

# Role of age and neuroinflammation in the mechanism of cognitive deficits in sickle cell disease

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## Impact statement

This study provides crucial information that could be helpful in the development of new or repurposing of existing therapies for the treatment of cognitive deficit in individuals with sickle cell disease (SCD). Its impact is in demonstrating for the first time that neuroinflammation and along with abnormal neuroplasticity are among the underlying mechanism of cognitive and behavioral deficits in SCD and that drugs such as minocycline which targets these pathophysiological mechanisms could be repurposed for the treatment of this life altering complication of SCD.

## Abstract

This study aims to determine whether sickle cell mice could recapitulate features of cognitive and neurobehavioral impairment observed in sickle cell patients and whether neuroinflammation could be a potential therapeutic target as in other non-sickle cell disease-related cognitive dysfunction. Cognitive (learning and memory) and behavioral (anxiety) deficits in 13- and later 6-month-old male Townes humanized sickle cell (SS) and matched control (AA) mice were evaluated using novel object recognition (NOR) and fear conditioning tests. Immunohistochemistry was performed to quantify peripheral immune cell (CD45<sup>+</sup>) and activated microglia (Iba1<sup>+</sup>) as markers of neuroinflammation in the dentate and peri-dentate gyrus areas. We evaluated cell fate by measuring 5'-bromodeoxyuridine and doublecortin fluorescence and phenotyped proliferating cells using either glial fibrillary acid protein (GFAP<sup>+</sup>), neuronal nuclei (NeuN<sup>+</sup>), CD45<sup>+</sup>, and Iba1<sup>+</sup>. In addition, Golgi-Cox staining was

used to assess markers of neuroplasticity (dendritic spine density and morphology and density of dendrite arbors) on cortical and hippocampal pyramidal neurons. Compared to matched AA controls, 13-month-old SS mice showed significant evidence of cognitive and behavioral deficit on NOR and fear conditioning tests. Also, SS mice had significantly higher density of CD45<sup>+</sup> and activated microglia cells (i.e. more evidence of neuroinflammation) in the dentate and peri-dentate gyrus area. Additionally, SS mice had significantly lower dendritic spine density, but a higher proportion of immature dendritic spines. Treatment of 13-month-old SS mice with minocycline resulted in improvement of cognitive and behavioral deficit compared to matched vehicle-treated SS mice. Also, treated SS mice had significantly fewer CD45<sup>+</sup> and activated microglia cells (i.e. less evidence of neuroinflammation) in the dentate and peri-dentate gyrus, as well as a significant improvement in markers of neuroplasticity.

**Keywords:** Sickle cell disease, cognitive impairment, neuroplasticity, neuroscience, neuroinflammation

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## Introduction

Cerebrovascular disease is one of the most common and dramatic complications of sickle cell disease (SCD) affecting both children and adults,<sup>1</sup> with cognitive and neurobehavioral abnormalities as possible sequelae. The prevalence of cognitive and neurobehavioral complications in individuals with SCD have been widely

reported.<sup>2–8</sup> Some studies have suggested that these abnormalities are a result of stroke and/or repeated silent cerebral infarctions (SCIs) observed in children, adolescents, and adults with SCD.<sup>9,10</sup> Other studies have also associated the presence of cognitive and neurobehavioral dysfunction with older age, anemia, overt stroke and SCIs, systemic inflammation as well as environmental stressors

(low socioeconomic status) in individuals with SCD.<sup>11–14</sup> However, recent studies indicate that cognitive and neurobehavioral abnormalities were present in individuals with SCD, even in the absence of magnetic resonance imaging (MRI) detectable infarcts or cerebral injury.<sup>2,8,14</sup> Andreotti et al., recently reported that children with SCD, in the absence of MRI evidence of cerebral infarct or injury, scored below the normative mean on cognitive testing. Further, in the same children, there was a significant negative correlation between plasma inflammatory cytokine levels and tests of executive function.<sup>2</sup> Note that a similar relationship existed in children with asthma, who also have high circulating levels of inflammatory cytokines,<sup>2</sup> suggesting a possible mechanistic role for inflammation and potentially neuroinflammation. Notwithstanding the abundance of epidemiological evidence, the mechanism of onset of cognitive and neurobehavioral deficits in SCD, as well as the underlying cellular and pathobiological mechanisms are largely unknown.<sup>15–18</sup>

The presence of cognitive and neurobehavioral deficits in individuals with SCD in the absence of overt cerebral injury<sup>2,14</sup> suggests the involvement of cellular mechanisms that are not detectable on clinical neuroimaging. The mechanisms of cognitive and neurobehavioral dysfunction in SCD in the absence of overt cerebral injury are largely unknown. But in non-sickle cell animal models, studies have shown that changes in the anatomy of neurons and neuroinflammation (local or as a result of cross-talk between systemic inflammation and central nervous system inflammation) are associated with cognitive and neurobehavioral dysfunction and deficits.<sup>19–24</sup> Furthermore, several studies have shown that cerebral ischemia results in abnormal remodeling of neuron dendrites (decreased arbors) and dendritic spines (decrease in number and maturation) and that decreased number of dendritic arbors and spines and proportion of mature dendritic spines correlate with cognitive and neurobehavioral dysfunction.<sup>25–28</sup> Also, there is evidence to suggest that neuroinflammation is the underlying mechanism of stress-induced cognitive deficit.<sup>19,20,22</sup> Taken together, the available data suggest that similar mechanisms as outlined above might be responsible for SCD-related cognitive and neurobehavioral dysfunction. While it has been suggested that the cognitive and neurobehavioral complications observed among individuals with SCD (with or without overt cerebral injury) could be attributed to pain and the chronicity of the disease, available studies have shown that individuals with SCD were 20% more likely than those with hemochromatosis and other chronic hematological diseases, to have anxiety, depression, or poor educational attainment.<sup>6</sup> Additionally, adult patients with SCD were more likely to be depressed and anxious after adjusting for pain frequency, pain and disease severity, and frequency of hospitalization.<sup>29,30</sup> This body of evidence suggests that SCD is associated with significant cognitive and neurobehavioral impairment that are independent of the individual burden of disease and/or overt central nervous system injury. Our hypothesis is that age-related neuronal remodeling (decreased density of dendrite arbors, dendritic spines and, proportion of mature dendritic

spines) and neuroinflammation are potential mechanisms underlying the development of cognitive and neurobehavioral dysfunction in SCD.

## Materials and methods

### Animal preparation

The Institutional Animal Care and Use Committee of Emory University approved this study. All research activities reported here were conducted in accordance with the National Research Council and National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th Edition*.<sup>31</sup> Reporting of methods, results, and all aspects of this article are in accordance with appropriate reporting standards (ARRIVE). Reporting of methods was based on the recommendations from the *Quality of methods reporting in animal models of colitis*.<sup>32</sup>

We utilized a combination of cross-sectional and cross-sectional prospective study designs. We also used the Townes mouse model, a humanized sickle cell (with HbSS) and corresponding control (with HbAA) mouse model (B6;129-Hba<sup>tm1(HbA)Tow</sup> Hbb<sup>tm2(HbG1,HbB\*)Tow</sup> / Hbb<sup>tm3(HbG1,HbB)Tow</sup> / J) throughout this study. Based on a recent report,<sup>33</sup> we estimated that an average sample size of 15 mice per group would be adequate for the cognitive and neurobehavioral studies. Furthermore, a sample size of four mice would be adequate to show statistically significant differences for the histological studies based on our prior published work in sickle cell mice.<sup>34–36</sup>

Mice for this study were bred at the Emory University managed breeding services, weaned at 3–4 weeks of age and were housed with a 12-h light/dark cycle with water and feed provided *ad libitum*. Mice were allowed to age naturally until ready for use in our experiments described below. Although the required sample size for the cognitive and neurobehavioral studies was 15 mice per genotype or treatment group, we started with 20 mice in each group to allow for a 10–20% attrition from mortality, which is usually higher in sickle cell mice compared to controls.

### Study design and overall methods

For this study, we used only male sickle cell mice (however, current and future studies includes both sexes). We first set out to determine whether aged (13 months old) sickle cell mice compared to age-matched controls show evidence of learning, cognitive, and neurobehavioral deficit. For this experiment aged sickle cell mice and matched controls (cohort I) were tested for the presence of learning, cognitive, and neurobehavioral deficits, compared to controls. We used the novel object recognition (NOR) test which consists of habituation (movement in an open field within an enclosure) and NOR. We later tested another cohort of 15 sickle cell mice and 12 matched controls (cohort II) that were six months old at the time of testing. This was to enable us to determine whether aging was an important factor in our observation in the first (cohort I) instance. Next, the cohort II mice were allowed to age until they were 13 months old and then retested using the same cognitive and neurobehavioral test paradigm as cohort I. We

later added an additional learning and memory test paradigm (fear conditioning) that is based on classical Pavlovian conditioning with a goal of ensuring that observations made in NOR experiments were not spurious and replicable by a different learning and memory test paradigm. We also wanted to determine the possible location of the lesion (amygdala vs. hippocampus or both) which the fear conditioning test can allow us to do. Based on emerging evidence,<sup>19,22,37</sup> we tested the hypothesis that neuroinflammation might be a mechanism for the observed differences in learning, cognitive, and neurobehavioral function between sickle cell mice and controls by assessing cellular markers of neuroinflammation and by blocking neuroinflammation using minocycline. Minocycline is a highly lipophilic drug capable of crossing the blood-brain barrier,<sup>38</sup> and was administered to a subset of previously aged mice (cohort II after habituation and NOR) and later to a larger sample of animals (cohort III,  $N = 15$  per genotype or treatment groups). Minocycline has been shown to have a neuroprotective effect in several neurological disorders<sup>39,40</sup> and its mechanism of action is proposed to be partly due to the inhibition of microglial activation<sup>41</sup> as well as mitigation of myeloid cell entry into the brain, thus reducing neuroinflammation. Studies also suggest that minocycline influences neuroplasticity by promoting dendritic spine development and maturation<sup>42</sup> and neurite outgrowth.<sup>43</sup>

All rodent neurobehavioral testing was performed by the Emory University Rodent Behavioral Core (RBC) using standard protocols. The RBC was blinded to the disposition and genotype of the mice. Unless where explicitly stated, all data, including image analysis was performed by individuals who were blinded to the genotype and/or disposition of the mice. After cognitive and neurobehavioral tests were completed, mice were sacrificed and the brain extracted for histological analysis. Immunohistochemical assay was performed to (1) ascertain/quantify the presence of inflammatory cells (activated microglia and bone marrow-derived microglial) and (2) quantify and phenotype proliferating cells in the dentate gyrus (DG; i.e. cell fate). Golgi-Cox impregnation was used to determine the density of dendrite arbors and dendritic spines, as well as dendritic spine morphology. The assignment of mouse brain for either immunohistochemistry or Golgi-Cox impregnation was done randomly within each treatment group and genotype.

### Minocycline treatment

To determine whether neuroinflammation was a mechanism for cognitive and neurobehavioral deficits in aged sickle cell mice, we administered minocycline at a dose of 90 mg/kg/day orally via drinking water for three weeks. The amount of water drank by the mice per cage was measured and the drug dosing was adjusted every day based on this value. This was to ensure consistency in the dosage of drug that the mice were getting each day. Sickle cell mice acting as controls were given plain drinking water. We had demonstrated in a pilot study that the presence or absence of minocycline in the drinking water did not affect the

pattern of drinking or the volume of water consumed by the mice per day. In every instant, minocycline treatment was started after the mice were aged to 13 months.

### Novel object recognition (NOR) test

This is a test of hippocampus-based learning and memory. It was adopted for our study because as in their human counterpart, sickle cell mice are also predisposed to developing pain crises, which could bias the results of a Morris water maze test or other paradigm that require additional effort more than that required for everyday movement and activity within a cage. This test is conducted in an open field made of transparent plexiglass and measuring 12"  $\times$  12"  $\times$  15" ( $L \times W \times H$ ). Initially, animals were allowed 2–3 days of habituation which consisted of allowing them to explore the open field in the enclosure for 5 min each day. The total distance covered during this time as well as the time spent in the center of the open field was recorded and provides a measure of the animal's behavior (anxiety/depression). On the third or fourth day, the mice were trained by placing two identical objects (test objects) in the enclosure and allowing the mice to explore it for 5 min. Following training, the mice were returned to their home cages for about 30 min. After this delay, the learning and memory of the mice were tested by replacing one of the test objects used during training with a novel object in the open field. The time spent investigating the familiar (test object) and the novel object was recorded over a 5-min period. Mice were removed and returned to their home cages at the end of the test session. The enclosure as well as the test and novel objects are wiped with alcohol between animals/tests. The time spent investigating the novel object on each encounter is recorded and the percent preference (preference ratio) for the novel object compared to the test object was calculated, and are presented in the results section and accompanying figures.

### Fear conditioning test

This test measured the ability of mice to form and retain the memory of an association between an aversive experience and environmental cues. We used a standard fear conditioning paradigm, conducted over three days. On the first day, the mice were placed in the fear conditioning apparatus (7"  $W \times 7" D \times 12" H$ , Coulbourn Instruments, Holliston, MA) and allowed to explore for 3 min. After the period of habituation, we presented the mice with three pairs of conditioned stimulus (CS)–unconditioned stimulus (US) with a 1-min inter trial interval between stimulus presentations. The CS was a 20-s long 85 dB tone and the US was 2 s of a 0.5 mA electric shock to the footpad/paw, timed to co-terminate with each CS presentation. It should be noted that this level of shock is relatively modest and very well tolerated by comparison to that in other studies such as seen in stress-induced drug reinstatement. The shock is delivered by a Precision Animal Shocker (Coulbourn Instruments, Holliston, MA) connected to each fear-conditioning chamber, with shock level set and verified before every training session. One minute after the presentation of the last CS–US presentation, the animals



were returned to their home cages. On day 2, the mice were presented with a context test, which involves placing them in the same chamber used for training on day 1, but this time, no shock is delivered. The percent freezing per minute was recorded over 9 min or 540 s. On day 3, the mice were presented with a tone or cued test by exposing them to the CS in a novel environment/compartment. Initially the animals were allowed to explore the novel environment for 2 min. Following this brief period of habituation, the 85 dB tone was presented to the mice every minute for 9 min and the amount of freezing behavior per minute was recorded. In both the contextual and cued fear test, freezing behavior indicates a memory for either the context in which the shock was delivered or for pairing the tone with a shock. More freezing indicates better learning of and memory for the context and/or the CS-US pairing, while lesser freezing behavior indicates the opposite.

### Immunohistochemistry

At the conclusion of all the cognitive and neurobehavioral tests, animals were transferred to their home cages and were sacrificed the next day for brain extraction and histology. Briefly, animals were sacrificed using an overdose of pentobarbital and then perfused fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Brains were extracted and post-fixed overnight at 4°C in 4% (w/v) PBS. In each mouse, the entire brain was sectioned at 50  $\mu\text{m}$  thickness. Using stereotactic coordinates,<sup>44</sup> the sections that contained the hippocampus (DG) were identified and used for immunohistochemistry in order to demonstrate the presence of inflammation (neuroinflammation) in the hippocampus (DG) and thus the possible role of neuroinflammation on the observed cognitive and/or neurobehavioral impairment or deficit in the sickle cell mice. We also attempted to map the predominant fate of new cells that were identified in the DG. To enable us accomplish all these, we developed four antibody panels, thus, panel 1: anti-mouse neuronal nuclei (NeuN; AB90, Millipore, Billerica, MA, 1:500), anti-mouse Iba1 (019-19741, Wako Chemicals, Richmond, VA, 1:500), anti-mouse CD45 (ab23910, Abcam, Cambridge, MA, 1:500); panel 2: anti-mouse 5'-bromodeoxyuridine (BrdU; ab8152, Abcam, Cambridge, MA, 1:500), anti-mouse Iba1 (1:500), anti-mouse CD45 (1:500); panel 3: anti-mouse BrdU (1:500), anti-mouse doublecortin (DCX; ab207175, Abcam, Cambridge, MA, 1:500); panel 4: anti-mouse NeuN (1:500), anti-mouse DCX (1:500), anti-mouse glial fibrillary acid protein (GFAP; ab4674, Abcam, Cambridge, MA, 1:1500) and starting with the first section showing the DG to the last section, we labeled every fourth brain slices with one of the antibody panels described above; i.e., slice 1 goes to panel 1, slice 2 to panel 2, slice 3 to panel 3, and slice 4 to panel 4, and then the process is repeated starting with panel 1. The general approach used for immunohistochemistry in our laboratory has already been published.<sup>34</sup> For primary antibody labeling, free-floating tissue sections (50  $\mu\text{m}$  thick) obtained from post-fixed brain samples were placed in a scintillating glass vial containing antibodies (diluted in antibody diluting solution for a total of 2 mL volume)

from one of the panels described above and after incubating for 16–24 h at room temperature on a nutating shaker, the sections were washed with PBS and then incubated for 2 h with the respective secondary antibody. Sections were mounted using Fluoromount G and imaged at 20X using a confocal microscope (Leica SP8, Leica Microsystems Inc., Buffalo Grove, IL).

*Immunohistochemistry and digital imaging analysis for Iba-1 and CD45.* This assay was done to determine the presence of peripherally derived inflammatory (CD45<sup>+</sup> or bone marrow-derived microglia) cells and/or activated microglia (Iba-1<sup>+</sup>) in and/or around (50 pixels on either side) the DG. The general approach for immunohistochemical labeling for Iba-1 and/or CD45 has been described above. For each mouse, images were taken from the DG of the hippocampus using a 20X objective. Image analysis was performed using NIH ImageJ software. For CD45, cells with positive labeling were counted in each DG section. Analysis of Iba-1 labeling for activated microglia was done using a combination of digital image analysis and visual confirmation of morphological features of activated microglia as described in the literatures.<sup>19,22,45</sup> In brief, a threshold for positive labeling was determined for each image that included all cell bodies and processes but excluded background staining. The number of CD45<sup>+</sup> cells and activated microglia was then expressed as a density per 1000  $\mu\text{m}^2$  of DG area.

*5'Bromodeoxyuridine and doublecortin labeling.* We examined the proliferation of actively dividing cells in the DG using BrdU labeling. BrdU (10 mg/mL; Sigma-Aldrich, St. Louis, MO) was dissolved in warm PBS and then filtered. On the last three days prior to cognitive and neurobehavioral testing, mice were injected intraperitoneally with 50 mg/kg BrdU at 9:00 AM. Brains were collected for BrdU immunohistochemistry after completion of cognitive and behavioral testing as described earlier. For quantification of BrdU positive (BrdU<sup>+</sup>) and doublecortin-positive (DCX<sup>+</sup>) cells, every fourth section throughout the hippocampus/DG was collected and stained with panel 3: anti-mouse BrdU (1:500), anti-mouse DCX (1:500) as described earlier. Sections were mounted on slides, cover-slipped with Fluoromount G (Beckman Coulter, Brea, CA), and stored at 4°C. Fluorescent images were captured using a 20X objective, on a confocal microscope (Leica SP8, Leica Microsystems Inc., Buffalo Grove, IL). The image analysis was performed using NIH ImageJ software for the total number of BrdU<sup>+</sup> cells, DCX<sup>+</sup> cells, and BrdU<sup>+</sup>/DCX<sup>+</sup> in each DG section. As was the case with Iba-1 and CD45 positive cell quantification, the total number of BrdU<sup>+</sup> cells, DCX<sup>+</sup> cells, and BrdU<sup>+</sup>/DCX<sup>+</sup> in the DG were normalized per 1000  $\mu\text{m}^2$  of DG area. This experiment enabled us to quantify the population of the proliferating cells (BrdU<sup>+</sup> cells) that are neural progenitor cells (NPCs; DCX<sup>+</sup>).

*Phenotyping BrdU<sup>±</sup> cells.* We then proceeded to determine the phenotype of proliferating (BrdU<sup>+</sup>) cells in the dentate and/or "peri-dentate" gyrus area. Thus, brain slices were obtained as described earlier and triple labeled as follows—panel 2: anti-mouse BrdU (1:500), anti-mouse Iba1 (1:500) and anti-mouse CD45 (1:500); panel 3:

anti-mouse BrdU (1:500), anti-mouse DCX (1:500); panel 4: anti-mouse NeuN<sup>+</sup> (1:500), anti-mouse DCX (1:500), anti-mouse GFAP (1:1500). Panel 2 enabled us to determine whether the proliferating cells are from the peripheral immune system or dividing microglia, panel 4 allowed us to determine whether NPCs (expressing DCX) are more likely young or mature neurons (NeuN<sup>+</sup>) vs. astrocytes (GFAP<sup>+</sup>). Fluorescent images were captured using a 20X objective on a confocal microscope (Leica SP8, Leica Microsystems Inc., Buffalo Grove, IL). The image analysis was performed using NIH ImageJ software for the total number of DCX<sup>+</sup>/NeuN<sup>+</sup> cells or DCX<sup>+</sup>/GFAP<sup>+</sup> cells in each DG section. As was the case with Iba-1 and CD45 positive cell quantification, the total number of DCX<sup>+</sup>/NeuN<sup>+</sup> cells or DCX<sup>+</sup>/GFAP<sup>+</sup> in the DG were normalized per 1000  $\mu\text{m}^2$  of DG area. A previous study was used as a reference for morphological comparison of immature and mature DCX<sup>+</sup> neurons.<sup>46</sup>

*Quantification of dendrites arbors, dendritic spine density and spine morphology.* This experiment was performed with the Golgi-Cox impregnation approach, using the FD Rapid GolgiStain<sup>TM</sup> Kit according to the manufacturer's instructions.<sup>25,26,28,47-49</sup> The experiment allowed us to visualize and characterize the remodeling/plasticity of pyramidal neurons in the cortical and hippocampal/DG regions of the brain. Brightfield images were acquired using a 60X oil immersion objective mounted on a fluorescent microscope.

The detailed approach for Golgi-Cox impregnation has already been published. Briefly, mice were sacrificed using an overdose of pentobarbital (150 mg/kg, intraperitoneally), and their brains were extracted, rinsed with Nanopure water (Millipore, Billerica, MA), and immersed in the impregnation solution composed of potassium dichromate, mercuric chloride, and potassium chromate. The brains were stored at room temperature for two weeks and then transferred and stored in the cryoprotectant solution for 2.5 days in the dark. Coronal sections of 100–200  $\mu\text{m}$  thickness were cut on a vibratome with the tissue bath filled with the cryoprotectant solution. Sections were mounted on gelatin-coated glass slides (Electron Microscopy Solution Inc., Hatfield, PA), and allowed to air dry at room temperature in the dark for 2–3 days. After drying, the sections were rinsed with Nanopure water, reacted in the working solution, and dehydrated with a 50%, 75%, 95%, and 100% graded ethanol series. Finally, the sections were defatted in xylene and cover-slipped using Permount (VWR International LLC., Radnor, PA).

Digitized images were acquired using a 60X (oil immersion) objective on an upright fluorescent microscope. Z-stacks of pyramidal neurons were obtained from the hippocampus/DG and the cerebral cortex in a clockwise fashion from the midline to ensure reproducibility. We quantified the dendritic spine density and morphology using manual visual rating combined with published criteria,<sup>50-52</sup> with the rater blinded to the genotype and disposition of the animals. The z-stacks were then projected using minimum intensity projection and the dendrite arborization pattern and thus branch number/density was quantified using an adaptation of the Sholl analysis ([http://imagej.net/Sholl\\_Analysis](http://imagej.net/Sholl_Analysis)).<sup>53-56</sup> Results of

dendritic spine quantification were expressed per 10  $\mu\text{m}$  dendrite segment and that for dendrite arbors were presented as number of inters as one moves outwards from the soma, up to distance of 20  $\mu\text{m}$ .

### Statistical consideration

A two-way analysis of variance (ANOVA) was used to compare groups on performance in respective neurobehavioral and cognitive tests that have a time component (time spent in the middle of the open field, total distance traveled and fear conditioning). A one-way ANOVA was used to compare the differences in percent preference for the novel object and the density of the different cells quantified in the dentate or "peri-dentate" gyrus area. Also, a one-way ANOVA was used to compare the differences in dendrite density and dendritic spine density as well as proportion of immature dendritic spines. When the data are not normally distributed, a non-parametric ANOVA was used instead. A *P* value of <0.05 was used as indicating a statistically significant difference between genotype groups and/or treatment groups. Unless otherwise stated, absolute values are presented as mean  $\pm$  SE.

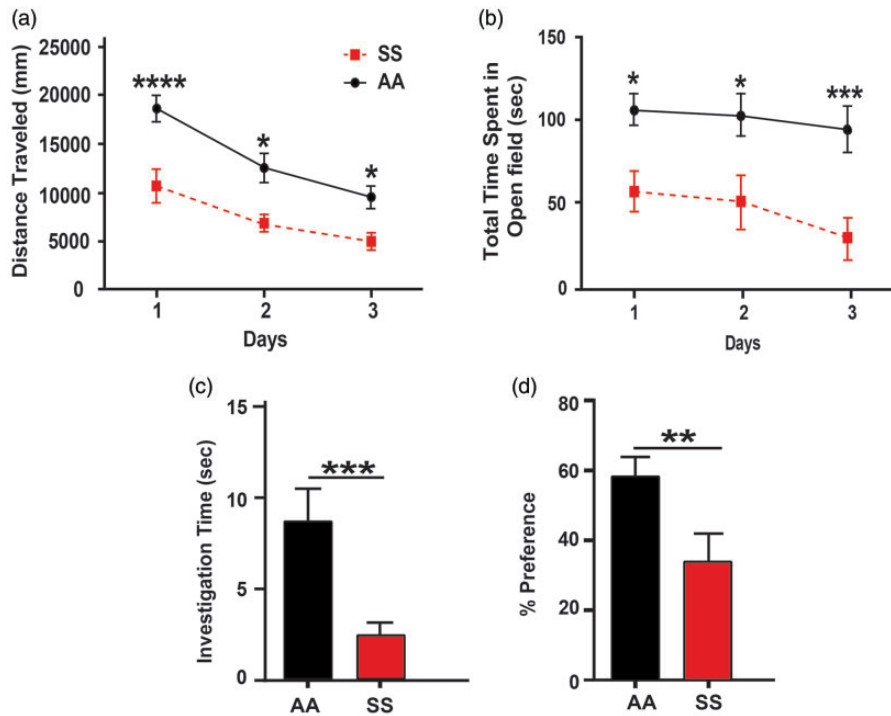
## Results

### Aged sickle cell mice displayed significant cognitive and neurobehavioral deficits compared to control mice

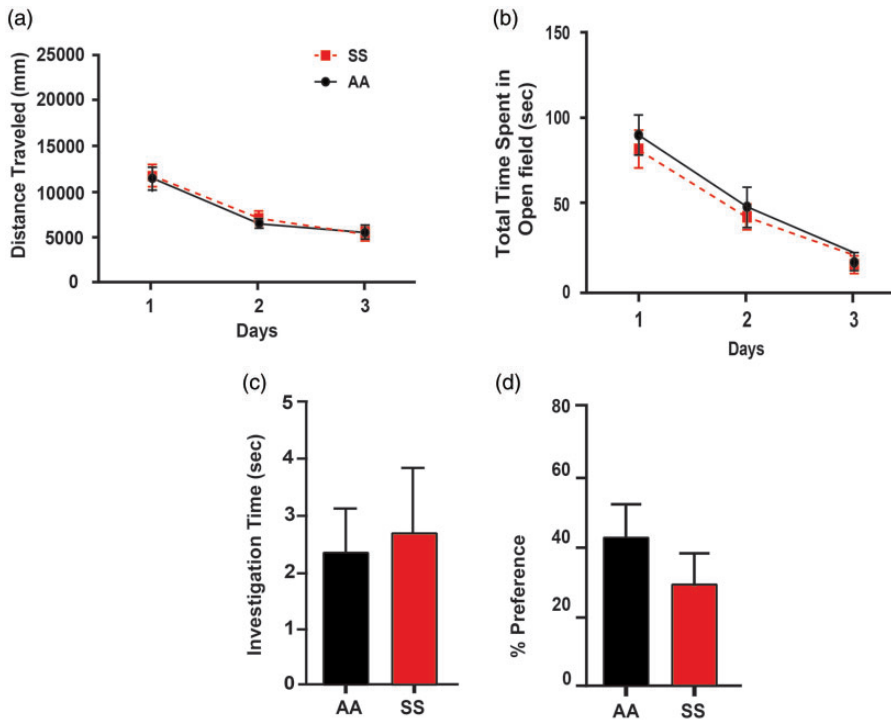
For this study, we examined the learning, memory, and anxiety/depression like behaviors in 13 months old Townes sickle cell and control mice using a NOR test. Sickle cell mice exhibited significant evidence of anxiety/depression like behaviors, indicated by the shorter distance traveled ( $P=0.0004$ , Figure 1(a)) and thigmotaxis ( $P=0.002$ ; Figure 1(b)) when compared to controls. Sickle cell mice spent significantly shorter investigation time ( $2.39 \pm 0.64$  s) on the novel object, compared to control ( $8.62 \pm 1.80$  s;  $P=0.007$ ; Figure 1(c)). Similarly, sickle cell mice showed learning and memory deficits indicated by a lower percent preference for the novel object ( $32.15 \pm 7.8\%$ ), compared to controls ( $58.3 \pm 9.9\%$ ;  $P=0.010$ ; Figure 1(d)).

### Aged sickle cell mice displayed cognitive and neurobehavioral deficits that were not seen in younger sickle cell mice

To demonstrate this, we examined the learning, memory, and anxiety/depression like behaviors in six months old Townes sickle cell and control mice using a NOR test paradigm, as was done for the original cohort of 13 months old sickle cell and control mice. There was no significant differences in neurobehavioral characteristics between sickle cell and controls mice as evidenced by similar measures for distance traveled ( $P=0.85$ ; Figure 2(a)) and thigmotaxis ( $P=0.55$ ; Figure 2(b)). There was also no significant difference in learning and memory between sickle cell and control mice at six months as they showed similar investigation time ( $2.64 \pm 1.20$  s vs.  $2.34 \pm 0.72$  s;  $P=0.25$ ; Figure 2(c)) on the novel object as well as percent preference for the novel object ( $28.39\%$  vs.  $45.92\%$ ;  $P=0.21$ ; Figure 2(d)). The

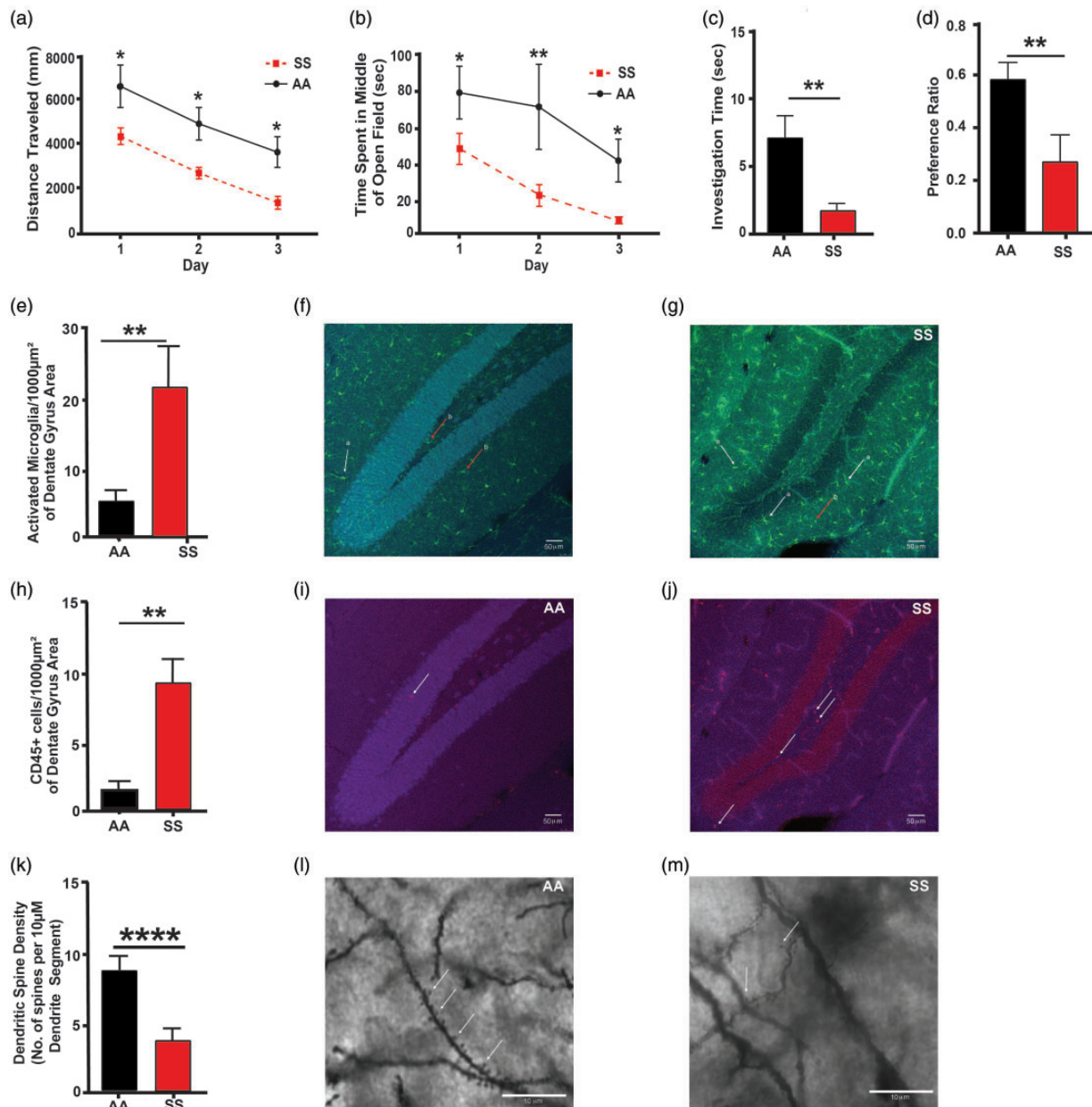


**Figure 1.** Abnormal cognition and behavior in 13 months old sickle cell compared to control mice. In the figure (a) distance traveled ( $P = 0.0004$ ), (b) time spent in the open field ( $P = 0.002$ ), (c) investigation time on the novel object ( $P = 0.007$ ), and (d) percent preference for the novel object ( $P = 0.01$ ) were significantly lower in sickle cell mice (SS) compared to controls (AA) indicating that aged (13 months) sickle cell mice have cognitive and neurobehavioral abnormalities compared to controls. Data are presented as mean  $\pm$  SEM.  $N = 10$  per group.



**Figure 2.** Similar levels of cognitive and neurobehavioral function in “younger” sickle cell compared to control mice. No significant cognitive or neurobehavioral abnormalities were seen in six month old sickle cell (SS) mice compared to controls (AA) as indicated by similar levels of (a) distance traveled, (b) time spent in the open field (second), (c) time in second spent investigating the novel object, and (d) percent preference for the novel object. Results are presented as mean  $\pm$  SEM.  $N = 15$  sickle cell mice and 12 AA or control mice.





**Figure 3.** Cognitive and neurobehavioral abnormalities reappeared when young (6 months old) sickle cell mice (from Figure 2) were aged to 13 months, compared to control mice. Aged sickle cell mice also had significant cellular evidence of neuroinflammation as well as lower dendritic spine density compared with controls. When aged to 13 months, sickle cell (SS) mice had significantly more cognitive and neurobehavioral abnormalities that were not seen at 6 months of age when compared to controls (AA). The (a) distance traveled ( $P=0.0008$ ); (b) time spent in the open field ( $P=0.0007$ ); (c) investigation time on the novel object ( $P=0.002$ ), and (d) percent preference for novel object where significantly lower when compared to AA mice ( $P=0.02$ ). We also saw a higher level of neuroinflammation (i.e. more pro neuro-inflammatory cells) in sickle cell mice when compared to controls. At baseline, there were more (e) activated microglia ( $P=0.005$ ), estimated using Iba1 labeling in combination with morphological characteristics of activated microglia cells and (h) peripherally (bone marrow) derived mononuclear cells ( $P=0.001$ ), measured via CD45 labeling, in the dentate and peri-dentate gyrus of area of sickle cell mice compared to controls. Furthermore, dendritic spine density was significantly reduced in sickle cell mice compared to controls (k). Aged (13 months old) SS mice had significantly lower dendritic spine density compared to AA mice ( $P=0.0002$ ). Results are presented as mean  $\pm$  SEM.  $N=9$  controls or AA mice, 8 SS treated mice, and 6 SS non-treated mice. The images to the right of the bar graphs of (e), (h), and (k) are representative images of the data plotted in the bar graph. The white arrows in (f) and (g) indicate microglia in the dentate and peri-dentate gyrus area. Arrows labeled (a) indicate activated microglia, while those labeled (b) are examples of normal or non-activated microglia. In (i) and (j), the white arrows show the presence of CD45+ peripherally (bone marrow) derived mononuclear cells in the dentate and peri-dentate gyrus area and shows the higher density of dendritic spines among SS compared to AA mice. (l) and (m) are representative images showing the significantly higher density of dendritic spines in AA compared to SS mice.

difference in behavior and cognition between sickle cell and control mice reappeared when the same mice were allowed to age to 13 months. The aged sickle cell mice exhibited more anxiety/depression-like behaviors indicated by significantly shorter distance traveled (Figure 3(a))

and thigmotaxis (Figure 3(b)) and spent significantly less time investigating the novel object compared to matched controls. Aged sickle cell mice also had significantly lower percent preference or preference ratio for the novel object compared to the matched control mice (Figure 3(d)).

### Aged sickle cell mice have more evidence of neuroinflammation compared to controls

Immunohistochemical analysis of brains from the 13 months old mice above shows that sickle cell mice had significantly more evidence of neuroinflammation, indicated by a significantly higher number of activated microglia per 1000  $\mu\text{m}^2$  of DG area ( $22.01 \pm 6.17$  vs.  $6.02 \pm 1.44$ ;  $P = 0.005$ ; Figure 3(e)), compared to controls. Also, SS mice had a significantly higher number ( $9.32 \pm 2.23$ ) of CD45<sup>+</sup> peripherally derived mononuclear (aka bone marrow-derived microglia) cells per 1000  $\mu\text{m}^2$  of DG area compared to controls ( $1.57 \pm 0.56$ ;  $P = 0.001$ ; Figure 3(f)).

### Aged sickle cell mice have lower dendritic spine density compared to controls

We assessed dendritic spine density in a subset of 13-month-old sickle cell and control mice in order to determine any underlying neuronal changes that could be responsible for the observed cognitive and neurobehavioral deficits. As shown in Figure 3(g) we observed a significantly lower dendritic spine density per 10  $\mu\text{m}$  of dendrite segment in sickle cell mice ( $5.62 \pm 0.45$ ) compared to control mice ( $8.46 \pm 0.37$ ;  $P = 0.0002$ ).

### Treatment with minocycline restored cognitive and neurobehavioral deficit in aged sickle mice

Next we randomized half of the 13-month-old sickle cell mice from the experiment in Figure 2 to either receive minocycline (90 mg/kg) in their drinking water or plain drinking water for three weeks. We had performed a pilot experiment to ensure that minocycline had no impact on the amount of water drank (data not shown), since better hydration can improve sickle cell outcomes in general. Cognitive function (learning and memory) and behavior in sickle cell mice that received minocycline improved to the same levels as non-sickle cell mice controls. On the other hand, cognitive function and behavior among sickle cell mice that did not receive minocycline were little changed or slightly worsened. The result for this specific cohort is presented in the online-only Supplementary results. We repeated this experiment in a larger of cohort of aged (13 months old) mice ( $n = 15\text{--}20$  mice per group) with an addition of five non-sickle cell (AA) mice group randomized to receiving minocycline (result not included). There was no significant difference between treated sickle cell and treated control mice and thus we have decided not to include treated controls in the presentation of our results. Our results from the open field test showed that treated sickle cell mice had reduced evidence of anxiety/depression similar to control levels, but better than non-treated sickle cell mice, indicated by a significantly longer distance traveled ( $P = 0.02$ ; Figure 4(a)) reduced thigmotaxis by spending more time in the middle of the enclosure ( $P = 0.04$ ; Figure 4(b)). Similarly, learning and memory for treated sickle cell mice measured as percent preference for the novel object ( $72.21 \pm 8.92\%$ ) were the same as that of controls, but significantly better than non-treated sickle cell mice ( $14.86 \pm 5.98\%$ ;  $P < 0.0001$ ; Figure 4(c)).

We further confirmed impaired cognitive (learning and memory) and neurobehavioral function as well as improvement with minocycline treatment using the fear conditioning test. In the acquisition phase, there were no significant differences between sickle cell mice, controls, or sickle cell mice on minocycline treatment. The percent freezing was 7.6%, 7.7%, and 10.2%, respectively, in sickle cell mice, control mice, and sickle cell mice on minocycline treatment, at the acquisition phase (Figure 4(d)). Thus percent freezing during the acquisition phase was essentially similar between all genotype and treatment groups except at the 340 seconds ( $P = 0.008$ ) and 420 seconds ( $P = 0.02$ ) time points where treated sickle cell mice or controls seemed to do better than non-treated sickle cell mice. On contextual fear testing, sickle cell mice treated with minocycline had significantly higher freezing percent across all time points, an indication of better learning and memory compared to sickle mice that were not treated with minocycline or that received plain drinking water. There was no significant difference in performance on this test between treated sickle cell mice and controls except at the 360 and 420 seconds time points (Figure 4(e)). Similarly, on cued fear testing, sickle cell mice that received minocycline also showed significantly better memory for the CS at all time points, compared to sickle cell mice that received plain drinking water. And as was the case with contextual fear testing, there were no significant differences between treated sickle cell mice and controls except at the 480 seconds time point (Figure 4(f)).

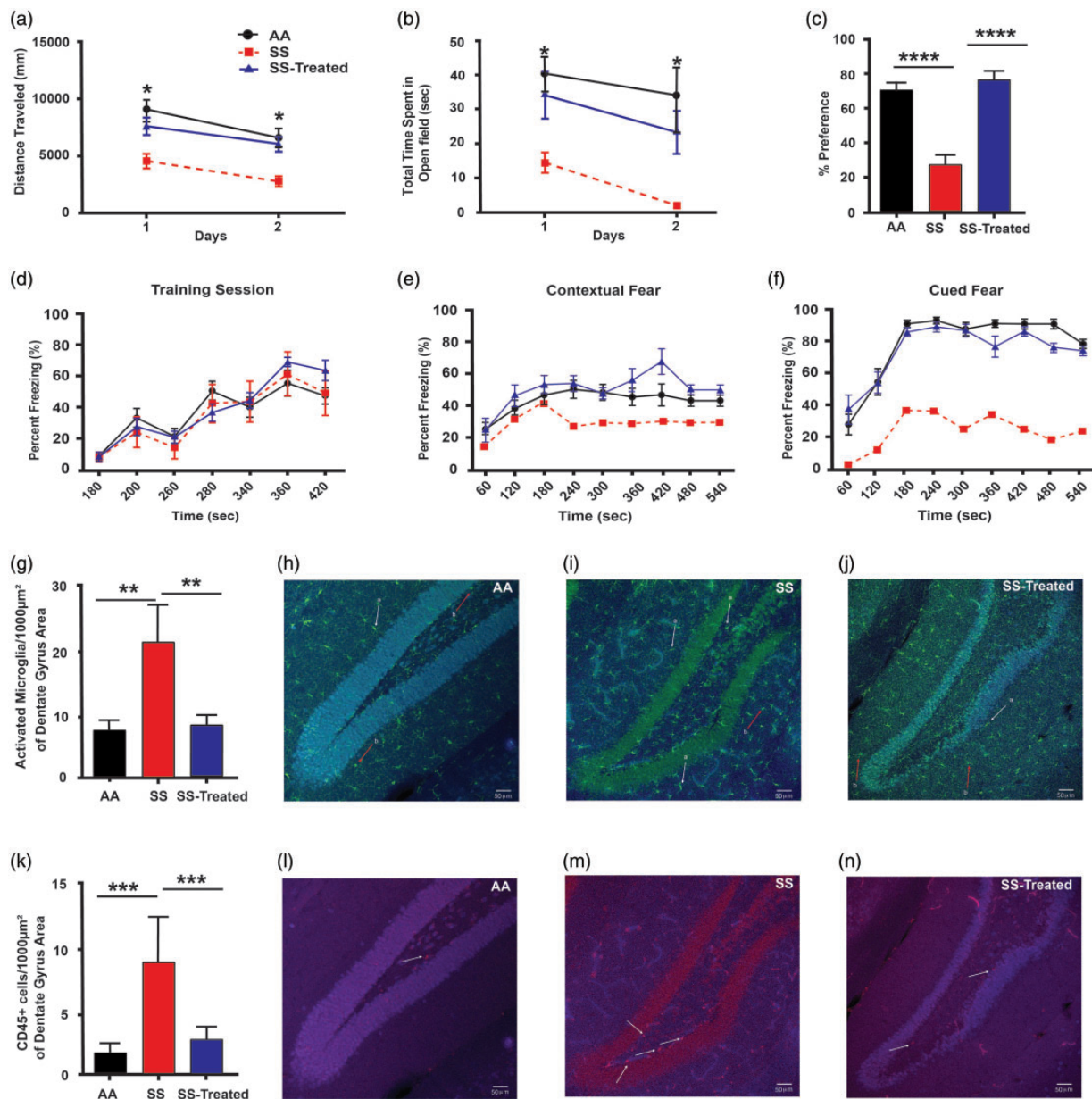
### Treatment with minocycline attenuates neuroinflammation in sickle cell mice

Consistent with previous results, the treatment of sickle cell mice with minocycline resulted in a significant reduction in evidence of neuroinflammation. Thus, sickle cell mice that received minocycline showed significant reduction in the number of activated microglia ( $8.21 \pm 1.93$  cells/1000  $\mu\text{m}^2$  vs.  $22.01 \pm 6.12$  cells/1000  $\mu\text{m}^2$ ;  $P = 0.007$ ) and CD45<sup>+</sup> or bone marrow-derived microglia or mononuclear cells ( $3.04 \pm 0.80$  cells/1000  $\mu\text{m}^2$  vs.  $9.32 \pm 2.23$  cells/1000  $\mu\text{m}^2$ ;  $P = 0.002$ ) compared to non-treated sickle cell mice. There was no statistically significant difference between treated sickle cell mice and controls, in the number of activated microglia or CD45<sup>+</sup> bone marrow-derived microglia in the dentate or peri-dentate gyrus area. However, similar to the observation between treated and non-treated sickle cell mice, the number of activated microglia ( $P = 0.010$ ) and CD45<sup>+</sup> bone marrow-derived microglia or mononuclear cells ( $P = 0.0006$ ) was significantly different between controls and non-treated sickle cell mice (Figure 4(g) and (h)).

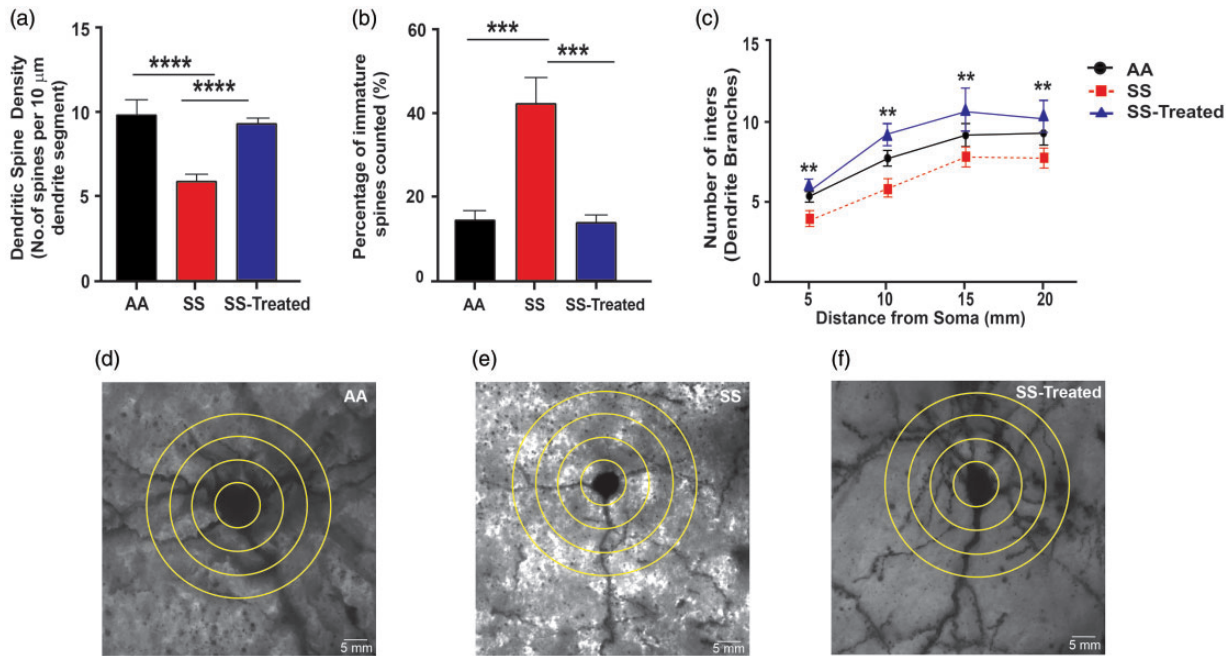
### Sickle cell mice treated with minocycline showed improvement in evidence of neuroplasticity and potentially neurogenesis

Finally, we sought to determine the structural neuronal mechanism for the observed cognitive and neurobehavioral benefit of minocycline. We observed that sickle cell mice that received minocycline had significantly higher dendritic spine density ( $9.31 \pm 0.69$  spines/10  $\mu\text{m}$  vs.





**Figure 4.** Minocycline treatment attenuates cognitive and neurobehavioral abnormalities and evidence of neuroinflammation in sickle cell mice compared to non-treated sickle cell mice. Figure shows that there was significant improvement in (a) distance traveled ( $P < 0.0001$ ), (b) time spent in open field ( $P = 0.008$ ), and (c) shows that percent preference for the novel object was significantly higher ( $P < 0.0001$ ) in treated sickle cell mice (SS-treated) compared to non-treated sickle cell mice (SS), non-treated sickle cell mice were not different from the data observed in our prior experiments in Figures 1 and 3(a) to (c). Cognitive and neurobehavioral performances were similar between SS-treated mice and controls. Also, fear conditioning test shows that treatment with minocycline attenuates cognitive and neurobehavioral deficits in treated sickle cell mice compared to non-treated sickle cell mice. The figure shows (d) comparable levels of performance on the acquisition phase of fear training, evidenced by similar percent freezing among non-treated sickle cell (SS), treated sickle cell (SS-treated), and control (AA) mice ( $P = 0.22$ ). In the testing phase, treated sickle cell mice performed significantly better on the (e) contextual fear testing with significantly higher percent freezing at all time points compared to non-treated sickle cell mice ( $P < 0.001$ ). Similarly, on (f) cued fear test, sickle cell mice treated with minocycline also had significantly higher percent freezing at almost all time points compared with non-treated sickle cell mice ( $P < 0.001$ ). Comparison of percent freezing between controls mice and non-treated sickle cell mice was also statistically significant, while there was no significant difference in percent freezing between treated sickle cell mice and controls. It should be noted that higher percent freezing indicates better memory for the conditioned and unconditioned stimulus and thus better learning, memory, and thus cognitive function. Additionally, we show that minocycline treatment attenuates levels of neuroinflammation in sickle cell mice when compared to non-treated sickle cell mice. Significantly fewer numbers of (g) activated microglia ( $P = 0.007$ ), estimated using the combination of Iba1 staining and morphological characteristics of activated microglia cells, and (h) peripherally derived mononuclear cells ( $P = 0.002$ ), measured via number of labeled CD45 cells, were observed in treated sickle cell mice (SS-treated) when compared to sickle cell mice (SS). Results are presented here as mean  $\pm$  SEM.  $N = 15$  mice per group for cognitive and neurobehavioral testing ((a) to (f)) and 9 per group for histological analysis ((g) to (h)). The images to the right of the bar graphs of (g) and (k) are representative images of the data plotted in the bar graph. The white arrows in (h), (i), and (j) indicate microglia in the dentate and peri-dentate gyrus area. Arrows labeled (a) indicate activated microglia, while those labeled (b) are examples of normal or non-activated microglia. In (l), (m), and (n), the white arrows show the presence of CD45<sup>+</sup> peripherally (bone marrow) derived mononuclear cells in the dentate and peri-dentate gyrus area and shows the higher density among SS compared to SS-treated and AA mice.



**Figure 5.** Minocycline treatment results in improvement in neuroplasticity evidenced by increased dendritic spine density and dendrite arbors, but a decreased proportion of immature spines in sickle cell mice treated with minocycline compared to non-treated sickle cell mice. In the figure, we see that (a) sickle cell mice treated (SS-treated) with minocycline had a significantly higher dendritic spine density ( $P < 0.0001$ ) per 10  $\mu\text{m}$  dendrite segment compared to non-treated sickle cell mice (SS). There were no significant differences in dendritic spine density between control (AA) and SS-treated mice. Also, (b) sickle cell mice that did not receive minocycline (non-treated sickle cell mice or SS) had a more than 2-fold higher proportion of immature dendritic spines compared to sickle cell mice that received minocycline, i.e. SS-treated ( $P < 0.0001$ ) and controls ( $P = 0.0002$ ). We used Sholl analysis to evaluate the number of dendrite branches (arbors) with 5  $\mu\text{m}$  interval between concentric circles (20  $\mu\text{m}$  distance total). Representative images are shown for (d) controls (AA), (e) non-treated sickle cell (SS), and (f) treated sickle cell mice (SS-treated). (c) shows that non-treated sickle cell (SS) mice had significantly fewer dendrite arbors compared to controls at 5  $\mu\text{m}$  ( $P = 0.004$ ) and 10  $\mu\text{m}$  ( $P = 0.005$ ) distance away from the soma. Similarly, SS mice had fewer dendrite arbors compared with treated sickle cell (SS-treated) mice at all distance from the soma, i.e. at 5  $\mu\text{m}$  ( $P < 0.0001$ ), 10  $\mu\text{m}$  ( $P = 0.0003$ ), 15  $\mu\text{m}$  ( $P = 0.0487$ ), and 20  $\mu\text{m}$  ( $P = 0.0280$ ) away from the soma. There was no significant difference seen between controls and treated sickle cell mice. The interval distance as well as the final distance of 20  $\mu\text{m}$  was chosen a priori based on the size of the image that could be generated from our fluorescent microscope. Results are presented as mean  $\pm$  SEM.  $N = 6$  per group. Note that these are the rest of the mice from the cognitive (learning/memory) and behavioral test presented in Figure 4(a) to (f).

$5.95 \pm 0.74$  spines/ $10 \mu\text{m}$ ;  $P < 0.0001$ , Figure 5(a)) compared to age-matched sickle cell mice that did not receive minocycline treatment or received plain drinking water. Consistent with previous results (Figure 3(c)), sickle cell mice that did not receive minocycline also had significantly fewer dendritic spines per  $10 \mu\text{m}$  of dendrite segment ( $5.95 \pm 0.74$  vs.  $9.85 \pm 1.87$ ,  $P = 0.0001$ ; Figure 5(a)) compared to controls. With regards to spine morphology, we also observed that sickle cell mice treated with minocycline had significantly (more than 2-fold) fewer proportion (%) of immature dendritic spines as a percentage of the total spines counted ( $12.08 \pm 1.57\%$  vs.  $32.08 \pm 4.81\%$ ,  $P < 0.0001$ ; Figure 5(a)) compared to age-matched sickle cell mice that received plain drinking water. Similarly, non-treated sickle cell mice also had a more than two-fold higher proportion of immature dendritic spines ( $32.08 \pm 4.81$  vs.  $14.34 \pm 2.48$ ,  $P = 0.0002$ ; Figure 5(b)) compared to controls. There was no statistically significant difference in the proportion of immature spines between treated sickle cell mice and controls. In further examining the impact of minocycline on neuroplasticity, we evaluated dendrite arborization using Sholl analysis. Compared to controls, non-treated sickle cell mice had significantly fewer dendrite arbors at 5  $\mu\text{m}$  (3.94 vs. 5.33;  $P = 0.004$ ) and 10  $\mu\text{m}$  (5.69 vs. 7.50;  $P = 0.005$ ) distance away from the soma (Figure 5(c), (d), and (e)). Also,

non-treated sickle cell mice also had significantly fewer dendrite arbors compared to sickle cell mice that received minocycline. This difference was present at all distances from the soma that was evaluated and  $P$  value at each distance was  $< 0.01$  (Figure 5(b), (c), (e), and (f)). These experiments show that the treatment of sickle cell mice with minocycline may have led to better neuroplasticity and our prior data above show that this is likely mechanistically linked to improvement in cognitive and neurobehavioral functions.

We sought to determine whether minocycline has any impact on cell fate for NPCs, due to its reported impact on inflammation and "spinegenesis". Our final sample size was too small for a robust assessment of neurogenesis and as such the analysis did not show statistically significant results (see online-only Supplementary results). However, we observed a trend where sickle cell mice treated with minocycline, having a higher number of dividing (BRDU<sup>+</sup>) cells when compared to non-treated sickle cell mice (Figure S2a). Also, while treated and non-treated sickle cell mice showed similar numbers of DCX<sup>+</sup> (NPCs) and BRDU<sup>+</sup>DCX<sup>+</sup> cells (Figure S2a). There seem to be a marked increase in the number of NPCs differentiating into mature or young neuronal (DCX<sup>+</sup>NeuN<sup>+</sup>) cells among sickle cell mice treated with minocycline, while

among sickle cell mice cells that did not receive minocycline, the NPCs seems to differentiate more into astrocytes (DCX<sup>+</sup>GFAP<sup>+</sup>) cells (Figure S2b).

## Discussion

In this study, we showed that aged (13 months old) Townes sickle cell mice have more severe cognitive and neurobehavioral deficits compared to age- and sex-matched controls, similar to what has been described in humans with SCD.<sup>14</sup> There was also significantly more evidence of neuroinflammation (evidenced by a higher density of activated microglia and bone marrow-derived proinflammatory mononuclear cells) as well as abnormal neuroplasticity (evidenced by lower dendrite and dendritic spine density and a higher proportion of immature dendritic spines) in aged sickle cell mice compared to age matched controls. We also noted that these neuroimmune and neuronal abnormalities tracked with the presence and severity of cognitive and neurobehavioral dysfunction and that treatment with minocycline (a drug which has been shown to reverse these changes<sup>19,20,42,57</sup>), restored cognitive and neurobehavioral function as well as led to a decrease in evidence of neuroinflammation and abnormal neuroplasticity.

In studies among individuals with SCD, evidence of cognitive and neurobehavioral (such as anxiety) deficits are observed in early childhood<sup>58</sup> and adolescents.<sup>3,4,6</sup> This, is in addition to the fact that children and adolescents with SCD also experience a high level of SCI (shown to result in cognitive deficits)<sup>59</sup> compared to their non-SCD counterparts.<sup>60–62</sup> This finding was instrumental in our decision to conduct this study.

However, our studies did not show early differences in cognitive and behavioral tests (at 6 months of age) but clearly show the presence of an age-related decline in cognitive and neurobehavioral function. The reason(s) for this apparent difference between age at development of cognitive decline in sickle cell mice compared to humans is not yet clear to us. However, we recognize that decline in cognitive function is generally an age-related phenomenon<sup>63,64</sup> and it has been reported that decline in cognitive function as well as its associated impact in individuals with SCD worsens with age.<sup>65,66</sup> And our study was able to capture this aspect of SCD-related cognitive impairment with the emergence of learning, memory, and neurobehavioral deficits by 13 months of age. Thus it was reasonable to conclude that our prior observations (in sickle cell mice) as well as reports in the literature, that the likelihood of development of cognitive and neurobehavioral abnormalities in SCD increases with age,<sup>14,67,68</sup> was supported by the results of our study.

It is well established that individuals with SCD are in a state of chronic systemic inflammation and that systemic inflammation is associated with increased vaso-occlusive crisis and stroke. Studies have also indicated that peripheral inflammation is associated with cognitive impairment in SCD<sup>2</sup> as well as neuroinflammation.<sup>19,22,37</sup> In our study, at baseline, evaluation of the hippocampus/DG revealed significant evidence of neuroinflammation in aged sickle cell mice. This was marked by the higher density of activated microglia and CD45<sup>+</sup> bone marrow-derived microglia

(mononuclear cells) compared to controls (Figure 3(e) and (f)). The finding of a higher density of bone marrow-derived microglia is of particular interest as it shows that in sickle cell disease, mononuclear cells from the peripheral blood migrate into the brain parenchyma in more numbers compared to controls and that these might be contributors to neuroinflammation. Additionally, studies have linked the presence of bone marrow-derived microglia in the amygdala and hypothalamus with increased anxiety,<sup>69,70</sup> suggesting that levels of bone marrow-derived microglia might play a role in the development of the anxiety-like behavior seen in sickle cell disease, although this study did not evaluate population of these cells in the amygdala. Further, we noted that dendritic spine density on pyramidal neurons was lower in aged sickle cell mice compared to controls (Figure 3(g)). Dendritic spine density is a component of neuroplasticity and has been shown to play a significant role in learning and behavior.<sup>71,72</sup> Our findings that both neuroinflammation and lower dendritic spine density are potential contributors to the presence of cognitive and behavioral deficits in SCD are strengthened by studies that indicate that the presence and interaction between the two factors are associated with cognitive decline.<sup>73,74</sup> These factors provide insight into the cellular and pathobiological mechanisms that contribute to cognitive and neurobehavioral decline in SCD.

Based on the above findings and reports in the published literature, we evaluated whether treatment with minocycline could influence cognitive and neurobehavioral changes as well as the neuronal and neuroinflammatory abnormalities observed in sickle cell mice. Minocycline is a drug (antibiotic) that is capable of crossing the blood-brain barrier and that is beneficial to hippocampal integrity.<sup>75,76</sup> Minocycline has been implicated in the reversal of neuronal cell death and the promotion of neurite outgrowth,<sup>39,43</sup> reduction of inflammation,<sup>77,78</sup> and the restoration of cognitive function.<sup>42,79</sup>

In our study, we first observed that when the sickle cell mice that developed cognitive and neurobehavioral deficits (see Figure 3(a) to (d)) after being aged from 6 (see Figure 2) to 13 months of age were randomized to treatment vs. no treatment with minocycline, the treated mice showed a complete reversal of established cognitive and neurobehavioral deficits (Figure S1a i–iii and S1b i–iii). The sickle cell mice that were randomized to not receive treatment with minocycline did not show any improvement in their performance on cognitive or neurobehavioral tasks or tests. Immunohistochemical analysis also shows that treatment with minocycline resulted in a decrease in evidence of neuroinflammation, indicated by a lower density of activated microglia as well as CD45<sup>+</sup> bone marrow-derived microglia (mononuclear cells) in the treated compared to the non-treated sickle cell mice (Figure S1c i–ii).

We repeated the above minocycline treatment study in a larger cohort of mice ( $N = 15$  per group, Figure 4(a) to (f)) to both confirm our findings and also allowed us to carry out histological analysis to gain more insight into the potential structural changes leading to better cognitive and neurobehavioral performance in the minocycline treated mice. Treatment with minocycline as before led to better



cognitive and neurobehavioral function in the treated compared to non-treated sickle cell mice. We also observed that sickle cell mice treated with minocycline had significantly lower density of activated microglia and CD45<sup>+</sup> bone marrow-derived microglia (mononuclear cells) in the hippocampus/DG compared to non-treated sickle cell mice (Figure 4(g) and (h)). Furthermore, treatment of sickle cell mice with minocycline resulted in a neuroplasticity pattern that is similar to that of the healthy controls and thus better than that of non-treated sickle cell mice (Figure 5(a) to (c)). For instance, treated sickle cell mice had significantly higher dendritic spine density (Figure 5(a)), but a significantly fewer proportion of immature dendritic spines (Figure 5(b)) on hippocampal and cortical pyramidal neurons compared to non-treated sickle cell mice. Additionally, analysis of dendrite arborization shows that sickle cell mice treated with minocycline had more dendrite arbors (branches for every 5 μm distance away from the soma compared with non-treated sickle cell mice (Figure 5(c) to (f)).

The results discussed above indicate that cognitive and neurobehavioral deficits in SCD are associated with neuroinflammatory and neuronal pathological changes. Although these are novel observations in SCD, they have been described in non-SCD-related cognitive and neurobehavioral deficits. As stated above, it has been shown that cognitive and neurobehavioral deficits as a result of stress from repeat social defeat are mediated by neuroinflammation seen as increased density of activated microglia and CD45<sup>+</sup> bone marrow-derived microglia (mononuclear cells) in the hippocampus/DG.<sup>19,20,22,80,81</sup> The use of minocycline to modify (improve) cognitive and neurobehavioral performance is also not in itself novel. However, this application to SCD-related cognitive and neurobehavioral deficit is the first time it has been used in this disease. Our results show that treatment of sickle cell mice with minocycline resulted in improvement in cognitive and neurobehavioral function in the treated compared to non-treated sickle cell mice. The mechanisms of this benefit seem to be a modulation of neuroinflammation and neuroplasticity as described in non-SCD models;<sup>19,42,82–85</sup> however, there could be other novel mechanisms. The sickle cell mice that were treated with minocycline displayed a lower level of cellular evidence of neuroinflammation (CD45<sup>+</sup> bone marrow-derived microglia (mononuclear cells) in the hippocampus/DG), this is thought to be due to the fact that minocycline decreases brain levels of inflammatory cytokines<sup>19</sup> by inhibiting matrix metalloproteases<sup>83,86,87</sup> and thus prevent the conversion of inactive forms of cytokines and chemokines to active ones. Another mechanism of action of minocycline by which sickle cell mice treated with the drug might benefit is via inhibition of sphingomyelinase activity<sup>88,89</sup> and thus reducing the negative impact of this enzyme on dendrite arborization, and proliferation and maturation of dendritic spines. Finally, it has also been documented that treatment with minocycline results in improved neurogenesis or at least a halting of the gliogenic effects of inflammatory cytokines.<sup>75,84,90,91</sup> Indeed, despite having similar density of neuron progenitor cells (NPCs or DCX and BrDU positive cells;

Figure S2a), in our study, we noted a tendency for NPCs in the hippocampus/DG of minocycline treated sickle cell mice, to differentiate to mature neurons as opposed to a tendency for differentiation into astroglia (gliogenesis) in the non-treated sickle cell mice (Figure S2b). Other potential mechanisms of cognitive and behavioral deficit that were not explored as part of this project are the roles of microinfarcts and cerebral microvasculopathy as described in our prior publication.<sup>34</sup> These mechanism will be examined as part of a currently ongoing, recently funded study, which will also examine whether these microinfarcts and cerebral microvasculopathy have any impact on neuroplasticity and also whether their burden (number)/severity or size (for microinfarcts) contributes to the severity of SCD-related cognitive and/or behavioral deficits.

In conclusion, we have shown that aged sickle cell mice have cognitive and neurobehavioral deficits akin to those described in children and adults with SCD. These deficits worsened with age and were associated with neuroinflammation and abnormal neuroplasticity. Finally, treatment with a neuroinflammation blocking drug such as minocycline reversed the observed deficits as well as the associated evidence of neuroinflammation and abnormal neuroplasticity. Thus minocycline and/or more novel drugs with similar mechanisms of action could represent a new line of treatment or prevention paradigm for cognitive and/or neurobehavioral deficits in individuals with SCD. These novel insights into a debilitating complication of SCD can now be studied further using our paradigms to investigate for instance, the role and interaction of specific cytokines and peripheral and central inflammatory pathways to dissect the pathogenesis and treatment of cognitive and behavioral deficits in individuals with SCD.

#### AUTHORS' CONTRIBUTIONS

HIH designed the experiment, HIH, RA, NAR, and JJ performed experiments and data analysis, HIH and RA wrote the manuscript and DRA, NAR, and JJ provided critical review. HIH and DRA performed final critical review. All authors endorsed the submission of this manuscript.


#### DECLARATION OF CONFLICTING INTERESTS

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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