

Characterizing the association between hippocampal reactive astrogliosis, anhedonia-like behaviors, and neurogenesis in a monkey model of stress and antidepressant treatment

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SUMMARY

With the intention to better understand the biological mechanism of Major Depressive Disorder (MDD) and antidepressants, we looked at the effects of stress and selective serotonin reuptake inhibitors (SSRIs) on a measure of astrocyte reactivity in non-human primate (NHP) models of stress. We focused on hippocampal astrocytes because they regulate both neural activity and neurogenesis and have been characterized as a crucial cell type in regulating inflammation in response to stress. During stress, astrocytes become reactive and lose the ability to support neuronal function. In addition, reactive astrocytes can increase inflammatory signals and contribute to behavioral deficits associated with stress. For this project, we hypothesized that stress would lead to astrocyte reactivity in the NHP hippocampus, and that SSRI treatment would be associated with decreased astrocyte reactivity. We also hypothesized that astrocyte reactivity would be positively correlated with anhedonia-like behaviors and reduced neurogenesis in the hippocampus. In this study we show that chronic separation stress in NHPs leads to increased signs of astrogliosis in the NHP hippocampus. Furthermore, we show that measures of increased astrogliosis inversely correlate with adult hippocampal neurogenesis in NHPs, as well as with higher anhedonia scores. Overall, our findings were consistent with our initial hypotheses that hippocampal astrogliosis is an important mechanism in stress-induced cognitive and behavioral deficits. By observing the association between adult separation stress, SSRI treatments, and astrocyte reactivity in the NHP hippocampus, we aimed to provide insight into the non-neuronal cellular changes that occur in the NHP hippocampus during stress and antidepressant treatment.

INTRODUCTION

More than 300 million people in the world are living with Major Depressive disorder (MDD), both in severe and mild forms, according to the World Health Organization (1). MDD is a type of mood disorder, simply known as depression, with symptoms including depressed mood, feelings of hopelessness and guilt, decreased concentration, insomnia or hypersomnia, decreased interest in pleasurable stimuli, and recurrent thoughts of death and suicide (2). Although epidemiologic studies show that genes contribute to the risk for depression to an extent, vulnerability to depression also comes from environmental causes, such as stress and emotional trauma (3). Stress, in particular, is deeply associated with the development of MDD, as episodes of depression can be triggered by some form of stress. Currently, the most prescribed method of treating MDD is with SSRIs. These antidepressants inhibit serotonin reuptake transporters in the synapse, increasing the amount of serotonin signal transmission between neurons; however, the exact mechanisms through which SSRIs relieve MDD symptoms are not well understood (4), and in many cases, MDD patients are resistant to SSRI treatment (3). Thus, a deeper understanding of how stress leads to the cellular changes in the brain that may underlie MDD symptoms is crucial to the development of future treatments for depression.

Recently, inflammatory response to stress has been implicated in the pathophysiology of MDD in both clinical human studies and preclinical work with animal models of depression (5). Stressful experiences stimulate the release of hormones such as corticotropin-releasing hormone from the hypothalamus and activate cytokines and other signaling molecules from specialized glial cell-types that monitor and respond to changes in the extracellular environment (6). These substances in turn activate complex inflammatory immune responses in many different cell types in the brain that can alter the overall functional neuronal output of the region and contribute to the behavior symptoms of psychiatric disease. Emerging evidence shows that astrocytes are a crucial cell type in regulating this inflammation response to stress (7). Astrocytes are star-shaped glial cells that provide homeostatic control and trophic support to neurons within the

brain (8). These cells regulate neural activity by modulating levels of neurotransmitters and other signaling molecules in the synapse, and can thereby influence behavior (9, 10). During inflammatory responses, astrocytes can become reactive in a process called astrogliosis, and show features of hypertrophy and increased proliferation, which can cause loss of normal astrocyte function (8). Prolonged astrogliosis can activate a chronic inflammatory response in the surrounding cells, leading to abnormal neuronal function or neurotoxicity and cell death (11). Through cell counting studies, it has been reported that glial cell number and density are altered in many brain regions in human MDD, including the anterior cingulate cortex, the prefrontal cortex, and the amygdala (11-13). The hippocampus, a brain region that mediates various aspects of cognition, behavioral flexibility, and affective responses, is particularly important for the behavioral dysfunction observed in MDD. The hippocampus is also the only area of the adult brain that features neurogenesis, in which new neurons are integrated into the hippocampal circuitry to support cognitive plasticity (14).

Previously, our laboratory has utilized a NHP model of social isolation stress-induced MDD called adult-separation stress to investigate the cellular changes that occur during stress in the monkey hippocampus (15, 16). NHPs have brain structures similar to those of a human in terms of cortical layers, nuclear organization, connectivity patterns, and overall evolutionary conservation of functional areas (17). Importantly, the highly social nature of NHP models enables researchers to use social environmental challenges in order to study the effects of stress (18). Furthermore, our NHP model is based on groups of female cohorts. This is important because it allows us to probe the effects of stress specifically in females, and epidemiology data shows that MDD rates are approximately two times higher in women vs. men (19, 20). In our previous studies with NHPs, we showed that social isolation stress leads to anhedonia behaviors in monkeys, as well as lower rates of neurogenesis in the dentate gyrus of the hippocampus, compared to controls (15, 16). In addition, we observed that administering the antidepressant treatment fluoxetine, an SSRI, to animals post-stress prevented anhedonia behaviors and led to increased neurogenesis in the hippocampus, compared to stressed animals given a placebo (16). However, the effects of stress or fluoxetine on other cell-types in this region that may also contribute to behavioral changes, specifically astrocytes, were not examined.

For this project, we hypothesized that adult separation stress would lead to astrocyte reactivity in the NHP hippocampus, and that SSRI treatment would be associated with decreased astrocyte reactivity. We have utilized immunohistochemistry images taken of the NHP hippocampus from three groups of animals: controls, stress-placebo, and stress-fluoxetine. We analyzed these images for astrocyte reactivity by counting the number of astrocytes that were expressing glial fibrillary acidic protein (GFAP), a protein that is often used as a marker for hypertrophic/reactive astrocytes

because it is known to be upregulated during stress response, particularly in the hippocampus (21, 22). We then compared the number of hippocampal GFAP+ astrocytes in each group to first determine the effects of stress on astrocyte reactivity vs. controls and correlated these astrocyte cell counts with behavioral results from previously performed colony room behavioral observations. We also assessed the effects of SSRI treatment on astrocyte reactivity in stress-fluoxetine vs. stress-placebo vs. control animals and correlated these counts with stress-related behaviors and neurogenesis rates. Overall, we found that numbers of hippocampal GFAP+ astrocytes were related to stress experience, measures of neurogenesis, and behavioral anhedonia. These data will provide insight into the non-neuronal cellular changes that occur in the NHP hippocampus during stress and following SSRI treatment.

RESULTS

Adult separation stress significantly increases GFAP+ astrocyte number within the dentate gyrus of NHP hippocampus vs. controls

In our previous study, we utilized an adult separation stress paradigm on a cohort of female NHPs to study the effects of social isolation stress. In this paradigm, subjects in the stress group were exposed to alternating periods of social isolation (2 days) in a single-housing cage and group housing (5 days) in their colony pen for a total duration of 15-weeks, while controls remained in their group housing pen for 7 days of the week. Since previous research has reported that astrogliosis is reflected by an increase in the number of GFAP+ reactive astrocyte cells, we utilized a GFAP+ immunohistochemistry IHC stain to quantify the number of astrocytes with a visible cell body inside multiple cell layers of the NHP dentate gyrus, including the hilus, subgranular zone, and the granule cell layer (**Figure 1A**). We chose this region because it includes the active zone of neurogenesis in the hippocampus and may reflect the impact of astrogliosis on neurogenesis processes. In untreated animals exposed to adult separation stress (stress-placebo), we found that the GFAP+ astrocyte counts of stressed monkeys showed significantly greater abundance in number (average of 27.37 astrocytes/mm²) than those of controls that were not exposed to stress (average of 11.71 astrocytes/mm²) (One-Way ANOVA, Main effect of treatment, $p = 0.01$ with Tukey's post-hoc test showing significant difference between control-saline and stress-saline, $p = 0.008$, $n = 3,3$) (**Figure 1B**). This finding indicates that stress is indeed associated with astrogliosis, which may contribute to dysfunction of the hippocampus in these animals.

We also wanted to determine if SSRI antidepressant treatment with fluoxetine affects the number of GFAP+ astrocytes post-stress. Previous reports in rodents and human patients have stated that astrocyte function is required for antidepressant efficacy, meaning astrocytes may be important to the mechanisms of MDD treatment (23). Interestingly, comparing the number of GFAP+ astrocytes in

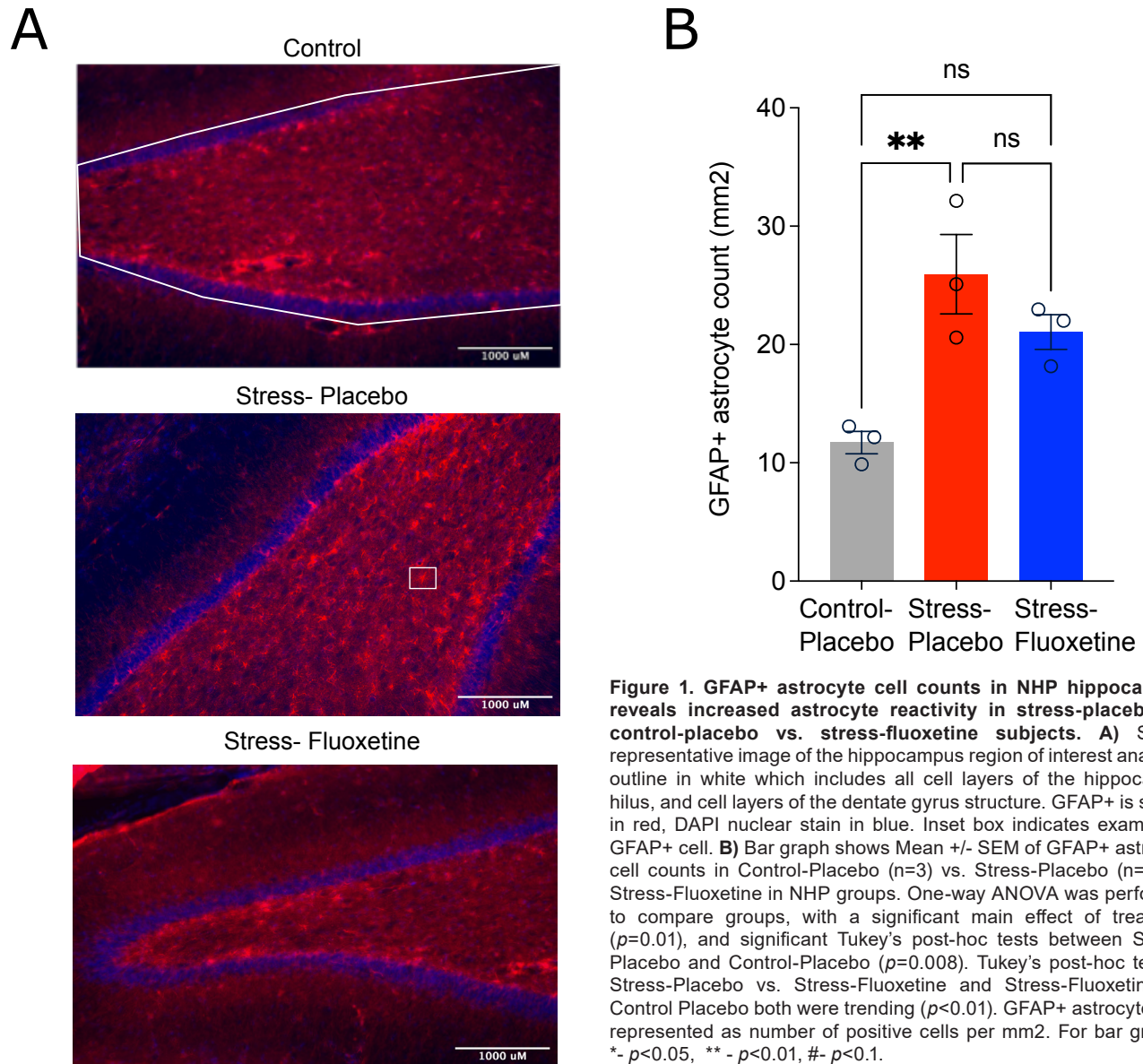


Figure 1. GFAP+ astrocyte cell counts in NHP hippocampus reveals increased astrocyte reactivity in stress-placebo vs. control-placebo vs. stress-fluoxetine subjects. **A)** Shows representative image of the hippocampus region of interest analyzed outline in white which includes all cell layers of the hippocampal hilus, and cell layers of the dentate gyrus structure. GFAP+ is shown in red, DAPI nuclear stain in blue. Inset box indicates example of GFAP+ cell. **B)** Bar graph shows Mean +/- SEM of GFAP+ astrocyte cell counts in Control-Placebo (n=3) vs. Stress-Placebo (n=3) vs. Stress-Fluoxetine in NHP groups. One-way ANOVA was performed to compare groups, with a significant main effect of treatment ($p=0.01$), and significant Tukey's post-hoc tests between Stress-Placebo and Control-Placebo ($p=0.008$). Tukey's post-hoc test for Stress-Placebo vs. Stress-Fluoxetine and Stress-Fluoxetine vs. Control Placebo both were trending ($p<0.01$). GFAP+ astrocytes are represented as number of positive cells per mm². For bar graphs, * - $p<0.05$, ** - $p<0.01$, # - $p<0.1$.

stressed animals treated with SSRIs vs. placebo, we found that antidepressant treatment does not have a significant effect on the astrocyte counts post-stress comparing directly to stress-placebo group. However, SSRI treatment still decreases the number of astrocytes to an extent, as the number of astrocytes in stress-fluoxetine groups is not significantly different from control groups via One-way ANOVA (**Figure 1B**). Overall, because the stress-fluoxetine group does not have a significantly higher number of astrocytes compared to controls (**Figure 1B**), the data still suggest that SSRI treatment may be associated with reduced astrogliosis.

GFAP+ astrocyte number is significantly correlated with neurogenesis rates in NHP hippocampus

Previous research has shown that astrocytes are key

components of the "neurogenic niche" that provides the necessary local microenvironment for the generation of neurons in the hippocampus (24). Astrocytes provide metabolic support to newly-generated neurons and promote their integration into hippocampal circuitry by releasing growth factors and other signaling molecules (25). Because astrocytes also can be regulated by stress, we hypothesized that increases in astrogliosis might also be correlated to changes in neurogenesis (26). We first compared numbers of doublecortin-positive (DCX+) cells in the dentate gyrus of the same animals that we measured GFAP+ astrocytes. DCX is a marker of immature neurons and is widely used as an index measure of neurogenesis rates (27). We assessed the differences in neurogenesis between experimental groups (**Figures 2A-B**). We confirmed that adult separation stress

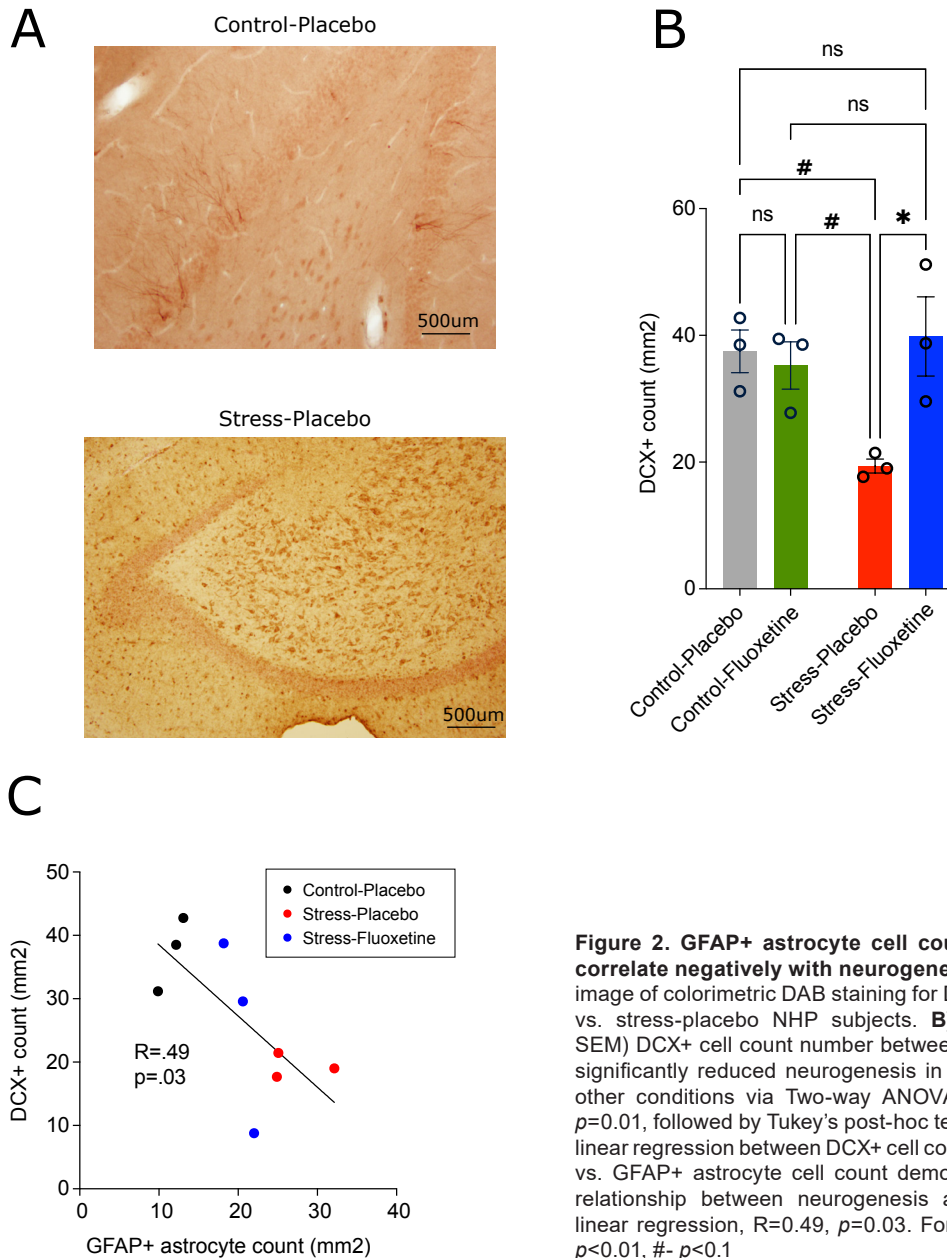


Figure 2. GFAP+ astrocyte cell counts in NHP hippocampus correlate negatively with neurogenesis rates. A) Representative image of colorimetric DAB staining for DCX+ cells in control-placebo vs. stress-placebo NHP subjects. **B)** Comparison of mean (+/- SEM) DCX+ cell count number between NHP groups demonstrates significantly reduced neurogenesis in stress-placebo condition vs. other conditions via Two-way ANOVA, main effect of interaction $p=0.01$, followed by Tukey's post-hoc test, $p=0.03$, $n=3,3$. **C)** Simple linear regression between DCX+ cell counts in the NHP hippocampus vs. GFAP+ astrocyte cell count demonstrates significant negative relationship between neurogenesis and astrogliosis, via simple linear regression, $R=0.49$, $p=0.03$. For bar graphs, * - $p<0.05$, ** - $p<0.01$, # - $p<0.1$

led to decreased neurogenesis in stress-placebo animals vs. stress-fluoxetine treated animals (via Two-way ANOVA, main effect of interaction $p = 0.01$, followed by Tukey's post-hoc test, $p = 0.03$, $n = 3,3$). Note that for this analysis, we included the control-fluoxetine group to determine if fluoxetine on its own was affecting neurogenesis rates; however, this condition was not available for further GFAP+ analysis. Interestingly, the neurogenesis rates did not reach statistical significance via post-hoc test for the control-placebo or control-fluoxetine vs. stress-placebo comparisons, though there was a trending effect of decreased neurogenesis in the stress-placebo group vs. both control conditions ($p < 0.1$ via Two-way ANOVA followed by Tukey's post-hoc test, $n = 3,3$). This result of

trending changes in neurogenesis indicates that fluoxetine treatment might be linked to reduced astrogliosis, and therefore may reduce the negative impact of astrogliosis on neurogenesis rates in the monkey hippocampus post-stress. However, further study is needed to confirm this hypothesis.

Next, when we examined the relationship between astrocyte counts and neurogenesis, we found that there is a significant negative relationship between neurogenesis and astrocyte counts (**Figure 2C**), ($p = 0.03$, via simple linear regression, $R = 0.49$). This result suggests that increased astrogliosis during stress may lead to loss of function of astrocytes, which in turn reduces their normal role during support of neurogenesis. Overall, this astrogliosis increase could lead

to fewer hippocampal neurons reaching advanced stages of maturity during stress. As normal hippocampal function and hippocampus-based cognitive processes depend on these newly integrated neurons, this reduced neurogenesis post-stress could contribute to compromised hippocampal function and behavioral deficits.

GFAP+ astrocyte number is significantly correlated with anhedonia scores in an NHP model of stress

Since reactive astrocytes are known to be involved in the stress response, we next investigated whether GFAP+ astrocyte cell numbers in the hippocampus were correlated with known stress-induced behaviors in our NHP adult separation stress model of MDD and antidepressant treatment vs. control animals. Anhedonia is one of the most treatment-resistant symptoms of MDD and therefore of high clinical relevance. In our previous study, we created an anhedonia score metric to quantify the number of times per behavioral observation session that each animal exhibited anhedonia-like behaviors such as slumped posture, disinterest in novel objects, and non-engagement with social activities. We first compared anhedonia scores of the groups, to assess if separation stress led to MDD-related behaviors, and if treatment with the SSRI fluoxetine rescued some of these effects (**Figure 3A**). For this analysis, we also included the control-fluoxetine group, to determine if the administration of fluoxetine in the absence of stress affected anhedonia (**Figure 3A**). Overall, NHP anhedonia scores were significantly higher for the stress-placebo group vs. any other group (e.g., control-placebo, control-fluoxetine, and stress-fluoxetine), via Two-Way ANOVA, main effects of stress ($p = 0.006$), treatment ($p = 0.008$), and interaction ($p = 0.01$), with significant Tukey's post-hoc tests between stress-placebo and all other three groups ($p < 0.01$ for each comparison).

We next aimed to determine if GFAP+ astrocyte numbers were related to average anhedonia scores in our NHP cohort. In correlating astrogliosis rates with the anhedonia subscale behaviors in both control-placebo, stress-placebo, and stress-fluoxetine NHP subjects, we found that GFAP+ astrocyte numbers correlated significantly with anhedonia behavior, with a significant positive correlation between astrocyte counts and anhedonia scores ($p = 0.03$ via simple linear regression, $R = 0.50$) (**Figure 3B**). These data support the hypothesis that hippocampal astrocytes are linked with behavioral responses to stress and suggest they may be associated with affective symptoms that are observed after chronic separation stress in monkeys. Furthermore, these data indicates that reduced astrogliosis may also be linked to improvements in anhedonia-like behavior after antidepressant treatment, as we see that GFAP+ astrocytes are reduced in stressed animals that have been treated with fluoxetine and show a behavioral rescue in their anhedonia scores, compared to stress-placebo subjects. Lastly, we investigated if GFAP+ astrocyte numbers correlated with other categories of observed behaviors in the NHP cohort. We did not detect any correlation between

average hierarchy behaviors and GFAP+ astrocytes (not significant via simple linear regression, $R = 0.005$, $p = 0.85$) (**Figure 3C**). These hierarchy behaviors include interactions that reflect both dominance and submissiveness within the cohort group. The lack of relationship with GFAP+ astrocytes suggests that behaviors related to hierarchy position may not be a significant factor in stress-responsiveness in the context of astrogliosis. Interestingly, when we examined the relationship between GFAP+ astrocytes and vigilance scores, which are considered to reflect a NHP model of anxiety-type behaviors, we also did not see any significant relationship, suggesting that anxiety-like behaviors and depression-like behaviors may be dissociable in their cellular mechanisms (not significant via simple linear regression, $R = 0.02$, $p = 0.65$) (**Figure 3D**). However, we did find that GFAP+ astrocyte counts did significantly correlate with affiliation scores for the NHP cohort (via simple linear regression, $R = 0.51$, $p = 0.02$) (**Figure 3E**). Affiliation scores in NHP social separation stress paradigms often increase in the stress groups, as the animals are separated from each other for a brief period (2-3 days) of each week. After being returned to the home cage, affiliation behaviors (e.g., grooming) will increase to re-establish social relationships. While the number of subjects in each group is not high enough to statistically analyze each group separately, in general the stress groups (with higher GFAP+ astrocyte counts) have higher levels of affiliation than the controls, possibly reflecting increased levels of social stress behaviors after separation.

DISCUSSION

Overall, our data indicate that chronic separation stress in NHPs leads to higher hippocampal GFAP+ astrocyte counts, indicating that this stress paradigm is associated with astrogliosis. Furthermore, GFAP+ astrocyte counts inversely correlate with adult hippocampal neurogenesis in NHPs, as well as with higher anhedonia scores, consistent with our initial hypotheses that hippocampal astrogliosis is an important mechanism in stress-induced cognitive and behavioral deficits.

Interestingly, opposite to our expected hypothesis, antidepressant with fluoxetine treatment did not directly lead to a statistically significant decrease in GFAP+ astrocyte counts post-stress compared to stress-placebo animals (**Figure 1B**). The fact that we do not see a direct difference between stress-placebo and stress-fluoxetine may be due to smaller sample size used in this study, as we may be underpowered to detect the effects of fluoxetine treatment. In addition, the treatment schedule used in this NHP study is slightly different than that used for MDD patients. The FDA-Labeled Indications and Target Adult Daily Dosage Range of fluoxetine for MDD patients is 20-80 mg daily for an average weight of 60-80 kg individual (or 3-4 mg/kg daily dose), compared to the once weekly dose of 13.5 mg/kg (equivalent to an effective daily dosage of 2 mg/kg) used for this animal study. This dosage schedule was implemented to mitigate the

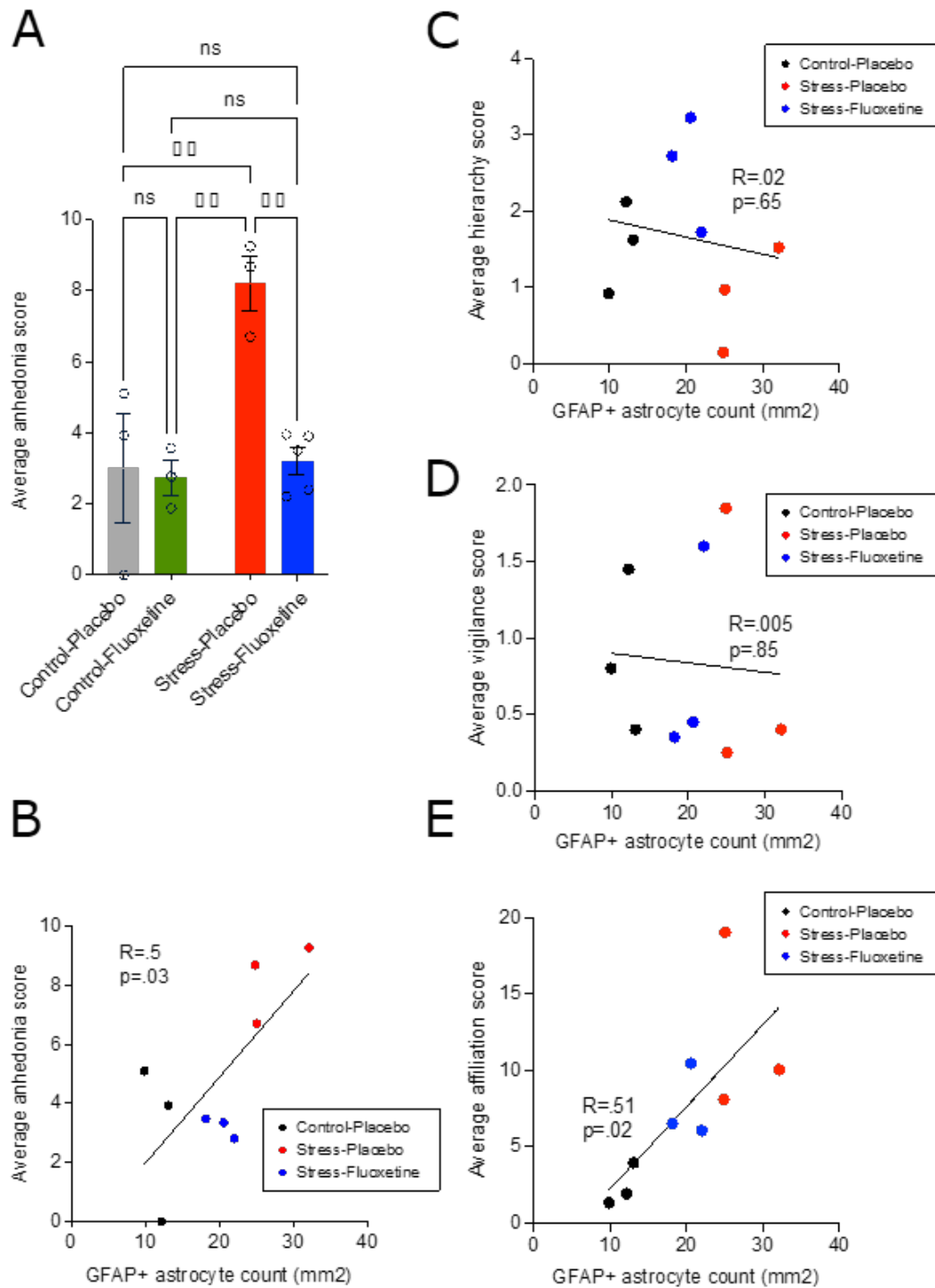


Figure 3. GFAP+ astrocyte cell counts in NHP hippocampus correlate positively with anhedonia behaviors. **A)** Bar graph shows mean \pm SEM of average anhedonia scores for each condition. Stress-placebo group displays significantly higher anhedonia scores than the other three conditions, via Two-Way ANOVA, main effects of stress ($p=0.006$), treatment ($p=0.008$), and interaction ($p=0.01$), with significant Tukey's post-hoc tests between stress-placebo and all other three groups ($p<0.01$ for each comparison), *, $p<0.05$, **, $p<0.01$, #, $p<0.1$. **B)** Simple Linear Regression of Average anhedonia scores for each group vs. GFAP+ astrocyte counts for each group, $R=0.5$, $p=0.03$, $n=3, 3, 3$. Anhedonia score represents the average number of anhedonia-type behaviors observed for each animal over 3 days of behavioral observations per week, for 3 weeks (at weeks 12-15, which is the post-stress timepoint). GFAP+ astrocytes are represented as number of positive cells per mm². **C)** Simple Linear Regression of Average hierarchy scores for each group vs. GFAP+ astrocyte counts for each group, $R=0.02$, $p=0.65$, $n=3, 3, 3$. **D)** Simple Linear Regression of Average vigilance scores for each group vs. GFAP+ astrocyte counts for each group, $R=0.005$, $p=0.85$, $n=3, 3, 3$. **E)** Simple Linear Regression of Average affiliation scores for each group vs. GFAP+ astrocyte counts for each group, $R=0.51$, $p=0.02$, $n=3, 3, 3$.

daily stressor of restraining and anesthetizing the animals for administration of saline placebo or fluoxetine. While there are also approved once weekly fluoxetine treatments for human patients, this different treatment schedule may also affect the timeline or efficacy of antidepressant effects in these animal subjects (28). One additional possibility for these results is that fluoxetine only moderately relieves the reactive astrogliosis phenotype, but fluoxetine treatment has a larger effect on other mechanisms that impact behavioral recovery from chronic stress (e.g., neurogenesis itself). In previous studies using rodents, for example, researchers have reported that fluoxetine treatment can reverse some aspects of astrogliosis, such as the decrease in number of astrocytes, while leaving other astrogliosis features unchanged, such as abnormal astrocyte morphology (29). Nevertheless, astrogliosis, as reflected by increased GFAP+ cell counts, is still significantly associated with lower neurogenesis rates and two distinct types of stress-related behaviors (anhedonia and affiliation), supporting the hypothesis that astrogliosis may negatively contribute to proper hippocampal function. In addition, it is still possible that fluoxetine antidepressant treatment lowers astrogliosis rates, but that the timescale of this rescue is longer than what was examined here. For example, there have been many studies on the delayed efficacy of SSRI treatments in humans and animal models of stress (30), and astrogliosis may take more time to recover from stress exposure than other processes.

The results from these experiments have demonstrated that stress and reactive astrocyte phenotypes are deeply interconnected, and that more research on astrocytes may be important in the development of novel therapeutic approaches for MDD that have a significant effect on astrogliosis. One limitation to note in this study is the small sample size, which is due to the prohibitive cost in performing monkey work and the availability of these tissues. One critical next step for this project is to expand the sample size to include more animals and increase the power in these experiments to confirm our hypotheses regarding astrogliosis and hippocampal function in stress and post-antidepressant treatment. We predict that adding more animals may help us to better detect these effects moving forward. In future studies, we would like to look at measures of alternative proteins to mark neurogenesis. For example, a mature neuronal marker can be used to see how many of these newly born cells are becoming functionally active under stress, such as Enolase 2/NSE and NeuN. Using alternative neuronal markers may help us better understand the mechanism of astrocyte reactivity on neurogenesis processes. Furthermore, it would be interesting to examine these same astrocyte reactivity measures in a monkey model of treatment with an antidepressant with a different mechanism than fluoxetine, such as ketamine. Ketamine is a relatively new type of antidepressant that does not work through serotonin signaling, as fluoxetine does, but instead acts at excitatory postsynaptic receptors to modulate neural activity (31). In addition, investigation of the effect of astrogliosis on

other symptoms of MDD would allow us to explore the role of astrocytes in more cognitive aspects of hippocampal function, such as through memory tasks or other spatial paradigms, since MDD affects cognitive processing functions as well. In conclusion, the findings of this project suggest that astrocytes and astrocyte reactivity are associated with stress and MDD-like symptoms in the NHP hippocampus, but further study is needed to understand how these cells interact with neurogenesis and SSRI mechanisms.

MATERIALS AND METHODS

NHP cohort

Note that all live animal work was previously completed prior to this project, and both neurogenesis and anhedonia data has been previously published (16). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research 2010). The SUNY Downstate Medical Center Institutional Animal Care and Use Committee (IACUC) approved the protocol.

Subjects

Adult female bonnet macaques were matched based on age, weight, and social rank, then randomized into groups based on intervention, as described in the previously published report (16). We used all female animals for this study because female macaques can be socially housed in colony settings, whereas males are singly housed due to social incompatibility. Female macaques form complex social hierarchies and thus are a better model to use for investigating the effects of social stressors (32). We used IHC images taken of postmortem hippocampus from the following NHP groups: control (n = 3), stress-placebo (n = 3), stress-drug (n = 3). Subjects in the stress group were exposed to the chronic separation stress paradigm, in which animals were subjected to alternating periods of social isolation (2 days) and group housing (5 days) for a total duration of 15-weeks. During this period, groups receiving the drug were treated with the selective serotonin reuptake inhibitor (SSRI), fluoxetine, at a dose of 13.5 mg/kg infused via nasogastric tube (NGT), once per week for 15-weeks, equivalent to a daily dose of 2 mg/kg of the drug. Once weekly treatment was performed to reduce the stress associated with fluoxetine administrations (notably, fluoxetine can be administered in once-weekly doses in human patients for maintenance of effect). The non-drug groups received the same treatment with saline. All groups were sacrificed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde (500 mg/kg) under deep anesthesia with pentobarbital (15 mg/kg, I.V.) on week 16 of interventions.

Immunohistochemistry

The left hippocampus was cut into 40 μ m sections and immuno-stained with rabbit anti-GFAP antibody (1:1000) and Alexaflour 568 anti-rabbit antibody (1:500), and counterstained with DAPI (1:10,000) to detect and quantify cell counts. Neurogenesis counts were performed previously

to this study; IHC was performed with goat anti-Doublecortin (DCX) (1:500) and Alexafluor 488 anti-goat antibody (1:500) and counterstained with DAPI (1:10,000) to detect and quantify cell counts. DCX is a protein expressed in immature neuronal cells, and is used as a marker of neurogenesis (15). DCX+ cells were counted in the dentate gyrus cell body layer.

Quantification of astrocytes

GFAP+ cells were manually quantified using the Photoshop “analysis” counting tool, while blinded to the condition of the animal. All GFAP+ cells inside the bounds of the dentate gyrus cell layer, including the hilus (**Figure 1**), with a clear cell-body visible in the plane of the image were counted within a single region-of-interest in the hippocampus, inclusive of the apex of the dentate gyrus. Total GFAP+ cell counts were divided by the area in mm² of the region of interest, measured by the area tool in ImageJ. GFAP+ cell counts are expressed as cells/mm². For each sample, three hippocampus image slices were quantified and averaged, for each group, an n = 3 was counted (control, stress-placebo, stress-fluoxetine).

Behavior

Reactive astrocyte counts were correlated with previously collected behavioral data. Briefly, home-cage behaviors for each animal were quantified through one-way mirrors by trained raters (inter-rater correlation coefficient (ICC) > 0.96) for three days a week. The frequency of 40 behaviors typical to bonnet macaques was scored for each animal at 30-second intervals per session (33). These behaviors were then collapsed into seven subscales, including anhedonia-like behaviors (including slumped posture, disinterest in novel objects, etc.), exploration behaviors, affiliation behaviors, and hierarchy behaviors. The average behavioral scores for the seven behavioral subscales were analyzed in three-week blocks to reduce fluctuations of individual sessions. For this project, we took the anhedonia behavior data from the last three weeks of this experiment (weeks 13-15) to correlate this measure with postmortem IHC astrocyte counts (animals sacrificed week 16).

Statistical analyses

All statistical analyses were performed in GraphPad Prism (version 9). To compare GFAP+ astrocyte counts between animal conditions, we used One-Way and Two-way ANOVA tests followed by Tukey’s multiple comparisons test. To correlate measures of GFAP+ astrocyte counts with behavioral scores and neurogenesis measures, we performed simple linear regression analyses and report the R value and p-value for the statistical test of slope being non-zero.

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