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Morphological Assessment Of The Impact

Of Stress On Primary

Somatosensory Cortical Microglia

A thesis submitted in partial
satisfaction of the requirements for the
degree of

MASTER OF SCIENCE

in

MOLLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

by

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December 2019

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MORPHOLOGICAL ASSESSMENT OF THE IMPACT OF STRESS ON S1BF MICROGLIA

by: **Jessica Keating**

ABSTRACT

Psychological stress has been implicated as a major risk factor in the nation's leading psychiatric disorders. Although much is known about how stress impacts brain regions such as the prefrontal cortex, amygdala, and hippocampus, little is known about its effects on the primary sensory cortex, such as the somatosensory cortex. Interestingly, previous work in the Zuo laboratory found that stress induces synaptic loss and dysregulated excitatory-inhibitory (E-I) balance in mouse primary somatosensory barrel field (S1BF). The collection of cells referred to as neuroglia work together with neurons to support and regulate central nervous system functioning. Amongst these cells, microglia are of particular interest due to their ability to make experience-dependent changes upon neuronal circuits. However, it remains unclear if glial-neuronal interaction may partake in such stress-induced synaptic and E-I balance defects. Due to the known role of microglia participating in synaptic stripping, the current study aimed to determine how microglia mediates stress induced defects in S1BF. To address this question, we examined the density and morphological changes in S1BF microglia in response to restraint stress, and how a known microglial suppressor, minocycline, affects stress-induced changes. The findings revealed that stress impacts S1BF microglia by increasing somatic size and decreasing process complexity, consistent with the morphological changes in microglial phagocytosis in inflammatory states. Minocycline administration in stressed animals blocked stress induced morphological changes in S1BF microglia. Our results suggest that microglia may be involved in stress-related defects, and manipulation of its states, such as prevention of microglial over-activation, may makes the brain more resistant to psychological stressors.

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CHAPTER 1
BACKGROUND

1.1. Brain Response to Environment

Brain development is guided by a convergence of genetic and external influences (Fox et al., 2010, Bae et al., 2015). The capacity for the brain to dynamically adapt to changes within environmental stimuli is referred to as neuroplasticity (Lundy-Ekman, 2013). While it is well known that genetic information can alter neuroplasticity and increase the risk of mental and neuropsychiatric disorders (Bray and O'Donovan, 2019, McClung and Nestler, 2008), input from the environment has also been shown to influence and regulate neuroplasticity (Sale et al., 2014). This is evidenced, in part, by research investigating activity- (or experience-) dependent neuroplasticity, which occurs in both young and adult mammals (Zito and Svoboda, 2002, Hofer et al., 2006) and refers to structural and functional brain changes in dendrites, axons, and synapses that are mediated through alterations in neuronal activities and or environmental stimuli (Trachtenberg et al., 2002, Holtmaat and Svoboda, 2009, Marik et al., 2010, Fu and Zuo, 2011).

In rodent experiments, experience-dependent neuroplasticity is measured through manipulation of sensory experiences, such as sensory enrichment and/or deprivation. Research has demonstrated that both sensory enrichment and deprivation have a profound effect on neuroplasticity through alteration of neuronal structures. For example, in enrichment studies, mice exposed to novel and stimulating environments exhibit increases within capillary density (He et al.,

2017), neurogenesis (Kempermann., et al 1997, Monteiro et al., 2014), and dendritic spine turnover (Jung and Herms, 2014). Alternatively, in the case of sensory deprivation, mice exhibited a decrease in capillary density and branching (Lacoste et al., 2014), dendritic spine pruning (Zuo et al., 2005), and decreased apical dendritic length (Chen et al., 2012b).

Sensory experiences have also been shown to impact the human brain, mirroring the changes that were noted in rodent experimentation. For example, in children with Autism Spectrum Disorder, environmental enrichment has been shown to induce significant improvements in cognitive aptitude, language abilities, and diminish atypical sensory responses (Woo et al., 2015). Moreover, research investigating prenatal exposure to maternal sounds has observed associated brain changes within auditory and language functioning (Beauchemin et al., 2010, Partanen et al., 2013). Collectively, these studies strongly suggest that significant life experiences have the capacity to induce both structural and functional brain changes.

1.2 Stress Impact on the Brain

Stress is a type of significant life experience that is defined as a nonspecific physiological response that makes it harder for the body to maintain homeostasis (Selye, 1973). While the physiological response to stress has been a key factor ensuring human survival across evolution, when experienced in excess, it can induce significant psychological damage. For example, stress is a significant risk factor in the nation's leading psychiatric disorders such as schizophrenia,

generalized anxiety disorder, major depressive disorder, bipolar disorder, and post-traumatic stress disorder (de Kloet et al., 2005, Schmitt et al., 2014, Donner et al., 2008, Kendler et al., 1999, Staufenbiel et al., 2013, Corcoran et al., 2002). Moreover, stress has been shown to increase anxiety (Pawlak et al., 2003, Holsboer, 1999), decrease memory (Lupien et al., 1997, Kuhlmann et al., 2005), alter sleeping patterns (Drake et al., 2004, Akerstedt et al., 2002), and induce musculoskeletal “pain” and “ache” (Lindegård et al., 2014).

In the brain, stress impacts neuroplasticity by altering neurotrophins, which are an important factor in regulating neuronal growth signals (McAllister et al., 1995, Alfonso et al., 2006, Chen et al., 2017). In addition to its impact on neurotrophins, stress has also been shown to impact neurotransmitters such as GABA, glutamate, and serotonin (Torres et al., 2002, Moghaddam et al., 1994, Jie et al., 2018) which can result in profound effects upon mood and cognitive functions (Nutt, 2008, Lener et al., 2017).

Dendritic spines are small neuronal protrusions that comprise the majority of excitatorysynaptic transmission (Nimchinsky et al., 2002) and impact the functioning of neuronal circuitry through changes in their size and density (Harris and Kater, 1994). Interestingly, Stress has been shown to alter dendritic spine morphology in a manner that has been correlated with altered behavior (Christoffel et al., 2011). Taken together, this demonstrates that spine morphology is a useful tool when assessing neuronal circuitry changes.

Stress research in the brain has been primarily focused on changes in

mPFC, hippocampus, and amygdala due to their roles in key mental processes such as cognitive flexibility, learning, memory, and fear. Stressful experiences induce functional changes through alteration of long-term potentiation (LTP) and long-term depression (LTD) (Kamal et al., 2014), which are believed to mediate learning and memory on a cellular level (Bliss and Collenridge, 1993). Stress has been shown to block the induction of LTP and enhance LTD in the hippocampus (Xu et al., 1997), impair LTP at mPFC neurons receiving hippocampal and thalamic projections (Cerqueira et al., 2007), and increase LTP at thalamic inputs in the amygdala (Suvrathan et al., 2014).

Gaps in Stress Research

While the neuroscience field has made many insightful discoveries about how stress impacts the brain, our current understanding is far from complete. For example, the current research has largely been characterizing the impacts of stress from the solitary perspective of the neuron, while neglecting their equally important counterparts, glial cells. This limited perspective provides an incomplete picture, for neurons and glia work together to support CNS functioning. Moreover, the function of glial cells is especially relevant within stress research, for previous studies have demonstrated that stress can induce a profound impact on the immune cells in the peripheral nervous system, while very little is known about how stress may impact the brain's immune system. In addition to the aforementioned shortcomings, the scope of this research has been further limited by the fact that the majority of this research has been conducted in

a few key brain regions, such as the prefrontal cortex (PFC), the hippocampus, and the amygdala, while other important brain regions are left largey unexplored.

1.2.1 Somatosensory Response to Stress

While the somatosensory cortex is not a region often associated with stress, recent research has demonstrated several sensorimotor abnormalities in psychiatric disorders such as major depressive disorder and PTSD, which highlight the importance of further characterizing how stress can impact this brain region (Schmaal et al., 2017, Badura-Brack et al., 2015). In humans, the primary somatosensory cortex (S1) is a region located on the postcentral gyrus that receives sensory information such as pain, temperature, and pressure, and integrates the incoming information to allow for active perception and interaction with the environment (Gallace and Spence, 2010, also see Figure 1A). In rodent research models, the barrel cortex is often used as a model to study somatosensation, and general cortical processing due to its somatotopic organization and presence of the barrel field, which correspond to facial whisker in a one-to-one fashion, and allow for targeted sensory manipulations (Petersen, 2007, also see Figure 1B). The barrel cortex has been shown to have a complex array of projections which mediate its function. While not a region often associated with stress induced changes, earlier work has shown stress can alter neuronal properties such as cFos expression, which is an immediate early gene marker indicating neuronal activities (Ryablin et al., 1995). While the synaptic and functional implications of this alteration were previously unclear, recent work

characterizing the impact of psychological stress in the barrel cortex has provided critical insight into the functional ramifications of these stress induced changes (Chen et al., 2018). For example, in response to restraint or unpredictable stress, mice have been shown to exhibit sensory perception deficits which were correlated with the progressive loss of dendritic spines and the decreased activity of parvalbumin-expressing interneurons. In response to recent work demonstrating the microglial role within synapse elimination (Tremblay et al., 2010), this thesis explores the potential role of microglia within the stress-induced synaptic and behavioral changes observed in the S1BF.

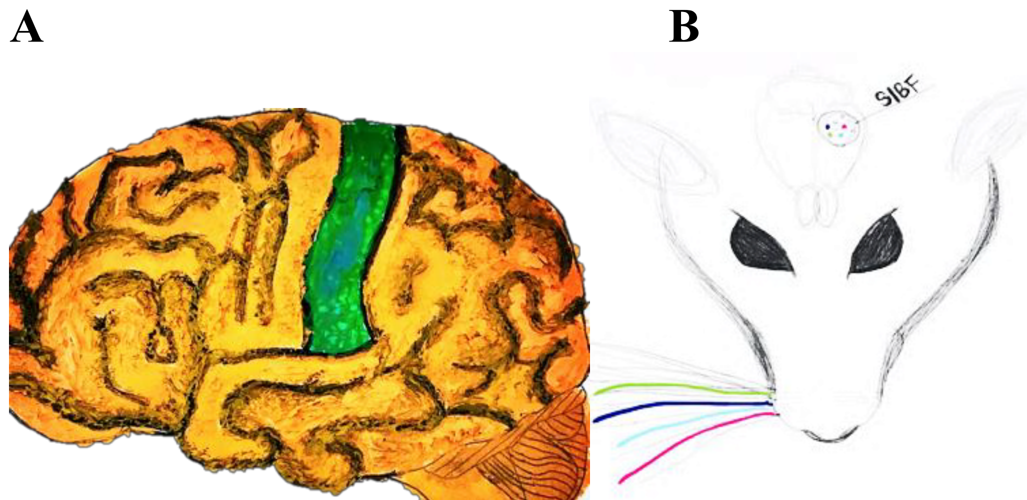


Figure 1. Painted Depictions of Somatosensory Cortex and S1BF. A. Green area depicts the primary somatosensory cortex. **B.** Drawing of the mouse barrel cortex where each barrel represents the receptive field of a single whisker. Illustrative credit: Jessica Keating, 2019.

1.3 Microglia

1.3.1 The Origin and Function of Microglia

The collection of cells referred to as neuroglia work together with neurons to support and regulate CNS functioning. For example, astrocytes participate in

synapse formation, function, and elimination (Chung et al., 2019), microglia participate in synaptic pruning (Xie et al., 2017), and oligodendrocytes wrap around neuronal axons to ensure the passage of information (Simons and Nave, 2016).

Microglia are principal immune cells derived from the mesodermal yolk sac which, excluding a few specific circumstances in which microglia can be derived from bone marrow, are primarily self-sustained through cell division with an average renewal rate of 4 years (Réu et al., 2017). The primary function of microglia is to protect and maintain CNS integrity (Banati and Graber, 1994) which is mediated through surveying the extracellular environment and scavenging for foreign or injured material (Kreutzberg, 1996, Batchelor et al., 1999, Aloisi, 2001), phagocytosis (Morsch et al., 2015), extracellular signaling (Cross and Woodroofe, 1999., Aloisi, 2001), antigen presentation (Aloisi et al, 1998., Gehrmann et al., 1995, Aloisi, 2001) cytotoxicity (Banati and Graber et al, 1994., Gehrmann et al.,1995, Medzhitov and Janeway, 2000), and synaptic stripping (Moran and Graber, 2004., Kettenmann et al., 2011).

1.3.2 Morphological States of Microglia

As suggested by their name, microglia are the smallest of all glial cells. They are composed of a soma and cellular processes which extend from the soma and utilize specialized ion channels to monitor for changes in the extracellular environment (Kettenman et al., 1990., Eder, 1998). While microglia were previously presumed to have little distinction, recent work has indicated that they

are largely heterogeneous population (Hammond et al., 2019) which dynamically respond to changes in the extracellular environment. For example, in contrast to homeostatic conditions in which microglia exhibit a complex and ramified morphology, during brain pathogenesis, microglia have been shown to exhibit an altered phagocytic-like phenotype (Morsh et al., 2015). Recent work has continued to elucidate the morphological distinctions in microglial morphology in response to various environmental stimuli. Several studies have observed the transition from a ramified morphology to an ameboid-like morphology (also see Figure 2) in response to inflammatory exposure. For example, saline injected animals displayed smaller somas and long, fine microglial processes, whereas animals injected with Lipopolysaccharide (LPS, an endotoxin that induces an inflammatory response) exhibited larger somas and shorter, thicker branches (Chen et al., 2012a).

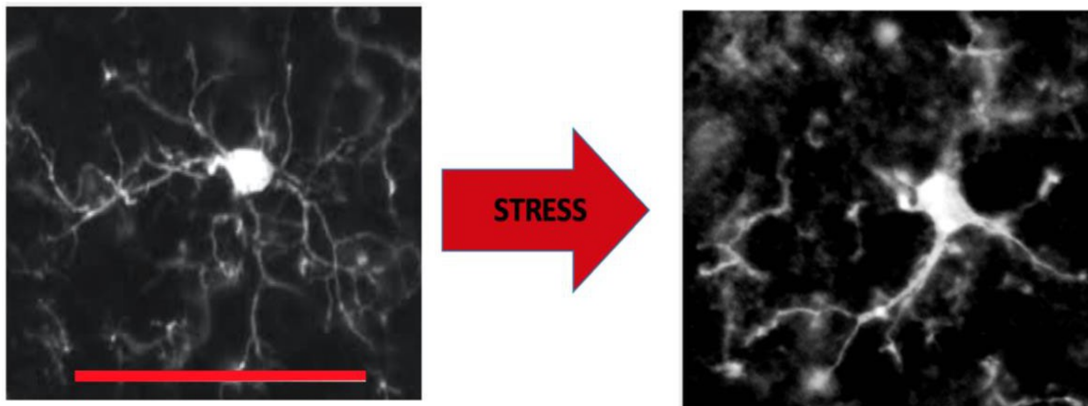


Figure 2. Schematic Depicting Microglial De-ramification and Somatic Enlargement in Response to Stress. Scale bar depicts 50 μm. Microglial cells were stained with Iba1 antibody and imaged with a 63X (NA=1.4) oil objective lens. Images were processed and inverted with Fiji (Version 1) software. Picture and figure illustrative credit: Jessica Keating,

Moreover, human research into aging- associated brain dysfunctions has indicated alterations in microglial morphology (Bachstetter et al., 2015, Mosher and Wyss-Coray, 2014). Taken together, these studies provided evidence to suggest that morphological changes in microglia can be used as a proxy to assess CNS associated changes.

1.3.3 Evidence That Microglia Participate in Neuroplasticity

As mentioned above, microglia are active within extracellular debris clearance. Electron microscopy work assessing the role of microglia within focal cortical inflammation found an increase in microglial density around the legion site and observed that microglial processes make direct contact with neuronal perikarya and apical dendrites which was followed by the displacement of a large portion of axosomatic synapses (Trapp et al., 2007). This microglia- neuron interaction indicated a potential role of microglia within neuroplasticity which was later supported by research showing that microglia exhibit experience-dependent participation of the elimination of postsynaptic structures (Tremblay et al., 2010).

1.3.4 Effects of Psychological Stress on Microglial Morphology

Stress has the capacity to induce morphological and functional microglial changes. For example, in response to the chronic stress RS paradigm, which involves 21 consecutive days of daily stress sessions in which mice are isolated from littermates and enclosed in a tight space that does not allow for normal movement, microglia exhibit increased branch complexity and an increased

expression of the inflammatory marker beta-1 integrin (Hinwood et al., 2013). Interestingly, these stress-induced alterations have been shown to vary between different brain regions, with only 9 out of the 15 characterized brain regions showing increased Iba1 immunoreactivity (Tynan et al., 2010), suggesting that microglia might exhibit regional specificity to stress-induced changes. While several studies have detailed the impacts of chronic stress on microglial morphology, it remains unclear how microglia are affected under acute stress conditions.

1.4. Thesis Overview

To address the current knowledge gaps in stress research, this study investigates how microglia in the barrel cortex (S1BF) respond to psychological stress. This was done through three primary means of investigation. The first of which assessed the microglial morphological alteration in response to psychological stress, which complements previously published work in the Zuo laboratory (Chen et al., 2018). Secondly, microglial activation was prevented through administration of the pharmacological agent (minocycline), to determine whether this treatment may block the deleterious effects induced by stress. Lastly, the potential microglial behavioral correlates were assessed by using minocycline in stressed mice to see whether microglial activity impacted stress-induced behavioral impairments. The Methods of this approach will be detailed in Chapter 2, Results can be found in Chapter 3, and Discussion in Chapter 4.

CHAPTER 2

MATERIASLS AND METHODS

2.1 Experimental Animals

Wildtype C57B6 mice around postnatal day 37-40 adolescent mice were used for all experiments. Mice were housed in UCSC animal facility which provided a 12-hour light-dark cycle and *ad libitum* access to food and water. All experiments were conducted in accordance with the Animal Care Use Committee (IACUC) of UCSC.

2.2 Restraint Stress (RS) Protocol

RS included both male and female mice. RS protocol was conducted by placing an individual mouse in a perforated 50 ml conical tube for 2 hours every day over the course of a week, as previously described (Chen et al., 2018). Littermates were randomly assigned to either the control or RS group, and identity of the mice were distinguished by ear clippings.

2.3 Minocycline Administration

Minocycline (Sigma-Aldrich USA, category #M9511) is a known microglial suppressor used in the current study. Minocycline was administered orally through the drinking water for 7 days at a concentration of 40mg/ml, which has been previously shown to be sufficient in the suppression of microglial activity (Hinwood et al., 2013). The only time mice do not have access to this minocycline infused water was during the daily 2h RS sessions. This cohort was also divided into two different groups (by random assignment): mice receiving

both minocycline + RS, and mice receiving minocycline without RS.

2.4 Whisker-Dependent Texture Discrimination Task

The whisker-dependent texture discrimination task was performed to assess sensory processing as previously described (Chen et al., 2018). In addition to habituation to the testing arena starting two days prior to behavioral testing, this test involves 3 phases: encoding (5 minutes), rest (5 minutes), testing (3 minutes). During the encoding phase mice were placed into the testing arena with two identical objects, which in this case, were two identical blocks coated with sandpaper exhibiting equal grit sizes. Mice were placed back into cages during the rest phase while the testing arena was prepared. During the testing phase, mice were placed back into the testing arena with a habituated object (block from encoding phase) and an equal sized block coated with sandpaper exhibiting a different sandpaper grit size (novel object). The amount of interactions, which were defined as direct contact with facial whiskers, was recorded and the amount and duration of those interactions were quantified. Statistical analysis was done with student's *t*-test to measure the amount of interactions between the novel and habituated object; anything below $p=0.05$ was considered significant.

2.5 Immunohistochemistry

Mice were transcardially perfused approximately 2.5 hours after the termination of the last RS session with 11 ml of 0.01M PBS and 20 ml of 4% PFA. Isolated brains were then stored overnight in 4% PFA solution in the fridge. Coronal brain slices were cut by a vibratome (Leica, VT1000S) at 50 μm and

stored in PBS at 4 degrees. Brain slice selection was standardized by selecting the first 2 coronal sections 250 microns posterior to the anterior commissure which allowed for maximum visualization of the Barrel Cortex (BC). The number of animals used and the number of cells and or density values collected varied between the density, somatic, and process analysis (for more details see Supplemental Table 1). The Allen Brain Atlas was then used to identify brain slices containing the barrel cortex. Selected slices were pretreated with hydrogen peroxide (70%), blocked with 7% normal donkey serum (Jackson Immuno) for 30 minutes, then incubated in goat-anti Iba1 (Abcam, 1:1000 dilution) on a cold rotator for 72 hours. Then, tissue was treated with a secondary antibody, biotinylated donkey-anti goat (Jackson Immuno, 1:200 dilution) for 2 hours at room temperature (RT), followed by 2 hours of ABC Elite solution (Vector labs). Lastly, the sections were incubated with 3,3' Diaminobenzidine substrate for 2.5 minutes, mounted on gelatin coated slides and left to dry overnight. Mounted sections were then dehydrated, defatted with SafeClear (Fisher Scientific) and cover-slipped with Permount (Fisher Scientific) and left to dry overnight at RT.

2.5.1 Imaging and Analyses

Immuno-stained sections were imaged using a Zeiss scope (Imager M2) microscope. Neurolucida software (version 11.0) was used to obtain and analyze images. Iba1+ density analysis was conducted by taking 2 single plane images with a 20X (NA=0.8) for BC cortical layers 2-5 which allowed for maximum visualization of Iba1+ cells. Process and somatic data were taken with a 63X

(NA=1.4) oil objective.

2.5.2 Defining Boundaries

Boundaries were defined by using Neurolucida 11.0 software under the Zeiss Imager M2 microscope. Once the cortex was clearly focused at 5X, anatomical structure observed under the microscope was matched with anatomical structure available in the Allen Brain Atlas (2011) to ensure the correct identification of the barrel cortex. Cortical layers 2-5 were then defined as separate boundaries within the cortical plate of the barrel cortex.

2.5.3 IBA1 + Density

Iba1+ density data was obtained using a 20X (0.8 NA) objective by taking a single plane image and counting the number of Iba1+ cells in which both the soma and process were visible within a defined boundary. Density analysis was done separately for each cortical layer. Density values were calculated as the total number of Iba1+ cells divided by the area boundary, expressed in Iba1+ cells per mm^2. Two images were taken for each cortical layer and separately analyzed resulting in two separate density values for each cortical layer per mouse (for more details see Supplemental Table 1). For each layer, the two obtained density values were averaged to result in a single density value for each experimental mouse.

2.5.4 Microglial Reconstruction

Microglia in cortical layers 2-5 of the S1BF were reconstructed with Neurolucida morphometric software and observed under 63X (NA=1.4) and

imaged by taking a z-stack at .28 μm increments that allowed for the visualization of an entire microglia including spanned out extracellular processes. Five individual microglia which exhibited intact processes that were not obscured by background cells were randomly selected and traced throughout the entirety of the obtained cortical section. Neurolucida was used to obtain morphological data for each cortical layer (also see Figure 3A, B). The number of included animals vary between the density, somatic, and process morphology analyses (for more details see Supplemental Table 1).

2.5.5. Generation of Morphological Data

Morphological metrics were obtained in Neurolucida explorer (version 11) which generated somatic and process data. For somatic data, the following metrics were used: 1. area, 2. perimeter, 3. feret maximum (the longest dimension of a cell body), 4. feret minimum, (longest somatic length perpendicular to feret maximum). For process data, the following were included: 1. process length, 2. branch quantity (amount of primary branches extending from soma), 3. process nodes (point at which primary branch splits into two or more branch points), 4. end quantity (amount of secondary and tertiary branches extending from each node), and 5. process length (the length total length divided by the total quantity of branches) (also see Figure 3C).

2.6. Statistics

Experimental group significance was analyzed with alpha-value set *a priori* at 0.05; all p- values less than 0.05 were considered statistically significant.

Microglial somatic and process statistics were performed using JMP (Version 14) software. Statistical significance was determined with a Student's *t*-test for density data between stressed and control conditions from the same layer and a mixed model Analysis of Variance (ANOVA) test was used for inter-group analysis between different S1BF layers. ANOVA testing was also used for process and somatic data. All significant ($p<0.05$) ANOVA metrics were followed up with a Tukey HSD test to determine which pairs were significant.

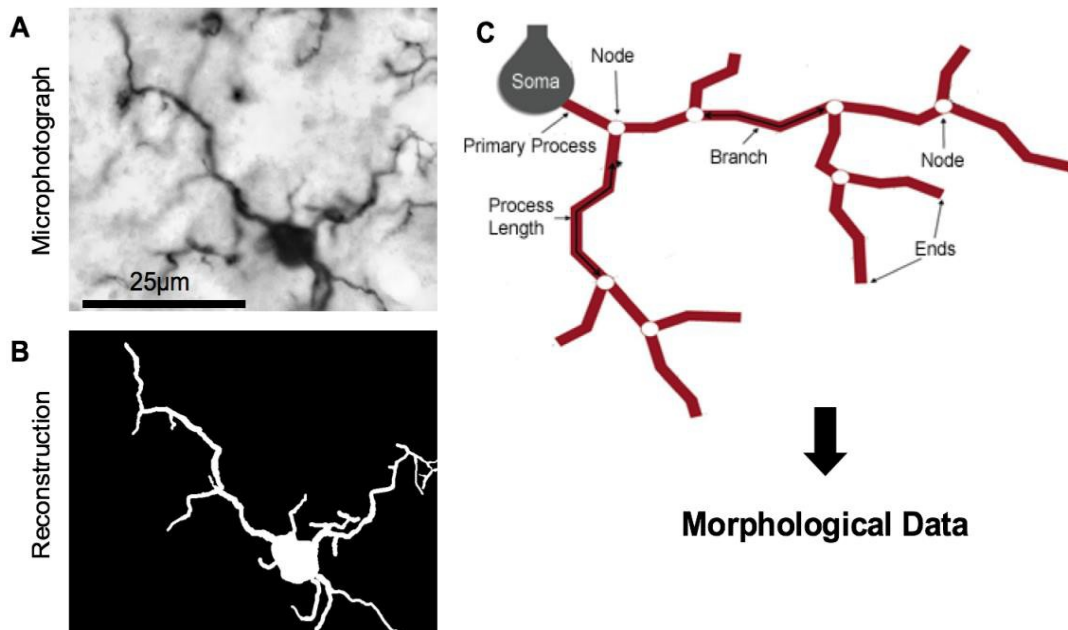
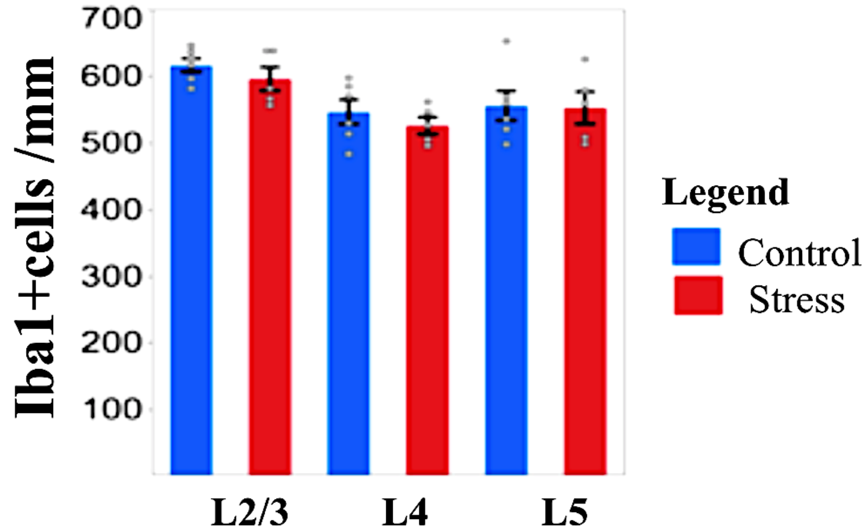


Figure 3. Neurolucida Reconstruction of Microglia. A. An Iba1+ cell in S1BF layer 4 taken under 63X (NA=1.4) objective. Scale bar= 50 µm **B.** Reconstruction of the same Iba1+ cell in 3A. **C.** Schematics of morphological metrics evaluated in this present study. Illustrative credit for **Figure 3 A-B**: Jessica Keating, 2019. Illustrative credit for **Figure 3.C**: adapted from Rietveld et al., 2015

CHAPTER 3
RESULTS

3.1. Stress Does Not Alter the Density of IBA1+ Cells in S1BF



We first wanted to see whether or not 7d RS is sufficient to induce a change in the quantity of S1BF microglia. We therefore measured Iba1+ cell densities. This analysis revealed that regardless of layer, 7d RS does not impact (p's>0.05) S1BF microglial density in cortical layers 2-5 (Figure 4, also see Table 1).

Figure 4. 7d RS Does Not Change S1BF Microglial Cell Density in Mouse Barrel Cortex Layers 2-5. Each dot represents the average score of two density recordings for an individual mouse. Error bars indicate SEM values.

	L2/3	L4	L5
Control (6)	616.153 (9.769)	546.217 (18.220)	555.598 (21.962)
7d RS (5)	595.616 (17.706)	525.822 (12.711)	552.400 (24.079)

Table 1. SBF1 Iba1+ Densities. Data expressed as Mean (SEM). The number of included animals is indicated to the right of the condition group on the left axis in parenthesis.

3.2 Stress Increases Microglial Soma Size While Minocycline Decreases it

Our next objective was to determine whether or not 7d RS impacted the size and shape of microglial somas, because previous work suggested that enlarged microglia somas could be a hallmark for microglial state changes (Jonas et al., 2012). We therefore traced microglia from each experimental group in S1BF cortical layers 2-5. Moreover, due to recent work showing a heterogeneous CNS microglial population (Hammond et al., 2019), we assessed distribution normality and morphological patterns from each experimental group across cortical layers 2-5.

3.2.1 Stress Increases Layer 2/3 Somatic Size and Elongation in S1BF and Minocycline Decreases Somatic Area

One-way ANOVA testing revealed that somatic perimeter (F_{3-716}= 21.26, $p<0.0001$) area ($F_{3,716}$= 16.12, $p<0.0001$), and feret maximum ($F_{3,716}$= 19.04, $p<0.0001$) were significantly impacted amongst experimental groups. Post-hoc testing (Tukey HSD) revealed that in comparison to controls 7d RS increased cell body size which was indicated by somatic perimeter ($p<0.0001$) and area ($p<0.01$). 7d RS also impacted microglial shape by increasing somatic elongation which was indicated by an increased feret maximum ($p<0.0001$), without affecting feret minimum, indicating that RS makes microglial somas longer following stress in comparison to controls. These changes are reflected in distribution plots which show that RS induces a wider and right-shifted distribution in comparison to controls.

All these effects induced by RS can be prevented by administration of

minocycline, as the RS animals that were treated with minocycline exhibited similar levels to control animals (p’s>0.05). 7d administration of minocycline by itself, *per se*, did not have a significant effect in impacting the somatic perimeter, or feret maximum/minimum values (p’s>0.05), though, there is a slight right-shift in distribution in comparison to controls. However, in terms of area, both unstressed and stressed animal groups treated with minocycline exhibited a decreased cell body size (for more details see Figure 5 and Table 2.

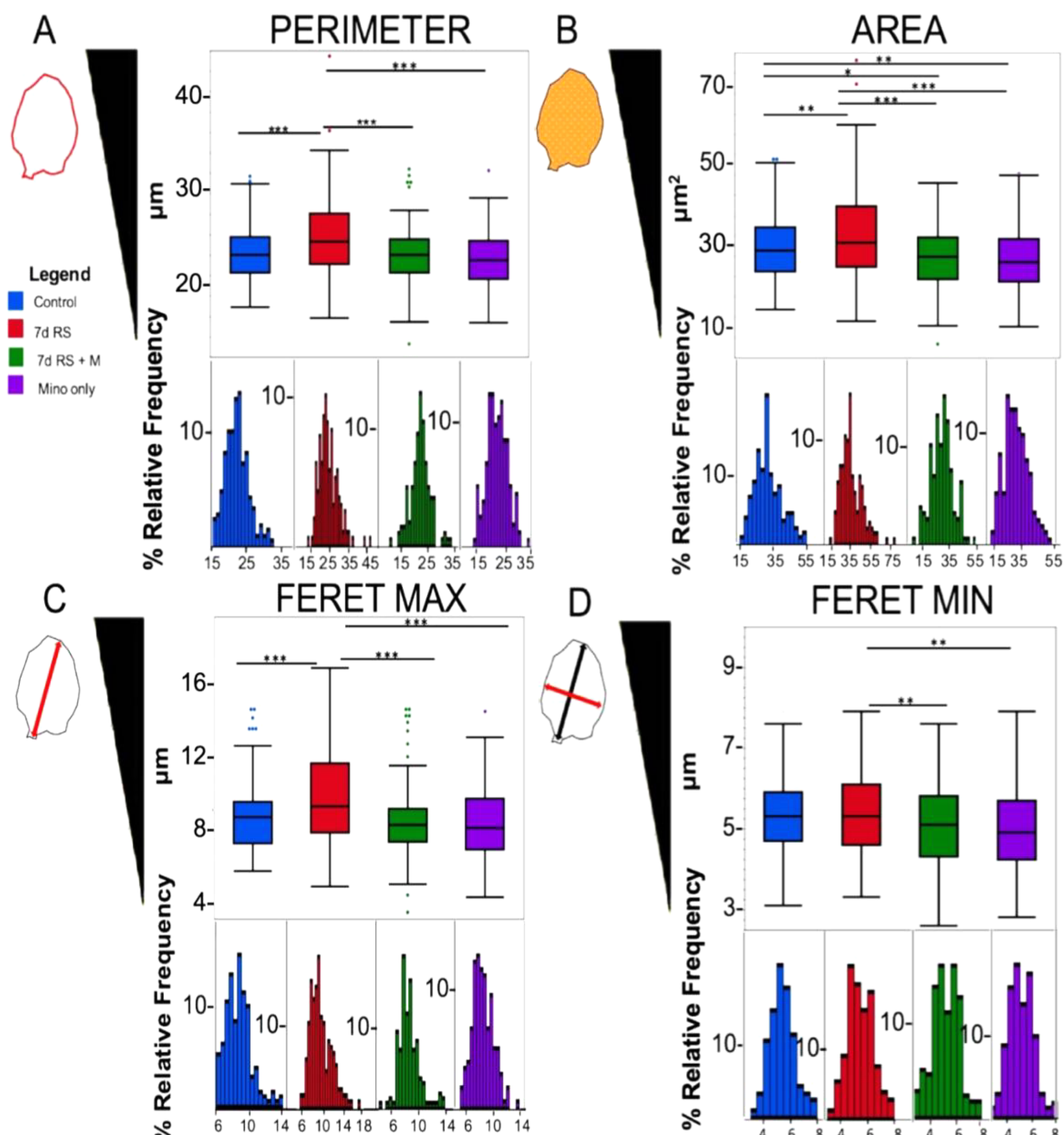


Figure 5. Quantitative Analysis of Layer 2/3 Microglial Somatic Morphology and Distribution. A. Perimeter (µm). **B.** Area (μm^2). **C.** Feret Maximum (µm). **D.** Feret Minimum (µm). Data shown as box and whiskers plot. Drawings to the left of the Y-axis represent the metric being quantified. Individual frequency distribution plots for each condition are directly below box and whisker plots. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the designated metric. The box represents the middle 50% of data points. Whiskers represent minimum and maximum data points. Colored dots above and below the whisker plot represent statistical outliers. Data quantified by means of ANOVA followed by Tukey HSD test. *p<0.05, **p<0.01, ***p<0.001

Somatic Metrics

	Control (3)	7D RS (3)	RS+M (3)	Mino Only (3)
Perimeter (μm)	22.695 (0.230)	24.833 (0.345)	22.359 (0.276)	21.948 (0.262)
Area (μm^2)	31.959 (0.576)	34.827 (0.759)	29.565 (0.640)	29.169 (0.602)
Feret Max (μm)	8.609 (0.107)	9.588 (0.151)	8.468 (0.124)	8.396 (0.121)
Feret Min (μm)	5.358 (0.065)	5.377 (0.070)	5.037 (0.084)	5.026 (0.073)

Table 2. Microglial Somatic Metrics Layer 2/3. Data expressed as Mean (SEM) The number of included animals is indicated to the right of each experimental group in parenthesis.

3.2.2 Stress Increases Layer 4 Somatic Area in S1BF and Minocycline Decreases Somatic Size and Elongation in RS+M Group

The multivariate analysis revealed that somatic area ($F_{3,233}=22.02$, $p<0.0001$), somatic perimeter ($F_{3,233}=16.91$, $p<0.0001$), feret maximum ($F_{3,308}=11.20$, $p<0.0001$), and feret minimum ($F_{3,308}=5.85, p<0.0001$) were significantly impacted amongst the included groups. Similar to what was found in layer 2/3, post-hoc testing revealed that RS increased somatic area ($p<0.05$) without impacting feret minimum, indicating that the increase in soma size was not from an increase in cell width. Interestingly, unlike in layer 2/3, RS did not induce significant effect upon somatic perimeter ($p=0.12$) and feret maximum ($p=0.16$). However, the noted increase in somatic area paired with the noticeable right-shifted distribution in feret maximum could indicate that while collectively layer 4 microglia are not exhibiting all of the RS typified changes observed in layer 2/3, certain subpopulations within this group are still exhibiting significant morphological changes.

Minocycline paired with RS exhibited a decreased somatic perimeter

(p<0.0001) which was not seen in the Minocycline only group. Somatic area was decreased in both RS+M (p<0.0001) and Mino Only (p<0.01) indicating that Minocycline induces a shrinking effect.

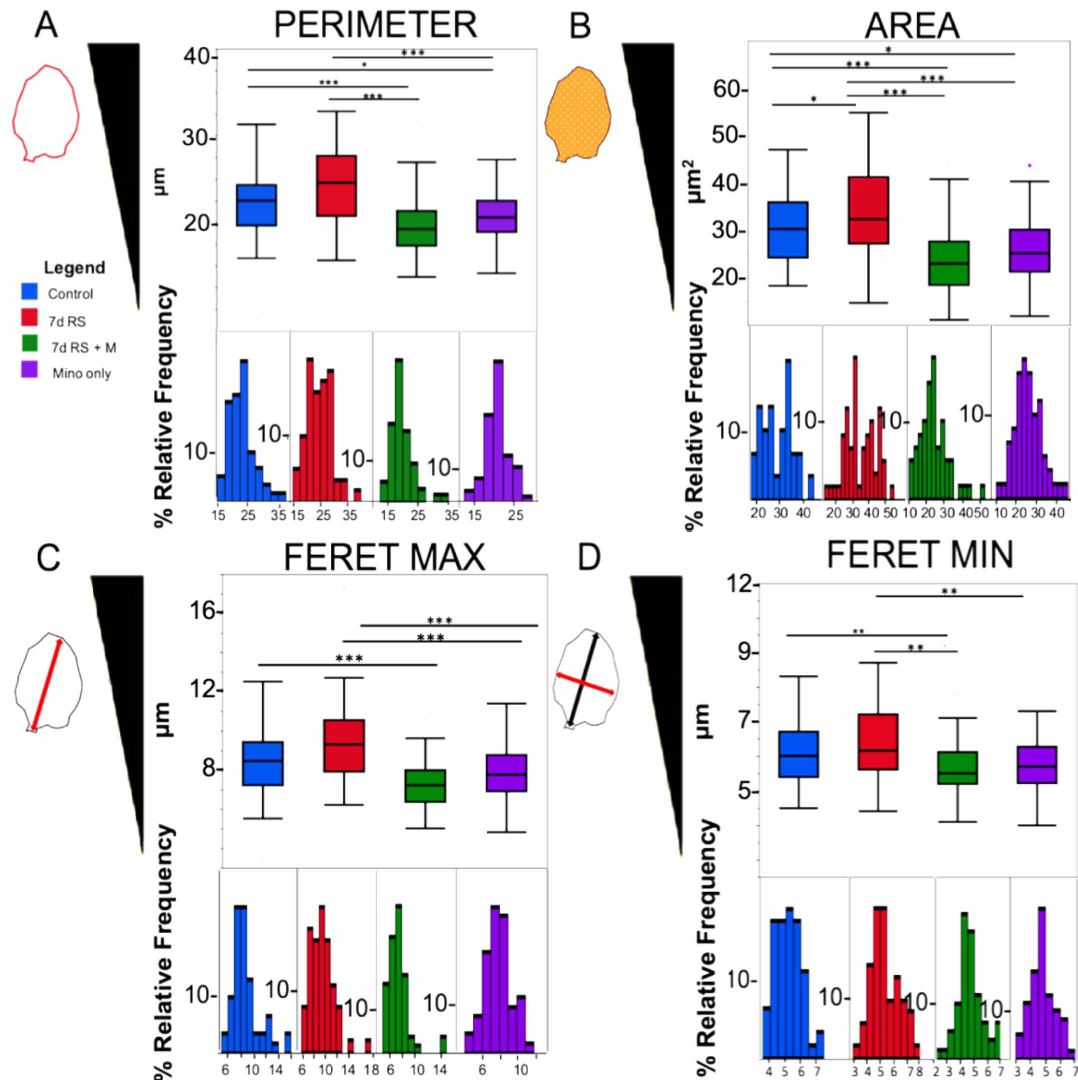


Figure 6. Quantitative Analysis of Layer 4 Microglial Somatic Morphology and Distribution. A. Perimeter (µm). **B.** Area (µm^2). **C.** Feret Maximum (µm). **D.** Feret Minimum (µm). Data shown as box and whiskers plot. Drawings to the left of the Y-axis represent the metric being quantified. Individual frequency distribution plots for each condition are directly below box and whisker plots. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the measured metric. The box represents the middle 50% of data points. Whiskers represent minimum and maximum data points. Colored dots above and below the whisker plot represent statistical outliers. Data quantified by means of ANOVA followed by Tukey HSD test *p<0.05, **p<0.01,***p<0.001

Somatic Metrics

	Control (3)	7D RS (3)	RS+M (3)	Mino Only (3)
Perimeter (μm)	22.824 (0.526)	24.430 (0.345)	19.710 (0.493)	20.870 (0.435)
Area (μm^2)	30.491 (0.991)	34.437 (0.759)	23.678 (1.063)	25.635 (0.854)
Feret Max (μm)	8.790 (0.272)	9.497 (0.269)	7.410 (0.224)	7.878 (0.187)
Feret Min (μm)	5.120 (0.110)	5.328 (0.132)	4.624 (0.124)	4.735 (0.101)

Table 3. Microglial Somatic Metrics Layer 4. Data expressed as Mean (SEM) The number of included animals is indicated to the right of each experimental group in parenthesis.

3.2.3 Stress Increases Layer 5 Somatic Perimeter and Elongation in S1BF and Minocycline+RS Decreases Somatic Area

The multivariate analysis revealed that somatic perimeter ($F_{3,308}=8.85$, $p<0.001$), area ($F_{3,308}=7.11$, $p<0.0001$), and feret maximum ($F_{3,308}=11.20$, $p<0.0001$) were significantly impacted amongst the included groups. Similar to layer 2/3, post-hoc testing revealed that RS increased microglial somatic perimeter ($p<0.05$), but surprisingly, does not impact overall somatic area. Moreover, as seen in layer 2/3, feret maximum values increased following stress ($p<0.01$) indicating that the increase in somatic perimeter likely came from an increase in somatic elongation. As seen in S1BF layers 2/3 and 4, minocycline paired with 7dRS decreased somatic area ($p<0.05$). This effect appears to be the result of a slight, but not significant decrease in feret minimum ($p=0.06$) which is evident in slight left-shifted distribution. (For more details see Figure 7 and Table 4)

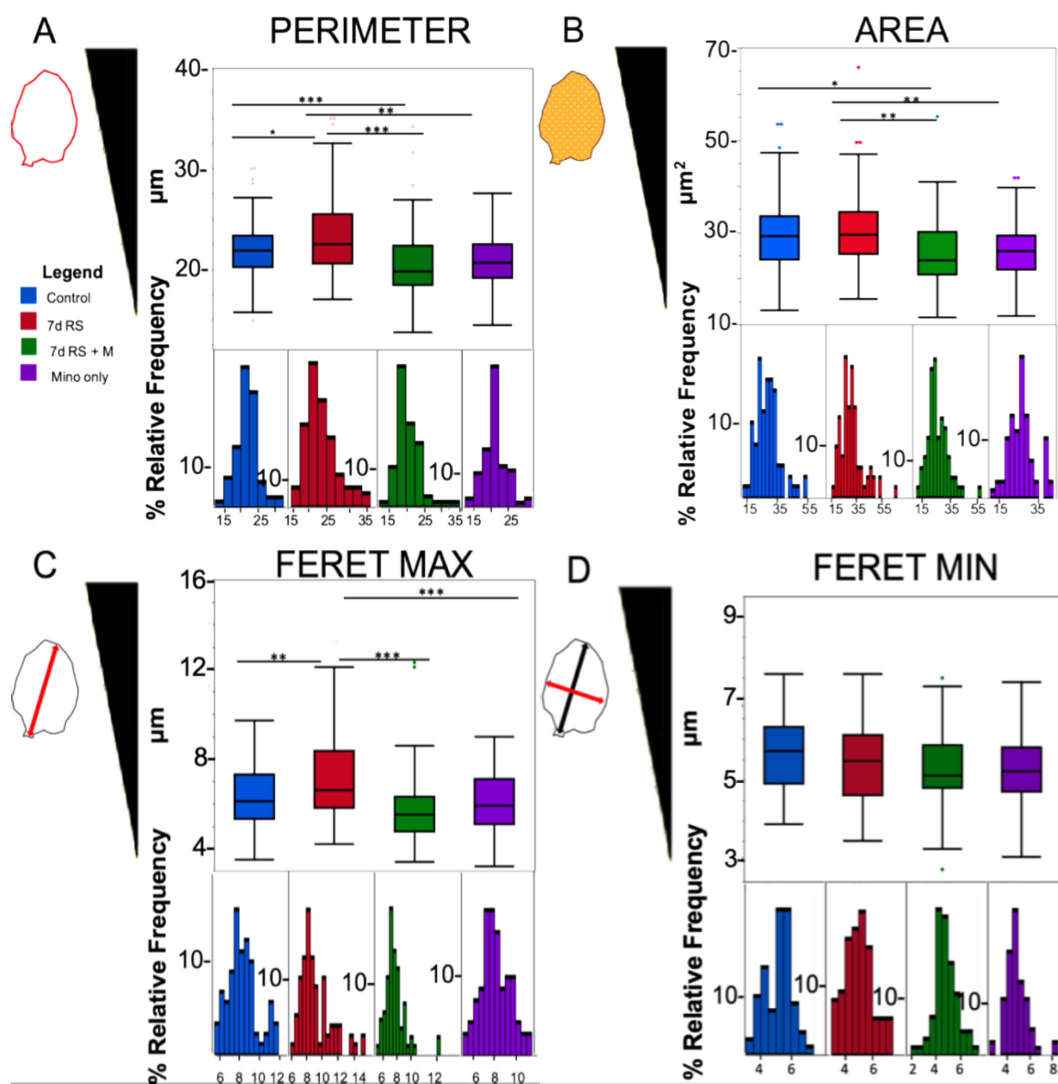


Figure 7. Quantitative Analysis of Layer 5 Microglial Somatic Morphology and Distribution. A. Perimeter (µm). **B.** Area (µm²). **C.** Feret Maximum (µm). **D.** Feret Minimum (µm). Data shown as box andwhiskers plot. Drawings to the left of the Y-axis represent the metric being quantified. Individual frequency distribution plots for each condition are directly below box and whisker plots. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the measured metric. The box represents the middle 50% of data points. Whiskers represent minimum and maximum values. Colored dots above and below the whisker plot represent statistical outliers. Data quantified by means of ANOVA followed by Tukey HSD test*$p<0.05$, **$p<0.01$, ***$p<0.001$

Somatic Metrics

	Control (3)	7D RS (3)	RS+M (3)	Mino Only (3)
Perimeter (μm)	21.918 (0.349)	23.390 (0.483)	20.622 (0.398)	21.139 (0.375)
Area (μm^2)	29.163 (0.896)	30.754 (1.032)	25.571 (0.902)	26.383 (0.750)
Feret Max (μm)	8.324 (0.169)	9.169 (0.222)	7.741 (0.184)	8.029 (0.153)
Feret Min (μm)	5.128 (0.096)	4.959 (0.113)	4.753 (0.104)	4.819 (0.104)

Table 3. Microglial Somatic Metrics Layer 5. Data expressed as Mean (SEM) The number of included animals is indicated to the right of each experimental group in parenthesis.

3.3 Stress Decreases Microglial Complexity

Our next objective was to determine whether or not 7d RS impacted the overall complexity of microglial processes because previous work suggested that changes in process morphology could indicate changes within functional activity (Chen et al., 2012a). We therefore traced microglia from each experimental group in S1BF cortical layers 2-5.

3.3.1 Stress Decreases Overall Microglial Complexity in S1BF Layer 2/3 Which is Blocked When Paired with Minocycline Administration

Previous studies have suggested that functional activity states of microglia may be indicated by the morphology of their cellular processes which span from the microglial somata (Hinwood et al., 2013). The multivariate analysis revealed that node quantity (F3,55= 18.16, p=0.0001), end quantity (F3,55= 12.51, p<0.0001), and process length (F3,55= 9.06, p<0.0001) were significantly affected between groups. Post-hoc testing revealed that while 7d RS did not impact the number of total processes spanning from the soma in comparison to controls, it did decrease the overall microglial complexity which was indicated by

decreases in node quantity (p<0.001), end quantity (p<0.01) and process length (p<0.001). Similar to what was seen in the somatic analysis, RS induced changes were reversed when paired with minocycline treatment resulting in similar process quantity, node quantity, end quantity, and process length compared to controls (p's>0.05). Interestingly, when administered to unstressed mice, minocycline by itself increased node quantity in comparison to control conditions (p<0.001). (for more details see Figure 8-9 and Table 5)

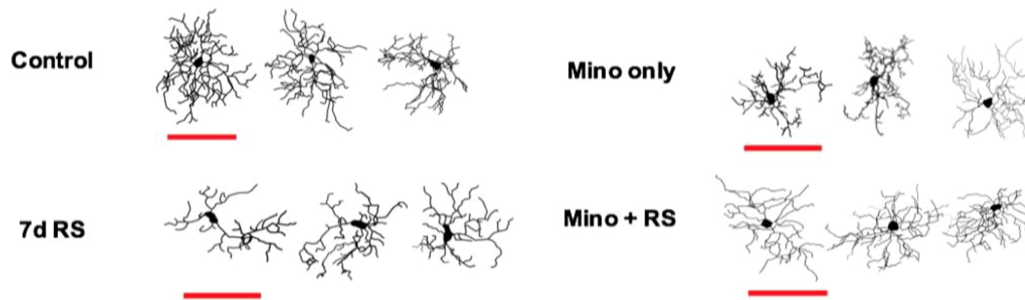


Figure 8. Representative S1BF Layer 2/3 Microglial Tracings Reconstructions

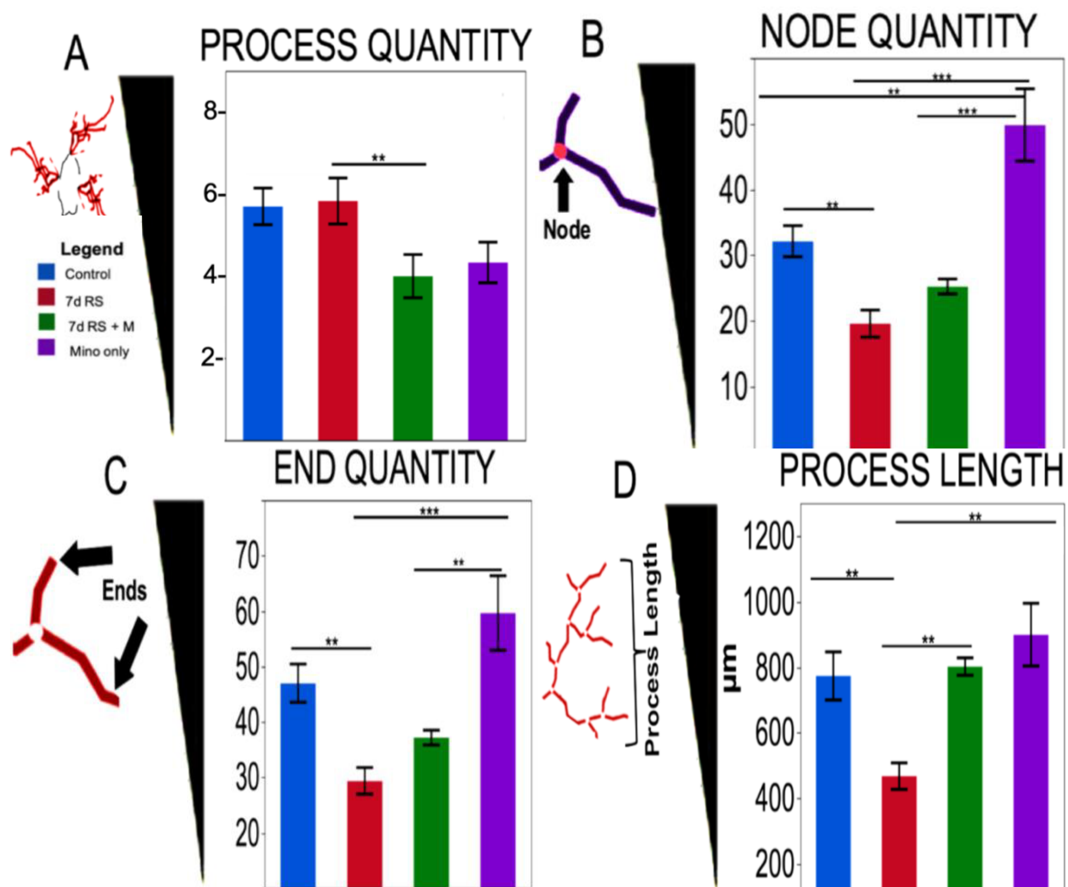


Figure 9. Bar Graph Depicting Layer 2/3 Microglial Process Complexity. A. Process quantity. **B.** Node quantity. **C.** End quantity. **D.** Process length (µm). Data shown as a bar graph. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the measured metric. Error bars represent SEM. Data quantified by means of ANOVA followed by Tukey HSD test. *p<0.05, **p<0.01, ***p<0.001

Process Metrics

	Control (5)	**7D RS (4)**	**RS+M (3)**	**Mino Only (3)**
Process Qty	7.700 (0.488)	7.833 (0.562)	6.000 (0.526)	6.333 (0.494)
Node Qty	32.150 (3.377)	19.611 (2.058)	25.267 (1.136)	49.833 (5.486)
End Qty	47.000 (3.436)	29.389 (2.388)	37.200 (1.332)	59.667 (6.721)
Process Length (µm)	773.425 (73.591)	467.983 (40.205)	802.100 (26.541)	899.433 (95.678)

Table 5. Microglial Process Metrics Layer 2/3. Data expressed as Mean (SEM) The number of included animals is indicated to the right of each experimental group in parenthesis.

3.3.2 Stress Decreases Overall Microglial Complexity in S1BF Layer 5 Which is Blocked When Paired with Minocycline Administration

Multivariate testing revealed that overall process complexity was impacted amongst groups which was indicated by significant alterations within node quantity ($F=3,43=20.95$, $p<0.0001$), end quantity ($F=3,43=24.55$, $p<0.0001$), and process length ($F=3,43=21.98$, $p<0.0001$). As seen in S1BF layer 2/3, post-hoc testing revealed that RS did not impact the number of primary processes spanning from the microglial soma ($p>0.05$), but decreased overall microglial complexity which was indicated by decreases in node quantity ($p<0.0001$), end quantity ($p<0.0001$), and process length ($p<0.0001$). Moreover, in comparison to controls, RS+M induced increases in process node ($p<0.05$) and end quantity ($p<0.01$).

While a different effect was seen in layer 2/3 process metrics, where the minocycline only group exhibited an increased process complexity (indicated by node and end quantity) in comparison with RS+M (p's<0.01), layer 5 RS+M microglia exhibited slight, but not significant ($p=0.87$) increase in the quantity of primary processes spanning from the microglial somata which could explain the increase in microglial complexity between both minocycline treated groups. (for more details see Figure 10 and Table 6).

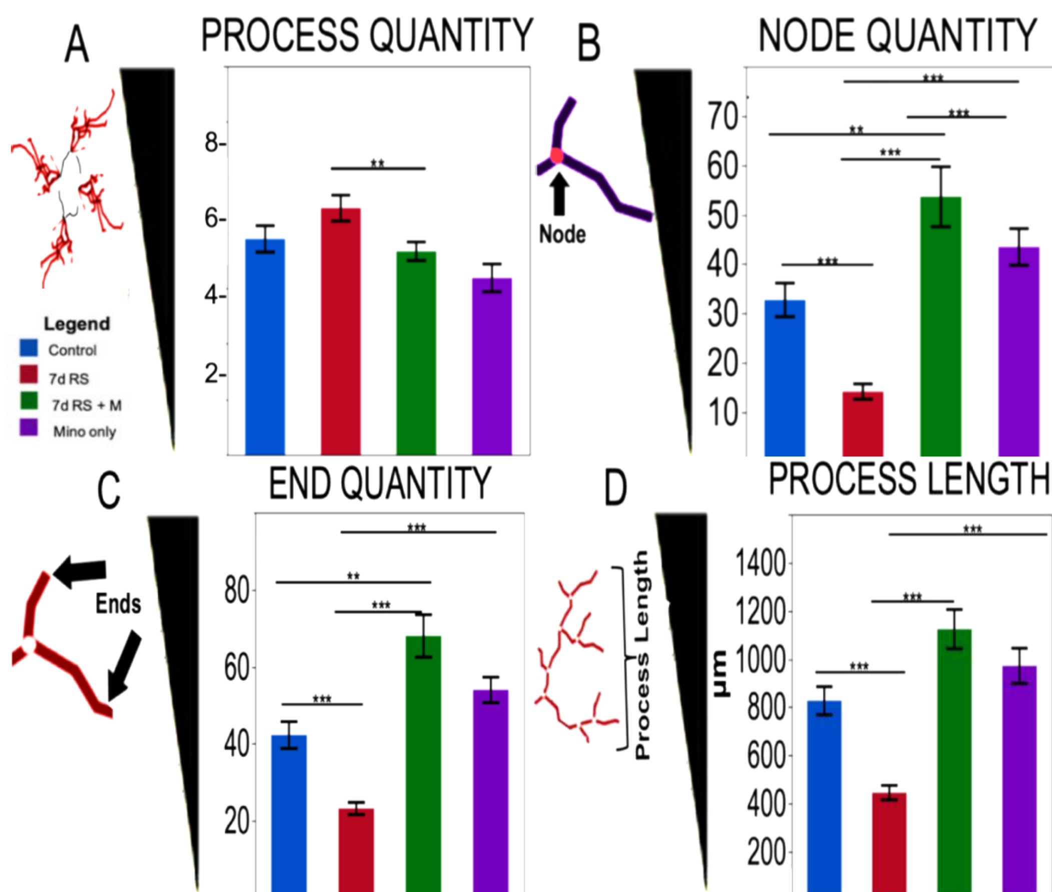


Figure 10. Bar Graph Depicting Layer 5 Microglial Process Complexity. A. Process quantity. **B.** Node quantity. **C.** End quantity. **D.** Process length (µm). Data shown as a bar graph. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the measured metric. Error bars represent SEM. Data quantified by means of ANOVA followed by Tukey HSD test. *p<0.05, **p<0.01, ***p<0.001

Process Metrics

	Control (5)	**7D RS (4)**	**RS+M (2)**	**Mino Only (3)**
Process Qty	7.778 (0.475)	8.889 (0.464)	7.333 (0.333)	6.375 (0.498)
Node Qty	32.778 (3.377)	14.222 (1.548)	53.667 (6.064)	43.500 (3.698)
End Qty	42.278 (3.477)	23.222 (1.577)	68.000 (5.508)	54.000 (3.290)
Process Length (µm)	827.417 (58.750)	446.028 (29.943)	1124.767 (81.076)	972.433 (95.513)

Table 5. Microglial Process Metrics Layer 5. Data expressed as Mean (SEM) The number of included animals is indicated to the right of each experimental group in parenthesis.

3.4 Stress Abolishes Whisker Dependent Discrimination Task Performance

While we knew from previous work (Chen et al., 2018) that 7d RS abolished the ability for whisker-dependent sensory discrimination ($p<0.001$), our next objective was to determine whether or not the noted behavioral change was associated with changes in microglial somatic and process morphology. To do this we assessed the performance of mice that were administered minocycline while undergoing 7dRS as well as mice administered with minocycline without 7d RS treatment.

3.4.1 Stress Abolishes Whisker Dependent Discrimination Task Performance Which is Blocked with Minocycline Administration

The behavioral results were consistent with previously published work showing that 7d RS abolished the ability for whisker dependent sensory discrimination (Chen et al., 2018). In comparison to control mice 7d RS significantly decreased whisker dependent sensory discrimination ($p<0.001$). Interestingly, 7d RS mice administered with Minocycline exhibited sensory discriminatory levels comparable to those of control mice ($p>0.05$) indicating potential microglial involvement within the stress induced discriminatory defects. No difference was observed between control mice and mice administered with minocycline without 7d RS treatment ($p>0.05$) suggesting that minocycline by itself does not have an impact on whisker dependent sensory discrimination.

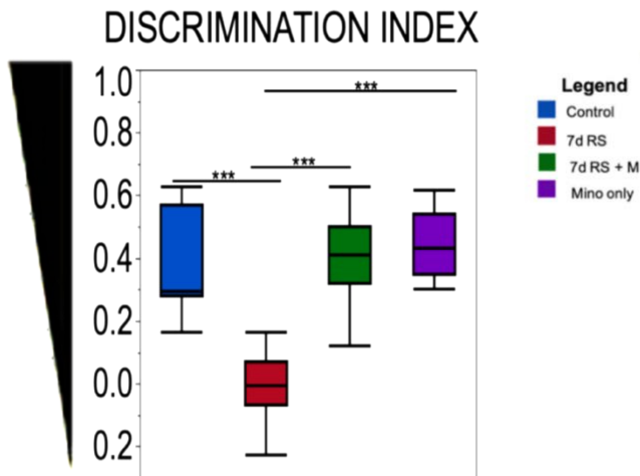


Figure 11 Minocycline Administration Prevent Stress-induced Whisker-discrimination Task Deficiencies. Data shown as box and whiskers plot. The Y-axis represents discrimination index scores and the X-axis indicates the experimental groups included for analysis. The box represents the middle 50% of data points. Whiskers represent minimum and maximum values. Data quantified by means of Student's t-test.***p<0.001

Behavioral Metrics

	Control (11)	**7D RS (13)**	**RS+M (11)**	**Mino Only (9)**
Discrimination index	0.389 (0.048)	0.002 (0.030)	0.393 (0.043)	0.719 (0.036)

Table 7. Discrimination Index Metrics. Data expressed as Mean (SEM). The number of included animals is indicated to the right of each experimental group in parenthesis.

CHAPTER 4
DISCUSSION

4.1 Summary and Interpretation of Results

To assess the potential role of microglia in the stressed-induced S1BF dendritic elimination and abolished sensory discrimination (Chen et al., 2018), we investigated how stress affects the microglial morphology and if prevention of microglial activity through minocycline administration could block the stress-induced microglial morphology changes. This analysis revealed that 7d RS impacts microglia in the following ways.

1) 7d RS impacted microglial somatic morphology across S1BF layers 2-5 by increasing somatic size, as measured by increases in somatic area and or perimeter. Moreover, with the exception of S1BF cortical layer 4, 7d RS also induced somatic elongation, which was indicated by an increased feret maximum. Taken together, these morphological changes are consistent with previous work relating the morphological alterations with inflammation-associated microglial activities, such as the engulfment of neuronal debris (Morsh et al., 2015). The stress induced somatic elongation is an especially interesting finding and is consistent with recent work characterizing a distinct microglial phenotype referred to as “rod- microglia”. This microglial type is known to be especially prominent in the S1BF and has been morphologically characterized to exhibit an elongated soma, little to no planar microglial primary processes, and a decrease in secondary process branching (Ziebell et al., 2012). They are also functionally characterized to play a significant

role in axonal damage and recovery (Taylor et al., 2014). Moreover, recent work in the S1BF observing the circuitry reorganization following diffuse brain injury noted the presence and accumulation of "rod" microglia, potentially implicating their involvement in the observed synaptic changes (Zeibell et al., 2017, Ziebell et al., 2012, Taylor et al., 2014, Cao et al., 2012). This association is further supported and made especially relevant to the focus of the current study, through a recent study showing that mice subjected to diffuse brain injury exhibited an accumulation of "rod" microglia in the S1BF which aligned to apical dendrites of excitatory pyramidal neurons and was correlated with axonal degeneration (Witcher et al., 2018). Additionally, investigation into protein expression on "rod" microglia following diffuse brain injury observed that these microglia express CD68, which labels the lysosomes and is used as a marker for phagocytosis (Ziebell et al., 2012), further implicating the potential role of this microglial phenotype in the engulfment of varied neuronal debris.

While it is currently unknown whether a specific neuron-microglia signaling pathway meditates the transition from a non-inflammation typified microglial phenotype to the aforementioned "rod" phenotype, several bi-directional generalized neuron-microglia crosstalk mechanisms have been identified. For example, neurons have been shown to activate microglia through the release of ATP during periods of neuronal firing (Fontainhas et al., 2011., Eyo et al., 2014), and microglia have been shown to regulate neuronal activity through direct projection of microglial processes to the neuronal soma (Li et al., 2012).

Another well studied neuron-microglia crosstalk mechanism involves CX3CR1 ubiquitously expressed on microglia) /CX3CL1 (ubiquitously expressed on neurons) fractalkine signaling (Lyons et al., 2009, Cardona et al., 2006). Whether or not the aforementioned neuron-microglia crosstalk mechanisms are utilized in "rod" microglial, or whether the method of crosstalk has yet to be identified, future studies would greatly benefit from further investigation into crosstalk mechanisms utilized amongst specific microglial phenotypes, such as "rod" microglia.

2) Administration of minocycline in stressed animals rescued the stress-induced microglial morphological changes, with the exception of S1BF layer 5, in which minocycline treated mice exhibited decreased somatic area in comparison to controls. Additionally, minocycline treatment by itself resulted in a decrease in overall cell body size (measured by area and or perimeter) which could indicate that one of the ways minocycline is acting on microglia is through a suppression of phagocytic activity, which alters somatic size due to the increased cellular debris taken into the microglial lysosome (Morsch et al., 2015).

3) Stress profoundly impacted microglial process morphology in the S1BF by decreasing overall process complexity. This impact was solely mediated through secondary and tertiary nodes, for the quantity of primary processes spanning from the microglial soma remained unchanged. Moreover, 7d RS also decreased overall process length in all cortical layers in S1BF. This stress-induced retraction and reduced complexity of microglial processes follows a similar

pattern with published work characterizing the morphological transformation occurring when microglia undergo phagocytosis or respond to brain injury (Cho et al., 2006, Davalos et al., 2005, Taylor et al., 2014).

4) Minocycline prevented stress-induced shortening of microglial processes, and in some instances, minocycline actually increased process length and complexity and decreased somatic size in comparison to controls. This indicates that the effect of minocycline can actually overpower the effects of stress. It may be informative for future research to conduct a study investigating how microglia morphology alters by varying minocycline dosages. Moreover, the noted morphology effects of minocycline may shine some light on the mechanism in which it is able to suppress microglial activity.

Additionally, the observed increase in branch complexity following minocycline administration could indicate that minocycline is increasing the "sensing" typified functions of microglia, such as the continuous extension and retraction of processes to monitor extracellular changes, and blocking the more "reactive" typified microglial activities, such as chemotaxis, which involves the retraction and decreased process ramification to allow for inflammation directed movement (Morsch et al., 2015).

5) Minocycline rescued stress-induced sensory processing deficits measured by. performance in whisker-discrimination testing. When stressed animals were treated with minocycline, they behaved comparably to control mice (see Figure 11). This suggests that microglia could be playing an instrumental role

in stress induced sensory processing deficits.

4.2 Comparison with Previous Studies and Potential Mechanisms

Microglial characterizations under healthy and normal conditions have been well-studied (for a review see Gomez-Nicola and Perry., 2015). However, with respect to microglial changes under stressed conditions, little is known. This lack of research is especially sparse in regions such as the primary somatosensory cortices. Current published indings have been largely focusing on other brain regions such as PFC and hippocampus (see Calcia et al., 2016). Our results characterizing the impact of stress on S1BF microglia add to the current understanding by indicating a microglial role in the stress induced neuronal and behavioral detriments previously observed in S1BF (Chen et al., 2018). This finding is further supported by the fact that when stress was paired with minocycline, a known microglial suppressor which works to block typified "reactive" microglial responses (He at al., 2001), both the stress-induced spine loss (current data in the lab, unpublished) and behavioral abnormalities (Supplemental Figure 4) were comparable to control mice.

While experimental techniques such as *in vivo* imaging are required to identify the specific mechanisms, one potential mechanism to explain these changes could be that stress is causing microglial processes to make direct contact with S1BF Layer 5 dendritic spines and that upon said contact, microglia participate within the engulfment of dendritic material which results in a decreased spine density. However, the current study does not provide any

information to indicate the order in which this stress-induced sequence might be taking place. For example, it could be possible that stress is acting first on neurons and causing them to express distress associated signals on their cellular matrix, which could then trigger the response and chemotactic movement of nearby microglia and facilitate the direct contact between microglial processes and pyramidal neurons and allow for the engulfment of neuronal debris. Moreover, another possibility could be that stress could be first acting on microglia by causing them to release cytotoxic chemicals into the extracellular environment which could lead to the expression of distress associated messengers on nearby neurons, and the subsequent recruitment of nearby microglia which could then facilitate the engulfment of neuronal debris.

Interestingly, microglial "activation" may come in different forms depending on the brain region. For example, in the mPFC, chronic stress (3 weeks) has been shown to lengthen microglial processes and result in a "hyper-ramified" typified morphology that was coupled with deficits in working memory which was measured by performance in T-maze testing. However, similar to the current study findings, minocycline administration was sufficient to block the stress induced microglial morphology changes which was coupled with an improvement in the stress-induced behavioral alteration (Hinwood, et al.,2013). While the reasons behind the contrasting microglial morphological changes in response to stress in the mPFC and S1BF remain unknown, this could be indicative of a "multiple-hit" model in which microglia follow a morphological

continuum. Thus, during acute settings, microglia undergo a priming period with which, during periods of chronic insult, can develop into an exaggerated hyper-response (Fiebich et al., 2014). Alternatively, it is also possible that stress could impact the PFC and somatosensory microglia differently. It will be especially interesting for future research to explore this question through systematic investigation of microglial morphological changes within these two brain regions during acute, intermediate, and chronic stress. While the mechanism facilitating the varied microglial morphology response has yet to be discovered, research examining the inhibition-excitation balance has shown that microglia "activated" by the inflammatory agebt LPS induce a different impact upon sodium channel densities in bipolar and pyramidal-neurons, thereby increasing their excitability (Klapal et al., 2016). The differential impact of microglia upon inhibitory and excitatory neurons could provide a mechanism to explain the noted shift in E-I balance occurring in synapses in the stressed brain. Moreover, recent work in the S1BF found that microglia are capable of engulfing neuronal synapses through CX3CR1-CX3CL1 signaling (Gunner et al., 2019) which provides a mechanism to explain the neuron-microglia communication suspected to occur in the stress induced synapse elimination in S1BF (Chen et al., 2018).

4.3 Methodological Consideration

While the methods in this study improve upon previous experimental shortcomings associated with microglial thresholding, which result in broad and largely qualitative findings, this approach is not without its own challenges. Firstly, due to the spatial organization of cortical microglia, it is not uncommon

for the process of one microglia to overlap with the process of another. This can make distinguishing and correctly identifying the origin of each intertwining microglial process challenging and is one of the reasons why computer-based auto-tracing software, which are known to be vulnerable to misidentification errors (Meijering, 2010), have largely not been utilized for the tracing of microglial processes, which decreases the ability for large- scale data generation. Additionally, the length of microglial processes limits the amount of traceable microglia contained within 50 µm cortical slices. Furthermore, due to the extended time of manual microglial tracings, separating the microglial population into morphological or functional subgroups is further complicated. The field as a whole would greatly benefit from the development of a computer-based program that relies upon algorithmic an algorithmic approach such as machine-learning which could match the accuracy of manual tracing while generating computer-generated program which could meet the accuracy of manual tracing while generating substantially larger data-sets.

4.4 Functional Implications of Results

The work done in this study, and in earlier work (Hinwood, et al.,2013, Morsh et al., 2015) highlight the connection between microglial structure and function. While we stipulate that the noted changes in microglial morphology are evident of underlying functional activity such as the elimination of neuronal structures (Tremblay et al, 2010), the connection between microglial structure and function have implications far beyond stress research. Due to the role of microglia

within overall brain maintenance and defense, understanding the mechanisms facilitating these functions could provide the resources to strategically manipulating them for CNS benefit. For example, understanding the circumstances that facilitate microglia engulfment of neuronal structures could provide insight into why, in the case of Alzheimer's disease, they are not able to effectively clear up the amyloid buildup that leads to the deterioration of neuronal connections and the demoralizing loss of memory and cognitive function (Solito and Sastre, 2012). This same concept could be applied to turn off microglial engulfment of neuronal structures in multiple sclerosis (MS), which damages the myelin sheath around nerve cells and harms the signaling connection between the body and brain (Hammond et al., 2018).

Moreover, the aforementioned ability for microglia to differentially impact sodium densities in bipolar and pyramidal-neurons (Klapal et al., 2016) suggests potential influence over E-I balance and could provide a new mechanism to explore psychiatric conditions such as schizophrenia and autism spectrum disorders which are, in part, characterized by an altered E-I balance (Gao and Penzes, 2015). Currently, it remains unresolved whether the changes in the E-I balance is regulating the state changes of microglia, or vice versa. Future studies can benefit to carefully dissect this cause-and-effect relationship between microglial activation and E-I balance.

4.5. Future Directions

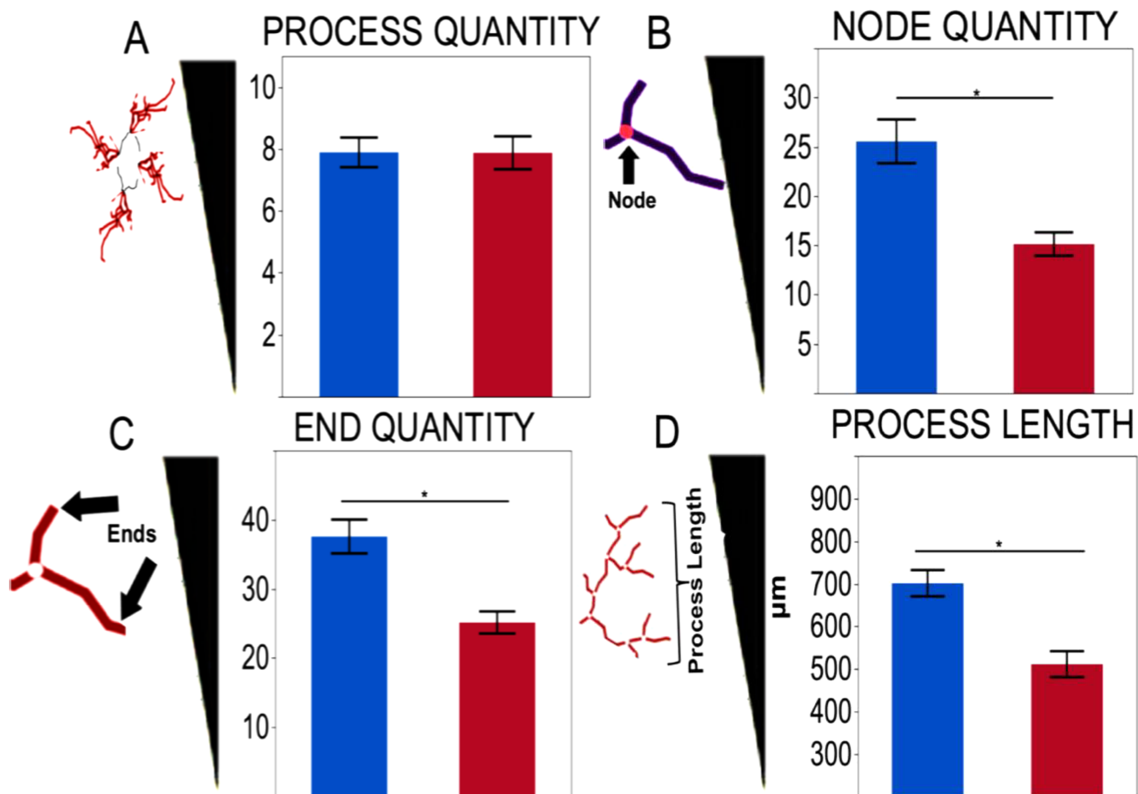
As previously mentioned, the large-scale changes required within microglial investigation are the creation of an advanced deep-learning typified computer program which can properly identify and track microglial morphological and immunoreactivity changes in real time for the generation of robust datasets. It is imperative that we are able to see the transition between microglial response and quiescence in order to develop a true understanding of microglial function, and the plethora of intricacies surrounding it. In regard to the current studies focus on assessing the role of microglia within stress induced synaptic and behavioral alterations, the next steps involve further investigation of the specific microglial mechanisms that could be facilitating the stressed induced changes. A particularly strong mode of approaching this could be through a combination of approaches such as FACS sorting to separate microglial subsets which could be differentially activated or prevalent under stressed conditions, multicolor brainbow labeling to assess whether or not stress alters the topology of the derived microglial subtypes, and genetic ablation to preferentially knock out particular microglial subtypes to determine their function within the stress induced synapse and behavioral changes. This approach would also address questions as to why the same stimulus does not necessarily evoke the same morphological response in microglial across different CNS regions and whether the variation in responses response is dependent upon the duration of the stressor (i.e. acute vs chronic), microglial sub-populations, or a combination of bo

APPENDIX

5.1 Supplementary Data and Figures

Analysis		Soma morphology			Branch analysis			Cell density analysis		
	Layer	Animal #	Soma #	Figure #	Animal #	Process #	Figure #	Animal #	Section #	Figure
Control	2/3	4	195	5	4	20	9	6	12	3
	4	3	59	6	4	18	Sup. 2	6	12	3
	5	3	80	7	4	18	10	6	12	3
RS	2/3	3	195	5	4	16	9	5	10	3
	4	3	60	6	3	16	Sup. 2	5	10	3
	5	3	80	7	3	17	10	5	10	3
RS+M	2/3	3	158	5	4	13	9	n/a	n/a	n/a
	4	3	60	6	n/a	n/a	Sup. 2	n/a	n/a	n/a
	5	3	80	7	2	3	10	n/a	n/a	n/a
Mino only	2/3	3	172	5	3	13	9	n/a	n/a	n/a
	4	3	60	6	n/a	n/a	Sup. 2	n/a	n/a	n/a
	5	3	80	7	3	7	10	n/a	n/a	n/a

Supplemental Table 1. Experimental Animal Information. Depiction of the included number of animals, microglial reconstructions, density counts per experimental group, and corresponding figure number.



Supplemental Figure 1. Bar graph Depicting Layer 4 Process Complexity. A. Process quantity**.** Node quantity. **C.** End quantity. **D.** Process length. Data shown as a bar graph. The Y-axis represents the relative population frequency (%) and the X-axis shows the range of values for the measured metric. Error bars represent SEM.*p<0.05

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