Acute minocycline treatment inhibits microglia activation, reduces infarct volume, and has domain-specific effects on post-stroke cognition in rats

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Abstract

Ischemic stroke affects millions of individuals worldwide and a high prevalence of survivors experience cognitive deficits. At present, the underlying mechanisms that drive post-stroke cognitive decline are not well understood. Microglia play a critical role in the post-stroke inflammatory response, but experimental studies show that an accumulation of chronically activated microglia can be harmful and associates with cognitive impairment. This study aimed to assess the effect of acute post-stroke minocycline treatment, a tetracycline derivative that readily crosses the blood-brain barrier and has been shown to inhibit microglia activation, on chronic microglia and astrocyte expression within both the infarct and remote white matter regions, as well as determine its effect on various domains of cognitive function post-stroke. Nine-month-old male rats received an injection of endothelin-1 into the right dorsal striatum to induce a transient focal ischemic stroke, and then were treated with minocycline or saline for 4 days post-stroke. Rats were tested using a series of lever-pressing tasks and the Morris water maze to assess striatal-based learning, cognitive flexibility, and spatial learning and reference memory. We found that minocycline-treated rats had smaller stroke-induced infarcts, less microglia activation in the infarct area and less microglia activation in remote white matter regions compared to saline-treated rats at 28 days post-stroke. The behavioural testing results differed according to the cognitive domain; whereas minocycline-treated rats trended towards improved striatal-based learning in a lever-pressing task, but cognitive flexibility was unaffected during the subsequent set-shifting task. Furthermore, minocycline treatment unexpectedly impaired spatial learning, yet it did not alter reference memory. Collectively, we show that post-stroke minocycline treatment can reduce chronic microglia activation even in remote brain regions, with domain-specific effects on cognitive function.

Introduction

Post-stroke cognitive impairment is highly prevalent and is associated with reduced quality of life[1–4] but the underlying mechanisms that drive post-stroke cognitive impairment and decline are poorly understood. Approximately 25% of ischemic strokes in humans are covert and produce lacunar infarcts located in subcortical regions such as the basal ganglia and deep white matter[5] and are associated with remote white matter abnormalities, including chronic pro-inflammatory microglia activation[6–8]. Subcortical covert strokes are also linked with increased risk of cognitive impairment and dementia clinically, and may affect multiple cognitive domains, including executive function and memory loss[9–12]. Further, experimental studies have identified an association between microglia activation in brain white matter and cognitive impairment, but this has not been sufficiently evaluated in the context of stroke[13,14]. Therefore, further interrogation of the post-stroke microglial response and its relationship with cognitive impairment is needed.

The post-stroke inflammatory response is characterized by activation and proliferation of microglia and astrocytes and infiltration of macrophages toward the stroke-induced infarct area[15–17]. Microglia activation may be important for early phagocytosis of debris and release of neuroprotective mediators[18],

however, activated microglia can take on a pro-inflammatory phenotype and exacerbate injury through production of cytokines, chemokines and reactive oxygen species and lead to blood-brain barrier breakdown[16,19]. If microglia remain chronically activated, they can be detrimental to the survival and function of neurons and limit tissue repair and recovery post-injury[20,21]. Further, post-stroke microglia activation is not contained to the infarct region and has also been detected in remote brain structures, particularly within the white matter[22–24]. Minocycline is a tetracycline derivative that can cross the blood-brain barrier and has previously been shown to inhibit microglia activation[25], serving as a promising therapeutic to inhibit post-stroke microglia activation. Previous experimental stroke studies using minocycline have largely focused on the microglial response in the infarct acutely [26–31], thus, our knowledge on its effect on chronic and remote microglia activation is limited.

Microglia activation in brain white matter is associated with worse cognitive outcomes in experimental studies [13,14, 32–34] and is also related to a loss of white matter integrity clinically[35]. Both preclinical and clinical studies have found that changes to white matter integrity are particularly associated with decreased executive functioning, in subdomains such as cognitive flexibility and working memory[13,14, 36–38]. To date, experimental stroke studies utilizing minocycline have employed behavioural tasks to assess hippocampal and motor function but have lacked testing in executive domains. Rodent models have observed improvements in hippocampal function with minocycline treatment[39–41] but, a study in healthy humans found that minocycline could impair spatial memory[42], emphasizing variability across species and the need for further cognitive testing. Overall, the possible differential effects of post-stroke minocycline treatment across cognitive domains are unknown.

Experimental stroke studies using minocycline have focused on acute infarct-related pathology when secondary injury may still be developing, emphasizing the need to evaluate chronic pathological outcomes. Further, we know that post-stroke microglia activation is not limited to the infarct, yet the effects of minocycline on microglia expression in remote brain regions post-stroke have not been investigated. Experimental studies performed in the acute phase post-stroke have observed smaller infarct sizes and reduced microglia expression in the infarct in minocycline-treated animals [26–31], thus, we hypothesized that these pathological changes would persist chronically and in remote brain structures. In the present study, we interrogated cognitive function across multiple domains, including a series of operant conditioning-based lever-pressing tasks to assess striatal-based learning and cognitive flexibility, and the Morris water maze to assess hippocampal-based spatial learning and reference memory. Previous work has identified a relationship between cognitive inflexibility and white matter microglia activation experimentally[13,14], thus, we hypothesized that inhibition of post-stroke microglia activation in the white matter could improve cognitive flexibility. Similarly, since we predicted a decrease in stroke-related pathology in the striatum of minocycline-treated rats, we also hypothesized that there would be an improvement in striatal-based learning. Although previous experimental studies using minocycline have observed improvements in hippocampal-based tasks[39–41], similar stroke models to ours have not reported hippocampal deficits[43,44], thus we did not expect to see treatment differences in

this cognitive domain. To address these hypotheses, we administered 4 days of minocycline treatment post-unilateral focal subcortical stroke and assessed its effect on chronic microglia and astrocyte expression in the infarct and remote brain regions and various domains of cognitive function.

Methods

Animals, Surgery, and Minocycline Treatment

Animal ethics and procedures used in this study were approved by the Animal Care Committee at Western University (protocol 2018 − 132). All rats in this study were housed in facilities maintained by Western University Animal Care and Veterinary Services on a 12-hour light/dark cycle with *ad libitum* access to food and water (unless otherwise stated). Male wildtype Fischer 344 rats were bred and aged in house to 9-months-old prior to induction of experimental stroke. Focal subcortical ischemia was modelled using a unilateral injection of endothelin-1 (ET-1) into the right dorsal striatum. Animals were anesthetized using 3% isoflurane and maintained at 1.5% isoflurane throughout surgery, with their body temperature maintained at 37°C. ET-1 (60 pmol dissolved in 3 µl sterile 0.9% saline) was administered using stereotaxic injection into the right dorsal striatum over 5 minutes. Post-surgery, rats were randomly divided into minocycline or saline treatment groups. Minocycline (Sigma-Aldrich; Oakville, ON) was diluted in sterile saline and administered intraperitoneally twice per day for four days after surgery (45 mg/kg on day of surgery and 22.5 mg/kg on subsequent 3 days as previously described[30]). Control rats received sterile saline injections administered intraperitoneally over the same timeline. To stay translational to the clinical utility of minocycline in stroke patients, non-stroke control animals were not included.

Lever-Pressing Tasks – Striatal-Based Learning and Cognitive Flexibility Testing

To assess striatal-based learning and cognitive flexibility, we utilized a series of operant conditioning-based lever-pressing tasks developed by Floresco et al.[45] and previously described by our lab in detail[13,46]. First, rats were food restricted and maintained at 85–87% of their free-feeding body mass to ensure motivation for 45 mg sucrose pellets (Dustless precision pellets, Bio-Serv; Burlington, ON). An operant conditioning chamber, housed within a sound-attenuating box, was equipped with two retractable levers located on either side of a food pellet receptacle and a cue light located above each lever. Customized software was used to operate the testing chamber (MED-PC IV, Med-Associates). Habituation to the operant chamber was followed by lever training in which the rats were required to press the lever that was randomly extended within 10 seconds to receive a sucrose pellet. To advance to testing, the rats had to press the lever on at least 85 out of 90 training trials. Following completion of lever training, rats' side bias was determined as previously described[45]. Briefly, over a series of trials both levers were simultaneously extended and the number of times each lever was pressed was recorded to determine whether the rat preferred the left or right lever.

Twenty-four hours after initial lever-press training and side bias determination, rats underwent a visual cue discrimination (VCD) task, in which they learned to press the lever that was associated with an illuminated cue light over 100 trials (Fig. 5A). A trial was initiated by the pseudo-random illumination of a cue light, followed by the extension of both levers. The rat would then have 10 seconds to press the lever located below the cue light to receive a sucrose pellet reward. A criterion of 8 consecutive correct lever presses was required to advance to the next phase of testing. Performance was assessed by tallying the number of errors committed over the 100 trials. Twenty-four hours after completing the VCD task, the rats underwent 20 more VCD trials to determine their memory retrieval of the “follow the light” strategy. A criterion of 15 out of 20 correct lever presses was required to advance to the next phase of testing (i.e., response discrimination (RD)). To assess cognitive flexibility (an important subdomain of executive function), on the 21st trial the task was switched to RD in which the rats were presented with a new rule: stay on one side and ignore the illuminated light (Fig. 5D). In the same manner as the VCD task, the light continued to pseudo-randomly alternate sides, but now, regardless of the light, only the side opposite to the rats’ side bias was always correct. Performance was assessed by the total errors committed across 120 trials. Rats that were not sufficiently motivated and failed to learn to lever press were excluded from all set-shifting results.

Morris Water Maze – Spatial Learning and Reference Memory Testing

After completing the set-shifting task, rats were taken off food restriction and allowed to return to their pre-food restriction body masses. A 2-day Morris water maze protocol adapted from Roof et al.[47] was used to assess hippocampal-based spatial learning and reference memory. In a dimly lit room, a circular tank (144 cm diameter) was filled with room temperature water (22–23°C) and dyed black with non-toxic acrylic paint. Cue signs were placed in the testing room on north (green cross), east (white triangle), and west (black square) walls (Fig. 6A). A target platform (12 cm diameter) was submerged 3 cm below the water surface in the centre of the northeast quadrant. On the first testing day, rats underwent 6 learning trials, each separated by 1 hour, to learn the location of the submerged platform. For each trial, rats were placed in the water in the south position facing the tank wall and allowed to swim until they found the platform. If a rat did not find the platform within 90 seconds, they were guided to the platform by the experimenter. The rats were allowed to stay on the platform for 30 seconds to observe their surrounding visual cues. Spatial learning performance was assessed by the amount of time required to find the hidden platform on each trial. The day after completing the 6 learning trials, the platform was removed from the tank and the rats underwent a single probe trial to assess reference memory. Rats were placed back in the water in the south position facing the tank wall and allowed to swim for 90 seconds. Their spatial reference memory was assessed by the time to reach the platform region (15 cm diameter corresponding to the previous location of the hidden platform), and time spent in the target quadrant. After MWM testing was complete, rats underwent 4 visually guided trials in which the spatial cues were removed from the walls and the platform was visibly marked with a flag placed on top of the platform to determine any differences in visual acuity or swim speed. Throughout all testing, time to locate the platform, average swim speeds, and swim paths were collected using ANYmaze tracking software

(Version 6.33, 267 Stoelting Company, Wood Dale, IL) with a webcam (C930e; Logitech, Switzerland) mounted on the ceiling above the water tank.

Immunohistochemistry (IHC)

Euthanasia and Brain Collection

Animals were euthanized 28 days post-ET-1 injection using an intraperitoneal injection of pentobarbital (Euthanyl, Bimeda MTC Animal Health Inc) and transcardiac perfusion was performed with 0.01M PBS followed by 4% paraformaldehyde (PFA). Brain tissue was collected and stored in 4% PFA for 24 hours at 4°C. Brains were then transferred into 30% sucrose solution and stored at 4°C prior to sectioning. Brain tissue was cut using a cryostat (CryoStar NX50, Thermo Fischer Scientific; Ottawa, ON) into 30 µm coronal brain sections and stored in cryoprotectant at -20°C until further processing.

IHC Staining: Standard diaminobenzidine (DAB)-mediated staining protocols were followed for free-floating sections. Brain tissue sections were initially rinsed in PBS and then incubated with H_2O_2 (Thermo Fischer Scientific; Ottawa, ON) in PBS for 10 minutes. Sections were then blocked for 1 hour at room temperature in 2% horse serum (Sigma-Aldrich; Oakville, ON) in 0.01M PBS and 0.2% Triton-X (Sigma-Aldrich; Oakville, ON). Next, sections were incubated at 4°C overnight with blocking solution and primary antibodies against NeuN (1:1000; MilliporeSigma; Burlington, MA), OX6/MHC-II (1:1000; BD Biosciences; Mississauga, ON), Iba1 (1:1000, Wako Chemicals; Richmond, VA), or GFAP (1:2000; Sigma-Aldrich; Oakville, ON). The next day, sections were incubated with horse anti-mouse (NeuN, MHC-II and GFAP) or goat-anti rabbit (Iba-1) biotinylated secondary antibody (1:500; Invitrogen; Waltham, MA, USA) in blocking solution for 1 hour at room temperature. Sections were processed using ABC kits (Thermo Fischer Scientific; Ottawa, ON) followed by incubation in DAB (Sigma-Aldrich; Oakville, ON). Stained sections were mounted onto slides using 0.3% gelatin and air dried overnight. Lastly, slides were dehydrated using progressive concentrations of ethanol and xylene and cover slipped using Depex mounting medium (Electron Microscopy Sciences; Hatfield, PA).

Image Analysis

All image analysis was conducted by an experimenter blinded to the treatment groups. Images were taken using an upright brightfield microscope (Nikon Eclipse Ni-E, Nikon DS Fi2 colour camera, NIS Elements Imaging; Mississauga, ON). Stitched images of anatomical regions of interest (forceps minor, corpus callosum, cingulum, internal capsule, hippocampus, and striatum) were captured using a 10x objective lens at Bregma levels + 3.00, + 2.00, + 0.00, and − 3.00 (Fig. 2A). White balance was automated using an off-tissue reference point and settings for light intensity, exposure, and aperture were kept consistent across images. Image processing of MHC-II$^+$ activated microglia, Iba1$^+$ microglia, and GFAP$^+$ astrocytes was conducted using ImageJ software. Our specific regions of interest were first outlined using the polygon tool, then the images were converted to 8-bit, the background was subtracted, and finally the images were thresholded with a fixed cut-off value. Cross-sectional area coverage was

recorded as a percentage for each region of interest. An average area coverage was calculated for the corpus callosum and cingulum, which spanned multiple coronal sections.

Stroke Infarct Volume Quantification

A series of anterior to posterior brain sections were stained with NeuN to visualize neuronal loss in the region of the stroke. Infarct regions were outlined, and areas were measured across the series of brain sections. To correct for edema, both the ipsilateral and contralateral hemispheres were also outlined, and the areas were measured. To calculate the infarct volume, the infarct area from each section was multiplied by the section thickness.

Data Analysis

All investigators were blinded to animal group identity during all aspects of the study and analyses. Statistical analyses were conducted using SPSS (Version 27; IBM Corporation, Armonk, NY, USA) and GraphPad Prism (Version 9; La Jolla, California, USA) software. Group comparisons were conducted using Welch's *t*-tests in which equal variances were not assumed. A two-way repeated measures ANOVA was used to assess learning performance across 6 trials in the MWM task. Significance was determined with = 0.05 and when required, Bonferroni's *post-hoc* correction was used. Methodology schematics were generated using BioRender (Biorender.com) and graphs were created using GraphPad Prism. All data are presented as mean values with error bars indicating standard error of the mean (SEM).

Results

Minocycline reduced stroke infarct volume and corresponding microglia and astrocyte expression

To assess the effects of post-stroke minocycline treatment on infarct volume, we conducted IHC to label for $NeuN^+$ neurons and identify regions of neuronal loss. As predicted, we found that minocycline-treated rats had significantly smaller infarct volumes than saline-treated rats (Fig. 1A, B; p = 0.0363). Similarly, we identified MHC-II^+ microglia in the infarct region and show that minocycline treatment significantly reduced the expression of chronic MHC-II^+ microglia activation in the infarct area (Fig. 1C, D; p = 0.0009). To assess the effects of minocycline treatment on total microglia numbers, we also measured $Iba1^+$ microglia expression and found that, compared to the saline controls, the minocycline-treated rats had significantly lower Iba1 expression within the stroke infarct area (Fig. 1E, F; p = 0.0354). Astrocytes also play an important role in the post-injury inflammatory response and can become activated in response to microglia activation[48,49]. Thus, we next identified $GFAP^+$ astrocytes in the infarct region and found that minocycline treatment significantly reduced $GFAP^+$ astrocyte expression in the infarct area (Fig. 1G, H; p = 0.0255). Overall, our collective findings were consistent with our predictions, as minocycline treatment not

only reduced infarct volume, but it also reduced the total microglia and the extent of activation of microglia and astrocytes in the stroke infarct region.

Minocycline reduced MHC-II$^+$ chronically activated microglia in white matter post-stroke but did not alter total microglia or astrocyte activation in remote brain regions

Previous work has found evidence of remote white matter injury and inflammation post-stroke[22], thus, we sought to determine the potential of minocycline to reduce chronic post-stroke microglia and astrocyte activation within remote white matter tracts. We analyzed the expression of MHC-II, Iba1, and GFAP in the forceps minor, corpus callosum, supraventricular corpus callosum (SVCC), internal capsule, and hippocampus (Fig. 2A). The SVCC, a subset of the corpus callosum (excluding the portion of the corpus callosum medial to the lateral ventricles), was analyzed separately due to particularly high levels of MHC-II$^+$ microglia in this region, and previous results suggesting a relationship between microglia activation in the SVCC and cognitive impairment in the executive function domain[13,14]. As predicted, we show that minocycline treatment significantly reduced MHC-II$^+$ microglia activation within the major white matter tracts including the forceps minor ($p = 0.0075$), corpus callosum ($p = 0.046$), SVCC ($p = 0.049$), and internal capsule ($p = 0.0048$) post-stroke (Fig. 2B-D, F). Minimal MHC-II$^+$ expression was observed in the cingulum and no difference between treatment groups was found (Fig. 2D). Given the important role of the hippocampus in spatial learning and reference memory, we also assessed microglia activation in this brain region but found low MHC-II$^+$ microglia expression and no difference between treatment groups was observed (Fig. 2F). Given that minocycline treatment reduced MHC-II$^+$ microglia activation in white matter regions post-stroke, we next determined if it also altered total microglia numbers. We show that minocycline treatment did not alter Iba1 expression in any white matter regions of interest or the hippocampus (Fig. 3A-F). To further interrogate the specificity of post-stroke minocycline treatment in these remote brain regions, we assessed astrocyte activation. Our results show that minocycline treatment did not alter the expression of GFAP$^+$ astrocytes in the white matter regions of interest or the hippocampus (Fig. 4A-F). Taken together, our results demonstrate that while minocycline does reduce microglia activation in remote white matter tracts post-stroke, it does so without altering total microglia numbers or astrocyte activation in these regions. Further, this subcortical stroke model did not induce a strong MHC-II$^+$ microglial response in the hippocampus where no treatment differences were observed.

Cognitive flexibility was unaffected but minocycline-treated rats trended towards improved striatal-based learning in series of lever-pressing tasks

To determine the effects of post-stroke minocycline treatment on cognitive flexibility and striatal-based learning, rats were tested in a series of operant conditioning-based lever-pressing tasks. First, rats were tested for their ability to learn visual cue discrimination (VCD); a striatal-based task in which they had to learn to press a lever associated with an illuminated light (Fig. 5A). In line with our prediction, minocycline-treated rats trended towards less errors over the 100 VCD trials compared to saline-treated rats (Fig. 5B; $p = 0.19$). This trend for performance enhancement did not carry over to the following day

when the rats were given 20 VCD retrieval trials, as both groups committed a similar number of errors (Fig. 5C). Immediately following the 20 VCD retrieval trials, the task rule was shifted from “follow the light” to “stay on one side” in which only the lever opposite the rats’ side bias was now considered correct (Fig. 5D). Despite the initial trend towards improvement in the minocycline group during the VCD task, both groups had a similar performance in the set-shift, as there was no significant difference in errors committed over the 120 trials (Fig. 5E); findings which were in contrast to our prediction that post-stroke minocycline treatment would improve cognitive flexibility.

Minocycline caused an initial spatial learning impairment but did not alter reference memory in the Morris water maze

Next, we sought to determine if minocycline treatment altered hippocampal-based spatial learning or reference memory in the MWM task (Fig. 6A). First, spatial learning was tested over 6 trials in which the rats learnt the location of a hidden platform. The effect of minocycline treatment on spatial learning was assessed using a 2-way repeated measures ANOVA and unexpectedly, we found that minocycline-treated rats took longer to locate the platform on trial 6 (Fig. 6B; Treatment x Trial interaction: $F_{(4,40)} = 3.31$, $p = 0.02$; $p_{bonf} = 0.017$), despite similar mean swim speeds (Fig. 6C). The following day, spatial reference memory was tested in a 90-second probe trial. Reference memory performance was assessed based on time to platform and time spent in the target quadrant; no differences between treatment groups were observed (Fig. 6D and E). To determine any differences in swim strategies, we measured the amount of time spent in the perimeter of the water maze, but again found no differences between groups (Fig. 6F). Lastly, both the minocycline- and saline-treated rats maintained similar mean swim speeds in the 90-second probe trial (Fig. 6G). Overall, minocycline treatment resulted in a mild spatial learning impairment, with no effect on spatial reference memory.

Discussion

In the present study, we used a rat model of focal subcortical ischemia to investigate for the first time if acute post-stroke minocycline treatment could reduce chronic microglia and astrocyte expression in remote white matter regions as well as the stroke infarct area. Furthermore, we sought to determine if minocycline treatment would produce differential effects on various cognitive behavioural tests performed post-stroke. In line with our hypotheses, we observed reduced microglia activation in both the infarct area and remote white matter regions at 28-days post-stroke in minocycline-treated rats. Additionally, minocycline differentially affected striatal-based learning, cognitive flexibility, and hippocampal-based spatial learning and reference memory. In the following sections, we further discuss our novel findings on the chronic expression of microglia and astrocytes and the domain-specific cognitive outcomes we observed following acute post-stroke minocycline treatment.

Four days of post-stroke minocycline treatment reduced infarct volume

Minocycline is a second-generation tetracycline first used as an antibiotic, but it has since been repurposed for a wide range of disorders as an anti-inflammatory, anti-apoptotic, and neuroprotective agent[50]. Based on an established treatment protocol[30], we sought to administer minocycline acutely (45 mg/kg X 2 on day of stroke and 22.5 mg/kg X 2 on subsequent 3 days) and confirm the effects on infarct volume in the chronic phase (28 days post-stroke). This minocycline treatment regimen has previously been shown to reduce infarct volume both acutely[30] and chronically[51], and as expected, we confirmed that minocycline was sufficient to reduce the infarct volume present at 28 days post-stroke in our rat model of focal subcortical ischemia. Other experimental studies have utilized varying minocycline treatment windows, doses, stroke models and species, producing variable neuroprotective effects. The results from previous preclinical stroke models using minocycline in the rat have varied from reduced infarct sizes[26–30, 51,52] to no effects[31,40,53,54], and minocycline even exacerbated injury in a mouse model of ischemia[55]. Despite some variability in the literature, we confirmed that minocycline reduced infarct volume in our stroke model, and we next investigated our central objective: to determine whether acute minocycline treatment yielded chronic changes in the expression of microglia and astrocytes in the infarct and remote brain regions.

Acute post-stroke minocycline treatment alters chronic microglia and astrocyte expression

Given that previous studies using minocycline post-stroke have largely focused on microglia in the infarct acutely, we sought to investigate the chronic anti-inflammatory effects of minocycline in both the infarct and remote brain regions. We found that post-stroke minocycline treatment reduced chronic total microglia (Iba1^{+}) expression in the infarct, aligning with previous studies that reported decreases in total microglia in the infarct acutely[26–29]. Our results differed from one study that found minocycline decreased Iba1 expression in the infarct at 1- and 2-weeks post-stroke but not 4-weeks[28]. Contrary to our 4-day treatment regimen, the previous study administered just one injection of minocycline at a much smaller dose (3 mg/kg), possibly explaining the contrasting results. Having found that minocycline reduced total microglia expression in the infarct area chronically, we next assessed total microglia in remote white matter regions and the hippocampus. In contrast to the infarct area, we did not observe any treatment differences in total microglia expression in any of the remote brain regions. Proliferation and recruitment of microglia are well defined in the infarct area post-stroke[17], but may be less prevalent in remote brain structures, perhaps explaining the lack of treatment differences in Iba1 expression remote from the site of injury. However, it is well established that post-stroke microglia activation increases in both the infarct and remote brain regions[16, 22–24], thus, we next assessed the effects of minocycline treatment on chronic microglia activation.

Although minocycline has been shown to reduce total microglia expression post-stroke, its anti-inflammatory effects are thought to occur through inhibition of microglia activation[25]. Since preclinical stroke studies using minocycline have identified a reduction in microglia activation markers in the infarct acutely (Cd11b[30], CD68[31], HMGB1[56]) we predicted that this effect would persist through the chronic

phase. Similar to the acute experiments[30,31,56], we found that acute post-stroke minocycline treatment can also reduce activated microglia (MHC-II$^+$) in the infarct at 28 days post-stroke. Given that microglia activation also increases in remote brain regions post-stroke, particularly in the white matter[22–24], we conducted a novel investigation into the efficacy of minocycline to reduce microglia activation in remote brain regions. In contrast to our findings for total microglia expression (i.e., a lack of treatment effect), we found that acute minocycline treatment post-stroke was effective at reducing chronic microglia activation in remote white matter regions but not the hippocampus. These novel findings highlight the potential for minocycline to be used in the future to interrogate chronic microglia activation in both the infarct and remote white matter regions.

In addition to characterizing the microglial profile post-stroke, we investigated astrocyte activation in the infarct and remote brain regions, as astrocyte proliferation and activation are also critical to the post-stroke inflammatory response, and astrocytes can become activated in response to microglia activation[48,49]. In line with our results for both total microglia and activated microglia, we found that minocycline reduced chronic astrocyte activation (GFAP$^+$) in the infarct area. Previous investigations into the effects of minocycline on astrocyte activation in the infarct have been performed in the acute phase and varying timelines have produced differing results, including no effects (3 days post-stroke)[30], increased astrocyte activation (3–7 days post-stroke)[31,57], and reduced astrocyte activation (7 days post-stroke)[29,52]. While our treatment regimen aligns with Yrjänheikki et al.[30] who found no effects of minocycline treatment on astrocyte activation in the infarct, their assessment occurred at just 3 days post-stroke. In contrast, we observed a reduction in astrocyte activation in the infarct at 28 days post-stroke, possibly revealing an interesting temporal profile to be considered in future studies. Next, our novel investigation into the effect of acute minocycline treatment on astrocyte activation in remote brain regions yielded results similar to that for total microglia expression (i.e., there were no treatment differences in astrocyte activation in remote white matter regions or the hippocampus). In contrast to microglia activation, which has been identified in remote brain regions post-stroke, astrocyte activation has largely been described in the infarct area[49], possibly explaining the lack of treatment differences in the remote brain structures. Having identified the chronic neurohistological outcomes of minocycline treatment in both the infarct and remote brain regions, we next investigated our second central objective: to determine whether our post-stroke minocycline treatment regimen would have differential effects on various cognitive-behavioural tests following stroke.

Minocycline produced cognitive domain-specific effects on behavioural performance

To date, preclinical stroke models using minocycline have assessed cognitive function mainly in hippocampal-dependent and motor domains. In the present study, we used a MWM task to assess hippocampal-based spatial learning and reference memory and found minocycline-treated rats presented with a mild deficit in spatial learning but not reference memory. Whereas rodent models of stroke and aging have previously reported improvements in hippocampal function with minocycline treatment[39–41],

our results align more closely with a clinical study that found minocycline impaired spatial memory performance[42]. Pathologically, we assessed microglia and astrocyte expression in the hippocampus but found no treatment differences, suggesting that other mechanisms are likely involved. Related to this possibility, it is worth noting that minocycline is nonspecific to microglia activation inhibition, and thus, it may exert other off-target effects that contributed to the observed impairment in spatial learning. Various versions of the MWM task have been used to assess hippocampal function in minocycline-treated rodents[39–41], but given the observed hippocampal deficit in our rats, future studies could consider including additional hippocampal-based cognitive tasks (e.g., novel object recognition or radial arm maze) when seeking to further explore the implications of post-stroke minocycline treatment on hippocampal function.

In addition to assessing hippocampal-dependent spatial learning and reference memory, we carried out the first investigation into the effect of post-stroke minocycline treatment on cognitive flexibility, a subdomain of executive function. Based on our previous work which identified a relationship between the extent of white matter microglia activation and cognitive inflexibility[13,14], we predicted that a reduction in white matter microglia activation would improve cognitive flexibility in the present study. Although we did detect significantly less microglia activation in the white matter of minocycline-treated rats as predicted, we observed no treatment differences in cognitive flexibility. One possibility for the lack of treatment effect in this study is because we did not induce sufficient impairment in cognitive flexibility to detect any treatment-related differences. Ultimately, looking beyond stroke models, it would be worthwhile for future research to evaluate whether minocycline can serve as a protective agent against deficits in cognitive flexibility, given that rodent models of aging and Alzheimer's disease have shown a direct relationship between cognitive inflexibility and the extent of microglia activation[13,14].

Related to our confirmed hypothesis that minocycline would reduce pathology in the infarct area (striatum), we also predicted that minocycline would improve the VCD learning component of the set-shifting task, as this behavioural task is heavily reliant on the striatum[45,58]. In line with the minocycline-related improvements in striatal pathology (i.e., reduced infarct volume, total microglia, as well as microglia and astrocyte activation), we also observed a trend towards improved striatal-based VCD learning in the minocycline-treated rats. Overall, this trend reveals the opportunity for more rigorous striatal-based testing (e.g., visuomotor associative learning tasks) in future studies, and confirms the importance of assessing cognition across multiple domains in studies utilizing minocycline. The collective cognitive-behavioural results of the present study (i.e., impaired spatial learning; no effect on cognitive flexibility; trend for improved VCD learning) highlight that the post-stroke effects of minocycline treatment on cognitive function are multi-faceted; findings which point to the need for additional investigation and scrutiny into minocycline as a putative post-stroke therapeutic strategy.

Experimental limitations and future directions

Women are at a greater risk of experiencing a stroke and have a higher prevalence of post-stroke cognitive decline[59,60]. Minocycline may also exert sex-specific effects given that an open-label clinical

trial utilizing post-stroke minocycline treatment improved neurological outcomes in males but not in females[61]. Sex-specific differences in microglia activation have also been identified[62], thus, an important limitation of this study was the exclusion of female rats. Subsequent studies using minocycline to assess post-stroke microglia activation and cognitive impairment should include females to better understand how sex may influence this relationship.

An increase in microglia that express MHC-II has been previously identified in the infarct and white matter tracts post-stroke, and represents a marker associated with a chronic activation state[22,24]. For this reason, we assessed the presence of MHC-II$^+$ activated microglia in our study. That said, recent single-cell transcriptomic studies have identified a wide range of microglia activation states and numerous microglia activation markers[63,64]. At this point, it is unknown how minocycline may differentially affect these various activation states. Experimental studies suggest that minocycline selectively inhibits M1-like (pro-inflammatory) microglia activation and may increase or have no effect on M2-like (anti-inflammatory) microglia[29,65]; however, we now know that microglia activation is much more complex than the bidirectional M1 versus M2. Future studies should consider the use of transcriptomics to elucidate the effects of post-stroke minocycline treatment on diverse microglia activation states.

Stroke-induced microglia activation causes the release of both neurotoxic and neuroprotective inflammatory mediators[18]. The temporal profile of the toxic versus protective microglial response is still not well understood and requires further investigation. Minocycline has mostly been administered acutely in experimental stroke models, but future studies may consider delaying treatment to a later timepoint. Delayed treatment could allow for early phagocytosis of debris and release of anti-inflammatory mediators to instigate injury recovery, prior to inhibition of the more pro-inflammatory and neurotoxic microglia phenotypes. Further, the mechanism by which minocycline inhibits microglia activation is unclear. Studies point to its inhibitory effects on enzymes such as iNOS and MMPs, as well as inhibition of caspase-1 and caspase-3 activation as potential targets[27, 66–68] but for a more mechanistic approach, future studies should consider the use of a more specific inhibitor of microglia activation, such as MCC950[69].

Conclusion

In the present study, we induced subcortical ischemia in a rat model to investigate the effects of post-stroke minocycline treatment on chronic microglia and astrocyte expression, as well as the rats' performance on behavioural tasks that required various domains of cognitive function. Overall, we revealed minocycline's capacity to reduce chronic microglia and astrocyte expression in the infarct, and for the first time, its ability to reduce microglia activation in remote white matter tracts post-stroke. These novel findings emphasize minocycline's use for interrogating inhibition of microglia activation in remote brain regions in future stroke studies. Moreover, minocycline produced domain-specific cognitive changes, confirming that there is a complex relationship between microglia activation and cognition, and providing a strong rationale for broad cognitive testing in future models using minocycline.

Declarations

Disclosures

Ethics Approval: All animal procedures were approved by the Animal Care Committee at Western University (protocol 2018-132). All rats used in this study were housed in facilities maintained by Western University Animal Care and Veterinary Services.

Consent for Publication: N/A

Availability of data and materials: The datasets analysed in this study are available from the corresponding author on reasonable request.

Competing Interests: The authors declare no competing interests.

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Author Contributions: SJM, VA, SVP performed in vivo experiments. SJM conducted immunohistochemistry experiments and completed data analysis and manuscript writing. SHH, BLA, LAS, and SNW contributed to research design, interpretation and manuscript editing.

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Figures

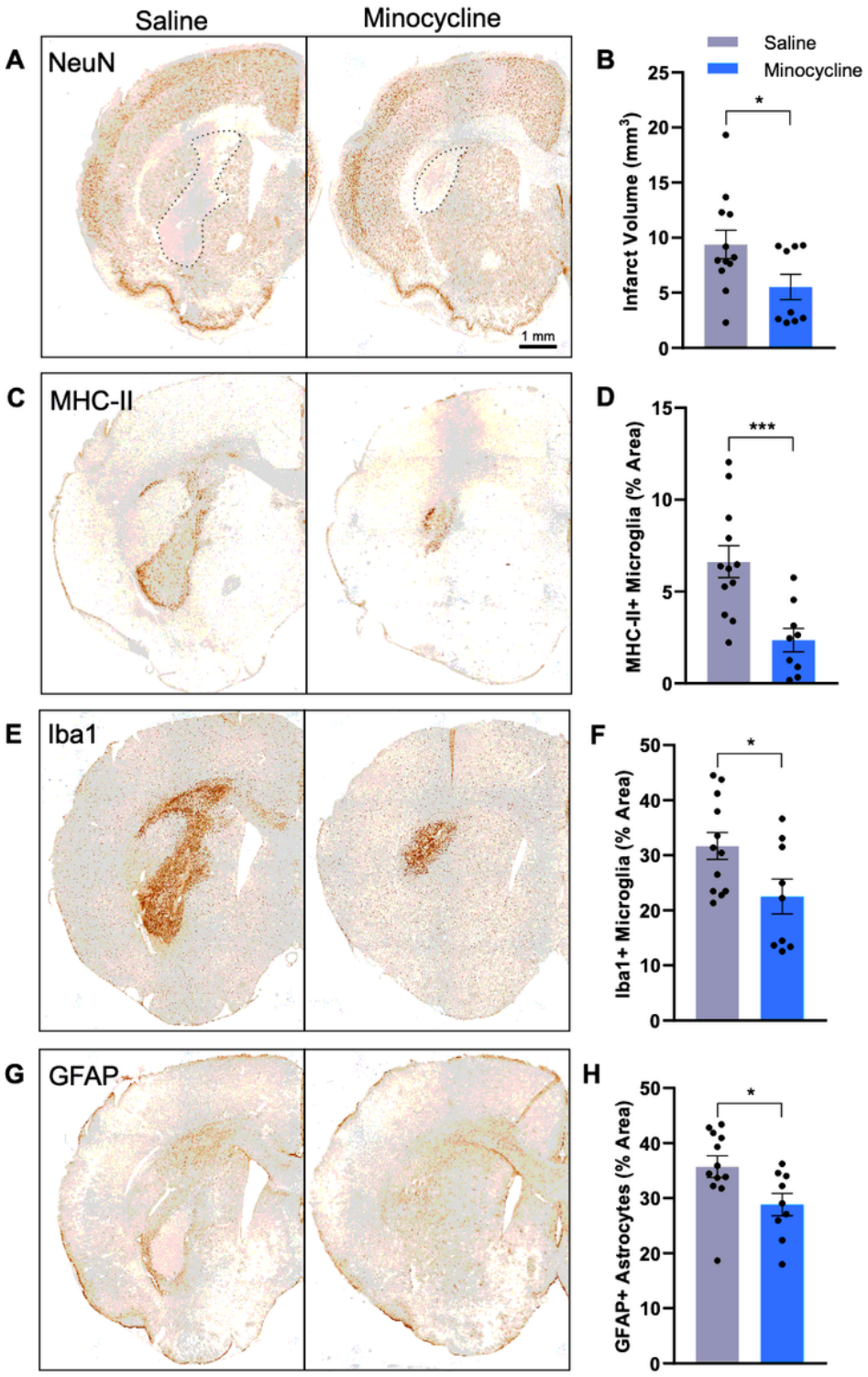

Figure 1

Minocycline treatment reduced infarct volume and corresponding microglia and astrocyte activation 28-days post-stroke. (A) Representative images of NeuN IHC staining in the infarct area of saline vs minocycline-treated rats. (B) There was a significant decrease in the infarct volume of minocycline-treated rats (Welch's *t*-test, *p < 0.05). (C) Representative images of MHC-II^{+} activated microglia IHC staining in the infarct area of saline- vs minocycline-treated rats. (D) There was a significant decrease in

the expression of MHC-II$^+$ activated microglia in the infarct area of minocycline-treated rats (Welch's *t*-test, ***p < 0.001). (E) Representative images of Iba1$^+$ microglia IHC staining in the infarct area of saline vs minocycline-treated rats. (F) There was a significant decrease in the expression of Iba1$^+$ microglia in the infarct area of minocycline-treated rats (Welch's *t*-test, *p < 0.05). (G) Representative images of GFAP$^+$ astrocyte IHC staining in the infarct area of saline vs minocycline-treated rats. (H) There was a significant decrease in the expression of GFAP$^+$ astrocytes in the infarct area of minocycline-treated rats (Welch's *t*-test, *p < 0.05). Scale bar = 1mm. Error bars represent SEM; n=12 saline and n=9 minocycline.

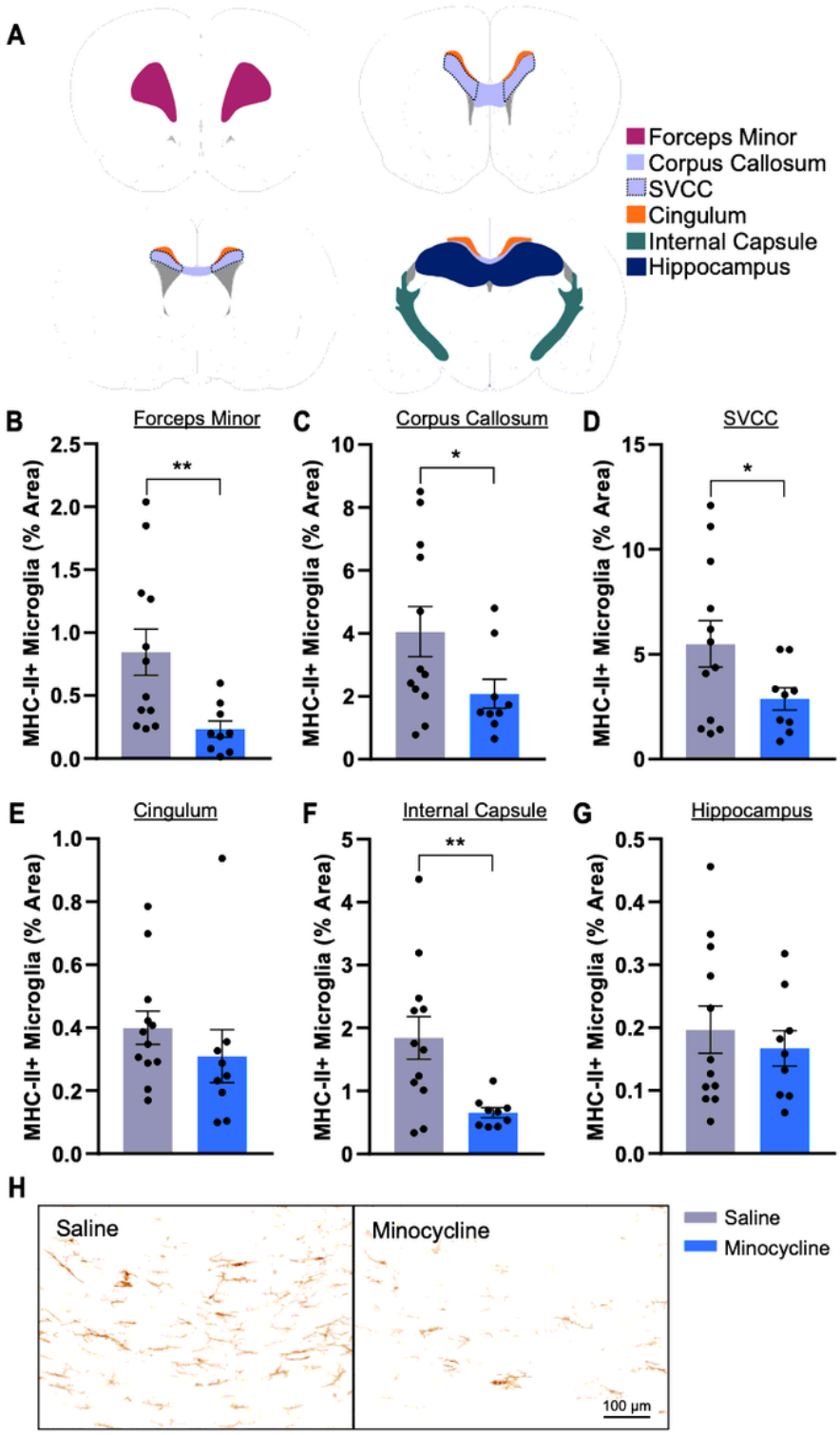

Figure 2

Minocycline treatment reduced MHC-II$^+$ microglia activation in remote white matter regions 28-days post-stroke. (A) Coronal sections and outlines of the regions of interest: forceps minor, corpus callosum, supraventricular corpus callosum (SVCC), cingulum, internal capsule, and hippocampus. (B-D, F) There was a significant reduction in MHC-II$^+$ microglia expression in the forceps minor, corpus callosum, SVCC, and internal capsule white matter regions in minocycline-treated rats (Welch's *t*-test, *p < 0.05, **p < 0.01).

(E, G) Minimal MHC-II^{+} expression was observed in the cingulum and hippocampus and no treatment differences were found. (H) Representative images of MHC-II^{+} activated microglia in the SVCC of saline and minocycline-treated rats at 28-days post-stroke. Scale bar = 100 μm. Error bars represent SEM; n=12 saline and n=9 minocycline.

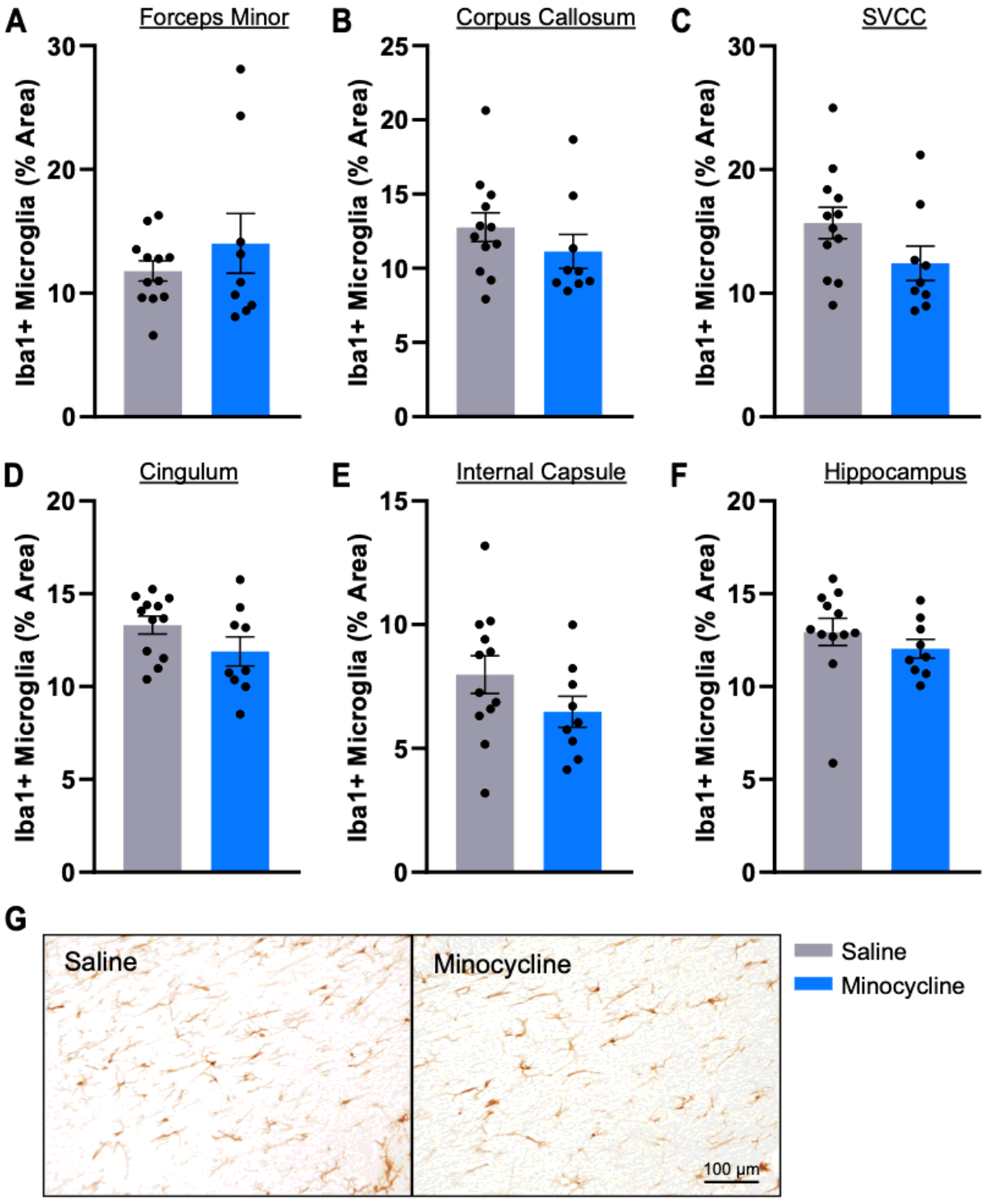

Figure 3

No treatment difference in total microglia expression in remote brain regions 28-days post-stroke. (A-F) There was no treatment difference in the expression of Iba1^{+} microglia in the white matter regions of interest or the hippocampus. (G) Representative images of Iba1^{+} activated microglia in the SVCC of saline and minocycline-treated rats at 28-days post-stroke. Scale bar = 100 µm. Error bars represent SEM; n=12 saline and n=9 minocycline.

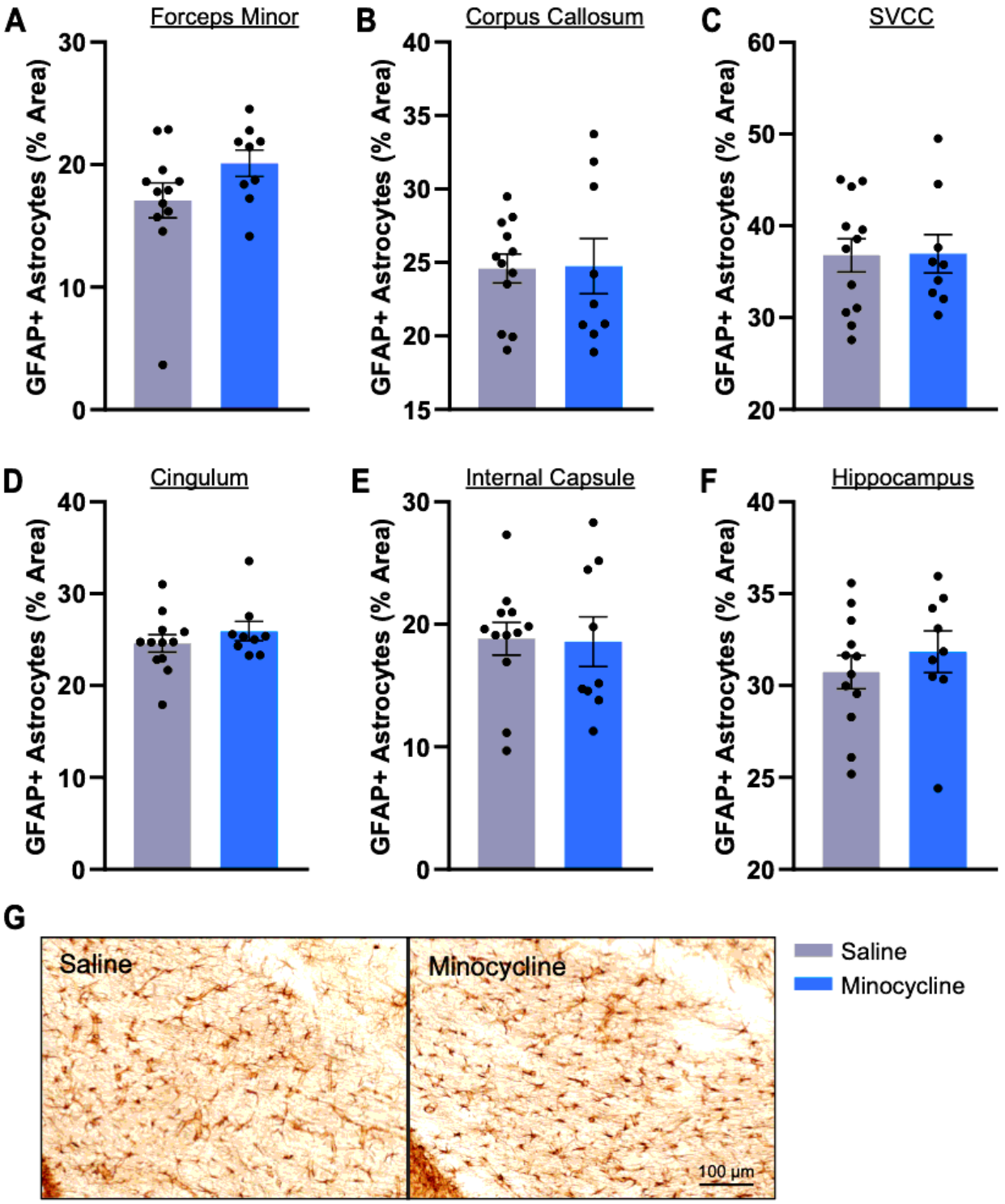

Figure 4

No treatment difference in astrocyte activation in remote brain regions 28-days post-stroke. (A-F) There was no treatment difference in the expression of $GFAP^+$ astrocytes in the white matter regions of interest or the hippocamps. (G) Representative images of $GFAP^+$ astrocytes in the SVCC of saline and minocycline-treated rats at 28-days post-stroke. Scale bar = 100 µm. Error bars represent SEM; n=12 saline and n=9 minocycline.

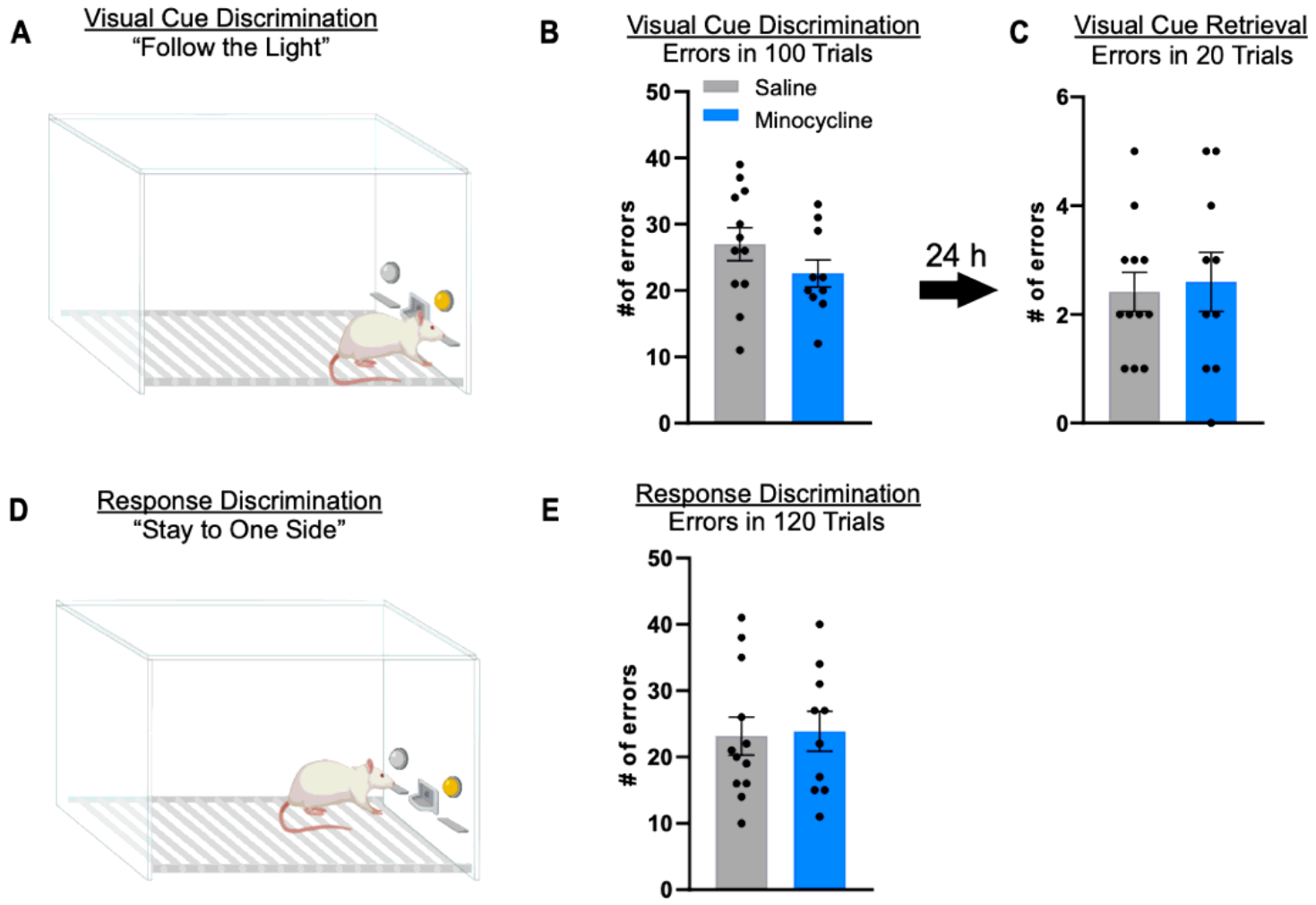

Figure 5

Cognitive flexibility was unaffected but minocycline-treated rats trended towards improved VCD learning. (A) The rats learned to press the lever associated with the illuminated light in the VCD task. (B) Minocycline-treated rats committed less errors over 100 trials in the VCD task (two-way ANOVA: main effect of treatment, *$p < 0.05$). (C) Twenty-four hours later the rats were given 20 VCD retrieval trials and there were no differences between groups. (D) Immediately following the VCD retrieval trials, cognitive flexibility was assessed by shifting the rule to "stay on one side" in which only the side opposite to the rats' side bias was correct. (E) Cognitive flexibility was unaffected by minocycline treatment as both groups committed a similar number of errors over 120 trials. Error bars represent SEM; n=12 saline and n=10 minocycline.

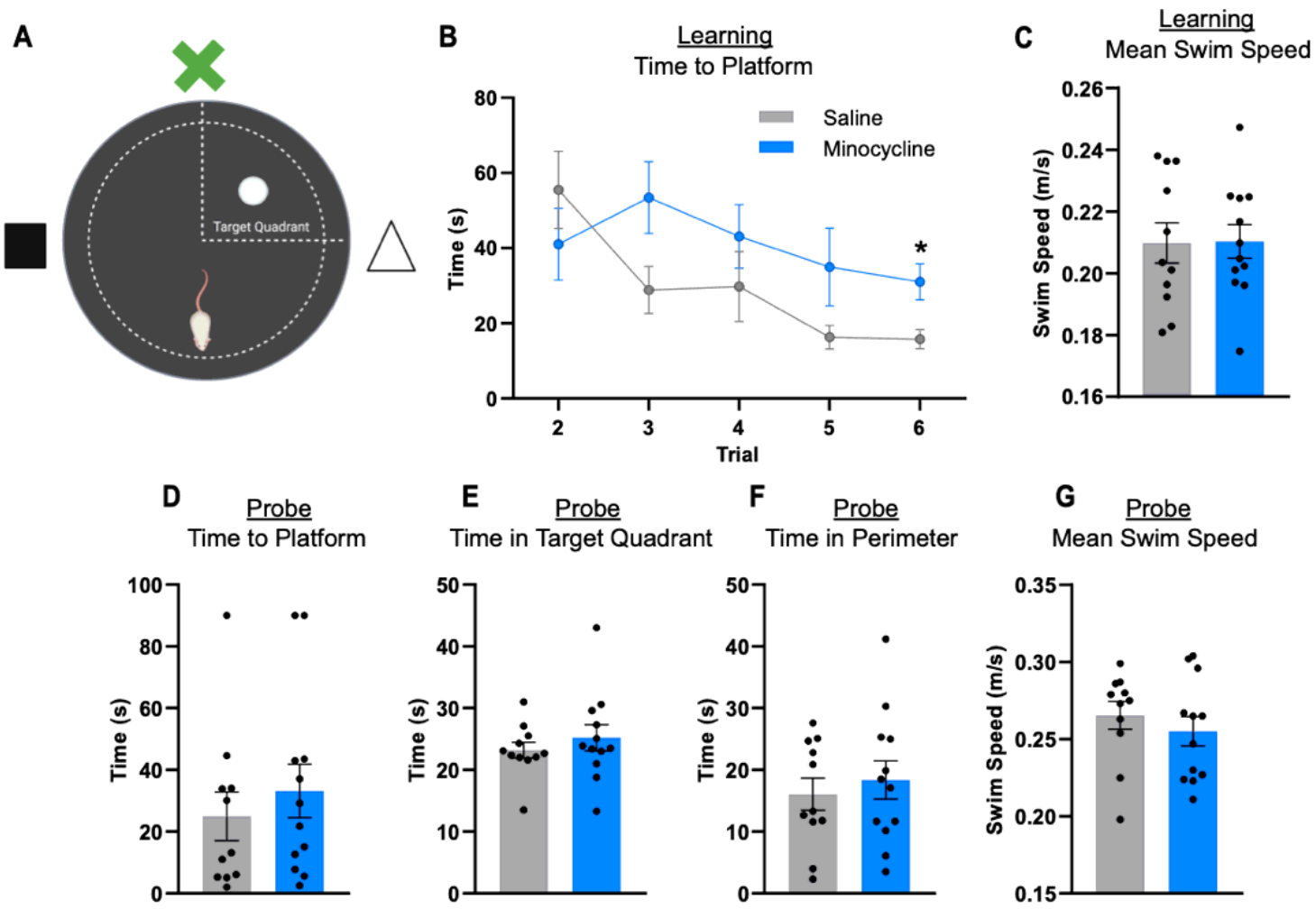

Figure 6

Minocycline treatment caused a spatial learning deficit but did not alter reference memory. (A) On the first day of testing, rats were given six trials to learn the location of a hidden platform using visual cues placed around the pool. (B) The minocycline-treated rats took longer to locate the hidden platform on the sixth learning trial (two-way repeated measures ANOVA, $*p_{bonf} < 0.05$). (C) The saline and minocycline-treated rats had similar swim speeds across the six learning trials. (D-G) Twenty-four hours later, the rats were placed back in the pool for a 90 s probe trial. (D, E) The saline and minocycline-treated rats took a similar amount of time to locate the platform region and spent a similar amount of time in the target quadrant. (F) No treatment differences in time spent in the perimeter of the pool were observed. (G) Similarly, the saline and minocycline-treated rats had similar swim speeds in the probe trial. Error bars represent SEM; n=11 saline and n=12 minocycline.

Supplementary Files

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