Research article

Stress-induced c-fos expression in the medial prefrontal cortex differentially affects the main residing cell phenotypes

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A B S T R A C T

Stress poses a challenge to the body's equilibrium and triggers a series of responses that enable organisms to adapt to stressful stimuli. The medial prefrontal cortex (mPFC), particularly in acute stress conditions, undergoes significant physiological changes to cope with the demands associated with cellular activation. The proto-oncogene c-fos and its protein product c-Fos have long been utilized to investigate the effects of external factors on the central nervous system (CNS). While c-Fos expression has traditionally been attributed to neurons, emerging evidence suggests its potential expression in glial cells. In this study, our main objective was to explore the expression of c-Fos in glial cells and examine how acute stress influences these activity patterns. We conducted our experiments on male Wistar rats, subjecting them to acute stress and sacrificing them 2 h after the stressor initiation. Using double-labelling fluorescent immunohistochemistry targeting c-Fos, along with markers such as GFAP, Iba-1, Olig2, NG2, and NeuN, we analyzed 35 μm brain slices obtained from the mPFC. Our findings compellingly demonstrate that c-Fos expression extends beyond neurons and is present in astrocytes, oligodendrocytes, microglia, and NG2 cells—the diverse population of glial cells. Moreover, we observed distinct regulation of c-Fos expression in response to stress across different subregions of the mPFC. These results emphasize the importance of considering glial cells and their perspective in studies investigating brain activity, highlighting c-Fos as a response marker in glial cells. By shedding light on the differential regulation of c-Fos expression in response to stress, our study contributes to the understanding of glial cell involvement in stress-related processes. This underscores the significance of including glial cells in investigations of brain activity and expands our knowledge of c-Fos as a potential marker for glial cell responses.

1. Introduction

Stress is a challenging condition that disrupts the homeostasis of the central nervous system (CNS) and activates behavioral and physiological responses to cope with the stressor [1,2]. Acute stress induces a rapid increase in neurotransmission, neuronal activation, and hormone release, ultimately culminating in profound alterations in the physiological parameters of the brain [3]. In the face of a stressor, distinct brain regions are activated, and among them, the medial prefrontal cortex (mPFC) [4] has emerged as a region of

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particular sensitivity to stress [5]. The mPFC is involved in various cognitive functions, including attention [6], decision-making [7], and working memory [8].

The assessment of cellular activation involved in the effects of stress on the mPFC and other brain regions can be accomplished through the examination of c-Fos protein expression, which is encoded by the proto-oncogene c-fos. This immediate early gene (IEG) possesses the capacity to modulate the expression of target genes (TGs) [9], acting as a component of various inducible enhancer pathways (CRE, SER, SIE) [10]. The low basal expression of c-Fos in the absence of stimulation renders it a reliable marker for quantifying neuronal activation in response to diverse stimuli [11]. Consequently, the detection of its nuclear protein has been extensively employed to investigate CNS activation and delineate neural pathways [12,13]. Experimental studies employing social stress [14–16], restraint stress [17], photoshock [18], electric shock [19], and exposure to environmental noise [20,21] have consistently demonstrated alterations in c-Fos protein activation. These findings underscore the potential of c-fos and its protein as valuable tools for investigating gene transcription in response to exogenous factors and for elucidating the neural networks implicated in stress responses.

Emerging research has brought attention to the involvement of glial cells in mediating brain activity, prompting the investigation of whether these cells are also subject to c-fos expression induced by stress [22]. Glial cells are no longer regarded solely as passive support cells but have been discovered to have crucial roles in modulating neural activity, information processing, and stress responses [23]. For instance, astrocytes have the ability to release neurotransmitters and regulate synaptic activity [24], while microglia are involved in regulating inflammation and synaptic pruning [25]. Oligodendrocytes and oligodendrocyte precursor cells (OPCs), on the other hand, are responsible for myelination and play critical roles in enhancing neural transmission speed and efficiency [26]. These findings suggest that glial cells significantly contribute to brain function and activity.

While c-Fos expression has primarily been associated with neurons, emerging evidence suggests that glial cells, including astrocytes, oligodendrocytes, microglia, and NG2 cells, may also express this immediate early gene [22]. In vitro studies have evidenced for example that cultured astrocytes may express c-Fos after addition of growth factors [27]. Activated microglia in the other hand may also react with c-Fos expression after LPS administration [28,29]. And, finally, the addition of growth factors to cultured cells, has been also proved to induce c-Fos in oligodendrocytes and OPCs [30]. Despite these findings, the expression of c-Fos in glial cells under stress and other aversive conditions remains unexplored.

Therefore, the present study aims to investigate the expression of c-Fos in different cell types in the brain, with a particular focus on glial cells, in response to acute stress. Additionally, the study seeks to examine these expression differences within the medial prefrontal cortex, given its high susceptibility to stress.

2. Materials and methods

2.1. Animals

Male Wistar rats, aged 90 days, were utilized for this study. The rats were housed in a controlled environment with a 12:12 light-dark cycle, maintained at a temperature of $22 \pm 2\,°C$, and a humidity level of 70 %. Prior to exposure to the stressful stimulus, the rats had unrestricted access to balanced food and water. The animals were procured from the in-house breeding facility at the West Center for Biomedical Research in Guadalajara, Mexico. All experimental procedures were conducted in accordance with the guidelines outlined in the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Institutional Ethics Commission, CI. 068–2014.

2.2. Varied acute stress protocol

To induce the expression of c-Fos, we used a combination of two different stressors, with a 10-min rest period between each stimulus. The first stressor consisted of a 30 min restraint stress protocol, where each rat was separately confined inside a PET plastic cylinder measuring 8×18 cm, equipped with 5 mm holes at each end to ensure proper ventilation. This setup allowed the rat to breathe comfortably while restricting its movement. The second stressor was the forced swimming paradigm, applying a modified Porsolt protocol [31]. The rats were individually placed in a plexiglass cylinder measuring 45 cm in height and 30 cm in diameter. They were then compelled to swim for a duration of 15 min, while maintaining the water temperature at a constant $10\,°C$ [32]. Taking as a reference previous studies on the c-Fos expression in rats [33,34], we employed four rats to evaluate the stress effects in this experiment. Meanwhile, the control group consisted of other four rats that were not subjected to any stressors and were kept under standard laboratory conditions.

2.3. Intracardiac perfusion

Two hours after exposure to the first stressful stimulus, the animals were administered a sublethal dose of sodium pentobarbital (60 mg/kg) via injection. Following the administration, the animals were perfused through the left ventricle with a 0.9 % sodium chloride solution, followed by a fixative solution consisting of 3.8 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The perfusion process ensured the proper fixation of the brain tissue. After perfusion, the brains were carefully extracted and left in post-fixation for a period of 2 h. Subsequently, the post-fixed brains were sliced into coronal sections with a thickness of 35 μm using a Leica VT1000E vibratome. Slices were taken at intervals of every four cuts, resulting in sections that were 140 μm apart. An average of 10 sections per cellular marker were obtained for each brain rat in total. This process was carried out until the medial prefrontal cortex of

the rat was completely covered. The stereotactic coordinates of Paxinos were employed as a reference guide during this procedure, specifically targeting the range of bregma +4.20 to +2.52 mm [35].

2.4. Fluorescence immunohistochemistry

Each brain was used to process the c-fos and the other five cellular markers of interest for our study. The tissue sections were initially washed in tris buffered saline with 1 % Tween (TBST) to remove any residual contaminants. Subsequently, blocking of non-specific binding sites was performed by incubating the sections in TBST containing 5 % goat serum for a duration of 1 h. The blocking step was carried out at 37 °C for 30 min, followed by an additional 30 min at room temperature. To detect the expression of c-Fos, the sections were incubated with either rabbit or mouse anti-Fos antibodies. Specifically, the antibodies used were cell signalling (E8) and Santa Cruz (SGIABO253), both at a dilution of 1:500 in TBST. The sections were incubated with the primary antibodies for a total of 54 h at 4 °C. For double labeling, additional antibodies were employed to detect specific cell types. These included mouse anti-NeuN (Millipore MAB377) at a dilution of 1:500, mouse anti-NG2 (Millipore 05–710) at a dilution of 1:100, rabbit anti-GFAP (Dako Z0334) at a dilution of 1:750, rabbit anti-Iba1 (Thermo Fisher 016–24461) at a dilution of 1:250, and rabbit anti-Olig2 (Millipore AB9610) at a dilution of 1:300. After 24 h of incubation with the primary antibodies, the sections were rinsed in TBST and subsequently incubated in secondary antibodies. The secondary antibodies used were anti-rabbit Alexa and anti-mouse Alexa, both at a dilution of 1:1000 in TBST. The incubation with the secondary antibodies was carried out for 2 h at a temperature of 20–25 °C. The secondary antibodies used were provided by Invitrogen and were labeled with Alexa 488 and Alexa 594, respectively. Following the incubation with the secondary antibodies, the sections were rinsed in tris buffered saline (TBS) to remove any unbound antibodies and other residues.

2.5. Image processing

The sections were carefully mounted onto slides and subsequently subjected to microscopic analysis. In order to ensure representative sampling, a systematic random sampling approach was employed, with a sampling interval of 5. The sections of the medial prefrontal cortex (mPFC) were further subdivided into specific regions, namely the dorsal Anterior Cingulate Cortex (ACC), Prelimbic (PL), and Infralimbic (IL) cortex. Additionally, the primary somatosensory cortex (Bregma 2.70–2.20 mm) was selected as an intra-subject control region, since there is no evidence linking this region to the acute stress response [15,19]. The microscopic analysis
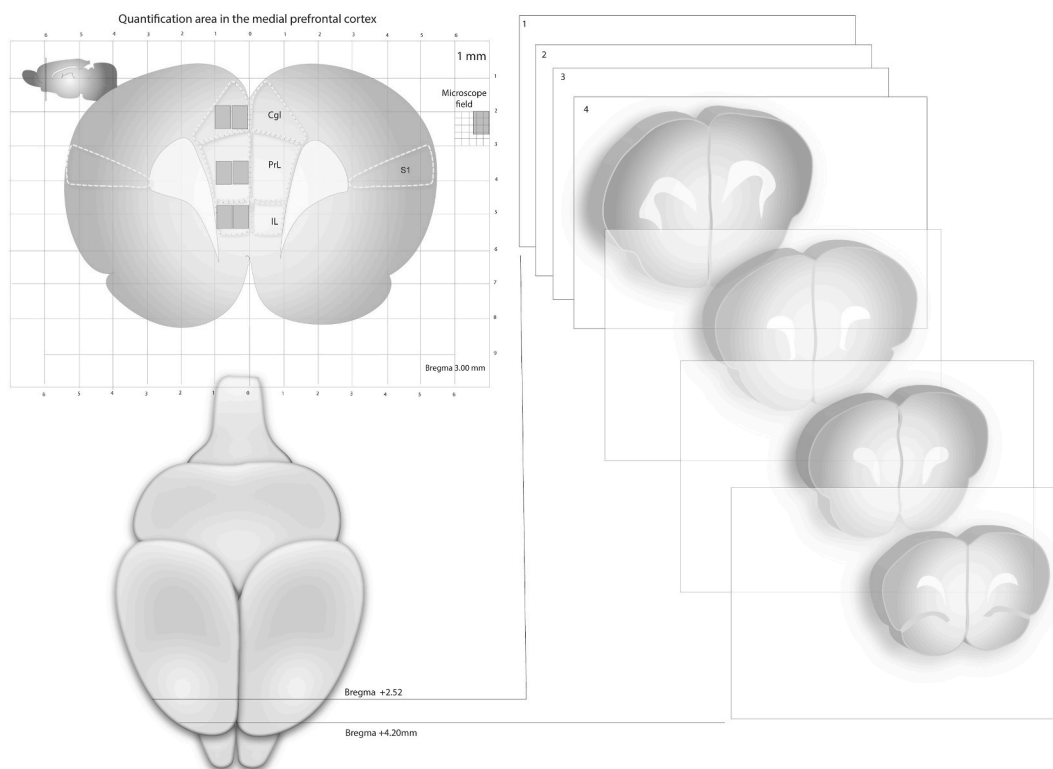
Fig. 1. Region of study and cell counting method. A) The figure shows a coronal section where our region of interest and the intra-subject control region were located. Pointed lines identify the medial prefrontal cortex (region of interest), and the primary somatosensory cortex (control region). Dark rectangle represents a microscopic field where the camera was posed. B) Systematic random sampling method for cell counting.

was performed using microphotographs captured with a Leica DFC 7000T camera, mounted on a DMi8 microscope at 20X magnification., which allowed for the visualization of an area measuring 622.18 x 466.55 μm. On average, approximately 650–720 microscope fields were counted per animal to ensure a comprehensive analysis. Two representative fields were considered per subregion on each hemisphere, and all cortical layers were included in the analysis.

2.5.1. c-Fos positive cell count

For this analysis, the captured microphotographs were processed using the freely available software ImageJ. This software facilitated the manipulation and enhancement of the images, enabling a clearer visualization of the marks. We used the "multi-point" tool for c-Fos measurement as an aid for the manual counting of completely stained spherical cells, which were considered c-Fos positive marks in each microscopic field (622.18 × 466.55 μm).

2.5.2. Double-label quantification

Merges from the c-Fos marked channel + specific cell type (NeuN, GFAP, Iba1, Ng2 or Olig2) were created using the Leica LCS
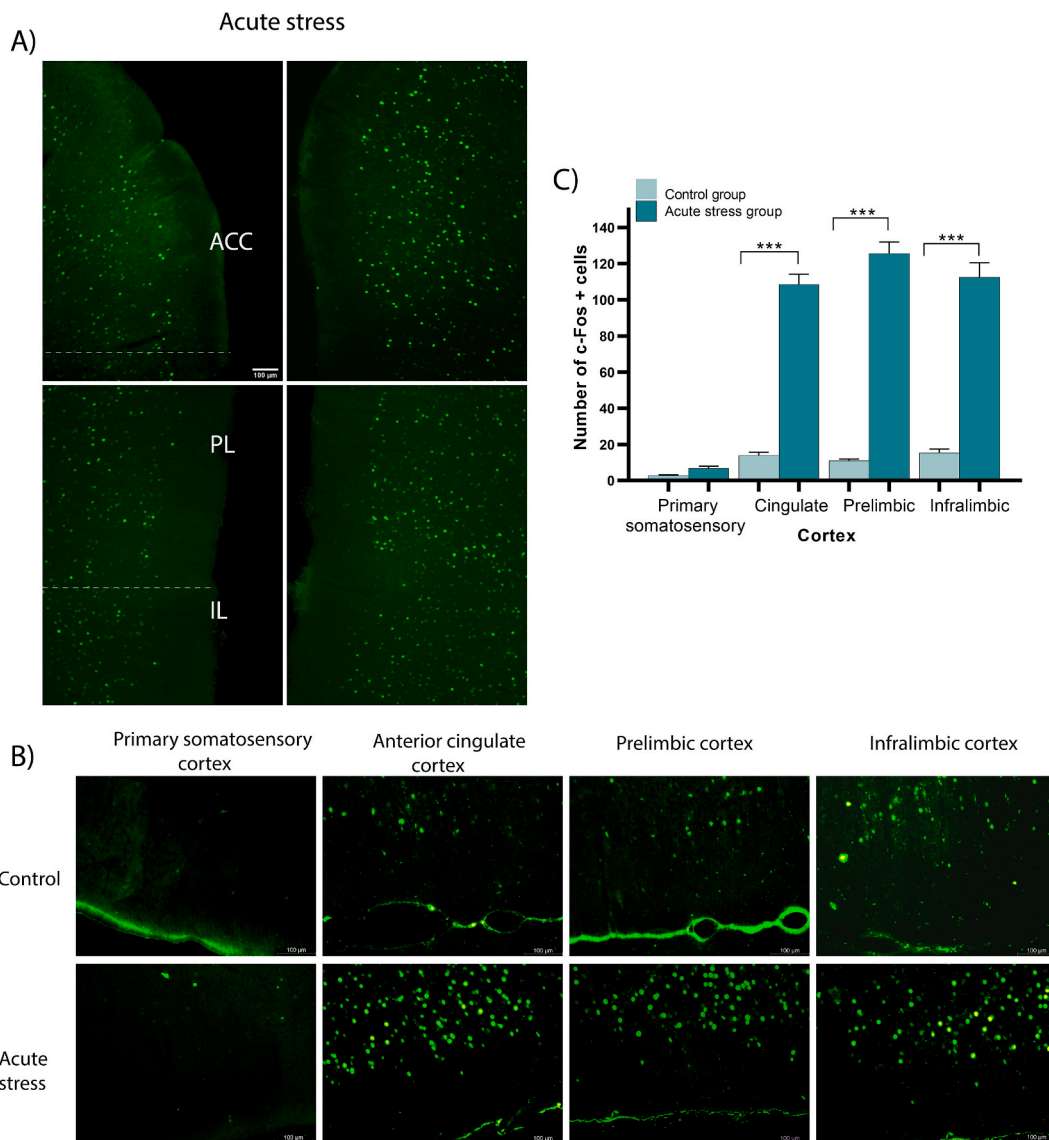

Fig. 2. c-Fos expression in medial prefrontal cortex of rats exposed to acute stress. A) the figure displays the representation of c-Fos expression in the ACC, PL and IL at a magnification of 10X B) Micrographs at a magnification 20X depict the expression of c-Fos in control and stress groups in every subregion of the mPFC and the primary somatosensory cortex of the rats. Fluorescent micrograph has a scale bar 100 μm. C) The graph corresponding to the count of Fos-positive cells in the control and acute stress groups is presented. The data represents the mean ± SEM. (One-way ANOVA; ***p < 0,001).

software tools "merge channels", "zoom" and "adjust of image". We used the c-Fos microphotographs as a reference to manually colocalize NeuN, GFAP, Iba1, Ng2 or Olig2 marks. Double labeled cells were corroborated on higher magnification (40x or 63x) and 50 % overlap among 3 z-stacks (0.9 μm each) was established as a minimum colocalization criteria. A Leica TCS SP2 confocal system was employed to establish colocalization criteria. The analysis was performed blind to treatment. Fig. 1 provides a visual representation of the regions of interest and the sampling method employed.

2.6. Statistical analysis

Descriptive statistical analysis was employed to examine the data, including measures of central tendency (mean) and dispersion (standard error of the mean SEM). These measures provided a summary of the quantitative variable under investigation. To compare the differences between the stress and non-stress conditions in every cell types, student's *t*-test were conducted. One-way analysis of variance (ANOVA) was performed to further explore and compare differences between regions and different types of brain lineage cells. To analyse expression differences between brain cell types after exposure to the stimuli we conducted a chi square test. As a complement, we conducted a mixed two-way ANOVA analysis to estimate the effect of factors implied on the experiment (stimuli and cell type) and its interaction. The impact of the effect was measured as partial eta squared and statistical significance was determined based on a predetermined threshold, typically set at $p < 0.05$.
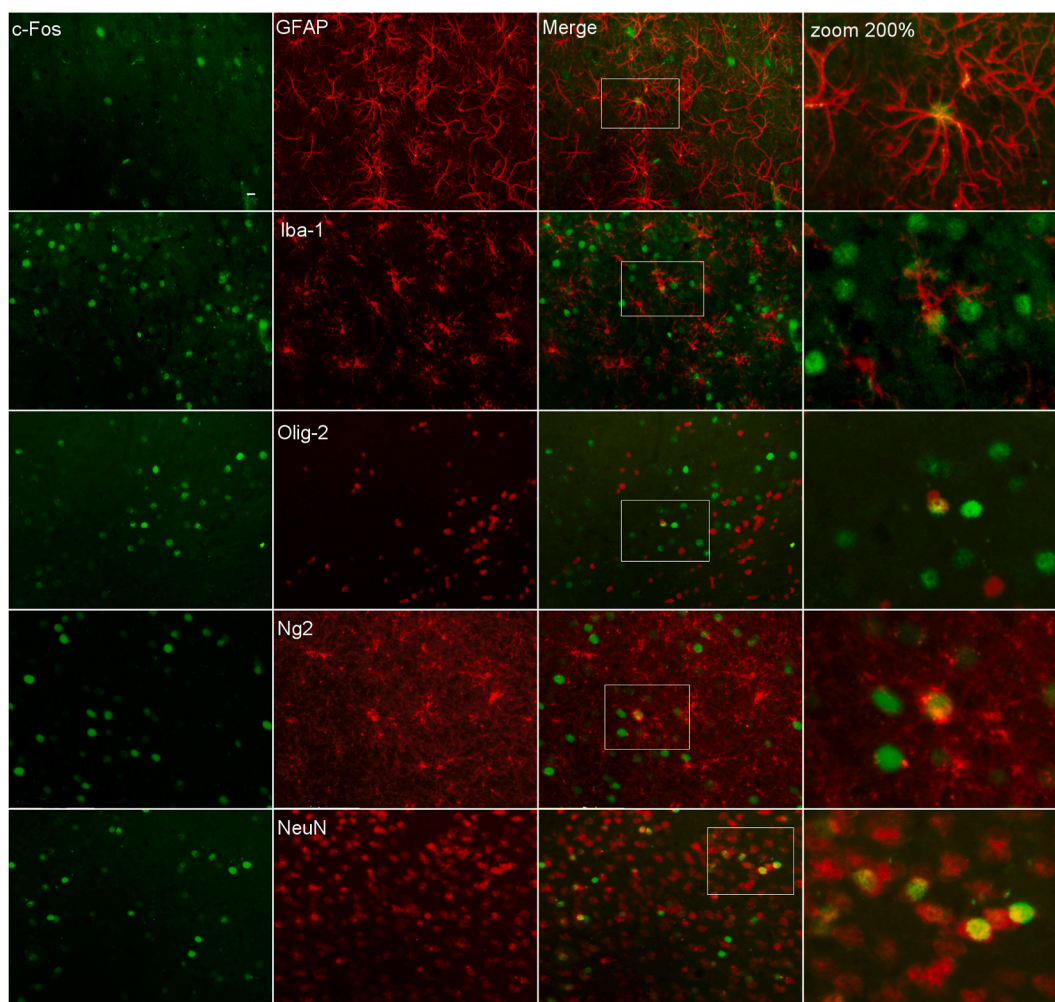

Fig. 3. c-Fos can be expressed by glial cells in addition to neurons in stressed brain. The images show evidence of double labeling in both neurons and glial cells with c-Fos in the brains of rats exposed to acute stress. Green signal correspond to positive c-Fos cells (first column) and the red signal (second column) indicates positive cell labeling for glia (GFAP, Iba-1, Olig2, NG2) and neurons. The third column show merges at 40X magnification. Scale bar 10 μm. The white rectangles indicate the area that was zoomed in by 200 % to illustrate a double labeling, which can be seen in the fourth column.

3. Results

3.1. Acute stress increases c-fos expression in mPFC

The number of c-Fos-positive cells was quantified in the Anterior Cingulate Cortex (ACC), Prelimbic Cortex (PL), and Infralimbic Cortex (IL) (Fig. 2A) of both the control and acute stress groups. The analysis revealed a significant increase in c-Fos expression in the mPFC of the stress group compared to the control group (Fig. 2B and C; $p < 0.001$). This enhanced expression was observed across all subregions of the mPFC, including the ACC, PL, and IL. Interestingly, there were no statistically significant differences detected between the subregions of the mPFC, indicating a similar level of c-Fos expression among these areas. These findings confirm the heightened expression of c-Fos, reflecting increased cellular activation, in response to acute stress within the medial prefrontal cortex,
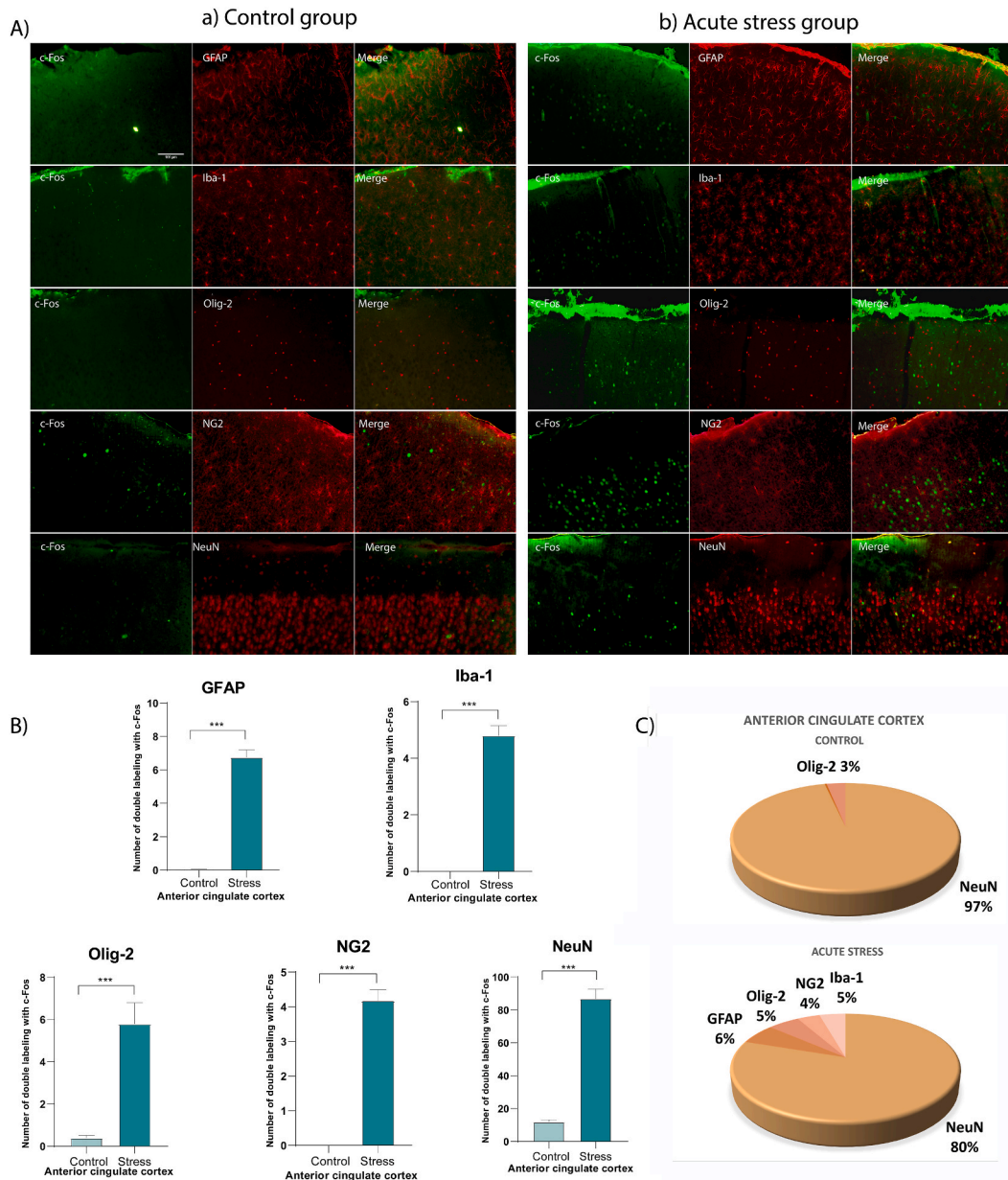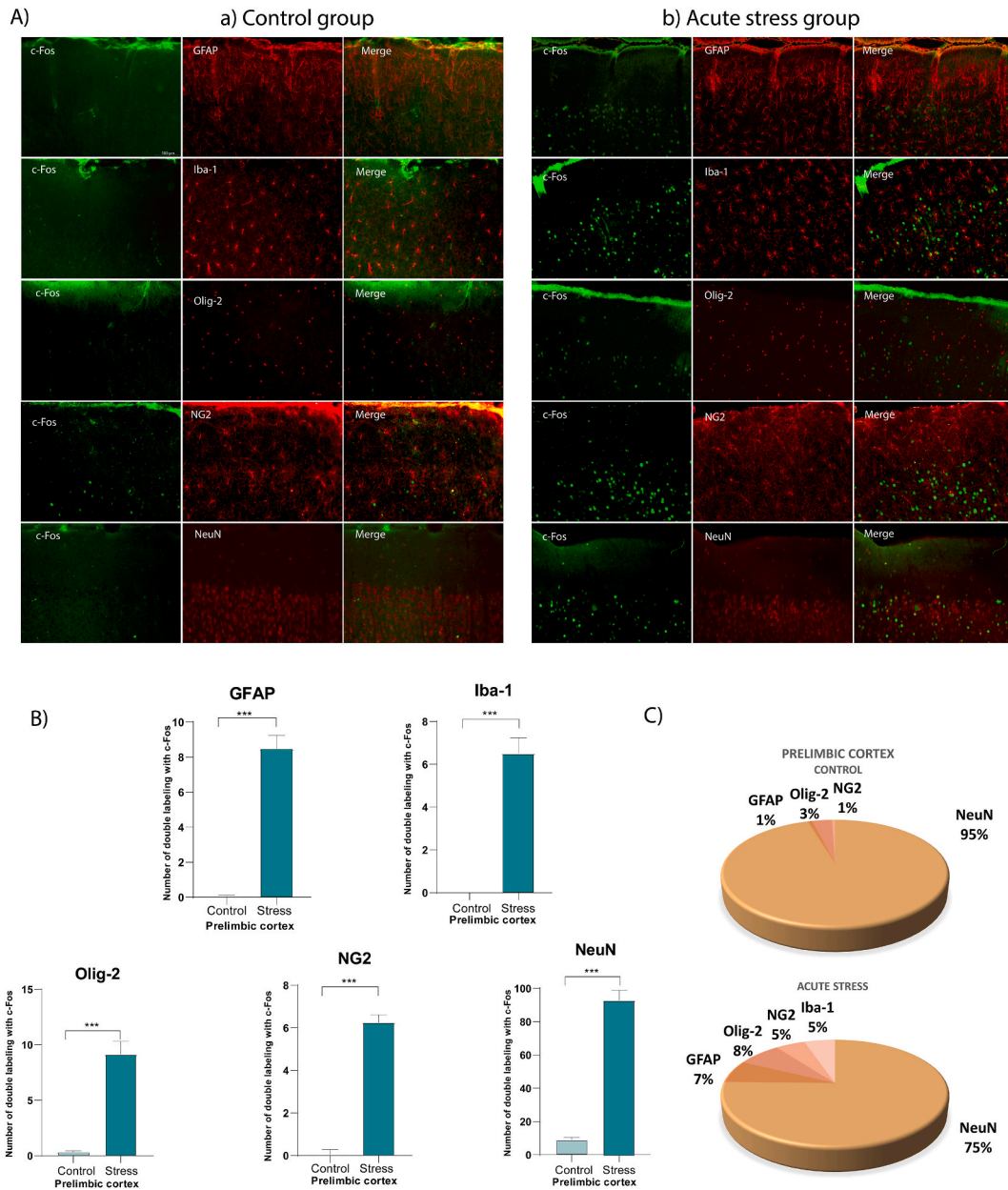

Fig. 4. c-Fos expression by cell type proportion is shifted in the Anterior Cingulate Cortex of rats exposed to acute stress. A) The images show the fluorescent micrographs of a) control and b) acute stress conditions. The green signal indicates the c-Fos + cells, the red signal indicates lineage cell+ (GFAP, Iba-1, Olig2, NG2, and NeuN), and the third column shows the corresponding merge. Magnification 20x, scale bar 100 μm. B) The graph corresponding to the count of double labeling of c-Fos/brain cell types in the control and acute stress groups is presented The data represents the mean ± SEM. (Student's *t*-test; ***p < 0,001.C) The proportions of double labeling of c-Fos/neurons and c-Fos/glia are illustrated.

as shown in Fig. 2B and C. Since no differences were found in the number of c-Fos-positive cells between the acute stress group and the control group within the primary somatosensory cortex, this region was excluded from further analyses.

3.2. All evaluated cell types expressed the c-fos proto-oncogene in mPFC

Furthermore, we conducted double labelling experiments to examine the co-localization of c-Fos with both neurons and glial cells. The analysis revealed the presence of c-Fos in Astrocytes, Microglia, Oligodendrocytes, NG2 cells and neurons examined. These findings provide evidence of c-Fos expression in glial cells under conditions of acute stress. Fig. 3 illustrates the five evaluated lineages and demonstrates the presence of c-Fos in each phenotype.
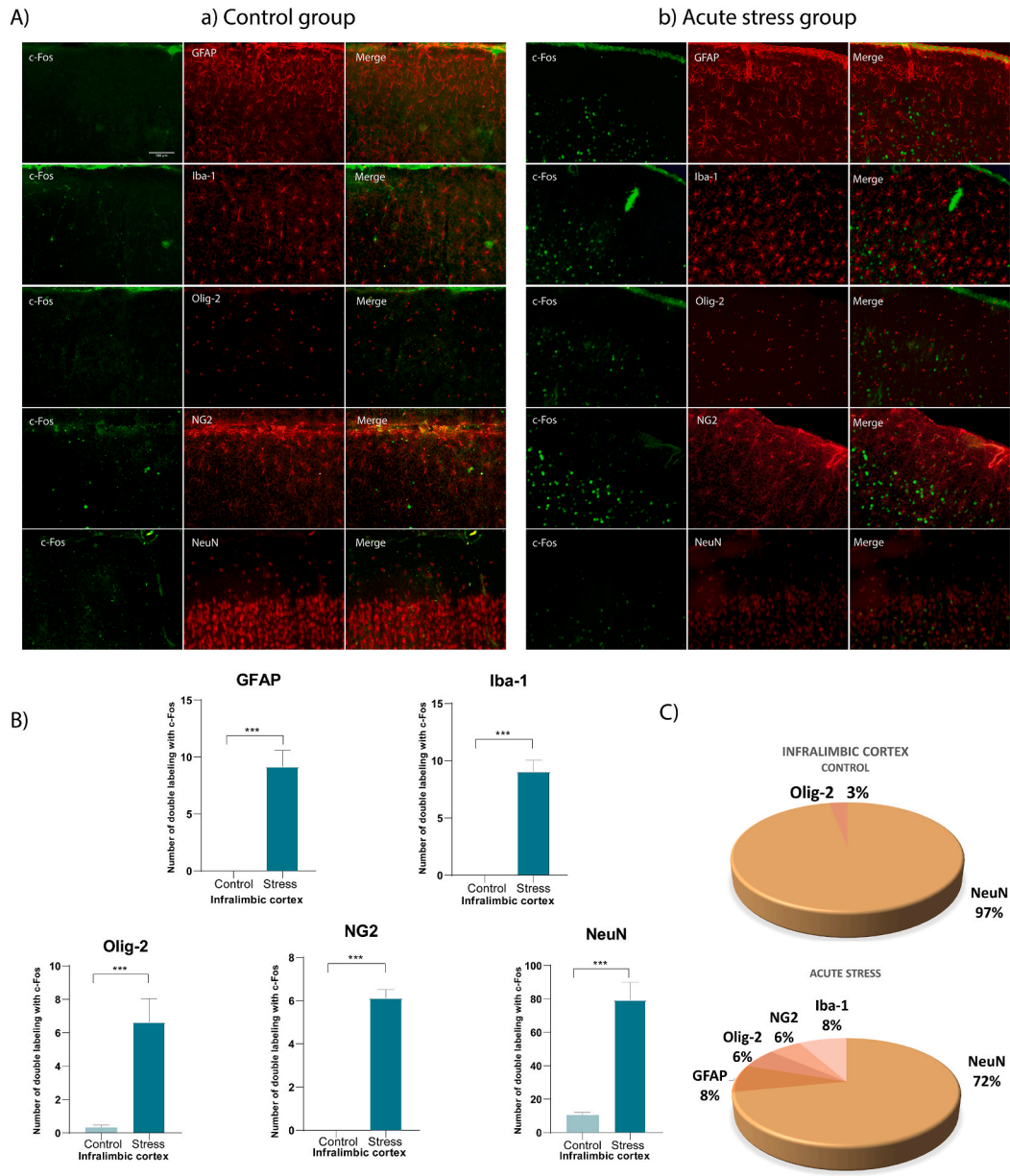

Fig. 5. c-Fos expression by cell type proportion is shifted in the Prelimbic cortex of rats exposed to acute stress. A) The images show the fluorescent micrographs of a) control and b) acute stress conditions. The green signal indicates the c-Fos + cells, the red signal indicates lineage cell+ (GFAP, Iba-1, Olig2, NG2, and NeuN), and the third column shows the corresponding merge. Magnification 20x, scale bar 100 μm. B) The graph corresponding to the count of double labeling of c-Fos/brain cell types in the control and acute stress groups is presented The data represents the mean ± SEM. (Student's t-test; *p < 0,05, **p < 0,01, ***p < 0,001). C) The proportions of double labeling of c-Fos/neurons and c-Fos/glia are illustrated.

3.3. Acute stress differentially affected neurons and glial cells in mPFC

Next, we conducted an analysis of double labeling in each subregion of the mPFC to evaluate the activation of neurons and glial cells. Statistical analysis revealed significant differences between the stressed and control groups in the Anterior Cingulate Cortex (ACC) (Fig. 4B; p < 0.001), Prelimbic Cortex (PL) (Fig. 5B; p < 0.001), and Infralimbic Cortex (IL) (Fig. 6B; p < 0.001).

In the Anterior Cingulate Cortex, the analysis showed that 97 % of the double-labeled cells in control group, corresponded to c-Fos/neuron, while only 3 % were identified as c-Fos/glia. However, in the stress group, 80 % of the labeled cells were c-Fos/neuron, and 20 % were identified as c-Fos/glia, this shift in proportion was confirmed by a chi square test ($\chi^2 = 11.89,4$ p = 0.0182). These findings indicate that acute stress induces a shift in the activation pattern, with a higher proportion of c-Fos expression observed in glial cells compared to the control condition. Fig. 4 presents the c-Fos/cell type co-labeling results within the ACC for both control and acute

Fig. 6. c-Fos expression by cell type proportion is shifted in the Infralimbic cortex of rats exposed to acute stress. A) The images show the fluorescent micrographs of a) control and b) acute stress conditions. The green signal indicates the c-Fos + cells, the red signal indicates lineage cell+ (GFAP, Iba-1, Olig2, NG2, and NeuN), and the third column shows the corresponding merge. Magnification 20x, scale bar 100 μm. B) The graph shows the mean of double labeling of c-Fos/brain cell types. The data represents the mean ± SEM. *p < 0,05 (Student's *t*-test; **p < 0,01, ***p < 0,001). C) The proportions of double labeling of c-Fos/neurons and c-Fos/glia are illustrated.

stress groups, illustrating the proportion of active cells from each lineage in this region.

In the Prelimbic Cortex, our analysis revealed that 95 % of the double-labeled cells in control group were identified as c-Fos/neuron, and only 5 % were classified as c-Fos/glia. However, under stress conditions, the proportions shifted to75 % of the labeled cells being c-Fos/neuron, and 25 % being identified as c-Fos/glia ($\chi^2 = 13.57,4$ p = 0.0088). Fig. 5 displays the quantification results of c-Fos double labeling across various cell lineages, along with activation proportions within the prelimbic region, for both control and acute stress groups.

In the Infralimbic Cortex, the control rats exhibited a predominance of c-Fos/NeuN cells, with 97 % of the double-labeled cells being identified as c-Fos/NeuN and only 3 % as glia. However, in the acute stress group, the proportions shifted, with 72 % of the labeled cells being c-Fos/NeuN and a notable increase to 28 % as c-Fos/glia ($\chi^2 = 20.54,4$ p = 0.0004). Fig. 6 illustrates the co-labeling proportions and c-Fos/cell type quantification results within the infralimbic cortex.

Then, we compared subregions of the mPFC and observed a higher expression of c-Fos/microglia-positive cells in the Infralimbic Cortex (IL) compared to both the Prelimbic Cortex (PL) and Anterior Cingulate Cortex (ACC). This indicates that microglia in the IL region may be particularly responsive to the acute stress stimulus compared to the other subregions of the medial prefrontal cortex (Fig. 7A). Furthermore, we also found a higher expression of c-Fos/NG2-positive cells in both the Prelimbic and Infralimbic cortices compared to the ACC. This shows that NG2 cells in the prelimbic and infralimbic regions may exhibit a greater response to acute stress (Fig. 7B) compared to the other glial types were not found significant (Fig. 7C–E). These findings highlight the regional differences in the activation of microglia and NG2 cells in response to acute stress within the medial prefrontal cortex.

Finally, we measured the impact of the effect for the factors involved in our experiment. We found statistical significance for all subregions examined: ACC (Stimuli p= <0.0001, partial eta squared = 14.23; Cell type p= <0.0001, partial eta squared = 51.86 and an interaction effect p= <0.0001, partial eta squared = 28.82), PL (Stimuli p= <0.0001, partial eta squared = 9.992; Cell type p= <0.0001, partial eta squared = 30.24 and an interaction effect p= <0.0001, partial eta squared = 19.75) and for IL (Stimuli p= <0.0001, partial eta squared = 18.14; Cell type p= <0.0001, partial eta squared = 52.76 and an interaction effect p= <0.0001, partial eta squared = 29.12). These results confirmed significant effect for both factors with a strong interaction between them.

4. Discussion

Our study provided evidence of the heightened sensitivity of the medial prefrontal cortex (mPFC) to acute stress, as indicated by a significant increase in c-Fos expression in animals exposed to the stressor. Notably, our findings went beyond neurons and revealed that glial cells also exhibited the expression of c-Fos, indicating their involvement in the stress response within this region. Furthermore, our data demonstrated that acute stress enhanced the proportion of glial cells capable of expressing this activity marker. It confirmed that glial cells play an active role in the response to acute stress and contribute to the overall physiological and cellular
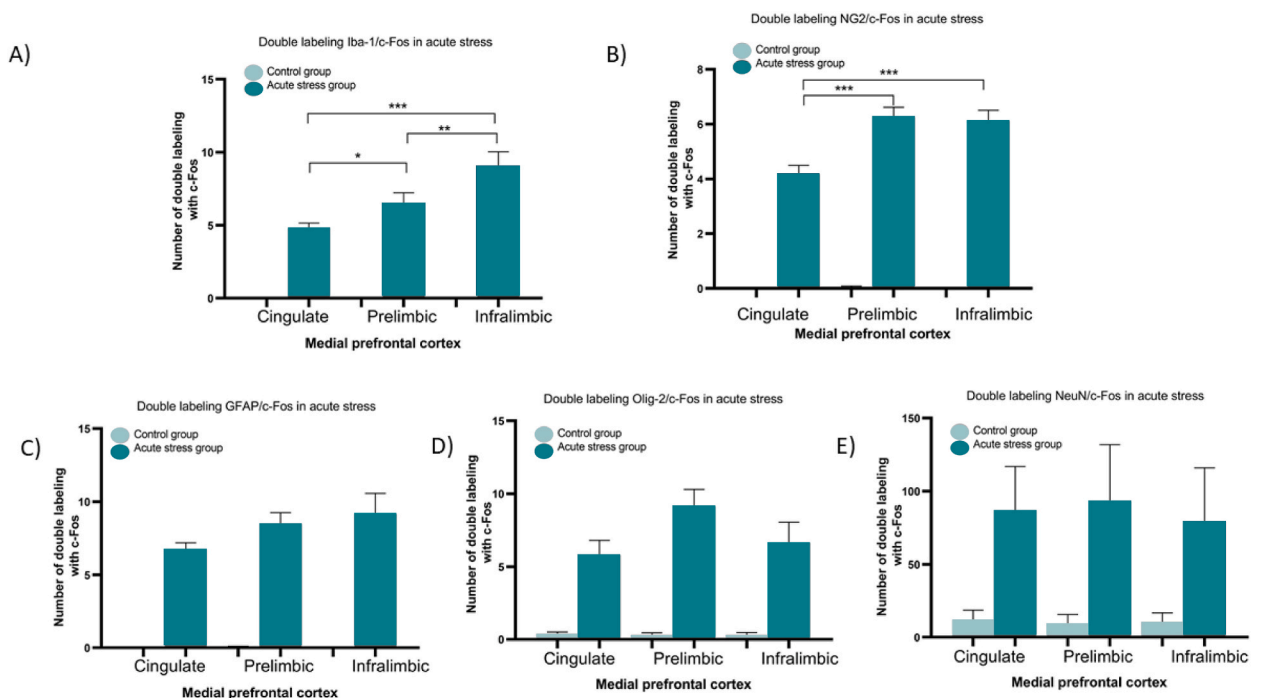
Fig. 7. c-Fos expression in rats exposed to stress varies between subregions of mPFC. The graph shows the differences of double-labeled c-Fos/cell types between subregions of the medial prefrontal cortex of the rat exposed to acute stress. The data represents the mean ± SEM. (One-way ANOVA; *p < 0,05 **p < 0,01, ***p < 0,001).

changes observed in the mPFC during stress.

4.1. - c-fos expression on the medial prefrontal cortex of stressed rats

It is known that transcriptional activation of the c-Fos gene in the brain is closely associated with neuronal depolarization. This process is influenced by the activation of specific channels, namely for example NMDA and VSCCs (voltage-sensitive calcium channels), which initiate signaling pathways that target response elements on the c-fos promoter. These response elements include the serum response element (SRE), cAMP response element (CRE), and SIF-inducible element (SIE) [36]. Under stressful conditions, additional signalling pathways associated with the NMDA receptor, such as the CREB/CRE and erk/MAPK/SER pathways, are also implicated in the upregulation of c-fos expression [37–40]. These pathways further contribute to the neuronal excitation and depolarization observed in response to stress. The upregulation of c-fos expression following extra-synaptic stimulation has been found to be more reliable and robust compared to other immediate early genes, such as Zif268 [41]. Therefore, c-Fos has become a widely used marker for studying neuronal activation and mapping neural circuits in response to various stimuli, including stress [19,42]. By utilizing c-Fos as an activation marker, we can gain valuable insights into the patterns of neuronal activation and the underlying neural circuits involved in stress responses. In this line of thought, the findings of this study align and confirm previous research indicating that the Anterior Cingulate Cortex (ACC), Prelimbic Cortex (PL), and Infralimbic Cortex (IL) are sensitive to acute stress. The specific activation patterns observed in different studies may vary depending on the intensity [43] as well as the permanence or nature of the stressors employed [4]. In this study, using a combined acute stress protocol, no statistically significant differences were found in c-Fos expression between the subregions of the medial prefrontal cortex (mPFC). Similar results have been reported for the acquisition of contextual fear responses [44], stimulus-response associations [45], assessment of controllability, behavioural and vegetative alert responses [46], cardiovascular adjustments [47], and control of avoidance and extinction responses [48]. The absence of significant differences between the subregions of the mPFC implies that they may function collectively to orchestrate various aspects of the stress response. This coordinated activation within the mPFC subregions likely contributes to the integration of emotional, cognitive, and physiological responses that occur during stressful situations.

4.2. Expression of c-fos in glia: possible roles and mechanisms associated with stress

Our findings evidenced that stress can induce the expression of c-Fos in non-neuronal cells of the brain, which do not typically undergo depolarization. This raises intriguing questions about the activation mechanisms and the potential role of c-Fos in glial cells under stress. While the effects of acute and chronic stress on neurons have been extensively studied, the scientific literature on the specific study of glial cells in the context of stress is still limited. Nonetheless, the effect of acute stress on glial cells has been recently explored, their involvement in the stress response is not as well understood as that of neurons. Prior to our study, the role of c-Fos in glia under stressful conditions had not been thoroughly investigated. Existing studies primarily focused on the relationship between Fos and glia in injury and disease models, often involving non-murine species [22].

4.2.1. Roles of c-fos in glia under stress conditions

Available studies have shown that astrocytes, undergo various changes in response to stress. These changes involve transcriptional regulation associated to morphology, establishment of contacts [49,50], release of gliotransmitters [51,52], growth factors, and interleukins [54]. Changes in these parameters have been reported to affect myelination [53], cognition (i.e learning and memory) [55] and cellular density [56,57]. Thus, according to a recent study, c-Fos expression in astrocytes could predict neuronal potentiation and memory enhancement [58] as stress studies previously reported. *In vitro* models showing increased c-Fos expression after growth factors induced proliferation/differentiation, suggest that c-Fos could play a key role in astrocyte proliferation/differentiation [27]. Moreover, a role in pathogenesis should be considered since increases in c-Fos expression have been reported in *in vitro* models of astrogliosis [59].

Microglia, the resident immune cells of the brain, also undergo modulation in response to acute stress. Microglial changes involve alterations in their activation state [60,61], morphological transformations [61–63], sensitization to subsequent proinflammatory stimuli [64,65], modulation of anti-inflammatory responses [61], dose-dependent increases in proliferation [66,67], and transcriptional regulation [64]. Stress could impact neuronal plasticity through microglia, as it regulates synapse formation [68] and neuronal activation [69]. Then, it is clear that acute stress can have profound effects on microglia, influencing their proliferation, morphology, and functional characteristics. Microglia undergo these changes to effectively respond to present and future challenges. Similar to astrocytes, the few *in vitro* available studies suggest that the c-fos gene may play a role in regulating proinflammatory responses and mediating morphological and functional alterations in microglia. BV2 microglial cultures showing c-fos expression in amoeboid/activated microglia after administration of paraquat and LPS sustain this [28,29]. Therefore, it can be hypothesized that c-fos is involved in the modulation of morphological, proliferative, and functional changes associated with the microglial response to acute stress.

Most studies examining the effects of stress on oligodendrocytes and NG2 cells have primarily focused on chronic stimulation. There is evidence linking glucocorticoids to the initiation and enhancement of myelin formation [70,71], increases in oligodendrogenesis [72], and higher cell density [73]. Also, NG2 cells have shown changes in density [74] and cellular activation [75] in response to chronic stress. While fewer studies have specifically investigated the acute responses of oligodendrocytes and NG2cells to stress, previous research has examined the effects of acute neuron activation on oligodendrocyte precursor cells (OPCs). Optogenetic stimulation of neurons has been shown to induce a proliferative response in OPCs and increase myelin thickness [76]. This suggests that

neuronal activation can influence the proliferation, differentiation, and myelination of OPCs, thereby affecting cellular plasticity. Recent *in vitro* studies confirmed that c-fos, along with c-jun, may contribute to the proliferation and differentiation towards oligodendrocytes [30]. These studies proposed PKC as a potential mediator of these processes [30,77]. Furthermore, c-Fos has been involved in the differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes [78]. Notably, the induction of c-Fos has been observed in oligodendrocyte progenitors stimulated by norepinephrine (NE) [79]. It has been also reported that c-Fos expresses in oligodendrocytes after excitatory stimulation by LSD in murine models [80]. Hence, it seems plausible that c-Fos regulate proliferation, differentiation, and maturation of these cell lineages under stressful conditions.

4.2.2. Mechanisms associated with the induction of c-fos in glia

The mechanisms underlying the induction of c-fos in glial cells remain unknown. While in neurons, c-fos is primarily induced by depolarization and changes in calcium concentration, serving as a marker of synchronous activation, similar mechanisms cannot be attributed to glia as they do not generate action potentials. Therefore, it would be valuable to explore in greater detail the specific mechanisms by which c-fos is induced in glial cells under conditions such as stress. One potential avenue to investigate is the integrative mechanisms involved in glia-to-glia and glia-to-neuron communication. For instance, the release of glutamate in excitatory synapses could activate glutamate receptors in glial cells, subsequently initiating signalling pathways that lead to the transcriptional activation of genes like c-fos. This hypothesis finds support in studies that have reported the induction of c-fos by glutamate in astrocytes through NMDA receptors [81], oligodendrocyte progenitors through AMPA-R and KA-R receptors [82], and microglia through ionotropic and metabotropic receptors [83]. In the case of microglia, their activation could be associated with the mitogen-activated protein kinases (MAPKs) pathways [29].

Another possibility to consider is the direct regulation of the c-fos gene by glucocorticoids through their interaction with glucocorticoid receptors (GR) in glial cells. This could involve the activation of various signalling pathways, as previous studies have reported the joint involvement of GR and NMDA receptor-Erk-MAPK pathways in the transcriptional regulation of genes like c-fos in neurons following psychological stress [84,85]. The direct regulation of c-fos by glucocorticoids in glial cells could imply the engagement of similar pathways.

It is important to note that these proposed mechanisms are based on our understanding of neuronal processes. However, it is plausible that the transcriptional induction of c-fos in glial cells occurs through distinct mechanisms. Therefore, further research is necessary to explore and elucidate the specific mechanisms by which c-fos transcription is induced in glial cells under different conditions, including stress.

Regarding the results presented in our study on the prefrontal cortex, the increased expression of c-Fos observed after acute stress could be associated, in the initial stages, with the negative feedback response of the medial prefrontal cortex (mPFC) to the initial elevation of glucocorticoids induced by acute stimulation [86]. Neuronal activation in the mPFC may be attributed to an increase in excitatory synaptic strength [87], while other neurotransmitters such as dopamine [88] and norepinephrine [89] are also involved in cell activation. These neurotransmitter systems likely contribute to the complex interplay of factors that influence c-Fos expression in the mPFC under acute stress conditions.

4.3. Increased proportion of glial cells capable of expressing c-fos in mPFC under acute stress

Our study evidenced the concurrent activation of glial cells in the medial prefrontal cortex (mPFC), which exhibited minimal c-Fos expression under control conditions but presented a significant 20–30 % increase in response to acute stress. This increase in glial c-Fos expression paralleled the observed neuronal activation, suggesting that glial cells may play a crucial role in meeting the demands of activated neurons. Specifically, we found statistically significant differences in the co-expression of c-fos in microglial cells within the prelimbic (PL) and infralimbic (IL) cortices. This suggests that microglia may directly contribute to the neuronal responses and alterations associated for example to working memory, as has been previously reported after chronic stress [90]. Furthermore, it is worth noting that microglia may become sensitized to future stimuli, potentially influencing the susceptibility of the IL cortex to pathological conditions including depression [91]. We also observed a higher expression of c-Fos in NG2 cells in the PL and IL regions compared to the ACC. This increased expression could be associated with transient elevations in activation, and the fact that NG2 cells are among the first glial cells to respond to damage and stress [74]. Both the IL and PL cortices may exhibit more sensitive changes in their immediate response to stressors, thus requesting a greater involvement of specific glial cell populations.

Overall, our findings shed light on the coordinated activation of glial cells within the mPFC under stress conditions, highlighting their potential contribution to neuronal responses and adaptive processes. These findings provide valuable insights into the complex interplay between neurons and glial cells in the mPFC and emphasize the importance of studying glial responses in the context of stress and related disorders.

5. Conclusions and perspectives

c-Fos has traditionally been used as a marker of neuronal activation in response to various stimuli, including stress. Its involvement in processes such as proliferation, transformation, differentiation, and cell death has been well-documented in neurons. However, the role of c-Fos in glial cells has remained elusive. This groundbreaking study represents the first investigation exploring c-Fos in glial cells under stress conditions. By exploring the mechanisms associated with non-neuronal cell activation, this study provides new insights and perspectives on the potential use of c-Fos as a marker of activation and plasticity/transformation. This study highlights its potential as an indicator of cellular responses to everyday stimuli, such as stress. While this represents an important initial step in

understanding the involvement of c-Fos in glia under stress, there are still numerous aspects to be explored. Further investigations are warranted to elucidate the mechanisms underlying gene induction, the regional and temporal dynamics of activation, and the specific roles of c-Fos in the acute and chronic stress responses of glial cells. Finally, despite the complexity associated with the analysis of numerous sections and cellular markers, we acknowledge, as a limitation in our study, the use of a relatively small sample size of animals. A larger sample size could be planned to strengthen our data and facilitate a more nuanced exploration of various aspects, including potential gender differences, time-related variations, and underlying molecular mechanisms.

Overall, this study pioneers the exploration of c-Fos as a glial plasticity/transformation marker under stress conditions, opening up new avenues for research and paving the way for a deeper understanding of the intricate interplay between glial cells and stress-related processes.

CRediT authorship contribution statement

Adriana Aguilar-Delgadillo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Fernando Cruz-Mendoza:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Sonia Luquin-de Andais teh:** Supervision, Project administration, Funding acquisition, Conceptualization. **Yaveth Ruvalcaba-Delgadillo:** Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Fernando Jáuregui-Huerta:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition.

Data availability statement

Data included in article/supplementary material is referenced in the article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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