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STRESS-ALTERED SYNAPTIC PLASTICITY AND DAMP SIGNALING IN THE HIPPOCAMPUS-PFC AXIS; ELUCIDATING THE SIGNIFICANCE OF IGF-1/IGF-1R/CAMKIIα EXPRESSION IN NEURAL CHANGES ASSOCIATED WITH A PROLONGED EXPOSURE THERAPY

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Abstract—Traumatic stress patients showed significant improvement in behavior after a prolonged exposure to an unrelated stimulus. This treatment method attempts to promote extinction of the fear memory associated with the initial traumatic experience. However, the subsequent prolonged exposure to such stimulus creates an additional layer of neural stress. Although the mechanism remains unclear, prolonged exposure therapy (PET) likely involves changes in synaptic plasticity, neurotransmitter function and inflammation; especially in parts of the brain concerned with the formation and retrieval of fear memory (Hippocampus and Prefrontal Cortex: *PFC*). Since certain synaptic proteins are also involved in danger-associated molecular pattern signaling (DAMP), we identified the significance of IGF-1/IGF-1R/CaMKIIα expression as a potential link between the concurrent progression of synaptic and inflammatory changes in stress. Thus, a comparison between IGF-1/IGF-1R/CaMKIIα, synaptic and DAMP proteins in stress and PET may highlight the significance of PET on synaptic morphology and neuronal inflammatory response. In behaviorally characterized Sprague–Dawley rats, there was a significant decline in neural IGF-1 ($p < 0.001$), hippocampal ($p < 0.001$) and cortical ($p < 0.05$) IGF-1R expression. These animals showed a significant loss of presynaptic markers (synaptophysin; $p < 0.001$), and changes in neurotransmitters (VGLUT2, Tyrosine hydroxylase, GABA, ChAT). Furthermore, naïve stressed rats recorded a significant decrease in post-synaptic marker (PSD-95; $p < 0.01$) and synaptic regulator (CaMKIIα; $p < 0.001$). As part of the synaptic response to a decrease in brain CaMKIIα, small ion conductance channel (KCa2.2) was upregulated in the brain of naïve stressed rats ($p < 0.01$). After a PET, an increase in IGF-1 ($p < 0.05$) and IGF-1R was recorded in the Stress-PET group ($p < 0.001$). As such, hippocampal ($p < 0.001$), but not cortical (ns) synaptophysin expression increased in Stress-PET. Although PSD-95 was relatively unchanged in the hippocampus and PFC, CaMKIIα ($p < 0.001$) and KCa2.2 ($p < 0.01$) were upregulated in Stress-PET, and may be involved in extinction of fear memory-related synaptic potentials. These changes were also associated with a normalized neurotransmitter function, and a significant reduction in open space avoidance; when the animals were assessed in elevated plus maze (EPM). In addition to a decrease in IGF-1/IGF-1R, an increase in activated hippocampal and cortical microglia was seen in stress ($p < 0.05$) and after a PET (Stress-PET; $p < 0.001$). Furthermore, this was linked with a significant increase in HMGB1 (Hippocampus: $p < 0.001$, PFC: $p < 0.05$) and TLR4 expression (Hippocampus: $p < 0.01$; PFC: ns) in the neurons. Taken together, this study showed that traumatic stress and subsequent PET involves an event-dependent alteration of IGF1/IGF-1R/CaMKIIα. Firstly, we showed a direct relationship between IGF-1/IGF-1R expression, presynaptic function (synaptophysin) and neurotransmitter activity in stress and PET. Secondly, we identified the possible role of CaMKIIα in post-synaptic function and regulation of small ion conductance channels. Lastly, we highlighted some of the possible links between IGF1/IGF-1R/CaMKIIα, the expression of DAMP proteins, Microglia activation, and its implication on synaptic plasticity during stress and PET. **© 2017 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: IGF-1R, CaMKIIα, IGF-1, synaptic morphology, DAMP, traumatic stress.

INTRODUCTION

Prolonged exposure therapy (PET) has been described as an effective method in the management of traumatic stress symptoms, which has led to enhanced quality of life in some patients (Yehuda et al., 2014; Castillo et al., 2016). PET and other traumatic stress interventions, are aimed at improving emotional, social and stress-related behaviors through fear-extinction learning (Fiorenza et al., 2012; Furini et al., 2014; Jerud et al., 2016; Larsen et al., 2016). Traumatic stress is associated with altered structural and functional connectivity in parts of the brain involved in fear memory processing, notably, the hippocampus, prefrontal cortex (PFC) and amygdala. The brains of traumatic stress patients are often characterized by abnormal white matter structure, reduced brain volume and hippocampal activity (Pang, 2015; Hayes et al., 2016). In this respect, the goal of fear extinction learning is to retrain the brain to prevent the retrieval of

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Abbreviations: CaMKIIα, calcium calmodulin-dependent kinase 2 alpha; HMGB1, high mobility group box protein 1; IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor type 1; KCa2.2, calcium-dependent potassium channel (SK2 or KCNN2 family); TLR4, toll-like receptor 4.

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aversive memories associated with the post-traumatic stress condition. However, the effectiveness of PET depends on multiple cellular mechanisms such as inflammation, neurotransmitter systems, and synaptic modifications in neural circuits associated with fear memory retrieval (Abraham et al., 2016; Novick et al., 2016).

Interestingly, in traumatic stress, synaptic IGF-1R signaling has been identified as a major player in the regulation of neurotransmitter activity and prevention of inflammation (Rubovitch et al., 2010; Zhao et al., 2012). Neurotrophic factors, such as insulin-like growth factor 1 and its type 1 receptor (IGF-1R), are known to modulate the synaptic activity of dopamine and glutamate in the developing and adult nervous systems (Mattson, 1990, 2008; Guevara-Aguirre, 1996; Pehar et al., 2010). Moreover, alterations in neural IGF-1 have been described in the cause and progression of several neuropsychiatric disorders associated with development, aging, degeneration and malformation of cytoskeletal proteins in neurons (Guevara-Aguirre, 1996; Pehar et al., 2010; Hwa et al., 2013). Additionally, other reports have shown that loss of neurotrophins, and associated receptors are pivotal to a compromised synaptic integrity, abnormal neurotransmitter signaling events and activation of inflammatory pathways in traumatic stress (Su et al., 2015; Finsterwald et al., 2015).

Generally, IGF-1R activation involves multiple signaling pathways. Notably, Akt/mTOR signaling facilitates an increase in the nuclear transcription of NF-κB. In a separate – but related – mechanism, *Ras* signaling promotes the activation of MAPK/ErK (Chetty et al., 2006; Rubovitch et al., 2010). In support of this proposition, a decrease in IGF-1R signaling has been shown to attenuates danger-associated molecular pattern (DAMP) signaling by blocking the activation of pro-inflammatory molecules such as MAPK/ErK, Akt/mTOR, NF-κB (Zhao et al., 2012; Yu et al., 2012). Additionally, IGF-1/IGF-1R signaling is known to regulate the nuclear translocation of HMGB1, while attenuating a significant part of DAMP signaling in the HMGB1-TLR4 pathway (Gontier et al., 2015). Several other divergent pathways, such as Wnt/β-Catenin signaling, involves IGF-1R and CaMKIIα alterations in synaptogenesis. Aside from its role in the regulation of long-term potentiation (LTP), CaMKIIα holds the ability to block some of the inflammatory and synaptic activities of MAPK/ErK (Bouallegue et al., 2009; Rosso and Inestrosa, 2013). Although previous studies have shown that IGF-1R signaling is involved in the regulation of inflammation and synaptic function, the significance of an event-dependent change in IGF-1R expression – as a response mechanism – in traumatic stress and PET remains unclear.

During stress events – such as predator exposure – a commensurate neural and psychological stress is induced. Since exposure therapy involves recreating the traumatic experience, an additional layer of neural stress is induced in the hippocampus-PFC axis during the retrieval of the associative memory. Thus, since IGF-1/IGF-1R directly modulate the activity of CaMKIIα in inflammation and synaptic function, this study sought to determine whether a change in IGF-1/IGF-1R/CaMKIIα expression may represent specific changes in neural morphology and DAMP signaling in stress, and modifications that may occur in PET.

EXPERIMENTAL PROCEDURES

Animal strain

Adult male Sprague–Dawley rats (Charles River Lab, Wilmington, MA) weighing between 250–300 gm was used for this study. The animals were kept under standard laboratory conditions of 12 h alternating dark and light cycle, and fed ad libitum. All animals handling procedures were in accordance with approved protocols by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University School of Veterinary Medicine.

Experimental model of traumatic stress

To induce traumatic stress, we adopted the model previously described by Zoladz et al. (2008). This method involves a combination of *acute* predator exposure events, with *chronic* psychosocial stress events (Fig. 1A). Rats *(n = 30)* were randomly assigned to traumatic stress *(n = 20)* or control *(n = 10)* groups. All animals were maintained in the animal holding facility for the duration of the experiment. Rats were kept in cylindrical holdings (Plexiglas containers) covered with cat chow and were placed in a separate metal cage (76 cm × 76 cm × 60 cm) with an adult cat (7 years old). This allowed for the free movement of the cat around the cylinder (food) but prevented the cat from touching the rat. Predator exposure duration of 1 h was adopted for each exposure event as previously described (Wilson et al., 2014). The first exposure was done on (Day 1) during the daylight cycle (07:00–19:00). After 10 days, a second exposure was done during the dark cycle (Day 11; 19:00–07:00). Between Day 1 to Day 31, rats were subjected to a random daily cohort cage rotation to eliminate any form of social support, and induce chronic psychosocial stress during the period of the experiment. It is important to note that no Cat or Cat material was allowed near the cage rotation cohort. Additionally, the last cage for a rotation was also the first cage, and represents the actual group of the rat (home cage). The control rats *(n = 10)* were kept in the same cages from Day 1-Day 31 and were not subjected to cage rotation or predator exposure events (Fig. 1A).

Prolonged exposure therapy (unrelated stimulus)

The underlying principle employs the re-exposure of naïve traumatic stress rats to an unrelated causative stimulus (Cat meow tone). The tone was played from a pre-recorded audio file through speakers positioned in the conditioning chamber. This is aimed at training the animal to facilitate the extinction of associative fear memory. While traumatic stress was induced through predator exposure and psychosocial stress, for the PET, we used a fear conditioning chamber, and pulses of cat meow tone in the dark for 5 min. As from Day 40, a group of *n = 10* stressed rats were subjected to the

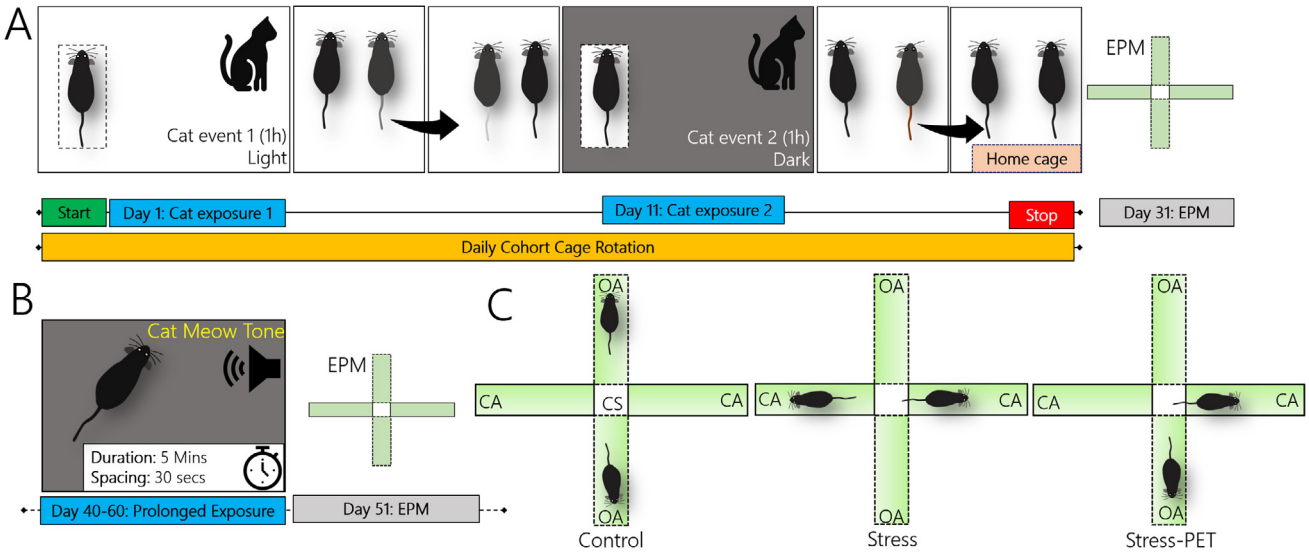


Fig. 1. (A) Schematic illustration of a combined acute predator (cat) exposure and chronic psychosocial stress (random cohort cage rotation) in a rat model of traumatic stress. (B) Schematic illustration of prolonged exposure therapy (PET). This involved the use of an unfamiliar stress stimulus (audio cat meow tone) spaced at 50 s for a maximum duration of 5 min. This was done for a total of 20 days after the final predator exposure (traumatic stress) event. (C) Graphic illustration of the typical outcome of an elevated plus maze (EPM) behavioral test for the Control, Stress and Stress-PET groups. Anxiety-linked behavioral changes in the stress rat involved an increase in avoidance of the open arm (OA), while spending more time in the closed arm (CA). After PET, the Stress-PET animals showed an improvement by exploring the OA to a significant level over the CA of the maze. *CS* depicts the center square and the starting point for each test event.

therapy (daily) for a total duration of 20 days. On Day 51, the animals were re-examined for anxiety-linked behavior in EPM (Fig. 1B).

Elevated plus maze (EPM; behavioral test for anxiety)

This was used to assess anxiety-linked behavioral change in naïve stressed rats, and stressed rats treated with PET. As such, we evaluated open space avoidance behavior (anxiety) as a function of percentage index time in the arm of the maze. The setup is composed of raised platform (55 cm high) with a cross-shaped maze; with each arm of the maze measuring *10 × 50 × 30 cm (WxLxH)*. Two opposite arms of the maze are enclosed while the other two are open. To determine anxiety-linked behavior in stressed rats, we measured the threshold at which a rat avoided the open arms of the maze. The threshold was derived as an estimate of the duration spent in the open and closed arms, and the frequency of entry into both arms. A score of entry or time spent was allotted only when the four paws of the animal was present in an arm. The EPM was set up in a soundproof room, equipped with adequate lighting and digital high-definition Camera to avoid sound distractions during the test. Twenty-four hours before the test, the animals were gently moved into the testing room for acclimatization to the testing area. At the commencement of the trial (Day 1), rats were placed in the center square of the EPM in the testing room. The movement of the animal within the maze was recorded on a computer outside the testing area (Fig. 1C). At the end of the test, we used Any Maze 5.1 (Wood Dale, IL, USA) to determine the score of arm entries, and arm duration for a 5-min test. Percentage time in the Open arm was calculated as:

$$\%\text{Time in Open Arm} = \frac{\text{Time in Open Arm}}{\text{Time in OA} + \text{Time in CA}} \times 100 \quad (1)$$

Likewise, percentage of Open Arm entries was determined as:

$$\%\text{Open Arm Entries} = \frac{\text{Score of OA Entries}}{\text{Score of OA Entires} + \text{Score of OA Entires}} \times 100 \quad (2)$$

Western blotting and protein quantification

For western blot analyses, 10-μl brain tissue lysate containing 10 μg of protein was processed for SDS–PAGE electrophoresis. After subsequent western blotting (wet transfer), Polyvinylidene fluoride membrane (PVDF) was incubated in Tris-buffered saline (with 0.01% Tween 20) for 15 min (TBST) at room temperature. Subsequently, the membrane was blocked in 3% Bovine serum albumin (prepared in TBST) for 50 min at room temperature. The protein of interest, and control (GAPDH) were detected using the following primary antibodies; mouse anti-CaMKIIα (Cell Signaling-#50049), mouse anti IGF-1 (abcam-#ab176523) and rabbit anti GAPDH (abcam-#ab37168). All primary antibodies were diluted in the blocking solution at *1:1,000*. Subsequently, the primary antibodies were detected using HRP-conjugated secondary antibodies (goat anti-rabbit-#65-6120 and goat anti-mouse-#65-6520; Invitrogen; dilution of *1:10,000* or *1:20,000*) following which the reaction was developed using a chemiluminescence substrate (Thermo Fisher-#34579).

Immunofluorescence

Animals were deeply anesthetized via inhalation of isoflurane in an enclosed chamber, then perfused transcardially through the left ventricle using 10 mM phosphate-buffered saline. Subsequently, 4% phosphate-buffered paraformaldehyde (PB-PFA) was introduced for perfusion fixation. The whole brain was harvested through cranial dissection and fixed in 4% PB-PFA overnight following which it was transferred to 4% PB-PFA containing 30% sucrose for cryopreservation. Free floating cryostat sections (40 μm) were obtained using a Leica Cryostat and stored in 10 mM PBS at 4 °C. The sections were washed three times (5 min each) in 10 mM PBS (pH 7.4) on a tissue rocker. Subsequently, blocking was performed in normal goat serum (Vector Labs), prepared in 10mMPBS + 0.03% Triton-X 100, for 2 h at room temperature. The sections were incubated in primary antibody solution overnight at 4 °C [Rabbit anti-IGF-1R (1:100; ThermoScientific-MA5-15148), Rabbit anti-HMGB1 (1:300; abcam-#ab79823), Rabbit anti-Tyrosine Hydroxylase (1:500; abcam-#ab6211), Rabbit anti-GABA (1:200; abcam-#ab8891), Rabbit anti-Synaptophysin (1:100; Cell Signaling-#5461), Rabbit anti-PSD-95 (1:100; Cell Signaling-#2507), Goat anti ChAT (EMD Millipore-#AB144P), Mouse monoclonal antibody to Rat CD11b/c (abcam-#1211), Mouse anti-CaMKIIα (Cell Signaling-#50049), Guinea pig anti-VGLUT2 (1:250; EMD Millipore-#AB2251), Mouse anti-SK2 (1:250; EMD Millipore-MABN1832), Mouse anti-TLR4 (1:250; abcam-#ab22048), Rabbit anti NeuN-Alexa 488 Conjugate (EMD Millipore-#MAB377X; 1:300)] diluted in 10 mM PBS, 0.03% Triton-X 100 and normal goat serum]. Subsequently, the sections were washed as previously described and incubated in secondary antibody solution [Goat anti Mouse 568, Goat anti Rabbit 594 and Goat anti Guinea Pig 568 (diluted at 1:1000) prepared in 10 mM PBS, 0.03% Triton X-100 and Normal Goat Serum] at room temperature (1 h). Immunolabeled sections (Table 1) were washed and mounted on gelatin-coated slides using a plain or DAPI containing anti-fade mounting medium (Vector Labs).

Confocal microscopy and cell counting

The distribution of immunolabeling in the hippocampus and PFC were imaged using an Olympus FluoView 10i confocal microscope (Olympus America, Center Valley, PA). Triple-labeling immunofluorescence was done in four steps. This involved acquisition of DAPI (405 nm), NeuN-Alexa 488 (488 nm), Alexa 568 or 594 (568 or 594 nm), followed by merging all the color channels. Laser intensity was normalized by using a preset configuration for all slides irrespective of the treatment. Protein and neurotransmitter expression was determined

Table 1. Supplemental table showing the combination of antibodies for immunofluorescence staining and confocal microscopy

Protein combination	Primary antibody	Secondary antibody
IGF-1R	Rabbit anti IGF-1R	Goat anti Rabbit Alexa 594
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
Synaptophysin	Rabbit anti Synaptophysin	Goat anti Rabbit Alexa 568
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
CaMKIIα	Mouse anti CaMKIIα	Goat anti Mouse Alexa 647
PSD-95	Rabbit anti PSD-95	Goat anti Rabbit 568
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
KCa2.2	Mouse anti KCa2.2	Goat anti Mouse 568
NeuN	Anti NeuN-Alexa 488	
VGLUT2	Guinea pig anti VGLUT2	Goat anti Guinea pig 568
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
Tyrosine	Rabbit anti Tyrosine hydroxylase	Goat anti Rabbit Alexa 594
hydroxylase	Anti NeuN-Alexa 488	
NeuN	DAPI	
DAPI		
GABA	Rabbit anti GABA	Goat anti Rabbit 594
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
ChAT	Goat anti ChAT	Rabbit anti Goat 568
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
CD11b/c	Mouse anti Cd11b/c	Goat anti Mouse 647
HMGB1	Rabbit anti HMGB1	Goat anti Rabbit 594
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	
TLR4	Mouse anti TLR4	Goat anti Mouse 568
NeuN	Anti NeuN-Alexa 488	
DAPI	DAPI	

from the confocal PFC and hippocampal images ($n = 7$ per group per protein) using Image J (NIH, USA). For each section, the count was determined per μm^2 for $n = 12$ different field of views. This was done for $n = 5$ successive sections taken at 50 μm apart – 2.2 mm behind the bregma (Franklin and Paxinos, 1997). We determined the borders of each region using cellular morphology or layer-dependent cell densities.

Statistics (immunoblotting and confocal cell count)

Protein expression (western blotting) was quantified using Image Lab Version 5.0 (BioRad, USA). After estimating the intensity of an immunolabeled protein band, the relative density of the protein was determined through normalization with the corresponding control protein band intensity (GAPDH). The relative densities were analyzed in *one-way* ANOVA (with *Tukey Post-hoc* test). The outcome was presented as bar chart with error bars representing the mean ± SEM. The average values for cell counting (confocal) and EPM parameters were compared for Control versus Stress, and Stress versus Stress-PET using One-Way ANOVA with Tukey *post hoc* test. All statistical comparisons were done in GraphPad Prism Version 5, and presented in bar graph showing the mean ± SEM.

RESULTS

Anxiety-linked response in traumatic stress and PET was linked with a depletion of IGF-1

Anxiety. Naïve stressed rats showed a significant increase in anxiety-linked behavior. This was seen as a decrease in OAD ($p < 0.001$), and an increase in CAD ($p < 0.001$) when compared with the control (open space avoidance). In a normalized index, the naïve stressed group recorded a significantly low percentage time spent in the open arm of the maze; when compared with the control ($p < 0.001$; Fig. 2A). Similarly, the naïve stressed rats recorded lower scores (percentage index) of entry into the open arm when compared with the control ($p < 0.01$). After PET, naïve stressed rats, generally, showed a reduction in open space avoidance (anxiety). This was characterized by an increase in percentage time in the open arm when compared with the naïve stress group ($p < 0.001$). In support of this outcome, the Stress-PET group also recorded an increase in percentage OA entries when compared with the naïve stressed rats (Fig. 2B; $p < 0.05$).

IGF-1: Protein quantification analysis showed that the expression of IGF-1 decreased significantly in the brain of behaviorally characterized traumatic stress rats (Fig. 2C, D) when the Stress group was compared with the control ($p < 0.001$). After a prolonged exposure therapy, the expression of IGF-1 increased ($p < 0.05$) versus the Stress group. However, neural IGF-1 remained relatively low when compared with the control value ($p < 0.001$). This suggests a significant loss of IGF-1 during the stress and the PET phase.

Stress signaling was associated with a depletion in brain IGF-1/IGF-1R, and synaptophysin

Similar to IGF-1, our results showed that IGF-1R expression was reduced significantly in the

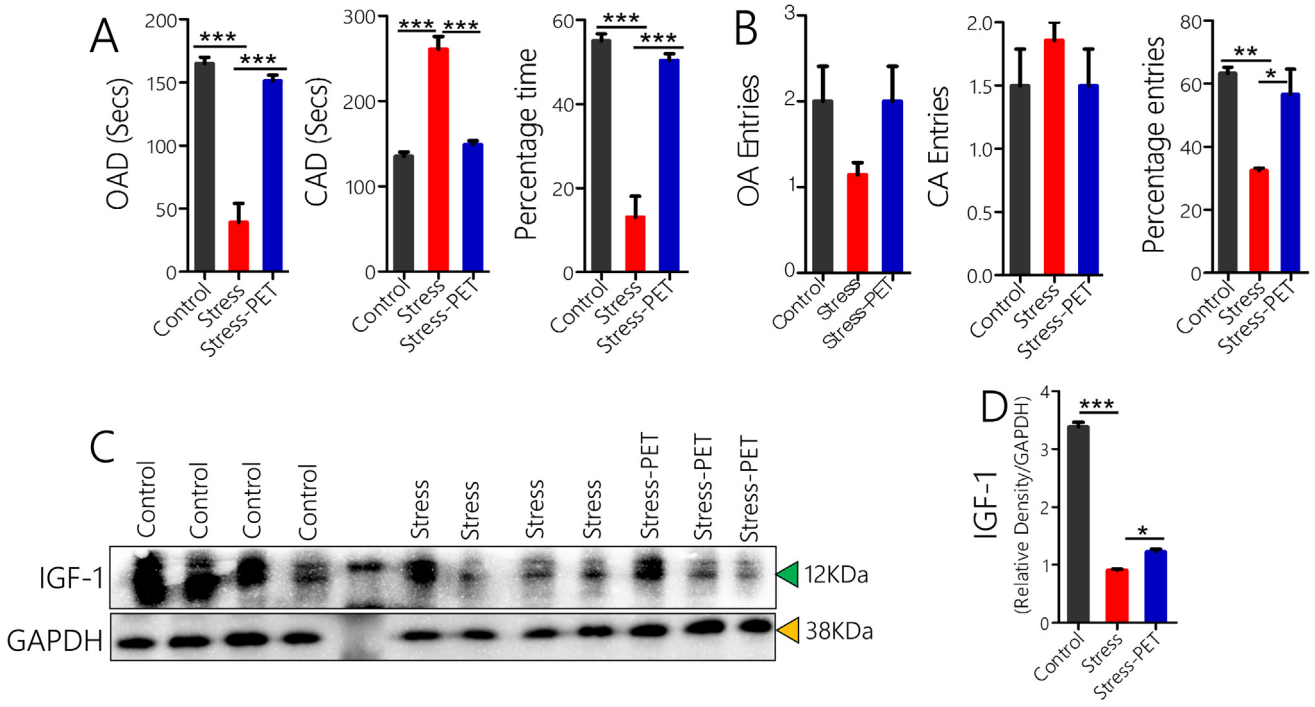
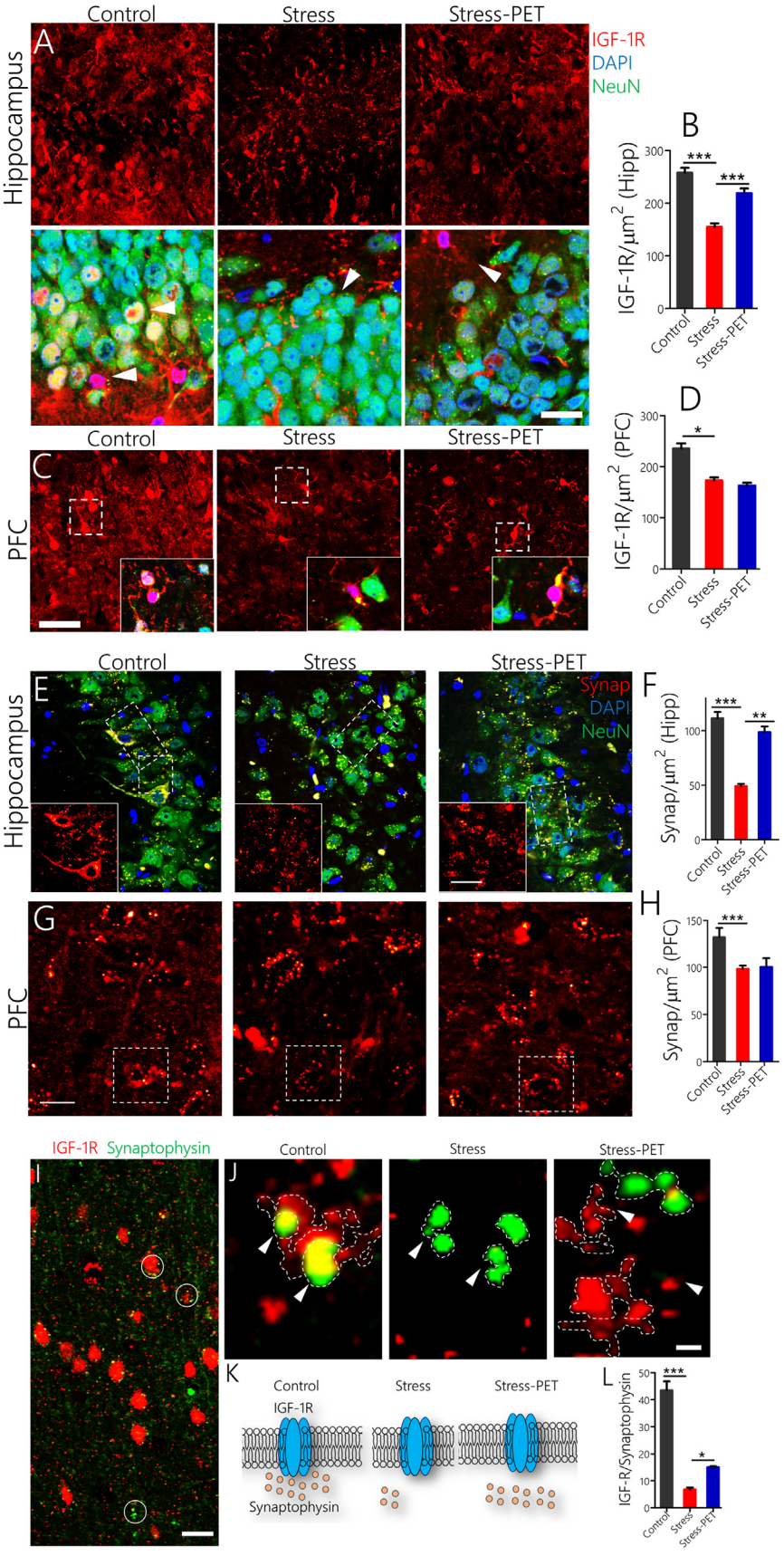


Fig. 2. (A) Percentage index for open arm duration (OAD) under each condition. (B) Percentage index for open arm entries (OAE). The Stress-PET group recorded an increase in OAD and OAE index when compared with the naïve stressed rats ($p < 0.001$ and $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hippocampus ($p < 0.001$; Fig. 3A, B) and PFC ($p < 0.05$; Fig. 3C, D) of behaviorally characterized naïve traumatic stress rats. Interestingly, in Stress-PET rats – with reduced anxiety-linked behavior – an increase in hippocampal ($p < 0.001$), but not cortical (ns) IGF-1R was observed. Since the role of IGF-1R signaling has been described extensively in the regulation of presynaptic calcium release, we examined the relationship between the change in IGF-1R, and synaptophysin in naïve stressed and Stress-PET rats. Down-regulation of IGF-1R in the hippocampus and PFC of naïve stressed rats was associated with a corresponding decrease in synaptophysin ($p < 0.001$ and $p < 0.001$; Fig. 3E–H). Interestingly, after a PET, the Stress-PET group showed an increase in hippocampal synaptophysin ($p < 0.01$; Fig. 3E, F). By contrast, like cortical IGF-1R, cortical synaptophysin did not change significantly in the Stress-PET group; when compared with the control (Fig. 3G, H; ns). In subsequent analysis, using high resolution confocal imaging, we co-localized IGF-1R and Synaptophysin in typical control neurons (Fig. 3I). Interestingly, in the Stress and Stress-PET groups, there was a displacement of the overlap when compared with the control. This ranged from an increase in the relative distance between IGF-1R and Synaptophysin, to a significant loss of both Synaptophysin and IGF-1R (Fig. 3J–L).

Post-synaptic changes were linked with a loss of CaMKIIα, and increased KCa2.2

Aside from its role in the modulation of presynaptic calcium release and synaptophysin, IGF-1R may affect synaptic function through its effect on CaMKIIα. Since alterations in IGF-1 and IGF-1R were associated with an increase in open space avoidance behavior, we probed further to determine the expression pattern of CaMKIIα, which is involved in the regulation of LTP, synaptic morphology and neurotransmitter function. In addition to a decrease in brain IGF-1(Fig. 2C, D), behaviorally characterized stressed rats recorded a significant reduction in hippocampal and cortical CaMKIIα when compared



with the control ($p < 0.001$, $p < 0.001$; Fig. 4A–G). This was also evident as a decrease in total brain CaMKIIα; as shown in immunoblotting (Fig. 4E–G; $p < 0.001$). Together, a decrease in IGF-1, IGF-1R and CaMKIIα expression was linked with a reduction in post-synaptic marker (PSD-95) in the hippocampus ($p < 0.01$; Fig. 5A, B) and PFC ($p < 0.01$; Fig. 5C, D) of behaviorally characterized stressed rats.

CaMKIIα is involved in the regulation of LTP and glutamatergic neurotransmission. As such, calcium movement during an LTP event activates calcium-dependent potassium channels; which are co-localized with CaMKIIα at post-synaptic densities. Since CaMKIIα, through calmodulin, regulates KCa2.2 activation, an overall decline in brain CaMKIIα may be associated with an increase in KCa2.2 activity; which is known to block LTP. In naïve stressed rats, we recorded a significant increase in hippocampal ($p < 0.01$) and cortical ($p < 0.01$) expression of KCa2.2; when compared with the control (Fig. 5E–H). After a prolonged exposure therapy, we observed an increase in hippocampal and cortical CaMKIIα ($p < 0.001$, $p < 0.001$; Fig. 4A-D). Also, there was a significant increase in brain CaMKIIα expression when the Stress-PET group was compared with the naïve stressed group ($p < 0.01$; Fig. 4E, F). Consequently, when assessed in EPM, the Stress-PET group showed a significant improvement in open space avoidance behavior (Fig. 1B, C, Fig. 2A, B). Although IGF-1, IGF-1R and CaMKIIα increased in Stress-PET, no significant change in cortical or hippocampal PSD-95 was seen in this group when compared with the Stress group (Fig. 5A–D). Despite an increase in neuronal KCa2.2 in naïve stressed brains, after a PET, KCa2.2 increased further in the hippocampus and PFC of the Stress-PET group ($p < 0.01$ and $p < 0.05$). This may represent a significant response mechanism involved in the inhibition of fear memory LTP (Fig. 1–2) by increasing intraneuronal K^+ (Fig. 4G).

Neurotransmitter synthesis and transport

As noted above, we found that changes in hippocampal and cortical IGF-1R expression was proportionate with alterations in synaptic morphology, which affected presynaptic vesicle release and post-synaptic morphology. In subsequent analysis, we further characterized the implication of the observed synaptic changes on neurotransmitter systems involved in hippocampal and cortical LTP formation, i.e. glutamate, tyrosine hydroxylase, GABA and acetylcholine.

VGLUT2. Since IGF-1R directly affects the presynaptic release of calcium, and vesicles (synaptophysin), we compared IGF-1R with the expression pattern of VGLUT2 in the hippocampus and PFC of naïve stressed rats, and stressed rats treated through a PET. Like the observations for synaptophysin (Fig. 3E, F), we found a significant decrease in hippocampal and cortical VGLUT2 expression when the naïve stressed group was compared with the control ($p < 0.001$ and $p < 0.001$; Fig. 6A–D). Interestingly, after a PET, the Stress-PET group showed normalized VGLUT2 in the hippocampus and PFC when compared with the naïve stressed group ($p < 0.001$ and $p < 0.001$).

Tyrosine hydroxylase. Like VGLUT2, the count of tyrosine hydroxylase (TH)-positive terminals was reduced significantly in the hippocampus of naïve stressed rats when compared with the control (Fig. 6E–H; $p < 0.001$). Subsequent analysis of the Stress-PET hippocampus showed a significant increase in TH-positive terminals when compared with the naïve stressed group ($p < 0.001$; Fig. 6E, F). By contrast, the distribution of TH terminals increased in the PFC of naïve stressed rats ($p < 0.001$; Fig. 6G-H) when compared with the control. In subsequent analysis of the Stress-PET group, we observed a significant reduction in cortical TH-positive terminals when compared with the naïve stressed group ($p < 0.001$; Fig. 6G, H).

GABA. Naïve stressed, and Stress-PET groups, showed a differential change in hippocampal and cortical GABA expression. The distribution of hippocampal GABA reduced significantly in naïve stressed rats when compared with the control ($p < 0.001$; Fig. 7A, B). However, in the PFC, the expression of GABA increased ($p < 0.05$; Fig. 7C, D); versus the control. After a PET, the Stress-PET group recorded a significant increase in hippocampal GABA ($p < 0.01$). Similarly, cortical GABA increased ($p < 0.01$; Fig. 7C, D); when compared with the Stress group.

ChAT. The expression of choline acetyl transferase (ChAT) decreased in the hippocampus of naïve stressed rats when compared with the control

Fig. 3. (A) IGF-1R expression decreased significantly in the hippocampus of naïve stressed animals when compared with the control ($p < 0.001$). After prolonged exposure therapy (PET), a significant increase in IGF-1R was observed when the Stress-PET group was compared with the Stress group ($p < 0.001$; scale bar = 20 μm). (B) Bar chart (mean ± SEM) representing the statistical changes observed in the DG. (C) Like the observations in the hippocampus, IGF-1R expression reduced significantly in the PFC of naïve stressed rats ($p < 0.05$) when compared with the control (scale bar = 20 μm; inset = 10 μm). However, no significant change was seen when the Stress group was compared with the Stress-PET. (D) Statistical analysis (*One-Way ANOVA*) depicting the change in IGF-1R expression in the PFC neurons. (E) Presynaptic vesicular trafficking protein (synaptophysin) was reduced in the hippocampus of naïve stressed rats; when compared with the control ($p < 0.001$). After a prolonged exposure therapy (Stress-PET), we observed an increase in synaptophysin expression; when compared with the Stress group ($p < 0.01$; scale bar = 20 μm; inset = 20 μm). (F) Bar chart depicting the change in synaptophysin for Stress and Stress-PET groups. (G) Like the observation in the hippocampus, synaptophysin decreased significantly in the PFC of traumatic stress rats when compared with the control ($p < 0.001$; scale bar = 10 μm). However, no significant change was recorded when the Stress-PET was compared with the Stress group. (H) Expression of synaptophysin in the PFC under each condition.

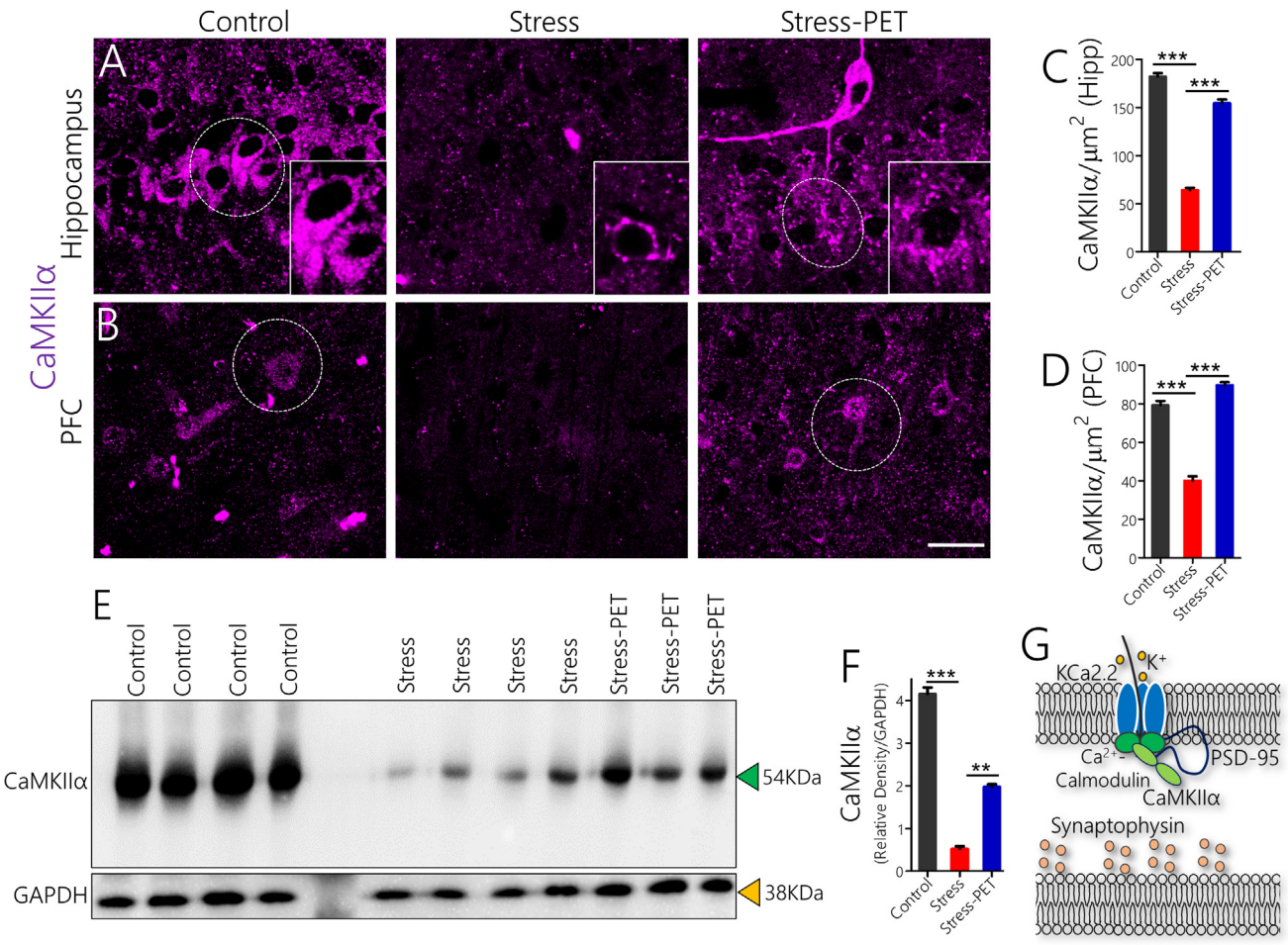


Fig. 4. (A) Representative confocal images showing the expression of hippocampal CaMKIIα (scale bar = 20 μm, inset = 10 μm). (B) Confocal images showing a decrease in the expression of CaMKIIα in the PFC of naïve stressed rats ($p < 0.001$). CaMKIIα expression was rescued after a prolonged exposure therapy ($p < 0.001$). (C) Hippocampal CaMKIIα levels during stress ($p < 0.001$) was rescued after a prolonged exposure therapy (Stress vs Stress-PET; $p < 0.001$). (D) Bar chart depicting a change in PFC CaMKIIα. (E) Protein immunoblotting and quantification of CaMKIIα in brain tissue lysate. (F) Bar chart depicting the significant reduction in CaMKIIα (relative density) in traumatic stress rats ($p < 0.001$ vs the control). Like the observation in confocal imaging, an increase was seen in the Stress-PET group ($p < 0.01$); after normalization with GAPDH. (G) Schematic summary of the interaction between CaMKIIα and IGF-1R at synaptic sites.

($p < 0.001$; Fig. 7E, F). Also, a significant reduction in cortical ChAT was seen in the Stress group ($p < 0.05$; Fig. 7G, H). After a PET, hippocampal ($p < 0.01$) and cortical ($p < 0.05$) ChAT expression increased when compared with the naïve stressed group.

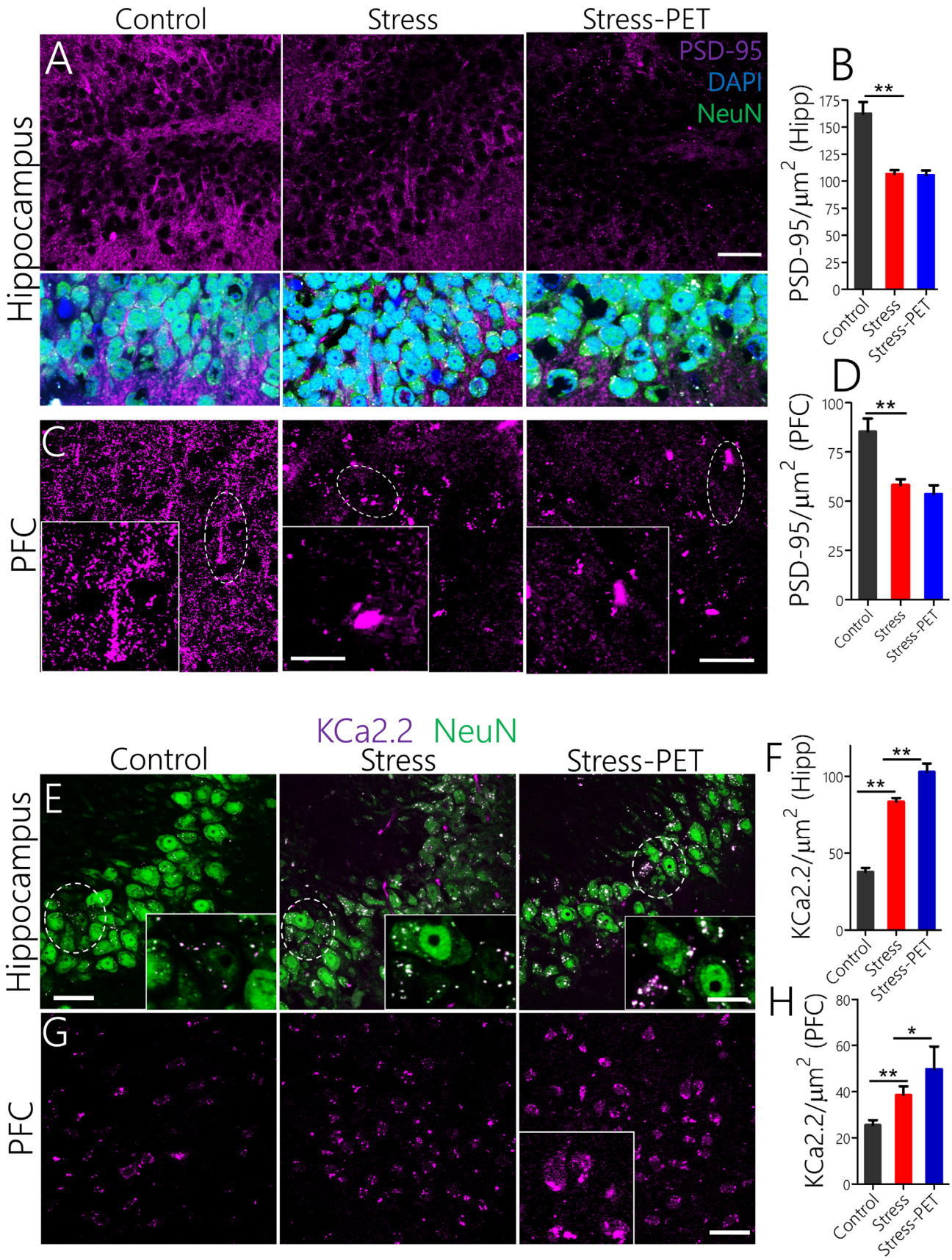
Phasic IGF-1/IGF-1R expression and a differential inflammatory response

A decline in brain IGF-1/IGF-1R expression was linked with an increase in hippocampal and cortical microglia activation (CD11b/c count) when comparing the Stress and Stress-PET groups with the control. Interestingly, while microglia activation increased after the stress period ($p < 0.05$), a further increase was seen after a PET ($p < 0.001$) for both hippocampus (Fig. 8A, B) and PFC (Fig. 8C, D)

In addition to a general increase in brain inflammation (microglia), the distribution of neuronal pro-inflammatory cytokines (HMGB1), and associated receptor (TLR4) – known to affect synaptic function and neurotransmitters – changed considerably in traumatic stress and after PET. As such, hippocampal HMGB1 expression increased considerably in naïve stressed rats when compared with the control ($p < 0.001$; Fig. 9A, B). By contrast, cortical neurons of the Stress group were characterized by a decrease in HMGB1 ($p < 0.05$) versus the control (Fig. 9C, D). After a PET, no significant change was seen in hippocampal HMGB1 (ns) when compared with the naïve stressed group. On the other hand, a significant increase in HMGB1 was recorded in the PFC of the Stress-PET group ($p < 0.05$); versus the Stress group. As part of the inflammatory response, naïve stressed rats also showed an increase in hippocampal neuronal TLR4 expression when compared with the control ($p < 0.01$; Fig. 9E, F). In subsequent analysis, we observed no significant change in cortical TLR4 expression (ns; Fig. 9G, H).

DISCUSSION

Taken together, the outcomes of this study showed that cortical and hippocampal IGF-1R expression were


Control
Stress
Stress-PET
A
PSD-95
DAPI
NeuN
Hippocampus
B
PSD-95/μm2 (Hipp)
**
175
150
125
100
75
50
25
0
Control
Stress
Stress-PET
C
PFC
D
PSD-95/μm2 (PFC)
**
100
75
50
25
0
Control
Stress
Stress-PET
KCa2.2 NeuN
Control
Stress
Stress-PET
E
Hippocampus
F
KCa2.2/μm2 (Hipp)
**
**
100
50
0
Control
Stress
Stress-PET
G
PFC
H
KCa2.2/μm2 (PFC)
**
*
80
60
40
20
0
Control
Stress
Stress-PET

altered in response to traumatic stress, and after a prolonged exposure therapy. This was also accompanied by varying synaptic and inflammatory changes in naïve stressed rats, and stressed rats treated with PET. IGF-1/IGF-1R signaling has been implicated in aging, neuroprotection and inflammation (Rubovitch et al., 2010; Dong and Zhang, 2015; Gontier et al., 2015). Additionally, CaMKIIα, in the IGF-1/IGF-1R signaling pathway, have been found to be involved in synaptic regulation and inflammation (Bouallegue et al., 2009; Rosso and Inestrosa, 2013). Thus, IGF-1R-mediated inflammation and synaptic response are not mutually exclusive. MAPK/ErK, which acts downstream of the IGF-1R-*Ras* pathway, is known to increase the release of vesicular glutamate (Sheng and Kim, 2002; Doyle et al., 2010a, 2010b), and phosphorylate CaMKIIα (Atkins et al., 1998; Soderling and Derkach, 2000). While the role of CaMKIIα has been extensively described in synaptic maturation, recent studies have highlighted its significance in long-term synaptic potentiation (LTP) during memory formation, and translocation of receptors at excitatory and inhibitory synaptic terminals (Barria et al., 1997; Atkins et al., 1998; Yamasaki et al., 2008). Additionally, CaMKIIα has been shown to attenuate the inflammatory signaling mechanism of Akt/TOR and MAPK/ErK (Bouallegue et al., 2009; Ma et al., 2012).

Similar to our results (Fig. 2C, D), previous studies have shown that a decrease in neural IGF-1 is associated with traumatic brain injury and stress. As such, post-stress administration of exogenous IGF-1 reduced the observed stress response (Kazanis et al., 2003). Although the mechanism of neural stress has been elaborated in aging and development, an important question yet to be addressed is the role of IGF-1/IGF-1R expression in the regulation of synaptic morphology, and the inflammatory response in traumatic stress, and a subsequent PET. Since there is a direct relationship between the depletion of IGF-1/IGF-1R, and the expression of CaMKIIα, we examined the significance of this change in synaptic function and neuronal inflammatory response after Stress and PET (Fig. 10).

Expression of IGF-1R proportionate with pre-synaptic function

The role of IGF-1R signaling has been described extensively in the control of synaptic activity in various brain areas. Thus, variations in IGF-1R may be attributable to synaptic modifications implicated in the pathophysiology of memory formation and retrieval in stress (Zhao et al., 1999; Freude et al., 2009). Additionally, IGF-1R governs neurotransmitter interactions involved in mood, emotion, addiction, reward, memory, and anxiety (Takeuchi et al., 2002; Pan et al., 2013; Zhang et al., 2014; Kleinridders et al., 2014; Gontier et al., 2015; Hu et al., 2016; Sun and Goodkin, 2016). Previous studies have shown that the activation IGF-1R promotes the presynaptic release of glutamate and the cellular synthesis of tyrosine hydroxylase; which favor dopaminergic neurotransmission (Dall'Igna et al., 2013; Duan et al., 2014). Furthermore, IGF-1R is known to directly regulate the activity of dopamine and glutamate at post-synaptic terminals by directly altering the activity of N-methyl-D-aspartate receptor (NMDAR R) and dopaminergic D2R (la Cour et al., 2011; Quesada et al., 2008; Kleinridders et al., 2014). Thus, some of the effect on IGF-1/IGF-1R on complex behaviors may be linked to its role in the combined regulation of dopaminergic and glutamatergic neurotransmission (Davis et al., 2000; Bouallegue et al., 2009; Gao et al., 2014; Dong and Zhang, 2015; Rusch et al., 2015; Hu et al., 2016).

Our results showed that a strong relationship exists between cortical and hippocampal IGF-1R expression, and the profile of presynaptic activity in traumatic stress, and after a PET. As such, a decrease in hippocampal and cortical synaptophysin in traumatic stress was associated with a decrease in IGF-1R expression. Furthermore, after a PET, an increase in IGF-1R was accompanied by a restoration of presynaptic (synaptophysin) function. Although our study addressed basic relationship between synaptophysin and IGF-1R expression (Fig. 3I–L), a recent study elaborately showed the role of hippocampal IGF-1R in real-time regulation of synaptic function and presynaptic vesicle release (Gazit et al., 2016). Their results demonstrate that pharmacological inhibition or a genetic knock down of IGF-1R reduced synaptophysin expression, and significantly reduced presynaptic activity. Furthermore, a blockade of IGF-1 reduced the activity of IGF-1R. This implied that a basal IGF-1R activity exist and is activated by intrinsic IGF-1 concentration during hippocampal synaptic function (Yamaguchi et al., 1990; Adams et al., 2000 as cited by Gazit et al., 2016). Since IGF-1R directly mediates the activity of synaptophysin, we investigated the significance of a change in IGF-1R on other presynaptic proteins involved in neurotransmitter synthesis and function. The outcome of this study showed that alterations in IGF-1R with response to stress, and subsequent PET created synaptic changes that were linked to modifica-

Fig. 5. (A) Significant reduction in postsynaptic densities (PSD-95) was observed in the hippocampus of Stress, and Stress-PET rats ($p < 0.01$ and $p < 0.01$; scale bar = 20 μm). (B) Bar chart depicting a statistical loss of PSD-95 in the hippocampus of naïve stressed rats, and after PET. (C) Similarly, the expression of PSD-95 was reduced in the prefrontal cortex of Stress and Stress-PET groups when compared with the control ($p < 0.01$; scale bar = 20 μm; inset = 5 μm). No significant difference was seen in PSD-95 expression in the Stress-PET group was compared with the Stress group. (D) Statistical analysis of change in PSD-95 distribution in the PFC. (E) Distribution of calcium-dependent potassium channels (KCa2.2) increased c in the hippocampus of naïve stressed rats when compared with the control ($p < 0.01$; scale bar = 20 μm, inset = 5 μm). Furthermore, a significant increase in KCa2.2 expression was recorded after PET was implemented (when the Stress-PET was compared with the Stress group) ($p < 0.01$). (F) Bar chart representing the statistical analysis for KCa2.2 re-distribution in the hippocampus of naïve stressed rats, and after PET (Stress-PET). (G) KCa2.2 expression increased in the PFC of stressed rats when compared with the control ($p < 0.01$). Like the observations in hippocampus, KCa2.2 expression increased when Stress-PET was compared with the Stress group ($p < 0.05$; scale bar = 20 μm). (H) Bar chart showing the differential expression of KCa2.2 in the PFC.

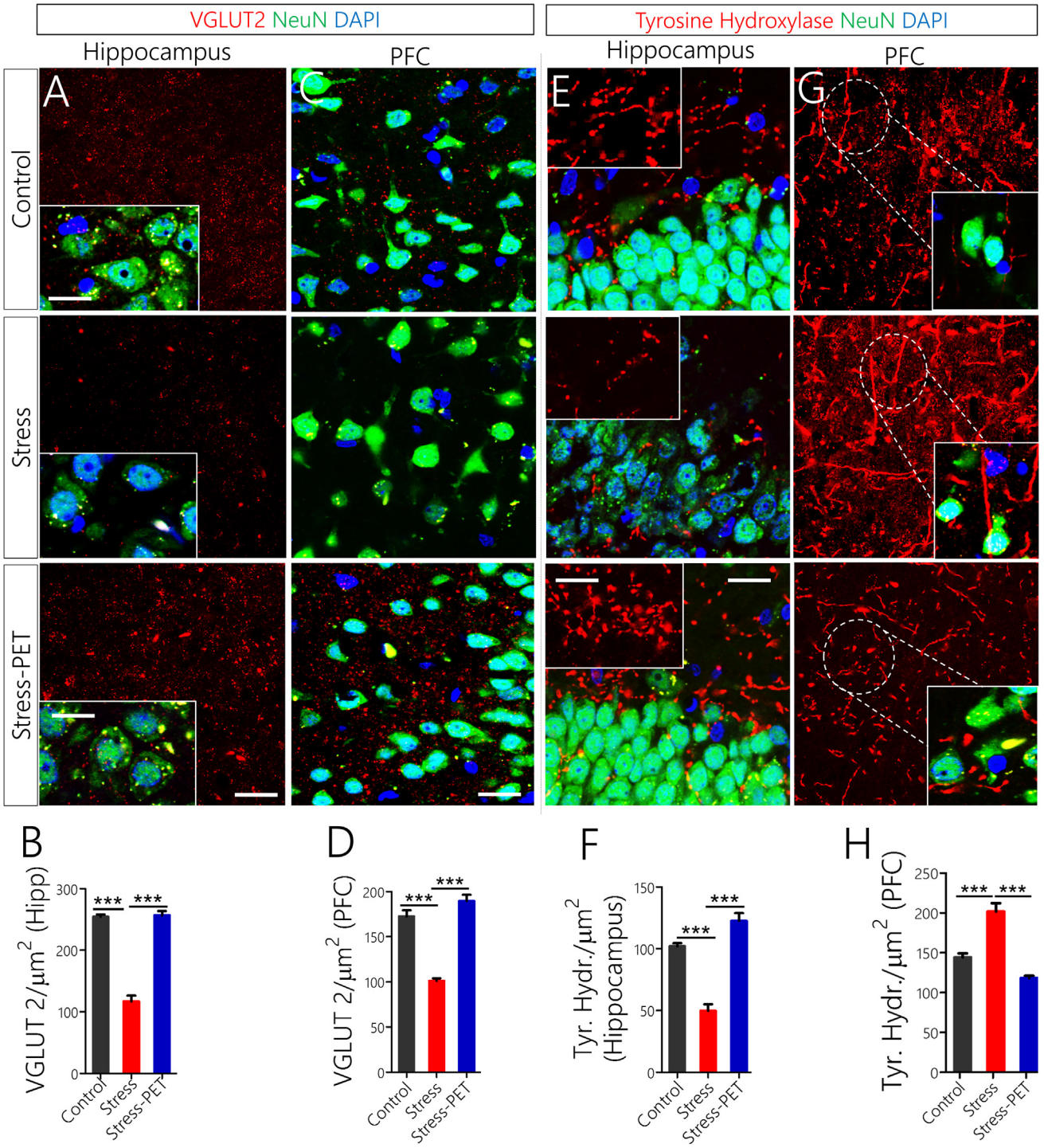


Fig. 6. (A) Vesicular glutamate transporter (VGLUT 2) expression decreased in the hippocampus of stressed rats when compared with the control ($p < 0.001$). VGLUT2 expression was rescued in the hippocampus of Stress-PET rats when compared with the stressed group ($p < 0.001$; scale bar = 20 μm; inset = 5 μm). (B) Hippocampal VGLUT 2 expression quantified in traumatic stress and PET. (C) VGLUT2 expression decreased in the PFC of stressed rats ($p < 0.001$) when compared with the control. A normalized cortical VGLUT2 expression was observed in stressed rats subjected to PET; when compared to naïve stressed rats ($p < 0.001$; scale bar = 20 μm). (D) Graphical representation of change in cortical VGLUT2 distribution. (E) Tyrosine hydroxylase (TH) expression decreased in the hippocampus of naïve stressed rats when compared with the control ($p < 0.001$). After the prolonged exposure therapy in the Stress group, the expression of tyrosine hydroxylase increased; when compared with the Stress group ($p < 0.001$; scale bar = 10 μm; inset = 10 μm). (F) Bar chart depicting a statistical change in hippocampal tyrosine hydroxylase expression for Stress and Stress-PET rats. (G) TH expression increased significantly in the PFC of naïve stressed rats when compared with the control ($p < 0.001$). After a phase of PET, the expression of tyrosine hydroxylase reduced significantly in the Stress group ($p < 0.001$); when compared with the naïve stressed group (scale bar = 20 μm). (H) Graphical representation of statistical change in cortical tyrosine hydroxylase in traumatic stress and PET.

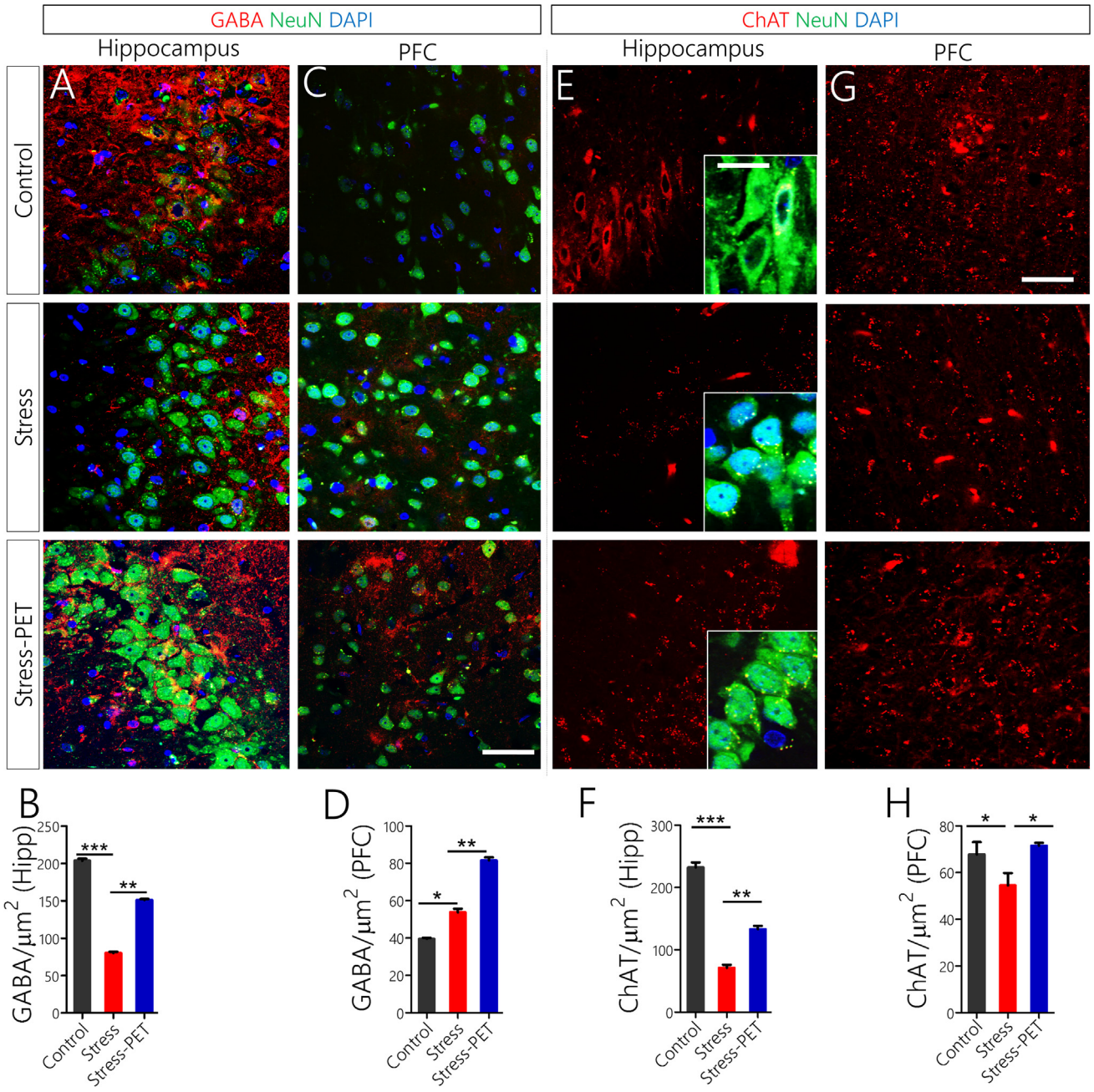


Fig. 7. (A) a decrease in GABA (inhibitory neurotransmitter) was observed in the hippocampus when the Stress group was compared with the control ($p < 0.001$). However, after the PE therapy, the distribution of GABA increased significantly in the hippocampus of Stress-PET rats ($p < 0.01$; scale bar = 20 μm). (B) Bar chart depicting the statistical change in hippocampal GABA expression. (C) An increase in GABA was recorded in the PFC of naïve stressed rats; when compared with the control ($p < 0.05$). Furthermore, in the Stress-PET group, the distribution of GABA increased significantly when compared with the naïve stressed group ($p < 0.01$; scale bar = 20 μm). (D) Bar chart depicting a statistical change in cortical GABA distribution in stress rats, and after a prolonged exposure therapy. (E) Expression of ChAT decreased in the hippocampus of naïve stressed rats when compared with the control ($p < 0.001$). Subsequent analysis showed that ChAT increased in the hippocampus of Stress-PET rats; when compared with the naïve stressed group ($p < 0.01$; scale bar = 20 μm; inset = 5 μm). (F) Statistical representation of a change in hippocampal ChAT expression. (G) ChAT expression reduced in the PFC of naïve stressed group; when compared with the control ($p < 0.05$). After a PET (Stress-PET), cortical expression of ChAT was upregulated versus the Stress group ($p < 0.05$; scale bar = 20 μm). (H) Bar chart showing the statistical change in cortical ChAT expression.

tions in cortical and hippocampal neurotransmitter synthesis. As such, the expression of markers for various neurotransmitter pathways varied between the hippocampus and PFC after traumatic stress, and PET events.

Interestingly, in the traumatic stress rats, a decrease in IGF-1R/Synaptophysin was associated with a significant decrease in hippocampal VGLUT2, Tyrosine hydroxylase, ChAT and GABA. While cortical VGLUT2 and ChAT showed a direct relationship with a reduction in IGF-1R, the distribution of tyrosine hydroxylase-positive terminals and GABA showed an inverse relationship. As such, cortical tyrosine hydroxylase

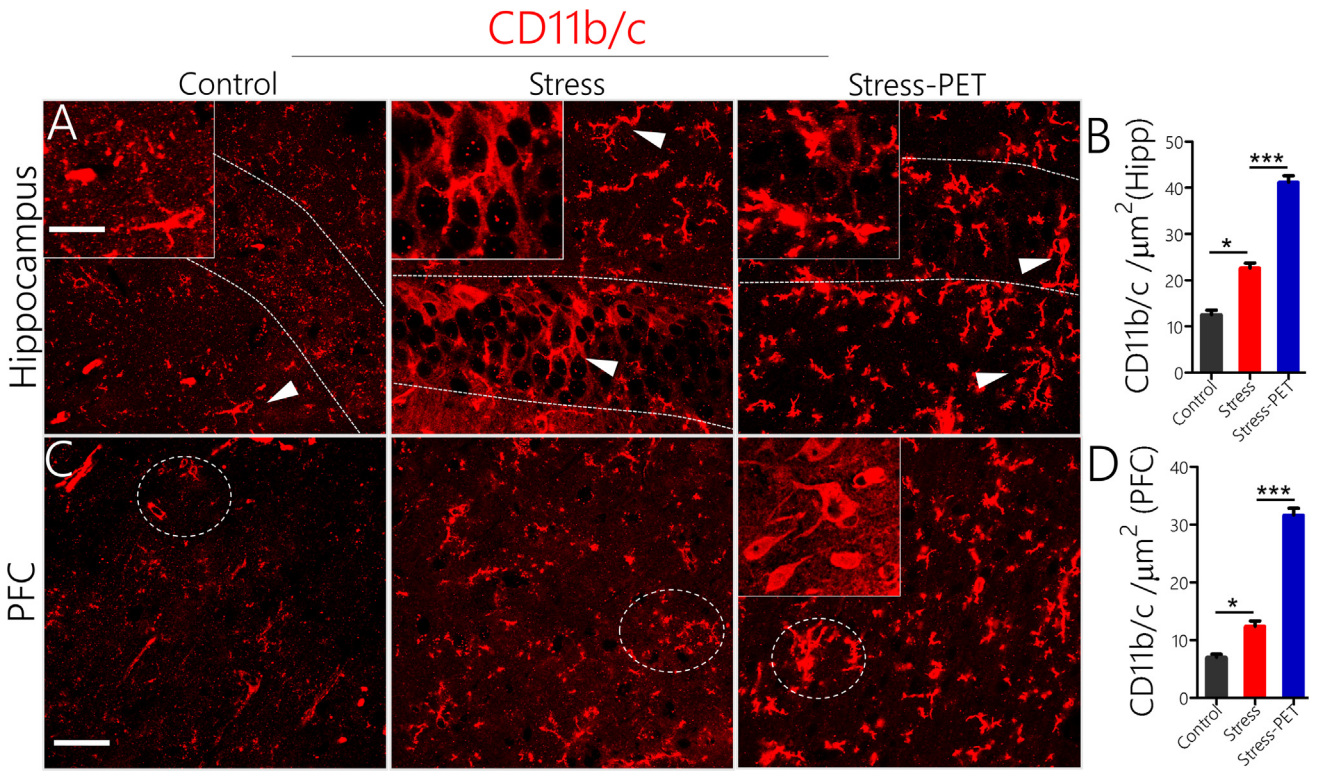


Fig. 8. (A) Representative confocal images showing the activation of microglia (CD11b/c) in the hippocampus of Stress and Stress-PET rats. Microglia activation increased in the Stress group when compared with the control ($p < 0.05$). Further increase in microglia was recorded in the Stress-PET group when compared with the Stress group ($p < 0.001$; scale bar = 20 μm, inset = 5 μm). (B) Bar chart showing statistical comparison of hippocampal microglia (CD11b/c) count in Control, Stress and Stress-PET groups. (C) Confocal images of activated microglia in the PFC. Like the hippocampus, an increase was observed in the Stress group ($p < 0.05$), while the Stress-PET showed a significant increase ($p < 0.001$; scale bar = 20 μm). (D) Bar chart depicting a statistical increase in cortical activated microglia in the Stress and Stress-PET group.

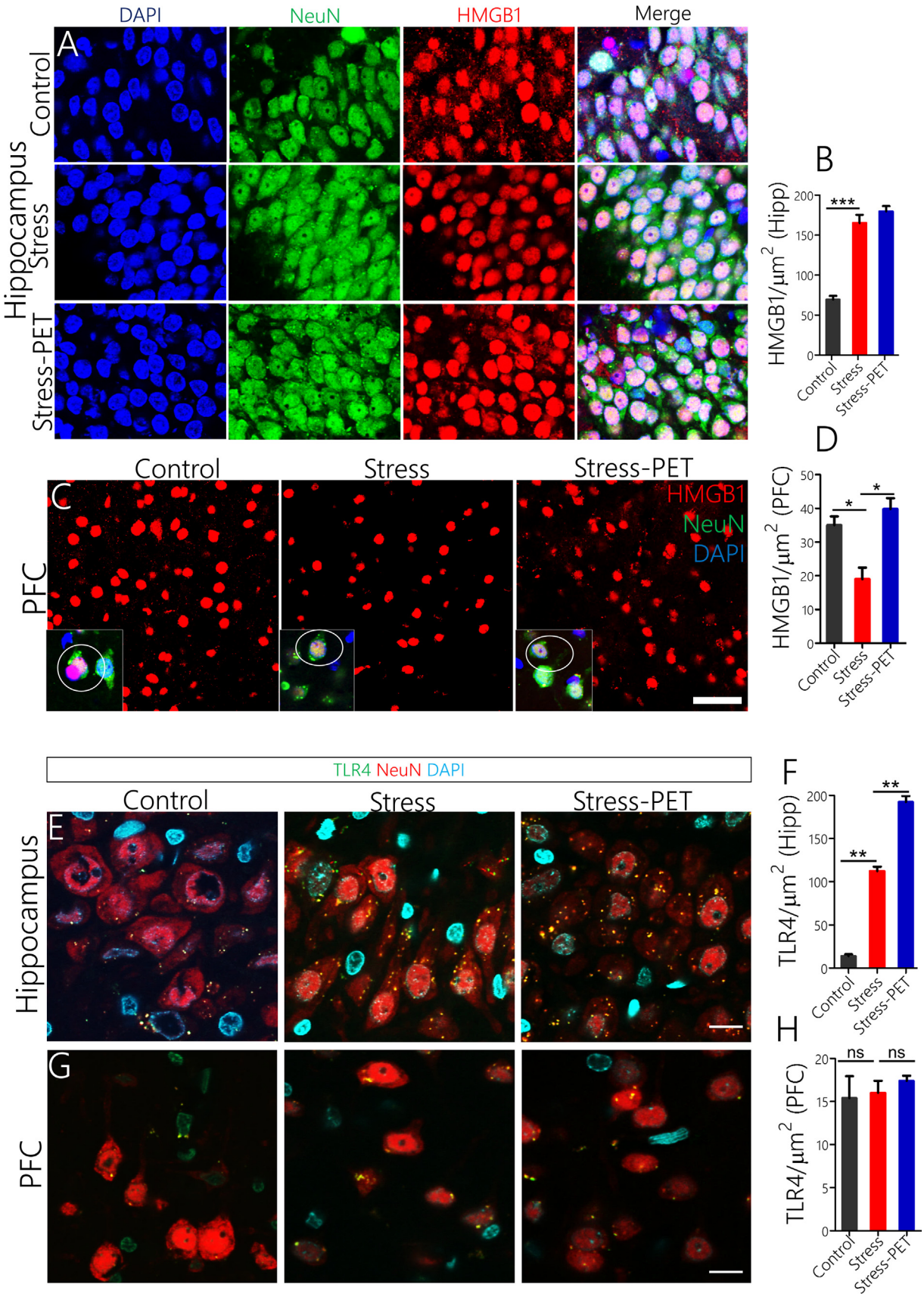
($p < 0.001$; Fig. 6G, H) and GABA ($p < 0.05$; Fig. 7G, H) expression increased significantly in traumatic stress. After PET, an increase in IGF-1R was associated with a significant restoration of hippocampal presynaptic function. In support of this outcome, hippocampal synaptophysin (Fig. 3E, F), VGLUT2, Tyrosine hydroxylase, ChAT and GABA increased above naïve stress values (Figs. 6–7). On the other hand, cortical IGF-1R showed no significant change after PET, as such no significant change was seen in synaptophysin. Surprisingly, the expression of cortical VGLUT2 and ChAT were rescued in PET, while GABA and tyrosine hydroxylase were restored to initial levels. Together we deduced that hippocampal, but not cortical IGF-1R showed the strongest association with presynaptic function in traumatic stress and PET.

In traumatic stress, the hippocampus and PFC recorded a decrease in ChAT expression. While the origin of hippocampal cholinergic neurons is relatively unclear, it is known that certain long axons expressing ChAT terminates in the hippocampus, and are involved in the modulation of hippocampal synaptic plasticity (Melander et al., 1985; Kaufer et al., 1998; Huang et al., 2013). Like the observations in other presynaptic excitatory system (VGLUT 2), hippocampal and cortical ChAT expression decreased significantly in naïve stressed rats, and was reversed after PET (increased). In support of this outcome, studies by other groups have shown cholinergic synaptic activity is decreased in stress. Furthermore, a change in cholinergic-glutamatergic response has been shown to alter anxiety-linked behavior in mice (Pavlovsky et al., 2012).

While hippocampal GABA expression was like the observations for IGF-1R, and synaptophysin (decrease in stress, and upregulated after PET), we recorded a significant increase in cortical GABA expression in the naïve stressed group. Owing to the role of hippocampal GABA in attenuation of fear memory, an increase in cortical GABA may represent a feedback mechanism preventing the formation of new memory associated with the fear stimulus (Saxe et al., 2006; Makkar et al., 2010.

Change in IGF-1/IGF-1R may affect post-synaptic morphology though CaMKIIα

Since IGF-1R signaling holds a regulatory role balancing MAPK/ErK and CaMKIIα (Zhao et al., 1999; Soderling and Derkach, 2000), and both proteins are required for the regulation of LTP (Atkins et al., 1998), it is logical to assume that a change in IGF-1R may entail alterations in LTP during traumatic stress, and PET. Mechanistically, down-regulation of IGF-1R during traumatic stress may imply the loss of its (IGF-1R-CaMKIIα) neuroprotective effect on cortical and hippocampal synaptic LTP. As such a significant depletion of brain CaMKIIα and MAPK/ErK


DAPI
NeuN
HMGB1
Merge
A
Hippocampus
Control
Stress
Stress-PET
B
HMGB1/μm2 (Hipp)
***
Control
Stress
Stress-PET
C
PFC
Control
Stress
Stress-PET
HMGB1
NeuN
DAPI
D
HMGB1/μm2 (PFC)
*
*
TLR4 NeuN DAPI
E
F
TLR4/μm2 (Hipp)
**
**
G
H
TLR4/μm2 (PFC)
ns
ns

signaling, creates a premise to suggest a loss of IGF-1R, in traumatic stress, creates a dysfunctional LTP (reduced synaptophysin and CaMKIIα), while permitting an alternative mechanism associated with the attenuation of pro-inflammatory response.

Physiologically, CaMKIIα regulates calcium-calmodulin – known to inhibit small ion conductance channels - in LTP (Grunnet et al., 2010; Mpari et al., 2010; Kuiper et al., 2012; Stackman et al., 2008). Together, CaMKIIα, and these small channels occupy postsynaptic densities in the hippocampus (González et al., 2012; Trimmer, 2015). In stressed rats, a decrease in CaMKIIα was associated with the loss of post-synaptic scaffolding protein (PSD-95). Although CaMKIIα increased after PET, PSD-95 was relatively unchanged. Interestingly, a decrease in CaMKIIα was also linked with an increase in the expression of KCa2.2 during traumatic stress response. Thus, tis outcome suggest that CaMKIIα-dependent regulation of KCa2.2, and post-synaptic function are targets of chronic stress exposure in the hippocampus.

Surprisingly, after PET, we recorded a further increase in KCa2.2. Since an increase in KCa2.2 is associated with elevated intraneuronal K^+, it facilitates a reduction in long-term potentiation and glutamatergic neurotransmission associated with contextual fear memory (Stackman et al., 2008; Lam et al., 2013; Murthy et al., 2015a,b). This may represent an activity-dependent change owing to the effect of KCa2.2 in glutamatergic excitatory post-synaptic currents (EPSCs) (Lin et al., 2008). Additionally, studies by other groups have shown that glutamate receptors, CaMKIIα and KCa2.2 are co-localized on the post-synaptic densities and extra-synaptic sites (Benke et al., 1998; Lin et al., 2008; Köhler et al., 1996), and are preferentially co-expressed in brain areas concerned with memory formation (Ishii et al., 1997; Hammond et al., 2006; Murthy et al., 2015a,b). In this study, our results demonstrate that an over expression of KCa2.2 in the PFC/hippocampus, coupled with a depletion of CaMKIIα and PSD-95, may represent a significant post-synaptic effect of fear-related traumatic stress. This outcome is further supported by the results of Murthy et al. (2015a,b), which suggests the involvement of KCa2.2 in specific aspects of fear memory formation, and the pathophysiology of hippocampal synaptic dysfunction. Interestingly, studies by other groups have shown that KCa2.2 expression in the hippocampus may be directly linked to contextual fear, and associative learning (McKay et al., 2012; Ishii et al., 1997; Hammond et al., 2006).

The significance of this outcome suggests a regulatory neural response to attenuate the formation or retrieval of fear memory when the animals were exposed to an unrelated stimulus. Since a loss of CaMKIIα represents a state of over-activated (unregulated) KCa2.2, an increased expression of KCa2.2 during stress and PET suggests its involvement in dampening of LTP associated with the fear memory formation, and retrieval. Ultimately, we deduced that an increase in KCa2.2, during the prolonged exposure period facilitates the inhibition of calcium-mediated action potentials governed through presynaptic glutamate release and CaMKIIα-mediated LTP. In support of these findings, other studies have shown that a direct activation of KCa2.2 may prevent fear memory retrieval by preventing hyperactivity in the neural circuits associated with fear memory. (Yu et al., 2012; Kleinridders et al., 2014).

Differential inflammatory response in stress and PET was linked with IGF-1R expression

Aside from its role in the modulation of synaptic changes relative to fear memory extinction, alterations in IGF-1R were reflective of neuroinflammatory changes relative to stress and PET. This was associated with an increase in hippocampal and cortical microglia activation after the stress phase ($p < 0.05$; Fig. 8). Interestingly, the distribution of microglia was further increased in the stress-PET PFC and hippocampus. Aside an increase in microglia, stress and PET caused an increase in inflammatory response associated with the distribution of HMGB1 and TLR4 in neurons. Extra-neuronal HMGB1 has also been reported in advanced stages of neuroinflammation (Tsung et al., 2007; Yu et al., 2012). Importantly, HMGB1 is a ligand for TLR4. Through the TLR4–MyD88 pathway, HMGB1 activates the release of pro-inflammatory cytokines such as IL-1β, and drives a downstream increase in mTOR and MAPK/ErK. Interestingly, quantification showed that the neuronal distribution of HMGB1 and TLR4 varied regionally. Although we have focused on the expression of TLR4 and HMGB1 in neurons, the distribution of these proteins in astrocytes and microglia are equally important and may contribute to the overall stress response mechanism as it relates to IGF-1R/CaMKIIα loss. To justify the interdependence of pro-inflammatory signaling and synaptic function, a study by Liu et al. (2008) suggests that CaMKIIα may be required for TLR4 activation in macrophages. Furthermore, an increase in TLR4 (and HMGB1) signaling has been shown to contribute to the recruitment of brain

Fig. 9. (A) Expression of HMGB1 increased significantly in the hippocampal neurons of naïve stressed rats when compared with the control ($p < 0.001$; scale bar = 15 μm). For the Stress-PET group, no significant change in HMGB1 was observed when compared with the naïve stressed group (ns). (B) Bar chart representing the change in hippocampal HMGB1 expression in naïve stressed rats, and after prolonged exposure therapy. (C) Decline in cortical HMGB1 expression was observed in naïve stressed rats when compared with the control ($p < 0.05$). However, for the Stress-PET group, the expression of HMGB1 increased in the cortex when compared with the naïve stressed group ($p < 0.05$; scale bar = 20 μm, 10 μm). (D) Quantification of changes in HMGB1 expression in the PFC. (E) Hippocampal TLR4 expression increased in naïve stressed rats ($p < 0.01$; versus the control). A significant increase was also observed after the PE therapy (Stress-PET), when compared with the naïve stressed group ($p < 0.01$; scale bar = 20 μm). (F) Statistical changes in TLR4 expression in hippocampus of stressed rats, and after a PET. (G) No significant change in TLR4 was seen in the PFC of naïve stressed, and Stress-PET groups when compared with the control (scale bar = 5 μm). (H) Statistical analysis for the expression of TLR4 in the PFC of Stress and Stress-PET animals.

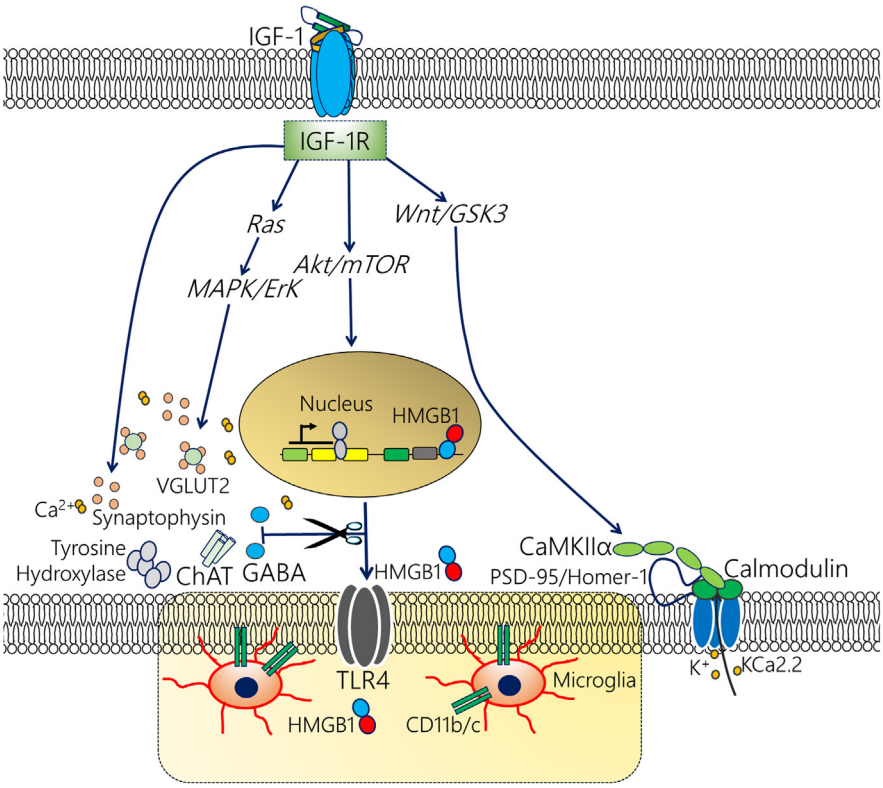


Fig. 10. Schematic summary of the possible link between IGF-1R signaling, synaptic changes, neurotransmitter function and inflammatory response. This represents multiple complex interacting pathways and potential areas for future investigation regarding the role of IGF-1R in neural stress response.IGF-1R has three major signaling pathways (*Ras, Wnt/GSK3 and Akt/mTOR*). While IGF-1R directly modulates synaptic activity (Synaptophysin, CaMKIIα and IGF-1), through its other signaling mechanisms, IGF-1R may regulate the pattern of inflammatory response (Microglia, HMGB1 and TLR4). Importantly, CaMKIIα, in the Wnt/GSK3 signaling pathway, can attenuate *Ras* and Akt/mTOR pathways. Furthermore, CaMKIIα has been shown to be directly linked to the regulation of LTP. Ultimately, through multiple interacting downstream pathways, a change in IGF-1R – as a response to stress - may modulate phasic synaptic and inflammatory response in the brain.

macrophages and production of inflammatory proteins in the brain in hypoxic (stress) conditions (Yao et al., 2013). A general increase in neuronal HMGB1 and TLR4, in the hippocampus of naïve stressed rats, caused significant changes in association with decreased IGF-1R expression (also IGF-1 and CaMKIIα). Furthermore, there was a significant increase in microglia activation in the hippocampus and cortex of the experimental groups (Stress and Stress-PET) when compared with the control (Fig. 8). After PET, the distribution of HMGB1 and TLR4 remained relatively high; thus, suggesting a persistent inflammatory response in the hippocampus during the PET phase (Fig. 9). Evidently, microglia activation was sustained in the hippocampus and cortex of the Stress-PET group when compared with the control and stress group (Fig. 8).

The significance of these outcomes cannot be overemphasized. Together we have shown that a prolonged exposure therapy did not only alter neurotransmitter function, but prompted an additional layer of stress; associated with a sustained inflammation. Furthermore, the trend in synaptic and inflammatory changes are not mutually exclusive through the IGF-1R/CaMKIIα signaling pathway. While PET slightly improved behavior – when assessed in EPM – there is a need for a stronger consideration of the underlying neurochemical changes and how this may influence long-term changes in brain inflammation and synaptic plasticity.

SUMMARY

Taken together, this study showed that traumatic stress response and subsequent PET involved an event-dependent alteration of IGF-1/IGF-1R/CaMKIIα. Since these proteins are involved in the regulation of inflammation and synaptic function, we showed that a change in the expression of these proteins was associated with both synaptic and inflammatory changes. We conclude that a phasic change in IGF-1/IGF-1R, in traumatic stress and subsequent PET, may represent a complex array of mechanisms required for synaptic modification, and regulation of inflammation in the hippocampus and PFC.

COMPETING INTERESTS

The author states that the present manuscript presents no conflict of interest.

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