Behavioral effects of MDMA ('ecstasy') on adult zebrafish

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3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is a potent psychedelic drug inducing euphoria and hypersociability in humans, as well as hyperactivity and anxiety in rodents. Adult zebrafish (*Danio rerio*) have become a widely used species in neurobehavioral research. Here, we explore the effects of a wide range (0.25–120 mg/l) of acute MDMA doses on zebrafish behavior in the novel tank test. Although MDMA was inactive at lower doses (0.25–10 mg/l), higher doses reduced bottom swimming and immobility (40–120 mg/l) and impaired intrasession habituation (10–120 mg/l). MDMA also elevated brain *c-fos* expression, collectively confirming the usage of zebrafish models for screening of hallucinogenic compounds. *Behavioural Pharmacology*

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is a popular recreational drug that modulates brain monoamines by inhibiting their reuptake (White *et al.*, 1996; Kalant, 2001; De la Torre *et al.*, 2004; Nagai *et al.*, 2007; Doly *et al.*, 2009) and degradation (Leonardi and Azmitia, 1994). The serotonergic system seems to be the primary target of MDMA action, although dopamine also plays an important role (Benturquia *et al.*, 2008; NIDA, 2010; Stove *et al.*, 2010).

Typical clinical effects of MDMA include euphoria, elation and sociability (Parrott, 2007; Bedi *et al.*, 2009; Stove *et al.*, 2010). MDMA can evoke adverse effects, including anxiety, depression, psychoses and cognitive deficits (Hall and Henry, 2006; Control, 2009; NIDA, 2010). The behavioral effects of acute MDMA have been extensively investigated in various animal models. In rodents, MDMA induces robust hyperlocomotion (Benturquia *et al.*, 2008; Colussi-Mas and Schenk, 2008; Stove *et al.*, 2010) and anxiety (Lin *et al.*, 1999; Ho *et al.*, 2004).

Earlier research has used the zebrafish as a model sensitive to various pharmacological manipulations, including the hallucinogens lysergic acid diethylamide (LSD) and salvinorin A (Braida *et al.*, 2007; Grossman *et al.*, 2010). As MDMA has not yet been tested in zebrafish, we examined its behavioral effects on this species. Finally, published rodent data show that psychedelic agents (e.g. LSD and MDMA), elevate the expression of *c-fos*, serving as a marker of neuronal activation that correlates with behavioral alterations (Salzmann *et al.*, 2003; Benturquia *et al.*, 2008; Reissig *et al.*, 2008). On the basis of earlier studies validating brain *c-fos* analyses in zebrafish (Baraban *et al.*, 2005; Wong *et al.*, 2010b), we also examined the effects of MDMA on *c-fos* expression.

Methods

Subjects and housing

A total of 142 adult (5–7-month-old) male and female wild-type (short-fin) zebrafish were obtained from a local commercial distributor (50 Fathoms, Metairie, Louisiana, USA). All fish were housed in groups of 20–30 fishes per 40 l tank. The tanks were filled with filtered system water and maintained at 25–27°C on a 14 : 10-h cycle. All fish used in this study were experimentally naive, and fed Tetramin Tropical Flakes twice daily. Animal experiments and care adhered to institutional and national regulations.

Behavioral testing

Behavioral testing was done between 11 : 00 and 15 : 00 h using tanks with water adjusted to the holding room temperature. To avoid the test battery effect, each experiment was carried out on a separate cohort. Zebrafish behavior was recorded by two trained observers (interrater reliability > 0.85) blinded to the treatments, and analyzed using Ethovision XT7 (Noldus IT, Wageningen, The Netherlands).

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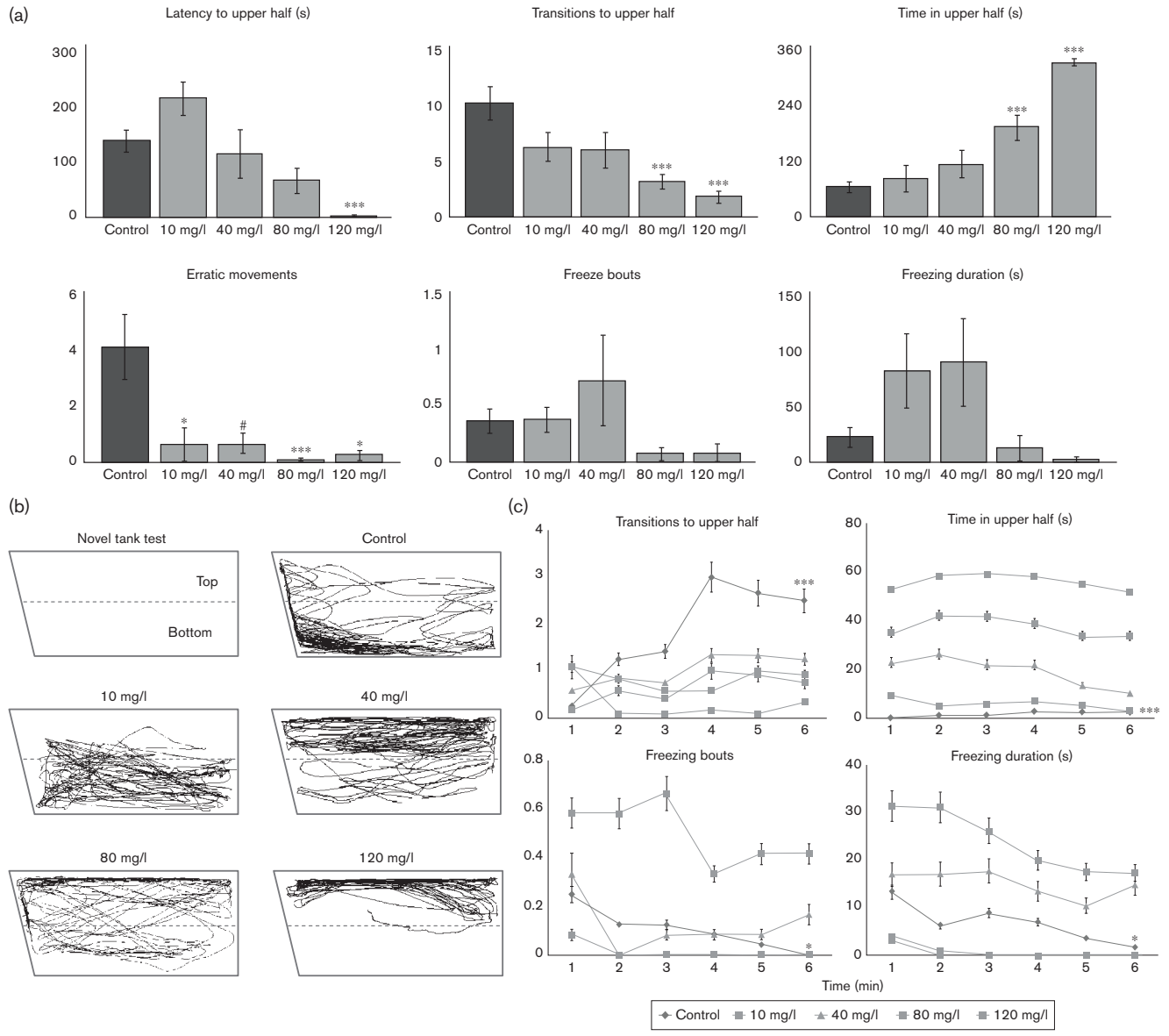
Experiment 1 tested zebrafish behavior in the standard, 6-min novel tank test following a 20-min pretreatment with varying doses (0.25–120 mg/l) of MDMA. Experiment 2 used mild-to-high effective doses (40, 80 and 120 mg/l, established in Experiment 1) and assessed rapid MDMA action, exposing zebrafish to a 30-min novel tank filled with drug-treated water, similar to the protocol described previously for LSD (Grossman *et al.*, 2010).

The novel tank test, used to assess zebrafish anxiety and locomotion (Levin *et al.*, 2007; Bencan *et al.*, 2009; Egan *et al.*, 2009), was a 1.5 l trapezoidal tank (15 height × 28 top × 23 bottom × 7 cm width; Aquatic Habitats, Apopka, Florida, USA; Fig. 1b) maximally filled with water and divided into two equal virtual horizontal portions [top and bottom (Egan *et al.*, 2009)]. The following endpoints were assessed in this paradigm: latency (s), entries to and

Fig. 1



Behavioral effects of acute 20-min exposure to 3,4-methylenedioxymethamphetamine (MDMA) on zebrafish tested in the standard 6-min novel tank test [Experiment 1; $n=27$ (controls), 28 (10 mg/l), 12 (40 mg/l), 27 (80 mg/l) and 12 (120 mg/l)]. (a) Manually recorded behavioral endpoints [$^{*}P<0.05$, $^{***}P<0.001$, $^{\#}P=0.05$–0.01 (trend) vs. control; post-hoc Tukey test for significant ANOVA data]. (b) Two-dimensional traces generated using Ethovision XT7 software and a side-view camera. Two-dimensional traces were examined for each experimental cohort, rated from 1 to n (based on similarity to each other), and the middle trace was selected as representative, to best illustrate the typical patterns of zebrafish locomotion. (c) Habituation (per-minute distribution) of zebrafish behavioral activity ($^{*}P<0.05$, $^{***}P<0.005$, minute 1 vs. minute 6 of the respective drug group, *U*-test).

time spent (s) in the upper portion, the frequency of erratic movements (sharp changes in direction, and unorganized spontaneous darting), as well as the frequency and duration (s) of freezing bouts [absence of movement, except for eyes and gills, for > 2 s (Egan *et al.*, 2009)]. Once manual data were generated for each minute of the test in Experiment 1, we examined intrasession habituation of zebrafish behavior by comparing behavioral scores for the first and last minutes of the test (Wong *et al.*, 2010a).

Video tracking

Recorded videos were analyzed using Ethovision XT7 (Noldus IT), as described previously (Cachat *et al.*, 2010a; Grossman *et al.*, 2010; Wong *et al.*, 2010a). The exported 'side view' two-dimensional traces were independently rated on a consensus basis from 1 to *n* (based on similarity to each other) by three trained observers blinded to the treatments. The middle trace was selected as representative for the group, to illustrate the two-dimensional spatial pattern of swimming activity (Grossman *et al.*, 2010).

Pharmacological manipulations

MDMA for this study was obtained through the NIDA Drug Supply Program. MDMA doses (40–120 mg/l) were chosen based on our pilot studies with a wide range of doses (0.25–120 mg/l). A standard 20-min pretreatment time was chosen here based on our pilot experiments with MDMA and other similar hallucinogenics (Grossman *et al.*, 2010; Stewart *et al.*, 2011a). Pilot testing of the dose range in the novel tank showed effects of MDMA at doses between 40 and 120 mg/l (Fig. 1), but an absence of effects at smaller doses (data not shown). Drug exposure was done by immersing individual zebrafish in a 1-l plastic beaker for 20 min before the testing (Experiment 1) or into a 1.5-l novel tank for 30 min during the testing (Experiment 2). Control fish were exposed to drug-free facility water for the same treatment time.

c-fos expression assay

Real time-PCR was performed for zebrafish *c-fos* mRNA in separate cohorts of animals exposed for 20 min to either drug-free water or to a behaviorally active dose (40 and 80 mg/l) of MDMA. The brains were dissected, with two brains combined per sample (six samples per group) for RNA extraction. cDNA was synthesized using random primers and iScript Select cDNA Synthesis Kit (Bio Rad, California, USA). For quantitative-PCR, cDNA was amplified with *c-fos* forward and reverse primers (Tang *et al.*, 2007).

Statistical analysis

Data were analyzed using one-way analysis of variance (factor: dose) followed by post-hoc Tukey testing for significance. Intra-session habituation (minute 1 vs. minute 6) data were tested using the paired *U*-test. Experiment 2 data were analyzed using one-way analysis of variance (factor: dose) with repeated measures (test minutes 1–30) followed by post-hoc Tukey testing (vs. respective minute in the control group) for significance. *c-fos* expression data were assessed using the nonpaired *U*-test (control vs. respective drug-treated group). Data were expressed as mean ± standard error of the mean. Significance was set at *P* value of less than 0.05.

Results

In Experiment 1, acute exposure to MDMA dose-dependently effected novel tank behavior, modulating latency to the top, top transitions, time spent in top and the number of erratic movements [$F(4,105) = 7.9$, 6.9, 23.3 and 5.9, respectively; $P < 0.001$] as well as freezing bouts and duration [$F(4,105) = 2.8$ and 3.5, $P < 0.05$; Fig. 1a]. There was a dose-dependent reduction in latency to top, top transitions and erratic movements, as well as an increase in time spent in top (Fig. 1a). These behavioral profiles were also evident in computer-generated two-dimensional traces of zebrafish swimming, showing a dose-dependent increase in top dwelling in MDMA-treated fish (Fig. 1b).

Interesting effects were observed for per-minute distribution of zebrafish activity that reflects intrasession habituation, particularly sensitive to various psychotropic drugs (Wong *et al.*, 2010a). Analysis of fish behavior in Experiment 1 showed robust intrasession habituation in control zebrafish, with significant minute 1 versus minute 6 differences for the number of top entries and time in top ($P < 0.0005$, *U*-test), as well as freezing bouts and duration ($P < 0.05$, *U*-test). There was no difference between 1 versus 6 min for erratic movements in control fish, consistent with earlier studies on the lack of intrasession habituation of this behavior (Wong *et al.*, 2010a). In contrast, MDMA exposure at 40, 80 and 120 mg/l markedly impaired zebrafish habituation, yielding no significant differences for minute 1 versus minute 6 data for the number of top entries, time in top, freezing bouts and freezing duration (Fig. 1c). Erratic movements, unaffected in controls, showed no habituation in any of the MDMA-treated fish cohorts (data not shown).

Experiment 2 examined the immediate effects of MDMA using a 30-min novel tank test filled with drug-treated water. Although most behaviors were similar to those observed in Experiment 1, Fig. 2 shows that MDMA at 40 and 80 mg/l rapidly affected zebrafish behavior, within 5–10 min evoking typical top dwelling responses reported in Experiment 2. Similarly, there were no anxiogenic effects or behavioral inhibition, as the drug-exposed fish displayed top dwelling and lower immobility throughout this test.

Finally, acute 20-min exposure to moderate behaviorally active doses of MDMA affected brain *c-fos* expression, causing a 12.3-fold (not significant) elevation at 40 mg/l

and a significant 26.6-fold increase at 80 mg/l ($P < 0.01$, *U*-test).

Discussion

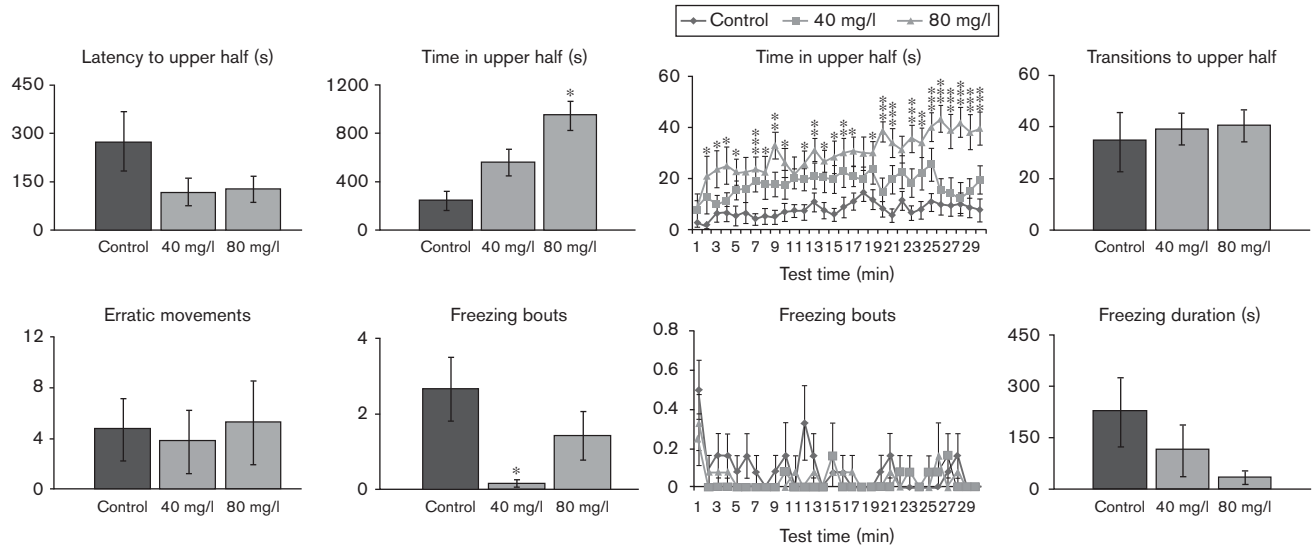
This study is the first report on the effects of MDMA in zebrafish, showing increased top dwelling, reduced immobility, impaired intrasession habituation and elevated brain *c-fos* expression. Although MDMA induces predominantly pleasant effects in humans (Liechti *et al.*, 2000; Parrott, 2007; Bedi *et al.*, 2009), it evokes hyperlocomotion and anxiety in rodents (Gurtman *et al.*, 2002; Navarro *et al.*, 2004; Sumnall *et al.*, 2004; Faria *et al.*, 2006). In the zebrafish novel tank paradigm, increased top dwelling typically implies reduced anxiety (Levin *et al.*, 2006; Egan *et al.*, 2009; Cachat *et al.*, 2010b). Similar to MDMA action in humans, our study did not find anxiety-like behavior in zebrafish (Figs 1 and 2). It is possible that other factors play a role in the reduced apparent anxiety of our zebrafish. For example, anxiolytic manipulations usually increase several 'top' behaviors, including both time spent and the number of entries to top (Levin *et al.*, 2006; Egan *et al.*, 2009; Cachat *et al.*, 2010b). In this study, the number of top entries was significantly reduced (Fig. 1), implying that top dwelling observed here may differ from a typical zebrafish anxiolytic response.

One possibility for this can be a serotonin syndrome-like state induced by serotonergic drugs in zebrafish (Stewart *et al.*, 2010). Similar 'surfacing' behavior was induced by other serotonergic drugs in this (Egan *et al.*, 2009; Grossman *et al.*, 2010; Sackerman *et al.*, 2010; Stewart *et al.*, 2011b) and other aquatic species (Abramson *et al.*, 1962). Alternatively, given the known properties of MDMA, increased top dwelling may also represent zebrafish disorientation and/or hallucinogenic-like states. For example, similar phenotypes were induced in zebrafish by other hallucinogens, such as LSD (Grossman *et al.*, 2010), salvinorin A (Braida *et al.*, 2007) and ketamine (Zakhary *et al.*, 2010), lending indirect support to this notion.

The behavioral effects of MDMA strikingly resemble the effects of LSD, another psychedelic drug previously tested in zebrafish (Grossman *et al.*, 2010). Effective doses of MDMA identified in this study (40–120 mg/l) were 450–800 times higher than the effective doses of LSD (50–250 μg/l) in zebrafish (Grossman *et al.*, 2010; Stewart *et al.*, 2011b). In humans, approximately 0.4–1 μg/kg LSD is generally sufficient to produce strong behavioral effects, compared with approximately 0.4–2 mg/kg (1000–2000 times higher) doses of a less potent MDMA (Control, 2007, 2009). Similar relative efficacy of these two drugs in zebrafish was close to that observed in humans, strongly supporting the translational value of zebrafish models for drug abuse research.

In line with earlier studies on zebrafish habituation (Wong *et al.*, 2010a), MDMA altered zebrafish habituation, maintaining locomotion at a constant level throughout the

Fig. 2



Immediate behavioral effects of 40 and 80 mg/l MDMA on zebrafish tested in the 30-min novel tank containing drug-treated water (Experiment 2; $n = 12$ in each group). Analysis of variance (ANOVA) analyses showed significant dose effect for time in top [$F(2,35) = 11.2$, $P < 0.0001$] and freezing bouts [$F(2,35) = 11.2$, NS, $P < 0.05$], but not for latency to top [$F(2,35) = 1.9$, not significant (NS)], transitions to top [$F(2,35) = 0.9$, NS], freezing duration [$F(2,35) = 1.6$, NS] or erratic movements [$F(2,35) = 0.1$, NS]. Line diagrams with per-minute distribution of activity are presented only for two endpoints with significant dose effect. Post-hoc Tukey test for significant ANOVA data; for bar diagrams $^*P < 0.05$ versus respective controls; for line diagrams $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ versus the respective minute's control value. Note marked behavioral effects (increased time in top) starting to appear in zebrafish within the first 5–10 min of the drug treatment.

test (Fig. 1). Although the ability of MDMA to impair habituation has also been reported in rodents (Gold and Koob, 1989; Kehne *et al.*, 1992; Scearce-Levie *et al.*, 1999), other factors (e.g. drug-induced hyperlocomotion) may also contribute to this phenotype.

Mounting experimental evidence links MDMA behavioral effects to altered expression of brain *c-fos*. For example, MDMA increases *c-fos* and Fos in multiple areas of rodent brain (Stephenson *et al.*, 1999; Salzmann *et al.*, 2003; Colussi-Mas and Schenk, 2008). Similar effects have been reported for LSD (Gresch *et al.*, 2002; Reissig *et al.*, 2008). In general, elevated zebrafish *c-fos* following the acute MDMA exposure reported here parallels rodent *c-fos* evidence, and zebrafish LSD data (own unpublished observations).

In summary, our study showed that 40–120 mg/l of MDMA evokes robust behavioral responses in zebrafish (Figs 1 and 2), paralleling some animal and clinical effects of this drug. MDMA also induced physiological responses in zebrafish, elevating brain *c-fos* expression, similar to its effects in rodents. Expanding previous zebrafish reports using various psychotropic agents, such as LSD, salvinorin A, ketamine and dizocilpine (Swain *et al.*, 2004; Braida *et al.*, 2007; Grossman *et al.*, 2010; Seibt *et al.*, 2010; Zakhary *et al.*, 2010), this MDMA study supports high sensitivity of this aquatic model to hallucinogenic drugs.

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