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Full Length Article

Developmental exposure to MDMA (ecstasy) in zebrafish embryos reproduces the neurotoxicity adverse outcome 'lower motor activity' described in humans

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ABSTRACT

The recreational use of MDMA (ecstasy) by pregnant women is associated with impaired neuromotor function in infants, but the Adverse Outcome Pathway behind this effect is not clear yet. We present for the first time the evaluation of developmental neurotoxic (DNT) effects of MDMA in zebrafish embryos. The aim of the study was to determine whether the zebrafish model reproduces the adverse outcome occurring in humans. We have studied the DNT effects of MDMA in zebrafish within a range of 5–250 μM performing different behavioural tests: spontaneous tail-coiling and light-dark locomotor response; after exposing the embryos to 4 different scenarios combining changes in pH, in starting exposure time and exposure duration. In these scenarios we evaluated the effects of MDMA in general embryonic development and compared the concentrations producing them with those inducing specific DNT effects. As a result, we have established the experimental conditions leading to the adverse outcome "lower motor activity" in zebrafish without producing general developmental delay or general toxicity. The experimental condition chosen opens the door to use this model in future mechanistic investigations to better characterize the Adverse Outcome Pathway associated with the adverse effects caused by MDMA prenatal exposure in humans.

1. Introduction

The use and abuse of recreational drugs during pregnancy causes concern due to the potential health risks for the developing child. Adverse effects of the consumption of legal drugs, such as tobacco and alcohol, on development have been widely substantiated by epidemiological and experimental studies. However, the knowledge of the hazards associated to the use of many illicit drugs during pregnancy is very limited (reviewed by Ross et al., 2015). One important group of illicit drugs with such limited information are the amphetamine derivatives like MDMA (3,4-methylenedioxymethamphetamine).

MDMA, commonly known as ecstasy, is a synthetic drug mainly consumed by teenagers and young adults in childbearing age for its psychotropic actions (EMCDDA, 2017). MDMA has both stimulant and hallucinogenic properties. It acts as a powerful releasing agent of serotonin, norepinephrine and dopamine and also acts as a reuptake inhibitor of their high-affinity transporters. Its psychostimulant and 'entactogen' effects enhance emotional empathy and prosocial behaviour and boost high risk sexual behaviours including casual and unprotected sex, increasing the likelihood of unwanted pregnancy (Castilla et al., 1999; Mattison et al., 2001; May and Parrott, 2015; Palamar et al., 2018). Although it is difficult to obtain exact information on drug consumption during pregnancy, a 0.1 % of the pregnant population was found to have consumed MDMA in a study from Pichini et al. (2005), while 1.4 % of mother requesting voluntary termination of pregnancy consumed it according to Falcon et al. (2010). Besides, the majority of pregnant MDMA consumers continue to use the drug during the first trimester, thus during the main period of the organogenesis, but they only continue consuming during the whole gestation in very rare cases (Ho et al., 2001; Moore et al., 2010).

Meconium analyses have confirmed that MDMA crosses the placenta and reaches the developing child after maternal intake (Pichini et al., 2005), and studies in pregnant rats demonstrated that MDMA reaches the fetal brain (Campbell et al., 2006). Concerning the postnatal period,

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it has been inferred that MDMA is probably found in breast milk as it is a low-molecular-weight hydrophobic molecule with a pKa of 9.9 (Cho et al., 2008). Transfer of methylamphetamine and amphetamine into human breast milk following recreational use of methylamphetamine has been demonstrated (Bartu et al., 2009).

According to our recent review about the developmental neurotoxicity (DNT) of MDMA (Barenys et al., 2020), the main adverse outcome reported in human epidemiological studies after intrauterine exposure to MDMA is a motor delay (Singer et al., 2012a, b). The effect is subtle at 1 month of age with a trend to lethargic behaviours and hypotonia, but becomes more evident three months later, with poorer motor quality, less coordination and more slow and delayed movements (Singer et al., 2012a). At 12 months of age, MDMA-exposed children show a lower Psychomotor Index (PDI) of the Bayley Development Scales, which accounts for gross and fine motor control and coordination. This lower PDI persists at 18 and 24 months of age in a group of heavily exposed children (Singer et al., 2012b). Besides, a lower Mental Development Index (MDI) is detected in children at 12 months of age (Singer et al., 2012b), but this effect is no longer observed at later ages, and no other effects on language, emotional regulation or attention are observed (Singer et al., 2016).

Many animal studies have also been conducted to study in detail the neurodevelopmental effects of MDMA. Most of these studies have been performed in rats and several of them study locomotor behaviour after pre- or postnatal MDMA exposure. In these studies, the exposure period appeared to be crucial for the adverse outcome: prenatal exposure either causes no adverse effects in locomotion or significant hyperactivity (Koprich et al., 2003; Thompson et al., 2009; Canales and Ferrer-Donato, 2014), while direct postnatal MDMA administration causes hypoactivity (Cohen et al., 2005; Vorhees et al., 2009; Skelton et al., 2012). Differences in MDMA effects and critical exposure windows between rodents and humans could be due to the relative shift in the pre- and postnatal brain maturation, as some brain developmental milestones in the prenatal human brain development overlap with postnatal rodent brain maturation (Semple et al., 2013; Workman et al., 2013).

Surprisingly, only few *in vitro* studies evaluated the effects of MDMA on the developing nervous system, and none of them was performed in whole organism models alternative to animal experimentation (Barenys et al., 2020). The zebrafish has long been applied as a model in the field of developmental biology and has also emerged as a popular tool for investigating the neurotoxicity of drugs and environmental chemicals (Lee and Freeman, 2014). Before 120 h post-fertilization (hpf) it is considered an alternative model to animal experimentation, and as it is a whole organism model, it allows the study of mechanisms of action as well as the evaluation of functional adverse outcomes like behaviour alterations. The high degree of genetic similarity with humans and other vertebrates and the similarity of neural development supports the application of the zebrafish as a complementary research tool to conventional vertebrate models for DNT assays. As zebrafish embryos and larvae show a wide repertoire of behaviour patterns that can be affected by neurological disorders (Orger and de Polavieja, 2017; Basnet et al., 2019; Vaz et al., 2019), it is reasonable to hypothesize that the zebrafish could be a good model to study the DNT effects of MDMA. Once established, this model could also be useful to evaluate the DNT effects of other amphetamine-derivatives, since their physicochemical properties and patterns of consumption are very similar.

In this work we have studied the DNT adverse outcomes of MDMA in zebrafish using different behavioural tests: spontaneous tail-coiling and light-dark locomotor response; and under different exposure conditions: pH (to increase the embryonic uptake of the drug; (Bittner et al., 2018)), testing time and exposure duration. Our aim was to determine whether the zebrafish reproduces the effects of MDMA observed in humans and rodents and under which exposure conditions, to finally establish a good alternative model for future MDMA DNT mechanistic assays.

2. Material and methods

2.1. Ethics statement

Experiments were conducted in accordance with the Ethics Committee for Animal Experimentation of the University of Barcelona (CCEA). The maintenance of the adult zebrafish colony was accepted with license number 334/18 of the Department of Environment and Housing of the Generalitat de Catalunya.

2.2. Zebrafish colony maintenance, egg harvest and selection

Adult zebrafish (Danio rerio; Piscicultura Iberica, Spain) were maintained in aquariums with a closed flow-through system in standardized water as specified in ISO 7346-3 (1996), at a temperature of 26 ± 1 °C, and a constant light:dark cycle of 14:10 h. Fish were daily fed with Artemia salina in the morning, and commercial flake food (Immunopro Mini) in the afternoon. Fish were placed overnight in a mating tank with artificial plants and marbles to stimulate spawning. Next morning, after lights turned on, eggs were collected by filtering the water with a sieve, and afterwards washed a minimum of three times with standardized ISO 7346–3 water diluted 1:5. Fertilized, non-coagulated, and synchronously divided eggs were selected using a stereomicroscope (Motic SMZ168, Motic China group, LTD., China) and were transferred to a 6-well plate (10 embryos/well).

2.3. Exposure conditions

After egg selection (maximum at 2 hpf) the water of each well was replaced with 5 mL of 0.3X Danieau's solution or freshly prepared test solutions of MDMA (CAS: 64057-70-1) in 0.3X Danieau's solution. MDMA, as (±)-3,4-Methylenedioxymethamphetamine hydrochloride was provided by the Government Delegate's Office of Catalonia (Health Department) and purified (99.5% purity) by the Organic Chemistry Laboratory from the University of Barcelona as follows: MDMA (50 g, 60 % purity) was dissolved in CH_2Cl_2 (600 mL) and the brown suspension obtained was stirred for 30 min. The mixture was filtered, and the solution was concentrated under reduced pressure to give 28.9 g of pure MDMA-base (57.8 % recovered) as a brown solid, which was identified by HNMR (400 MHz). The brown solid was dissolved in a mixture of EtOH–Et_2O (60 mL-10 mL) and the solution was stirred at 50 °C until total dissolution of the solid (20 min). The organic solution was acidified by slow addition of a 1.25 M solution of ethanol-HCl (30 mL, acid pH) observing the appearance of a white solid. The solution was concentrated under reduced pressure to give the hydrochloride of MDMA as a white solid, which was washed with ether several times. Et_2O was decanted and white solid was dried in a desiccator in the presence of KOH and phosphorus pentoxide, getting 18.3 g (36.6 % recovered) of MDMA-HCl (melting point 148−149 °C). Embryos were incubated at 26 ± 1 °C with a light:dark cycle of 14:10 h in one of the following four different exposure scenarios (Figs. 1 and 2). In these fours scenarios, assessors were not blind to treatment conditions, but two different assessors participated in the evaluations.

2.3.1. Developmental exposure (2−72 hpf) at pH 7.4 for general developmental evaluation

Zebrafish embryos were exposed to 0, 5, 25 or 50 μM MDMA from 2 to 72 hpf under semi-static conditions (solutions were renewed at 24 and 48 hpf). General developmental landmarks like morphological development of different structures, spontaneous movements and hatching time, as well as presence of specific dysmorphogeneses were evaluated as follows. Morphological development and dysmorphogenesis were evaluated at 24, 48, and 72 hpf using a score system previously established (Beekhuijzen et al., 2015), and described in brief in Table 1:

The frequency of spontaneous tail coiling was evaluated at 24 hpf (see spontaneous tail coiling section). Hatching of eggs was visually

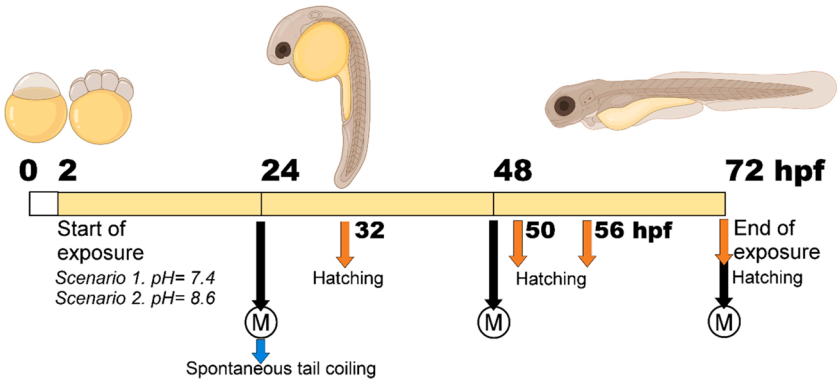

Fig. 1. Schematic representation of exposure scenarios 1 and 2. Exposure duration and endpoints evaluated were the same in both scenarios. The only difference between scenario 1 and 2 was the pH of the buffered medium (scenario 1: pH = 7.4; scenario 2: pH = 8.6). Blue arrow indicates evaluation of spontaneous tail coiling at 24 hpf, black arrows indicate evaluation of morphology at 24, 48 and 72 hpf and red arrows indicate hatching evaluation at 32, 50, 56 and 72 hpf. M: morphological evaluation; hpf: hours post-fertilization.

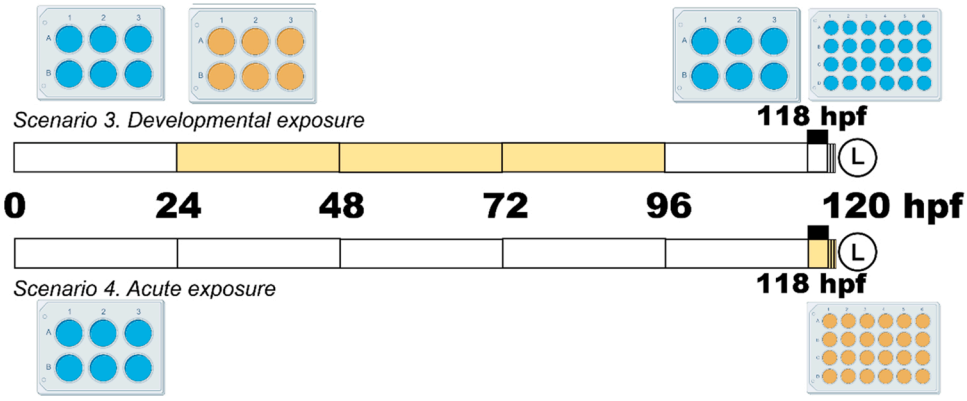

Fig. 2. Schematic representation of exposure scenarios 3 and 4. In both scenarios exposure solutions had a pH value of 7.4, and locomotor response to light/dark stimuli was evaluated at 120 hpf. The difference between scenario 3 and 4 was the moment of starting the exposure and the duration of the exposure to MDMA. L: locomotor response to light/dark stimuli evaluation. Orange-colored wells and time-periods indicate MDMA exposure. Blue-colored wells and transparent time-periods indicate embryos were grown in Danieau's solution 0.3X without exposure.

Table 1
Summary of the general morphological score system used at 24, 48 and 72 hpf established by (Beekhuijzen et al., 2015).

Hours post-fertilization (hpf)	24 hpf	48 hpf	72 hpf
Detachment of the tail	2	3	3
Somite formation	1	1	1
Eye development	2	3	3
Movement	1	1	1
Circulation	1	1	1
Heartbeat	0	1	1
Pigmentation of the body	0	1	1
Pigmentation of the tail	0	1	1
Pectoral fin	0	0	1
Protruding mouth	0	0	1
Hatching	0	0	1
Total Morphological Score (TMS)	7	12	15

evaluated at 32, 50, 56 and 72 h under a stereomicroscope. At 72 hpf the length of larvae was measured under a stereomicroscope with an ocular micrometre while keeping the larvae in a buffered tricaine (0.01% w/v) solution.

2.3.2. Developmental exposure (2−72 hpf) at pH 8.6 for general developmental evaluation

This experimental scenario was performed under the same conditions of exposure, at the same time-points and evaluating the same endpoints than in condition 1, excluding that all solutions were prepared at pH 8.6 by replacing HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAS: 7365-45-9) from Danieau's solution 0.3X by TAPS buffer (N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; CAS: 29915-38-6) as previously described (Bittner et al., 2018).

2.3.3. Developmental exposure (24-96 hpf) at pH 7.4 for locomotor response evaluation at 120 hpf

Zebrafish embryos were grown in 6-well plates (10 larvae/well in 5 mL) in Danieau's solution 0.3X for 24 h and then exposed to 0, 5, 25 or 50 μM MDMA (10 larvae/well in 10 mL) until 96 hpf, when they were transferred to a new 6-well plate with Danieau's solution 0.3X (10 larvae/well in 5 mL). At 118 hpf, larvae were transferred to 24-well plates (1 larvae/well in 2 mL), and kept under dark conditions for 2 h until their locomotor response to light/dark stimuli was evaluated at 120 hpf (see locomotor response to light/dark stimuli section).

2.3.4. Acute exposure (118–120 hpf) at pH 7.4 for locomotor response evaluation at 120 hpf

Zebrafish embryos were grown in 6-well plates in Danieau's solution 0.3X for 118 h (10 larvae/well in 5 mL from 0 to 24 hpf, 10 larvae/well in 10 mL from 24 to 96 hpf, and 10 larvae/well in 5 mL from 96 to 118 hpf). At 118 hpf, larvae were transferred to a 24-well plate (1 larvae/well in 2 mL) and were exposed to 0, 5, 25 or 50 μM MDMA for 2 h in a dark environment. At 120 hpf, their locomotor response to light/dark stimuli was evaluated (see locomotor response to light/dark stimuli section).

2.4. Spontaneous tail coiling

Videos for the evaluation of spontaneous tail coiling under exposure conditions 1 and 2 were recorded at 24 hpf. Ten embryos per concentration were recorded for 45 s at 7.5 frames/second (camera DFK 72AUC02, The Imaging Source Europe GmbH, Germany) under a stereomicroscope using the IC Capture software (The Imaging Source Europe GmbH, Germany). The frequency of spontaneous tail coiling was analyzed with the KNIME Analytics Platform using and adapting the open source workflow (https://kni.me/w/ICVpP1wntrdj5xvJ) previously described in (Ogungbemi et al., 2020). Minor changes introduced

to the workflow are described in Supplementary file Table S1.

2.5. Locomotor response to light/dark stimuli

At 120 hpf, the 24-well plates containing larvae from exposure conditions 3 or 4 (larvae which were acclimated to the plate and to the darkness environment for 2 h in all cases) were introduced in a methacrylate thermoregulated box (27.5 ± 0.5 °C) under a camera (DMK 23UX236, The Imaging Source Europe GmbH, Germany) in a custom-made locomotor assay set-up as described in (Teixidó, 2013). Briefly, the light source was placed at the bottom and provided both infrared and visible light. Video was captured at a rate of 25 frames per second. We used a modified version of the locomotor response test consisting of a five cycles of four minutes, alternating light (3x) and dark (2x) periods and lasting 20 min in total (Ali et al., 2012). Swam distance of each larvae per cycle was analyzed using ImageJ. Videos were cut to clips of 4 min and analyzed to track individual larval movement (Supplementary file Table S2). Larval trajectories consisting on a CSV list of X and Y coordinates were exported to a MS Excel template to calculate the total distance swam per minute, and the total distance swam in the whole light period (3 cycles of 4 min: total 12 min) and whole dark period (2 cicles of 4 min: total 8 min). As positive control, ethanol 2 % v/v acute exposure between 118 and 120 hpf was included.

2.6. Statistical analysis

Statistical analysis was performed with GraphPad PRISM v.8. The statistical unit was considered to be the exposure unit, meaning each well containing 10 pooled embryos. At least 3 independent replicates were performed for each experiment, leading to a total n≥ 3 including a total of 30 embryos per concentration. Statistical comparison was performed using One-way ANOVA with Dunnett multiple comparison's tests for all concentration-effect analysis. Two-way ANOVA test was performed to evaluate the effects of time and concentration exposure on hatching. Two-way ANOVA test was performed to evaluate the effect of concentration and time over the entire testing period on the light/dark assay and to evaluate the effect of concentration and time on locomotion response within each time-block. Unpaired *t*-test was performed to

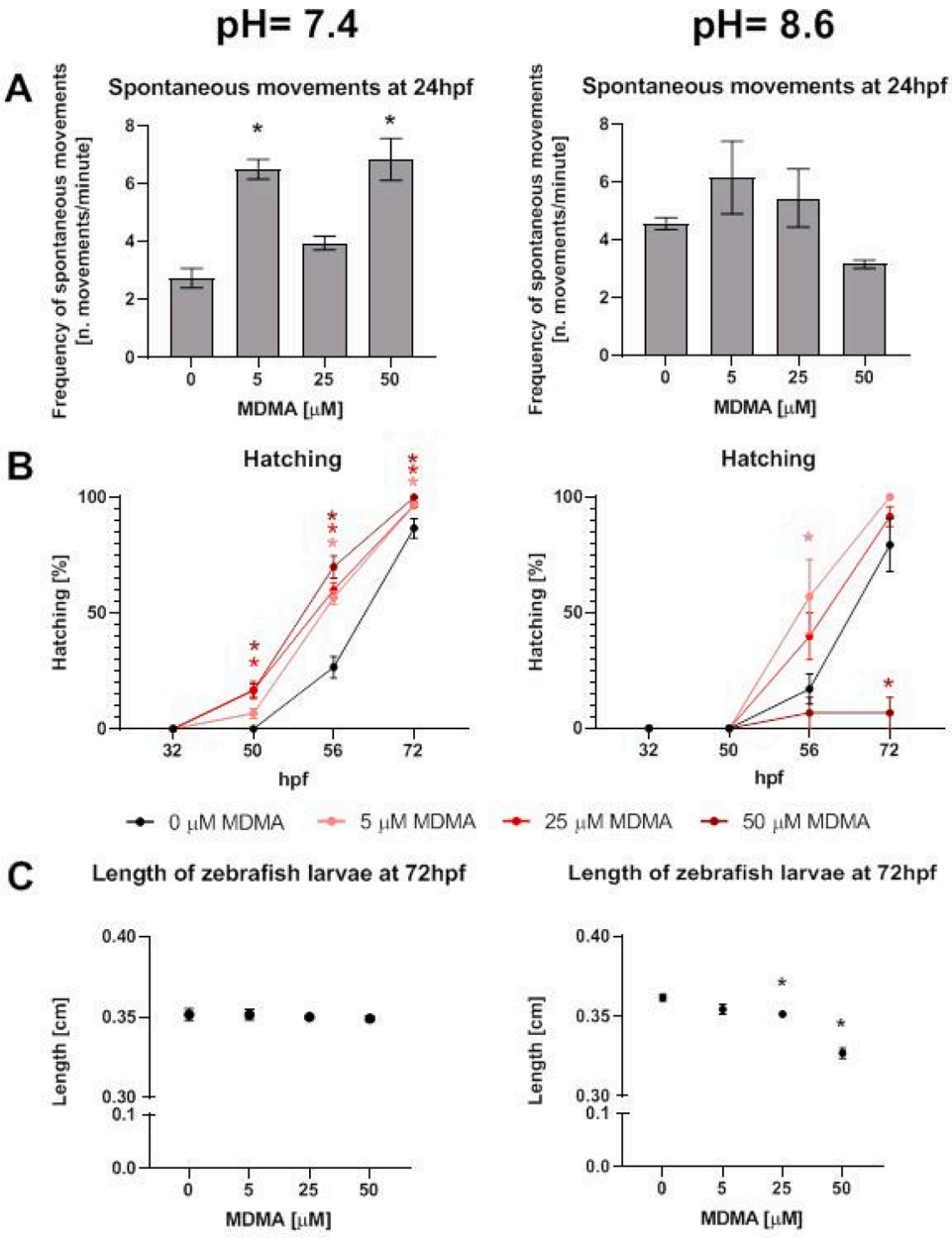

Fig. 3. MDMA significantly increases spontaneous tail coiling and hatching rate at pH = 7.4, while at pH = 8.6 induces developmental delay. A) Frequency of spontaneous tail coiling of zebrafish embryos exposed to MDMA. **B)** Hatching percentage monitored at 32, 50, 56 and 72 hpf. **C)** Length of zebrafish larvae at 72 hpf. All results are presented as mean ± SEM of three independent experiments including 10 embryos per concentration and experiment (n = 3 including a total of 30 embryos per concentration group). * indicates $p < 0.05$ compared to control group by one-way ANOVA and Dunnett's multiple comparison tests in A) and C) and $p < 0.05$ *versus* control group at the same time point by Two-way ANOVA and Tukey's multiple comparison tests in B).

analyze differences between control and the positive control ethanol 2% v/v. Threshold for significant differences was established at $p < 0.05$ in all statistical analysis. Error bars presented in all figures represent SEM (standard error of the mean).

3. Results

3.1. Developmental exposure to MDMA at pH = 7.4 significantly increases spontaneous tail coiling and hatching rate

The first behaviour exhibited by zebrafish embryos appears at approximately 17 hpf and is a spontaneous movement of the musculature characterized by repeated vigorous tail coils (Saint-Amant and Drapeau, 1998). MDMA exposure starting at 2 hpf (Fig. 1; Condition 1) increased spontaneous tail coiling in zebrafish embryos at 24 hpf, and the effect was significant in two of the three tested concentrations (Fig. 3A). This early behaviour is assumed to be important for the hatching of the embryo from its chorion, although the relationship has not yet been proved (Ogungbemi et al., 2019). According to that assumption, hatching rate was monitored at 32, 50, 56 and 72 hpf to detect if MDMA induced also a faster hatching rate. Indeed, the hatching rate was significantly faster at all MDMA tested concentrations. At 25 and 50 μM, the hatching rate was significantly higher than in control embryos already at 50 hpf, and at 5 μM, from 56 hpf on (Fig. 3B). This faster hatching rate was not a consequence of an accelerated development since the length of the larvae (Fig. 3C) or the total morphological score of the embryos (Table 2) were not increased. Since MDMA exposure from 2 to 72 hpf at pH = 7.4 did not produce any increase in lethality or dysmorphogenesis in the embryos (Table 2), we can determine that this exposure scenario induces specific effects on the nervous system of the embryos, but since spontaneous movement effects presented a biphasic response while the faster hatching rate presented a concentration-dependent response, the relationship between both effects is not clear.

3.2. Adverse effects of MDMA in zebrafish embryos are pH-dependent

While increasing the pH of the medium to 8.6 had no deleterious effects on the development of control zebrafish, as previously reported (Bittner et al., 2018), it had an influence in the spontaneous movements at 24 hpf and in the outcome observed after MDMA exposure. At 24 hpf control embryos presented a significantly higher frequency of spontaneous movements ($p = 0.0096$). MDMA exposed embryos presented a non-significant increase in spontaneous tail coiling at 5 and 25 μM, and a non-significant decrease at 50 μM (Fig. 3A). According to these results, hatching rate was significantly faster only at the lowest concentration tested, while at the highest concentration, a significant delay in hatching was observed (Fig. 3B). In this case, this significant delay was accompanied by a significant reduction in body length (Fig. 3C) and a reduction in the total morphological score of the embryos at 72 hpf (Table 2), indicating a general developmental delay and not a specific behavioural effect. Again, this exposure scenario did not induce any increase in lethality or dysmorphogeneses in the embryos (Table 2).

Table 2
Summary of general developmental endpoints at 72 hpf after MDMA exposure.

MDMA	pH = 7.4 Lethality [%]	Dysm. [%]	TMS [points]	pH = 8.6 Lethality [%]	Dysm. [%]	TMS [points]
0 μM	3.3 ± 3.3	6.7 ± 3.3	14.9 ± 0	0 ± 0	0 ± 0	14.8 ± 0.1
5 μM	0 ± 0	0 ± 0	14.9 ± 0.1	10 ± 5.8	0 ± 0	15 ± 0
25 μM	0 ± 0	0 ± 0	14.9 ± 0.1	6.7 ± 3.3	10 ± 5.8	14.8 ± 0.1
50 μM	3.3 ± 3.3	0 ± 0	15 ± 0	0 ± 0	3.3 ± 3.3	10.9* ±1.5

Results presented as mean ± SEM of three independent experiments including ten embryos per concentration and experiment. TMS was calculated according to the score defined by Beekhuijzen et al. (2015), where the maximum score possible at 72 hpf is 15 points. * indicates $p < 0.05$ compared to control by One-way ANOVA with Dunnett's multiple comparison test. Dysm.: dysmorphogeneses; TMS: total morphological score.

3.3. Embryonic exposure to MDMA leads to decreased larval motor activity

Based on the results of exposure conditions 1 and 2, the condition with pH = 7.4 (condition 1) was chosen for further behavioural studies. Condition 2 (pH = 8.6) was discarded because a general developmental delay was detected and that could interfere with the investigation of specific developmental neurotoxic effects assessed by swimming activity. In zebrafish development, organized swimming is achieved few days later than spontaneous tail coiling (Saint-Amant and Drapeau, 1998) and the evaluation of swimming activity in response to a visual stimulus is normally evaluated from day 4 on (Padilla et al., 2011). Therefore, in this case zebrafish behaviour was evaluated at 120 hpf measuring the light-dark locomotor response, a test in which zebrafish larvae have been proved to be sensitive to neuroactive drugs and to respond to these drugs increasing or decreasing their locomotor response following similar patterns than mammals (reviewed by Basnet et al., 2019). To distinguish between the developmental neurotoxic and the acute effects of MDMA in locomotion, in the third scenario MDMA exposure (which lasted 72 h but started at 24 hpf to adapt to practical possibilities of the animal facility) was stopped 24 h before the behavioural assay (Fig. 2). As expected after the results of scenario 1, in scenario 3 general developmental endpoints (lethality %, dysmorphogeneses %) were not altered by MDMA exposure. However, developmental exposure to MDMA significantly decreased larval motor activity at all tested concentrations during light cycles (Fig. 4), and at the two highest concentrations under dark conditions.

3.4. Decreased larval motor activity is more severe after developmental exposure than after acute exposure

To compare the developmental and the acute effects of MDMA on motor function, in the last condition, larvae were exposed only during the 2 h before the performance of the light/dark assay (from 118 to 120 hpf). In this case, only larvae exposed to the highest MDMA concentration swam a significantly shorter distance than the controls, and that effect was present in both, light and dark cycles (Fig. 4). This milder effect compared to the one observed under condition 3 cannot be explained by the differences in chorion presence (condition 3: with chorion; condition 4: hatched), but by means of the difference in exposure duration. However, internal levels of MDMA or metabolites in the embryos should be measured for confirmation. Acute larval exposure to ethanol was used as positive control, and it induced a significant increase in locomotor activity during the light phases (Supplementary Fig. S3), as previously described in the literature (Guo et al., 2015).

4. Discussion

In this study we describe for the first time the developmental neurotoxic effects of MDMA in zebrafish embryos. The aim of the study was not to simply describe the adverse effects of MDMA observed after developmental exposure, but to determine whether the zebrafish model reproduces a decreased motor function-like behavior similar to the adverse outcome occurring in humans (Singer et al., 2016), and to find under which experimental conditions, to establish a good alternative model for future MDMA DNT mechanistic assays. Our results show that depending on the exposure conditions and behavior assay used, MDMA induces hyper- or hypo-activity in zebrafish embryos. A similar situation has been described in rodents *in vivo*, were prenatal/postnatal exposure

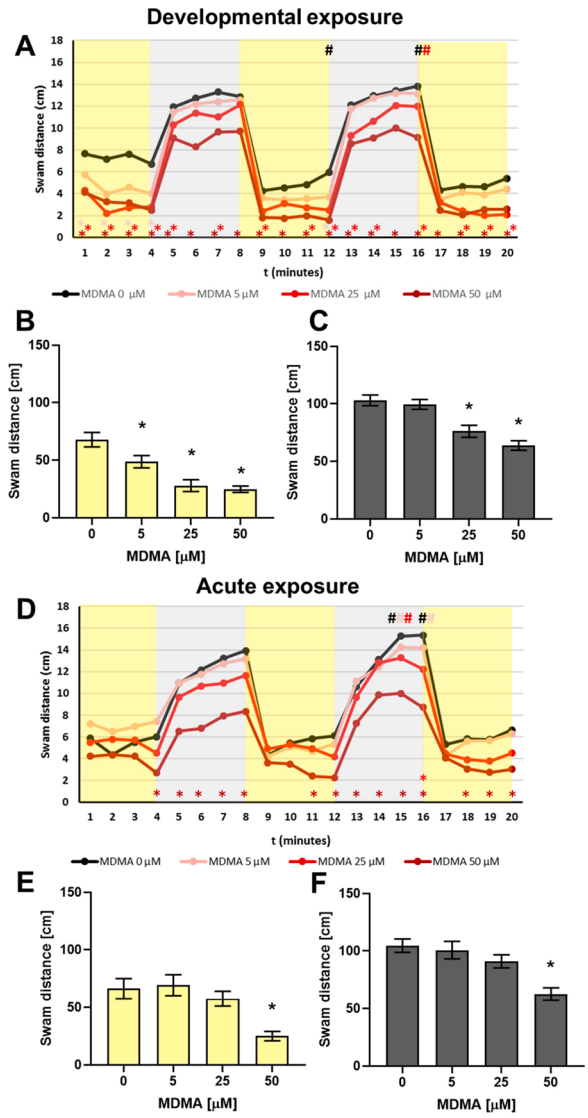

Fig. 4. MDMA significantly decreases locomotor activity in zebrafish larvae. Locomotor activity of zebrafish larvae at 120 hpf after different patterns of exposure to MDMA (conditions 3, developmental exposure: A, B and C; and condition 4, acute axposure: D, E and F). Results are presented as mean incremental swam distance per minute (A and D) or in the whole light period (B and E; 12 min in total) or in the whole dark period (C and F; 8 min in total) of 3 independent experiments including 10 embryos per concentration and experiment (n = 3 including a total of 30 embryos per concentration group). Gray and yellow areas behind graphs A and D represent dark and light cycles respectively. In A and D, a two-way ANOVA test was performed to evaluate the effect of concentration and time over the entire testing period. In both cases the interaction p value obtained was <0.0001, therefore the two-way ANOVA was performed within each time block. The result is indicated as * for $p < 0.05$ compared to control group at the same time-point by Two-way ANOVA with Tukey's multiple comparison test, and as # for $p < 0.05$ compared to the first minute of the same group and same light/dark period by Two-way ANOVA with Tukey's multiple comparison test. In B, C, E and F, * indicates $p < 0.05$ compared to control group by One-way ANOVA with Dunnett's multiple comparison test.

induce hyper-/hypo-activity in pups, respectively (reviewed by Barenys et al., 2020). Since MDMA developmental exposure in zebrafish induced hyperactivity when tested at 24 hpf after 20 h of exposure (scenario 1), and considering the dual effects described in rodents, a later evaluation time-point with a later exposure onset was tested (scenario 3 and 4) and hypoactivity could be observed at 120 hpf after 72 of exposure (scenario 3: from 24 to 96 hpf) or after 2 h of exposure (scenario 4: from 118 to 120 hpf). Although a direct correlation of the interpretation of the light/dark locomotor assay to humans is not possible, in this test zebrafish larvae present dose-response patterns similar to the ones obtained in mammals for neuroactive drugs with well-known effects in humans (Basnet et al., 2019). Since the neurological system of zebrafish is well studied and key genes and pathways are conserved between zebrafish and other species including humans, the observation that MDMA developmental exposure results in an altered behavior following the pattern observed in humans indicates that developmental exposure to MDMA in zebrafish also disrupts the underlying circuits controlling the development of locomotor function and supports the use of zebrafish embryos to study the DNT effects of MDMA. These effects could be DNT specific since adult zebrafish acutely exposed to MDMA display a constant locomotion in other tests at this concentration range (Stewart et al., 2011; Ponzoni et al., 2016).

Besides finding a suitable evaluation time-point, it was also important to define working concentrations reflecting the human MDMA recreational consumption situation. Although it is not easy to establish a correspondence between the concentrations in zebrafish experiments and the concentration in humans in utero, a few criteria were clear in order to enable the comparison: the concentrations should not produce any increase in lethality, nor in the percentage of dysmorphogeneses, and should not produce general developmental delay, since in humans the decrease in motor function occurs in absence of these effects. The concentrations tested in this study (5–50 µM) include concentrations in the range of those described in humans after MDMA exposure (Elliott, 2005; Farré et al., 2015) and concentrations reported in studies analyzing the kinetics of MDMA in pregnant animals (Campbell et al., 2006). Randomized control trials in humans giving two MDMA doses of 100 mg 4 h apart have described a mean C_{max} in plasma of 458 ng/mL (equivalent to 2.4 µM; (Farré et al., 2015)). For ethical reasons, higher doses have not been tested in controlled trials, but in real cases of intoxications, much higher plasma concentrations have been found (4.33 mg/L; equivalent to 22.4 µM, (Elliott, 2005)). Besides, after a single dose of 15 mg/kg in pregnant rats, a dose very often used in DNT studies in rodents, plasma C_{max} was 3980 ng/mL (equivalent to 20.6 µM; (Campbell et al., 2006)). In our experiments, we could identify the expected specific DNT adverse outcome at the lowest concentration tested (5 µM), which is in the human relevant exposure range. At higher concentrations there was a concentration-dependent increase in the adverse effect, still in absence of lethality, dysmorphogeneses and developmental delay.

These criteria of absence of non-DNT specific effects were fulfilled under pH = 7.4, but not under pH = 8.6 (scenario 2). Since the pKa value of MDMA is 9.9 (PubChem PubChem, 2021), initial experiments were performed under pH = 8.6 to increase the proportion of internal/-external concentration, to see if the toxicity increased compared to the pH = 7.4 scenario 1, when the non-ionized fraction in solution was increased, as it was demonstrated before for pharmaceutical drugs in water samples (Alsop and Wilson, 2019). Indeed, the effects observed at pH = 8.6 were more severe than at 7.4, and included general developmental delay, detected by shorter length, delayed hatching, and significantly lower TMS. For this reason, this experimental setting was discarded for further behavioural evaluations.

Another important criterion to consider for the establishment of the experimental conditions was to set a washout period between MDMA exposure and behavioural evaluation, to allow the possibility to distinguish DNT from acute effects and to confirm that MDMA produces lasting alterations in the developing organism. A washout period of 24 h was selected, to still include a late onset (at 24 hpf) +72 h of exposure +24 h hours of washout within the 120 hpf period when zebrafish are considered an alternative model to animal experimentation (scenario 3). The duration of exposure was also a key factor: after 72 h of exposure the effect was more severe than after 2 h (scenario 4). In this case the effect observed was the same, but it was only significant at the highest concentration, which is not within the human relevant concentration-range.

For all these reasons, we can conclude that we have found the best conditions to study MDMA-induced DNT in the zebrafish embryo, mainly, exposure from 24 to 96 hpf at pH = 7.4 and behavioural evaluation at 120 hpf (scenario 3). These conditions produce a significant reduction in motor activity in both light and dark periods, without producing a general developmental delay. According to the literature, two hypothetical AOPs have been proposed for MDMA leading to this lower motor activity: one involving disturbances of the serotonergic and one involving disturbances of the dopaminergic system (Barenys et al., 2020). Since serotonin and dopamine also play a key role in the establishment of locomotion in zebrafish (Brustein et al., 2003; Areal and Blakely, 2020), and alterations in these neurotransmitters and in other related behaviours can also be measured during zebrafish early development (Legradi et al., 2015; Tufi et al., 2016), the model and the conditions we selected open the door to study the existing knowledge gaps in the MDMA-induced DNT AOP and to perform a risk assessment based on mechanistic understanding.

Credit authorship contribution statement

Marta Barenys, **Elisabet Teixidó**, and **Jesús Gómez-Catalán** were responsible for the study concept and design. **Marta Barenys**, **Shami Álvarez** and **Ariadna Santamaria** contributed to the acquisition of experimental data. **Marta Barenys**, **Shami Álvarez**, **Elisabet Teixidó** and **Ariadna Santamaria** performed the data analysis. **Marta Barenys** and **Jesús Gómez-Catalán** assisted with data analysis and interpretation of findings. **Marta Barenys**, **Shami Álvarez** and **Jesús Gómez-Catalán** drafted the manuscript. **Elisabet Teixidó** and **Jesús Gómez-Catalán** provided critical revision of the manuscript for important intellectual content. **Marta Barenys**, **Shami Álvarez**, **Ariadna Santamaria**, **Elisabet Teixidó**, **Jesús Gómez-Catalán** critically reviewed content and approved final version for publication.

Conflict of interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

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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.2021.11.001.

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