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Increased response to 3,4 methylenedioxymethamphetamine (MDMA) reward and altered gene expression in zebrafish during short- and long-term nicotine withdrawal

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Increased response to 3,4 methylenedioxymethamphetamine (MDMA) reward and altered gene expression in zebrafish during short- and long-term nicotine withdrawal

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Abstract

An interactive effect between nicotine and 3,4 methylenedioxymethamphetamine (MDMA) has been reported but the mechanism underlying such interaction is not completely understood. This study used zebrafish to explore gene expression changes associated with altered sensitivity to the rewarding effects of MDMA following 2 week exposure to nicotine and 2-60 days of nicotine withdrawal. Reward responses to MDMA were assessed using a conditioned place preference (CPP) paradigm and gene expression was evaluated using quantitative real time PCR of mRNA from whole brain samples from drug-treated and control adult zebrafish. Zebrafish pre-exposed for 2 weeks to nicotine showed increased conditioned place preference in response to low dose, 0.1 mg/kg, MDMA compared to un-exposed fish at 2, 7, 30 and 60 days withdrawal. Pre-exposure to nicotine for two weeks induced a significant increase of c-Fos and vasopressin receptor expression but a decrease of D_3 dopaminergic and oxytocin receptor expression at 2 days of withdrawal. C-Fos mRNA increased also at 7, 30, 60 days of withdrawal. Nicotine pre-exposed zebrafish submitted to MDMA-induced CPP, showed an increase in expression of p35 at day 2, α4 at day 30, vasopressin at day 7, and D_3 dopaminergic receptor at day 7, 30, and 60. These gene alterations could account for the altered sensitivity to the rewarding effects of MDMA in nicotine pre-exposed fish, suggesting that zebrafish have an altered ability to modulate behaviour as a function of reward during nicotine withdrawal.

Keywords

Psychostimulants, Conditioned Place Preference, reward, receptors

Introduction

Nicotine is the main psychoactive substance contained in cigarette smoke and is able to cause both physical and psychological dependence. As with other addictive drugs, nicotine acts on the brain's reward circuit to cause dopamine release in the nucleus accumbens (NAc) [1]. Smoking behaviour and psychostimulant use are highly comorbid [2]. Compared to the general population, psychostimulant users report extremely high rates of cigarette smoking usage [3,4]. Indeed, tobacco use occurs in about 90% of methamphetamine, cocaine and MDMA users [5-7bis]. Moreover, individuals who quit smoking are more likely to remain abstinent from illicit stimulants than people that still smoke.

Among different psychostimulants, an interactive effect between nicotine and 3,4 methylenedioxymethamphetamine (MDMA), also known as ecstasy, has been reported [9]. MDMA is a synthetic drug with stimulant and prosocial effects widely abused in young smokers [8]. About 90% of American adolescents who consume MDMA are also tobacco smokers [10]. Early initiation of marijuana use or of both smoking and drinking, increases the risk for ecstasy use initiation [11]. In addition, in MDMA abusers, regular tobacco use may exacerbate the neurotoxic effects of MDMA [12,13]. Although no findings are available for MDMA in humans, adolescent exposure to nicotine influences the likelihood of other psychostimulant use, including cocaine and methamphetamine [14]. A study of a cohort representative of the U.S population revealed that users who initiated cocaine after having smoked cigarettes showed higher rates of dependence (20.2 percent), and the rate of dependence was much lower among compared to those who initiated cocaine before smoking (6.3 percent) [14] .Indeed, cocaine-dependent patients achieving smoking abstinence in response to smoking cessation treatment showed not only improved smoking outcomes but improved cocaine-use outcomes as well [7] giving further evidence for an interaction between nicotine and psychostimulants during withdrawal. In addition to the above co-morbidity, studies in rodents have reported that behavioural effects of psychostimulants are often enhanced by previous nicotine exposure. Chronic pretreatment with nicotine enhances cocaine reward-related behaviours, including locomotor sensitization, conditioned place preference (CPP) and self-administration [15-24].

Further, early-life exposure to nicotine [19] as well as nicotine exposure and withdrawal [21], is associated with increased stimulant use in later life. Similarly, pre-exposure for 14 days to nicotine significantly increased MDMA-induced reward in mice [25]. Nicotine exposure also significantly increased MDMA reward in rodents assessed using CPP paradigm [25]. However, neither the behavioural and age specificity of this increase in psychostimulant reward by nicotine nor the role of nicotinic receptor subtypes involved have been clearly identified.

From a molecular point of view, psychostimulant-induced increases in dopamine neurotransmission are affected by nicotine. Indeed, co-administration of nicotine and cocaine or amphetamine induces additive or synergistic effects on dopamine release in the NAc [26-28]. Nicotine and cocaine have been found to produce changes in synaptic plasticity in several brain regions, including the ventral tegmental area (VTA), NAc, hippocampus and amygdala [29,30]. Pre-treatment with nicotine induces a plastic effect in the core of the NAc [29] and in the amygdala [30] indicating that nicotine serves as a priming and enhancing agent for the formation of Long Term Potentiation after subsequent exposure to cocaine. In addition, it has been recently shown that nicotine and alcohol induce a permissive epigenetic environment for cocaine-induced gene expression [32], however, how identified epigenetic changes affect specific pathways associated with psychostimulant reward and how this may effect sensitivity to psychostimulants during withdrawal is yet to be explored.

Over the past two decades, in the field of behavioural neuroscience, several zebrafish models have been developed to evaluate rewarding effects of psychoactive compounds [33]. As in rodents, CPP, which is assumed to reflect the motivational properties of drugs and their potential for abuse [34,35], has been adopted in zebrafish neurobehavioral research [36-39]. Amphetamine, cocaine, ethanol, morphine, salvinorin A, and nicotine induce robust CPP in zebrafish, often following a single administration. These studies demonstrate the potent rewarding properties of such drugs of abuse in zebrafish and underscore the translational value of the zebrafish CPP model of drug reward [33]. In zebrafish the dopaminergic pathway that goes from the posterior tuberal nucleus to the dorsal nucleus is reminiscent of the dopaminergic pathway of mammals from the VTA to the NAc [40]. This dopamine projection is involved in zebrafish cocaine-induced CPP [35], suggesting conservation of dopaminergic reward circuitry in zebrafish and mammals.

While in rodents the effect of pre-exposure to nicotine/alcohol on subsequent cocaine addiction has already been reported [19, 40-43], few studies have examined the effect of nicotine dependence on subsequent psychostimulant-induced reward in zebrafish. However, recently Pisera-Fuster et al., [45] found a greater nicotine-induced CPP response in zebrafish that had been pre-exposed to nicotine and subjected to short term withdrawal compared to drug-free controls.

On this basis, the aim of the present work was twofold: *i*) to evaluate the effects of short and long term nicotine withdrawal on zebrafish responses to the rewarding effects of MDMA; *ii*) to correlate the behavioural alterations during

nicotine cessation and in response to MDMA-induced reward with changes in gene expression. MDMA reward response was assessed using CPP and changes in gene expression evaluated using quantitative real time PCR (qpcr) of mRNA of whole brain samples from drug-treated and control zebrafish. Among the genes known to be affected by chronic drug abuse exposure, we investigated the immediate early gene c-Fos and p35, a downstream target of immediate early gene activation, previously shown to be linked to physiological and behavioural changes in rodents and to vary after psychostimulants exposure [46-48]. We also analyzed expression of other genes belonging to members of pathways and processes implicated in drug dependence in mammalian models [47]: nicotinic and adenosinic receptors involved in the addictive properties of drugs of abuse; D_1, D_2 and D_3 dopaminergic receptors which are selectively expressed in important areas of the brain implicated in addiction-regulated centres; and receptors for oxytocin and vasopressin, hormones which are involved in MDMA-induced reward (49), whose homologues in teleost fish are isotocin and vasotocin.

Materials and Methods

Animals and housing

Adult male and female (approximately 50%:50%) AB wild type (short fin) zebrafish (Danio rerio) were obtained from Università Politecnica delle Marche, Dipartimento di Scienze della Vita e dell'Ambiente, Ancona, Italy. All fish were housed in groups of 30 in a 96 L home tank maintained under standard conditions at 28 (±2) °C, 14–10 h day/night cycle (lights on at 7:00 AM) for at least two weeks before the experiments. Fish were habituated to the CPP apparatus for 1 h a day the week before the start of the experiments. Behavioural testing took place during the light phase between 9.00 and 14.00 h. Tank water, containing sea salts (Instant Ocean, Aquarium System, Sarrebourg, France) at a concentration of 0.6 g/10L, was obtained by reverse osmosis filter system. Water quality was maintained at optimal levels and checked daily for pH (6.5-7.5) and every 3 days for nitrates (<0.02 ppm). Fish were fed twice a day with commercial flakes (tropical fish food, Consorzio G5, Italy) supplemented with live brine shrimp. All the fish were drug naive, and each fish was used only once. Ten fish per group were used. A total of 160 fish were used in the present study. All protocols were approved by the National Ethics Committee for the care and use of laboratory animals and by the National Ministry of Health with the Italian Government Decree N° 323/2018 PR. Care was taken to minimize the number of animals used and their suffering in accordance with the ARRIVE guidelines from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (http://www.nc3rs.org.uk/page.asp?id=1357). Animals were euthanized using terminal anesthesia with tricaine methanesulfonate (MS-222, Sigma-Aldrich). The experimental design for the behavioural and gene expression analysis is reported in Figure 1.

Exposure to nicotine

Chronic treatment with nicotine (1mg/L) (Sigma-Aldrich, St. Louis, MO, USA) was carried out in groups of 20 fish at a time in the home tank before inducing withdrawal. 50% of the water in all tanks was replaced every other day, to maintain relatively constant nicotine concentrations during the 2 weeks of treatment. The use of zebrafish has only recently entered the field of nicotine research thus scarce are the findings on drug metabolism. The $t_{1/2}$ of nicotine in zebrafish is unknown and, as nicotine is in constant exogenous supply throughout the exposure period, clearance is also undetermined. Although several zebrafish cytochrome P450 enzymes have been characterized, a zebrafish equivalent of the human CYP2A6 has not been identified [50]. Nicotine dose was chosen according to Stewart et al., 2015 [51]. The duration of chronic treatment and withdrawal was selected based on our previous unpublished study confirming the lack of non-specific toxic/sedative effects of nicotine. The withdrawal intervals were the same as those used in other nicotine studies in mice [52]. After the last treatment (14^{th} day) fish were placed in drug-free water in their home tank for the entire period of the withdrawal syndrome. The CPP was performed at 2, 7, 30 and 60 days post-withdrawal in different groups of fish (N = 10 per end-point). Drug-free control fish were placed into tank water with no drugs added.

Treatment

Body weight was measured according to Ponzoni et al. [49]. Fish were removed from their tank using a net, momentarily blot dried on gauze, and placed in a container containing tank water, positioned on a digital scale. 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. The fish were placed in anaesthetic solution of tricaine methanesulfonate (Sigma Aldrich, St. Louis, MO, USA) (150 mg/L). Upon loss of response to touch, fish were injected intramuscularly (i.m.) in the caudal musculature with a volume depending on the weight of the fish (2 µl/g) using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland).

Conditioned Place Preference

The fish were tested in a two-chamber tank (10 cm × 20 cm × 15 cm) as previously described [49]. The tank was divided into two halves (10 cm × 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, one hour after habituation to the apparatus, 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 group was split in half: a subset of fish was injected i.m. with MDMA (0.1 or 10 mg/kg i.m.) and after 1-2 min (in order to allow the anaesthetic effect of tricaine to wear off). confined to their least preferred side for 30 minutes; the remaining half received the vehicle and were confined to their most preferred side. Twenty four hours later, each fish, previously injected with vehicle, received MDMA and those injected with MDMA received vehicle. Then they were confined in the opposite compartment for 30 min. Drug-texture

pairings were always counterbalanced. On the third day (post-conditioning phase: POST), fish had free access to both sides for 15 min. The time spent in each compartment and the number of transitions between the two sides was recorded. The change (Δ) in preference, obtained by subtracting the baseline value from the final value in the drug-paired compartment, reflected rewarding or aversive properties.

RNA extraction and quantitative real-time PCR

Five brains per group (drug-free with or without CPP with vehicle or MDMA, and nicotine-exposed plus CPP with vehicle or MDMA) were extracted 2, 7, 30 or 60 days after nicotine cessation one hour following CPP and snap frozen at −80 °C until later use. Total RNA from brains was extracted using TRIzol reagent (Thermo Scientific) as suggested by the manufacturer. Briefly, samples were homogenized and lysed with TRizol reagent and chloroform, and RNA separated by centrifugation. RNA was precipitated with isopropanol, washed with 70% ethanol, air dried and solubilized with RNase-free water. RNA yield and quality were determined using a Thermo NanoDrop 2000 (ThermoFisher). Then, RNA extracts were treated with DNase I (ThermoFisher) and cDNA libraries created using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs) as suggested by the manufacturer. Resulting cDNA yield and quality were also evaluated using Nanodrop. Relative gene expression mRNA levels were quantified using the LightCycler 480 qPCR system (Roche) based on our previously published protocols [53-58] which are MIQE compliant [57]. Briefly, thermocycling begins with 95ºC for 30s prior to 45 cycles of amplification at 95ºC for 5s, 60ºC for 5s, 72ºC for 5s, 78ºC for 1s (data acquisition). A 'touch-down' annealing temperature intervention (66ºC starting temperature with a step-wise reduction of 0.6ºC/cycle; 8 cycles) was introduced prior to the amplification step to maximise primer specificity. Melting analysis (95ºC for 10s, 75ºC for 10s then heating from 75 to 95ºC at a ramp rate of 0.57ºC/s with continuous data acquisition) was performed at the end of qPCR amplification to validate single product amplification in each well. Relative quantification of mRNA transcripts was calculated based on the second derivative maximum algorithm (Roche). Reference genes for all the qPCR analyses were β-actin, ef1α and rpl13α based on previous studies [59,60]. Accession numbers and primer sequences for the genes can be found in Supplementary Table 1.

Statistical analysis

Data were expressed as mean values ± the standard error of the mean (SEM). Between-group differences were assessed using one-way or two-way analysis of variance (ANOVA) for repeated measures followed by Tukey's or Bonferroni *post hoc* test. Place preference was calculated by subtracting the time spent in the drug-paired compartment before drug conditioning from the time spent after drug conditioning. Relative mRNA expression in qPCR was calculated against reference gene cycle-threshold (Ct) values, and then subjected to one-way ANOVA. Relative mRNA expression

obtained from the group of fish exposed to water and then treated with vehicle or MDMA at 2, 7, 30 and 60 days from nicotine withdrawal were pooled after having verified no significant differences among the time intervals using one-way ANOVA, followed by Tukey's test. To account for multiple testing a Bonferroni correction was applied. Data were analysed using Prism 7 software (GraphPad, USA).

Results

MDMA induced a significant CPP only in zebrafish pre-exposed to nicotine

To investigate if nicotine exposure might increase the risk for drug reward after short and long-term withdrawal, we treated zebrafish, pre-exposed to nicotine or water for two weeks, with MDMA at a dose without rewarding properties *per se* as previously shown [49]. Each zebrafish spent an equivalent average amount of time in each of the two outer compartments during the pre-conditioning phase (PRE) either after water exposure (compartment $_{\text{three black polka dots}}$ =453.50 ± 22.70 s; compartment $_{\text{no three black polka dots}}$ = 446.50 ± 22.70 s, t_{18}=0.22,$p > 0.05$) or nicotine exposure (compartment $_{\text{three black polka dots}}$ = 445.40 ± 30.30s; compartment $_{\text{no three black polka dots}}$ = 454.60 ± 30.30s, t_{18}=0.21, $p > 0.05$). The effect of pre-exposure to nicotine (1 mg/L) for 2 weeks on subsequent MDMA-induced CPP is reported in Figure 2. Analysis of time difference spent during pre- and post-conditioning after MDMA treatment showed main effects of pre-exposure factor ($F(7, 144) = 5.15$, $p < 0.02$) and of treatment ($F(1, 144) = 26.83$ $p < 0.0001$) and a significant treatment x exposure interaction ($F(7, 144) = 22.67$, $p < 0.0001$). Post hoc analysis revealed a significant increase of the time spent in the drug-paired compartment in zebrafish pre-exposed to nicotine and treated with MDMA at all the tested time intervals. Two days after nicotine cessation there was the greatest place preference which significantly decreased at 2, 7, 30 and 60 days. MDMA did not induce any significant effect on the time spent in the drug-paired compartment in animals pre-exposed only to water. After 2 days from withdrawal the time in the MDMA-paired compartment in zebrafish pre-exposed to nicotine was significantly higher than that shown in the following days (7, 30 and 60).

Treatment with MDMA did not alter the swimming activity

To assess whether the greater effect on MDMA-induced CPP found in zebrafish pre-exposed to nicotine compared to drug-free zebrafish was influenced by motor alterations, the number of transitions between the two compartments of CPP apparatus was measured during the post-conditioning phase (Figure 3). The total number of crossed lines, whose change is a measure of altered distance travelled between the two compartments, was not different among groups (pre-exposure factor: [$F(7, 144) = 7.22$; $p > 0.133$], treatment: [$F(1, 144) = 0.73$; $p > 0.72$] and interaction : [$F(7, 144) = 1.24$, $p > 0.96$].

mRNA gene expression is altered in nicotine exposed zebrafish *per se* and following MDMA treatment compared to drug-free fish

Some significant changes in gene expression between drug-free and treated zebrafish was found. First of all we checked in a group of fish not exposed to CPP nor withdrawn, if the sub-threshold dose of MDMA (0.1 mg/kg) induced, *per se* (in the absence of CPP), some mRNA changes in zebrafish brains. No alterations were found for mRNA expression for any gene tested. When the dose was increased to 10 mg/kg α6 nicotinic receptor mRNA level was significantly decreased [$F(2,12)= 5.21$; $p < 0.05$] (Figure 4). No significant differences in levels of gene expression in brains from drug-free (non-nicotine exposed) animals subjected to CPP with MDMA or vehicle alone were found at any time point (data not shown). Thus for the purpose of all further analysis and graphical representation, data for levels of expression in brains from drug-free animals (i.e. those not pre-treated with nicotine and subject to CPP with either vehicle or MDMA) are compressed across days (Statistical data are reported in supplementary Table 2).

Two way ANOVA for treatment and days withdrawal showed main effects of treatment and days withdrawal on c-Fos expression and a treatment x day interaction [F_{days} (3,112) = 7.66; $p < 0.001$; $F_{treatment}$ (3,112) = 4.23, $p < 0.01$]; [$F_{interaction}$ (9,112) = 1.67; $p > 0.05$] after MDMA treatment (Figure 5). *Post hoc* comparison revealed that pre-exposure to nicotine for two weeks induced a significant increase of c-Fos at 2, 7 and 30 days of the withdrawal period compared with unexposed controls. No changes were found after MDMA-induced CPP. Two-way ANOVA indicated that there was a difference among groups of p35 mRNA level [F_{days} (3,188) = 0.45; $p > 0.05$; $F_{treatment}$ (3,188) = 0.48, $p > 0.05$; $F_{interaction}$ (9,188) = 3.71; $p < 0.001$] (Figure 5). *Post hoc* analysis indicated that p35 level remained unchanged immediately after nicotine exposure but significantly increased in nicotine pre-exposed zebrafish submitted to MDMA-induced CPP at day 2 and slowly decreased across the days.

Further significant changes were observed in animals pre-exposed to nicotine and subjected to MDMA during CPP (α4: [F_{days} (3,64) = 3.25; $p < 0.05$; $F_{treatment}$ (3,64) = 0.361; $p > 0.05$; $F_{interaction}$ (9,236 = 0.35; $p > 0.05$]; α6: [$F_{treatment}$ (3,236) = 0.19; $p > 0.05$]; F_{days}(3,236) = 3.44; $p < 0.02$; $F_{interaction}$ (9,64) = 2.43; $p <0.01$] (Figure 6). *Post hoc* analysis revealed an increase of α4 mRNA level at day 30 and of α6 mRNA level at day 2 followed by a decrease at day 30 and day 60. No changes for the subunits α2, α5, α7 and β2, β3, β4 mRNA level (data not shown) were found.Two-way ANOVA indicated significant changes of D_3 mRNA level among groups [$F_{treatment}$ (3,220) = 4.36; $p<0.01$; F_{days} (3,220) = 0.55; $p<0.01$; $F_{interaction}$ (9,220) = 0.21; $p<0.05$.

Post hoc analysis indicated a slight decrease of D_3 dopaminergic receptor at day 2 after nicotine cessation (Figure 6). After MDMA-induced CPP a progressive increase was found for mRNA level of D3 dopaminergic receptor at day 7, 30, and 60.

A similar trend was found also for mRNA of A_{2a} adenosinic receptor [$F_{treatment}$ (3,192) = 3.23; $p < 0.05$; F_{days} (3,192) = 0.25; $p > 0.05$; $F_{interaction}$ (3,192) = 0.202; $p > 0.05$] (Figure 7). *Post hoc* analysis revealed a significant increase at day 30 after inducing CPP with MDMA.

Finally, changes among groups in mRNA level of oxytocin/vasopressin receptors were found: Oxytocin: [$F_{treatment}$ (3,220) = 6.20, $p < 0.001$; $F_{interaction}$ (9,220) = 1.56, $p > 0.05$; F_{days} (3,220) = 1.89; $p > 0.05$; vasopressin: [$F_{treatment}$ (3,190) = 4.16; $p < 0.01$; $F_{interaction}$ (9,190) = 1.58; $p < 0.05$; F_{days} (3,190) = 3.45; $p < 0.05$] (Figure 7). *Post hoc* analysis revealed a significant increase, after nicotine cessation and treatment with MDMA, at day 30 and day 60. An increase only at day 7 was found for mRNA level of vasopressin receptors.

Discussion

In the present study we used adult zebrafish as a model to explore the interaction between nicotine withdrawal and subsequent psychostimulant-induced reward. We used MDMA as a psychostimulant and characterised changes in expression of candidate genes predicted to be involved in reward responses based on findings in previous studies [49,59].

We show that nicotine withdrawal led to an increased sensitivity to MDMA-induced reward in zebrafish as it does in other species [25] and this increased sensitivity lasts over a period of 2-60 days. Differences in behavioural sensitivity to MDMA are associated with sensitivity to MDMA-induced changes in c-Fos and D3 receptor expression not evident in the absence of nicotine withdrawal. These findings suggest altered regulation of dopaminergic signalling may underlie the observed behavioural response.

Behavioural changes seen in zebrafish following nicotine exposure are in agreement with previous findings. Nicotine withdrawal induced a slight conditioned place aversion at least till Day 30 as seen previously in withdrawn rats [61,62] . Nicotine withdrawal is associated with increased anxiety (negative affective state) in all species studied [63]. The slight conditioned aversion to the least preferred side seen here may reflect this negative affective state as suggested in rodent studies [64-65].

A sub-threshold dose of MDMA, unable to induce reward by itself, produced a significant CPP in nicotine withdrawn animals at all time points tested (2-60 days). Although we cannot formally exclude the possibility, a direct interaction between residual nicotine (or nicotine catabolites) present in the fish is unlikely to account for the differences in behaviour as nicotine is rapidly metabolised in fish as it is in other animals [50] and would not be present after 2 days of withdrawal. Further, it is unlikely that the observed increase in MDMA-induced CPP is related to the alteration of

swimming activity since the total number of transitions between the two compartments was not different among the groups.

This is the first time that responses to MDMA were studied during nicotine withdrawal in zebrafish. However, our findings agree with those reported by Ciudad-Roberts et al., 2013 [25] in which 3 mg/kg MDMA induced CPP in animals that had been pre-treated with 2 mg/kg nicotine for 14 days but not in nicotine naive controls. Similarly, we previously demonstrated that a sub-threshold dose of Δ^9-THC was very effective in inducing CPP, after early and late nicotine abstinence, in mice pre-exposed to e-cigarette vapour or cigarette smoke for 7 weeks [66]. Notably, nicotine pretreatment in mice strongly increased CPP induced by 2 mg/kg cocaine, which was similar to the CPP induced by cocaine alone at 20 mg/kg [67].

We correlated the behavioural alterations during nicotine cessation and after MDMA-induced CPP with gene expression using qPCR of whole brain samples from nicotine-treated and drug-free zebrafish. A sub-threshold dose of MDMA (0.1 mg/kg) did not induce, *per se*, changes in expression of any of the genes analysed, confirming that this dose was ineffective. However, when the dose was increased to 10 mg/kg α6 nicotinic receptor mRNA level was significantly decreased. No comparative data are available in literature. It is somewhat surprising that 10 mg/kg per se did not induce detectable changes in c-Fos expression. Published data on rodent show that psychedelic agents (e.g., LSD and MDMA), elevate the expression of c-fos, acting as a marker of neuronal activation that correlates with behavioral alterations [68-70]. Some studies have validated brain c-fos analyses in zebrafish [71,72]. It is possible that tissue heterogeneity within brain tissue might have prevented us observing region-restricted differences or that the increase of c-Fos was not maximal at the time animals were culled: animals were killed one hour after MDMA exposure which might not reflect the peak of increased expression of c-Fos. However, as an increase in c-Fos was seen following CPP with 0.1mg/kg MDMA the lack of change seen following 10mg/kg in the absence of CPP remains to be determined. Perhaps, measuring deltaFosB protein (known to inhibits c-Fos activation [73] may give some insight. However, these deltaFosB experiments were outside the scope of this study as there is currently no available antibody that detects zebrafish deltaFosB available. Nicotine withdrawn fish showed increased expression of the immediate early gene c-Fos following CPP with vehicle alone. Fos family proteins are transcription factors which are activated in response to different kinds of stimulation. The observed increase in c-Fos expression in withdrawn animals is in agreement with studies in rodents where nicotine withdrawal enhanced neuronal activity, as measured by c-Fos expression, across selective midbrain and limbic brain regions during nicotine withdrawal [74]. No further change in c-Fos expression was observed after MDMA treatment in nicotine exposed zebrafish. On the contrary, the increase in c-Fos was reduced in MDMA conditioned animals. Thus the increased c-Fos may reflect the enhanced place aversion as a result of increased negative affective state, a state that, on the basis of c-Fos expression, is overcome by exposure to

MDMA. The ability of MDMA exposure to counteract withdrawal-induced place aversion may contribute to the increased MDMA-induced CPP.

Cyclin-dependent kinase 5 (Cdk5) and p35 regulate synaptic plasticity, neurotransmitter release, and dopamine signaling, which could regulate processes implicated in drug abuse and neuropsychiatric disorders [74]. mRNA expression of p35, the neuron-specific activator of Cdk5, significantly increased after treatment with MDMA, 2 days after nicotine cessation. Similarly, in mice with a history of repeated THC treatment, an increase of expression of Cdk5 and its regulatory protein p35 has been reported [75]. Notably, several ΔFosB target genes, including Cdk5 and its coactivator p35, are increased in the NAc of transgenic ΔFosB overexpressing- or repeated cocaine-treated mice.

During early and late withdrawal, reverse transcription-qPCR analysis revealed no significant changes in the expression of any nicotine receptor subunits in nicotine-paired zebrafish brains. However, an increase of α4 at 2 and of α6 at 30 days after MDMA CPP, was found. Nicotinic acetylcholine Receptors (nAchRs) are pharmacological targets for MDMA which directly interacts with the subunit α4β2 in mouse brain membranes and cultured PC12 cells [76]. Moreover, MDMA potentiates the regulatory effects of nicotine in rat brain [77]. MDMA is known also to modulate the dopaminergic system by acting on the heteromeric nicotinic receptors reducing the inward current caused by nicotine application at the level of the soma of midbrain dopaminergic cells [78]. In previous studies in adult zebrafish, chronic and intermittent exposure to nicotine induced long-term changes in the transcriptional expression of α7-, α6- and ß2-nAChR subunits which was associated with long-term changes in the transcriptional expression of epigenetic factors [45]. These findings appear in contrast with our results. However, the concentration used in the previous study [45] was ten times higher than that used here. This discrepancy could account for the lack of nicotinic receptor subunit up-regulation seen here.

We found a progressive increase in mRNA gene expression of A_{2a} adenosine receptors in zebrafish treated with nicotine and MDMA which reached significance at 30 days post withdrawal. Adenosine A_{2A} receptors have been shown to be involved in the control of dopamine release and are putative therapeutic targets in addiction [79]. Notably, MDMA reinforcing effects have been shown to completely disappear in mice lacking the A_{2a} adenosine receptor, as evidenced by a lack of self-administration response in A_{2a} mutant mice [80]. In addition, mice lacking A_{2a} adenosine receptors, exhibit a reduction of THC-induced rewarding effects and rimonabant-precipitated THC withdrawal syndrome [81]. In Ruiz-Medina et al., 2011 [80] it has been suggested that adenosine A1 and A2A receptor blockade may account for the caffeine-induced exacerbation of the MDMA effect on dopamine and serotonin release and may aggravate MDMA toxicity.

A significant increase in mRNA expression of D_3 dopaminergic receptor was found in zebrafish pre-exposed to nicotine and treated with MDMA compared to both withdrawn animals subject to CPP with vehicle alone, and, at

days withdrawal, to drug free zebrafish subject to CPP with MDMA. Increase in D_3 dopaminergic receptor density in cocaine addicts and metamphetamine polydrug users [82] has generated interest in developing selective antagonists as potential pharmacotherapeutics for addiction [83]. Indeed, blockade of D_3 dopaminergic receptor attenuated the rewarding and incentive motivational effects of methamphetamine in rats and may have pharmacotherapeutic potential in the treatment of methamphetamine addiction. Even if D_1 and D_2 dopaminergic receptors are involved in addiction we found only a slight increase in D_1 and a slight decrease of D_2 dopamine receptors which however were not significant (data not shown). Future studies on gene expression, performed in specific brain areas, could help to reveal potential site-specific increases.

Finally, mRNA gene expression of isotocin/vasotocin receptors, the analogues of oxytocin/vasopressin receptors (particularly isotocin receptors) was increased after nicotine and MDMA treatment, suggesting the functional importance of isotocin receptors on MDMA behavioral activation and long-term neuroplasticity. Oxytocin is known to play a role in several neuropsychiatric disorders, including alcohol and drug addiction [84]. Studies in rodents have shown that chronic exposure to nicotine, methamphetamine, cocaine, and morphine generally result in an up-regulation of oxytocin receptors binding and decrease in oxytocin peptide content in various brain regions [85-87]. After 3 weeks of abstinence, alcohol-dependent rats showed increased oxytocin receptor mRNA and protein levels in different brain regions [88].

We found an increase of mRNA gene expression of vasopressin receptor in zebrafish exposed to nicotine and evaluated during early withdrawal and after MDMA treatment compared to water and MDMA group. Vasopressin has different functions within the brain such as social behaviour regulation, stress and anxiety responses, all of which are altered by drugs of abuse [89]. Little is known about the role of vasopressin receptors on drug abuse. In rodents [77 godino] protracted (14-days) withdrawal from chronic escalating dose of cocaine produced persistent elevations of vasopressin mRNA levels in the brain. Consistent with our findings supporting an interaction between nicotine- pre-exposure, vasopressin and psychostimulant reward, microinjections of vasopressin in the lateral ventricles produced an increase in locomotor activity, and animals treated with vasopressin had a higher psychomotor response to amphetamine, indicating cross□sensitization between vasopressin and amphetamine. In a CPP model with a priming injection during the reinstatement phase, systemic treatment with a vasopressin receptor subunit type B antagonist during the extinction phase diminished methamphetamine□seeking reinstatement behaviour in rodents [90].

In summary, nicotine pre-treatment increased behavioural sensitivity to MDMA-induced CPP. MDMA-CPP alone had no effect on expression of any of the genes assessed. However, MDMA CPP induced subtle differences in expression of genes associated with MDMA-induced reward in nicotine-withdrawn animals. Thus increases in expression of a4 nAChR, a6AChR, A2a, D3 and oyxtocin receptors were seen at different stages of withdrawal. The increased sensitivity

to MDMA-induced changes in gene expression is consistent with previous reports that nicotine pre-treatment generates a permissive environment for subsequent stimulant-induced gene expression [23] and suggest that the kinetics of the permissive environment may vary for different genes. Further, our findings suggest that such changes in sensitivity persist long after nicotine exposure and, if seen in humans, may contribute to the observed increased stimulant use following early-life exposure to nicotine [20] as well as nicotine exposure and withdrawal [21]. Taken together the findings on gene expression reveal a series of changes in zebrafish brain which could account for the altered sensitivity to the rewarding effects of MDMA in nicotine pre-exposed fish. These suggest that nicotine exposed zebrafish have diminished ability to modulate behaviour as a function of reward during nicotine withdrawal sharing an homology across humans and rodents and providing a strong translational framework. Future studies are necessary to investigate if this phenomenon is shared by other drugs of abuse.

Author contribution

MS designed the study and supervised the project. DB wrote the manuscript and analyzed the data. LP performed the experiments, contributed to designing experimental procedures and analyzed the data. CHB and JTP designed gene expression analysis experiments and interpreted data. JTP performed RNA extraction, generated cDNA libraries and analysed expression data. MTT conducted qPCR and analysis. All authors revised the manuscript.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Experimental design for the behavioural and molecular analyses.

Figure 2. Effect of nicotine (1 mg/L) pre-exposure (for 2 weeks) on MDMA-induced CPP evaluated 2, 7, 30 and 60 after nicotine withdrawal. MDMA (0.1 mg/kg) was given i.m. Preference was calculated by subtracting the time spent in the drug-paired compartment before drug conditioning from the time spent after drug conditioning. (n = 10 zebrafish for each group). Data are mean ± SEM. *$p < 0.05$, ****$p < 0.001$ compared to the corresponding drug-free group; # $p< 0.05$, ###$p < 0.001$, #### $p < 0.0001$ compared to the corresponding vehicle; $$$$p < 0.0001$, compared to the corresponding treatment at 7, 30 and 60 days. Comparisons were performed using two-way ANOVA followed by post-hoc Bonferroni test.

Figure 3. Effect of nicotine (1 mg/L) pre-exposure for 2 weeks on the number of transitions evaluated for 5 min, during the post-conditioning phase of MDMA-induced CPP, on day 2, 7, 30 and 60 after nicotine cessation. (n = 10 zebrafish for each group). Data are mean ± SEM. Comparisons were performed using two-way ANOVA followed by post-hoc Bonferroni test.

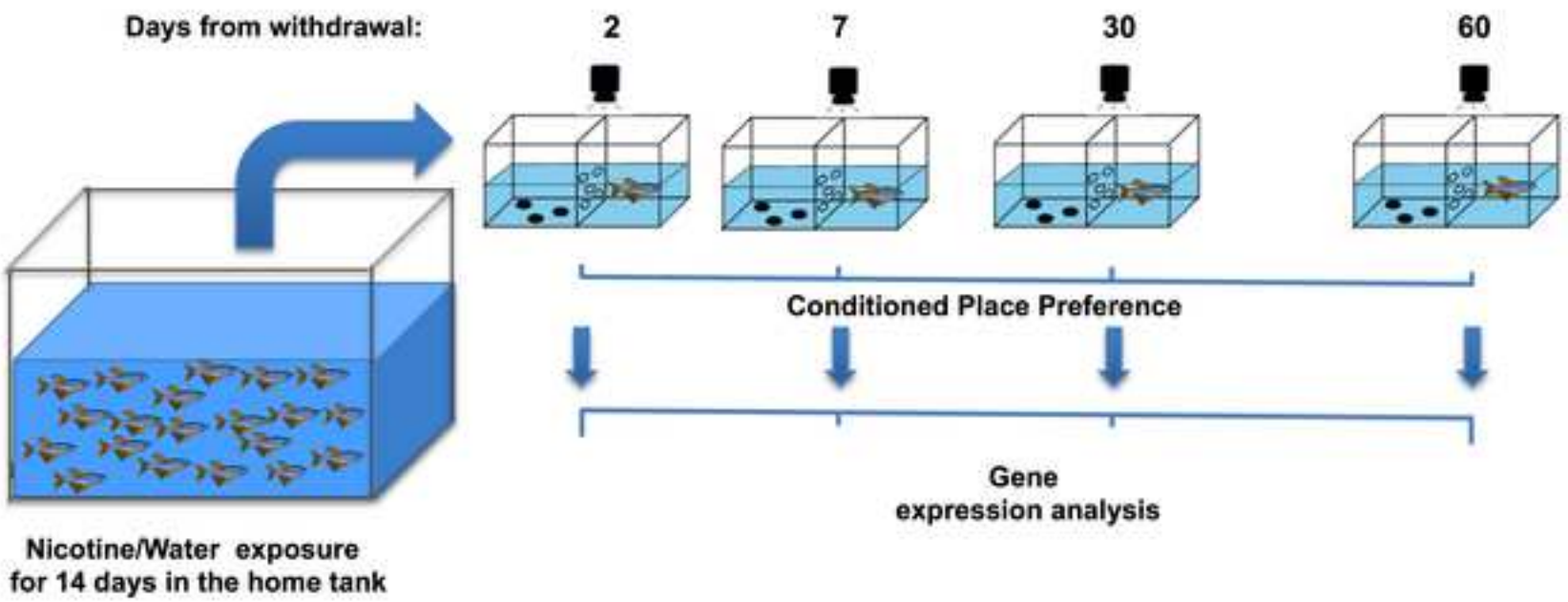
Figure 4. Quantitative real-time PCR analysis of gene expression following intramuscular treatment of MDMA at an ineffective (0.1 mg/kg) and at a rewarding dose (10 mg/kg). Data are mean ± SEM of 5 zebrafish per group.*$p < 0.05$, compared to vehicle group (one-way ANOVA followed by Tukey's test).

Figure 5. Quantitative real-time PCR analysis of different immediate early genes evaluated 1 h after CPP on day 2, 7, 30 and 60 after nicotine cessation. Data are mean ± SEM of 5 zebrafish per group.*$p < 0.05$, **$p < 0.01$ compared to vehicle group; & $p < 0.05$, && $p < 0.01$ compared to nicotine, same day (two-way ANOVA followed by Bonferroni's test).

Figure 6. Quantitative real-time PCR analysis of nicotinic and dopaminergic receptors signalling pathway evaluated 1 h after CPP on day 2, 7, 30 and 60 after nicotine cessation. Data are mean ± SEM of 5 zebrafish per group. *$p < 0.05$, **$p < 0.01$ compared to vehicle group; & $p < 0.05$, && $p < 0.01$ compared to nicotine withdrawn group, same day (one-way ANOVA followed by Tukey's test).

Figure 7. Quantitative real-time PCR analysis of members of adenosinic and oxytocin receptors signalling pathway evaluated 1 h after CPP on day 2, 7, 30 and 60 after nicotine cessation. Data are mean ± SEM of 5 zebrafish per group. *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$ compared to vehicle group; & $p < 0.05$ compared to nicotine, same day (one-way ANOVA followed by Tukey's test.

Figure 1



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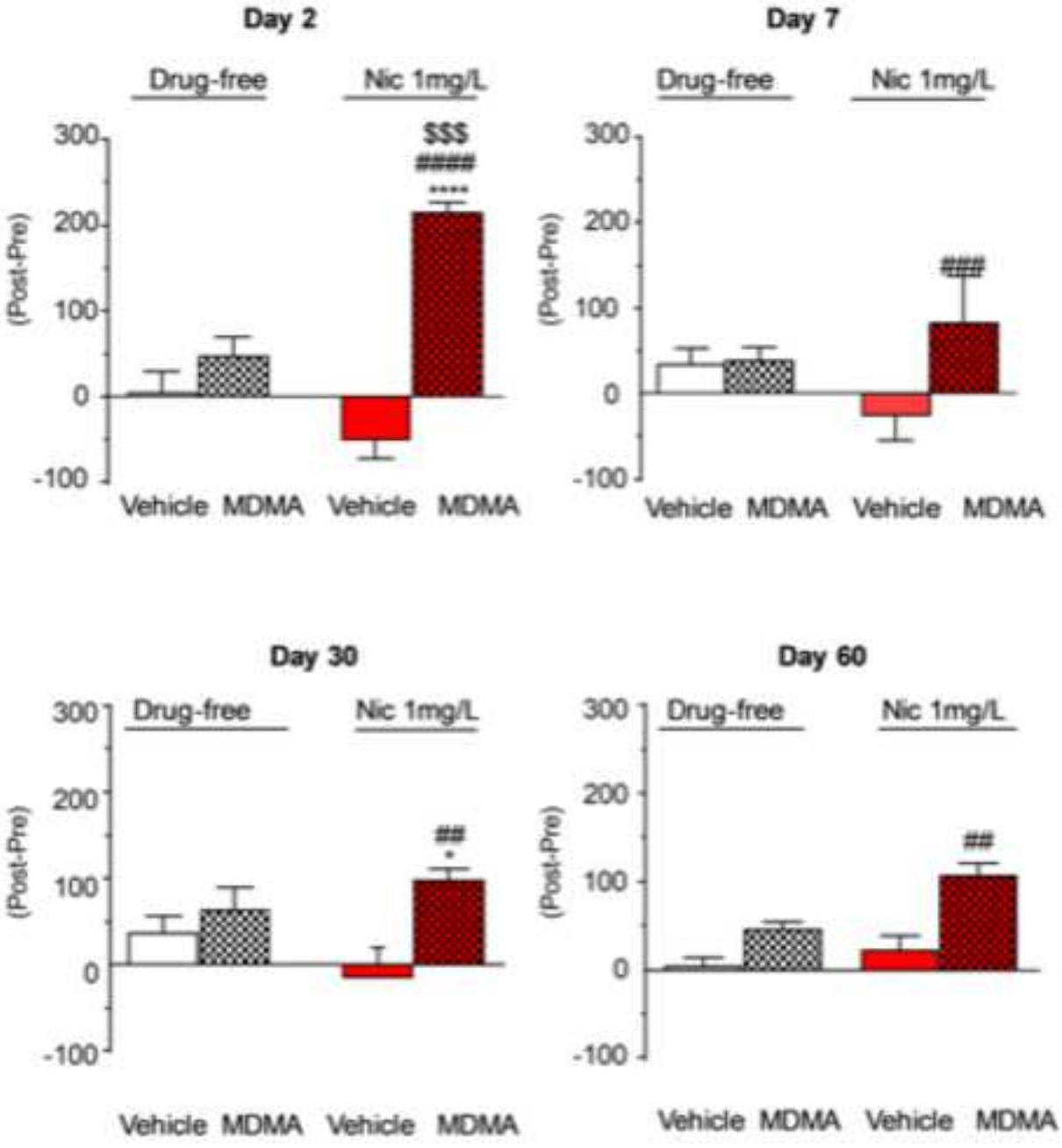

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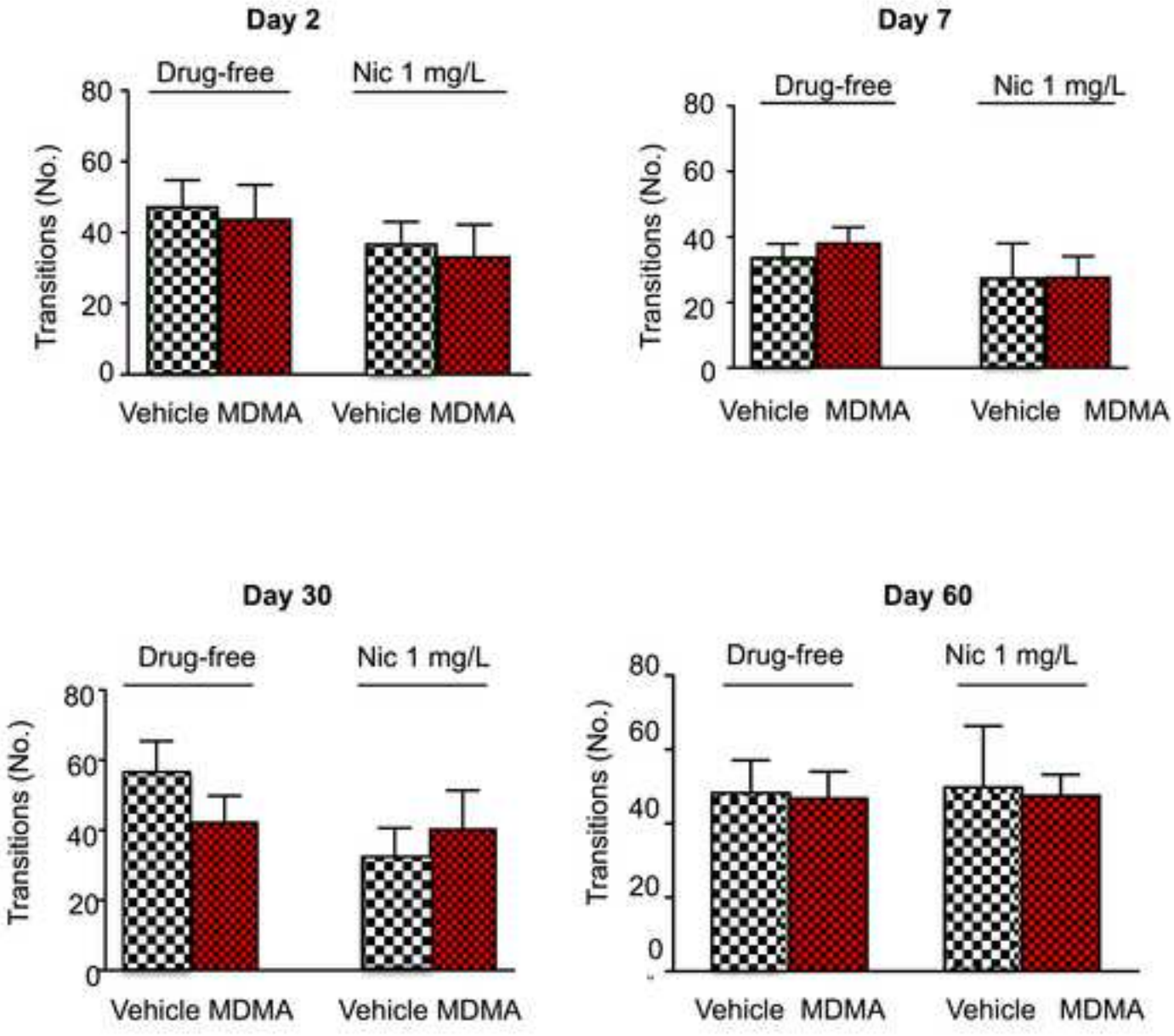

Figure

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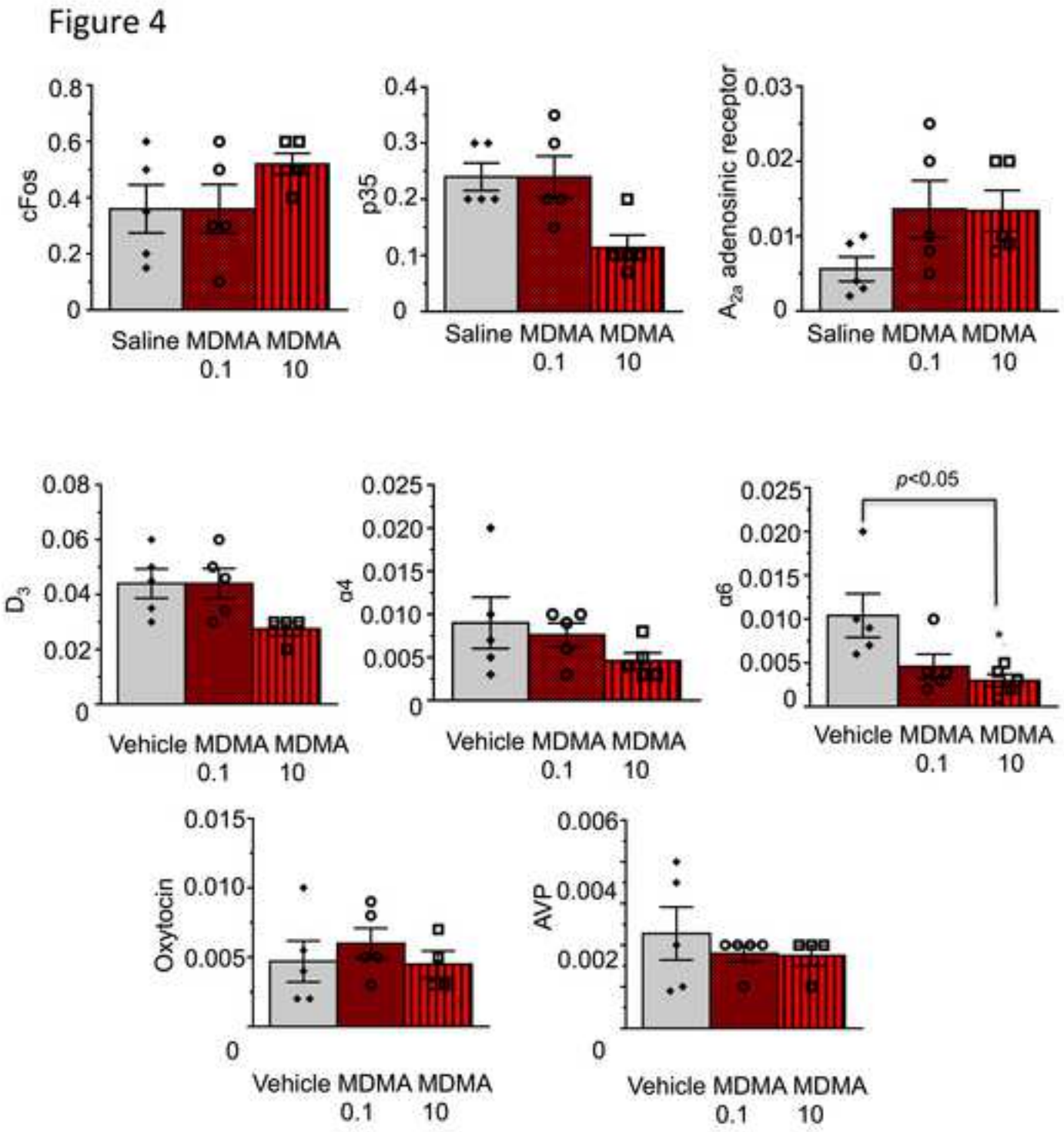

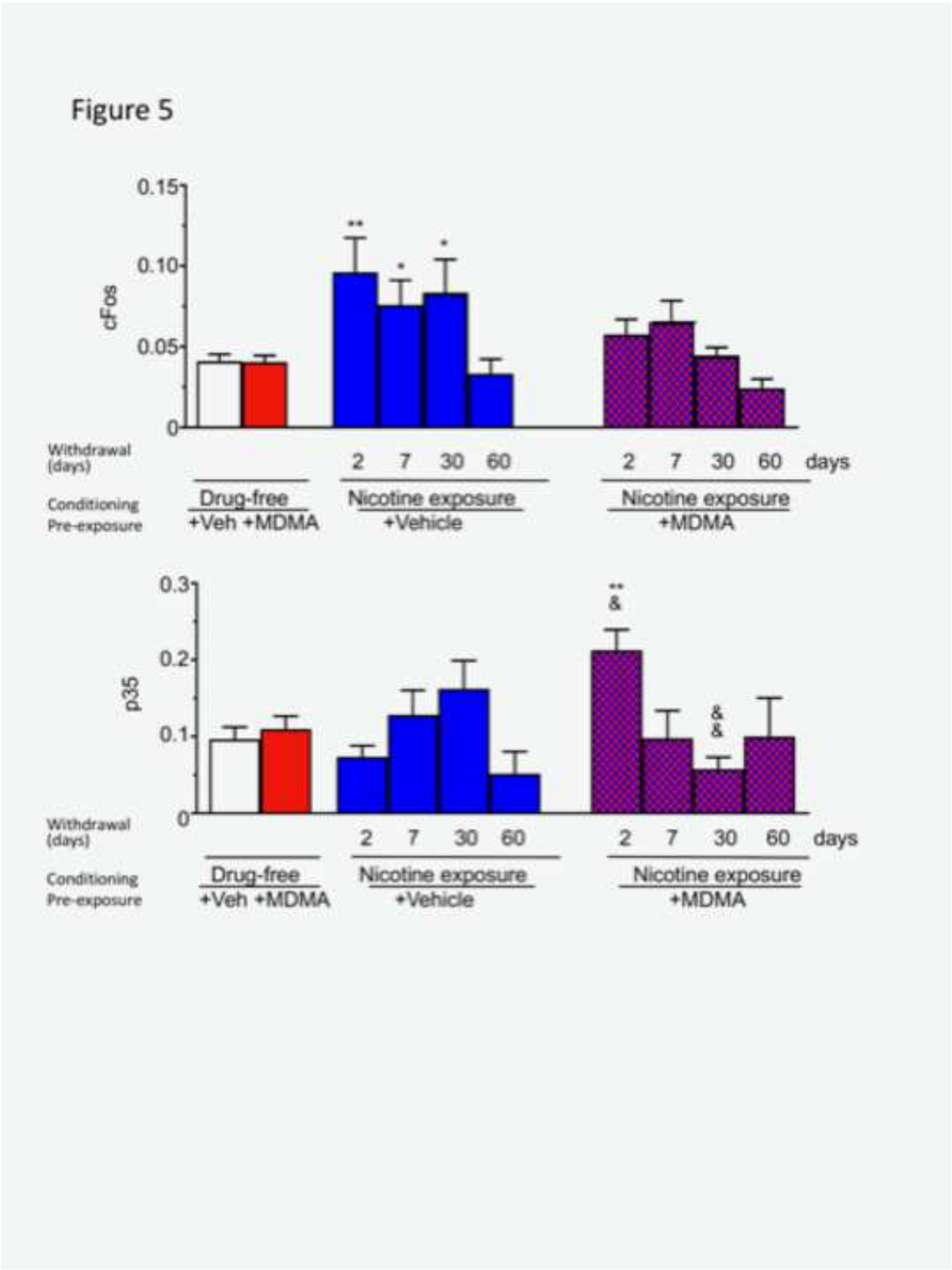

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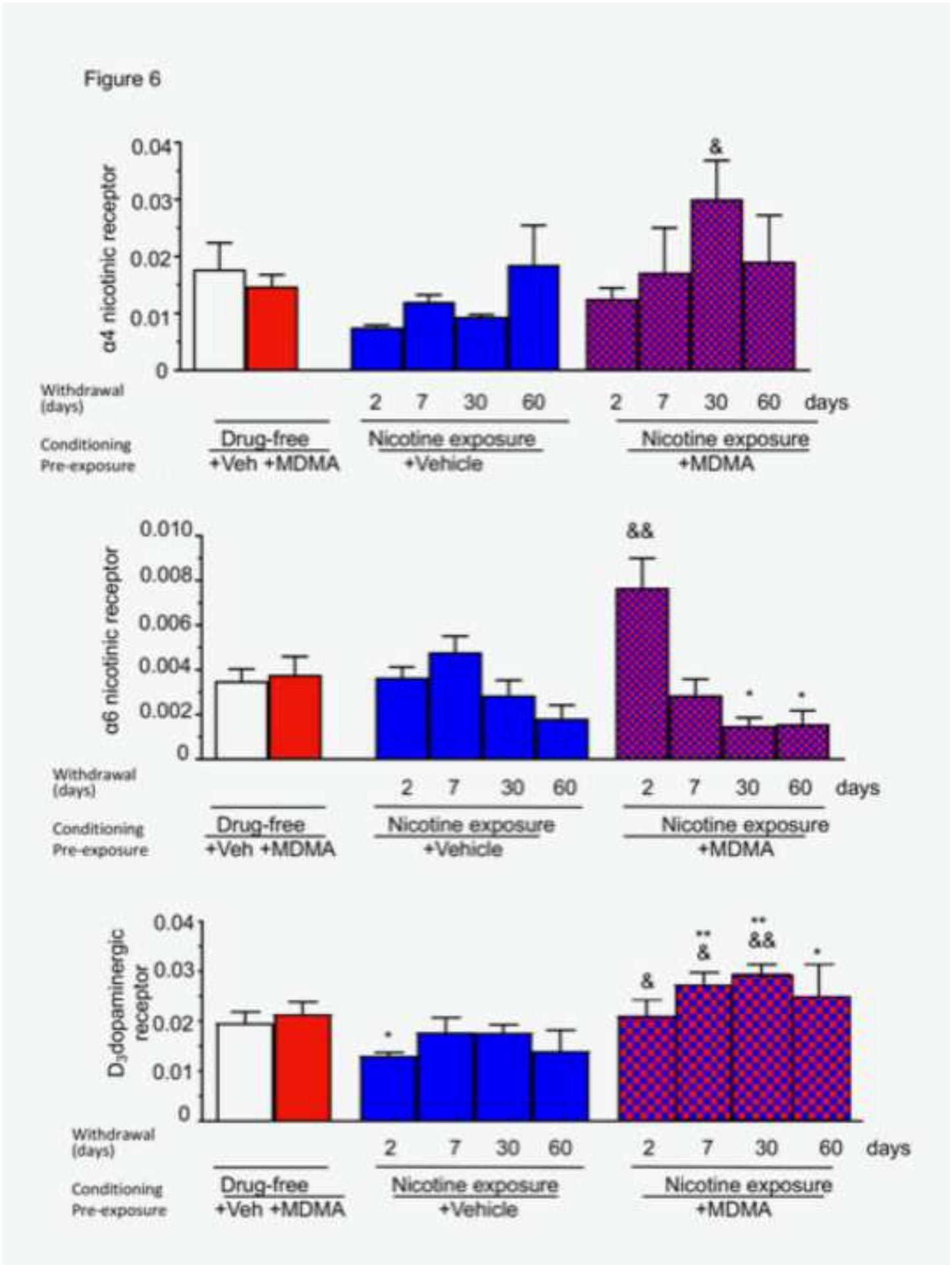

Figure

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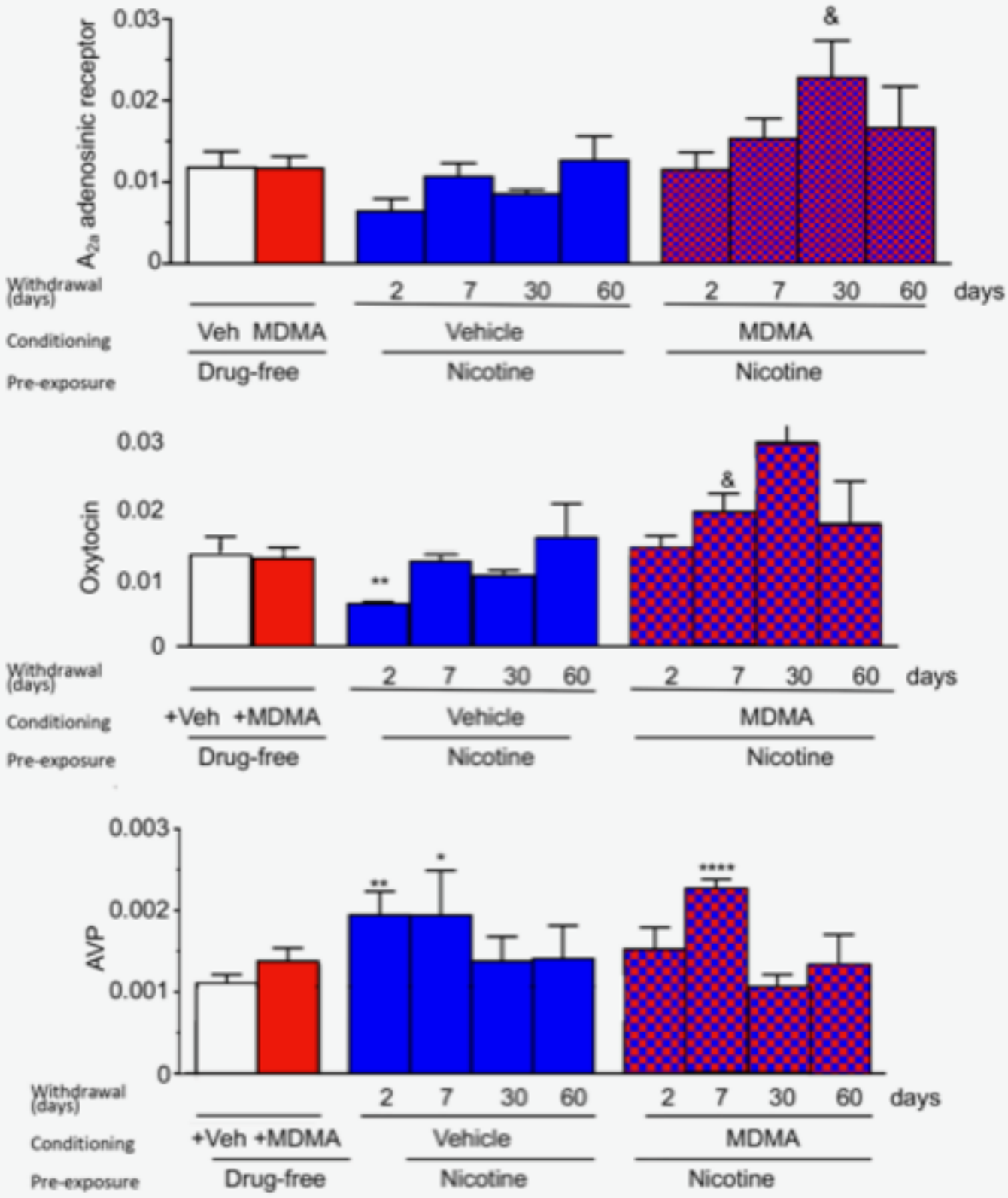
Figure 7



Supplementary Table 2