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Rapid GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury

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Rapid GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury

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Authors contributions: JAP - writing—original draft preparation; MM - conducted the experiments and substantially contributed to draft preparation; CAG - conducted the experiments, reviewed and the edited the paper; KAK - writing—review and editing; GM and GA-B helped in the methodology, formal analyses and final revision; AC - conceived the study, obtained and administered the funding, revised the manuscript.

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RE: Response to reviewers' comments for submission NRR-D-20-00870

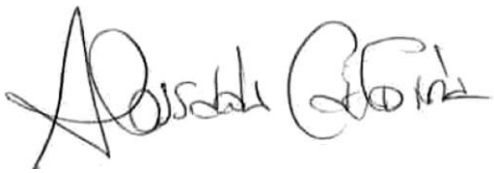
Dear Prof. Kwok-fai So and Xiao-Ming Xu,

Please find enclosed with the present letter a copy of the revised version of original article entitled "Early GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury" submitted by Mawj Mandwie, Jordan A. Piper, Catherine A. Gorrie, Kevin A. Keay, Giuseppe Musumeci, Ghaith Al-Badri and Alessandro Castorina to be reconsidered for publication in Neural Regeneration Research. The work has not been published elsewhere and is not under review with another journal. The authors have agreed to the re-submission of the revised version of the manuscript.

In the revised work, we carefully considered all the recommendations given by the two expert reviewers and revised the methodology to reduce the percentage of similarity as reported by the Editorial office. We put all the efforts to correctly incorporate the suggested changes in the text. All the revisions in the paper were performed using the Microsoft Word tracked-changes option. Below you will find an itemised list with the responses to each reviewers' concerns. We are grateful to you and the reviewers for your support and hope that the revised submission, if accepted, will further contribute to the growth of the journal.

I look forward to hearing back from you soon.

Kind regards,



Associate Professor Alessandro Castorina

Responses to each of the issues raised by reviewers are shown in blue.

Reviewer #1:
In this study, the authors studied the mRNA and protein expression of two cell-type specific markers GFAP and Iba1 in a few cognition-related brain regions in the first 24 hours following spinal cord injury, which may be helpful to identify possible changes of astrocytes and microglia within a short period after SCI. In general, the data are well organized and the paper is well written. I believe the dysfunction of high cognition such as emotion, decision-making and working memory after SCI is a valuable aspect with big potential for neuroscientist to look into. Based on that, I have two major concerns on the present work.

Response: We would like to thank the reviewer for the nice comments on the quality of the work and on the writing style.

1. The authors used female animals to collect the data. However, the changes of GFAP and Iba1 could be different in male animals considering there are prominent sexual differences in many aspects of high cognitive functions. The authors should do some experiments on male animals to draw a conclusion or should at least add some speculations in the discussion if they have.

Response: As the reviewer correctly pointed out, there is documented evidence of sexual dimorphism in the behavioural responses to acute stress and definitely in several types of high order cognitive and affective functions. Apparently, these differences account for hormonal, sex chromosomes and their interaction with the environment (Rubinow and Schmidt, 2019), with reports showing that females, as opposed to males, respond with a cooperative-like behaviour whereas males show aggressiveness when exposed to stress (Youssef et al., 2018). Interestingly, in a recent work it has been demonstrated that stressed-susceptible brain regions such as the prefrontal cortex or the hippocampus exhibit higher activation patterns in male *versus* female in rats exposed to acute immobilisation stress, but not in the forced swimming test (Sood et al., 2018). These results pinpoint that characteristic sexual dimorphism in response to acute stress raised by the reviewer, which may indeed be dependent on the specific nature of the stressor. In this exploratory study we utilised female rats as these are conventionally used in spinal cord injury models due to their enhanced ability to recover and tolerate the surgical procedure compared with males (Datto et al., 2015). A further reason was related to ethical concerns, as in our experience male rats subjected to SCI often develop a severe form of autotomy (self-mutilation) directed to their hind-limbs and sometimes genitalia that we have never observed in female rats. The exaggerated autotomy seen in male SCI rats seems to also occur after neurectomy of peripheral nerves, as shown in other studies (Wagner et al., 1995).

In order to include these observations in the text and clarify the reasons behind our choice to opt for female rats in this study, we added a paragraph in the discussion section. We also modified the title of the manuscript as follows: “Rapid GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury” to specify that the study was conducted using female rats.

Supporting references:

Rubinow DR, Schmidt PJ. Sex differences and the neurobiology of affective disorders. Neuropsychopharmacology. 2019 Jan;44(1):111-128.

Youssef FF, Bachew R, Bissessar S, Crockett MJ, Faber NS.
Sex differences in the effects of acute stress on behavior in the ultimatum game. Psychoneuroendocrinology. 2018 Oct;96:126-131.

Sood A, Chaudhari K, Vaidya VA.
Acute stress evokes sexually dimorphic, stressor-specific patterns of neural activation across multiple limbic brain regions in adult rats. Stress. 2018 Mar;21(2):136-150.

Datto JP, Bastidas JC, Miller NL, Shah AK, Arheart KL, Marcillo AE, Dietrich WD, Pearse DD.
Female Rats Demonstrate Improved Locomotor Recovery and Greater Preservation of White and Gray Matter after Traumatic Spinal Cord Injury Compared to Males. J Neurotrauma. 2015 Aug 1;32(15):1146-57.

Wagner R, DeLeo JA, Coombs DW, Myers RR. Gender differences in autotomy following sciatic cryoneurolysis in the rat. Physiol Behav. 1995 Jul;58(1):37-41.

2. It is an attractive topic how the changes of high cognitive functions occur after SCI. But the mechanisms underlying these changes must be long-term modulations occurring at molecular, cellular and systematic levels. Thus, the changes of astrocytes and microglia within a short period such as 24 hours after SCI are very likely just some acute responses, and may not be tightly relevant to high cognitive dysfunction which normally takes longer time to occur. The authors could do some experiments at later time window and compare with their findings at 24 hours after SCI, or at least address this issue in the discussion.

Response: We agree with the reviewer's comments pinpointing how changes in high cognitive functions following a traumatic injury may require long term in order to produce those structural changes in neuronal circuitry that would justify the appearance of clinically relevant behavioural alterations. Nonetheless, as indicated in the discussion section, we believe that such striking and early adaptive responses seen in astrocyte and microglia across the different CNS regions we investigated (despite their physical distance from the injury site) may still reflect an early neurochemical occurrence that would later translate into subsequent behavioural comorbid dysfunctions. It is also postulated that the alterations we observed here may not be effective in triggering behavioural alterations in a subsets of rats, witnessing an individual resilience as it happens in people afflicted by this debilitating condition. However, this topic goes beyond the goal of the present investigations, but will be certainly explored in future studies.

We added a short paragraph in the Conclusions section to highlight the importance of additional studies aimed at verifying this theory in a long-term setting that will also involve behavioural appraisals, which will be the goal of our future investigations.

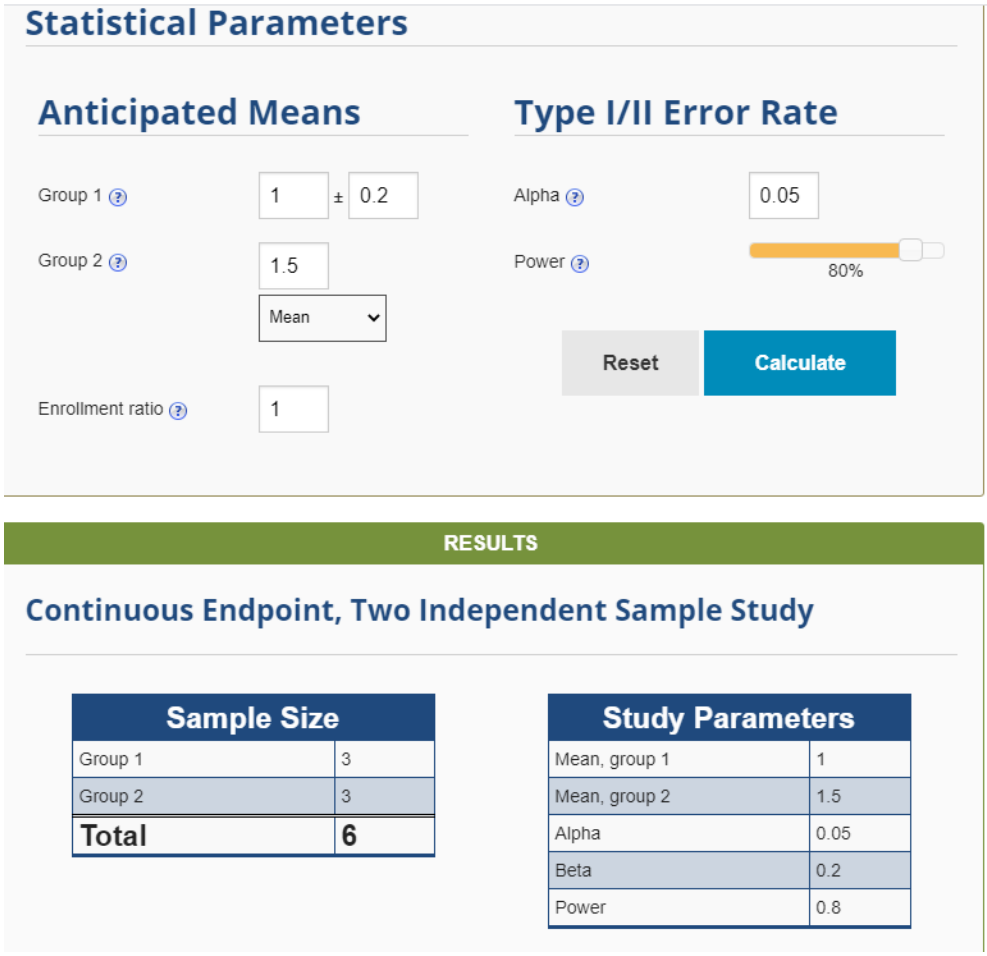
Reviewer #2: In this study, the authors investigated mRNA and protein expression of GFAP and iba1 in different locations of brain after traumatic thoracic spinal cord injury. Actually, this is an interesting study and straightforward. I have some major comments, listed below, needs to be addressed:

Response: We thank reviewer #2 for the nice comments on the manuscript. We also appreciate the efforts made to further improve the quality are of this work.

1. At the acute stage after spinal cord injury, the mRNA and protein show diverse and dramatic changes. In this study, the authors only have 3 animals per group. A larger n number (at least 6 per group) is suggested for this study;

Response: As mentioned above, this is an exploratory study. Given the intrinsic nature of these types of studies, we could not predict *a priori* for any obvious changes in the pattern of gene and protein expression for any of the tested markers. Therefore, we conducted *a priori* power calculation to calculate the right sample size on the assumption that a gene or protein expression fold change of >= 1.5 would have been considered biologically relevant. Based on this assumption and by estimating an inter-experiment standard deviation of 0.2 (20% variation), using a power of 80% and an alpha value of 0.05, our power calculations revealed that n=3 per group was big enough to provide sufficient statistical power. As the reviewer suggests, since the effect size was pretty remarkable, and also given the potential future implications of these findings, we thought these data was solid enough to be published, as done in previous work (Castorina et al., 2019).

Snapshot showing results of power calculations using the online tool ClinCalc.com (https://clincalc.com/stats/samplesize.aspx)



Supporting reference:

Castorina A, Vogiatzis M, Kang JWM, Keay KA.
PACAP and VIP expression in the periaqueductal grey of the rat following sciatic nerve constriction injury. Neuropeptides. 2019 Apr;74:60-69.

2. Activation of astrocyte and microglia shows typical morphological change. The mRNA and protein expression are not sufficient to show the alteration of these glia cells, the author should provide histological evidence as well;

Response: As correctly stated by this reviewer, astrocyte and microglial polarisation states are associated with the occurrence of characteristic morphological features (amoeboid shape in M1 microglia and reactive astrocytes with thick processes and enlarged soma); however, the neurochemical alterations that precede glial morphological changes are sequential events. Whilst we agree that demonstrating the distribution of reactive glia in each of the brain regions may provide some complementary support to our findings, we still believe that portraying the global mRNA and protein expression of GFAP and Iba1 in several stress-sensitive brain regions immediately after SCI (at 24 hours) still provides remarkable evidence to capture scientific interest in supraspinal glial activation following SCI and foster novel investigations in the field.

3. For the microdissection of prefrontal cortex, amygdala, lateral thalamus, dorsal and ventral hippocampus,

hypothalamus and periaqueductal gray regions, the references listed in this paper is not sufficient and clear. Considering this is the key point of the manuscript, the author should describe it in detail, for example, they can include some images of the procedure;

Response: All the authors agreed that the methodology used to describe the landmarks and protocols used to microdissect such discrete rat brain regions should be provided in more detail. Therefore, we included an additional Section entitled “2.2 Microdissections” where we provide a detailed description of the methodology used to obtain the tissue blocks containing the regions of interest that were used in this study. For additional clarification, we prepared a new figure (**Figure 2A-F**) that includes schematics showing how sections were cut and how tissue blocks were excised from each section. Subsequent figures were renumbered accordingly and a new caption was added. We are now more confident that such additional efforts will increase the reproducibility of microdissections experiments by other research groups. We are grateful to this reviewer for the suggestion.

4. More timepoints of the assessment is suggested at the acute stage after spinal cord injury in this study.

Response: As in the response to Reviewer #1 above, this study aimed at addressing the early changes in GFAP and Iba1 expression following spinal cord injury. Future studies will aim at investigating the temporal profile of these markers at later time points. This was indicated in the conclusion section. Thanks to the reviewer for the insightful comment.

To conclude, we really appreciate the efforts made by both reviewers to improve the quality of the manuscript by raising important issues which I hope we satisfactorily addressed. We would also like to thank the Editorial Office for providing their invaluable help. We hope that the revised manuscript will be positively received.

Best regards,

Alessandro Castorina

Rapid GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) is a devastating condition often associated with sleep disorders, mood change and depression. Evidence suggests that rapid changes to supporting glia may predispose individuals with SCI to such comorbidities. Here, we interrogated the expression of astrocyte- and microglial-specific markers glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) in the rat brain in the first 24 hours following spinal cord injury (SCI). Female Sprague Dawley rats underwent thoracic laminectomy; half of the rats received a mild contusion injury at the level of the T10 vertebral body (SCI group), the other half did not (Sham group). Twenty-four hours post-surgery the rats were sacrificed, and the amygdala, periaqueductal grey, prefrontal cortex, hypothalamus, lateral thalamus, hippocampus (dorsal and ventral) were collected. GFAP and Iba1 mRNA and protein levels were measured by real-time qPCR and Western blot.

In SCI rats, GFAP mRNA and protein expression increased in the amygdala and hypothalamus (*p<0.05). In contrast, gene and protein expression decreased in the thalamus (**p<0.01) and dorsal hippocampus (*p<0.05 and **p<0.01, respectively). Interestingly, Iba1 transcripts and proteins were significantly diminished only in the dorsal (*p<0.05 and **p<0.01, respectively) and ventral hippocampus, where gene expression diminished (*p<0.05 for both mRNA and protein). Considered together, these findings demonstrate that as early as 24 hours post-SCI there are region-specific disruptions of GFAP and Iba1 transcript and protein levels in higher brain regions.

Keywords: Glial fibrillary acidic protein; Ionized calcium binding adaptor molecule 1; spinal cord injury; neurotrauma; Microglia; Astrocytes

1. Introduction

Spinal cord injuries (SCI) can result in long-term and permanent deafferentation of cortical circuits of the central nervous system (CNS) (Wall and Egger, 1971; Ziemann et al., 1998). Such changes can result in a substantial reorganization of cortical maps, exemplifying the plastic properties of the CNS (Aguilar et al., 2010). Sleep disturbance, anxiety, depression and cognitive dysfunction is highly prevalent in SCI patients (Davidoff et al., 1990; Kennedy and Rogers, 2000; Biering-Sørensen and Biering-Sørensen, 2001). This suggests strongly that following spinal cord trauma, in addition to changes in cortical circuits, other brain regions critical for the regulation of sleep, mood and cognition are also significantly impacted. For a complete understanding of the neurochemical bases of these changes in complex behaviors, it is essential to understand the changes in the brain triggered during the earliest stages of spinal cord injury, from which these long-term changes evolve.

Glial cells are the supporting cells of the CNS (He and Sun, 2007). Alterations within the astrocyte and microglia compartments play significant roles in the onset and progression of several pathophysiological processes that can lead to a spectrum of affective dysfunctions (Öngür and Heckers, 2004; Pav et al., 2008), as well as synaptic alterations (Honer et al., 1999; Coyle and Schwarcz, 2000; Cotter et al., 2001; Scholz and Woolf, 2007). Both astrocytes and microglia play major roles in shaping these CNS functions, and are likely to be the first cell populations primed following trauma, such as is associated with SCI. Glial fibrillary acidic protein (GFAP) is well established as the primary filament present in mature astrocytes within the CNS, where it is involved in modulating the structural stability, shape, and motility of the cells, as well as the cell-to-cell interactions with neurons (Eng, 1985; Eng and Shiurba, 1988; Eng et al., 2000; Li et al., 2020). Ionized calcium binding adaptor molecule 1 (Iba1), is expressed in the cells of several tissues,

including brain, testis, spleen and, to a lesser extent, in the kidneys and lungs. In the brain, Iba1 is expressed uniquely by microglia (Ito et al., 2001; Hwang et al., 2008), where it elicits actin-bundling activity and participates in membrane ruffling and phagocytosis when the microglia are activated (Ohsawa et al., 2004).

In a number of studies examining mood change and cognitive dysfunction identical to that seen in individuals with SCI there are reports of regionally specific reductions in glial cell populations and/or glial activities in the amygdala, prefrontal cortex, hippocampus and periaqueductal gray (Öngür et al., 1998; Bowley et al., 2002; Imbe et al., 2012). Examining changes in GFAP and Iba1 transcript and protein levels may provide important insights into the temporal and topographical responses of glial cells occurring in higher brain regions after spinal cord injury.

In this study, we evaluated the hypothesis that within the initial 24 hours after an injury, SCI leads to a rapid mRNA and protein changes in glial cells of discrete brain regions critical for the regulation of mood/emotion, stress responsivity, memory and decision-making. To answer this question, we investigated the gene and protein expression of GFAP and Iba1 in the amygdala, periaqueductal gray, prefrontal cortex, hypothalamus, thalamus and dorsal and ventral hippocampus of female rats 24 hours after SCI.

2. Materials and Methods

All procedures were carried out with the approval of the institutional Animal Care and Ethics Committee (UTS ACEC13-0069), according to the guidelines set out by the National Health and Medical Research Council code of conduct for the use of animals in research (Nguyen et al., 2017).

2.1 Animals

Six adult female Sprague Dawley rats (9 weeks old, 250-300g) were acquired from the Animal Resource Centre (Perth, WA, Australia). Rats were housed in cages on a 12-hour dark-light cycle with unlimited access to food and water. Each cage was provided with environmental enrichment. Animals were assigned randomly to either; (1) mild contusion spinal cord injury (SCI) group (SCI; *n* = 3), or (2) sham surgery group (Sham; *n* = 3) (see **Figure 1**).

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2.2 Surgery and euthanasia

Rats were anaesthetised with 2% isoflurane in O_2 (flow rate of 1L/min), once a surgical plane of anaesthesia was established, the fur above the thoracic region was shaved and iodine applied to the exposed skin. A subcutaneous injection of local anaesthetic (0.2ml Bupivacaine) was administered at the site of SCI or sham surgery. Each rat was given analgesics (buprenorphine hydrochloride -*Temgesic* 0.03mg/kg, s.c), antibiotics (cephazolin sodium 33mg/kg, s.c) and Hartman's replacement solution (compound sodium lactate 15ml/kg, s.c). A midline incision was made from the mid to lower thoracic region and subcutaneous tissues cleared from the spinous process of the T10 vertebral body. A bilateral laminectomy of the T10 vertebrae exposed the dorsal surface of the spinal cord.

Using a NYU/MASCIS weight-drop impactor, the vertebral column of each rat was stabilised with clamps attached to the T9 and T11 vertebrae and the exposed spinal cord subjected to a mild weight-drop contusion injury (6.5mm, 10g, 2.5 mm impactor head diameter). The surgical incision was closed in layers and sutured, and the animals were returned to a warmed cage where they were observed closely during recovery. During the next 24 hours, each rat received two further doses of analgesics (buprenorphine hydrochloride -Temgesic 0.03mg/kg, s.c), antibiotics (cephazolin sodium 33mg/kg, s.c) and Hartman's replacement solution and underwent manual bladder expression (Nguyen et al., 2017).

At the end of this 24 hour period, the rats were deeply anaesthetised and euthanized using pentobarbital sodium (Lethabarb, 1ml/kg i.p.). The brain of each rat was carefully removed and transferred to HBSS buffer before being snap frozen in liquid nitrogen. The brains were stored at -80 degrees until microdissection.

2.3 Microdissections

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-The prefrontal cortex, amygdala, lateral thalamus, dorsal and ventral hippocampus, hypothalamus, and periaqueductal gray regions were microdissected using our previously described methods (Chiu et al., 2007; Castorina et al., 2019), and with reference to ~~Paxinos and Watsons~~a stereotaxic atlas of the rat brain (Paxinos and Watson, 2006). The brain was sectioned into smaller tissue blocks by making~~Briefly~~, three complete coronal ~~brain sections~~cuts ~~(2 mm thick) were cut~~ at specific ~~different~~ antero-posterior (AP) levels using a steril~~eised~~, ~~pre-~~chilled razor blade, cleaned in "RNA-ase Away". The first coronal section was made at the anterior border of the optic chiasm (+ 0.3 mm anterior to bregma), the second at the posterior border of the interpeduncular fossa, and the third immediately posterior to the inferior colliculi as the midbrain aqueduct opens in the fourth ventricle

(approx. -4.6 mm to -7.8 mm caudal to bregma). Each section created a tissue block that included one or more of the regions of interest, as detailed below:

Prefrontal cortex: ~~The~~In the most anterior tissue block we isolated the medial prefrontal cortex ~~anterior level of the optic chiasm (bregma level: + 0.3 mm) was used to orientate along the AP axis. To obtain the prefrontal cortex~~, the first coronal section was cut between bregma levels +4.68mm and +2.52mm (**Figure 2A**). Thereafter, we removed the anterior olfactory nucleus which occupies the ventral 1.5mm of this section ~~(which contains the anterior olfactory nucleus)~~ (**Figure 2B**) and then ~~performed~~made two ~~vertical~~parasagittal cuts ~~at about~~approximately 1 mm lateral to the midline abutting the ~~(using the~~ forceps minor ~~as a reference for the lateral boundary)~~.

Amygdala: Similar~~ly~~ to the prefrontal cortex, a 2-mm thick coronal section~~s were~~was cut between -1.92mm and -3.96 caudal to bregma (**Figure 2C**). To obtain a block that included the entire amygdaloid complex, ~~we~~ we used the opening of the lateral ventricle as a reference point to further dissect~~ed a~~the triangular~~le-shaped~~ shaped area of the amygdala ~~4mm~~ from each side ~~at~~located ~~about~~approximately 4mm from the midline ~~(~~ ~~(we used the opening of the lateral ventricle as a reference) (~~**Figure 2D**).

Thalamus: To obtain ~~tissue blocks that grossly contained the major thalamic nuclei~~lateral thalamus samples, ~~we utilised~~we used the same ~~sections~~tissue blocks used to ~~dissect~~isolate the amygdala~~, as these nuclei extend roughly across the same AP levels~~. ~~Once the amygdala blocks were excised, we further removed the ventral 2mm to exclude the~~The hypothalamus was isolated from the remaining tissue block and using the internal capsule as the lateral boundary and the dorsal opening of the 3rd ventricle as the upper boundary ~~hypothalamic formation. Thereafter,~~ we micro-dissected the lateral thalamus from each side as two semi-rectangular tissue~~-shaped blocks~~samples (~~width =~~ ~8mm ~~and~~x ~~height =~~ 2mm) ~~using the internal capsule as the lateral boundary and the dorsal opening of the 3rd ventricle as a reference for the upper boundary~~ (**Figure 2D**).

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Dorsal hippocampus: was isolated from the same tissue block used ~~We used the same sections used~~ to ~~excise~~isolate the amygdala and lateral thalamus ~~to obtain this specific ROI~~. The right and left dorsal hippocampi~~us is~~are easily identified and isolated. The tissue was ~~not difficult to identify under a microscope, with its typical butterfly shape. By using a small scalpel, we~~ separated from the cortical layer above ~~surrounding the upper portion of the dorsal hippocampus~~ and the corpus callosum ~~, then carefully collected the tissue from left and right hemispheres~~ (**Figure 2D**).

Ventral Hippocampus: The ventral ~~part of the~~ hippocampus was dissected from the most caudal tissue block and was isolated ~~after cutting a further coronal~~from a smaller tissue block ~~section (3-mm thick) taken at~~approx. ~~-~~ ~~=~~4.6 mm to -7.8mm caudal to bregma ~~and the caudal boundary at approximately −7.8 mm caudal to bregma~~ (**Figure 2E**). ~~In these sections, the~~The ventral hippocampus ~~is~~was ~~separated~~isolated from the surrounding cortex under a dissecting microscope using the thin layer of white matter surrounding the lateral boundary for reference, the tissue was removed ~~from the surrounding cortex~~ with ~~curved-tip~~ Dumont tweezers (12cm, 0.17x0.1mm curved-tip~~tips~~) ~~and using the thin layer of white matter surrounding the lateral boundary as a visual reference, as shown in~~see **Figure 2F**.

PAG: ~~The PAG is a tubular-shaped region of the midbrain surrounding the cerebral aqueduct. Using the 3-mm section utilised to dissect~~The tissue block used to isolate the ventral hippocampus was used to isolate the PAG. The PAG is a tubular-shaped region of the midbrain surrounding the cerebral aqueduct which resulted in ~~we isolated the tissue around the aqueduct, obtaining~~ a ~~block~~tissue sample of about 2 mm diameter (**Figure 2F**).

Each of the brain regions were weighed, and immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

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2.~~3~~ RNA extraction and cDNA synthesis

Each of the brain regions obtained from SCI and Sham groups were processed for RNA extraction, following the manufacturer's protocol, with minor modifications (Sigma-Aldrich). To ~~RNA was~~ extract~~ed~~ RNA ~~from each~~ sample~~s~~ we used~~using~~ 1ml TRI reagent (Sigma-Aldrich) and 0.2ml chloroform. We then ~~to obtain three distinct phases: (the upper aqueous phase containing RNA, the interphase containing DNA and the organic phase containing proteins). The aqueous phase was collected and placed in a new RNase-free tube and~~ precipitated the RNA with 0.5 ml 2-propanol at 12~~.~~000×g for 15 min at 4°C (Castorina et al., 2014). We~~The supernatant was discarded, and the~~ pellet ~~was~~ washed the pellet with 75% ethanol, ~~and~~ left to air dry and re-dissolved in 30µL milliQ H_2O. Final RNA concentration~~s~~ was~~ere~~ measured with a ~~calculated using~~ spectrophotometer~~ry~~ (Nanodrop ND-1000® spectrophotometer, Wilmington, DE, USA). To obtain s~~S~~ingle-stranded cDNAs we used ~~were synthesized using~~ the Tetro cDNA synthesis kit (Bioline, Sydney, NSW, Australia). We incubated ~~Total RNA (~~1 µg~~)~~ of total RNA ~~was incubated~~ with ~~the Tetro~~ reverse transcriptase (200 U/µl); Oligo-$(dT)_{18}$ primer (100 nM); 0.5 mM dNTP mix, RNase-inhibitor (10 U/µL) at 45 °C for 40 min in a final volume of 20 µL. Temperature was finally increased to 85 °C for 5 min ~~The reaction was~~to terminate the reaction ~~terminated by incubation of samples at 85 °C for 5 min~~.

2.~~5~~4 ~~Quantitative R~~real time ~~polymerase chain reaction (~~qPCR~~)~~ analysis

To analyze changes in steady-state levels of GFAP and Iba1 transcripts between SCI and Sham rats we used the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Gladesville, NSW, Australia). The ribosomal protein 18S was used as the housekeeping gene. qPCR experiments were carried out by following a modified protocol, adapted from

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our previous study (Castorina et al., 2013). 3µl of diluted cDNA (10ng/µl), 5µl of SensiFAST SYBR®No-ROX master mix (Bioline), 0.8 µl of 5µM forward primer, 0.8µl of 5µM reverse primer and 0.4 µl of MilliQH_2O were added to a final volume of 10 µl per reaction. Differentially expressed genes were analysed using the ΔΔCt method and are expressed as mean fold change ~~To investigate the different expression levels, we analysed the mean fold change values of each sample calculated using the ΔΔCt method described by Schmittgen and Livak~~ (Schmittgen and Livak, 2008). The ΔΔCt of each sample~~cDNA~~ was obtained~~calculated~~ by subtracting the calibrator (Sham) ΔCt to the target sample ΔCt and then applying th~~. e~~The formula $2^{-\Delta\Delta Ct}$. ~~was then used to calculate the fold-change.~~ Baseline measurements ~~for each calibrator sample~~ were set to 1. PCR product specificity was assessed by melting curve analysis, with each gene displaying an individual peak. The sequences of the genes used in this study are listed in **Table 1**.

PLACE TABLE 1 ABOUT HERE

2.6~~5~~ Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western blot

To obtain our protein lysates, brain tissues taken from different regions were homogenised using a sterilized conical pestle in RIPA Buffer (1:5 w/vol, Sigma-Aldrich, Castle Hill, NSW, Australia) containing a Protease Inhibitor cocktail (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, Castle Hill, NSW, Australia). Samples were then cleared by centrifugation at 12000×g for 10 minutes. Protein quantification was ~~determined~~ performed using the ~~bicinchoninic acid assay (~~BCA~~)~~ assay ~~(Pierce BCA Protein Assay Kit)~~ (ThermoFisher Scientific).

Denatured proteins (30µg) ~~Samples~~ were prepared by adding ~~3.75µL of~~ 4× Laemmli buffer (Bio-Rad, Gladesville, NSW) and ~~containing~~ β-mercaptoethanol (Sigma-Aldrich~~, Castle Hill,~~

~~NSW, ,~~ Australia) and heating ~~mixture, (ratio 1:9 vol/vol) to 30µg protein in a final volume of 15µL. Samples were denatured~~ for 10 min ~~minutes~~ at 70°C. Samples~~Proteins~~ were run on a gradient Tris-glycine ~~separated by SDS-polyacrylamide~~ gel ~~electrophoresis (SDS-PAGE) using~~ (4-20% ~~mini gels (~~, Bio-Rad~~, Criterion 15-well Mini-Protean SFX~~), ~~alongside~~ with a ~~5µl of the~~ molecular weight ladder~~/marker~~ (Bio-Rad ~~Prestained HyperLadder Precision Plus Protein™~~). Gels were transferred to a PVDF membrane using the Trans-Blot Turbo instrument (Bio-Rad) (Giunta et al., 2010). Once ~~terminated~~transfer was completed, membranes were ~~immediately placed~~washed thoroughly ~~in a container filled~~ with TBS + 0.1% Tween 20 (Sigma-Aldrich, Castle Hill, NSW, Australia) (TBST 1x) ~~to wash out any residues during transfer~~. Membranes were then ~~To block non-specific binding sites, membranes were~~ blocked ~~for 1 hour~~ in 5% dry non-fat skim milk in TBST with slow agitation (50-60 rpm) for 1 hour at room temperature (RT).

Membranes were incubated with either GFAP (Abcam, Cat# ab68428; dilution 1:2000) or Iba1 primary antibodies (Abcam,Cat# ab178846; dilution 1:500) in blocking buffer overnight at 4°C with slow agitation. Thereafter, m~~M~~embranes were washed 3× with ~~then placed in a container with 1×~~ TBST, ~~and washed rapidly three times,~~ followed by 3×~~three further~~ 5 minutes long washes. Finally, membranes were incubated with a ~~in~~ secondary antibody (horse radish peroxidase~~HRP~~-conjugated goat anti-rabbit IgG) for 1 hour at RT~~room temperature~~, diluted at 1:10000 in blocking buffer. M~~The m~~embranes were finally ~~then~~ washed ~~once again~~ to remove excess secondary antibody (Bucolo et al., 2012). Blots were revealed by chemiluminescence method (Clarity Western ECL, Bio-Rad) using ~~Imaging was then performed on~~ the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad). ~~To detect bands, we utilized Clarity Western ECL Blotting Substrate (Bio-Rad).~~

2.~~6~~7 Statistical analysis

~~All~~ D~~d~~ata are reported as mean ± S.E.M. Comparisons between groups were assessed using the unpaired Student's *t*-test. P ~~values~~ ≤.05 was~~ere~~ considered statistically significant. Data analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. Results

3.1. Surgical Procedures

There were no adverse events during our surgical procedures and all rats recovered well after surgery. Rats with SCI showed signs of hind limb movement impairment, consistent with the location and severity of the injury. Sham rats (controls) did not show any signs of locomotor impairment as reported in our previous work (Nguyen et al., 2017).

3.2 Acute changes in GFAP mRNA and protein expression in the rat brain following SCI

The acute effects of SCI on the expression of GFAP in the amygdala and periaqueductal gray (PAG), two regions pivotal in mediating emotional coping behaviours and that play important roles in the development of the behavioural dysfunction comorbid with injury and trauma (Keay and Bandler, 2001; Phelps and LeDoux, 2005; Mor et al., 2015; Motta et al., 2017), were evaluated. **Figures 32A & 43A** show that GFAP mRNA and protein expression levels were significantly upregulated in the amygdala compared to Sham controls (t_{10}=2.261 & t_6=3.061, *p≤.05, as determined by Student *t*-test), whereas in the PAG, the expression of GFAP mRNA and proteins were not significantly affected 24 hours after SCI (**Figure 32B & 43B**; t_{10}=1.144 & t_6=0.872, p>.05 for GFAP). **Figures 32C & 43C** show that GFAP expression in the prefrontal cortex was unchanged 24 hours after SCI (t_{10}=0.630 for mRNA and t_6=0.936, p>.05 for GFAP), whereas hypothalamic GFAP mRNA and protein expression was significantly increased at this time (**Figure 32D & 43D**, t_{10}=2.490 and t_6=3.123, *p≤.05, Students *t*-test).

PLACE FIGURE 32 and 43 ABOUT HERE

The thalamus receives a substantial spinal input and is the critical relay for somatosensory inputs to the cerebral cortex (Yuan et al., 2016); it also receives substantial inputs from

spinal recipient brainstem regions, including projections from the ventro-lateral portion of the PAG carrying deep noxious inputs (Floyd et al., 1996), therefore we sought to determine if SCI altered GFAP expression in the thalamus. We report a surprising, and robust decline in GFAP transcript levels at this acute 24hr time point (**Figure 32E**, t_{10}=3.488, **p≤.01, Student *t*-test), further confirmed by protein analyses (**Figure 43E**, t_6=2.684, **p≤.01).

GFAP expression in the hippocampus was also significantly reduced in the dorsal hippocampus both at the mRNA (**Figure 32F**, t_{10}=2.500, *p≤.05, as determined by Student *t*-test) and even more robustly at the protein level (**Figure 43F**, t_6=4.029, **p≤.01), but not in the ventral hippocampus of SCI rats (**Figure 32G & 43G**, t_{10}=1.474 & t_6=1.659, p>.05).

PLACE FIGURE 54 and 65 ABOUT HERE

3.3 Acute changes in Iba1 mRNA and protein expression in the rat brain following SCI

24hrs post SCI the mRNA and protein expression of the microglial marker Iba1 were unaffected in the amygdala (**Figure 54A & 65A**, t_{10}=0.514 & t_6=0.936, p>.05); the PAG (**Figure 45B & 65B**, t_{10}=0.186 & t_6=1.059, p>.05); the prefrontal cortex (**Figure 54C & 65C**; t_{10}=1.342 & t_6=0.216, p>.05); the thalamus (**Figure 54E & 65E**, t_{10}=1.148 & t_6=0.406, p>.05) and the hypothalamus (**Figure 54D & 65D**, t_{10}=0.248 & t_6=0.307, p>.05). In contrast, Iba1 mRNA and protein expression levels in the hippocampus were reduced in both the dorsal hippocampus (**Figure 54F & 65F**, t_{10}=2.292 & t_6=3.739, *p≤.05 and **p≤.01, respectively) and the ventral hippocampus (**Figure 54G & 65G**, t_{10}=2.251 & t_6=2.453, *p≤.05).

4. Discussion

In this study, we identified early changes in GFAP and Iba1 mRNA and protein expression levels in the female rat brain following a mild spinal cord injury (SCI). We interrogated astrocyte- and microglial-specific cell markers, as our main goal was to detect early disruptions within the glial compartment following SCI. Given the complex architectural organization of the brain and the differential involvement of specific brain regions in the development of the comorbidities associated with spinal cord injury our focus was on brain areas controlling the affective, cognitive and sensory responses to traumatic stressors, for these measurements.

There is documented evidence of sexual dimorphism in the behavioral responses to acute stress and as in several types of high order cognitive and affective functions (Rubinow and Schmidt, 2019). Apparently, these differences account for hormonal, sex chromosomes and their interaction with the environment (Rubinow and Schmidt, 2019), with studies indicating that female rats, as opposed to males, develop distinct coping strategies in response to stress (Youssef et al., 2018). Interestingly, in a recent work it has been demonstrated that stressed-susceptible brain regions such as the prefrontal cortex or the hippocampus exhibit higher activation patterns in male *vs.* female in rats exposed to acute immobilisation stress, but not in the forced swimming test (Sood et al., 2018). These data pinpoint the sexually dimorphic response strictly depends on the specific nature of the stress, a topic that warrants further investigations. In this exploratory study, we utilised female rats as these are conventionally used in spinal cord injury studies due to their better compliance to the surgical procedure compared with male rats (Datto et al., 2015). A further reason was ethical, as in our experience male rats subjected to SCI often develop a severe form of autotomy (self-mutilation) directed to their hind-limbs and sometimes genitalia, an adverse event that we have never observed in female rats. Interestingly, the

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exaggerated autotomy in male SCI rats also seems to occur after neurectomy of peripheral nerves, as shown in other studies (Wagner et al., 1995).

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Our analyses identified regionally specific changes in GFAP gene and protein expression in several supraspinal structures. To contrast, changes in Iba1 expression were restricted to the dorsal and ventral hippocampus, brain regions critical in integrating memory formation, spatial navigation and emotional regulation (Schultz and Engelhardt, 2014). To our knowledge, this is the first evidence describing acute changes in supraspinal GFAP and Iba1 mRNA and protein regulation, 24hrs post-SCI.

4.1 SCI and GFAP expression in the brain

Accumulating evidence shows that the activity of astrocytes is crucial in determining the behavioral outputs of both the amygdala and hypothalamus, via a process that involves the selective regulatory activity of specific synapses by activated astrocytes (Martin-Fernandez et al., 2017; Chen et al., 2019). At the cellular level, astrocytes express receptors for both noradrenaline (β2-adrenergic receptors [βARs]) and glucocorticoids (GRs) (Hertz et al., 2010; Jauregui-Huerta et al., 2010), each of which play different roles in modulating the calcium influx and ATP release of individual astrocytes (Chen et al., 2019).

During the initial response to an acute stressor such as a traumatic injury, noradrenaline release precedes that of the glucocorticoids, cortisol and/or corticosterone (Pearson-Leary et al., 2015; Chen et al., 2019). This suggests that increased noradrenergic activity likely predominates in the immediate phase of the response to traumatic spinal cord injury. In view of this, and considering the different temporal activation patterns and anatomical distributions of astrocytic βARs and GRs in the brain (Gao et al., 2016), it is possible that

the regionally distinct patterns of GFAP mRNA and protein regulation that we observed after SCI might be linked to differential exposure of these discrete brain regions to increasing NE and glucocorticoids after the spinal cord injury.

GFAP gene and protein levels were significantly increased in the stress-responsive amygdala and hypothalamus, each of which receives strong afferent drive from the noradrenergic locus coeruleus (Palkovits et al., 1980; Kawakami et al., 1984). The locus coeruleus is reliably activated by acute stressors and it is tempting to suggest that a strong activation of this noradrenergic region immediately following SCI, could lead to significant release of NE in the amygdala and hypothalamus, leading to increased astrocyte activity, as reflected by the induction of GFAP expression reported here.

In contrast, GFAP transcripts and proteins were reliably decreased in the lateral thalamus. The lateral thalamus, encompassing key somatosensory thalamic relays, is a critical source of somatosensory inputs both between different subcortical areas and the cortex (Herrero et al., 2002). Unlike the amygdala and hypothalamus, the thalamus is not strongly regulated by ascending noradrenergic pathways (Simpson et al., 2006). It is however particularly sensitive to the effects of deafferentation triggered by SCI, and many populations of thalamic neurons respond to SCI by immediately increasing their firing activities (Alonso-Calvino et al., 2016). It is therefore possible that the decline in GFAP mRNAs and proteins we report reflects a compensatory mechanism in which the astrocytes surrounding hyperactive thalamic neurons diminish their activity in an effort to dampen the effects of deafferentiation.

The hippocampus is particularly vulnerable to both acute and chronic stressors, including those triggered by physical trauma (Jing et al., 2017). This brain structure has a highly conserved architectural organisation along its dorso-ventral axis (Schultz and Engelhardt,

2014), with the dorsal hippocampus critical for spatial navigation and memory and the ventral hippocampus regulating emotional processing and expression (Amaral and Witter, 1989). Despite architectural similarities, several studies have pinpointed significant differences in the transcriptional and proteomic profiles of the dorsal and ventral sub-regions of the hippocampus in response to stress (Maggio and Segal, 2009; Pierard et al., 2017; Floriou-Servou et al., 2018). An important observation of this study was that SCI significantly reduced both gene and protein expression of the astrocytic marker GFAP in the dorsal, but not the ventral part of the hippocampus. Reduced GFAP expression has been previously reported in the hippocampus and prefrontal cortex in a rat model of depression (Eldomiaty et al., 2020). By contrast, mild cortical contusion has shown to increase hippocampal GFAP mRNA levels as early as after 12hrs post-injury (Hinkle et al., 1997). In line with our hypothesis that a spinal trauma can predispose an individual to the development of comorbid behavioral dysfunctions, it is perhaps not surprising that GFAP expression is reduced.

4.2 SCI and Iba1 expression in the brain

Iba1 is a microglia-specific calcium binding protein both *in vitro* and *in vivo*, whose expression reflects cellular polarization state (Ito et al., 1998). In this study, we observed that Iba1 mRNA and protein expression were selectively reduced in the dorsal and ventral regions of the hippocampus after SCI. At first consideration these findings appear counterintuitive, however, the 'shock' suffered by these vulnerable brain regions as a consequence of the physical trauma of spinal cord injury, might well-reflect the impacts of the shock evoked in animals models evaluating the central effects of electroconvulsive therapy (Jinno and Kosaka, 2008). Our data suggest that the spinal cord trauma triggers immediate plastic changes in the hippocampus that are associated with attenuated microglia activity, in this acute post injury phase. Considering the recently identified role of

microglia in synapse turnover (Wang et al., 2020), it is not unreasonable to suggest that attenuated Iba1 expression might reflect the pathological increase in neuronal plasticity that occurs after a traumatic experience such as SCI.

PLACE TABLE 2 ABOUT HERE

5. Conclusions

In summary, our data provides evidence for early changes in glial activity in several brain regions involved in the development of behavioral comorbidities following SCI. Glial activity changes show clear regional specificity, and it is the activity of astrocytes that is most strongly affected during this period. We also identified attenuated Iba1 mRNA and protein expression in the hippocampus, which is consistent with rapid and adaptive neuroplasticity in this region. However, whilst the changes in the expression of the glial markers were remarkable, it should be noted that the associated comorbid changes in higher order cognitive functions and affective behaviors may require long-term modulations occurring at molecular, cellular and systemic levels. Therefore, additional investigations addressing the changes in glial activity over time are warranted. Nonetheless, ~~Taken together,~~ these findings provide the first evidence of early supraspinal glial expression changes following spinal cord injury which could lay the foundations for the subsequent development of affective and cognitive dysfunction that is comorbid with SCI in many individuals (summarized in **Table 2**).

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Figure Legends

Figure 1. Flowchart of the experimental procedure. Female, Sprague-Dawley rats, 9 weeks of age were divided into 2 groups- Sham (N=3) and SCI (N=3). A small incision was made in the thoracic region above the T10 vertebrae and a laminectomy performed (Krishna et al., 2013) . The SCI group then received a weight drop contusion, by dropping a 10g weight from 6.25mm height and an impact head diameter of 2.5mm onto the exposed dura of the spinal cord (Nguyen et al., 2017). Sham rats received the same surgical procedure but not the weight-drop procedure. All rats were euthanized 24hrs after surgery/injury and each brain was microdissected into the required identified regions. Each region was then processed for RNA extraction and downstream real-time qPCR analyses.

Figure 2. Schematic depicting rat brain microdissection procedures. 2-mm (Sections 1 and 2) or 3-mm thick coronal brain sections (Section 3) were cut from either Sham-operated or SCI rats as indicated in (**A**, **C** and **E**) and tissue blocks containing the prefrontal cortex (**B**), the dorsal hippocampus, thalamus and amygdala (**D**), or the ventral hippocampus and PAG (**F**) were microdissected under a stereoscopic microscope

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(magnification 10×) using the Paxinos and Watson rat brain atlas as a reference (Paxinos and Watson, 2006).

Figure 3~~2~~. Real-time qPCR data showing the differential mRNA expression of GFAP in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus in Sham and spinal cord injured rats (SCI).

Real-time qPCRs~~Amplifications~~ were carried out ~~performed~~ using selected primer~~s~~ pairs that were designed and o~~-o~~ptimised to amplify small fragments~~for qPCR analyses~~ (≤150 bp length) ~~which recognize fragments~~ within the coding sequence of the gene of interest (~~for details refer to~~please see **Table 1**). Results ~~are presented as~~show mean fold changes ± SEM obtained from two independent experiments ~~which were each~~ run in duplicate. Fold changes for the genes of interest~~of each gene~~ were were calculated using the comparative ΔΔCt method ~~obtained~~ after normalization to the reference gene 18S ~~and were calculated using the comparative ΔΔCt method~~. Baseline gene expression ~~levels~~ of the Sham groups was~~ere~~ set to 1.

-*p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Figure 4~~3~~. Western blots analyses of GFAP protein expression in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured (Sham) and spinal cord injury rats (SCI). (**A-G**) Representative GFAP immunoblots and semi-quantitative densitometric analyses are shown for the (**A**) amygdala, (**B**) periaqueductal grey, (**C**) prefrontal cortex, (**D**) hypothalamus, (**E**) thalamus, dorsal and ventral hippocampus (**F** & **G**). Data are the mean ± SEM of two separate experiments. *p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Figure 5~~4~~. Real-time qPCR data showing the differential mRNA expression of Iba1 in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured (Sham) and spinal cord injury rats (SCI). Target-specific a~~A~~mplificons~~ations~~ were obtained ~~performed~~ using custom-designed~~selected~~ primer~~s~~ pairs optimised for qPCR analyses (≤150 bp length). Sequences are shown ~~which recognize fragments within the coding sequence of the gene of interest (for details refer to~~in **Table 1~~)~~**. Results ~~are presented as~~shown are the mean fold changes ± SEM obtained from two independent experiments, which were each run in duplicate. Fold changes for the genes of interest were were calculated using the comparative ΔΔCt method after normalization to the reference gene 18S. Baseline gene expression of the Sham groups was set to 1. ~~Fold changes of each gene were obtained after normalization to the reference gene 18S, and were calculated using the comparative ΔΔCt method. Baseline expression levels of the SHAM groups were set to 1.~~ *p≤.05 *Vs* SHAM, using Student's *t*-test. Ns = not significant.

Figure 6~~5~~. Western blots analyses of Iba1 protein expression in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured (Sham) and spinal cord injury rats (SCI). (**A-G**) Representative Iba1 immunoblots and semi-quantitative densitometric analyses are shown for the (**A**) amygdala, (**B**) periaqueductal grey, (**C**) prefrontal cortex, (**D**) hypothalamus, (**E**) thalamus, dorsal and ventral hippocampus (**F** & **G**). Data are the mean ± SEM of two separate experiments. *p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Rapid GFAP and Iba1 Expression Changes in the Female Rat Brain following Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) is a devastating condition often associated with sleep disorders, mood change and depression. Evidence suggests that rapid changes to supporting glia may predispose individuals with SCI to such comorbidities. Here, we interrogated the expression of astrocyte- and microglial-specific markers glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) in the rat brain in the first 24 hours following spinal cord injury (SCI). Female Sprague Dawley rats underwent thoracic laminectomy; half of the rats received a mild contusion injury at the level of the T10 vertebral body (SCI group), the other half did not (Sham group). Twenty-four hours post-surgery the rats were sacrificed, and the amygdala, periaqueductal grey, prefrontal cortex, hypothalamus, lateral thalamus, hippocampus (dorsal and ventral) were collected. GFAP and Iba1 mRNA and protein levels were measured by real-time qPCR and Western blot.

In SCI rats, GFAP mRNA and protein expression increased in the amygdala and hypothalamus (*$p<0.05$). In contrast, gene and protein expression decreased in the thalamus (**$p<0.01$) and dorsal hippocampus (*$p<0.05$ and **$p<0.01$, respectively). Interestingly, Iba1 transcripts and proteins were significantly diminished only in the dorsal (*$p<0.05$ and **$p<0.01$, respectively) and ventral hippocampus, where gene expression diminished (*$p<0.05$ for both mRNA and protein). Considered together, these findings demonstrate that as early as 24 hours post-SCI there are region-specific disruptions of GFAP and Iba1 transcript and protein levels in higher brain regions.

Keywords: Glial fibrillary acidic protein; Ionized calcium binding adaptor molecule 1; spinal cord injury; neurotrauma; Microglia; Astrocytes

1. Introduction

Spinal cord injuries (SCI) can result in long-term and permanent deafferentation of cortical circuits of the central nervous system (CNS) (Wall and Egger, 1971; Ziemann et al., 1998). Such changes can result in a substantial reorganization of cortical maps, exemplifying the plastic properties of the CNS (Aguilar et al., 2010). Sleep disturbance, anxiety, depression and cognitive dysfunction is highly prevalent in SCI patients (Davidoff et al., 1990; Kennedy and Rogers, 2000; Biering-Sørensen and Biering-Sørensen, 2001). This suggests strongly that following spinal cord trauma, in addition to changes in cortical circuits, other brain regions critical for the regulation of sleep, mood and cognition are also significantly impacted. For a complete understanding of the neurochemical bases of these changes in complex behaviors, it is essential to understand the changes in the brain triggered during the earliest stages of spinal cord injury, from which these long-term changes evolve.

Glial cells are the supporting cells of the CNS (He and Sun, 2007). Alterations within the astrocyte and microglia compartments play significant roles in the onset and progression of several pathophysiological processes that can lead to a spectrum of affective dysfunctions (Öngür and Heckers, 2004; Pav et al., 2008), as well as synaptic alterations (Honer et al., 1999; Coyle and Schwarcz, 2000; Cotter et al., 2001; Scholz and Woolf, 2007). Both astrocytes and microglia play major roles in shaping these CNS functions, and are likely to be the first cell populations primed following trauma, such as is associated with SCI. Glial fibrillary acidic protein (GFAP) is well established as the primary filament present in mature astrocytes within the CNS, where it is involved in modulating the structural stability, shape, and motility of the cells, as well as the cell-to-cell interactions with neurons (Eng, 1985; Eng and Shiurba, 1988; Eng et al., 2000; Li et al., 2020). Ionized calcium binding adaptor molecule 1 (Iba1), is expressed in the cells of several tissues,

including brain, testis, spleen and, to a lesser extent, in the kidneys and lungs. In the brain, Iba1 is expressed uniquely by microglia (Ito et al., 2001; Hwang et al., 2008), where it elicits actin-bundling activity and participates in membrane ruffling and phagocytosis when the microglia are activated (Ohsawa et al., 2004).

In a number of studies examining mood change and cognitive dysfunction identical to that seen in individuals with SCI there are reports of regionally specific reductions in glial cell populations and/or glial activities in the amygdala, prefrontal cortex, hippocampus and periaqueductal gray (Öngür et al., 1998; Bowley et al., 2002; Imbe et al., 2012). Examining changes in GFAP and Iba1 transcript and protein levels may provide important insights into the temporal and topographical responses of glial cells occurring in higher brain regions after spinal cord injury.

In this study, we evaluated the hypothesis that within the initial 24 hours after an injury, SCI leads to a rapid mRNA and protein changes in glial cells of discrete brain regions critical for the regulation of mood/emotion, stress responsivity, memory and decision-making. To answer this question, we investigated the gene and protein expression of GFAP and Iba1 in the amygdala, periaqueductal gray, prefrontal cortex, hypothalamus, thalamus and dorsal and ventral hippocampus of female rats 24 hours after SCI.

2. Materials and Methods

All procedures were carried out with the approval of the institutional Animal Care and Ethics Committee (UTS ACEC13-0069), according to the guidelines set out by the National Health and Medical Research Council code of conduct for the use of animals in research (Nguyen et al., 2017).

2.1 Animals

Six adult female Sprague Dawley rats (9 weeks old, 250-300g) were acquired from the Animal Resource Centre (Perth, WA, Australia). Rats were housed in cages on a 12-hour dark-light cycle with unlimited access to food and water. Each cage was provided with environmental enrichment. Animals were assigned randomly to either; (1) mild contusion spinal cord injury (SCI) group (SCI; *n* = 3), or (2) sham surgery group (Sham; *n* = 3) (see **Figure 1**).

PLACE FIGURE 1 ABOUT HERE

2.2 Surgery and euthanasia

Rats were anaesthetised with 2% isoflurane in O_2 (flow rate of 1L/min), once a surgical plane of anaesthesia was established, the fur above the thoracic region was shaved and iodine applied to the exposed skin. A subcutaneous injection of local anaesthetic (0.2ml Bupivacaine) was administered at the site of SCI or sham surgery. Each rat was given analgesics (buprenorphine hydrochloride -*Temgesic* 0.03mg/kg, s.c), antibiotics (cephazolin sodium 33mg/kg, s.c) and Hartman's replacement solution (compound sodium lactate 15ml/kg, s.c). A midline incision was made from the mid to lower thoracic region and subcutaneous tissues cleared from the spinous process of the T10 vertebral body. A bilateral laminectomy of the T10 vertebrae exposed the dorsal surface of the spinal cord.

Using a NYU/MASCIS weight-drop impactor, the vertebral column of each rat was stabilised with clamps attached to the T9 and T11 vertebrae and the exposed spinal cord subjected to a mild weight-drop contusion injury (6.5mm, 10g, 2.5 mm impactor head diameter). The surgical incision was closed in layers and sutured, and the animals were returned to a warmed cage where they were observed closely during recovery. During the next 24 hours, each rat received two further doses of analgesics (buprenorphine hydrochloride -Temgesic 0.03mg/kg, s.c), antibiotics (cephazolin sodium 33mg/kg, s.c) and Hartman's replacement solution and underwent manual bladder expression (Nguyen et al., 2017).

At the end of this 24 hour period, the rats were deeply anaesthetised and euthanized using pentobarbital sodium (Lethabarb, 1ml/kg i.p.). The brain of each rat was carefully removed and transferred to HBSS buffer before being snap frozen in liquid nitrogen. The brains were stored at -80 degrees until microdissection.

2.3 Microdissections

The prefrontal cortex, amygdala, lateral thalamus, dorsal and ventral hippocampus, hypothalamus, and periaqueductal gray regions were microdissected using our previously described methods (Chiu et al., 2007; Castorina et al., 2019), and with reference to a stereotaxic atlas of the rat brain (Paxinos and Watson, 2006). The brain was sectioned into smaller tissue blocks by making, three complete coronal cuts at specific antero-posterior (AP) levels using a sterile, chilled razor blade, cleaned in "RNA-ase Away". The first coronal section was made at the anterior border of the optic chiasm (+ 0.3 mm anterior to bregma), the second at the posterior border of the interpeduncular fossa, and the third immediately posterior to the inferior colliculi as the midbrain aqueduct opens in the fourth ventricle (approx. -4.6 mm to -7.8 mm caudal to bregma). Each section created a tissue block that included one or more of the regions of interest, as detailed below:

Prefrontal cortex: In the most anterior tissue block we isolated the medial prefrontal cortex, the first coronal section was cut between bregma levels +4.68mm and +2.52mm (**Figure 2A**). Thereafter, we removed the anterior olfactory nucleus which occupies the ventral 1.5mm of this section (**Figure 2B**) and then made two parasagittal cuts approximately 1 mm lateral to the midline abutting the forceps minor.

Amygdala: Similar to the prefrontal cortex, a 2-mm thick coronal section was cut between -1.92mm and -3.96 caudal to bregma (**Figure 2C**). To obtain a block that included the entire amygdaloid complex, we used the opening of the lateral ventricle as a reference point to further dissect the triangular shaped area of the amygdala from each side located approximately 4mm from the midline (**Figure 2D**).

Thalamus: To obtain lateral thalamus samples, we used the same tissue blocks used to isolate the amygdala. The hypothalamus was isolated from the remaining tissue block and using the internal capsule as the lateral boundary and the dorsal opening of the 3rd ventricle as the upper boundary we micro-dissected the lateral thalamus from each side as two semi-rectangular tissue samples (~8mm x ~2mm) (**Figure 2D**).

Dorsal hippocampus: was isolated from the same tissue block used to isolate the amygdala and lateral thalamus. The right and left dorsal hippocampi are easily identified and isolated. The tissue was separated from the cortical layer above and the corpus callosum (**Figure 2D**).

Ventral Hippocampus: The ventral hippocampus was dissected from the most caudal tissue block and was isolated from a smaller tissue block approx. -4.6 mm to -7.8mm caudal to bregma (**Figure 2E**). The ventral hippocampus was isolated from the surrounding cortex under a dissecting microscope using the thin layer of white matter surrounding the lateral boundary for reference, the tissue was removed with Dumont tweezers (12cm, 0.17x0.1mm curved-tip) see **Figure 2F**.

PAG: The tissue block used to isolate the ventral hippocampus was used to isolate the PAG. The PAG is a tubular-shaped region of the midbrain surrounding the cerebral aqueduct which resulted in a tissue sample of about 2 mm diameter (**Figure 2F**).

Each of the brain regions were weighed, and immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

PLACE FIGURE 2 ABOUT HERE

2.4 RNA extraction and cDNA synthesis

Each of the brain regions obtained from SCI and Sham groups were processed for RNA extraction, following the manufacturer's protocol, with minor modifications (Sigma-Aldrich). To extract RNA from samples we used 1ml TRI reagent (Sigma-Aldrich) and 0.2ml chloroform. We then precipitated the RNA with 0.5 ml 2-propanol at 12000×g for 15 min at 4°C (Castorina et al., 2014). We washed the pellet with 75% ethanol, left to air dry and re-dissolved in 30µL milliQ H_2O. Final RNA concentration was measured with a spectrophotometer (Nanodrop ND-1000® spectrophotometer, Wilmington, DE, USA). To obtain single-stranded cDNAs we used the Tetro cDNA synthesis kit (Bioline, Sydney, NSW, Australia). We incubated 1 µg of total RNA with reverse transcriptase (200 U/µl); Oligo-$(dT)_{18}$ primer (100 nM); 0.5 mM dNTP mix, RNase-inhibitor (10 U/µL) at 45 °C for 40 min in a final volume of 20 µL. Temperature was finally increased to 85 °C for 5 min to terminate the reaction.

2.5 Real time qPCR analysis

To analyze changes in steady-state levels of GFAP and Iba1 transcripts between SCI and Sham rats we used the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Gladesville, NSW, Australia). The ribosomal protein 18S was used as the housekeeping

gene. qPCR experiments were carried out by following a modified protocol, adapted from our previous study (Castorina et al., 2013). 3µl of diluted cDNA (10ng/µl), 5µl of SensiFAST SYBR®No-ROX master mix (Bioline), 0.8 µl of 5µM forward primer, 0.8µl of 5µM reverse primer and 0.4 µl of MilliQH_2O were added to a final volume of 10 µl per reaction. Differentially expressed genes were analysed using the ΔΔCt method and are expressed as mean fold change (Schmittgen and Livak, 2008). The ΔΔCt of each sample was obtained by subtracting the calibrator (Sham) ΔCt to the target sample ΔCt and then applying the formula $2^{-\Delta\Delta Ct}$. Baseline measurements were set to 1. PCR product specificity was assessed by melting curve analysis, with each gene displaying an individual peak. The sequences of the genes used in this study are listed in **Table 1**.

PLACE TABLE 1 ABOUT HERE

2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western blot

To obtain our protein lysates, brain tissues taken from different regions were homogenised using a sterilized conical pestle in RIPA Buffer (1:5 w/vol, Sigma-Aldrich, Castle Hill, NSW, Australia) containing a Protease Inhibitor cocktail (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, Castle Hill, NSW, Australia). Samples were then cleared by centrifugation at 12000×g for 10 minutes. Protein quantification was performed using the BCA assay (ThermoFisher Scientific).

Denatured proteins (30µg) were prepared by adding 4× Laemmli buffer (Bio-Rad, Gladesville, NSW) and β-mercaptoethanol (Sigma-Aldrich, Australia) and heating for 10 min at 70°C. Samples were run on a gradient Tris-glycine gel (4-20%, Bio-Rad), with a molecular weight ladder (Bio-Rad). Gels were transferred to a PVDF membrane using the Trans-Blot Turbo instrument (Bio-Rad) (Giunta et al., 2010). Once transfer was completed, membranes

were washed thoroughly with TBS + 0.1% Tween 20 (Sigma-Aldrich, Castle Hill, NSW, Australia) (TBST 1x). Membranes were then blocked in 5% dry non-fat skim milk in TBST with slow agitation (50-60 rpm) for 1 hour at room temperature (RT).

Membranes were incubated with either GFAP (Abcam, Cat# ab68428; dilution 1:2000) or Iba1 primary antibodies (Abcam,Cat# ab178846; dilution 1:500) in blocking buffer overnight at 4°C with slow agitation. Thereafter, membranes were washed 3× with TBST, followed by 3×5 minutes long washes. Finally, membranes were incubated with a secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG) for 1 hour at RT, diluted at 1:10000 in blocking buffer. Membranes were finally washed to remove excess secondary antibody (Bucolo et al., 2012). Blots were revealed by chemiluminescence method (Clarity Western ECL, Bio-Rad) using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad).

2.7 Statistical analysis

Data are reported as mean ± S.E.M. Comparisons between groups were assessed using the unpaired Student's *t*-test. P≤.05 was considered statistically significant. Data analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. Results

3.1. Surgical Procedures

There were no adverse events during our surgical procedures and all rats recovered well after surgery. Rats with SCI showed signs of hind limb movement impairment, consistent with the location and severity of the injury. Sham rats (controls) did not show any signs of locomotor impairment as reported in our previous work (Nguyen et al., 2017).

3.2 Acute changes in GFAP mRNA and protein expression in the rat brain following SCI

The acute effects of SCI on the expression of GFAP in the amygdala and periaqueductal gray (PAG), two regions pivotal in mediating emotional coping behaviours and that play important roles in the development of the behavioural dysfunction comorbid with injury and trauma (Keay and Bandler, 2001; Phelps and LeDoux, 2005; Mor et al., 2015; Motta et al., 2017), were evaluated. **Figures 3A &** 4**A** show that GFAP mRNA and protein expression levels were significantly upregulated in the amygdala compared to Sham controls (t_{10}=2.261 & t_6=3.061, *p$\leqslant$.05, as determined by Student *t*-test), whereas in the PAG, the expression of GFAP mRNA and proteins were not significantly affected 24 hours after SCI (**Figure 3B & 4B**; t_{10}=1.144 & t_6=0.872, p>.05 for GFAP). **Figures 3C & 4C** show that GFAP expression in the prefrontal cortex was unchanged 24 hours after SCI (t_{10}=0.630 for mRNA and t_6=0.936, p>.05 for GFAP), whereas hypothalamic GFAP mRNA and protein expression was significantly increased at this time (**Figure 3D & 4D**, t_{10}=2.490 and t_6=3.123, *p$\leqslant$.05, Students *t*-test).

PLACE FIGURE 3 and 4 ABOUT HERE

The thalamus receives a substantial spinal input and is the critical relay for somatosensory inputs to the cerebral cortex (Yuan et al., 2016); it also receives substantial inputs from

spinal recipient brainstem regions, including projections from the ventro-lateral portion of the PAG carrying deep noxious inputs (Floyd et al., 1996), therefore we sought to determine if SCI altered GFAP expression in the thalamus. We report a surprising, and robust decline in GFAP transcript levels at this acute 24hr time point (**Figure 3E**, t_{10}=3.488, **p≤.01, Student *t*-test), further confirmed by protein analyses (**Figure 4E**, t_6=2.684, **p≤.01).

GFAP expression in the hippocampus was also significantly reduced in the dorsal hippocampus both at the mRNA (**Figure 3F**, t_{10}=2.500, *p≤.05, as determined by Student *t*-test) and even more robustly at the protein level (**Figure 4F**, t_6=4.029, **p≤.01), but not in the ventral hippocampus of SCI rats (**Figure 3G & 4G**, t_{10}=1.474 & t_6=1.659, p>.05).

PLACE FIGURE 5 and 6 ABOUT HERE

3.3 Acute changes in Iba1 mRNA and protein expression in the rat brain following SCI

24hrs post SCI the mRNA and protein expression of the microglial marker Iba1 were unaffected in the amygdala (**Figure 5A & 6A**, t_{10}=0.514 & t_6=0.936, p>.05); the PAG (**Figure 5B & 6B**, t_{10}=0.186 & t_6=1.059, p>.05); the prefrontal cortex (**Figure 5C & 6C**; t_{10}=1.342 & t_6=0.216, p>.05); the thalamus (**Figure 5E & 6E**, t_{10}=1.148 & t_6=0.406, p>.05) and the hypothalamus (**Figure 5D & 6D**, t_{10}=0.248 & t_6=0.307, p>.05). In contrast, Iba1 mRNA and protein expression levels in the hippocampus were reduced in both the dorsal hippocampus (**Figure 5F & 6F**, t_{10}=2.292 & t_6=3.739, *p≤.05 and **p≤.01, respectively) and the ventral hippocampus (**Figure 5G & 6G**, t_{10}=2.251 & t_6=2.453, *p≤.05).

4. Discussion

In this study, we identified early changes in GFAP and Iba1 mRNA and protein expression levels in the female rat brain following a mild spinal cord injury (SCI). We interrogated astrocyte- and microglial-specific cell markers, as our main goal was to detect early disruptions within the glial compartment following SCI. Given the complex architectural organization of the brain and the differential involvement of specific brain regions in the development of the comorbidities associated with spinal cord injury our focus was on brain areas controlling the affective, cognitive and sensory responses to traumatic stressors, for these measurements.

There is documented evidence of sexual dimorphism in the behavioral responses to acute stress and as in several types of high order cognitive and affective functions (Rubinow and Schmidt, 2019). Apparently, these differences account for hormonal, sex chromosomes and their interaction with the environment (Rubinow and Schmidt, 2019), with studies indicating that female rats, as opposed to males, develop distinct coping strategies in response to stress (Youssef et al., 2018). Interestingly, in a recent work it has been demonstrated that stressed-susceptible brain regions such as the prefrontal cortex or the hippocampus exhibit higher activation patterns in male *vs.* female in rats exposed to acute immobilisation stress, but not in the forced swimming test (Sood et al., 2018). These data pinpoint the sexually dimorphic response strictly depends on the specific nature of the stress, a topic that warrants further investigations. In this exploratory study, we utilised female rats as these are conventionally used in spinal cord injury studies due to their better compliance to the surgical procedure compared with male rats (Datto et al., 2015). A further reason was ethical, as in our experience male rats subjected to SCI often develop a severe form of autotomy (self-mutilation) directed to their hind-limbs and sometimes genitalia, an adverse event that we have never observed in female rats. Interestingly, the

exaggerated autotomy in male SCI rats also seems to occur after neurectomy of peripheral nerves, as shown in other studies (Wagner et al., 1995).

Our analyses identified regionally specific changes in GFAP gene and protein expression in several supraspinal structures. To contrast, changes in Iba1 expression were restricted to the dorsal and ventral hippocampus, brain regions critical in integrating memory formation, spatial navigation and emotional regulation (Schultz and Engelhardt, 2014). To our knowledge, this is the first evidence describing acute changes in supraspinal GFAP and Iba1 mRNA and protein regulation, 24hrs post-SCI.

4.1 SCI and GFAP expression in the brain

Accumulating evidence shows that the activity of astrocytes is crucial in determining the behavioral outputs of both the amygdala and hypothalamus, via a process that involves the selective regulatory activity of specific synapses by activated astrocytes (Martin-Fernandez et al., 2017; Chen et al., 2019). At the cellular level, astrocytes express receptors for both noradrenaline (β2-adrenergic receptors [βARs]) and glucocorticoids (GRs) (Hertz et al., 2010; Jauregui-Huerta et al., 2010), each of which play different roles in modulating the calcium influx and ATP release of individual astrocytes (Chen et al., 2019).

During the initial response to an acute stressor such as a traumatic injury, noradrenaline release precedes that of the glucocorticoids, cortisol and/or corticosterone (Pearson-Leary et al., 2015; Chen et al., 2019). This suggests that increased noradrenergic activity likely predominates in the immediate phase of the response to traumatic spinal cord injury. In view of this, and considering the different temporal activation patterns and anatomical distributions of astrocytic βARs and GRs in the brain (Gao et al., 2016), it is possible that

the regionally distinct patterns of GFAP mRNA and protein regulation that we observed after SCI might be linked to differential exposure of these discrete brain regions to increasing NE and glucocorticoids after the spinal cord injury.

GFAP gene and protein levels were significantly increased in the stress-responsive amygdala and hypothalamus, each of which receives strong afferent drive from the noradrenergic locus coeruleus (Palkovits et al., 1980; Kawakami et al., 1984). The locus coeruleus is reliably activated by acute stressors and it is tempting to suggest that a strong activation of this noradrenergic region immediately following SCI, could lead to significant release of NE in the amygdala and hypothalamus, leading to increased astrocyte activity, as reflected by the induction of GFAP expression reported here.

In contrast, GFAP transcripts and proteins were reliably decreased in the lateral thalamus. The lateral thalamus, encompassing key somatosensory thalamic relays, is a critical source of somatosensory inputs both between different subcortical areas and the cortex (Herrero et al., 2002). Unlike the amygdala and hypothalamus, the thalamus is not strongly regulated by ascending noradrenergic pathways (Simpson et al., 2006). It is however particularly sensitive to the effects of deafferentation triggered by SCI, and many populations of thalamic neurons respond to SCI by immediately increasing their firing activities (Alonso-Calvino et al., 2016). It is therefore possible that the decline in GFAP mRNAs and proteins we report reflects a compensatory mechanism in which the astrocytes surrounding hyperactive thalamic neurons diminish their activity in an effort to dampen the effects of deafferentiation.

The hippocampus is particularly vulnerable to both acute and chronic stressors, including those triggered by physical trauma (Jing et al., 2017). This brain structure has a highly conserved architectural organisation along its dorso-ventral axis (Schultz and Engelhardt,

2014), with the dorsal hippocampus critical for spatial navigation and memory and the ventral hippocampus regulating emotional processing and expression (Amaral and Witter, 1989). Despite architectural similarities, several studies have pinpointed significant differences in the transcriptional and proteomic profiles of the dorsal and ventral sub-regions of the hippocampus in response to stress (Maggio and Segal, 2009; Pierard et al., 2017; Floriou-Servou et al., 2018). An important observation of this study was that SCI significantly reduced both gene and protein expression of the astrocytic marker GFAP in the dorsal, but not the ventral part of the hippocampus. Reduced GFAP expression has been previously reported in the hippocampus and prefrontal cortex in a rat model of depression (Eldomiaty et al., 2020). By contrast, mild cortical contusion has shown to increase hippocampal GFAP mRNA levels as early as after 12hrs post-injury (Hinkle et al., 1997). In line with our hypothesis that a spinal trauma can predispose an individual to the development of comorbid behavioral dysfunctions, it is perhaps not surprising that GFAP expression is reduced.

4.2 SCI and Iba1 expression in the brain

Iba1 is a microglia-specific calcium binding protein both *in vitro* and *in vivo*, whose expression reflects cellular polarization state (Ito et al., 1998). In this study, we observed that Iba1 mRNA and protein expression were selectively reduced in the dorsal and ventral regions of the hippocampus after SCI. At first consideration these findings appear counterintuitive, however, the ‘shock’ suffered by these vulnerable brain regions as a consequence of the physical trauma of spinal cord injury, might well-reflect the impacts of the shock evoked in animals models evaluating the central effects of electroconvulsive therapy (Jinno and Kosaka, 2008). Our data suggest that the spinal cord trauma triggers immediate plastic changes in the hippocampus that are associated with attenuated microglia activity, in this acute post injury phase. Considering the recently identified role of

microglia in synapse turnover (Wang et al., 2020), it is not unreasonable to suggest that attenuated Iba1 expression might reflect the pathological increase in neuronal plasticity that occurs after a traumatic experience such as SCI.

PLACE TABLE 2 ABOUT HERE

5. Conclusions

In summary, our data provides evidence for early changes in glial activity in several brain regions involved in the development of behavioral comorbidities following SCI. Glial activity changes show clear regional specificity, and it is the activity of astrocytes that is most strongly affected during this period. We also identified attenuated Iba1 mRNA and protein expression in the hippocampus, which is consistent with rapid and adaptive neuroplasticity in this region. However, whilst the changes in the expression of the glial markers were remarkable, it should be noted that the associated comorbid changes in higher order cognitive functions and affective behaviors may require long-term modulations occurring at molecular, cellular and systemic levels. Therefore, additional investigations addressing the changes in glial activity over time are warranted. Nonetheless, these findings provide the first evidence of early supraspinal glial expression changes following spinal cord injury which could lay the foundations for the subsequent development of affective and cognitive dysfunction that is comorbid with SCI in many individuals (summarized in **Table 2**).

Author Contributions: JAP - writing—original draft preparation; MM - conducted the experiments and substantial contributed to original draft preparation; CAG - conducted the experiments, reviewed and the edited the paper; KAK - writing—review and editing; GM and GA-B helped in the methodology, formal analyses and final revision; AC - conceived the study, obtained and administered the funding, revised the manuscript.

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Figure Legends

Figure 1. Flowchart of the experimental procedure. Female, Sprague-Dawley rats, 9 weeks of age were divided into 2 groups- Sham (N=3) and SCI (N=3). A small incision was made in the thoracic region above the T10 vertebrae and a laminectomy performed (Krishna et al., 2013) . The SCI group then received a weight drop contusion, by dropping a 10g weight from 6.25mm height and an impact head diameter of 2.5mm onto the exposed dura of the spinal cord (Nguyen et al., 2017). Sham rats received the same surgical procedure but not the weight-drop procedure. All rats were euthanized 24hrs after surgery/injury and each brain was microdissected into the required identified regions. Each region was then processed for RNA extraction and downstream real-time qPCR analyses.

Figure 2. Schematic depicting rat brain microdissection procedures. 2-mm (Sections 1 and 2) or 3-mm thick coronal brain sections (Section 3) were cut from either Sham-operated or SCI rats as indicated in (**A**, **C** and **E**) and tissue blocks containing the prefrontal cortex (**B**), the dorsal hippocampus, thalamus and amygdala (**D**), or the ventral hippocampus and PAG (**F**) were microdissected under a stereoscopic microscope (magnification 10×) using the Paxinos and Watson rat brain atlas as a reference (Paxinos and Watson, 2006).

Figure 3. Real-time qPCR data showing the differential mRNA expression of GFAP in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus in Sham and spinal cord injured rats (SCI).

Real-time qPCRs were carried out using selected primer pairs that were designed and optimised to amplify small fragments (≤150 bp length) within the coding sequence of the gene of interest (please see **Table 1**). Results show mean fold changes ± SEM obtained

from two independent experiments run in duplicate. Fold changes for the genes of interest were were calculated using the comparative ΔΔCt method after normalization to the reference gene 18S. Baseline gene expression of the Sham groups was set to 1.

*p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Figure 4. Western blots analyses of GFAP protein expression in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured (Sham) and spinal cord injury rats (SCI). (**A-G**) Representative GFAP immunoblots and semi-quantitative densitometric analyses are shown for the (**A**) amygdala, (**B**) periaqueductal grey, (**C**) prefrontal cortex, (**D**) hypothalamus, (**E**) thalamus, dorsal and ventral hippocampus (**F** & **G**). Data are the mean ± SEM of two separate experiments.

*p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Figure 5. Real-time qPCR data showing the differential mRNA expression of Iba1 in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured (Sham) and spinal cord injury rats (SCI). Target-specific amplificons were obtained using custom-designed primer pairs optimised for qPCR analyses (≤150 bp length). Sequences are shown in **Table 1**. Results shown are the mean fold changes ± SEM obtained from two independent experiments, which were each run in duplicate. Fold changes for the genes of interest were were calculated using the comparative ΔΔCt method after normalization to the reference gene 18S. Baseline gene expression of the Sham groups was set to 1.

*p≤.05 *Vs* SHAM, using Student's *t*-test. Ns = not significant.

Figure 6. Western blots analyses of Iba1 protein expression in the amygdala, PAG, PFC, hypothalamus, thalamus, ventral & dorsal hippocampus of sham-injured

(Sham) and spinal cord injury rats (SCI). (**A-G**) Representative Iba1 immunoblots and semi-quantitative densitometric analyses are shown for the (**A**) amygdala, (**B**) periaqueductal grey, (**C**) prefrontal cortex, (**D**) hypothalamus, (**E**) thalamus, dorsal and ventral hippocampus (**F** & **G**). Data are the mean ± SEM of two separate experiments. *p≤.05 or **p≤.01 *Vs* Sham, using Student's *t*-test. Ns = not significant.

Figure 1

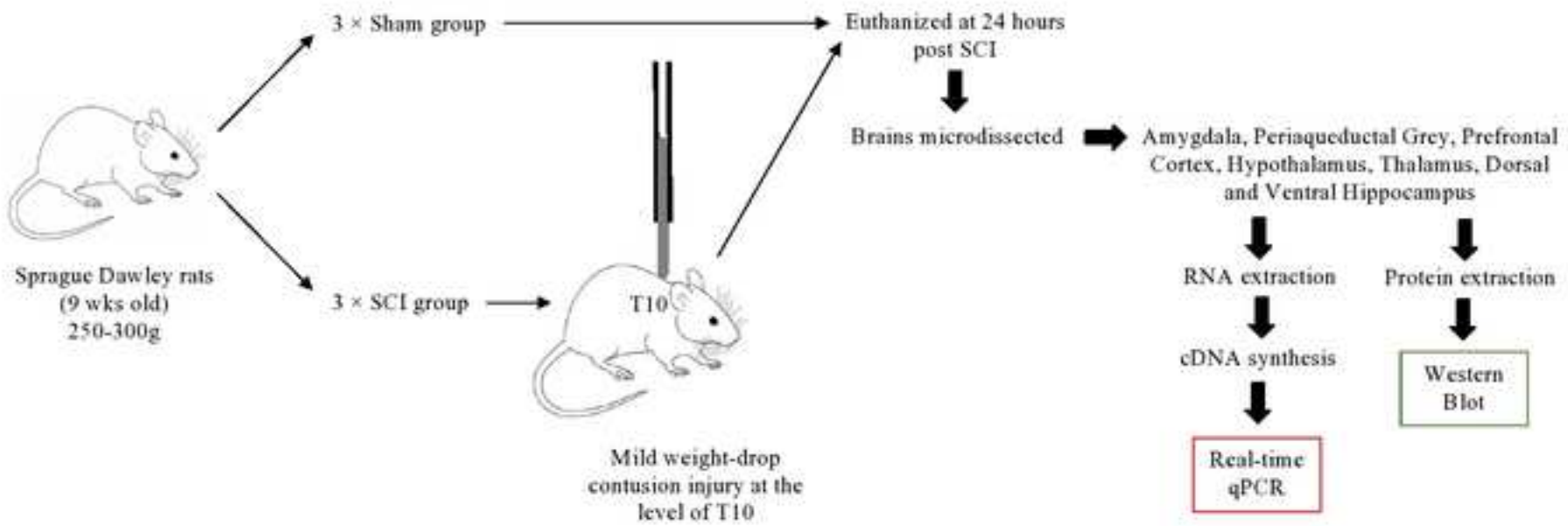

Figure 2A-F (new Figure)

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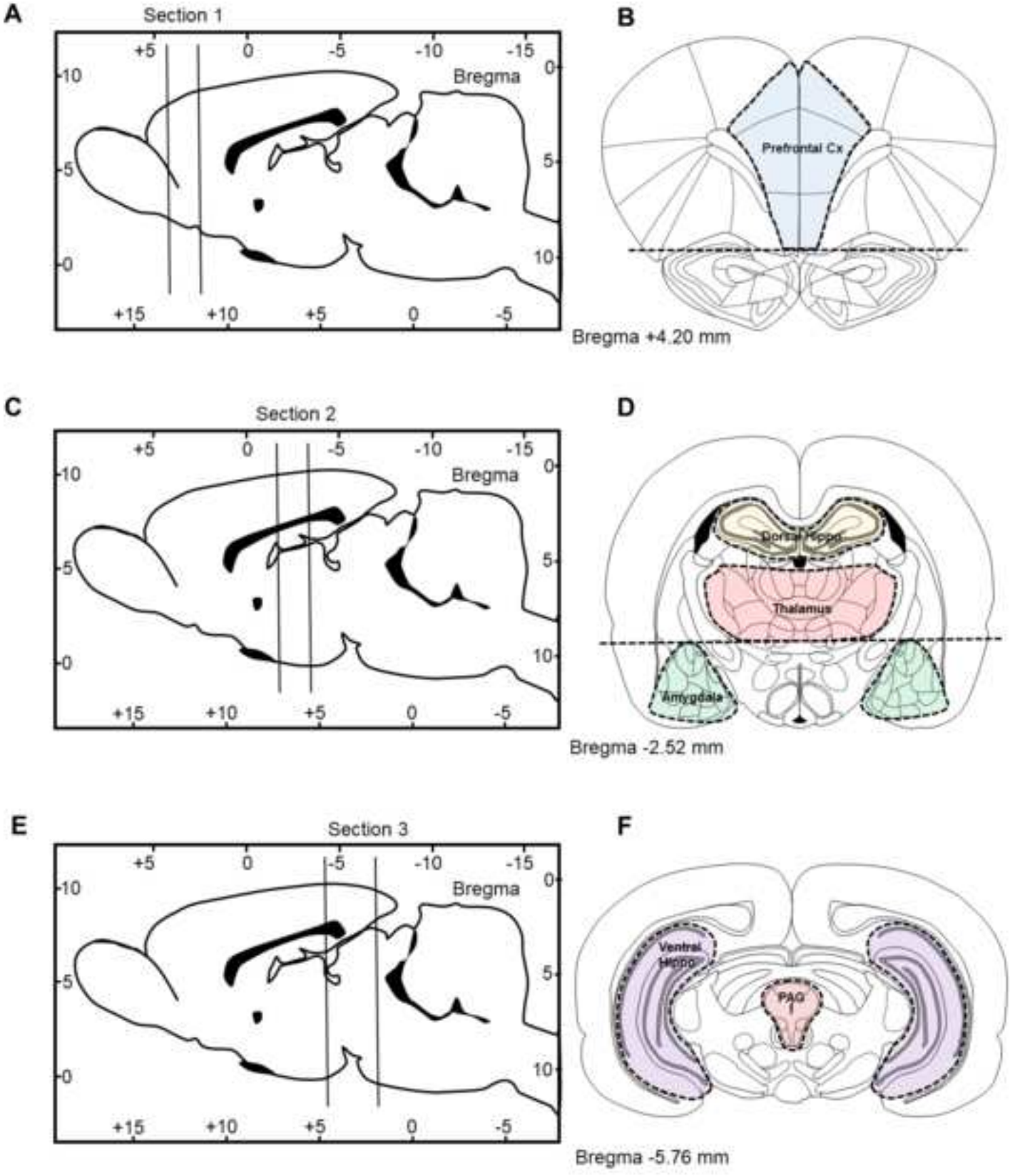

Figure 3A-G

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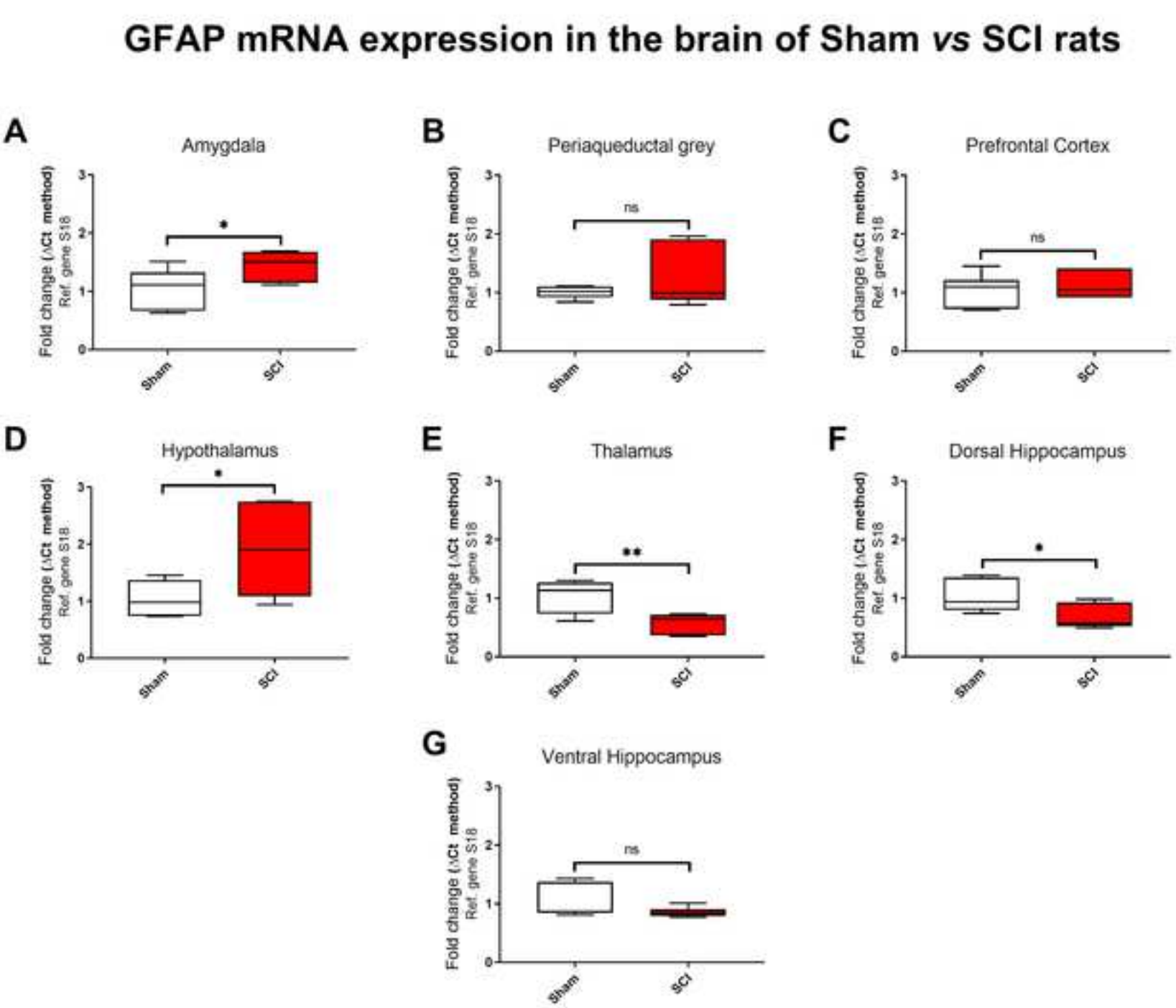

Figure 4A-G

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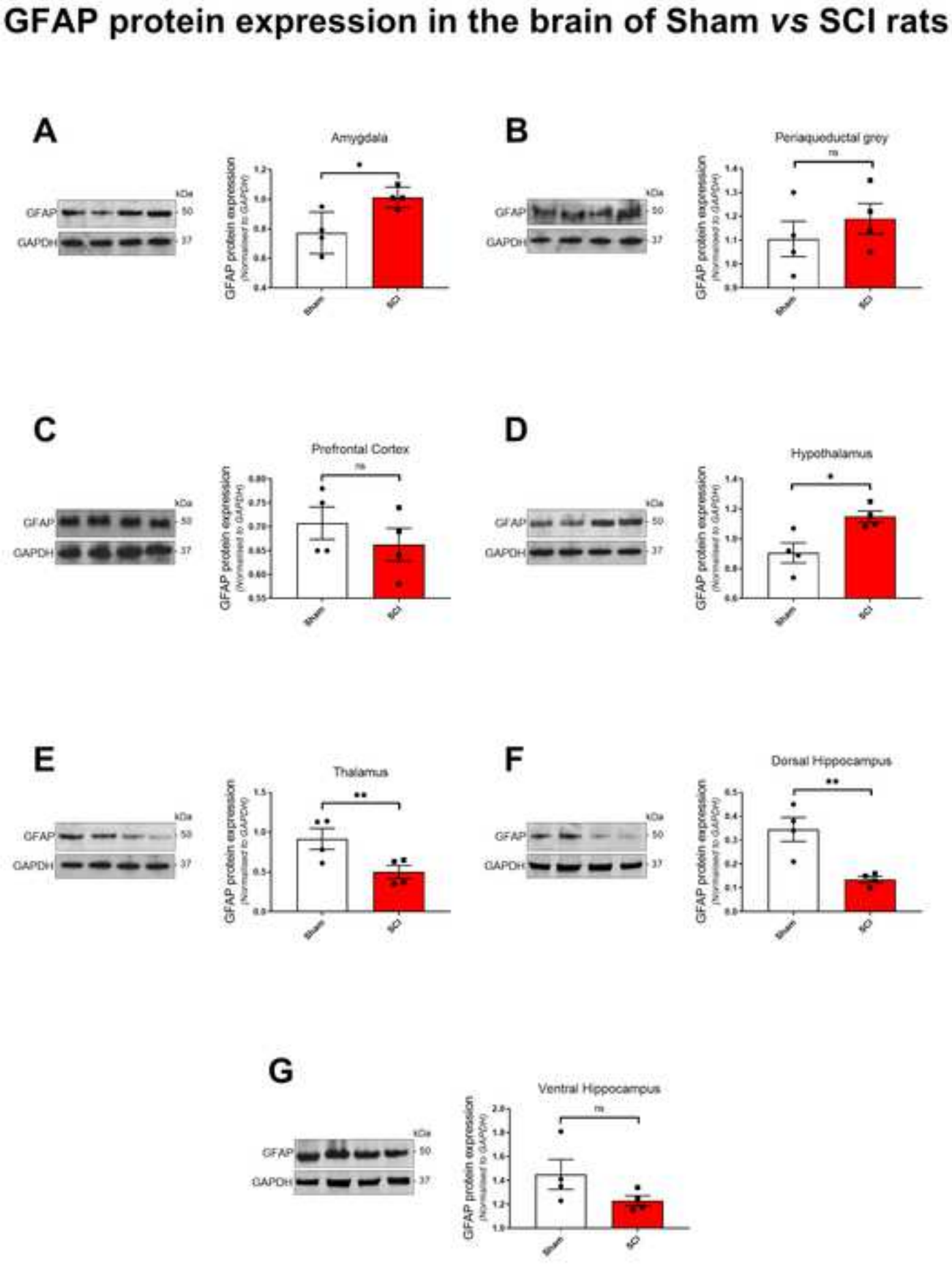

Figure 5A-G

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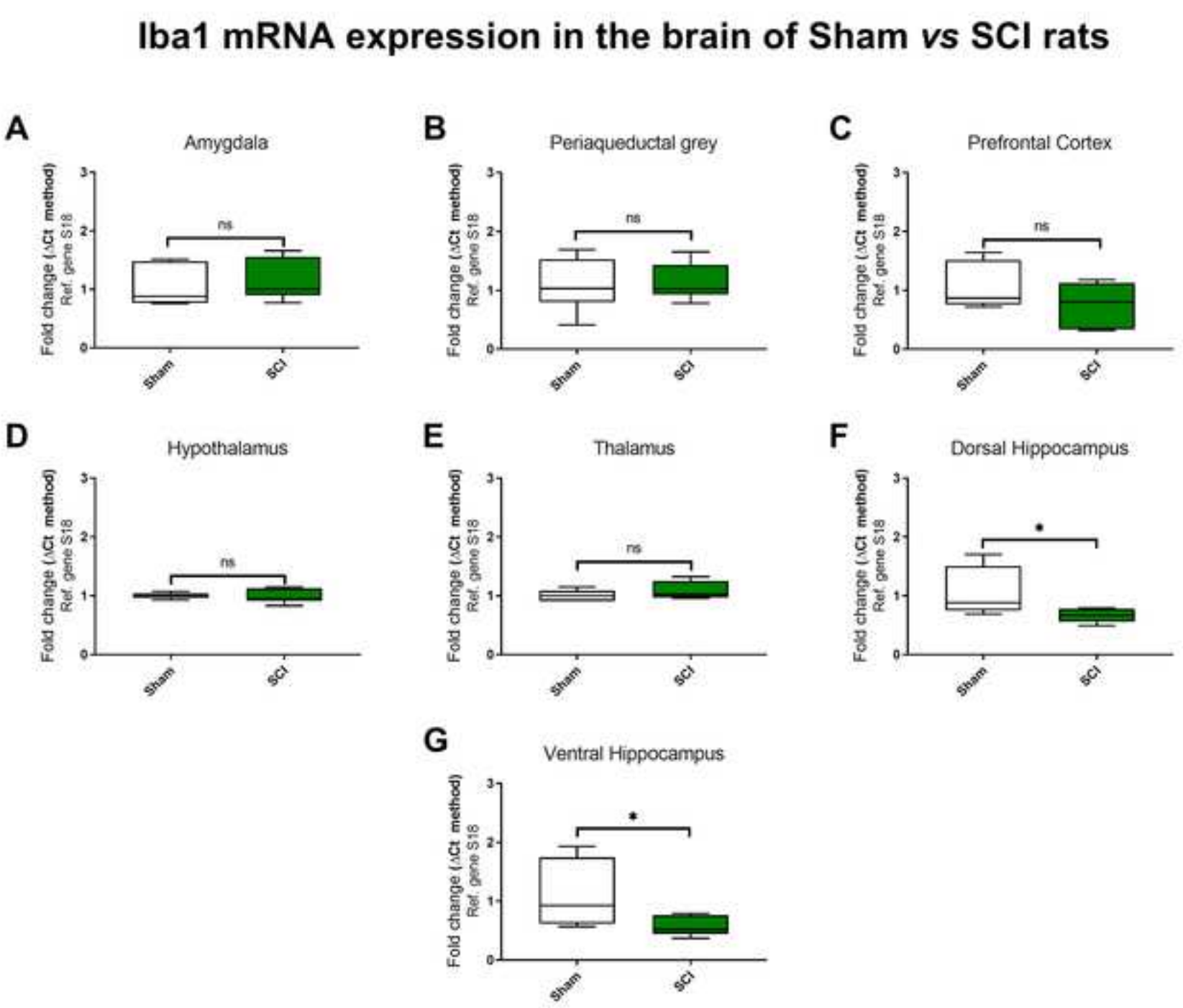

Figure 6A-G

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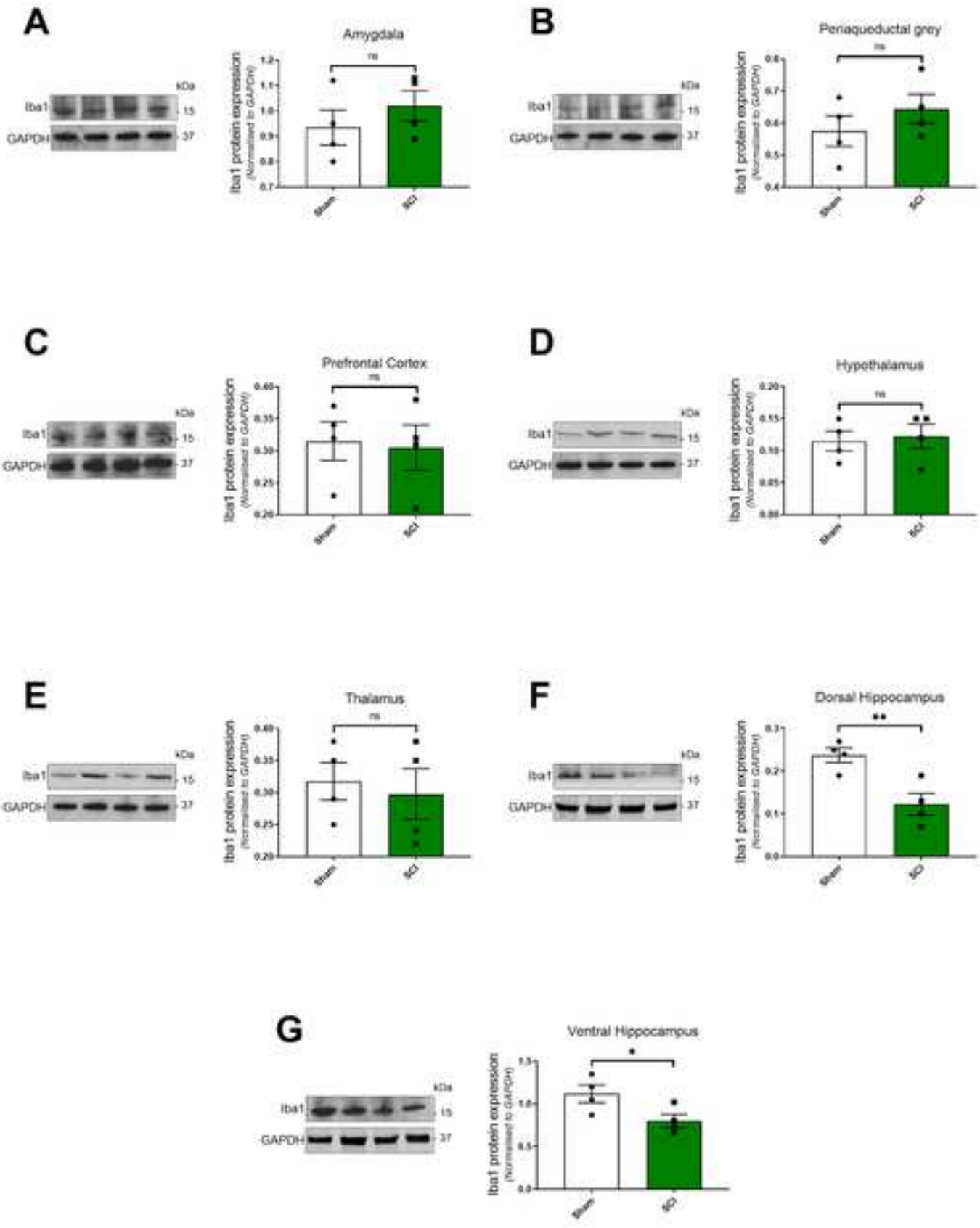

Table 1. Primer sequences targeting the rattus norvegicus GFAP and Iba1 genes, optimised for real-time PCR.

Gene (Ref. Seq.)	Primers	Location of primers	Tm (°C)	Length (bp)
GFAP (NM_017009.2)	5'-GCGAAGAAAACCGCATCACC-3’	1189	60.01	150
	3’-TCTGGTGAGCCTGTATTGGGA-5’	1338	61.12	
Aif1 (NM_017196.3)	5’- AGCAAGGATTTGCAGGGAGG-3’	108	60.32	143
	3’- TTGAAGGCCTCCAGTTTGGAC-5’	250	60.48	
18S Ribosomal protein subunit (NM_213557.1)	5’-GGCGGAAAATAGCCTTCGCT-3’	113	61.1	101
	3’-AGCCCTCTTGGTGAGGTCAA-5’	213	60.77	

Table 2. Table summarizing the topographical disruptions of GFAP and Iba1 mRNA and protein expression levels seen in response to SCI after 24hrs. Arrows indicate the direction (upregulation or downregulation) and statistical significance (one arrow indicates *p≤.05, two arrows if *p≤.01 *Vs* sham) of the observed changes.

Brain Region	GFAP mRNA	GFAP protein	Iba1 mRNA	Iba1 protein
Amygdala	↑	↑	No change	No change
Periaqueductal Gray	No change	No change	No change	No change
Prefrontal Cortex	No change	No change	No change	No change
Hypothalamus	↑	↑	No change	No change
Thalamus	↓↓	↓↓	No change	No change
Dorsal Hippocampus	↓	↓↓	↓	↓↓
Ventral Hippocampus	No change	No change	↓	↓