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13. SUPPLEMENTARY NOTES

14. ABSTRACT The goal of this project is to evaluate the hypothesis that traumatic brain injury induces alterations in the brain's reward circuitry which may make an injured brain more susceptible to the rewarding effects of opioids. We are currently conducting experiments to evaluate the hypothesis that TBI causes changes in the analgesic response to opioids following acute and repeated drug administration. We are secondly in the midst of testing the hypothesis that moderate TBI increases the susceptibility for opioid abuse as measured by an alteration in the rewarding properties of oxycodone. We have completed the first year of experimentation and thus far have found that the mean duration of transient unconsciousness in the animals that received TBI is consistent with a moderate injury. A trend toward increase tail withdrawal latencies was observed in the TBI group, but the number of animals per group is yet not sufficient for complete analysis. We have also observed a trend for differences between potency of oxycodone administration between TBI and sham rats. Trends for between groups differences were also seen in self-administration experiments. All studies are on-going.

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None

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Table of Contents

Introduction:

Progress Report for DoD Peer Reviewed Medical Research Program of the Office of the Congressionally Directed Medical Research Program FY10 Investigator-Initiated Research Award: Partnering PI Option Application entitled "Opioid Abuse after TBI"

This report was prepared by Candace L. Floyd, Ph.D. and Katherine L. Nicholson, DVM, Ph.D.

Our progress in completion of the aims is detailed below.   A brief description of the aims, the work conducted, and the data collected respective to the statement of work is provided after the original text of the aims and statement of work, which are denoted by bolded and italicized font, respectively.

Aim 1: Evaluate the hypothesis that moderate TBI causes changes in the analgesic response to opioids following acute and repeated drug administration.

Aim 2: Investigate the hypothesis that moderate TBI increases the susceptibility for opioid abuse as measured by an alteration in the rewarding properties of oxycodone.

Aim 3: Evaluate the propensity for development of physical dependence to opioids following moderate TBI.

Body:

Update on Tasks from Year 1 Statement of Work:

This project evaluating the impact of traumatic brain injury on the risk of opioid drug abuse will be completed in 3 years. Given the labor-intensive nature of certain components of the study, work on multiple specific aims will occur concurrently to utilize resources most efficiently. Tasks in year one will focus on preparation of the laboratory; testing of antinociception and tolerance production using the warm water tail withdrawal (WWTW) procedure; and determining acquisition of SA. The rats will be randomly distributed to TBI condition/behavioral procedure and oxycodone dose upon arrival

- *Dr. Floyd will travel to VCU and perform the injury procedures over 2 to 3 day periods on five occasions during the first year and on 3 occasions during subsequent years.*

     Dr. Floyd made 4 trips to VCU to perform injury procedures in the first year. The 5th trip was scheduled and then cancelled due to the fact that she broke her scaphoid bone in a fall wherein she landed on her outstretched hand. She is expected to be able to return to normal

hand/ wrist function (i.e. grasping and performing surgery) by Sept. 2012 but was unable to perform the injury procedure for 8 weeks that her hand was in a cast.

- *In the first year visits, Dr. Floyd will conduct the TBI procedure and train personnel at VCU to perform the procedure in order to permit larger numbers of animals to be prepared during years 2 and 3 as detailed below.*

Dr. Floyd conducted all the TBI procedures in the first year and has begun the training of the faculty and staff in the laboratory of Dr. Nicholson.

- *During the first two years, we will generate TBI subjects for studies to evaluate tolerance production using two pain models as well as acquisition of oxycodone self-administration.*

The team has this task well underway and the details of the animals conducted thus far are listed below.

- *The first 2 months were allotted for purchase of supplies, preparation of the laboratory and purchase, acclimation and catheter or infusion pump implantation in the first cohort, we will begin generating traumatically injured rats in groups of 21 to 27 every two months. The animal numbers described are based on a loss of 5% of subjects following sham brain injury and 20% loss following moderate injury.*

All necessary approvals (i.e. IACUC, occupational health) are in place. Additionally, the acclimation and catheter/infusion pump implantation protocols have been optimized and several subjects in the first cohort of animals have undergone the experimental testing paradigm using these techniques.

- *For the tolerance and dependence procedures, the goal is to generate 10 subjects per treatment condition as outlined in the research design for a total of 120 test subjects completing evaluation of the antinociceptive effects of oxycodone following acute and chronic administration and 60 subjects completing assessment of development of physical dependence.*

Many animals in this cohort have been included in the experiments thus far and details of the group numbers to data as well as a summary of the preliminary data are listed below.

- *The 10 subjects completing each treatment condition will be euthanized following the final oxycodone exposure for collection of brains for analysis. The total time required for acclimation, food training, injury and behavioral evaluation of these subjects is approximately 6 to 8 weeks.*

- Many animals in this cohort have been included in the experiments thus far and details of the group numbers to data as well as a summary of the preliminary data are listed below.

5

- *Each cohort of self-administration (SA) animals will require 35 to 60 days to complete testing depending on the aspect of SA being assessed. This includes time for acclimation to the laboratory and handling, catheterization surgery and recovery, brain injury and evaluation of acquisition, reinforcing efficacy or reinstatement to oxycodone SA. This time frame is based on exclusive use of 4 self-administration chambers in 2 or 3 runs/day = 8 to 12 animals/day. Therefore, 8 to 12 rats can undergo injury and be designated to a SA procedure every 6 to 8 weeks. For all SA studies, the numbers shown below reflect the number of animals entering the different SA paradigms. With an anticipated loss of ~20% of subjects due to premature loss of catheter patency (acquisition) and failure to acquire the baseline behavior (PR and reinstatement procedures) this will result in a total of 10 subjects/treatment group.*

Many animals in this cohort have been included in the experiments thus far and details of the group numbers to data as well as a summary of the preliminary data are listed below.

- *Analysis of structural change in brain regions associated with reward/risk circuitry including the nucleus accumbens, amygdala, hippocampus, and prefrontal-parietal white matter tracts ACUTELY after TBI in rats*

We have induced the TBI in these animals and are currently collecting serial sections of the brains in preparation for histochemical processing.

- *Begin histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor numbers and growth factors from rodent brains received from VCU relating to aims 1 and 2.*

Brains have been collected from VCU and are currently being sectioned for subsequent analysis.

- *Lead preparation of peer-reviewed manuscript(s) to report scientific discoveries obtained from analysis of TBI-induced alteration in reward circuitry, opioid neurotransmission and neurotrophic factors at ACUTE post-TBI time point*

These experiments are on-going and we expect completion of data sufficient for a manuscript in the upcoming months.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rats undergoing TBI	Laboratory/ study setup		21		21		21		21		21	
Rats Surviving TBI			~18		~18		~18		~18		~18	
Rats→ SA Acquisition			8		8		8		8		8	
Rats→ WWTW/ TOL			10		10		10		10		10	

By end of year one:

- o 50 rats will have completed testing in the antinociception/tolerance study. At the end of the study, these subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB for analysis.

- o 40 subjects will have entered evaluation of acquisition of oxycodone self-administration, with an anticipated 32 completing acquisition assessment.

<u>*1. Overview of milestones completed and in progress*</u>

Table 1. Shown is the distribution of animals entered into the study during project Y1(from 07/01/12 through 06/30/12).

Total # subjects entered into protocol	Total number catheterized	Total number undergoing sham injury	Total entering acquisition procedure	Total completing acquisition assessment
	=55	=17	=17	=14
		Total number undergoing TBI	Total entering acquisition procedure	Total completing acquisition assessment
=88		=38	=23	=21
	Total number implanted with infusion pumps	Total number undergoing sham injury	Total number entering antinociceptive testing (WWTW)	Total completing dosing
	=33	=18	=18	=18
		Total number undergoing TBI	Total number entering antinociceptive testing (WWTW)	Total completing dosing
		=14	=14	=13

Animal loss included:

- 1 subject removed/destroyed subcutaneous pump just prior to scheduled injury
- 15 subjects lost during fluid percussion injury
- 4 subjects due to loss of catheter patency
- 1 subject due to pump malfunction

<u>Animal use relative to SOW designated milestones:</u>

- Total number of subjects entered into project = 87 (goal 105)
- Total number surviving head injury procedure = 74 (goal 90)
- Completed testing in SA = 35 (goal 48 enter, 32 finish)
- Completed WWTW = 25 completed testing and 31 completed dosing (goal 50)

As mentioned early in this report, Dr. Floyd's final visit to VCU in Y1 was canceled due to an injury precluding her performing the craniectomies. While Drs. Nicholson and Shelton have undergone training over the course of Y1, their level of skill/experience was not sufficient at this time to permit produce TBI in animals for inclusion in the study. We anticipate being able to compensate for this small setback in the coming year through:

- Drs. Shelton and Nicholson being able to contribute to the numbers injured during each visit. We have multiple stereotaxic frames available at VCU and can set up multiple surgical stations to permit them to perform the procedures concurrently with the oversight of Dr. Floyd.
- As a commitment to this project and this collaborative research, the Department of Pharmacology/Toxicology has purchased a fluid percussion device for Dr. Nicholson. This will permit Dr. Floyd to fly versus transport the device with her as in the original proposal and make more frequent short visits in year 2 to get the numbers back on track.

2. Subjects:

Adult male Sprague Dawley rats were purchased from Charles River at age/size ranges predicted to result in body weights of 300-350 g at time of injury. Self-administration subjects were purchased approximately 2 weeks prior to injury permitting time for 7 days of handling and acclimation before intravenous catheterization was performed (see below). Subjects to be used in the warm water tail withdrawal procedure (WWTW) were delivered minimally 3 weeks prior to scheduled injuries in order to fully acclimate them to the restraint tubes.

3. Fluid percussion Injury:
All subjects underwent fluid percussion injury following handling, training and surgical instrumentation for subsequent behavioral procedures. Lateral fluid percussion will be induced in adult rats as previously described (Floyd et al., 2002).Anesthesia was induced and maintained with 4% isoflurane. The subjects were surgically prepared and transferred to a stereotaxic device for craneictomy and continued to be maintained under isoflurane anesthesia.

Description of Procedure. An incision (~8mm) was made in scalp and fascia scraped from the skull. A point mid-way between Bregma and Lambda and central suture/lateral ridge was marked on the medial skull surface with sterile tissue marker. A 4.8mm craniotomy was cut with a trephine by hand over the right motor cortex. An injury cannula was fashioned from the hub of a female leur-lock 20g needle by affixing the plastic tube to the skull with glue and securing with dental acrylic. After the acrylic hardened (15 minutes), the injury cannula was filled with sterile saline, and the brain injury induced by compressing the sterile saline with the fluid percussion device(Custom Design and Fabrication, VCU, Richmond, VA) controlled to deliver an equivalent impact to each animal of moderate (2.5ATM) severity. After induction of TBI, the

scalp was sutured w/ 4-0 PDS and the animal was returned to a clean, warmed, home cage when ambulatory. Sham control animals underwent all procedures with the omission of the fluid percussion pulse.

Post-TBI analysis of transient loss of consciousness. Analysis of righting reflex suppression is an indicator of duration of loss of consciousness after TBI. Counting of time until return of consciousness began immediately after the percussion injury.   When a conscious rat is placed on its back, it will flip to its feet or "right" itself (Floyd et al., 2002).   Time to return of righting reflex after TBI was recorded and used as an indicator of loss of consciousness, a valid measure of injury severity. As shown below (Table 2, Figure 1), the loss of consciousness for the subjects undergoing a lateral fluid percussion injury of moderate severity was ~2-fold longer than that for sham injured subjects. Comparison using the student's t test verified a significant difference between injury groups ($p < 0.001$).

Table 2. Righting times across injury groups with SD and SEM calculations. Data for all subjects was within 2 standard deviations of the mean for their respective groups.

	Moderate Injury	Sham
Mean Righting time (sec)	740.9	300.7
St Dev	270.1	77.9
SEM	44.4	12.6

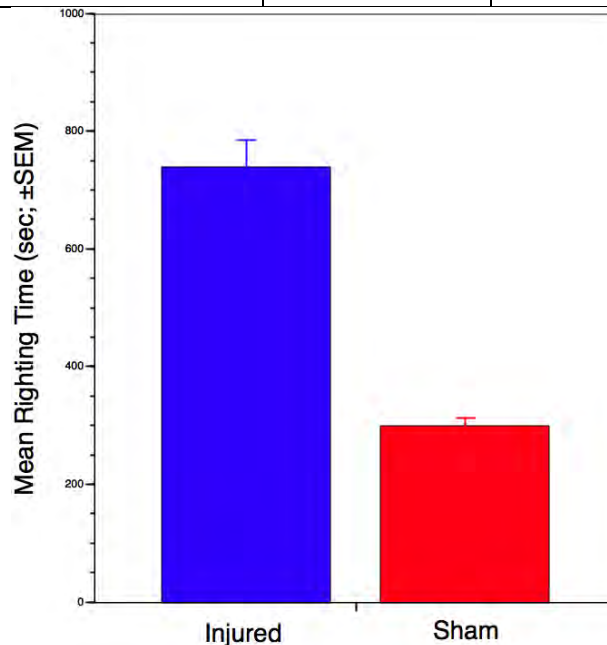Figure 1. Shown are the mean righting times (sec; ±SEM) for all subjects undergoing either a lateral fluid percussion injury of moderate severity (Injured) or a sham injury (Sham).

Following injury, subjects were monitored closely and recovery performance recorded for 5 days. All subjects received 3 ml saline containing enrofloxacin SC, daily for 3 days. After 5 days, subjects began evaluation for the behavioral effects of oxycodone towards completion of Aims 1 and 2 as described below.

4. Tasks performed specific to Aim 1 – evaluation of acute antinociceptive effects of oxycodone and development of tolerance using a WWTW procedure

The first slated studies towards achieving this aim involved use of a warm water tail withdrawal procedure. Basically, the subjects were habituated to placement in a specially designed rodent restraint tube (Braintree Scientific, Braintree, MA) with their tails hanging freely out the caudal end. The distal 5 cm of the tail was immersed in containers of water of different temperatures. To qualify for testing the rat must leave his tail immersed in 40^0 C water for 15 sec during 3 repeated exposures with 2 min between exposures. This is a non noxious stimulus and once the animals have been habituated to the restraint and to the sensation of tail immersion, almost all subjects qualify. During testing, the subjects were exposed to noxious water temperatures of 50^0 and 55^0, consistent with our previous work (Morgan and Nicholson, 2011) and latency to withdraw the tail recorded. Through the use of cumulative dosing, an entire oxycodone dose effect curve was determined over approximately 2 hours.   To achieve the goals of the grant, the subjects were slated to undergo the following in order:

1. Habituation and training in the procedure.
2. Implantation of preprogrammed mini pumps.
3. Sham or lateral fluid percussion injury.
4. Determination of an oxycodone dose effect curve.
5. Repeated daily dosing with oxycodone via the mini pump.
6. Redetermine the oxycodone dose effect curve after 5 days of repetaed dosing.
7. Continue repeated dosing for an additional 5 days.
8. Determine a final oxycodone dose effect curve.
9. Collect brain tissue for histology and biochemistry at UAB.

Table 3. Shown are the numbers of subjects assigned to different injury and chronic dosing conditions across Y1

Total number implanted with infusion pumps =33	Total number undergoing sham injury	Chronic Dosing Assignments		Total completing testing		Total Completing Dosing	
		Saline	Oxycodone	Chronic Saline	Chronic Oxycodone	Chronic Saline	Chronic Oxycodone
	=18	12	6	10	4	12	6
	Total number undergoing TBI	Chronic Dosing Assignments		Total completing testing		Total Completing Dosing	
		Saline	Oxycodone	Chronic Saline	Chronic Oxycodone	Chronic Saline	Chronic Oxycodone
	=14	6	8	5	6	6	7

As shown in Table 3, not all subjects completed testing at all time points across the 17-day dosing period. Despite several years of previous experience with this model using the same methods, we experienced unexpected complications during the course of this study. Basically, a significant number of the subjects developed lesions on the distal 0.5cm of their tails following the initial dose effect curve determination. These did not appear until 72-96 hours after the tail immersion and prevented many of the subjects from testing in the subsequent scheduled dose-effect curve determination(s). The hyperalgesia induced by the tail inflammation resulted in ~50% of subjects failing to qualify for testing on day 11 post-TBI. Most subjects were able to test at the completion of the study on day 17 post-TBI and they account for the numbers in Table 3. Regardless of qualifying for WWTW testing, all subjects were dosed equivalently, e.g., any subject that did not qualify for testing remained in the restraint tubes and received injections of oxycodone, but did not have tail immersion performed. In this way, the drug exposure remained constant across all groups and brain tissue could still be collected and used for histology and biochemistry at the end of the study. At no time other than during tail immersion did the subjects appear affected by the tail lesions, all remained active and gained wait and were amenable to handling. We have used different approaches to mitigate the development of the inflammation and have improved the recovery rate and thereby limited the loss of subjects from the final dose effect curve determination. We suspect the problems we are having may be due to the priming of the immune/inflammatory response by the preceding surgeries/brain injury and the presence of the subcutaneous pump resulting in an enhanced inflammatory reaction to what is typically a very limited level of injury to the tail. Given this, in order to ensure appropriate assessment of the antinociceptive effects of oxycodone, we believe it may be necessary to modify our approach. Several options we have considered are:

- Decreasing the cut off time to 12 sec to further reduce any potential tissue damage
- Lengthen then time between cumulative dose-effect curve determinations.
- Use a between subjects design.

Any changes will be thoroughly discussed with the project officer and the animal use protocol amended prior to instituting any changes.

<u>Pump programming and implantation.</u> Five days prior to induction of TBI, a programmable microinfusion pump (iPrecio system, Data Sciences International, St. Paul, MN) was implanted subcutaneously under isoflurane anesthesia. The guidance provided by the manufacturer for surgical implantation, in our hands, was not optimal. Over the progress of several cohorts of rats, we have evaluated different approaches and believe we have found the best location and procedure for 1) ensuring pump integrity and 2) minimizing seroma development. The rats were induced and maintained on 3% isoflurane anesthesia. They were placed in sternal recumbency and a 2-3cm surgical incision was made longitudinally through the skin just caudal to the scapulae. A pocket for the infusion pump was made using blunt dissection directed craniad. The pump was inserted in the pocket and the subcutaneous catheter tubing extending from the pump reservoir attached to a small trocar. The trocar was routed caudad and exteriorized trough a

small (2mm) skin incision in the lumbar region. This distal end of the tubing was disconnected from the trocar and allowed to retract under the skin. The pump itself was secured to the surrounding fascia and musculature with two 4-0 PDS stay sutures. The dorsal skin incision was closed with wound clips. The pumps were programmed to run with a continuous flush of saline at a rate of 0.2 µl/hour from the time of implantation until chronic dosing beginning on day 5 post-TBI. Following determination of the oxycodone dose effect curve on that day, the pump reservoir was filled with saline (control group) or an oxycodone solution which provided the ED_{50} dose determined earlier in the day in a 30 µl volume. Every 6 hours (approximately 0600,1200,1800 and 0000 hours), 30 µl of saline or oxycodone solution was released in order to mimic clinical exposure. During the intervening hours, the pump continued a low level (0.2µl/hour) flush to maintain patency of the pump tubing. The one exception to this schedule was on day 11 post-TBI, the 1200 dose was deleted from the program in order not to confound determination of the second dose effect curve. Dosing stopped after the 0600 dose on day 17 post-TBI to permit determination of the final dose effect curve. Pump reservoirs were readily palpated and refilled as needed across the 17-day period.

Results to date. Figure 2 presents the baseline tail withdrawal latencies for the brain injured and sham injured subjects at the two water temperatures. It should be noted that because subjects are added across groups randomly, we have not achieved our final group sizes for any of the test conditions. Therefore, limited statistical comparisons have been performed and no final conclusions can be drawn at this point. Baseline latencies were all within normal ranges across all points. While there was variability at the 11-day post TBI time point, this is likely a reflection of the limited number of subjects that qualified for testing at that point. There were no significant differences between baselines latencies within each injury condition across the three test days. Latencies were significantly different between groups at the 11 days post-TBI test day, this in part may reflect the smaller number of subjects that qualified for testing at this point. As predicted, the latencies at 55^0 were significantly shorter than the corresponding latencies at 50^0 reflecting the greater intensity of the noxious stimulus. Baseline latencies were used to calculate %MPE = [(test latency - control latency)/ (cut-off time - control latency)] X 100% for each test point. Within each injury group, there was no significant difference between baseline latencies across test days, although the latency was significantly different between the sham and the TBI groups during determination of the second dose effect curve. Because so few subjects qualified for testing after the first 5 days of chronic dosing, the data are not presented in the remaining graphs.
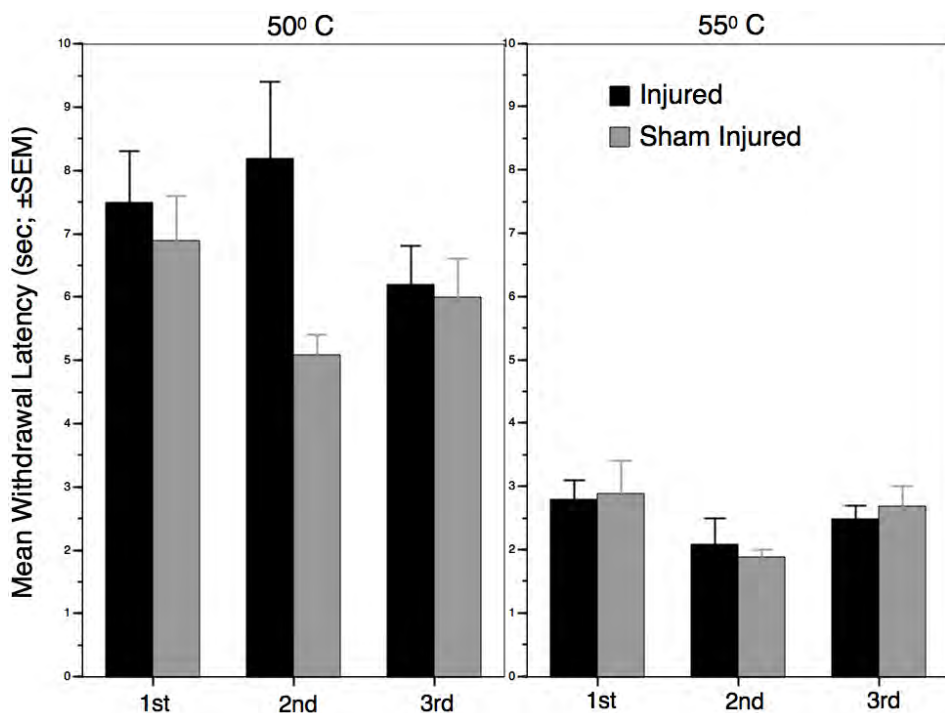
Figure 2. Shown are the baseline tail withdrawal latencies for injured and sham injured subjects 50^0 and 55^0 C water. The latencies are measured 15 min after a saline injection and serve as control values for determination of the % maximum possible effect for all test points following oxycodone administration. The data were collected during determination of an oxycodone dose effect curve on days 5 post TBI (1st - acute dosing); and on days 11 (2nd) and 17 (3rd) after exposure to 5 and 10 days of oxycodone dosing, respectively. Only 12 of 31 subjects met criteria for testing on day 11 post TBI and 25 of 31 subjects met criteria for testing on day 17.

Firgure 3 presents the oxycodone dose effect curves generated for rats before (post-TBI day 5; blue squares) and after (post-TBI day 17) chronic dosing with either saline (red circle) or oxycodone (open diamonds). The ED_{50} dose of oxycodone at 55^0 C stimulus (based on the initial dose effect curve) was administered SC via programmed iPrecio mini pumps 4 times daily. As can be seen, for all treatment/injury conditions oxycodone produced a dose-dependent antinociceptive effect at both stimulus intensities. What does vary is the relative potency in producing these effects. As expected, across all treatment/injury conditions, the potency of oxycodone in producing these effects was greater at the lower intensity stimulus. ED_{50} values for oxycodone under baseline conditions (before chronic dosing) were generated using nonlinear regression analysis of the linear portion of the curves. The ED_{50} at the 55^0 stimulus intensity, 1.2 mg/kg (see Table 4) was used for chronic dosing. Following chronic dosing with oxycodone, the dose effect curves were shifted to the right indicating that oxycodone was less potent in producing its antinociceptive effects and suggesting tolerance had developed. However, the dose effect curves following saline dosing were similarly shifted to the right. It's uncertain why this occurred. An initial concern is that this is a reflection of the tail inflammation problems we are seeing, that the rats are experiencing hyperalgesia and therefore even the saline treated subjects require higher doses of oxycodone to block nociception. However, while slightly lower, baseline

14

latencies were not significantly lower than the baseline latencies thereby arguing against a hyperalgesic state. Another possibility is that neuroadaptation occurred even after the limited exposure to oxycodone. Acute tolerance has been reported in humans and nonhuman subjects and is often referred to as acute opioid-induced hyperalgesia (Chu et al., 2008, Lee et al., 2011). However, typically this phenomenon dissipates over approximately 24 hours. Testing of the remaining subjects will tell us whether this effect will persist. Preliminary statistical comparison of ED_{50} values (Table 4) shows that at this point in the study, the diminished potency was significant following oxycodone administration in the brain injured subject and after saline administration in the sham injured subjects. Testing in sham injured subjects after oxycodone administration only showed a significant shift when tested at 55^0. The lack of significance for the 50^0 stimulus is likely a reflection of a high level of variability in responding in this group. When comparing across injury condition, the oxycodone dose effect curves were remarkably similar as shown in Figure 4. Only under acute (baseline) testing at 50^0 C was there a significant difference in the potency of oxycodone with sham injured subjects demonstrating greater response to oxycodone doses. Overall, based on the data generated to date, there does not appear to be a difference in the acute antinociceptive effects of oxycodone or in the development of tolerance to those effects between sham and brain injured subjects.

Table 4. Shown are the ED_{50} values for oxycodone's antinociceptive effects across both injury conditions prior to and after repeated dosing with oxycodone (1.2 mg/kg) or saline.

Test Time point	Baseline		Post Chronic Saline		Post Chronic Oxycodone	
Water Temp (^{0}C)	50^0	55^0	50^0	55^0	50^0	55^0
ED_{50} in Injured Subjects (mg/kg)	0.55[#]	1.25[&]	1.24	2.10	1.33*	2.87*[&]
95% CL	0.43-0.69	1.06-1.47	0.69-2.21	1.44-3.08	0.83-2.14	2.21-3.73
ED_{50} in Sham Subjects (mg/kg)	0.35	1.15[&]	0.97*	2.00*[&]	1.00	3.66*
95% CL	0.32-0.39	1.01-1.30	0.70-1.34	1.35-2.98	0.37-2.77	2.74-4.89

*significantly different from baseline value at p <0.05.
significantly different from sham injury at p <0.05.
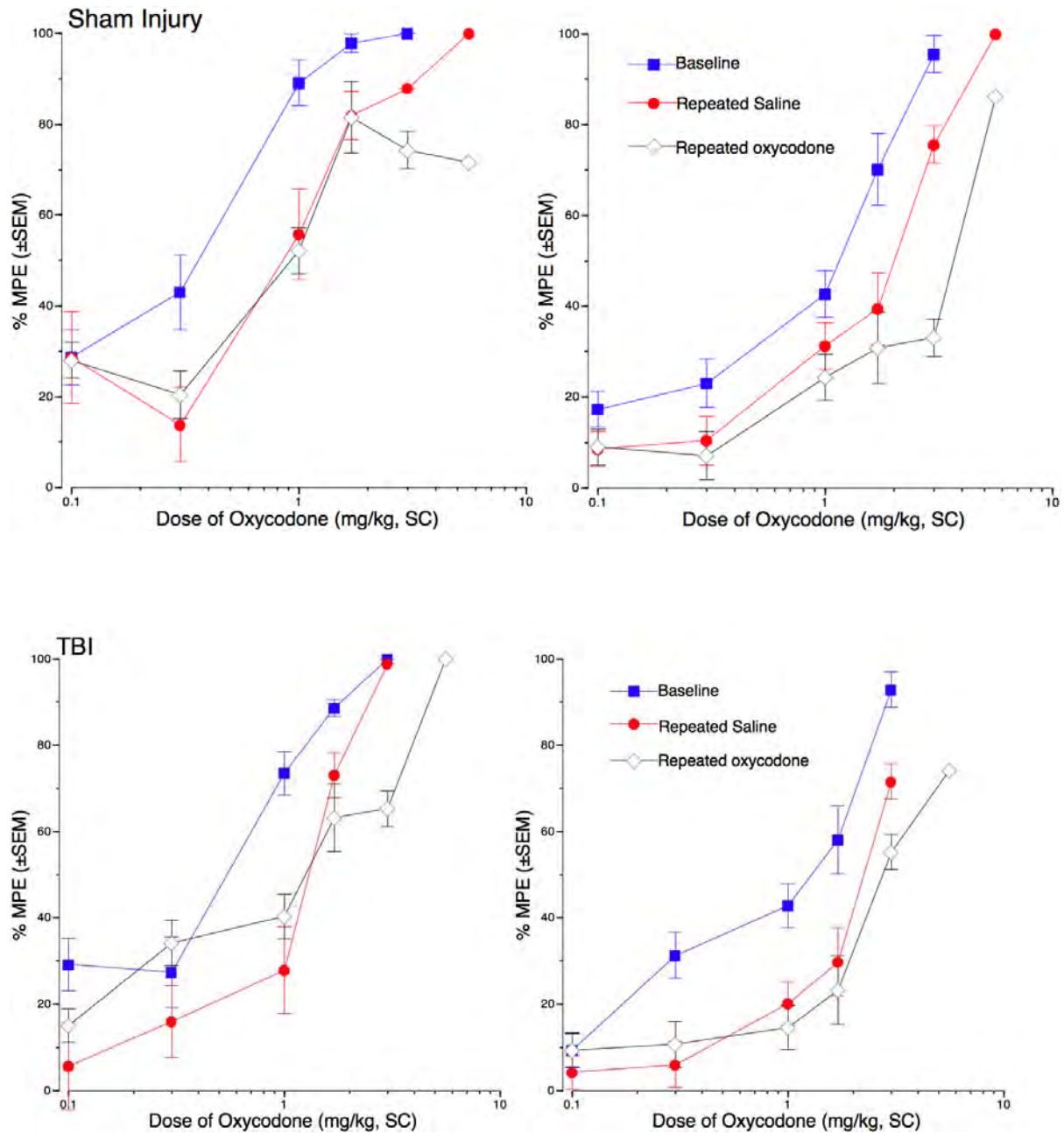& significantly different from 500 C at p <0.05

Figure 3. Shown is the percent maximum possible effect for oxycodone antinociceptive effects in sham injured (top panels) and moderately brain injured (bottom panels) rats at 50^0 (left panels) and 55^0 (right panels) C.
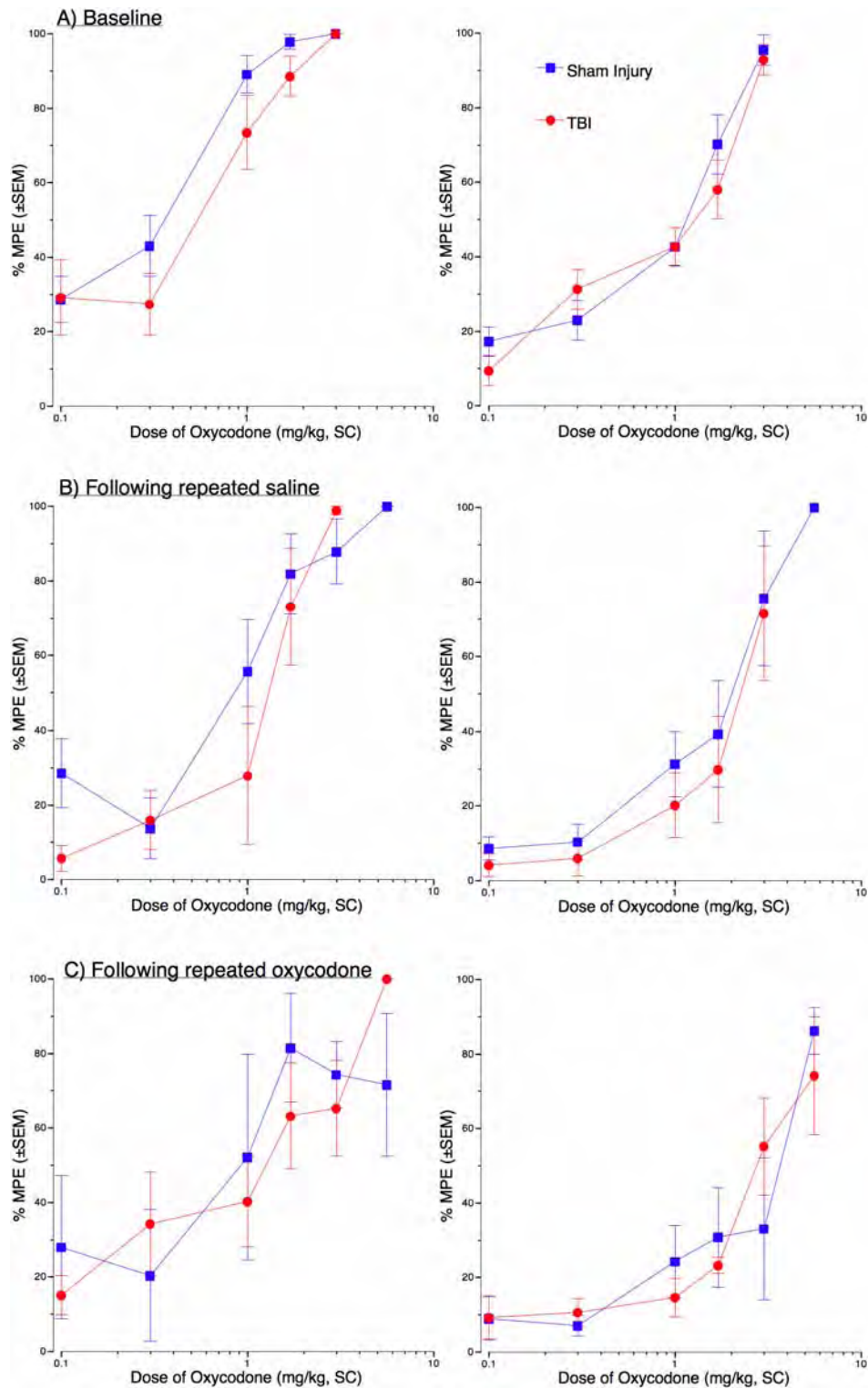
Figure 4. Shown are dose effect curves for oxycodone antinociceptive effects for sham injured (blue squares) and brain injured (red circles) across different dose effect curve determinations at 50^0 and 55^0)

Tissue sample collection. 48 hours after the final dose effect curve determination approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining 50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 5. Details of samples prepared and sent to UAB:

Injury Condition	Chronic Treatment	Preparation	Number
Sham injured	Saline	Frozen	4
Sham injured	Saline	Perfused	5
Sham injured	Oxycodone	Frozen	2
Sham injured	Oxycodone	Perfused	4
Injured	Saline	Frozen	4
Injured	Saline	Perfused	2
Injured	Oxycodone	Frozen	6
Injured	Oxycodone	Perfused	4

5. Tasks performed specific to Aim 2 assessing the rewarding properties of oxycodone in injured versus sham injured subjects

The first slated studies towards achieving this aim assessed acquisition of oxycodone self-administration. Upon arrival animals were acclimated to handling and the laboratory environment. Five days prior to injury, animals underwent surgical implantation of a chronic indwelling venous catheter under isoflurane anesthesia with morphine pretreatment. A surgical incision was made longitudinally through the skin above the jugular area. The underlying fascia was bluntly dissected and the external jugular vein isolated and ligated with 5-0 silk suture. A small cut was made into the vein using an iris scissors and the catheter introduced into the vein to a level near but not into the right atrium. The vein encircling the catheter was then tied with 5-0 silk suture. A second suture was used to anchor the catheter to surrounding fascia. The rat was then placed ventral side down on the surgical table and a 2 cm incision was made 1 cm lateral from mid-scapula. A second 0.3 cm incision was then made mid-scapula. The distal end of the catheter was passed subcutaneously from the ventrum (vein cannulation area) to the larger dorsal incision and attached to the cannula/connector. The cannula/connector was then inserted subcutaneously through the larger incision while the upper post portion of the connector/cannula exits through the smaller mid-scapular incision. All incisions were sprayed lightly with a gentamicin/ betamethasone valerate topical antibiotic and the larger dorsal incision and ventral neck incision closed with michel wound clips. Catheters were flushed daily with amoxicillin/subalactam (20/10 mg/kg) in a saline/glycerol/heparin solution to enhance catheter

longevity. Periodic infusion of 7.5mg/kg ketamine IV was used to verify catheter patency by presence of immediate onset of sedation.

As shown in Table 5 below, 35 subjects completed testing in the self-administration acquisition procedure. Five days following TBI or sham injury described above, subjects began daily self-administration testing conducted in standard operant chambers housed inside isolated and ventilated enclosures (Med Associates). Each chamber is equipped with two response levers with a white stimulus light above each lever, a 5-watt house light in the rear wall and an adjustable Sonalert (Model ENV-223AM, Med Associates) in the upper left wall.   During each session, infusion tubing, protected by a stainless steel spring tether (Plastics One), was connected to the back-mounted cannula pedestal. Infusions were delivered via a peristaltic pump located outside each chamber.   Schedule parameters were controlled by MED-PC IV software (Med Associates) running on a PC compatible computer. Rats were brought to the laboratory daily (7 days/week) and allowed to acclimate for 15-30 min before being connected to the infusion tether and placed in the chamber for the 2-hour acquisition session. During the session, a single response, fixed ratio (FR) 1, on the right lever resulted in the delivery of a 0.1-ml, 3-sec infusion of one of the three oxycodone doses as shown in Table 5. Responding on the left lever had no scheduled consequence but was recorded as a measure of behavioral activity in the chamber. Criteria for acquisition was three consecutive days of receiving > 15 infusions and responses on the active lever > responses on the inactive lever. Subjects were permitted up to 21 sessions to achieve criteria.

Forty subjects began acquisition testing and 35 completed testing, the remaining 5 (3 sham, 2 injured) lost catheter patency rpior to 21 days Of the 35 (21 injured, 14 sham) that completed acquisition testing, 7 (3 injured, 3 sham) failed to acquire self administration behavior. As shown in Figure 5, a greater percentage of injured than sham subjects acquired self-administration behavior at the intermediate and high dose of oxycodone. However, given the low number of subjects in the sham injured groups, the differences at this point are not significant. As expected, the 0.03 mg/kg/infusion dose showed the highest percent acquisition in both groups. The lowest dose, 0.003 mg/kg/infusion, resulted in higher percent of subjects acquiring the behavior than we had predicted, we had estimated 40-50% but as shown the acquisition rate was in the 60-80% range. What did not show considerable difference between the injury conditions or between the infusion doses was the mean rate of acquisition as shown in Figure 6. Analysis of the daily cumulative acquisition percent for the sham and injured subjects as shown in Figures 7 and 8 provides additional insight into the patterns for acquisition. At this point in testing, the primary difference exist at the intermediate dose, addition of the necessary subjects as scheduled in year 2 will be important to determine if this effect persists.

Table 5. Shown are the numbers of subjects assigned to different injury and dosing conditions across Y1

Total number catheterized	Total number undergoing sham injury	Total entering acquisition procedure	Total completing acquisition assessment	Oxycodone Dose (mg/kg/infusion)		
				0.003	0.01	0.03
=55 (24+25+6)	=17 (8+9)	=17 (8+9)	=14	4	4	6
	Total number undergoing FPI	Total entering acquisition procedure	Total completing acquisition assessment	Oxycodone Dose (mg/kg/infusion)		
				0.003	0.003	0.003
	=38 (16+16+6)	=23 (11+8+4)	=22	6	8	7

Following acquisition, the rats were allowed to continue self-administering the acquisition dose until performance was stable. Stability was defined as 3 days in which number of infusions did not differ from the mean for the 3 days by more than 20% with no increasing or decreasing trends. The mean number of infusions across those three days is shown in Figure 9. Dose dependent differences are present between the two injury groups with the sham injured animals maintaining highest levels of responding at intermediate dose of oxycodone. In addition, there was an inverted U-shaped dose-effect curve relating dose per infusion to infusion rate for the sham injured rats. This type of dose-effect relationship is typical of drugs effective as positive reinforcers (Young and Herling, 1986). For the brain injured subjects there was a dose-dependent increase in infusions across the three oxycodone doses. While this does not demonstrate the inverted U-shaped distribution, it is very possible we are seeing the ascending limb of the inverted-U. Such would be the case if oxycodone were less potent in producing it reinforcing effects in the brain injured subjects. While that might seem to suggest that the injured animals are less vulnerable to oxycodone abuse, studies have shown the exact opposite to be true for many drugs of abuse. A decreased sensitivity to a drug of abuse has been associated with 1) decreased potency for production of aversive effects which may limit intake 2) increased levels of self administration (clinical and preclinical) in order to achieve intoxication resulting in higher levels of receptor occupancy and greater occurrence of drug induced neuroadaptations. Thus a decreased sensitivity to a drug of abuse can actually be associated with an increased vulnerability to abuse and addiction. However, additional subjects, as scheduled for Year 2, must be added to the study to verify the current findings and increase the statistical strength of the comparison.
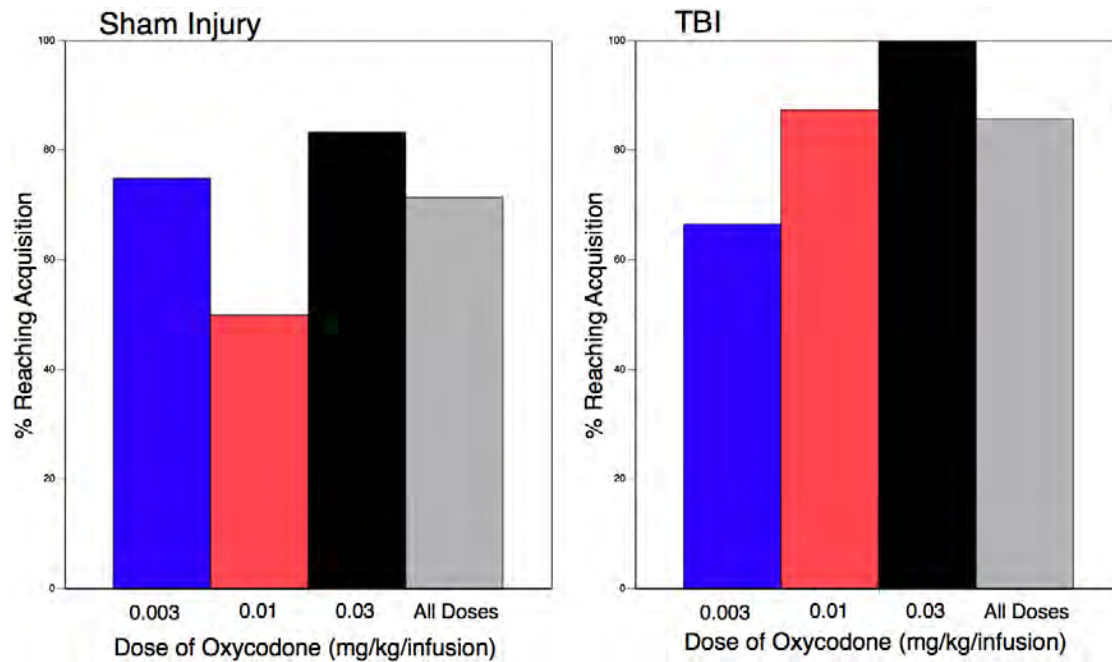
Figure 5. Shown are the percent of subjects in each injury group acquiring self-administration behavior across the different oxycodone doses and for all doses. (n=14 for sham injured subjects, n=21 for TBI subjects).
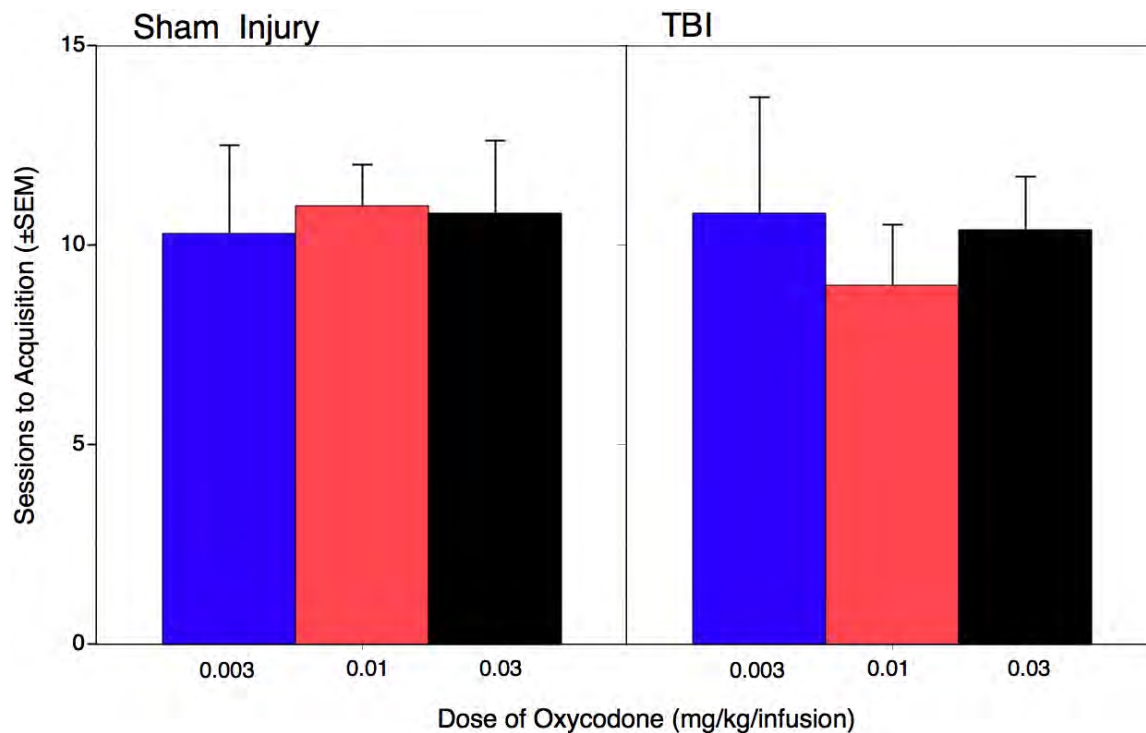

Figure 6. Shown are the mean number of days to achieve self administration acquisition criteria for sham (left panel) and brain injured (right panel) subjects subjects across the three oxycodone doses.
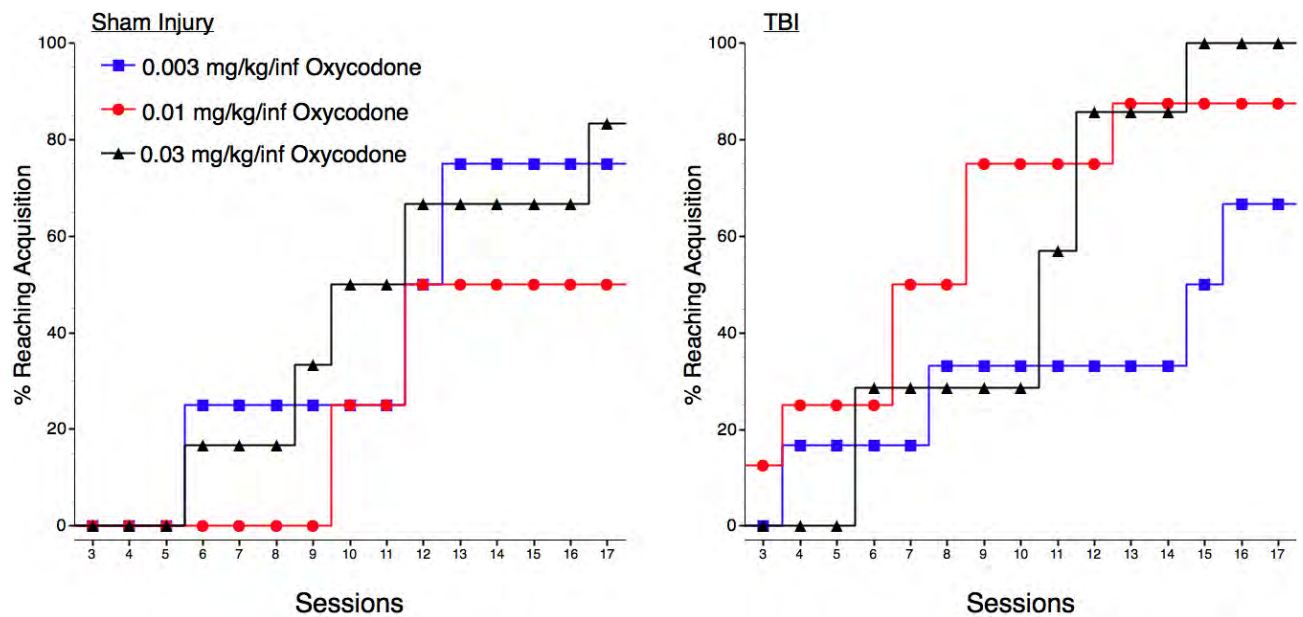
Figure 7. Shown are the cumulative percentages of subjects acquiring self-administration behavior across sessions for sham injured (left panel) and brain injured (right panel) rats across the three oxycodone doses.
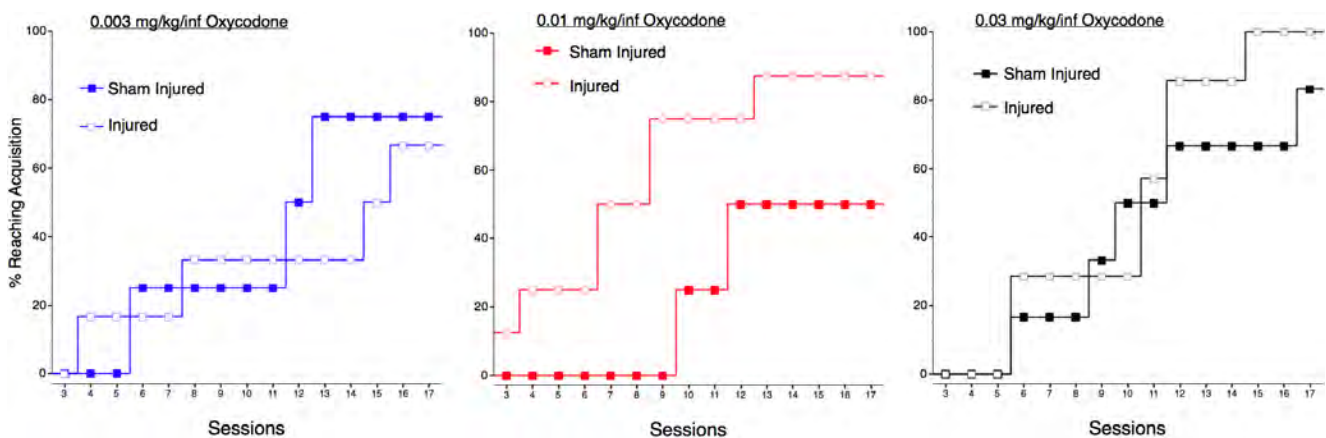

Figure 8. Shown are the cumulative percentages of subjects acquiring self-administration behavior across sessions for sham injured and brain injured rats for each of the three oxycodone doses.

The primary dependent measures collected during acquisition testing included the number of days to meet the acquisition criterion, the percentage of rats per group meeting the criteria, and the number of infusions received following stabilization of responding. However, data regarding a number of other parameters were also routinely collected. For example, responding on the

22

inactive lever was recorded and was found to be higher for the brain injured subjects than for the sham injured subjects (Figure 10). This could reflect a couple potential important differences between our injury groups. This could suggest that the brain injured subjects have a higher general activity level. Higher levels of nonspecific behavior (behavior not directed towards oxycodone self-administration) result in increased inactive lever responding either. If this is the case, the generally higher activity level in the injured subjects may be due to higher baseline activity or could be due to a greater drug-induced increase in activity levels in brain injured subjects. Another potential cause for the higher inactive lever responding is a deficit in learning. During acquisition, subjects must learn that responding on one lever results in drug infusion and presentation of infusion-associated cues whereas responding on the other lever has no consequence. In rats that ultimately acquire self-administration behavior, typically at the onset of acquisition, high levels of responding are emitted on both levers and over days, responding will shift more and more to the reinforced lever. Given that brain injury can result in impaired learning and memory, it may be that the injured rats do not learn to distinguish that one lever is reinforced and one is not as readily as the sham injured subjects and thus continue to exhibit higher levels of incorrect behavior. Further analysis of response patterns across the session, response levels on the three days defining acquisition and responding on the levers during the timeout period immediately after infusion delivery is ongoing and may provide additional insight into subtle but important differences in self-administration behaviors induced by brain injury.
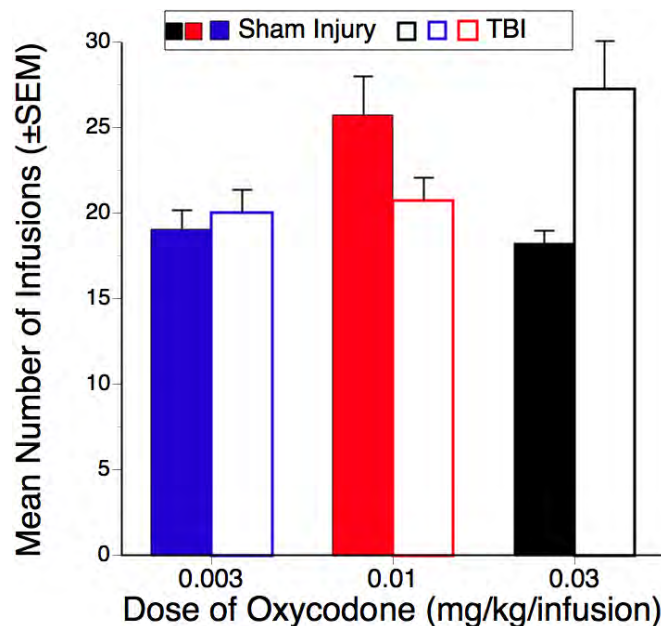

Figure 9. Shown are the mean number of oxycodone infusions self-administered across three days of stable responding comparing levels for sham injured (filled bars) to brain injured (open bars) rats across the three acquisition doses.
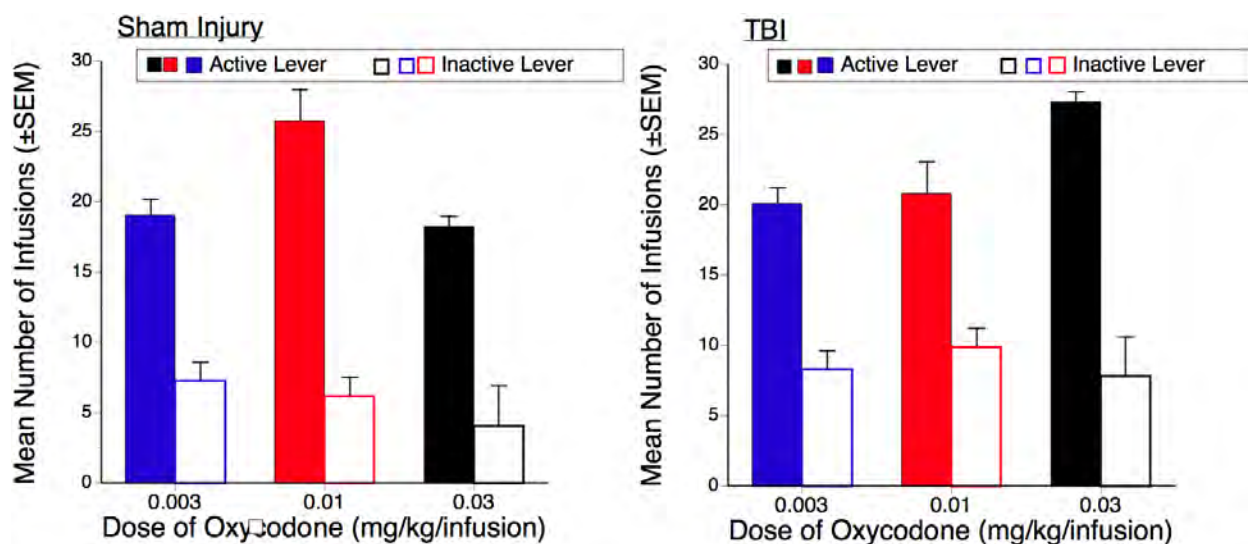
Figure 10. Shown are the mean number of lever responses emitted on the active (closed bars) and inactive (open bars) levers during three days of stable responding for oxycodone self administration for sham injured (left panel) and brain injured (right panel) rats.

Tissue sample collection. 48 to 72 hours after the final self-administration, session approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining 50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 6. Details of tissue samples prepared and sent to UAB

Injury Condition	Preparation	Number
Sham injured	Frozen	7
Sham injured	Perfused	7
Injured	Frozen	11
Injured	Perfused	10

Progress on year 1 tasks to be conducted exclusively at UAB:

YEAR 1:

- Task 1: Optimization of techniques for analysis of structural change in brain regions associated with reward/risk circuitry including the nucleus accumbens, amygdala, hippocampus, and prefrontal-parietal white matter tracts after TBI in rats
  - o Obtain and optimize protocols for the analysis of cell death/ gliosis, DA signaling, opioid receptor number and growth factors in rat brain tissue.

24

This task is on-going. We are in the process of completing this task and have made good progress. We have collected brain tissue from the regions associated with reward/risk circuitry including the nucleus accumbens, amygdala, hippocampus, and prefrontal-parietal white matter tracks in uninjured rats and those that received a TBI. Tissue has been collected at 24 hours post-TBI using micropunches.

With regard to cell death, we have first been examining markers of programmed cell death, apoptosis. The markers of apoptosis that we have been evaluating include procaspase 3, caspase 3, and caspase 9. These markers are catalytically active and thus often difficult to detect in brain tissue. Our key issue at this time is the signal to noise ratio. We are working to decrease the non-specific binding by increasing the blocking, the wash times, and adjusting the primary and secondary antibody concentrations. Another area that we are working to optimize is separation of bands in transfer. We are optimizing this by evaluating different gradients of gels and transfer parameters. Examples of Western blots are included below:
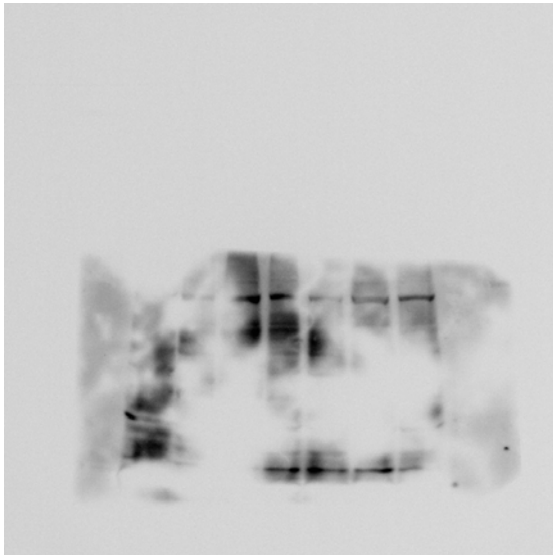

Figure 1: Example of Western Blot in Optimization: transfer

This is a Western Blot using an antibody against caspase 3. There were issues with transfer. These have been addressed in subsequent development and optimization steps.
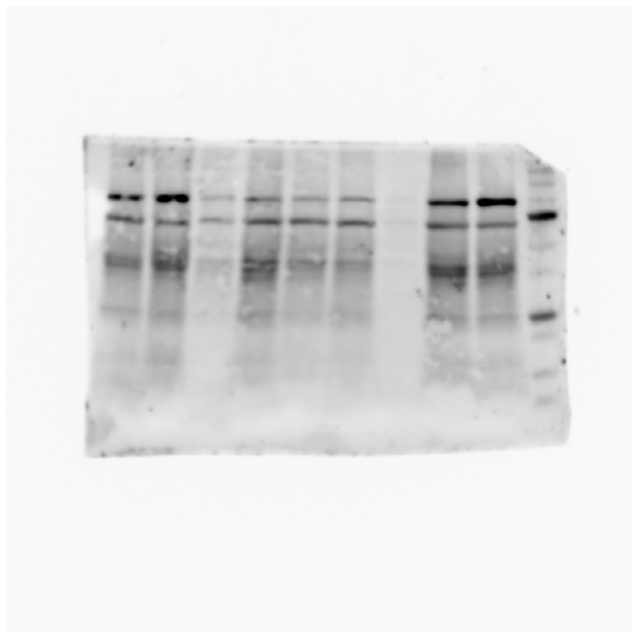
Figure 2: Example of Western Blot in Optimization: Non-specific binding

This is a Western Blot using an antibody against caspase 3. There were issues with non-specific binding and signal to noise ratio. These issues are currently being addressed in subsequent development and optimization steps.
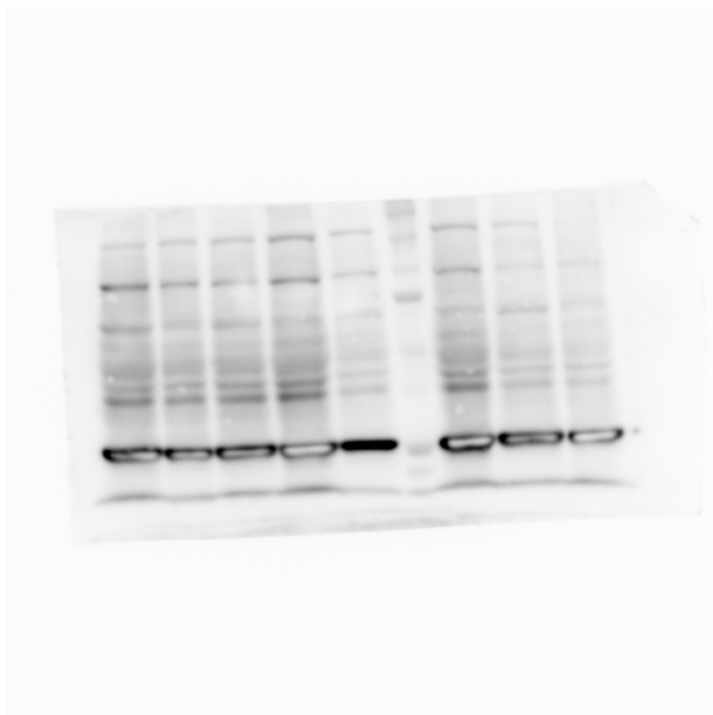

Figure 3: Example of Western Blot in Optimization: Non-specific binding

This is a Western Blot using an antibody against caspase 3. There were issues with non-specific binding and signal to noise ratio. As illustrated, this is a better blot with regard to transfer and signal clarity, however, there is still a high level of non-specific binding. These issues are currently being addressed in subsequent development and optimization steps.

Next we have been examining protein markers of necrosis in the various brain regions. We are examining the high-mobility group protein B1 (HMGB1). This is an intracellular protein which is known to interactive with p53 and is highly up-regulated in necrosis.
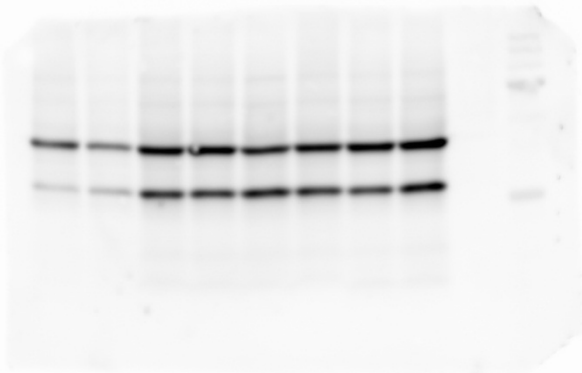

Figure 4: Example of Western Blot in Optimization: Non-specific binding

This is a Western Blot using an antibody against HMGB1. There were issues with non-specific binding and signal to noise ratio. We are currently examining altering the primary antibody concentration to increase the signal to noise ratio.
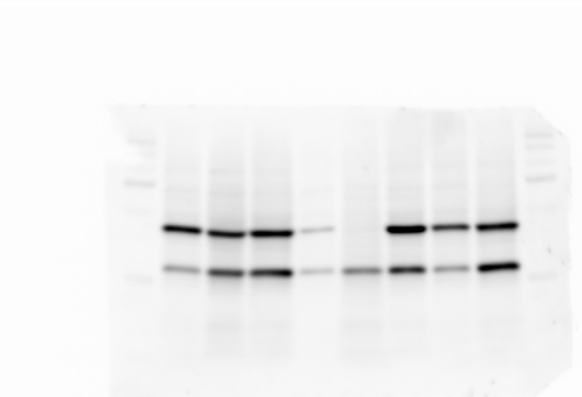

Figure 5: Example of Western Blot in Optimization: Non-specific binding

This is a Western Blot using an antibody against HMGB1. This blot for HMGB1 expression was conducted at a 1:500 dilution. The signal to noise ratio is much better than with the 1:1000 concentration (above). Also, we see differences between moderate TBI (first 3 lanes) and uninjured (middle 2 lanes) and severe TBI (last 3 lanes). Thus, we are now ready to analyze

- Task 2: Travel to VCU to induce lateral fluid percussion TBI in adult rats in months 3, 5, 7, 9 and 11 of year 1, as described above

As also detailed in the VCU section of this report, Dr. Floyd made 4 trips to VCU to perform injury procedures in the first year. The 5th trip was scheduled and then cancelled due to the fact that she broke her scaphoid bone in a fall wherein she landed on her outstretched hand. She is expected to be able to return to normal hand/ wrist function (i.e. grasping and performing surgery) by Sept. 2012 but was unable to perform the injury procedure for 8 weeks that her hand was in a cast.

- Task 3: Begin histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor numbers and growth factors from rodent brains received from VCU.

Here are the procedures which are currently being carried out. We are currently processing brains for analysis and have not completed any groups yet.

Tissue preparation

At 24 hours post-TBI, animals were deeply anaesthetized with Fatal Plus (100mg/kg i.p.; Vortech Pharmaceuticals, Dearborn, MI) and perfused intracardially with cold 0.1M phosphate buffer saline, pH 7.4, followed by cold 4% paraformaldehyde for twenty minutes. Brains were harvested and post-fixed for 24 hours at 4°C in 4% paraformaldehyde, then subsequently cryoprotected in an increasing gradient of 10%–30% sucrose for 24 hours at 4°C. The brains were marked with tissue dye over the right hemisphere, blocked and trimmed at 5 mm rostrally and 8 mm caudally, then embedded in OCT-Compound (TissueTek; Fisher Scientific, Pittsburg, PA) and frozen in ice-cold isopentane. Tissue was stored at -80°C until serial random sectioning. Serial 50 µm slices were sectioned on a cryostat (Leica Instruments, Nusloch, Germany) and collected from Bregma -0.8mm to -4.8mm, encompassing the cortical region at the injury epicenter as well as the entire hippocampal formation (Paxinos and Watson, 2005). The sections were mounted on 1% gelatin-coated slides and stored at -20°C until further histological analysis.

Cresyl violet histochemistry

Cresyl violet histological processing of tissue stains Nissl substance, which is composed mostly of rough endoplasmic reticulum and is lost after neuronal injury or axonal degeneration (Carson, 1990). For cresyl violet histochemistry, tissue was rinsed and dried overnight before staining. Sections were dehydrated through graded alcohol to xylene for two changes of 5 min each, and then rehydrated through graded alcohol to water. Sections were then submerged in 0.1% aqueous cresyl fast violet (EM Science, Gibbstown, NJ) in a sodium acetate buffer for four minutes, followed by differentiation in 95% ethanol with 0.2% HCl for five minutes. Differentiation was timed such that both Nissl substance and cell nuclei were clearly visible. Slides were washed in graded alcohol and xylene and coverslipped with Permount mounting media (Fisher Scientific).

Glial fibrillary acidic protein (GFAP) immunoreactivity

Reactive glial response was determined by measuring the luminance intensity of GFAP staining. Slide-adhered sections were washed in 0.1M phosphate buffer (3 times for 10 min) and then blocked in an endogenous peroxidase treatment (0.5% hydrogen peroxide in 0.1M phosphate buffer) for 30 min. Following washes in 0.1M phosphate buffer and phosphate-buffered saline (3 times for 5 min each), non-specific background was blocked with a solution of 3% normal goat serum, 3% bovine serum albumin, 0.3% Triton X, and 0.05M phosphate buffered saline. Tissue was rinsed in 0.1M phosphate buffered saline and incubated in a diluent mixture (1% normal goat serum + 2% bovine serum albumin + 0.3% Triton X + 0.05M phosphate buffered saline) containing anti-GFAP (Dako) at a 1:4,000 titre for 30 min at 37°C, then overnight at 4°C. Next, tissue was washed in 0.1M phosphate-buffered saline (9 times for 10 min), then incubated for 24 hrs at 4°C in the diluent mixture (described above) containing secondary antibody serum at a 1:400 titre (goat anti-rabbit Alexa Fluor 488; Invitrogen). Sections were rinsed in 0.1M phosphate buffered saline (3 times for 10 min) and 0.1M phosphate-buffered saline (6 times for 10 min), then slides were coverslipped with DPX mountant (Electron Microscopy Sciences Inc., Hatfield, PA).

Fluoro-Jade B immunohistochemistry

Fluoro-Jade B is an anionic fluorescein derivative that binds to degenerating neurons (Schmued and Hopkins, 2000). Briefly, sections were rehydrated through graded ethanol to distilled water, then incubated in 0.06% potassium permanganate for 15 minutes to reduce background. Tissue was rinsed in distilled water and stained with 0.006% Fluoro-Jade B in 0.1% acetic acid for 30 min at room temperature, then sections were washed with distilled water (3 times for 1 min) and dried for 30 min at 37°C, followed by drying at room temperature overnight. Finally, sections were rinsed in xylene (2 times for 5 min) and coverslipped with DPX mounting media (Electron Microscopy Sciences Inc., Hatfield, PA).

Caspase-3 immunohistochemistry

Caspase 3 acts as an effector caspase following activation via autoproteolytic cleavage or cleavage by other proteases as part of the programmed cell death cascade and is an indicator of extrinsic or intrinsic apoptosis. Briefly, slide-adhered sections were first washed in 0.1M phosphate buffer, then in 0.1M phosphate buffered saline (2 times for 10 minutes each.) Non-specific background was blocked with a solution of 3% normal goat serum, 0.3% Triton X, 3% bovine serum albumin and 0.05M phosphate buffered saline and tissue was incubated for 40 minutes at 37°C followed by 20 minutes at room temperature. Sections were rinsed briefly in 0.1M phosphate buffered saline, then incubated in Caspase-3 primary antibody diluent for 48 hours at 4°C which consisted of 1% normal goat serum, 0.3% Triton X, 2% bovine serum albumin, Caspase 3 antibody at a 1:4,000 titre (cleaved; mouse monoclonal IgG; Santa Cruz Biotechnology Inc.) and 0.05M phosphate buffered saline. After primary antibody incubation, tissue was rinsed in 0.1M phosphate buffered saline (6 times for 15

minutes), then placed in secondary antibody diluent containing 1% normal goat serum, 0.3% Triton X, 2% bovine serum albumin, Alexa 568 secondary antibody at a 1:500 titre (goat anti-rabbit IgG; Invitrogen) and incubated for 24 hours at 4°C. Following incubation with secondary antibody, sections were washed in 0.1M phosphate buffered saline (3 times for 15 minutes) and slides were coverslipped with DPX mountant (Electron Microscopy Sciences Inc., Hatfield, PA).

CD11b immunohistochemistry

CD11b is a cell marker found on the plasma membrane of activated microglia. Slide-adhered sections were rinsed in 0.1M phosphate buffer (3 times for 10 min), then an endogenous peroxidase block was performed for 30 min. After two 10 min rinses in 0.1M phosphate buffered saline, non-specific background was blocked with a solution of 3% normal horse serum, 0.3% Triton X, 3% bovine serum albumin and 0.1M phosphate buffered saline and tissue was incubated for 40 min at 37°C followed by 20 min at room temperature. Sections were washed in 0.1M phosphate buffered saline for 5 min and then incubated in primary antibody (1.5% horse serum, 0.3% Triton X, 2% bovine serum albumin, and CD11b at a dilution of 1:20,000 in 0.1M phosphate buffered saline) overnight at 4°C. 24 hours later, tissue was rinsed in 0.1M phosphate buffered saline (4 x 10 min) and then biotinylated secondary antibody (Vectastain Elite ABC Mouse IgG kit; 3 drops normal blocking serum, 1 drop biotinylated secondary antibody and 0.1% Triton X per 10 mL of 0.1M phosphate buffered saline) was applied for 30 minutes at room temperature. Sections were again washed in 0.1M phosphate buffered saline (4 x 10 min) and then the ABC reagent (4 drops each Reagent A and B with 0.1% Triton X per 10 mL of 0.1M phosphate buffered saline) was applied for 30 min at room temperature. Tissue was rinsed in 0.1M phosphate buffered saline (4 x 10 min) and then sections were developed in Vector SG (6 drops of Chromagen SG, 6 drops of hydrogen peroxide, and 0.1% Triton X per 10 mL of 0.1M phosphate buffered saline) for 5 min. Tissue was rinsed in 0.1M phosphate buffered saline (3 x 5 min) and 0.1M phosphate buffer (3 x 5 min) and slides were allowed to dry for one hour before being coverslipped with Permount mounting media (Fisher Scientific).

Unbiased stereological quantification of histology

Stereological counting was conducted on an Olympus BX-51 microscope linked to a MicroFire® true color CCD digital camera (Optronics, Goleta, CA) using StereoInvestigator software (Microbrightfield Inc., Williston, VT) at 200X-400X magnification. In the regions of interest, the optical fractionator probe was used to quantify the total number of neurons and the Cavalieri probe was used to quantify luminance intensity. For analysis of cresyl violet histochemistry, only neurons possessing a soma diameter greater than 10µm and a clearly defined nucleus and/or nucleolus were counted. In order to assess Fluoro-Jade B immunohistochemistry, only cells with fluorescence intensity twice that of background were counted. GFAP, CD11b and caspase 3 –positive cells were quantified using relative luminance intensity. Beginning at a randomly chosen first section near Bregma -0.8mm, cells were counted in every 10th section throughout the rostral-caudal extent of the

lesion, ending approximately at Bregma -4.8mm (~4mm total tissue). All assessments were performed by investigators naïve to the treatment of the animal.
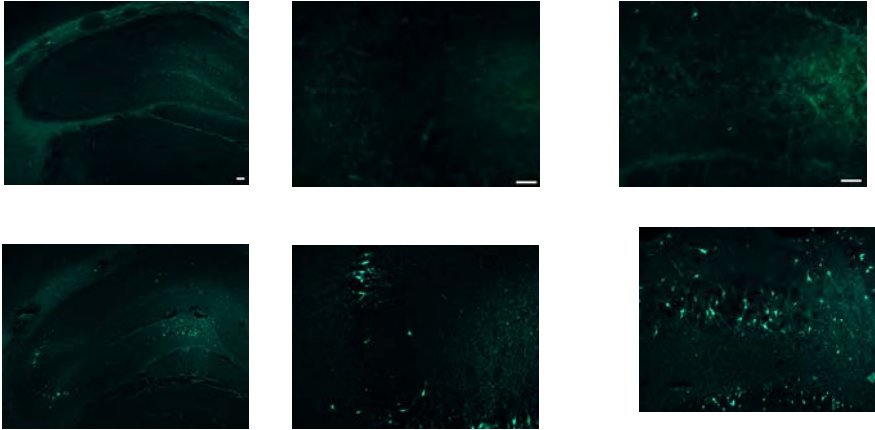

Figure 6: Example of Fluoro-Jade histochemistry to identify degenerating neurons after TBI as previously described. Representative micrographs from serial coronal sections processed for FJ histochemistry are shown. No degenerating neurons were identified in the sham-injury groups as illustrated by the micrographs in the upper panels. TBI caused extensive neuronal degeneration in the ipsilateral cortex and hippocampus as seen in the lower panels.

- Task 4: Lead preparation of abstracts / posters to report scientific discoveries obtained from analysis of TBI-induced alteration in reward circuitry, opioid neurotransmission and neurotrophic factors

    We assisted with submission of an abstract to the Society for Neuroscience Meeting. We are make good progress on the project and anticipate more scientific reports next year.

Key Research Accomplishments:

For testing of acute antinociceptive effects and tolerance development using the WWTW procedure, based on the data collected to date, no differences have been detected in response to oxycodone between subjects who had undergone lateral fluid percussion injury versus those that had undergone sham injury. Given the relatively small group sizes, differences may still evolve. In addition, chronic dosing with the ED80 dose of oxycodone may uncover differences between the brain and sham injury subjects. Similarly, Year 2 will incorporate testing in the hotplate test of antinociception. WWTW involves spinally mediated nociception, it remains to be seen if assessment for antinociception and tolerance development using a supraspinally mediated model of pain may uncover differences in response to oxycodone between injured and sham injured rats.

At this time, we have completed testing for acquisition of oxycodone self-administration in 35 subjects. While sham injury group sizes are currently insufficient to demonstrate a significant difference, the data suggest that the high (0.03 mg/kg/infusion) and, in particular, the intermediate (0.01 mg/kg/infusion) doses of oxycodone resulted in faster rate and a higher total percentage of subjects acquiring the self-administration behavior. Comparison of the levels of responding for oxycodone under stable self administration behavior were similar for brain injured and sham injured subjects however the brain injured subjects appeared to be less sensitive to oxycodone's effects taking the highest number of infusions at the 0.03 mg/kg/infusion dose as compared to the sham injured subjects. Decreased sensitivity to the reinforcing effects has been suggested to result in increased drug taking and a higher risk for abuse and addiction. Completion of the remaining acquisition subjects in the next few months and assessment of drug taking under a progressive ratio schedule will demonstrate if the injured subjects are potentially at greater risk.

We are also well underway with the processing of brains to assess the pathophysiology and differences between treatment groups. The immunoblotting and immunohistochemical techniques are nearly completely optimized.

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