Ponzoni et al. (2014), with slight modifications. The tank was positioned in front of the webcam for optimal video recording for later video-aided analysis. Each video was evaluated by trained observers blind to treatment.

Treatment

Zebrafish body weight was measured as previously described (Braida et al. 2007). Briefly, fish were removed from their tank using a net and placed in a container containing tank water, positioned on a digital balance. Fish weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurements was recorded. Each fish was injected i.m. in the caudal musculature along the posterior axis. We chose to use intramuscular injection in order to control the amount of drug each fish received more precisely than to dissolve the drugs in the tank water. This type of injection did not alter fish behavior as previously described for social behavior (Braida et al. 2012). The site of injection was constantly maintained at the area below the caudal fin on the left side of each fish. Each volume, depending on the fish's weight (2 μL/g), was given using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). Fish were individually pulled out of the water tank with the use of a net. Then, each fish was kept immobilized through the net with two fingers of the left hand. Immediately after, the needle was positioned at a 45° angle in relation to the back of the fish with the needle pointing towards the head. The injection was in the largest portion of the caudal muscle, immediately posterior to the caudal fin. The needle was inserted into the muscle just beyond the bevel of the needle. Total time out of water was approximately 10 s. Each fish was removed from the net and immediately dipped in the tank water.

Drugs

MDMA (0.5–160 mg/kg), DOB (0.1–20 mg/kg), and PMA (0.0005–2 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile saline and administered i.m. immediately before each test. For the antagonism studies, ritanserin (0.025–2.5 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), the serotonin $5HT_2$ subtype receptor antagonist was put in the same syringe of the agonists to avoid potential tissue trauma as previously described (Ponzoni et al. 2014). Vehicle group received saline (2 μl/g). Ten fish per treatment were used. The doses of the drugs were calculated as salt. All drugs were prepared fresh daily. The dose range of MDMA was chosen on the basis of its ability to induce CPP in mice (Daza-Losada et al. 2007) and to produce significant subjective effects in humans (Harris et al. 2002). Due to the lack of data on PMA and DOB in zebrafish, a wide range of doses was used.

Statistical analysis

To objectively and reliably quantify trance-like behavior, inter-observer differences in scoring were tested with three observers who independently, through the videos, processed the scored behavior. Apart occasional exceptions, the three independent observers measured the same event. Inter-observer variability due to difference in animal handling and scoring was determined from the results of three separate drug studies carried out simultaneously but independently by two observers on a blind randomized treatment basis. Each observer contributed half of the data. The results were statistically analyzed using the appropriate statistical test and if no significance ($p > 0.05$) was obtained data were put together. One- or two-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni's post hoc tests was used to assess the differences among groups. The number of animals showing trance-like behavior was analyzed by Fisher's exact probability test. For CPP, data were analyzed by linear regressions and the ED_{50} (mg/kg) with its confidence limits, for the first part of

the curve, was calculated. ED50 values with non-overlapping confidence limits were considered significantly different according to Robertson et al. (1992). All the statistical analysis was performed using Prism 6 software (Prism version 6, GraphPad Inc, La Jolla, CA).

Results

CPP

The effect of increasing doses of MDMA, DOB, and PMA injected i.m. on CPP is shown in Fig. 1. Each zebrafish spent an equivalent average amount of time in each of the two outer compartments during the conditioning phase (425 ± 21 s in one with the polka dots and 475 ± 18 s in the one without the polka dots, $p = 0.1002$, not significant). Analysis of time spent during pre- and post-conditioning after different drugs showed main effects of treatment, time, and interaction: MDMA: treatment ($F(6, 126) = 5.75$, $p < 0.0001$), time ($F(1, 126) = 72.13$, $p < 0.0001$) and treatment × time ($F(6, 126) = 5.34$, $p < 0.0001$) (Fig. 1a); DOB: treatment ($F(5, 108) = 4.44$, $p = 0.001$), time ($F(1, 108) = 26.2$, $p < 0.0001$), and treatment × time ($F(5, 108) = 4.07$, $p = 0.002$) (Fig. 1b); PMA: treatment ($F(6, 126) = 7.35$, $p < 0.0001$), time ($F(1, 126) = 22.93$, $p < 0.0001$), and treatment × time ($F(6, 126) = 6.41$, $p < 0.0001$) (Fig. 1c). Post hoc revealed a significant increase of post-conditioning time after MDMA (from 0.5 to 10 mg/kg), DOB (0.25 to 1 mg/kg), and PMA (0.01 to 0.1 mg/kg) treatment. The progressive increase of CPP was interpolated by linear regression lines (Fig. 1d), statistically significant ($R^2_{MDMA} = 0.92$, $P = 0.04$; $R^2_{DOB} = 0.91$, $P = 0.04$; $R^2_{PMA} = 0.89$, $P = 0.05$). The ED_{50} (mg/kg) and confidence limits for the different drugs are reported in Fig. 2. PMA resulted the most potent and MDMA the less potent in modifying CPP. Notably, PMA resulted about 200 times more potent ($ED_{50} = 0.03 \pm 1.1$) compared to MDMA ($ED_{50} = 5.79 \pm 1.1$) and 13 times more active than DOB ($ED_{50} = 0.38 \pm 1.2$) in producing rewarding effect.

Hallucinatory behavior

DOB and PMA, but not MDMA, treatment modified normal swimming activity in zebrafish compared to saline group in terms of appearance of trance-like behavior (Fig. 3a–c). Fisher's exact probability test revealed a significant difference among groups, except for MDMA (Fig. 3a), Compared to saline group, which did not show any alteration, fish treated with DOB (2 mg/kg) (Fig. 3b) and PMA (0.1 mg/kg) (Fig. 3c) showed a significant increase in the number of animals showing trance-like behavior (Supplementary Video S1). Treatment with high doses of DOB (10 and 20 mg/kg) and PMA (0.25, 0.5, and 2 mg/kg) did not show trance-like behavior compared to saline group. The number of crossed lines (Fig. 3d–f) did not change after MDMA treatment ($F(8, 81) = 0.75$, $p = 0.65$), when compared to saline group. Instead, a significant decrease in swimming activity was observed after DOB ($F(7, 72) = 3.33$, $p = 0.004$) and PMA treatment ($F(7, 72) = 3.9$, $p < 0.005$), at 2 and 0.1 mg/kg, respectively.

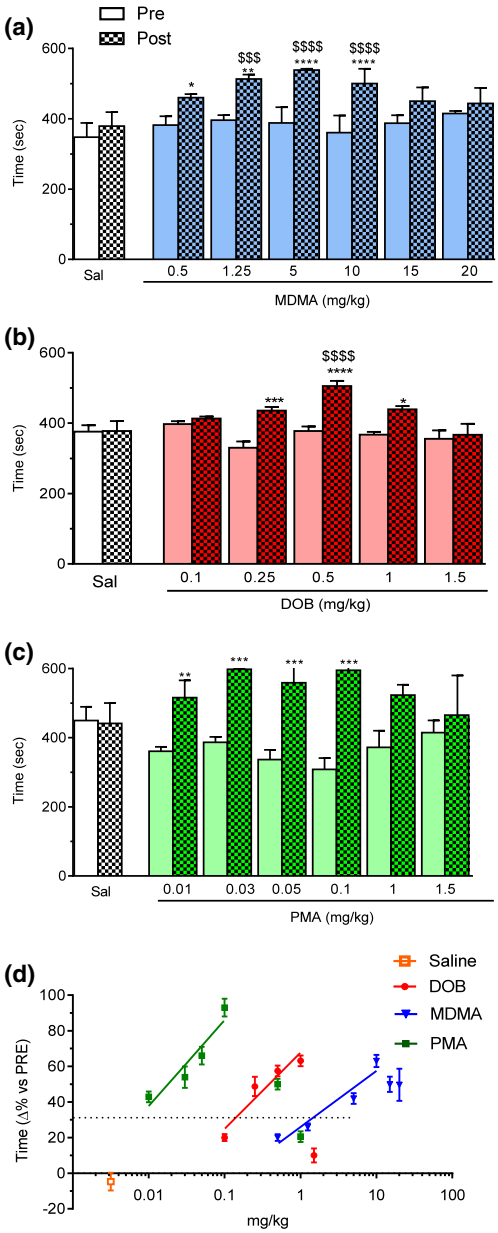

Fig. 1 Effect of MDMA (**a**), DOB (**b**), and PMA (**c**) on CPP in terms of time spent in the drug-paired compartment during pre- and post-conditioning. All the compounds elicited CPP following a biphasic effect. **d** Linear regression of the difference in time spent by each zebrafish in the drug-paired compartment between pre- and post-conditioning time expressed as Δ% versus PRE. All the drugs were given i.m. immediately before the conditioning session ($n = 10$ zebrafish for each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the corresponding pre-conditioning; $$$p < 0.001$, $$$$p < 0.0001$ compared with saline group during post-conditioning (Bonferroni post hoc test)

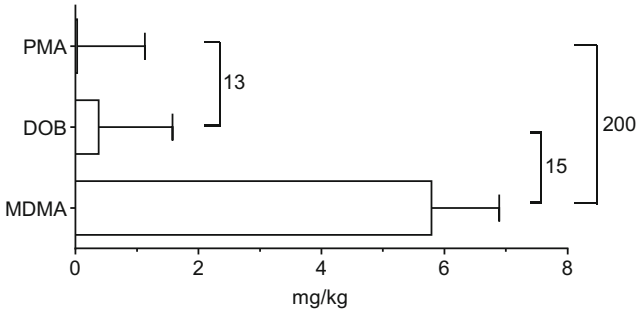

Fig. 2 ED_{50} (mg/kg) (± confidence limits) calculated on the basis of regression lines depicted in Fig. 1 for CPP following MDMA, DOB, and PMA treatment. The *numbers* in the figure represent the ratio

Antagonism study

To investigate the effects of serotonin $5HT_2$-type receptors on MDMA, DOB, and PMA behaviors, we used ritanserin. Analysis of the difference time spent during pre- and post-conditioning, after different drug treatments, combined with different doses of the antagonist showed main effects of agonist, antagonist, and agonist × antagonist interaction (Fig. 4): MDMA: agonist factor ($F(1, 54) = 21.78$, $p < 0.0001$), antagonist factor ($F(2, 54) = 9.44$, $p = 0.001$), and agonist × antagonist interaction ($F(2, 54) = 35.93$, $p < 0.0001$); DOB: agonist factor ($F(1, 54) = 1.94$, $p = 0.03$), antagonist factor ($F(2, 54) = 48.24$, $p < 0.0001$), and agonist × antagonist interaction ($F(2, 54) = 29.05$, $p < 0.0001$); and PMA: agonist factor ($F(1, 54) = 40.94$, $p < 0.0001$), antagonist factor ($F(2, 54) = 7.03$, $p = 0.002$), and agonist × antagonist interaction ($F(2, 54) = 24.12$, $p < 0.0001$).

Ritanserin antagonized trance-like behavior induced by DOB at 0.25 mg/kg while starting from 0.025 mg/kg blocked that induced by PMA (Fig. 5). Ritanserin, per se, did not induce any change.

Discussion

In the present study, we report the first direct comparison of the rewarding properties of MDMA and of its two derivatives, DOB and PMA, in zebrafish. In addition, DOB and PMA evoked a trance-like behavior resulting PMA the most potent drug. These effects were mediated through $5HT_2$-like receptor.

The CPP paradigm is assumed to reflect the motivational properties of drugs and their potential for abuse (Tzschentke 2007; Aguilar et al. 2009). Our findings on MDMA rewarding effects agree with previous reports in which the drug produced

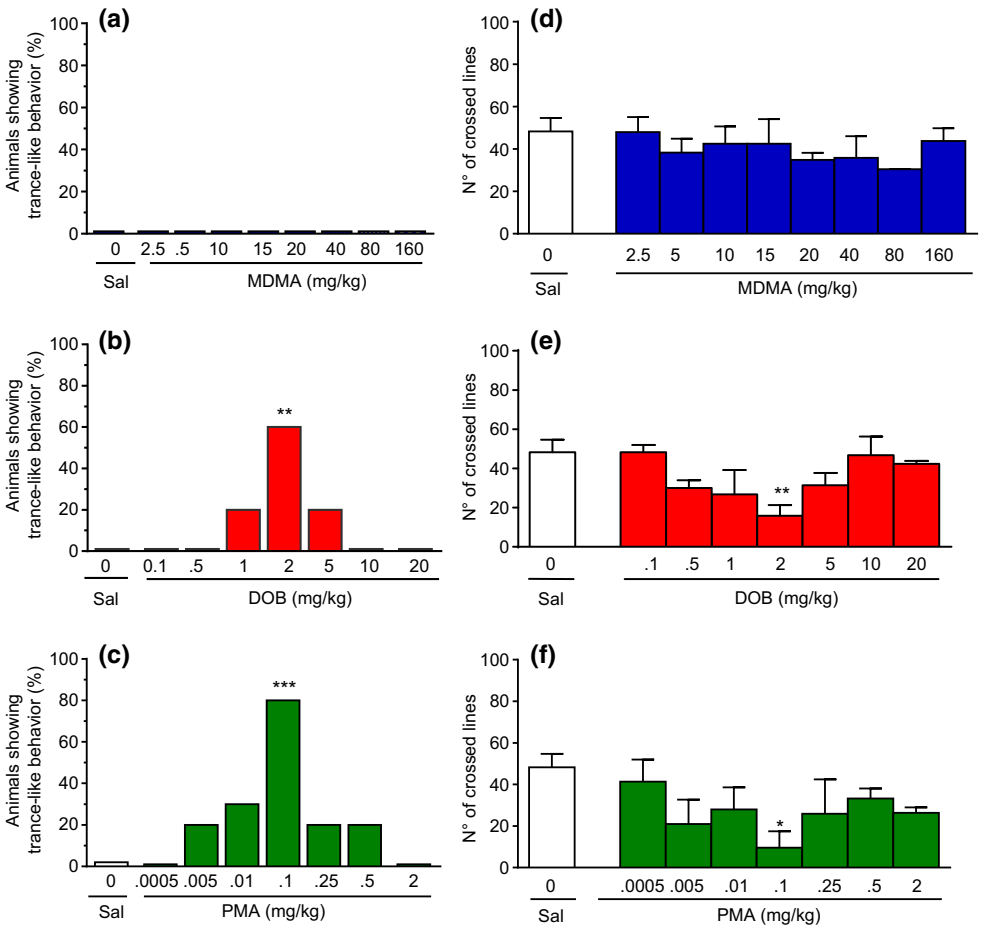

Fig. 3 Effect of increasing doses of MDMA (**a**), DOB (**b**), and PMA (**c**) on the appearance of trance-like effect and on the number of crossed lines (**d**–**f**) evaluated for 5 min after treatment. Each compound was given i.m immediately before the test. Data were expressed as percent or mean (± SEM). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the corresponding saline group (*Sal*) (Fisher's exact probability test or Tukey's test). ($n = 10$ zebrafish for each dose)

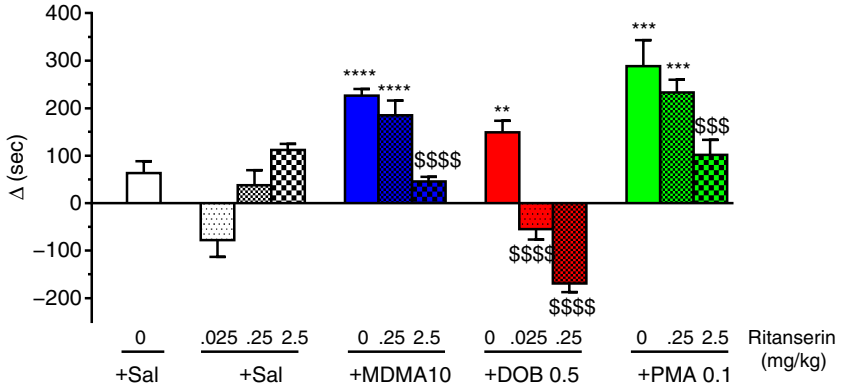

Fig. 4 Effect of ritanserin (mg/kg) on rewarding properties induced by MDMA, DOB, and PMA. Data were expressed as mean difference (± SEM) of time spent in the drug-paired compartment between post- and pre-conditioning. Ritanserin was co-administered with each drug or saline (*Sal*) i.m. immediately before the test. ($n = 10$ zebrafish for each group). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with the corresponding Sal group; $\$\$\$p < 0.001$, $\$\$\$\$p < 0.0001$ compared with the corresponding drug alone (Bonferroni's test)

CPP in mice (Daza-Losada et al. 2007; Rodríguez-Arias et al. 2010; Ribeiro Do Couto et al. 2012) and increased intravenous self-administration in rats (Fantegrossi et al. 2013; Vandewater et al. 2015). For DOB and PMA, this is the first report demonstrating their rewarding effects in animals. PMA and DOB showed hallucinatory behavior while MDMA did not, according to their structural chemistry belonging to methoxylated phenetylamine derivatives (Araújo et al. 2015). DOB has been shown to produce in humans strong hallucinogenic properties (Owens et al. 1991), while no reports are available for PMA whose only high toxicity has been described. Thus, this is the first evidence of PMA-induced hallucinatory behavior in animals.

About MDMA, we cannot exclude a hallucinatory behavior at higher doses than 160 mg/kg. However, in our study, we employed a range wider than that used for recreational use in humans (1.0–3.0 mg/kg) (Baylen and Rosenberg 2006; Dumont and Verkes 2006; Parrott 2013). Employing the Positive and Negative Affect Scales, an increase in negative mood, but not in hallucinations, following 100 mg oral MDMA (Parrott et al. 2011a, b) was found. Our finding agrees with the fact that MDMA is generally not considered a hallucinogen and hallucinations are not frequently reported in studies with human subjects, even if subtle visual alterations, such as color changes, have been reported as an effect of pills that were found to contain exclusively MDMA (de la Torre et al. 2004). Furthermore, few volunteers reported mild hallucinogen-like effects with 1.5 mg/kg. However, it must be noted that these volunteers had a previous history with MDMA (Harris et al. 2002). Since hallucinogens alter sensory functions (Winkelman 1991), it can be argued that the rewarding effects observed in zebrafish can be due to the hallucinogenic properties of the drugs. However, DOB and MDMA induced CPP at doses devoid of hallucinatory behavior. Due to the high potency of PMA, the influence of hallucinatory behavior on the other effects cannot be excluded.

We found that the number of crossed lines after DOB and PMA treatment decreased probably due to the appearance of trance-like behavior while no change in the locomotor response, after MDMA treatment, was observed. This finding

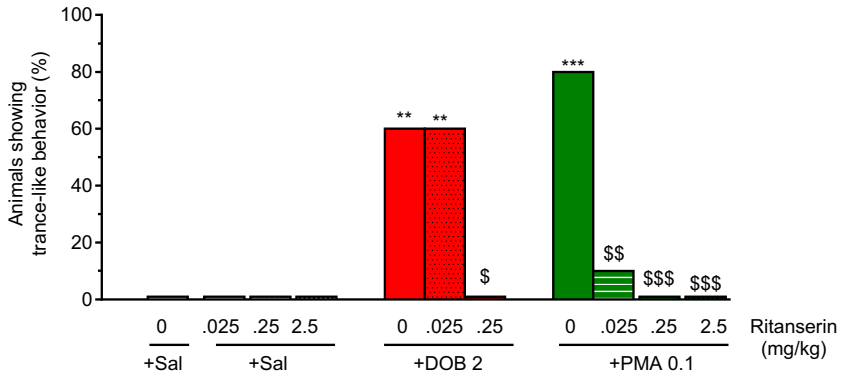

Fig. 5 Effect of PMA and DOB on the number of fish (%) showing trance-like behavior scored for 5 min after treatment. Ritanserin was co-administered with each drug or saline (*Sal*) i.m. immediately before the test. $n = 10$ zebrafish for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the corresponding saline group; $\$p < 0.05$, $\$\$p < 0.01$, $\$\$\$p < 0.001$ compared with the corresponding drug alone (Fisher's exact probability test)

appears in line with previous reports indicating that MDMA did not significantly alter zebrafish motor activity (Stewart et al. 2011).

Interestingly, in the CPP task, the compounds showed a biphasic pattern, being low doses progressively rewarding and high doses progressively ineffective. The inverted U-shaped curve can be readily found in animal models and in some human studies. In zebrafish, a biphasic effect for Salvinorin A, cocaine, and amphetamine on CPP, for neurohypophyseal hormones on social behavior and anxiety, and for ethanol on locomotion (for reference, see Stewart et al. 2011) was shown. In addition, a similar trend has also been observed in rodents when given different substances of abuse. For instance, MDMA produced CPP in rats at a dose of 5 mg/kg, but at 10 mg/kg, there was a return to baseline (control) performance levels (Diller et al. 2007). Methamphetamine produced biphasic effects either on locomotion (Huang et al. 2012) or on CPP paradigm with low doses producing reward and high doses aversion (Cunningham and Noble 1992). It is difficult to find a common mechanism of action shared by many drugs of abuse to explain the inverted U-shaped curve. The common neural substrate mediating the rewarding properties of different drugs of abuse is the increase of dopamine efflux in the mesolimbic dopaminergic system (Koob and Le Moal 2001). MDMA acts as a monoaminergic agonist that induces presynaptic release of 5-HT and DA (Kankaanpää et al. 1998; Green et al. 2003; Escobedo et al. 2005), mainly by reversing the functioning of the serotonin transporter. Moreover, MDMA increases DA in the nucleus accumbens primarily through serotonergic stimulation (Kankaanpää et al. 1998; Robledo et al. 2004; O'Shea et al. 2005; Cadoni et al. 2005). Thus, dopaminergic (Bilsky et al. 1998; Daniela et al. 2004; Brennan et al. 2009; Schenk et al. 2011; Vidal-Infer et al. 2012) and serotonergic (Roger-Sánchez et al. 2013a, b, c; Müller and Homberg 2015) mechanisms contribute to the rewarding effects of MDMA. On the other hand, in vivo microdialysis experiments have found that PMA markedly influences DA and 5-HT release in rat brain (Gołembiowska et al. 2016), while for DOB, no data are available on dopamine activity. Dopamine action in nucleus accumbens is mediated predominantly via activation of D1 and D2 dopamine receptors. These two subtypes exert opposite effects on behavior with activation of D1-type neurons promoting positive reinforcement and activation of D2-type neurons being aversive and decreasing reward (Kravitz et al. 2012). Notably, dopamine D1 and D2 subtype receptors have all been identified in zebrafish brain (Boehmler et al. 2004; Li et al. 2007). Thus, the lack of CPP at high doses could be the result of D1 but also D2 autoreceptor activation which in turn decrease dopaminergic excitation (Tran et al. 2015).

About the biphasic effect obtained for the trance-like effect after drug treatment, our findings agree with those of Fantegrossi et al. (2010) who found a biphasic dose–response effect for the phenethylamine hallucinogen *R*(−)-2,5-dimethoxy-4-iodoamphetamine (DOI) on the mouse head twitch, a typical response for hallucinogenic effect in rodents. A modulation of serotonin 5-HT_{2A} and 5-HT_{2C} receptors has been proposed. That is, activation of the 5-HT_{2A} receptor at low doses of the drug might be responsible for the ascending limb of the dose–response curve, whereas activation of serotonin 5-HT_{2C} receptor by higher doses might be responsible for the descending limb of the curve.

Notably, either on CPP or hallucinatory behaviors, PMA resulted the most active compound. Although there are no data regarding the effect of PMA on the behavior we observed, PMA and MDMA have markedly different effects on core body temperature and locomotor activity, resulting in PMA the most hyperthermic and the less stimulant on locomotion (Daws et al. 2000). It is well known that serotonin 5-HT_{2A} receptor agonists have typically been referred to "hallucinogens" and some of them (LSD, mescaline) have been tested also in zebrafish (Neelkantan et al. 2013). The antagonistic effect found with ritanserin likely agrees with the characteristic activation of serotonin 5-HT_2 receptors in brain (for reference, see Araújo et al. 2015). Notably, serotonin 5-HT_{2C} subtype receptor has been recently sequenced and cloned from zebrafish brain showing to be highly homologous to human and mouse sequences (Schneider et al. 2012).

Conclusions

Taken together, these findings demonstrate, for the first time, an abuse potential for MDMA and its two derivatives DOB and PMA, in zebrafish, being PMA the most active compound. The rewarding and hallucinatory effect was blocked by serotonin 5-HT_2 receptor subtype, suggesting the involvement of serotonergic system. Collectively, the observed effects of MDMA and the hallucinogens, in fish, strikingly resemble those in humans, further supporting the translational value of zebrafish model for psychedelic drug research.

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Compliance with ethical standards The experiments were performed in compliance with the recommendations of the European Community Council Directive No. 86/609/EEC and the subsequent Italian Law on the Protection of animals used for experimental and other scientific reasons. The experimental protocol was approved by the Italian Governmental Decree No. 18/2013. All efforts were made to minimize the number of animals used and their discomfort.

Conflict of interest The authors do not declare any conflict of interest.

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