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Methamphetamine binge administration dose-dependently enhanced negative affect and voluntary drug consumption in rats following prolonged withdrawal: Role of hippocampal FADD

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ABSTRACT

While prior studies have established various interacting mechanisms and neural consequences (i.e., monoaminergic nerve terminal damage) that might contribute to the adverse effects caused by methamphetamine administration, the precise mechanisms that mediate relapse during withdrawal remain unknown. This study evaluated the long-term consequences of binge methamphetamine administration (3 pulses/day, every 3 h, 4 days, i.p.; dose-response: 2.5, 5, 7.5 mg/kg) in adult Sprague-Dawley rats at two behavioral levels following 25 days of withdrawal: (1) negative affect (behavioral despair – forced-swim test, and anhedonia –1% sucrose consumption, two-bottle choice test) and (2) voluntary methamphetamine consumption (20 mg/l, two-bottle choice test). Striatal and hippocampal brain samples were dissected to quantify monoamines content by HPLC and to evaluate neurotoxicity (dopaminergic and serotonergic markers) and neuroplasticity markers (i.e., cell fate regulator FADD) by Western blot. The results showed that methamphetamine administration induced dose-dependent negative effects during prolonged withdrawal in adult rats. In particular, rats treated repeatedly with methamphetamine (7.5 mg/kg) showed: (1) enhanced negative affect – increased anhedonia associated with behavioral despair, (2) increased voluntary methamphetamine consumption, (3) enhanced neurotoxicity – decreased dopamine and metabolites in striatum and decreased serotonin in hippocampus, (4) altered neuroplasticity markers – decreased FADD protein and increased p-FADD/FADD balance selectively in hippocampus, and (5) higher consumption rates of methamphetamine were associated with lower FADD content in hippocampus. These results confirm that methamphetamine withdrawal dose-dependently induced negative affect and decreased monoamines content, while also increased voluntary methamphetamine consumption and

suggested a role for hippocampal FADD neuroplasticity in these drug-withdrawal adaptations.

Keywords:

Methamphetamine withdrawal; negative affect; voluntary consumption

INTRODUCTION

The psychostimulant methamphetamine is a widely abused drug (UNODC, 2015) and thus a major health problem. Although the initial neurobiological mechanisms that underlie the acute effects of methamphetamine are well understood (i.e., reinforcing effects through activation of the reward system; e.g., Nestler, 2005), its recreational use is often characterized by a pattern of repeated frequent drug administrations during a short period of time (i.e., binge exposure) that likely leads to high dependence rates (e.g., Krasnova and Cadet, 2009; Moszczynska and Callan, 2017). Methamphetamine interacts in the central nervous system with monoamine transporter sites with different affinities – with more potent actions on dopamine (DA) and noradrenaline (NE) release than serotonin (5-HT) (reviewed in Teixeira-Gomes et al., 2015). The potential for methamphetamine-induced persistent deficits in monoaminergic systems is well recognized in adult rodents (e.g., Krasnova and Cadet, 2009; Carvalho et al., 2012). For example, high-dose binge administrations of methamphetamine decreased DA and its principal metabolites concentrations in striatum (Ricaurte et al., 1982) as well as 5-HT in hippocampus (e.g., Morgan et al., 1972). Moreover, there are other well established interacting mechanisms and neural consequences (i.e., excitotoxicity, oxidative stress and metabolic compromise) that might contribute to the known adverse effects caused by repeated methamphetamine (reviewed in Skeinkellner et al., 2011; Teixeira-Gomes et al., 2015). Interestingly, methamphetamine neurotoxicity is dose-dependent in experimental animals (see revision, Moszczynska and Callan, 2017) and its effects can last from days to several months. Moreover, during methamphetamine withdrawal, many behavioral consequences emerge such as intense drug craving which is likely linked to the propensity to drug relapse (see revision, Moszczynska and Callan, 2017).

Although the most prominent behavioral symptoms during long-term methamphetamine withdrawal are cognitive impairments and negative affect (e.g., behavioral despair, anhedonia), which are similar to the symptoms of major depressive disorder (Barr et al., 2002), the precise mechanisms leading to these withdrawal symptoms remain unknown.

The neurotoxic effects of methamphetamine on monoaminergic systems has been proposed to be mediated, at least in part, by the induction of neuronal apoptosis (see revision, Krasnova and Cadet, 2009). In particular, methamphetamine-induced neuronal apoptosis has been linked to stimulation of the FasL/Fas-mediated cell death pathway, such as eliciting the expression of FasL in striatal neurons and causing cleavage of downstream caspases 8 and 3 (e.g., Jayanthi et al., 2005). However, the regulation of indispensable FADD (Fas-associated protein with death domain), a cell fate regulator that binds to Fas receptor and mediates either cell death or survival depending on its phosphorylation state and cellular localization (e.g., Alappat et al., 2005) by methamphetamine is unknown. Interestingly, accumulating evidence suggests that FADD forms have an important role in the pathophysiology of depression (García-Fuster et al., 2014; García-Fuster and García-Sevilla, 2016), drug addiction (e.g., Ramos-Miguel et al., 2012) and cognition (Ramos-Miguel et al., 2017; Hernández-Hernández et al., 2017), making this multifunctional marker, that could balance cell death with cell survival and/or plasticity (i.e., p-FADD/FADD balance as a postulated index of neuroplasticity; Ramos-Miguel et al., 2012), a most relevant candidate for mediating some of the mechanisms driving methamphetamine withdrawal symptoms.

As the age of methamphetamine exposure seems to be crucial for its neurotoxic outcome (e.g., Teixeira-Gomes et al., 2015), with adult rats being more susceptible than adolescents (e.g., García-Cabrerizo and García-Fuster, 2016a), and the dosage regimen and species are

major variables to the negative drug effects (e.g., Carvalho et al., 2012), this study utilized adult Sprague-Dawley rats to ascertain the dose-response consequences of methamphetamine administration. In particular, the effects of binge methamphetamine administration were evaluated in a dose-dependent manner (2.5, 5 and 7.5 mg/kg, i.p., 3 pulses per day for 4 days) following prolonged withdrawal at different levels: (1) negative affect (i.e., behavioral despair and anhedonia), (2) voluntary methamphetamine consumption (i.e., oral self-administration), (3) ex vivo monoamines content, (4) dopaminergic and serotonergic markers and (4) dysregulation of FADD protein forms. A preliminary report of a portion of this work was presented at the 10th FENS Forum of Neuroscience (García-Cabrerizo and García-Fuster, 2016b).

MATERIAL AND METHODS

Animals

This study utilized a total of 47 adult male Sprague-Dawley rats bred at the University of the Balearic Islands in 2 separate experimental waves (25 and 22 rats respectively), which were performed in consecutive months and combined for analysis. Rats were housed in controlled environmental conditions (22 °C, 70% humidity, and 12-h light/dark cycle) with *ad libitum* access to a standard diet and tap water in groups of 3 until experimental Day 32 (D32), when they were housed individually (see Fig. 1a and further experimental details below). Rats were habituated to the experimenter by being handled for a couple of days prior to any manipulations. All animal care and experimental procedures complied with the ARRIVE guidelines (Kilkenny et al., 2010), were conducted according to standard ethical guidelines (European Communities Council Directive 86/609/EEC and Guidelines for the Care and Use

of Mammals in Neuroscience and Behavioral Research, National Research Council 2003) and were approved by the Local Bioethical Committee (UIB-CAIB). All efforts were made to minimize the number of rats used and their suffering.

Drug treatment

Groups of randomly allocated adult rats (350-400 g) were treated with (+) methamphetamine hydrochloride (Sigma-Aldrich, MO, USA) following a binge paradigm (dose-response: 2.5, 5 or 7.5 mg/kg, every 3 h, 3 times per day, i.p.) during 4 consecutive days (total of 12 pulses). The number of rats for each experimental group was as follows: wave 1 (2.5 mg/kg, n = 7; 5 mg/kg, n = 6; 7.5 mg/kg, n = 4) and wave 2 (5 mg/kg, n = 9; 7.5 mg/kg, n = 6), thus rendering a total n of 7, 15 and 10 rats per dose respectively. Control rats received the same number of saline injections (0.9% NaCl, 1 ml/kg, n = 15 rats total – 8 and 7 per wave) at the indicated times (see Fig. 1a). The dose range was chosen based on prior studies in which methamphetamine induced changes in brain neurochemistry and/or neurotoxicity in Sprague-Dawley rats (e.g., Teixeira-Gomes et al., 2015; García-Cabrerizo and García-Fuster, 2016a; Moszczynska and Callan, 2017). Rats were weighted every day during the course of drug treatment (D1-D4) and were left undisturbed (forced withdrawal) for 25 days prior to any behavioral testing (see Fig. 1).

Behavioral testing

The effects of prolonged methamphetamine withdrawal were evaluated on negative affect at two levels: (1) behavioral despair – time spent immobile in the forced swim test (FST) (e.g., Barr et al., 2002; Slattery and Cryan, 2017), and (2) anhedonia – sucrose consumption in a

two-bottle choice test (e.g., Slattery et al., 2007). Finally, drug withdrawal effects on eliciting oral voluntary methamphetamine consumption were evaluated with a two-bottle choice (e.g., Wheeler et al., 2009).

FST

As previously described in detail (García-Cabrerizo et al., 2015), during the pre-test (D31, see Fig. 1a) each rat was placed in a tank (41 cm high x 32 cm diameter) for 15 min filled with water (25 ± 1 °C) to a depth of 25 cm. Fresh water tanks were used for each rat and temperature was monitored constantly. On test day (D32, Fig. 1a), rats were videotaped while being in the water tank for 5 min. An investigator blind to the experimental groups scored the videos by measuring the time spent immobile vs. active (climbing and swimming) utilizing Behavior Tracker software (Version 1.5, CA, USA). Immediately after the FST (D32, see Fig. 1), rats were single housed for the remaining of the experimental design.

Sucrose consumption

On D35 rats were weighted (Fig. 1a). Then, following a standard protocol (e.g., Slattery et al., 2007), rats were trained to drink from two water bottles placed on the extreme sides of the cage for 24 h (D35-36). The next day, one bottle was randomly switched for an identical bottle but containing 1% of sucrose solution (diluted in tap water), a concentration shown in a preliminary study to provide a robust but not maximal sucrose consumption per day (dose-response study: from 0.1-2% sucrose; see García-Cabrerizo and García-Fuster, 2016b), and was left undisturbed for 24 h (D36-37). Then, bottles were reversed – to avoid bias towards any one side – and rats were allowed to drink for another 24 h (D37-38). Finally, the bottle

containing 1% sucrose was replaced with a water bottle and drinking from both bottles was measured for 24 h (D38-39) before one water bottle was removed from the cage (D39). Throughout the experiment, all bottles were weighted daily and the amount of sucrose or water consumed (g) per day was calculated for each rat (i.e., sum of total g of either sucrose or water consumed every 24 h period: D36-37 and D37-38, and divided by 2 days).

Methamphetamine consumption

On D42 rats were weighted and a second bottle of water was introduced in each cage for 24 h before the test began (D42-43, Fig. 1a). The next day, one bottle was randomly switched with a bottle that contained 20 mg/l of methamphetamine solution (diluted in tap water and daily prepared; Wheeler et al., 2009). This concentration of drinking methamphetamine induced aversion in naïve rats (i.e., preliminary dose-response study: dose range from 5 mg/l to 20 mg/l; see García-Cabrerizo and García-Fuster, 2016b), as methamphetamine is known to have a bitter flavor and to induce condition taste aversion (Wheeler et al., 2009). Rats were allowed to drink for a total of 6 days (D43-49) during which bottles were reversed every 24 h to avoid bias towards any one side. On D49, rats were weighted for the last time (see Fig. 1a). Finally, the bottle of methamphetamine was replaced with a water bottle and drinking from both bottles was measured for 24 h (D49-50) before one bottle was removed from the cage (D50). All bottles were weighted daily and the amount of voluntary methamphetamine consumed (g) per day was calculated for each rat (i.e., sum of total g of either methamphetamine or water consumed every 24 h period: D43-44, D44-45, D45-D46, D46-47, D47-48 and D48-49, and divided by 6 days).

Tissue collection

On D51, rats were killed by rapid decapitation and their brains removed for neurochemical analyses (Fig. 1a). The extracted brains were cut sagittally on an ice-cold plate and the striatum and hippocampus were freshly dissected from each hemisphere and were immediately frozen in liquid nitrogen, and stored at -80 °C until further use. The left-side brain regions were used to evaluate monoamines levels using a High-Performance Liquid Chromatography (HPLC) assay, while the right-side regions were used for Western blot analysis (e.g., dopaminergic, serotonergic and neuroplasticity markers).

HPLC

The levels of monoamine neurotransmitters (DA, NE, 5-HT) and metabolites (mainly 3,4-dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; 5-hydroxyindoleacetic acid, 5-HIAA) were determined on D51 by HPLC as previously described (Moranta et al., 2009). Before doing so, total protein concentrations (μg) were calculated using a small aliquot for each sample by BCA assay (Thermoscientific, Rockford, IL, USA). Then, the rest of each striatal or hippocampal sample was placed individually into cold tubes containing 1 ml of 0.4 M $HClO_4$, 0.01% K_2EDTA, and 0.1% $Na_2S_2O_5$ and then homogenized and centrifuged at 40000 xg for 15 min at 4 °C. The resulting supernatant was filtered with a 0.45 μm syringe (Spartan-3, Sigma-Aldrich) and various aliquots (10 μl for striatal and 30 μl for hippocampal samples) were injected into the HPLC system (further details in Moranta et al., 2009). The compounds were detected electrochemically and the current produced was monitored by use of an interphase (Waters busSAT/IN Module) connected to a digital PC. The contents of DA, NE, 5-HT and metabolites in a given sample were calculated by interpolating the

corresponding peak height into a parallel standard curve using the software Millennium[32] (Waters) and corrected by the total amount of protein (ng monoamines/μg of protein).

Western blot

Total homogenates of striatal or hippocampal samples from the right brain hemisphere were prepared (García-Cabrerizo et al., 2015) and brain proteins (40 μg protein) were resolved by electrophoresis on 10% SDS–PAGE minigels (Bio-Rad Laboratories, Hercules, CA, USA), followed by immunoblotting standard procedures (García-Fuster et al., 2007). Membranes were incubated overnight at 4 °C with the appropriate primary antibody: (1) Santa Cruz Biotechnology (CA, USA): anti-FADD (H-181) (1:5000; sc-5559), anti-DARPP-32 (H-62) (1:1000; sc-11365), anti-D2 receptor (H-50) (1:1000; sc-9113), anti-5-HT_{2C} (D-12) (1:500; sc-17797), anti-5-HT_{2A} (A-4) (1:1000; sc-166775); (2) Cell Signaling (MA, USA): anti-phospho-Ser191-FADD (1:750; no. 2785), (3) Chemicon International (CA, USA): anti-phospho-Ser31-TH (1:1000; AB5423); anti-DAT (1:5000; MAB369); and (4) Sigma-Aldrich (MO, USA): anti-ß-actin (1:10000; clone AC-15). The next day, membranes were incubated with the secondary antibody, horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (1:5000 dilution; Cell Signaling), immunoreactivity of target proteins was detected with ECL reagents (Amersham, Buckinghamshire, UK) and the signal of bound antibody was visualized by exposure (1-60 min) to autoradiographic film (Amersham ECL Hyperfilm). Autoradiograms were quantified by densitometric scanning (GS-800 Imaging Calibrated Densitometer, Bio-Rad). The content of target proteins in the striatum or hippocampus of rats treated with methamphetamine was compared in the same gel with that of control rats. This procedure was repeated until each sample was quantified at least 3 times in different gels

(each gel with different samples from all experimental groups). Finally, percent changes in immunoreactivity with respect to control samples (100%) were calculated for each rat in the various gels, and the mean value was used as a final estimate. ß-actin was used as a loading control.

Data and statistical analysis

All data were analyzed with GraphPad Prism, Version 6 (GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as mean values ± standard error of the mean (SEM). For the statistical evaluation of changes in rat body weight a two-way repeated measures ANOVA followed by Dunnett's or Tukey's multiple comparisons tests was used, in which Treatment (control, 2.5 mg/kg, 5 mg/kg and 7.5 mg/kg of methamphetamine) and Day (D1 to D42) were treated as independent variables. D49 was not included in the analysis, as rats from wave 2 were not weighted on that day. When evaluating changes in the FST a two-way ANOVA (post-hoc Dunnett's test) was used with Treatment (control, 2.5 mg/kg, 5 mg/kg and 7.5 mg/kg of methamphetamine) and Behavior (immobile vs. active) as independent variables. For the two-bottle choice tests, a two-way ANOVA with Treatment (control, 2.5 mg/kg, 5 mg/kg and 7.5 mg/kg of methamphetamine) and Bottle (water vs. 1% sucrose or water vs. 20 mg/l methamphetamine) as independent variables were used. Multiple t-tests were utilized to ascertain differences in bottle liquid consumption among treatment groups. Finally, when evaluating the effects of methamphetamine withdrawal on the content of monoamines and their metabolites (by HPLC) or on the regulation of neurochemical markers (by Western blot) a one-way ANOVA (post-hoc Dunnett's test) was used. Pearson's

correlation coefficients were calculated to test for possible association between variables. The level of significance was set at $p \leq 0.05$.

RESULTS

Methamphetamine binge administration dose-dependently induced anorexigenic effects in rats

As shown in Fig. 1b, methamphetamine administration induced changes in body weight as demonstrated by a significant Treatment x Day interaction ($F_{15,215} = 12.23$, $p < 0.001$), and an effect of Treatment ($F_{3,43} = 21.35$, $p < 0.001$). In particular, post-hoc analysis revealed reduced weight gain during repeated methamphetamine exposure in a dose-response manner when compared to control treated rats: doses of 5 and 7.5 mg/kg (D2, D3 and D4, at least *$p < 0.05$) and dose 2.5 mg/kg (D4, at least $p < 0.05$) (Fig. 1b). Moreover there was a significant effect of Day ($F_{5,215} = 44.47$, $p < 0.001$) suggesting the induction of tolerance to methamphetamine anorexigenic effects (i.e., smaller change in body weight: D4 vs. D2 for doses 5 and 7.5 mg/kg; at least $p < 0.05$, Tukey's multiple comparisons test; significance not represented in Fig. 1b). These results agreed with prior data describing the anorexigenic effects of methamphetamine administration (Carvalho et al., 2012; Steinkellner et al., 2011) in relation to the dose administered (see García-Cabrerizo and García-Fuster, 2016a for similar results – dose regimen: 5 mg/kg). Interestingly, the effects of repeated methamphetamine on body weight did not persist over time, as there were no significant weight differences following 25 days of withdrawal and during the remaining of the experimental design (see D35, Fig. 1b), along with the expected increase in food intake to a

level similar to controls during withdrawal (e.g., Torney and Lasagna, 1960; Kraeuchi et al., 1984).

Methamphetamine binge administration dose-dependently enhanced negative affect in rats following prolonged withdrawal

The effects of prolonged methamphetamine withdrawal on inducing negative affect were evaluated at two levels: (1) behavioral despair (i.e., increased immobility in the FST) and (2) anhedonia (i.e., decreased sucrose intake by the two-bottle choice test). The results for the FST (D32) showed that although there was a significant Treatment x Behavior interaction ($F_{3,86} = 3.94$, $p = 0.01$) and an effect of Behavior ($F_{1,86} = 4.45$, $p = 0.038$), there was no effect of Treatment ($F_{3,86} = 0.01$, $p > 0.05$) (Fig. 2a). On the other hand, the two-bottle choice test (D36-38, 2 days exposure to 1% sucrose), showed no significant Treatment x Bottle interaction ($F_{3,86} = 1.66$, $p = 0.181$), no effect of Treatment ($F_{3,86} = 0.86$, $p = 0.465$), but showed a significant effect of Bottle ($F_{1,86} = 246.8$, $p < 0.001$). Thus, multiple t-tests revealed that rats treated with 7.5 mg/kg of methamphetamine showed a significant decrease in the amount (g/day) of 1% sucrose consumed paired with an increase in water consumption (at least $*p < 0.05$ for each bottle vs. control rats; Fig. 2b). Additionally, rats treated with 7.5 mg/kg of methamphetamine showed decreased preference for the bottle containing 1% sucrose (-12 ± 3%, $p < 0.001$) as measured during the last 24 h of the test (D37-38) (data not shown). Moreover, Pearson's correlation coefficients were calculated to test for possible associations between the two behavioral variables used to measure negative affect (i.e., immobility in the FST and g of 1% sucrose consumption per day in the two-bottle choice).

Interestingly, there was a significant negative correlation between both variables for rats treated with 7.5 mg/kg of methamphetamine ($r = -0.605$, $n = 10$, $p < 0.05$) (Fig. 2c).

Methamphetamine binge administration dose-dependently elicited voluntary drug consumption in rats following prolonged withdrawal

The effects of prolonged methamphetamine withdrawal on eliciting voluntary drug consumption (i.e., increased methamphetamine intake) were evaluated by the two-bottle choice (D43-49, 6 days exposure to 20 mg/l methamphetamine, Fig. 2d). The results showed no significant Treatment x Bottle interaction ($F_{3,92} = 0.41$, $p = 0.752$) and no effect of Treatment ($F_{3,92} = 2.25$, $p = 0.088$). However, there was a significant effect of Bottle ($F_{1,92} = 15.05$, $p < 0.001$), and multiple t-tests revealed that rats treated with 7.5 mg/kg of methamphetamine showed a significant increase in the amount (g/day) of 20 mg/l methamphetamine consumption (at least $^{*}p < 0.05$ vs. control rats; Fig. 2d).

Methamphetamine binge administration dose-dependently reduced monamines content following prolonged withdrawal in rat brain

As shown in Fig. 3a, methamphetamine induced dose-dependent significant changes in the contents of DA ($F_{3,39} = 6.99$, $p < 0.001$) and its metabolites (DOPAC: $F_{3,39} = 6.79$, $p < 0.001$ and HVA: $F_{3,37} = 4.02$, $p = 0.014$) in striatum following prolonged withdrawal. In particular, post-hoc analyses revealed that rats treated with 5 or 7.5 mg/kg of methamphetamine showed decreased contents for DA, DOPAC and HVA ($^{**}p < 0.01$ and $^{*}p < 0.05$ vs. control rats, Fig. 3a). No changes were observed in the content of 5-HT ($F_{3,40} = 0.73$, $p = 0.540$) and 5-HIAA ($F_{3,40} = 0.33$, $p = 0.327$) in striatum (data not shown). When evaluating the effects of

methamphetamine withdrawal on hippocampus, the results showed dose-dependent significant changes in the contents of 5-HT ($F_{3,42} = 2.56$, $p = 0.068$), 5-HIAA ($F_{3,42} = 3.25$, $p = 0.031$) and NE ($F_{3,41} = 3.57$, $p = 0.022$). Post-hoc analyses revealed that methamphetamine administration decreased 5-HT (*$p < 0.05$ for dose of 7.5 mg/kg) and NE (*$p < 0.05$ for dose of 2.5 mg/kg) when compared to control rats (Fig. 3b).

Methamphetamine binge administration dose-dependently regulated neurochemical markers following prolonged withdrawal in rat brain

To further evaluate the effects of methamphetamine in rat brain, some key striatal dopaminergic markers (i.e., D2 receptor, DAT, p-TH and DARPP-32) as well as two hippocampal serotonergic markers (i.e., 5-HT_{2A} and 5-HT_{2C} receptors) were evaluated by Western blot. As shown in Table 1, prolonged methamphetamine withdrawal did not induce significant changes in any of the selected markers in striatum and/or hippocampus (vs. control-treated rats). However, when evaluating the effects of methamphetamine withdrawal on the cell fate regulator FADD, there were region-specific changes in FADD protein forms, with more robust changes observed in hippocampus (Fig. 4). In particular, while a one-way ANOVA did not detect significant changes in striatum for FADD protein ($F_{3,42} = 0.34$, $p = 0.793$), the analysis was significant for p-FADD ($F_{3,38} = 3.07$, $p = 0.039$) and p-FADD/FADD balance ($F_{3,38} = 2.88$, $p = 0.048$). Yet, post-hoc comparisons did not detect any significant changes among experimental groups (Fig. 4a-b). On the other hand, when evaluating the effects of methamphetamine withdrawal in hippocampus, the results showed significant changes for all protein forms (FADD: $F_{3,39} = 4.81$, $p = 0.006$; p-FADD: $F_{3,41} = 5.67$, $p = 0.002$; p-FADD/FADD balance: $F_{3,37} = 9.64$, $p < 0.001$). Post-hoc analyses revealed

that rats treated with 7.5 mg/kg of methamphetamine had decreased FADD content and increased p-FADD/FADD balance (**$p < 0.01$ and *$p < 0.05$ vs. control rats; Fig. 4c-d) during prolonged withdrawal in hippocampus. Moreover, rats treated with 2.5 mg/kg of methamphetamine showed a significant reduction in p-FADD content (*$p < 0.05$ vs. control rats; Fig. 4c-d). Methamphetamine binge administration did not alter the content of β-actin ($F_{3,41} = 2.18$, $p = 0.105$), and thus it was used as a loading control (see representative immunoblots in Figs. 4b and d).

Increased methamphetamine consumption following prolonged withdrawal is associated with reduced hippocampal FADD protein content

Pearson's correlation coefficients were evaluated to test for possible associations between voluntary methamphetamine consumption, the content of brain monoamines and the regulation of neurochemical markers following prolonged withdrawal. The main results showed that in rats treated with 7.5 mg/kg of methamphetamine the amount of methamphetamine consumed during withdrawal negatively correlated with FADD protein content in hippocampus ($r = -0.720$, $n = 9$, $p = 0.014$) (Fig. 5).

DISCUSSION

The main results of this study showed that repeated binge methamphetamine administration induced dose-dependent negative effects during prolonged withdrawal in adult rats. In particular, following 25 days of forced withdrawal, rats treated repeatedly with methamphetamine (7.5 mg/kg) showed: (1) enhanced negative affect – increased anhedonia associated with behavioral despair, (2) increased voluntary methamphetamine consumption,

(3) enhanced neurotoxicity – decreased DA and metabolites in striatum and decreased 5-HT in hippocampus, (4) altered neuroplasticity markers – decreased FADD protein and increased p-FADD/FADD balance selectively in hippocampus, and (5) higher consumption rates of methamphetamine during withdrawal were associated with lower FADD content in hippocampus.

It is well accepted that withdrawal from repeated methamphetamine treatment precipitates in animals behavioral and physiological symptoms similar to the ones described for major depressive disorder, such as despair and anhedonia (Barr et al., 2002). In this regard, the present results showed that binge methamphetamine dose-dependently induced negative affect in rats during prolonged withdrawal by showing combined increased time spent immobile in the FST (i.e., a measure of behavioral despair) with reduced sucrose consumption in the two bottle choice test (i.e., a measure of anhedonia or loss of pleasant stimulus). Prior experiments showed that withdrawal from many drugs, including methamphetamine, led to depressive-like behavior (Iijima et al., 2013; Ren et al., 2015; Hajheidari et al., 2015) and anhedonia in rodents (e.g., Koob, 2013). In fact, psychostimulant withdrawal in rodents has been proposed as a model in which to study depressive-like behavior (e.g., Barr et al., 2002). In this line of thought, the present results reinforced this notion, while presented a paradigm to further ascertain the consequences (i.e., rate of voluntary drug consumption) and/or putative neurochemical correlates (i.e., role of FADD) associated with methamphetamine withdrawal.

Prior experiments validated the oral route of methamphetamine self-administration (i.e., two-bottle choice) as a viable alternative to intra-venous (i.v.) self-administration procedures for investigating the motivational effects to consume the drug (Wheeler et al., 2009). In fact,

although the absorption of orally administered methamphetamine occurs more slowly in the intestines (peak plasma levels being reached 180 minutes after dosing; Schepers et al., 2003) as compared to more rapid routes (i.v.: cerebral circulation in 10-15 seconds; reviewed in Rawson, 2013), prior studies suggested that oral administration resulted in similar enhanced levels of use as the i.v. route (Cruickshank and Dyer, 2009). Thus, this study utilized a two-bottle choice test (20 mg/l of drinking methamphetamine over water; e.g., Shabani et al., 2016) to ascertain the rate of voluntary drug consumption in methamphetamine-withdrawn rats. In particular, rats with a history of methamphetamine intake (dose of 7.5 mg/kg) showed increased voluntary methamphetamine consumption, which suggested either increased sensitivity to the conditioned rewarding properties of the drug (unlikely due to decreased general reward sensitivity as measured by decreased 1% sucrose consumption) or insensitivity to its aversive effects. Similarly, mice bred for high methamphetamine intake, a genetic selective-breeding model of binge-level drug intake and addiction vulnerability (Shabani et al., 2016), showed a profound reduction in sensitivity to the aversive effects of methamphetamine (Shabani et al., 2012). In summary, the present results extend recent reports, which showed increased voluntary oral methamphetamine consumption following 2 weeks of withdrawal (Doyle et al., 2015; Hajheidari et al., 2015), to suggest even more prolonged effects observed up to 45 days following the last methamphetamine administration (i.e., from D4 to D49).

In terms of the neurotoxic effects observed in methamphetamine-withdrawn rats, the present results broaden the prior literature demonstrating drug-induced negative effects in rat brain (e.g., see revisions, Krasnova and Cadet, 2009; Moszczynska and Callan, 2017) by showing persistent, long-lasting and dose-dependent decreases in striatal contents of DA (and its main

metabolites DOPAC and HVA) and decreases in hippocampal 5-HT and NE as measured by HPLC 47 days after the last exposure to methamphetamine. It is worth mentioning that the observed decreases in total monoamines (and metabolites) levels are not sufficient to establish any changes to neurotransmission, as *in vivo* microdialysis will be the minimum requisite to understand these effects (e.g., Yamamoto et al., 2017). To further evaluate the possible molecular mechanisms behind these neurotoxic effects, although the literature in experimental animals has proven parallel reductions in dopaminergic (e.g., DAT, TH) and serotonergic (e.g., SERT) markers (see revision, Moszczynska and Callan, 2017), the present results showed no changes in any of the protein markers evaluated (dopaminergic: D2 receptor, DAT, p-TH, DARPP-32; serotonergic: 5-HT_{2A} and 5-HT_{2C} receptors) in rat brain following prolonged methamphetamine withdrawal. These results propose that while the contents of brain monoamine neurotransmitters were decreased, some of the key receptors and molecules that mediate their effects were intact, suggesting that changes in neurotransmission *per se* may not be involved in the observed behavioral changes or proposing a different time-course in the recovery from the damage caused by methamphetamine. In fact, possible adaptive mechanisms might take place (i.e., see role of FADD below), such as increased synthesis of monoamine neurotransmitters markers and upregulation of function of its signaling in an attempt to compensate for the prior neurotoxic damage (see detail discussion in revision by Moszczynska and Callan, 2017).

In this regard, repeated administration of methamphetamine (dose of 7.5 mg/kg) resulted in decreased FADD protein content and increased p-FADD/FADD balance (i.e., a putative neuroplasticity marker; Ramos-Miguel et al., 2012) selectively in hippocampus of withdrawn rats. Similarly, neuroprotective pathways (decreased Fas) were activated following voluntary

methamphetamine oral consumption (Wheeler et al., 2009). Thus, although differences in the pathways related to apoptosis are known to be involved in methamphetamine-induced neurotoxicity (e.g., Cadet et al., 2003), the effects seem to be regulated in a time-course manner, with neurotoxicity observed up to 24 h post methamphetamine administration, but normalized 1 week later (Jayanthi et al., 2005). Moreover, experiments done with a different amphetamine-like psychostimulant such as MDMA also showed reduced content of FADD, Bax and cytochrome c in rat hippocampus following repeated administration (García-Cabrerizo and García-Fuster, 2015), which suggested, as apoptosis was not triggered, the induction of neurochemical changes. Accordingly, the present results replicated previously accepted neurotoxicity effects (i.e., decreased DA and metabolites in striatum and decreased 5-HT and NE in hippocampus) paired with normalized monoaminergic neurotransmitter signaling but decreased FADD (and increased p-FADD/FADD balance) content selectively in hippocampus, suggesting the induction of neural plasticity and/or repair mechanisms to a prior drug insult (i.e., neurochemical adaptations). The regulation of FADD adaptor during methamphetamine withdrawal further supports the role of this multifunctional protein outside of the apoptotic pathway, thus reinforcing its function as a major signaling step in adaptive cell responses (see prior studies, e.g., García-Fuster et al., 2007; 2014 and García-Fuster and García-Sevilla, 2016; Ramos-Miguel et al., 2012, 2017). Future studies will attempt to ascertain a possible mechanistic link between the observed behaviors and changes in hippocampal FADD adaptor. In this regard, a recent study also showed a combination of depressive-like behavior (including anhedonia) and increased BDNF (i.e., a marker of neural plasticity) content during prolonged methamphetamine withdrawal in mice (Ren et al., 2015). Moreover, in terms of the observed region-specific FADD regulation, prior studies also

showed similar results, with specific effects observed in hippocampus as compared to other brain regions (e.g., striatum and/or prefrontal cortex, see García-Cabrerizo et al., 2015; Hernández-Hernández et al., 2017). Interestingly, methamphetamine-induced hippocampal changes are particularly relevant given the role of this brain region in learning, memory and executive functioning and given the well known deficits in cognitive performance that occur during methamphetamine withdrawal (see Moszczynska and Callan, 2017). In this context, two recent studies from our group showed an association between loss of FADD protein and cognitive decline either in an elderly human population (Ramos-Miguel et al., 2017) or in rats (Hernández-Hernández et al., 2017). The present results showed that rats with higher voluntary intake of methamphetamine showed less hippocampal FADD suggesting that hippocampal FADD regulation paralleled the long-lasting behavioral abnormalities taking place during methamphetamine withdrawal such as cognitive decline, and therefore, drugs that manipulate FADD (e.g., pro-cognitive drugs increase FADD in parallel to improving cognitive performance, see Hernández-Hernández et al., 2017) might confer beneficial effects against this symptom.

In summary, the present data demonstrated that binge methamphetamine exposure dose-dependently induced persistent negative affect during withdrawal as observed by two core symptoms of depression (i.e., behavioral despair and anhedonia), together with deficits in striatal DA and hippocampal 5-HT contents, thus validating this drug paradigm as a good model in which to further study methamphetamine withdrawal syndrome. In particular, rats exposed to a prior history of methamphetamine (7.5 mg/kg) showed increased voluntary methamphetamine consumption in association with region-specific changes in hippocampal FADD protein forms, suggesting a possible role, that deserves further mechanistic

exploration, for this neuroplasticity marker in the adaptations taking place in this brain region during prolonged methamphetamine withdrawal.

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Authors Contribution

MJG-F and RG-C were responsible for the study concept and design. RG-C conducted the experiments. RG-C and MJG-F analyzed the data and MJG-F drafted the manuscript. Both authors contributed to and have approved the final version for publication.

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Table 1. Effects of prolonged methamphetamine withdrawal (25 days) on striatal dopaminergic and hippocampal serotonergic protein markers in rats by Western blot analysis.

Striatum	Control	2.5 mg/kg	5 mg/kg	7.5 mg/kg	ANOVA: $F_{DFn,DFd}$, *p* value
D2	100±6	95±6	105±7	110±15	$F_{3,30} = 0.40$, $p = 0.752$
DAT	102±6	84±4	102±6	104±11	$F_{3,35} = 1.34$, $p = 0.279$
p-TH	102±8	85±5	94±9	92±8	$F_{3,36} = 0.58$, $p = 0.633$
DARPP-32	102±10	104±2	107±7	114±8	$F_{3, 38} = 0.30$, $p = 0.824$

Hippocampus	Control	2.5 mg/kg	5 mg/kg	7.5 mg/kg	ANOVA: $F_{DFn,DFd}$, *p* value
5-HT_{2A}	100±3	108±6	85±6	107±8	$F_{3,43} = 3.80$, $p = 0.017$
5-HT_{2C}	101±5	100±12	119±11	111±6	$F_{3, 42} = 1.24$, $p = 0.307$

D2: dopamine receptor; DAT: dopamine transporter; p-TH: phosphorylated tyrosine hydroxylase; DARPP-32: dopamine- and cyclic AMP-regulated neuronal phosphoprotein, Mr 32 kDa; 5-HT_{2A}: serotonin 2A receptor; 5-HT_{2C}: serotonin 2C receptor. Data are means ± SEM of at least 3 experiments per group, and expressed as percentage of the corresponding control-treated group. ANOVAs did not detect significant changes after the various treatments with methamphetamine followed by 25 days of spontaneous withdrawal, except for 5-HT_{2A} regulation on hippocampus, although post-hoc comparisons did not detect any significant changes among experimental groups.

Figure Legends

Fig. 1. (a) Experimental design. **(b)** Methamphetamine binge administration dose-dependently induced anorexigenic effects in rats. The graph represents the change in rat body weight (g) per day of treatment. Groups of treatment: Control (saline-treated rats, n = 15) and methamphetamine (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg x 3, i.p., 4 days, n = 7, 15 and 10 respectively). Data represent mean ± SEM of body weight (g). Two-way repeated measures ANOVA followed by Dunnett's or Tukey's multiple comparisons tests: at least *$p < 0.05$ when compared to control-treated rats. D: Day; METH: methamphetamine.

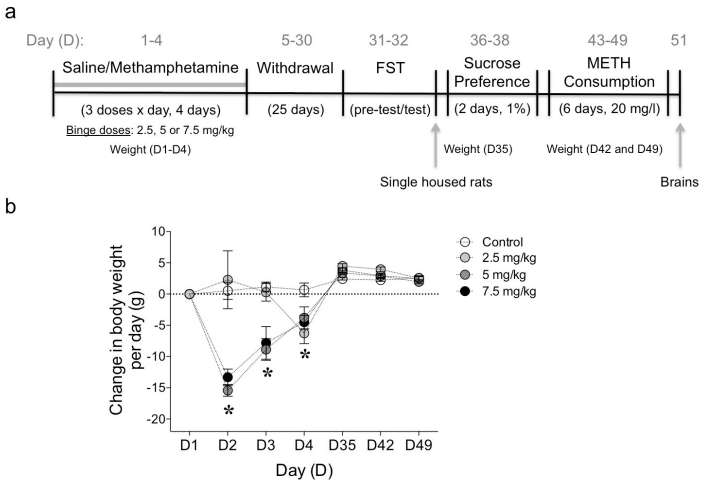

Figure 1
García-Cabrerizo and García-Fuster

Fig. 2. Methamphetamine binge administration dose-dependently enhanced negative affect in rats following prolonged withdrawal. **(a)** Forced-swim test (FST, D32). Groups of treatment: Control (saline-treated rats, n = 15) and methamphetamine (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg x 3, i.p., 4 days, n = 7, 15 and 10 respectively). Data represent mean ± SEM of the time (sec) spent immobile vs. active (climbing + swimming). **(b)** 1% sucrose preference (two-bottle choice test, D36-38). Data represent mean ± SEM of the amount of water or 1% sucrose consumed (g) per day. **(c)** Association between variables. Scatter plot depicting a significant negative correlation between the time spent immobile in the FST with the amount of 1% sucrose consumed for rats treated with 7.5 mg/kg of methamphetamine. Each circle represents a different treated rat. The solid line is the best fit for the correlation ($r = -0.605$, $n = 10$, $p < 0.05$). The dotted curves indicate the 95% confidence interval for the regression line. **(d)** Methamphetamine binge administration dose-dependently elicited voluntary drug consumption in rats following prolonged withdrawal (two-bottle choice test, D43-49). Data represent mean ± SEM of the amount of water or 20 mg/l of methamphetamine consumed (g) per day. Two-way ANOVA followed by multiple t-tests comparisons: $*p < 0.05$ when compared to the corresponding control group.

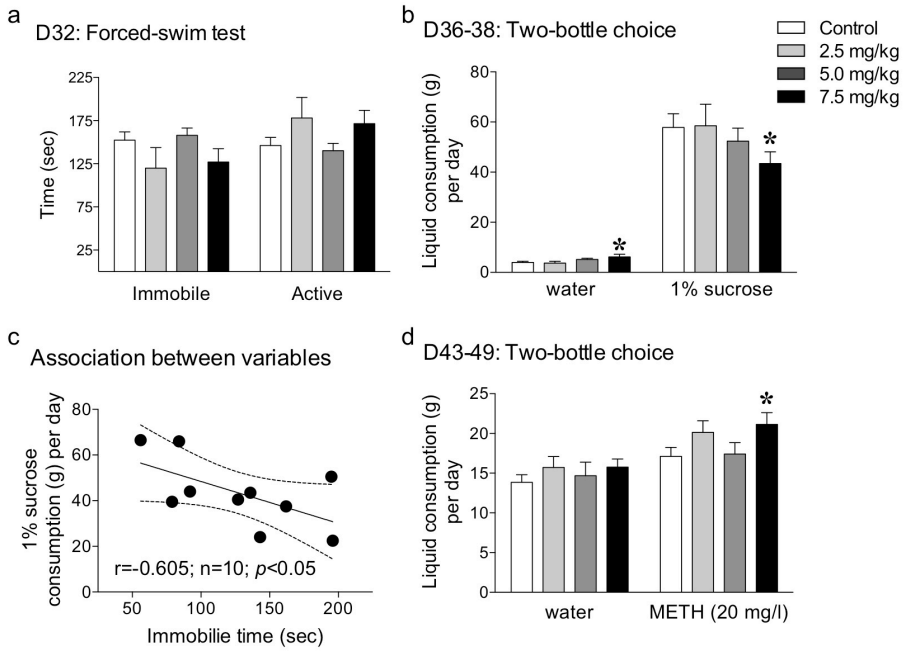

Figure 2
García-Cabrerizo and García-Fuster

Fig. 3. Methamphetamine binge administration dose-dependently reduced the content of monoamines following prolonged withdrawal in rat **(a)** striatum and **(b)** hippocampus. Groups of treatment: Control (saline-treated rats, n = 15) and methamphetamine (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg x 3, i.p., 4 days, n = 7, 15 and 10 respectively). Columns represent mean ± SEM of ng of DA, DOPAC, HVA, 5-HT, T-HIAA and NE/μg protein. One-way ANOVA followed by Dunnett's multiple comparisons test: *$p < 0.05$ and **$p < 0.01$ when

compared to the corresponding control group. DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; 5-HT: serotonin; 5-HIAA: 5-hydroxy-indole acetic acid; NE: noradrenaline).

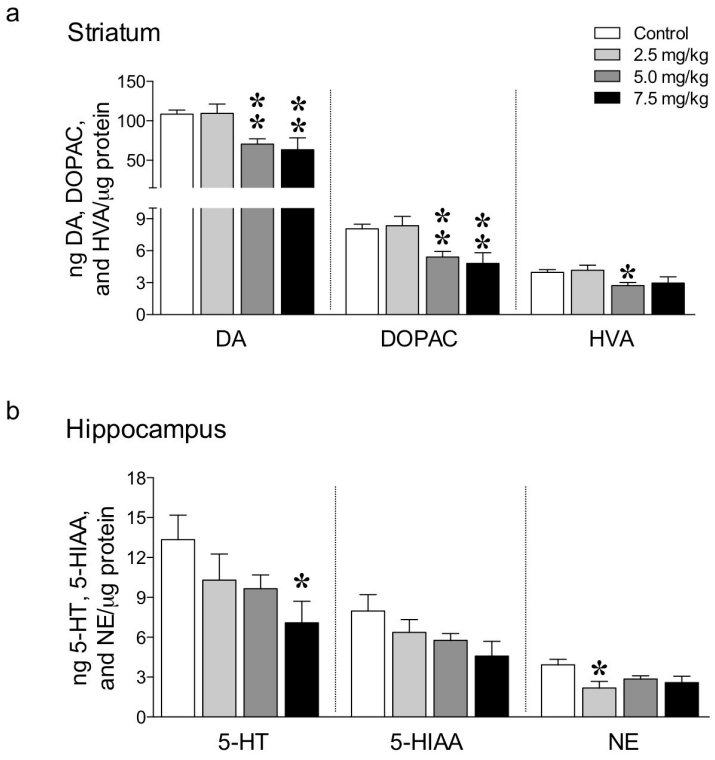

Figure 3
García-Cabrerizo and García-Fuster

Fig. 4. Methamphetamine binge administration dose-dependently regulated FADD forms following prolonged withdrawal in rat **(a-b)** striatum and **(c-d)** hippocampus. Groups of

treatment: Control (saline-treated rats, n = 15) and methamphetamine (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg x 3, i.p., 4 days, n = 7, 15 and 10 respectively). Columns represent mean ± SEM of n experiments per group and expressed as a percentage of control-treated rats. One-way ANOVA followed by Dunnett's test: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared to the corresponding control group. **(b-d)** Representative immunoblots depicting labeling of total FADD, p-FADD and the corresponding for β-actin as a loading control in striatum and hippocampus respectively.

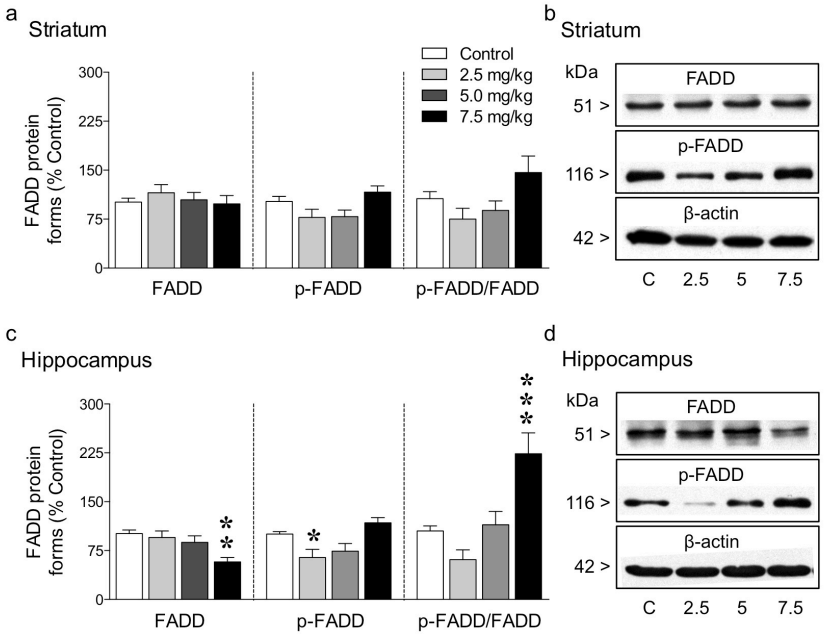

Figure 4
García-Cabrerizo and García-Fuster

Fig. 5. Increased methamphetamine consumption following prolonged withdrawal is associated with reduced hippocampal FADD protein content. Scatter plot depicting a significant negative correlation between the amount of oral methamphetamine consumed (20 mg/l) and FADD content in hippocampus for rats treated with 7.5 mg/kg of methamphetamine. Each circle represents a different treated rat. The solid line is the best fit for the correlation (r = -0.720, n = 9, p = 0.014). The dotted curves indicate the 95% confidence interval for the regression line.

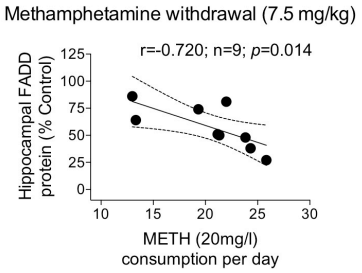

Figure 5
García-Cabrerizo and García-Fuster