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Brain pattern of histone H3 phosphorylation after acute amphetamine administration: Its relationship to brain c-fos induction is strongly dependent on the particular brain area

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ABSTRACT

Recent evidence strongly suggests a critical role of chromatin remodelling in the acute and chronic effects of addictive drugs. We reasoned that Immunohistochemical detection of certain histone modifications may be a more specific tool than induction of immediate early genes (i.e. c-fos) to detect brain areas and neurons that are critical for the action of addictive drugs. Thus, in the present work we studied in adult male rats the effects of a high dose of amphetamine on brain pattern of histone H3 phosphorylation in serine 10 ($pH3S_{10}$) and c-fos expression. We firstly observed that amphetamine-induced an increase in the number of $pH3S_{10}$ positive neurons in a restricted number of brain areas, with maximum levels at 30 min after the drug administration that declined at 90 min in most areas. In a second experiment we studied colocalization of $pH3S_{10}$ immunoreactivity ($pH3S_{10}$-IR) and c-fos expression. Amphetamine increased c-fos expression in medial prefrontal cortex (mPFC), dorsal striatum, nucleus accumbens (Acb), major Island of Calleja (ICjM), central amygdala (CeA), bed nucleus of stria terminalis lateral dorsal (BSTld) and paraventricular nucleus of the hypothalamus (PVN). Whereas no evidence for increase in $pH3S_{10}$ positive neurons was found in the mPFC and the PVN, in the striatum and the Acb basically all $pH3S_{10}$ positive neurons showed colocalization with c-fos. In ICjM, CeA and BSTld a notable degree of colocalization was found, but an important number of neurons expressing c-fos were negative for $pH3S_{10}$. The present results give support to the hypothesis that amphetamine-induced $pH3S_{10}$-IR showed a more restricted pattern than brain c-fos induction, being this difference strongly dependent on the particular brain area studied. It is likely that those nuclei and neurons showing $pH3S_{10}$-IR are more specifically associated to important effects of the drug, including neural plasticity.

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1. Introduction

The induction of immediate early genes, mainly c-fos, has been a valuable tool for the characterization of brain areas activated by particular stimuli, i.e. specific sensorial signals, stress and addictive drugs (Herrera and Robertson, 1996; Pacak and Palkovits, 2001). However, in response to emotional stressors and certain drugs of abuse such as cocaine and amphetamine, a wide range of brain areas shows enhanced c-fos expression; for instance, medial prefrontal cortex (mPFC), dorsal striatum, nucleus accumbens (Acb), some amygdala regions and the paraventricular nucleus of the hypothalamus (PVN) (Armario, 2006; Cullinan et al., 1995: Miyamoto et al., 2004; Rotllant et al., 2010). It is likely that this wide brain induction of c-fos after some stressors and amphetamine reflects, in part, processes of general arousal, making it difficult to use c-fos to identify those neurons that are actually critical for the processing of a particular stimulus and those that are not.

In recent years, accumulating evidence has shown that epigenetic mechanisms such as DNA methylation and histone modifications could play a pivotal role in the long-term effects of perinatal events as well as in the consequences of exposure to stressors and addictive drugs (Meaney et al., 2007; Reul and Chandramohan,

Abbreviations: Acb, nucleus accumbens; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; BSTld, bed nucleus of the stria terminalis, lateral dorsal; CeAm, central amygdala, medial division; CPu, Caudate-Putamen; CPuDLa, Caudate-Putamen, dorsal-lateral part anterior; CPuDLp, Caudate-Putamen, dorsal-lateral part posterior; CPuDMa, Caudate-Putamen, dorsal-medial part anterior; CPuDMp, Caudate-Putamen, dorsal-medial part posterior; CPuVLa, Caudate-Putamen, ventral-lateral part anterior; CPuVLp, Caudate-Putamen, ventral-lateral part posterior; CPuVMa, Caudate-Putamen, ventral-medial part anterior; CPuVMp, Caudate-Putamen, ventral-medial part posterior; $pAcH3S_{10}L_{14}$, phospho-(Ser-10) Acetyl- (Lys14) H3 histone; $pH3S_{10}$, phospho-(Ser-10) H3 histone; $pH3S_{10}$-IR, phospho-(Ser-10) H3 histone immunoreactivity; ICjM, major island of Calleja; IHC, immunohistochemistry; ISH, in situ hybridization; mPFC, medial prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus.

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2007; Tsankova et al., 2007). Epigenetic mechanisms can either enhance or reduce expression of particular genes modifying the structure of chromatin and the accessibility of transcriptional machinery to DNA. More particularly, phosphorylation of histone H3 and acetylation of histone H3 and H4 are associated with enhancing gene expression (Berger, 2001). By western-blot analysis, increases in histone H3 and/or H4 acetylation have been reported in some brain regions after stress, cocaine or ethanol (Chwang et al., 2007; Hollis et al., 2010; Levenson et al., 2004; Miller et al., 2008; Pandey et al., 2008; Wang et al., 2010). Unfortunately, western-blot does not allow identifying particular populations of neurons showing histone modifications.

We then reasoned that those neurons most critically involved in the impact of the drug may experience some of the above histone modifications in an important number of genes to the point that these neurons can be identified by classical immunohistochemistry (IHC) procedure. In this regard, a high number of neurons positive for acetylation of histone H3 at lysine 14 or histone H4 at lysine 5 ($AcH3L_{14}$ or $AcH4L_5$, respectively) has been detected by IHC under control (vehicle) conditions, with modest increases in $AcH4L_5$, but not $AcH3L_{14}$, in the striatum after acute cocaine administration (Brami-Cherrier et al., 2005). In contrast, a low number of neurons positive for phosphorylation of histone H3 in serine 10 ($pH3S_{10}$) or phosphoacetylation of histone H3 in serine 10 and lysine 14 ($pAcH3S_{10}L_{14}$) are detected under control conditions that are increased by stress and drugs of abuse. More specifically, increases in the number of $pH3S_{10}$ positive neurons have been reported after exposure to stress in the dentate gyrus (Bilang-Bleuel et al., 2005), and after cocaine administration in the dorsal and ventral striatum (Bertran-Gonzalez et al., 2008; Brami-Cherrier et al., 2005; Sanchis-Segura et al., 2009; Stipanovich et al., 2008). Similarly, increases in the number of $pAcH3S_{10}L_{14}$ positive neurons have been reported after stress in the dentate gyrus (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007, 2008) and cocaine in the striatum (Brami-Cherrier et al., 2005; Sanchis-Segura et al., 2009).

The pattern of a low number of $pH3S_{10}$ or $pAcH3S_{10}L_{14}$ positive neurons under control conditions, together with a good sensitivity to acute stimuli is ideal to characterize putative brain areas and neuronal populations that could be important for the effects of drug or stress. Although both $pH3S_{10}$ and $pAcH3S_{10}L_{14}$ show similar patterns after stress (Bilang-Bleuel et al., 2005) or cocaine administration (Brami-Cherrier et al., 2005), we were unable to obtain satisfactory IHC results using available commercial antibodies against $pAcH3S_{10}L_{14}$ modification. For these reasons, the present work focused on amphetamine-induced changes in $pH3S_{10}$ positive neurons. It is important to note that a neuron showing no $pH3S_{10}$ immunoreactivity ($pH3S_{10}$-IR) can still have enhanced $pH3S_{10}$ at the promoter regions of some specific genes. For instance, cocaine-induced c-fos expression is linked to an enhanced $pH3S_{10}$ at the c-fos gene promoter (Brami-Cherrier et al., 2009), but this does not mean that positive $pH3S_{10}$-IR has to be detected in those neurons expressing c-fos after the drug if such phosphorylation only affect c-fos or a few number of other genes.

In order to demonstrate that the pattern of $pH3S_{10}$ is more restricted than that of c-fos and to help delineating possible areas of interest in which further to study long-term amphetamine-induced epigenetic changes, we decided to map amphetamine-induced brain pattern of $pH3S_{10}$ using IHC and compare this pattern with the expression of c-fos. In a first study we showed that the increase in the number of $pH3S_{10}$ positive neurons after amphetamine was restricted to a few telencephalic and diencephalic brain regions, in contrast with published data regarding c-fos. Then, in a second experiment we studied c-fos and $pH3S_{10}$ colocalization in some of the above areas. As we observed brain areas and neurons where c-fos expression was not associated to $pH3S_{10}$-IR we wonder whether fos positive neurons could manifest a similar pattern of changes after amphetamine (low number of positive neurons in basal conditions and increases after amphetamine) in other histone modifications. Using different antibodies against $AcH3L_9$, $AcH3L_{9/14}$, $AcH4L_5$ and $AcH4L_8$, in all cases, a high number of positive neurons were observed with modest, if any, increases after amphetamine administration (unpublished), suggesting that the pattern observed with $pH3S_{10}$, is clearly distinct from that observed with histone acetylation.

2. Experimental procedures

2.1. Animals

Male, 2-month-old, Sprague-Dawley rats obtained from the Animal Facility Service of the Universitat Autònoma de Barcelona were used. The animals were housed two per cage under standard conditions of temperature (22 ± 1 °C) and a 12 h–12 h light–dark schedule (lights on at 7:00), with *ad libitum* access to food and water. The experimental treatments were carried out in the morning and the animals were allowed at least one week to acclimate themselves to the housing conditions before the beginning of the experiments. Prior to the experimental day, all animals were handled and received two habituation sessions of subcutaneous injection. This work has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by Ethical Committee for Animal Experimentation of the Universitat Autònoma de Barcelona.

2.2. Experiment 1: time course of histone H3 phosphorylation

Previous studies with cocaine indicated that the number of $pH3S_{10}$ + neurons was greatly enhanced at 15–30 min after cocaine administration in the dorsal and ventral striatum (Bertran-Gonzalez et al., 2008; Brami-Cherrier et al., 2005; Sanchis-Segura et al., 2009). However, the possibility that changes in $pH3S_{10}$ may follow a different time-course depending on the particular brain area evaluated prompted us to firstly study the dynamics of the response. On the basis of some pilot studies we chose 30 and 90 min as post-injection times. Animals were randomly assigned to two groups: VEH, receiving sterile saline solution (NaCl 0.9%, 0.5 mL/kg s.c.) or AMP, receiving 5 mg/kg of d-amphetamine sulphate (Sigma, Barcelona, Spain, 0.5 mL/kg s.c). The dose is expressed in terms of the base. After injection, the animals were returned to their home cages and sacrificed at 30 min (VEH-30, $n = 4$; AMP-30, $n = 8$) or 90 min (VEH-90, $n = 4$; AMP-90, $n = 8$) post-injection.

Rats were anesthetized with isofluorane and perfused with saline solution (4 °C) for 2 min and with 4% paraformaldehyde and 3.8% borax (4 °C) for 12 min. Then brains were removed and post-fixed overnight at 4 °C. After that, brains were changed to a cryoprotectant solution (0.2 M NaCl, 43 mM potassium phosphate (KPBS) containing 30% sucrose) for 48 h at 4 °C. Then, brains were frozen on dry ice and stored at −80 °C. From each brain, four series of 20 μm sections from bregma 4.00 to −0.40 mm were obtained and stored at −20 °C in an anti-freeze solution (30% ethyleneglycol, 20% glycerol in 0.25 mM phosphate buffer at pH 7.3). Then, $pH3S_{10}$-IR was evaluated in telencephalic and diencephalic brain regions.

2.3. Experiment 2: c-fos and $pH3S_{10}$

The previous experiment demonstrated that maximum amphetamine-induced $pH3S_{10}$-IR was observed at 30 min post-injection. Since maximum levels of *c-fos* mRNA are found 30–60 min after amphetamine administration (Day et al., 2005;

Wang et al., 1995), 30 min is the optimal time point for double labelling of c-fos mRNA levels and pH3S$_{10}$-IR. Thus, a new experiment was specifically done for this purpose, using a perfusion procedure that allows tissue processing for both IHC and in situ hybridization (ISH). Animals were assigned to VEHICLE ($n = 4$) or AMPH ($n = 4$) groups. After injection, the animals were returned to their home cages to be sacrificed 30 min later. Perfusion and further brain processing were performed as described for Experiment 1, except that solutions were DEPC treated to prevent RNA degradation. Four series of 20 μm sections from bregma 4.00 to −3.30 mm were obtained from each animal. Despite the lack of pH3S$_{10}$-IR, the PVN nucleus was included in the double labelling analysis for purposes of comparison as it shows a high level of c-fos expression after amphetamine administration (Rotllant et al., 2010).

2.4. pH3S$_{10}$ immunohistochemistry

pH3S$_{10}$ IHC was performed on floating sections. Endogenous peroxidase was blocked with a solution at 0.3% H_2O_2, 0.1% sodium azide in KPBS and non-specific binding was reduced by incubating with a blocking solution containing 0.4% Triton X-100 and 3% non fat milk in KPBS. Sections were incubated in a polyclonal rabbit antiserum (sc-8656R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500 overnight at 4 °C. Then, sections were incubated for 1 h at room temperature with an anti-rabbit IgG labelled with biotin (Southern Biotechnology Assoc., Birmingham, AL, USA) at a dilution of 1:200. Finally, streptavidin labelled with horseradish peroxidase (Southern Biotechnology Assoc.) at a dilution of 1:400 was added and the incubation maintained for 1 h at room temperature. The pH3S$_{10}$ antibody–peroxidase complex was revealed using 0.05% diaminobenzidine (Sigma) and 0.01% H_2O_2 in KPBS. Sections were mounted onto poly-L-lysine-coated slides, dehydrated with ethanol series and cover slipped with DPX (Sigma).

2.5. Combined immunohistochemistry and in situ hybridization

Sections were first processed for pH3S$_{10}$ detection, placing the tissue directly in contact with the primary antibody (Sc-8656R, Santa Cruz Biotechnology, dilution 1:500) in a blocking solution (0.04% Triton X-100, 2% bovine albumin serum and 0.25% heparin in KPBS) overnight at 4 °C. Then, sections were incubated for 1 h at room temperature with an anti-rabbit IgG labelled with biotin (Southern Biotechnology Assoc.) at a dilution 1:200. Finally, streptavidin labelled with horseradish peroxidase (Southern Biotechnology Assoc.) at a dilution of 1:400 was added and the incubation maintained for 1 h at room temperature. The pH3S$_{10}$ antibody–peroxidase complex was revealed using 0.05% diaminobenzidine (Sigma) and 0.01% H_2O_2 in KPBS. All solutions were DEPC treated to prevent RNA degradation. Sections were mounted onto SuperFrost Ultra Plus slides (Fisher Scientific, Madrid, Spain) and vacuum dried prior to the *c-fos* mRNA detection by ISH. First, sections were post-fixed in 4% paraformaldehyde, digested with protease K (Roche, Sant Cugat del Vallès, Spain) and acetylated in 0.25% acetic anhydrous in 0.01 M triethanolamine. Several washes were done between each step. Finally, sections were washed in 2× saline-sodium citrate (SSC), dehydrated in ethanol series and air-dried. Thereafter, sections were incubated for 16–18 h at 60 °C with the labelled probe (1×10^6 cpm/slice). After hybridization, the slides were washed in 4× SSC containing 1 mM DTT, digested with RNase A (GE Healthcare, Cerdanyola, Spain), washed in descending concentrations of SSC containing 1 mM DTT, dehydrated through a series of ethanol solutions and air-dried. The slides were then exposed to a XAR-5 Kodak Biomax MR autoradiography film (Kodak, Madrid, Spain). After autoradiography film exposure, slides were defatted in xylenes, and dipped in LM-1 nuclear emulsion (GE Healthcare). Slides were exposed for 15 days and developed in D19 developer (Kodak) for 3.5 min at 14 °C, dehydrated with ethanol series and cover slipped with DPX (Sigma).

Plasmid and probe preparation. The *c-fos* probe was generated from EcoRI fragment of rat *c-fos* cDNA (Dr. I. Verma, The Salk Institute, La Jolla, CA, USA), subcloned into pBluescript SK-1 (Stratagene, La Jolla, CA, USA) and linearised with SmaI. Radioactive antisense cRNA copies were generated using α^{-35}S UTP (GE Healthcare) and a transcription kit (Roche) following the protocol recommended by the supplier.

2.6. Image analysis

2.6.1. pH3S$_{10}$ immunohistochemistry

Grey scale images were taken with a digital camera (NIKON, DMX 1200) coupled to a bright-field microscope (NIKON, Eclipse E400), using a 40× objective, with no further modifications. Sample images were captured by an experimenter blind to the treatment groups from different areas at the same coordinates for each animal, using as a reference the stereotaxic atlas of Paxinos and Watson (1998). The average of four sections taken at 80 μm intervals was used for the analysis of each structure, including both hemispheres; that is, eight measures per area and animal. Due to the large size of selected brain nuclei captured image only represents a part of the nuclei. Both major divisions of the Acb (Shell, AcbSh; Core, AcbC) and the Major Island of Calleja (ICjM) were quantified from Bregma 1.70 to 0.70 mm. The quantification of the Caudate-Putamen (CPu) was assessed drawing two imaginary axis that divided this area in four quadrants, namely dorsal-medial (CPuDM), ventral-medial (CPuVM), dorsal-lateral (CPuDL) and ventral-lateral (CPuVL). Additionally, the CPu were separated in anterior (CPuDMa, CPuVMa, CPuDLa, CPuVLa; from Bregma 1.70 to 0.70 mm) and posterior (CPuDMp, CPuVMp, CPuDLp, CPuVLp; from Bregma 0.70 to −0.26 mm) parts. The lateral dorsal division of the bed nucleus of the stria terminalis (BSTld) was quantified from Bregma 0.20 to −0.40. To select pH3S$_{10}$ + immunopositive nuclei as targets for quantification, PC-based software (Scion Image, Scion Corporation, Frederick, MD, USA) was used and targets were subsequently identified in the captured images by grey level thresholding. For each brain area, the threshold for the labelled signal was defined according to the background staining. Size criteria were applied to exclude structures other than pH3S$_{10}$ immunopositive nuclei from measurement therefore, only particles with a minimum area of 100 pixels were considered as a cell nucleus. The pH3S$_{10}$ nuclei per mm^2 were calculated as the average number obtained of all analyzed images.

2.6.2. Double-labelling histochemistry

Colour images were taken with a digital camera (NIKON, DMX 1200) coupled to a bright-field microscope (NIKON, Eclipse E400), using a 40× objective, with no further modifications. Sample images were captured by an experimenter blind to the treatment groups from different areas at the same coordinates for each animal, using as a reference the stereotaxic atlas of Paxinos and Watson (1998). The average of three sections taken at 80 μm intervals was used for the analysis of each structure, including both hemispheres; that is, six measures per area and animal. The analysis included the AcbSh and AcbC (both from Bregma 1.70 to 0.70 mm), the ICjM, (from Bregma 1.70 to 0.70 mm), the BSTld (from Bregma 0.20 to −0.40 mm) and the medial division of the central amygdala (CeAm, from Bregma −2.12 to −3.00 mm). In the CPu only the CPuVMa (from Bregma 1.70 to 0.70 mm) was selected for quantification due to the fact that all the CPu divisions showed similar pH3S$_{10}$ levels 30 min after amphetamine administration,

whereas *c-fos* mRNA expression was higher in the medial region. The number of cell nuclei showing $pH3S_{10}$-IR the number of neurons expressing c-fos mRNA and the number of neurons showing both $pH3S_{10}$-IR and *c-fos* mRNA expression was determined over the captured images. Measurements were performed placing individual $pH3S_{10}$ nuclei within a circle of fixed diameter and counting the number of silver grains inside the area. Background grain densities were estimated from 20 sampling circles per section per rat in the corpus callosum. Neurons were considered double-labelled when they showed both brown nuclear deposits and a silver grain density exceeding 3 times the background. Isolated silver grain clusters not showing a $pH3S_{10}$ + nucleus were counted as c-fos mRNA single labelled neurons. The single and double labelled neurons per mm^2 were calculated for each particular area and animal as the average number obtained from all analyzed images.

2.7. Statistical analyses

The statistical analysis was performed using the "Statistical Package for Social Sciences" (SPSS, version 17). All samples to be statistically compared were processed in the same assay to avoid inter-assay variability. If needed, data were log-transformed to achieve homogeneity of variances. Dynamics of $pH3S_{10}$-IR was analyzed by two-way ANOVA with treatment (VEHICLE, AMPHETAMINE) and time (30, 90 min) as the main factors. When significant interaction between treatment and time was found, further decomposition of the interaction was done. Double-labelling histochemistry results comparing the effect of the treatment (VEHICLE, AMPHETAMINE) were analyzed by the Student *t*-Test.

3. Results

3.1. Dynamics of histone H3 phosphorylation after amphetamine administration

No evidence for an effect of amphetamine to increase $pH3S_{10}$ positive neurons was found in the mPFC or PVN in any of the times evaluated. Conversely, an amphetamine-induced increase in the number of $pH3S_{10}$ positive neurons was observed in AcbC and AcbSh, ICjM, all subdivisions of CPu and BSTld, but not in the lateral ventral division of the BST. Precise statistical analysis is summarized in Table 1. In the AcbSh the two-way ANOVA revealed significant effects of treatment and time, whereas the interaction treatment by time was not significant (Figs. 1 and 2). In the AcbC, the ICjM and all the subdivisions of the CPu analyzed, the two-way ANOVA revealed a significant effect of treatment; although, an effect of time was observed in AcbC, ICjM, CPuDLa, CPuVLa, CPuVMp, CPuDLp and CPuVLp, but not in CPuVMa and CPuDMp, in all cases a significant interaction treatment by time was observed. Further comparisons showed that in AcbC, ICjM and every CPu subdivision the number of $pH3S_{10}$ positive neurons was elevated in amphetamine treated animals 30 min after injection ($p < 0.001$, all areas). In AcbC, ICjM and in every CPu division, amphetamine administration produced a higher increase in $pH3S_{10}$ positive neurons at 30 min than at 90 min ($p < 0.001$, all areas) (Figs. 1 and 2), although in some of these areas the number of $pH3S_{10}$ positive neurons remained still higher than in vehicle at 90 min post-injection (AcbC, $p < 0.01$; ICjM, $p < 0.05$; CPuVMa, $p < 0.001$; CPuVMp, $p < 0.001$; CPuDLp, $p < 0.05$; CPuVLp, $p < 0.001$). In the BSTld the two-way ANOVA revealed significant effects of treatment, but not of time or the interaction treatment by time (Figs. 1 and 2).

3.2. $pH3S_{10}$ and c-fos mRNA double labelling

Amphetamine administration showed the expected pattern of c-fos induction, with greatly enhanced c-fos signal in cortical areas, including the mPFC (not shown) and the PVN. Representative images of *c-fos* mRNA expression and $pH3S_{10}$ positive neurons after saline or amphetamine administration in selected brain areas can be seen in Fig. 3. In the CPu, both c-fos mRNA and $pH3S_{10}$-IR showed a striosome-matrix induction pattern as described by Graybiel et al. (1990). Note the lack of $pH3S_{10}$-IR in the PVN despite the strong c-fos mRNA expression.

The *t*-tests showed that amphetamine administration increased the number of $pH3S_{10}$ positive neurons in the AcbSh ($t(6) = 3.97$; $p < 0.01$), the AcbC ($t(6) = 22.65$; $p < 0.001$), the ICjM ($t(6) = 11.92$; $p < 0.001$), the CPuVMa ($t(6) = 15.53$; $p < 0.001$), the BSTld ($t(6) = 8.02$; $p < 0.001$) and the CeAm ($t(6) = 5.96$; $p < 0.001$). The *t*-tests also showed an effect of amphetamine in the number of double labelled neurons in the AcbSh ($t(6) = 5.04$; $p < 0.01$), the AcbC ($t(6) = 15.73$; $p < 0.001$), the ICjM ($t(6) = 12.62$; $p < 0.001$), the CPuVMa ($t(6) = 14.03$; $p < 0.001$), the BSTld ($t(6) = 7.99$; $p < 0.001$) and the CeAm ($t(6) = 6.17$; $p < 0.001$) (Fig. 4, panel A). In all these areas, basically all $pH3S_{10}$ positive neurons also showed *c-fos* mRNA expression and therefore data corresponding to the number of $pH3S_{10}$ positive neurons and double labelled neurons were basically identical. Finally, *t*-tests showed that amphetamine administration slightly reduced the number of neurons expressing c-fos mRNA but not $pH3S_{10}$ positive signal in the AcbC ($t(6) = 3.01$; $p < 0.05$), whereas increased them in the ICjM ($t(6) = 4.01$; $p < 0.01$), the BSTld ($t(6) = 7.59$; $p < 0.001$) and the CeAm ($t(6) = 4.48$; $p < 0.01$) (Fig. 4, panel B).

Table 1
Results of the ANOVA of dynamics of histone H3 phosphorylation after amphetamine administration.

Two-way ANOVA:	Treatment		Time		Treatment × Time	
Brain Area	F (1,23)	Significance	F (1,23)	Significance	F (1,23)	Significance
Accumbens nuc Core	54.63	$p < 0.001$	16.70	$p < 0.001$	4.86	$p < 0,05$
Accumbens nuc, Shell	8.86	$p < 0,01$	31.31	$p < 0.001$		NS
Major Island of Calleja	65.21	$p < 0.001$	22.45	$p < 0.001$	20.70	$p < 0.001$
Caudate-Putamen, dorsal-lateral ant	31.53	$p < 0.001$	23.41	$p < 0.001$	25.00	$p < 0.001$
Caudate-Putamen, dorsal-medial ant	18.59	$p < 0.001$	5.68	$p < 0,05$	20.78	$p < 0.001$
Caudate-Putamen, ventral-medial ant	90.48	$p < 0.001$		NS	13.17	$p < 0,01$
Caudate-Putamen, ventral-lateral ant	18.88	$p < 0.001$	12.54	$p < 0,01$	12.39	$p < 0,01$
Caudate-Putamen, dorsal-lateral post	37.33	$p < 0.001$	7.81	$p < 0,05$	8.39	$p < 0,01$
Caudate-Putamen, dorsal-medial post	53.77	$p < 0.001$	49.69	$p < 0.001$	31.16	$p < 0.001$
Caudate-Putamen, ventral-medial post	207.69	$p < 0.001$		NS	54.40	$p < 0.001$
Caudate-Putamen, ventral-lateral post	237.26	$p < 0.001$	15.32	$p < 0.001$	15.32	$p < 0.001$
Bed nuc stria terminalis, lateral dorsal	52.79	$p < 0.001$		NS		NS

Efect of treatment (vehicle, amphetamine) and time (30 min, 90 min) on phosphorilated histone H3 immunoreactivity in several brain areas. NS = Not Significant.

4. Discussion

The present results demonstrate for the first time that amphetamine administration increases the number of neurons showing pH3S$_{10}$-IR in some particular brain areas. The pattern was clearly more restrictive than that of c-fos and appears to be at its maximal level at 30 min after drug administration, with a further progressive decline in most areas. A notable exception was the BSTld where activation was maintained at a high level 90 min after the drug administration. Neurons showing pH3S$_{10}$-IR were always c-fos positive, but the opposite was not true, with the percent of neurons showing colocalization changing dramatically among the different brain areas.

In a first experiment we studied the pattern of pH3S$_{10}$-IR in a wide range of brain regions at 30 and 90 min after amphetamine administration. We found that some areas where a marked induction of immediate early genes (i.e. c-fos) is consistently observed after amphetamine administration, such as the mPFC or the PVN (i.e. Colussi-Mas et al., 2007; Badiani et al., 1998; Engber et al., 1998; Rotllant et al., 2010), showed no increase in the number of pH3S$_{10}$-IR positive neurons either at 30 or 90 min. These data thus clearly confirm our initial hypothesis that the pattern of pH3S$_{10}$-IR is more restricted than that of c-fos expression. In general, the increase in the number of pH3S$_{10}$ positive neurons was

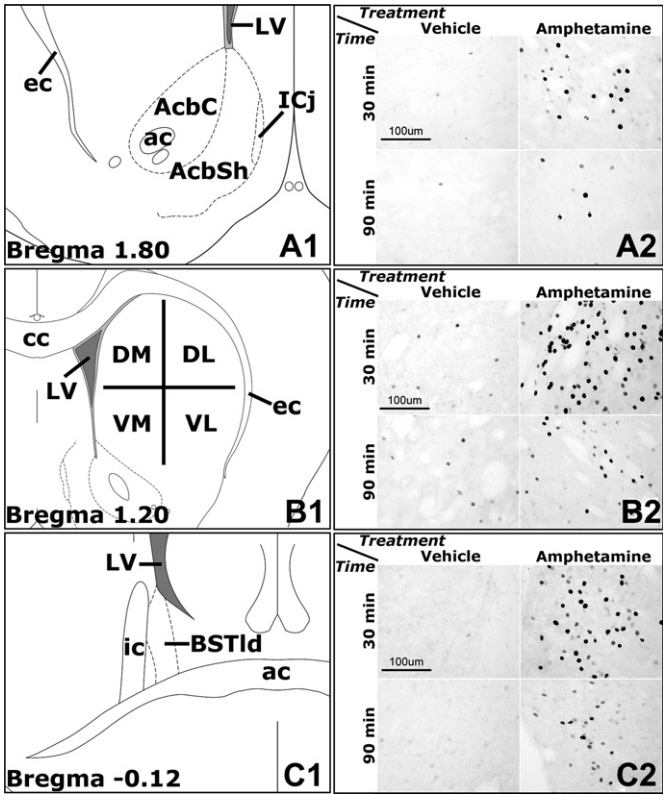

Fig. 1. Representative images of phospho-(Ser-10)-histone H3 (pH3S$_{10}$) immunoreactivity in the core of the nucleus accumbens (panels A1-A2), the ventral-medial part of the Caudate-Putamen (panels B1-B2) and the lateral-dorsal division of the bed nucleus of the stria terminalis (panels C1-C2) induced 30 and 90 min after the administration of a single dose of amphetamine (5 mg/kg) or vehicle. Abbreviations: ac = anterior commissure; AcbC = nucleus accumbens core; AcbSh = nucleus accumbens shell; BSTld = bed nucleus of the stria terminalis, lateral dorsal; cc = corpus callosum; DL = Caudate-Putamen, dorsal-lateral part; DM = Caudate-Putamen, dorsal-medial part; ec = external capsule; ic = internal capsule; ICj = island of Calleja, mayor; LV = lateral ventricle; VL = Caudate-Putamen, ventral-lateral part; VM = Caudate-Putamen, ventral-medial part.

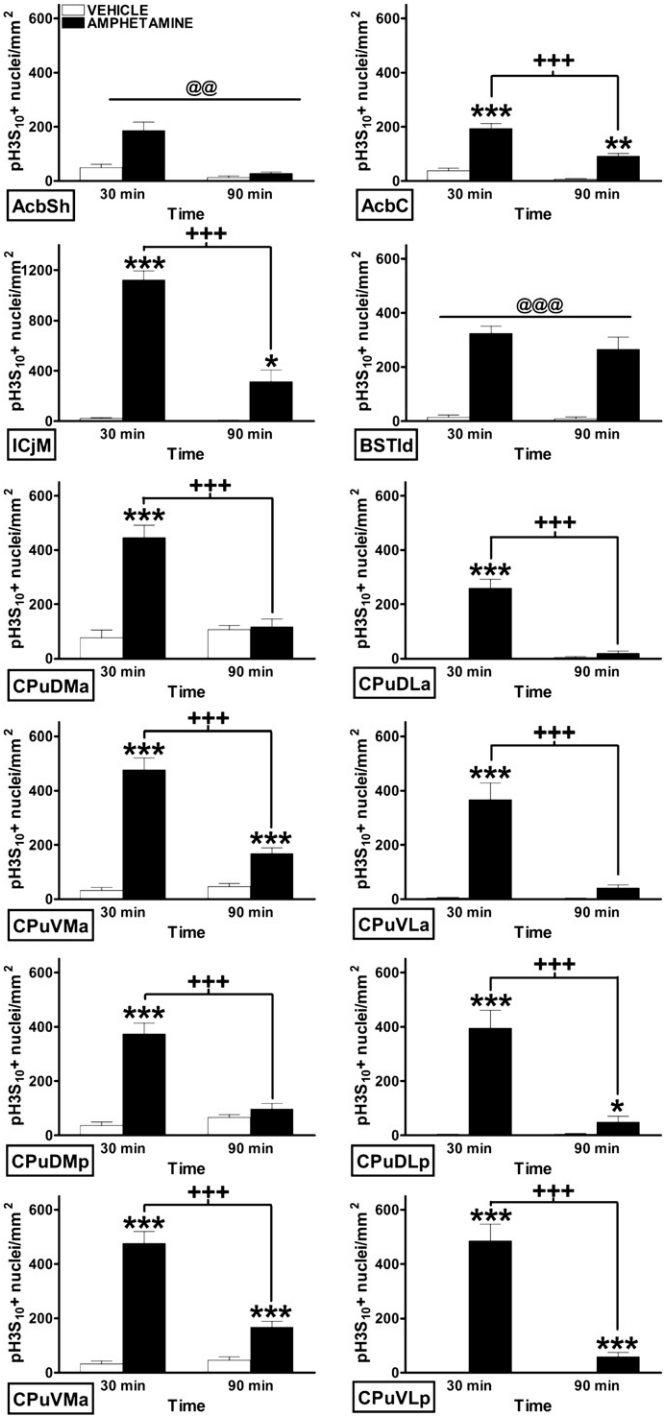

Fig. 2. Dynamics of histone H3 phosphorylation in serine 10 (pH3S$_{10}$) in different brain regions 30 and 90 min after the administration of a single dose of amphetamine (5 mg/kg; $n = 8$ by time) or vehicle ($n = 4$ by time). Means and SEM of the number of nuclei per mm^2 showing positive pH3S$_{10}$ nuclei are represented. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs respective vehicle values. $+++p < 0.001$ 30 min vs 90 min values, between the same treatment. $@@p < 0.01$; $@@@p < 0.001$ amphetamine vs vehicle group, regardless of time. Abbreviations: AcbC = nucleus accumbens core; AcbSh = nucleus accumbens shell; BSTld = bed nucleus of the stria terminalis, lateral dorsal; CPuDLa = Caudate-Putamen, dorsal-lateral part anterior; CPuDLp = Caudate-Putamen, dorsal-lateral part posterior; CPuDMa = Caudate-Putamen, dorsal-medial part anterior; CPuDMp = Caudate-Putamen, dorsal-medial part posterior; CPuVLa = Caudate-Putamen, ventral-lateral part anterior; CPuVLp = Caudate-Putamen, ventral-lateral part posterior; CPuVMa = Caudate-Putamen, ventral-medial part anterior; CPuVMp = Caudate-Putamen, ventral-medial part posterior, ICjM = major island of Calleja.

higher in the striatum than in the Acb, whereas in the BSTld the increase was close to that observed in the striatum. Interestingly, the most dramatic increase in the number of $pH3S_{10}$ positive neurons was observed in the ICjM.

When the time-course of the changes in $pH3S_{10}$-IR was studied, maximum induction was observed at 30 min after amphetamine administration in all brain regions. This corroborates previous results after cocaine administration that focused on striatal regions (Brami-Cherrier et al., 2005). In Acb, CPu and ICjM the pattern of $pH3S_{10}$-IR dramatically decreased 90 min as compared to 30 min after amphetamine, but a notable exception was observed in the BSTld. In this particular area, $pH3S_{10}$-IR was essentially maintained

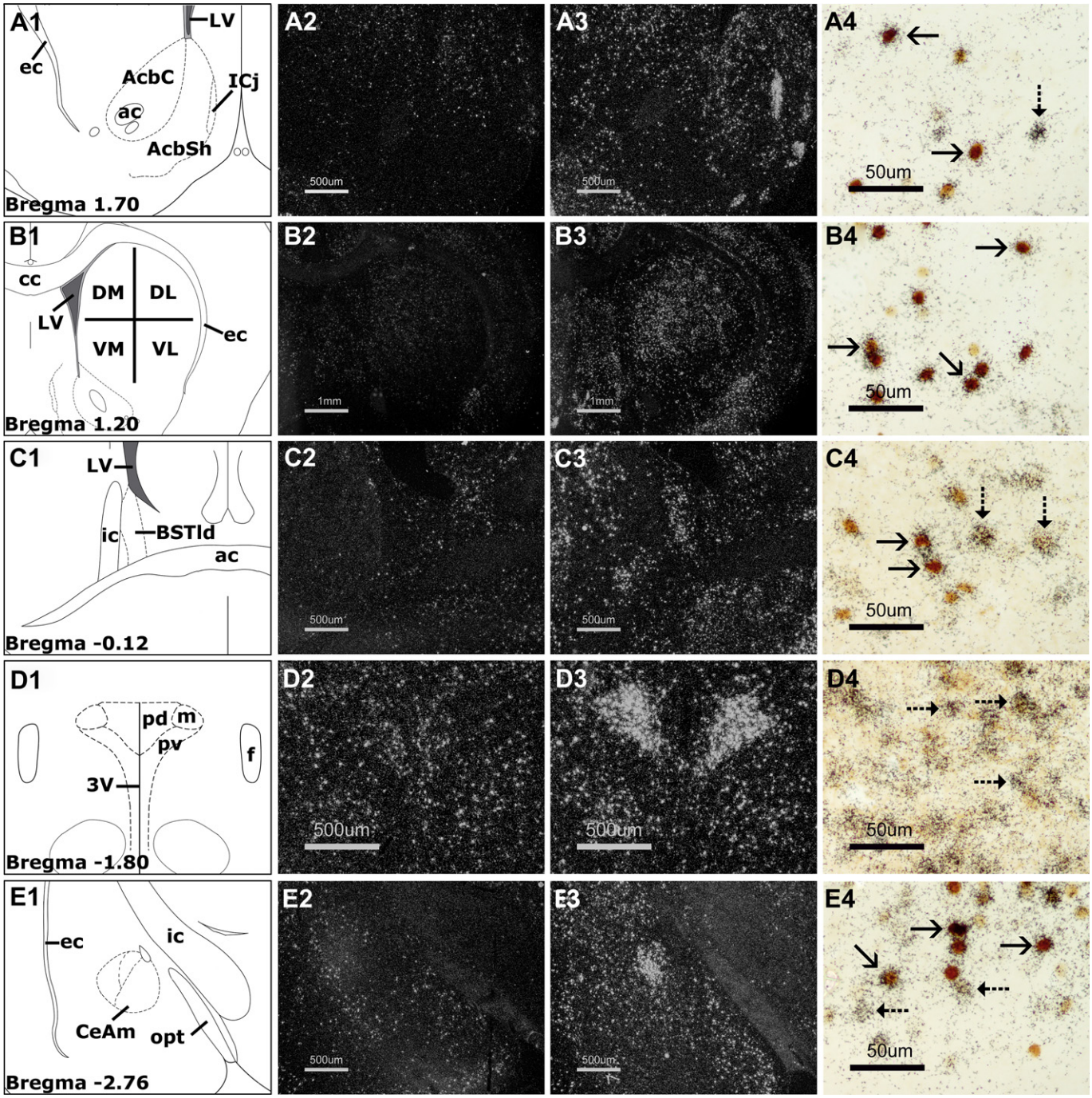

Fig. 3. Representative images showing double labelling histochemistry for c-fos mRNA and phospho-(Ser-10)-histone H3 ($pH3S_{10}$) immunoreactivity in the nucleus accumbens (panels A1-A4), the caudate-putamen (panels B1-B4), the bed nucleus of the stria terminalis (panels C1-C4), the paraventricular nucleus of the hypothalamus (panels D1-D4) and the central amygdala (panels E1-E4). Schematic drawings (panels A1, B1, C1, D1 and E1) and dark-field photomicrographs of c-fos mRNA expression in rats given saline (panels A2, B2, C2, D2 and E2) or amphetamine (panels A3, B3, C3, D3 and E3). Bright-field photomicrographs of the same sections are depicted (panels A4, B4, C4, D4 and E4) showing neurons labelled for both c-fos mRNA and $pH3S_{10}$ (black arrow) or only for c-fos mRNA (broken arrow) in amphetamine treated animals. Note that virtually all the $pH3S_{10}$ + nuclei are also labeled for c-fos mRNA. Abbreviations: 3V = third ventricle; ac = anterior commissure; AcbC = nucleus accumbens core; AcbSh = nucleus accumbens shell; BSTld = bed nucleus of the stria terminalis, lateral dorsal; cc = corpus callosum; CeAm = central amygdala, medial division; DL = Caudate-Putamen, dorsal-lateral part; DM = Caudate-Putamen, dorsal-medial part; ec = external capsule; f = fornix; ic = internal capsule; ICj = island of Calleja, mayor; LV = lateral ventricle; m = paraventricular nucleus of the hypothalamus, magnocellular part; opt = optic tract; pd = paraventricular nucleus of the hypothalamus, parvocellular dorsal part; pv = paraventricular nucleus of the hypothalamus, parvocellular ventral part; VL = Caudate-Putamen, ventral-lateral part; VM = Caudate-Putamen, ventral-medial part.

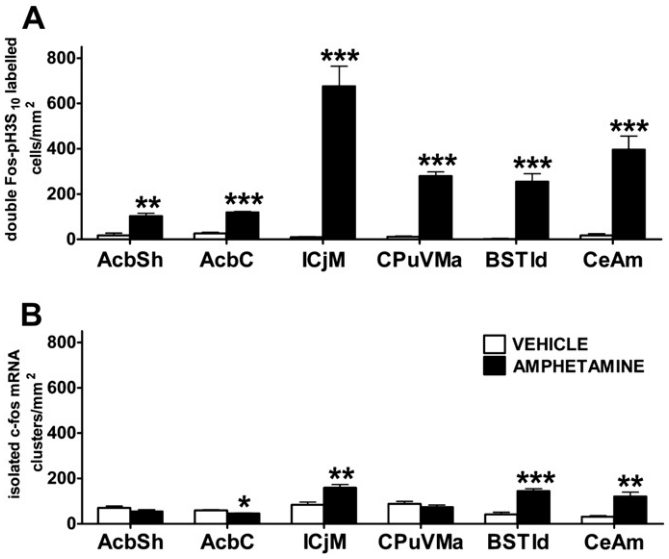

Fig. 4. Phospho-(Ser-10)-histone H3 ($pH3S_{10}$) immunoreactivity and c-fos mRNA expression in different brain regions after amphetamine administration. Graphs show the means and SEM ($n = 4$ Rats per group) of the number of $pH3S_{10}$ + neurons also showing c-fos mRNA expression (panel A) and the number of c-fos silver grain clusters not associated to $pH3S_{10}$ (panel B). *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ vs saline group (VEHICLE), *t*-test. Abbreviations: AcbC = nucleus accumbens core; AcbSh = nucleus accumbens shell; BSTld = bed nucleus of the stria terminalis, lateral dorsal; CeAm = central amygdala, medial division; CPuVMa = striatum, ventral-medial part anterior; ICjM = major island of Calleja.

to the same level 90 min after amphetamine. The time-course of changes in $pH3S_{10}$-IR has not been previously studied after cocaine or any other drug of abuse, but after kainate administration in the hippocampus its induction reaches a maximum at 30 min and dramatically decreases at 3 h (Sng et al., 2006). Therefore, the sustained activation of $pH3S_{10}$ in the BSTld is quite interesting and suggest this area to be an important locus of amphetamine-induced epigenetic changes.

Once characterized the regional pattern and time-course of changes in $pH3S_{10}$-IR, we wanted to determine colocalization with c-fos expression at 30 min after the drug. Amphetamine administration induced the expected pattern of c-fos expression in all cortical and subcortical regions selected for the present study, but a more restricted pattern of changes in $pH3S_{10}$-IR. For instance, no changes in $pH3S_{10}$-IR were observed in the mPFC (data not shown) or the PVN where c-fos induction was marked. In addition, we analyzed changes in the CeAm, where we observed both c-fos expression and a huge increase in the number of $pH3S_{10}$ positive neurons. In those areas showing both c-fos induction and increases in $pH3S_{10}$-IR after amphetamine, colocalization studies revealed that virtually all $pH3S_{10}$ positive neurons also expressed c-fos, but the opposite was not always true: (i) in the striatum and the Acb all neurons that responded to the drug increasing c-fos expression were also $pH3S_{10}$ positive; (ii) in the ICjM, CeAm and BSTld some of them were not; and (iii) in the PVN no evidence for increased $pH3S_{10}$-IR was found despite a marked induction of c-fos.

Although the effects of amphetamine on $pH3S_{10}$-IR have not yet been studied, there are data demonstrating an increase in the number of $pH3S_{10}$ positive neurons after cocaine administration in dorsal and ventral striatum (Bertran-Gonzalez et al., 2008; Brami-Cherrier et al., 2005; Sanchis-Segura et al., 2009; Stipanovich et al., 2008), a process mediated by extracellular signal-regulated kinase (ERK)/mitogen- and stress-activated protein kinase (MSK) pathways and DARPP-32 (Bertran-Gonzalez et al., 2008; Brami-Cherrier et al., 2005; Stipanovich et al., 2008). A critical role of ERK/MSK1 in the induction of $pH3S_{10}$ and c-fos has also been demonstrated in striatal neuron cultures after activation of glutamate receptors (Brami-Cherrier et al., 2007). In vivo, enhanced $pH3S_{10}$-IR was restricted to D1-expressing striatonigral neurons (Bertran-Gonzalez et al., 2008). In the present study, all striatum and Acb neurons expressing c-fos were also $pH3S_{10}$ positive, suggesting common regulatory processes. In fact, it has been demonstrated that kainate administration increased $pH3S_{10}$ and c-fos expression in the hippocampus and this is associated with and increase in $pH3S_{10}$ at the c-fos promoter (Crosio et al., 2003).

The present findings showing that after amphetamine administration all neurons expressing c-fos were also $pH3S_{10}$ positive in dorsal striatum and accumbens support a critical role of these areas in the effects of addictive drugs. Previous work has demonstrated enhanced histone H3 and H4 acetylation in the striatum after acute and chronic cocaine administration that affects the promoter regions of some important genes such as c-fos, fosB, bdnf and cdk5 (Kumar et al., 2005; Levine et al., 2005; Renthal et al., 2008). More particularly, acute cocaine administration increased phosphoacetylation of histone H3 in the promoter of c-fos (Kumar et al., 2005) and chronic amphetamine administration reduced c-fos expression in the striatum through mechanisms that involved ΔFosB-induced recruitment of histone deacetylase 1 and an increase of H3 methylation at the the c-fos promoter (Renthal et al., 2008). These two latter processes result in reduced c-fos transcription. The fact that epigenetic changes have important consequences on behaviour is supported by the findings that histone deacetylase inhibitors (HDACi) decreased initial cocaine self-administration (Romieu et al., 2008), but increased it after longer experience with the drug (Wang et al., 2010). Similarly, HDACi enhances drug-induced locomotor sensitization and place preference (Sanchis-Segura et al., 2009) as well as place preference extinction (Malvaez et al., 2010).

In contrast to the results observed in striatal regions, in ICjM, CeAm and BSTld an important number of neurons expressing c-fos were devoid of $pH3S_{10}$ positive signal. The latter results, together with the fact that in the mPFC and in the PVN the increase in c-fos expression was not accompanied by $pH3S_{10}$-IR, indicate dissociation between both processes. In fact, acute cocaine administration was found to induce $pH3S_{10}$-IR exclusively in D1R positive striatal neurons, whereas induction of c-fos was also observed in some D2R positive neurons (Bertran-Gonzalez et al., 2008), supporting dissociation between both changes. There are two possibilities to explain such dissociation. First, detection of $pH3S_{10}$ by IHC is likely to require simultaneous phosphorylation of H3 histones in an important number of genes; therefore, the present results are compatible with the possibility that in areas such as the mPFC and the PVN amphetamine enhances $pH3S_{10}$ in some neurons, but the modification was restricted to a few genes, for instance c-fos. Second, under some circumstances, activation of c-fos expression may not be associated with enhanced $pH3S_{10}$. In this regard, Aurora kinase has been found to participate in $pH3S_{10}$. In the pineal gland, inhibition of Aurora kinase markedly reduced norepinephrine-induced $pH3S_{10}$ while increasing c-fos expression (Price et al., 2009), suggesting alternative pathways for the control of c-fos. Further studies using chromatin immunoprecipitation procedures to directly demonstrate a putative region-dependent $pH3S_{10}$ in the c-fos promoter are needed.

If we assume that histone modifications may reveal brain areas and neurons where plastic changes associated to amphetamine are more marked, evaluation of changes in $pH3S_{10}$ and other chromatin remodelling processes may be valuable to more precisely identify some of the critical brain areas and neurons associated to addictive drugs and other stimuli. In this regard, evidence for a critical role of striatum, Acb, CeAm and BSTld in amphetamine-induced

conditioned place preference and self-administration is compelling (Chevrette et al., 2002; Hsu et al., 2002; O'Dell et al., 1999). Lack of $pH3S_{10}$ increase in the mPFC after amphetamine may be at first sight surprising, but there is negative evidence for a role of mPFC on amphetamine-induced conditioned place preference and self-administration (Tzschentke, 2000), suggesting a less important role of the mPFC as compared with other brain areas. It may be of interest to study to what extent the brain pattern of $pH3S_{10}$ caused by cocaine differs from that of amphetamine. It is also noteworthy that the highest induction of c-fos and increases in $pH3S_{10}$ positive neurons were found in the ICjM. This ventral striatal region (Fallon et al., 1978) is a poorly studied structure that is greatly activated by different types of addictive drugs, including amphetamine derivatives (Moorman and Leslie, 1996; Stephenson et al., 1999) and appear to be involved in rewarding effects of electrical self-stimulation (Nakajima and Patterson, 1997) and sexual attraction (Lanuza et al., 2008). Our present data give support to an important role of ICjM in reward-related behaviours.

In conjunction with other previous data, the results presented herein reveal a much more restricted pattern of changes in $pH3S_{10}$ positive neurons as compared to the induction of c-fos after amphetamine administration, thus supporting the hypothesis that changes in histones may affect a lower number of regions and neurons than changes in c-fos expression. Identification of $pH3S_{10}$ positive neurons may give valuable information about an important role of certain brain areas in the longer-lasting effects of the drug. However, we cannot rule out that amphetamine administration is inducing other important yet not characterized histone modifications in areas such as the mPFC and the PVN.

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