EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 31, pp. 2043–2052, 2010 doi:10.1111/j.1460-9568.2010.07242.x

BEHAVIORAL NEUROSCIENCE

Immediate-early gene response to repeated immobilization: Fos protein and *arc* mRNA levels appear to be less sensitive than *c-fos* mRNA to adaptation

Sheila Ons,* David Rotllant,* Ignacio J. Marín-Blasco and Antonio Armario
Department of Cellular Biology, Physiology and Immunology, Institute of Neuroscience and Animal Physiology Unit, School of Biosciences, Autonomous University of Barcelona, Bellaterra, Barcelona, Spain

Keywords: adaptation, chronic stress, paraventricular hypothalamic nucleus, rat

Abstract

Stress exposure resulted in brain induction of immediate-early genes (IEGs), considered as markers of neuronal activation. Upon repeated exposure to the same stressor, reduction of IEG response (adaptation) has been often observed, but there are important discrepancies in literature that may be in part related to the particular IEG and methodology used. We studied the differential pattern of adaptation of the IEGs *c-fos* and *arc* (activity-regulated cytoskeleton-associated protein) after repeated exposure to a severe stressor: immobilization on wooden boards (IMO). Rats repeatedly exposed to IMO showed reduced *c-fos* mRNA levels in response to acute IMO in most brain areas studied: the medial prefrontal cortex (mPFC), lateral septum (LS), medial amygdala (MeA), paraventricular nucleus of the hypothalamus (PVN) and locus coeruleus. In contrast, the number of neurons showing Fos-like immunoreactivity was only reduced in the MeA and the various subregions of the PVN. IMO-induced increases in *arc* gene expression were restricted to telencephalic regions and reduced by repeated IMO only in the mPFC. Double-labelling in the LS of IMO-exposed rats revealed that *arc* was expressed in only one-third of Fos+ neurons, suggesting two populations of Fos+ neurons. These data suggest that *c-fos* mRNA levels are more affected by repeated IMO than corresponding protein, and that *arc* gene expression does not reflect adaptation in most brain regions, which may be related to its constitutive expression. Therefore, the choice of a particular IEG and the method of measurement are important for proper interpretation of the impact of chronic repeated stress on brain activation.

Introduction

When repeatedly exposed to the same (homotypic) stressor, animals often show a progressive reduction of some physiological responses, that of plasma adrenaline and adrenocorticotropic hormone (ACTH) being particularly consistent (Martí & Armario, 1998). Because the response to stressors is orchestrated by the brain, a lower impact of repeated exposure to a stressor on the brain is expected. Thus, repeated exposure to the same stressor results in lower levels of *c-fos* mRNA levels, an immediate-early gene (IEG) widely used as a marker of neuronal activation, although the extent to which this reduction is widespread or restricted to particular brain regions appears to depend on the characteristics and length of exposure to the stressors (Melia *et al.*, 1994; Bonaz & Rivest, 1998; Kollack-Walker *et al.*, 1999; Campeau *et al.*, 2002; Girotti *et al.*, 2006). The paraventricular nucleus of the hypothalamus (PVN), an area critical for the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, is one of the areas showing a more consistent reduction of *c-fos* expression (Umemoto *et al.*, 1994a,b, 1997; Watanabe *et al.*, 1994; Bonaz & Rivest, 1998; Kollack-Walker *et al.*, 1999; Carter *et al.*, 2004; Girotti *et al.*, 2006), in accordance with the consistent adaptation of ACTH. However, adaptation is less consistent and involves a lower number of brain areas when Fos-like protein instead of *c-fos* mRNA levels has been studied (for review, see Armario, 2006).

Not all IEGs are equally sensitive to adaptation. Whereas widespread brain reduction of *c-fos* and other IEGs mRNA levels was observed after repeated restraint, no reduction at all (Melia *et al.*, 1994; Umemoto *et al.*, 1994a, 1997) or a lower reduction (Girotti *et al.*, 2006) has been observed with *zif-268*. Because *zif-268* is constitutively expressed in some brain areas such as the cortex (i.e. Cullinan *et al.*, 1995), it is possible that IEGs that are constitutively expressed may be less sensitive to adaptation than those that are not. Within this framework, we are interested in *arc*, an IEG that is constitutively expressed in some brain areas, codes for a protein that exhibits similarities to spectrin, and is involved in synaptic plasticity and learning. We and others have demonstrated that *arc* expression is activated in telencephalic areas after exposure to several stressors (Ons *et al.*, 2004; Trneckova *et al.*, 2007; Kozlovsky *et al.*, 2008). However, whether or not *arc* expression is sensitive to adaptation to repeated stress is not known.

Correspondence: Dr A. Armario, as above.
E-mail: Antonio.Armario@uab.cat

*S.O. and D.R. contributed equally to this work.

Received 8 April 2009, revised 25 March 2010, accepted 25 March 2010

Thus, the present work tested three main hypotheses. First, the degree of IEG adaptation may be greater in those areas whose IEG expression after stress is related to the intensity of stressors, assuming that adaptation implies lower stimulatory inputs to the latter areas. In the remaining areas, the stimulatory inputs are not related to the degree of emotional reaction to the situation and therefore they would not reduce IEG expression after repeated stress. Second, *c-fos* mRNA levels may better reflect adaptation to chronic repeated stress than Fos-like protein. Third, the constitutively expressed *arc* gene may be less sensitive to adaptation than *c-fos*.

Materials and methods

Animals

Male, 2-month-old, Sprague–Dawley rats supplied by the animal service of the Autonomous University of Barcelona were used. The animals were housed two per cage under standard temperature conditions (22 ± 1°C), and maintained on a 12 h light: 12 h dark schedule (lights on at 07:00 h) with *ad libitum* access to food and water. The animals were allowed to acclimatize to the housing conditions for at least 1 week before beginning the experimental treatments, which were carried out in the morning. 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 the Ethical Committee for Animal Experimentation of the Autonomous University of Barcelona.

Rationale for the selection of the stressor and the brain areas studied

We studied changes in IEGs after chronic repeated exposure to a severe stressor, immobilization on wooden boards (IMO), because adaptation of the HPA axis to this stressor has been consistently found (Martí & Armario, 1998). In addition, we used a protocol previously found to result in adaptation of the ACTH response (Marquez *et al.*, 2004). We chose to study several brain areas that are critically involved in the control of the response to stress (Herman *et al.*, 2003) and are representative examples of those areas sensitive to the intensity of stressors [medial amygdala (MeA), lateral septum (LS), PVN and locus coeruleus (LC)], and those that are not [medial prefrontal (mPFC) and piriform (Pir) cortices] in terms of *c-fos* expression (Campeau *et al.*, 2002; Ons *et al.*, 2004; Pace *et al.*, 2005).

General procedure

Experiment 1

Animals were randomly assigned to controls (CONT), handled every day for 11 days ($n = 8$), or chronic immobilization (IMO12; $n = 7$), immobilized 1 h daily for 11 days by taping their four limbs to metal mounts attached to a wooden board (Marti *et al.*, 2001). Head movements were restricted with two metal loops around the neck. On the last experimental day (Day 12), all animals were immobilized for 1 h. Blood samples (300 μL) were taken by tail-nick procedure before IMO (basal levels, BAS), immediately after IMO and at 30, 60 and 90 min after the end of IMO (R30, R60 and R90). Blood samples were collected in ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain), and the plasma obtained after centrifugation was stored at −20°C until further analysis of ACTH and corticosterone by radioimmunoassay (RIA).

Experiment 2

Animals were divided into CONT ($n = 20$) and IMO12 ($n = 20$) groups, and treated as in Experiment 1. On the last experimental day (Day 12), rats were assigned to the various acute treatments: BAS, left undisturbed until perfusion ($n = 6$ for each group), IMO, immobilized for 1 h and immediately perfused ($n = 7$ for each group), and IMO + 1 h, immobilized for 1 h and returned to their home cages for one additional hour before perfusion ($n = 7$ for each group). We chose these particular time points because both *c-fos* and *arc* mRNA levels achieve a maximum after 1 h of exposure to IMO (Trneckova *et al.*, 2007), and *c-fos* mRNA levels have been found to be sensitive to a single previous experience with IMO in the PVN and other brain areas at 1 h post-IMO (Vallès *et al.*, 2006). Before perfusion, rats were anaesthetized with 80 mg/kg of ketamine (Merial Laboratories, Barcelona, Spain) and 10 mg/kg of xylazine (Bayer, Barcelona, Spain) administered i.p. Then, they were transcardially perfused firstly with sterilized saline solution (0.9% NaCl, 4°C) for 2 min, and then with 4% paraformaldehyde (PFA) + Borax for 8–10 min (4°C). After perfusion, brains were removed, submerged in PFA + Borax, and stored at 4°C for 24 h. Then, they were cryoprotected for 48 h with a solution containing 30% sucrose in potassium phosphate-buffered saline (0.2 M NaCl, 43 mM potassium phosphate, KPBS). The brains were then frozen at −80°C until 30-μm coronal sections were obtained with a cryostat. Sections were collected in cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethyleneglycol, 20% glycerol) and stored at −20°C. Fos immunohistochemistry (IHC) as well as *in situ* hybridization histochemistry (ISHH) of *c-fos* and *arc* were performed on different sections from the same animals. Fos-like immunoreactivity (FLI) was visualized in free-floating sections, whereas ISHH sections were previously mounted on poly-L-lysine-coated slides.

Experiment 3

To study colocalization of *arc* mRNA and FLI, a pilot experiment was performed. Rats were perfused under non-stressful conditions (BAS, $n = 2$) or immobilized for 1 h and returned to their home cages for one additional hour before perfusion (IMO + 1 h, $n = 2$). This time point allows to obtain maximum levels of protein *c-fos* and an almost maximum level of *arc* mRNA level in the LS (Trneckova *et al.*, 2007), thus optimizing the conditions for detecting double-labelling. Perfusion and further brain processing were performed as described for Experiment 2.

RIA

Plasma ACTH and corticosterone levels were determined by double-antibody RIA, as previously described (Marquez *et al.*, 2006). In brief, ACTH RIA used ^{125}I-ACTH (GE Healthcare, Cerdanyola, Spain) as the tracer, rat synthetic ACTH 1–39 (Sigma) as the standard and an antibody raised against rat ACTH kindly provided by Dr W.C. Engeland (Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA). Corticosterone RIA used ^{125}I-carboximethyloxime-tyrosine-methyl ester (GE Healthcare), synthetic corticosterone (Sigma) as the standard and an antibody raised in rabbits against corticosterone–carboximethyloxime–bovine serum albumin (BSA) kindly provided by Dr G. Makara (Institute of Experimental Medicine, Budapest, Hungary). We followed the RIA protocol recommended by Dr G. Makara (plasma corticosteroid-binding globulin was inactivated by low pH; Bagdy & Makara, 1995). The intra-assay coefficient of variation was 6.0%. The sensitivity of the assay was 17 pg/mL for ACTH and 0.05 μg/dL for corticosterone.

ISHH

Plasmids and probes 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; Van Beveren *et al.*, 1983), subcloned into pBluescript SK-1 (Stratagene, La Jolla, CA, USA) and linearized with SmaI. *arc* probe was generated from the full-length rat *arc* cDNA (Lyford *et al.*, 1995), subcloned into EcoRI-XhoI site of pBluescriptII SK+ (Stratagene), and linearized with EcoRI. The *c-fos* and *arc* plasmids were generously provided by Dr S. Rivest (Laval University, Quebec, Canada, for previous work with this probe see Rivest & Rivier, 1994) and Dr P.F. Worley (John Hopkins University, Baltimore, MD, USA, for previous work with this probe see Lyford *et al.*, 1995), respectively. Radioactive anti-sense cRNA copies were generated using a transcription kit (Promega, Madison, WI, USA). Once digested, linearized plasmids were incubated in a transcription buffer (in mM: Tris–Cl, 40, pH 7.9; $MgCl_2$, 6; spermidine, 2; NaCl, 10), 10 mM dithiothreitol (DTT), 0.2 mM GTP/ATP/CTP, 200 μCi [α^{-35}S] UTP (specific activity > 1000 Ci/mmol; GE Healthcare), 40 U RNasin and 20 U of T7 RNA polymerase for 60 min at 37°C. The DNA template was digested with RNase-free DNase (Promega; 1 U DNase in 0.25 μg/μL tRNA and 9.4 mM Tris/9.4 mM $MgCl_2$) and extracted with phenol–chloroform–isoamylalcohol 25 : 24 : 1. The cRNA was precipitated with the ammonium acetate method, re-suspended in 10 mM Tris/1 mM EDTA, pH 8.0, and stored at −20°C.

General protocol

The protocol used for ISHH was adapted from Simmons *et al.* (1989). All the solutions were pre-treated with diethylpyrocarbonate (DEPC) and sterilized before use. Sections were post-fixed in 4% PFA + Borax rinsed in KPBS, digested with protease K (Roche, Sant Cugat del Vallès, Spain; 0.01 mg/mL in 100 mM Tris–HCl pH 8.0 and 50 mM EDTA pH 8), rinsed in DEPC-treated water and 0.1 triethanolamine pH 8.0 (TEA) and acetylated in 0.25% acetic anhydrous in 0.01 M TEA. Finally, they were washed in 2× saline-sodium citrate (SSC), dehydrated through a graded concentration of ethanol and then air-dried. Thereafter, 90 μL of hybridization buffer (50% formamide, NaCl 0.3 M, Tris–Cl 10 mM pH 8.0, EDTA 1 mM pH 8.0, 1× Denhardts, 10% dextrane sulphate, yeast tRNA 500 μg/mL and 10 mM DTT) containing the labelled probe (1×10^6 cpm/90 μL) were spotted onto each slide and sealed with a coverslip. Sections were incubated for 16–18 h in a humid chamber at 60°C. After hybridization, the slides were washed in 4× SSC containing 1 mM DTT, digested with RNase A (GE Healthcare; 0.02 mg/mL in 0.5 M NaCl, 10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0), washed in descending concentrations of SSC containing 1 mM DTT, dehydrated through a series of ethanol solutions (50, 70, 95 and 2 × 100%) and air-dried. The slides were then exposed to a XAR-5 Kodak Biomax MR autoradiography film (Kodak) for 24–72 h, depending on the intensity of the signal in each zone. That is, exposure time varied in function of the IEG and the brain area studied in order to be within the linear portion of the intensity of radioactive signal–optical density curve, as evaluated with the ^{14}C micro-scales (GE Healthcare). After developing the films, the slides were counterstained with 0.25% thionin for histological control.

IHC

Endogenous peroxidase was blocked with a solution of 2% H_2O_2, 80% methanol in KPBS, and non-specific binding was reduced by incubating with a blocking solution containing 0.04% Triton X-100 and 3% non-fat milk in KPBS. Sections were incubated in a polyclonal rabbit antiserum (Sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 500 for 2 h at 37°C. Then, sections were incubated with an anti-rabbit IgG labelled with biotin (Southern Biotechnology, Birmingham, AL, USA) at a dilution of 1 : 200, 1 h incubation at room temperature. Finally, streptavidin labelled with horseradish peroxidase (Southern Biotechnology) at a dilution of 1 : 400 was added and the incubation maintained for 1 h at room temperature. The *c-fos* 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 coverslipped with DPX (Electron Microscopy Sci., Washington DC, USA). The Sc-52 antiserum was raised against an epitope of 14 amino acids located within the first 50 amino acids of the Fos protein. There is no significant sequence homology between Fos protein epitope used and other members of the Fos family (FosB, Fra1 and Fra2), and incubation with the peptide that contains the same sequence of the epitope (Sc-52p; Santa Cruz Biotechnology) eliminated all brain FLI.

Combined IHC and ISHH

Combined immunoperoxidase and isotopic hybridization histochemical localization for FLI and *arc* mRNA, respectively, was performed using a modification of protocols described by Chan *et al.* (1993). Sections were first processed for *c-fos* protein detection, placing the tissue directly in the primary antibody (Sc-52; Santa Cruz Biotechnology, dilution 1 : 500) in blocking solution (0.04% Triton X-100, 2% BSA and 0.25% heparin in KPBS) overnight at 4°C. After that, we used the protocol for IHC described above, revealing the *c-fos* antibody–peroxidase complex with diaminobenzidine. Additionally, all solutions were DEPC treated to prevent RNA degradation. Sections were mounted onto poly-L-lysine-coated slides and vacuum-dried. ISHH for *arc* mRNA detection was performed using the standard protocol described above. After autoradiography film exposure, slides were defatted in xylenes and dipped in LM-1 nuclear emulsion (GE Healthcare). Slides were exposed for 60 days and developed in D19 developer (Kodak) for 3.5 min at 14°C, dehydrated with ethanol series and coverslipped with DPX (Electron Microscopy Sci.).

Image analysis

The analyses of *c-fos* mRNA, FLI, *arc* mRNA and double-labelling histochemistry were conducted in the same coordinates for each particular area using the stereotaxic atlas of Paxinos & Watson (1998) as a reference. The cingulate, (Cg), prelimbic (PrL), infralimbic (IL) and Pir cortices were quantified between bregma 3.20 and 2.70 mm. The LS was quantified between bregma 1.20 and 0.20 mm. All the PVN divisions were quantified between bregma −1.70 and −1.90 mm. The MeA and basolateral (BLA) amygdala were both quantified between −2.56 and −2.80 mm. Finally, the LC was quantified between bregma −9.30 and −10.04 mm. Sections out of the defined coordinates for a particular area were not included, and in some cases some sections were lost for technical reasons. Then, the number of animals per area and group varied from five to six for the BAS animals, and from five to seven for the IMO rats (IMO and IMO + 1 h).

ISHH

Densitometric analyses were done on the autoradiography films. The mRNA levels were semi-quantitatively determined by measuring the optical density and the number of pixels in defined areas with a Leica Q 500 MC system. The average of three–four different sections per

area of interest and animal (two–three in the case of the PVN), each one including both hemispheres, were used for the analysis. *c-fos* and *arc* mRNA values are expressed in arbitrary units (number of pixels × optic density).

c-fos *IHC*

Grey-scale images were taken with a digital camera (NIKON, DMX 1200) coupled to a bright-field microscope (NIKON, Eclipse E400), using a 10× or 40× lens, with no further modifications. The average of three–four different sections per area of interest and animal (two–three in the case of the PVN), each one including both hemispheres, was used for the analysis. To select *c-fos*+ nuclei as targets for quantification, a 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 *c-fos*+ nuclei from measurement; therefore, only particles with a minimum area of 100 pixels were considered as a cell nucleus. The FLI nuclei per mm^2 were calculated as the average number of all analysed images.

Double-labelling histochemistry

Four sections were used for the analysis (including both hemispheres). The number of cell nuclei showing FLI and the number of cells showing both FLI and *arc* mRNA expression were determined by a blind experimenter over the captured images (NIKON, DMX 1200 – Eclipse E400 system). Measurements were performed placing individual FLI cells 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. Cells were considered double-labelled when they showed both brown nuclear deposits and a silver grain density exceeding three times the background (Herman, 1995; Kovács & Sawchenko, 1996).

Statistical analysis

All samples to be statistically compared were processed in the same assay to avoid inter-assay variability. Where necessary, data were log-transformed to achieve homogeneity of variances. Plasma ACTH and corticosterone data were analysed by mixed ANOVA, with treatment (CONT, IMO12) as the between-subject factor, and blood sampling time (BAS, IMO, R30, R60 and R90) as the within-subject factor. *arc* ISHH data were analysed by two-way ANOVA, with chronic treatment (CONT, IMO12) and time (BAS, IMO, IMO + 1 h) as the main factors. In the case of *c-fos* ISHH data, both BAS groups (corresponding to CONT and IMO12) were not included in the statistical analysis as basal levels were usually low in these animals. Due to the time course of protein expression and the low basal levels, FLI was analysed by *t*-test comparing CONT vs. IMO12 only at IMO + 1 h. If interaction between the two main factors was found to be significant with ANOVA in any variable, further decomposition of the interaction was done.

Results

Plasma ACTH and corticosterone

The ANOVA of plasma ACTH levels (Fig. 1) revealed effects of treatment ($F_{1,13} = 36.5$, $P < 0.001$), sampling time ($F_{4,52} = 67.3$, $P < 0.001$) and the interaction treatment by sampling time

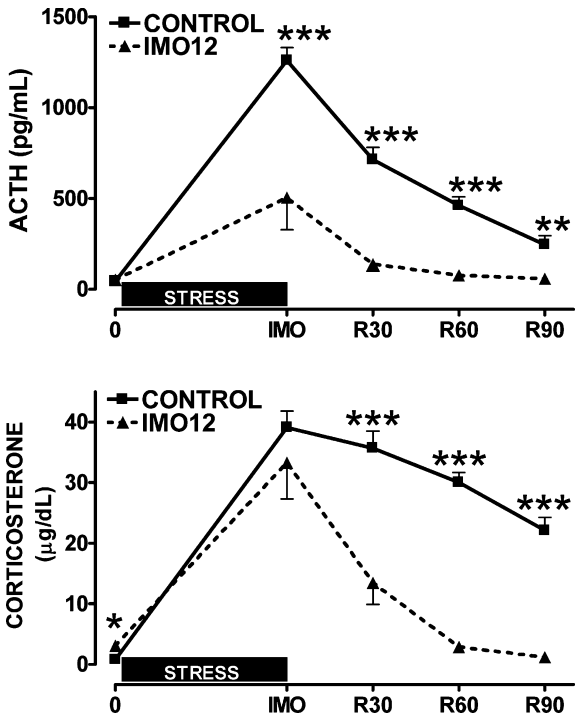

FIG. 1. Plasma adrenocorticotropic hormone (ACTH) and corticosterone response to acute immobilization on wooden boards (IMO) on Day 12 in both stress-naive (CONT, $n = 8$) rats and rats previously exposed to IMO for 11 days (IMO12, $n = 7$). Means and SEM are represented. Rats were blood sampled before IMO (0), just after IMO and at 30, 60 and 90 min after the termination of IMO (R30, R60 and R90, respectively); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. respective control values.

($F_{4,52} = 14.5$, $P < 0.001$). Further decomposition of the interaction showed that ACTH levels of IMO12 and CONT groups differed just after IMO ($P < 0.001$) and at all times post-IMO: R30 ($P < 0.001$), R60 ($P < 0.001$) and R90 ($P < 0.01$), but not under basal conditions. The ANOVA of plasma corticosterone levels (Fig. 1) revealed effects of treatment ($F_{1,13} = 41.4$, $P < 0.001$), sampling time ($F_{4,52} = 60.3$, $P < 0.001$) and the interaction treatment by sampling time ($F_{4,52} = 14.0$, $P < 0.001$). Further decomposition of the interaction showed that corticosterone levels of IMO12 and CONT groups differed under basal conditions (higher levels in IMO12 group, $P < 0.05$), were similar just after IMO and lower in the IMO12 group at all time post-IMO: R30 ($P < 0.001$), R60 ($P < 0.001$) and R90 ($P < 0.001$).

c-fos *mRNA levels*

Whereas a low or non-detectable signal for *c-fos* mRNA was observed throughout the brain in basal conditions, a widespread induction of *c-fos* mRNA was observed after exposure to IMO stress in all major brain regions from the cerebral cortex to the medulla, including the cerebellum (Fig. 2). Quantification was only done in a few selected brain areas for the reasons outlined in the Materials and methods.

The two-way ANOVA revealed a similar behaviour of *c-fos* mRNA levels in the three regions of the mPFC. Thus, significant effects of chronic treatment were found in Cg ($F_{1,19} = 7.4$, $P < 0.05$), PrL ($F_{1,20} = 5.0$, $P < 0.05$) and IL ($F_{1,19} = 7.9$, $P < 0.05$) cortices, with no effects of time or the interaction chronic treatment by time (Fig. 3), indicating that previous exposure to IMO resulted in lower *c-fos* mRNA levels after acute challenge with the same stressor. In the Pir cortex there was no effect of either chronic treatment or time.

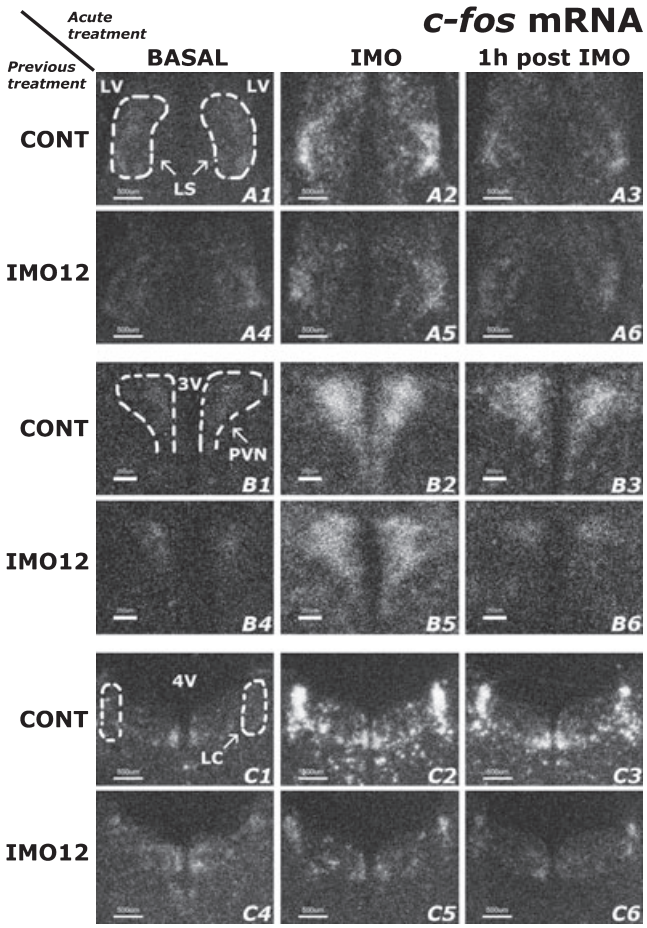

FIG. 2. Representative photomicrographs of brain film autoradiographies of *c-fos* mRNA in the lateral septum (A1–6), the paraventricular nucleus of the hypothalamus (B1–6) and the locus coeruleus (C1–6) for the six experimental groups: stress-naive rats killed under resting conditions (CONT–BASAL: A1, B1, C1); stress-naive rats killed immediately after 1 h exposure to immobilization on wooden boards (IMO) (CONT–IMO: A2, B2, C2); stress-naive rats killed 1 h after the termination of IMO (CONT–1 h post IMO: A3, B3, C3); chronic IMO rats killed under resting conditions (IMO12–BASAL: A4, B4, C4); chronic IMO rats killed immediately after 1 h exposure to IMO (IMO12–IMO: A5, B5, C5); and chronic IMO rats killed 1 h after the termination of IMO (IMO12–1 h post-IMO: A6, B6, C6). Abbreviations: 3V, third ventricle; 4V, fourth ventricle; LC, locus coeruleus; LS, lateral septum; LV, lateral ventricle; PVN, paraventricular nucleus of the hypothalamus.

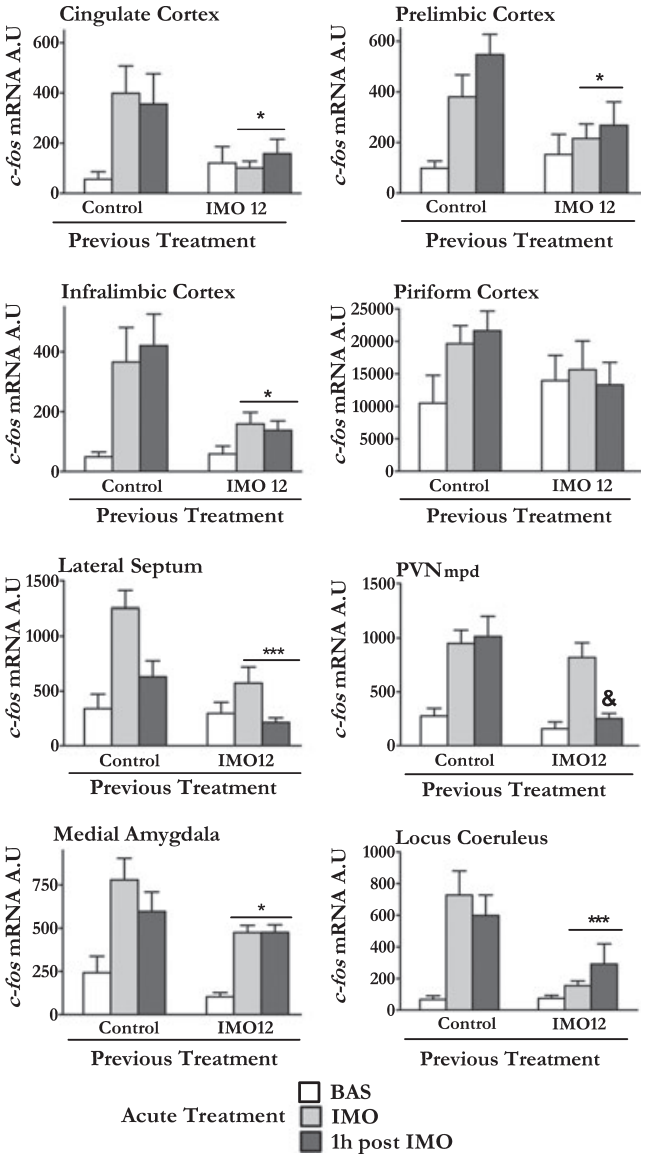

FIG. 3. *c-fos* mRNA levels in the different brain areas analysed. Both stress-naive (CONT) rats and rats daily exposed to immobilization on wooden boards (IMO) for 11 days (IMO12) were killed on Day 12 under resting conditions (BAS, $n = 5–6$ for each group), immediately after 1 h of IMO (IMO, $n = 5–7$ for each group) or 1 h after the termination of IMO (1 h post-IMO, $n = 5–7$ for each group). Means ± SEM are represented. Due to the low basal *c-fos* mRNA levels, only the IMO and the 1 h post-IMO data from both groups (CONT and IMO12) were included in the statistical analysis (see text); $^{\&}P < 0.001$; $^{*}P < 0.05$; $^{***}P < 0.001$ vs. corresponding acute stress condition in control rats. PVNmpd, paraventricular hypothalamic nucleus, medial parvocellular, dorsal part.

In the LS, the ANOVA revealed that previous exposure to IMO significantly reduced the *c-fos* response to acute IMO ($F_{1,24} = 17.9$, $P < 0.001$) and *c-fos* levels declined over time ($F_{1,24} = 12.3$, $P < 0.01$), whereas the interaction chronic treatment by time was not significant (Fig. 3). In the MeA (Fig. 3), a significant effect of previous IMO ($F_{1,23} = 5.9$, $P < 0.05$), but not of time, was found.

Figure 4 shows delineation of the main subdivisions of the PVN where *c-fos* mRNA levels and FLI were measured. In the former case, mRNA levels were quantified in the medial parvocellular dorsal part of the PVN (PVNmpd), and the ANOVA revealed a significant effect of chronic IMO ($F_{1,24} = 11.1$, $P < 0.01$), non-significant effect of time and a significant interaction chronic IMO by time ($F_{1,24} = 5.6$, $P < 0.05$; Fig. 3). Further comparisons showed that *c-fos* mRNA levels were similar in CONT and chronic IMO groups as measured just after IMO, but lower mRNA levels were found in chronic IMO rats at IMO + 1 h ($P < 0.001$), indicating a faster post-IMO decline of *c-fos* expression in chronic IMO rats (Fig. 3). In the LC, the ANOVA revealed that chronic IMO resulted in lower *c-fos* mRNA levels after exposure to IMO ($F_{1,21} = 16.9$, $P < 0.001$), but no effect of time or the interaction chronic IMO by time was found (Fig. 3).

FLI

FLI in the BAS groups was relatively low and not altered by chronic IMO (Fig. 4). No effect of chronic IMO was observed in any of the four cortical regions studied (Fig. 5). Similarly, the effect in the LS did not reach statistical significance ($P = 0.096$). In contrast, chronic IMO resulted in lower levels of FLI in the MeA ($t_{11} = 2.42$, $P < 0.05$), as

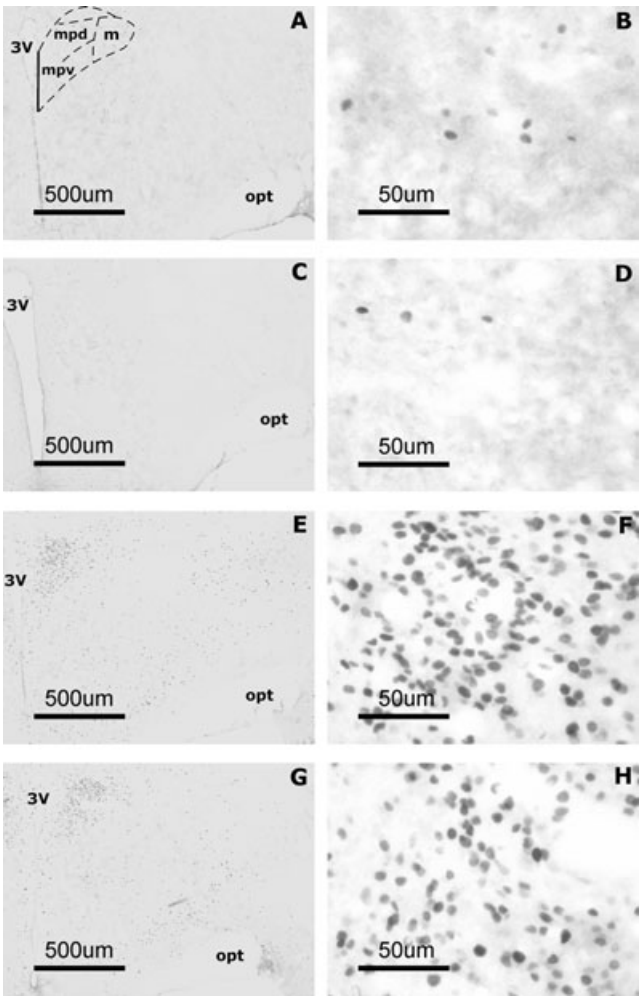

FIG. 4. Representative images (4× and 40×) of Fos+ neurons in the PVN of both stress-naive (CONT) and chronic IMO rats (IMO12) killed on Day 12 under resting conditions (BAS) or 1 h after the termination of IMO (1 h post-IMO). The groups are as follows: CONT–BAS (A and B), IMO12–BAS (C and D), CONT–1 h post-IMO (E and F) and IMO12–1 h post-IMO (G and H). (A) The main subdivisions can be seen in which FLI was measured (m, magnocellular; mpd, medial dorsal parvocellular; mpv, medial ventral parvocellular); 3V, third ventricle; opt, optic tract.

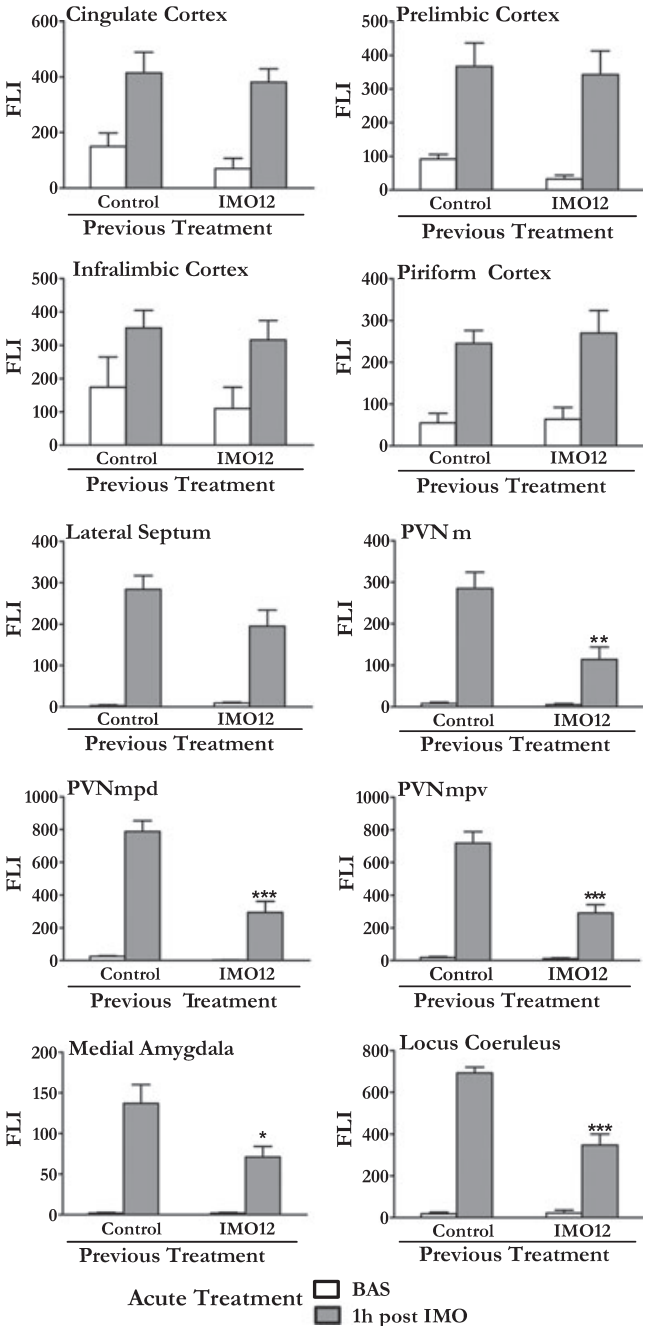

FIG. 5. Number of Fos-like immunoreactive (FLI) neurons in the different areas analysed. Both stress-naive (CONT) rats and rats daily exposed to immobilization on wooden boards (IMO) for 11 days (IMO12) were killed on Day 12 under resting conditions (BAS, n = 5–6 for each group) or 1 h after the termination of IMO (1 h post-IMO, n = 5– for each group). Means ± SEM are represented. Due to the low FLI basal levels only the 1 h post-IMO animals from both groups (CONT and IMO12) were compared in the analysis (see text). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding control group. PVNm, paraventricular hypothalamic nucleus (PVN), magnocellular; PVNmpd, PVN, medial dorsal parvocellular; PVNmpv, PVN, medial ventral parvocellular.

well as in the different subregions of the PVN (see Fig. 4 for delineation of the PVN): magnocellular (PVNm; $t_{12} = 3.63$, $P < 0.01$), PVNmpd ($t_{12} = 5.40$, $P < 0.001$) and medial parvocellular ventral (PVNmpv; $t_{12} = 5.23$, $P < 0.001$; Fig. 5). Finally, acute IMO-induced FLI was also reduced, and also in the LC of IMO12 compared with CONT ($t_8 = 5.02$, $P < 0.001$; Fig. 5).

arc *mRNA levels*

Confirming our previous results, constitutive *arc* gene expression was observed in several brain areas, and acute exposure to IMO increased *arc* mRNA levels only in telencephalic regions (Fig. 6).

The two-way ANOVA revealed a similar behaviour of *arc* mRNA levels in the three regions of the mPFC (Cg, PrL, IL): no significant effect of chronic IMO or time, but a significant effect of the interaction chronic IMO by time (Cg: $F_{2,25} = 11.8$; Prl: $F_{2,25} = 14.8$; IL: $F_{2,25} = 17.1$; $P < 0.001$ in all cases; Fig. 7). Further comparisons showed that *arc* expression increased in CONT but not chronic IMO rats, thus resulting in lower *arc* mRNA levels in chronic IMO than CONT rats immediately after acute IMO exposure (Cg; PrL, IL; $P < 0.001$ in the three areas), but no differences at 1 h post-IMO, when their levels had returned to basal conditions in all groups. In the other regions studied (Pir, LS, MeA, BLA; Fig. 7), no effect of chronic IMO on *arc* gene expression was found, despite the fact that in all of them IMO increased *arc* gene expression, as revealed by the

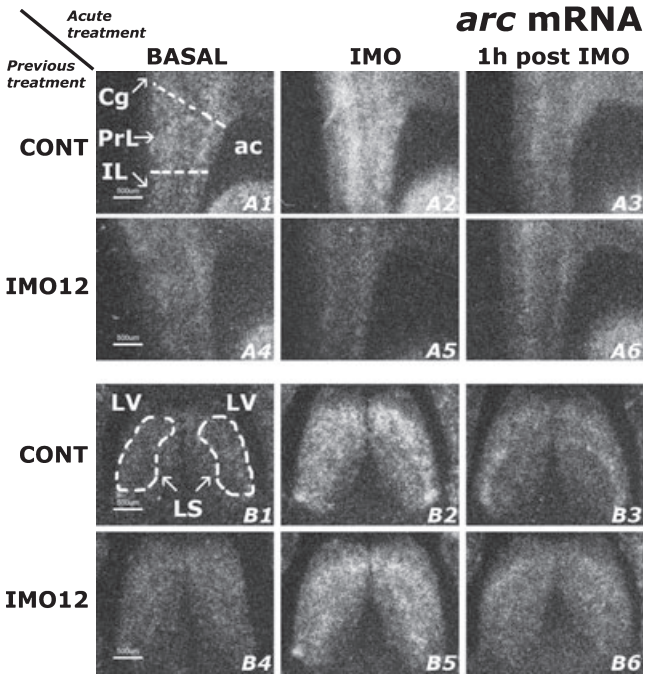

FIG. 6. Representative photomicrographs of brain film auto-radiographies of *arc* mRNA in the different subregions of the mPFC (A1–6) and the lateral septum (B1–6) for the six experimental groups: stress-naive rats killed under resting conditions (CONT–BASAL: A1, B1); stress-naive rats killed immediately after 1 h exposure to immobilization to wooden boards (IMO) (CONT–IMO: A2, B2); stress-naive rats killed 1 h after the termination of IMO (CONT–1 h post-IMO: A3, B3); chronic IMO rats killed under resting conditions (IMO12–BASAL: A4, B4); chronic IMO rats killed immediately after 1 h exposure to IMO (IMO12–IMO: A5, B5); and chronic IMO rats killed 1 h after the termination of IMO (IMO12–1 h post-IMO: A6, B6). Abbreviations: ac, anterior commissure; Cg, cingulate cortex; IL, infralimbic cortex; LS, lateral septum; LV, lateral ventricle; PrL, prelimbic cortex.

significant effects of time: Pir ($F_{2,30} = 8.2$, $P < 0.001$); LS ($F_{2,30} = 25.2$, $P < 0.001$); MeA ($F_{2,31} = 7.4$, $P < 0.01$); BLA ($F_{2,31} = 12.7$, $P < 0.001$).

FLI and arc *mRNA double-labelling*

The distribution of FLI and *arc* mRNA expression were similar to that observed within the single-labelling studies. Quantification was performed in the LS, which showed high levels of FLI and *arc* mRNA after IMO + 1 h. The percentage of FLI neurons also showing *arc* mRNA signal was 33%. Furthermore, virtually all clusters of silver grains observed were associated to a FLI nucleus (Fig. 8).

Discussion

The present results reveal some important differences between the evaluation of *c-fos* mRNA levels and FLI regarding the effects of repeated exposure to IMO in some brain regions. These differences were more evident in the mPFC and LS, where a marked reduction of *c-fos* mRNA response to the homotypic stressor was found in chronic IMO rats with no changes in the number of Fos-like+ neurons. In addition, chronic exposure to IMO resulted in lower levels of *arc* gene induction by the homotypic stressor only in mPFC areas, suggesting a more restricted pattern of adaptation than *c-fos*. Therefore, conclusions about the sensitivity of different brain areas to adaptation to repeated stress appear to be markedly dependent on the variable measured.

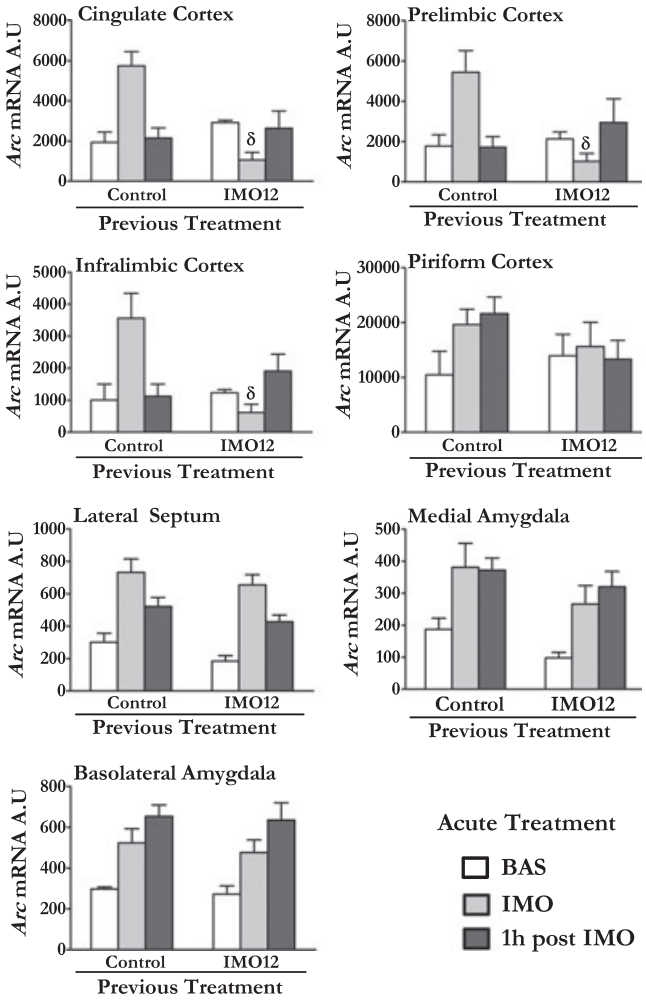

FIG. 7. *arc* mRNA levels in the different brain areas analysed. Both stress-naive (CONT) rats and rats daily exposed to immobilization on wooden boards (IMO) for 11 days (IMO12) were killed on Day 12 under resting conditions (BAS, $n = 4$–6 for each group), immediately after 1 h of IMO (IMO, $n = 5$–7 for each group) or 1 h after the termination of IMO (1 h post-IMO, $n = 5$–7 for each group). Means ± SEM are represented. $^{\delta}P < 0.05$ vs. corresponding acute stress condition in control rats.

Although IMO is a severe stressor, we have previously found that 1 h of daily exposure to the stressor for more than 4 days resulted in a reduction of the ACTH response to the homotypic stressor (Marquez *et al.*, 2004), and these results have been repeatedly found in our lab (unpublished data). Therefore, we chose this particular schedule to study IEG response. In addition, we studied IEG expression just after IMO but also at 1 h after the termination of IMO as our previous results with the long-term effects of a single exposure to IMO revealed differences in *c-fos* expression during the post-IMO that were not found immediately after the stressor (Vallès *et al.*, 2006). Under these conditions, we found again that prior chronic exposure to IMO reduced plasma ACTH and corticosterone responses to the same stressor, particularly during the post-IMO period. In parallel, *c-fos* expression induced by the acute IMO was substantially reduced by previous daily exposure to the stressor in most of the areas studied. Present data thus confirm previous reports showing that *c-fos* mRNA levels are very sensitive to repeated stress, although some differences between experiments are observed, which may be related to the type of stressor, the length of daily exposure and the number of exposures (Melia *et al.*, 1994; Bonaz & Rivest, 1998; Kollack-Walker *et al.*, 1999; Campeau

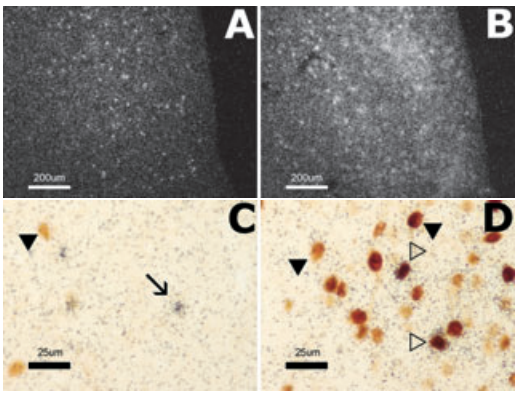

FIG. 8. Representative images (10× and 40×) showing double-labelling histochemistry for *arc* mRNA and FLI. Dark-field photomicrographs showing *arc* mRNA expression in the LS of an animal killed under resting conditions (A) and another one killed 1 h after the termination of 1 h IMO (B). Brightfield photomicrographs of the same sections are depicted in (C and D), and show neurons labelled for both *arc* and FLI (white triangle), only for FLI (black triangle) and only for *arc* (black arrow). Note that only under resting conditions, but not after IMO, neurons that were positive only for *arc* could be detected.

et al., 2002; Girotti *et al.*, 2006). It is important to note that adaptation to daily repeated stress appears to follow the rules of habituation and, therefore, the degree of adaptation is expected to be positively related to the duration and number of exposures to the stressor.

Acute IMO-induced *c-fos* expression was reduced in a wide range of brain regions in those animals having repeated experience with the stressor. This is theoretically relevant regarding putative mechanisms involved in adaptation to repeated stress as we and others have consistently found that *c-fos* mRNA levels in the mPFC (and in an important number of other brain regions) are not sensitive to the intensity of stressors and respond to the same extent to minor stressful procedures and severe stressors (Campeau *et al.*, 2002; Ons *et al.*, 2004; Pace *et al.*, 2005). When rats were repeatedly exposed to IMO, an important degree of adaptation of HPA hormones was observed in the present work. Similar results have been previously found with HPA hormones and other stress indices (e.g. Marquez *et al.*, 2004). However, it is clear that the level of activation of the HPA axis was still much higher than that observed for instance after a first exposure to a novel environment (see Rotllant *et al.*, 2007). Consequently, we would expect no reduction of *c-fos* expression after chronic IMO in those areas where *c-fos* expression is clearly insensitive to stress intensity (e.g. the mPFC). As this was not the case, alternative explanations are needed. We have hypothesized that daily repeated exposure to the same stressor may result in the progressive reinforcement of some inhibitory pathways that superimpose the stimulatory pathways, thus partially reducing the final response to the situation (Armario, 2006). These inhibitory pathways may reach both the areas sensitive and insensitive to the intensity of the stressor and, consequently, would reduce the stimulation caused by the last acute stressor in both types of brain areas. Because generalized adaptation of *c-fos* expression after repeated restraint is an early event and it is observed even in thalamic and cortical areas involved in sensorial processing (Girotti *et al.*, 2006), it can be assumed that inhibitory processes, whatever the region in which they are generated, rapidly spread to all brain regions involved in the processing of the stressor, thus resulting in a lower level of activation. The origin and nature of these general inhibitory pathways are not known, but may be related to some kind of memory (familiarity) about the set of sensory signals generated by the stressor, as the *c-fos* response normalizes when the stressors are changed (i.e. Melia *et al.*, 1994).

When FLI instead of mRNA levels were evaluated, we found some apparent discrepancies between the two measures, the most striking contrast being observed in the mPFC. There, repeated exposure to IMO resulted in almost no increase in *c-fos* mRNA levels after the homotypic stressor, whereas the number of neurons showing FLI was maintained high and unchanged with respect to that of stress-naive rats. A similar pattern was found in the ventral region of the LS (LSv). In contrast, in the other regions studied, reduction of *c-fos* gene expression was associated with lower levels of FLI. How can we explain such dissociation between *c-fos* mRNA and protein levels? One possibility is that repeated exposure to the same stressor could reduce the amount of *c-fos* expression per cell, affecting the number of activated cells less. This would result in lower total levels of mRNA levels in a particular region, with little changes in the number of Fos+ neurons. A second, non-mutually exclusive, possibility is that optimum levels of synthesis of Fos protein in those regions may be achieved with modest increases in mRNA levels. Finally, it is possible that the lower increases in *c-fos* mRNA levels of chronic IMO rats would be translated into a less sustained time course of Fos protein synthesis and then would be noted beyond the 2 h period studied in the present work.

To our knowledge, there is no previous report comparing, in quantitative terms, FLI in the mPFC of repeatedly stressed rats confronted to the homotypic stressor. Nevertheless, qualitative analysis of FLI after daily repeated foot-shock also suggests a lack of adaptation of FLI in this particular region (Li & Sawchenko, 1998). Regarding the LSv, reduced FLI has been observed after repeated restraint and social defeat (Chen & Herbert, 1995; Martinez *et al.*, 1998; Stamp & Herbert, 1999), but it should be taken into account that in function of all indices of stress intensity, IMO is a very severe stressor (Armario *et al.*, 1991; Marquez *et al.*, 2002) and this may explain the discrepancies.

There are also inconsistent results in the literature regarding IEG adaptation to repeated stress in other brain areas. In general, careful inspection of the data reveals that adaptation was less consistent and involved a lower number of brain areas when FLI instead of *c-fos* mRNA levels were used as an index of *c-fos* expression (Chen & Herbert, 1995; Li & Sawchenko, 1998; Martinez *et al.*, 1998; Stamp & Herbert, 1999). This applies to the PVN, where data regarding adaptation are very consistent with mRNA levels (Umemoto *et al.*, 1994a,b, 1997; Watanabe *et al.*, 1994; Bonaz & Rivest, 1998; Kollack-Walker *et al.*, 1999; Campeau *et al.*, 2002; Girotti *et al.*, 2006), but less consistent with FLI (Chen & Herbert, 1995; Li & Sawchenko, 1998; Martinez *et al.*, 1998; Stamp & Herbert, 1999; Dumont *et al.*, 2000; Viau & Sawchenko, 2002). It is of interest that in our hands, chronic exposure to IMO resulted in reduced FLI response to the homotypic stressors in two different subregions of the parvocellular PVN and in the PVNm. These data reflect the consistent adaptation of the hypophysiotropic region of the PVN to chronic repeated stress, but also the putative involvement of magnocellular PVN neurons in some of the physiological changes associated to stress.

What is clear from the present results is that measurement of *c-fos* mRNA levels or the Fos protein is not equivalent in all brain areas, some of them showing a good relationship between both variables (e.g. PVN and LC) and others an important discrepancy (mPFC and LSv). In any case, the overall picture is that measurement of *c-fos* mRNA levels may be more sensitive to adaptation to repeated stress than FLI, at least when only one time point is evaluated. If we are interested in how repeated exposure to IMO reduces net stimulatory inputs to some neurons, *c-fos* mRNA levels may be better than Fos protein. On the contrary, the amount of Fos protein is likely to be a better marker of putative transcriptional changes associated to *c-fos* induction, although the actual downstream changes in Fos-related

cellular transcription induced by previous repeated experience with IMO remain unexplored.

We also studied expression of an effector IEG, *arc*, that has been found to be activated by predominantly emotional (novel environment, predator odour) and mixed, systemic and emotional, stressors (forced swim and IMO) in a wide range of telencephalic regions, including mPFC, LSv, MeA and BLA (Ons *et al.*, 2004; Trneckova *et al.*, 2007; Kozlovsky *et al.*, 2008). The present results confirm our previous results and extend them by studying the effects of chronic exposure to stress. We found that, among the regions quantified, *arc* expression was sensitive to previous experience with IMO only in the mPFC, where both *c-fos* and *arc* expression were reduced in chronic IMO rats. In the other areas studied (LSv, MeA and BLA), *arc* expression was not modified by previous repeated exposure to the same stressor. These data indicate that *arc* gene expression may be more resistant than that of *c-fos* to generalized reduction after repeated stress. This may be due to a greater sensitivity of *arc* gene expression to low levels of neuronal activation as compared with *c-fos*, as is the case of zif268 (Wisden *et al.*, 1990; Worley *et al.*, 1993). In fact, it has been reported that *arc* expression is activated *in vivo* by an intensity of stimulation of the dentate gyrus lower than that needed to activate *c-fos* expression (Waltereit *et al.*, 2001). Whether this lower stimulatory threshold is the basis of constitutive telencephalic expression shared by zif268 (e.g. Cullinan *et al.*, 1995) and *arc* (e.g. Link *et al.*, 1995; Lyford *et al.*, 1995; Pinaud *et al.*, 2001; Ons *et al.*, 2004) is not known. Alternatively, assuming that chronic stress enhanced inhibitory signals, as discussed above, the control of *arc* expression may be less affected by superimposed inhibitory signals than *c-fos*.

One important question regarding IEGs is their colocalization. Because *arc* IHC did not yield a good signal with several different antibodies, we needed to combine ISH for *arc* with detection of FLI, but this was not possible in the already analysed sections because they were not in good enough condition when double-labelling was tried. Then, we performed a pilot experiment in two control and two acute IMO rats, killed 1 h after the termination of IMO to compare with our previous FLI data. The LSv was chosen because in this region *arc* mRNA levels are maintained for 1 h after the termination of IMO (Trneckova *et al.*, 2007). We found that the number of *arc*+ neurons was only about one-third of the number of Fos+ neurons, and that virtually all *arc*+ neurons also expressed *c-fos*. Although these data may suggest that the expression of *arc* during stress is restricted to a subset of FLI neurons, some technical limitations should be considered. A partial degradation of *arc* mRNA throughout the double-labelling procedure may have resulted in loss of some *arc*+ neurons. Similar results may be obtained if *arc* mRNA was moving out of the soma to dendrites. Therefore, additional studies are needed for a more precise answer to the degree of *c-fos* and *arc* coexpression. If partial colocalization proved to be true, this opens up the possibility that *arc*+ neurons may be phenotypically different from those only expressing *c-fos*, and this could explain the differential sensitivity to stimulation and perhaps their different behaviour after repeated stress.

Recently formed *arc* mRNA are transported to dendrites (Wallace *et al.*, 1998), and more particularly to active synapsis, in a *N*-methyl-D-aspartate (NMDA)-dependent process (Steward & Worley, 2001). Local synthesis of *arc* protein then takes place at these active sites where *arc* plays an important role in synaptic plasticity and learning (Guzowski *et al.*, 2000, 2001). What may be the functional consequences of this different regional pattern of adaptation to repeated IMO? At first sight, enhanced *arc* expression may be considered positive for the development of synaptic plasticity. However, it has been reported that *arc* expression was increased in several brain regions (e.g. mPFC and hippocampal formation) when an operant lever-pressing task was being learned, but the correlation between *arc* expression and learning performance was negative (Kelly & Deadwyler, 2002). Therefore, it is possible that a reduced level of *arc* expression is the reflection of using more specific (precise) circuits in good learners as opposed to bad learners. If this is the case, the greater reduction of *arc* expression in mPFC regions as compared with subcortical areas may reflect a progressive refinement of circuits activated by IMO in mPFC as compared with subcortical regions.

In conclusion, the present data support previous findings that repeated exposure to the same stressor resulted in a reduction of *c-fos* expression in a wide range of brain areas as evaluated by mRNA levels, which is not necessarily reflected in a reduced number of Fos+ neurons. The functional consequences of such a dissociation between *c-fos* mRNA and protein remain to be established. In addition, mRNA levels of the effector IEG *arc* appear to be more resistant to adaptation than those of *c-fos*, with the notable exception of the mPFC, suggesting differential regulation and function under stress. In fact, *arc*+ neurons represent only a subset of those neurons expressing *c-fos* after stress. Whether or not maintenance of *arc* expression after chronic stress in some regions with respect to others reflects a different degree of synaptic plasticity is not known.

Acknowledgements

This work was supported by grants SAF2005-00358 and SAF2008-01175 (Ministerio de Educación y Ciencia) and RD06/0001/0015 (Instituto de Salud Carlos III, Redes temáticas de Investigación Cooperativa en Salud). We thank Roser Nadal for her contribution to statistical analysis.

Abbreviations

ACTH, adrenocorticotropic hormone; BAS, basal; BLA, basolateral amygdala; BSA, bovine serum albumin; Cg, cingulate prefrontal cortex; CONT, control group; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; FLI, Fos-like immunoreactivity; HPA, hypothalamic-pituitary-adrenal; IEG, immediate-early gene; IHC, immunohistochemistry; IL, infralimbic prefrontal cortex; IMO, immobilization on wooden boards; ISHH, *in situ* hybridization histochemistry; KPBS, potassium phosphate-buffered saline; LC, locus coeruleus; LS, lateral septum; LSv, lateral septum, ventral region; MeA, medial amygdala; mPFC, medial prefrontal cortex; PFA, paraformaldehyde; Pir, piriform; PrL, prelimbic prefrontal cortex; PVN, paraventricular hypothalamic nucleus; PVNm, paraventricular hypothalamic nucleus, magnocellular región; PVNmpd, paraventricular hypothalamic nucleus, medial parvocellular, dorsal part; PVNmpv, paraventricular hypothalamic nucleus, medial parvocellular, ventral part; RIA, radioimmunoassay; SSC, saline-sodium citrate; TEA, triethanolamine.

References

Armario, A. (2006) The contribution of immediate early genes to the understanding of brain processing of stressors. In Pinaud, R. & Tremere, L. (Eds), *Immediate Early Genes in Sensory Processing, Cognitive Performance and Neurological Disorders*. Springer Science+Business Media, New York, pp. 199–221.

Armario, A., Gil, M., Marti, J., Pol, O. & Balasch, J. (1991) Influence of various acute stressors on the activity of adult male rats in a holeboard and in the forced swim test. *Pharmacol. Biochem. Behav.*, **39**, 373–377.

Bagdy, G. & Makara, G.B. (1995) Paraventricular nucleus controls 5-HT2C receptor-mediated corticosterone and prolactin but not oxytocin and penile erection responses. *Eur. J. Pharmacol.*, **275**, 301–305.

Bonaz, B. & Rivest, S. (1998) Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. *Am. J. Physiol.*, **275**, R1438–R1449.

Campeau, S., Dolan, D., Akil, H. & Watson, S.J. (2002) *c-fos* mRNA induction in acute and chronic audiogenic stress: possible role of the orbitofrontal cortex in habituation. *Stress*, **5**, 121–130.

Carter, R.N., Pinnock, S.B. & Herbert, J. (2004) Does the amygdala modulate adaptation to repeated stress? *Neuroscience*, **126**, 9–19.

Chan, R.K., Brown, E.R., Ericsson, A., Kovács, K.J. & Sawchenko, P.E. (1993) A comparison of two immediate-early genes, c-fos and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. *J. Neurosci.*, **13**, 5126–5138.

Chen, X. & Herbert, J. (1995) Regional changes in *c-fos* expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. *Neuroscience*, **64**, 675–685.

Cullinan, W., Herman, J., Battaglia, D., Akil, H. & Watson, S. (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience*, **64**, 477–505.

Dumont, E.C., Kinkead, R., Trottier, J.F., Gosselin, I. & Drolet, G. (2000) Effect of chronic psychogenic stress exposure on enkephalin neuronal activity and expression in the rat hypothalamic paraventricular nucleus. *J. Neurochem.*, **75**, 2200–2211.

Girotti, M., Pace, T.W., Gaylord, R.I., Rubin, B.A., Herman, J.P. & Spencer, R.L. (2006) Habituation to repeated restraint stress is associated with lack of stress-induced *c-fos* expression in primary sensory processing areas of the rat brain. *Neuroscience*, **138**, 1067–1081.

Guzowski, J., Lyford, G., Stevenson, G., Houston, F., McGaugh, J., Worley, P. & Barnes, C. (2000) Inhibition of activity-dependent *Arc* protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J. Neurosci.*, **20**, 3993–4001.

Guzowski, J., Setlow, B., Wagner, E. & McGaugh, L. (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes *Arc*, *c-fos*, and zif268. *J. Neurosci.*, **21**, 5089–5098.

Herman, J.P. (1995) In situ hybridization analysis of vasopressin gene transcription in the Paraventricular and Supraoptic Nuclei of the rar: regulation by stress and glucocorticoids. *J. Comp. Neurol.*, **363**, 15–27.

Herman, J., Figueiredo, H., Mueller, N., Ulrich-Lai, Y., Ostrander, M., Choi, D. & Cullinan, W. (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front. Neuroendocrinol.*, **24**, 151–180.

Kelly, M. & Deadwyler, S. (2002) Acquisition of a novel behavior induces higher levels of *Arc* mRNA than does overtrained performance. *Neuroscience*, **110**, 617–626.

Kollack-Walker, S., Don, C., Watson, S.J. & Akil, H. (1999) Differential expression of *c-fos* mRNA within neurocircuits of male hamsters exposed to acute or chronic defeat. *J. Neuroendocrinol.*, **11**, 547–559.

Kovács, K.J. & Sawchenko, P.E. (1996) Sequence of stress-induced alterations in indices of synaptic and trascriptional activation in parvocellular neurosecretory neurons. *J. Neurosci.*, **16**, 262–273.

Kozlovsky, N., Matar, M.A., Kaplan, Z., Kotler, M., Zohar, J. & Cohen, H. (2008) The immediate early gene *Arc* is associated with behavioral resilience to stress exposure in an animal model of posttraumatic stress disorder. *Eur. Neuropsychopharmacol.*, **18**, 107–116.

Li, H. & Sawchenko, P. (1998) Hypothalamic effector neurons and extended circuitries activated in ''neurogenic'' stress: a comparison of footshock effects exerted acutely, chronically and in animals with controlled glucocorticoid levels. *J. Comp. Neurol.*, **393**, 244–266.

Link, W., Konietzko, U., Kauselman, G., Krug, M., Schwanke, B., Frey, U. & Kuhl, D. (1995) Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proc. Natl Acad. Sci. USA*, **92**, 5734–5738.

Lyford, G., Yamagata, K., Kaufmann, W., Barnes, C., Sanders, L., Copeland, N., Gilbert, D., Jenkins, N., Lanahan, A. & Worley, P. (1995) *Arc*, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*, **14**, 433–445.

Marquez, C., Belda, X. & Armario, A. (2002) Post-stress recovery of pituitary-adrenal hormones and glucose, but not the response during exposure to the stressor, is a marker of stress intensity in high stressful situations. *Brain Res.*, **926**, 181–185.

Marquez, C., Nadal, R. & Armario, A. (2004) The hypothalamic-pituitary-adrenal and glucose response to daily repeated immobilization stress in rats: individual differences. *Neuroscience*, **123**, 601–612.

Marquez, C., Nadal, R. & Armario, A. (2006) Influence of reactivity to novelty and anxiety on hypothalamic-pituitary-adrenal and prolactin responses to two different novel environments in adult male rats. *Behav. Brain Res.*, **168**, 13–22.

Martí, O. & Armario, A. (1998) Anterior pituitary response to stress: time-related changes and adaptation. *Int. J. Dev. Neurosci.*, **16**, 241–260.

Marti, O., Garcia, A., Valles, A., Harbuz, M. & Armario, A. (2001) Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. *Eur. J. Neurosci.*, **13**, 129–136.

Martinez, M., Phillips, P. & Herbert, J. (1998) Adaptation in patterns of *c-fos* expression in the brain associated with expossure to either single or repeated social stress in male rats. *Eur. J. Neurosci.*, **10**, 20–33.

Melia, K., Ryabinin, A., Schroeder, R., Bloom, F. & Wilson, M. (1994) Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *J. Neurosci.*, **14**, 5929–5938.

Ons, S., Martí, O. & Armario, A. (2004) Stress-induced activation of the immediate early gene *Arc* (activity-regulated cytoskeleton associated protein) is restricted to telencephalic areas in the rat brain: relationship to *c-fos* mRNA. *J. Neurochem.*, **89**, 1111–1118.

Pace, T.W., Gaylord, R., Topczewski, F., Girotti, M., Rubin, B. & Spencer, R.L. (2005) Immediate early gene induction in hippocampus and cortex as a result of novel experience is not directly related to the stressfulness of that experience. *Eur. J. Neurosci.*, **22**, 1679–1690.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego.

Pinaud, R., Penner, M., Robertson, H. & Currie, W. (2001) Upregulation of the inmediate early gene Arc in the brains of rats exposed to enviromental enrichment: implications for molecular plasticity. *Brain Res. Mol. Brain Res.*, **91**, 50–56.

Rivest, S. & Rivier, C. (1994) Stress and interleukin-1beta -induced activation of c-fos, NGFI-B and CRF gene expression in the hypothalamic PVN: comparison between Sprague-Dawley, Fischer-344 and Lewis rats. *J. Neuroendocrinol.*, **6**, 101–117.

Rotllant, D., Nadal, R. & Armario, A. (2007) Differential effects of stress and amphetamine administration on Fos-like protein expression in corticotropin releasing factor-neurons of the rat brain. *Dev. Neurobiol.*, **67**, 702–714.

Simmons, D., Arriza, J. & Swanson, L. (1989) A complete protocol for In Situ Hibridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *J. Histotechnol.*, **12**, 169–181.

Stamp, J. & Herbert, J. (1999) Multiple immediate-early gene expression during physiological and endocrine adaptation to repeated stress. *Neuroscience*, **94**, 1313–1322.

Steward, O. & Worley, P. (2001) Selective targeting of newly synthesized *Arc* mRNA to active synapses requires NMDA receptor activation. *Neuron*, **30**, 227–240.

Trneckova, L., Rotllant, D., Klenerova, V., Hynie, S. & Armario, A. (2007) Dynamics of immediate early gene and neuropeptide gene response to prolonged immobilization stress: evidence against a critical role of the termination of exposure to the stressor. *J. Neurochem.*, **100**, 905–914.

Umemoto, S., Kawai, Y. & Senba, E. (1994a) Differential regulation of IEG in the rat PVH in single and repeated stress models. *Neuroreport*, **6**, 201–204.

Umemoto, S., Noguchi, K., Kawai, Y. & Senba, E. (1994b) Repeated stress reduces the subsequent stress-induced expression of Fos in rat brain. *Neurosci. Lett.*, **167**, 101–104.

Umemoto, S., Kawai, Y., Ueyama, T. & Senba, E. (1997) Chronic glucocorticoid administration as well as repeated stress affects the subsequent acute immobilization stress-induced expression of immediate early genes but not that of NGF1-A. *Neuroscience*, **80**, 763–773.

Vallès, A., Marti, O. & Armario, A. (2006) Long-term effects of a single exposure to immobilization: a *c-fos* mRNA study of the response to the homotypic stressor in the rat brain. *J. Neurobiol.*, **66**, 591–602.

Van Beveren, C., van Straaten, F., Curran, T., Müller, R. & Verma, I.M. (1983) Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular fos gene products have different carboxy termini. *Cell*, **32**, 1241–1255.

Viau, V. & Sawchenko, P. (2002) Hypophysiotropic neurons of the paraventricular nucleus respond in spatially, temporally, and phenotypically differentiated manners to acute vs. repeated restraint stress. *J. Comp. Neurol.*, **445**, 293–307.

Wallace, C., Lyford, G., Worley, P. & Steward, O. (1998) Differential intracellular sorting of immediate early gene mRNAs depends on signals in the mRNA sequence. *J. Neurosci.*, **18**, 26–35.

Waltereit, R., Dammermann, B., Wulff, P., Scafidi, J., Staubli, U., Kauselmann, G., Bundman, M. & Kuhl, D. (2001) Arg3.1/*Arc* mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. *J. Neurosci.*, **21**, 5484–5493.

Watanabe, Y., Stone, E. & McEwen, B. (1994) Induction and habituation of *c-fos* and zif/268 by acute and repeated stressors. *Neuroreport*, **5**, 1321–1324.

Wisden, W., Errington, M.L., Williams, S., Dunnett, S.B., Waters, C., Hitchcock, D., Evan, G., Bliss, T.V. & Hunt, S.P. (1990) Differential expression of immediate early genes in the hippocampus and spinal cord. *Neuron*, **4**, 603–614.

Worley, P.F., Bhat, R.V., Baraban, J.M., Erickson, C.A., McNaughton, B.L. & Barnes, C.A. (1993) Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. *J. Neurosci.*, **13**, 4776–4786.