It is accepted that processing of information concerning particular stimuli is mainly done in specific brain pathways and is associated to electrophysiological activation of particular sets of neurons. This electrophysiological activation is accompanied by parallel activation of intracellular signaling pathways, some of them resulting in the prompt, protein-synthesis independent, activation of the transcriptional activity of a number of genes, called immediate early genes (IEG), the best characterized being c-fos (Sheng and Greenberg 1990). Most IEGs, including c-fos, are themselves transcription factors, which regulate the expression of multiple genes assumed to be important for proper response to stimuli. However, there are a few IEGs that are not transcription factors, but instead are involved in particular cellular functions (Steward and Worley 2002). Among them, arc (activity-regulated cytoskeleton-associated protein) is particularly interesting because its mRNA is distributed along dendrites, where the protein is locally synthesized. Arc encodes for a protein associated to actin, likely to be related to N-methyl-D-aspartate receptor scaffolding (Link et al. 1995; Lyford et al. 1995; Husi et al. 2000; Steward and Worley 2001) and involved in synaptic plasticity and
learning (Steward et al. 1998; Guzowski et al. 2000, 2001; Moga et al. 2004). Recently, we have also demonstrated that arc expression is activated under stress, with the special feature of such enhanced expression being restricted to telencephalic regions (Ons et al. 2004).

IEGs, particularly c-fos, have been a major tool for the characterization of brain areas involved in the processing of stressors. This enhanced expression can be evaluated by quantifying the number of neurons demonstrating positive Fos-like immunoreactivity or the levels of c-fos or other IEG mRNA levels by in situ hybridization. On the basis of the information generated by IEG expression studies and other complementary approaches, it is well accepted that there are at least two broad categories of stressors: systemic (physical) and emotional (processive). Whereas the specific pathways activated by systemic stressors (i.e. hypoglycemia, hypovolemia, drugs) markedly differ (Pacak and Palkovits 2001), exposure to different types of emotional stressors commonly activate a wide range of brain areas, from the cortex to the brainstem (Cullinan et al. 1995; Kovacs 1998; Ons et al. 2004), and the information finally converges in several effector areas such as the paraventricular nucleus of the hypothalamus (PVN), the key area in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis.

Despite the widespread use of IEGs as a tool to map brain regions activated by stressors, the relationship between the dynamics of IEG expression and the length of exposure to stressors is at present unclear. It is assumed that c-fos expression is transient, apparently due to the self-inhibition caused by the presence of an AP1 region in the regulatory regions of the c-fos gene (Sassone-Corsi et al. 1988); accordingly, c-fos expression in the PVN markedly declines despite maintenance of exposure to stress (IMO or restraint) (Imaki et al. 1992; Senba et al. 1994; Umemoto et al. 1997). In contrast, sustained (3–6 h) levels of c-fos mRNA expression in the PVN have been reported after adrenalec-tomy (Brown and Sawchenko 1997), endotoxin administration (Rivest and Laflamme 1995) or colloid-induced hypovolemia (Tanimura et al. 1998). A similar pattern has been reported for immature (hnRNA) nuclear CRF RNA levels. Thus, sustained exposure to restraint caused only a transient peak of CRF gene transcriptional activity, the subsequent inhibition being independent of glucocorticoid feedback inhibition and likely to be related to the action of the inducible cAMPy early repressor, ICER (Shepard et al. 2005). In contrast, colloid-induced hypovolemia resulted in high levels of CRF hnRNA levels for at least 6 h (Tanimura et al. 1998). Since the half life of c-fos mRNA is about 10–15 min (Zangenehpour and Chaudhuri 2002) and that of CRF hnRNA is probably shorter on the basis of poststress dynamics (Imaki et al. 1996; Kovacs and Sawchenko 1996; Ogilvie et al. 1998; Ma et al. 1999), maintenance of high levels for several hours imply in both cases sustained transcriptional activity.

Whether these marked discrepancies between stressors in the time-course of c-fos or CRF gene expression are related to the nature of the stressors, to their severity or to both is at present unclear. In addition to this major theoretical question related to the dynamics of stress-induced transcriptional changes, a better knowledge of such dynamics is important for several reasons. Firstly, IEG expression is usually measured at one single time point after exposure to stressors and it is therefore important to know which are the optimum time points for its measurement, particularly taking into account that the length of exposure to stressors varies extraordinarily in the literature (from minutes to several hours) and therefore an inappropriate time point may be often chosen. Secondly, if it is assumed that the amount of synthesized IEG proteins would determine, at least to a certain extent, their impact on the cell (either transcriptional or functional), it would be difficult to evaluate such an impact without a precise knowledge of the time course of IEG expression triggered by stressors. Thirdly, it is currently unknown whether this transient response is an universal property of all IEGs or can be restricted to a set of IEGs.

Finally, the impact of the termination of exposure to stress on the dynamics of biochemical changes (IEGs and neuropeptides) triggered during exposure to stressors is poorly characterized. This is in contrast to our knowledge of poststress dynamics of HPA hormones and other physiological variables, in that it is known that the termination of exposure to stress specifically accelerates the decline in ACTH levels as compared to continuous exposure (Garcia et al. 2000; Belda and Armario, unpublished) and that maintenance of poststress levels of hormones is positively related to the intensity of stressors (Garcia et al. 2000; Marquez et al. 2002). These data suggest that termination of exposure to stressors triggers some kind of signal to reduce the ongoing activation, but that these termination signals are opposed by the intensity of activational signals caused by previous exposure to the stressor. However, it is not known whether the observed changes in hormone secretion are paralleled by similar changes in IEG or neuropeptide gene transcription in the PVN and other brain regions. The main purposes of the present experiments were thus to characterize the dynamics of IEG (c-fos and arc) and CRF gene responses to prolonged exposure to a severe stressor (IMO) and the specific effect of termination of exposure to the stressor on ongoing transcriptional activity. We quantified IEG expression in a restricted number of areas that are fully representative of the overall response to stressors, either because they are important for the integration of the response to emotional stressors or because they appear to be sensitive to the intensity of stressors, or both: mPFC, LSv, MeA, PVN and LC (Herman et al. 2003; Ons et al. 2004). In addition, CRF gene expression was only studied in the PVN because it is the only brain area where hnRNA levels can be presently detected.
Methods

Animals
Thirty-seven male, 2-month-old, Sprague-Dawley rats (average body weight 350 g) were used. The animals were housed two per cage under standard conditions of temperature (22 ± 1°C) and maintained on a 12 h light–12 h dark schedule (lights on at 07:00 hours) with ad libitum access to food and water. The animals were allowed to acclimatize to the housing conditions for at least 1 week before the beginning of the experimental treatments, which were carried out in the morning. The Ethical Committee for Animal and Human Experimentation of the Universitat Autònoma de Barcelona approved the experimental procedures used in this work.

General procedure
The animals were randomly assigned to the following groups: Controls (CO), immobilization (IMO-0.5) for 30 min, IMO for 1 h (IMO-1), IMO for 2 h (IMO-2) and IMO for 4 h (IMO-4). An additional group of rats exposed to IMO for 1 h but sacrificed 1 h after the termination of exposure to IMO was also included (IMO + R). Immediately after the termination of exposure to the stressor (or under basal conditions for the controls) animals were anesthetized with 80 mg/kg ketamine (Merital Laboratories, Barcelona, Spain) and 10 mg/kg xylazine (Bayer, Barcelona, Spain). Then, they were transcardially perfused with saline solution (0.9% NaCl) for 2 min, and with 4% paraformaldehyde (PFA) for 10 min. After perfusion, brains were removed, submerged in PFA and stored at 4°C until the termination of exposure to IMO was also included (IMO + R).

In situ hybridization assay
The c-fos probe was generated from EcoRI fragment of rat c-fos cDNA (Dr I. Verma, The Salk Institute), subcloned into pBluescript SK-I (Stratagene, La Jolla, CA) and linearized with Smal. Arc probe was generated from the full length rat arc cDNA, subcloned into EcoRI-XhoI site of pBluescriptII SK + (Stratagene), and linearized with EcoRI. The c-fos and arc probes were generously provided by Dr S. Rivest (Laval University, Quebec, Canada) and Dr P. F. Worley (Johns Hopkins University, Baltimore, MD, USA), respectively. Radioactive antisense cRNA copies were generated using a transcription kit (Promega, Madison, WI, USA). Once digested, linearized plasmids were incubated in a transcription buffer (40 mM Tris-Cl pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol (DTT), 0.2 mM GTP/ATP/CTP, 200 μCi [α-35S]UTP (specific activity > 1000 Ci/mmol, Amersham Pharmacia Biotech, London, UK), 40 U RNA inhibitor and 20 U of T7 RNA polymerase for 60 min at 37°C. The DNA template was digested with RNase-free DNase (Promega, Madison, WI) and 1 U DNase in 0.25 μg/μL tRNA and 9.4 mM Tris/9.4 mM MgCl2 and extracted with phenolchloroform-isooamyl alcohol 25 : 24 : 1 (Sigma, Barcelona, Spain). 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.

The protocol used for in situ hybridization histochemistry was adapted from Simmons et al. (1989). All the solutions were pretreated with diethylpyrocarbonate and sterilized before use. Sections were postfixed in 4% PFA + Borax rinsed in potassium phosphate-buffered saline, digested with proteinase K (Roche, Penzberg, Germany; 0.01 mg/mL in 100 mM Tris-HCl pH 8.0 and 50 mM EDTA pH 8), rinsed in diethylpyrocarbonate-treated water and 0.1 triethanolamine pH 8.0 (Sigma) and acetylated in 0.25% acetic anhydride in 0.01 M triethanolamine. Finally, they were washed in 2× saline-sodium citrate, 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 sulfate, yeast tRNA 500 μg/mL and 10 mM DTT containing the labeled probe (1 × 105 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× saline-sodium-citrate containing 1 mM DTT (Sigma), digested with RNase A (Amersham Pharmacia Biotech; 0.02 mg/mL in 0.5 mM NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0), washed in descending concentrations of saline-sodium citrate 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, Madrid, Spain) for 24–96 h, depending on the intensity of the signal in each zone.

Image analysis
Densitometric analyses were done on the films. The hnRNA and mRNA levels were semiquantitatively determined in two to three sections per brain area and animal by setting up the best threshold to avoid detecting any background signal and measuring the optical density and the number of pixels in defined areas with a Leica Q 500 MC system. c-fos and arc mRNA levels are expressed in arbitrary units (number of pixels × optic density). All the samples to be statistically compared were processed in the same assay to avoid interassay variability. Analyses were performed in the following brain areas: prelimbic (PrL) and infralimbic (IL) region of prefrontal cortex (mPFC), lateral septum ventral (LSv), medial amygdala (MeA), parvocellular and magnocellular regions of paraventricular nucleus of hypothalamus (PVN), and locus coeruleus (LC).

Statistical analysis
The purpose of the experiment was two-fold: to study the effect of length of exposure to IMO (factor time) and to study the specific contribution of the release of animals from the situation to gene expression. For these reasons, two statistical analyses were performed. For the first purpose, a one-way ANOVA was used, including the control group for the study of arc expression and omitting the control group in the case of c-fos and CRF gene expression due to the low levels of expression under non-stress conditions. When appropriate, post hoc comparisons were done using the Student-Newman-Keuls (SNK) test (α = 0.05) to show statistical differences between particular groups. For the second purpose, IMO-2 and IMO + R groups were compared using the t-test.

Results
Undetectable or low levels of c-fos mRNA levels were observed in unstressed (control) animals and therefore this
group was not included in the statistical analysis. In contrast, constitutive arc expression was found in some brain areas, including those which were selected for quantification; consequently, the control group was included in the statistical analysis (see Fig. 1 for representative images).

The one-way ANOVAs of c-fos mRNA levels revealed a marginally significant effect of time on c-fos expression in the PrL mPFC (p < 0.056), but a significant effect in all other brain regions analyzed: IL mPFC (p < 0.007), LSv (p < 0.001), MeA (p < 0.001), ppPVN (p < 0.001), mPPVN (p < 0.001) and LC (p < 0.02). In all regions except the MeA and LC, maximum mRNA levels were observed after 30 min of IMO. In the MeA and LC maximum was found after 1 h of IMO. Post-hoc comparisons revealed that in the PrL and IL, significant declines in mRNA levels were only observed after 4 h of IMO (Fig. 2). In contrast, in the LSv, the decline was faster than in the mPFC, being significant after 1 h of IMO, with a further significant decline after 4 h as compared to 1 h of IMO (Fig. 2). In the MeA, maximum response was observed after 1 h of IMO with a significant decline after 2 and 4 h of IMO as compared to both 30 min and 1 h of IMO (Fig. 2). In the pPVN and the mPVN, the pattern of c-fos expression was similar: high levels of mRNA were maintained for 2 h, with a significant decline after 4 h of IMO (Fig. 3). Finally, in the LC, the peak of mRNA levels was delayed as compared to most other areas and was found after 1 h of IMO; mRNA levels after 30 min of IMO were lower and of similar magnitude as those observed after 2 or 4 h of IMO (Fig. 3).

When c-fos mRNA levels of rats exposed to 1 h of IMO and left undisturbed in the animal room for an additional hour (IMO + R group) were compared with those exposed for 2 h (Figs 2 and 3) no differences were observed in any of the studied areas, except in the LSv; in this area the release of the animals from the board resulted in lower mRNA levels than the maintenance of IMO exposure (t-test, p < 0.03).

The transcriptional activity of the CRF gene was studied measuring hnRNA levels, which were almost undetectable under unstressed conditions, but sharply increased after IMO (Fig. 3). The ANOVA revealed a significant effect of time (p < 0.05). Post hoc comparisons showed a moderate but significant decrease of the transcript from 30 min to 1 h of IMO, with maintenance of these intermediate levels even after 4 h of IMO. Again, no differences in hnRNA levels were found between 2 h IMO and IMO + R groups.

The one-way ANOVAs of arc mRNA levels that included the unstressed group revealed a significant effect of time in all regions studied: PrL mPFC (p < 0.001), IL mPFC (p < 0.003), LSv (p < 0.002) and MeA (p < 0.001). Maximum expression was already found after 30 min of IMO in all areas (Fig. 4); however, the dynamics of arc expression was dependent on the particular area: arc mRNA levels were still at maximum levels after 4 h of IMO in mPFC regions, but significantly declined after 2 h of IMO in the LSv and the MeA, reaching resting levels. Similarly to c-fos, arc expression in the IMO + R group was not different from that found in the 2 h IMO group in any studied region.

Discussion
To our knowledge, this is the first study specifically aimed at characterizing the dynamics of CRF hnRNA and brain IEG response after continuous exposure to a stressor and the specific contribution of the release of the animals from the situation. Our data indicate that in most brain areas, c-fos expression progressively declined despite continuous exposure to IMO, although the precise dynamics was dependent on the region and the particular IEG. Arc expression was in general more sustained than that of c-fos, although regional differences were also observed. With the exception of c-fos expression in the LSv, the decline of IEG expression was indistinguishable when comparing the levels achieved after 2 h of IMO and those achieved 1 h after the termination of 1 h of IMO (post-IMO), suggesting that interpretation of poststress changes in IEGs as evidence for a shutting off of the ongoing response associated to termination of exposure to the stressor may be in most cases inappropriate. Similarly, the decline in CRF hnRNA levels in the pPVN was not related to the termination of exposure to IMO.

Dynamics of c-fos response to continuous exposure to IMO
A widespread c-fos induction was observed after IMO, with a pattern similar to that previously reported using the same stressor (Ons et al. 2004) or after exposure to other, predominantly emotional, stressors (Cullinan et al. 1995; Bonaz and Rivest 1998). However, quantification was restricted to some particular areas for the reasons already outlined.

Time-related changes in c-fos mRNA levels during continuous exposure to IMO were dependent on the brain region studied. Peak levels of mRNA levels were observed at 30 min in most regions (PrL, IL, LSv, PVNp, PVNm), but later on (60 min) in the MeA and, particularly, in the LC. The
data concerning the LC are in accordance with some other previous reports (Watanabe et al. 1994; Cullinan et al. 1995; Bonaz and Rivest 1998) and suggest that c-fos activation in the LC may be secondary to previous activation of other areas. To our knowledge, there are no previous data reporting a delayed maximal expression of c-fos in the MeA as compared to other telencephalic regions. After maximum levels were reached, c-fos mRNA levels then declined in all regions, although the precise dynamics also was region-dependent. Thus, c-fos expression only declined in mPFC and PVN after 4 h of IMO, whereas the sharpest decline after peak expression was observed in the LSv and MeA. On the basis of the present results, optimum time to evaluate maximal c-fos response to a continuous severe stressor, although region-dependent, may be within 30–60 min after initial exposure to the stressor. Previously, a more marked decline of c-fos expression in the PVN has been reported, despite continuous exposure to another method of IMO (Imaki et al. 1992; Umemoto et al. 1997), but it is possible that the procedure was less severe than that used in the present experiment and the intensity of the stressor may have contributed to slowing down the rate of poststress decline in c-fos mRNA levels.

The mechanisms involved in the area-dependent differences in the dynamics of c-fos expression are unclear, although the presence of local factors regulating c-fos and

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**Fig. 2** Quantification of c-fos mRNA levels in medial prefrontal cortex (mPFC), lateral septum and medial amygdala after different periods of IMO. Values are expressed as the means and SEM (n = 4–7 per group) of arbitrary units (AU). Numbers under the bars indicate the period of continuous exposure to IMO and 1 + R indicates the group exposed to 1 h of IMO and sacrificed 1 h after the termination of IMO (closed bars). The latter group was only compared to the 2 h IMO group (t-test, & p < 0.05 between the signaled groups). The remaining groups (excluding 0 h) were compared with ANOVA and post hoc SNK test: Δ indicates significant difference vs. peak values and # vs. both 0.5 h and 1 h IMO groups.

**Fig. 3** Quantification of c-fos mRNA levels in the PVN and locus coeruleus and of CRF hnRNA levels in the PVN. Values are expressed as the means and SEM (n = 4–7 per group) of arbitrary units (AU). Numbers under the bars indicate the period of continuous exposure to IMO and 1 + R indicates the group exposed to 1 h of IMO and sacrificed 1 h after the termination of IMO (closed bars). The latter group was only compared to the 2 h IMO group (t-test). The remaining groups (excluding 0 h) were compared with ANOVA and post hoc SNK test: Δ indicates significant difference vs. peak values and # significant difference vs. 0.5 h, 1 h and 2 h IMO groups.
other IEG mRNA stability (Guhaniyogi and Brewer 2001) should be considered. However, at least a partial decline was found in all regions. The mechanisms involved in the generalized progressive decline of c-fos expression after prolonged exposure to IMO are unclear. A possibility is that c-fos expression was progressively reduced by a specific self-inhibition process through the AP1 complex (Sassone-Corsi et al. 1988). Alternatively, the decline may have been due to other self-inhibition process or to the incapability of neurons to maintain an intense and prolonged c-fos expression. It should be taken into account that a similar progressive decline during continuous exposure to severe stressors is observed in plasma levels of ACTH (Rivier and Vale 1987; García et al. 2000), in part due to the incapability of corticotrope cells to maintain sustained ACTH synthesis and release (Rivier and Vale 1987). The finding that some stimuli are able to maintain c-fos expression at a high level for hours (Rivest and Laflamme 1995; Tanimura et al. 1998) and that the dynamics of c-fos expression was region-dependent strongly suggest that regulation of c-fos expression over time is unlikely to be due to an incapability of neurons to maintain sustained c-fos expression, but rather to specific repressor mechanisms.

Because expression of c-fos and other genes appears to decline despite the persistence of exposure to the stressor, the specific contribution of the termination of exposure to the situation is unclear. For this reason, in the present work we studied the decline of c-fos mRNA 1 h after the termination of exposure to IMO for 1 h, which would allow us to compare this group with those animals continuously exposed to IMO for 2 h. Levels of c-fos mRNA were similar in the two groups in all regions except in the LSv, where the decline was greater in those animals released from the board. Therefore, it appears that in most brain areas the decline in c-fos mRNA levels cannot be interpreted as evidence for the shutting off of the response associated to the termination of IMO. The LSv is the only area, among those studied, where the release of animals from the stressful situation appears to induce an specific blockade of c-fos expression. It is also noteworthy that this specific effect of the release of animals from the board was observed despite the fact that LSv was the region showing the fastest decline after continuous exposure to IMO. Thus, the putative role of this area in the control of behavioral and physiological changes associated to the poststress period deserves to be studied.

Only a few studies have investigated the levels of c-fos mRNA after termination of exposure to the stressors. Cullinan et al. (1995) reported a fast decline all over the brain of c-fos and also of another IEG (zif268) at 1 h after the termination of exposure to restraint or forced swimming. In contrast, we have observed no significant decline in a subset of areas, including the lateral and medial preoptic area, the PVN, the paraventricular thalamic nucleus, the LC and the nucleus of the solitary tract (Valle`s et al. 2006). Because restraint and forced swim are less severe than IMO in terms of HPA activation and other physiological changes (Armario and Jolin 1989; Marti et al. 2001), the intensity of stressors may be also important to determine the poststress dynamics of c-fos expression.

Dynamics of arc expression

The overall pattern of arc expression was clearly different from that of c-fos in some respects:

- stress-induced c-fos expression was restricted to telencephalic regions, in accordance with previous report (Ons et al. 2004);
- in all regions studied, peak levels of arc mRNA levels were already observed at 30 min after initial exposure to IMO;
in PrL and IL cortical areas, no decline of mRNA levels was observed even after 4 h of IMO, in contrast to the decline in c-fos mRNA levels.

Whereas the half-life of c-fos mRNA levels is around 10–15 min (Zangenehpour and Chaudhuri 2002), the half-life of arc mRNA levels is not known. However, the decline over time observed in arc expression in the LSv and MeA suggests that the half-life is not longer than 30–60 min. The existence of marked differences between c-fos and arc in a particular brain regions regarding the maintenance of expression over time may reflect a different half-life or, alternatively, the existence of particular regulatory mechanisms affecting the maintenance of expression of each IEG. That expression of arc is more sustained than that of c-fos is also supported by the constitutive expression of the former in some brain regions such as the cortex and the hippocampal formation.

The dynamics of arc mRNA levels, like that of c-fos, was strongly dependent on the brain region studied. Thus, whereas no decline was found in prefrontal cortical areas, significant declines were observed at 2 and 4 h after initial exposure to IMO in the MeA and LSv, again suggesting a differential regulation of IEGs among different brain areas. Interestingly, MeA and LSv were areas where the decline of c-fos expression over time was stronger; neurons in these areas appear to be unable to maintain a sustained expression of different IEGs despite the persistence of the stressor. Because IEG expression in MeA and LSv is more sensitive to the intensity of stressors (Ons et al. 2004) than are PrL and IL areas, it might argued that a progressive reduction of activational properties of IMO may only affect those regions which are also sensitive to the intensity of the stressor. However, this does not appear to be the case of c-fos in the LC and the PVN. Regarding arc, there is evidence for regional heterogeneity in the time-course after exposure to a lever-press learning task (Kelly and Deadwyler 2003).

When the influence of termination of exposure to IMO was specifically studied, we found no evidence in any region for an accelerated decline of arc expression due to the release of animals from the situation, supporting the results with c-fos. This lack of specific effect of termination of exposure to the stressor was also observed in the LSv, although the fast time-dependent decline of arc expression in this area even after continuous exposure to IMO may have masked an additional contribution of the release from the stressor.

CRF gene expression

Whereas basal levels of CRF hnRNAs were almost undetectable, exposure to IMO induced a strong increase, a peak being reached at 30 min. After that, a small decline was observed at 1 h, but the levels were essentially maintained thereafter even after 4 h of IMO. In the post-IMO group, no decline was found as compared with those which were maintained immobilized, suggesting maintenance of CRF expression at the same level as that observed in the animals that still continued to be immobilized. The present results thus indicate that during continuous exposure to a severe stressor, CRF neurons are able to maintain submaximum levels of CRF gene expression. The maintenance of submaximum levels of CRF hnRNA for hours in IMO animals appears to be in contrast to a recently published paper showing that restraint-induced transcriptional activation of CRF gene was transient, with a return to prestress levels after 30 min despite the persistence of exposure to the stressor (Shepard et al. 2005). Interestingly, this time-course was found to be independent of stress-induced glucocorticoid release and likely to be linked to intracellular repression of CRF transcription. As increases in hnRNA levels without concomitant changes in CRF mRNA levels have been reported, for instance, after exposure to ether or ethanol (Kovacs and Sawchenko 1996; Rivier and Lee 1996), it is possible that the duration of the enhanced CRF gene transcription rather than the peak may be dependent on the intensity of the stressors. Consequently, the increase in mRNA levels should be consistent only when the duration of transcriptional activation is above a certain level. Our findings of a similar level of transcriptional activity in those rats exposed to 2 h of IMO and those exposed for 1 h and then maintained in their home cages for an additional hour suggest that, at least in stress-naive animals, there are no specific signals associated to the termination of exposure to the stressor to shutting off transcription of CRF gene, in accordance with the response of c-fos and arc in most brain regions.

General conclusions

The present results demonstrate marked differences in the pattern of response of two clearly distinct IEGs (c-fos and arc) to sustained exposure to a severe stressor. In addition, even with the same IEG, marked regional differences were observed in the dynamics of response. It appears that c-fos expression is not maintained for a long period at the maximum level in any brain region after prolonged exposure to a stressor, whereas arc expression is quite well maintained, at least in some areas, thus reflecting a differential impact of biochemical signals associated to stress on the regulation of these two IEGs. This is particularly important because there is evidence, regarding c-fos and other IEGs different from arc, that stress activates various IEGs within the same neurons (Chan et al. 1993; Papa et al. 1993; Guldenaar et al. 1994; Wang et al. 1997; Swank 1999). It is therefore possible that divergences between different IEGs may emerge after prolonged exposure rather than during the initial response to the stressor.

Unexpectedly, the study of the dynamics of stress-induced IEG and CRF gene transcription after the termination of...
exposure to IMO revealed that putative signals associated to the termination of exposure to the stressor did not specifically influence the rate of decline of gene expression in most cases, with the exception of the c-fos in the LSv. Therefore, at least under the conditions used in the present study, which are similar to those used to characterize the poststress dynamics of peripheral HPA hormones (García et al. 2000), poststress changes in IEG and CRF gene expression would not be appropriate to study specifically the shutting-off of the stress response after the termination of exposure to stressors. As we have previously observed that the post-IMO dynamics of both peripheral HPA hormones and c-fos expression in the pPVN are sensitive to a single previous experience with the stressor, in that recovery of prestress levels was accelerated (Valles et al. 2003), it is possible that signals associated with the termination of exposure to severe stressor may become progressively more potent as a function of the degree of previous experience with the situation.

The present results and others from the literature can be easily reconciled assuming that the period of time over which the expression of IEG and other genes (i.e. CRF) induced by stress can be maintained is positively related to the intensity and duration of exposure to the stressor, these two factors partially overcoming inhibitory processes such as ICER-associated blockade of CRE transcription. Whereas during a first exposure to the stressor, gene expression may not be sensitive to release of the animals from the stressful situation, signals associated with such a release may become progressively more important as a function of the previous experience with the situation.

The functional consequences of the present findings are difficult to predict because of our poor knowledge of the quantitative relationship between gene expression and further cellular processes (including protein synthesis) regarding IEGs and neuropeptides. The finding that c-fos expression declines despite persistence of the stressor suggests that optimum Fos protein synthesis could be achieved with submaximum levels of the corresponding mRNA. Similarly, it is unclear whether beyond certain levels of Fos protein, gene transcription regulated by c-fos is actually affected by the amount of the protein. However, the existence of regional differences in IEG expression (or its mRNA stability) suggests a functional role of differential maintenance of mRNA levels. To answer some of these questions, it may be of interest to study, under conditions similar to those used in the present experiment but over a longer time-course, the level of Fos protein and the expression of some genes known to be regulated by c-fos (e.g. preproenkephalin), or the relationship between arc expression and some markers of synaptic plasticity. It is unlikely that the dynamics of changes in CRF gene expression would contribute to explaining the dynamics of peripheral changes in HPA hormones as these are two clearly different phenomena, one linked to intracellular signals and the other to electrophysiological activation of CRF neurons, but the amount of CRF mRNA and protein may reflect the capability of the HPA axis to respond to further challenges.

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