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Chronic MDMA induces neurochemical changes in the hippocampus of adolescent and young adult rats: Down-regulation of apoptotic markers

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ABSTRACT

While hippocampus is a brain region particularly susceptible to the effects of MDMA, the cellular and molecular changes induced by MDMA are still to be fully elucidated, being the dosage regimen, the species and the developmental stage under study great variables. This study compared the effects of one and four days of MDMA administration following a binge paradigm (3 × 5 mg/kg, i.p., every 2 h) on inducing hippocampal neurochemical changes in adolescent (PND 37) and young adult (PND 58) rats. The results showed that chronic MDMA caused hippocampal protein deficits in adolescent and young adult rats at different levels: (1) impaired serotonergic (5-HT_{2A} and 5-HT_{2C} post-synaptic receptors) and GABAergic (GAD2 enzyme) signaling, and (2) decreased structural cytoskeletal neurofilament proteins (NF-H, NF-M and NF-L). Interestingly, these effects were not accompanied by an increase in apoptotic markers. In fact, chronic MDMA inhibited proteins of the apoptotic pathway (i.e., pro-apoptotic FADD, Bax and cytochrome c) leading to an inhibition of cell death markers (i.e., p-JNK1/2, cleavage of PARP-1) and suggesting regulatory mechanisms in response to the neurochemical changes caused by the drug. The data, together with the observed lack of GFAP activation, support the view that chronic MDMA effects, regardless of the rat developmental age, extends beyond neurotransmitter systems to impair other hippocampal structural cell markers. Interestingly, inhibitory changes in proteins from the apoptotic pathway might be taking place to overcome the protein deficits caused by MDMA.

1. Introduction

Adolescence is a period of great vulnerability to the neurochemical effects of specific drugs of abuse (Spear, 2007), even though some reports agree that during adolescence animals are less susceptible than in adulthood to the effects of amphetamines (reviewed at Teixeira-Gomes et al., 2015). Interestingly, the amphetamine derivative 3,4-methylene dioxymethamphetamine (MDMA) is one of the most commonly abused drugs among adolescents and young adults (SAMHSA, 2002). There is a large body of evidence showing that MDMA produces degeneration of 5-hydroxytryptamine (5-HT) nerve endings in multiple brain regions, including hippocampus (HC), in experimental animals and humans (for review, see Green et al., 2003). Remarkably, the 5-HT system undergoes substantial maturational change during adolescence (Chen et al., 1997), such as differential expression of 5-HT receptors with age (Li et al., 2004), which may contribute to the sensitivity on how adolescents respond to MDMA. In fact, the literature supports the notion that the long-lasting behavioral and neurochemical effects promoted by MDMA are dependent on the developmental age of drug exposure (see review, Teixeira-Gomes et al., 2015).

The cellular and molecular mechanisms by which MDMA induces neurochemical changes are still to be fully elucidated, being the dosage regimen and the species under study a great variable (Green et al., 2003). Besides MDMA inducing deficits in 5-HT markers, which may or may not reflect neuronal loss or axonal degeneration (Capela et al., 2009; Biezonski and Meyer, 2011), it also induces a broader neuronal damage (see for example the modulation of other gene markers in HC, Weber et al., 2014) suggesting MDMA effects in the brain are complex and deserve further exploration. Especially, the neural adaptations taking place in HC, which is a brain region known to be critical for learning and memory and particularly susceptible to MDMA effects (Steinkellner et al., 2011).

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Therefore, this study is a direct comparison of the neurochemical alterations in HC following the same MDMA dosing regimen at different developmental stages (early adolescence vs. young adulthood). Neurotransmitter systems beyond 5-HT axon terminals, such as 5-HT post-synaptic receptors (i.e., 5-HT_{2A}, 5-HT_{2C}) and GABA neuronal markers (i.e., glutamate decarboxylase enzymes, GAD1 and GAD2) were evaluated to corroborate MDMA deficits on neurotransmitter systems. The main goal of the study was to evaluate MDMA effect on structural proteins such as neurofilament (NF) proteins (NF-H, NF-M, and NF-L for high, medium and low molecular weights), known to play a crucial role in neuronal shape organization and function and modulated by drugs of abuse (Beitner-Johnson et al., 1992). Moreover, enolase-2, an enzyme found in mature neurons which is a marker of neuronal damage (Hatfield and McKerman, 1992), and glial fibrillary acidic protein (GFAP), an indicative of brain toxicity shown to be either increased (Green et al., 2003) or unaltered by MDMA in HC (Wang et al., 2004) were also evaluated. Finally, to better understand MDMA effect on structural proteins and given that prior studies reported increased apoptotic cell death markers in cultured hippocampal neurons in vitro by MDMA (Capela et al., 2013), the last goal of the study evaluated MDMA effects on cell death markers. In particular, c-Jun N-terminal kinase (p-JNK1/2) which initiates cell death signaling (Dhanasekaran and Reddy, 2008) by regulating proteins from both the extrinsic (i.e., cell fate adaptor Fas-associated death domain, FADD) and intrinsic (i.e., Bax, cytochrome c) apoptotic pathways leading to poly (ADP-ribose) polymerase 1 (PARP-1) cleavage (i.e., cell death/plasticity) were evaluated. A preliminary report of a portion of this work was presented at the 27th European College of Neuropsychopharmacology Congress (García-Cabrerizo and García-Fuster, 2014).

2. Materials and methods

2.1. Rats

For this study 35 adolescent male Sprague–Dawley rats were purchased from Charles River (L'Ambresle, France) at weaning (PND 21, $n = 18$) or mid-late adolescence (PND 42, $n = 17$) (see Teixeira-Gomes et al., 2015 for rat characterization of developmental stages). Rats were housed with ad libitum access to a standard diet and tap water in controlled environmental conditions (22 °C, 70% humidity, and 12-h light/dark cycle). Prior to any experimental procedure, rats were habituated to the experimenter by being handled daily for two days. All animal care and experimental procedures were conducted according to standard ethical guidelines (UK Animals, Scientific Procedures, Act, 1986 and European Communities Council Directive 86/609/EEC) and approved by the Local Bioethical Committee (UIB-CAIB). All efforts were made to minimize the number of rats used and their suffering.

2.2. Drug treatment and tissue collection

Rats were weighted prior to any drug treatment (PND 27-29 and PND 48-50, see Fig. 1a) and also daily throughout the whole experiment (PND 33-36 and PND 54-57). Rats were treated following a binge paradigm for 4 consecutive days (3 pulses per day, every 2 h) with either saline (0.9% NaCl, 1 ml/kg, i.p.) or MDMA (5 mg/kg, i.p.; kindly provided by 'Agencia Española de Medicamentos y Productos Sanitarios, Ministerio de Sanidad, Política Social e Igualdad', Spain). Control rats received saline during all treatment days (PND 33-36 or PND 54-57). Acute MDMA rats received 3 days of saline (PND 33-35 or PND 54-56) and 1 day of MDMA (PND 36 or PND 57; a total of 15 mg/kg of MDMA). Chronic MDMA rats received 4 days of MDMA (PND 33-36 or PND 54-57; a total of 60 mg/kg of MDMA). Doses were chosen based on previous studies in which MDMA was shown to induce changes in brain neurochemistry (Weber et al., 2014) in comparison to higher doses (20 mg/kg or more for several days, for a total of 160 mg/kg) which are required in Sprague–Dawley rats to produce neurotoxic damage (i.e., long-term 5-HT deficits) in several brain regions (see reviewed in Green et al., 2003). Core body temperature was recorded under normal room temperature conditions (22 °C) immediately before the first MDMA or saline injection on PND 33 or PND 54 (baseline temperature) and also daily 30 min after the last MDMA or saline pulse (PND 33-36 or PND 54-57; see Fig. 1a) by a rectal probe connected to a digital thermometer (Compact LCD display thermometer, SA880-1M, RS, Corby, UK). Rats were killed by decapitation without anesthesia 24 h after the

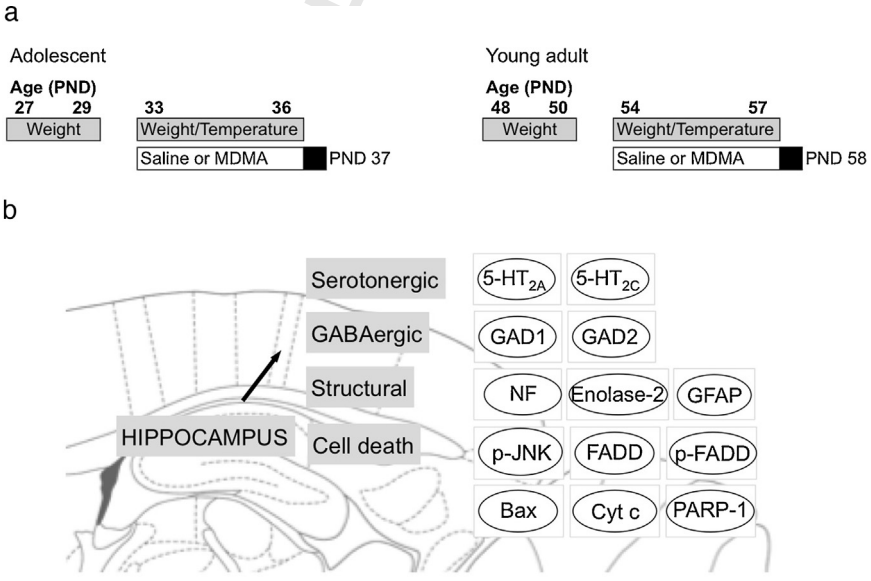

Fig. 1. Experimental design. (a) Adolescent and young adult rats were manipulated (i.e., weight-, rectal temperature) and treated at the indicated post-natal days (PND). Control rats received saline (0.9% NaCl, 1 ml/kg, every 2 h, i.p., for 4 days) while other rats received either 1 day (acute) or 4 days (chronic) of MDMA (3 × 5 mg/kg, every 2 h, i.p.) (PND 33-36 or PND 54-57). Rats were sacrificed 24 h after the last dose (PND 37 or PND 58). (b) Schematic representation of the molecular markers evaluated in HC by Western blot (WB) analysis.

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last treatment dose (PND 37 or PND 58) (Weber et al., 2014), and their brains removed and processed to allow the investigation of hippocampal markers by western blot (WB) analysis (see Fig. 1b).

2.3. Western blot (WB) analysis

Total homogenate of rat HC was prepared for immunoblotting standard procedures (García-Fuster et al., 2009) and brain proteins (40 μg protein) were resolved by electrophoresis on 10–15% SDS-PAGE minigels (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated with appropriate primary antibody whose vendors and dilution conditions were the following: (1) Santa Cruz Biotechnology (CA, USA): anti-5-HT_{2C} (D-12) (1:500), anti-FADD (H-181) (1:5000), anti-Bax (N-20) (1:1000); (2) Cell Signaling (MA, USA): anti-GAD1 (#5305) (1:1000), anti-GAD2 (#3988) (1:1000), anti-enolase-2 (D20H2) (1:1000), anti-phospho-Ser191 FADD (1:750), anti-PARP-1 (#9542) (1:1000), anti-phospho-Thr183/Tyr185 JNK1/2 (1:2000); (3) BD Biosciences (CA, USA): anti-cytochrome c (1:5000); (4) Neuromics (MN, USA): anti-5HT_{2A} (RA24288) (1:666); (5) Covance (CA, USA): anti-NF-H and M (clone SMI-32) (1:1000); (6) Novocastra-Leica (NCL, UK): anti-GFAP (1:1000); and (7) Sigma–Aldrich (MO, USA): anti-NF-L (N5139) (1:1000), anti-β-actin (clone AC-15) (1:10000), anti-α-tubuline (clone B-5-1-2) (1:2000). The secondary antibody (anti-rabbit or anti-mouse IgG linked to horseradish peroxidase) was incubated for 1 h at room temperature (1:5000 dilution; Cell Signaling). Immunoreactivity of target proteins was detected with ECL reagents (Amersham, Buckinghamshire, UK) and signal of bound antibody was visualized by exposure to autoradiographic film (Amersham ECL Hyperfilm) for 1–60 min, which was quantified by densitometric scanning (GS-800 Imaging Calibrated Densitometer, Bio-Rad). For each developmental stage (PND 37 or PND 58), the amount of target proteins in hippocampal brain samples of rats under different treatment groups (acute and chronic MDMA) was compared in the same gel with that of their respective age control rats. Percent changes in immunoreactivity (MDMA-treated rats) with respect to control samples (100%) at each age time-point (PND 37 or PND 58) were calculated for each treated rat in various gels, and the mean value was used as a final estimate. As the content of β-actin was slightly decreased by chronic MDMA in HC of adolescent rats (PND 37: $11 \pm 2\%$, $p < 0.05$), α-tubulin was quantitated and used as a loading control as it was not altered by any treatment conditions (data not shown).

2.4. Data and statistical analysis

Data were analyzed with GraphPad Prism, Version 6. Results are expressed as mean values ± standard error of the mean (SEM). Each developmental age (PND 37 and PND 58) was evaluated in separate WB experiments and one-way ANOVA followed by Dunnett's multiple comparison test (control, acute, chronic) or Student's *t*-test (control vs. chronic) analysis were used to ascertain statistical differences. This was done to avoid signal saturation in WB experiments given the basal differences observed in FADD protein content with age (i.e., increased FADD in early adolescence as compared to later adolescence or adulthood; unpublished data from our group; also see FADD immunoblot results, Fig. 6b). The level of significance was $p \leq 0.05$.

3. Results

3.1. Effect of MDMA treatment on body weight and core body temperature in adolescent and young adult rats

Rat's weight gain did not change throughout the duration of the experimental treatment by MDMA administration (acute or chronic) when compared to control treated rats as measured by a two-way ANOVA in adolescent (effect of PND: $F_{6,105} = 63.12$, $p < 0.001$; effect of treatment: $F_{2,105} = 0.82$, $p > 0.05$; interaction PND × treatment: $F_{12,105} = 0.22$, $p > 0.05$) or young adult rats (effect of PND: $F_{6,102} = 73.73$, $p < 0.001$; effect of treatment: $F_{2,105} = 11.18$, $p < 0.001$; interaction PND × treatment: $F_{12,105} = 0.65$, $p > 0.05$) (see Fig. 2a).

When analyzing the effect of MDMA on rat's rectal temperature, a two-way ANOVA detected a significant interaction between age (PND) and treatment (control, acute and chronic MDMA) for both the adolescent ($F_{6,60} = 4.981$, $p < 0.001$) and young adult ($F_{6,57} = 23.84$, $p < 0.001$) treated rats. Post hoc analysis revealed that acute exposure to MDMA induced hyperthermia in adolescent and young adult rats (see PND 36 and PND 57 for acute MDMA groups and PND 54 for first day of chronic MDMA treatment). However, chronic exposure to MDMA in adolescent and young adult rats showed tolerance (PND 36 and PND 57 for chronic groups, black circles; Fig. 2b) to the acute induced hyperthermia (see Piper et al., 2005 for similar results; see Green et al., 2003 for revision).

3.2. Chronic MDMA induces hippocampal neurochemical changes in adolescent and young adult rats

3.2.1. Neurotransmitter systems

MDMA effect on proteins regulating neurotransmitter systems (i.e., serotonergic and GABAergic, see Fig. 1b) was studied in HC by WB analysis. 5-HT_{2A} and 5-HT_{2C} receptors were evaluated as serotonergic markers. Chronic MDMA, but not acute, reduced hippocampal 5-HT_{2A} receptor in adolescent rats (PND 37: $F_{2,15} = 6.06$, $p < 0.05$) (acute MDMA: $36 \pm 12\%$ decrease, $p > 0.05$; chronic MDMA: $59 \pm 13\%$ decrease, $p < 0.01$) but did not reach statistical significance in young adult rats (PND 58: $F_{2,12} = 0.80$, $p > 0.05$) (acute MDMA: $18 \pm 14\%$ decrease; chronic MDMA: $23 \pm 11\%$ decrease) (Fig. 3a). Contrarily, chronic MDMA administration had no effect on hippocampal 5-HT_{2C} receptor in adolescent rats (PND 37: $F_{2,15} = 0.37$, $p > 0.05$) (chronic MDMA: $11 \pm 22\%$) but decreased its content in young adult rats (PND 58: $F_{2,14} = 4.04$, $p < 0.05$) (chronic MDMA: $57 \pm 7\%$, $p < 0.05$) (Fig. 3b).

GAD1 and GAD2 enzymes were evaluated as GABAergic markers in the HC as they regulate the conversion of glutamate to GABA and are thought to be involved in synaptic transmission. Acute and chronic treatments with MDMA did not modulate GAD1 in HC of adolescent (PND 37: $F_{2,15} = 0.98$, $p > 0.05$) (acute MDMA: $3 \pm 3\%$ decrease; chronic MDMA: $8 \pm 2\%$ decrease) or young adult (PND 58: $F_{2,14} = 0.37$, $p > 0.05$) (acute MDMA: $8 \pm 7\%$ decrease; chronic MDMA: $13 \pm 10\%$ decrease) rats (Fig. 3c). However, chronic, but not acute, treatments with MDMA reduced GAD2 in HC of adolescent (PND 37: $F_{2,15} = 3.61$, $p = 0.05$) ($24 \pm 6\%$, $p < 0.05$) and young adult (PND 58: $F_{2,14} = 3.64$, $p = 0.05$) ($16 \pm 2\%$, $p < 0.05$) rats (Fig. 3d). These results suggest that chronic MDMA induces, both in adolescent and young adult rats, a neurochemical effect in HC by decreasing the content of serotonergic (i.e., 5-HT_{2A} and 5-HT_{2C}) and GABAergic (i.e., GAD2) neurotransmitter markers.

3.2.2. Structural proteins

MDMA effect on structural proteins (see Fig. 1b) was studied in HC by WB analysis. NF proteins (NF-H, NF-M, and NF-L for high, medium and low molecular weights) play a crucial role in neuronal shape organization and function and were therefore evaluated as neuronal cytoskeleton markers. One-way ANOVA detected significant differences between the groups of treatments for NF proteins in HC of adolescent (PND 37: NF-H, $F_{2,15} = 3.49$, $p = 0.05$; NF-M, $F_{2,15} = 4.52$, $p < 0.05$; NF-L, $F_{2,15} = 2.19$, $p > 0.05$) and young adult (PND 58: NF-H, $F_{2,14} = 2.63$, $p > 0.05$; NF-M, $F_{2,14} = 4.69$, $p < 0.05$; NF-L, $F_{2,14} = 10.20$, $p < 0.01$) rats. In particular, chronic MDMA

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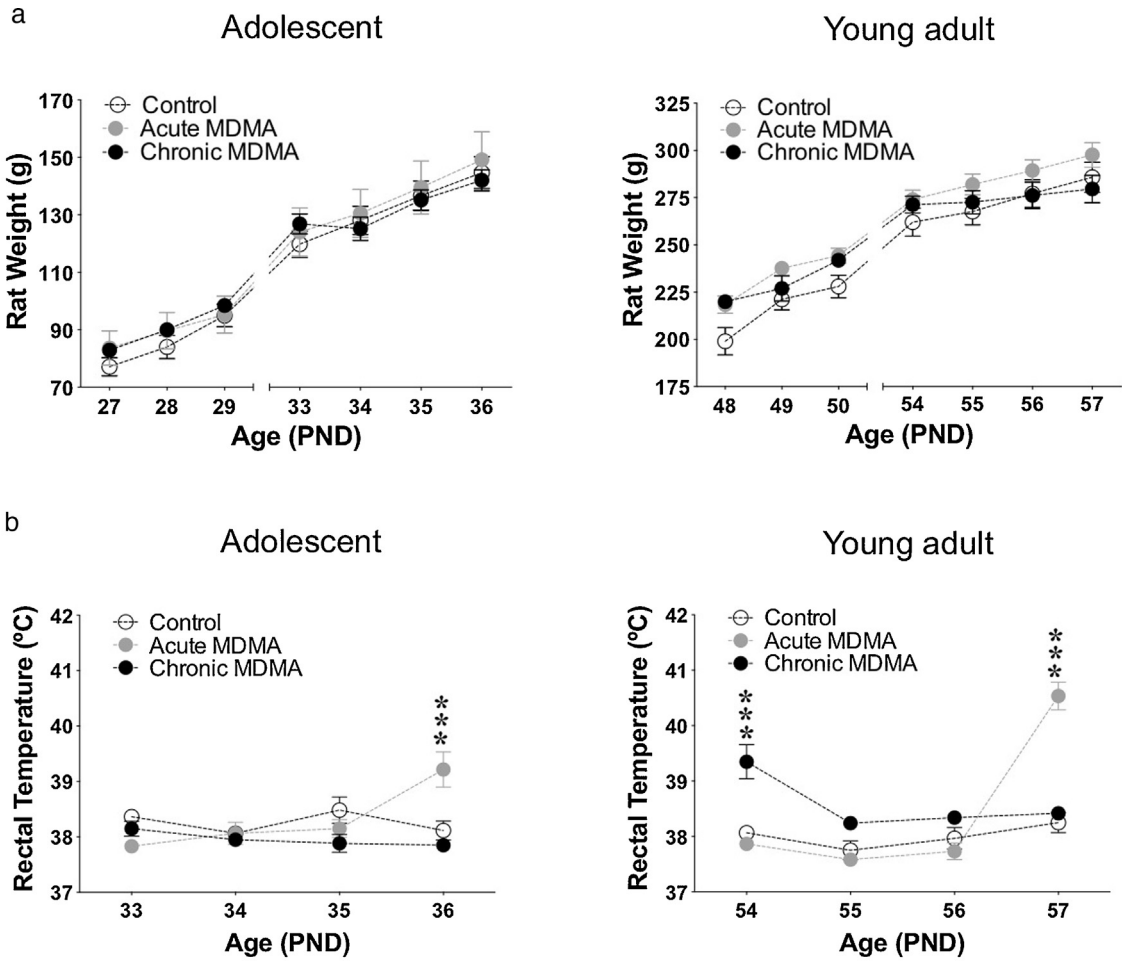

Fig. 2. Effect of MDMA treatment on rat body weight (a) and rectal temperature (b) in adolescent and young adult rats. (a) Rat weight (g) was similar for all experimental groups prior to any drug treatment (PND 27-29 or PND 48-50) and following MDMA administration (PND 33-36 or PND 54-57). (b) Acute MDMA induced the expected increase in body temperature (PND 36 and PND 57) while chronic MDMA led to tachyphylaxis to the hyperthermic response. Two-way ANOVA detected a significant interaction between age (PND) and treatment (control, acute and chronic MDMA) for both the adolescent ($F_{6,60} = 4.981$, $p < 0.001$) and young adult ($F_{6,57} = 23.84$, $p < 0.001$) treated rats. $^{***}p < 0.001$ when compared with the corresponding age-control group.

reduced the content of all NF proteins in HC of adolescent (PND 37) (NF-H: 31 ± 9%, $p < 0.05$; NF-M: 45 ± 5%, $p < 0.05$; NF-L: 12 ± 3%, $p < 0.05$, Student's *t*-test when compared to control group) and young adult (PND 58) (NF-H: 24 ± 3%, $p < 0.05$, Student's *t*-test when compared to control group; NF-M: 34 ± 6%, $p < 0.05$; NF-L: 12 ± 1%, $p < 0.01$) rats (Fig. 4a–d). However, acute MDMA only reduced NF-M (30 ± 11%, $p < 0.05$) and NF-L (9 ± 3%, $p < 0.01$) in HC of young adult (PND 58) rats (Fig. 4b–d).

Two other structural proteins were also evaluated, enolase-2 as a neuronal marker, and GFAP as a glial and neurotoxic marker. Remarkably, acute and chronic MDMA did not modulate enolase-2 (Fig. 5a) or GFAP (Fig. 5b) in HC of adolescent (PND 37: enolase-2, $F_{2,15} = 0.94$, $p > 0.05$; GFAP, $F_{2,15} = 0.62$, $p > 0.05$) and young adult (PND 58: enolase-2, $F_{2,14} = 1.50$, $p > 0.05$; GFAP, $F_{2,14} = 1.07$, $p > 0.05$) rats. These results suggest that chronic MDMA induces, both in adolescent and young adult rats, a neurochemical effect in HC by decreasing the content of structural NF proteins independently of GFAP toxicity.

3.3. Chronic MDMA dampens hippocampal pro-apoptotic proteins in adolescent and young adult rats

MDMA effect on cell death markers (see Fig. 1b) was studied in HC by WB analysis. As mentioned earlier, p-JNK1/2 plays a critical role in cell death as it can engage the activation of both the extrinsic and intrinsic apoptotic pathways (Dhanasekaran and Reddy, 2008). Interestingly, acute and chronic treatments with MDMA did not modulate p-JNK1/2 in HC of adolescent (PND 37: $F_{2,14} = 0.83$, $p > 0.05$) or young adult (PND 58: $F_{2,14} = 2.45$, $p > 0.05$) rats (Fig. 6a). The overall effects observed for p-JNK1/2 are a sum of the effects observed for p-JNK1 and p-JNK2 separately (data not shown). From the extrinsic apoptotic pathway, FADD adaptor forms (i.e., pro-apoptotic FADD; anti-apoptotic p-FADD, and the index of neuroplasticity p-FADD/FADD) were evaluated. Chronic MDMA, but not acute, reduced pro-apoptotic FADD in HC of adolescent (PND 37: $F_{2,15} = 3.80$, $p < 0.05$) (31 ± 10%, $p < 0.05$) and young adult (PND 58: $F_{2,14} = 2.80$, $p > 0.05$) (31 ± 5%, $p < 0.05$, Student's *t*-test when compared to control group) rats (Fig. 6b), without altering anti-apoptotic p-FADD content (PND 37: $F_{2,15} = 0.66$, $p > 0.05$; PND 58: $F_{2,14} = 0.17$, $p > 0.05$) (Fig. 6c). The index of neuroplasticity p-FADD/FADD (see Ramos-Miguel et al., 2012) resulted in non-statistically significant increases at both ages (PND 37: $F_{2,15} = 2.05$, $p > 0.05$) (2.19-fold) (PND 58: $F_{2,14} = 1.08$, $p > 0.05$) (1.18-fold). Moreover, two pro-apoptotic markers from the intrinsic apoptotic pathway (Bax, cytochrome c) were also decreased by chronic MDMA administration: Bax (PND 37: $F_{2,15} = 5.24$, $p < 0.05$) (26 ± 4%, $p < 0.05$) (PND 58: $F_{2,14} = 3.04$, $p > 0.05$) (16 ± 2%, $p < 0.05$, Student's *t*-test when compared to control group; Fig. 6d) and cytochrome c (PND 37: $F_{2,13} = 17.57$, $p < 0.001$) (42 ± 2%, $p < 0.001$) (PND 58: $F_{2,14} = 3.77$, $p < 0.05$) (20 ± 3%, $p < 0.05$; Fig. 6e). The

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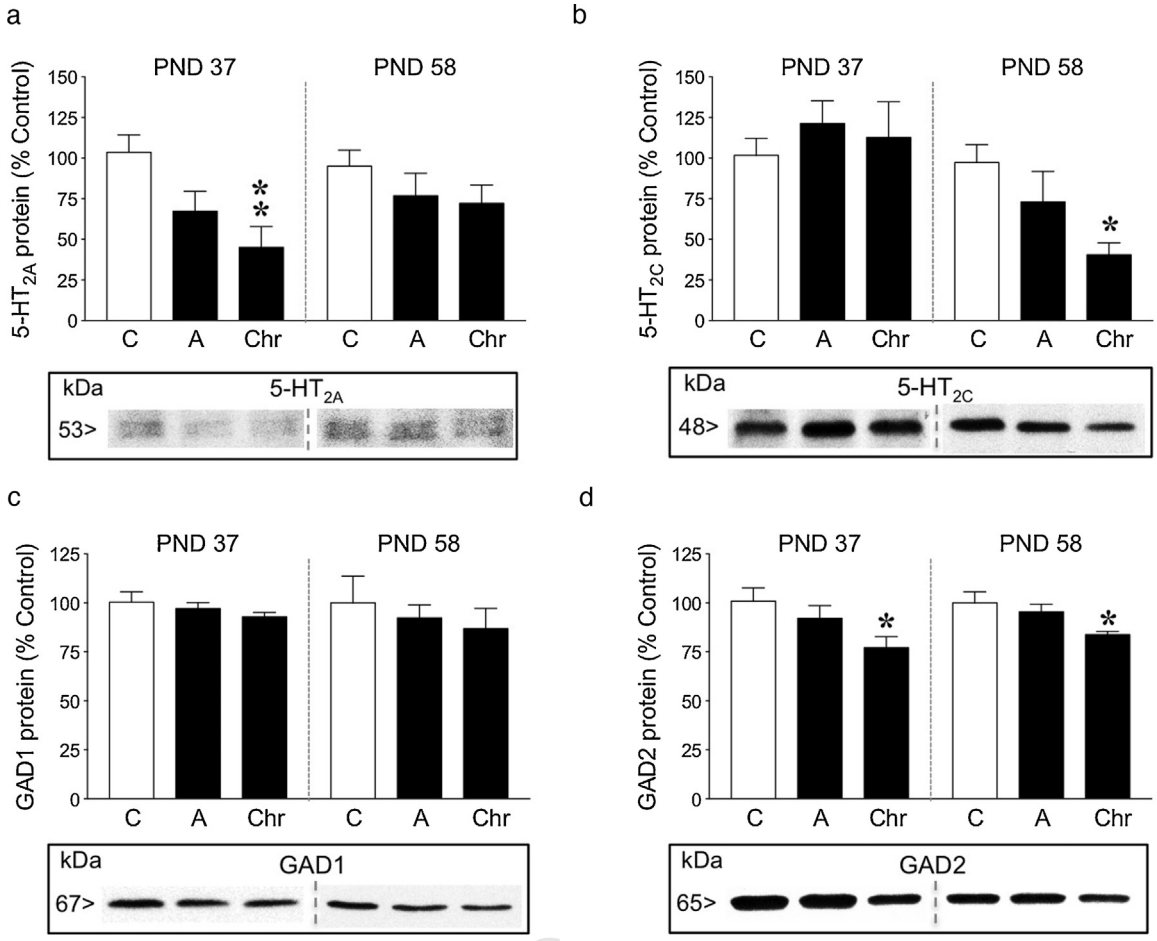

Fig. 3. Acute and chronic effects of MDMA on (a) 5-HT_{2A}, (b) 5-HT_{2C}, (c) GAD1 and (d) GAD2 protein content in rat HC of adolescent (PND 37) and young adult (PND 58) rats. Groups of treatment: control (C, $n = 6$), acute MDMA (A, $n = 6$), chronic MDMA (Chr, $n = 6$-5). Columns are means ± SEM of n experiments per group and expressed as a percentage of control (C)-treated rats for each age of study (PND 37 or PND 58). One-way ANOVA detected significant differences between the groups of treatments for 5-HT_{2A} (PND 37: $F_{2,15} = 6.06$, $p < 0.05$), 5-HT_{2C} (PND 58: $F_{2,14} = 4.04$, $p < 0.05$) and GAD2 (PND 37: $F_{2,15} = 3.61$, $p = 0.05$; PND 58: $F_{2,14} = 3.64$, $p = 0.05$). $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the corresponding age-control group (ANOVA followed by Dunnett's test). Bottom panels: representative immunoblots depicting labeling of 5-HT_{2A}, 5-HT_{2C}, GAD1 and GAD2 are shown for each set of experiments.

fragment/PARP-1 ratio (i.e., a hallmark of apoptosis and/or altered plasticity), which is calculated dividing the immunodensity of the cleaved fragment band (85 kDa) by its native form (116 kDa) for each rat, was altered by MDMA (PND 37: $F_{2,11} = 2.66$, $p > 0.05$; PND 58: $F_{2,14} = 21.01$, $p < 0.001$). Remarkably, the fragment/PARP-1 ratio was decreased by chronic MDMA at PND 37 (0.44-fold, $p < 0.05$, Student's *t*-test when compared to control group) and by acute and chronic MDMA at PND 58 (acute: 0.53-fold, $p < 0.001$; chronic: 0.51-fold, $p < 0.001$; Fig. 6f). These results suggest that chronic MDMA induces, both in adolescent and young adult rats, a neurochemical effect in HC by inhibiting apoptotic markers (i.e., pro-apoptotic FADD, Bax and cytochrome c, as well as decreased cleavage of PARP-1).

4. Discussion

Taken together the results suggest that chronic MDMA induces neurochemical changes in HC of both adolescent and young adult rats at different biochemical levels (i.e., neurotransmitter systems and NF structural proteins) while inhibiting the apoptotic cell death machinery possibly as an adaptive mechanism to the drug induced-deficits.

The current results suggest a role for 5-HT_{2A} receptors in the neurochemical effects mediated by chronic MDMA in HC of adolescent rats while associates 5-HT_{2C} receptors with its effects in young adult rats. Little is known about MDMA effects on post-synaptic 5-HT receptors (i.e., 5-HT_{2A}, 5-HT_{2C}) in rat brain. For example, chronic MDMA induced a transient down-regulation of 5-HT_{2A} receptors in several brain regions including HC (Scheffel et al., 1992) and cortex (Reneman et al., 2002), while another study found hippocampal 5-HT_{2A} unaltered by MDMA (Yau et al., 1997). More recently, chronic MDMA reduced 5-HT_{2A} receptor mRNA in cortex while increased 5-HT_{2C} receptor in cortex and hypothalamus (Kindlundh-Högberg et al., 2006). Similarly, acute MDMA decreased 5-HT_{2C} mRNA levels in specific hippocampal subfields (Yau et al., 1997) while chronic MDMA increased 5-HT_{2C} expression in CA3 pyramidal neurons (Yau et al., 1994). As mentioned earlier, the 5-HT system is known to undergo substantial development during adolescence (Chen et al., 1997) such as 5-HT_{2A} and 5-HT_{2C} receptor expression patterns in brain change in a regionally specific manner with age (Li et al., 2004). The functional difference between these receptor subtypes, together with the fact that 5-HT_{2C} receptors are present at higher densities than 5-HT_{2A} in adult HC (reviewed in Berumen et al., 2012) could contribute to the differential results observed for adolescent and young adult rats in this study. There is little evidence that MDMA produces deficits in other neurotransmitter systems in rat beyond the serotonergic system. For instance, chronic MDMA in HC reduced not only 5-HT but also GABA levels 7 days after last drug injection (Perrine et al., 2010), damaged GABAergic terminals (Armstrong and Noguchi, 2004), and decreased the number of

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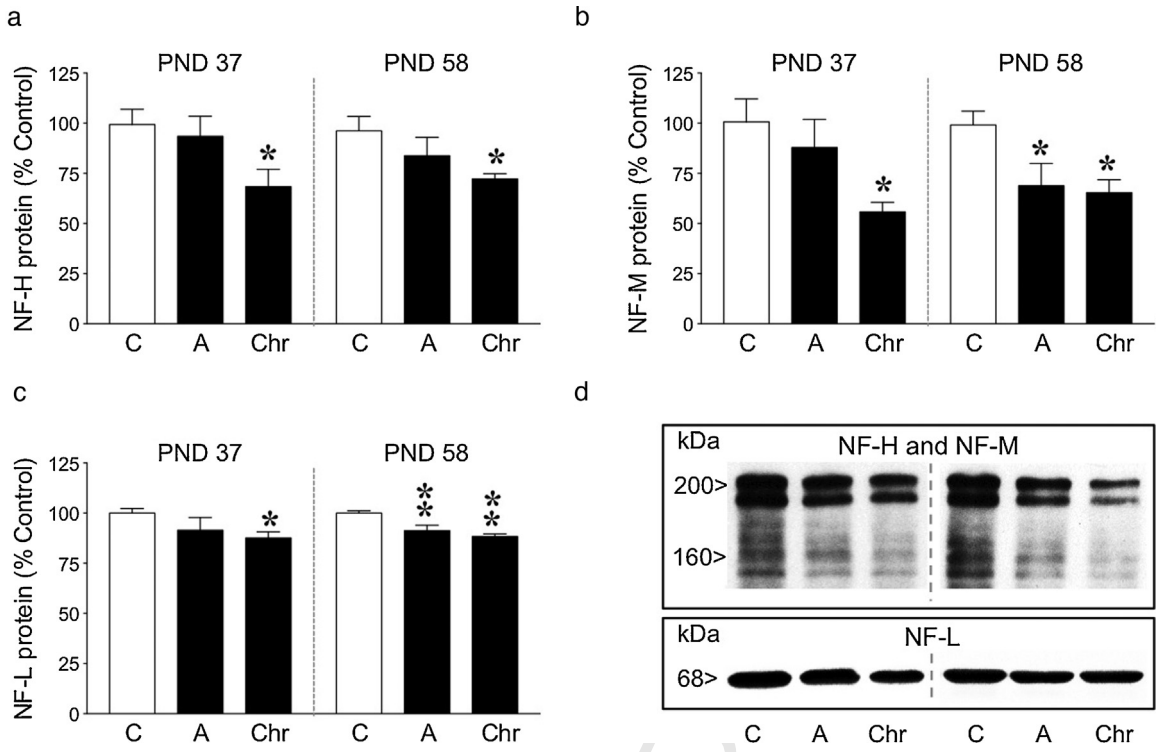

Fig. 4. Acute and chronic effects of MDMA on (a) NF-H, (b) NF-M, and (c) NF-L protein content in rat HC of adolescent (PND 37) and young adult (PND 58) rats. Groups of treatment: control (C, $n = 6$), acute MDMA (A, $n = 6$), chronic MDMA (Chr, $n = 6–5$). Columns are means ± SEM of n experiments per group and expressed as a percentage of control (C)-treated rats for each age of study (PND 37 or PND 58). One-way ANOVA detected significant differences between the groups of treatments for NF-H (PND 37: $F_{2,15} = 3.49$, $p = 0.05$), NF-M (PND 37: $F_{2,15} = 4.52$, $p < 0.05$; PND 58: $F_{2,14} = 4.69$, $p < 0.05$) and NF-L (PND 58: $F_{2,14} = 10.20$, $p < 0.01$). $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the corresponding age-control group (ANOVA followed by Dunnett's test). Student's t-test detected significant differences (at least $^{*}p < 0.05$) between chronic MDMA and control groups for NF-H (PND 58) and NF-L (PND 37). (d) Representative immunoblots depicting labeling of NF-H and NF-M as well as NF-L are shown for each set of experiments.

parvalbumin-positive GABA cells in the DG (Anneken et al., 2013). Interestingly, in the present study, the GABAergic neuronal marker GAD2 is shown to be reduced by chronic MDMA in HC of both adolescent and young adult rats, suggesting and confirming together with the observed decreases in 5-HT post-synaptic receptors (5-HT_{2A} and 5-HT_{2C}), that this binge paradigm of repeated treatment with MDMA decreases neurotransmitter neuronal markers.

Besides MDMA producing deficits in neurotransmitter markers, the cellular and molecular changes induced by MDMA need to be elucidated (Green et al., 2003), especially in a brain region particularly vulnerable to MDMA effects and critical for learning and memory such as HC (Steinkellner et al., 2011). In this regard, NF proteins are intermediate filaments that compose the cytoskeleton in mature neurons and provide integrity and associated functions (e.g., axonal transport, axonal plasticity and neuronal morphology) (e.g., Hoffman and Lasek, 1975; Lee and Cleveland, 1996). A number of studies had examined drug-induced effects on NF proteins in brain areas relevant to drug reward. For example, chronic administration of either morphine

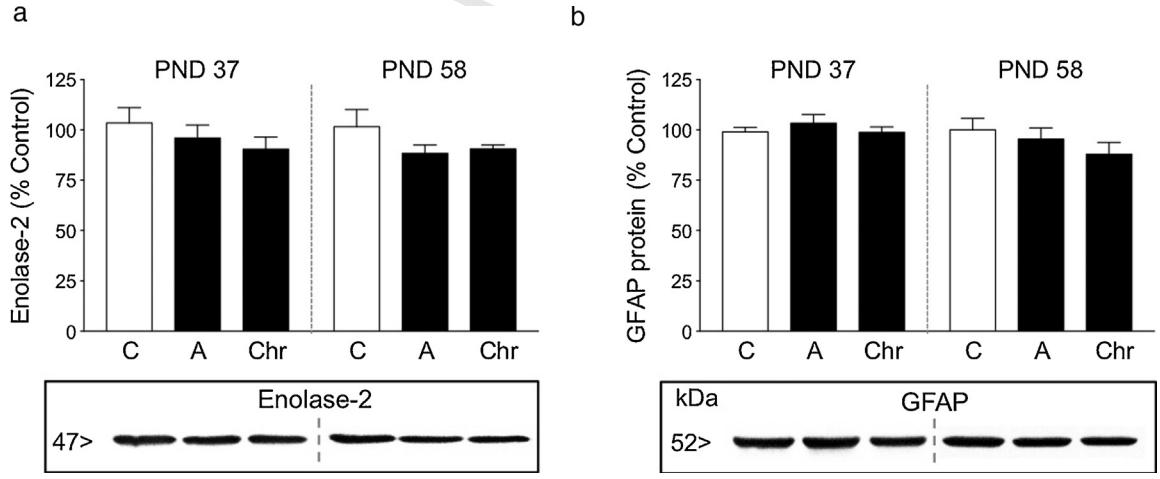

Fig. 5. Acute and chronic effects of MDMA on (a) enolase-2 and (b) GFAP protein content in rat HC of adolescent (PND 37) and young adult (PND 58) rats. Groups of treatment: control (C, $n = 6$), acute MDMA (A, $n = 6$), chronic MDMA (Chr, $n = 6–5$). Columns are means ± SEM of n experiments per group and expressed as a percentage of control (C)-treated rats for each age of study (PND 37 or PND 58). One-way ANOVA did not detect significant differences between the groups of treatments. Bottom panels: representative immunoblots depicting labeling of enolase-2 and GFAP are shown for each set of experiments.

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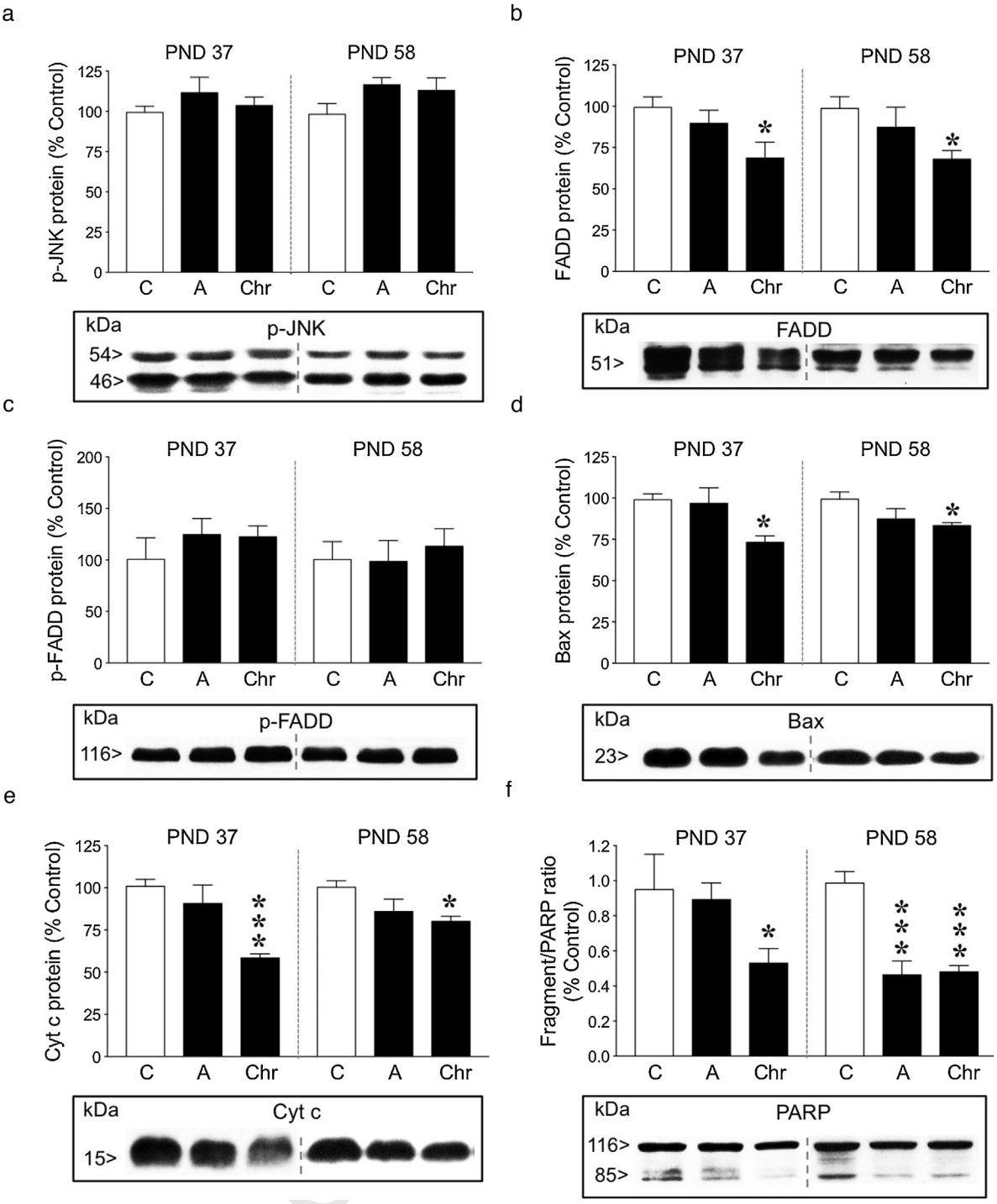

Fig. 6. Acute and chronic effects of MDMA on (a) p-JNK1/2, (b) FADD, (c) p-FADD, (d) Bax, (e) cytochrome c (Cyt c) and (e) fragment/PARP-1 ratio (85 kDa cleaved fragment divided by 116 kDa native PARP-1) protein content in rat HC of adolescent (PND 37) and young adult (PND 58) rats. Groups of treatment: control (C, $n = 6$), acute MDMA (A, $n = 6$), chronic MDMA (Chr, $n = 6–5$). Columns are means ± SEM of n experiments per group and expressed as a percentage of control (C)-treated rats for each age of study (PND 37 or PND 58). One-way ANOVA detected significant differences between the groups of treatments for FADD (PND 37: $F_{2,15} = 3.80$, $p < 0.05$), Bax (PND 37: $F_{2,15} = 5.24$, $p < 0.05$), cytochrome c (PND 37: $F_{2,13} = 17.57$, $p < 0.001$; PND 58: $F_{2,14} = 3.77$, $p < 0.05$) and fragment/PARP-1 ratio (PND 58: $F_{2,14} = 21.01$, $p < 0.001$). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with the corresponding age-control group (ANOVA followed by Dunnett's test). Student's t-test detected significant differences (at least $^{*}p < 0.05$) between chronic MDMA and control groups for FADD (PND 58), Bax (PND 58) and fragment/PARP-1 ratio (PND 37). Bottom panels: representative immunoblots depicting labeling of p-JNK1/2 (p-JNK1: 54 kDa, p-JNK2: 46 kDa), FADD, p-FADD, Bax, cytochrome c and PARP-1 (native PARP-1: 116 kDa and fragment: 85 kDa) are shown for each set of experiments.

or cocaine decreased the levels of NF-L, NF-M and NF-H in rat ventral tegmental area (Beitner-Johnson et al., 1992). Seven days after methamphetamine administration NF-L was reduced in mice striatum (Sanchez et al., 2003). Moreover, chronic nicotine treatment decreased NF-M and NF-H immunoreactivity in rat brain (Bunnemann et al., 2000). Interestingly, marked reductions in total NF proteins were observed in the prefrontal cortex of chronic opioid addicts (García-Sevilla et al., 1997; Ferrer-Alcón et al., 2000). However, to the best of our knowledge, this is the first study to examine MDMA effects on NF proteins in rat brain, and in particular in HC. Similarly to the effects already described for other drugs of abuse, the present results showed decreased NF protein

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levels (NF-H, NF-M and NF-L) in HC in response to chronic MDMA, giving further evidence for a common substrate following an addictive drug exposure in different brain regions (see also Beitner-Johnson et al., 1992). Interestingly, acute MDMA also decreased NF proteins (NF-M and NF-L) but only in HC of young adult (PND 58) rats, in line with previous reports showing that adolescent animals are less susceptible than adults to the neurochemical changes induced by MDMA (see review, Teixeira-Gomes et al., 2015), as observed in the current experiment where a chronic treatment was needed to promote the observed neurochemical changes in adolescent rats. The difference observed in the magnitude of change caused by MDMA for NF-H (up to 31%) and NF-M (up to 45%) as compared to NF-L (up to 12%) may relate to differences in assembly states and ratios of NF subunits that could be regulated by the local concentration and phosphorylation of individual subunits. While the triplet proteins are co-assembled in vivo, NF-L appears to be an indispensable polypeptide for the formation of these intermediate filaments (Lee et al., 1987). Distinctive ratios of the three proteins are found in different tissues and their expressions, which are not fixed, vary during growth and differentiation (Nixon and Shea, 1992). Moreover, all NF polypeptides are phosphorylated in vivo in a proportion relative to their mass, which determines important biochemical properties of each subunit (Julien and Mushynski, 1998). For example, the phosphorylation of NF-H appears to be a relevant mechanism in the induction of cross-bridges between NF, which could be essential for the maintenance of the neuronal cytoskeletal structure (Shaw et al., 1986). Therefore, MDMA might be differentially affecting this complex hetero-polymeric structure as well as the dynamically changing phosphate topography of NF proteins in HC of adolescent and young adult rats. Interestingly, the regulation of NF proteins by MDMA could play a role in modifying neuronal morphology such as the ability to reorganize patterns of synaptic connectivity (i.e., morphology of dendrites and dendritic spines) in brain regions altered following repeated exposure to psychostimulants (Robinson and Kolb, 1999). In fact, a binge administration of MDMA reduced spine density in CA1 region of HC in adolescent rats (Abad et al., 2014). Moreover, changes in cytoskeletal proteins may be part of the mechanism participating in drug-induced neurotransmitter changes. In fact, eliminating NF proteins from the CNS profoundly disrupted synaptic plasticity without altering the structural integrity of synapsis, suggesting additional roles for NF proteins beyond static structural support of axon caliber, and therefore proposing NF proteins as integral components of synapses and as modulators of in vivo neurotransmission (Yuan et al., 2015).

Remarkably, no other structural proteins analyzed (i.e., enolase-2 and GFAP) were altered by MDMA treatments. The content of enolase-2, an enzyme found in mature neurons which is a marker of neuronal damage (Hatfield and McKerman, 1992), was not altered by MDMA exposure in HC of adolescent and young adult rats. There is no previous data on MDMA effects on this marker, yet, similar negative results were observed in cortical samples of cocaine addicts (Álvaro-Bartolomé and García-Sevilla, 2013). Astrocyte hypertrophy can occur as a result of neuronal injury leading to increases in GFAP expression. Several studies have shown increased GFAP content following MDMA administration in mice and rats (Green et al., 2003). However, and similar to the current results, MDMA-pretreated rats which showed the expected impaired in serotonergic function did not report changes in hippocampal GFAP (Wang et al., 2004). An absence of glial activation suggests that the use of indirect methods (i.e., relied on quantifying protein expression) for detecting selective neurotoxicity may have limitations and therefore proposes that the current paradigm of chronic MDMA administration (low dose of MDMA, one point of analysis at 24 h) induces neurochemical changes in protein content (e.g., neurotransmitter systems, NF proteins) rather than neurotoxicity (discussed in Green et al., 2003; Steinkellner et al., 2011; Biezonski and Meyer, 2011) in HC. In any case, the administration of higher doses of MDMA in Sprague-Dawley rats and the evaluation of molecular changes at later time points following injections (e.g., 7 days) where 5-HT deficits are still present (Green et al., 2003) might allow to observe neural damage.

As disruption of cytoskeletal elements can induce apoptotic cell death (e.g., Kothakota et al., 1997), the observed decrease in hippocampal NF proteins by chronic MDMA may reflect some form of neural injury (i.e., increased apoptosis). In fact, prior studies reported that MDMA increased apoptotic cell death markers in cultured hippocampal neurons in vitro by the stimulation of post-synaptic 5-HT_{2A} receptors (Capela et al., 2013). Moreover, in rat HC, a single dose of MDMA increased cell death by TUNEL staining (Riezzo et al., 2010), while chronic MDMA increased pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 mRNA levels as measured 7 days after the last MDMA administration (Soleimani Asl et al., 2012). On the contrary, and unexpectedly, the present results showed that chronic MDMA did not modulate p-JNK1/2, which initiates cell death signaling by up-regulating pro-apoptotic markers from the extrinsic and intrinsic apoptotic pathways (Dhanasekaran and Reddy, 2008). In fact, chronic MDMA reduced the extrinsic pro-apoptotic marker FADD as well as the intrinsic pro-apoptotic markers Bax and cytochrome c in HC of adolescent and young adult rats as measured 24 h after the last MDMA administration. The discrepancy in apoptotic markers regulation (e.g., increased Bax in Soleimani Asl et al., 2012 vs. decreased Bax in the present results) could be related to the dosage regimen of MDMA administered (number of days: 7 days vs. 4 days for the present study; daily MDMA dose: up to 40 mg/kg vs. 15 mg/kg for the present study) and to the time point of analysis following MDMA administration (7 days vs. 24 h for the present study). These methodological differences allowed the evaluation of neurotoxicity markers (increased apoptotic markers; Soleimani Asl et al., 2012) vs. neurochemical changes (decreased apoptotic protein markers; current study) induced by MDMA administration. Interestingly, the same time point of analysis following MDMA administration (24 h) was shown to modulate several genes (e.g., decreased expression of genes related to axon sheaths and tissue remodeling) in HC (Weber et al., 2014), which together with the protein deficits observed in the current study, suggest early neurochemical adaptations (mRNA and protein level) in this brain region following MDMA administration. Moreover, the current results showed that chronic MDMA decreased PARP-1 cleavage suggesting the inhibition of apoptotic mechanisms in response to the neurochemical deficits caused by MDMA. In this context, multiple apoptosis-regulatory proteins also mediate a wide range of non-apoptotic functions (Galluzzi et al., 2012). In particular, the neuroplasticity role of the cell fate adaptor FADD on other drugs of abuse (i.e., opiates, cocaine, cannabinoids) has been greatly studied in rat, mouse and postmortem human brain (see review, Ramos-Miguel et al., 2012). Moreover, PARP-1 has been recently shown to not only lead cells to death (DNA damage; Cagnol et al., 2006) but to also enhance behavioral responses to cocaine (Scobie et al., 2014). Moreover, the present results showed decreases in 5-HT_{2A} receptors following chronic MDMA and therefore one would expect decreases in the apoptotic signals mediated by this receptor (Capela et al., 2013). Interestingly, the reduction of NF proteins did not seem to depend on loss of neurons, since there was no activation of enolase-2 or GFAP and apoptosis was down-regulated and therefore suggesting either the induction of neural plasticity or repair mechanisms to a prior drug insult.

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5. Conclusions

The results from this study, together with the lack of GFAP activation, support the view that chronic MDMA effects, regardless of the rat developmental stage, extend beyond 5-HT axon terminals to impair other hippocampal cell markers (i.e., GABAergic system, and NF proteins). Interestingly, inhibitory changes in proteins from the apoptotic pathway might be taking place to overcome the protein deficits caused by MDMA.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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