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PRINCIPAL INVESTIGATOR: Candace L. Floyd, Ph.D., and Katherine L. Nicholson, Ph.D., D.V.M.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294-0111

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E-Mail: clfloyd@uab.edu	

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14. ABSTRACT

The goal of this project is to evaluate the hypothesis that traumatic brain injury (TBI) induces alterations in the brain's reward circuitry which may make an injured brain more susceptible to the rewarding effects of opioids. We conducted experiments to evaluate the hypothesis that TBI causes changes in the analgesic response to opioids following acute and repeated drug administration. We secondly tested 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 experimentation and found that TBI induces alteration in duration of transient unconsciousness, the rewarding properties of oxycodone, and the brain circuitry related to reward signaling. We also found that TBI does not significantly change the analgesic properties of oxycodone or the rewarding effects of food. We conclude from our study that TBI induces alterations in the brain reward circuitry which lead to a significant increase in the rewarding effects of oxycodone.

15. SUBJECT TERMS

Nothing listed

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Introduction:

Final 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 specific aims as outlined by the statement of work is detailed below. As this was a highly collaborative project and for the sake of completeness, the entire statement of work is listed followed by sections detailing the specific tasks that were completed at the University of Alabama at Birmingham (UAB). Also note, the Statement of Work was revised and approved in 5/16/2013, and the goals related to the revised Statement of Work are presented.

The specific aims of the research project were:

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.**

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. 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. 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. In Dr. Floyd's visits during years 2 and 3, she will perform the TBI procedure and evaluate performance by VCU staff in the procedure in order to maintain consistency. 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. Testing of subjects in other aspects of self-administration behavior (reinstatement and reinforcing efficacy) and development of physical dependence will begin late in year two and extend into year three. Within these scheduling parameters, subject groups based on the level of TBI, the behavioral procedure and oxycodone dose will be randomly generated for each cohort of subjects purchased across the study timeline in order to minimize any order effects.

Tasks to be conducted at VCU:

The following tables outline the proposed work schedule for the duration of the study. Following 2 months 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 30 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.

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. 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.

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. 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.

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.

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:

Task 1: 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.

Task 2: 40 subjects will have entered evaluation of acquisition of oxycodone self-administration, with an anticipated 32 completing acquisition assessment.

Year Two – Continued testing of antinociception and tolerance production with WWTW and hotplate test. Complete testing of acquisition of self-administration of oxycodone across test groups (4 doses: 0.003, 0.01, 0.03 and 0.056 mg/kg/infusion), begin assessing relapse to oxycodone self-administration. Begin assessing acquisition of food maintained behavior and locomotor effects of oxycodone. These rats will be randomly distributed to TBI condition/behavioral procedure and oxycodone dose upon arrival.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rats undergoing TBI			22		24				30	28	48	34
Rats Surviving TBI			17		22				26	23	42	29

Rats→ SA			0		22				26	11	16	10
Rats→TOL			17		0				0	12	15	10
Rats → Food/LA											11	9

By end of year two:

Task 1: 67 additional rats will have completed testing of the antinociceptive effects of oxycodone and the development of tolerance using the WWTW (24 rats) and hotplate test (41 rats) completing all saline controls in both injury conditions as well as all subjects under the ED80 repeated dosing condition (89 subjects out of the total 120). These subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB following the final oxycodone testing.

Task 2: 85 additional subjects will have been entered into evaluation of acquisition of oxycodone self-administration. With a potential loss of 20%, this will provide 10 subjects/treatment condition completing the study (50 subjects total completing acquisition assessment). Once the optimum dose for acquisition has been determined, 19 TBI subjects will be generated to evaluate relapse to oxycodone self administration, with ~14 subjects completing testing.

We will also evaluate sham and brain-injured subjects for acquisition of food maintained behavior. The same subjects will be used for assessing the effects of oxycodone on locomotor activity. Twenty-six subjects will undergo either sham or moderate injury to provide 20 subjects completing food acquisition.

Year Three – Complete testing in all subjects and all evaluation of cellular and histological changes by month 9. All behavioral analysis will be completed by month 9 allowing four months for final data analysis and manuscript preparation.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rats undergoing TBI	28		28		28		27		14	All behavioral testing complete. Final data analysis and		

Rats Surviving TBI	~24		~24		~24		~23		~12	manuscript preparation.
Rats→TOL	8		7							
Rats→ SA	4		5		12		11			
Rats→ Dep	12		12		12		12		12	

By end of year three:

Task 1: 15 additional rats will have completed testing of the antinociceptive effects of oxycodone and the development of tolerance completing all testing in both injury conditions. These subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB following the final oxycodone testing.

Task 2: 6 additional subjects (requiring ~8 subjects entering into relapse procedure) will have completed evaluation for relapse to oxycodone SA and 20 subjects (requiring ~24 subjects entering into relapse procedure) will have completed testing the relative reinforcing efficacy of oxycodone in the PR self administration procedure. This will provide the desired 10 subjects/treatment condition.

Task 3: 60 rats will have completed evaluation of production of physical dependence to provide 10 subjects/condition. These subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB.

Tasks to be conducted 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
  - Obtain and optimize protocols for the analysis of cell death/ gliosis, DA signaling, opioid receptor number and growth factors in rat brain tissue.
- 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
- 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.
- Task 4: Lead preparation of abstracts / posters to report scientific discoveries obtained from analysis of TBI-induced alteration in reward circuitry and opioid neurotransmission

YEAR 2:

- Task 1: Travel to VCU to induce lateral fluid percussion TBI in rats in months 3, 7, and 11 of year 2, as described above
- Task 2: Continue histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor number from rodent brains received from VCU
- Task 3: Lead preparation of peer-reviewed manuscript(s) to report results.

YEAR 3:

- Task 1: Travel to VCU to induce lateral fluid percussion TBI in rats in months 1, 3 and 5 of year 3, as described above
- Task 2: Continue histological and biochemical analysis of cell death/ gliosis, DA signaling, and opioid receptor expression from rodent brains received from VCU
- Task 3: Lead preparation of peer-reviewed manuscript(s) to report results.

BODY

Update on Tasks from Year 1 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
  - Obtain and optimize protocols for the analysis of cell death/ gliosis, DA signaling, opioid receptor number and growth factors in rat brain tissue.

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:
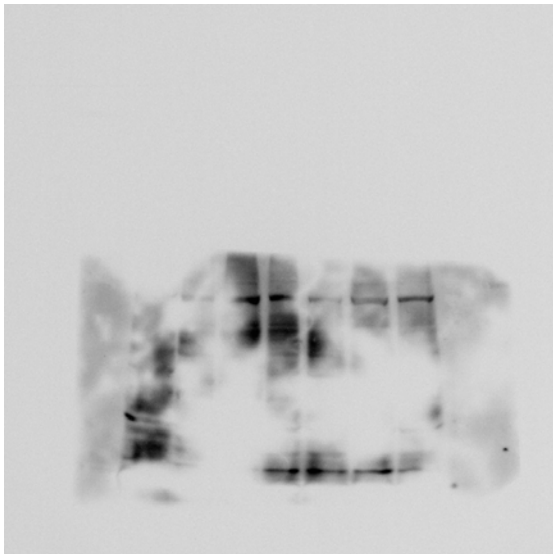

Figure 1: Example of Western Blot in Optimization: transfer

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.
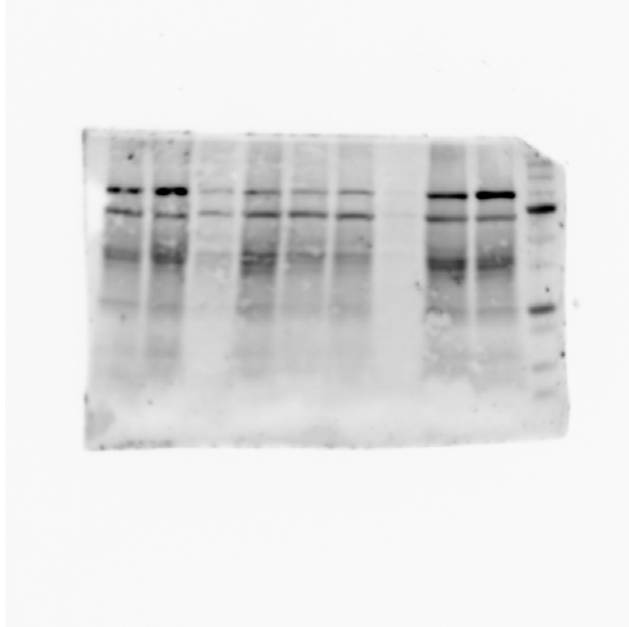
Figure 2: Example of Western Blot in Optimization: Non-specific binding

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.
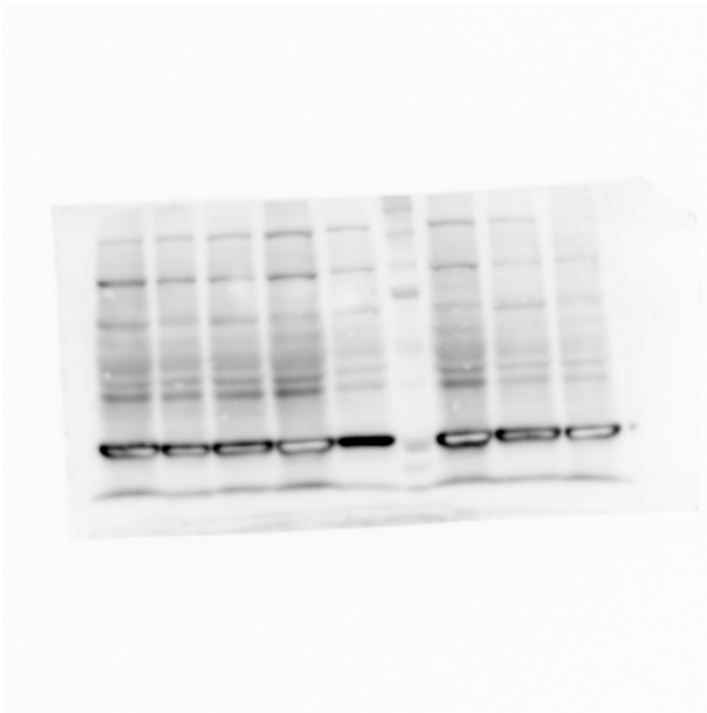

Figure 3: Example of Western Blot in Optimization: Non-specific binding

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.
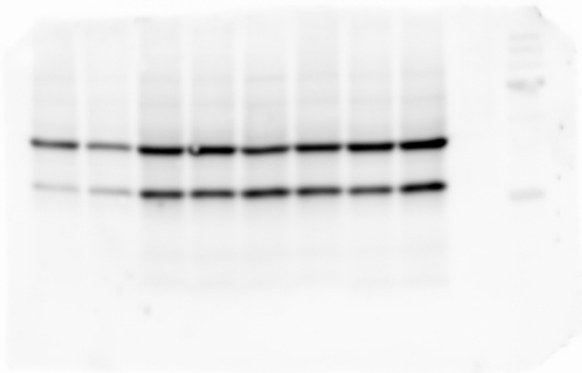

Figure 4: Example of Western Blot in Optimization: Non-specific binding

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.
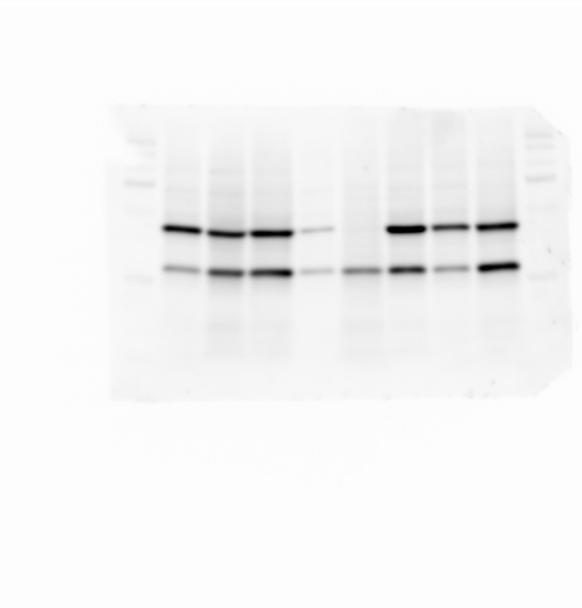

Figure 5: Example of Western Blot in Optimization: Non-specific binding

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 experimental conditions using this method. Examining altering the primary antibody concentration to increase the signal to noise ratio.

- 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.
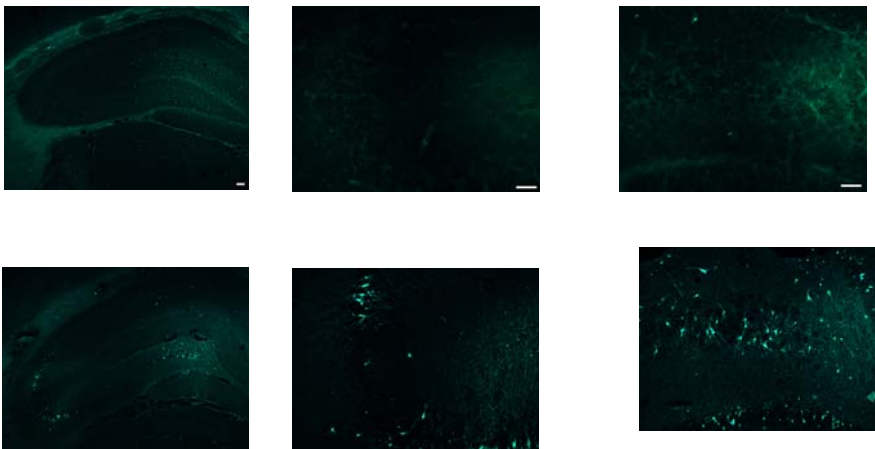

Figure 6: Example of Fluoro-Jade histochemistry to identify degenerating neurons after TBI as previously described. Representative micrographs from serial coronal sections processed for FJ histochemistry are shown. No degenerating neurons were identified in the sham-injury groups as illustrated by the 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.

Key Research Accomplishments for Year 1:

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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Progress on year 2 tasks to be conducted exclusively at UAB:

YEAR 2:

- *Task 1: Travel to VCU to induce lateral fluid percussion TBI in rats in months 3, 5, and 9, 10, 11 and 12 of year 2, as described above*

Completed as described above.

- *Task 2: Continue histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor number and growth factors from rodent brains received from VCU*

This task is on-going for year 2 and utilized the same methodologies as in described above for year 1. The main focus was quantification of Mu Opiod Receptor and Dopamine Receptors as detailed below.

Immunoblotting for quantification of Mu Opiod Receptor and Dopamine receptors

Immunoblotting was conducted using standard techniques as we have previously described. [3] Briefly, brains were extracted and regions rapidly dissected out, collected with a 2mm tissue puhcn, and immediately flash frozen on dry ice. Tissue was kept at -80°C until generation of tissue lysate. Hippocampi were mechanically homogenized in ice cold lysis buffer (100 mM Tris, pH 7.5, 1% sodium dodecyl sulfate (SDS)) containing protease inhibitors (Complete Mini Protease Cocktail Tablets, Roche Diagnostics, Indianapolis, IN) and then sonicated for 10 seconds followed by centrifugation at 14,000g for 5 minutes at 4°C to pellet debris. Protein quantification of the supernatant was performed with the Bio-Rad DC protein assay kit (Hercules, CA) and protein diluted to a final concentration of 2μg/μL. An equal volume of 2x Laemmli sample buffer (Sigma-Aldrich Co., St. Louis, MO) was added to the protein and samples were placed in boiling water for 5 minutes. Protein was then loaded into a 10% gradient pre-cast SDS gel (Bio-Rad Mini-PROTEAN® TGX precast gel, Hercules, CA) and run at 100V for 2 hours, then transferred at 100V for 1 hour. Membranes were blocked overnight at 4°C in blocking buffer (5% milk in TBS-T), then incubated in primary Ab {anti-mu opioid receptor (ab134054) 1:5000; anti-D1 (ab81293)1:35,000; anti-D2 1:3000 (ab21218); anti-α-tubulin loading control] at room temperature for 1 hour. Following 3 x 10-minute rinses, membranes were incubated in horseradish peroxidase-conjuated secondary Ab for 1 hour at room temperature (goat anti-rabbit IgG, 1:2000, Bio-Rad, Hercules, CA; goat anti-mouse IgG, 1:5000,

Santa Cruz Biotechnology, Santa Cruz, CA), then washed again (3 x 10 minutes), developed with enhanced chemiluminescence (SuperSignal® West Femto Maximum Sensitivity Substrate kit, Thermo Scientific, Pittsburgh, PA), and imaged with the Kodak Image Station 4000 MM. Protein was quantified with UN-SCAN-IT gel™ Version 6.1 software (Silk Scientific Inc., Orem, UT) and total protein expression is normalized to α-tubulin expression in the same lane. Relative protein expression is reported in arbitrary units.
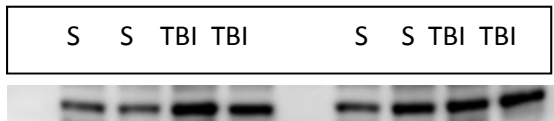
Figure 6: Representative immunoblot for mu opioid receptor from amygdala and VTA at 5 days post-TBI. Lanes (left to right): amygdala sham, sham, TBI , TBI; VTA sham, sham TBI, TBI,
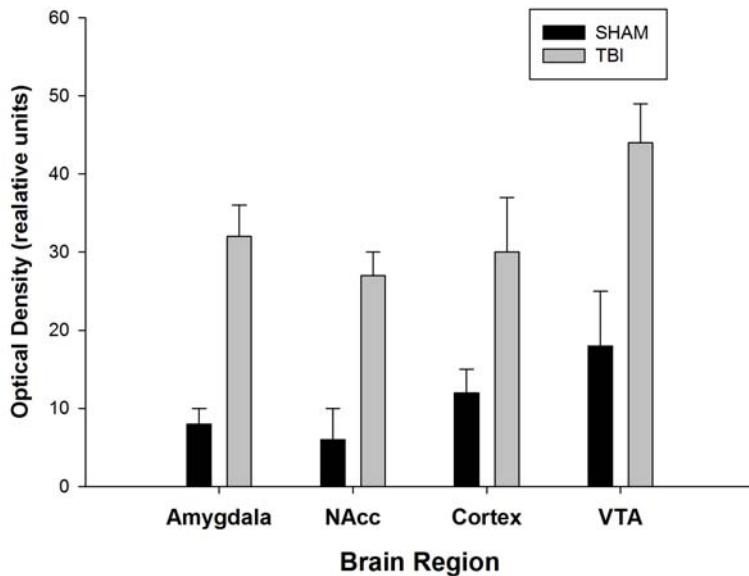
Figure 7: Quantification of expression of mu opioid receptor expression at 5 days post-TBI. The brain regions examined were amygdala, nucleus accumbens (NAcc), frontal cortex, and ventral tegmental area (VTA). Black bars are from sham animals and gray are from animals that received TBI.
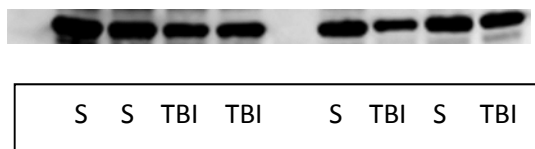

Figure 8: Representative immunoblot for dopamine receptor 1from amygdala and VTA at 5 days post-TBI. Lanes (left to right): amygdala sham, sham, TBI , TBI; VTA sham, TBI, sham, TBI
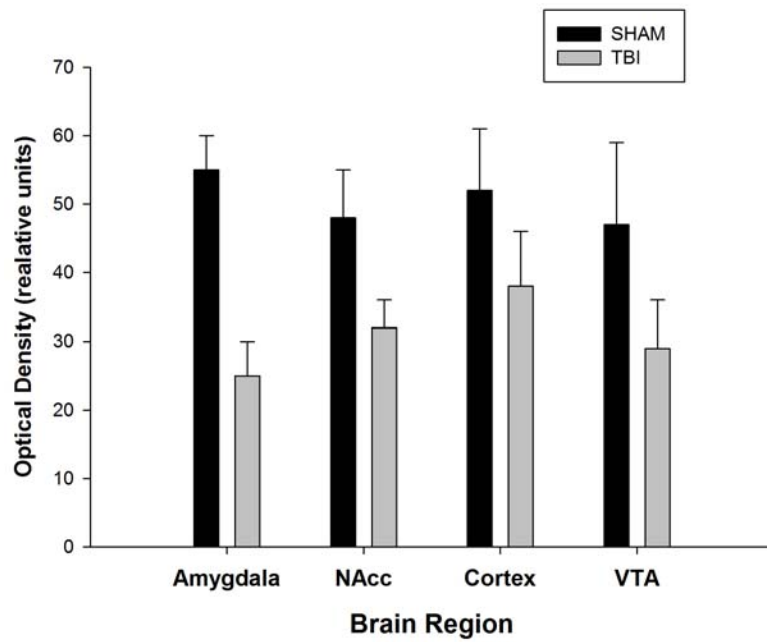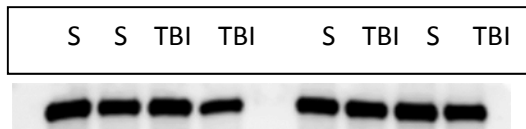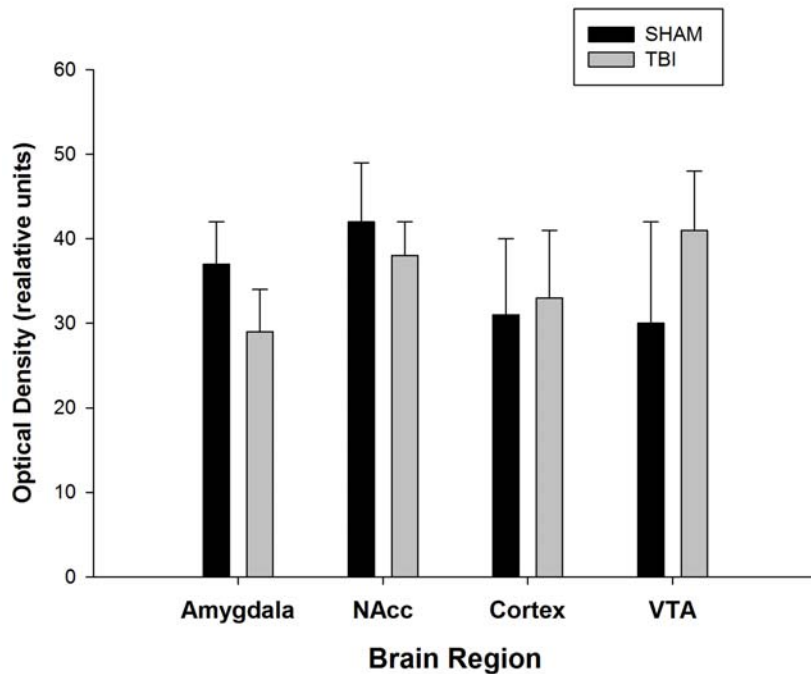
Figure 9: Quantification of expression of dopamine receptor expression at 5 days post-TBI. The brain regions examined were amygdala, nucleus accumbens (NAcc), frontal cortex, and ventral tegmental area (VTA). Black bars are from sham animals and gray are from animals that received TBI.

Figure 10: Representative immunoblot for dopamine receptor 2 from amygdala and VTA at 5 days post-TBI. Lanes (left to right): amygdala sham, sham, TBI , TBI; VTA sham, TBI, sham, TBI

Figure 11: Quantification of expression of dopamine subtype 2 receptor expression at 5 days post-TBI. The brain regions examined were amygdala, nucleus accumbens (NAcc), frontal cortex, and ventral tegmental area (VTA). Black bars are from sham animals and gray are from animals that received TBI.

- *Task 3: 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 in 2012. Additionally, we recently presented data at the 2013 National Neurotrauma Society meeting and that poster/abstract is below.

1. Overview of milestones completed and in progress

- Production goals reached as to total number of subjects entered into project
- Presentation of data by Dr. Nicholson at the Society for Neuroscience Annual meeting in 2012
- Presentation of data to date by Dr. Floyd at the National Neurotrauma Society Annual meeting in 2013

Key Research Accomplishments:

- TBI group showed an increased sensitivity to oxycodone's reinforcing effects as compared to sham group

- TBI group appeared to be less sensitive to oxycodone's "use-limiting" effects compared to the sham injured subjects

- TBI group exhibited higher timeout responding suggestive of increased impulsivity

- No differences were detected between groups in baseline nociception or in response to the acute anti-nociceptive effects of oxycodone

- Preliminary data indicate changes in expression of mu opioid receptor and dopamine subtype 1 receptor after TBI.

- **Taken together, these data indicate that TBI may enhance self-administration of oxycodone, particularly at the moderate dose.**

Reportable Outcomes:

- **Presentation of data at 2013 National Neurotrauma Society Annual Meeting**

Conclusion:

The data thus far suggest that moderate/severe traumatic brain injury induces a change in the response to oxycodone such that injured subjects are more likely to abuse oxycodone and less sensitive to the negative effects the drug. This is likely due to changes in the brain reward circuitry induced by injury.

Progress on year 3 tasks to be conducted exclusively at UAB:

A. Progress on Year 3 Tasks completed by UAB personnel (Dr. Floyd):

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

Dr. Floyd conducted all the TBI procedures in year three such that nearly all the animals need to meet the production goals were collected. As a few of the animals still need to be completed, please see the table in the VCU report, Drs. Floyd and Nicholson requested a 1 year no cost extension to complete the few remaining animals. This will likely require 1-2 more trips for Dr. Floyd to VCU to induce the TBI.

Briefly as we have previously described[1, 2], traumatic brain injury was induced in adult, male Sprague Dawley rats by lateral fluid percussion. Uninjured controls (sham group) received all surgical procedures with the omission of the fluid percussion impact, as we have previously described. Data from some of these animals is shown below and behavioral data components from all animals in the study are listed in Dr. Nicholson's report.

- *Task 2: Continue histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor expression and growth factors from rodent brains received from VCU*

This task was on-going and experiments and results are continuing to be collected in support of this task. The data are described below.

For these data, a 2 mm tissue punch was used to extract the brain regions of interest which included the ventral tegmental area (VTA), amygdala (AMG), cortex (CTX), nucleus accumbens (NAC), and hippocampus (HIP). Tissue samples were homogenized and then immunoblotting was conducted to assess expression of the mu opioid receptor, dopamine receptor subtype 1, dopamine receptor subtype 2, dopamine transporter, and tyrosine hydroxylase. Twenty-five micrograms of protein was used for all immunoblots and all blots were incubated in the primary antibody overnight at $4°C$ and blocked for 1 hour in 5% bovine serum albumin at room temperature. The immunoblots were incubated in secondary antibodies for 1 hour at room temperature. The following antibodies were used:

Primary Antibodies:

Mu Opioid receptor: Alomone Labs (catalog #AOR-001) 1:200 dilution in 1% BSA

D1 receptor: Alomone Labs (catalog #ADR-001) 1:200 dilution in 1% BSA

D2 receptor: Alomone Labs (catalog #ADR-002) 1:200 dilution in 1% BSA

Dopamine transporter: Alomone Labs (catalog #AMT-003) 1:200 dilution in 1% BSA

Tyrosine Hyroxylase: Life Tech (catalog #36990) 1:1000 dilution in 1% BSA

β-Actin: Cell Signaling (catalog #3700P) 1:4000 dilution in 1% BSA

Secondary Antibodies:

(For all blots except actin) Goat anti-rabbit: Biorad : (catalog #170-6515) 1:2000 dilution in 1% BSA

(For actin blots only) Goat anti-mouse: Biorad : (catalog #170-6516) 1:4000 dilution in 1% BSA

Immunoblots were annalyzed in the liner range by realative optical density of each band. All comparison groups were evaluted on the same blot and optical density values were normalized to β-actin as a loading control. Normalized optical density values were averaged for comparisons across immunoblots.
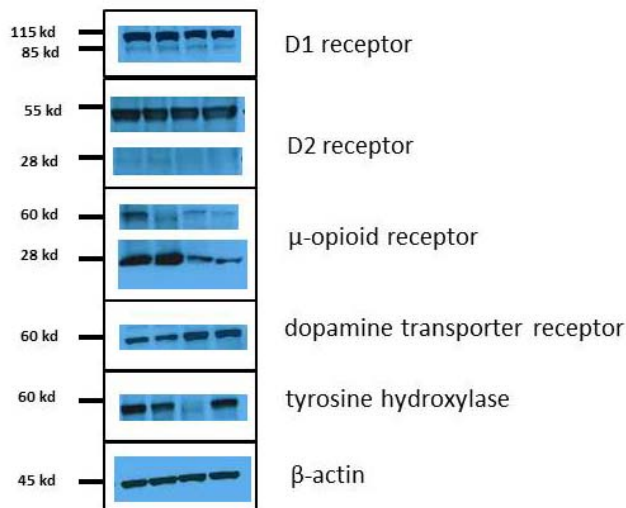
Figure 12: Representative immunoblots from regions of rodent brains taken from subjects who received moderate lateral fluid percussion TBI (two right lanes) or uninjured control (two left lanes). D1= dopamine receptor subtype 1, D2= dopamine receptor subtype 2.
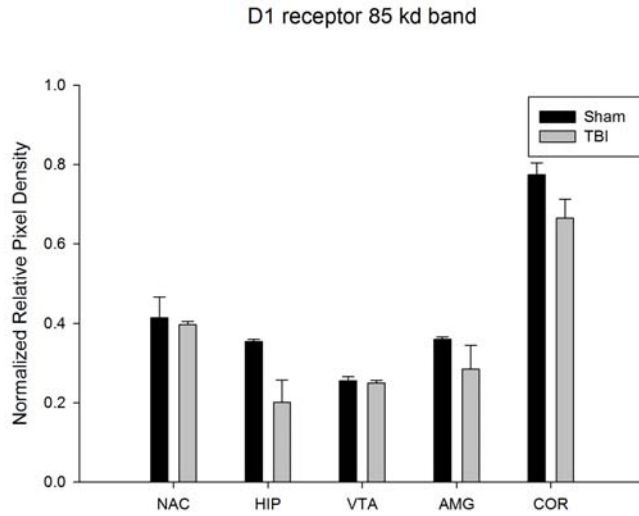
D1 receptor 85 kd band

Figure 13: Quantification of the dopamine 1 receptor subtype at the 85kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).
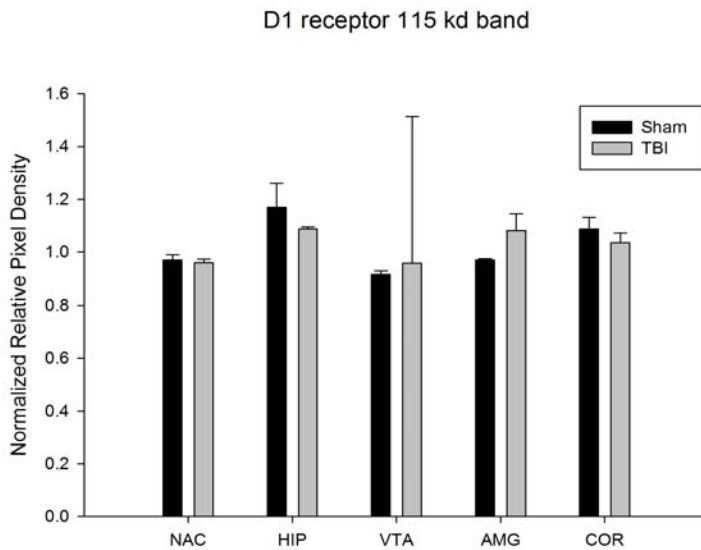
D1 receptor 115 kd band

Figure 14: Quantification of the dopamine 1 receptor subtype at the 115 kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).

With regard to the dopamine receptor subtype 1 at 5 days post-TBI, we found modest trends toward reduction of expression in the brains of subjects that received TBI as compared to uninjured controls, particularly in the hippocampus and cortex. Also, we observed a trend toward increased expression of DA1 In the amygdala and large variability in the VTA. However after analysis of variance, none of these trends reached a level of statistical significance. Taken together, these data suggest that at 5 days post-TBI,

there are not robust changes in the expression of the dopamine receptor 1
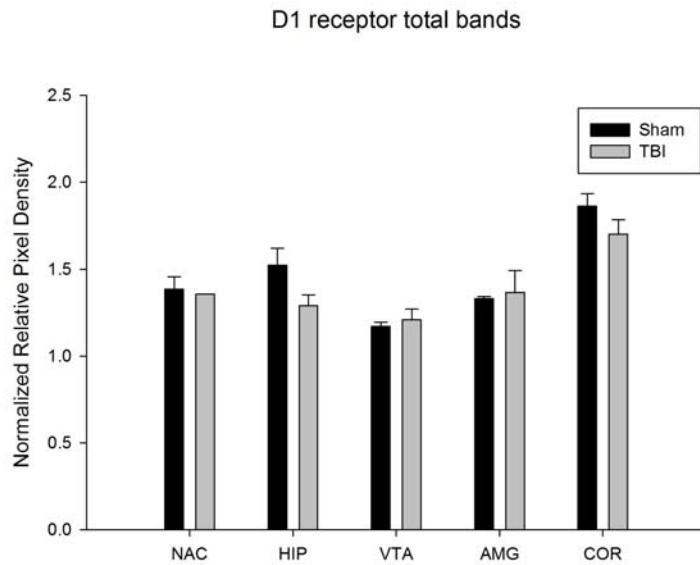subtype.

D1 receptor total bands

Figure 15: Quantification of the dopamine 1 receptor subtype averaged from the 85 kd and 115 kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).

Next, we evaluated the effect of TBI on expression of the dopamine receptor 2
subtype from the same brain samples as described above.
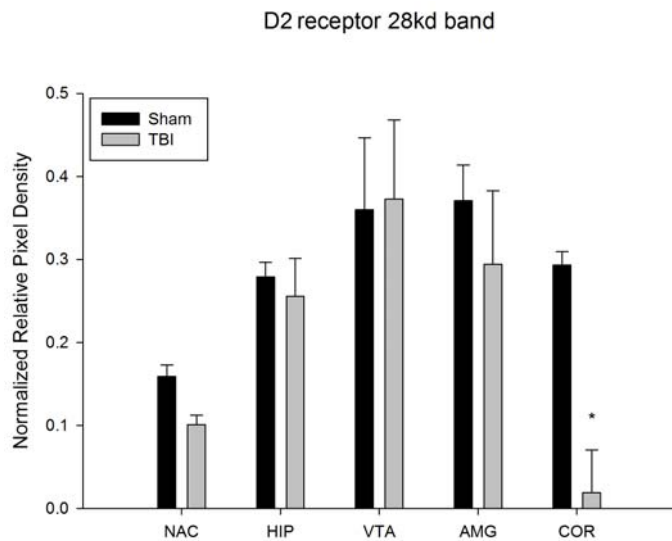

D2 receptor 28kd band

Figure 16: Quantification of the dopamine 2 receptor subtype at the 28kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR). *=p<0.05.
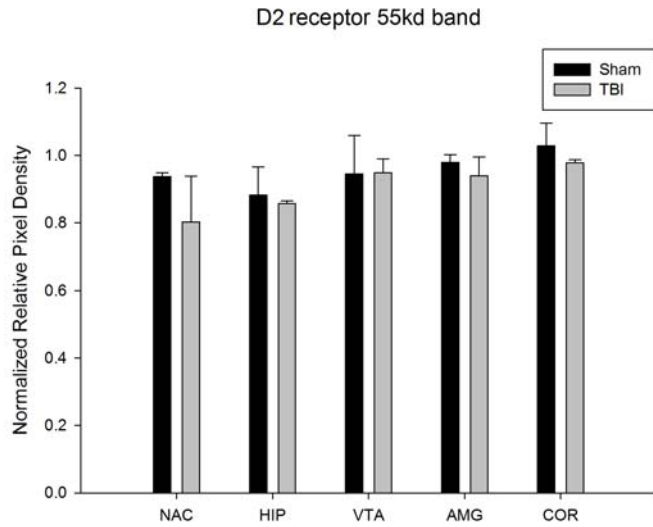
D2 receptor 55kd band

Figure 17: Quantification of the dopamine 2 receptor subtype at the 55kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).
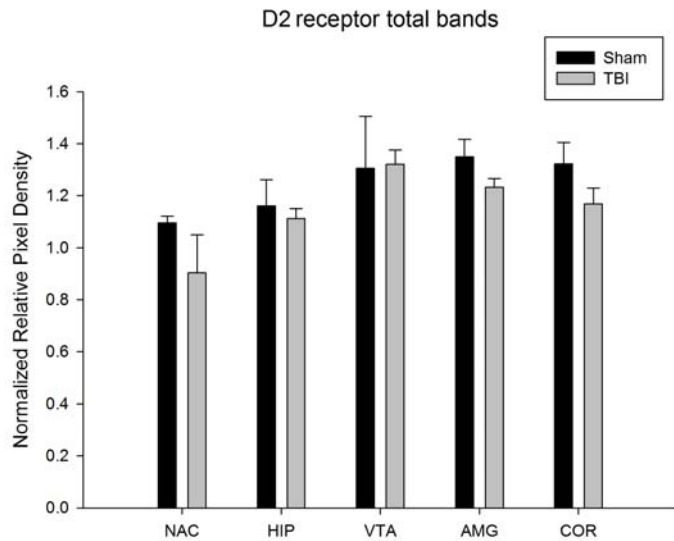

D2 receptor total bands

Figure 18: Quantification of the dopamine 2 receptor subtype averaged from the 28kd and 55kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).

With regard to the dopamine receptor subtype 2, analysis of the band at the 28kd molecular weight revealed robust decreases in the expression nucleus accumbens and cortex in subjects that received TBI. This reached statistical

significance in the cortex. However, analysis of the 55kd molecular weight band the reduction in expression of D2 in the injured brains was much less robust. When densitometry of both molecular bands was averaged, no robust differences in the expression of the D2 receptor were observed, likely due to the influence of the 55kd band values. Thus, when taken together, robust decreases in the D2 receptor subtype were observed in the cortex (statistically significant) and nucleus accumbens when only the 28 kd molecular weight band was considered.
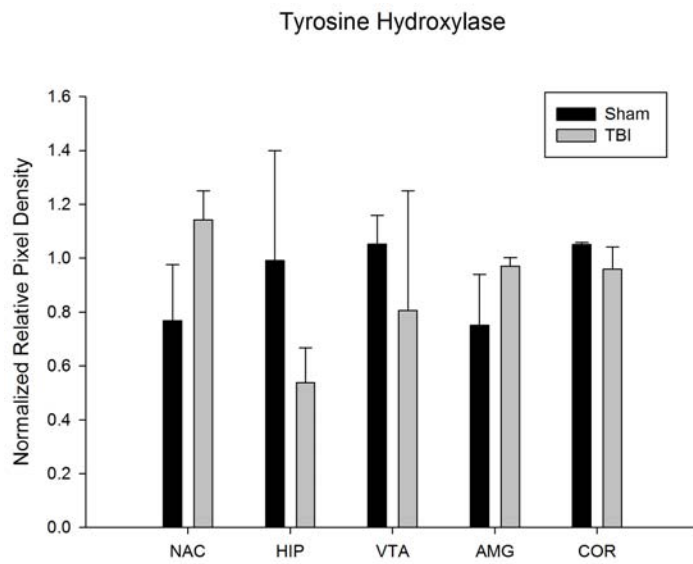

Figure 19: Quantification of the expression of tyrosine hydroxylase levels comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).

Next, we evaluated the effects of TBI on the expression of tyrosine hydroxylase (TH), the enzyme responsible for catalyzing the conversion of tyrosine to L-DOPA and a key component in dopamine synthesis. Interestingly, the trends in TH expression varied across the brain region. We observed a trend to increased TH in the nucleus accumbens and reduced TH in the hippocampus in the TBI group as compared to the uninjured control. Other brain regions were either more variable in the expression of TH after TBI (i.e. VTA) or we did not observe a robust difference in TH expression after TBI (i.e. amygdala and cortex). Taken together, these data suggest that TBI induces increases in dopamine synthesis in the nucleus accumbens and decreases in the hippocampus, however these trends did not reach statistical significance and more evaluation is needed.

Our next evaluation was the expression of the mu opioid receptor after TBI across these brain regions.
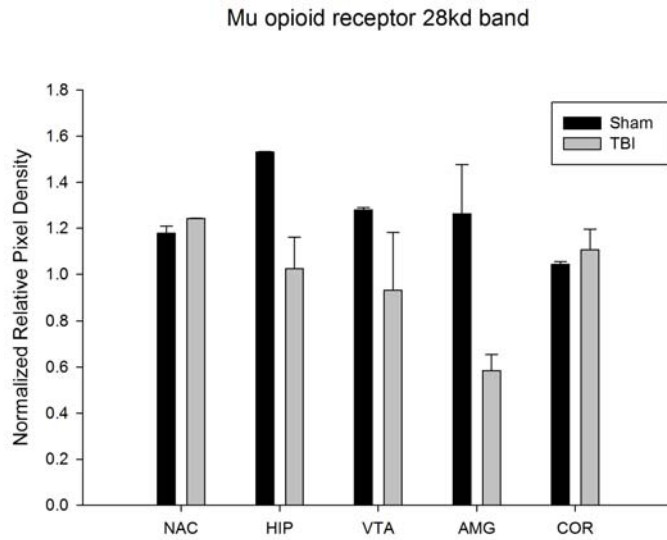

Mu opioid receptor 28kd band

Figure 20: Quantification of the mu opioid receptor at the 28kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).

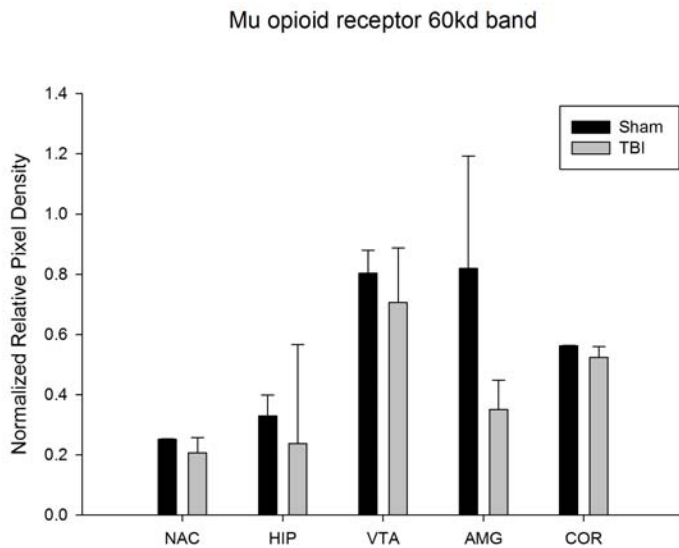
Mu opioid receptor 60kd band

Figure 21: Quantification of the mu opioid receptor at the 60kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR).
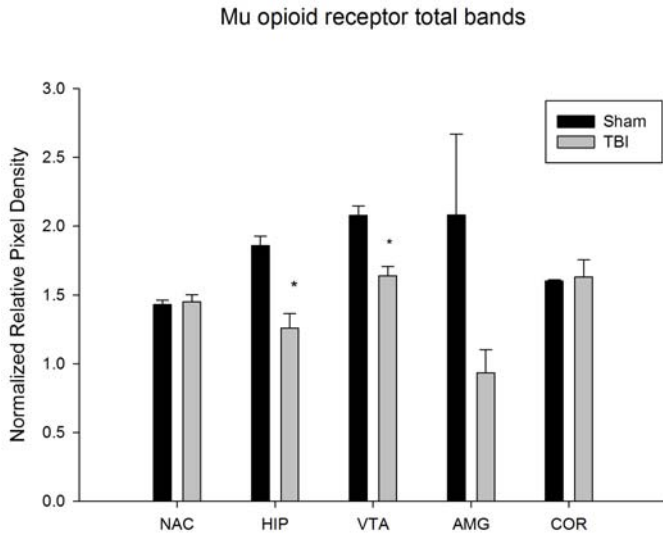
Mu opioid receptor total bands

Figure 22: Quantification of the mu opioid receptor subtype averaged from the 28kd and 60kd molecular weight comparing the uninjured control (sham) to TBI groups at 5 days post-injury. The ipsilateral side was evaluated in the following brain regions: nucleus accumbens (NAC), hippocampus (HIP), ventral tegmental areas (VTA), amygdala (AMG), and cortex (COR). *=$p<0.05$.

With regard to the expression of the mu opioid receptor after TBI, we found that there were trends for reduction in expression after TBI in the hippocampus, VTA, and amygdala when assessing the 28kd molecular weight. However, these trends did not reach statistical significance. Similar trends were observed for analysis of the 60kd molecular weight band in that TBI reduced expression of the mu opioid receptor in the hippocampus, VTA, and amygdala. These trends did not reach statistical significance. When the data from the 28kd band and the 60kd band were averaged, a statistically significant reduction in expression of the mu opioid receptor was observed in the hippocampus and VTA. Also, a strong non-significant trend for TBI-induced reduction in the mu opioid receptor expression was observed in the amygdala. Taken together, these data suggest that TBI induces a reduction in the expression of the mu opioid receptor in key brain regions associated with reward circuitry, including that hippocampus, VTA and amygdala. Subsequent work is needed to further evaluate these trends.

- *Task 3: Lead preparation of peer-reviewed manuscript(s) to report results.*

This task is also on-going. We are in preparation of the associated manuscript.

Key Research Accomplishments:

- **Taken together, these data indicate that TBI may alter brain reward circuitry, particularly dopaminergic signaling and expression of the mu opioid receptor.**

Reportable Outcomes:

- Presentation of data by Dr. Floyd in the Associated in Emergency Medical Education and Alliance for Global Narcotics Training Conference at the Defense Health Headquarters in March 2014
- Submission of abstract for presentation of data to Military Health System Research Symposium in 2014 (abstract not accepted for presentation)

Conclusion:

The data for the project (combining both VCU and UAB efforts) thus far suggest that moderate/severe traumatic brain injury induces a change in the response to oxycodone such that injured subjects are more likely to abuse oxycodone and less sensitive to the negative effects the drug. This is likely due to changes in the brain reward circuitry induced by injury, particularly with dopaminergic signaling and expression of mu opioid receptor.

Reference List

(1) Day NL, Floyd CL, D'Alessandro TL, Hubbard WJ, Chaudry IH. 17beta-estradiol confers protection following traumatic brain injury in the rat and involves activation of G Protein-coupled estrogen receptor 1 (GPER). *J Neurotrauma* 2013.

(2) Floyd CL, Golden KM, Black RT, Hamm RJ, Lyeth BG. Craniectomy position affects morris water maze performance and hippocampal cell loss after parasagittal fluid percussion. *J Neurotrauma* 2002;19(3):303-316.

Update on Tasks in NCE Year 4 Conducted Exclusively at UAB:

The work in the NCE was focused largely obtaining additional data to support the completion of task 2.

> *Task 2: Continue histological and biochemical analysis of cell death/ gliosis, DA signaling, opioid receptor expression and growth factors from rodent brains received from VCU*

We continued our examination of neuronal cell death in the dentate gyrus of the hippocampus of animals in the various experimental group. An example of these data is figure 23. Using stereological quantification, we counted the number of neurons in the dentate gryus of brains from animals that received a sham procedure (black bar) or a moderate TBI (gray bar). There were not significant differences between the sham and TBI group on the side of the brain contralateral to the injury. However, in the dentate gyrus ipsilateral to the injury, we saw a significant reduction in the total neuronal cell number, which is consistent with our previous results. We next evaluated the effect of TBI on cell death in the animals that received oxycodone for the warm water tail withdrawal (WWTW) and hot plate (HP) tests. Similar to the results with animals that received food reward, we found no significant differences in the number of neurons in the dentate gyrus of rats from the Sham or TBI group, regardless of oxycodone administration, on the contralateral side. We found that TBI induced a significant decrease in the number of neurons in the dentate gyrus, and that this reduction in cell number was not different between the oxycodone groups. These data suggest that the TBI caused ipsilateral loss of neurons in the hippocampus, a key component of the reward circuit.
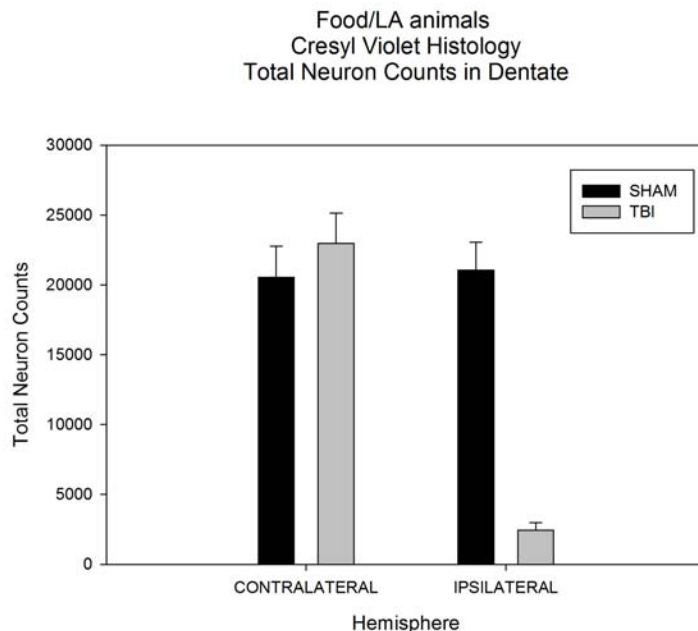

Figure 23: Quantification of neuronal cells in the dentate gyrus of the hippocampus in animals that received either TBI or Sham and then were assessed for food responding or locomotor activity. We found no significant difference in the number of neurons on the contralateral side comparing TBI to Sham groups. We found a significant reduction in the number of neurons when comparing the TBI group to the sham group on the ipsilateral side.

WWTW/HP animals
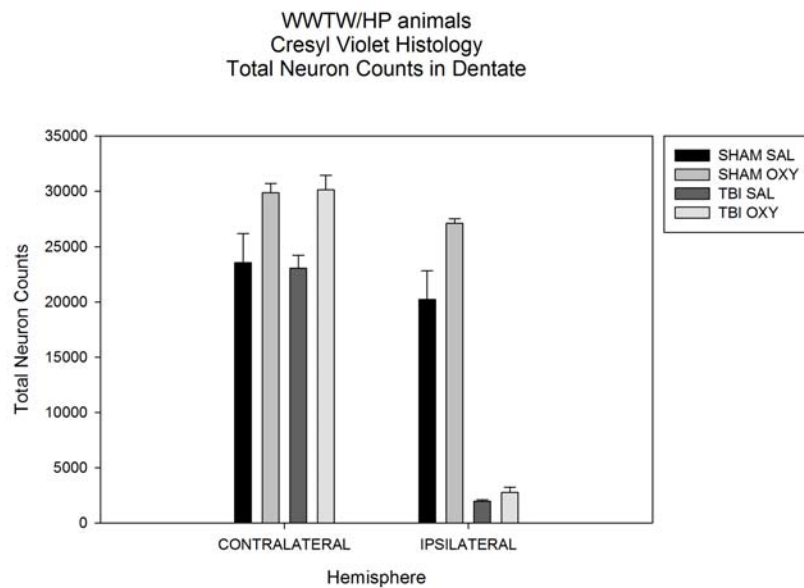Cresyl Violet Histology
Total Neuron Counts in Dentate

Figure 24: Quantification of neuronal cells in the dentate gyrus of the hippocampus in animals that received either TBI or Sham and then were administered oxycodone for subsequent assessment in the warm water tail withdrawal task and the hot plate task. We found no significant difference in the number of neurons on the contralateral side comparing TBI to Sham groups. We found a significant reduction in the number of neurons when comparing the TBI group to the sham group on the ipsilateral side, regardless of oxycodone administration.

We examined the key elements of reward circuitry in brain regions from animal that were administered oxycodone in the laboratory in Virginia. We examined the expression of the dopamine receptor 1 (D1) in the brain regions associated with reward circuitry including the nucleus accumbens (NAC), ventral tegmental area (VTA), and the frontal cortex. We compared the changed in receptor protein expression to the expression from the sham saline group, first evaluating the 85kd band. We found that both oxycodone and TBI induced a modest reduction in the expression of D1 in the NAC. We also found that both sham and oxycodone induced a robust increase in expression of D1 in the VTA and a more modest increase in the cortex.

D1 Dopamine Receptor 85kd band
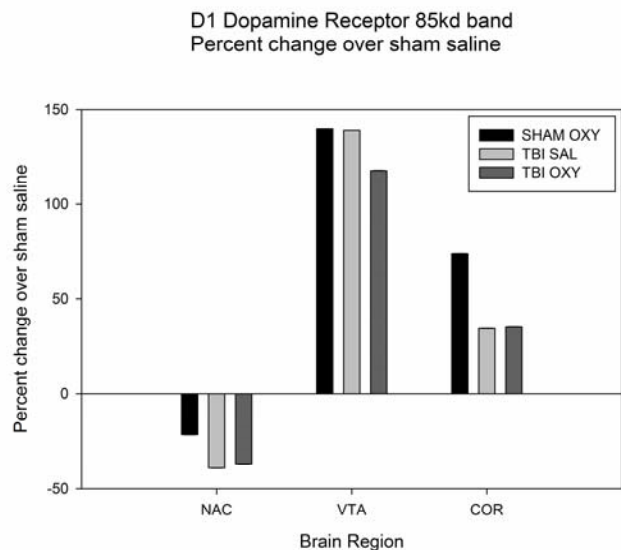Percent change over sham saline

Figure 25: Quantification of D1 receptor expression. The 85kd band of the D1 receptor expression was quantified using immunoblotting in 3 brain regions, nucleus accumbens (NAC), ventral tegmental area (VTA), and the frontal cortex (COR) in animals that receive a TBI or sham control $\pm$ oxycodone administration. Values are compared to the sham saline group. See text for more details.

D1 Dopamine Receptor 115kd band
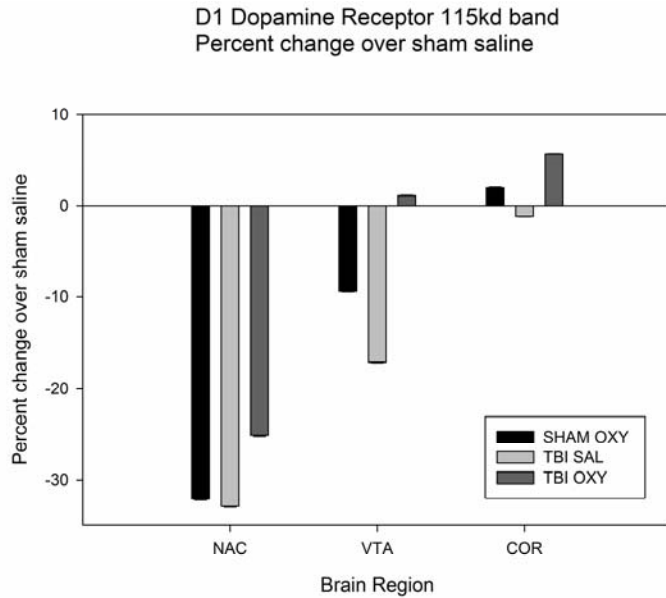Percent change over sham saline

Figure 26: Quantification of D1 receptor expression. The 115kd band of the D1 receptor expression was quantified using immunoblotting in 3 brain regions, nucleus accumbens (NAC), ventral tegmental area (VTA), and the frontal cortex (COR) in animals that receive a TBI or sham control ± oxycodone administration. Values are compared to the sham saline group. See text for more details.

We found that both oxycodone and TBI induced a robust reduction in the expression of D1 in the NAC. We found mixed effect of injury and oxycodone induced in expression of D1 in the VTA and a modest increase in the cortex.

We next evaluated the expression of the Dopamine receptor type D2, in the same regions and using the same techniques. When the 28kd band was considered, we found that both injury and oxycodone exposure increased expression in the NAC. We found variable expression in the VTA and COR for injury and oxycodone exposure.

D2 Dopamine Receptor 28kd band
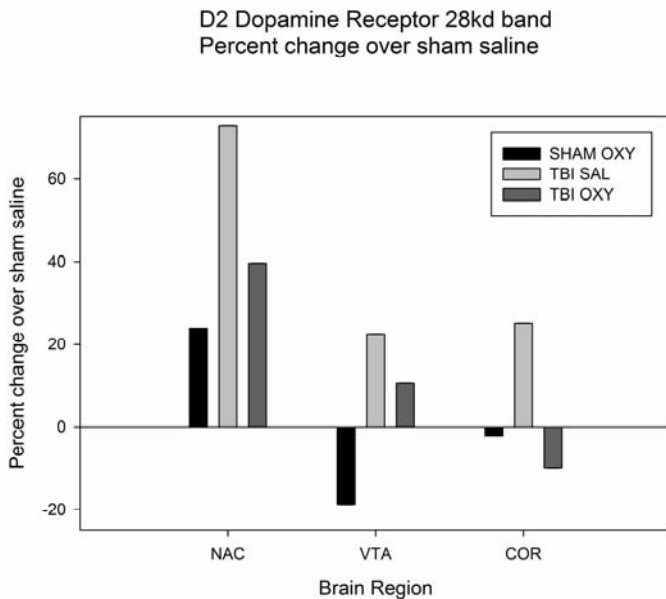Percent change over sham saline

Figure 27: Quantification of D2 receptor expression. The 28kd band of the D2 receptor expression was quantified using immunoblotting in 3 brain regions, nucleus accumbens (NAC), ventral tegmental area (VTA), and the frontal cortex (COR) in animals that receive a TBI or sham control ± oxycodone administration. Values are compared to the sham saline group. See text for more details.

Key Research Accomplishments:

- Demonstrated cell death in the ipsilateral hippocampus following moderate TBI and that this was not altered by post-injury administration of oxycodone.

- Demonstrated alterations between sham and TBI animals in the D1 and D2 receptor expression in the NAC, VTA, and Cortex.

Conclusion:

Taken together, the data from this project show that moderate TBI induces alterations in oxycodone self-administration and in the brain circuitry associated with reward.

Reportable Outcomes:

1. Publications:

In progress, but none submitted or published at this time.

2. Meeting Abstracts:

National Neurotrauma Society 2013, see appendix 1.

3. Personnel:

Candace Floyd, Ph.D.

Kelly Bradley

Tracy Niedzielko

Effects of oxycodone following traumatic brain injury in rats

Candace Floyd, Marie Greenwood, Kelley Bradley, Keith Shelton, Katherine Nicholson

Introduction: 50 words
Epidemiological data indicate that drug abuse rates increase following traumatic brain injury (TBI), but the underlying reasons remain unclear. There is overlap in reward pathways and regions commonly damaged in TBI suggesting that TBI could alter risk for drug abuse.

Methods: 150 words
Adult male Sprague Dawley rats underwent sham (control) or lateral fluid percussion injury of moderate severity followed by assessment of tolerance development and intravenous self -administration behavior. The antinociceptive effects of oxycodone were evaluated using hot plate-induced paw withdrawal and warm water tail withdrawal models both acutely and following chronic administration of oxycodone. The reinforcing effects of oxycodone were examined using acquisition of oxycodone self-administration behavior. Upon conclusion of the behavioral testing, brains were collected and reward circuitry examined. In a second cohort of rats, TBI was induced and acute changes in mu opioid receptor, and dopamine receptors in the ipsilateral nucleus accumbens (NuAc), ventral tegmental area (VTA), hippocampus, and amygdala were assessed via immunoblotting.

Results: 300 words
No consistent differences have been detected in the antinociceptive effects of oxycodone between TBI subjects and sham injured subjects. However the data suggest that the high (0.03 mg/kg/infusion) and intermediate (0.01 mg/kg/infusion) doses of oxycodone resulted in a faster rate and a higher total percentage of TBI subjects acquiring self-administration. Additionally, the TBI subjects appeared to be less sensitive to oxycodone's effects, self-administering the highest number of infusions at the 0.03 mg/kg/infusion dose as compared to sham injured subjects. At 24 hours post TBI, expression of mu opioid receptors was increased in the NuAc and hippocampus, but reduced in the VTA. In contrast, dopamine receptors subtype 1 and 2 expression was elevated in the VTA at 24 hours after injury, but not changed in other regions.

Conclusions: 150 words
These data indicate that moderate TBI induces acute changes in the reward circuitry. Also, animals subjected to moderate brain injury will self-administer oxycodone to a greater degree than uninjured controls while being less responsive to the effects of oxycodone.   Taken together, these data indicate that TBI may alter the brain reward circuitry and enhance self-administration of oxycodone.