April 2014, Vol. 8, No. 4, pp. 295-305
Journal of Life Sciences, ISSN 1934-7391, USA

EAE (Experimental Autoimmune Encephalomyelitis), Corticotropin-Releasing Factor and the Blood Brain Barrier

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Received: December 03, 2013 / Accepted: January 07, 2014 / Published: April 30, 2014.

Abstract: EAE (experimental autoimmune encephalomyelitis) is an established, inducible animal model employed in the study of MS (multiple sclerosis) characterized by inflammation, BBB (blood brain barrier) malfunction, demyelination and neuronal disruption. CRF (corticotropin releasing factor) is a neuropeptide critically associated with immune function, BBB permeability, and the hypothalamic-pituitary-adrenal axis. Potential CRF targets in the brain include astrocytes, as well as endothelial cells of cerebral microvessels, since they have been reported to express CRFR (CRF receptors). Further, both of these cell types function critically in regulating BBB permeability. CRF-BP (CRF binding protein) is also expressed in both neurons and glial cells. Changes in the cortical CRF system could be a contributing factor to the BBB disruption associated with MS/EAE and has been suggested to play a protective role against cytokine-induced inflammation. The current study assessed alterations associated with the C57BL/6 mouse model of EAE in the cortical CRF system and correlated these events with changes to the microvascular unit. Immunohistochemical confocal microscopy was used to analyze the distribution of CRF, CRF-BP, and CRFR in the mouse cerebral cortex. The authors observed a reduction in detectable CRF immunofluorescence in the EAE motor cortex, an increase in CRFBP immunoreactivity in EAE astrocytes and a concurrent reduction in astrocytic CRFR immunofluorescence. Staining techniques were used to visualize astrocytes/microvessels to document alterations in BBB integrity. Changes in the CRF system were associated with a modification of the blood brain barrier as manifested by a poorly defined astrocytic barrier in EAE microvessels. Evidence suggests that manipulation of CRF signaling pathways offers an intriguing target for interventional therapies designed to modify BBB permeability that may be beneficial for treating disease states such as MS.

Key words: EAE (experimental autoimmune encephalomyelitis), corticotropin-releasing factor, blood brain barrier, astrocytes.

1. Introduction

The authors' preliminary findings suggest that GABAergic function in NAGM (normal appearing grey matter) from MS motor cortex is compromised [1]. Specifically, a subset of GABAergic inhibitory interneurons are selectively affected, particularly those identified as expressing the calcium binding protein parvalbumin [2]. This type of interneuron has also been shown to co-localize CRF [3], a neuropeptide critically associated with immune function, BBB (blood-brain barrier) permeability, and the endocrine system. CRF acts as a neurohormone and has been reported to be produced in the PVN (paraventricular nucleus) of the hypothalamus, as well as, expressed in other cells throughout the brain, including the cerebral cortex with expression patterns displaying species variability [4]. The releasable pool of CRF is known to be stored in nerve terminals and can be detected immunohistochemically [5]. CRF binds to G-protein coupled receptors CRF-R1/CRF-R2 and stimulates increases in cAMP levels [6]. These receptors have been identified on neurons where CRF, released from GABAergic inhibitory interneurons, has been shown to target pyramidal cells in the neocortex and increase

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network excitability [7]. In addition, CRF receptors have been localized to astrocytes [8] and other cells that are integral to BBB integrity [9, 10]. CRF has anxiolytic effects in the frontal cortex, inhibits exploratory/locomotor activity and inhibits excitatory synaptic transmission [11]. Acute emotional responses and a potential link to human affective disorder have been associated with overactive CRF systems [12]. CRF binds to a 37 kDa binding protein which has been linked with inhibition of CRF endocrine activity. The CRF-BP is expressed in neurons and glia and the ratio of free to bound CRF critically regulates receptor binding availability [13]. CRF-BP is stored in secretory granules, released upon stimulation in cortical neurons [14] and can be immunohistochemically detected. CRF-induced increases in cortisol release have been linked to decreases in BBB permeability [15], while others have suggested that CRF directly protects against an impaired BBB [16, 17]. Interestingly, CRF mRNA is reduced during peak clinical signs in EAE [18]. CRF may impact disease progression via modification of the BBB by direct effects on mast cells [19], astrocytes, macrophages [20] and microglia [21]. However, to date no studies have investigated the link between cortical CRF immunoreactivity and its association with BBB functionality. This may prove to be an important mechanism associated with inflammatory-mediated CNS dysfunction. The aim of the current study was to use immunohistochemical techniques to analyze the motor cortex CRF system in EAE mice including analyzing CRF, CRF-BP, and CRF receptor localization within astrocytes and correlate these changes with alterations in the blood brain barrier.

2. Materials and Methods

2.1 Animals

Brains frozen on dry ice from animals with experimentally induced autoimmune encephalomyelitis and control animals were kindly provided by Dr. Yong from the University of Calgary using established protocols described elsewhere [22]. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and strictly conformed to guidelines established by the Canadian Council on Animal Care. Briefly, 8 weeks old C57BL/6 mice (Charles River, Montreal, QC) were subcutaneously injected with 50 μg MOG_{35-55} in CFA (Complete Freund's Adjuvant) and pertussis toxin was injected intraperitoneally on days 0 and 2. Control animals received saline injections of CFA instead of MOG as well as the pertussis toxin days 0 and 2. This method produces a monophasic disease, with a peak clinical severity (paralysis of tail and hind limbs, and paresis of fore-limbs) at around day 16 following MOG immunization, plus a long-lasting paresis of both tail and limbs for months thereafter. All animals used were from the same experiment at peak clinical severity and sacrificed on days 16-19 after MOG immunization. EAE and control tissue for the current and unrelated experiments were removed, snap frozen in Dr. Yong's lab and transported on dry ice to Kent State University. Tissue was not immediately fixed at the University of Calgary since samples were used for a variety of experiments including those incompatible with fixation. Five control and five EAE brains from animals with disability scores of 4 using the following scale were analyzed; (1) flaccid tail; (2) moderate hind-limp paralysis; (3) complete hind-limp paralysis; (4) forelimb paralysis; (5) death.

2.2 Tissue Processing

Frozen brains were fixed in 4% paraformaldehyde in PBS for 48 h, after which they were placed in PBS and stored at 4 °C in PBS until slicing. Frozen brains were fully intact prior to fixation with no observable effects on tissue integrity. Furthermore, any effects would be similarly apparent in both control and EAE brains permitting relevant comparisons between the samples. Sectioning was completed within 4 days of placing in PBS, and media replaced with fresh buffer

after the first 24 h. Individual brains were sliced on a media cooled vibratome at a thickness of 100 microns and stored in PBS multi-well dishes at 4 °C until used for the staining procedure.

2.3 Staining and Analysis

Eight adjacent slices per animal were used in the current study that contained similar regions of the motor cortex for each experiment. Tissue was immunofluorescently processed to stain for astrocytes using GFAP (glial fibrillary acidic protein), microvessels using IGG (immuno gamma globulin), and CRF, CRFR1/2 or CRF-BP using appropriate antibodies. Slices 1 and 4 were stained for CRF, 2 and 5 were stained for CRFR1/2, 3 and 6 were stained for CRF-BP, slice 7 was stained for anti-factor VIII (AFVIII, or von Willebrand Factor) to label endothelial cells, slice 8 was stained for mouse IGG. The procedure utilized dual (CRF, CRFR1/2, CRF-BP) and single (GFAP, Mouse-IGG, Anti-factor-VIII) antibody labeling procedures. Rabbit polyclonal anti-CRF, CRFR1/2 and CRF-BP (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a concentration of 1:50 in PBS, 3% donkey serum and 0.5% Triton-X and slices incubated for 3 days at 4 °C in one of the 3 antibodies. After 3 × 15 min washes in PBS, slices 1-6 were incubated in Topro3 iodide (1:1000, Invitrogen, Carlsbad, California), donkey anti-rabbit alexa fluor 488 (1:200, Invitrogen, Carlsbad, California) and mouse anti-human GFAP-CY3 (1:500, Sigma, St. Louis, MO.) in PBS for 48 h. Slice 7 was incubated in donkey antibody to AFVIII FITC conjugated (1:100, ABCAM Cambridge, MA) and mouse anti-human GFAP-CY3 (1:500, Sigma, St. Louis, MO.) in PBS for 48 h. Slice 8 was incubated in donkey anti-mouse alexa fluor 633 (1:200, Invitrogen, Carlsbad, California). After 3 × 15 min washes, sections were dried on charged slides and coverslipped with Vectashield antifading mounting media (Vector Laboratories, Burlingame, CA). Non-specific binding was assessed using the staining procedures outlined above omitting the primary antibody (where applicable) confirming that the dual antibody procedure was required, labeling known constituents and not causing significant background staining. In addition, fluorescently stained structures displayed morphological characteristics congruent with those reported in the literature. Imaging was performed with an Olympus laser scanning confocal microscope and associated Fluoview software (Olympus America, Melville, NY) equipped with 3 laser lines. Signals acquired from each fluorophore were sequentially scanned to prevent any bleed through from adjacent channels. All image data stacks were acquired using sequential confocal scanning in order to prevent fluorescent channel bleed-through and analyzed using ImageJ (NIH, Bethesda, MD).

3. Results and Analysis

Images acquired from the motor cortex were summed along the z-axis using ImageJ's projection algorithm assessed for fluorescence signal intensity and samples statistically compared. Data was analyzed using ImageJ and in house software for visualizations. CRF, CRF-BP and CRFR intensity was acquired from each sample and compared across subjects. Five astrocytes from each slice were individually scanned and the amount of CRF, CRF-BP and CRFR co-localization analyzed. Five microvessel segments were scanned from the anti-factor VIII stained slices per animal and used to analyze the structure of the blood brain barrier (a total of 50 segments from all animals).

Statistics: Normal distribution of data was verified and data analyzed using ANOVA, and a P value of < 0.05 considered statistically significant.

Native immune gamma globulin immunoreactivity was compared between EAE and control motor cortex to provide evidence for a disruption of the blood brain barrier associated with EAE and an activated immune system. Control animals displayed significantly less IgG immunofluorescence as compared to EAE tissue

slices (Fig. 1). The increased staining observed with the anti-mouse IgG in EAE animals was associated with a profound increase in staining (as measured by average cortical intensity) within and around motor cortex microvessels (Fig. 1) indicating the presence of significantly increased native antibody molecules in the EAE brain.

Adjacent brain slices within the motor cortex were also stained for Anti-factor VIII (AFVIII) immunoreativity in order to assess the impact of EAE on the state of microvessel endothelial cells. AFVIII immunoreactivity was confined to a subpopulation of microvessels and displayed a punctate appearance. Cortical sections from EAE animals displayed a significant increase in the number of immunoreactive-labeled microvessels compared to controls. Detectable microvessel segments were analyzed for fluorescence and an increase in intensity was observed in EAE motor cortex (Fig. 2).

Accompanying the increase in AFVIII staining intensity was a change in microvessel staining patterns. In control animals, the distribution of AFVIII staining in the brain appeared to be confined within the endothelial cells of the vessel wall that maintained a

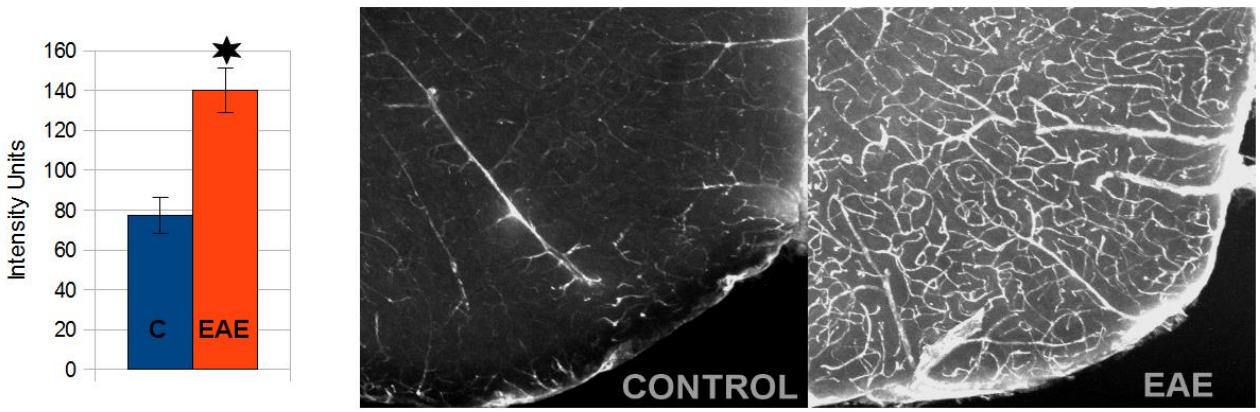

Fig. 1 The integrity of the blood brain barrier. In order to assess the integrity of the blood brain barrier, the presence of immunogammaglobulins in the brain was analyzed using a rabbit anti-mouse fluorescently conjugated antibody to directly visualize native IGG's within the brain. EAE brains displayed a profound and significant increase in cortical fluorescent staining in and around microvessels in the motor cortex. This clearly demonstrates the breakdown of the blood brain barrier associated with EAE onset.

★ Significant at $P < 0.05$, data represented as mean ± SEM from 5 control /5 EAE animals.

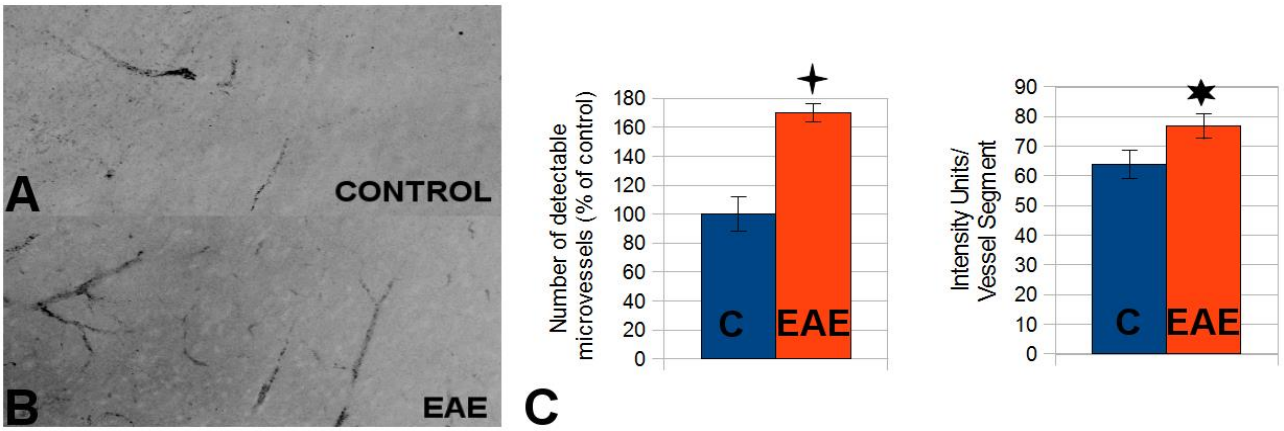

Fig. 2 Control and EAE brains immunofluorescently stained for AFVIII and GFAP to analyze the blood brain barrier. EAE was associated with an increase in detectable AFVIII positive micro vessels (A, B), as well as an increase in AFVIII staining intensity in positive vessels (C). Data analyzed from 5 microvessel segments per animal with 5 control and 5 EAE animals per group.

★ Significant at $P < 0.05$, Significant at $P < 0.05$, data is represented as mean ± SEM from 50 micro vessel segments.

close approximation with the astrocytic endfeet surrounding wall of the microvessels. Brain slices from EAE animals displayed a more diffuse staining pattern that suggests that the relationship between endothelial cells and astrocytes are modified potentially due to more loosely associated astrocytic processes within the microvessel wall and/or changes in microvessel size (Fig. 3). In order to quantitate this observed difference, the extent (width) of AFVIII immunofluorescence was measured using ImageJ's measuring tool divided by the width of the GFAP vessel staining. The calculated AFVIII/GFAP width ratio was significantly different between control and EAE groups (Fig. 3).

Along with the changes in cortical capillary integrity, brains from EAE mice exhibited significant decreases in CRF levels in the motor cortex when compared to controls without any concomitant alterations in the global immunofluorescence signal intensity for CRFR or CRFBP (Fig. 4).

In order to investigate if the CRF system of specific cellular subtypes was differentially affected, we analyzed astrocytes from the motor cortex in control and EAE mice. Multichannel fluorescence data staining astrocytes (GFAP) and CRFR or CRFBP were analyzed for co-localization (including a total of 100 astrocytes, 50 CRFR stained and 50 CRFBP stained). GFAP staining elucidated astrocyte cell bodies and extensive branching patterns, and the CRFR and CRFBP displayed a punctate appearance (Fig. 5).

Z-projected confocal stacks containing the scanned astrocytes were digitally circumscribed to ensure only single cells were analyzed and a threshold operation used to create an image mask. The thresholded image mask was used to clip the CRFR/CRFBP images and pixels within the unclipped region were summed. This technique measures the amount of fluorescent stain collected from within individual astrocytes. Analysis of these images revealed that induction of EAE resulted in a significant increase in CRFBP immunostaining and a coincident reduction in CRFR immunofluorescence in astrocytes within the motor cortex (Fig. 6).

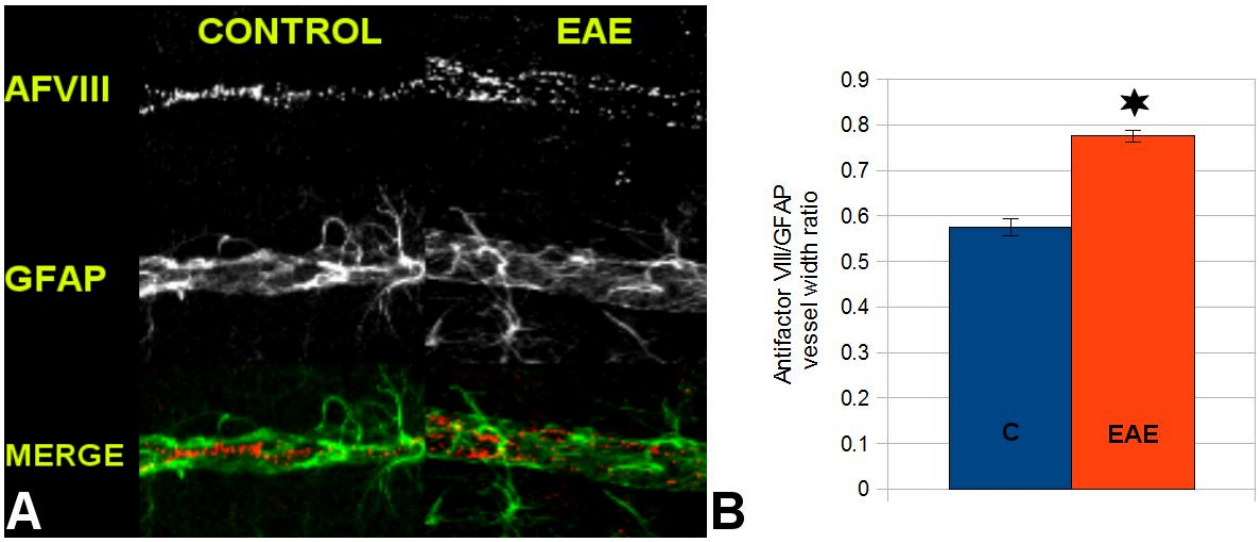

Fig. 3 Control and EAE brain microvessels were characterized by different AFVIII staining patterns. (A) Summed confocal image stacks displaying AFVIII (red) and GFAP (green) staining. Control microvessel AFVIII staining was limited to central regions and clearly outlined by astrocytic processes. EAE animals displayed more diffuse AFVIII immunofluorescence with a less defined astrocytic barrier. (B) The width of AFVIII to GFAP staining ratio was analyzed to quantitate the differential staining patterns and was significantly higher in EAE brains compared to controls.

★ Significant at $P < 0.01$, data is represented as mean ± SEM and collected from 2 width ratio measurements from 25 control and

25 EAE microvessel segments.

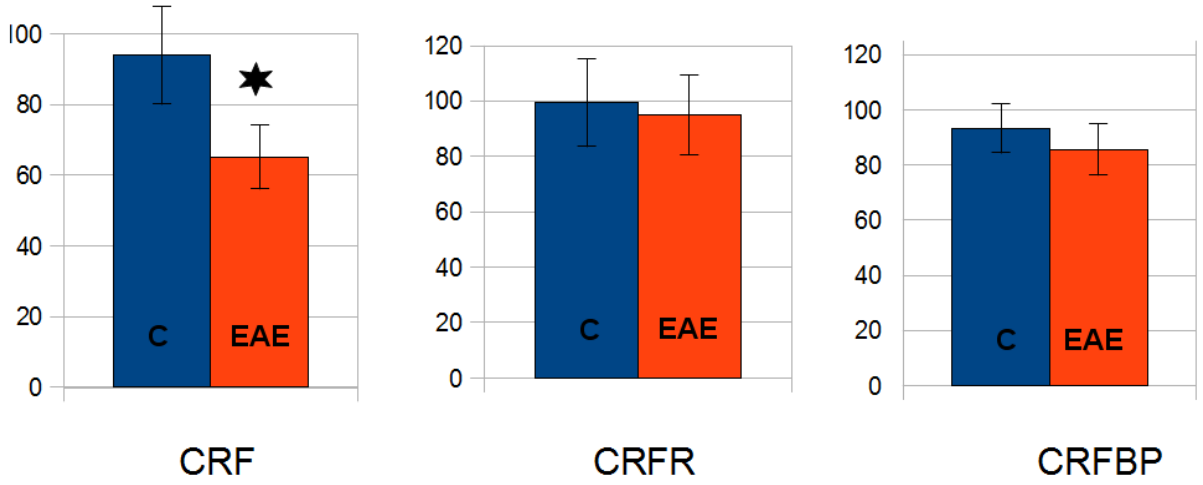

Fig. 4 Quantitation of CRF, CRF-BP, and CRFR immunofluorescence in all cortical cell types. EAE and control tissue sections spanning all layers of the motor cortex were fluorescently stained for CRF, CRF-R and CRF-BP. In the above figures controls are blue and EAE data is red, with mean values for each group displayed (bars; ± SEM). The data indicates that EAE is associated with a significant reduction in cortical CRF immunostaining profiles, which is accompanied with no changes in CRFR or CRF-BP immunofluorescence.

★ Significant at $P < 0.05$, data collected from 5 control and 5 EAE mice.

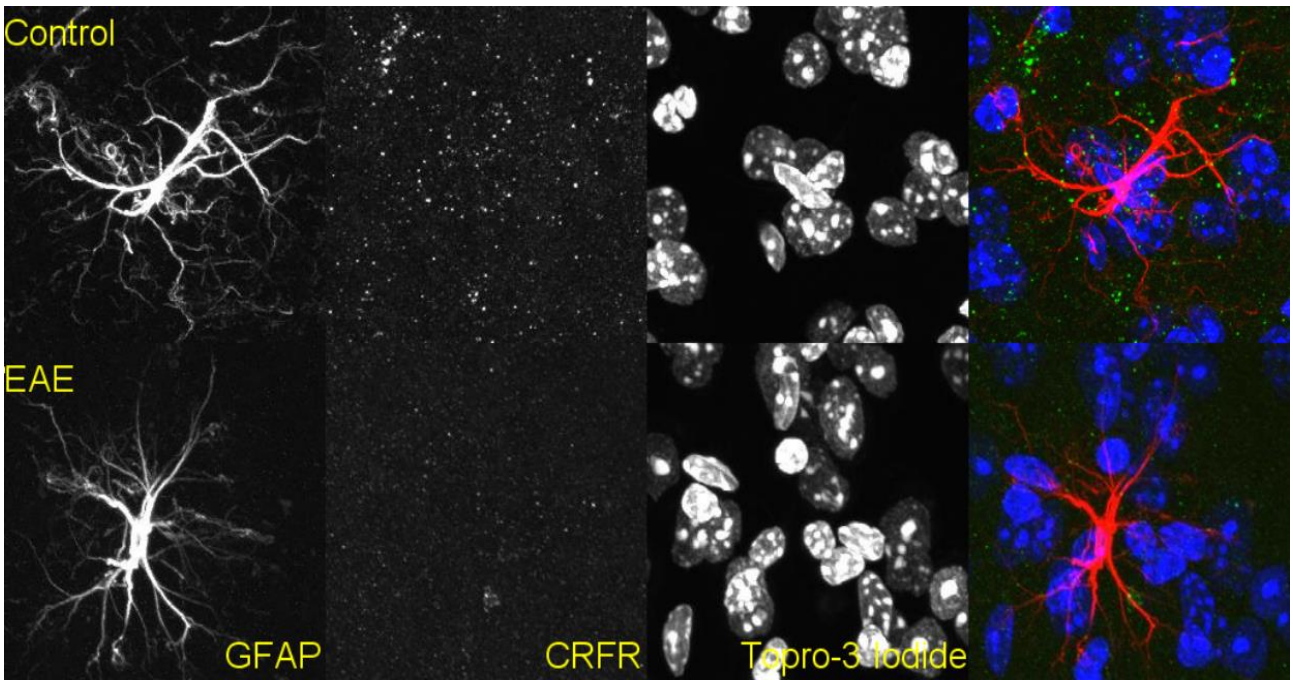

Fig. 5 Imaging the cortical CRF system in astrocytes. The images above are representative z-projected confocal image stacks from control and EAE motor cortex. The tissue was stained for GFAP (first column), CRFR (second column) and DNA (topro-3 iodide third column).

Astrocyte size was also analyzed to ensure that differences in GFAP staining did not contribute to the observed differences in astrocytic CRFR/CRFBP immunofluorescence. Using Image J's Area Fraction metric the percentage of pixels occupied by the thresholded astrocyte was measured (Fig. 7). This analysis indicated that there were no significant differences in the size of analyzed astrocytes, and that

this did not contribute to the observed significant differences in CRFR/CRFBP immunostaining.

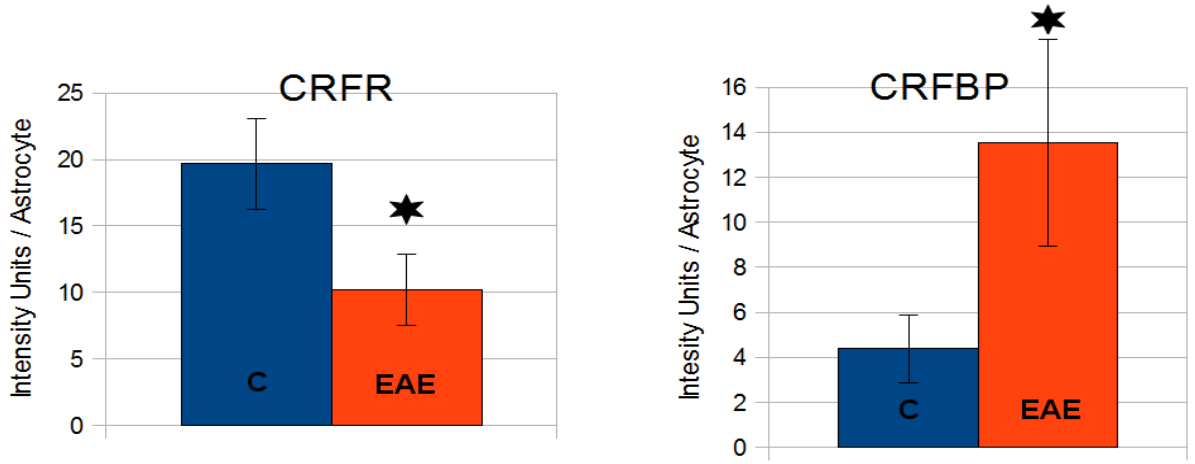

Fig. 6 Quantitation of CRF-BP and CRFR immunofluorescence colocalized to astrocytes. EAE and control tissue sections spanning the motor cortex were fluorescently stained for GFAP, CRF-R and CRF-BP. In the above figures controls are blue and EAE data is red, with mean values for each group displayed (bars; ± SEM). The data indicates that EAE is associated with a significant reduction in astrocyte CRFR profiles, which was accompanied with an increase in CRF-BP immunofluorescence. 10 astrocytes per animal were imaged and analyzed for either CRFR or CRFBP immunofluorescence, totaling 50 CRFR and 50 CRFBP stained astrocytes.

★ Significant at $P < 0.05$, data collected from 5 control and 5 EAE animals.

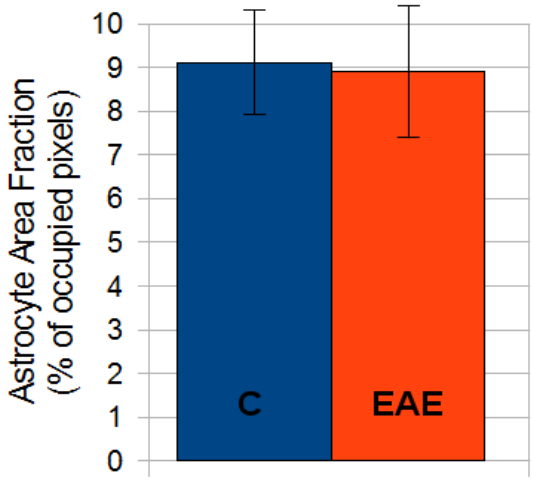

Fig. 7 Astrocytic size did not differ between groups. The EAE and Control astrocytes from motor cortex were immunofluorescently stained with GFAP and used to analyze CRFR and CRFBP levels were analyzed for size. The area fraction indicates the amount of the image occupied by the thresholded astrocyte. No significant differences were observed between EAE/control astrocyte area fraction.

Data from 50 control and 50 EAE astrocytes and represented as mean ± SEM.

4. Discussion

The authors propose that EAE is associated with a modification of the cortical CRF system correlating with immunoreactive and structural changes in the blood brain barrier at the level of the endothelial cell and astrocyte. Motor cortex from mice with disability scores of 4 were selected at peak clinical severity for direct comparisons to our existing studies in human/mouse motor cortex and maximize chances of detecting cortical disruption. We detected a significant reduction in cortical immunoreactive CRF staining compared to controls. This is consistent with previous reports demonstrating reductions in CRF mRNA in the hypothalamic PVN during peak clinical signs [18], when glucocorticoids are high. This is a potentially important relationship, since glucocorticoid induced negative feedback may underlie the reductions in cortical CRF observed in the current study. However, it is also known that the nature of GC feedback on CRF producing cells is region specific and can be stimulatory or inhibitory [23]. In any event, a subset of cortical inhibitory interneurons express CRF and it is released as a neuromodulator increasing excitability

in pyramidal cells [7]. CRF release requires high neuronal activity and possibly sustained elevations in intracellular calcium[5] and perturbations in calcium levels in both EAE and MS are apparent [24]. Further, calcium channel blockers ameliorate symptoms in mouse models of multiple sclerosis [25] and blockade of receptor mediated calcium toxicity is neuroprotective in EAE [26]. In addition, the authors have reported reductions in calcium binding protein (parvalbumin) interneurons in MS motor cortex [2]. These data may indicate that increases in intracellular calcium associated with EAE causes increased release of CRF from motor cortex interneurons with a concurrent reduction in releaseable intracellular pools of CRF reducing detectable immunoreactivity.

Changes in CRF were not definitively localized to neurons, may represent changes in multiple cell types, and it is unknown if changes were caused by increased release or reduced production. We observed significant decreases in CRF receptor expression and increases in CRF binding protein in areas coincident with the cerebral microvasculature and localized to astrocytes in EAE motor cortex. Changes in astrocytic structures consistent with endfeet were also adjacent to AFVIII-expressing microvessels. Astrocytes displayed increased CRFBP and reduced CRFR immunoreactivity as analyzed by GFAP/CRFR or GFAP/CRFBP co-localization. This difference was not due to changes in astrocytic size since the area fraction between control/eae astrocytes did not differ. Astrocytes express CRF-BP mRNA [27, 28], CRFR1 [29] and are CRF targets [21]. Alterations in CRF-BP and CRF receptor expression may play a role in inflammatory disease, and CRF targeted astrocytes (as well as microglia) participate in inflammatory responses [21].

The reason for the changes in CRF-BP and CRFR is unclear but it is possible that changes in CRF and CRFBP levels in the vicinity of analyzed astrocytes resulted in changes in receptor expression and CRF signaling. CRFR expression is CRF controlled. Konishi et al., [20] demonstrated that cultured hypothalamic neurons increased CRFR1 mRNA expression after CRF treatment, while Pozzoli et al [31] reported reduced CRFR1 mRNA in pituitary cultures after treatment. The current results are the first to correlate simultaneous reduction in both cortical CRF and its receptor in astrocytes.

Increased BBB permeability is a hallmark of MS/EAE that frequently precedes clinical signs, in the current study changes in the CRF system were accompanied by modified endothelial cell staining and structural differences in the BBB. An increased thickness of endothelial cell staining compared to microvascular width was apparent. Increased endothelial width was associated with a less defined astrocytic barrier surrounding the ECs indicating a disruption of the BBB, and a modification of EC structure in EAE animals. EAE microvessel ECs also displayed an increase in the extent of AFVIII staining compared to GFAP staining when compared to controls. The expression of Anti factor VIII (von Willebrand factor), a glycoprotein important in platelet adhesion [32], is increased in EAE spinal cords [33]. AFVIII is synthesized and stored in secretory granules following exposure to inflammatory mediators [34] suggesting a role in inflammation and is an adhesive surface that binds leukocytes combining critical steps in recruitment [35]. Increased AFVIII could augment brain monocyte infiltration, a hallmark of both MS and EAE lesions. In MS, enhanced leukocyte migration across endothelial cells is observed [29] and monocyte infiltration into the CNS is detectable in both MS and EAE. Increases in detectable AFVIII staining in EAE associated with structural changes in the blood brain barrier are consistent with studies showing that AFVIII release from ECs, the formation of endothelial fenestrations, and increased venular permeability contribute to increases in BBB permeability in MS [36-38].

The authors' data are consistent with studies

demonstrating disruptions of BBB astrocytes as well as EC's in inflammatory diseases. Astrocytes and the blood brain barrier are dramatically modified during MS and EAE, and changes in CRF signaling could be a major contributing factor that causes the observed alterations. It has been proposed that CRF signaling in the brain plays an important role in BBB integrity. Astrocytes, and EC's function in regulating BBB permeability [39] and known to express CRF receptors. Changes in cortical CRF could contribute to increased BBB infiltrate trafficking due to greater microvessel permeability and augmented inflammatory cytokine signaling. We observed that changes in the CRF signaling axis coincide with significant changes in microvascular structure. Following recovery from EAE, levels of CRF return to normal and signs of disability disappear [18]. This occurs in response to many changes in the mice, however, one potential mechanism may include re-establishment of BBB integrity via CRF signaling. Similar hypotheses have been suggested in MS. It has been shown that the number of cells expressing CRF immunoreactivity increases with disease duration in the MS hypothalamus and that this response represents a potential mechanism to protect against future inflammatory attacks [40, 41].

5. Conclusions

The current study indicates reduced immunodetectable CRF in the EAE motor cortex. Deficits in brain CRF content are associated with several neurodegenerative disorders (including Alzheimer's, Huntington's and Parkinson's disease [6]). However, CRF may contribute to the peripheral inflammatory effects of autoimmune disorders. EAE and MS are both characterized by auto-immune and neuro-degenerative components and as such could be impacted by a combination of alterations to different CRF pools (central vs. peripheral). In conclusion, EAE is associated with a modification of the cortical CRF system manifested in reductions of detectable total motor cortex CRF content, reductions in astrocytic CRF receptors and a co-incident increase in CRF-BP. EAE was associated with structural changes in the blood brain barrier that may be causally related to the observed changes in the CRF system. Future studies will be needed to isolate the specific cause of the changes in astrocyte CRFR/BP but it is likely that these changes occurred due to local increases in CRF. Further, CRF signaling is a component of the complex cellular crosstalk that occurs in MS and EAE resulting in BBB breakdown, cellular infiltration and ultimately disease progression. Evidence suggests that manipulation of these pathways offers an intriguing area for interventional therapies designed to modify BBB permeability that may not only be beneficial for treating disease states such but also for temporarily allowing pharmacological access to the brain for drug delivery into central regions.

Acknowledgments

The authors would like to kindly acknowledge Dr. Wee Yong from the University of Calgary for kindly providing control and diseased mouse tissue.

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