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Background: Due to the changing nature of war, there is a pressing need for new treatments for traumatic brain injury (TBI). The grantee previously found screened that the combination of minocycline (MINO) and N-acetyl cysteine (NAC) synergistically improved brain function when dosed one hour following closed cortical impact (CCI) in rats. The overall objective of this proposal is that MINO/NAC synergistically improves brain function following TBI. Three tasks will be done to achieve this objective: 1) Differing doses of MINO/NAC will be tested for the ability to improve behavior and histology following closed head injury in mice (CHI). 2) MINO/NAC was effective when dosed one hour after injury. Longer intervals between injury and drug dosing will be tested. 3) A restoration of cognitive function will be tested three months after CHI. The grantee has shown that MINO plus NAC improves cognition in two models of TBI, a single impact closed head injury (CHI) in mice and the CCI model in rats. The drug combination improves cognition and limits white matter injury in two models and two species. These data attest to the potency of the drug combination.					
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Introduction

There is a pressing need for new treatments for TBI because of the increased frequency of head injuries during Operation Iraqi Freedom and Operation Enduring Freedom. Multidrug treatment is one of many therapeutic approaches that are being tested. The applicant previously performed a screen of drug pairs to treat TBI ("Multidrug treatment of traumatic brain injury", PT073028) from the Fiscal Year 2007 CDMRP program for Psychological Health/Traumatic Brain Injury. Drug pairs were screened that: (1) limited TBI as monotherapy in preclinical or early clinical trials and (2) have FDA approval for uses other than TBI. The drug screen used a novel behavioral assessment with high cognitive demand to find drug combinations that best restored brain function following moderate or mild closed cortical impact (CCI) (1). The applicant found that MINO and NAC improved both cognitive and histological outcomes following CCI (2). As monotherapy, MINO-treated rats learned, but had no 24-hour retention of an active avoidance task. NAC-treated rats were greatly impaired in both acquisition and retention. The combination of MINO plus NAC, given one hour after injury produced a large improvement in both acquisition and retention. NAC alone had no effect indicating a synergistic drug interaction with MINO in improving cognitive function. In addition, MINO plus NAC-treated rats had better sparing of both white and grey matter.

These observations provide the justification for the central hypothesis that: MINO plus NAC synergistically improve brain function following TBI. MINO plus NAC has the potential to rapidly get a safe and effective combination therapy into clinical trials. The combination is likely to be safe. Both drugs have been used in the clinic for decades with well-known pharmacokinetics, pharmacodynamics and drug interactions. The current project is producing much of the preliminary data needed to begin testing MINO plus NAC in the clinic.

The CCI model of TBI was responsible for the slow progress of the applicant, and switching models led to more rapid progress. As new lab members in the applicant's laboratory learned the CCI model, approximately 25% of the rats receiving sham injury showed varying amounts of impairments in the active place avoidance task (P. Bergold, unpublished observation). This became a serious setback since the proposal used this behavioral task as the most sensitive determinant of drug efficacy. As the applicant was working on this problem, there were mutterings in the TBI community of difficulties with the craniotomy used in the CCI model. These concerns were confirmed when a recent study showed that the craniotomy triggered neuroinflammation that potentially damaged the brain [3]. The craniotomy used in the CCI model is likely responsible for the behavioral deficits in the rats that received sham-CCI.

As a result, the applicant switched to an alternative model, a closed head injury (CHI) model that does not require a craniotomy. Most CHI models injure the brain by dropping a weight. Weight drop models are difficult to control and tend to have greater injury heterogeneity than with CCI [4]. An impact needed to produce behavioral deficits frequently led to skull fracture [4]. Recently, investigators have used the impactor used in the CCI model to directly strike the skull in multiple hit model of repeated concussion injury. This has led to rapid progress in the project.

Body

The statement of work of this project was revised due to technical difficulties with the CCI model of TBI (Figure 1). Despite these difficulties, MINO plus NAC was shown to be effective in the CCI model at two doses of MINO. A new TBI model has been developed, the closed head impact (CHI) model has been developed and MINO has been shown to be effective at four different doses with the optimum MINO concentration between 22.5 and 10mM. The CHI model has also been shown to produce long-term behavioral deficits.

A revised statement of work for this project describes three tasks:

Task 1. Optimizing the dosing of minocycline (MINO) plus N-acetyl cysteine (NAC) in the Closed Head Impact (CHI) model of TBI

Task 2. Evaluation of the Therapeutic Window of MINO plus NAC in the CHI model.

Task 3. Examination whether MINO plus NAC provide long-term behavior improvements

Regulatory approval has been obtained for the both the CHI and CCI models have been approved by the SUNY-Downstate Institutional Animal Care and Use Committee as well as the USAMRMC Animal Care and Use Review Office. These approvals cover the three tasks in the statement of work.

Task 1 Optimization of the dosing of MINO and NAC in both the CCI and CHI models of TBI

Dosing of MINO (45mg/kg) plus NAC (150mg/kg) to rats one hour after moderate CCI has been previously shown to synergistically improved performance on the active place task (2). Task 1 tests different doses of MINO and NAC separately to obtain a more complete dose-response. Preliminary data from this task is shown in figure 2.

Rats received either sham- or moderate CCI The moderately injured group did increase the number of entrances during training since there was a significant treatment effect ($F_{(3, 17)} = 4.54$ $p < 0.02$, ANOVA). There was a strong, but not significant trend for both MINO (45mg/kg) plus NAC (150mg/kg), and MINO (90mg/kg) plus NAC (150mg/kg) to reduce the number of entrances. The number of entrances, however, did not appear different between the two treated groups. These data repeat earlier finding that MINO plus NAC improves cognition after moderate CCI, but increasing the MINO concentration does provide a further increase in efficacy. Additional doses of MINO plus NAC will be tested to determine the optimal dosing of both drugs

There was an ongoing problem in the CCI model because approximately 25% of the rats receiving sham injury showed varying amounts of impairment in the active place avoidance task. Many changes were made in the behavioral task were made to address this problem. These changes included:

- 1) Changing room and visual cues in the behavioral apparatus
 - 2) Increasing the time for habituating rats to the laboratory, laboratory workers and behavioral apparatus
 - 3) Testing sham-injured rats on other active avoidance set-ups at SUNY Downstate
 - 4) Testing multiple drills for craniotomy
 - 5) Purchasing Sprague-Dawley rats from alternative vendors
 - 6) Placed rats on reversed day-night cycle to ensure that the rats were fully awake when tested during the day
 - 7) Titrated amount of foot shock for each rat
- A. Switched from the albino Sprague-Dawley rat strain to the non-albino Long-Evans strain.

None of the changes significantly decreased the number of rats that received sham-injury that showed impairments in the active place avoidance task. This suggested that the craniotomy used in the controlled cortical impact model produced sufficient brain injury to impair a subset of rats. As a result, Dr. Bergold decided to switch from the controlled cortical impact model to a closed head injury that does not require a craniotomy. Accompanying the change in the model was also changing the species used from rat to mouse. Mice were selected due to the thinner skull that increased the probability that a single blunt blow to the head could injure the brain.

Closed head injury was done on C57/BL6 mice (4.0 to 4.5 months old). A baseline weight was obtained on all mice prior to sham-closed head injury (CHI) or sham-CHI. Deep anesthesia was induced with isoflurane (3% in oxygen (1.0 L/min)) and maintained via a nose cone and isoflurane (2% in oxygen (1.0 L/min)). The head of the mouse was fixed in a stereotaxic frame using soft foam pads (David Kopf,

Tujunga, CA). Foam pads avoided the tendency of conventional ear bars to break the bones of the inner ear during the impact.

Rectal temperature was maintained at 36.5–37.5°C using a circulating warm water heating pad. Isoflurane anesthesia was maintained throughout the surgery. A cortical contusion was produced using an electromagnetic contusion device (Myneurolab, St. Louis, MO). A 5.0 mm diameter impactor tip was placed at a 10° angle 5mm off the midline and 2mm from the eyes of the mouse. Closed head injury was produced with by a single 6.3 m/s impact to the skull that compressed the skull 3 mm. After the impact, the mouse was removed from the device and allowed to recover in its home cage. A mouse was determined to have recovered from anesthesia if it has regaining its ability to right itself and ambulate.

Injured mice were divided into two groups based upon whether apnea developed immediately after CHI. One group with no apnea and recovered immediately after CHI. The other group developed apnea after injury. These mice needed 20 seconds to one minute of cardiopulmonary respiration in the 100% O₂. The mice were then observed for 5 minutes to ensure that the mice had completely recovered from apnea. Sham-CHI mice underwent the same procedure without the impact. Sham-CHI mice had no apnea. Of 13 mice injured, 18 had apnea and 6 did not. The mice without apnea were studied separately from the study of the effects of MINO plus NAC.

Mice received three injections of MINO (90 or 45mg/kg) plus NAC (150mg/kg) one hour, one and two days after injury. A second set of injured mice received three injections of saline with the same injection schedule. One week after injury, behavioral assessment was done in a rectangular room (4m by 3m) that contained a behavioral apparatus containing a 40-cm diameter metal disc with prominent visual landmarks on the walls. A computer tracked the position of the mouse using a computer controlled infrared Firewire camera mounted 1.2 m above the arena. The signal from the camera was analyzed by a spot-tracker (BioSignal Group, Brooklyn, NY). The outer part of the arena was marked with a light emitting diode (LED). The positions of the mouse and the arena LED was determined every 33 ms and used to calculate the movement of the animal relative to the arena. In some determinations, the computer defined a 60° segment of the arena as a do-not-enter shock zone. A 500 ms shock was produced 500 ms after entry into the shock zone. Additional shocks were administered every 1.5 s until the mouse has vacated the shock zone. The shock consisted of a brief constant current (500 ms, 60 Hz, 0.2 mA) delivered through the grid floor. The number of times a mouse entered the shock zone was computed by Track Analysis software (Bio-Signal Group Corp., Brooklyn, NY).

Beginning 7 days after CHI or sham-CHI, mice was habituated to handling and the training environment. All training sessions were 10 minutes long with a 50-minute intertrial interval. Mice first had a pretraining session in consisting of a arena rotating 1rpm with the shock zone turned off. Total distance traveled was assessed. The mice then had 3 sessions with the shock zone turned on and number of shock zone entrances and the distance assessed (Figure 3).

Development of a single-hit CHI model: Post-injury resuscitation predicts future behavioral deficits

Injury heterogeneity has hampered the use of CHI models (ref). The applicant tested whether a single impactor hit could produce reproducible deficits in the active place avoidance task. Initial studies repeated the substantial injury heterogeneity that confounds many closed head TBI models (ref). The problem of injury heterogeneity was solved with the discovery that mice with a post-CHI apnea that needed cardiopulmonary resuscitation consistently developed behavioral deficits (Fig 3). The production of CHI and the behavioral assessment are described in the detailed methods section. Resuscitation is needed in approximately 75% of the mice receiving CHI. These mice showed behavioral deficits while the remaining 25% of the mice were no different than sham-CHI uninjured mice. Limiting the study to the 75% of the mice that required resuscitation allowed testing of MINO plus NAC in the CHI model (Fig. 4). CHI has many advantages over CCI. CHI neither exposes the

skull or performs a craniotomy. These surgical procedures have been implicated in behavioral deficits in rats and mice on the active place avoidance task. At present, 100% of sham-CHI mice who avoided craniotomy have normal behavior. In contrast, injured mice that need resuscitation uniformly had behavioral deficits (Fig. 3). The unambiguous difference between sham-CHI and CHI needing resuscitation drastically simplified the assessment of drug efficacy using the active place avoidance task.

Titration of the MINO dose in the CHI model

Mice received three injections, 1 hour, 1 and 2 days after injury, of MINO (2.5, 10, 22.5 or 45 mg/kg) combined with a fixed dose of NAC (150mg/kg). A second set of injured mice received the same schedule of three saline injections. A control group received sham-CHI and saline injections. One week after injury, behavior was assessed with open field and active place avoidance as described in the detailed methods section. Performance of the sham and injured groups did not differ on open field and passive place avoidance (data not shown). This suggests that that CHI did not produce long-lasting motor or sensory deficits. Performance on the active place avoidance task as measured by shock zone entrances result from differences in cognition and memory rather than sensory or motor deficits. On the active place avoidance task, there was a significant group effect on the number of entrances into a shock zone of MINO dose (ANOVA, $F_{5,33} = 5.63$, $p < 0.001$). The rank order of entrances was shown to be Sham-CHI, saline = CHI, MINO 10 = CHI, MINO 22.5 < CHI, MINO 45 < CHI, Saline ($p < 0.05$, Student Neuman Keuls Post-Hoc). Surprisingly, the number of entrances for two of the MINO doses tested (10 and 22.5 mg/kg) did not significantly differ from sham-CHI. This suggests that the injured mice treated with MINO (10 or 22.5mg/kg) plus NAC (150 mg/kg) had similar performance to uninjured mice. It is extremely unusual for a drug or drug combination to completely reverse cognitive deficits after experimental TBI. This attests to the high potency of the drug combination. The titration of MINO is largely completed with the optimum dose laying between 22.5 and 10mg/kg.

The dosing of MINO also revealed the lowest toxic dose when combined with NAC. Administration of MINO (90mg/kg) with NAC (150mg/kg) was fatal to 7 out of 10 mice in the first week after CHI injury. The 3 mice that did not die, however, had significantly improved performance on the active place avoidance task (40.3 ± 3.4 entrances). The applicant measured a therapeutic effect without toxicity when rats were dosed with MINO (90 mg/kg) plus NAC (150 mg/kg) in the CCI model of TBI. Administration of MINO (90 mg/kg) plus (NAC 150 mg/kg) to rats receiving moderate CCI improved performance on the active place avoidance task without any toxicity (Fig. 2).

MINO plus NAC protects white matter in the CHI model

Fourteen days after CHI or sham-CHI, sagittal brain sections were prepared and stained with luxol fast blue. The width of the corpus callosum was measured in seven locations equally spaced across the structure (Fig. 5). Only injured mice needing resuscitation were compared to mice receiving sham-CHI. CHI induced white matter damage since the CHI, saline group showed a strong trend toward a significantly thinner corpus callosum than the sham-CHI group. MINO (45mg/kg) plus NAC (150mg/kg) prevented this damage suggesting that brains treated with the drug combination show histological improvement. This study provides the justification for the additional histological studies in tasks 1 and 2 to optimize dosing and time window of MINO plus NAC. In all the studies of MINO plus NAC performed by the applicant, histological improvement in white matter have always tracked with behavioral improvements.

CHI produces long-term deficits in the active place avoidance task.

Animal models that show persistent behavioral deficits are needed to development of drugs to treat TBI since drugs can be shown to reverse deficits. Many drugs that have failed clinical trials

have only been shown to increase the rate of improvement that would occur regardless if the drug were present. Mice received either sham-CHI or CHI that required resuscitation after injury. One month later, the mice were tested on an active place avoidance task. The injured mice had significantly more entrances than sham-injured mice ($t_{11} = -2.21$, $*p < 0.05$) (Figure 6). These data suggest that long-term deficits arise from CHI.

MINO plus NAC improves cerebral circulation after CHI

After one month, the sham-CHI or CHI mice were placed into deep anesthesia, and transcardially perfused with phosphate-buffered saline to exsanguinate the brain. The brains were then transcardially fixed with 4% paraformaldehyde. The brains were removed from the skull and photographed. The brains of sham-CHI mice or mice of CHI without resuscitation showed efficient exsanguination as seen by removal of vascular blood from brain regions. In contrast, injured mice that needed resuscitation showed abnormal exsanguination from the rostral brain and the cerebellum. This abnormal exsanguination was not seen in a second group of injured mice treated with MINO 45mg/kg plus NAC 150 mg/kg. There are many potential reasons for this effect, but it suggests either a direct or indirect effect of the drug combination on the brain vasculature.

Key research accomplishments

MINO plus NAC has been shown to be effective in two animal models of TBI - controlled cortical impact and closed head impact.

MINO (90 mg/kg) plus NAC (150 mg/kg) is effective and safe in rats but can be lethal to mice. MINO 90 mg/kg is no more effective than MINO 45 mg/kg when combined with NAC 150 mg/kg in the CCI model of TBI in rats.

The optimum MINO dose combined with NAC 150mg/kg likely lays between 22.5 and 10 mg/kg.

The applicant has developed a CHI model in which a single impact causes long lasting behavioral deficits in the active place avoidance task similar to those seen after moderate CCI (Abdel-Baki, et al., 2010).

The majority of mice receiving CHI develop a fatal apnea unless they receive cardiopulmonary resuscitation. The mice that develop long-lasting apnea develop behavioral deficits while the mice that have transient apnea have no behavioral deficits. This is one of the only closed head models in which behavioral and histological damage can be induced by a single blow.

The brains of mice that need resuscitation have long-term retention of blood in the rostral portions of the cortex and the cerebellum. This suggests a long-term impairment in the blood brain barrier.

Reportable outcomes

Dr. Bergold presented a portion of this work at the 2012 National Neurotrauma Society Meeting in Phoenix, AZ 7/22/2012-7/25/2012.

Ms. Margalit Haber, a graduate student of Dr. Bergold's presented a portion of this work at the 2012 Society for Neuroscience Annual Meeting in New Orleans, LA 10/13/2012-10/17/2012

Ms. Margalit Haber, a graduate student of Dr. Bergold's presented a portion of this work at the 2013 National Neurotrauma Meeting in Nashville, TN 8/4/2013- 8/7.

A portion of this work has been published:

Margalit Haber, BS; Samah G Abdul-Baki, MD; Natalia M Grin'kina, MD, PhD; Rachel Irizarry, BS; Alina Ershova, BS; Sara Orsi, BS; Raymond J Grill, Ph.D.; Pramod Dash, PhD; Peter J Bergold, PhD; Minocycline plus N-acetylcysteine synergize to modulate inflammation and prevent cognitive and memory deficits in a rat model of mild traumatic brain injury." *Exp Neurol.* 249:169-77. 2013. A copy of this paper is included in the appendix.

Conclusions

MINO plus NAC improves cognition in the CCI and CHI models of TBI.

MINO plus NAC limits white matter injury in both the CCI and CHI models of TBI.

MINO (90mg/kg) plus NAC is tolerated by rats but is toxic to mice.

The CHI model produces two levels of injury that correlates with the need for an immediate need for resuscitation after injury.

The optimum MINO dose is lays between 22.5 and 10mg/kg. These doses improve performance in the active avoidance task to levels similar to sham-injured animals.

The CHI produces persistent vascular impairment that can be reversed by MINO plus NAC treatment.

References

1. Abdel Baki, S.G., et al., Minocycline synergizes with N-acetylcysteine and improves cognition and memory following traumatic brain injury in rats. *PLoS One*, 5: e12490. 2010.
2. Abdel Baki, S.G., et al., A hierarchy of neurobehavioral tasks discriminates between mild and moderate brain injury in rats. *Brain Res.* 1280: 98-106. 2009.
3. Cole, et al., Craniotomy: True Sham for Traumatic Brain Injury, or a Sham of a Sham? *J. Neurotrauma* 28: 359-369 2011.
4. Xiong, Y., A. Mahmood, and M. Chopp, Animal models of traumatic brain injury. *Nat Rev Neurosci.* 14: 128-42. 2013.

Appendix

Figures

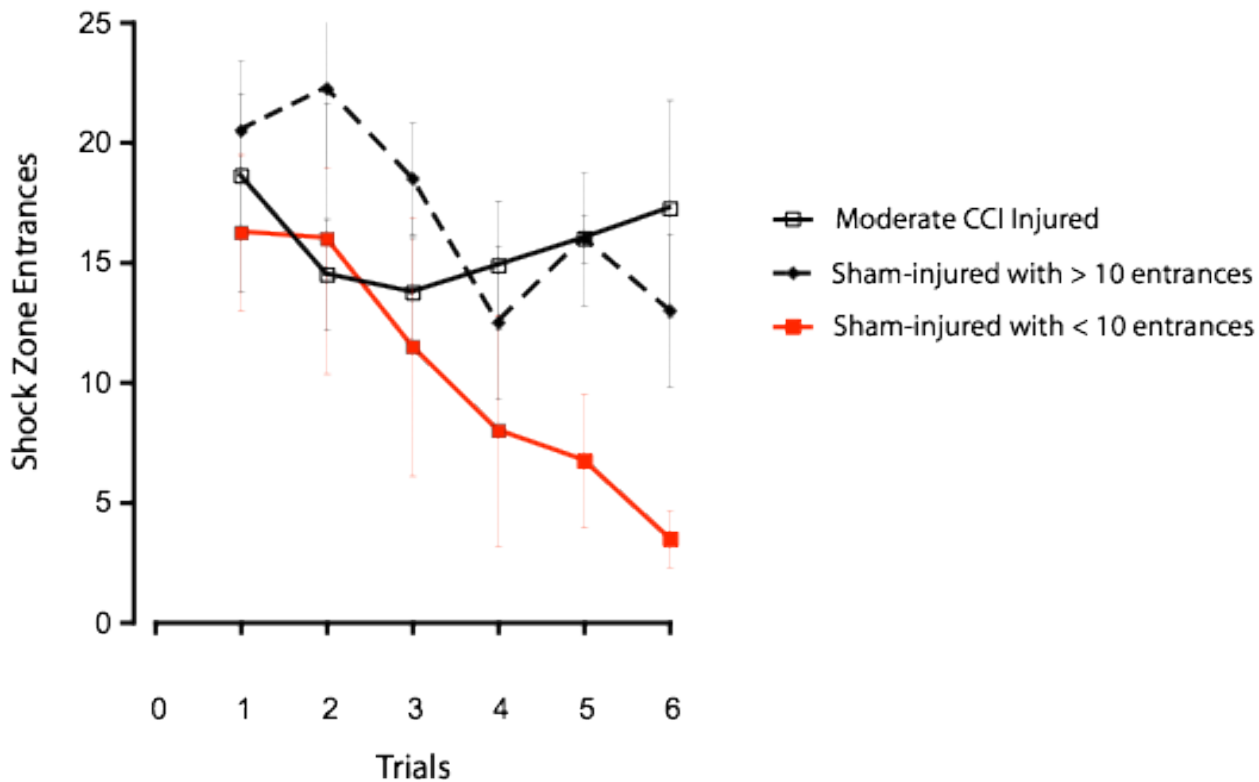


Figure 1 Two groups of sham-injured rats that have difference performance on the active avoidance task. Rats received either sham-CCI, consisting of a craniotomy; or moderate CCI. Rats were returned on their home cages for one week. They were then tested on open field and passive place avoidance. The following day the rats received six ten-minute trials of active place avoidance and the number of entrances in the shock zone assessed. The sham-injured group respond as a uniform group to the task was subdivided into two based on the number of entrances. The moderately injured group did not decrease the number of entrances during the six trials since there was no significant effect of trial ($F_{(5,35)}=0.87$, $p > 0.5$). This suggested that the injured rats could not acquire the task. In the sham-injured group, four out of 10 of the sham-injured rats reduced the number of shock-zone entrances to fewer than 5 entrances on trial 6; the remaining 6 rats had more than 10 shock zone entrances on trial 6. Thus, this sham-injured group was further subdivided into two groups (sham, >10; sham, >5) based upon the number of trial 6 shock-zone entrances. There were significant effects of trial in both groups (<10, ANOVA, $F_{(5,15)} = 5.0188$, $p < 0.01$; >10, $F_{(4,25)}=3.401$, $p < 0.02$) This suggests that both sham groups could acquire the task. Surprisingly, there was a significant effect of treatment among the three groups ($F_{(5,75)} = 2.848$, $p < 0.02$) suggesting that the two sham subgroups differed in their ability to acquire the active avoidance task. These data suggest that the craniotomy used in the CCI model damages the brain in some of the sham-CCI rats.

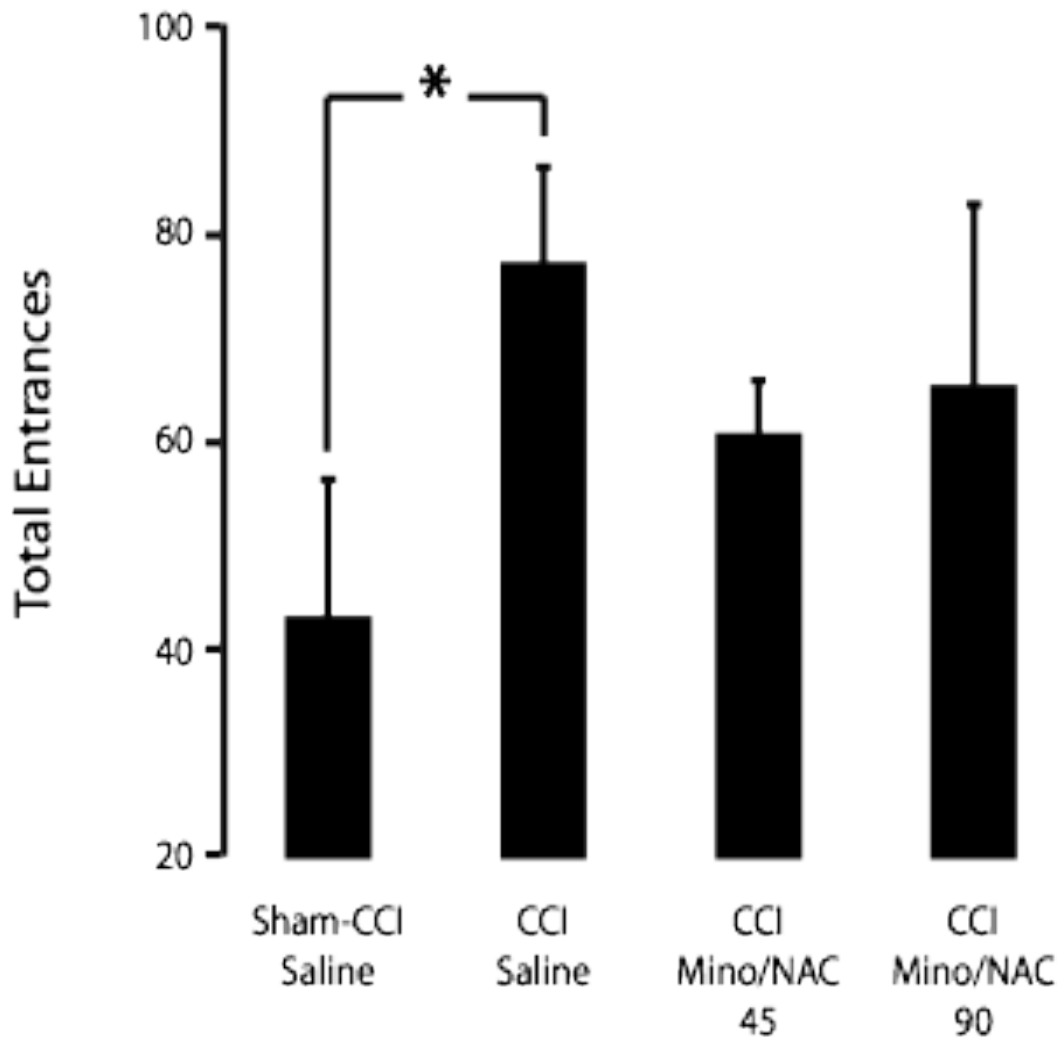


Figure 2 MINO plus NAC improves cognition after moderate CCI Four groups of rats were tested: sham-CCI, saline; moderate CCI, saline; moderate CCI, MINO (45mg/kg) NAC (150mg/kg), and moderate CCI, MINO (90mg/kg) NAC (150 mg/kg). There were four rats per group except for sham-CCI which had 5 rats. Rats received either sham- or moderate-CCI. The rats were returned to their home cages and dosed with drugs 1 hour, 1 and 2 days after surgery. Seven days after surgery, rats received 2 days of behavioral testing that included six ten-minute trials of active place avoidance. The experimental outcome was total number of entrances into the shock zone (Post-Hoc, * $p < 0.01$, Student Neuman Keuls Post-test).

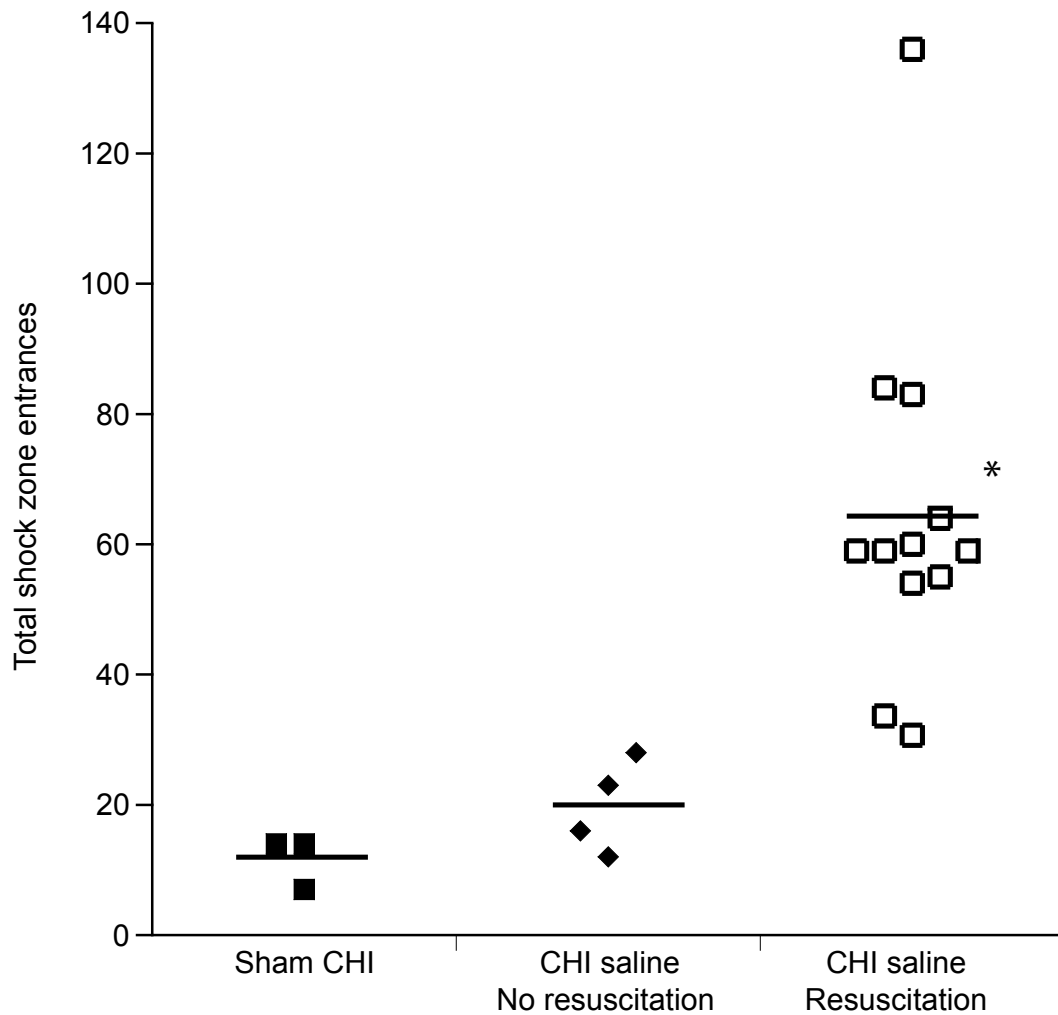


Figure 3 A need for resuscitation predicts subsequent behavioral deficits in the CHI model of TBI.

C57/Bl mice received either a sham-CHI or CHI. Immediately after injury, they could be divided into a group either had no apnea or a transient apnea of < 20 seconds (CHI saline, No resuscitation) or an apnea lasting more than 30 seconds that required cardiopulmonary resuscitation while breathing 100% O₂ (CHI saline, Resuscitation). One week after injury, all groups received behavioral testing including an active place avoidance task. Total number of entrances was assessed. There was a significant group effect (ANOVA, $F_{2,18} = 8.98$, $p < 0.005$) with the injured group needing resuscitation significantly different from Sham-CHI or the injured group needing no resuscitation (Student-Newman-Keuls post-hoc, $*p < 0.01$). These data suggest that post-injury apnea is a surrogate marker of post-injury cognitive deficits.

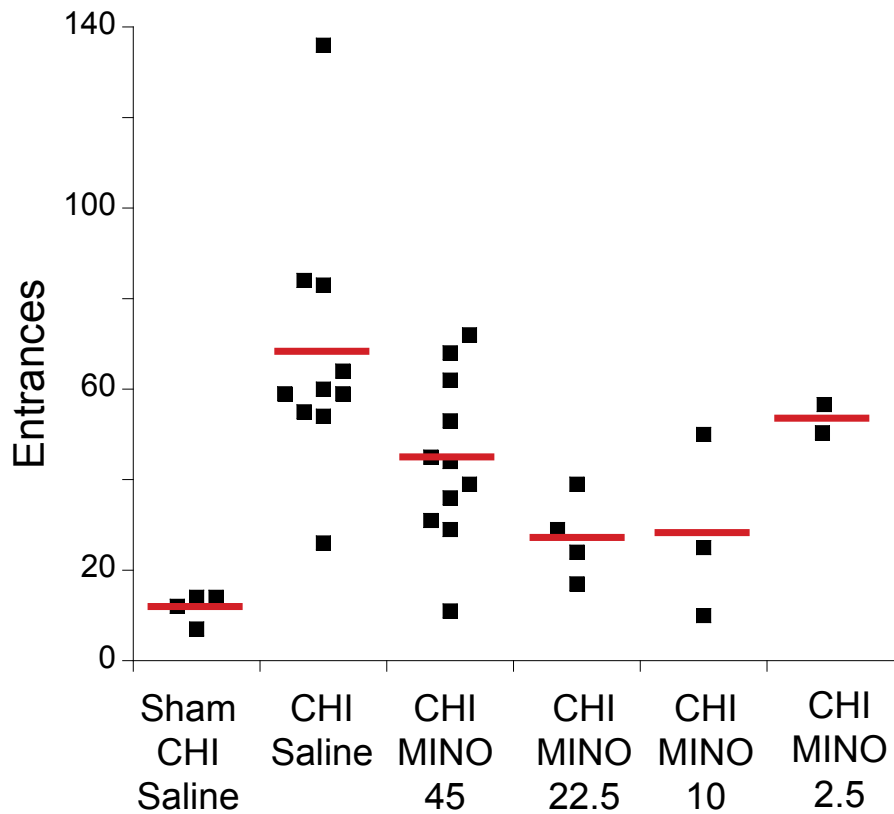
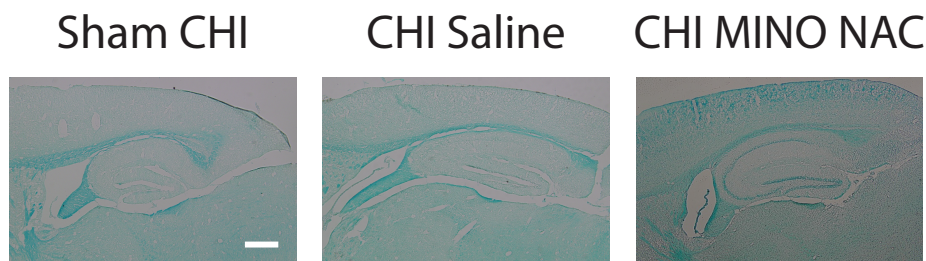


Figure 4 Determination of an optimum MINO dose against a fixed dose of NAC Sham-CHI mice received saline treatment (Sham-CHI, Saline). All injured mice required resuscitation within 30 second after injury. Injured mice received saline (CHI, Saline) or a fixed NAC dose (150mg/kg) with differing doses of MINO (2.5, 10, 22.5, or 45 mg/kg). Drug efficacy was assessed using the number of total shock zone entrances at 7 days after injury. There was a significant effect of treatment (ANOVA, $F_{5,33} = 5.63$, $p < 0.001$). The rank order of entrances was shown to be Sham-CHI, saline = CHI, MINO 10 = CHI, MINO 22.5 < CHI, MINO 45 < CHI, Saline ($p < 0.05$, Student Neuman Keuls Post-Hoc). There were only two mice in the CHI, MINO 2.5 so it was not included in the post-hoc analysis. These data suggest that the optimum MINO dose lies between 22.5 and 10mM.

A) Luxol blue stained sagittal sections



B) Summary

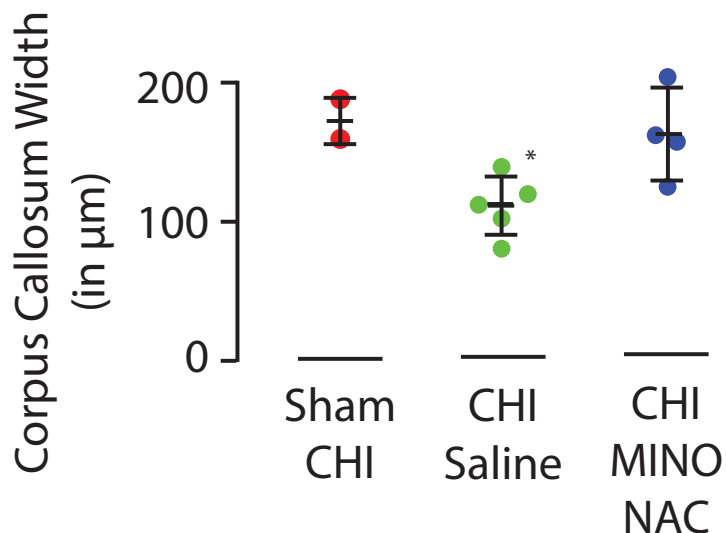


Figure 5 MINO plus NAC limits white matter injury after CHI Mice received either Sham-CHI or CHI. The CHI group was treated with saline or MINO (45mg/kg) plus NAC (150mg/kg). Fourteen days after either sham-CHI or CHI, mice were sacrificed, and stained with luxol fast blue. Panel A, Representative micrographs of sagittal sections of hippocampus, corpus callosum and cortex. The width of the corpus callosum was assessed in parasagittal sections by measuring its width in 7 equally spaced locations. Panel B, Summary of the corpus callosum thickness. There was a group effect on the thickness of the corpus callosum (ANOVA, $F_{2,9} = 4.82$, $p < 0.05$). CHI saline strongly trended to decrease thickness as compared to Sham-CHI (Student Newman Keuls, $p = 0.07$). CHI treated with MINO (45 mg/kg) plus NAC (150 mg/kg) was significantly different than CHI, saline (Post-hoc, $p < 0.05$) but not from Sham-CHI ($p > 0.5$). Data is shown as a scatter plot $\pm$ SEM. Scale bar, 500 μm .

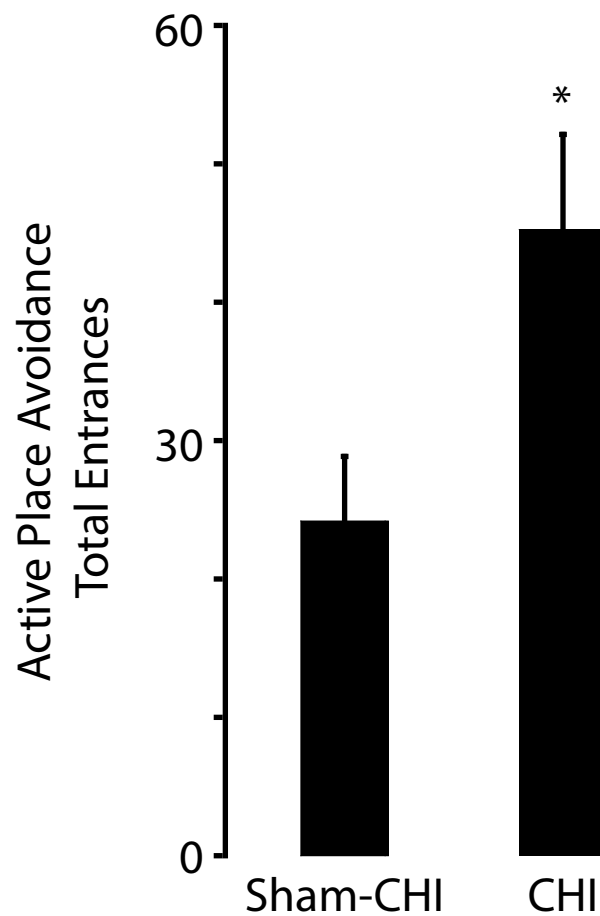
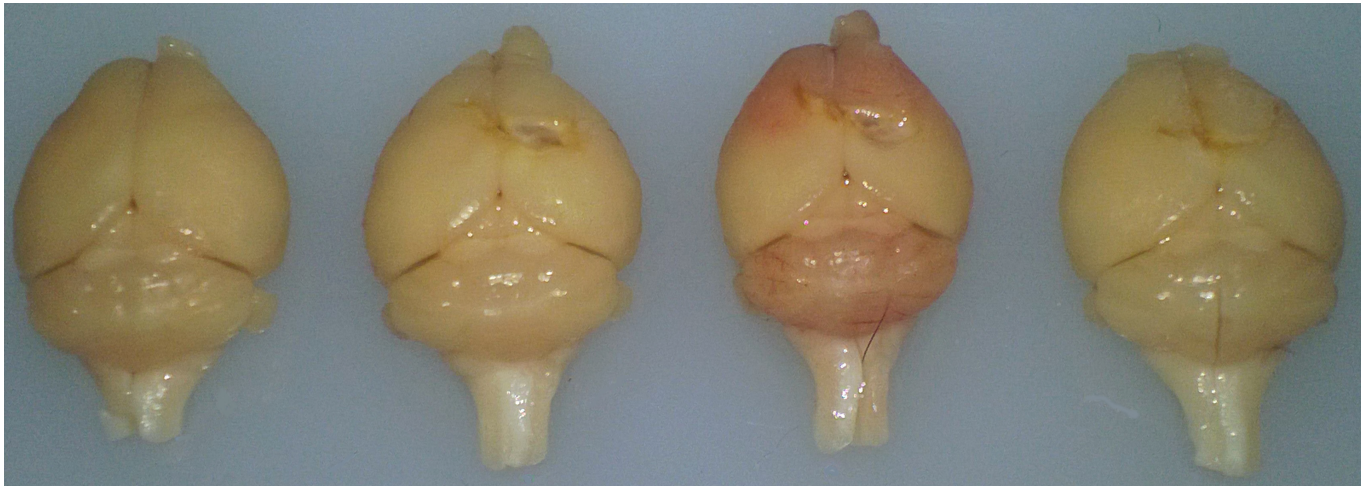


Figure 6 Mice remain impaired one month after closed head injury. Mice received either Sham-CHI (n=5) or CHI (n=8). All mice receiving sham-CHI spontaneously began breathing while all those receiving CHI required resuscitation within 30 seconds after injury. All mice were returned to their home cages for one month before tested on the active place avoidance task. Mice receiving CHI had significantly more shock zone entrances than those receiving sham-CHI ($t_{11} = -2.21$, $*p < 0.05$). These data suggest that long-term deficits arise from CHI. These data also show the feasibility of testing whether the drug combination of MINO plus NAC produce long-term improvements after CHI.



Sham-CCI
Saline

CCI
Saline
No resuscitation

CCI
Saline
Resuscitation

CCI
MINO plus NAC
Resuscitation

One month after injury

Figure 7 External Anatomy of the Brain after Sham-CHI or CHI Mice received either Sham-CHI or CHI. Approximately 25% of the mice spontaneously began breathing within 30 seconds after CHI; the remainder required resuscitation. Brains receiving sham-CHI, or CHI without resuscitation were able to be exsanguinated. In contrast, the brains of injured mice that needed resuscitation retained blood in the rostral portion of the cortex and cerebellum suggesting impairments in the brain vasculature. Injured mice treated with MINO plus NAC did not show retained blood suggesting a vasculature that can be exsanguinated. Similar results were observed in three brains from each group.



Minocycline plus N-acetylcysteine synergize to modulate inflammation and prevent cognitive and memory deficits in a rat model of mild traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) differs in severity from severe to mild. This study examined whether a combination of the drugs minocycline (MINO) plus N-acetylcysteine (NAC) produces behavioral and histological improvements in a mild version of the controlled cortical impact model of TBI (mCCI). Following mCCI, rats acquired an active place avoidance task by learning the location of a stationary shock zone on a rotating arena. Rats acquired this task with a training protocol using a 10-minute intertrial interval. Mildly injured rats had an apparent deficit in long-term memory since they did not acquire the task when the intertrial interval was increased to 24 h. Mildly injured rats also had an apparent deficit in set shifting since, after successfully learning one shock zone location they did not learn the location of a second shock zone. MINO plus NAC synergistically limited these behavioral deficits in long-term memory and set shifting.

mCCI also produced neuroinflammation at the impact site and at distal white matter tracts including the corpus callosum. At the impact site, MINO plus NAC attenuated CD68-expressing phagocytic microglia without altering neutrophil infiltration or astrocyte activation. The drugs had no effect on astrocyte activation in the corpus callosum or hippocampus. In the corpus callosum, MINO plus NAC decreased CD68 expression yet increased overall microglial activation as measured by Iba-1. MINO plus NAC acted synergistically to increase Iba-1 expression since MINO alone suppressed expression and NAC alone had no effect. Despite the known anti-inflammatory actions of the individual drugs, MINO plus NAC appeared to modulate, rather than suppress neuroinflammation. This modulation of neuroinflammation may underlie the synergistic improvement in memory and set-shifting by the drug combination after mCCI.

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Introduction

Approximately one million mild traumatic brain injury (TBI) cases occur in America (Glasgow Coma Score 13–15) (Faul et al., 2010). Other than palliative care, there are no treatments for mild TBI (Loane and Faden, 2010). The drug combination of minocycline (MINO) plus N-acetylcysteine (NAC) was selected by their ability to synergistically improve acquisition and long-term retention on an active place avoidance task in the moderate controlled cortical impact model of TBI (Abdel Baki et al., 2010). Rats treated with MINO alone could acquire active place avoidance, but had no 24-hour retention. NAC had no effect on acquisition; yet restored 24-hour task retention when combined

with MINO (Abdel Baki et al., 2010). The controlled cortical impact (CCI) method, however, models both moderate and mild TBI (Abdel Baki et al., 2009; Yu et al., 2009). Mild CCI produces deficits in set-shifting, in which a rat cannot learn a second shock zone location (Abdel Baki et al., 2009). Thus, this study addresses the important question of whether MINO plus NAC limits behavioral deficits produced by CCI.

Rats or mice injured by moderate CCI exhibit a rapid and large loss of gray and white matter at the proximal impact site as well as a more delayed white and gray matter damage distal to the impact site (Dixon et al., 1991; Hall et al., 2008). This suggests that moderate CCI that was originally thought to be a model of focal injury damages the brain at a distance from the impact site. The histological consequences of mild CCI are not as well characterized but studies using diffusion tensor magnetic image resonance imaging have shown that TBI selectively damages longitudinal and midline white matter tracts (Niogi and Mukherjee, 2010).

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As single drugs, minocycline (MINO) and N-acetylcysteine (NAC) have shown efficacy in a variety of animal models of brain disease (Ramon Diaz-Arrastia et al., 2013). MINO is sufficiently lipophilic to cross the blood brain barrier. In addition to its anti-microbial action, MINO has a variety of anti-inflammatory, anti-apoptotic and antioxidant activities (Kim and Suh, 2009). MINO inhibits a variety of pro-inflammatory enzymes that are associated with brain injury including poly-ADP ribose polymerase-1, cyclooxygenase 2, inducible nitric oxide synthase, metalloproteinases and NADP oxidase. MINO also decreases the expression of interleukin 1 β , interleukin 6, tumor necrosis factor α , macrophage inflammatory protein 1 α , interferon-inducible protein 10, and the chemokine receptors CCR5, and CXCR3. After injury, the major effect of MINO is suppression of microglial activation (Kim and Suh, 2009). In vitro studies have suggested that MINO acts directly on microglia (Kobayashi et al., 2013).

NAC treatment reduces a variety of neurological symptoms when dosed within 24 h after blast TBI suggesting its efficacy as a single drug (Hoffer et al., 2013). NAC is a potent antioxidant that is used clinically to treat acetaminophen overdose and as a mucolytic to treat cystic fibrosis (Ramon Diaz-Arrastia et al., 2013). NAC also modulates glutamatergic neurotransmission (Bridges et al., 2012). In animal models of TBI, NAC has shown anti-inflammatory activity by reducing levels of interleukin 1 β and tumor necrosis factor α (Chen et al., 2008; Hicdonmez et al., 2006). It is not yet clear if these anti-inflammatory effects are a direct or indirect effect of NAC.

In this study, we examined changes in behavior and neuro-inflammation following mild CCI (mCCI). MINO plus NAC synergistically limits behavioral deficits when dosing began 1 h following mCCI. The drugs retained efficacy when dosed 24 h after injury. The effect of the drug combination on neutrophils, astrocytes and microglia was also examined.

Materials and methods

Production of mild CCI

Sprague–Dawley rats (250–300 g, Charles River, Wilmington, MA) were anesthetized using isoflurane (3–5%) in oxygen (0.8 L/min) and a unilateral craniotomy (5.0 mm) was made midway between lambda and bregma. Mild CCI was produced using a 3.0 mm diameter tip with a velocity of 4 m/s and depth of 1.5 mm (Leica, St. Louis, MO). Rats received three intraperitoneal injections of MINO (45 mg/kg) in NaPO₄ (10 mM), NaCl (138 mM) and KCl (2.7 mM); NAC (150 mg/kg) in physiological saline (0.9% NaCl (w/v)); saline alone; or MINO plus NAC in saline 1 h and 1 and 2 days after surgery. The highly hydrophobic MINO readily dissolves into aqueous buffers when combined with the weak acid NAC (150 mM), pH 1.25. Therefore, all drugs were prepared in weakly acidic conditions. All drugs were from Sigma (St. Louis, MO). The Institutional Animal Care and Use Committee at the State University of New York–Downstate Medical Center has approved this study (Protocol ID: 08-877-10).

Behavioral analysis

Rats were tested on four behavioral tasks described in detail in Abdel Baki et al. (2009). The first three tasks of the four were open field, followed by passive place avoidance, and then either the massed or spaced versions of active place avoidance. Rats performed similarly on the first two tasks ensuring similar sensory and motor skills (Table 1). Thus, cognitive differences were responsible for differences in performance in the subsequent tasks. Active place avoidance involves segregating relevant from irrelevant sensory cues. Conflict active place avoidance also involves set-shifting, segregating relevant and irrelevant memories (Abdel Baki et al., 2009). These tasks place higher cognitive demand on the rat than more commonly used rodent behavioral tasks (Abdel Baki et al., 2009). Massed and spaced active place avoidance

uses different intertrial intervals and tests either short-term or long-term memory (Figs. 1A,B). If a rat could acquire massed active place avoidance, they were tested on the fourth test, conflict active place avoidance. Testing began seven days after surgery in a circular arena with a transparent wall that allowed the rat to view prominent visual room cues. The open-field test was done on a stationary arena and no shock zone. This task habituated the rats to the arena and assessed gross motor deficits. Total distance traveled was assessed. Immediately after open-field testing, rats were tested on the passive place avoidance test where a 60° shock zone was added to the stationary arena. Total distance traveled and shock zone entrances were assessed. The following day, rats were tested on an active place avoidance task using a massed or spaced training protocol. Both massed or spaced active place avoidance used the same apparatus as before except the arena rotated at 1 rpm while a 60° shock zone remained stationary. In the massed version of active place avoidance, rats received six 10-minute trials with a 10-minute intertrial interval. Shock zone entrances on all trials were measured. Speed, linearity, shocks/entrance and time to first entrance were assessed on the final trial. If a rat could learn massed active place avoidance, the next day, the rat received conflict active place avoidance. Conflict training which assesses set-shifting was identical to massed active place avoidance except the shock zone was shifted 180°. Shock zone entrances were assessed. Spaced active place avoidance was the alternative to massed training. In spaced training, rats received identical open field and passive place avoidance on day 7. Beginning on day 8 after surgery, rats were tested on spaced active place avoidance with the same rotating arena and stationary shock zone as massed active place avoidance, except rats received a single 20-minute training session daily for 15 days. Shock zone entries and time to first entrance were assessed. Speed, linearity, shocks/entrance and time to first entrance were assessed on the fifteenth trial.

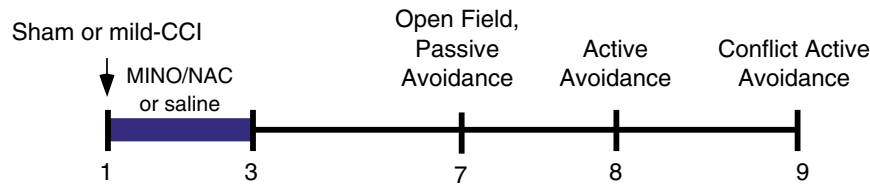
Statistical analysis

Behavioral parameters were analyzed using 2-way repeated-measures ANOVA by comparing treatment of groups across trials. A primary goal was to measure asymptotic behavior differences between groups. Therefore, 1-way ANOVA planned comparisons evaluated group differences for conflict active place avoidance on the 12th trial, the final massed trial; and for spaced active place avoidance on the 15th trial, the final spaced trial. Immunoblots, immunohistochemistry and immunofluorescence were analyzed by 1-way ANOVA. When appropriate, group differences were compared by Student–Newman–Keuls post-hoc tests. Student's *t* test was used to examine group differences for long-term behavioral deficits. Statistical significance was set at 0.05.

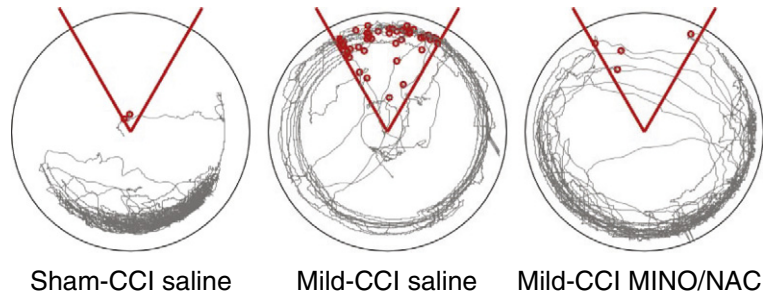
Immunofluorescence and immunohistochemistry

Serial frozen sections of brain, cut in the coronal or sagittal plane, were examined from samples collected 2 days after sham or mild injury and were immunolabeled with antibodies recognizing antigenic markers of microglia. Iba-1 (Abcam) is a pan-selective marker of microglia. Microglial activation is accompanied by increased Iba-1 expression (27). CD68 (Abcam) immunoreactivity increases in phagocytic microglia (Ramprasad et al., 1996). Initially coronal sections were prepared, later studies used parasagittal sections that allowed for simultaneous assessment of more white matter regions. Percent fluorescent area was measured using NIH image J software (Inman et al., 2005). Infiltrating neutrophils were identified via myeloperoxidase (MPO) (DAKO) immunolabeling. MPO-immunoreactive cells were visualized using diaminobenzidine as a chromagen and were imaged under bright field conditions. Bright field and fluorescence micrographs were obtained on Olympus BX-51 microscope with an Olympus DP70 camera.

A) Experimental Design



B) Representative tracks from trial 14



C) Summary of active and conflict active place avoidance

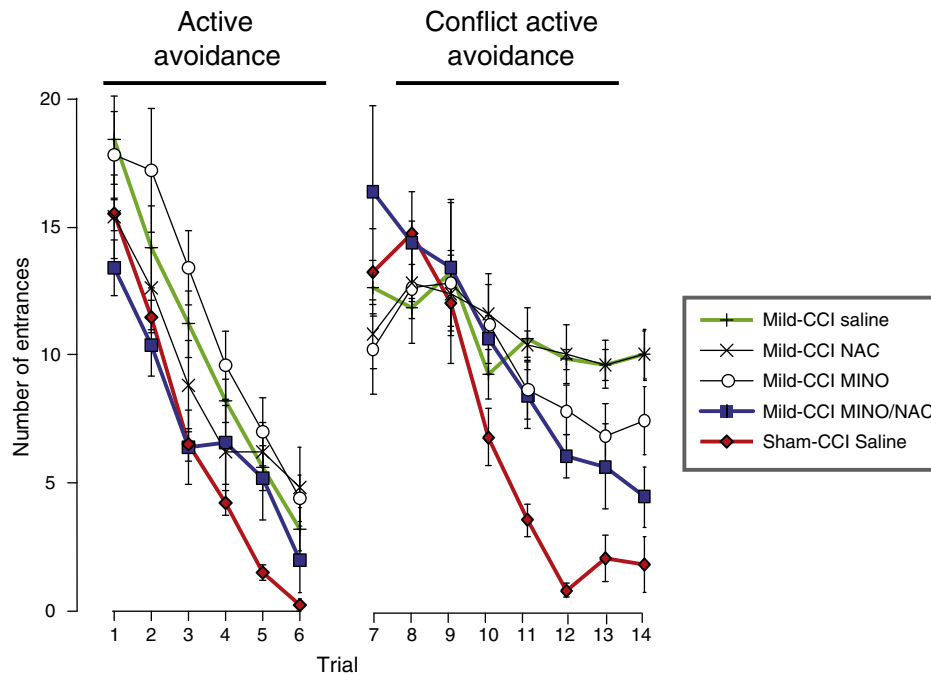


Fig. 1. MINO plus NAC improve performance on the conflict active place avoidance task. Panel A, Experimental design. Rats were subjected to mCCI or sham-mCCI. Rats received three injections of saline, MINO, NAC, or MINO plus NAC 1 h, 1 day and 2 days after surgery. Seven days after surgery, all groups were tested on open field and passive place avoidance (Table 1). The following 2 days, all groups were tested on active place avoidance and conflict active place avoidance. Panel B, Representative tracks of rats on the 14th and final trial of conflict active place avoidance. Panel C, Summary of conflict active place avoidance. Injured animals treated with MINO plus NAC had fewer entrances than those treated with either MINO or NAC.

Protein extraction and immunoblot analysis

Rats were deeply anesthetized with isoflurane at various times after sham- or mild-CCI. The head was removed and the brain rapidly extracted. The corpus callosum and hippocampus were isolated and the tissue was snap frozen on dry ice. Proteins in both gray and white matter were extracted in Tris-HCl (40 mM, pH 7.4), Urea (5 M), SDS (2% (w/v)), 2-mercaptoethanol (1% (v/v)), phenylmethylsulfonyl fluoride (0.1 mM), benzamide (6.5 mM), aprotinin (5 μ M), and leupeptin (0.11 mM).

Extracts (2 μ g) were electrophoresed on 15% acrylamide gels and transferred to a nitrocellulose membrane. Protein bands immunoreactive to glial fibrillary acidic protein (GFAP, Abcam) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore) were detected using appropriate secondary antibodies conjugated to alkaline phosphatase (Sigma). GAPDH immunoreactivity provided a control for the amount of protein loaded. The intensity of immunoreactive bands of the appropriate molecular weight was measured using NIH image J and presented as the ratio of the intensity of GFAP to GAPDH.

Results

mCCI produced cognitive and memory deficits that are limited by MINO plus NAC

Five groups (Sham-CCI, saline; mCCI, saline; mCCI, MINO; mCCI, NAC; mCCI, MINO plus NAC) tested whether MINO plus NAC improved acquisition of the conflict active place avoidance task. Beginning 7 days after sham-CCI or mCCI, rats were tested in open field, passive place avoidance, massed active place avoidance, and conflict active place avoidance (Abdel Baki et al., 2009). There was no significant difference in the total distance traveled on open-field testing, distance traveled or shock zone entrances in passive place avoidance (Table 1). These results suggest that all groups retained similar motor ability as well as a similar ability to sense and avoid shock and to initiate and inhibit exploration. Eight days after surgery, all groups were tested on a massed active place avoidance task (Fig. 1A). All groups showed a similar ability to avoid the shock zone since there was a significant effect of trial (ANOVA, $F_{5,125} = 196.59$, $p < 0.001$) but not group (ANOVA, $F_{4,25} = 3.83$, $p > 0.1$) and no interaction of group and trial (ANOVA, $F_{5,50} = 2.03$, $p > 0.1$). On day 9, the rats received conflict active place avoidance testing with the shock zone shifted 180° from the previous day's location (Fig. 1A). On the final trial of conflict active place avoidance, sham-CCI saline-treated rats avoided the shock zone while mildly injured saline-treated rats had repeated entrances (Fig. 1B). Treatment with MINO plus NAC resulted in fewer shock zone entrances suggesting that treated rats learned the location of the new shock zone (Fig. 1B). Some groups reduced the number of entrances across trials since there was a significant effect of group (ANOVA, $F_{4,25} = 146.43$, $p < 0.01$) and trial (ANOVA, $F_{5,125} = 226.22$, $p < 0.01$), without an interaction between group and trial (ANOVA, $F_{5,50} = 6.89$, $p > 0.05$). On trial 14, the groups had large differences in shock zone entrances with a rank order of: Sham-CCI, saline < mCCI, MINO plus NAC < mCCI, MINO < mCCI, NAC = mCCI, saline (Fig. 1C; post-hoc, $p < 0.05$). These data suggest that MINO plus NAC acts synergistically to improve performance on conflict active place avoidance.

Rats did not acquire the conflict active place avoidance task one month after mild injury

Cognitive deficits in most mTBI patients resolve within 1–2 weeks, the remaining patients have long-lasting deficits (Levin and Robertson, 2013). We therefore examined whether the deficits in conflict active place avoidance were long lasting. Rats received Sham-CCI or mCCI followed by three injections of saline at 1 h, 1 day and 2 days after surgery (Fig. 2A). Twenty-nine days after surgery, sham-CCI saline-treated and mCCI saline-treated rats performed similarly in open field and passive place avoidance (Supplemental Table 1). On the following day, the two groups showed similar learning on massed place avoidance since there was a significant effect of trial, but not of group (ANOVA,

trial, $F_{5,20} = 92.15$, $p < 0.0001$; group, $F_{1,4} = 7.34$, $p > 0.05$) (Fig. 2B). Thirty-one days after surgery, the same groups were tested on conflict active place avoidance. Sham-CCI, saline-treated rats acquired the location of the new shock zone significantly better than injured rats since there was a significant effect of trial and group, with no interaction between group and trial (Fig. 2B) (ANOVA, trial, $F_{5,20} = 23.03$, $p < 0.001$; group, $F_{1,4} = 25.55$, $p > 0.01$, interaction, $F_{5,20} = 0.70$, $p > 0.5$). These data suggest that mildly injured rats are impaired in acquiring conflict active place avoidance one month after mCCI.

MINO plus NAC limited memory deficits after mild CCI

Unlike the massed version, spaced active place avoidance requires 24-hour retention for task acquisition (Abdel Baki et al., 2010). Five groups (Sham-CCI saline; mCCI, saline; mCCI, MINO; mCCI, NAC; mCCI, MINO plus NAC) were prepared. These groups had similar performance on open field and passive place avoidance (Supplemental Table 1). Seven days after surgery, rats were tested on spaced active place avoidance. Some groups reduced the number of entrances across trials since the effects of group (ANOVA, $F_{4,15} = 27.37$, $p < 0.0001$) and trial (ANOVA, $F_{14,350} = 81.13$; $p < 0.01$) were significant, without a group and trial interaction (ANOVA, $F_{56,350} = 2.79$; $p > 0.3$). The rank order of the groups was: sham-CCI, saline < mCCI, MINO plus NAC < mCCI, saline = mCCI, MINO = mCCI, NAC (post-hoc, $p < 0.05$). The remaining groups were unable to lower their shock zone entrances after the fifth trial (Fig. 3B). Time to first entrance was also assessed that measures the ability of the rat to avoid without using a shock to cue latent memories (Abdel Baki et al., 2009). Comparisons among groups were performed on the 15th day since all groups had reached asymptote on the number of entrances while the sham-CCI, saline group reached asymptote on time to first entrance. The effect of group was significant (ANOVA, $F_{4,25} = 41.28$, $p < 0.01$). Sham-CCI, saline had the longest time to first entrance followed by mCCI, MINO plus NAC. mCCI, MINO plus NAC had a far longer time to first entrance than either the mCCI, saline; mCCI, NAC; or mCCI, MINO groups (post-hoc, $p < 0.01$). Analysis of shock zone entrances and time to first entrance data strongly suggest drug synergy since the MINO plus NAC improved performance even though the individual drugs had no effect. The long-lasting improvement in memory was still evident 23 days after mCCI (Fig. 3C).

Microglial activation following mCCI was restricted to the impact site and distal white matter tracts

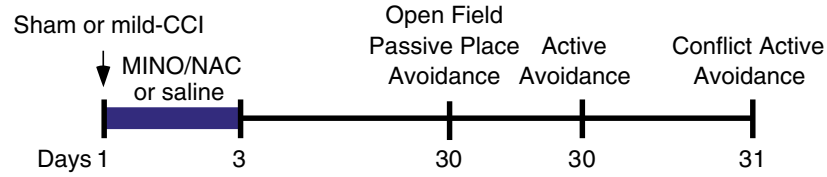
CCI produces injury at impact site as well as distal white and gray matter (Hall et al., 2008). CCI also induces neuroinflammation (Kumar and Loane, 2012). MINO and NAC have anti-inflammatory action; therefore, antigenic markers of neuroinflammation were examined both proximal and distal to the impact site. Five groups of rats were prepared (Sham-CCI, saline; mCCI, saline; mCCI, MINO; mCCI, NAC; mCCI, MINO plus NAC). Two days after sham-injury or injury, parasagittal sections

Table 1
Measurements of rat behavior during massed active place avoidance.

Task	Parameter	Sham-CCI saline	mCCI, saline	mCCI, MINO	mCCI, NAC	mCCI, MINO plus NAC
Open field	Total distance	25.4 ± 1.3	22.8 ± 2.5	29.0 ± 4.3	26.9 ± 0.2	21.5 ± 2.9
Passive place avoidance	Shock zone entrances	14.3 ± 2.9	16.3 ± 3.8	12.5 ± 4.6	15.5 ± 3.2	13.8 ± 2.1
	Total distance	9.7 ± 2.2	12.9 ± 3.5	12.0 ± 3.8	13.9 ± 3.3	13.9 ± 2.3
Active place avoidance	Speed	5.6 ± 0.1	5.3 ± 0.2	5.3 ± 0.3	5.1 ± 0.2	5.4 ± 0.3
	Linearity	0.65 ± 0.12	0.68 ± 0.02	0.68 ± 0.04	0.70 ± 0.02	0.67 ± 0.01
	Shocks per entrance	0.62 ± 0.12**	1.2 ± 0.13	1.2 ± 0.18	1.4 ± 0.08	1.25 ± 0.2
	Time to first entrance	432.7 ± 130.6*	39.3 ± 7.0	33.8 ± 5.8	38.0 ± 9.3	185.8 ± 71.5*

Rats received either sham-CCI or mCCI. Beginning 1 h after surgery, rats received either saline, MINO, NAC or MINO plus NAC. Seven days later all groups were tested on open field and passive place avoidance. On the open field test, there was no significant effect of treatment ($F_{4,29} = 0.59$, $p > 0.5$). On passive place avoidance, there was no effect of treatment on distance traveled ($F_{4,29} = 1.00$, $p > 0.4$) or total shock zone entrances ($F_{4,29} = 0.58$, $p > 0.5$). On active place avoidance, there was no significant effect of treatment on speed and linearity (Speed, $F_{4,29} = 1.7$, $p > 0.1$; Linearity, $F_{4,29} = 2.67$, $p > 0.05$). Shocks per entrance, however, showed a significant effect of treatment with uninjured rats having fewer shocks per entrance than the injured groups ($F_{4,29} = 20.20$, $p < 0.001$, post-hoc, ** $p < 0.001$). There was also a significant effect on time to first entrance with a rank order of Sham-CCI, saline > mCCI, MINO plus NAC > mCCI, MINO = mCCI, NAC = mCCI, saline (post-hoc, * $p < 0.05$).

A) Experimental Design



B) Summary of active and conflict active place avoidance

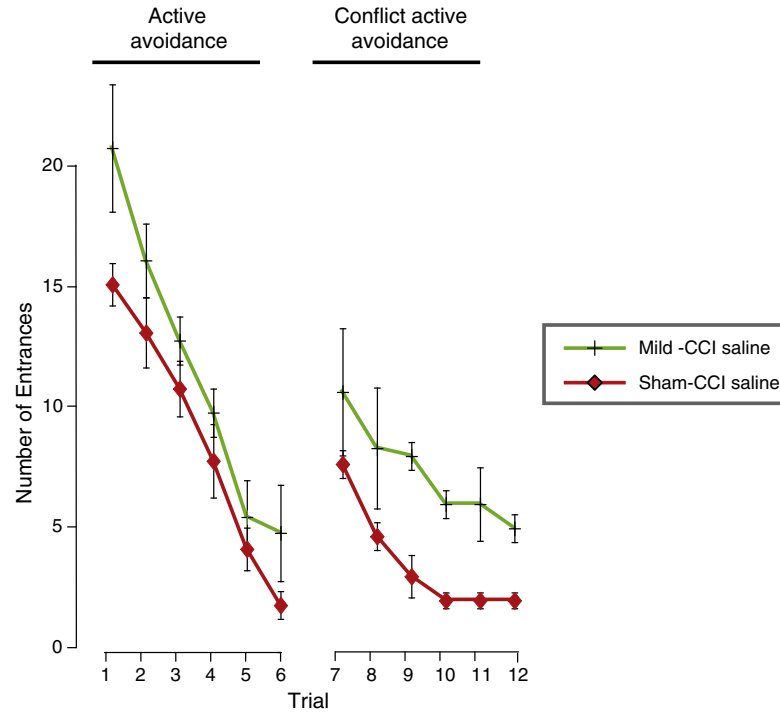


Fig. 2. Long-lasting deficits in conflict place avoidance after mild injury. Panel A, Experimental Design, Sham injured and injured rats received three saline injections 1 h, and 1 and 2 days after surgery. Twenty-nine days after injury rats received a hierarchy of behavioral tasks ending with conflict active place avoidance. Panel B, Summary of shock zone entrances. These data suggest that mCCI produces long-lasting cognitive deficits.

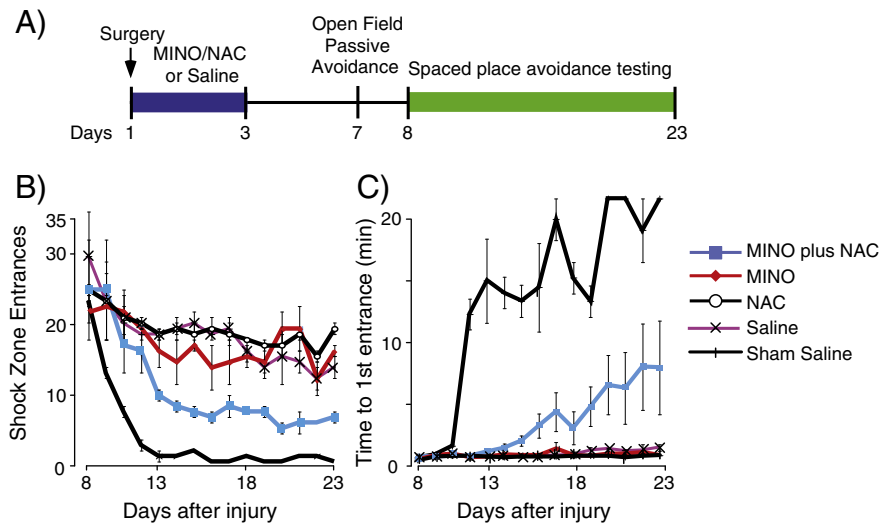


Fig. 3. MINO plus NAC improve performance on the spaced active place avoidance task Panel A, Experimental Design. Rats received either sham-mCCI or mCCI. One hour after surgery, the rats received three injections of either saline, MINO, NAC or MINO plus NAC. Seven days after surgery, all groups were tested on open field and passive place avoidance (Supplemental Table 2). The following day, all groups were tested for 15 days on spaced active place avoidance that consisted of a single 20-minute trial with a 24-hour intertrial interval. Panel B, Summary of shock zone entrances. Panel C, Summary of time to first entrance.

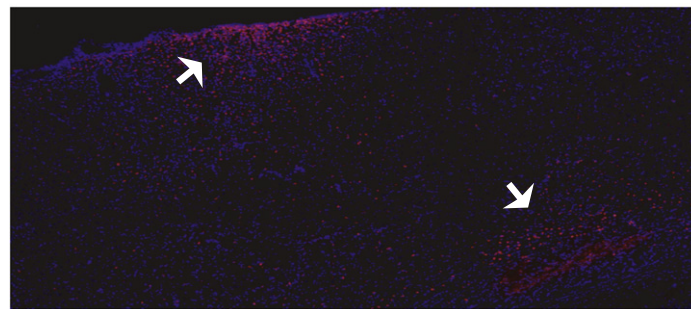
containing the impact site were stained with Iba-1 and CD68; two antigenic markers that increase in activated microglia (Kurushima et al., 2000; Lynch, 2009). Two days post-injury was selected since microglial and astrocyte activation begins within minutes after injury and persists for many days (Li et al., 2009; Suma et al., 2008).

At the impact site, there was a strong effect of treatment on the number of CD68⁺ immunoreactive cells ($F_{2,9} = 21.30$, $p < 0.002$). Few CD68⁺ cells were observed at the impact site of Sham-CCI, saline-treated rats that greatly increased in mCCI, saline-treated rats ($p < 0.01$, post-hoc). MINO plus NAC greatly suppressed the increase in CD68⁺ cells ($p < 0.01$, post-hoc). In the corpus callosum, CD68⁺ immunoreactivity had a significant effect of group ($F_{2,11} = 6.48$, $p < 0.02$).

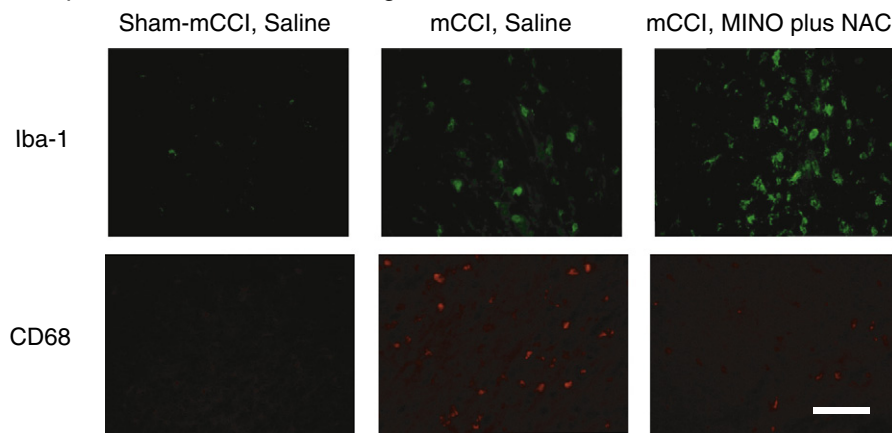
CD68⁺ immunoreactivity was significantly increased in mCCI, saline as compared to sham-CCI, saline or mCCI-MINO plus NAC ($p < 0.02$, post-hoc) (Figs. 4B,C). These data suggest that neuroinflammation differed between the impact site and distal white matter since the drug combination decreased CD68⁺ immunoreactivity in the corpus callosum but not at the impact site.

There was a significant effect of treatment on Iba-1⁺ cells in the corpus callosum (ANOVA, $F_{5,25} = 9.58$, $p < 0.0002$). mCCI significantly increased Iba-1⁺ reactivity as compared to sham-CCI ($p < 0.02$, post-hoc). MINO treatment strongly trended toward decreasing the number of Iba-1⁺ cells in injured rats as saline treatment ($p = 0.06$, post-hoc) while NAC had no effect. Unexpectedly, Iba-1⁺ immunoreactivity in

A) CD68⁺ cells in the cortex and corpus callosum



B) MINO plus NAC modulates microglial activation



C) Summary of modulation of microglia by MINO plus NAC

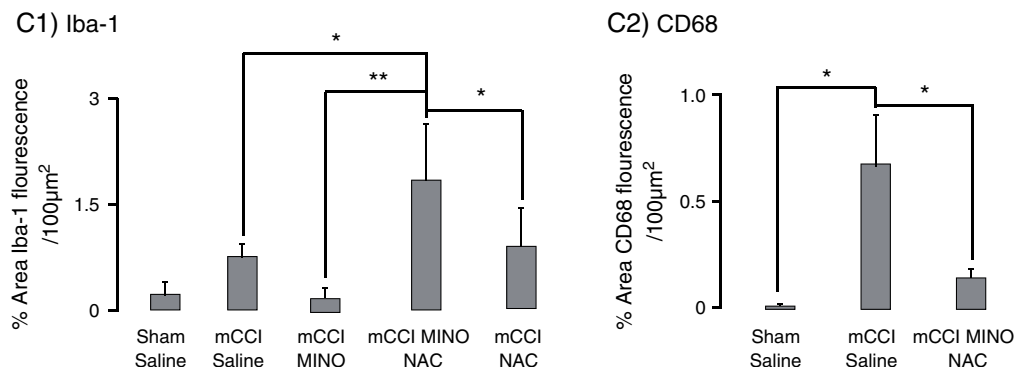


Fig. 4. MINO plus NAC synergistically modulate microglial activation 2 days after mCCI. Panel A, Microglial activation in a representative sagittal section of brain stained for CD68. CD68⁺ cells were found predominantly at the impact site (rightward arrow) and in the cingulum and corpus callosum (leftward arrow). Panel B, Microglia labeled with Iba-1 and CD68 in the corpus callosum of rats receiving sham-CCI, saline; mCCI, saline; or mCCI MINO plus NAC. Panel C1, Summary of changes in Iba-1 immunoreactivity. MINO plus NAC significantly increased Iba-1 immunoreactivity as compared to mildly injured rats treated with saline ($*p < 0.05$), MINO ($**p < 0.002$), or NAC ($*p < 0.05$). Panel C2, Summary of changes in CD68 immunoreactivity. mCCI significantly increased CD68 immunoreactivity that was attenuated by MINO plus NAC ($*p < 0.05$). Scale bar, 20 μm.

MINO plus NAC-treated injured rats was significantly higher than injured rats treated with either saline, MINO or NAC (saline, $p < 0.05$; MINO, $p < 0.002$; NAC, $p < 0.05$, post-hoc). Others have reported a similar decrease by MINO using the marker CD11b (Homsí et al., 2010). MINO plus NAC acted synergistically on Iba-1⁺ cells after mCCI since MINO plus NAC increased Iba-1⁺ immunoreactivity; as individual drugs, MINO decreased Iba-1 immunoreactivity while NAC had no effect (Fig. 4C).

MINO plus NAC does not alter neutrophil infiltration or astrocyte activation

We also examined whether MINO plus NAC modulates astrocyte activation or neutrophil infiltration after mild CCI. Coronal brain sections containing the impact site were prepared from rats 2 days after injury and stained with the neutrophil marker myeloperoxidase (MPO). MPO⁺ cells were observed at the impact site in mCCI, saline-treated but not sham-CCI, saline-treated rats (Supplementary Fig. 1). MPO⁺ stained cells were not observed in brain regions distal from the impact site (data not shown). These data suggest that neutrophils present at the impact site were unaffected by MINO plus NAC. MINO alone has been previously reported to have no effect on neutrophil infiltration (Bye et al., 2007).

At 2 days, mCCI increased GFAP immunoreactivity at the impact site (Fig. 5) and in underlying white matter including corpus callosum since there was a significant group effect of GFAP immunoreactivity at the impact site (ANOVA, $F_{2,7} = 27.81$, $p < 0.005$, $p < 0.001$) (Supplemental Table 3). MINO plus NAC did not alter the increased GFAP expression 2 days after injury (Fig. 5). GFAP immunoreactivity also increased in the corpus callosum 2 days after injury; this increase was also unaffected by MINO plus NAC. At 4, 7, or 14 days after injury, GFAP expression was no longer increased in hippocampus and corpus callosum; MINO plus NAC had no effect at these later time points (Supplemental Table 3).

Discussion

This study shows that mCCI produces long-lasting behavioral and histological impairments that are partially alleviated by MINO plus NAC. Deficits were observed in set shifting as assessed in conflict active

place avoidance, and in long-term memory as tested by spaced active place avoidance. MINO alone improved set-shifting, while NAC had no effect (Fig. 1). The drug combination acted synergistically since combining MINO with NAC significantly improved set-shifting more than MINO alone. The drugs also synergized in improving long-term memory since the drug combination was effective while the individual drugs had no effect (Fig. 2). Moderate CCI produced deficits in long-term memory that were also improved synergistically by MINO plus NAC (Abdel Baki et al., 2010). Siopi, et al. reported that MINO (90 mg/kg) dosed 5 min after injury limited memory deficits in mice on the novel object recognition task (Siopi et al., 2012). In contrast, MINO (45 mg/kg) given 1 h after CCI had no effect on the memory deficit in spaced active place avoidance (Fig. 3). Differences in the behavioral task, the MINO dose or time to first dose may underlie why MINO improved memory in the object recognition task but ineffective in the spaced active place avoidance task.

How MINO improves behavior is unclear, but recent findings about the pharmacology of NAC provides insight into how it may enhance memory. (Figs. 1, 3) (Abdel Baki et al., 2010). In addition to its well-known anti-oxidant action, NAC also modulates glutaminergic neurotransmission. After administration, NAC remains unchanged or is deacylated to cysteine. Cysteine can then be further oxidized to cystine. Cystine enters astrocytes cells via the cystine–glutamate antiporter (system x_c[−]) with a concomitant release of glutamate that alters the size of the excitatory post-synaptic potential (EPSP) (Bridges et al., 2012). NAC or cysteine also enters the brain through cysteine transporters where they enhance cystine uptake by the cystine–glutamate antiporter. Low dose NAC decreases extracellular postsynaptic potential (EPSP); high doses of NAC, such as the one used in this study, increase the EPSP (Kupchik et al., 2012). High dose NAC also elevates extracellular glutamate levels in the hippocampus (De Bundel et al., 2011). The doses of NAC used in this study are sufficiently high to alter glutaminergic neurotransmission (Kupchik et al., 2012). Thus, NAC may restore memory by modulation of glutaminergic neurotransmission (Fig. 3).

mCCI induced neuroinflammation proximally at the impact site and at distal white matter sites. At the impact site, mCCI activated astrocytes, neutrophils and microglia while distal sites showed predominantly microglial activation (Figs. 4, 5 and Supplemental Fig. 1) at the time

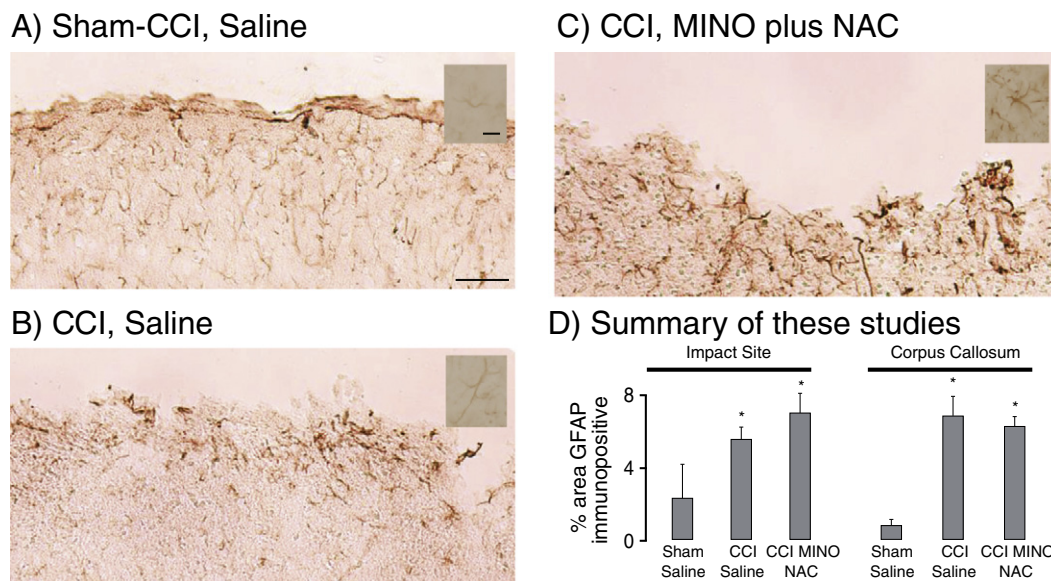


Fig. 5. Activation of astrocytes by mild CCI is unaffected by MINO plus NAC. Representative images of the impact site immunostained for GFAP in rats 2 days after surgery that received: (A) sham-CCI, saline; mCCI, (B) saline or mCCI, or (C) MINO plus NAC. Summary of GFAP immunostaining (D). Insets for each panel show a representative cell showing cell morphology. Scale bar, 10 μ m. At the impact site, mildly injured rats receiving saline or MINO plus NAC had significantly more GFAP immunoreactivity than sham-injured rats receiving saline (** $p < 0.01$, post-hoc). Right, in the corpus callosum, mildly injured rats receiving saline or MINO plus NAC had significantly more GFAP immunoreactivity than sham-injured rats receiving saline. These data suggest that MINO plus NAC had little effect on astrocytes activation (** $p < 0.01$, post-hoc). Scale bar, 100 μ m.

points examined. MINO plus NAC did not appear to influence astrocyte activation or neutrophil infiltration, but profoundly affected microglial activation. Dosing of the drug combination begins 1 h after injury. The lack of an effect on astrocyte activation or neutrophils infiltration may be a result of the drug concentrations chosen in this study and/or the timing of administration. Dosing the drugs after neuroinflammation has been initiated by the injury may alter the anti-inflammatory effects of the drug combination. In contrast, MINO plus NAC acted upon microglia to modulate neuroinflammation. The drug combination had no effect at the impact site while suppressing CD68 immunoreactivity in the corpus callosum. The drug combination also increased Iba-1 immunoreactivity in the corpus callosum in injured rats (Fig. 4). As individual drugs, MINO decreased and NAC had no effect on Iba-1 immunoreactivity. This drug effect could result from altering the activation of resident microglia. An alternative possibility is that the drug combination altered the population of circulating macrophages infiltrating the brain. This possibility is less likely since blood brain barrier was likely intact since no neutrophils were observed in the parenchyma of mildly injured rats.

The ability of the drugs to increase Iba-1 immunoreactivity was unexpected since MINO and NAC have well-known anti-inflammatory actions. MINO suppresses microglial activation in animal models of TBI, spinal cord injury, stroke, Parkinson's disease, Alzheimer's disease, multiple sclerosis and amyelolateral sclerosis (Cai et al., 2010; Kim and Suh, 2009; Kovessi et al., 2012) (Bye et al., 2007). NAC has known antioxidant activity, but it decreases pro-inflammatory cytokine expression after experimental TBI and blocks microglial activation in vitro (Chen et al., 2008; Lijia et al., 2012). Thus, it was unexpected that Iba-1 expression was synergistically increased by MINO plus NAC while CD68 expression was suppressed (Fig. 4).

Upon activation, microglia rapidly differentiate into a variety of forms broadly defined as M1 and M2 (Kumar and Loane, 2012; Yong and Rivest, 2009). M1 microglia release proinflammatory cytokines and produce large amounts of reactive oxygen and reactive nitrogen species. M1 microglia may also phagocytose injured and apoptotic tissue. M2 microglia release anti-inflammatory cytokines leading to repair and remodeling of damaged tissue (Kumar and Loane, 2012). Iba-1 is expressed by both M1 and M2 microglia (Perry et al., 2010). MINO plus NAC modulate microglial activation as seen by increased Iba-1 expression that is concomitant with a decrease in CD68⁺ phagocytic microglia (Fig. 4). Each individual drug can modulate microglial activation. In a mouse model of amyelolateral sclerosis, MINO inhibited differentiation of M1, but not M2, microglia (Kobayashi et al., 2013). Injection of A β 1–42 or lipopolysaccharide into the brain produced a local activation of predominantly M1 microglia that express NADPH oxidase. NAC altered microglial differentiation from the M1 toward the M2 phenotype by scavenging of superoxide anion produced by NADPH oxidase (Choi et al., 2012). Microglial modulation may be an additional site of drug synergy between MINO and NAC due to the many ways that microglial biology is regulated by redox (Brune et al., 2013).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.expneurol.2013.09.002>.

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References

- Abdel Baki, S.G., et al., 2009. A hierarchy of neurobehavioral tasks discriminates between mild and moderate brain injury in rats. *Brain Res.* 1280, 98–106 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19464271).
- Abdel Baki, S.G., et al., 2010. Minocycline synergizes with N-acetylcysteine and improves cognition and memory following traumatic brain injury in rats. *PLoS One* 5 (8), e12490 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20824218).

- Bridges, R., et al., 2012. Thinking outside the cleft to understand synaptic activity: contribution of the cystine–glutamate antiporter (system xc[−]) to normal and pathological glutamatergic signaling. *Pharmacol. Rev.* 64 (3), 780–802 (<http://www.ncbi.nlm.nih.gov/pubmed/22759795>).
- Brune, B., et al., 2013. Redox control of inflammation in macrophages. *Antioxid. Redox Signal.* 19 (6), 595–637 (<http://www.ncbi.nlm.nih.gov/pubmed/23311665>).
- Bye, N., et al., 2007. Transient neuroprotection by minocycline following traumatic brain injury is associated with attenuated microglial activation but no changes in cell apoptosis or neutrophil infiltration. *Exp. Neurol.* 204 (1), 220–233 (<http://www.ncbi.nlm.nih.gov/pubmed/17188268>).
- Cai, Z.Y., Yan, Y., Chen, R., 2010. Minocycline reduces astrocytic reactivation and neuroinflammation in the hippocampus of a vascular cognitive impairment rat model. *Neurosci. Bull.* 26 (1), 28–36 (<http://www.ncbi.nlm.nih.gov/pubmed/20101270>).
- Chen, G., et al., 2008. Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine. *Mediat. Inflamm.* 716458 (<http://www.ncbi.nlm.nih.gov/pubmed/18483565>).
- Choi, S.H., et al., 2012. Inhibition of NADPH oxidase promotes alternative and anti-inflammatory microglial activation during neuroinflammation. *J. Neurochem.* 120 (2), 292–301 (<http://www.ncbi.nlm.nih.gov/pubmed/22050439>).
- De Bundel, D., et al., 2011. Loss of system x(c)–does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. *J. Neurosci.* 31 (15), 5792–5803 (<http://www.ncbi.nlm.nih.gov/pubmed/21490221>).
- Dixon, C.E., et al., 1991. A controlled cortical impact model of traumatic brain injury in the rat. *J. Neurosci. Methods* 39 (3), 253–262 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1787745).
- Faul, M., Xu, L., Wald, M.M., Coronado, V.G., 2002–2006. Traumatic brain injury in the United States: emergency department visits, hospitalizations, and deaths. (Atlanta (GA)). (http://www.cdc.gov/traumaticbraininjury/pdf/blue_book.pdf).
- Hall, E.D., et al., 2008. Evolution of post-traumatic neurodegeneration after controlled cortical impact traumatic brain injury in mice and rats as assessed by the de Olmos silver and fluorojade staining methods. *J. Neurotrauma* 25 (3), 235–247 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18352837).
- Hicdonmez, T., Kanter, M.C., Tiryaki, M., Parsak, T.S., Cobanoglu, S., 2006. Neuroprotective effects of N-acetylcysteine on experimental closed head trauma in rats. *Neurochem. Res.* 31, 473–481.
- Hoffer, M.E., et al., 2013. Amelioration of acute sequelae of blast induced mild traumatic brain injury by N-acetyl cysteine: a double-blind, placebo controlled study. *PLoS One* 8 (1), e54163 (<http://www.ncbi.nlm.nih.gov/pubmed/23372680>).
- Homsí, S., et al., 2010. Blockade of acute microglial activation by minocycline promotes neuroprotection and reduces locomotor hyperactivity after closed head injury in mice: a twelve-week follow-up study. *J. Neurotrauma* 27 (5), 911–921 (<http://www.ncbi.nlm.nih.gov/pubmed/20166806>).
- Inman, C.F., et al., 2005. Validation of computer-assisted, pixel-based analysis of multiple-colour immunofluorescence histology. *J. Immunol. Methods* 302 (1–2), 156–167 (<http://www.ncbi.nlm.nih.gov/pubmed/15992812>).
- Kim, H.S., Suh, Y.H., 2009. Minocycline and neurodegenerative diseases. *Behav. Brain Res.* 196 (2), 168–179 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18977395).
- Kobayashi, K., et al., 2013. Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis.* 4, e525 (<http://www.ncbi.nlm.nih.gov/pubmed/23470532>).
- Kovessi, E., et al., 2012. Acute minocycline treatment mitigates the symptoms of mild blast-induced traumatic brain injury. *Front. Neurol.* 3, 111 (<http://www.ncbi.nlm.nih.gov/pubmed/22811676>).
- Kumar, A., Loane, D.J., 2012. Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. *Brain Behav. Immun.* 26 (8), 1191–1201 (<http://www.ncbi.nlm.nih.gov/pubmed/22728326>).
- Kupchik, Y.M., et al., 2012. The effect of N-acetylcysteine in the nucleus accumbens on neurotransmission and relapse to cocaine. *Biol. Psychiatry* 71 (11), 978–986 (<http://www.ncbi.nlm.nih.gov/pubmed/22137594>).
- Kurushima, H., et al., 2000. Surface expression and rapid internalization of macrophage (mouse CD68) on elicited mouse peritoneal macrophages. *J. Leukoc. Biol.* 67 (1), 104–108 (<http://www.ncbi.nlm.nih.gov/pubmed/10648004>).
- Levin, H.S., Robertson, C.S., 2013. Mild traumatic brain injury in translation. *J. Neurotrauma* 30 (8), 610–617 (<http://www.ncbi.nlm.nih.gov/pubmed/23046349>).
- Li, B., et al., 2009. Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1 β level after traumatic brain injury. *Neurosurgery* 65 (1), 179–185 (discussion 185–6. <http://www.ncbi.nlm.nih.gov/pubmed/19574840>).
- Lijia, Z., et al., 2012. A self-propelling cycle mediated by reactive oxide species and nitric oxide exists in LPS-activated microglia. *Neurochem. Int.* 61 (7), 1220–1230 (<http://www.ncbi.nlm.nih.gov/pubmed/23000131>).
- Loane, D.J., Faden, A.L., 2010. Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. *Trends Pharmacol. Sci.* 31 (12), 596–604 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21035878).
- Lynch, M.A., 2009. The multifaceted profile of activated microglia. *Mol. Neurobiol.* 40 (2), 139–156 (<http://www.ncbi.nlm.nih.gov/pubmed/19629762>).
- Niogi, S.N., Mukherjee, P., 2010. Diffusion tensor imaging of mild traumatic brain injury. *J. Head Trauma Rehabil.* 25 (4), 241–255 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20611043).
- Perry, V.H., Nicoll, J.A., Holmes, C., 2010. Microglia in neurodegenerative disease. *Nat. Rev. Neurol.* 6 (4), 193–201 (<http://www.ncbi.nlm.nih.gov/pubmed/20234358>).
- Ramon Diaz-Arastia, R., Kochanek, P.M., Bergold, P.J., Kenney, K., Marx, C.E., Grimes, J.B., Loh, Y., Adam, G.E., Oskvig, D., Curley, K.C., Salzer, W., 2013. Pharmacotherapy of traumatic brain injury: state of the science and the road forward report of the

- Department of Defense Neurotrauma Pharmacology Workgroup. *J. Neurotrauma* (Epub ahead of print 22 Aug 2013) (<http://www.ncbi.nlm.nih.gov/pubmed/23968241>).
- Ramprasad, M.P., et al., 1996. Cell surface expression of mouse macrophage receptors for oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 93 (25), 14833–14838 (<http://www.ncbi.nlm.nih.gov/pubmed/8962141>).
- Siopi, E., et al., 2012. Evaluation of late cognitive impairment and anxiety states following traumatic brain injury in mice: the effect of minocycline. *Neurosci. Lett.* 511 (2), 110–115 (<http://www.ncbi.nlm.nih.gov/pubmed/22314279>).
- Suma, T., et al., 2008. Effects of in situ administration of excitatory amino acid antagonists on rapid microglial and astroglial reactions in rat hippocampus following traumatic brain injury. *Neurol. Res.* 30 (4), 420–429 (<http://www.ncbi.nlm.nih.gov/pubmed/18248696>).
- Yong, V.W., Rivest, S., 2009. Taking advantage of the systemic immune system to cure brain diseases. *Neuron* 64, 55–60 (<http://www.ncbi.nlm.nih.gov/pubmed/19840549?dopt=Abstract&holding=npg>).
- Yu, S., et al., 2009. Severity of controlled cortical impact traumatic brain injury in rats and mice dictates degree of behavioral deficits. *Brain Res.* 1287, 157–163 (<http://www.ncbi.nlm.nih.gov/pubmed/19573519>).