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Dynamics of stress-induced *c-fos* expression in the rat prelimbic cortex: lessons from intronic and mature RNA and protein analyses

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ABSTRACT

Despite the extensive use of *c-fos* as a marker of stress-induced neuronal activation, key aspects regarding its dynamics of expression remain poorly characterized. In the present study, we assessed in the prelimbic cortex of adult male rats the immediate transcriptional response of *c-fos* by measuring the heteronuclear (hn)RNA and mature (m)RNA expression by double fluorescent *in situ* hybridization as well as the c-Fos protein using immunofluorescence (FOS). We quantified in three different experiments the number of *c-fos* hnRNA+, mRNA+ and FOS+ neurons under basal conditions, immediately after different periods of immobilization stress (IMO), and after a recovery period. Our results indicate that stress induced a large increase in the number of positive neurons for all markers analyzed, each displaying a different time course. Moreover, our findings indicate that measuring the intensity of signal per neuron also provides relevant information. In addition, we report an increased number of FOS+ neurons after only 8–15 min of IMO, suggesting a surprisingly fast initiation of protein translation. Finally, the maturation from *c-fos* hnRNA+ to mRNA+ might depend on the duration and/or intensity of stress-induced activation. Our findings contribute to a better understanding of the dynamics of stress-induced *c-fos* expression and underscore the importance of examining multiple molecular components when using *c-fos* as a proxy of neuronal activation.

1. Introduction

Immediate early genes (IEGs) are genes whose expression in response to stimuli does not require *de novo* protein synthesis and therefore their transcription is very fast (Herdegen and Leah, 1998). IEGs are ubiquitous in neurons and respond to a variety of stimuli, and among them, *c-fos* is the most broadly used and extensively studied. In the brain, c-fos expression is linked to neuronal activation, allowing the characterization of brain areas and neuronal populations activated in response to a wide range of exteroceptive and interoceptive stimuli (Sheng and Greenberg, 1990; Hughes and Dragunow, 1995; Herrera and Robertson, 1996). In this regard, c-fos expression has been fundamental for understanding neuronal responses to emotional and systemic stressors and mapping the brain areas relevant to stress processing, such as the paraventricular nucleus of the hypothalamus and the medial prefrontal cortex (mPFC) (Kovács, 1998; Hoffman and Lyo, 2002; Armario, 2006). These studies have predominantly relied on the quantification of the number of positive neurons for c-fos using immunohistochemistry to detect the c-Fos protein (herein FOS) or using *in situ* hybridization to detect *c-fos* mature RNA (mRNA) levels. Although these approaches have provided critical insights into the brain processing of systemic and emotional stressors, some caveats need to be considered.

Abbreviations: dFISH, double *in situ* fluorescent hybridization; FOS, c-Fos protein; hnRNA, heteronuclear RNA; IEG, immediate early gene; IF, immunofluorescence; IMO, immobilization stress; mPFC, medial prefrontal cortex; mRNA, mature RNA; PrL, prelimbic cortex.

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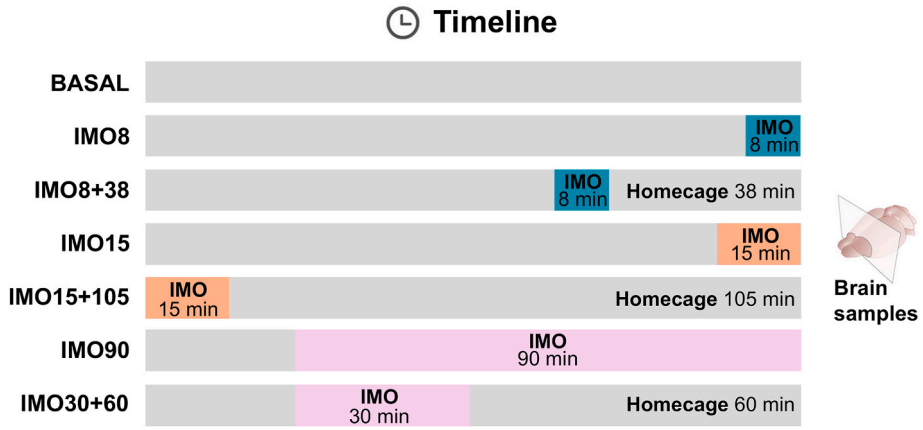

Fig. 1. Experimental design. Schematic representation of the experimental groups for the study of *c-fos* RNA expression and FOS protein after different times of immobilization stress (IMO). Grey represents no stress exposure (home cage conditions). Animals were euthanized by intracardiac perfusion, and their brains were obtained for histological analyses.

The time course of stress-induced changes in *c-fos* mRNA and FOS is quite different. Focusing on emotional or predominantly emotional stressors such as stress immobilization (IMO), it is widely assumed that *c-fos* mRNA levels can be reliably detected even after a brief stress exposure (5–15 min) (Sharp et al., 1991; Senba et al., 1994; Imaki et al., 1995, 1996; Umemoto et al., 1997). Instead, peak FOS expression is generally considered to be reached around 2 h after stress onset. Nevertheless, after 30 min of acute restraint stress, maximum number of FOS+ neurons was observed at 60 rather than 90 min (Yokoyama and Sasaki, 1999). In addition, another study using different times of continuous exposure to restraint or IMO also suggests that the peak levels of FOS+ neurons can be achieved at 1 h rather than 2 h (Chowdhury et al., 2000). In contrast, in another study using 60 min restraint, the maximum response was at 2 rather than 1 h (Fevurly and Spencer, 2004). Immunohistochemistry has been more extensively used than *in situ* hybridization, and the impact of the exposure to brief stressors (e.g., 5 min in a novel environment) is typically quantified 2 h after initial exposure (e.g., Duncan et al., 1996; Leite Silveira et al., 2001). However, no study has investigated the time course of FOS expression assessed immediately after a brief exposure to emotional stress. Thus, the differences in timeline and methodologies employed in the study of *c-fos* expression complicate the interpretation of results of neuronal activation, particularly for brief stress exposures.

Considering that the half-life of *c-fos* mRNA is considerably shorter than that of the FOS protein (Sheng and Greenberg, 1990; Zangenehpour and Chaudhuri, 2002), it is obvious that quantifying mRNA levels is more appropriate than FOS to study the dynamics of the response to stressors when comparing different types of emotional stressors or different periods of exposure. Notably, *c-fos* mRNA levels decline progressively over time (a few hours), despite the persistence of the emotional stressor (Imaki et al., 1992; Trnecková et al., 2007; Marín-Blasco et al., 2018). The mechanisms involved are not entirely clear, but it appears to be in great part due to the progressive reduction of stimulatory inputs when no major harm occurs rather than to an inability of neurons to sustain *c-fos* transcription (Marín-Blasco et al., 2018).

The interpretation of stress-induced FOS in terms of the number of neurons activated is strongly dependent on the assumption that the initial activation of transcription, reflected in the presence of hnRNA, is always followed by the maturation and transport of mature RNA to the cytoplasm, which progresses into the synthesis of the protein. If this is not the case, quantification of FOS-positive neurons can underestimate the number of activated neurons. Nevertheless, a previous study showed that the longer the restraint stress (15–120 min), the higher the number of FOS+ neurons in several brain areas 2 h after stress onset (Crane et al., 2005). These results can be explained by the recruitment of new neurons beyond the initial 15-min exposure, but an alternative explanation is that the duration of stress increases the probability that neurons in which *c-fos* transcription was initially triggered (hnRNA+) complete the necessary steps for mRNA formation and eventual protein synthesis. In this scenario, FOS protein detection would not fully reflect the number of neurons engaged during brief exposures, as not all neurons in which intronic RNA is being transcribed may reach the threshold needed for RNA maturation and translation. Unfortunately, to our knowledge, this potential problem has not been previously addressed.

Therefore, in the present work, we address this issue by studying the transition between initial *c-fos* transcription and RNA maturation as well as the transition between *c-fos* mRNA and protein in male rats after exposure to different durations of IMO. We focus on the prelimbic region (PrL) of the mPFC, as it has been pointed out as a key center for stress processing and coordination of the stress response (revised by McKlveen et al., 2015). Here, using double fluorescent *in situ* hybridization (dFISH) with intronic and exonic probes to detect *c-fos* hnRNA and mRNA levels, respectively (Guzowski et al., 1999; Lin et al., 2011; Kondoh et al., 2016), along with immunofluorescence (IF) to detect FOS protein, we provide new insights into the dynamics of stress-induced *c-fos* expression and its use as a marker of neuronal activation.

2. Materials and methods

2.1. Animals and general procedure

Adult male Sprague-Dawley rats (2 months old) purchased from Janvier were used. The animals were housed in pairs in transparent cages (1000 cm^3; 57 × 27 × 14.5 cm, Panlab SLU) with absorbent bedding. They were maintained under constant temperature (22 °C), relative humidity (40–60 %), and a 12-h light/dark cycle. Standard maintenance food (A-04 diet, Panlab SLU) and water were provided *ad libitum*. The experimental procedures were approved by the Animal and Human Experimentation Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, in accordance with European Union Directive (2010/63/EU) and Spanish legislation (RD53/2013). The animals were acclimatized to the animal facility for one week before the experiment. Additionally, before the experiment, 3–4 handling sessions were performed to reduce the stress associated with experimental procedures.

2.2. Immobilization stress

Rats were immobilized in a prone position by taping their four limbs to metal mounts attached to a board (e.g. Gagliano et al., 2008). Head

movements were restricted with two plastic pieces (7 × 6 cm) placed at each side of the head and the body was additionally subjected to the board by a piece of plastic cloth (10 cm wide) attached with Velcro® surrounding the trunk of the animal. The experimental design and different timelines for each experiment are detailed below and represented in Fig. 1.

2.2.1. Experiment 1: Time-course of c-fos response following 8-min IMO

Rats were randomly assigned to the following experimental groups (n = 4 per group): basal, IMO8, exposed to 8 min IMO and immediately euthanized; IMO8+38, exposed to 8 min IMO and euthanized after a resting period of 38 min.

2.2.2. Experiment 2: Time-course of c-fos response following 15-min IMO

Rats were randomly assigned to the following experimental groups (n = 4 per group): basal, IMO15, exposed to 15 min IMO and immediately euthanized; IMO15+105, exposed to 15 min IMO and euthanized after a resting period of 105 min. One rat for the IMO15 group had to be excluded for technical reasons.

2.2.3. Experiment 3: Comparison of FOS + neurons between two time-matched IMO paradigms: sustained exposure versus recovery phase

Rats were assigned to the following groups (n = 4 per group): basal (n = 4), IMO30+60 (n = 4), exposed to IMO for 30 min and euthanized after a resting period of 60 min, and IMO90 (n = 5), exposed to IMO for 90 min and immediately euthanized.

2.3. Perfusion and histological processing

The two animals in the same home cage were euthanized simultaneously to reduce distress and avoid altering basal RNA expression in the brain samples. Animals were anesthetized by inhalation of isoflurane (Laboratorios Esteve) within a maximum of 30 s after they were removed from the animal room or from the room in which they were exposed to stress. They were transcardially perfused, firstly with sterile saline solution (0.9 % NaCl) for 1 min, and then with 3,7-4 % paraformaldehyde (PFA, Casa Álvarez Material Científico S.A., Spain) for 10 min. After perfusion, their brains were extracted, post-fixed in PFA and stored at 4 °C overnight (O/N). Then, they were embedded in a cryoprotectant solution containing 30 % sucrose in potassium phosphate-buffered saline (KPBS; 0.2 M NaCl, 43 mM potassium phosphate). The brains were then frozen in dry ice-cooled isopentane and preserved at −80 °C until sectioning. Coronal brain sections of 20 μm thickness were obtained serially with a cryostat (Ref. CM3050-S, Leica Microsystems), collected in anti-freeze solution (0.05 M sodium phosphate buffer, pH 7.3, 30 % ethylene glycol, 20 % glycerol) and stored at −20 °C until further processing. Prelimbic cortex sections were mounted on positively charged slides (Superfrost Plus, Thermo Scientific) between Bregma 3.20 and 2.70 mm according to the reference stereotaxic atlas by Paxinos and Watson (2014).

2.3.1. Double fluorescent in situ hybridization (dFISH) probes

The *c-fos* mRNA antisense probe was generated from the EcoRI fragment of rat *c-fos* DNA (Dr. I. Verma, The Salk Institute, La Jolla, CA), subcloned into a pBluescript SK-1 (Stratagene, La Jolla, CA) and linearized with SmaI (Ref. R0141S, New England BioLabs). The intronic *c-fos* probe was a kind gift from Dr Lin (California Institute of Technology, Caltech, CA) and it was linearized with SalI (Ref. R0138S, New England BioLabs). The *c-fos* intronic probe targets specifically the first intron of the *c-fos* gene and hence, only detects immature or nuclear intronic *c-fos* RNA (i.e., hnRNA). In contrast, the *c-fos* mRNA antisense probe was directed against the first three exons and half four exons of the *c-fos* gene. In each transcription to produce the probes, 1 μg of digested plasmid was used as DNA template and UTP labeled with Digoxigenin or Fluorescein (DIG/Fluorescein RNA Labelling Mix 10X conc, Roche) was used as labeled ribonucleotide for *c-fos* intronic RNA and *c-fos* mRNA antisense probes, respectively. After transcription, the cDNA template was digested with RNase-free DNase I (T7 transcription Kit, Roche). Then 45 μL of a sodium chloride-Tris-EDTA buffer solution (STE, 0.1 NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 8.0) was added and the mixture was incubated at 65 °C for 5 min to inactivate enzymes. The probes were isolated through gel filtration columns (mini–Quick Spin RNA Columns, Ref. 11814427001, Roche) and stored at −20 °C.

2.3.2. dFISH procedure

The dFISH protocol used was adapted from Simmons et al. (1989). All the solutions used in the pre-hybridization and hybridization steps were pre-treated with diethylpyrocarbonate (DEPC; Sigma-Aldrich) and sterilized. Tissue was first post-fixed in 3.7–4 % PFA for 20 min, washed in KPBS and then incubated with proteinase K (Roche) at a concentration of 0.01 mg/mL in 100 mM Tris-HCl pH 8.0, and 50 mM EDTA, pH 8.0, for 15 min at 37 °C. After digestion, sections were washed with DEPC-treated water and then in 0.1 M triethanolamine pH 8.0 (TEA; Sigma) and acetylated with 0.25 % acetic anhydride (Sigma) in 0.1 M TEA pH 8.0. Then sections were washed in 2x saline-sodium citrate solution (SSC; Sigma) which contained 0.3 M NaCl, 0.03 M sodium citrate tribasic, and dehydrated through graded increasing concentrations of ethanol air-dried.

After, 150 μL of hybridization buffer (50 % formamide, 0.3 M NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 1x Denhardt's solution, 10 % dextran sulphate, yeast tRNA 500 μg/mL, and 10 mM dithiothreitol [DTT]), containing the DIG-labeled *c-fos* intronic RNA probe (1:2000) and the Fluorescein-labeled *c-fos* mRNA probe (1:2000) were added onto each slide. Then slides were covered with a coverslip and incubated in a humid chamber for 18h at 60 °C. After hybridization, sections were washed in 4x SSC at 37 °C and digested with RNase A (Roche) at 200 μg/mL in an appropriate buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) at 37 °C for 30 min. Then sections were washed at room temperature (RT) in descending concentrations of SSC, heated at 60 °C in 0.1x SSC for 30 min and rinsed in 0,1x SSC at RT. Then sections were stored in a Tris-buffered saline with Tween 20 [T-TBS; 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05 % Tween 20 (Sigma)].

Sections were incubated with H_2O_2 (Sigma) at 3 % in a Tris-buffered saline (TBS: 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) for 30 min at RT to eliminate endogenous peroxidase (POD). Then, slides were incubated in 2 % bovine serum albumin (BSA; Sigma) and 3 % fetal calf serum (FCS) in T-TBS for 1h at RT, to block non-specific binding. After that, slides were incubated with an anti-Digoxigenin antibody (Anti-DIG-POD, Ref. 11093274910, Roche at 1:2000) in 1 % BSA in T-TBS, using incubation chambers (CoverWell, Grace Bio-Labs), O/N at 4 °C. Signal was then amplified using the tyramide signal amplification kit (TSA-Plus Cyanine 5, Akoya Biosciences, 1:50). The day after, the same procedure of POD and non-specific blocking was followed and then slides were incubated with an anti-Fluorescein antibody (Anti-Fl-POD, Ref. NEF710007EA, PerkinElmer, 1:500) O/N at 4 °C. Signal was amplified using the tyramide signal amplification kit (TSA-Plus Fluorescein, Akoya Biosciences, 1:50). After amplification, nuclei were counterstained with Hoechst 33258 pentahydrate (Invitrogen) at 1:10000 in TBS. Finally, slides were washed with TBS and rinsed with deionized water and cover-slipped with an aqueous mounting medium (Fluoromount™, Sigma). Slides were stored at 4 °C in an opaque box to avoid exposure to light and were used for confocal microscopy within 1 month after performing the dFISH for optimal image quality.

2.3.3. Immunofluorescence (IF)

Sections were washed in 1x TBS and then incubated in blocking solution (5 % normal donkey serum, NDS in 1x TBS with 0.4 % Triton X-100) for 1.5h at RT. Then sections were incubated with FOS protein antibody (sc-52-G, Santa Cruz Biotechnology, 1:500) in blocking solution O/N at 4 °C. After that, sections were incubated with the secondary antibody (Alexa Fluor 488-conjugated anti-goat) in a blocking solution for 2h at RT. The slices were then washed in 1x TBS and counterstained

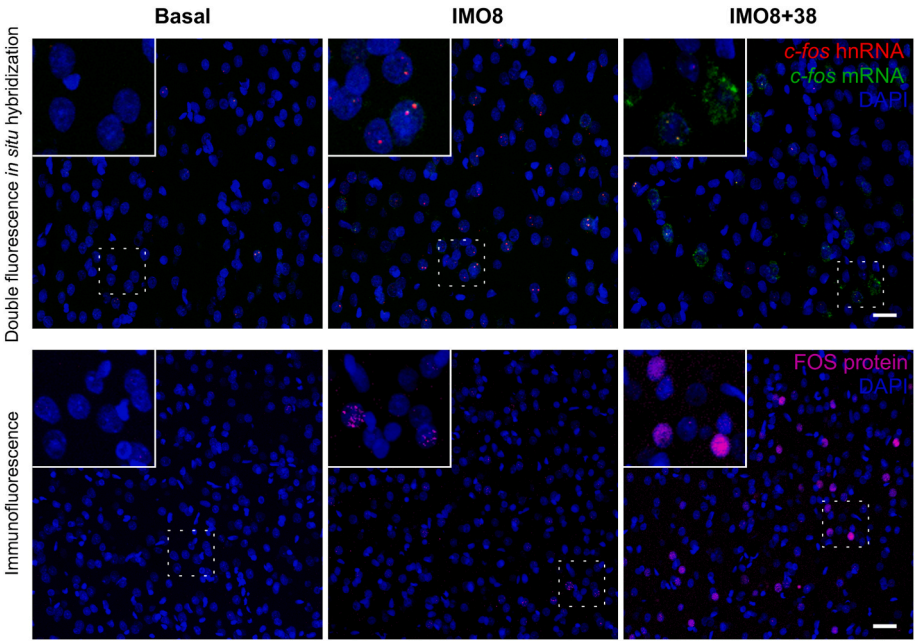

Fig. 2. Representative images of *c-fos* RNA and FOS protein staining in the PrL **after IMO exposure or in basal conditions.** Confocal images of double fluorescent *in situ* hybridization (dFISH) and simple immunofluorescence (IF) assays for c-fos hnRNA and mRNA (upper panels, red and green signal, respectively) and FOS protein (lower panels, magenta signal). The white square highlights a magnified view of the dotted region for greater detail. Rats were exposed to 8 min immobilization stress (IMO) and euthanized either immediately after (middle) or following a 38-min recovery period (right) or under basal conditions (left). Scale bar = 20 μm.

with Hoechst 33258 (1:10000 in 1x TBS), washed twice in 1x TBS and mounted onto slides. All dry-mounted sections were stored at 4 °C in an opaque box until image acquisition.

2.4. Image capture and analysis

For dFISH procedures, images of the PrL were captured using confocal microscopy (Leica TCS SP5 at the Microscopy Service of the Autonomous University of Barcelona). For FOS IF, the prelimbic cortex was captured using the Olympus Fluoview 1000 confocal microscope (Microscopy Service of the Autonomous University of Barcelona). Six images were taken (from 6 different sections) for each animal with the same settings (laser power, gain, and offset) with a 20x objective. Quantification of cells positive for heteronuclear and mature *c-fos*, and FOS protein as well as the signal intensity (integrated density, IntDen) was performed using the image processing software ImageJ (FIJI, version 1.51) and region of interest (ROI) tool. In all cases, the number of cells and IntDen shown for each group was calculated as the average obtained from all the analyzed images for each animal and then averaged for all the animals of the experimental group. The experimenter was always blind to the experimental group.

2.5. Statistics

The statistical package for social science' (SPSS) program was used for statistical analysis (version 23 for Windows). For experiments 1 and 2, statistical analysis was performed using the generalized linear model (GzLM; McCulloch and Searle, 2001). This model does not require homogeneity of variances or normal distribution. Nevertheless, log. transformation of the data was done when this procedure improved the homogeneity of variances. The significance of the effects was determined by the Wald chi-square statistic (χ^2) and where appropriate, *post hoc* pairwise comparisons using sequential Bonferroni's correction were conducted. In experiment 3, the two relevant experimental groups were compared using the Student's *t*-test. In all cases, the criterion for statistical significance was set at $p < 0.05$. The data were graphically represented using GraphPad Prism 9.0 (La Jolla, California, USA).

3. Results

3.1. Time-course of c-fos response following 8-min IMO

In the first experiment, we assessed the dynamics of *c-fos* response to 8 min IMO evaluating the number of hnRNA+, mRNA+ and FOS+ neurons in the PrL immediately after stress exposure and after a 38-min recovery period (Figs. 2 and 3). The GzLM analysis revealed a significant time effect for the number of hnRNA+ ($\chi^2(2) = 270$, $p < 0.001$), mRNA+ ($\chi^2(2) = 84.6$, $p < 0.001$) and FOS+ ($\chi^2(2) = 140$, $p < 0.001$) neurons. *Post-hoc* comparisons of the number of hnRNA+ neurons showed a pronounced increase after 8 min IMO compared to the basal group ($p < 0.001$), which markedly declined during the 38 min post-stress period ($p < 0.001$ vs IMO8) (Fig. 3A). However, the number of hnRNA+ neurons still were higher than basal levels ($p = 0.001$). *Post-hoc* comparisons of mRNA+ neurons showed a significant increase after 8 min IMO ($p < 0.001$ vs basal), with a further additional increase during the post-stress period ($p < 0.043$ vs IMO8) (Fig. 3B). Interestingly, the number of hnRNA+ neurons immediately after IMO was higher than that of mRNA+ neurons after the 38 min recovery period. *Post-hoc* comparisons of the number of FOS+ neurons showed a significant increase after 8 min IMO ($p < 0.001$), followed by a further marked increase after the recovery period ($p < 0.001$ vs IMO8 and basal) (Fig. 3C).

To know whether assessing the level of activation of individual neurons could also add valuable information about stress-induced *c-fos* expression in the PrL, we also measured the total fluorescent signal as well as the intensity of signal per neuron in mRNA+ and FOS+ neurons. For mRNA+ neurons, both total fluorescence intensity and intensity per neuron showed a significant time effect ($\chi^2(2) = 109$ and $\chi^2(2) = 113$, respectively, $p < 0.001$ in both cases) (Fig. 3B). *Post-hoc* comparison of the total mRNA signal revealed an increase after 8 min IMO ($p < 0.015$) and a further rise during the recovery period ($p < 0.001$ vs basal and IMO8). Similarly, the intensity of signal per neuron showed a marked increase in the IMO8 group ($p < 0.001$ vs basal) and an additional

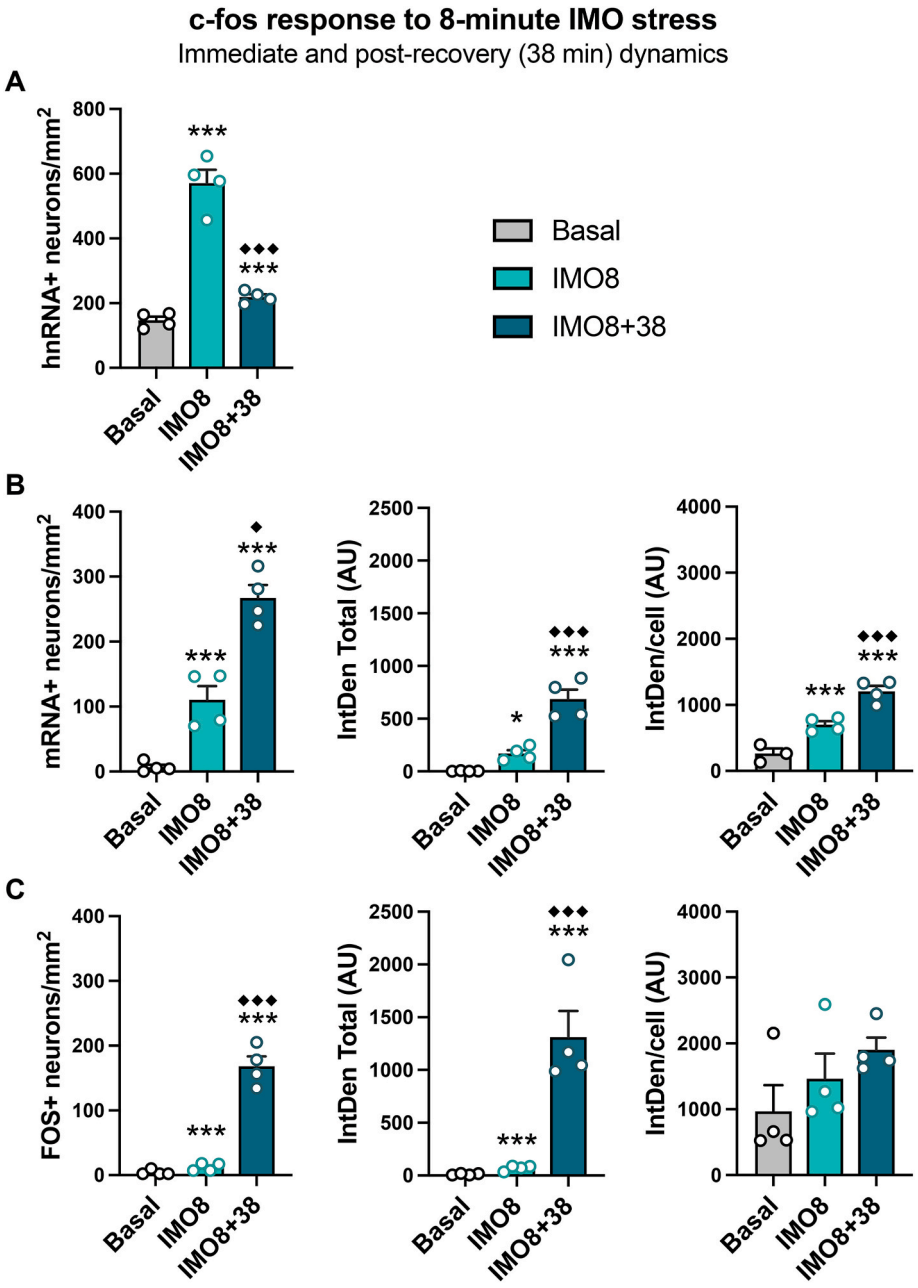

Fig. 3. ***c-fos*** **response to 8 min immobilization (IMO) stress.** Data represented as mean and SEM (n = 4/group). Rats were euthanized under basal conditions or after exposure to 8 min IMO stress, either immediately after or following a 38-min recovery period. Panel A shows the number of hnRNA+ neurons per mm2. Panel B shows the number of mRNA+ neurons per mm2 (left), the total intensity of mRNA signal (middle) and the intensity of signal per neuron (right). Panel C shows the number of FOS+ neurons per mm2 (left), the total intensity of FOS signal (middle) and the intensity of signal per neuron (right). *p < 0.05, ***p < 0.001 vs basal; ◆ p < 0.05, ◆◆◆ p < 0.001 vs IMO8.

increase after the recovery period (p < 0.001 vs basal and IMO8). For FOS+ neurons, significant effects for the total FOS signal ($\chi^2(2) = 197$, p < 0.001) were found, but not for the signal per neuron (Fig. 3C). *Post-hoc* comparisons showed that the total FOS signal increased after 8 min IMO (p < 0.001 vs basal), with a strong additional rise during the recovery period (p < 0.001 vs IMO8 and basal).

3.2. Time-course of c-fos response following 15 min IMO

In a second study, we examined the *c-fos* response immediately after a longer stress exposure, 15 min IMO, and following a 105-min recovery period (Fig. 4).

The statistical analysis revealed a significant time effect for the number of hnRNA+ ($\chi^2(2) = 109$, p < 0.001), mRNA+ ($\chi^2(2) = 183.4$, p < 0.001) and FOS+ ($\chi^2(2) = 98.0$, p < 0.001) neurons. *Post-hoc* comparisons of hnRNA+ neurons showed a marked increase after 15 min IMO compared to the basal group (p < 0.001) that declined during the recovery period to basal levels (p < 0.001 vs IMO15) (Fig. 4A). *Post-hoc* comparisons of mRNA+ neurons showed a significant increase after 15 min IMO (p < 0.001 vs basal), which was sustained during the post-stress period (p < 0.001 vs basal) (Fig. 4B). Remarkably, the number of hnRNA+ neurons immediately after IMO was similar to that of mRNA+ neurons after the recovery period. *Post-hoc* comparisons of FOS + neurons showed a significant increase after 15 min IMO (p < 0.05 vs basal), followed by a marked increase after the recovery period (p < 0.001 vs basal) (Fig. 4C).

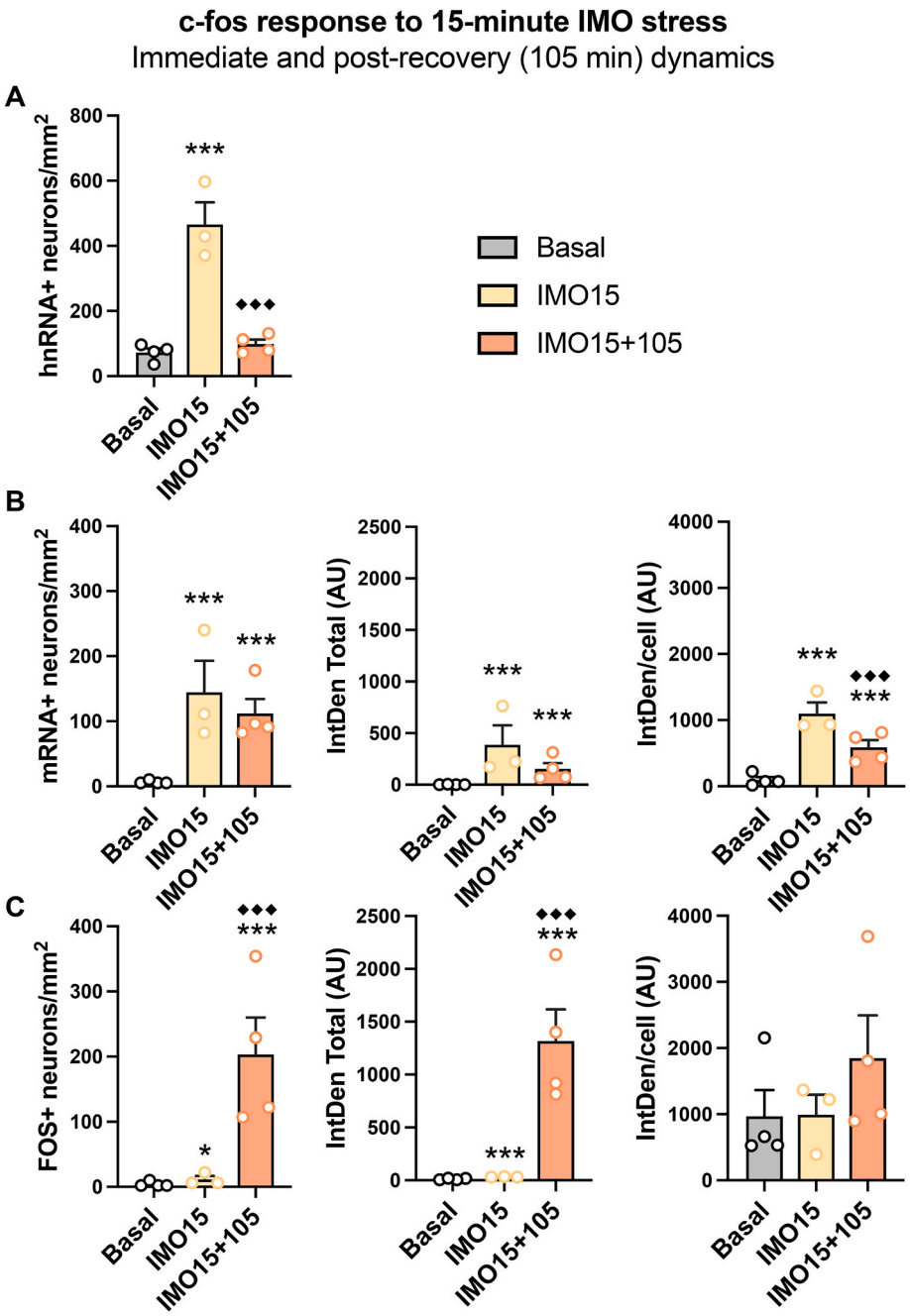

Fig. 4. ***c-fos* response to 15 min immobilization (IMO) stress.** Data represented as mean and SEM (n = 3–4/group). Rats were euthanized under basal conditions or after 15 min IMO stress, either immediately after or following a 105-min recovery period. Panel A shows the number of hnRNA+ neurons per mm2. Panel B shows the number of mRNA+ neurons per mm2 (left), the total intensity of mRNA signal (middle) and the intensity of signal per neuron (right). Panel C shows the number of FOS+ neurons per mm2 (left), the total FOS signal and the integrated signal per neuron. *p < 0.05, ***p < 0.001 vs basal; ◆◆◆ p < 0.001 vs IMO15.

Regarding the quantification of the activation levels of the neurons, a significant time effect for the total mRNA signal intensity and mRNA signal intensity per neuron was found ($\chi^2(2) = 108$, $p = 0.001$, and $\chi^2(2) = 56.0$, $p < 0.001$, respectively) (Fig. 4B). *Post-hoc* comparison of the total mRNA signal showed an increase at 15 min ($p < 0.001$) that was still maintained after the recovery period despite a trend to decrease ($p < 0.001$ vs basal and IMO15). *Post-hoc* comparison of the signal per neuron showed a sharp increase in the IMO15 group ($p < 0.001$ vs basal), with a partial decline after the recovery period ($p < 0.001$ vs IMO15 and basal). Significant effects were observed for the total FOS + signal ($\chi^2(2) = 214$, $p < 0.001$), but not for the signal per neuron (Fig. 4C). *Post-hoc* comparisons revealed a higher total FOS signal at 15 min ($p < 0.001$), with a strong additional increase after the recovery period ($p = 0.001$ vs IMO15 and basal).

3.3. *Comparison of FOS + neurons between two time-matched IMO paradigms: sustained exposure versus recovery phase*

To determine the impact of prolonged stress, as well as the influence of recovery after stressor exposure, we compared FOS expression in rats subjected to 90 min of sustained IMO and rats exposed to 30 min of IMO followed by 60 min of recovery (Fig. 5). Analysis of the number of FOS+ neurons (Fig. 5A), as well as the intensity per cell (Fig. 5B) indicated that both parameters were higher in the IMO90 than in the IMO30+60 group ($t = 2.88$, $p = 0.012$; $t = 4.11$, $p = 0.002$, respectively).

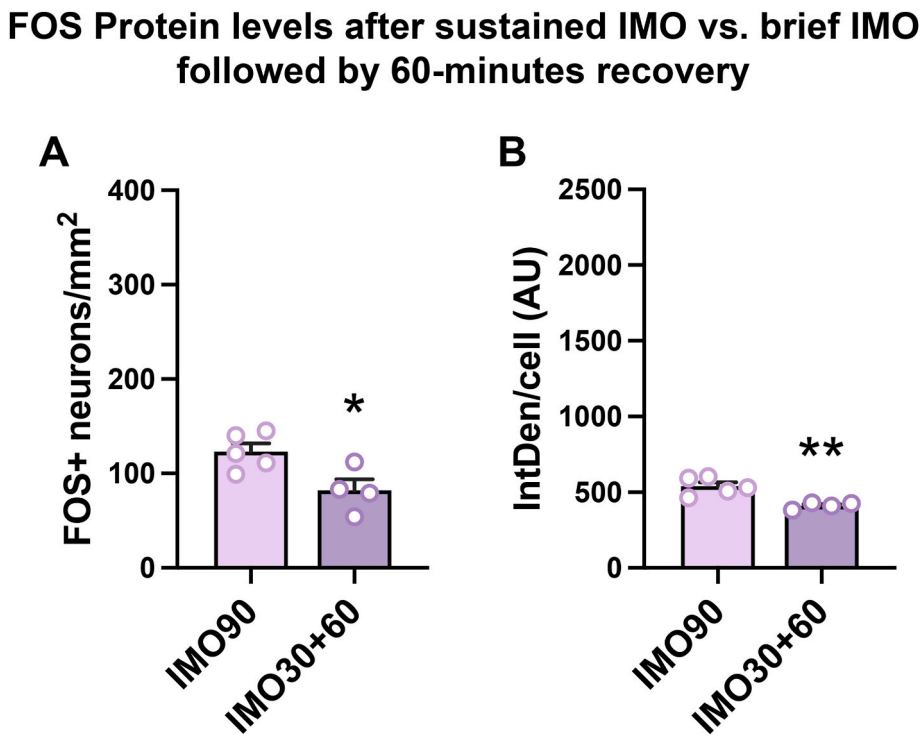

Fig. 5. FOS protein levels in response to 90 min immobilization (IMO) compared with 30 min IMO followed by a 60 min-recovery period. Data represented as mean and SEM (n = 4–5/group). Panel A shows the number of FOS+ neurons per mm2. Panel B shows the total intensity of FOS signal per neuron. *p < 0.05, **p < 0.01 vs IMO90.

4. Discussion

By combining dFISH of intronic and exonic *c-fos* probes alongside IF for FOS protein, the present study reveals novel interesting insights into the dynamics of stress-induced *c-fos* expression in the brain. A key finding is that some threshold level appears to be required for the transition from immature to mature *c-fos* RNA. Therefore, studies relying solely on the measurement of a single *c-fos* form, particularly FOS protein, might only partially represent the neuronal populations activated by stressors, at least with brief exposures (less than 15 min).

In the first experiment, we evaluated *c-fos* expression in response to a brief (8 min) exposure to IMO and included a recovery period of 38 min as a compromise between maximum *c-fos* mRNA levels and detection of FOS (Cullinan et al., 1995; Imaki et al., 1996; Chowdhury et al., 2000; Pace et al., 2005). Under basal conditions, the number of mRNA+ and FOS+ neurons was very low. However, a much higher number of hnRNA+ than mRNA+ neurons were detected in these conditions. This is probably due to the extremely fast activation of *c-fos* transcription (hnRNA), likely triggered by animal transport, anesthesia and perfusion needed to obtain brain samples. A minimum time of 2.5 min is required to take the animals and start the *in vivo* brain fixation procedure, a period that could be sufficient to initiate *c-fos* transcription. These findings underscore the need for careful experimental design when evaluating the hnRNA of IEGs in response to stimuli. Despite this fact, a marked rise in the number of hnRNA+ neurons was still observed after 8 min IMO. After imposing a 38-min recovery period, the number of hnRNA+ neurons markedly declined, although remaining above basal levels, suggesting that transcription was maintained in some neurons. This rapid *c-fos* transcription aligns with previous studies that reported increases in the hnRNA of *c-fos* and other IEGs such as *Arc* and *Zif268* after 2–5 min stress exposure (Guzowski et al., 1999; Lin et al., 2011).

Regarding mRNA+ neurons, a more than 15-fold increase with respect to basal levels was already found after 8 min IMO. Interestingly, measurement of the intensity of the signal per cell showed a nearly 3-fold increase from basal to 8 min IMO. As a result, the total integrated signal for mRNA increased about 50-fold after 8 min IMO, indicating that not only the number of positive neurons but also the intensity of the signal should be considered. After recovery, the number of mRNA+ neurons increased further, concomitantly with a higher signal intensity per neuron. However, the number of mRNA+ neurons observed after recovery was lower than the number of hnRNA+ neurons after 8 min IMO. Considering that the half-life of mature *c-fos* transcript is about 10–15 min (Sheng and Greenberg, 1990; Zangenehpour and Chaudhuri, 2002) and that *c-fos* mRNA levels reach a maximum in about 30 min even after a short exposure to emotional stressors (Imaki et al., 1993; Emmert and Herman, 1999), it is unlikely that some neurons were not detected because of the degradation of previously formed mRNA. The most plausible explanation is that a certain threshold level of nuclear transcription is needed for RNA maturation. The subsequent detection of FOS protein in a similar number to mRNA+ neurons supports the hypothesis that in general, in all activated neurons, *c-fos* mRNA transitions to FOS protein.

Somewhat unexpectedly, we found a significant increase (3-fold) in the number of FOS+ neurons already 8 min after stress, indicating an extremely fast stress-induced protein synthesis. To our knowledge, this phenomenon has not been explored in the mPFC, but other studies have detected FOS protein as early as after 5 min IMO in the locus coeruleus using Western blot (Hebert et al., 2005), or after 10 min of tactile stimulation in the somatosensorial cortex by immunohistochemistry (Bisler et al., 2002). Additionally, 15 min restraint increased FOS in several brain areas, including the mPFC (Kellogg et al., 1998) and 20 min of stress also increased the number of FOS+ neurons in the lateral septum (Marín-Blasco et al., 2018). It thus appears that the synthesis of FOS is an outstanding example of fast protein synthesis whose underlying mechanisms remain to be studied.

How the duration of stress exposure affects the number of recruited neurons and the expression levels of *c-fos* is at present unclear. In this regard, Crane et al. (2005) showed a progressive increase in the number of FOS+ neurons in several brain areas with increasing times of exposure to restraint (15–120 min), when measured always 2 h after stress onset.

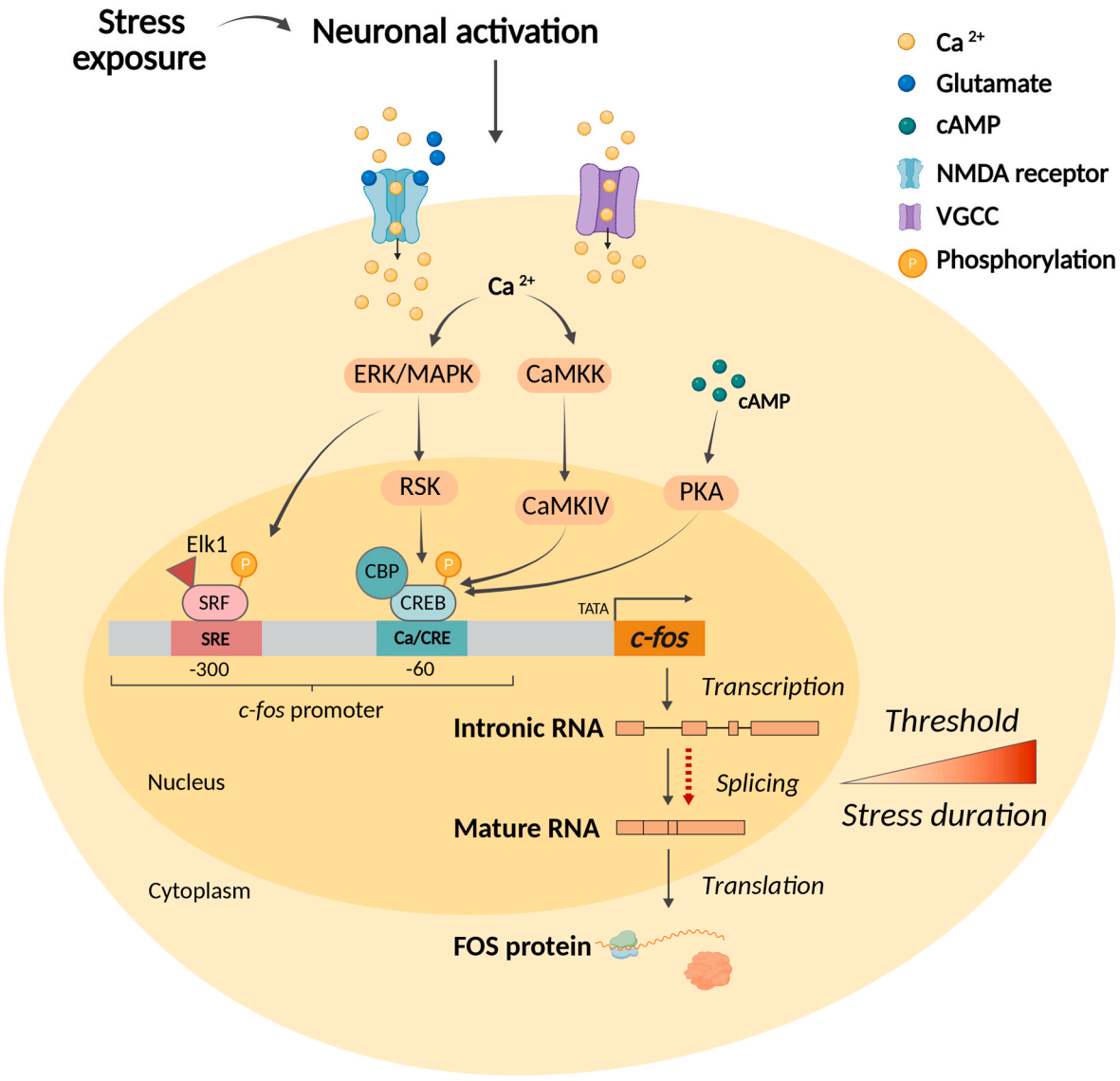

Fig. 6. Schematic diagram of the molecular mechanisms regulating stress-induced c-fos expression in the PrL. After stress exposure, calcium influx through N-methyl-D-aspartate receptors (NMDAR) and voltage-gated calcium channels (VGCC) leads to the activation of calcium-regulated signaling proteins, including extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and calcium-calmodulin dependent kinases (CAMK). Furthermore, increases in cyclic adenosine monophosphate (cAMP) activate protein kinase A (PKA). ERK/MAPK activate the ribosomal S6 kinase (RSK), which phosphorylates the cAMP-response element-binding protein (CREB) that is also the target of CAMKIV and PKA. CREB, together with CREB binding protein (CBP) binds to the calcium/cAMP response element (Ca/CRE) in the *c-fos* promoter, a regulatory element critical for activity-dependent *c-fos* transcription. ERK/MAPK activation also phosphorylates Elk-1, which binds to the serum response factor (SRF) that, in turn, binds to the serum response element (SRE). These processes induce the rapid transcription of *c-fos* intronic RNA in the nucleus. The intronic RNA undergoes splicing to produce mature RNA, which is then exported to the cytoplasm for translation of FOS protein. Our findings indicate that the transition from intronic to mature RNA in the PrL seems to require sustained transcriptional activity (indicated by the dashed red arrow), which is directly associated to stress duration. Part of the scheme is based on Kovács (1998); Cruz et al. (2015).

Two possible explanations for these findings are that new neuronal populations are recruited when stress exposure is prolonged or that longer stress durations can favor the transition from *c-fos* hnRNA to FOS protein. To assess these hypotheses, we studied in another set of animals the *c-fos* response to 15 min IMO, imposing a resting period of 105 min to follow the typical design in the literature of *c-fos* when evaluating FOS+ neurons.

After 15 min IMO, the number of both hnRNA+ and mRNA+ neurons increased, but again a lower number of mRNA+ than hnRNA+ was found, confirming the results obtained after 8 min IMO. In addition, after the 105-min recovery period, the number of hnRNA+ neurons was indistinguishable from controls, indicating that IMO-induced transcription was no longer maintained. The number of mRNA+ neurons after recovery did not differ from those observed after 15 min IMO, although a trend to decrease the number and a significant decrease in intensity of signal per neuron were found. Again, the number of FOS+ neurons did not differ from that of mRNA+ neurons at 15 min, suggesting that in those neurons that maintain detectable levels of mRNA, the protein was also detectable. It thus appears that increasing the duration of stress favors the transition from *c-fos* hnRNA to mRNA and supports the hypothesis that all mRNA+ neurons become FOS+. In view of these results, the apparent recruitment of neurons beyond the 15-min period reported by Crane et al. (2005) might be better explained by an increased probability that neurons in which *c-fos* transcription was activated (hnRNA+) for a certain time would complete the transition to FOS protein synthesis.

To further demonstrate that the final number of FOS+ neurons is dependent on the duration of the stressor, we compared in a third

experiment the number of FOS+ neurons in rats maintained immobilized for 90 min with another group exposed to the stressor for 30 min followed by a 60-min recovery period. A higher number of FOS+ neurons as well as higher signal intensity per neuron were found in the continuous 90-min IMO group compared with the group allowed to recover. Since the maximum levels of the FOS protein are reached between 1 and 2 h after stress onset and its estimated half-life is 1h (Kruijer et al., 1984), it is unlikely that the lower number of FOS+ neurons after recovery was due to lack of detection of neurons in which *c-fos* protein was initially expressed. We did observe that the integrated signal per neuron was significantly lower after recovery than after continuous IMO exposure, suggesting partial degradation of *c-fos* protein. Overall, these results confirm our hypothesis that sustained stress enhances the likelihood of the transition from transcriptional initiation to protein synthesis and the fact that relying solely on FOS protein expression may underestimate the true extent of neuronal activation, especially after short stress exposures.

Although our study provides novel insights into the temporal dynamics of c-fos expression in response to acute stress, several limitations should be acknowledged. First, the relatively small sample sizes (n = 4 per group) might constrain the statistical power and generalizability of our results, although our data were consistent across experiments, and the statistical significance of the comparisons conducted was high. Second, we employed dFISH because it allowed us to detect and distinguish between different c-fos expression stages (intronic and mature *c-fos* transcripts) with a high spatial and subcellular resolution in intact brain tissue architecture. However, dFISH is a time-consuming technique that requires specific probes for target sequences as well as accurate image acquisition and quantification. More recent techniques such as single-cell RNA-sequencing and spatial transcriptomics offer higher sensitivity to quantify RNA changes, greater sensitivity to detect low-abundance targets, and broader gene multiplexing, although with a higher cost and in some cases without spatial context (e.g. single-cell RNA sequencing). For our purposes, dFISH provided us with a clear temporal snapshot of transcriptional activity with a high spatial resolution (even nuclear vs cytoplasmic), which was critical for interpreting the progression of c-fos expression. Finally, our study was restricted to the PrL subdivision of the rat prefrontal cortex given its implication in stress processing and its relevance in stress-related disorders (McKlveen et al., 2015). While it is plausible that the dynamics of expression described here may apply to other brain regions, some studies have indicated that c-fos expression can reach its peak at different time points after stress exposure across diverse brain regions (Cullinan et al., 1995; Trnecková et al., 2007; Bonapersona et al., 2022). Thus, future studies should explore whether the c-fos expression dynamics and molecular transitions described in the present study are conserved across different stress-responding brain regions and in response to different modalities or durations of stress.

In conclusion, the present work highlights the complex temporal dynamics of *c-fos* expression, from transcription initiation to protein production (Fig. 6). By combining for the first time the measurement of the three main stages of *c-fos* expression in response to stress, we demonstrate an extremely fast protein synthesis and that the transition between transcription initiation and protein production has certain threshold requirements, probably related to the duration of stress exposure. Future studies are needed to elucidate in more detail the key molecular mechanisms underlying these processes in the brain and their relationship with stress duration.

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CRediT authorship contribution statement

Patricia Molina: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xavier Belda:** Writing – review & editing, Methodology, Investigation, Data curation. **Sandra Beriain:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sara Serrano:** Writing – review & editing, Data curation. **Gentzane Compte:** Writing – review & editing, Data curation. **Raül Andero:** Writing – review & editing, Supervision, Conceptualization. **Antonio Armario:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interest or potential conflict of interest.

Data availability

Data will be made available on request.

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