

Deficits in Discrimination after Experimental Frontal Brain Injury Are Mediated by Motivation and Can Be Improved by Nicotinamide Administration

Cole Vonder Haar, William R. Maass, Eric A. Jacobs, and Michael R. Hoane

Abstract

One of the largest challenges in experimental neurotrauma work is the development of models relevant to the human condition. This includes both creating similar pathophysiology as well as the generation of relevant behavioral deficits. Recent studies have shown that there is a large potential for the use of discrimination tasks in rats to detect injury-induced deficits. The literature on discrimination and TBI is still limited, however. The current study investigated motivational and motor factors that could potentially contribute to deficits in discrimination. In addition, the efficacy of a neuroprotective agent, nicotinamide, was assessed. Rats were trained on a discrimination task and motivation task, given a bilateral frontal controlled cortical impact TBI (+3.0 AP, 0.0 ML from bregma), and then reassessed. They were also assessed on motor ability and Morris water maze (MWM) performance. Experiment 1 showed that TBI resulted in large deficits in discrimination and motivation. No deficits were observed on gross motor measures; however, the vehicle group showed impairments in fine motor control. Both injured groups were impaired on the reference memory MWM, but only nicotinamide-treated rats were impaired on the working memory MWM. Nicotinamide administration improved performance on discrimination and motivation measures. Experiment 2 evaluated retraining on the discrimination task and suggested that motivation may be a large factor underlying discrimination deficits. Retrained rats improved considerably on the discrimination task. The tasks evaluated in this study demonstrate robust deficits and may improve the detection of pharmaceutical effects by being very sensitive to pervasive cognitive deficits that occur after frontal TBI.

Key words: animal models; controlled cortical impact; operant learning

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a serious problem affecting society today. In the United States, 1.7 million TBIs occur annually and as many as 10 million worldwide.^{1,2} These numbers represent a strain on society and a fiscal burden of more than \$60 billion.³ Currently, there are no Food and Drug Administration-approved pharmaceutical treatments for TBI.⁴

Many pharmaceutical agents have been screened in clinical trials, but all have failed, highlighting the large need for the development of therapeutic compounds. Many potential reasons have been suggested for these failures. Some of the top reasons put forth are poor design of clinical trials, narrow therapeutic mechanisms of drugs, and inadequate preclinical behavioral characterization.^{4–6} As a field, all of these must be addressed when considering the development of a novel therapeutic agent. This means that preclinical scientists should consider multiple injury models and/or locations when screening therapeutics, that drugs should be picked

based on their potential to address multiple mechanisms of injury, and that clinical trials should be designed in such a way as to maximize the ability to detect drug effects.

Recently, nicotinamide (NAM; niacin, vitamin B₃) has shown considerable efficacy in a number of preclinical studies, both in fluid percussion injury (FPI) and controlled cortical impact (CCI) injury as well as across multiple injury locations.^{7–11} It is considered to have multimodal action; it works to reduce cell death from energy loss, reduce free radicals, inhibit poly(adenosine diphosphate-ribose)polymerase-1, and inhibit sirtuins.^{12–14} Despite the amount of preclinical testing that has been done with NAM, there have been very few assessments looking at cognitive functioning. In fact, in some studies, the cognitive outcomes measures have not always shown improvement, especially at lower doses.^{10,15} As is true of many TBI studies, the primary cognitive dependent measure for NAM has been the Morris water maze (MWM), which only assesses one aspect of cognition: spatial learning. The results from these studies suggest that either NAM treatment does not necessarily

provide the same benefits to cognitive recovery as it does to sensorimotor recovery or that the cognitive behavior that has been tested is insufficient to capture all of the effects of NAM.

The use of other cognitive measures for assessing TBI has been gaining interest in the field. Many mouse models have begun to use more social, naturalistic, or emotional measures (e.g., novel object, resident-intruder, elevated plus maze) as a means of evaluating cognitive capabilities,^{16,17} and both rat and mouse studies have begun to investigate even more complex measures such as discrimination and fear learning.^{16–21} In rats, the bilateral frontal model of brain injury, originally developed by the Stein laboratory,²² has proven to be very useful in evaluating cognitive behaviors because it induces a “pure” cognitive deficit (i.e., very little sensorimotor damage). The frontal regions that are affected by this injury in the rat have been suggested to be homologous in terms of behavioral function to those in the human prefrontal cortex.^{23,24} This is especially important because cognitive deficits in humans have been shown to be pervasive after TBI and are often the most difficult for the patient to adapt to because they may not be aware of the extent of them.^{25,26} In rats, models of frontal TBI have recently been used to induce cognitive deficits across a range of tasks, including discriminations, MWM, set shifting, and fear learning.^{20,21} Many of these measures are borrowed from the field of the experimental analysis of behavior where they have been used for many years to assess deficits from frontal lesions.^{27–30}

In particular, the deficits seen on previous discrimination tasks after TBI appear promising for the assessment of therapeutic agents. Each study that used a discrimination task showed a robust deficit, which produced a very large injury window between sham and injured performance.^{18,20} Previously, operant tasks such as these have not been widely used for assessment in the field of TBI and have never been used to assess a therapeutic agent. As of this writing, only three studies have used actual operant chambers to determine deficits after injury.^{20,31,32}

The goal of the current study was to use a discrimination task for the evaluation of the efficacy of NAM in treating TBI. Further, it also evaluated potential contributions of motor deficits or motivational deficits in performance on the discrimination task. We used several measures to obtain a robust picture of function after injury. These included accuracy of discrimination and the MWM as a measure of cognitive capability, locomotor activity monitoring, and lever hold duration as a measure of motor function, and the break point (the point at which rats stop responding) in the progressive ratio (PR) as a measure of motivation.

Methods

Animals

Twenty-seven male Long-Evans rats, approximately 350 g at the time of surgery, were used in this study. The procedures conducted in this study were reviewed and approved by the Southern Illinois University Institutional Animal Care and Use Committee, and the study was conducted in a laboratory certified by the American Association for the Accreditation of Laboratory Animal Care. Rats were housed singly in standard cages on a 12-h light: dark cycle. Behavioral testing took place during the light cycle. Rats were maintained at 340–350 g; water was available *ad libitum*.

Operant training

Apparatus. Testing occurred in a bank of four standard modular operant chambers measuring 29×29×24 cm (Coulbourn Instruments, Whitehall, PA). Each chamber was positioned inside a

sound-attenuated chamber with white noise in the background. Operant chambers were interfaced with a computer via a PCI-PDISO-16 board (Measurement Computing, Norton, MA) and controlled by custom programs written in the VisualBasic.net programming language (Microsoft, Redmond, WA). One side of the chambers was blank. The other side was equipped with three levers (2 cm long×3.5 cm wide), each with a panel of three light-emitting diode lights above them. A dipper (Coulbourn) was located below the center lever to deliver reinforcer (0.04 mL sweetened condensed milk per delivery; diluted 3:1, water:milk). There was a Sonalert audio signal (Coulbourn; 2000 Hz, 10 dB) as well as a house light located at the top of the chamber for general illumination.

Discrimination training. Rats were put through the steps listed below to shape their behavior to perform the two-choice discrimination. The time spent on each training step varied from rat to rat, but the average training times were not different across the groups. Sessions lasted approximately 1–1.5 h and were conducted 7 days per week (Fig. 1).

Lever-pressing behavior was shaped through the method of successive approximations.³³ Initially, rats were food restricted and placed into chambers, with each of the three levers set to deliver reinforcer on being pressed. Milk was presented for 3 sec, followed by a 6 sec intertrial interval (ITI). Once rats were reliably pressing levers, differing fixed ratio schedules (ranging from FR-1–FR-10) were presented across the levers to distribute pressing behavior roughly equally across the three levers. Sessions lasted 1 h. Once responses were distributed evenly, rats were moved on to discriminative stimulus training.

During discriminative stimulus training, an FR-1 schedule of reinforcement was in effect for a given lever when the light panel above it was illuminated (solid light). Pressing of an unlit lever caused all lights to go out and a 15 sec ITI to begin. Correct responses resulted in reinforcer being presented for 3 sec followed by a 12 sec ITI. Sessions initially lasted 200 trials and were tapered down to 100 trials once rats began responding robustly and then a maximum trial time of 30 sec was instituted, after which it went straight to the ITI. Once rats reached 85% accuracy on this phase, they were moved on to behavioral chain training.

The behavioral chain training was exactly the same as the discriminative stimulus training, except that each trial began with the light above the center lever illuminated and a press on the center lever randomly turned on one of the side stimuli. Only presses to the lever on the lit side were reinforced. Presses on the lever on the unlit side resulted in a 15 sec ITI. Sessions lasted 100 trials. Once the chain was acquired, the FR-1 center response was gradually stepped up to FR-3, and then a 30 sec maximum trial duration was imposed. Once an 85% accuracy criterion was reached, rats were moved onto training on the PR schedule.

PR training. This task was conducted in the same apparatus as the discrimination, but used a slowly blinking house light as well as a slowly blinking center stimulus light to distinguish the contexts of the tasks (700 ms pulse; 0.71 Hz). Procedures for this task were adapted from previously published protocols,³⁴ but changed to preferentially sample the lower range of response requirements (see below). Only presses to the center lever were reinforced; the side levers had no programmed consequences. Initially, under the PR schedule of reinforcement, two responses were needed to the center lever for reinforcer to be delivered. After the delivery of reinforcer, the response requirement increased by two responses. Thus, the second reinforcer required four presses and the third reinforcer required six presses, and so on. The step size was increased to five when the response requirement equaled 20. Thus, the 11th reinforcer required 25 presses, the 12th required 30, and so on. When the response requirement equaled 50, the step size was increased again up to 10. Thus the 17th reinforcer required 60 presses, the 18th, 70 presses, and so on. The final sequence of requirements was

as follows: (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70...*n*).

Because break points have been shown to be relatively independent of step size,^{35,36} this sequence allowed for differential sampling at the low end of break points and thus increased sensitivity to injury effects in those ranges. If the rat did not press the center lever for a 5-min period, the session was terminated. Any press to the center lever reset this interval. After 2 days of learning on this task, rats were moved into pretesting.

Pretesting (baseline)

Discrimination and PR. To establish a baseline for comparison post-surgery, on alternating days, rats were given a session of two-choice discrimination testing and a session of PR testing for 12 days (six sessions on each task). Baseline accuracy on the two-choice discrimination was recorded as were baseline break points on the PR schedule.

Motor assessment. Gross motor ability was assessed on the last day of pretesting by evaluating spontaneous motor activity in a square Plexiglas open field (Photobeam Activity System, San Diego Instruments, San Diego, CA) measuring 40.5 cm wide by 40.5 cm long by 38 cm high according to a previously published protocol.³⁷ Rats were placed and allowed to explore freely for 10 min. Sixteen crossing infrared beams recorded position and locomotor behavior to a computer database. A separate set of eight beams tracked rearing behavior. Distance traveled and rears, measured in number of beams broken, were the primary dependent measures.

Baseline fine motor assessments in the operant chamber were also made by recording the lever hold duration throughout the PR task. The PR hold durations were chosen because there is a fairly regular pattern of responding and higher number of overall responses in this task compared with the discrimination. Hold duration has been used previously for the evaluation of motor dysfunction in Parkinson disease and Huntington disease models.^{38,39} After all pretesting, rats were moved on to surgery.

Surgery

Surgical procedures were performed according to previous studies under aseptic conditions.^{7,11} Rats were anesthetized under a mixture of isoflurane (2–4%) and oxygen (0.8 L/min) and then placed into a stereotaxic frame on a heated surgical stage. A bilateral CCI procedure or sham procedure was performed. A midline incision was made through the skin and fascia. A 6.0 mm diameter craniotomy centered at 3.0 mm from bregma (AP +3.0, ML +0.0) was created using a microdrill. Special care was taken to avoid any damage to the meninges or cortex. The cortical region containing the prefrontal cortex was exposed. A circular, flat-faced stainless steel impactor tip with a diameter of 5.0 mm was then used to induce the injury. It was attached to an electromagnetic impactor device (Leica Biosystems, St. Louis). To induce a severe brain injury, the tip impacted the cortex at a rate of 3.0 m/sec for 0.5 sec to a depth of 2.5 mm. After injury, bleeding was stopped and the incision sutured closed. Rats were then placed in a heated recovery chamber until locomotor behavior returned. Sham procedures followed an “intact sham” procedure.^{18,40} This consisted of a midline incision, suturing of the incision, and then placement in the recovery chamber.

Drug administration

Rats were given injections of NAM (150 mg/kg, intraperitoneally [i. p.]) or 0.9% sterile phosphate-buffered saline (PBS; 1 mL/kg, i. p.) starting at 2 h post-surgery and then at 12, 24, 36, 48, 60 and 72 h according to previous protocols.^{41,42} Rats were matched for

pre-injury performance and then randomly assigned to one of three groups. Group 1 received a CCI and was given NAM injections (NAM, *n* = 10). Group 2 received a CCI and was given saline injections (vehicle, *n* = 9). Group 3 received sham procedures and was given saline injections (sham, *n* = 8).

Experiment 1

The purpose of the first experiment was to characterize the performance of rats on the discrimination task and compare with the motor measures, progressive ratio task, and MWM to determine if NAM administration improved performance. A secondary goal was to determine if motivation or motor impairments contributed to discrimination performance. After surgery, rats were allowed 6 days to recover before testing resumed on day 7. The two-choice discrimination was assessed on days 7–27 with a break every 5 days to assess the PR motivation task. The gross motor abilities of rats were assessed on days 7, 14, and 27 in the locomotor activity monitors. On days 15–18 post-surgery, rats were tested on the reference memory paradigm of the MWM. On days 21–23, rats were tested on the working memory paradigm of the MWM (Fig. 1).

Discrimination. Discrimination testing followed the procedures described above. Rats were required to press the center lever three times to turn on a light on either the left or the right lever, then were required to make a correct choice to receive the reinforcer. Accuracies were recorded for each day and averaged into bins of 4 days of testing for analysis.

PR. The PR testing followed the procedures described above and was tested every fifth day. The break points of rats were recorded for analysis.

Motor assessment. The beams broken in the activity monitors were recorded for gross movement as well as rears and recorded for analysis of gross locomotor ability. The durations of the lever holds in the operant chamber during PR sessions were also recorded and averaged for analysis of fine locomotor ability.

MWM. The MWM testing followed a previously published protocol in a 1.5 m diameter circular tank with two phases of testing: reference memory and working memory.⁷ Consistent reference cues were present on the three walls surrounding the tank throughout testing. During the reference memory paradigm, a clear platform (10 cm × 10 cm) was submerged in 32 cm of 22°C water in the center of the northeast quadrant of the tank. Rats were lowered into the water at pseudorandomized start points (one trial starting from each cardinal direction) facing the wall of the tank. Once in the water, rats were allowed to freely explore until they located the submerged platform and climbed onto it or 90 sec elapsed. If a rat was unable to locate the platform after 90 sec, it was guided by hand to the platform. Rats were allowed to remain on the platform for 10 sec before being removed and placed in a heated cage to dry. The latencies were recorded by hand. There were four trials per day. The latencies from the four trials were averaged to form a score for the day.

The working memory paradigm of the MWM was the same as the reference memory paradigm, except that on each day, the platform was placed in a new pseudorandom quadrant (northwest, southwest, southeast). Testing occurred as before, except that the first trial was considered a learning trial and not included in the analysis. The latencies from the last three trials were averaged to form a score for the day.

Experiment 2

The purpose of the second experiment was to evaluate how effective retraining was for injured rats. It used the same animals and started immediately after Experiment 1 was completed. After 16

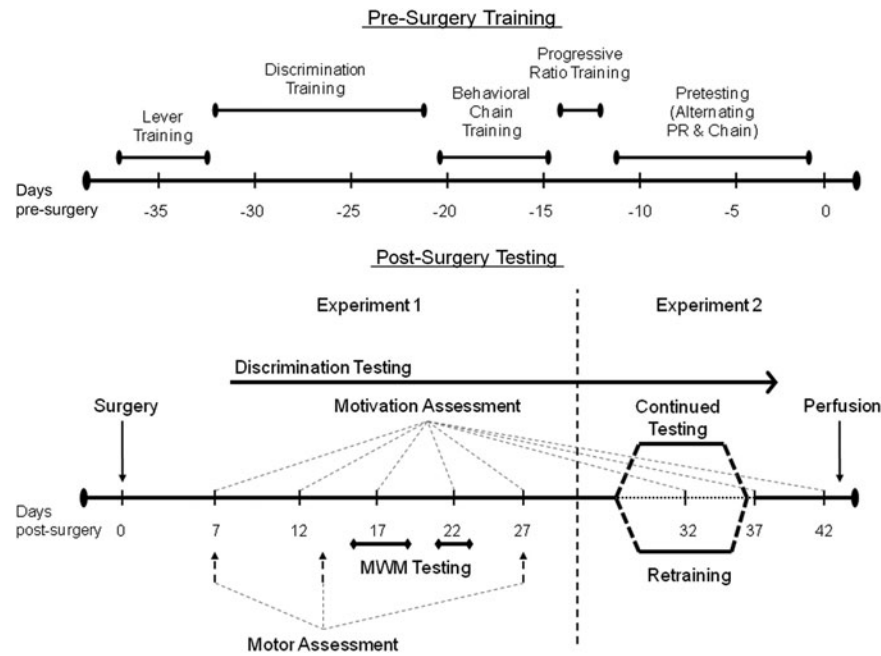


FIG. 1. The top part of this figure shows the steps of training for the discrimination and progressive ratio tasks. The bottom part shows the timeline of behavior after injury, divided by experiments 1 and 2. Rats that were not responding at least 25% of the time by the end of experiment 1 were retrained in experiment 2. Testing continued normally for those that were responding at least 25% of the time. PR, progressive ratio; MWM, Morris water maze.

sessions of discrimination testing in Experiment 1, rats that were not completing at least 25% of the response chains were stepped back to the previous training step—a simple discrimination in which the stimulus lights appeared automatically without requiring a centering response. These rats were given eight sessions of retraining and then placed back on the chain to reassess their responding. Rats that were completing greater than 25% of the discrimination chains continued testing under the conditions from Experiment 1 (Fig. 1).

Discrimination. Discrimination testing continued as described above with a centering response for the rats that had been responding more than 25% of the time. The rats that were not responding at a high rate were stepped back a training step and the left or right light appeared without a centering response. The accuracies and response rates were collected for analysis.

PR. The PR task continued to be assessed every 5 days while the retraining occurred. The break points were recorded for analysis.

Histology and lesion analysis

Forty-three days after injury, rats were anesthetized with a lethal dose of sodium pentobarbital (Euthasol; Virbac Animal Health, Fort Worth, TX; 0.3 mL, i.p.) and transcardially perfused with ice-cold (5°C) PBS, followed by 10% phosphate-buffered formalin. The brain was then removed from the skull and post-fixed in formalin for 24 h, then placed in a 30% sucrose solution until saturated and sliced frozen, coronally, on a sliding microtome at 40 μ m. After slicing, brain sections were mounted to gel-subbed slides for staining.

Brain sections were stained with cresyl-violet to visualize and analyze the extent of the lesion according to a previous protocol.⁷ An Olympus microscope (BX-51; Center Valley, PA) with an Olympus 13.5 megapixel digital camera (DP-70) attached to it was used to capture images of the coronal sections transversing the

lesion cavity (+5.0, +4.0, +3.0, +2.0, +1.0 from bregma, see Fig. 2). ImageJ (NIH, Bethesda, MD) was used to measure individual brain slices. The lesion volume was then calculated according to previous studies.¹⁸ The area of five slices was averaged together using the Cavalieri method, then multiplied by the thickness and number of sections to estimate the volume (formula: $0.04 * 5 * \text{average area}$).⁴³

Analysis

Means and standard error of the mean were collected for all data and analyzed using SPSS 15. Repeated measures analysis of variance (ANOVAs) were used to examine differences in performance between groups on measures with multiple time points. Univariate ANOVAs were used to examine differences on single point outcome measures. Tukey Honestly Significant Difference (HSD) was used to conduct pairwise *post-hoc* comparisons. The Huynh-Feldt correction was used to correct for violations of sphericity. Pearson correlation was used to examine the relationship between lesion size and performance. Statistical significance was determined by a p value < 0.05.

Results

Experiment 1

Discrimination. There were no differences between the groups before surgery, $F(2, 24) = 1.39, p = 0.269$. After surgery, the accuracies were analyzed in a 3×4 (Group \times Bin) repeated measures ANOVA. There was no main effect of bin, $F(1.54, 36.84) = 2.71, p = 0.092$. There was a significant difference between the groups, $F(2, 24) = 53.04, p < 0.001$. The vehicle group was significantly impaired compared with the sham group, $HSD(15) = 92.59, p < 0.001$, and NAM group, $HSD(17) = 32.49, p = 0.003$. The NAM group was impaired compared with the sham group, $HSD(16) = 60.10, p < 0.001$. There was no significant interaction, $F(3.07, 36.84) = 0.92, p = 0.442$ (Fig. 3).

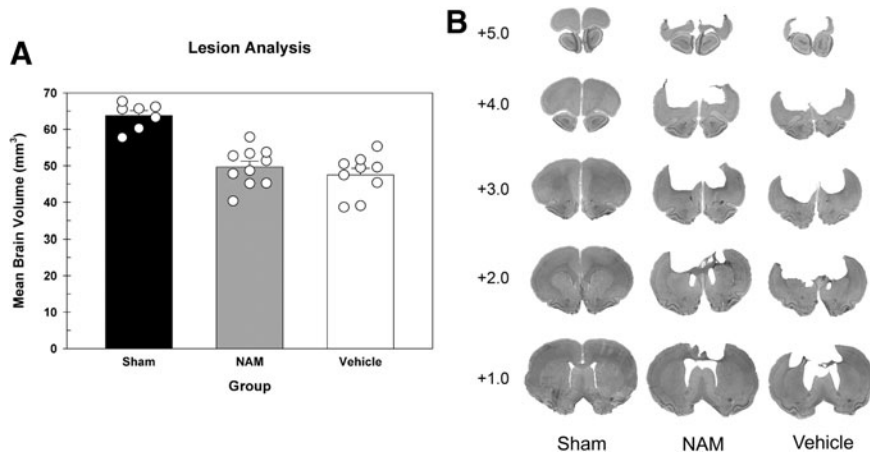


FIG. 2. Panel A shows the remaining brain volume (+SEM) after surgery. Both NAM and vehicle groups had significantly less brain volume than the sham group. Superimposed dots represent the volumes of individual rats. Panel B shows representative brain sections, demonstrating the extent of the injury. Numbers are the location relative to bregma in mm.

PR. Rats were normalized to their own baseline for evaluation. After surgery, the break points were analyzed in a 3×5 (Group×Day) repeated measures ANOVA. There was no significant main effect of day, $F(2.77, 66.45) = 2.77, p = 0.236$. There was a significant difference between the groups, $F(2, 24) = 6.90, p = 0.004$. The vehicle group was significantly impaired compared with the sham group, $HSD(15) = 76.94, p = 0.005$, and NAM group, $HSD(17) = 56.37, p = 0.030$. There was no difference between the NAM and sham groups, $HSD(16) = 20.57, p = 0.603$. There was no significant interaction, $F(5.54, 66.45) = 0.34, p = 0.900$ (Fig. 3).

Motor assessment

Pre-surgery. In the activity chambers, there were no differences between the groups before surgery in gross movement, $F(2, 24) = 0.10, p = 0.903$, or in rears, $F(2, 24) = 1.60, p = 0.223$. There was also no difference in lever hold durations between the groups before surgery, $F(2, 24) = 0.06, p = 0.943$ (Fig. 4).

Gross movement. After surgery, the overall movement was analyzed in a 3×3 (Group×Day) repeated measures ANOVA. There was a significant main effect of day, $F(2, 48) = 19.33,$

$p < 0.001$. There was no significant difference between the groups, $F(2, 24) = 1.52, p = 0.240$. There was, however, a significant interaction, $F(4, 48) = 3.80, p = 0.009$. Simple main effects revealed that the vehicle group, $F(2, 16) = 7.65, p = 0.005$, and NAM group, $F(2, 18) = 22.94, p < 0.005$, changed across days, while the sham group did not. The groups were significantly different from each other on the first test day, $F(2, 24) = 5.29, p = 0.013$. Specifically, the NAM group moved significantly more than the sham group, $HSD(16) = 617.43, p = 0.009$ (Fig. 4).

Rears. After surgery, the overall rears were analyzed in a 3×3 (Group×Day) repeated measures ANOVA. There was a significant main effect of day, $F(2, 48) = 6.78, p = 0.003$. There was no significant difference between the groups, $F(2, 24) = 0.35, p = 0.710$. There was no significant interaction, $F(4, 48) = 2.09, p = 0.096$ (Fig. 4).

Fine motor control. The difference in lever hold durations between pre- and post-surgery were compared in a 3×2 repeated measures ANOVA (Group×Phase). There was no significant effect of phase, $F(1, 24), p = 0.532$ or significant effect of group, $F(2, 24), p = 0.144$. There was, however, a group×phase interaction, $F(2, 24), p = 0.002$. This interaction was because of the sham group

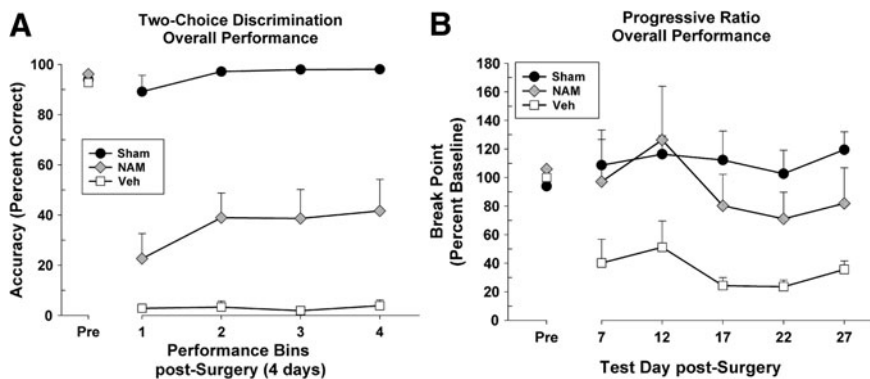


FIG. 3. Panel A shows the average accuracy (+standard error of the mean [SEM]) on the two-choice visual discrimination. Injured rats performed significantly worse than the sham group. The nicotinamide (NAM) group had significantly improved performance compared with the vehicle group. Panel B shows the average break points (+SEM; as a percent of baseline) on the progressive ratio schedule (motivation). The vehicle group performed significantly worse than the sham group. The NAM group had improved performance compared with the vehicle group and was not significantly different than the sham group.

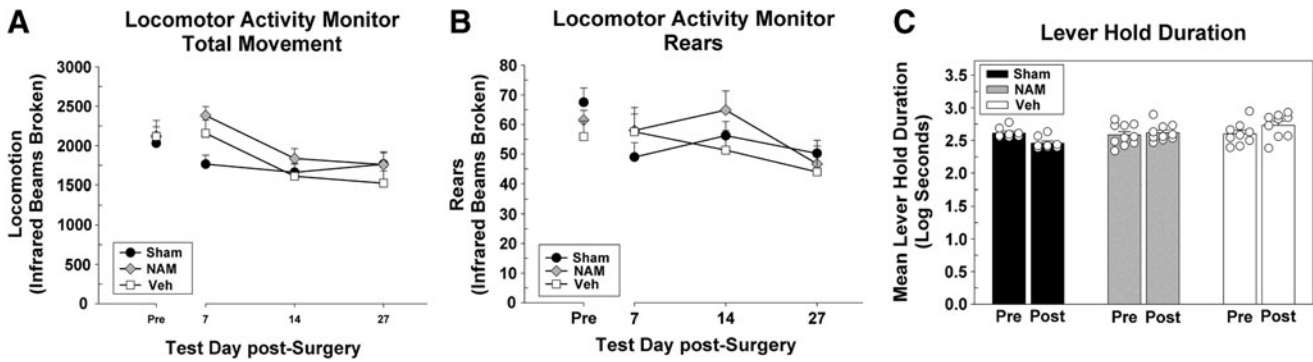


FIG. 4. Panel A shows the average distance traveled (+standard error of the mean [SEM]) in the activity monitoring chambers. The nicotinamide (NAM) and vehicle groups showed a transient increase in locomotion, which returned to baseline levels. Panel B shows the average number of rears (+SEM) in the activity chamber. There was no difference between the groups in rearing. Panel C shows the average log hold time (duration of press) of the lever (+SEM). Sham rats reduced their average hold time from pre-surgery to post-surgery, while the vehicle group increased their average hold time. The vehicle had significantly longer hold times post-injury than the sham group, but the NAM group was not significantly different from either. Superimposed dots represent the hold times for individual rats.

reducing their average hold time from pre- to post-surgery, $t(7) = 11.99$, $p < 0.001$ and the vehicle group increasing their hold time, $t(8) = 2.32$, $p = 0.049$. The NAM group did not change, $t(9) = 0.86$, $p = 0.413$. In addition, in the post-surgery phase, there was a significant difference between the groups, $F(2, 24) = 7.92$, $p = 0.002$. The vehicle had significantly higher hold times than the sham group, $HSD(15) = 0.27$, $p = 0.002$, but was not significantly different from the NAM group, $HSD(17) = 0.11$, $p = 0.215$. The NAM group was not significantly different from the sham group, $HSD(16) = 0.16$, $p = 0.063$ (Fig. 4).

MWM. The reference memory paradigm was analyzed in a 3×4 (Group \times Day) repeated measures ANOVA. There was a significant main effect of day, $F(2.82, 67.58) = 128.71$, $p < 0.001$. There was a significant difference between the groups, $F(2, 24) = 9.00$, $p = 0.001$. The vehicle group was significantly impaired compared with the sham group, $HSD(15) = 11.59$, $p = 0.049$, but not compared with the NAM group, $HSD(17) = 7.51$, $p = 0.219$. The NAM group was significantly impaired compared with the sham group, $HSD(16) = 19.10$, $p = 0.001$. There was also a significant interaction, $F(5.63, 67.58) = 2.95$, $p = 0.015$. Simple main effects revealed that there was only a significant difference between the groups on the first day of testing, $F(2, 24) = 14.36$, $p < 0.001$ with the vehicle group taking significantly longer compared with the sham group, $HSD(15) = 25.62$, $p = 0.003$, and the NAM group taking significantly longer compared with the sham group, $HSD(16) = 35.49$, $p < 0.001$ (Fig. 5).

The working memory paradigm was analyzed in a 3×3 (Group \times Day) repeated measures ANOVA. There was no significant main effect of day, $F(1.83, 43.95) = 2.54$, $p = 0.095$. There was a significant difference between the groups, $F(2, 24) = 3.75$, $p = 0.038$. The vehicle group was not significantly different from the sham group, $HSD(15) = 3.51$, $p = 0.142$, or the NAM group, $HSD(17) = 1.13$, $p = 0.782$. The NAM group, however, was impaired compared with the sham group, $HSD(16) = 4.65$, $p = 0.035$. There was no interaction, $F(3.66, 43.95) = 0.82$, $p = 0.508$ (Fig. 5).

Experiment 2

Subgroup determination and analysis. Rats not responding at least 25% of the time by the 16th session (4th bin) of discrimi-

nation testing were stepped back a stage in the discrimination training so that no centering response was needed. This lasted for eight sessions. This measure was required for 100% of the vehicle rats, 50% of the NAM rats, and 0% of the sham rats. This formed four groups for the second experiment: vehicle ($n = 9$), NAM-Retraining ($n = 5$), NAM ($n = 5$), and sham ($n = 8$). Rats that were responding at least 25% of the time continued the testing regimen from experiment 1. After the retraining period, all rats were reassessed on the testing regimen from experiment 1 for four sessions.

Responses. To determine if the retraining increased responding and compare performance in experiment 1 with experiment 2, the responses from experiment 1 of the rats in the retraining group were compared with the responses from experiment 2 and

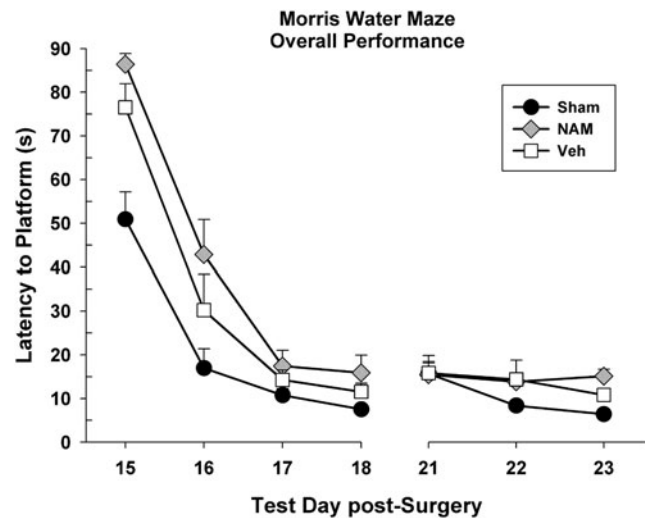


FIG. 5. This figure shows the average latency (+standard error of the mean) to locate the platform. On the reference memory paradigm, both the vehicle and nicotinamide (NAM) groups performed significantly worse than the sham group. On the working memory paradigm, the NAM group performed significantly worse than the sham group. There was no difference between vehicle and sham.

TABLE 1. EFFECTS OF RETRAINING AND CROSS-EXPERIMENT COMPARISON*

	Retraining required	Discrimination responses			Discrimination accuracy			Progressive ratio break point (% baseline)		
		Testing 1	Retraining	Testing 2	Testing 1	Retraining	Testing 2	Testing 1	Retraining	Testing 2
NAM-retraining	50%	11%	94%	46%	10%	79%	42%	55%	48%	79%
Vehicle	100%	4%	83%	23%	3%	69%	21%	35%	43%	51%

*This table shows the effect of retraining (subgroups NAM-retrain and Vehicle) on several performance measures and compares differences in performance between experiment 1 and experiment 2. Retraining increased responding and accuracy on the discrimination, but progressive ratio break points were not significantly different. There were no group differences in the animals that received retraining.

analyzed in a 2x3 (GroupxPhase [Testing 1, Retraining, Testing 2]) repeated measures ANOVA. There was a significant main effect of phase, $F(2, 24)=66.02, p<0.001$. There was no significant difference between the groups, $F(1, 12)=1.97, p=0.186$. There was no significant interaction, $F(2, 24)=0.71, p=0.501$ (Table 1).

Discrimination. To determine if the retraining increased accuracy and compare performance in experiment 1 with experiment 2, the accuracies from experiment 1 of the rats in the retraining group were compared with the accuracies from experiment 2 and analyzed in a 2x3 (GroupxPhase [Testing 1, Retraining, Testing 2]) repeated measures ANOVA. There was a significant main effect of phase, $F(2, 24)=36.58, p<0.001$. There was no significant difference between the groups, $F(1, 12)=1.95, p=0.188$. There was no significant interaction, $F(2, 24)=0.54, p=0.587$ (Table 1).

To analyze the specific effects within experiment 2, the accuracies of the rats in the retraining groups were compared in a 2x3 (Group x Bin) repeated measures ANOVA. There was a significant main effect of bin, $F(1.53, 18.37)=22.38, p<0.001$. There was no significant difference between the groups, $F(1, 12)=1.71, p=0.216$. There was no significant interaction, $F(1.531, 18.37)=0.38, p=0.635$. The accuracies of the rats that did not need retraining were analyzed in a 2x3 (GroupxBin) repeated measures ANOVA. There was no significant main effect of bin, $F(2, 22)=1.79, p=0.191$. There was a significant difference between the groups,

$F(1, 11)=6.73, p=0.025$. There was no significant interaction, $F(2, 22)=1.62, p=0.220$ (Fig. 6).

PR. PR testing continued once every five sessions as described in experiment 1. To determine if the retraining increased break points and compare performance in experiment 1 with experiment 2, the average break points from experiment 1 of the rats in the retraining group were compared with the break points from experiment 2 and analyzed in a 2x3 (GroupxPhase [Testing 1, Retraining, Testing 2]) repeated measures ANOVA. There was no significant main effect of phase, $F(2, 24)=2.99, p=0.069$. There was no significant difference between the groups, $F(1, 12)=1.5, p=0.244$. There was no significant interaction, $F(2, 24)=0.82, p=0.452$ (Table 1).

To analyze specific effects within experiment 2, the break points of the rats in the retraining groups were analyzed in a 2x3 (GroupxDay) repeated measures ANOVA. There was a significant main effect of day, $F(2, 24)=3.76, p=0.038$. There was no significant difference between the groups, $F(1, 12)=0.65, p=0.436$. There was no significant interaction, $F(2, 24)=1.67, p=0.210$. The break points of the rats that did not require retraining were analyzed in a 2x3 (GroupxDay) repeated measures ANOVA. There was no significant main effect of day, $F(2, 22)=1.57, p=0.231$. There was no significant difference between the groups, $F(1, 11)=0.32, p=0.584$. There was no significant interaction, $F(2, 22)=1.22, p=0.313$ (Fig. 6).

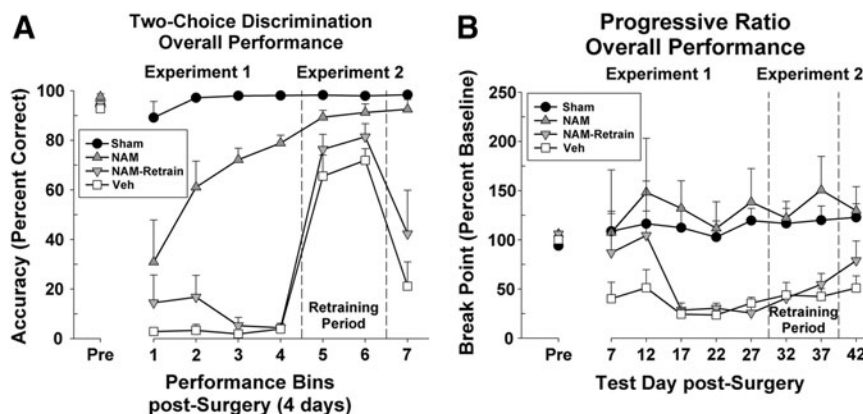


FIG. 6. Panel A shows the average accuracy (+standard error of the mean [SEM]) on the two-choice visual discrimination after dividing into subgroups during experiment 2. The retrained animals (nicotinamide [NAM]-Retrain, Veh) were not significantly different than each other, but did improve their accuracy during retraining. The animals that did not require retraining (NAM, Sham) were significantly different from each other and did not change significantly over time. Panel B shows the average break points (+SEM; as a percent of baseline) on the progressive ratio schedule (motivation) after dividing into subgroups during experiment 2. The retrained animals (NAM-Retrain, Veh) were not significantly different than each other, but their break points significantly increased during the retraining phase. The animals that did not require retraining (NAM, Sham) were not significantly different from each other and did not change significantly across time.

Lesion analysis

The remaining brain volumes were compared in a one-way ANOVA. There was a significant difference between the groups, $F(2, 24) = 24.24, p < 0.001$. The vehicle group had significantly less brain volume than the sham group, $HSD(15) = 16.30, p < 0.001$, but was not significantly different from the NAM group, $HSD(17) = 2.17, p = 0.352$. The NAM group had significantly less brain volume than the sham group, $HSD(16) = 14.13, p < 0.001$ (Fig. 2).

Given the bimodal split in discrimination performance in the NAM-treated group, a brief analysis was conducted to determine if lesion size was the major determinant of performance. There was no significant difference in the NAM and NAM-Retraining groups, $t(8) = -0.49, p = 0.638$. There was also no significant correlation between lesion size and discrimination performance, $r = -0.30, p = 0.397$, or PR, $r = -0.02, p = 0.953$.

Discussion

The results of this study demonstrate that discrimination tasks can be successful in detecting large deficits after frontal TBI. Based on the results of this study, however, these deficits appear to be largely driven by deficits in motivation. The evidence for motivational impairments as the driving factor can be seen in the lack of responding from injured animals on the discrimination as well as the lower break points on the PR motivation task. It can also be seen in the immediate improvement in responding during experiment 2 when the response requirement was lowered.

The lack of gross motor deficits suggests that the bilateral frontal injury had very little effect on spontaneous locomotion or rearing. Mild fine motor deficits were observed in the vehicle-treated TBI rats, however, which may need to be considered for some behaviors. Further, NAM administration at the current dose was sufficient to improve function on both discrimination and motivation tasks. Some caution should be taken, however, when interpreting the abilities of NAM to improve performance. In the current study, the NAM-treated group was bimodal, with one half performing near sham levels and the other half performing near vehicle-treated TBI levels. This suggests that NAM may have limited efficacy or that there are other factors, not measured in this study, that play a role in promoting NAM-mediated recovery of function. This is further emphasized by the lack of gross anatomical sparing in the NAM group, potentially pointing to more subtle factors underlying sparing or reorganization.

The results seen in this study align with previous TBI research and lesion studies of the prefrontal cortex. Previous TBI studies have found deficits in discrimination ability after frontal TBI.^{18,20} The deficits seen in this study, however, were considerably more pronounced than in previous work. This is likely primarily due to the implementation of a centering response on the center lever (intended to reduce side biases). The increased response requirement emphasized the motivational deficits in injured rats, and this deficit was immediately reduced when the centering response was removed (experiment 2, Fig. 5). Discrimination deficits have also been seen with electrolytic or excitotoxic lesions to the medial prefrontal cortex.^{28,44,45} There are marked similarities in the deficits seen in TBI animals; however, the deficits associated with TBI tend to be much more severe and pervasive, likely from the damage to multiple other regions.

Motivational deficits have not been commonly studied in the field of experimental TBI, although clinical patients have shown amotivational and anhedonic states an injury.^{46–48} In other fields, most motivational lesion studies have focused on the nucleus accumbens

and the dopaminergic pathways connecting it to the prefrontal cortex as the primary physiological mediator of motivation.^{49,50} The traumatic injury seen in the current study does not cause focal damage to the nucleus accumbens, but does affect a number of its regional pathways through the prelimbic cortex, infralimbic cortex, and anterior cingulate cortex, which could lead to dopaminergic dysfunction and motivational deficits.

One component that was dissimilar from previous studies was the outcome in the MWM. The frontal injury had a relatively small effect on performance—there were only significant impairments in injured animals on the first day of reference memory testing and only in the NAM group in working memory testing. It is particularly interesting that NAM did not improve function on the MWM, especially compared with much more robust MWM effects seen in a number of previous TBI studies.^{7,9,11} This suggests two possibilities. The first is that Long-Evans rats (used in the current study) do not suffer as large deficits as Sprague-Dawley rats (used in numerous previous works) in the MWM from this frontal injury location. The second is that the MWM may not be the absolute barometer of cognitive function that it is frequently assumed to be in the TBI field. Specifically, the MWM may have considerably less relevance for frontal injuries compared with hippocampal injuries. Both of these explanations warrant further investigation, in particular because the MWM is so ubiquitous in the field of TBI.

This study emphasizes a growing need for the field of experimental TBI to use additional behavior assessments, which could improve the behavioral test battery and therefore improve the assessment of therapeutic agents. A prime example of this comes from the current study in the bimodal split of the NAM-treated group. Clearly, NAM treatment alone is not sufficient for all animals to improve function on these cognitive measures. This increases our knowledge of drug capabilities and could be used to inform future experimental studies or to optimize clinical trial design. Without sensitive tests such as these, this may not have been realized until much later.

The literature on operant behavioral tests in TBI has been sparse for many years, with only a few articles using these robust techniques.^{31,32} Other researchers, however, have also begun to show interest in diversifying the test battery for TBI. Additional measures such as discrimination, attentional set shifting, fear learning, and social interactions could reveal novel deficits or novel treatment effects not observed previously.^{16–18,20,21} It should also be noted, however, that tests such as these can be considerably more training-intensive (35 days in the current study) than many other common behavioral assessments used in the experimental TBI field. While this may be seen as a large barrier for some researchers, the amount of data that can be collected, robustness of TBI-induced deficits, and high relevance to the human condition give strong reasons for pursuing these types of studies. In particular, this type of behavioral assessment may be useful once a treatment has been screened in other, faster tasks to verify improvements in cognition.

The results from this study can be expanded on in a number of ways. Because these operant learning tasks have shown considerable efficacy, other operant tasks could be investigated. Simple schedule-control, a hallmark of behavioral pharmacology and basic operant behavior, may reveal interesting deficits in these animals that have not previously been evaluated as well as provide the framework for more complex behaviors. Further, using these types of tasks will establish a stronger link between neuropsychological tests that are used in human patients with TBI and the behavioral assessments that are used in animal models.

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Address correspondence to:

Michael R. Hoane, PhD

Restorative Neuroscience Laboratory

Department of Psychology

Life Science II, MC 6502

Southern Illinois University

Carbondale, IL 62901

E-mail: mhoane@siu.edu