ADAPTIVE PLASTICITY IN THE HIPPOCAMPUS OF YOUNG MICE INTERMITTENTLY EXPOSED TO MDMA COULD BE THE ORIGIN OF MEMORY DEFICITS

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Concise title: Adaptive plasticity in the hippocampus of young mice exposed to MDMA

ABSTRACT

(±)3,4-methylenedioxymethamphetamine (MDMA) is a relatively selective dopaminergic neurotoxin in mice. This study was designed to evaluate whether MDMA exposure affects their recognition memory and hippocampal expression of plasticity markers. Mice were administered with increasing doses of MDMA once per week for 8 weeks (three times in one day, every 3h) and sacrificed two weeks (2 w) or three months (3 m) later. The treatment did not modify hippocampal tryptophan hydroxylase 2, a serotonergic indicator, but induced an initial reduction in dopaminergic markers in substantia nigra, which remained stable for at least 3 months. In parallel, MDMA produced a decrease in dopamine (DA) levels in the striatum at 2 w, which were restored 3 months later, suggesting dopaminergic terminal regeneration (sprouting phenomenon). Moreover, recognition memory was assessed using the object recognition test. Young (2 w) and mature (3 m) adult mice exhibited impaired memory after 24 h but not after just 1 h retention interval. Two weeks after the treatment, animals showed constant levels of CREB but an increase in its phosphorylated form and in c-Fos expression. BDNF and especially *Arc* overexpression was sustained and long lasting. We cannot rule out the absence of MDMA injury in the hippocampus being due to the generation of BDNF. The levels of NMDAR2B, PSD-95 and synaptophysin were unaffected. In conclusion, the young mice exposed to MDMA showed increased expression of early key markers of plasticity, which sometimes remained for three months, and suggests hippocampal maladaptive plasticity that could explain memory deficits evidenced here.

KEYWORDS:

MDMA; mice; recognition memory; BDNF; ARC; synaptic plasticity

Highlights

- Intermittent repeated MDMA exposure reduces mouse TH-immunoreactivity in the SN
- MDMA treatment induces a transient decrease in the striatal DA levels

- Mice exhibit a delay-dependent memory impairment that last for 3 months
- The animals show a significant increase in p-CREB, c-Fos, BDNF and *Arc* expression
- We suggest that hippocampal maladaptive plasticity explains the memory deficits

Introduction

(±)3,4-methylenedioxymethamphetamine (MDMA: “ecstasy”) is one of the most popular recreational psychoactive substances. Repeated MDMA administration at doses that are not thought to have any persistent effect on the dopaminergic system [1] produces long-term deficits in neurochemical indices of serotonergic function in the rat brain [2]. Conversely, it is generally agreed that in mice, high doses of MDMA induce a relatively selective dopaminergic terminal injury in the striatum [3, 4] and only transiently disrupts 5-HT neurochemistry in the frontal cortex [3, 5], depending on the administration schedule and dose [6, 7].

Several studies in rats report acute effects of MDMA on learning and spatial memory functions [8–10]. Moreover, impaired memory has been reported 3 months after MDMA administration in rats [11].Studies focusing on long-term consequences of MDMA in mouse memory are sparse; probably due to the fact that only a few studies have focused on the mouse hippocampus as a potential target for the effects induced by MDMA [12–14]. Moreover, none of those studies focuses on plasticity markers and memory with either a short or a long delay after MDMA exposition.

The importance of such research comes from the fact that MDMA is considered an addictive substance. Addictive drugs, including amphetamines, produce forms of structural plasticity that can be observed in the nucleus accumbens and prefrontal cortex. This plasticity reflects reorganization of patterns of synaptic connectivity [15], involving stable changes in the brain that are responsible for addiction, a life-long condition. In addition to the mesolimbic dopamine (DA) pathway, the plasticity may affect structures that mediate learned or conditioned responses, such as the amygdala, the hippocampus and the cerebral cortex [16].

MDMA in particular has been associated with stable adaptations in the dendritic structures of cortico-striatal neurons, based on a large increase in the spine density in both areas [17].

Alterations in intracellular messenger pathways, transcription factors and immediate early genes seem to be of fundamental importance for the development of plasticity, which is associated with addiction and cognition. Moreover, it is known that phospho-CREB (p-CREB) regulates the transcription of genes that contain a CRE (cAMP response element) site within their regulatory regions. This upregulation initiates some of the long-term changes in neuronal circuit functions that in turn promote the transcription of many genes, among them *Arc, c-Fos, and* expression of the NMDA receptor NR1 subunits *NR1* and *NR2. c-Fos is* an immediate-early gene (IEG). Its expression by individual neurons can be used as a marker of cell activation. *Arc* (the activity-regulated cytoskeleton-associated gene) is also an IEG which is translated to a cytosolic protein involved in the mechanism of synaptic plasticity associated with long-term potentiation (LTP) and learning [18]. Expression of *Arc* mRNA and/or protein is increased by neuronal excitation induced by electrical stimulation or by activation of glutamatergic, dopaminergic or serotonergic receptors [19–21].

Another DA-dependent change, strongly associated with drug-induced neuroplasticity, is activation of the brain-derived neurotrophic factor (BDNF) expression. BDNF belongs to the class of psychostimulant-regulated IEGs [22]. It is well known that BDNF promotes forms of excitatory synaptic plasticity, such as early- and late-phase LTP, and also promotes dendritic spine formation [23]. Until now, there were no studies of the impact of MDMA on the expression of this neurotrophin in the hippocampus of mice, which could provide useful information regarding the plasticity developed by the amphetamine derivative.

The effects of MDMA on learning and memory depend on the drug schedule and on the age at which the drug is administered [24]. Although a great deal of research has been devoted to elucidating the molecular mechanisms responsible for the cognitive deficits induced by

MDMA, to date not a single molecule or pathway has been identified. In the present paper we focus on MDMA exposure in young mice and its consequences for the young and mature adult brain. We attempt to simulate the pattern of human recreational consumption of MDMA through the dosage schedule we apply. We use mice, not rats, because this treatment schedule seems more likely to produce behavioral deficits in mice without inducing hippocampal toxicity.

Therefore, this study was designed to evaluate whether repetitive and intermittent MDMA exposure affects recognition memory and the expression of markers related with this cognitive process in a short period after ecstasy exposure. We also aimed to determine whether any such changes remain and become chronic, and to identify the type and nature of the factors that are mainly responsible for the impact of MDMA on recognition memory.

MATERIALS AND METHODS

Animals

Adolescent male C57BL/6 mice (4-5 weeks old at the beginning of the experiment) (Charles River Laboratories, France) were kept under controlled temperature, humidity and light conditions with food and water provided ad libitum. They were treated according to European Community Council Directive 86/ 609EEC and the procedure registered at the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

Drug treatment

To model recreational MDMA use, we considered appropriate to simulate the widespread practice of "boosting" (taking supplemental doses over time in order to maintain the drug's effect) [25] MDMA was administered three times in a day, every 3h, once a week for eight weeks. The treatment schedule started with a standard dose of MDMA (5 mg/kg) or saline (5

ml/kg) [26] and drug increased over the treatment period (up to 10 mg/kg) imitating the classic consumption of a reinforcing compound. Accordingly, the doses were: 5 mg/kg (two weeks), 7.5 mg/kg (the following three weeks) and 10 mg/kg (the last three weeks). Taking into account that the neurotoxic dose in mice is 25 mg/kg, three times in a day, every 3h (Colado et al. 2001), the highest dose selected was 10mg/kg. Exposition was at high ambient temperature (26ºC) as described elsewhere [27]. After the end of the treatment (ATET), mice were killed at two different time points: two weeks ATET (3.5 months old, n=31) and three months ATET (6 months old, n=36). At 3-6 months of age, mice have completed their development, but are not affected by senescence yet; we referred to them as young (3.5 months old) and mature (6 months old) adults.

Therefore, in the present study, four groups were considered:

- Saline treated mice, killed two weeks ATET: Saline 2w
- MDMA treated mice, killed two weeks ATET: MDMA 2w
- Saline treated mice, killed 3 months ATET: Saline 3m
- MDMA treated mice, killed 3 months ATET: MDMA 3m

Object recognition test

One week before death, the recognition memory of the animals was tested using the object recognition test (ORT). The test consisted of a familiarization session (three consecutive days) in which mice explored an arena (a circular area measuring 40 cm diameter) without objects for 10 min. During the first familiarization session, the animals were monitored to assess locomotor activity (Smart 3; Panlab SL, Barcelona, Spain). On the 4th day, the mice were trained with two identical objects, (two objects “A”; 10 min). Novel object recognition was tested 1 h and 24 h after the training session, when mice were exposed to the familiar object A and a novel object B (1 h) or C (24 h) for 10 min. The time spent by the mice at each

of the objects was measured in seconds. Exploration of the objects was defined as sniffing, touching and having moving vibrissae whilst directing the nose towards the object at a distance of less than 1 cm. The data were expressed as measures of discrimination between the new and familiar object, thus correcting the difference in the exploratory time of each object by the total exploration time.

Tyrosine hydroxylase (TH) - positive neurons in substantia nigra (SN)

Mice were anaesthetized by i.p. injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg) and were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the brains were removed. Coronal sections of 30 µm were obtained. Free-floating sections were rinsed in 0.1 M phosphate buffer, pH 7.2 and preincubated in a blocking solution (10% fetal bovine serum (FBS), 0.2 mol/l of glycine, Triton X-100 0.2% in 0.2% PBS-gelatin). Then, the sections were incubated for 48h at 4 °C with mouse anti-tyrosine hydroxylase antibody (1:200 BD Biosciencies). After that, the sections were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:200; Sigma-Aldrich) for 2 h at room temperature. Finally, the slices were mounted on glass slides using Fluoromount (EMS). To quantify the total number of TH-positive neurons in the SN, unbiased counting frame was positioned on each photomicrograph taken at a magnification of 10x, according to the atlas of [28]. TH-neurons were counted in both hemispheres using the Image J software, and the density (cells/area) was calculated in relation to the area delimited by the frame.

Measurement of neurotransmitters

For analysis of neurotransmitter levels, the mice were killed by cervical dislocation and decapitation, the brains rapidly removed and the striatum dissected out on ice. Samples were prepared by sonication in 10 volumes of 0.1M perchloric acid, centrifuged for 30 min at 12,000 × *g* after which 40 µl of filtered supernatant was injected into HPLC system equipped with a Waters 2465 electrochemical detector set to a potential +0.70 V, and a column Nova

Pack C18 4µm 3.90 x 150 mm (Waters, Milford, MA). The mobile phase consisted of purified water with 10% methanol, 1.92 mM 1-octanesulfonic acid, 0.1 mM EDTA and 10 mM phosphoric acid. Column temperature was set at 37 °C and a flow rate of 1 ml/min. The retention times for norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), DA, homovanillic acid (HVA) and serotonin (5-HT) were 2.74, 4.46, 6.95, 8.13, 11.24 and 20.66 min, respectively.

Hippocampal lysate preparation

The hippocampus was quickly dissected out, frozen and stored at −80 °C until use. When required, tissue samples were thawed and homogenated at 4 °C in lysis buffer (Tris-HCl 20mM, NaCl 137 mM, Nonidet P40 1%, EDTA 2mM, 4.5 µg/µl of aprotinin, 0.1 mM of phenylmethylsulfonyl fluoride, 1 mM of sodium orthovanadate, and phosphatase inhibitor cocktail 1 (Sigma-Aldrich, St.Louis, MO, USA), lysated samples were mixted in an orbital for 2 h at 4 °C. The protein samples were then centrifuged at 15,000 xg for 30 minutes. The supernatant was recovered and protein content was determined using the Bio-Rad Protein Reagent.

Western blotting and immunodetection

Western blotting (WB) and immunodetection protocol was used to determine the proteins levels as described (Pedrós et al., 2014). Primary antibodies are detailed in supplementary material. All results are normalized to GAPDH or Actine, unless stated otherwise.

RNA extraction and quantification

Total RNA was isolated from the hippocampus of mice, using the RNeasy Lipid Tissue Mini Kit, according to manufacturer's protocol (Qiagen). RNA pellet was reconstituted in RNAse-free water, with the RNA integrity determined by Agilent 2100 Bioanalyzer.

Quantitative RT-PCR

First-strand cDNA was reverse transcribed from 1 µg of total RNA from the hippocampus, using the High Capacity cDNA Reverse Transcription kit, according to manufacturer's protocol (Applied Biosystems). Equal amounts of cDNA of each individual animal were subsequently used for qRT-PCR, and each sample was analyzed in duplicate for each gene. The PCR reaction contained 15 ng of reverse-transcribed RNA, 2 x IQ^{TM} 2SYBRGreen Supermix (BioRad, Barcelona, Spain) and 100 nM of each primer. The PCR assays were performed on a StepOnePlus Real Time PCR system (Applied Biosystems), and normalized to the average transcription levels of actin, using the delta–delta Ct method [30]. Primer Express Software (Applied Biosystems, Foster City, CA) was used to design the primers (supplementary material).

Statistical analysis

Unless otherwise indicated, we used Student's t-test for comparing the means of two treatments (saline or MDMA). All data are presented as mean ± SEM, and the P values less than 0.05 were considered as significant.

RESULTS

Object Recognition test

On the first day of the familiarization of the ORT, locomotor activity was registered. Student's t-test for distance travelled and locomotor speed showed a significant effect of treatment when assayed short time after finishing drug exposure (2w) (distance: 3081.3 ± 160.5 cm saline *vs* 2604.0 ± 132.9 cm MDMA, p <0.05; speed: 5.14 ± 0.24 cm/s saline *vs* 4.45 ± 0.23 cm/s MDMA, p <0.05; n=26). This effect disappeared three months after finishing exposure.

Memory results were analyzed using two-way ANOVA and post-hoc comparisons by Tukey's procedure, as we wanted to assess the effect of two variables: treatment and delay time. Both young and mature adult mice previously exposed to MDMA exhibited impaired object

recognition memory, measured as reduced novel preference, after a retention interval of 24 h but not after just 1 h. Statistical analysis revealed a significant effect of delay time (2w $F_{1,24}$ = 83.78, p <0.001, n=26; 3m: $F_{1,21}$ = 33.23, p <0.001, n=23) and of the interaction treatment x delay time (2w: $F_{1,24}$ = 4.27, p <0.05, n=26; 3m: $F_{1,21}$ = 60.95, p <0.001, n=23) (see Fig. 1 A and B). These results indicate a delay-dependent deleterious effect of the drug on memory.

As glucocorticoids impair performance of spatial memory [31], we determined the effect of our chronic MDMA regimen on this receptor protein in this area. Neither, the 2w group nor the 3m group showed any effect of MDMA exposure (2w: 100 ± 7.90% saline *vs* 91.88 ± 9.46% MDMA, n=7, n.s.; 3m: 100 ± 11.76% saline *vs* 120.96 ± 15.47% MDMA, n=7, n.s.)

Effects of MDMA on neuronal injury markers

To assess the neurotoxicity we measured neurotransmitter levels in striatum, also counting TH-positive cells in SN. To confirm that the treatment did not damage serotonergic terminals in the hippocampus, we also evaluated tryptophan-hydroxylase 2 (TPH2), a marker of 5-HT terminals.

Two weeks after the treatment, MDMA exposure caused a significant decrease of striatal DA levels (42.1 ± 4.1 nmol/g wet tissue (wt) saline *vs* 24.8 ± 4.7 nmol/g wt MDMA, n=8 , p=0.057) and its main metabolite, DOPAC (14.3 ± 1.0 nmol/g wt saline *vs* 9.31 ± 0.98 nmol/g wt MDMA, n=8, p<0.05), without changing norepinephrine (0.66 ± 0.07 nmol/g wt saline *vs* 0.67 ± 0.19 nmol/g wt MDMA, n=8) or 5-HT levels (1.78 ± 0.37 nmol/g wt saline *vs* 1.77 ± 0.33 nmol/g wt MDMA, n=8). In parallel, we found a slight MDMA-induced decrease in the number of TH-positive neurons in SN (0.26 ± 0.02 positive cells/area saline *vs* 0.20 ± 0.01 positive cells/area MDMA, n=12, p<0.05)(Fig. 2). However, no changes were observed for TPH2 expression in the hippocampus (100 ± 9.67% saline, 102.54 ± 7.98 % MDMA, n=7).

When the same parameters were evaluated 3 months ATET, the levels of DA (37.20 ± 2.65 saline *vs* 37.70 ± 6.96 MDMA, n=15) and DOPAC (14.48 ± 1.16 saline *vs* 12.83 ± 1.34

MDMA, n=15) were restored in mice exposed to MDMA. As expected, the MDMA effect on TH-positive cells in the SN was maintained (0.23 ± 0.01 positive cells/area saline *vs* 0.18 ± 0.01 positive cells/area MDMA, n=8, $p<.0.05$)(Fig. 3) and no changes in TPH2 expression in hippocampus were found (100 ± 13.54% saline, 92.18 ± 8.90% MDMA, n=8).

Effects of MDMA on plasticity markers (P-CREB/CREB) in the hippocampus

Two weeks after the chronic administration of MDMA, no changes in the total level of CREB protein were detected (100 ± 21.37% saline, 97.35 ± 8.57% MDMA, n=9), but an increase in its phosporylated form was observed (100 ± 21.37% saline, 165.8 ± 14.62% MDMA; $p<0.05$). As expected, this increase disappeared 3 months after the last dose of the drug (100 ± 13.95% saline, 89.95 ± 15.72% MDMA, n.s., n= 8) (Fig. 4).

Early genes/proteins

We performed PCR and WB assays to determine changes in genes or proteins strongly associated with neuroplasticity [22]. Of the CREB-regulated genes, we chose *Arc* and *c-Fos.*

Results showed that both *c-Fos* mRNA and c-FOS protein in the hippocampus were significantly induced by MDMA shortly ATET (mRNA: 100 ± 23.77% saline n=4; 391.1 ± 46.8% MDMA, n=9, $p<0.01$) (protein: 100 ± 7.92% saline, 144.0 ± 8.7% MDMA, n=8, $p<0.01$), but returned to basal values when assayed 3 months ATET (Fig. 5; mRNA: 100 ± 12.24% saline, 117.1 ± 17.17% MDMA, n.s., n=9, protein: 100 ± 16.68% saline, 96.74 ± 16.68% MDMA, n.s., n=9). The overexpression of *c-Fos* mRNA and c-FOS protein that lasts two weeks indicates a very strong signal during drug exposure.

Regarding ARC, repeated administration of MDMA to C57BL/6 mice induced significant expression of *Arc* mRNA (100 ± 11.72% saline, 190.2 ± 22.47% MDMA, $p<0.05$, n= 9) and ARC protein (100 ± 5.96% saline, 126 ± 4.61% MDMA, $p<0.05$, n=8) when assayed 2 weeks ATET (Fig. 6A and B). Surprisingly, the overexpression of this gene was sustained and long lasting (100 ± 11.17% saline, 163.7 ± 20.67% MDMA, $p<0.05$, n=9), as shown in MDMA-

treated mice, 3 months ATET. Nevertheless, no changes were found in ARC protein levels (100 ± 4.43% saline, 114.0 ± 11.32% MDMA, n.s., n=7) in mature adult mice (Fig. 6C and D).

BDNF

Despite of in the present study MDMA did not modify 5-HT parameters in hippocampus, we sought to measure its effect on the expression of BDNF, as our group reported increased hippocampal BDNF levels depending on cognitive training in rats exposed to MDMA [27].

Figure 7 summarizes the effects of MDMA on BDNF levels. A significant increase in *BDNF* mRNA transcript expression was found shortly after drug withdrawal (100 ± 8.01% saline, 133.6 ± 7.07% MDMA, $p<0.05$, n=10). Likewise, MDMA-treated animals showed a slight increase in BDNF protein levels, although it did not reach statistical significance (100 ± 10.06% saline, 126.8 ± 10.19% MDMA, n.s., n=8)

In mature adult mice (3 months ATET), a delayed expression of BDNF protein was observed in MDMA-trated animals (100 ± 5.57% saline, 138.35 ± 11.08 MDMA, $p<0.05$ n=9), although mRNA showed non-significant changes (100 ± 11.61% saline, 124.2 ± 11.08% MDMA, n.s., n=10). The changes in BDNF expression related with the amphetamine derivative exposure are thus independent of the impact of MDMA on hippocampal 5-HT neurochemistry, absent from our study.

Regulation of synaptic plasticity-related proteins

Finally, other CREB-regulated genes are those encoding glutamate receptors. Synaptic plasticity associated with addiction and cognition relies on the normal integration of glutamate receptors at the post-synaptic density (PSD). Therefore, we studied the effects of MDMA exposure in the levels of four synaptic proteins: PSD-95, NR1, NR2B and synaptophysin (2w n=7-9, 3m n=8-11).

The effects of the assayed MDMA schedule on these proteins are shown in Fig. 8, and are presented as relative protein expression. Although an apparent decrease was observed for NR1 expression in both young and mature adults, statistical significance was not reached (2w: 100 ± 24.18% saline, 71.91 ± 8.52% MDMA, n.s., n= 7; 3m: 100 ± 27.83% saline, 62.55 ± 12.70% MDMA, *p*=0.2835, n=10) (Fig 8A and E). Moreover, MDMA failed to induce any change in NR2B expression (Fig 8B and F).

Studies have shown that interactions between the PSD-95 protein and NMDA receptors located at the spine tip may also regulate dendritic spine morphology. Thus, we analyzed the expression of this protein in the hippocampus of the treated animals. According to our results with NR2B, MDMA did not significantly modify the levels of this scaffolding protein (Fig 8C and G)(2w: 100 ± 15.24% saline, 71.28 ± 6.11% MDMA, n.s., n= 8; 3m: 100 ± 5.58% saline, 86.52 ± 6.42% MDMA, n.s., n=9).

Finally, to explore the relationship between the cognitive changes observed in MDMA-treated mice and synaptic modifications, we studied the effect of a recent or a long earlier exposure in synaptophysin. Once again, our results revealed no relationship in this sense (Fig. 8D and H)(2w: 100 ± 14.67% saline, 96.45 ± 16.62% MDMA, n.s., n= 9; 3m: 100 ± 4.79% saline, 108.67 ± 14.34% MDMA, n.s., n=8)

DISCUSSION

MDMA is an amphetamine derivative that acts as a psychostimulant by increasing catecholamine concentration in synapses. In comparison with methamphetamine, MDMA has enhanced potency for 5-HT release and reduced potency for DA release [32]. It also acts as an agonist of 5-HT2A receptors [33] and, at high doses, as a 5-HT or DA neurotoxin in non-human primates, rats and mice, often associated with depletion of 5-HT or DA terminal markers [2].

It is well established that MDMA has a different pharmacology in mouse compared to rat. In contrast to its selective 5-HT neurotoxicity in rats, after a binge schedule in mice (20-30 mg/kg, t.i.d, 3 h intervals), it is a relatively selective DA neurotoxin without long-lasting effects on the 5-HT content. Long-term studies in rats demonstrated that after MDMA-induced neurotoxicity, significant (even complete) recovery may occur from 8 weeks to a whole year [34]. Nevertheless, other studies have shown incomplete recovery in certain brain areas or in particular animals after 12-18 months post-treatment [35, 36]. Another important finding from those time course studies in rats was the late appearance of a serotonergic hyperinnervation of certain subcortical brain areas, a phenomenon thought to reflect axonal/terminal sprouting following synaptic loss.

In the present study, we applied a dosage schedule that imitated classic weekend recreational use of MDMA. The highest dose used was notably lower than the dose established as toxic in binge regimens [6], but we used a more extended treatment. Costa et al. [37] also used a prolonged and intermittent exposure (10 mg/kg two times in a day, twice per week during 9 weeks); nevertheless, we thought that once per week and administering increasing doses simulates better the pattern of consumption in adolescents. This treatment produced an initial reduction in TH-immunoreactivity in the SN, which agreed with the results of Costa et al. There were no previous reports of longer-term effects of MDMA on mice, but our results here indicate an irreversible effect on dopaminergic neurons in SN or,

alternatively, suggest that recovery from dopaminergic damage in mice takes more than three months.

In the striatum, neurotransmitter analysis revealed initial major decrements in DA levels, which recovered three months later. This suggests an increase in catecholamine synthesis or a compensatory DA axonal sprouting and branching after synaptic loss; or both. Acute activation of striatal dopamine synthesis after MDMA could be a compensatory response to the carrier-mediated efflux of transmitter [38]. Regarding dopaminergic terminal regeneration (sprouting), it was reported in mice 30 days after MDMA exposure [39], and involves plasticity events such as reactive synaptogenesis and rerouting of axons to unusual locations. In parallel, MDMA-exposed mice significantly reduced the distance travelled and speed when assayed shortly after finishing the treatment. These results agree with the reduction of DA neurons in SN and with DA depletion in the striatum; and this depressant effect disappeared three months later, correlating with the restoration of striatal DA levels.

In contrast, analysis of the hippocampus collected in 2w and 3m groups yielded no changes in the serotonergic marker (TPH2). In the same groups, previously to sacrifice, recognition memory was tested using the ORT. When this task was tested after a short retention interval (1h), the MDMA-treated mice performed similarly to control animals. However, when memory was tested after a long retention interval (24h), for which a key role of the hippocampus has been reported, the MDMA group showed a very significant deficit, which was maintained for a prolonged time ATET. We ruled out changes in the glucocorticoid receptor as the origin of these deficits. These results indicate a delay-dependent deleterious effect of the drug on recognition memory, and that the hippocampus is the structure mainly responsible. The hippocampus is involved in object recognition memory regardless of retention interval, but parahippocampal structures (e.g., perirhinal cortex) are sufficient to support object recognition memory over short retention intervals [40].

On the other hand, it has been suggested that structural plasticity associated with exposure to drugs of abuse reflects a reorganization of the patterns of synaptic connectivity which contributes to some of the persistent effects associated with drug use, including addiction. Exposure to amphetamine, cocaine, nicotine or morphine produces persistent changes in the structure of dendrites and dendritic spines on neurons in some brain regions, such as those associated with incentive motivation, reward and judgment [15]. Furthermore, persistent changes, such as those associated with learning and memory, are thought to be due to the reorganization of synaptic connections in brain circuits. Therefore MDMA, which induces anomalous neuroplasticity as a consequence of its addictive properties [41], could affect memory processes. This only could be possible if the plasticity changes are present in brain structures related to memory, such as hippocampus. Following rat developmental MDMA exposure, Williams et al. [42] found neuronal cytoarchitectural changes, which are long-lasting and are in regions consistent with the learning and memory deficits observed in such animals. Eight weeks after chronic administration of MDMA to rats, van Nieuwenhuijzen et al. [43] reported residual changes in hippocampal proteins implicated in learning-related neuroplasticity, and our group reported increased hippocampal BDNF levels and an effect on spine density depending on cognitive training [27]. However, previously there were no data on the effects on mice, where the consequences of MDMA are known to be different.

The cAMP response element-binding protein binds to CRE sites as a dimer and only activates transcription when both subunits are phosphorylated at their Ser133 residue (p-CREB). As CREB can be phosphorylated at Ser133 by protein kinase A (PKA), p-CREB can be detected after DA type1 receptor stimulation by classical psychostimulants. P-CREB may initiate some long-term changes in neuronal circuit functions, thereby promoting the transcription of many genes, among them *Arc, c-Fos, NR1* and *NR2B.* Therefore, CREB may be a universal modulator of processes required for memory formation [44]. Mice exposed to the assayed MDMA schedule showed constant levels of CREB but an increase in its phosphorylated form. It is worth stressing that these results were obtained in animals whose

MDMA exposure finished 2 weeks previously, indicating an important phosphorylation stimulus. This was confirmed by measuring c-Fos, a marker of cell activation that behaves similarly to p-CREB, which increased shortly after treatment but had then reverted 3 months ATET.

Activity-regulated gene 3.1 (*Arg3.1*), also known as *Arc*, is an IEG that is regulated by BDNF- and CREB-dependent signaling [45, 46]. Arc protein acts as a stabilization factor for filamentous-actin, which results in the regulation of dendritic spine morphology [47, 48]. Arc could be considered a key regulator of protein synthesis-dependent forms of synaptic plasticity, which are thought to underlie memory storage [49]. In rats, single or repeated cocaine treatment, as well as long-term abstinence (48 days) following drug administration, all increase Arc expression [50–52], although it is associated, at the same time, with alterations in the finely tuned mechanisms that regulate Arc degradation [52]. In our experiments, MDMA increased *Arc* transcript and protein expression after two weeks of withdrawal, which was also evident, although reduced, after 3 months of withdrawal. These results indicate an intense effect of MDMA on synaptic plasticity particularly intense in the early stages of withdrawal which fades with time.

In the same way, we assayed the effects of MDMA on BDNF. The mRNA related to BDNF gene and to that of *Arc* are induced by cellular activity and transported into dendrites, thereby promoting plasticity [22]. The changes in BDNF accumulate and rise with increasing periods of abstinence [53]. Martínez-Turrillas et al. [54] studied the effects of an acute administration of MDMA to rats. They found that after MDMA, BDNF mRNA levels were increased in frontal cortex, but reduced in hippocampus after 48 h, in spite of the marked increase at this time point in the levels of the transcription factor pCREB. Those authors attributed this variation to the high vulnerability of the rat hippocampus to the neurotoxic effects of the amphetamine. However, because hippocampus serotonergic neurotransmission is not affected by MDMA in mice, the consequences for BDNF levels are probably different. In neurons, the expression

of BDNF mRNA is enhanced when the AMPA-type glutamate receptor is activated [55, 56], and requires an increase in intracellular calcium concentrations [57, 58]. We found [59] that treatment with MDMA significantly disrupted calcium homeostasis, favoring glutamate release in the hippocampus. These results allowed us to speculate about a possible effect of MDMA on BDNF expression. Indeed, in this area, we observed that few days after treatment, BDNF mRNA levels were increased with no significant effects at protein levels. Conversely, BDNF mRNA levels were not affected 3 months after exposure, while it significantly increased protein levels. This suggests that prolonged exposure to MDMA differently affects BDNF transcription and translation. It is known [60] that after an appropriate stimulus, a homogenous increase of BDNF mRNAs is carried out in the cell body, and it can be selectively targeted to the active synapse or non-selectively translocated to dendrites, but trapped by an active spine, where it would be quickly translated to BDNF protein. Therefore BDNF mRNA and BDNF protein may be non-simultaneously stored in different locations inside the neurons [61], which would explain the discrepancy between mRNA and protein levels found in the present study. We cannot dismiss the idea that in our model, the absence of injury in the hippocampus is due to the generation of BDNF in this area [62].

In addition to being involved in learning plasticity and neural cell death [63], the glutamate/glutamatergic system is involved in addiction to several drugs of abuse [64]; but knowledge of glutamate receptor regulation following MDMA administration is limited. Kindlundh-Högberg et al. [65] investigated in adolescent rats the immediate effects of repeated intermittent MDMA administration upon gene-transcript levels of glutamatergic receptors. In contrast to other brain areas, they did not found changes in hippocampal NR1 or NR2B expression, 10 h after the last injection. In our study, the evaluation of NR2B, PSD-95 and synaptophysin also yielded constant levels, with only NR1 showing a tendency to decrease, which was not statistically significant.

Drugs of abuse, such as psychostimulants, usurp the normal basic reward function, essentially hijacking molecular and cellular processes, such as those involved in addiction, leading to persistent adaptive behavioral responses [64]. It is not completely understood to what extent these maladaptive changes in the reward circuit expand and affect other brain areas. In the present study, young mice exposed to intermittent and repeated doses of MDMA showed increased expression of key early markers of plasticity, which sometimes persisted for 3 months. Considering that the neuronal injury was detected in the SN but not in the hippocampus, we suggest that hippocampal maladaptive plasticity could explain the memory deficits evidenced here.

Conclusions

Mice exposed to an intermittent and repetitive schedule of MDMA for 8 weeks initially showed lower locomotor activity, concordant with the reduction of DA neurons in the SN and the depletion of DA in the striatum. Consistent with what happens with DA in the striatum, 3 months after treatment, this hypolocomotor effect disappeared. Mice also exhibited deficits in recognition memory that persisted at least 3 months ATET. However, in the hippocampus, MDMA administration did not cause any changes in 5-HT terminals or in NMDA subunits, indicating that other mechanisms underlie MDMA-elicited memory deficits. These behavioral changes correlate with significant overexpression of hippocampal plasticity markers downstream of CREB phosphorylation, which could be the result of DA D1 receptor stimulation. It is especially worth stressing the long-lasting increase in BDNF protein after drug exposure.

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FIGURE LEGENDS

Figures were created using GraphPad Prism program

Fig. 1: Assessment of recognition memory in animals exposed to saline or MDMA and tested one week (A, n=26) or three months (B, n=23) after the end of the treatment. The time spent exploring new object was registered 1 h and 24 h after the familiarization session, and expressed as measures of discrimination between the new and familiar object. Data are presented as mean ± SEM. Two-way ANOVA revealed a significant effect of delay time (2w $F_{1,24}$ = 83.78, P <0.001; 3m: $F_{1,21}$ = 33.23, P <0.001) and of the interaction treatment x delay time (2w: $F_{1,24}$ = 4.27, P <0.05; 3m: $F_{1,21}$ = 60.95, P <0.001). Post-hoc comparisons by Tukey's procedure yielded the significances that are displayed in the graph. * P <0.05 *vs* time-matched saline.

Fig. 2: Exposure to MDMA significantly decreased the TH positive neurons in the SN. Bar graph show the number of TH positive neurons in SN, two weeks after the last exposure to the drug. Data represent the mean ± SEM. Post-hoc Turkey test: * P<0.05 *vs* saline. Panels show representative photomicrographs of the effects of Saline or MDMA on TH immunofluorescence in the SN, after 2 weeks of withdrawal. Scale bar: 50μM.

Fig. 3: Long lasting effect of exposure to MDMA on TH positive neurons in the SN. Bar graph show the number of TH positive neurons in SN, 3 months after the last exposure to the drug. Data represent the mean ± SEM. Post-hoc Turkey test: * P<0.05 *vs* saline. show

representative photomicrographs of the effects of MDMA on TH immunofluorescence in the SN, after 3 months of withdrawal. Scale bar: 50µM.

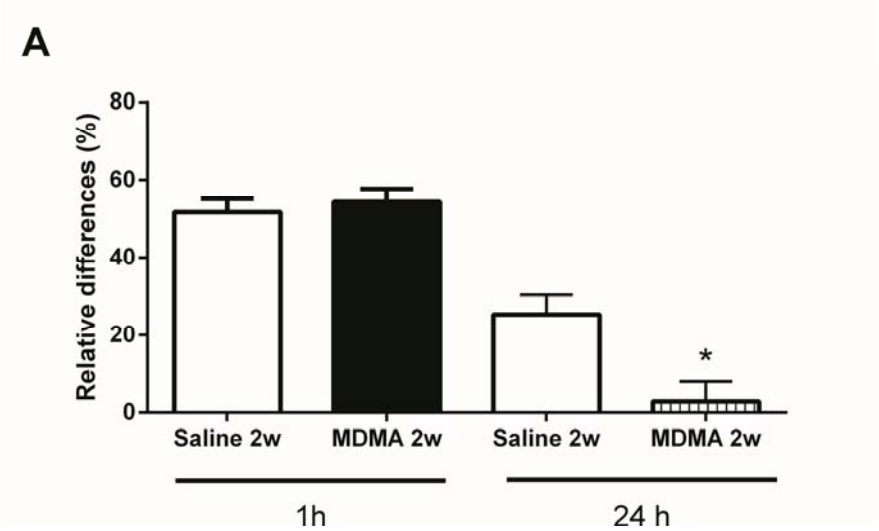
Fig. 4: Effect of MDMA exposure on the expression of mouse hippocampal proteins CREB (A, C) and p-CREB (B, D). This was assessed two weeks (2w: A, B) or three months (3m: C, D) after the end of the treatment. Data are presented as mean ± SEM; * *P* <0.05 *vs* saline.

Fig. 5: Effect of MDMA exposure on the expression of mouse hippocampal *c-Fos* mRNA transcript (A, C) and protein (B, D) tested two weeks (2w) or three months (3m) after the end of the treatment (A, B; and C, D, respectively). Data are presented as mean ± SEM; * *P* <0.05 *vs* saline.

Fig. 6: Consequences of repeated and intermittent MDMA exposure for mouse hippocampal *Arc* mRNA transcript (A, C) and protein expression (B, D) assessed two weeks (2w: A, B) or three months (3m: C, D) after the end of the treatment. Data are presented as mean ± SEM; * *P* <0.05 and **P<0.01 *vs* saline.

Fig. 7: Effect of MDMA exposure on mouse hippocampal *BDNF* mRNA transcript (A, C) and protein expression (B, D) tested two weeks (2w) or three months (3m) after the end of the treatment (A, B; and C, D, respectively). Data are presented as mean ± SEM; * *P* <0.05 *vs* saline.

Fig. 8: Lack of specific regulation of synaptic plasticity-related proteins by repeated and intermittent MDMA treatment, in mouse hippocampus. Expression of the: NR1 (A, E), NR2 (B, F), PSD-95 (C, G) and synaptophysin (D, H) proteins was evaluated two weeks (2w: A, B, C, D) or three months (3m: E, F, G, H) after the end of the treatment. Data are presented as mean ± SEM.


A
Relative differences (%)
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60
40
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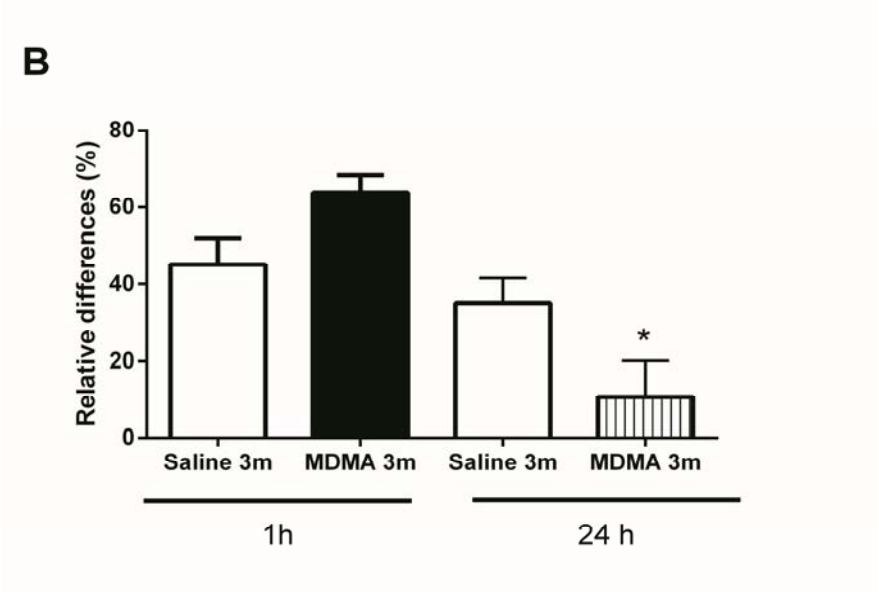
Saline 2w
MDMA 2w
Saline 2w
MDMA 2w
1h
24 h


B
Relative differences (%)
80
60
40
20
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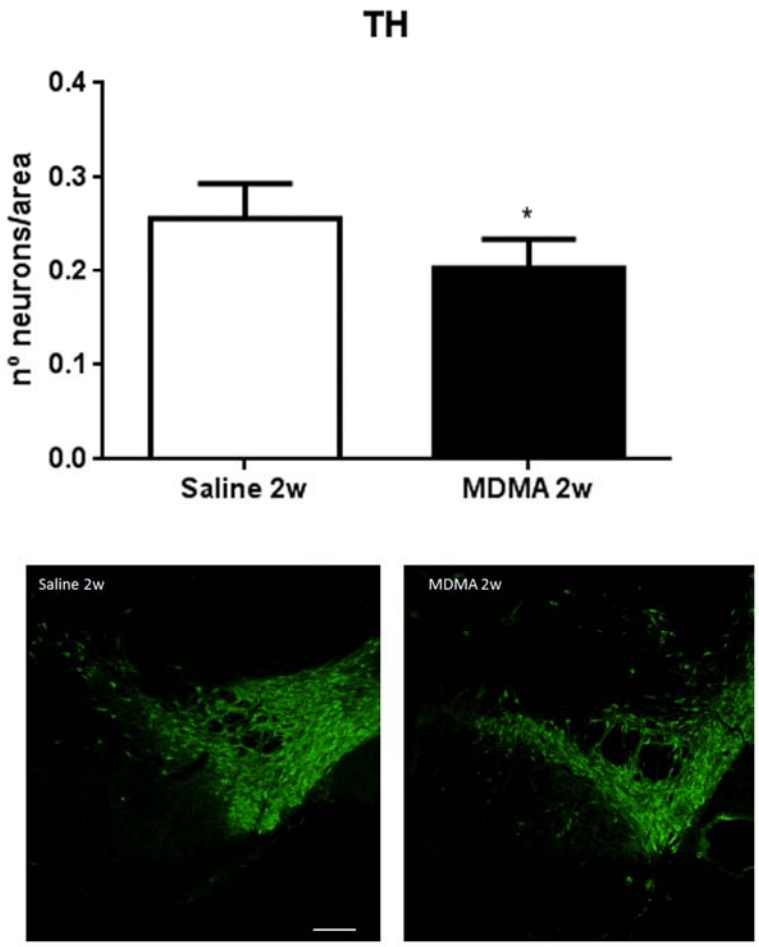
Saline 3m
MDMA 3m
Saline 3m
MDMA 3m
1h
24 h


TH
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0.3
0.2
0.1
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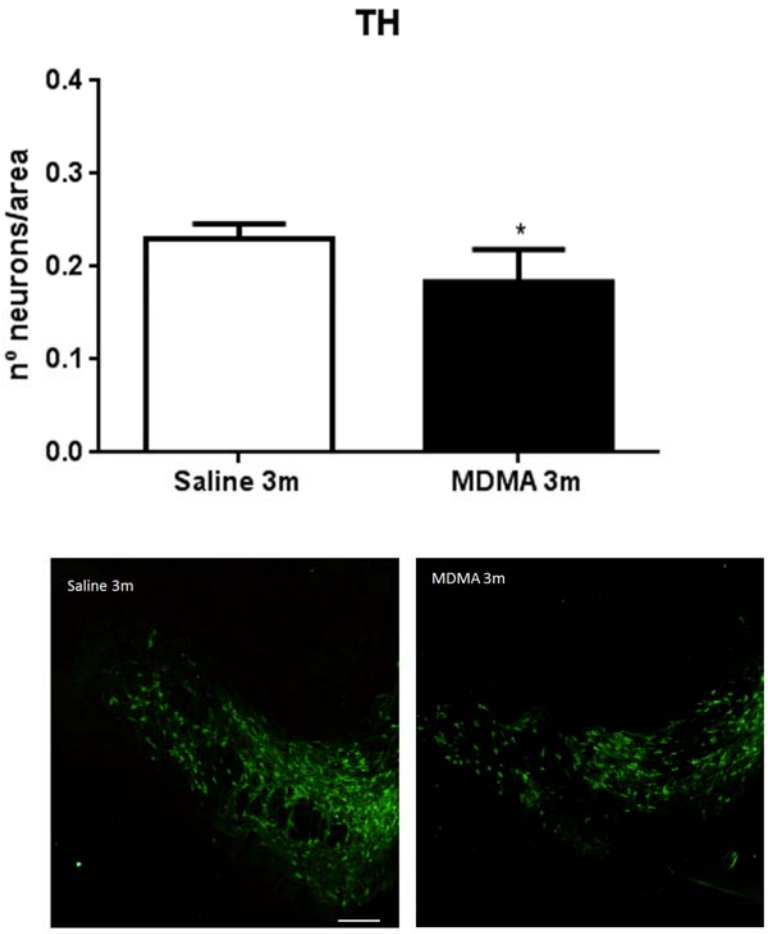
n° neurons/area
*
Saline 2w
MDMA 2w
Saline 2w
MDMA 2w


TH
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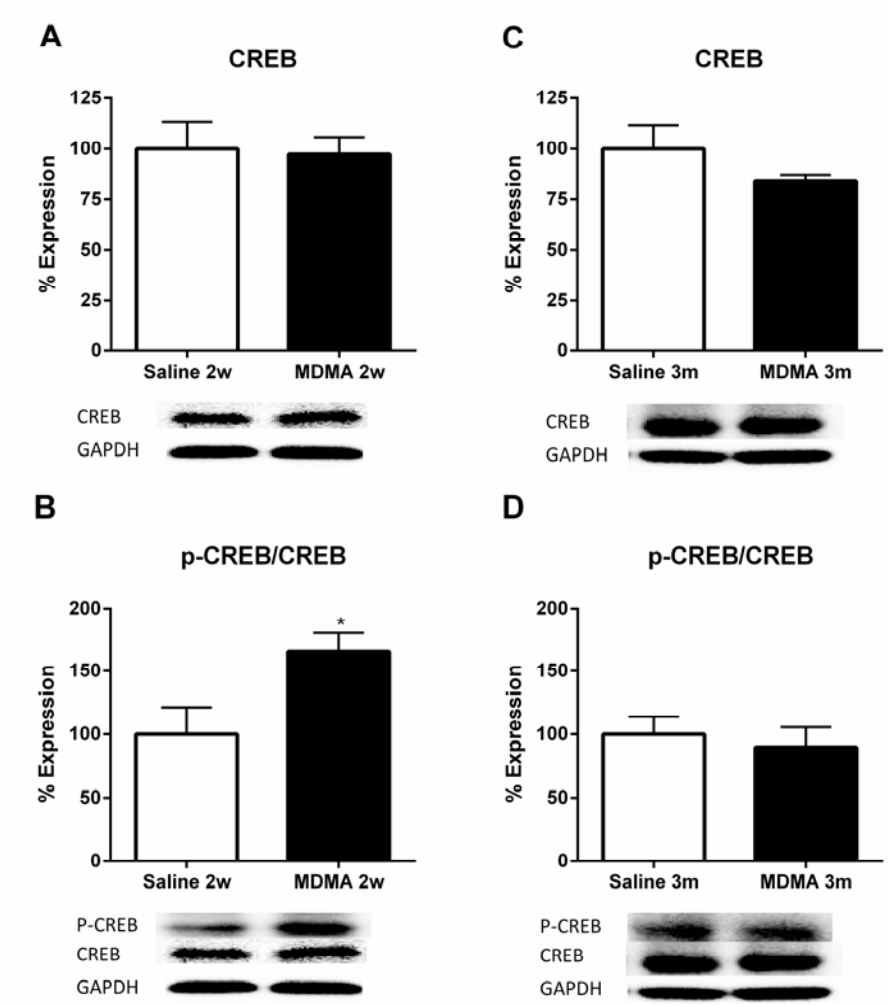
MDMA 3m


A
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Saline 2w
MDMA 2w
CREB
GAPDH
C
CREB
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Saline 3m
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CREB
GAPDH
B
p-CREB/CREB
% Expression
200
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Saline 2w
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CREB
GAPDH
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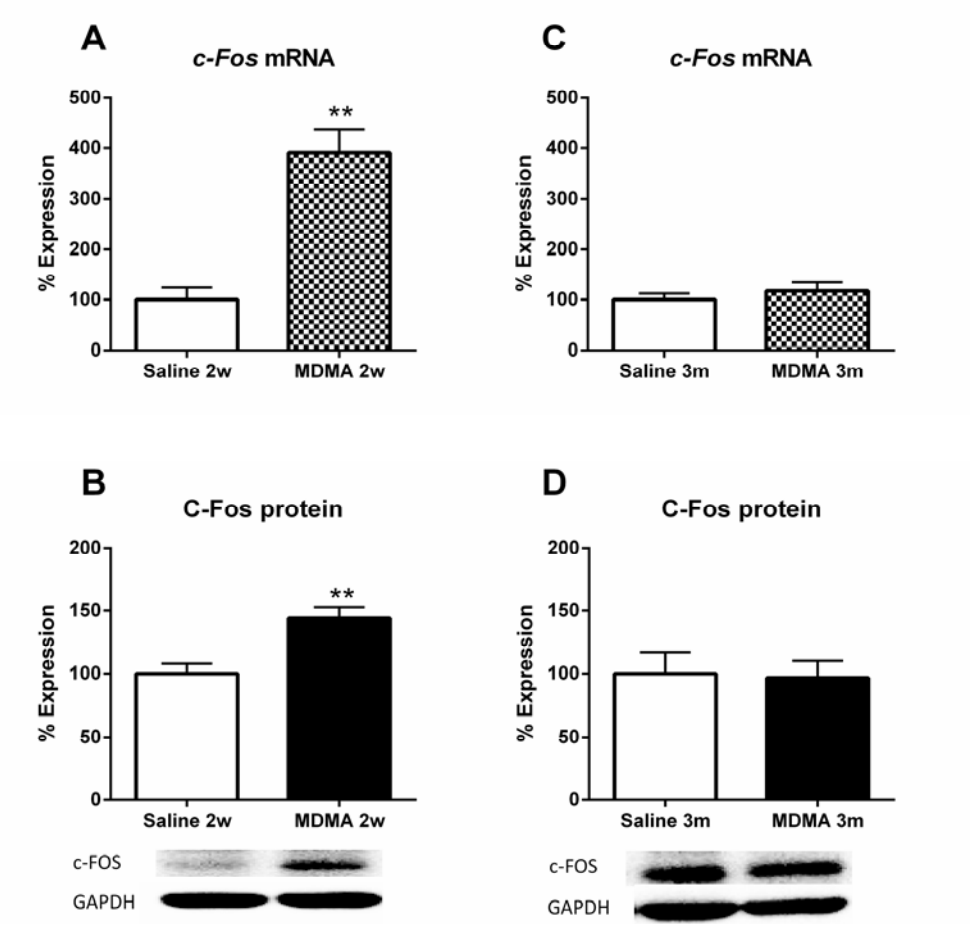
MDMA 3m
P-CREB
CREB
GAPDH


A
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100
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Saline 2w
MDMA 2w
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c-Fos mRNA
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Saline 3m
MDMA 3m
B
C-Fos protein
% Expression
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Saline 2w
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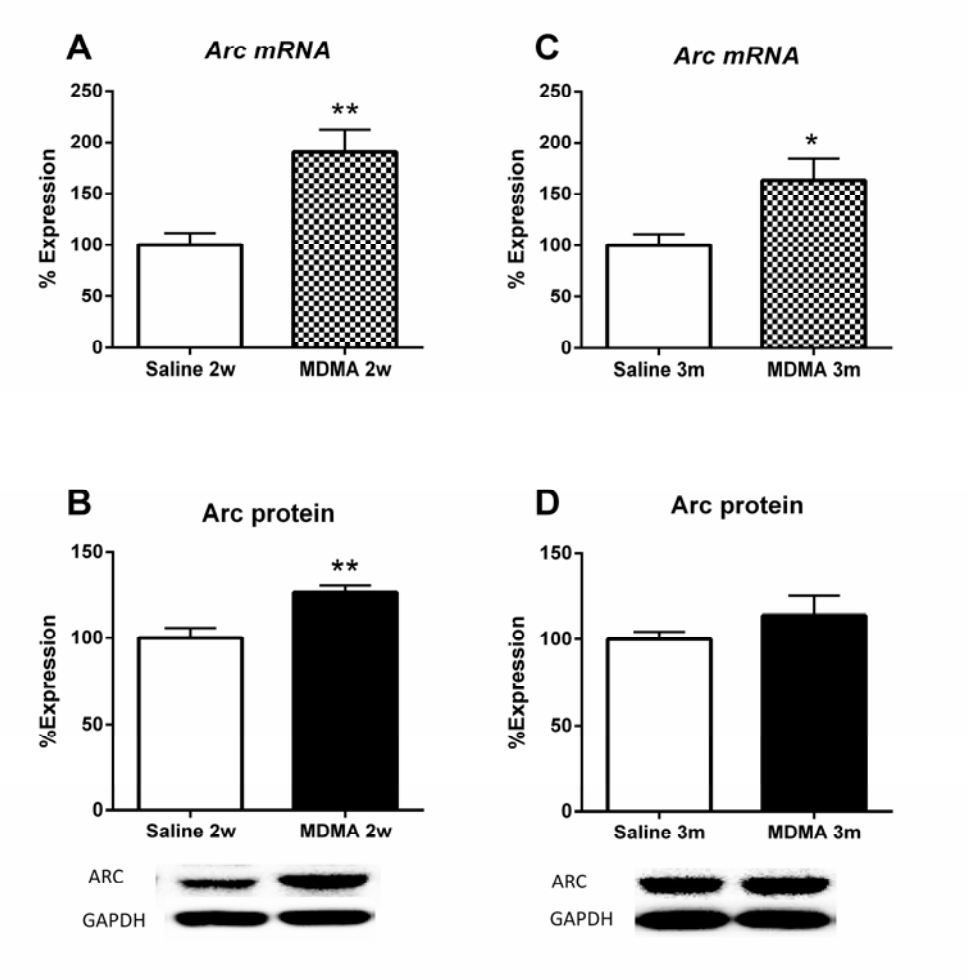
GAPDH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Arc mRNA
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Saline 2w
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Arc mRNA
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MDMA 3m
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Arc protein
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0
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Saline 2w
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ARC
GAPDH
D
Arc protein
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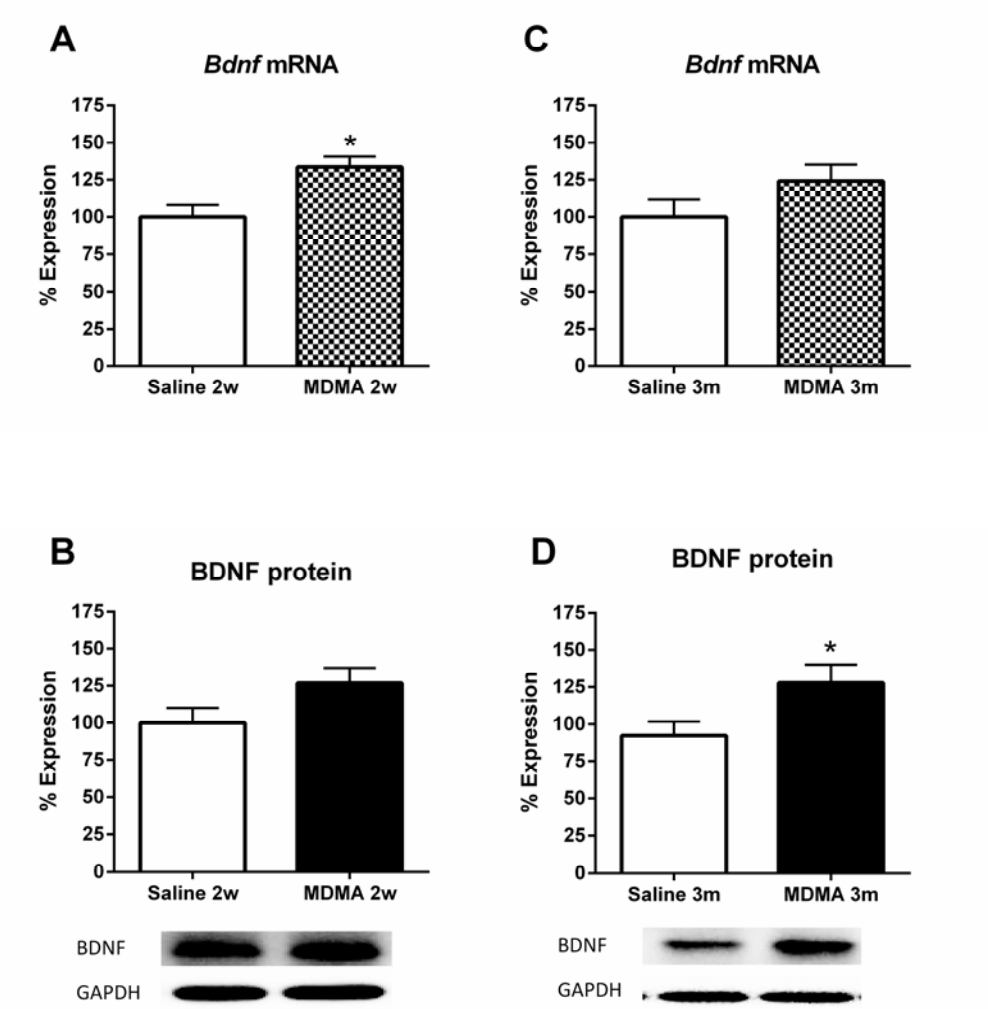
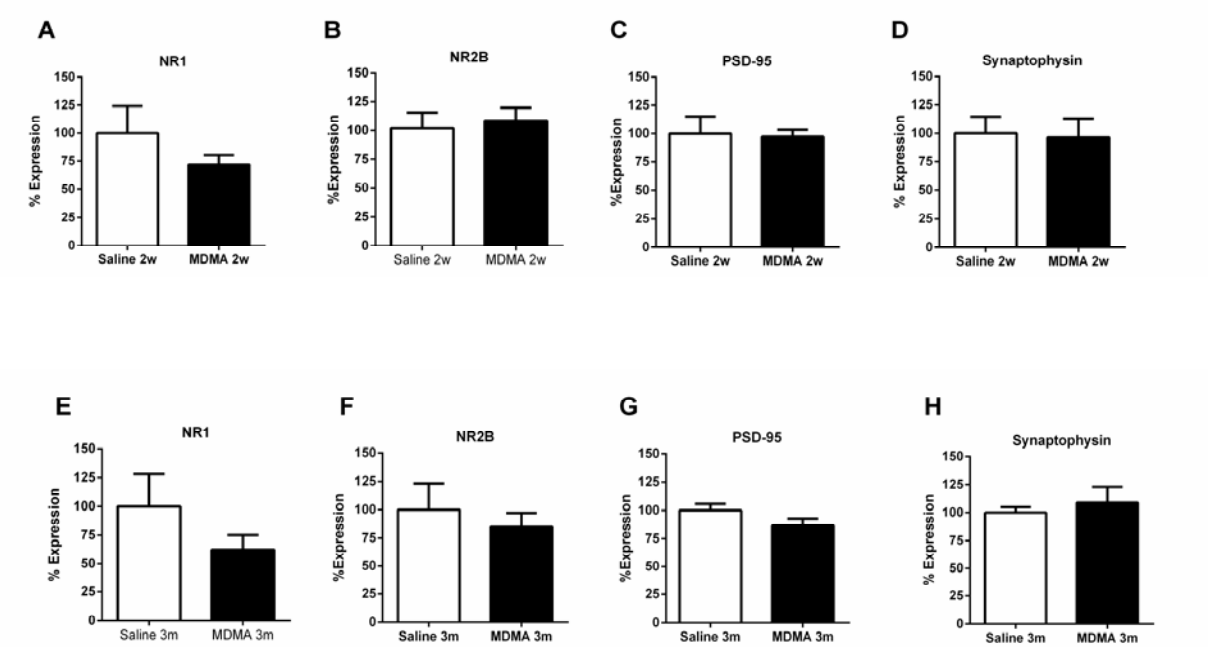
Saline 3m
MDMA 3m
ARC
GAPDH


A
Bdnf mRNA
% Expression
175
150
125
100
75
50
25
0
*
Saline 2w
MDMA 2w
C
Bdnf mRNA
% Expression
Saline 3m
MDMA 3m
B
BDNF protein
% Expression
Saline 2w
MDMA 2w
BDNF
GAPDH
D
BDNF protein
% Expression
*
Saline 3m
MDMA 3m
BDNF
GAPDH


A
NR1
% Expression
150
125
100
75
50
25
0
Saline 2w
MDMA 2w
B
NR2B
%Expression
Saline 2w
MDMA 2w
C
PSD-95
%Expression
Saline 2w
MDMA 2w
D
Synaptophysin
% Expression
Saline 2w
MDMA 2w
E
NR1
% Expression
Saline 3m
MDMA 3m
F
NR2B
%Expression
Saline 3m
MDMA 3m
G
PSD-95
%Expression
Saline 3m
MDMA 3m
H
Synaptophysin
% Expression
Saline 3m
MDMA 3m