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Acute or delayed treatment with anatabine improves spatial memory and reduces pathological sequelae at chronic timepoints after repetitive mild TBI

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Abstract

Traumatic brain injury (TBI) has chronic and long term consequences for which there are currently no approved pharmacological treatments. We have previously characterized the chronic neurobehavioral and pathological sequelae of a mouse model of repetitive mild TBI (r-mTBI) through to two years post-TBI. Despite the mild nature of the initial insult, secondary injury processes are initiated which involve neuroinflammatory and neurodegenerative pathways persisting and progressing for weeks and months post-injury and providing a potential window of opportunity for therapeutic intervention. In this study we examined the efficacy of a novel anti-inflammatory compound, anatabine, in modifying outcome after TBI.

Our model of r-mTBI involves a series of 5 mild impacts (midline impact at 5 m/s, 1mm strike depth, 200ms dwell time) with an interval of 48 hours. Anatabine treatment was administered starting 30 minutes after injury and delivered continuously through in the water. At 6 months after TBI, anatabine treatment improved spatial memory in injured mice. Nine months after TBI, a cohort of mice were euthanized for pathological analysis which revealed reductions in astroglial (GFAP) and microglial (IBA1) responses in treated, injured animals. Treatments for the remaining mice were then crossed-over to assess the effects of late treatment administration and effects of treatment termination. 9 months following crossover the remaining mice showed no effect of injury on their spatial memory, and while pathological analysis showed improvements in mice who had received delayed treatment, corpus callosum IBA1 increased in post-crossover placebo r-mTBI mice.

These data demonstrate efficacy of both early and late initiation of treatment with anatabine in improving long term behavioral and pathology outcomes after mild TBI. Future studies will characterize the treatment window, the time course of treatment needed, and the dose needed to achieve therapeutic levels of anatabine in humans after injury.

Keywords: ANIMAL STUDIES, INFLAMMATION, TRAUMATIC BRAIN INJURY, THERAPEUTIC APPROACHES FOR THE TREATMENT OF CNS INJURY

Introduction

In the US traumatic brain injury (TBI) results in approximately 2.5 million emergency department visits per year, of which 75% are mild TBI.[1] Although these injuries are clinically classified as mild, exposure to repetitive injury is associated with chronic neurodegeneration and deficits which affect the long term recovery and outcome of the patient.[2] Although studies have shown that mild TBI can result in reduced productivity and impacts the quality of life of the patient, the pathophysiology driving these effects is unclear, hence strategies for therapeutic intervention have been slow to advance.[3]

Traumatic brain injury is a physical injury to the central nervous system that can result in the initiation of downstream secondary injury mechanisms in the hours, days, and weeks following injury.[4-7] After human TBI, neuropathology studies have shown evidence of inflammation and progressive white matter degeneration following just a single moderate or severe TBI,[8] with imaging studies showing evidence of persistent neuroinflammation and reduced radial diffusivity and fractional anisotropy, even after mild injury. [9-11] Consistent with these findings, animal studies have shown persistent inflammation and axonal damage in the corpus callosum of mice exposed to repetitive mild TBI (r-mTBI) at chronic timepoints after injury, [12, 13] with white matter disruption and an increase in radial diffusivity 60 days after repetitive mild TBI in rats.[14]

Pro-inflammatory genes and nuclear factor -kappa B cells(NF-κB) have been reported to remain activated for up to a year in a rat model of closed head injury (CHI). [15, 16] In mice, chronic microglial activation in combination with progressive lesion expansion has been seen after moderate cortical injury up to 1 year after TBI and up to at least a month after mild closed head injury. [17, 18] Our strategy has been to target neuroinflammation in the hope of reducing secondary injury and improving the neuropathological and behavioral outcomes after TBI.

Anatabine is a naturally occurring minor alkaloid that is present in solanaceous plants such as potatoes, tomatoes, and eggplants,[19] which inhibits NF-kB activation in HEK293 and SH-SY5Y cells in a concentration-dependent manner and in APP over-expressing 7W CHO cells.[20, 21] In in vivo studies of the effects of anatabine in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis, anatabine improved the clinical score and reduced hind limb paralysis.[22] Given its excellent bioavailability and anti-inflammatory actin, anatabine might have utility as a therapeutic

agent after TBI. This study explores the behavioral and neuropathological outcomes of anatabine in a mouse model of closed, mild TBI.

Materials and Methods

All mice were male wild type C57BL/6J (Jackson Laboratories) male mice 10 weeks old at the time of injury. 48 mice were divided into groups of 24 mice receiving repetitive injury (5 hits with an inter-mTBI injury interval of 48 hours), 24 receiving repetitive sham injury (anesthesia alone) and 12 mice from each group assigned to anatabine treatment, 12 to placebo. The injury protocol was performed as previously described.[12] Briefly, mice were anesthetized with 1.5 liter/min O_2 and 3% isoflurane delivered continuously by a nose cone. The animal was mounted in a stereotactic frame in a prone position secured by ear and incisor bars. Anesthesia controls were matched for the time spent under anesthesia by the r-mTBI mice. An electromagnetic impactor (Leica, Impact One™ Stereotaxic Impactor, Richmond, IL) generated a midline mTBI on the mouse scalp with a 5.0mm diameter flat face tip, at a 5m/s strike velocity, with a 1.0mm strike depth, and a 200msec dwell time. Sham injured animals underwent the same procedures and anesthesia duration as the r-mTBI mice on each occasion, in order to control for the effects of repeated anesthesia.

30 minutes after each injury or sham procedure, mice received an intraperitoneal injection (IP) of either phosphate buffered saline or anatabine (2mg/kg) (provided by Rock Creek Pharmaceuticals). This dose was administered via IP in order to control the timing and dose of the first administration after injury as the mice do not immediately return to drinking from their water bottles after surgery. After the first dose, for the anatabine treatment group, normal water was substituted with anatabine treated water, and was continuously re-filled for continuous treatment over 9 months (without the need for further intraperitoneal injections) at an estimated dose of 20mg/kg/day (placebo mice received regular water). Water intake was estimated given previous research showing that C57BL/6J mice consume water at a rate of approximately 7.4 ml/30g of weight.[23] At 9 months post TBI, the treated and untreated groups were switched. Previously untreated mice began receiving anatabine in their water at an estimated dose of 20mg/kg/day, while mice previously treated with anatabine began receiving regular water alone.

Experimenters were blinded to group assignments during all neurobehavioral testing. Rotarod testing was performed as previously described with modifications.[12] Briefly, baseline testing preceded the administration of r-mTBI or r–sham procedures, but only one day of baseline testing was performed. One day prior to the start of the injury or sham procedure, mice were pre-trained and baseline tested for Rotarod performance. All mice were given 3 trials to acclimate to the Rotarod at a constant speed of 5 revolutions per minute (RPM). For all subsequent tests, the Rotarod was set to an accelerating speed of 5-50 RPM over a period of 5 minutes. 3 trials were given with a 3 minute rest period between trials. Latency to fall was recorded and averaged over each day. Baseline testing occurred one day prior to the start of the r-mTBI or r-sham procedure, and one day after the administration of the last r-mTBI or r-sham procedure, Rotarod testing was resumed. Mice were then tested every other day after surgery starting on day 1 and ending on day 7. This testing was repeated again 6 months after TBI. Post-surgery testing occurred at an accelerating speed of 5-50 RPM over a period of 5 minutes. 3 trials were given with a 3 minute rest period between trials. Latency to fall and the speed of the Rotarod at the moment of the fall were recorded.

Following Rotarod, Barnes maze testing was administered as previously described immediately after the first rotarod test and again at 6 months after TBI.[12] The Barnes maze was 1.2 meters in diameter and had 18 holes around the perimeter. Each mouse was given 1.5 minutes to explore the maze and enter the goal box. Each mouse received 30 seconds in the goal box at the end of the trial period. Each mouse received 4 trials starting in front of each of 4 randomly selected cardinal holes for a period of 6 days. On the 7th day the goal box was removed and the mouse was given a 60 second probe trial starting in the center of the table. Each mouse's orientation and movement was recorded by Noldus Ethovision XT software. Rotarod testing was performed acutely in the first week after the final injury. Barnes maze testing was performed on days 8-15 after the final injury, then again at six months after TBI, the Barnes maze protocol was repeated (both acquisition and probe trials) as described above. Following the cross-over of treatment, Barnes maze acquisition and probe testing was again repeated at 12 and 18 months post-TBI (3 and 9 months after treatment crossover).

Pathology was assessed at 9 and 18 months post-TBI. Four mice per group were euthanatized for neuropathological analyses 9 months after TBI/sham procedures. All remaining mice were euthanized 18 months after TBI, with 4 per group processed for neuropathological analyses at this final timepoint. All animals were deeply anesthetized with

isofluorane before being intracardially perfused by gravity drip with a heparinized PBS solution pH-7.4 for 3 min, followed by an overnight fixation of brain samples in 4% paraformaldehyde and paraffin embedding. Separate series of 5-6 μm-thick sections were cut throughout the extent of the cortex and hippocampus and associated areas using a microtome (2030 Biocut, Reichert/Leica) and mounted on positively charged glass slides (Fisher, Superfrost Plus). Sections were stained in entire batches with antibodies (cell markers) raised against: Glial Fibrillary Acid Protein (GFAP) (rabbit anti-GFAP, 1:10,000, Dako) for astrocytosis, ionized calcium-binding adapter molecule 1 (IBA1) for microglia, amyloid precursor protein (APP) for axonal injury, phosphorylated signal transducer and activator of transcription 3 (p-STAT3) (rabbit anti-pSTAT3, 1:500) or Luxol fast blue for myelin.

Sections were deparaffinized in Histo-Clear (National Diagnostics) and rehydrated in 95% and 80% ethanol before the immunohistochemical procedure. Sections were then rinsed in water and treated with 0.3% hydrogen peroxide for 5 minutes to block endogenous peroxidase. After rinsing, sections were heat treated in target retrieval solution for 8 minutes using a pressure cooker for antigen retrieval. Sections were incubated overnight with primary antibodies, followed by rinsing with PBS, and were then stained with a complementary secondary antibody (from the Vectastain Elite ABC Kit or Vector Mouse on Mouse (M.O.M.™) Kit) for 1hr and finally incubated with avidin-biotin-horseradish peroxidase solution (Vectastain Elite ABC kit; Vector Laboratories) for a further hour.

A solution of 3, 3'-diaminobenzidine (DAB) chromogen and hydrogen peroxide was used to develop the sections. Chromogen development was kept at a constant 5 minutes to limit variability for quantification. The reaction was terminated by rinsing sections in distilled water. Finally, sections were dehydrated using 80% and 95% ethanol followed by Histo-Clear and were then coverslipped. Immunoreacted sections were viewed using an Olympus (BX63) light microscope and photos were taken using an Olympus DP72 camera.

For GFAP and IBA1 staining, immunoreactivity for cell markers was measured quantitatively using optical segmentation. Three non-overlapping 100 μm^2 regions were selected for each section (n=4). Quantification was performed by blind assessment using ImageJ (with each slide analyzed blind with respect to marker or animal group). Images were separated into individual color channels using the color deconvolution plugin (hematoxylin counter stain and DAB chromogen). Each stain was quantified within the cortex, hippocampus and corpus callosum. The area of positive immunoreactivity was calculated for each section and expressed as a percentage of the total area. For APP and p-STAT3

staining, the total number of positive profiles were quantified throughout the corpus callosum on each section. For Luxol fast blue thickness was measured along the center of the body of the corpus callosum.

Statistical analysis was performed using JMP 12.0 (SAS, Cary, NC). Normality tests were conducted using the Shapiro-Wilk W test and the data were transformed using square root or natural log transformation when not normally distributed. If the distribution was still not normal after transformation, non-parametric methods were used. Normally distributed data were analyzed using ANOVA (with repeated measures where appropriate) and t-tests. P values of $p < 0.05$ were considered as statistically significant and marked with an asterisk in the figures.

Results

Behavioral analyses

At the acute evaluation of the Rotarod testing, placebo treated sham mice had a higher fall latency than placebo treated r-mTBI mice on days 1 through 7 ($p<0.01$) (figure 1). Although anatabine treated sham mice had a higher fall latency than anatabine treated r-mTBI mice on day 1 ($p<0.01$), on days 3, 5, and 7 there were no differences between the anatabine treated sham and TBI mice. Anatabine treated sham mice were not different from placebo treated shams on day 1, but by day 7 there was a difference between the two shams ($p<0.05$). Upon re-testing at 6 months post-TBI, there were no differences between any group's fall latency on any day of testing (data not shown).

Barnes maze testing was performed on days 8 through 14 after TBI (figure 2a) and again 6 months post-TBI. The Barnes maze is a 1.8 m diameter board, with 18 holes positioned around the perimeter, situated in a room rich in visual cues. Acquisition trial testing showed an effect of injury on spatial learning ($p<0.05$) but not treatment (figure2c). Treatment with anatabine did not show any effect on the probe trial latency at the acute time point (figure 2a).

At the 6 month post-TBI probe trial, anatabine treated r-mTBI mice showed an improved latency to the target hole compared to placebo treated r-mTBI mice ($p<0.05$) indicating a positive effect of anatabine treatment (figure 2b). At this time point, the anatabine treated r-mTBI mice located the target hole as quickly as their respective shams (11.0 and 9.9 seconds respectively, $p>0.05$) as well as placebo treated shams (11 and 10.6 seconds respectively, $p>0.05$), showing no effect of injury on their probe trial performance.

Post-Crossover Behavioral Data

At the 12 month post-TBI probe trial (3 months post-crossover), anatabine treated r-mTBI mice that were originally treated with regular water showed no improvement compared to their shams in spite of treatment with anatabine (p<0.05) (figure 3a). Mice in the r-mTBI group originally treated with anatabine continued to show no differences compared to their respective sham group despite the cessation of treatment three months prior (p>0.05).

Six months later, at the 18 month post-TBI timepoint (9 months post-crossover), there were no differences between r-mTBI mice and sham mice in either the original treatment or delayed treatment groups, nor were there any differences between the two r-mTBI groups (p>0.05) (figure 3b).

Neuroinflammatory Response

At 9 months after TBI, evaluation of astrocytosis showed that hippocampal GFAP staining was increased in the placebo treated r-mTBI compared to sham animals (2.9 % area and 1.5% area respectively, p<0.05) (figure 4) but not in mice treated with anatabine compared to either sham group (p>0.05). However, TBI-induced astrocytosis in the corpus callosum was increased in both placebo and anatabine treated mice compared to their respective shams (p<0.01).

At 18 months post-injury, 9 months after the treatment crossover, GFAP staining in the hippocampus was increased for all groups. Animals treated with anatabine after the crossover continued to show an increase in GFAP staining within the hippocampus after r-mTBI (p<0.05). Interestingly, the situation was reversed in the late placebo treatment group, with late placebo treated sham animals showing more GFAP staining than their r-mTBI counterparts (p<0.05).

Microglia staining by IBA1 in the hippocampus showed that at 9 months post-injury there was a small increase in the placebo treated r-mTBI mice compared to sham mice (1.8% area and 1.3% area respectively, p<0.05) (figure 5), which was not seen in anatabine treated r-mTBI mice compared to their respective shams (1.3% and 1.4% area respectively, p>0.05). In the corpus callosum the anatabine treated r-mTBI exhibited less IBA1 staining than placebo treated r-mTBI mice (p<0.05), but still showed an increased level of microglial activity compared to sham controls (3.4% and 1.5% area respectively, p<0.01).

18 months after TBI, post-crossover placebo treated TBI animals had a higher level of microglial staining in the corpus callosum than post-crossover placebo treated shams (19.3% vs 6.7% area, p<0.05) despite the improvement seen in early anatabine treated animals at the 9 month time point. Injured mice treated late with anatabine during the post-

crossover phase showed no difference compared to their corresponding shams (p>0.05). Injured mice treated late with anatabine showed less microglial staining in the corpus callosum than their placebo treated counterparts (p<0.01).

White Matter Degeneration

At 9 months post-TBI, Luxol fast blue staining (LFB) showed less density within the body of the corpus callosum in TBI mice compared to sham mice (p<0.05) but treatment with anatabine did not affect this TBI-induced reduction. This difference was maintained post-crossover at the 18 month time point with the corpus callosum thickness showing reductions in both late anatabine and late placebo treated groups compared to their respective shams (p<0.05) but with no differences between the treatment groups (figure 6).

At 9 months after TBI, Amyloid Precursor Protein staining showed an increase in the number of APP positive axons throughout the body and splenium of the corpus callosum in both the placebo treated and anatabine treated TBI groups compared to their shams, with no difference between the treated groups. Placebo treated TBI mice had an average of 30 positive axons compared to an average of 2 for placebo treated sham mice (p<0.05). Anatabine treated TBI mice had an average 38 positive axons compared to an average of 2 for anatabine treated sham mice. There were no differences between placebo treated and anatabine treated sham animals (figure 7). 18 months after TBI, 9 months after crossover, this pattern was maintained with TBI mice showing a greater number of APP positive axons (P<0.05) regardless of treatment, and no differences between anatabine and placebo mice.

STAT3 Phosphorylation

At 9 months post-TBI we evaluated STAT3 phosphorylation to determine whether anatabine acts on STAT3 signaling to reduce inflammation in TBI. Staining for p-STAT3 revealed the corpus callosum was the only brain region to exhibit positively stained cells. Cell counts of positive cells throughout the corpus callosum revealed a higher number in placebo treated TBI mice than in placebo treated sham animals (64.2 on average vs 6.3, p<0.05). Anatabine treated TBI mice also had an elevated number of positive cells compared to their corresponding shams (33.5 vs 13) but fewer than in placebo treated TBI mice (p<0.05) (figure 8). At the 18 month time point the p-STAT3 positive cell counts were elevated in all groups, with post-crossover placebo sham mice at 222.5 positive cells in the corpus callosum on average, post-crossover

placebo TBI at 381.9, post-crossover anatabine treated sham at 261.5, and post-crossover anatabine treated TBI at 392.5 positive cells. Both post-crossover TBI groups showed elevated levels above shams regardless of treatment.

At completion, our treatment study did not include any naïve (untreated) mice as all groups had at some point during the crossover design received anatabine, thus confounding our ability to separate treatment-dependent from age-dependent effects on STAT3 phosphorylation. However, we had tissue available from a previously published separate cohort of r-mTBI or r-sham mice (using the same paradigm) and analyzed at chronic timepoints[13]. Therefore, for comparison with the current study, we conducted p-STAT3 staining on slides from these r-sham or r-mTBI animals analyzed at 12 and 24 months post-TBI, timepoints flanking the final timepoint of our current study.. Batches of samples from all four timepoints from both studies combined were processed simultaneously for p-STAT3 to allow for direct comparison of data from the two studies. The treatment naive r-sham animals had an average of 286 p-STAT3 positive cells in the corpus callosum at the 12 month timepoint, and an average of 2725.8 at the 24 month timepoint. Naive r-mTBI mice had averages of 894.25 and 2616.4 at 12 and 24 months post-TBI respectively. Thus at 12 and 24 months post r-mTBI treatment naïve mice had higher numbers of p-STAT3 positive cells than either the early or late anatabine treated r-mTBI animals at the 18 month time point (figure 7).

Discussion

In this study we examined the effect of anatabine on neurobehavioral and pathological outcomes in a mouse model of repetitive mild traumatic brain injury. Our results showed no effect of treatment at acute timepoints after injury, but that treatment with anatabine improved the spatial memory and reduced the neuroinflammation of mice at a chronic timepoint after injury. A cross-over study of these mice showed that even with a 9 month delay, anatabine treatment can still reduce IBA-1 staining and STAT3 phosphorylation, but a 9 month cessation of treatment begins to reverse the IBA-1 signal within the corpus callosum of the mice. Treatment with anatabine appears to reduce chronic inflammation in a regionally-specific manner and improve the spatial memory in this model of TBI. Though previous human clinical studies targeting neuroinflammation have historically failed, this study reveals that more chronic timepoints and treatments may be necessary to reduce sequelae of the disease.

Clinical trials of potential treatments targeting secondary injury after TBI have so far largely been unsuccessful. Neuroprotective compounds that have been administered to reduce secondary injury have all failed in phase 3 clinical trials thus far.[24-26] Anti-inflammatory treatments have had no success thus far either, and the Corticosteroid Randomisation After Significant Head Injury (CRASH) study showed increased risk of death in patients who received methylprednisolone for 48 hours after TBI.[27] Even treatments with multifarious drugs, such as erythropoietin and progesterone, intended to target the pathology through multiple mechanisms of action have failed in phase III clinical trials. Often these negative results are determined to have been due to difficulties arising from the heterogeneity of the patient population, delays in clinical trial enrollment before treatment, as well as uncertainties in the effective treatment window, dose, and bioavailability data from pre-clinical studies. The studies cited here were designed to treat patients with severe TBI, but with mild TBI most human studies have looked at pharmacological compounds to treat the cognitive and emotional consequences without actually impacting the progression of pathological damage within the brain. These treatments have sought to alleviate the symptoms commonly reported in mTBI cases, but our study highlights the importance of the chronic phase after TBI and how a drug targeting neuroinflammation may not show acute improvement, and even chronic treatment may not provide sustained benefits if the treatment is halted for a prolonged period of time.

As in our previous studies,[12, 13] we found that among the placebo treated mice the r-mTBI mice exhibited worse motor coordination, spatial learning, and spatial memory in the Rotarod and Barnes maze tests. Neuropathological analysis in the current study also revealed the same chronic, persistent neuroinflammation and white matter damage within the r-mTBI mice as we saw in our previous studies. At an acute time point of two weeks there was no improvement of the anatabine treated r-mTBI mice over placebo treated r-mTBI mice, but at a chronic timepoint 6 months after TBI we saw improvements in spatial memory during the probe trial of the Barnes maze which correlated with brain region specific improvements seen by immunohistochemical analysis. Hippocampal pathology, although not as prominent as white matter pathology in the corpus callosum, may be important to our neurobehavioral results. IBA1 was increased in the hippocampus and corpus callosum of placebo treated r-mTBI mice at 9 months post TBI, but anatabine treated mice showed no elevation of IBA1 in the hippocampus at the 9 month timepoint. It is important to note that the corpus callosum shows the largest amount of pathology of any brain region within our model of TBI.[12, 13] We have also observed extensive optic nerve and retinal pathology in this model of TBI which may be affecting visual based memory

task results as well,[28] and future studies will examine the effect anatabine may have on the functional visual outcome of mice in this model.

GFAP staining showed an increase in the hippocampus of placebo treated r-mTBI mice, but not in anatabine treated mice. Although these differences in the hippocampal pathology are small, they may also contribute to the spatial memory differences that were seen in the Barnes maze 6 months after TBI. Interestingly, GFAP staining remained low in the hippocampus of post-crossover placebo r-mTBI mice, but became higher in post-crossover placebo sham animals at this time point, while post-crossover anatabine treated r-mTBI mice continued to show higher levels of GFAP staining. Within the r-mTBI group, early treatment appears to be necessary to prevent injury-dependent increases in GFAP staining, but the reason for the increase in post-crossover placebo treated sham animals is unclear. Prevention of chronic inflammation within the hippcampal region appears to be important in rescuing spatial memory, even though the hippocampus exhibits far less inflammation at chronic timepoints than the corpus callosum. More work needs to be done to characterize the region specific changes after TBI as well as following experimental treatment to determine which pathological hallmarks are the most important in determining outcome and therapeutic benefits.

Despite the reduction of IBA1 staining, neither GFAP nor APP staining were reduced in the corpus callosum of anatabine treated r-mTBI mice at 9 months post-TBI. Additionally, staining with LFB showed a reduction in the thickness of the corpus callosum, but this was not abated by treatment with anatabine. This suggests that anatabine is not acting to reduce the structural changes and persistent white matter loss occurring after TBI. At the 18 month post-TBI timepoint, 9 months after treatment crossover, IBA1 staining had increased in the post-treatment placebo animals compared to sham animals but had decreased to sham levels in post-crossover anatabine treated r-mTBI mice. This suggests that the chronic inflammatory sequelae are only temporarily reduced by anatabine treatment. Axonal damage is not being prevented, and may be driving these prolonged inflammatory sequelae. The cessation of treatment then results in a return of the chronic inflammatory phenotype.

It has been suggested that previous animal studies failed in translation to human application often due to the failure to perform appropriate pre-clinical studies to establish the bioavailability and treatment window of their compounds.[29] In human mild TBI cases, the initial injury may go undiagnosed for long periods of time until secondary clinical manifestations begin appearing at chronic timepoints.[30] This is especially true in settings where repetitive mild head

injury is common such as in athletes.[31] In order to address the issue of delayed diagnosis and delayed treatment, our study utilized a cross-over design, allowing us to examine the effect of delayed treatment administration as well as the effects of treatment cessation.

Prior to the crossover, we witnessed an improvement in spatial memory during the probe trial of the Barnes maze which correlated with regionally-specific reductions in IBA1 and GFAP staining. After the crossover, we saw an increase of IBA1 staining in the corpus callosum of post-crossover placebo treated r-mTBI mice while late-anatabine treated r-mTBI saw a reduction to sham levels. Although we did not see TBI-dependent spatial memory impairments at the 18 month timepoint, pathologically we begin to see a return of TBI-dependent neuroinflammation following the 9 month cessation period, suggesting that anatabine is not targeting the underlying causes of the inflammation, but is instead modulating the inflammatory response only for the duration of treatment.

Cessation of treatment did not result in reductions in spatial memory in the originally treated r-mTBI mice, but a non-significant trend of increasing latency to find the target hole was observed. In particular, the originally treated r-mTBI mice showed an increase in latency to find the target hole between the 3 month and 9 month post-crossover timepoints (12 and 18 months post-TBI respectively). This combined with pathological data showing a resurgence of microglial staining in the corpus callosum, suggests that sustained treatment may be required. A reduced n of 8 at the post-crossover timepoints may have also obscured the statistical significance of the neurobehavioral trend towards a return of cognitive decline. It is interesting to note that there were some mice which performed *nearly* 2 standard deviations worse than the average. If we were to eliminate those mice from the data as outliers, there would be a very consistent pattern to the data showing both sham groups and the post-crossover treatment group all performing at the same level while the post-crossover placebo group starts to show a return of the TBI effect. This shows that even delayed administration of anatabine may ameliorate TBI-dependent cognitive dysfunction at chronic timepoints if it is administered for a chronic length of time, but prolonged cessation of treatment may result in the return of cognitive dysfunction.

In order to better understand the mechanism behind these changes, we examined STAT3 phosphorylation histologically as well. Anatabine has been shown to reduce phosphorylation of STAT3 and NF-kB,[20, 21] which may be influencing microglial activation downstream from the axonal damage. Prior to treatment crossover, anatabine treated r-mTBI had

fewer p-STAT3 positive cells in the corpus callosum than placebo treated r-mTBI mice, correlating well with their neuroinflammatory and spatial memory profiles. Following the treatment crossover there was no difference between p-STAT3 in the post-crossover treated and untreated r-mTBI animals, nor were there any differences compared to their shams. We noted a strong aging effect on STAT3 phosphorylation, which may have obscured the TBI-dependent signal, but inclusion of treatment naïve cohorts of mice was beyond the scope of the current study. Thus in order to place our findings in the context of aging-dependent STAT3 phosphorylation we analyzed tissue collected from treatment naïve mice in a previous study using the same r-mTBI/r-sham paradigm and euthanized at timepoints flanking the current final timepoint, namely 12 or 24 months post-injury/sham. By bracketing the 18 month time point in this manner we were able to note a dramatic decrease in STAT3 phosphorylation in the anatabine treated r-mTBI animals at 18 months post-injury compared to the treatment naïve r-mTBI animals at 12 or 24 months post injury, regardless of whether anatabine was administered in the first 9 or last 9 months of the study. Both pre- and post-crossover treated r-mTBI mice had lower levels of STAT3 phosphorylation within the corpus callosum than untreated r-mTBI mice from the 12 month post-TBI timepoint. Though this suggests that the lack of a TBI-dependent signal in STAT3 phosphorylation at the 18 month timepoint is due to a treatment effect rather than aging, by the 24 month timepoint in the untreated cohort the levels had increased to a point of saturation and obscured any TBI-dependent increase. This is consistent with previous reports of STAT3 activation's role in senescent macrophages.[32] Even if aging is obscuring a TBI-dependent signal in the post-crossover cohort, early or delayed treatment with anatabine appears to reduce the age-dependent increase observed in the sham animals as well when compared to the untreated cohort, supporting its potential utility regardless of its effect on injury-dependent inflammation.

Although we do not have any naïve tissue from an 18 month time point for comparison, it is possible that anatabine treatment of the sham animals has also delayed the onset of the age dependent increase seen in the naïve animals at the 24 month time point. We hypothesize that anatabine treatment may have delayed the increase which was probably continuous and ongoing in the untreated animals between the 12 and 24 month timepoints. This treatment-related reduction in STAT3 phosphorylation appears to be ongoing at chronic time points after TBI and perseveres even after the cessation of anatabine administration. This may be important in reducing neurodegenerative chronic inflammation driving at least some of the neurobehavioral deficits seen in this model of mild TBI.

Anatabine shows potential as a method of reducing chronic inflammation after TBI, though the reductions seem to be regionally specific and more complete in less severely affected regions. A wide variety of animal models as well as human studies have shown a common theme of chronic inflammation after TBI.

 This study shows that chronic inflammation may be a key factor in mediating spatial memory loss after TBI, and may be amenable to treatment. Ideally, a potential therapeutic should be effective against the long-term consequences of head injury, and be able to reduce the chronic pathological and behavioral consequences. Previous problems in clinical trials have arisen due to difficulties in early recruitment as well as bioavailability and treatment dose and timing.   Anatabine has a history of safe use as a dietary supplement, and as it is well-characterized with a low risk of adverse events, it shows potential as a compound for future clinical trials. It can be safely administered in a chronic treatment paradigm, which may be necessary in order to provide efficacy at long term time points after injury. Future studies will seek to quantify the minimum effective dose, examine the effect of delayed treatment, and examine time points after the cessation of treatment in preparation for future human clinical trials.

References

1. Marin, J.R., Weaver, M.D., Yealy, D.M. and Mannix, R.C. (2014). Trends in visits for traumatic brain injury to emergency departments in the United States. Jama 311, 1917-1919.
2. Dean, P.J. and Sterr, A. (2013). Long-term effects of mild traumatic brain injury on cognitive performance. Frontiers in human neuroscience 7, 30.
3. Boake, C., McCauley, S.R., Pedroza, C., Levin, H.S., Brown, S.A. and Brundage, S.I. (2005). Lost productive work time after mild to moderate traumatic brain injury with and without hospitalization. Neurosurgery 56, 994-1003; discussion 1994-1003.
4. Faden, A.I., Demediuk, P., Panter, S.S. and Vink, R. (1989). The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244, 798-800.
5. Morganti-Kossmann, M.C., Rancan, M., Otto, V.I., Stahel, P.F. and Kossmann, T. (2001). Role of cerebral inflammation after traumatic brain injury: a revisited concept. Shock 16, 165-177.

6. Ates, O., Cayli, S., Gurses, I., Yucel, N., Iraz, M., Altinoz, E., Kocak, A. and Yologlu, S. (2006). Effect of pinealectomy and melatonin replacement on morphological and biochemical recovery after traumatic brain injury. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 24, 357-363.

7. Nonaka, M., Chen, X.H., Pierce, J.E., Leoni, M.J., McIntosh, T.K., Wolf, J.A. and Smith, D.H. (1999). Prolonged activation of NF-kappaB following traumatic brain injury in rats. Journal of neurotrauma 16, 1023-1034.

8. Johnson, V.E., Stewart, J.E., Begbie, F.D., Trojanowski, J.Q., Smith, D.H. and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain : a journal of neurology 136, 28-42.

9. Ramlackhansingh, A.F., Brooks, D.J., Greenwood, R.J., Bose, S.K., Turkheimer, F.E., Kinnunen, K.M., Gentleman, S., Heckemann, R.A., Gunanayagam, K., Gelosa, G. and Sharp, D.J. (2011). Inflammation after trauma: microglial activation and traumatic brain injury. Annals of neurology 70, 374-383.

10. Mayer, A.R., Ling, J., Mannell, M.V., Gasparovic, C., Phillips, J.P., Doezema, D., Reichard, R. and Yeo, R.A. (2010). A prospective diffusion tensor imaging study in mild traumatic brain injury. Neurology 74, 643-650.

11. Rutgers, D.R., Toulgoat, F., Cazejust, J., Fillard, P., Lasjaunias, P. and Ducreux, D. (2008). White matter abnormalities in mild traumatic brain injury: a diffusion tensor imaging study. AJNR. American journal of neuroradiology 29, 514-519.

12. Mouzon, B., Chaytow, H., Crynen, G., Bachmeier, C., Stewart, J., Mullan, M., Stewart, W. and Crawford, F. (2012). Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes. Journal of neurotrauma 29, 2761-2773.

13. Mouzon, B.C., Bachmeier, C., Ferro, A., Ojo, J.O., Crynen, G., Acker, C.M., Davies, P., Mullan, M., Stewart, W. and Crawford, F. (2014). Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. Annals of neurology 75, 241-254.

14. Donovan, V., Kim, C., Anugerah, A.K., Coats, J.S., Oyoyo, U., Pardo, A.C. and Obenaus, A. (2014). Repeated mild traumatic brain injury results in long-term white-matter disruption. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 34, 715-723.

15. Crack, P.J., Gould, J., Bye, N., Ross, S., Ali, U., Habgood, M.D., Morganti-Kossman, C., Saunders, N.R. and Hertzog, P.J. (2009). The genomic profile of the cerebral cortex after closed head injury in mice: effects of minocycline. J Neural Transm 116, 1-12.

16. Sun, S.C., Ganchi, P.A., Ballard, D.W. and Greene, W.C. (1993). NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 259, 1912-1915.

17. Loane, D.J., Kumar, A., Stoica, B.A., Cabatbat, R. and Faden, A.I. (2014). Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation. Journal of neuropathology and experimental neurology 73, 14.

18. Venkatesan, C., Chrzaszcz, M., Choi, N. and Wainwright, M.S. (2010). Research Chronic upregulation of activated microglia immunoreactive for galectin-3/Mac-2 and nerve growth factor following diffuse axonal injury.

19. Andersson, C. (2003). Nicotine alkaloids in Solanaceous food plants. Nordic Council of Ministers.

20. Paris, D., Beaulieu-Abdelahad, D., Abdullah, L., Bachmeier, C., Ait-Ghezala, G., Reed, J., Verma, M., Crawford, F. and Mullan, M. (2013). Anti-inflammatory activity of anatabine via inhibition of STAT3 phosphorylation. European journal of pharmacology 698, 145-153.

21. Paris, D., Beaulieu-Abdelahad, D., Bachmeier, C., Reed, J., Ait-Ghezala, G., Bishop, A., Chao, J., Mathura, V., Crawford, F. and Mullan, M. (2011). Anatabine lowers Alzheimer's Abeta production in vitro and in vivo. European journal of pharmacology 670, 384-391.

22. Paris, D., Beaulieu-Abdelahad, D., Mullan, M., Ait-Ghezala, G., Mathura, V., Bachmeier, C., Crawford, F. and Mullan, M.J. (2013). Amelioration of experimental autoimmune encephalomyelitis by anatabine. PloS one 8, e55392.

23. Bachmanov, A.A., Reed, D.R., Beauchamp, G.K. and Tordoff, M.G. (2002). Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behavior genetics 32, 435-443.

24. Doppenberg, E.M., Choi, S.C. and Bullock, R. (2004). Clinical trials in traumatic brain injury: lessons for the future. Journal of neurosurgical anesthesiology 16, 87-94.

25. Skolnick, B.E., Maas, A.I., Narayan, R.K., van der Hoop, R.G., MacAllister, T., Ward, J.D., Nelson, N.R., Stocchetti, N. and Investigators, S.T. (2014). A clinical trial of progesterone for severe traumatic brain injury. The New England journal of medicine 371, 2467-2476.

26. Robertson, C.S., Hannay, H.J., Yamal, J.M., Gopinath, S., Goodman, J.C., Tilley, B.C., Epo Severe, T.B.I.T.I., Baldwin, A., Rivera Lara, L., Saucedo-Crespo, H., Ahmed, O., Sadasivan, S., Ponce, L., Cruz-Navarro, J., Shahin, H., Aisiku, I.P., Doshi, P.,

Valadka, A., Neipert, L., Waguspack, J.M., Rubin, M.L., Benoit, J.S. and Swank, P. (2014). Effect of erythropoietin and transfusion threshold on neurological recovery after traumatic brain injury: a randomized clinical trial. Jama 312, 36-47.

27. Thompson, H.J. and Bakshi, A. (2005). Methylprednisolone was associated with an increase in death after head injury. Evidence-based nursing 8, 51.

28. Tzekov, R., Quezada, A., Gautier, M., Biggins, D., Frances, C., Mouzon, B., Jamison, J., Mullan, M. and Crawford, F. (2014). Repetitive mild traumatic brain injury causes optic nerve and retinal damage in a mouse model. Journal of neuropathology and experimental neurology 73, 345-361.

29. Narayan, R.K., Michel, M.E., Ansell, B., Baethmann, A., Biegon, A., Bracken, M.B., Bullock, M.R., Choi, S.C., Clifton, G.L., Contant, C.F., Coplin, W.M., Dietrich, W.D., Ghajar, J., Grady, S.M., Grossman, R.G., Hall, E.D., Heetderks, W., Hovda, D.A., Jallo, J., Katz, R.L., Knoller, N., Kochanek, P.M., Maas, A.I., Majde, J., Marion, D.W., Marmarou, A., Marshall, L.F., McIntosh, T.K., Miller, E., Mohberg, N., Muizelaar, J.P., Pitts, L.H., Quinn, P., Riesenfeld, G., Robertson, C.S., Strauss, K.I., Teasdale, G., Temkin, N., Tuma, R., Wade, C., Walker, M.D., Weinrich, M., Whyte, J., Wilberger, J., Young, A.B. and Yurkewicz, L. (2002). Clinical trials in head injury. Journal of neurotrauma 19, 503-557.

30. Thurman, D. (2001). The epidemiology and economics of head trauma. Head trauma: basic, preclinical, and clinical directions. New York, NY, Wiley and Sons 327, 347.

31. Buck, P.W. (2011). Mild traumatic brain injury: a silent epidemic in our practices. Health Soc Work 36, 299-302.

32. Nakamura, R., Sene, A., Santeford, A., Gdoura, A., Kubota, S., Zapata, N. and Apte, R.S. (2015). IL10-driven STAT3 signalling in senescent macrophages promotes pathological eye angiogenesis. Nature communications 6, 7847.
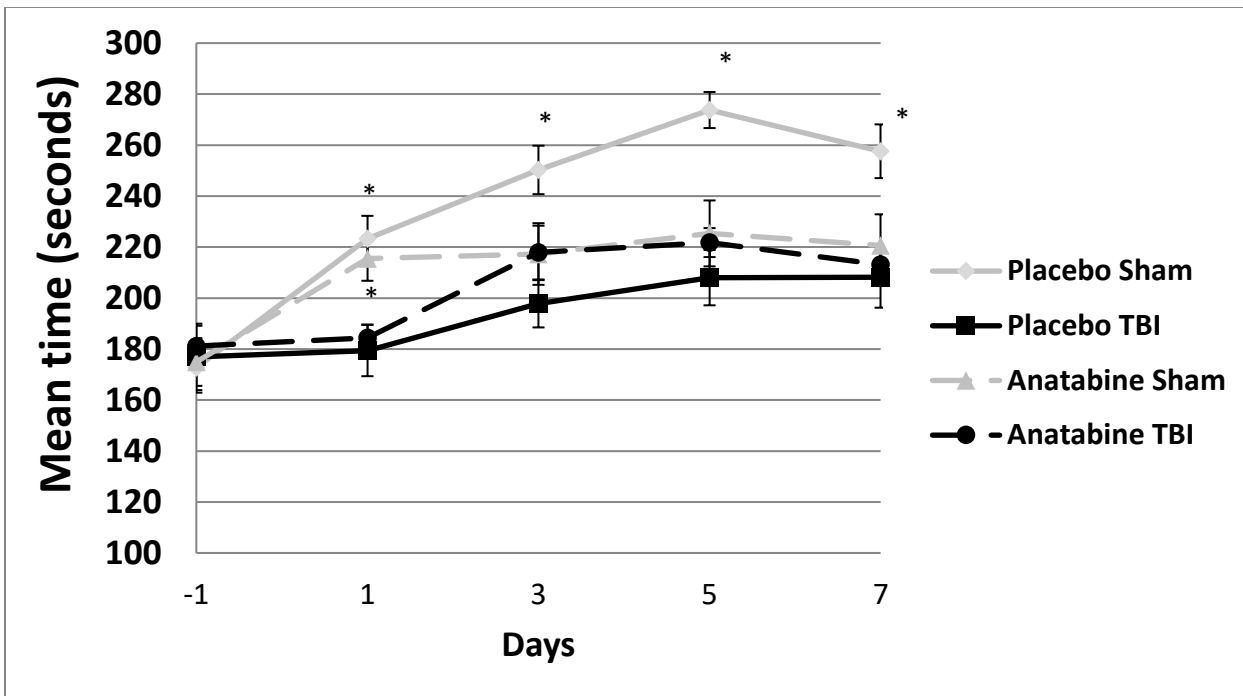
Figure 1 – Rotarod testing showed a significantly higher fall latency of placebo treated sham mice compared to placebo treated r-mTBI mice throughout the first week after the completion of the injury procedure (p<0.05). Anatabine treated sham animals performed significantly better than r-mTBI mice on day 1 after injury, but performed at similar levels to r-mTBI mice on all subsequent days. Bars represent standard error.
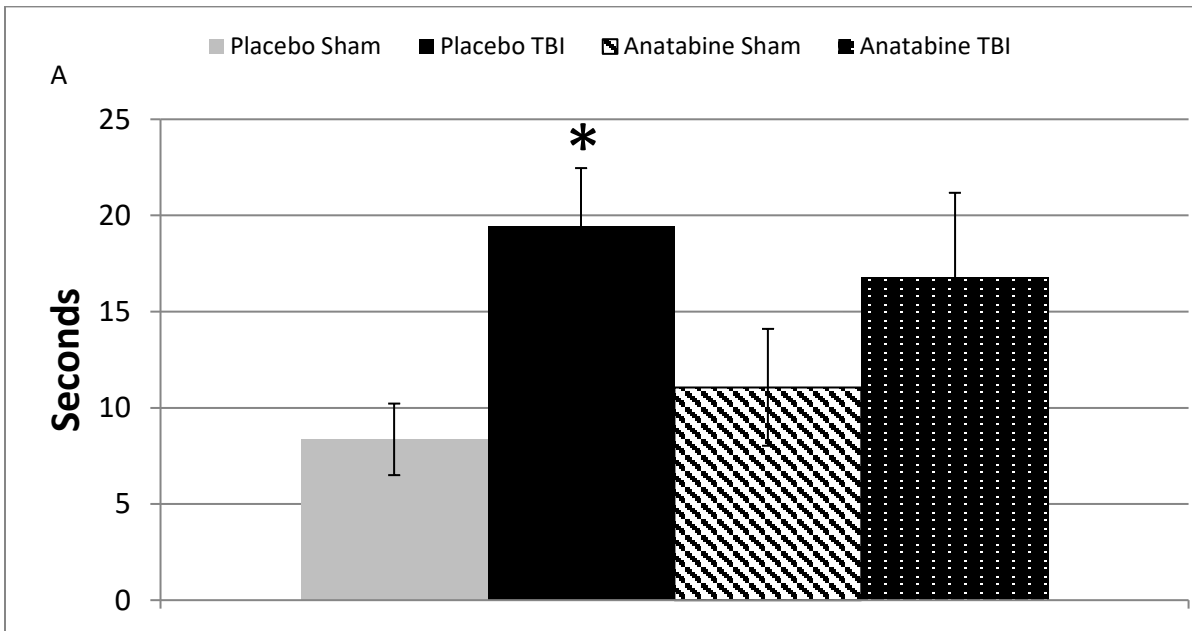
Figure 2a – The Barnes maze probe trial at the acute time point two weeks after the final injury showed a significantly increased latency to find the target hole in the placebo treated r-mTBI group, but no significant difference between the placebo and Anatabine treated TBI groups. Bars represent standard error.
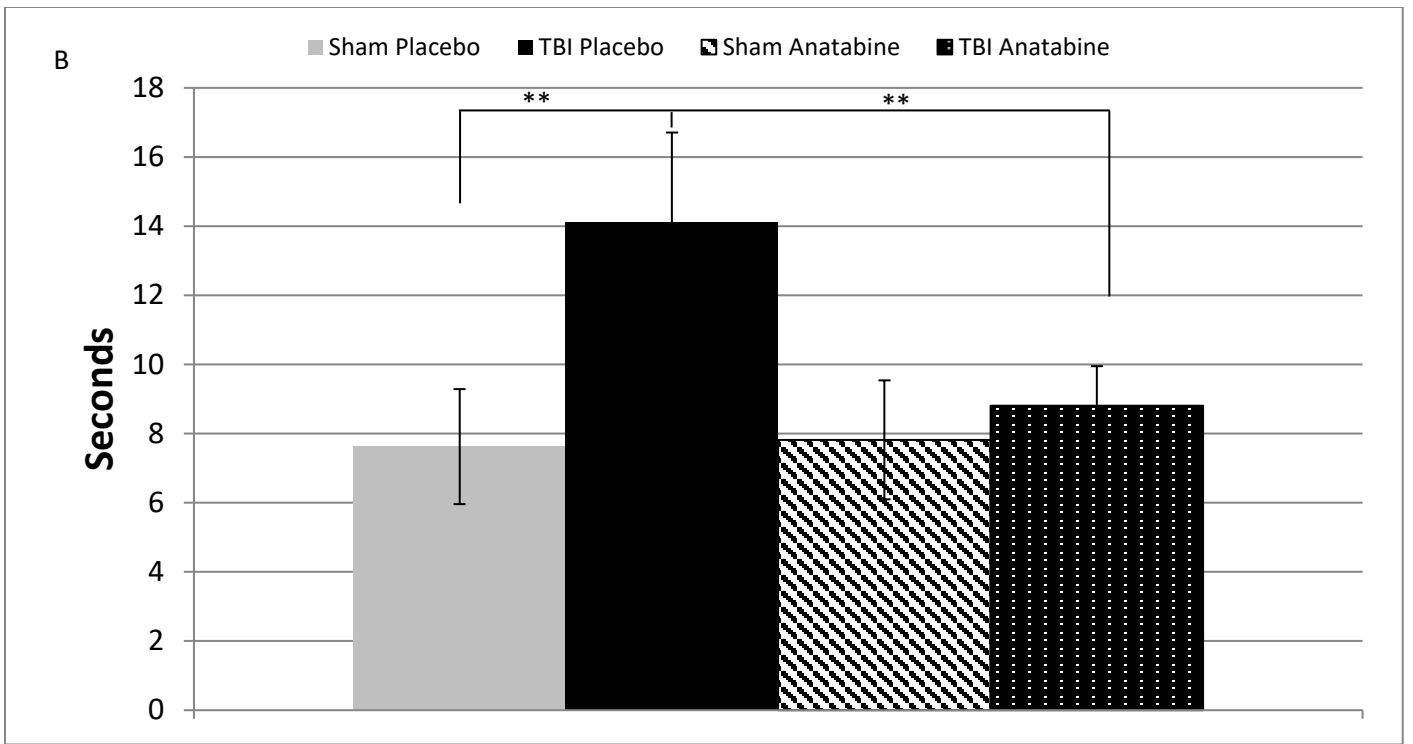
Figure 2b – The Barnes maze probe trial 6 months after the final injury showed a significantly increased latency to find the target hole in the placebo treated r-mTBI group, while anatabine treated r-mTBI mice had a significantly lower significant latency to find the target hole than their placebo treated counterparts (p<0.05). Anatabine treated r-mTBI mice showed no significant difference compared to either sham group. Bars represent standard error.
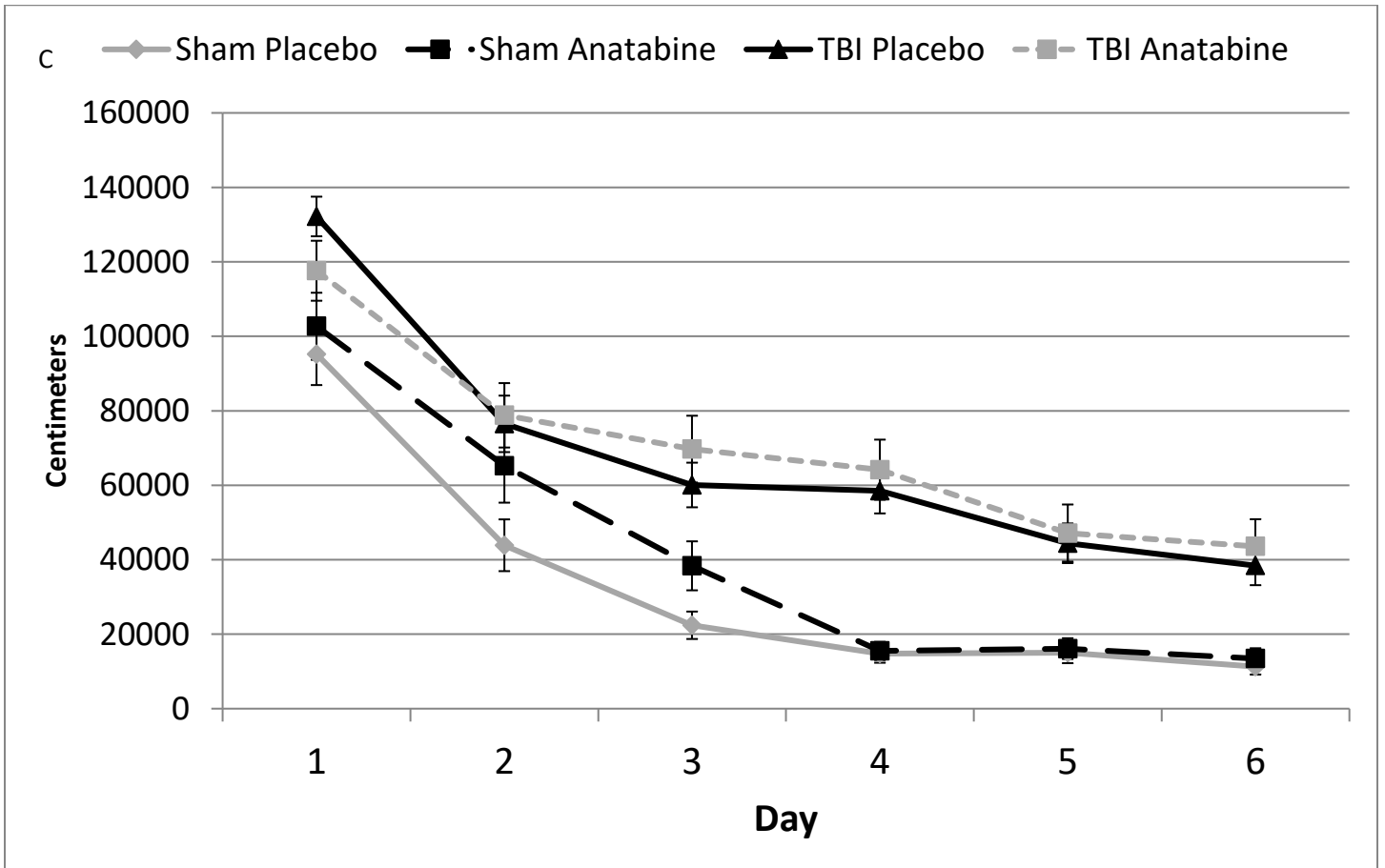
Figure 2c – Cumulative distance during Barnes maze acquisition trials 6 months after the final injury showed a significantly increased cumulative distance to the target hole in both r-mTBI groups compared to their sham controls, regardless of treatment (p<0.05). Cumulative distance was measured as the total sum of the distance of the center point of the mouse to the center of the target hole for each sample of the trial. Bars represent standard error.
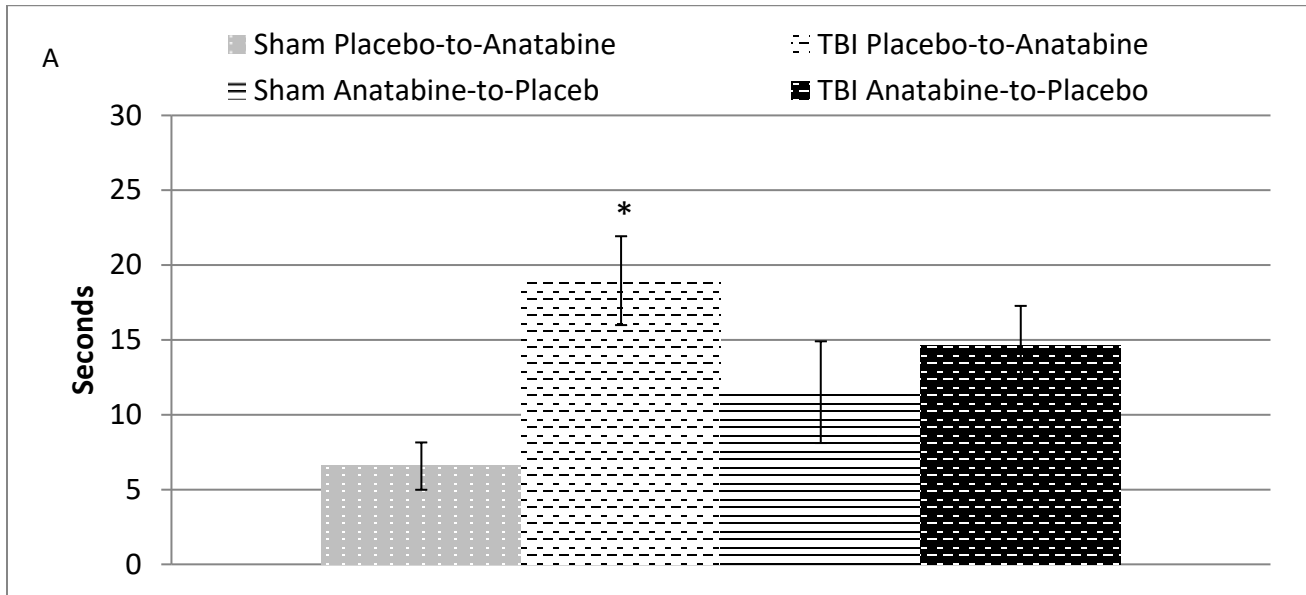
Figure 3a – Barnes maze probe trial at the 12 month post-injury time point (3 months post-crossover). R-mTBI mice that were originally treated with regular water continued to exhibit a significantly higher latency to find the target hole compared to their shams in spite of treatment with anatabine (p<0.05). Post-crossover anatabine-to-placebo treated r-mTBI mice (treated with anatabine during the pre-crossover period) continued to show no significant difference compared to sham animals. Placebo-to-anatabine indicates late treatment post-crossover, anatabine-to-placebo indicates the absence of treatment post-crossover. Bars represent standard error.
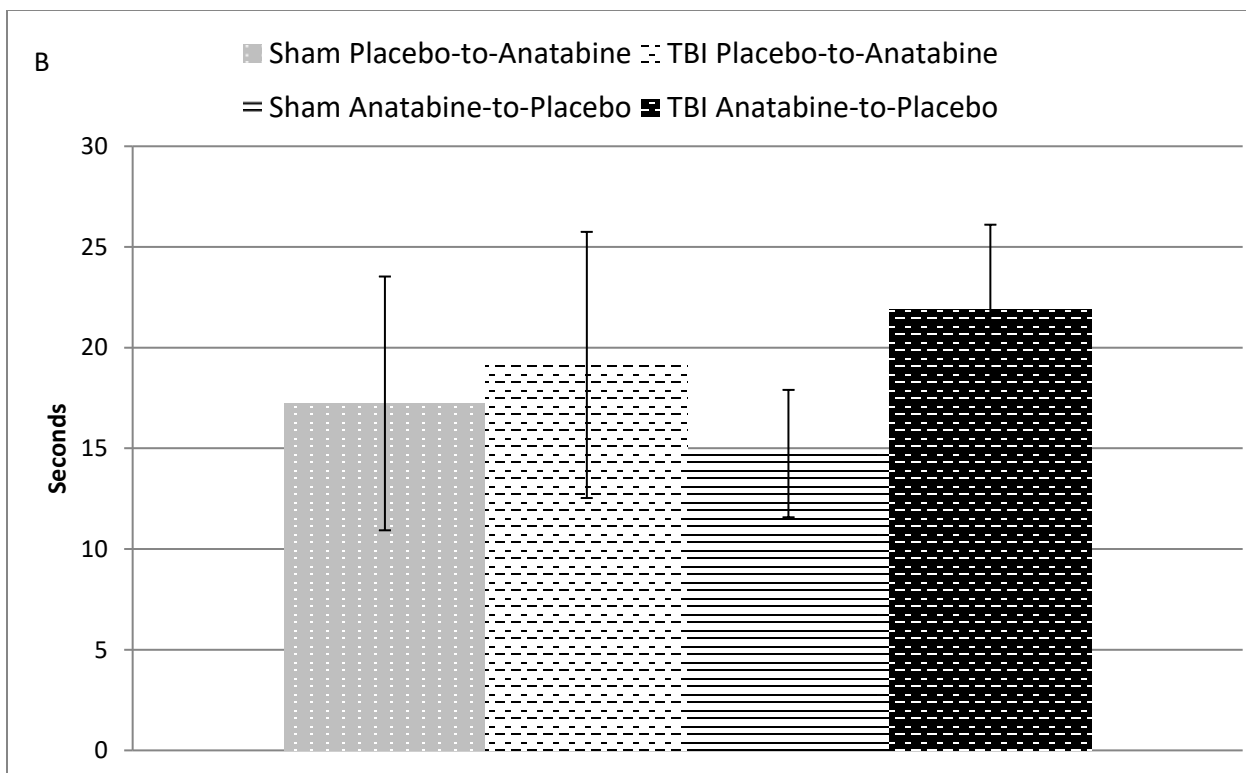
Figure 3b – Barnes maze probe trial at the 18 month post-injury time point (9 months post-crossover). At this time point there were no significant differences between r-mTBI mice and sham mice in either the original treatment or delayed treatment groups (p>0.05) indicating a possible ablation of the TBI effect by anatabine. One TBI placebo-to-anatabine and one sham anatabine-to-placebo animal died between the 12 and 18 month timepoints due to natural causes unrelated to this study. Age-related effects increased the variation in performance between animals and also elevated sham animal latency up to the level held by r-mTBI mice at the 12 month post-injury time point. Bars represent standard error.

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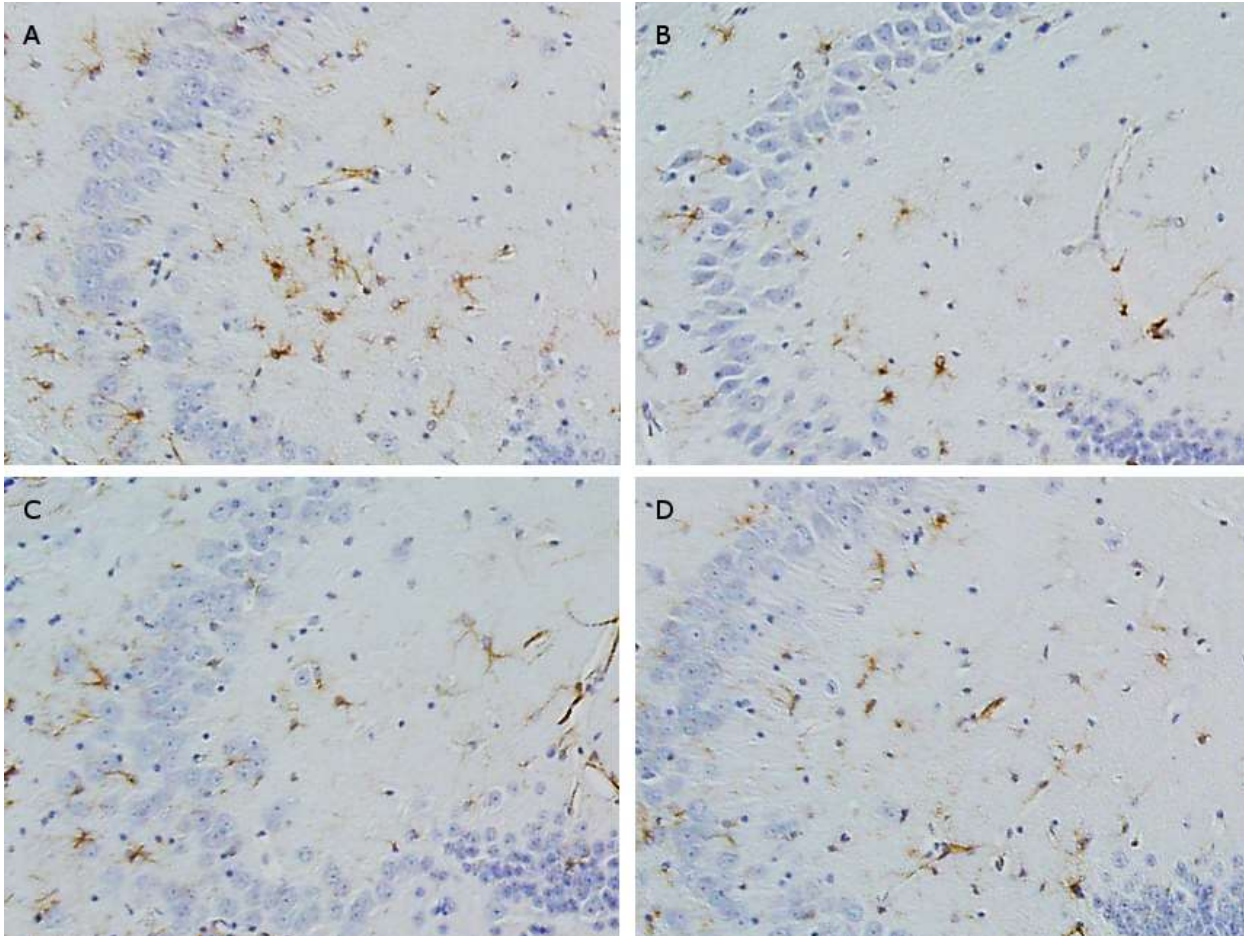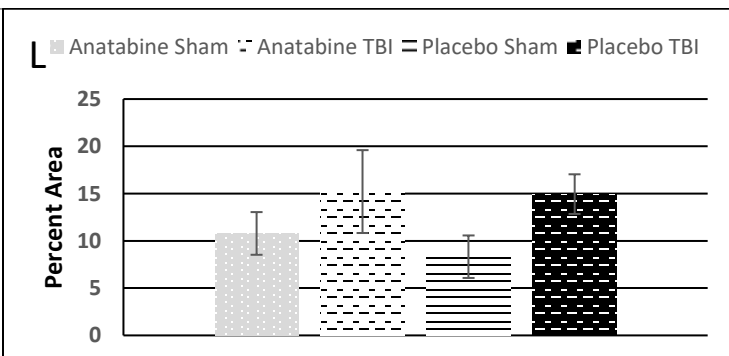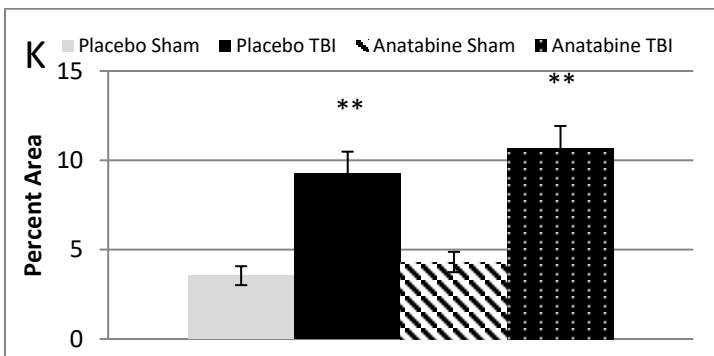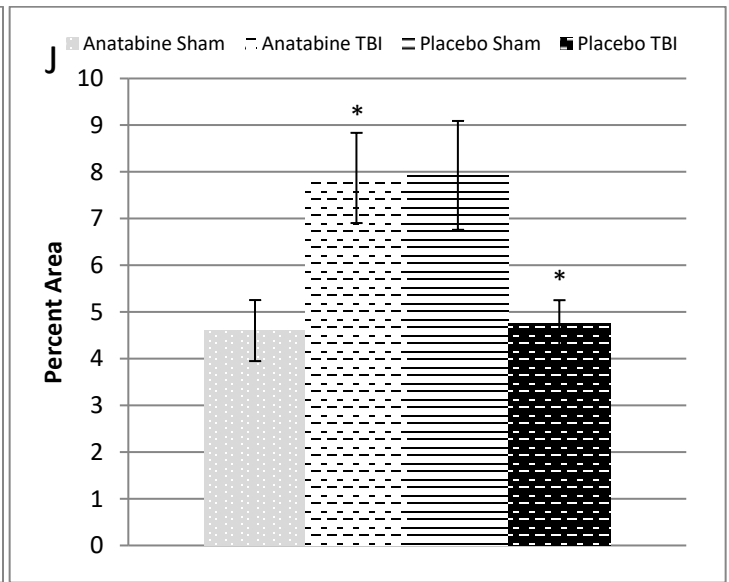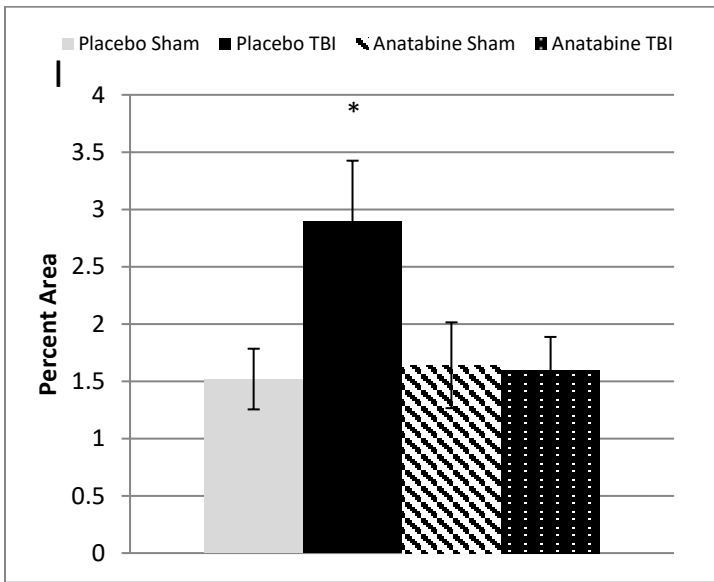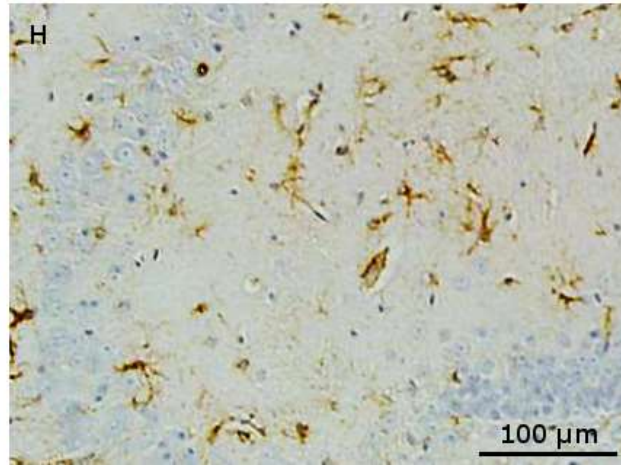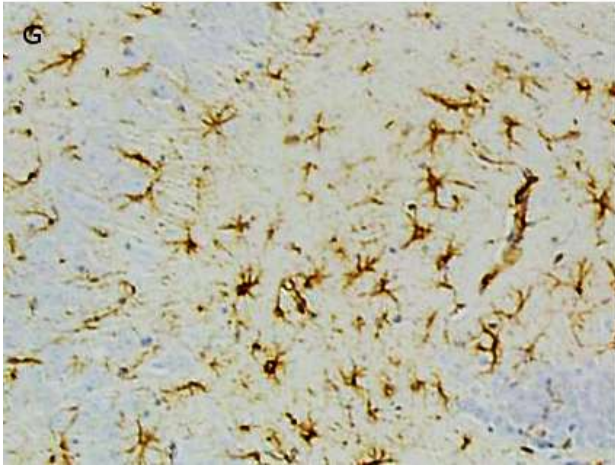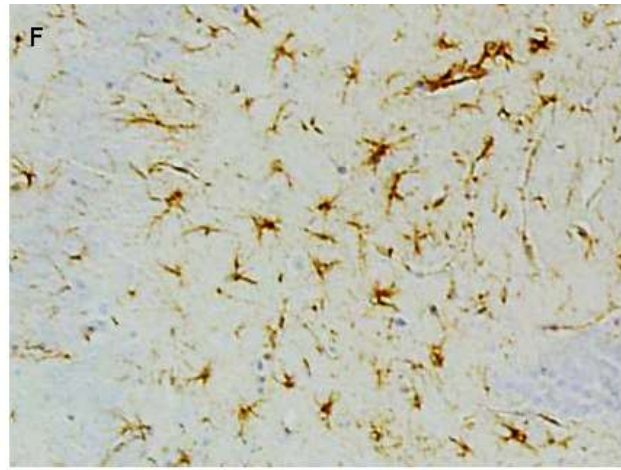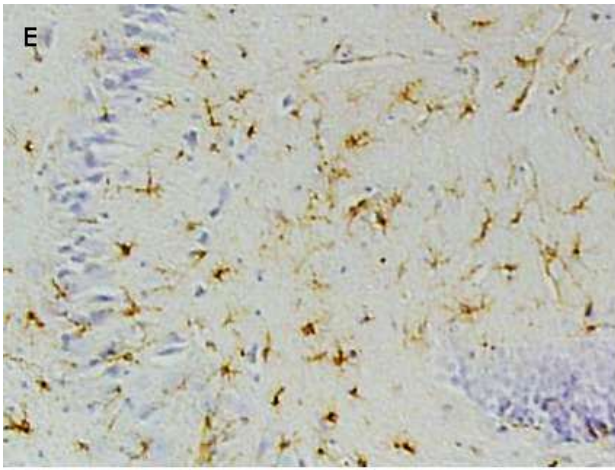
Figure 4 – Hippocampal GFAP A) Placebo treated r-mTBI 9 months post-TBI B) Anatabine treated r-mTBI 9 months post-TBI C) Placebo treated sham 9 months post-TBI D) Anatabine treated sham 9 months post-TBI E) Post-crossover placebo treated r-mTBI 18 months post-TBI F) Post-crossover anatabine treated r-mTBI 18 months post-TBI G) Post-crossover placebo treated sham 18 months post-TBI H) Post-crossover anatabine treated sham 18 months post-TBI. I) GFAP percent area quantification in the hippocampus at 9 months post-TBI. J) GFAP percent area quantification in the hippocampus at 18 months post-TBI. K) GFAP percent area quantification in the corpus callosum at 9 months post-TBI. L) GFAP percent area quantification in the corpus callosum at 18 months post-TBI. Placebo or anatabine refers to the treatment being received at the time of euthanasia for pathological analysis. Bars represent standard error.
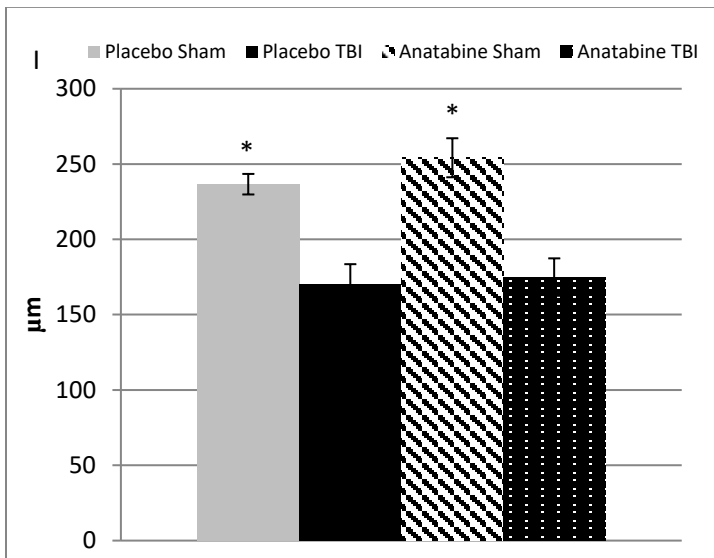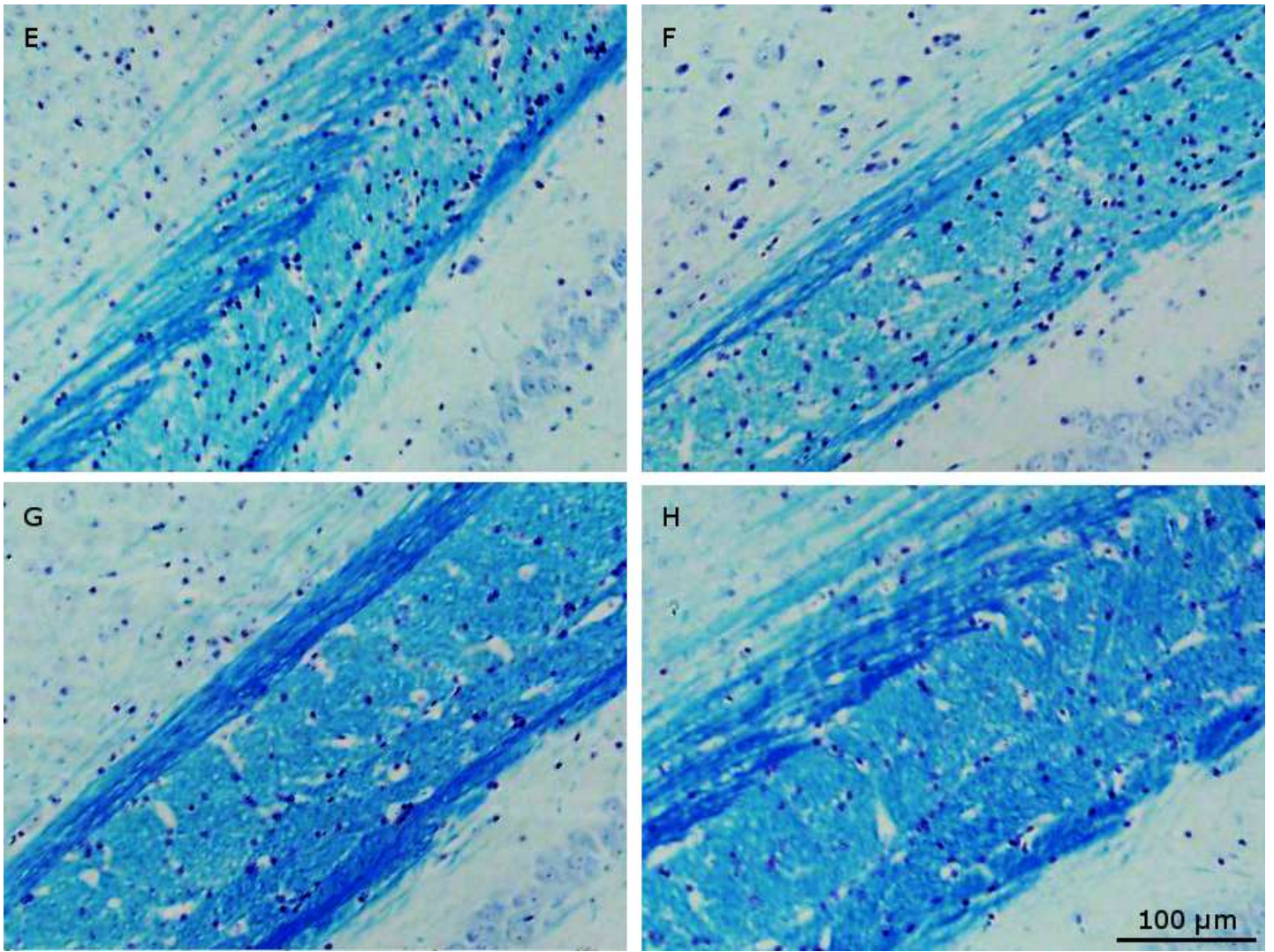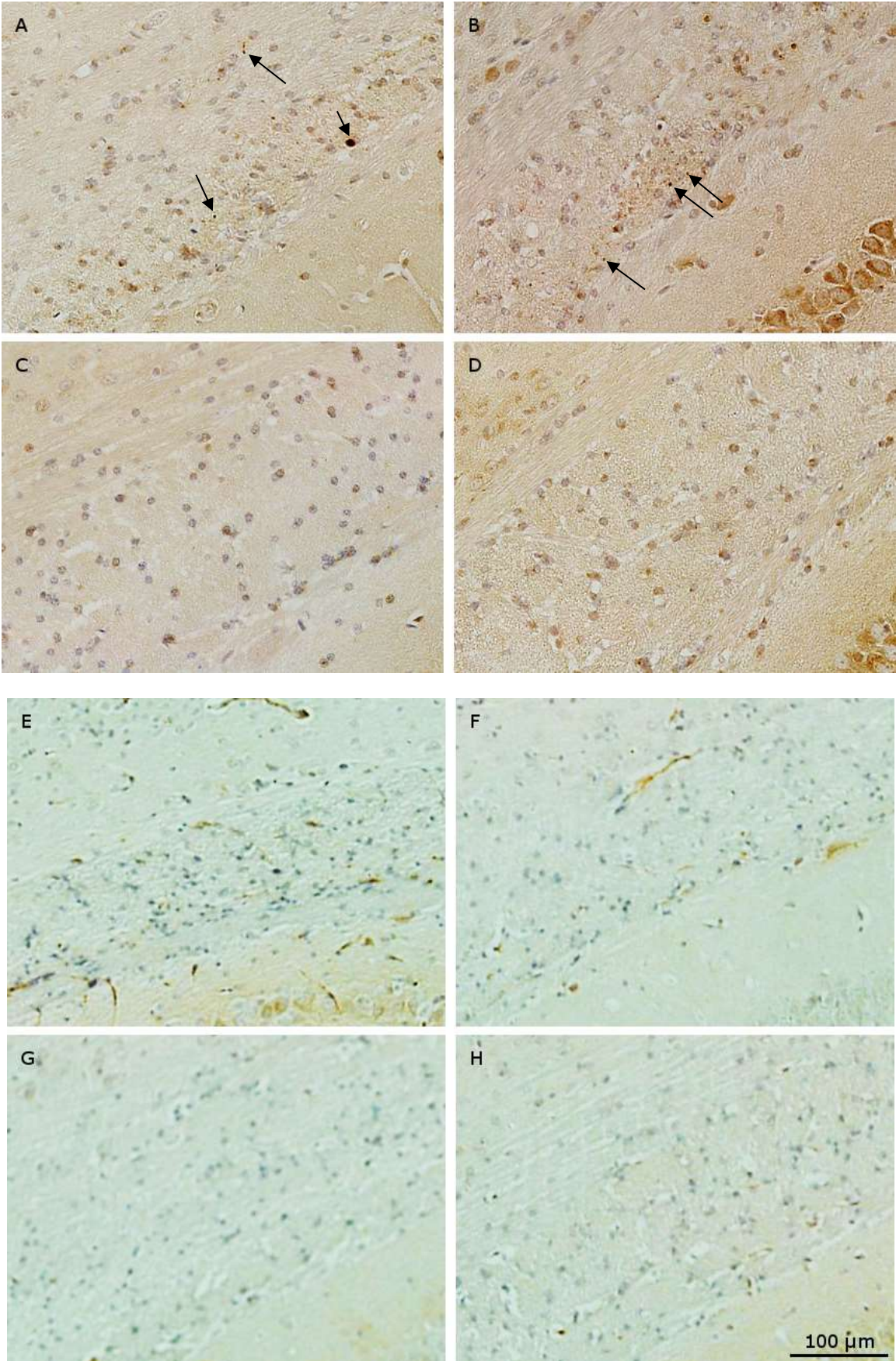
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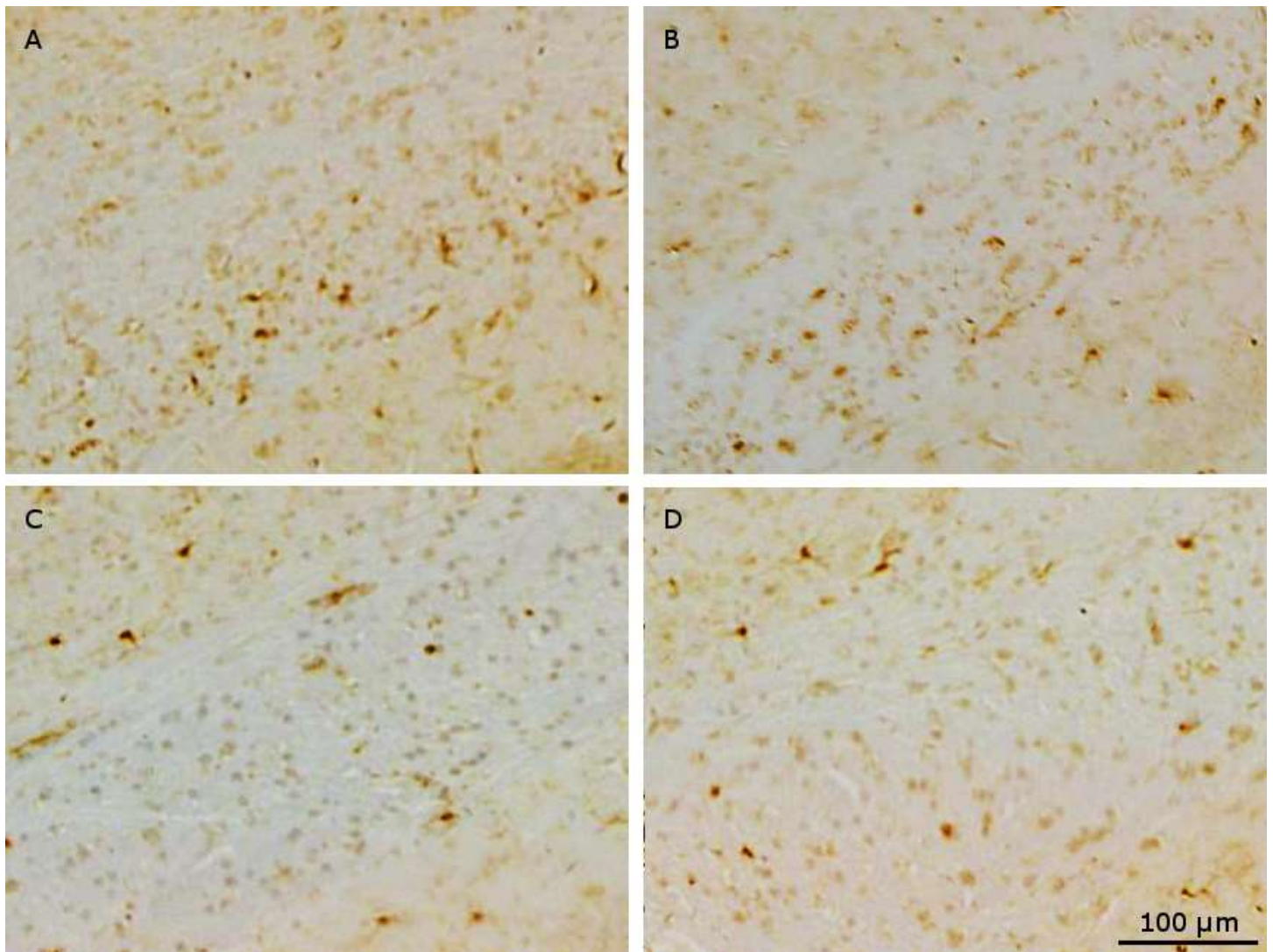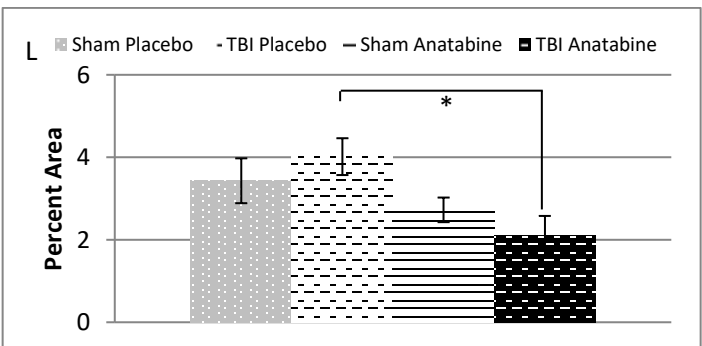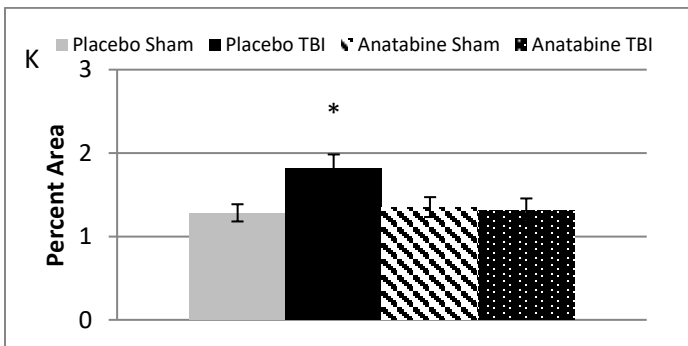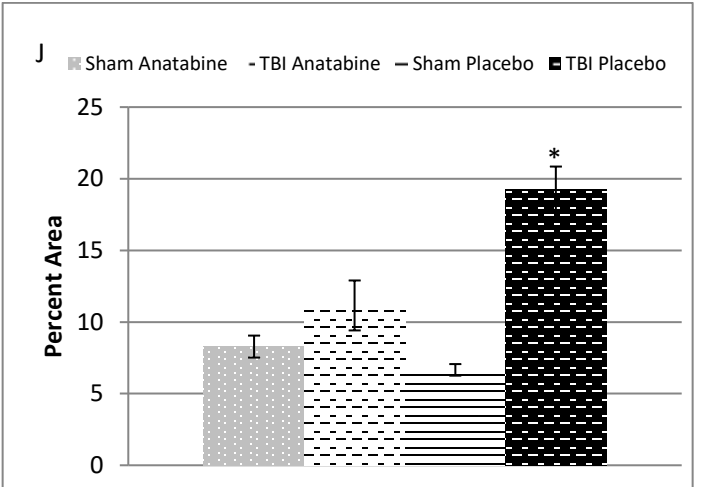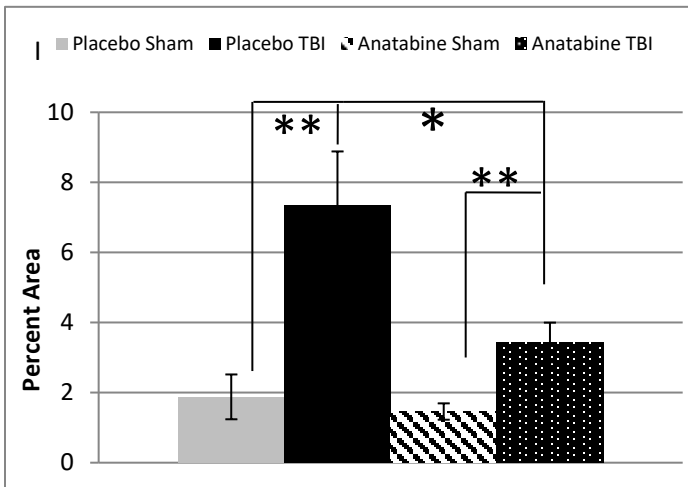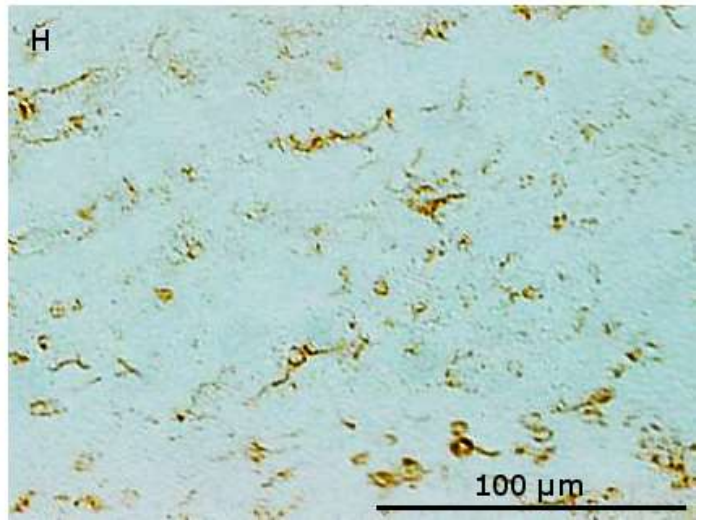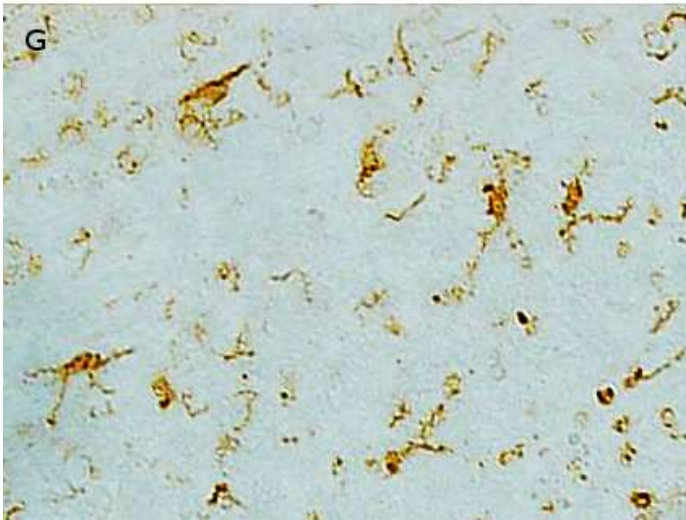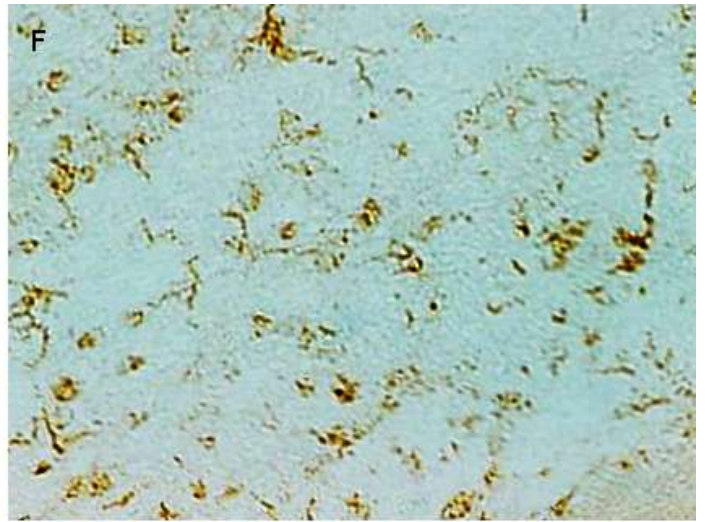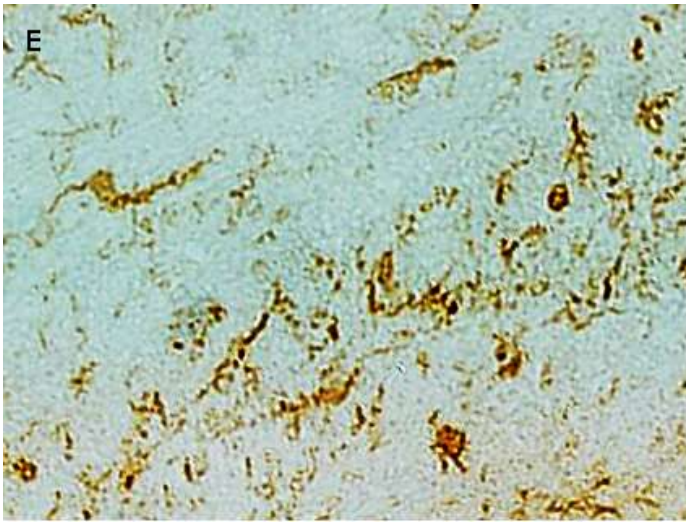
Figure 5 – Corpus callosum IBA1 A) Placebo treated r-mTBI 9 months post-TBI B) Anatabine treated r-mTBI 9 months post-TBI C) Placebo treated sham 9 months post-TBI D) Anatabine treated sham 9 months post-TBI E) Post-crossover placebo treated r-mTBI 18 months post-TBI F) Post-crossover anatabine treated r-mTBI 18 months post-TBI G) Post-crossover placebo treated sham 18 months post-TBI H) Post-crossover anatabine treated sham 18 months post-TBI.   I) IBA1 percent area quantification in the hippocampus at 9 months post-TBI. J) IBA1 percent area quantification in the hippocampus at 18 months post-TBI. K) IBA1 percent area quantification in the corpus callosum at 9 months post-TBI. L) IBA1 percent area quantification in the corpus callosum at 18 months post-TBI. Placebo or anatabine refers to the treatment being received at the time of euthanasia for pathological analysis. Bars represent standard error.
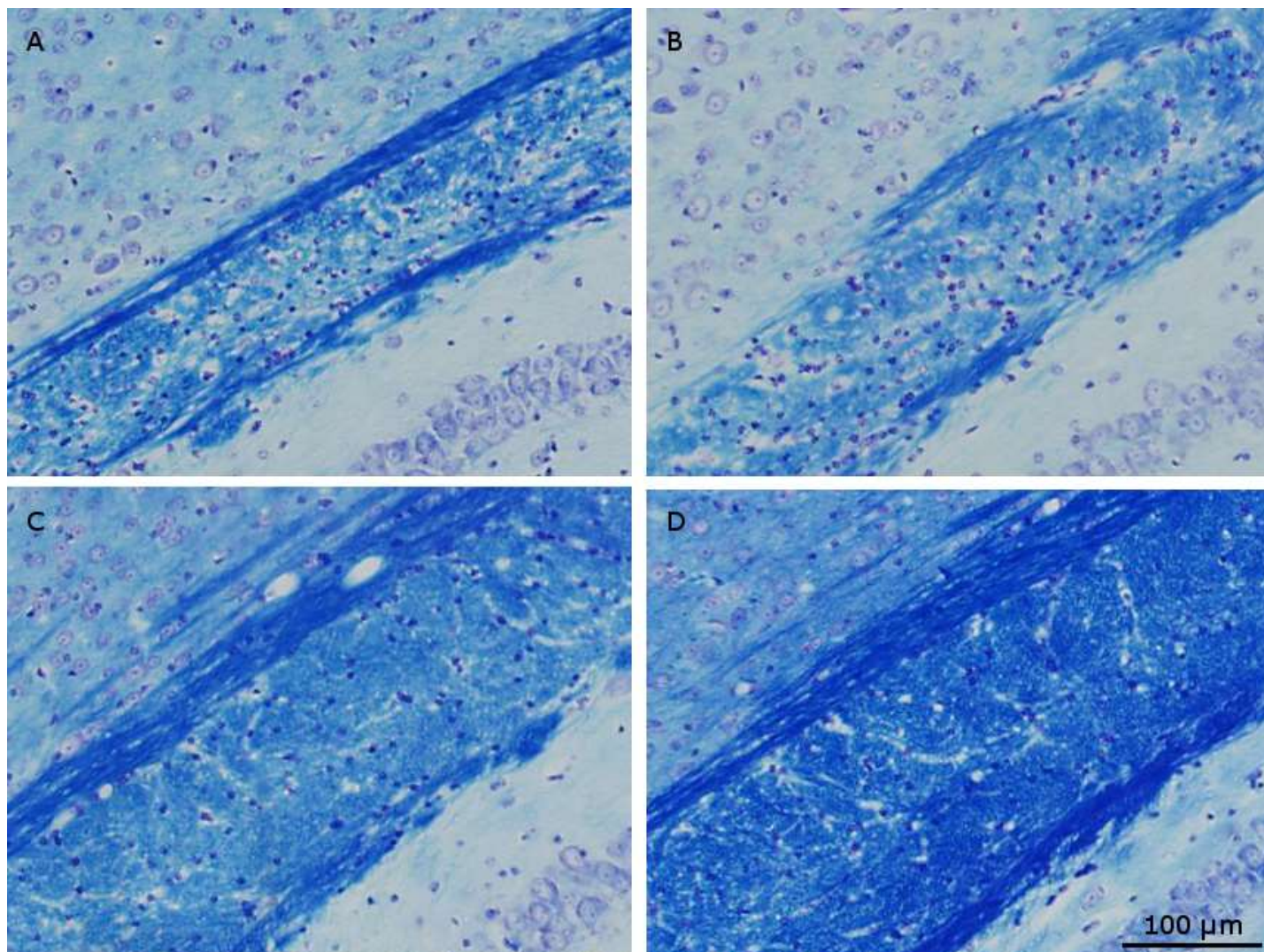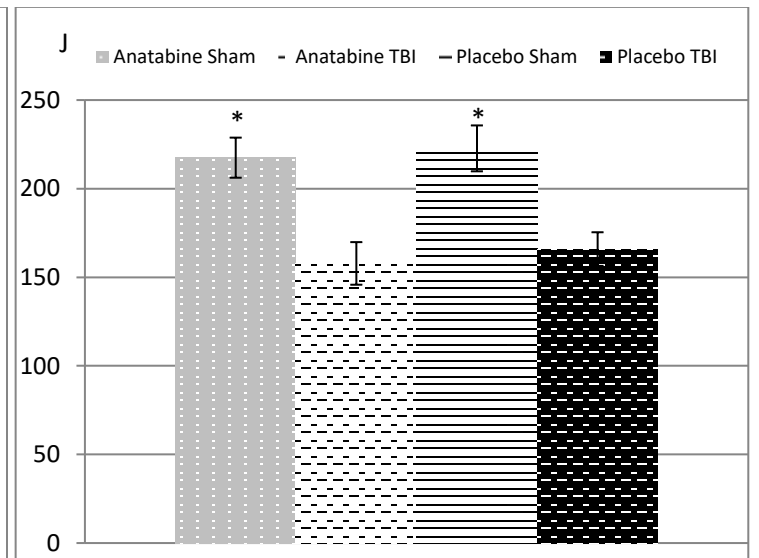
Figure 6 – Luxol Fast Blue staining of the corpus callosum A) Placebo treated r-mTBI 9 months post-TBI B) Anatabine treated r-mTBI 9 months post-TBI C) Placebo treated sham 9 months post-TBI D) Anatabine treated sham 9 months post-TBI E) Post-crossover placebo treated r-mTBI 18 months post-TBI F) Post-crossover anatabine treated r-mTBI 18 months post-TBI G) Post-crossover placebo treated sham 18 months post-TBI H) Post-crossover anatabine treated sham 18 months post-TBI. I) Thickness of the corpus callosum at 9 months post-TBI. J) Thickness of the corpus callosum at 18 months post-TBI. Placebo or anatabine refers to the treatment being received at the time of euthanasia for pathological

analysis. Quantification of the thickness of the corpus callosum shows a significant effect of injury (p<0.05) regardless of treatment with anatabine. Bars represent standard error.
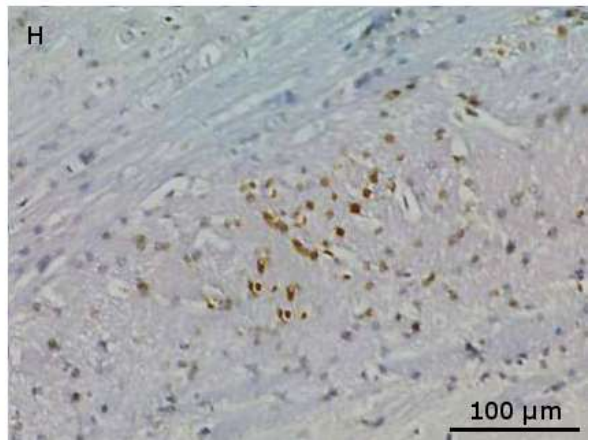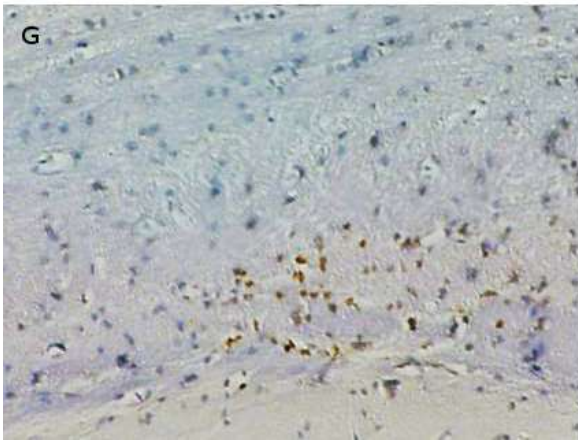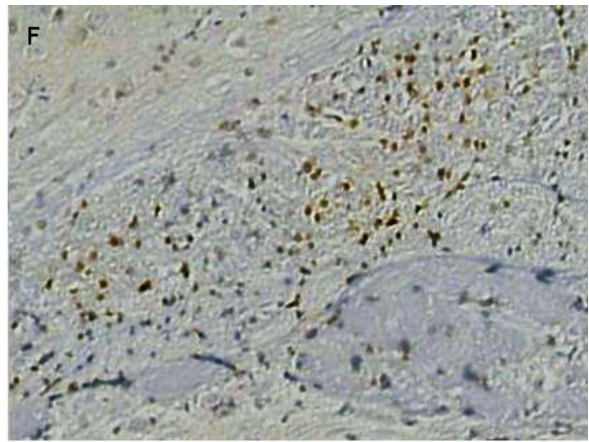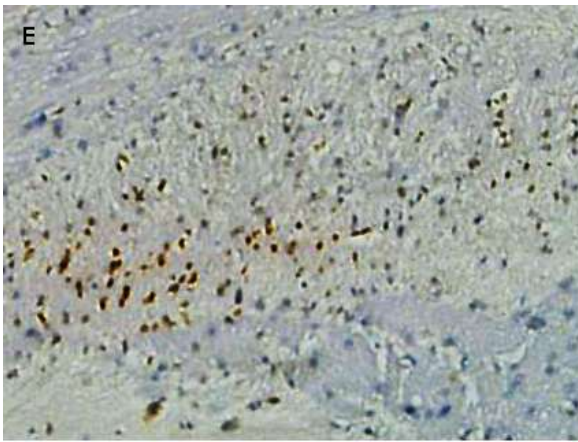

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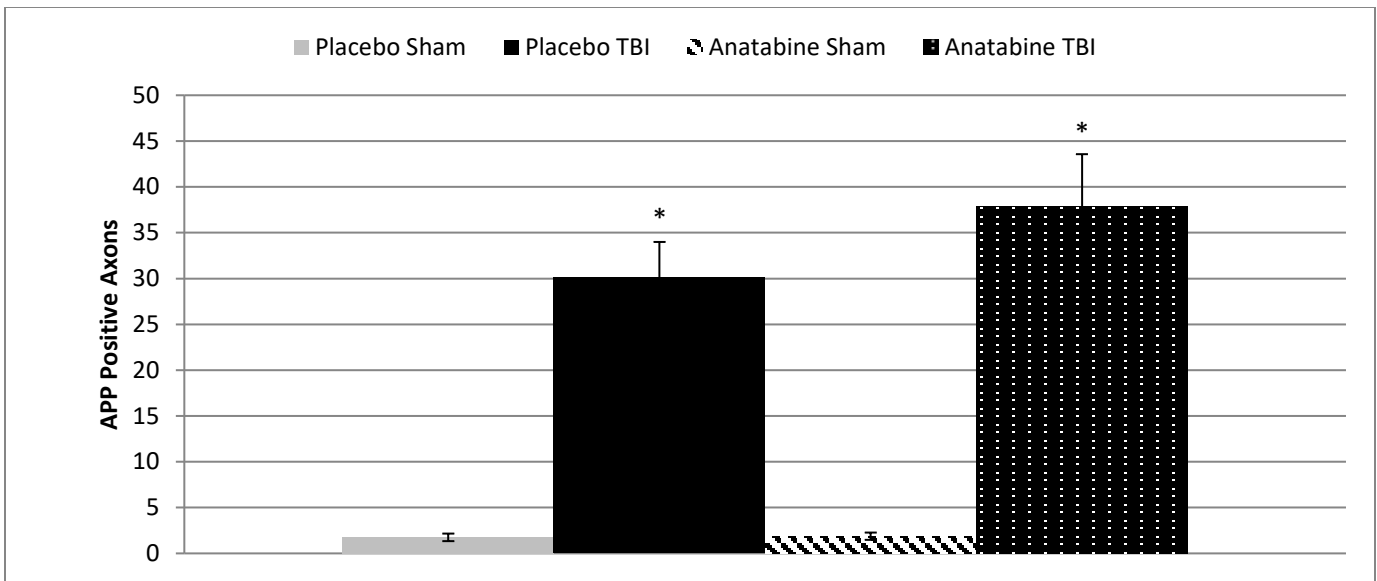
Figure 7 – APP staining of the corpus callosum A) Placebo treated r-mTBI 9 months post-TBI B) Anatabine treated r-mTBI 9 months post-TBI C) Placebo treated sham 9 months post-TBI D) Anatabine treated sham 9 months post-TBI E) Post-crossover placebo treated r-mTBI 18 months post-TBI F) Post-crossover anatabine treated r-mTBI 18 months post-TBI G) Post-crossover placebo treated sham 18 months post-TBI H) Post-crossover anatabine treated sham 18 months post-TBI. Quantification of the number of APP positive axons within the corpus callosum shows a significant effect of injury (p<0.05) regardless of treatment with anatabine. Bars represent standard error.
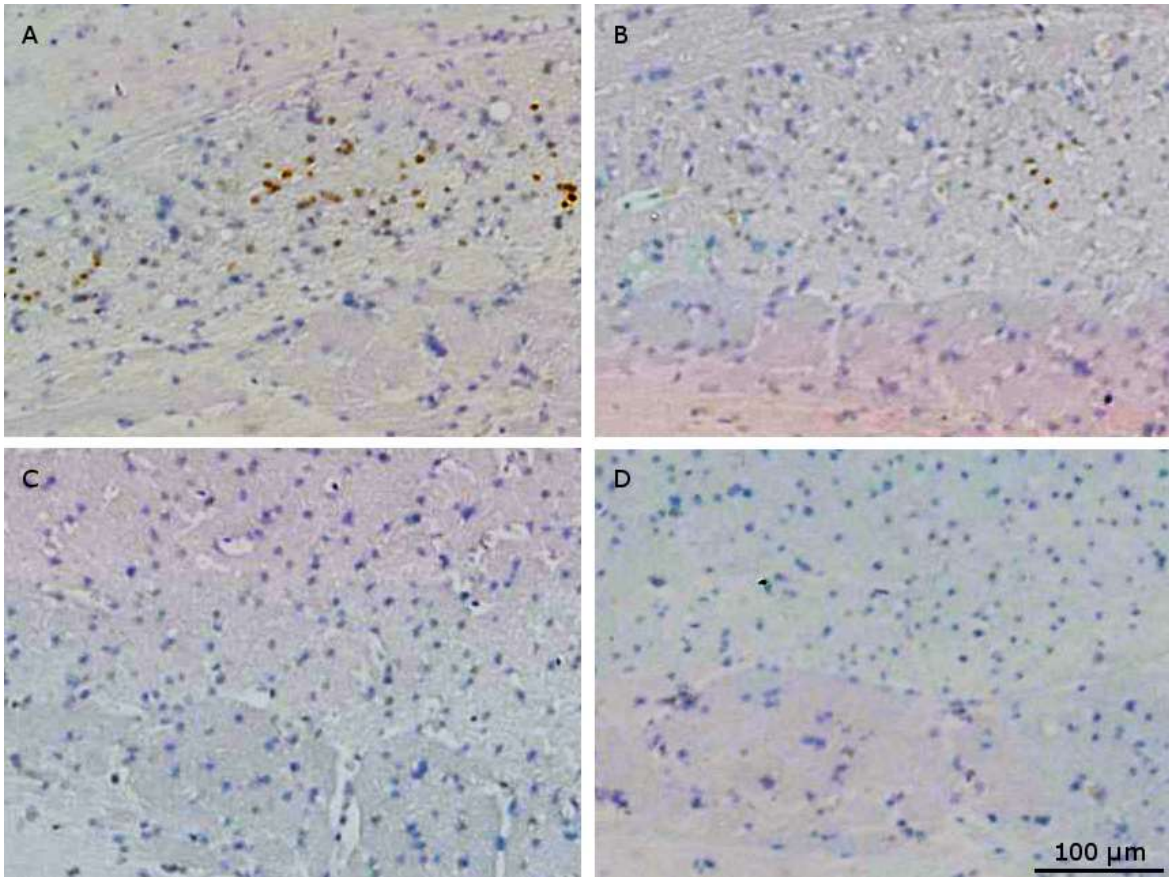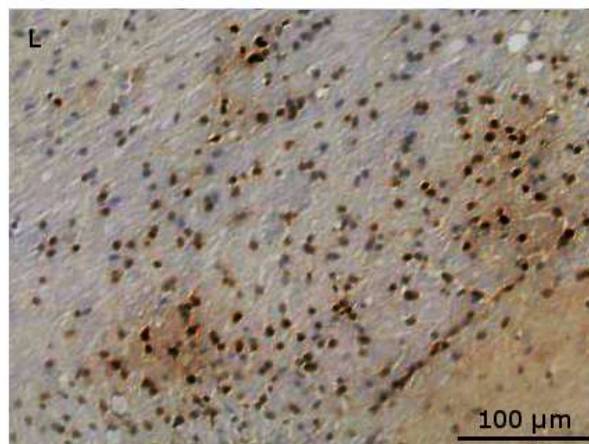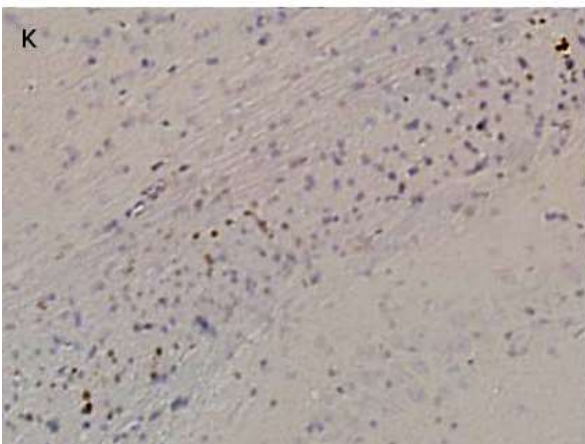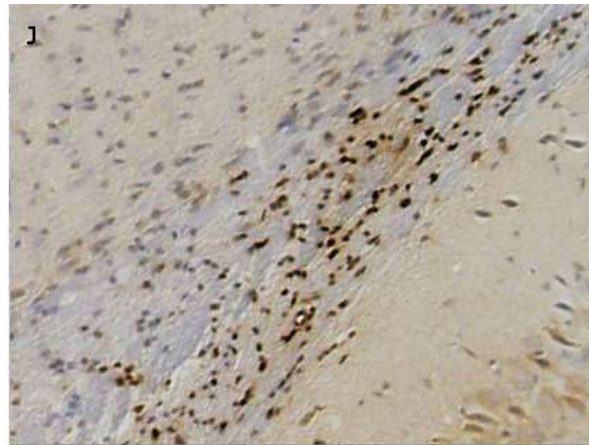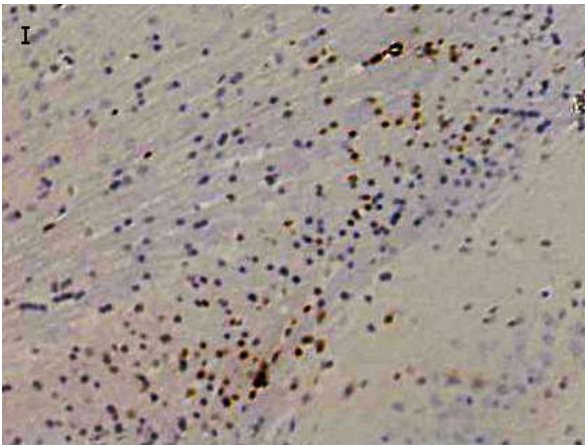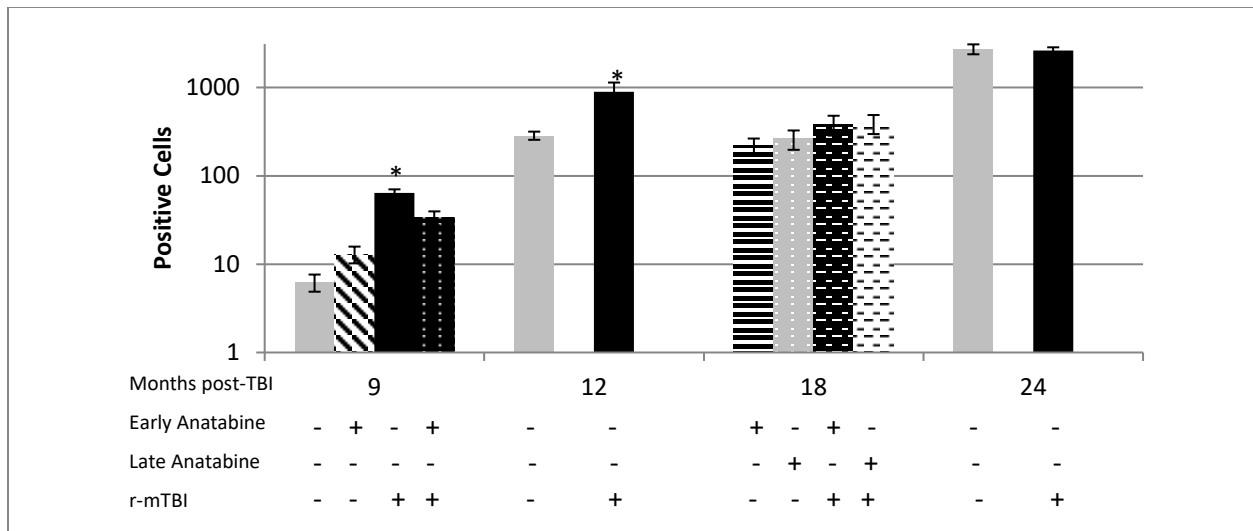
Figure 8 – Phospho STAT3 staining of the corpus callosum A) Placebo treated r-mTBI 9 months post-TBI B) Anatabine treated r-mTBI 9 months post-TBI C) Placebo treated sham 9 months post-TBI D) Anatabine treated sham 9 months post-TBI E) Post-crossover placebo treated r-mTBI 18 months post-TBI F) Post-crossover anatabine treated r-mTBI 18 months post-TBI G) Post-crossover placebo treated sham 18 months post-TBI H) Post-crossover anatabine treated sham 18 months post-TBI. Placebo or anatabine refers to the treatment being received at the time of euthanasia for pathological analysis. I) Untreated r-mTBI at 12 months post-TBI J) Untreated r-mTBI at 24 months post-TBI K) Untreated sham at 12 months post-TBI L) Untreated sham at 24 months post-TBI. M) Quantification of p-STAT3 staining showed a significant increase of p-STAT3 positive cells in the r-mTBI groups of untreated mice both 9 and 12 months after TBI. STAT3 phosphorylation also increased significantly with age, but this appears to have been reduced by either early or late treatment with anatabine at the 18 month post-injury timepoint. Quantification is displayed on a logarithmic scale. Early anatabine refers to the group treated for the first 9 months post-TBI, late anatabine refers to the group treated from 9 to 18 months post-TBI. Bars represent standard error.