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ABSTRACT OF DISSERTATION

Thomas Matt Woodcock

The Graduate School

University of Kentucky

2009

MODULATION OF THE ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTOR FOLLOWING EXPERIMENTAL RAT BRAIN INJURY IMPROVES CELLULAR AND BEHAVIORAL OUTCOMES

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy
at the University of Kentucky

By

Thomas Matt Woodcock

Lexington, Kentucky

Director: Dr James R Pauly

Lexington, Kentucky

2009

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MODULATION OF THE ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTOR FOLLOWING EXPERIMENTAL RAT BRAIN INJURY IMPROVES CELLULAR AND BEHAVIORAL OUTCOMES

Traumatic brain injury (TBI) is a leading cause of death and long-term disability worldwide, and survivors are often left with cognitive deficits and significant problems with day to day tasks. To date, therapeutic pharmacological treatments of TBI remain elusive despite numerous clinical trials. An improved understanding of the molecular and cellular response to injury may help guide future treatment strategies. One promising marker for brain injury is the translocator protein (TSPO), which is normally expressed at a low level, but is highly expressed following brain damage and is associated with neuroinflammation. The isoquinoline carboxamide PK11195 binds selectively to the TSPO in many species, and has therefore become the most-studied TSPO ligand. To characterize the time-course of TSPO expression in the controlled cortical injury (CCI) model of TBI we subjected Sprague-Dawley rats to CCI and euthanatized them after 30 minutes, 12 hours, 1, 2, 4, or 6 days. Autoradiography with radiolabelled PK11195 was used to assess the time-course of TSPO binding following CCI. Autoradiographs were compared to adjacent tissue slices stained with the microglia/macrophage marker ED-1, with which a moderate positive correlation was discovered. PK11195 autoradiography was used as a tool with which to assess neuroinflammation following CCI and the administration of an α7 nAChR antagonist, methyllycaconitine (MLA). We hypothesized that blocking the calcium permeable α7 nAChR after brain injury would have a neuroprotective effect by attenuating excitotoxicity in the short-term. Our study revealed clear dose-dependent tissue sparing in rats administered MLA after trauma and a modest improvement in functional outcome. The relatively modest recovery of function with MLA, which could be due to prolonged α7 nAChR blockade or downregulation lead us to explore the potential of α7 nAChR partial agonists in treating TBI. The α7 nAChR partial agonists tropisetron, ondansetron, and DMXB-A produced a moderate attenuation of cognitive deficits, but did not have a neuroprotective effect on tissue sparing. These studies show that following TBI, α7 nAChR modulation

can have neuroprotective effects and attenuate cognitive deficits. Whether this modulation is best achieved through partial agonist treatment alone or a combination antagonist/agonist treatment remains to be determined.

KEYWORDS: Traumatic Brain Injury, PK11195, Nicotinic, Antagonist, Partial Agonist, Microglia

Thomas Woodcock

Student's Signature

22 January 2010

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DISSERTATION

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The Graduate School

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DISSERTATION

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1. CHAPTER 1

Introduction & Background

1.1 An overview of traumatic brain injury

Traumatic brain injury (TBI) is one of the leading causes of death and long-term disability worldwide. At least 10 million people sustain a TBI that results in death or hospitalization every year (Langlois et al., 2006), a conservative estimate since many TBIs go unreported. Survivors of TBI are frequently left with long-term or life-long cognitive impairments that have a significant impact on quality of life (Pagulayan et al., 2006; Strandberg, 2009). In addition to these cognitive impairments, people who have sustained a TBI are at significantly higher risk of developing other health problems. Compared to the general population, TBI victims are twice as likely to binge drink (Horner et al., 2005), several times more likely to develop epilepsy (Frey, 2003), and 7.5 times as likely to die 1-3 years after the initial injury (Selassie et al., 2005). Furthermore, moderate and severe TBI increases the probability of developing depression and Alzheimer's disease (AD) in later life (Plassman et al., 2000).

In the United States civilian population TBI is responsible for about 1.5 million new hospital visits every year (Rutland-Brown et al., 2006), and it has been estimated that there are currently over 5.3 million TBI patients (Langlois et al., 2006). The most frequent causes of moderate to severe TBIs are road traffic accidents, followed by falls and assaults. The incidence of TBI is highest in people between 15 and 24 years of age, and those persons over the age of 75 (Kraus et al., 1984). The direct cost of care associated with TBI is estimated to be greater than $30 billion, and additional lifetime costs associated with TBI are estimated to be another $30 billion. Despite the huge amount of money spent on treating TBI, a recent study found that as many as 40% of those hospitalized experienced significant unmet needs one year later (Corrigan et al., 2004). Needs most often cited were improvement in memory and problem solving, managing stress and emotional upset, controlling one's temper, and improving

one's job skills. It is also important to note that the true number of people who sustain a TBI is difficult to determine, since large numbers of victims of mild TBI's may not visit the emergency room.

1.1.1 Epidemiology and financial burden of TBI in the United States

An increased awareness of TBI has been brought about in the United States of America by various reports from warzones in Afghanistan and Iraq. Those in military service have always had a higher incidence of TBI than the general population, and while penetrating injuries are common, the incidence of closed head injury is known to be much higher (Galarneau et al., 2008). Most of these injuries are the result of an explosion (e.g. an improvised explosive device), and the term 'blast injuries' is used to describe them. The injury is caused by large and sudden changes in air pressure as a direct result of an explosion (Taylor and Ford, 2009; Wolf et al., 2009). However, brain injuries can also be sustained from impact of objects put in motion by the blast, or by impact of the head against another object. Because of the nature of blast injuries the incidence of TBI in military personnel fighting in the "War on Terror" is difficult to estimate as they can often go unrecognized. A recent survey of military personnel returning from the theatre of war revealed a 12% incidence of mild TBI, a condition that can significantly affect quality of life (Schneiderman et al., 2008). The increased awareness of TBI has lead to the Department of Defense and the Department of Veterans Affairs instituting routine screening of veterans for TBI (Affairs, 2007), and medical professionals are now being trained to recognize and treat these brain injuries (Martin et al., 2008).

1.1.2 Classification of TBI: closed versus penetrating head injuries

There are many different ways in which a person can sustain a TBI, including, but not limited to, motor vehicle collisions, falls, and gun-shots. In order to develop treatment strategies for TBI it is important to understand how these injuries vary, and therefore, it is necessary to categorize these injuries based on similarities. The easiest way of classifying injuries is to determine whether they are skull penetrating brain injuries, or closed skull injuries.

Penetrating brain injuries are most often caused by gunshots associated with combat or other acts of violence (e.g. stab wounds). Closed head injuries are significantly more common (Styrke et al., 2007), and are caused when static or dynamic forces are applied to the head. The rapid acceleration, deceleration, and rotational forces involved in vehicular collisions are the most common cause of this injury (Denny-Brown and Russell, 1941; Ivancevic, 2008; Krave et al., 2005). Injuries caused by dynamic forces can be further sub-divided into injuries involving an impact, and those that do not (impulsive). In many instances injuries will be the result of both inertial forces and of an impact. Obviously, the amount of force acting on the brain is an important factor in influencing the severity of the TBI.

1.1.3 Classification of TBI: primary and secondary injuries

It is clear that there is significant variation in the types and severity of TBIs and it is easy to presume that following the initial insult, the brain healing process begins. Unfortunately this is not the case, and it is widely recognized that brain injuries can become more severe with time (Anderson et al., 2005; Himanen et al., 2006; Sato et al., 2001; Shiozaki et al., 2001). This is due to the evolution of a secondary injury that occurs in the minutes, hours, days, and even months after the initial (primary) injury. The primary injury causes a disruption in the homeostasis of the brain; axons are damaged or sheared, cells are ruptured, blood vessels are damaged, and tissue may be lost (Gaetz, 2004; Gennarelli, 1993; Gennarelli and Graham, 1998; Hammoud and Wasserman, 2002; Strich, 1961; Strich, 1956). An immediate and significant effect of this damage is often ischemia, which contributes significantly to secondary damage (Bramlett and Dietrich, 2004; Graham et al., 1978; Graham et al., 1988; Nortje and Gupta, 2006; Zauner et al., 2002). Damaged or ruptured cells release a multitude of different messenger molecules, including pro-inflammatory cytokines and the excitatory neurotransmitters glutamate and acetylcholine (ACh). Increased levels of these molecules lead to activation of their respective ionotrophic receptors and an influx of calcium (Ca^{2+}) and sodium (Na^{+}) into neurons. Under normal conditions ACh and glutamate are released in precisely controlled areas, and are

then rapidly degraded (ACh) or taken up into presynaptic neurons or astrocytes via energy dependant transporters (glutamate). However, following an injury the haphazard nature of neurotransmitter release combined with the amount released leads to the activation of many cellular pathways and damaging enzymes. The end result of this is cellular dysfunction and cell death via apoptosis or necrosis.

1.1.4 Classification of TBI: injury severity

TBI patients can be categorized as having a severe, moderate, or mild TBI when admitted to the hospital. The most widely used tool for assessment of the severity of injury is the Glasgow Coma Score (GCS). The GCS consists of a battery of tests for eye response (1-4 points), verbal response (1-5 points), and motor response (1-6 points). The test therefore rates patients on a scale of 3-15, 3 being deep coma/death, and 15 being fully awake (Teasdale and Jennett, 1974). It is generally accepted that a score of ≥13 is a mild injury, 9-12 is moderate, and a score ≤8 is classed as a severe injury (Jennett, 1998; Parikh et al., 2007). In addition to the GCS, doctors may take into account measures such as the duration of post-traumatic amnesia and loss of consciousness to make a final assessment of injury severity. While this scale has been shown to be of prognostic value (Narayan et al., 2002), a recent workshop convened by the Veterans Brain Injury Center and the National Institute of Disability and Rehabilitation criticized its use in recruiting for clinical trials because of the lack of assessment of factors such as age, extra-cranial injury, and pre-existing neurological impairment that may override treatment effects (Saatman et al., 2008). Furthermore, the GCS does not discriminate well in less severely injured patients, which comprise the majority of cases.

Since these clinical assessments are clearly not applicable in animal models of TBI the injury severity is frequently defined by the parameters of the injury-producing device (Cernak, 2005; Morales et al., 2005). Because of this discrepancy, it is important to realize that while a mild/moderate/severe injury in a

rodent paradigm models some aspects of the human condition it is not necessarily fully equivalent (see below for a more detailed discussion).

1.1.5 The secondary injury cascade

Excitotoxicity is the term used to describe the process by which excitatory neurotransmitters can adversely affect the survival of neurons by over-stimulating their respective ionotrophic receptors. T. Hayashi is credited with being the first to report the excitotoxic effects of glutamate in 1954, when he observed that glutamate applied directly to the cortex induced convulsions (Hayashi, 1954). But it was not until 1969 that J. Olney coined the term excitotoxicity when he noticed that monosodium glutamate fed to mice or monkeys produced lesions in the brain (Olney, 1969; Olney and Sharpe, 1969). Since these early studies the mechanisms by which glutamate causes neuronal death have been thoroughly elucidated.

Once released into the synaptic cleft, glutamate can bind to and activate a family of glutamate receptors. Activation of ionotrophic receptors leads to depolarization of the post-synaptic membrane by facilitation of cation flow into the neuronal cytoplasm. In total there are 11 types of glutamate receptor, 8 are metabotrophic and 3 are ionotrophic. The three types of ionotrophic glutamate receptors are the kainite receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and the N-methyl-D-aspartic acid (NMDA) receptor (Dingledine et al., 1999; Liu and Zhang, 2000; Paoletti and Neyton, 2007). AMPA and NMDA receptors are found on post-synaptic neurons in intimate proximity to presynaptic nerve terminals (Antal et al., 2008; Kharazia et al., 1996). Whereas activation of AMPA receptors is dependant only on glutamate binding, NMDA receptors require the binding of co-agonist glycine (Kleckner and Dingledine, 1988), depolarization-induced removal of magnesium ion blockade of the ion channel (Nowak et al., 1984; Ruppersberg et al., 1994) and binding of glutamate. The initial activation of AMPA receptors by glutamate leads to sodium influx and membrane depolarization, which facilitates the removal of the magnesium block in nearby NMDA receptors and allows for

NMDA receptor activation by glutamate when the co-agonist glycine is present. Once activated NMDA receptors are selectively permeable to calcium ions (Jahr and Stevens, 1993; MacDermott et al., 1986), further depolarizing the post-synaptic membrane and leading to activation of intracellular signaling pathways. However, sustained high levels of glutamate can lead to prolonged activation of excitatory glutamate receptors. It is the NMDA receptor that is believed to be the major contributor to the toxic effects of glutamate due to its relatively high permeability to calcium.

Calcium is an important secondary messenger that has various physiological actions inside a cell (Berridge et al., 2003; Petersen et al., 2005). Under normal physiological conditions the extracellular calcium concentration is 10,000 fold higher than the intracellular concentration (approximately 1 mM versus 100 nM, respectively). This high degree of control is maintained by the elimination of calcium from the cell via the sodium/calcium exchanger (Yu and Choi, 1997), calcium ATPase (Jensen et al., 2004), or by sequestration in calcium 'sinks' such as the endoplasmic reticulum and the mitochondria (Pizzo and Pozzan, 2007). However, when there is an excess of extracellular glutamate, prolonged activation of NMDA receptors may lead to the accumulation of calcium in the cell (Dubinsky, 1993). The mitochondria can take up a lot of calcium via the mitochondrial calcium uniporter (Kirichok et al., 2004), which has a much higher activity than the extrusion pathway via $2Na^{+}/Ca^{2+}$ exchange. In this way the mitochondria play a role in maintaining cellular calcium homeostasis (Carafoli, 2003), but when too much calcium is present adenosine-5'-triphosphate (ATP) synthesis is inhibited and reactive oxygen species (ROS) including superoxide and peroxonitrite are produced (Brookes et al., 2004; Hall et al., 2004). Peroxidation of lipids by ROS is damaging to the cell and could result in cell death. Furthermore, the lack of ATP will have many detrimental effects in the cell; including failure of energy-dependent glutamate transporters. Normally, glutamate is cleared from the extracellular space by active transport across the plasma membrane; therefore a failure of these transporters will lead to a reduced clearance of glutamate and exacerbated NMDA receptor stimulation. Calcium

overload can lead to the formation of the permeability transition pore (Crompton, 1999) and subsequent release of cytochrome *c (Luetjens et al., 2000).* In the cytoplasm, cytochrome c can activate the intrinsic apoptosis pathway via activation of caspase 9, which then activates caspases 3 and 7 (Cohen, 1997). The excess calcium produced by over-stimulation of NMDA receptors is very different from normal physiological circumstances where the effects of calcium on the cell are regulated by discrete, localized increases. These large-magnitude global increases in calcium concentration within the cell activate numerous signaling cascades and enzymes, including phospholipases, proteases, and endonucleases. Once activated, these enzymes lead to membrane damage, loss of membrane potential, cytoskeletal damage, and deoxyribonucleic acid (DNA) damage. The disruption of cellular homeostasis in a disease state can lead to programmed cell death via apoptosis, or to a disorderly cell death via necrosis. Taken together this demonstrates the damage that excitotoxicity can cause and the integral role of the mitochondria in secondary injury and neuronal dysfunction. Studies in rodent models of TBI have demonstrated the early onset of mitochondrial dysfunction and lipid peroxidation (Singh et al., 2006).

Although many studies focus on the role of glutamate it is important to realize that several other excitatory neurotransmitters could potentially contribute to calcium-related excitotoxicity. Acetylcholine is one such neurotransmitter best known for its prominent role at the neuromuscular junction (NMJ) in the peripheral nervous system (PNS). It is now well established that ACh also has a critical role in the CNS, where it acts at G-protein coupled muscarinic acetylcholine receptors (mAChR) as well as a number of ionotrophic receptors. Of particular interest with regard to excitotoxicity is the α7 nicotinic acetylcholine receptor (nAChR); a ligand-gated ion channel with high permeability to calcium (Delbono et al., 1997; Sands et al., 1993). Also of note is the serotonin receptor 5-HT_3 (5-hydroxytryptamine), since unlike other serotonin receptors it is a sodium-permeable ion-channel rather than a G-protein coupled receptor. While the 5-HT_3 receptor is not selectively permeable to calcium like the NMDA and α7 nAChR (Mochizuki et al., 1999), it is of interest because it is unique among

serotonin receptors, sharing a great deal of homology with the α7 nAChR receptor, and is found in areas of the brain known to be especially vulnerable to injury such as the hippocampus (Fletcher and Barnes, 1999; Ge et al., 1997; Parker et al., 1996).

In addition to excitotoxicity, the contribution of inflammation to secondary injury is an important consideration. In the periphery, the inflammatory response has numerous roles including phagocytosis of dead cells and invading pathogens, and the promotion of recovery. However, in the brain an inflammatory response could contribute to post-injury damage and loss of function. The brain has long been considered to be an immunologically privileged organ (Murphy and Strum, 1923; Shirai, 1921), able to tolerate introduction of antigen without eliciting an immune response. However, it is now becoming apparent that immune cells play a central role in the pathogenesis of secondary injury (for a review see Galea et al., 2007). Microglia are the main immune cell in the brain, accounting for an estimated 10-20% of all non-neuronal cells in the central nervous system (CNS) (Lawson et al., 1990). Microglia are known to have multiple phenotypes that are seen depending on their environment (Gehrmann et al., 1995). In normal brain, resting microglia have small cell bodies and numerous long processes that constantly elongate and shorten giving the impression that they have a surveillance role (Kreutzberg, 1996; Nimmerjahn et al., 2005). Several factors are known to lead to microglial activation, including pro-inflammatory cytokines and cell necrosis factors. Such conditions can be found in the brain under neurodegenerative or injury states, which leads to the transformation of microglia to an activated state (Ransohoff and Perry, 2009). The activation of microglia can be very rapid; several studies have shown that activation can be detected within hours or minutes of an insult (Kelley et al., 2007; Liu et al., 2009; Raghavendra Rao et al., 2000; Suma et al., 2008). The transformation of quiescent microglia to a fully activated phagocytic cell is a graded response. Non-phagocytic activated microglia exhibit fewer, thicker, retracted processes and larger cell bodies. Activated microglia begin to proliferate and secrete cytotoxic factors and pro-inflammatory cytokines. When

fully activated they are phagocytic, taking on a completely amoeboid appearance (Gehrmann et al., 1995). These phagocytic microglia migrate to the site of injury and engulf foreign material and cellular debris, while also secreting pro-inflammatory cytokines to amplify the immune response. Therefore, modulation of microglial cell activation may be one possible way to decrease brain inflammation and secondary neuropathology following TBI.

1.1.6 Current treatment strategies for TBI patients

In individuals that suffer a TBI the primary focus is on stabilizing the patient to improve their chances of a favorable clinical outcome. This can be achieved by first immobilizing the patient to lessen the risk of further CNS injury. To reduce the risk of cerebral ischemia healthcare workers must ensure the patient has adequate airway patency, breathing and oxygenation. Close attention is paid to insure a proper oxygen supply to the brain by maintaining blood flow and blood pressure. In the hospital careful monitoring of intracranial pressure (ICP), and mean arterial blood pressure provides an estimate of the cerebral perfusion pressure (CPP). The CPP is maintained at a satisfactory level to prevent additional brain damage (Rosner, 1995; van Santbrink et al., 2002). ICP can be continuously monitored by surgically implanting a catheter into one of the lateral ventricles. Maintenance of ICP can be achieved by administering sedatives, paralytics, and if necessary by draining cerebrospinal fluid (CSF) from a lateral ventricle. More drastic measures may be taken in severe cases, including a decompressive craniectomy to allow the brain to swell until ICP is resolved (Schirmer et al., 2008).

When ready, moderately and severely injured patients discharged from the acute care setting will receive physical/occupational, speech/language therapy, or be referred to a psychiatrist, or social worker as required. Rehabilitation and therapy for survivors of TBI focuses on helping the patient to function independently at home and at work. However, the services received often fail to address the emotional, cognitive, and behavioral needs of the patient (Martin et al., 2001). Even victims of mild injuries can experience long-lasting

cognitive deficits that include memory loss, problems with concentration and attention, and deficits in executive functions such as judgment, planning, problem-solving and reasoning.

1.2 Modeling TBI in the laboratory

1.2.1 Overview of in vivo TBI paradigms

In the clinic a wide range of types and severity of head injury are seen that often result in vastly different outcomes. Due to this heterogeneity in TBI there is no one model that can mimic the full range of injury severities and clinical sequelae seen in human TBI (Park et al., 2007; Saatman et al., 2008). For this reason several models are widely used in pre-clinical TBI research to model the full range of injury severities and pathological characteristics seen in TBI patients. Although *in vitro* models of TBI facilitate the evaluation of pharmacological treatments in a rapid and cost-efficient manner, they lack face validity. *In vivo* models, summarized below, allow for assessment of the whole animal response to injury, including measurement of cognitive and behavioral deficits after experimental brain injury. This makes for a more relevant model at the expense of increased cost, variability, and difficulty in sampling.

One of the first standardized *in vivo* models of TBI was the weight-drop model in which a free-falling weight impacts the exposed skull of an anesthetized rat to produce what is usually referred to as a ‘focal’ injury (Shapira et al., 1988). The model has since been adapted for use on mice (Chen et al., 1996; Flierl et al., 2009). A key feature of this model is its simplicity; minimal surgical preparation required and the severity of injury can be easily altered by adjusting the mass and height of the impacting weight. Weight drop injury leads to immediate and brief unconsciousness, cortical contusion, selective neuronal death in the cortex, hippocampus and thalamus, and behavioral deficits (Chen et al., 1996). The model does have disadvantages, including the likelihood of rebound injury and a high variability, necessitating larger group sizes to see effects of a comparable magnitude as those seen in similar models.

Furthermore, this model has a relatively high mortality rate, again increasing the number of animals that must be used.

A related model is the impact acceleration model (Foda and Marmarou, 1994; Marmarou et al., 1994), in which the injury is again produced by a free falling weight. However, in this model a stainless steel plate is glued to the skull to distribute the force of impact over a wide area, minimizing the probability of skull fracture and producing a more diffuse injury. A second critical difference between this model and the weight-drop model is that the animal is unrestrained, and its head is placed on a piece of foam of known spring constant to allow movement of the head after impact. The physical properties of the foam, combined with the mass and height from which the weight is dropped, determine the severity of injury (Piper et al., 1996). This model produces measurable neurological and cognitive deficits in rats, and more severe injuries can be obtained by increasing the height at which the weight is dropped from or by the addition of a hypoxic insult (Beaumont et al., 1999). More recently the impact-acceleration model has been modified to produce a diffuse axonal injury (DAI) (Kallakuri et al., 2003), or to produce mild TBIs (Ucar et al., 2006) since the model is predominantly used to produce severe injuries. Like the weight-drop model, since this model also utilizes a free-falling weight to produce injury there remains the possibility of a second rebound injury.

Fluid percussion injury (FPI) is one of the most commonly used and best characterized *in vivo* models of TBI. Before performing the injury, the rat is anaesthetized, its skull exposed, and a craniotomy performed. Like the previous two models, the energy used to produce injury comes from a falling weight, here in the form of a pendulum. Unlike the other models the pendulum strikes a saline-filled syringe that forces a saline bolus through the sealed craniotomy to impact the dural membrane causing a pressure wave that spreads throughout the epidural space producing a diffuse injury (McIntosh et al., 1989). The injury severity can be adjusted by changing the height of the pendulum, and therefore the force of the pressure pulse that is transmitted through the saline reservoir.

The model produces damage with characteristics consistent with that of a diffuse brain injury. However, a significant drawback of the FPI model is the high mortality rate associated with injury to the brainstem, especially at higher injury severities (McIntosh et al., 1989; Thibault et al., 1992).

The controlled cortical injury (CCI) model of TBI was developed in 1988 for use by General Motors and was originally designed for ferrets (Lighthall, 1988). It was modified for use in rodents (Dixon et al., 1991; Hamm et al., 1992) and is now commonly used in rats and mice to produce fairly consistent focal injuries (relative to other TBI models). The model utilizes a pneumatically driven impactor to administer a single mechanical deformation of the exposed brain without rebound injury. Due to the precision machinery used the injury severity and type can be adjusted by using different shape and size impactor tips, adjusting the impactor velocity, or altering the depth of cortical deformation. In our laboratory the surgery is performed on anesthetized rats that have been immobilized in a Kopf stereotaxic frame (David Kopf Instruments, CA). This allows for the precise positioning of a craniotomy midway between bregma and lambda (bregma -4.5 mm), and 5-7 mm laterally over the somatosensory cortex. Injury in this location on the somatosensory cortex leads to cortical tissue loss and damage to the hippocampus either through direct energy transfer or via the perforant pathway following damage to the entorhinal cortex. Work in our laboratory and others have shown that this placement produces measurable cognitive deficits (Dixon et al., 1995b; Guseva et al., 2008; Verbois et al., 2003a). In addition, this model benefits from a lower rate of mortality when compared to other models of brain injury (Kochanek et al., 1995).

1.2.2 Measuring CNS damage following experimental TBI

Well established methods to assess the short and long-term neuropathological effects of the experimental TBI and potential pharmacotherapies are needed. Post-mortem assessments of gross tissue loss/disruption, individual neuronal or glial cell loss, and expression of cell or cell cycle specific enzymes and genes help provide a more detailed characterization

of the injury. Post-injury assessments can measure markers such as cell death via apoptosis or necrosis, or could utilize markers of reactive gliosis. Reactive gliosis refers to the accumulation of activated glial cells including microglia, macrophages and astrocytes immediately after CNS injury. The term 'neuroinflammation' is now more commonly used to describe this process. Astrocytes synthesize more glial fibrillary acidic protein (GFAP) and undergo some morphological changes including the elongation of more pseudopodia that form a dense mesh to fill space left by dead neurons. Microglia, the CNS equivalent of macrophages, rapidly become activated and secrete cytokines, neurotropic factors, and other biologically active factors. Macrophages are difficult to delineate from microglia, being from the same lineage. They are known to cross the blood brain barrier after brain injury and are thought to play a similar role to microglia.

For models that result in a visible loss of cortical tissue the simplest and quickest assessment that can be made is that of cortical tissue sparing analysis (Scheff and Sullivan, 1999). The analysis is carried out on nissl-stained (e.g. Cresyl violet) coronal brain sections. This makes it easy to distinguish viable tissue so that measurements can be made of the area of tissue remaining, i.e. spared. For unilateral injuries measurements of area can be taken for both hemispheres, allowing for a calculation of the lesion's area by subtracting the ipsilateral area from the contralateral 'control' area in each section. When studying the neuroprotective effects of certain treatments it makes more sense to express these numbers as 'tissue spared' as opposed to lesion size. This is easily achieved by dividing the ipsilateral area by the contralateral area (and multiplying by 100) to yield a percentage of tissue spared. While this method is a good measure of the severity and extent of injury, it does not give any indication of functional injury or cell loss that could be occurring in other areas of the brain, or indeed in regions of the ipsilateral cortex distal to the site of impact. More sensitive techniques are required to quickly assess and quantify these more subtle, but potentially significant changes.

To assess changes that occur on a cellular level after injury, radioligands or antibodies can be employed to mark specific receptors or antigens expressed by certain cell types. For example, neuronal survival can be assessed with antibodies against NeuN (neuronal nuclei), a DNA binding protein found in most neuronal nuclei (Mullen et al., 1992). Degenerating neurons long been known to be argyrophilic and silver staining methods have developed to visualize the cell bodies and neuronal processes of these neurons (Tenkova and Goldberg, 2007). A more straightforward and recent technique to label degenerating neurons is fluoro-jade, an anionic fluorescein derivative. Fluoro-jade is thought to bind to polyamines that are found in degenerating cells (Schmued and Hopkins, 2000a, b). Additionally, apoptotic cells can be visualized by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). To be useful in identifying the cell types that are dying TUNEL is often carried out as a double or triple staining with antibodies against specific cell types such as microglia/macrophages (e.g. anti-CD68, OX-42, F4/80, iba-1), astrocytes (e.g. anti-GFAP), or neurons (e.g. NeuN). In addition, the identification of microglial activation and macrophage infiltration by immunohistochemical techniques can provide a good assessment of the degree of cellular damage resulting from a particular injury or damaging agent (Nixon et al., 2008). A drawback of each of these assessments is the difficulty of obtaining an accurate quantification. Precise quantification of cell numbers can be very time-consuming and little information on expression on individual cells can be acquired. To this end, another useful tool to assess damage and inflammation after TBI may be autoradiography using the ligand [^{3}H]-PK11195 (Banati et al., 1997; Sitte et al., 2001; Vowinckel et al., 1997). Autoradiography allows for a rapid assessment of ligand binding density in multiple discrete regions within the brain. Studies that have utilized [^{3}H]-PK11195 autoradiography have demonstrated its potential as a sensitive marker of inflammation in models of neurodegenerative disease and brain injury (Chen and Guilarte, 2006; Grossman et al., 2003; Pedersen et al., 2006; Roberts et al., 2009). Although the precise antigen and cell types to which PK11195 binds have not been fully elucidated, it appears to be associated with

mitochondrial cholesterol transport in inflammatory cell types (Banati et al., 1997; Giannaccini et al., 2000). Further evaluation of the binding profile of [^{3}H]-PK11195 in a model of TBI could help to establish the technique as a valuable tool in the assessment of post-traumatic inflammation.

1.2.3 Assessing functional deficits

To be clinically relevant, models of TBI should be able to produce functional and cognitive deficits that are measureable by well defined tests in the laboratory. To this end several methods have been developed to characterize the deficits seen after injury in rodent models. The rotorod is one such test in which a mouse or rat is placed on a rotating drum in a semi-enclosed chamber. The drum slowly accelerates and the latency of the animal to fall is recorded, giving an indication of motor coordination, balance and stamina. Another test is the ledged tapered beam, in which animals must traverse a suspended ledged beam which begins wide enough to walk comfortably, and tapers to a point. The number of times each paw slips onto the ledge and at what point on the beam can be recorded as an indication of balance and motor function. Other assessments include locomotor activity monitoring and open field testing. More complete assessments of overall neurological functioning can be made using a battery of simple tests. An example of such a scoring modality is the composite "neuroscore", which takes into account forelimb flexion when suspended by the tail, resistance to pulsion, hindlimb splay, toe-spread and toe-extension, and ability to stand on an inclined surface facing up, down, left or right (Gruner et al., 1996; McIntosh et al., 1989). Another battery of tests is the neurological severity score (NSS) which assesses hemiparesis, ability to traverse beams of various widths, ability to balance on round or square sticks, failure to exit a circle, startle reflex, and seeking behavior (Beni-Adani et al., 2001; Panikashvili et al., 2005). While useful in assessing acute motor deficits it should be remembered that a large number of people suffer mild injuries in which cognitive and other neuropsychiatric changes cause the most damage to the routines of daily living. Fortunately, there are some well established tests of cognitive performance in the rodent, including the T-maze and the commonly used Morris water-maze (MWM).

The MWM was developed in 1984 by Richard Morris to test spatial memory in rats (Morris, 1984). The testing apparatus consists of a circular pool 1.5-2 m in diameter and about 60 cm deep, filled with water to a depth of about 30 cm. The water is made opaque by the addition of a pigment to obscure the location of a 15 cm diameter escape platform hidden approximately 1 cm below the water level, and to provide contrast against the color of the animal being tested. Visual cues are placed around the outside of the pool in plain sight of the test subject. The rat or mouse is carefully placed in the pool rear end first facing the edge of the pool to reduce stress and bias respectively. The animal then searches the pool for an exit, and its escape from the pool and rescue by the experimenter reinforces its desire to find the escape platform swiftly in subsequent trials. Improvement in performance in the MWM comes about because the rat/mouse has learned the position of the escape platform in the pool relative to the visual cues around the test room. The entire experiment is recorded using a video camera mounted above the pool so that a more thorough evaluation of each rats' search pattern can be made.

Various training paradigms exist that include acquisition training and memory retention testing (Brandeis et al., 1989; Wenk, 1998). Acquisition training usually consists of a block of 4 swim trials in which the animal starts at each of 4 randomly chosen positions around the pool. If the animal successfully finds the platform it must remain there for a short amount of time (e.g. 15 s) before being rescued. If a pre-determined amount of time passes without the animal finding the platform (e.g. 60 s) it is manually guided or placed on the platform. The data from each of the 4 trials in the block can then be averaged. Further blocks of training trials can be performed on consecutive days or even on the same day (Kraemer et al., 1996). Following the acquisition testing, a memory retention test is usually performed in which the escape platform is removed from the pool. Each animal is allowed to swim in the pool for a set amount of time (e.g. 30 s, 60 s, 120 s). During this memory retention test the animals' spatial ability can be assessed by measuring the amount of time it spends searching in the vicinity of the escape platform, the number of times it crosses into the

platform area, or the number of times it crosses into the target quadrant. The ability of researchers to make accurate assessments of these parameters has been greatly improved by the availability of electronic tracking systems such as Videomex (Columbus Instruments, Columbus, OH).

Since the first description of the water maze by Morris in 1984, which utilized hooded rats, there have been thousands of studies utilizing the technique with a variety of both rat and mouse strains. It is therefore important to consider whether these or indeed other factors could influence MWM performance when comparing studies. For example, differences in spatial performance between men and women have been well documented (Astur et al., 1998; Duff and Hampson, 2001; Newhouse et al., 2007; Rizk-Jackson et al., 2006), and similar sex-differences have been reported in rodents (Frye, 1995; Galea et al., 1996). These differences could obviously be due to sex hormones as suggested in several studies (Daniel et al., 1999; Roof, 1993; Warren and Juraska, 1997). In addition to sex differences significant differences have been found in the performance of different rodent species, specifically mice in the MWM (Lipp and Wolfer, 1998). Mice have a tendency to float or swim along the edge of the pool (thigmotaxis) more than rats, and often perform quite poorly in comparison. A comparison study has found similar performance for mice and rats in 'dry' spatial learning tasks but not for the water maze task, indicating that a difference in spatial ability is not the factor at play (Whishaw and Tomie, 1996). Another major factor to consider is the age of animals within a study, as it is well established that MWM performance declines with age (Brandeis et al., 1989; Gower and Lamberty, 1993). Furthermore, it has been recognized that the MWM causes a stress response in rodents that could potentially confound results (Engelmann et al., 2006).

The MWM is known as a hippocampal dependant task because the hippocampal formation is clearly essential for spatial learning, as demonstrated by impaired performance in animals with hippocampal lesions (Morris, 1984; Morris et al., 1982; Moser et al., 1993; Pearce et al., 1998). However, the

involvement of other brain regions cannot be ruled-out. In addition to the involvement of the hippocampal formation in spatial learning other brain regions including the caudate putamen (a.k.a. stiatum) (Whishaw et al., 1987), basal forebrain (Mandel et al., 1989), cortical regions (Liu and Bilkey, 1998; Mogensen et al., 1995; Roof et al., 1993), and cerebellum (Lalonde, 1994) have been implicated. This demonstrates that spatial learning requires the coordinated action of multiple brain regions and is a complex process. Despite the apparent complexity the process, long-term potentiation (LTP), is considered to be one of the major cellular/synaptic mechanisms involved in learning (Lynch, 2004). LTP is process by which the efficacy of signaling between two neurons is increased following repeated stimulation. This effect is long-term and mediated by changes in gene expression and protein synthesis. In the hippocampus spatial learning is thought to involve place cells, which exhibit a high rate of firing when an animal is in a specific position within and environment corresponding to the cell's 'place field' (Buzsaki, 2005; O'Keefe and Dostrovsky, 1971; Poucet et al., 2000). In the rat hippocampus there are hundreds of thousands of pyramidal cells in close proximity with a high degree of connectivity (Li et al., 1994). The fields of place cells within this population are distributed evenly across the environment (Muller et al., 1987), so any pair of fields in the environment could be represented by hundreds of connected cells. Therefore, because the firing frequency of each place cell varies as a function of the distance between them, place cells with overlapping place fields could undergo LTP and thus encode spatial distance (Isaac et al., 2009; Muller et al., 1996). The importance of LTP in spatial learning is highlighted by studies in which NMDA antagonists are administered. Morris *et al.* originally demonstrated impaired water maze performance of rats administered the NMDA antagonist (2R)-amino-5-phosphonovaleric acid (AP5) via intraventricular infusion (Morris et al., 1986), and subsequent studies have highlighted the role of NMDA receptors in spatial learning (McNamara and Skelton, 1993). As well as NMDA involvement it has long been known that the cholinergic system plays a significant role in spatial localization (Sutherland et al., 1982).

The neuropharmacology of spatial learning continues to be studied, but the involvement of cholinergic and glutamatergic neurotransmitter systems are well established (Hagan and Morris, 1987; McNamara and Skelton, 1993). Several studies have shown that NMDA receptor blockade impairs MWM performance (Davis et al., 1992; Hauben et al., 1999; McLamb et al., 1990; Morris et al., 1986; Upchurch and Wehner, 1990). It has also been shown that acute treatment with an NMDA agonist (D-cycloserine) can improve spatial memory following lesioning of the hippocampus in rats (Schuster and Schmidt, 1992). The water maze has been used extensively in the study of neurotrauma because of the need for a treatment for the cognitive impairments reported after TBI in humans. The MWM has been shown to be capable of testing for cognitive deficits in rodents following experimental trauma (Kraemer et al., 1996; Scheff et al., 1997; Smith et al., 1995; Yamaki et al., 1998). The precise mechanisms leading to these deficits are unclear, but glutamatergic and cholinergic disruption has been demonstrated (Dixon et al., 1999).

1.2.4 Acetylcholine receptors

Acetylcholine is a major neurotransmitter in the mammalian nervous system where it acts on a diverse family of receptors. Sir Henry Dale first discovered that there are 2 distinct subclasses of cholinergic receptors in 1914 when he observed that crude extracts of *Amanita muscarina* (muscarine) or *Nicotinana tabacum* (nicotine) mimicked the effects of 'esters of choline' (now known as acetylcholine) in different tissues (Dale, 1914). It is now known that the mAChRs are G-protein coupled receptors that function via activation of secondary messenger molecules, and nAChRs are ligand-gated ion channels permeable to sodium and calcium ions (Millar and Gotti, 2009).

The mAChRs can be excitatory or inhibitory depending on the secondary messenger system that they are coupled with. The M1, M3, and M5 subtypes activate phospholipase C via the $G_{\alpha q}$ protein (Guo and Schofield, 2003), which produces phosphatidylinositol trisphosphate (IP_3) that leads to release of calcium from intracellular stores. In contrast, the M2 and M4 subtypes inhibit adenylyl

cyclase via $G_{\alpha i/o}$ thereby giving ACh an inhibitory action (Caulfield and Birdsall, 1998; Ishii and Kurachi, 2006). Although mAChRs are more abundant in the brain and have been implicated in several neurological conditions, compared to nAChRs they are not as diverse in their subtypes and pharmacological properties.

Relative to mAChRs, nAChRs elicit a fast response by directly facilitating the flow of cations across the plasma membrane, thereby leading to a rapid depolarization. The first nAChR to be characterized was from the electric organ of the marine ray *Torpedo*. The natural abundance of receptors in this species made it well suited to studies of nAChR structure and dynamics (Kosower, 1987; Mishina et al., 1984). Since these early studies nAChRs have been found throughout the CNS as well as the PNS (Sargent, 1993). Electron microscopic techniques have made it clear that nAChR subunits form pentamers around a central ion channel (Miyazawa et al., 1999). However, the lack of crystallographic data has precluded the acquisition of more detailed atomic structures. In the well studied NMJ the nicotinic receptor is composed of two α subunits (termed "α1"), a β subunit (termed "β1"), a δ subunit, and a γ or ε subunit (Green and Millar, 1995). In contrast, the stoichiometry of neuronal nAChRs is still being elucidated. Two subtypes of nAChR subunits have been identified in the CNS: alpha (α) subunits of which 9 are known (α2-α10), and beta (β) subunits of which 3 are known (β2-β4). Although the number of subunit types presents the possibility of a huge array of nAChR compositions it appears that the assembly of nAChRs is tightly regulated and results in a limited number of channel types. In general, nAChR subunits form heteromeric receptors made up of three alpha and two beta subunits ($\alpha_{(3)}$ $\beta_{(2)}$) (Anand et al., 1991; Boorman et al., 2000; Cooper et al., 1991), and are selectively permeable to sodium. Exceptions to the rule are the α7, α8, and α9 subunits, which are able to form functional homomeric receptors *in vivo* (Elgoyhen et al., 1994; Gerzanich et al., 1994; Gotti et al., 1994). The predominant nAChR compositions in the mammalian brain are thought to be α4β2 (Lindstrom et al., 1995; Papke et al., 1989; Picciotto et al., 1998; Zoli et al., 1998), and the homomeric α7 receptor

(Chen and Patrick, 1997; Couturier et al., 1990; Orr-Urtreger et al., 1997). Unlike other nAChRs, the α7 nAChR has been found to be much more permeable to calcium than sodium (Delbono et al., 1997; Sands et al., 1993). This high permeability to calcium is intriguing because calcium is an important secondary messenger in the CNS, and plays a major role in excitotoxicity as discussed earlier.

1.2.5 Evidence for cholinergic disruption following TBI

It is now well established that the cholinergic system is significantly impaired following brain injury. Impaired choline transport (Dixon et al., 1994a) and decreases in the activity of the ACh synthesizing enzyme choline acetyltransferase (ChAT) (Leonard et al., 1994), contribute to a widespread disruption of the cholinergic system. Earlier receptor binding studies focused on the expression of mAChRs, and found variable effects of injury, including increased, decreased and unchanged expression in animal models of neurotrauma (DeAngelis et al., 1994; Jiang et al., 1994; Lyeth et al., 1994; Sihver et al., 2001). A reduction in nAChR expression following brain injury could contribute to impaired cognitive ability in these animals (Verbois et al., 2002; Verbois et al., 2000). Indeed, improvements in cognitive functioning have been correlated with increased levels of nAChRs (Kadir et al., 2006). Further support for the role of nAChRs in cognition and cholinergic disruption in TBI comes from studies in our laboratory that have demonstrated a modest recovery of MWM performance in animals given chronic intermittent nicotine injections (Verbois et al., 2003a). Interestingly, this nicotine treatment regimen did not attenuate the reduction in α7 nAChRs suggesting that the stimulation of the remaining receptors may be sufficient for cognitive enhancement.

Further information on cholinergic disruption after injury comes from some interesting parallels between the pathophysiology of TBI patients and sufferers of chronic neurodegenerative diseases. For instance, TBI has some parallels with schizophrenia, in that deficits in auditory and sensory gating are found in both schizophrenics (Adler et al., 1982; Braff and Geyer, 1990) and TBI patients

(Arciniegas et al., 1999; Arciniegas et al., 2000). Furthermore, TBI patients with sensory gating deficits have shown improvement when administered an acetylcholinesterase (AChE) inhibitor (Arciniegas et al., 2000; Arciniegas et al., 2002), implicating the involvement of AChRs. The α7 nAChR is of special interest since decreased levels of the receptor in post-mortem brains may explain the lack of pre-pulse inhibition of sensory stimuli in schizophrenics compared to controls (Freedman et al., 1995). This hypothesis is bolstered by studies that determined that the deficit in sensory gating in schizophrenics can be linked to a genetic mutation of the α7 nAChR gene (Freedman et al., 1997; Raux et al., 2002). Several studies have demonstrated a normalization of auditory gating in schizophrenics after nicotine administration (Adler et al., 1993; Adler et al., 1992), as well as more general improvements (Myers et al., 2004; Smith et al., 2002). Furthermore, the rate of smoking is much higher amongst schizophrenics (around 80%) than in the general population (de Leon et al., 1995), raising the possibility that schizophrenics are self-medicating with nicotine to treat their symptoms. It should also be noted that one of the treatments for schizophrenia is clozapine. Clozapine is pharmacologically non-selective drug that acts on serotinergic, dopaminergic, and cholinergic receptors. It is clozapines' agonist activity on α7 nAChRs which is thought to normalize sensory gating in schizophrenics (Martin et al., 2004a).

Another neurodegenerative condition with some similarities to TBI is AD, where those inflicted have a significantly impaired cholinergic system (Terry and Buccafusco, 2003). Furthermore, TBI has been identified as a positive risk-factor for developing AD (Jellinger et al., 2001). The decline of cholinergic activity is hypothesized to contribute significantly to the devastating cognitive deficits seen in AD patients. A loss of α4β2 nAChRs has been well documented in AD (Perry et al., 2000; Schmaljohann et al., 2004; Wevers et al., 1999). The role of the α7 nAChR in AD is less clear, some studies have reported a modest increase (Chu et al., 2005; Counts et al., 2007; Dineley et al., 2001), and some a decrease (Wevers et al., 1999). Studies in our laboratory have consistently found similar reductions in α7 nAChR binding following brain injury (Guseva et al., 2008; Kelso

et al., 2006; Verbois et al., 2002; Verbois et al., 2000), but only modest reductions in α4 nAChR binding (Verbois et al., 2002). In addition, people with AD have a significant reduction in high affinity choline uptake sites (Rodriguez-Puertas et al., 1994) and a reduction in ChAT activity (Waller et al., 1986). Current therapies seek to maintain cholinergic signaling by inhibiting AChE, and evidence gathered from rodent studies suggests that nAChR agonists may enhance cognition (Arendash et al., 1995a; Boess et al., 2007; Kem, 2000). Taken together these findings demonstrate a deficit in nAChR expression in both AD and TBI that could be liked to cognitive deficits.

1.2.6 Agonists, antagonists and partial agonists of nAChRs

The study of nAChRs has been significantly aided by the discovery and molecular characterization of selective pharmacological antagonists and agonists. Since nicotinic cholinergic transmission regulates the NMJ and somatic nervous system a large number of predatory animals have evolved potent venoms that target the α1 nAChR. One of the first of such toxins discovered was α-bungarotoxin (α-BTX) from the banded krait (*Bungarus multicinctus*) which is an irreversible antagonist at the NMJ nAChR (Changeux et al., 1970). Shortly after, α-BTX was found to bind in brain fractions (Salvaterra and Moore, 1973), and autoradiographic characterization of its binding in the brain was carried out (Polz-Tejera et al., 1975). Later the α7 nAChR was shown to be the site to which α-BTX binds in the CNS (Couturier et al., 1990). In addition to snake venom, venoms from the many species of predatory cone snails are now of great interest and since they have been shown to contain an abundance of selective nAChR ligands (Cartier et al., 1996; Luo et al., 1998; Luo et al., 1999; McIntosh et al., 1999; McIntosh et al., 1994; Terlau and Olivera, 2004). While only a small proportion of these toxins have been purified and characterized their value and utility in studying the structure and function of specific nAChR subtypes is clear (Nicke et al., 2004). It is not only animal venom derived toxins that are of interest with relation to nAChRs. Aside from nicotine there is a range of plant alkaloids that act upon various nAChR subtypes, including curare, and methyllycaconitine (MLA, see appendix Figure 7.1). MLA is an alkaloid isolated from the tall

larkspur (*Delphinium brownii*) and is responsible for numerous cattle poisonings in the American Midwest. It causes paralysis in cattle by antagonizing the NMJ nAChRs (Pfister et al., 1999). In addition to its NMJ effects MLA has been shown to be a potent and competitive antagonist at CNS α7 nAChRs (Macallan et al., 1988). This property makes MLA a valuable tool for use in studying the function and localization of α7 nAChR subunits in the brain (Ward et al., 1990). Furthermore, unlike the large polypeptide chains of α-bungarotoxins and α-conotoxins, MLA has some ability to cross the blood-brain barrier (BBB) in rodents (Navarro et al., 2002; Stegelmeier et al., 2003; Turek et al., 1995). Combined, these properties make MLA a potential therapeutic agent with which to inhibit the calcium-mediated effects of ACh in the CNS.

In addition to full agonists and antagonists a range of nicotinic receptor partial agonists are available. Similar to a full agonist, a partial agonist binds to and activates the target receptor. However, unlike a full agonist the partial agonist has limited efficacy, meaning that a smaller proportion of the target receptors are in an activated state when saturated with ligand. For example, varenicline is a partial agonist of α4β2 nAChRs, and is now marketed as a tool for smoking cessation. As a partial agonist it can alleviate the symptoms of nicotine cravings and withdrawal, while also inhibiting the effects of nicotine exposure. α7-nAChR partial agonists such as 3-2,4 dimethoxybenzylidene anabaseine (DMXB-A, code-name GTS-21), tropisetron and ondansetron (for molecular structures see appendix, Figure 7.1) have been tested, and continue to be tested for therapeutic value in conditions such as AD and schizophrenia (Freedman et al., 2008; Kem, 2000; Koike et al., 2005; Oliveira et al., 2008). The utility of these α7 nAChR partial agonists in TBI remains to be determined. However, the potential for partial agonists to treat TBI has been demonstrated in a rat model, in which an NMDA partial agonist (D-cycloserine) or mAChR partial agonist (Lu 25-109-T) improved cognitive performance following injury (Pike and Hamm, 1997b; Temple and Hamm, 1996).

1.3 Specific aims and hypotheses

Evidence to-date suggests that following TBI acute increases in ACh could contribute to excitotoxicity, and more long-term deficits in ACh may contribute to cognitive deficits and other neuropsychiatric aspects of recovery. The α7 nAChR is of particular interest because it is widely expressed in the brain, is highly permeable to calcium (Fucile et al., 2003; Sands et al., 1993; Seguela et al., 1993), and its expression is significantly affected by brain injury (Verbois et al., 2002; Verbois et al., 2000). Previous studies in our laboratory have shown that post-traumatic nicotine can attenuate cognitive deficits in rats (Verbois et al., 2003a). In a separate study chronic nicotine treatment reversed α7 nAChR binding deficits (Verbois et al., 2003b). Furthermore, we have demonstrated that dietary supplementation with the selective agonist choline results in a modest degree of improvement in the MWM, spares cortical tissue, and reduces neuroinflammation in a rat model of TBI (Guseva et al., 2008).

Since nicotine has numerous central and peripheral effects a more selective drug is needed to target the post-injury excitotoxic cascade and to treat long-term cognitive deficits. Therefore, we hypothesize that administration of a selective antagonist immediately after injury will attenuate excitotoxicity and consequently lead to measurable improvements in cognition. While attenuating the excitotoxic effects of ACh may be neuroprotective, it does not directly treat the long-term cognitive deficits frequently reported by TBI patients. Another strategy might be to administer a partial agonist, thereby forgoing the efficacy of a full agonist or antagonist but eliminating some concerns over the timing of administration. Administration of a competitive partial agonist immediately after injury may attenuate α7 nAChR activation, potentially reducing the excitotoxic effects of elevated acetylcholine levels. Furthermore, administration of partial agonists beyond the initial period of increased cholinergic activity may still be therapeutic by improving cholinergic tone and thereby potentially improving cognitive outcomes.

In order to easily quantify the level of brain injury and make assessments of neuroprotective effects of experimental treatments it is important to have new, innovative tools. Although tissue sparing is frequently used it is only relevant if the injury is severe enough to produce tissue loss, and animals must be euthanized at a time point long enough to allow tissue encavitation. To this end we propose the use of labeled PK11195 as a sensitive tool for assessment of brain damage. Previous studies in both rodents and humans have demonstrated the ability of PK11195 to reveal subtle brain damage likely attributable to a microglial response (Banati et al., 1997; Chen and Guilarte, 2006; Grossman et al., 2003; Pedersen et al., 2006; Roberts et al., 2009; Sitte et al., 2001; Vowinckel et al., 1997). Therefore, we aim to develop this tool for easy assessment of brain inflammation that can be used to evaluate the pharmacological studies.

With these aims in mind we hypothesize that the characterization of a post-CCI time-course of neuroinflammation will aid in the assessment of the efficacy of α7 nAChR antagonist and α7 nAChR partial agonist treatments following experimental rat brain injury. Additionally, post-CCI treatment with a α7 nAChR antagonist or α7 nAChR partial agonists will lead to a significant improvement in cellular and functional outcomes.

This hypothesis will be tested by performing a series of experiments that will (1) characterize the time course of post-traumatic neuroinflammation; (2) assess the ability of an α7 nAChR antagonist to produce a neuroprotective effect and improve functional recovery; and (3) determine the therapeutic potential of α7 nAChR partial agonists in alleviating CCI-induced cognitive deficits and improving cellular outcomes.

- **Hypothesis #1** – PK11195 binding to TSPO can be used to detect differences in the timing and magnitude of microglial activation and macrophage infiltration following brain injury

- **Specific Aim 1:** Conduct a detailed time-course of [^{3}H]-PK11195 autoradiography in a CCI model of TBI and make assessments of the changes in binding that occur in multiple brain regions
- **Specific Aim 2:** Compare the timing and magnitude of changes in [^{3}H]-PK11195 binding to a marker of activated microglia/macrophages and to a marker of neuronal death

- **Hypothesis #2** – Administration of the α7 nAChR antagonist MLA after experimental brain injury will spare cortical tissue, reduce neuroinflammation, and improve cognitive outcomes
  - **Specific Aim 1:** Evaluate cognitive performance in rats administered either MLA or saline following CCI
  - **Specific Aim 2:** Assess the neuroprotective effects of MLA by conducting a cortical tissue sparing analysis
  - **Specific Aim 3:** Determine the effect of MLA on post-CCI α7 nAChR binding and measure the magnitude of post-CCI neuroinflammation using [^{3}H]-PK11195 autoradiography

- **Hypothesis #3:** - Administration of a α7 nAChR partial agonist following brain injury will improve spatial learning in the MWM, spare cortical tissue and attenuate neuroinflammation
  - **Specific Aim 1:** Compare the post-CCI cognitive performance of rats treated with either tropisetron or saline
  - **Specific Aim 2:** Determine whether post-CCI tropisetron is neuroprotective, and resolve regional differences in neuroinflammation
  - **Specific Aim 3:** Assess the effect of tropisetron on CCI-induced α7 nAChR binding deficits
  - **Specific Aim 4:** Evaluate two other α7 nAChR partial agonists as potential treatments for post-CCI cognitive impairments

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2. CHAPTER 2

[^{3}H]-PK11195 Autoradiography as a Tool to Assess TBI-Induced Brain Inflammation

2.1 Introduction

Over one and a half million people sustain a TBI every year in the United States alone, and the majority of these people will be left with motor and/or neuropsychiatric impairments that significantly affect sufferers' quality of live. The majority of TBI cases are the result of motor vehicle accidents, yet while great advances have been made in automobile safety and accident prevention there has been no significant improvement in the pharmacological interventions available clinically for those that suffer a TBI. The lack of drugs specifically tailored to TBI reflects the complexity and heterogeneity of the condition, and comes despite intensive research in the area. Several promising candidate pharmacological strategies and lead compounds have all failed in human clinical trials for neuroprotection and functional recovery following TBI (Bolland et al., 1998; Harders et al., 1996; Marmarou et al., 1999; Marshall et al., 1998; Morris et al., 1999; Muizelaar, 1994; Muizelaar et al., 1993; Stewart et al., 1999; Ward, 1950).

A large proportion of the research conducted in TBI to date has focused on the idea of excitotoxicity. The primary insult causes shearing and tearing of axons and cell bodies that releases non-physiological amounts of the excitatory neurotransmitter glutamate. Glutamate binds to and activates NMDA receptors, which are highly permeable to calcium; therefore, over-stimulation of these receptors will lead to calcium overload, mitochondrial dysfunction, free radical formation, endonuclease, protease, and phospholipase activation, culminating in neuronal cell death via apoptotic or necrotic mechanisms. Thus blockade or inhibition of NMDA receptors could protect cells that survive the primary insult from an excitotoxic fate. Unfortunately NMDA antagonists have lacked efficacy in human trials despite promising results in animal studies. The failure of these

trials has prompted much discussion and speculation as to the possible reason for discrepancy between animal and human data (Albensi et al., 2004; Muir, 2006). One issue is the lack of a well defined time-course of cellular and molecular events that occur after TBI in animals and humans. New markers of tissue damage could provide a better understanding of the pathogenesis of TBI, and help to define windows for possible therapeutic intervention.

In recent years the role played by reactive gliosis has garnered increasing attention as inflammation is now though to play a significant role in many neurodegenerative conditions. For example, the activation of brain-resident microglia has been implicated in the pathogenesis of AD (Eikelenboom et al., 1994; Moore and O'Banion, 2002; Rogers, 1995), Parkinson's disease (Gao et al., 2002; McGeer and McGeer, 2004), stroke (Denes et al., 2007; Wiart et al., 2007), amyotrophic lateral sclerosis (ALS) (Almer et al., 2001; Weydt et al., 2002) and TBI (Adelson et al., 1998; Morganti-Kossman et al., 1997). Activation of glial cells occurs as a graded response to insult, and is typically related to the degree of injury (Raivich et al., 1999). Therefore, a selective and sensitive marker to facilitate the characterization of neuroinflammation in models of neurodegeneration and brain injury may be very useful.

One promising candidate for such a marker is the peripheral benzodiazepine receptor (PBR). As its name suggests, the PBR was initially found to be expressed in the peripheral tissues of the heart, liver, adrenal gland, testis, lymphatic cells, and hemopoietic cells (reviewed in Woods and Williams, 1996). Subsequent studies however, demonstrated that the brain (primarily olfactory bulb) also expresses PBRs (Benavides et al., 1984; Marangos et al., 1982; Schoemaker et al., 1983), eventually leading to a change of nomenclature to the translocator protein 18kDa (TSPO) (Papadopoulos et al., 2006a). The potential for the TSPO as a marker of inflammation is demonstrated by marked increases in expression following CNS damage. Increased TSPO expression has been reported following chemical-induced injury, such as trimethyltin (Guilarte et al., 1995; Kuhlmann and Guilarte, 2000), domoic acid (Kuhlmann and

Guilarte, 1997), and MPTP (Kuhlmann and Guilarte, 1999). Increases are also seen in ischemic stroke models (Stephenson et al., 1995) and patients (Gerhard et al., 2000; Gerhard et al., 2005; Pappata et al., 2000). Other studies have demonstrated increases in TSPO expression in neurodegenerative disease (Cagnin et al., 2001; Gerhard et al., 2003; Versijpt et al., 2003), and brain trauma (Miyazawa et al., 1995; Raghavendra Rao et al., 2000). Changes in the levels of TSPO expression are therefore thought to reflect the progression of neuroinflammation.

A growing body of evidence suggests that the TSPO is localized primarily on the outer mitochondrial membrane (Anholt et al., 1986; Antkiewicz-Michaluk et al., 1988; Bribes et al., 2004; Garnier et al., 1994). However, early evidence of TSPO expression on erythrocytes (Olson et al., 1988), which lack mitochondria, and studies in other tissues (O'Beirne et al., 1990; Woods and Williams, 1996) suggest a possible plasma-membrane population of TSPO. In addition, some evidence exists for nuclear localization of TSPOs in glial cells (Kuhlmann and Guilarte, 2000). Several different functions have been attributed to the TSPO such as porphyrin transport (O'Hara et al., 2002; Taketani et al., 1994), explaining the mitochondrial localization (porphyrin is needed for hemoglobin synthesis in mitochondria). Other functions suggested include cholesterol transport and regulation of steroid synthesis (Costa et al., 1994; Jamin et al., 2005; Lacapere and Papadopoulos, 2003; Li et al., 2001; Papadopoulos et al., 2006b), regulation of apoptosis (Maaser et al., 2001) or proliferation (Galiegue et al., 2004), and others (Faure et al., 2003; Gavish et al., 1999; Lacapere and Papadopoulos, 2003; Larcher et al., 1989; Ostuni et al., 2004; Torres et al., 2000; Veenman and Gavish, 2006).

The most widely used radioligand for tissue localization of the TSPO is the isoquinoline carboxamide PK11195, possibly because of its high affinity for the TSPO (Le Fur et al., 1983) in animals and humans (Casellas et al., 2002). PK11195 has already been used to image inflammation in a number of brain inflammatory disorders including ischemia (Dubois et al., 1988), encephalitis

(Vowinckel et al., 1997), thiamin deficiency (Leong et al., 1994), and closed head injury (Raghavendra Rao et al., 2000). Using [^{3}H]-PK11195 autoradiography it is possible to track the evolution of brain damage over time in multiple brain regions. In injured animals significant increases in [^{3}H]-PK11195 binding have been observed in the ipsilateral cortex, hippocampus and thalamus (Grossman et al., 2003; Raghavendra Rao et al., 2000). These increases are thought to reflect the activation of brain-resident microglia or the presence of infiltrating peripheral macrophages. Comparisons of PK11195 with markers of microglia and macrophages support this idea, but until now the time-course of changes in [^{3}H]-PK11195 binding have not been carefully evaluated in a well-controlled animal model of TBI. Therefore, the purpose of the present study is to establish the time course of microglial infiltration into rat brain regions following a cortical contusion injury.

2.2 Materials and methods

2.2.1 Animals and surgery

All experiments were conducted in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee (IACUC) that follows the guidelines set forth by the National Institutes of Health guide for the care and use of laboratory animals. Experiments were designed to minimize animal suffering and the number of animals required for the study. For all experiments, male Sprague-Dawley rats weighing 275-300 g housed 3 per cage were obtained from Harlan Breeding Laboratories (Indianapolis, IN) and randomly assigned into treatment groups. Surgical manipulations were carried out as previously described (Verbois et al., 2003a; Verbois et al., 2002, 2003b; Verbois et al., 2000). Briefly, animals were anaesthetized with 3% isoflorane and immobilized in a stereotaxic frame. A 6 mm craniotomy was performed using a Michele trephine just above the somatosensory cortex (Bregma –2.8, 2.5 mm lateral) and an electronically controlled piston (5 mm diameter) was used to administer a 1.5 mm cortical deformation at a velocity of 3.5 m/s. Following the injury the skull disc was replaced and sealed with dental acrylic.

2.2.2 [^{3}H]-PK11195 autoradiography

Animals were euthanatized 30 minutes, 12 hours, 2, 4 or 6 days following trauma and the brains immediately removed and frozen in isopentane. Brain sections (16 μm) were prepared using a Lecia CM1850 cryostat and were mounted onto Super-Frost Plus™ slides (Fisher Scientific, Pittsburgh, PA). The slides were stored at -80°C until radioligand binding studies could be carried out. Neuroinflammation was assessed by [^{3}H]-PK11195 autoradiography on a set of slides from each animal. For [^{3}H]-PK11195 autoradiography, slides were warmed to room temperature and pre-incubated in buffer for 15 minutes, then transferred to fresh buffer containing 1 nM [^{3}H]-PK11195 for 2 hours, then washed in buffer, and dried overnight. The slides were exposed to RayMax Beta High Performance Autoradiography Film (ICN Biomedicals Inc., Aurora, OH) to visualize the areas of ligand binding. Radioactive rat brain tissue standards were included with each film X-ray cassette in order to determine the response of the film to different amounts of radioactivity. A Northern Lights illuminator with a digital camera (a CFW-1310M, Scion Corp) was used to capture images that were analyzed with ImageJ (v1.39) on an iMac. Molar quantities of bound ligand were determined by constructing a standard curve from radioactivity tissue standards fitted to an exponential curve. To test the specificity of [^{3}H]-PK11195 binding a parallel binding assay was carried out in the presence of a saturating amount of unlabelled Ro5-4864, which also has high affinity for binding to the TSPO. Inclusion of an excess amount of Ro5-4864 reduced [^{3}H]-PK11195 binding to control levels, indicating that these ligands are competing for the same binding sites (data not shown). Changes in [^{3}H]-PK11195 binding following CCI were quantified in several regions of the cortex, hippocampus and thalamus. Binding density was measured in both the ipsilateral and contralateral cortex to give an indication of the inflammatory response in tissue directly affected by the impact. This was achieved by outlining the cortex from its dorsal side, ventral to the entorhinal rhinal fissure. In the hippocampus [^{3}H]-PK11195 was measured in the CA1 and CA2/3 fields, the hilus, the lateral blade of the dentate gyrus, and the ventral blade of the dentate gyrus. In addition to the cortex and

hippocampus, thalamic nuclei have numerous projections into the cerebral cortex (Castro-Alamancos and Connors, 1997), which could be affected by CCI. Therefore, binding was assessed in the lateral dorsal (LD) thalamus, and the ventral posterolateral (VPL) thalamic areas.

2.2.3 Cortical tissue sparing analysis

Tissue sparing analysis was carried out in each of the animals to follow the progression of tissue encavitation at each time point and to facilitate comparisons with binding data. Analysis of the amount of tissue lost following injury was carried out as previously described (Scheff and Sullivan, 1999). To summarize, 8 Cresyl violet stained sections from each animal were included in the analysis, each evenly spaced throughout the lesion site. For each section the area of the ipsilateral cortex and contralateral cortex was measured separately. The average area of each side of the cortex was calculated and by dividing the ipsilateral area by the contralateral area, then multiplying by 100, a percentage of cortical tissue remaining is produced.

2.2.4 Fluoro-Jade B staining

Fluoro-Jade B (FJ-B) staining was used to assess neuronal degeneration following CCI. Fluoro-Jade (A) is an anionic fluorochrome that was first described in 1997 as sensitive marker for neuronal degeneration (Schmued et al., 1997). In this study we utilized FJ-B; a related compound with higher specificity (Schmued and Hopkins, 2000a). Dying and dysfunctional cells were visualized by FJ-B staining as described previously (Anderson et al., 2005). Briefly, fresh-frozen sections mounted on Super-Frost Plus™ slides (Fisher Scientific, Pittsburgh, PA) were post-fixed in 4% paraformaldehyde for 10 minutes and washed in water prior to immersing in a solution of 1% sodium hydroxide in 80% ethanol for 5 minutes. Slides were then hydrated in a series of ethanol washes and placed in a solution of 0.06% potassium permanganate for 10 minutes. Next, slides were rinsed in distilled water for 2 minutes and incubated in a 0.0004% solution of FJ-B (Histo-Chem Inc., Jefferson, AR) for 20 minutes. Lastly, the sections were then rinsed in distilled water, air-dried and

cover slipped. FJ-B-positive neurons were visualized using an Olympus BX-51 microscope with a fluorescein isothiocyanate (FITC) filter cube. Individual stained cells were manually counted throughout specific regions of interest in 6 equally spaced sections. Regions of interest were the cerebral cortex (Bregma -2.5 mm to Bregma -4.5 mm), the dentate gyrus (dorsal and ventral blades individually), the CA1 and CA2/3 fields of the hippocampus, the hilus, and the LD thalamus (Bregma -3.0 mm to Bregma -3.8 mm).

2.2.5 Immunohistochemistry

Immunohistochemistry for activated microglia and macrophages was carried out with a monoclonal mouse anti-rat CD68 [ED-1] antibody (Abcam, Cambridge, MA). CD68 is a transmembrane protein expressed on macrophages and activated microglia, which is thought to be involved in cellular phagocytosis (Holness and Simmons, 1993). Fresh-frozen tissue sections mounted on slides were post-fixed by immersion in 4% paraformaldehyde for 10 minutes. Sections were washed in phosphate-buffered saline (PBS) containing 0.1% Triton-X and blocked in the same solution containing 3% normal horse serum for 1 hour. Endogenous peroxidases were quenched by incubating sections is PBS containing 0.3% hydrogen peroxide. Next, the ED-1 antibody was diluted 1:100 in blocking solution, applied to sections in a humidified chamber and incubated overnight at 4˚C. Sections were then washed in PBS and incubated with biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA) for 60 minutes. Following further washing, the secondary antibodies were detected using a Vector Avidin: Biotinylated enzyme Complex (ABC) Elite kit (Vector Labs, CA), and visualized by the addition of Nickel and Cobalt enhanced 3,3'-Diaminobenzidine (DAB). Finally, the sections were dehydrated and coverslipped prior to analysis. ED-1 stained cells were counted in the same regions as FJ-B positive cells so that direct comparisons could be made.

2.2.6 Statistics

[^{3}H]-PK11195 binding was measured in 6 equally-spaced sections for each region of interest and an average value was calculated. Data from each

animal was analyzed by a 2-way analysis of variance (ANOVA) in which time-point and hemisphere were independent variables, and the [^{3}H]-PK11195 binding density was the dependant variable. Individual group comparisons were assessed by carrying out a Tukey's honestly significant difference (HSD) post-hoc test.

Tissue sparing analysis was analyzed with a one-way ANOVA, with time-point as the independent variable and tissue sparing as the dependant variable. Group comparisons were analyzed using the Tukey's HSD post-hoc test.

For FJ-B staining and CD68 immunohistochemistry cells were counted within a defined region of interest in 6 equally spaced sections. The cell count was averaged for each animal and data were analyzed using a Kruskal-Wallis test with time-point as the independent variable and number of cell profiles as the dependent variable. Dunn's multiple comparisons were used as the post-hoc test to address individual group differences. All statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc, La Jolla, CA).

2.3 Results

2.3.1 [^{3}H]-PK11195 binding is a sensitive marker of tissue damage

In the cortex increases in [^{3}H]-PK11195 binding were visible after 2 days but only became significant 4 days after injury (Figure 2-1). Data were analyzed by 2-way ANOVA, which indicated a significant effect of day ($F_{(5,32)} = 4.83$, $p < 0.005$) and hemisphere ($F_{(1,32)} = 14.1$, $p < 0.001$), with a significant interaction ($F_{(5,32)} = 3.83$, $p < 0.01$). Interestingly [^{3}H]-PK11195 binding appears to be elevated throughout the entire dorsal-ventral span of the cerebral cortex (Figure 2-3).

In the hippocampus increased [^{3}H]-PK11195 binding is initially limited to the ipsilateral dentate gyrus 12 hours after injury, and by 24 hours is increased in other regions of the hippocampus (Figure 2-2). In the dentate gyrus, CA1, CA2/3, and hilus a 2-way ANOVA revealed a significant effect of time-point ($F_{(5,32)} = 33.3$, $p < 0.001$; $F_{(5,32)} = 23.9$, $p < 0.001$; $F_{(5,32)} = 36.5$, $p < 0.001$; $F_{(5, 32)} =$

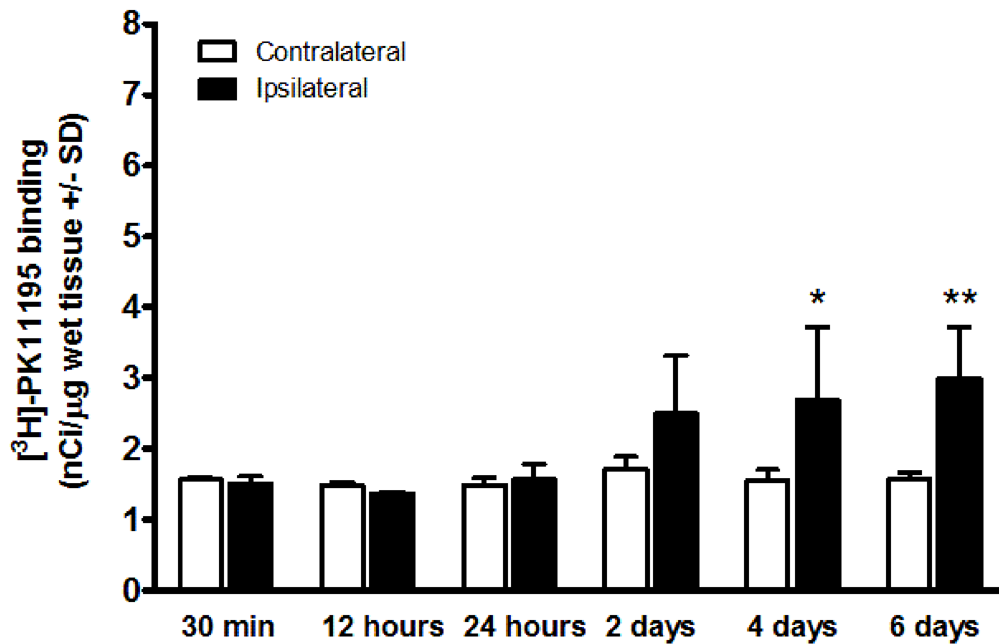

Figure 2-1 - The time-course of [^{3}H]-PK11195 binding in the rat cerebral cortex following a 1.5 mm CCI

In the ipsilateral cerebral cortex, increases in TSPO expression following injury began 2 days after injury and reached significance by the fourth day. Mean binding densities +/- standard deviation are plotted for ipsilateral and contralateral regions of the cortex (n = 4). Asterisks denote significant differences from binding in the contralateral hemisphere as found by Tukey's HSD ($p < 0.05$, **$p < 0.01$).*

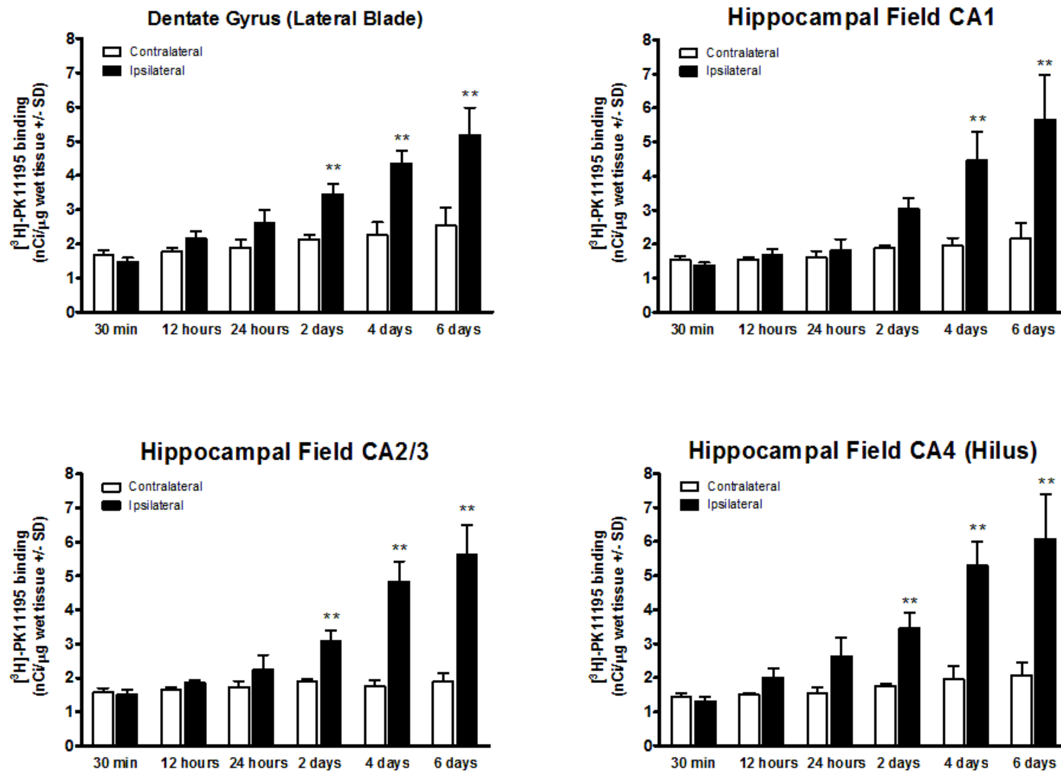

Figure 2-2 - [^{3}H]-PK11195 binding in regions of the rat hippocampus following a 1.5 mm CCI

*Changes in [^{3}H]-PK11195 binding were seen starting at 12 hours in some hippocampal regions, but significant increases were not found until 2 days after injury in this study. Binding densities were measured in each of the dentate gyrus, CA1 field, CA2/3 field, and in the hilus at selected time-points following CCI (n = 4). Data presented are for ipsilateral and contralateral areas and are mean binding densities +/- standard deviation. Asterisks denote significant differences from binding in the contralateral hemisphere (**p < 0.01).*

26.5, $p < 0.001$ respectively), hemisphere ($F_{(1,32)} = 82.2$, $p < 0.001$; $F_{(1,32)} = 47.4$, $p < 0.001$; $F_{(1,32)} = 128$, $p < 0.001$; $F_{(1, 32)} = 96.3$, $p < 0.001$ respectively), and a significant interaction term ($F_{(5,32)} = 12.7$, $p < 0.001$; $F_{(5,32)} = 13.3$, $p < 0.001$; $F_{(5,32)} = 29.7$, $p < 0.001$; $F_{(5, 32)} = 15.2$, $p < 0.001$ respectively). Tukey's HSD test was used for individual group comparisons. In the dentate gyrus, CA2/3 field and hilus binding was significantly elevated in the ipsilateral hemisphere on day 2, 4, and 6 ($p < 0.01$), and in the CA1 field significantly increased binding was revealed on days 4 and 6 ($p < 0.01$).

Interestingly, data for the contralateral dentate gyrus seemed to show an increase in binding at later time points. When the contralateral dentate gyrus data was analyzed by one-way ANOVA a significant effect of time post-CCI was found ($F_{(5,16)} = 3.91$, $p < 0.05$). A small increase can be seen after 4 days (Figure 2-3), and Tukey's HSD post-hoc revealed a significant elevation 6 days after injury when compared to levels measured immediately after injury ($p < 0.05$).

In addition to the cortex and hippocampus, localized increases in binding density were observed in the thalamus (Figure 2-4). The thalamus is 3.5 mm distal from the surface of the impacted cortex (Paxinos and Watson, 1998). Despite this, significant increases in binding were seen in the ipsilateral side versus contralateral following a 1.5 mm CCI in the LD thalamus ($F_{(5,32)} = 38.4$, $p < 0.001$), and in the VPL thalamus ($F_{(5,32)} = 43.5$, $p < 0.001$).

Tukey's HSD multiple comparisons were performed to reveal significant increases in binding to ipsilateral regions of the thalamus versus contralateral beginning 2 days after injury. At 4 and 6 day time-points [^{3}H]-PK11195 binding continued to increase in both thalamic regions (Figure 2-4, 2-5).

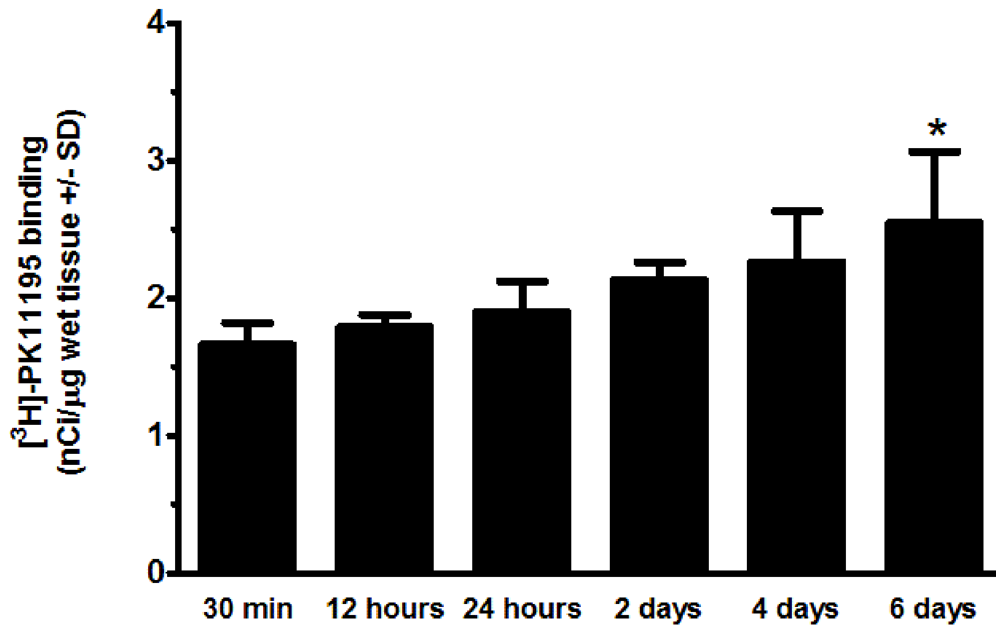

Figure 2-3 - [^{3}H]-PK11195 binding in the contralateral dentate gyrus

In the contralateral dentate gyrus, increases in [^{3}H]-PK11195 binding were found versus ipsilateral measurements that became significant 6 days after CCI ($p < 0.05$). This could represent a delayed damage to this region, possibly mediated by disruption of commissural fibers connecting the two hemispheres. Data plotted are means +/- standard deviations ($n = 4$).*

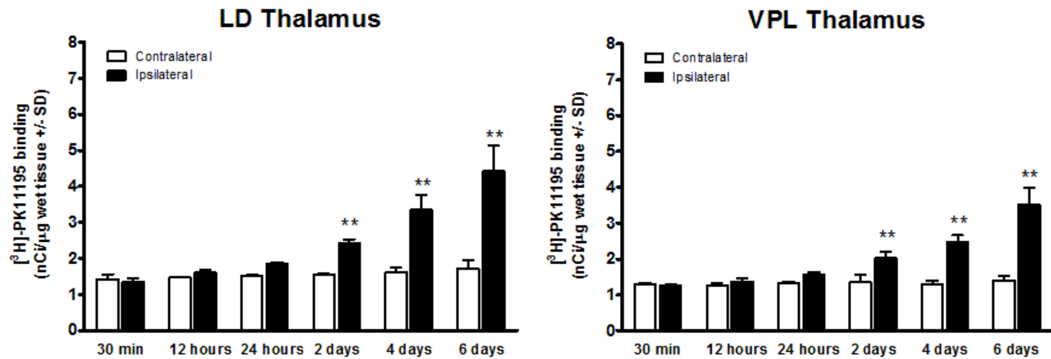

Figure 2-4 - [^{3}H]-PK11195 binding in the lateral-dorsal and ventral-posterolateral thalamus at selected time-points after CCI

*Increases in [^{3}H]-PK11195 binding in the thalamus began at 2 days after injury and steadily increased at 4 and 6 days (n = 4). These data for the LD and VPL thalamus were analyzed by two-way ANOVA, and individual comparisons were made using Tukey's HSD. Significant increases in binding were found between hemispheres in both regions at 2, 4, and 6 day time-points (**$p < 0.01$).*

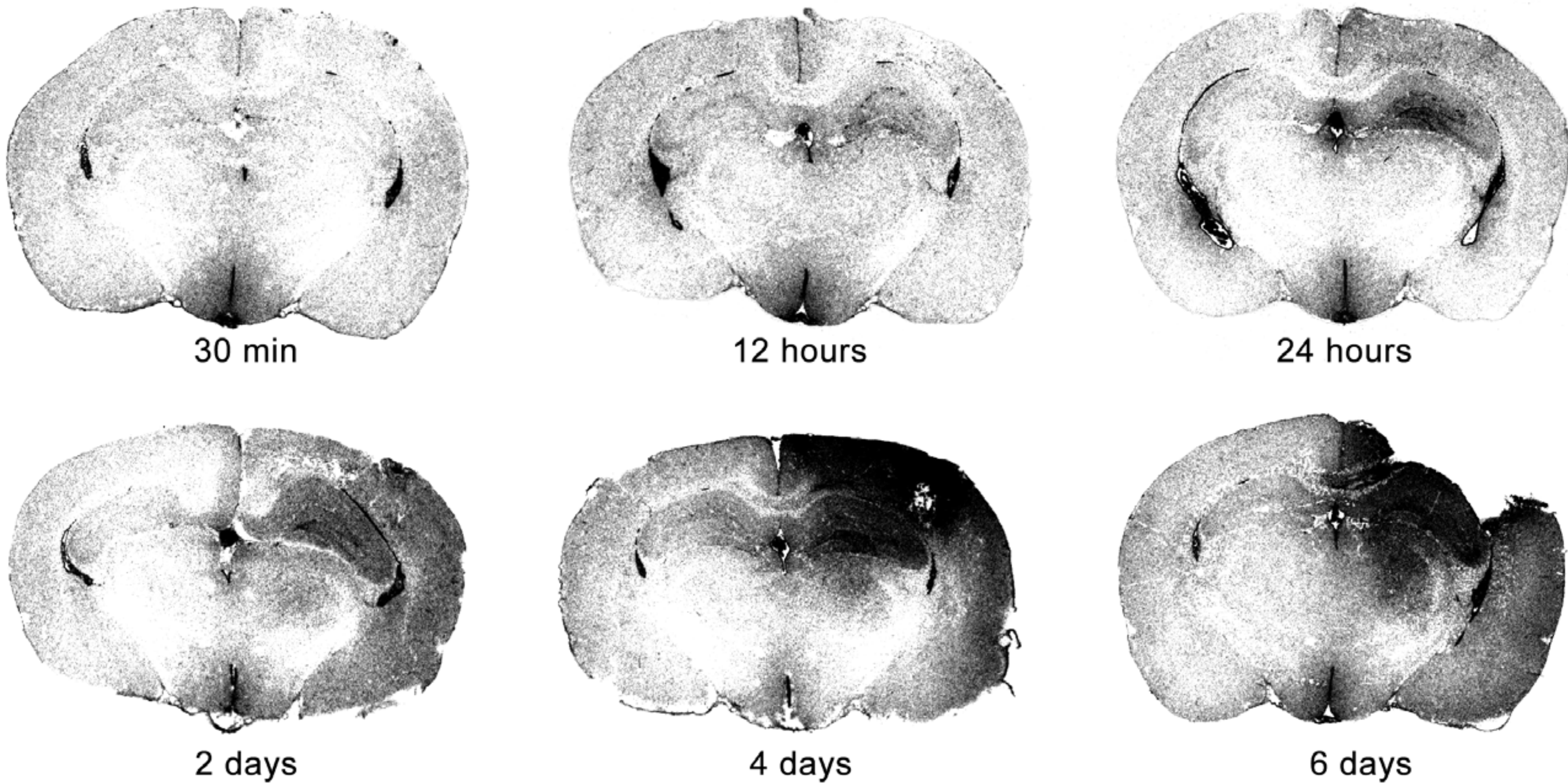

Figure 2-5 - Images of coronal brain sections representative of [^{3}H]-PK11195 binding at each time-point post-CCI

This figure shows the time-course of increases in binding following a 1.5 mm CCI. Note the early increases in the dentate gyrus at 12 hours, and the increases in the thalamic regions at 4 and 6 days post-injury.

2.3.2 Cortical tissue loss following CCI

There was no apparent tissue loss on days 1 to 4 post-CCI, and an 83% tissue sparing observed 6 days after injury (Figure 2-6). The data were analyzed by one-way ANOVA, which demonstrated an overall effect of time post-CCI on cortical tissue loss ($F_{(17,5)} = 7.15$, $p < 0.001$). Subsequent Tukey's HSD post-hoc tests revealed a significant loss of tissue at day 6 post-CCI compared to other time-points ($p < 0.05$).

2.3.3 Increases in FJ-B positive cells occur early after CCI

Numerous FJ-B positive cells were observed throughout the ipsilateral cortex at 12 hours and 24 hours after injury. The majority of these cells are clustered around the impact site (Figure 2-13). By 2 days there were fewer dying cells, and FJ-B positive cells continued to decrease in number through day 6 (Figure 2-7). This is in direct contrast to [^{3}H]-PK11195 binding density, which only begins to increase at 2 days and is significantly elevated versus control at 4 and 6 days post-CCI.

Cell count data were analyzed using a Kruskal-Wallis test, which reported a significant effect of time-point on the number of FJ-B-positive cells (H = 17.64, df = 5, p = 0.0034, N = 30). Comparisons of each group to the 30 minute control group were made with a Dunn's multiple comparisons test. Significant increases in FJ-B positive cells were found in the 12 hour and 1 day time-point groups ($p < 0.01$, $p < 0.05$ respectively).

In the hippocampus FJ-B positive neurons were first observed at the 12 hour time-point, and were also most numerous at this time-point (Figure 2-8). In the dentate gyrus a Kruskal-Wallis test determined that the group means did not significantly differ. However, a Dunn's multiple comparison found significant increases in FJ-B-positive cells at 12 hours, 1 day and 2 days. In the CA1, CA2/3 and hilus there was a significant effect of time-point on the number of FJ-B positive cells ([H = 11.77, df = 5, p = 0.0381, N = 30], [H = 14.75, df = 5, p = 0.0115, N = 30], [H = 13.22, df = 5, p = 0.0214, N = 30] respectively). For each region Dunn's multiple comparisons were made between the 30 minute time-

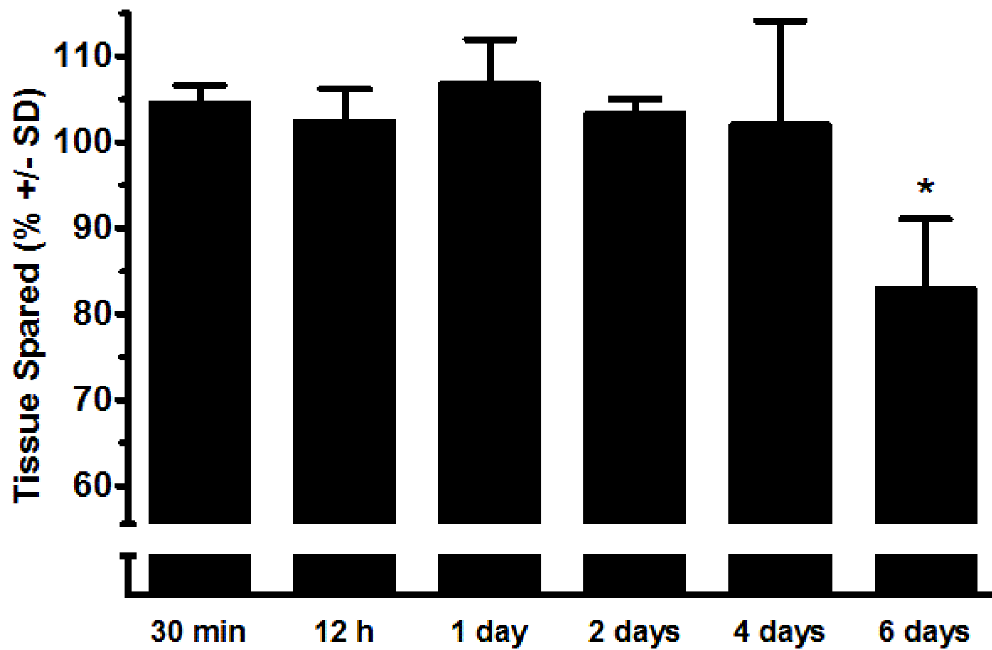

Figure 2-6 - Tissue sparing analysis performed at various time-points after a 1.5 mm CCI in the rat

*There was no apparent tissue loss at time-points up to 4 days post-CCI, but an 83% tissue sparing was found on day 6 post-CCI. Data were analyzed by one-way ANOVA with Tukey's HSD post-hoc (*p < 0.05). Data plotted are means plus or minus standard deviation (n = 4).*

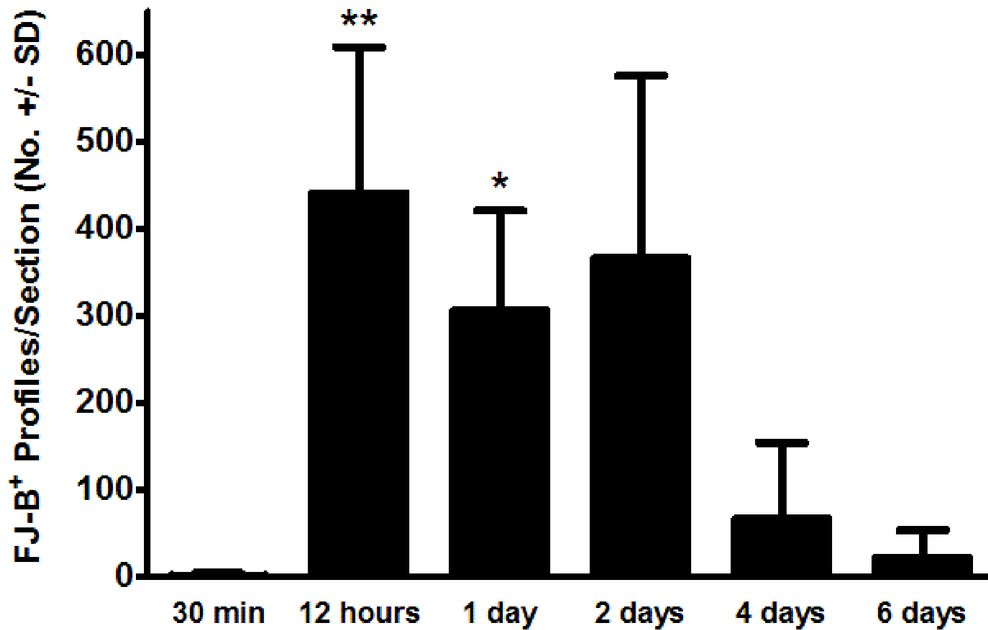

Figure 2-7 - The time-course of FJ-B positive cells in the cerebral cortex following CCI in the rat

*FJ-B cells were abundant in the cortex at 12 hours post-CCI, with fewer positively stained cells counted in the region at subsequent time points. Data for the ipsilateral cortex were analyzed by Kruskal-Wallis test, and Dunn's multiple comparison was used to determine significant differences from the 30 minute post-CCI group (asterisks denote a significant difference versus 30 minute time-point [*p < 0.05, **p < 0.01]). Data are plotted as means plus or minus standard deviation (n = 4).*

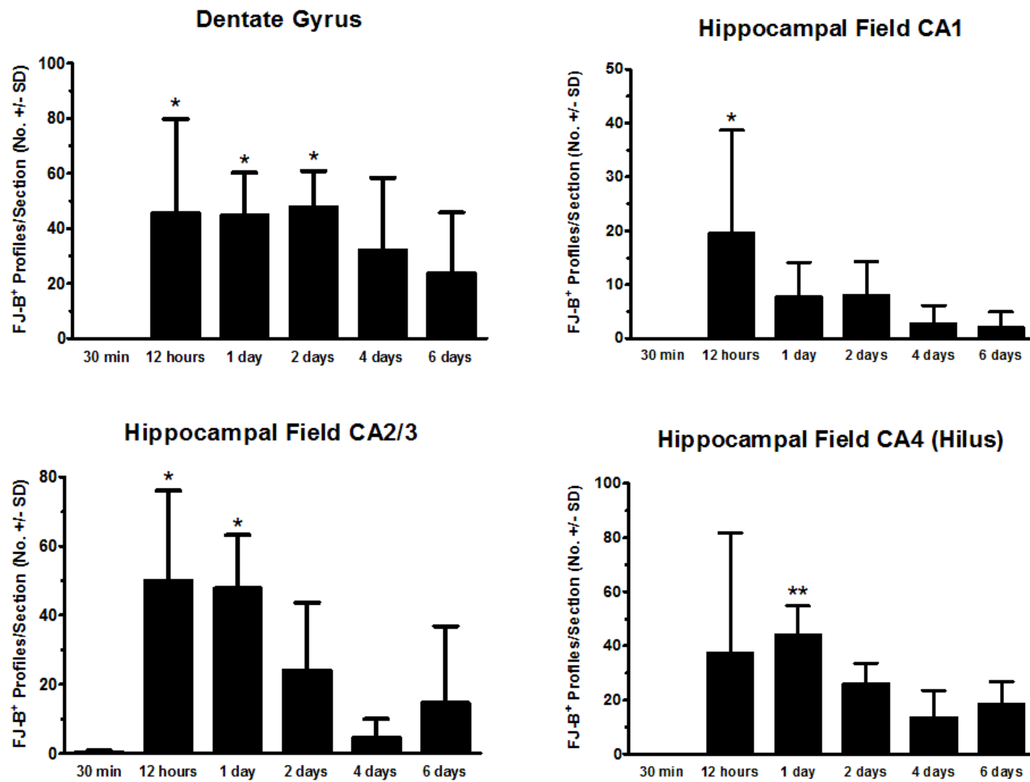

Figure 2-8 - Time-course of FJ-B staining in the hippocampus following CCI

FJ-B positive cells were counted in the dentate gyrus, CA1 field, CA2/3 fields and the hilus following CCI. Data are plotted as means plus or minus standard deviation (n = 4). Asterisks denote a significant difference from the 30 minute group as determined with a Dunn's multiple comparisons test (p < 0.05).

point and each of the other groups. In the CA2/3 fields and in the hilus significant numbers of FJ-B positive cells were found up to 24 hours after injury ($p < 0.05$). Interestingly, in the medial portion of the CA1 field of the hippocampus at the 12 hour time point a small but significant ($p < 0.05$) number of FJ-B cells were seen.

In the LD thalamus a Kruskal-Wallis test found a significant effect of time on the numbers of FJ-B positive cell profiles ($H = 18.81$, $df = 5$, $p = 0.0021$, $N = 30$). Significant numbers of FJ-B positive cell profiles were not quantified until 4 days after injury, and significant numbers remained at the 6 day time-point (Figure 2-9, and Figure 2-14).

2.3.4 The time-course of changes in CD68 positive cells following trauma

The spatial and temporal pattern of CD68 positive microglia and macrophages was used to make comparisons to [^{3}H]-PK11195 autoradiographs from adjacent brain sections. CD68 positive cells were found in all regions where [^{3}H]-PK11195 binding was elevated. In the ipsilateral cortex CD68 positive cell profiles were observed in small numbers in the areas surrounding the contusion starting at 12 hours, and were present in large numbers by 4 days post-injury (Figure 2-10, and Figure 2-13). A Kruskal-Wallis test revealed significant group differences ($H = 17.61$, $df = 5$, $p = 0.0035$, $N = 30$), and a Dunn's post-hoc test showed that the numbers of CD68 positive cell profiles per section were significantly more in the 4 and 6 day post-CCI groups versus the 30 minute post-CCI group ($p < 0.005$, and $p < 0.05$ respectively).

In the dentate gyrus, hilus, and CA2/3 fields of the hippocampus CD68 stained cell profiles were seen beginning at 12 hours post-CCI and were widespread and numerous by 1 day after injury (Figure 2-11). Large amounts of staining persisted in these areas at all subsequent time points. A Kruskal-Wallis test carried out on these data did not find any significant differences ([$H = 7.531$, $df = 5$, $p = 0.1840$, $N = 30$], [$H = 9.079$, $df = 5$, $p = 0.1060$, $N = 30$], and $H = 8.754$, $df = 5$, $p = 0.1193$, $N = 30$, respectively. The CA1 was unique in that only a few CD68 positive cell profiles were found in the region beginning at 1, 2 and 4

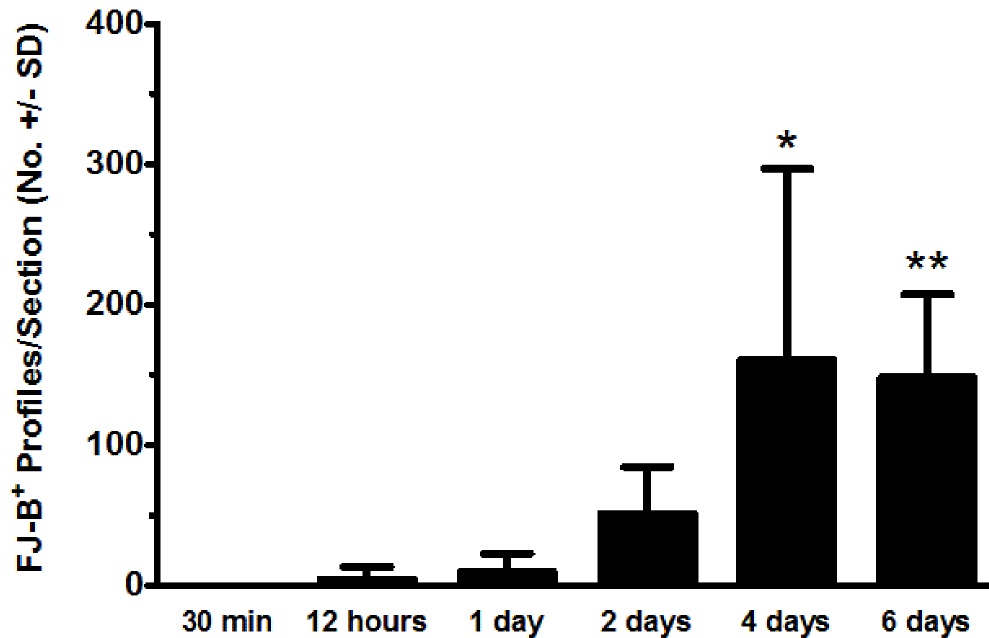

Figure 2-9 – FJ-B positive cell profiles in the lateral dorsal thalamus following CCI

*Very few FJ-B positive cells were seen in the thalamus up to 2 days after injury, but statistically significant numbers were observed in the region at 4 and 6 day time-points (Dunn's multiple comparisons, *$p < 0.05$, **$p < 0.01$). Data are plotted as means plus or minus standard deviation ($n = 4$).*

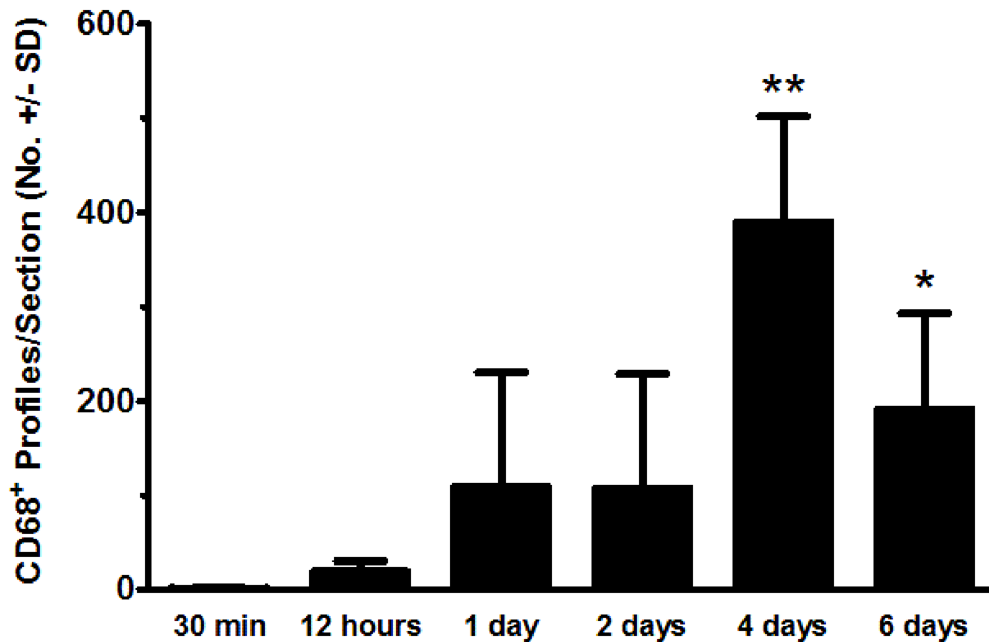

Figure 2-10 - CD68 immunohistochemistry for activated macrophages in the cortex following CCI

Activated macrophages and microglia were stained using ED-1 antibodies against the marker CD68. Positively stained cell profiles were counted in the ipsilateral cortex and data are presented as means for each time-point plus or minus standard deviation (n = 4). A significant increase versus data from the 30 minute post-CCI group is denoted by an asterisk (p < 0.01).

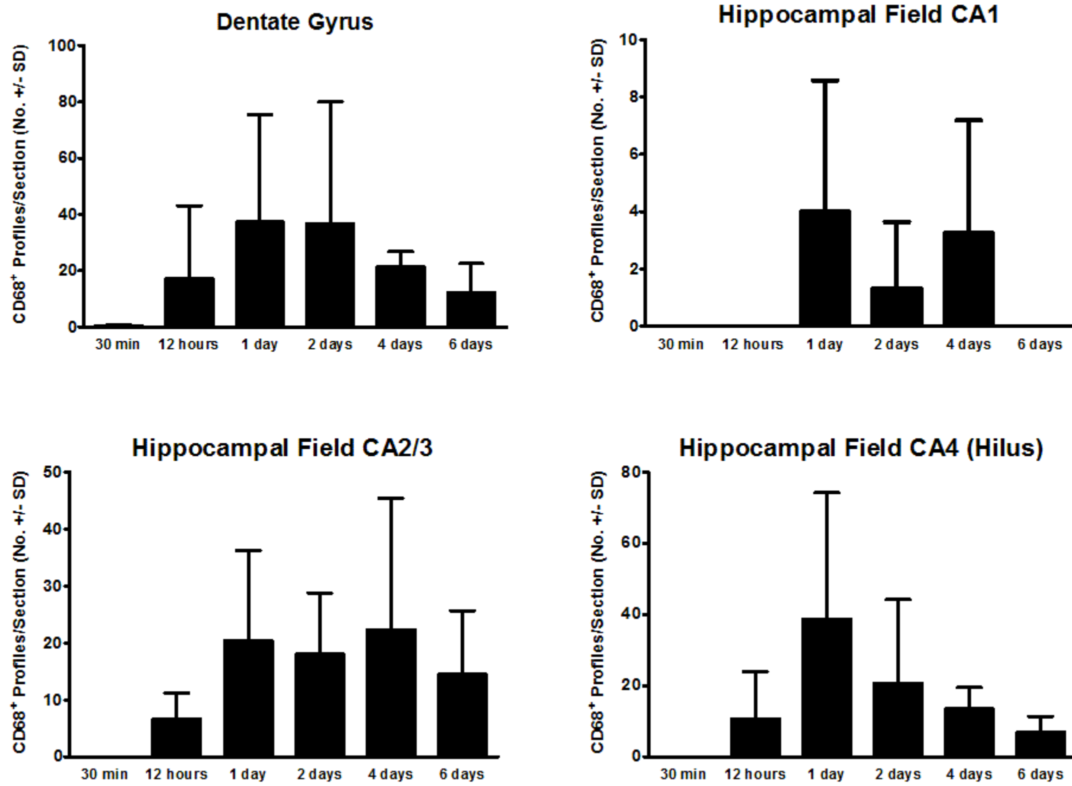

Figure 2-11 - The time-course of CD68 positive cells in hippocampal regions following CCI

CD68 positive cells were not seen in any hippocampal region 30 minutes after injury. In the CA1 field a small number of cells were found at 1, 2 and 4 days post injury, but there was no significant effect. In other regions CD68 positive cells were present from 12 hours and up to the 6 day time-point. Data are plotted as means plus or minus standard deviation (n = 4).

days post-injury, and at no point was there a significant effect of time-point ($H = 7.760$, $df = 5$, $p = 0.1700$, $N = 30$).

The LD thalamus contrasts the cortex and hippocampus in that changes in the numbers of $CD68^+$ cell profiles do not seem to mirror changes in $[^3H]$-PK11195 binding density. Analysis of $[^3H]$-PK11195 autoradiographs showed significant increases in binding density beginning at 2 days (Figure 2-4). In comparison, very few $CD68^+$ cells were observed in the LD thalamus at any time point (Figures 2-12), and although there appears to be a trend to increased numbers between 2 and 6 days post-injury, these changes were small and not significant ($H = 5.731$, $df = 5$, $p = 0.3332$, $N = 30$).

2.3.5 Numbers of $CD68^+$ cell profiles correlate with $[^3H]$-PK11195 binding

The amount of $[^3H]$-PK11195 binding, CD68 staining, and FJ-B staining vary widely with time in the cortex. As the largest area, and as the area most affected by the contusion, data for this region were the most robust and therefore used for correlation analysis (Figures 2-1, 2-7, 2-10). Therefore, all three of these assessments were carried out in the same area of the cortex in each animal and correlations of CD68 and FJ-B were made to $[^3H]$-PK11195 binding density (Figure 2-15). Analysis of the data using a Spearman's test revealed a moderate positive correlation between $[^3H]$-PK11195 binding density and CD68 staining, ($r = 0.687$ (19), $p_{(two\text{-}tailed)} = 0.0012$). In contrast there was a negative correlation between $[^3H]$-PK11195 binding and FJ-B staining, ($r = -0.4357$ (21), $p_{(two\text{-}tailed)} = 0.0483$).

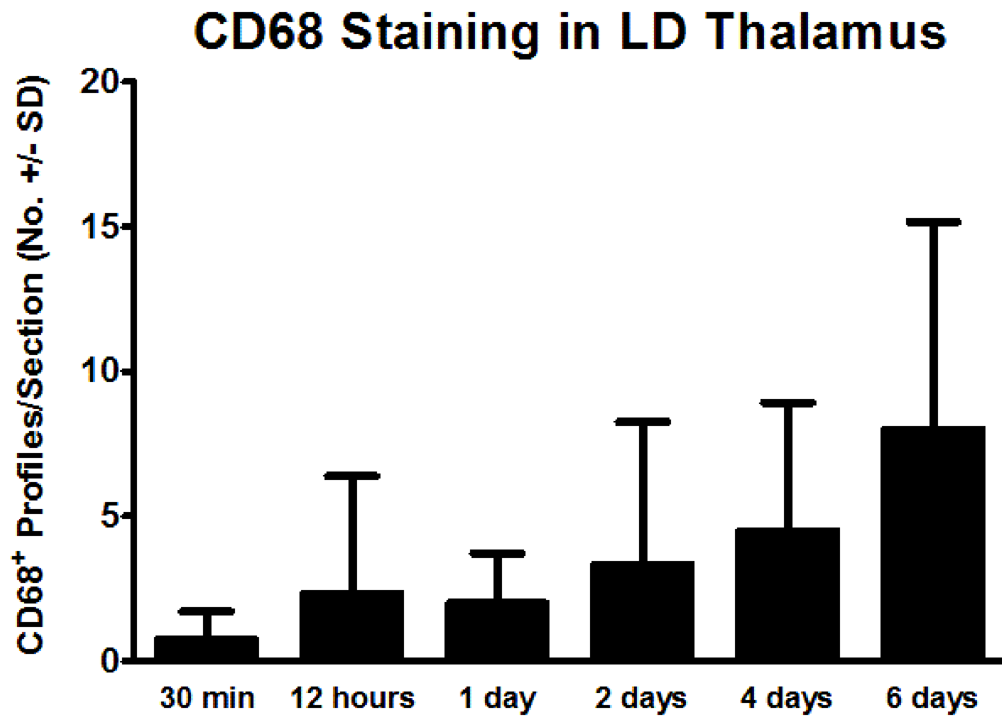

Figure 2-12 - CD68 positive cells in the thalamus at selected time-points after CCI

Very few cells stained positive for CD68 in the thalamus at any time-point following CCI. A Kruskal-Wallis test did not report a significant effect of time on CD68 cell count, and no significant differences were found between any time-point. Data are plotted as means plus or minus standard deviation (n = 4).

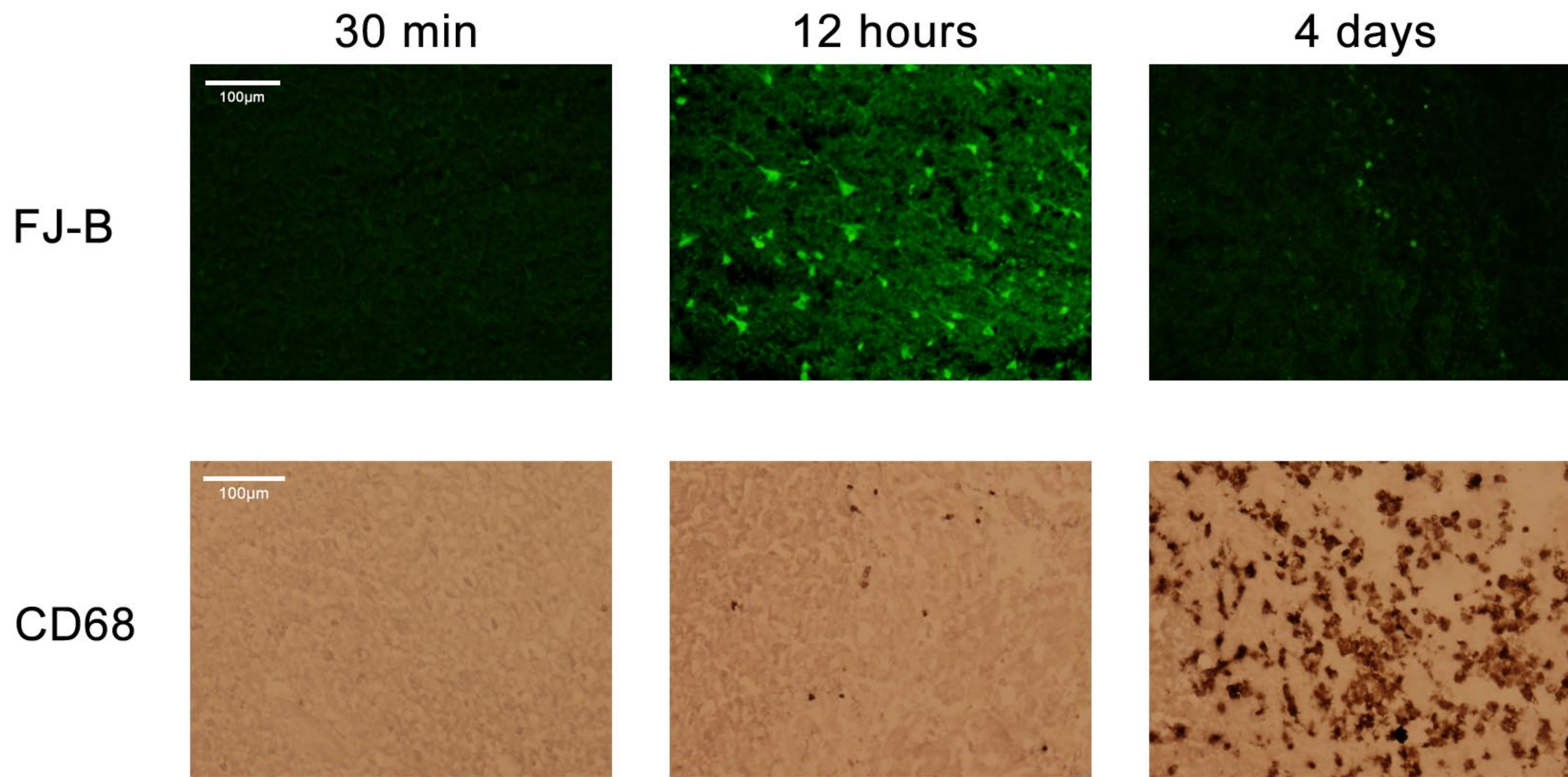

Figure 2-13 - Representative images of FJ-B and CD68 staining in cortex following CCI

No FJ-B or CD68^{+} cell profiles were seen in any animals at the 30 minute time-point. By 12 hours FJ-B staining was widespread in the cortex, while relatively few CD68 cell profiles were counted. Four days after the injury the number of FJ-B cell profiles had reduced significantly from their peak at 12 hours, while the number of CD68 positive cell profiles had increased significantly.

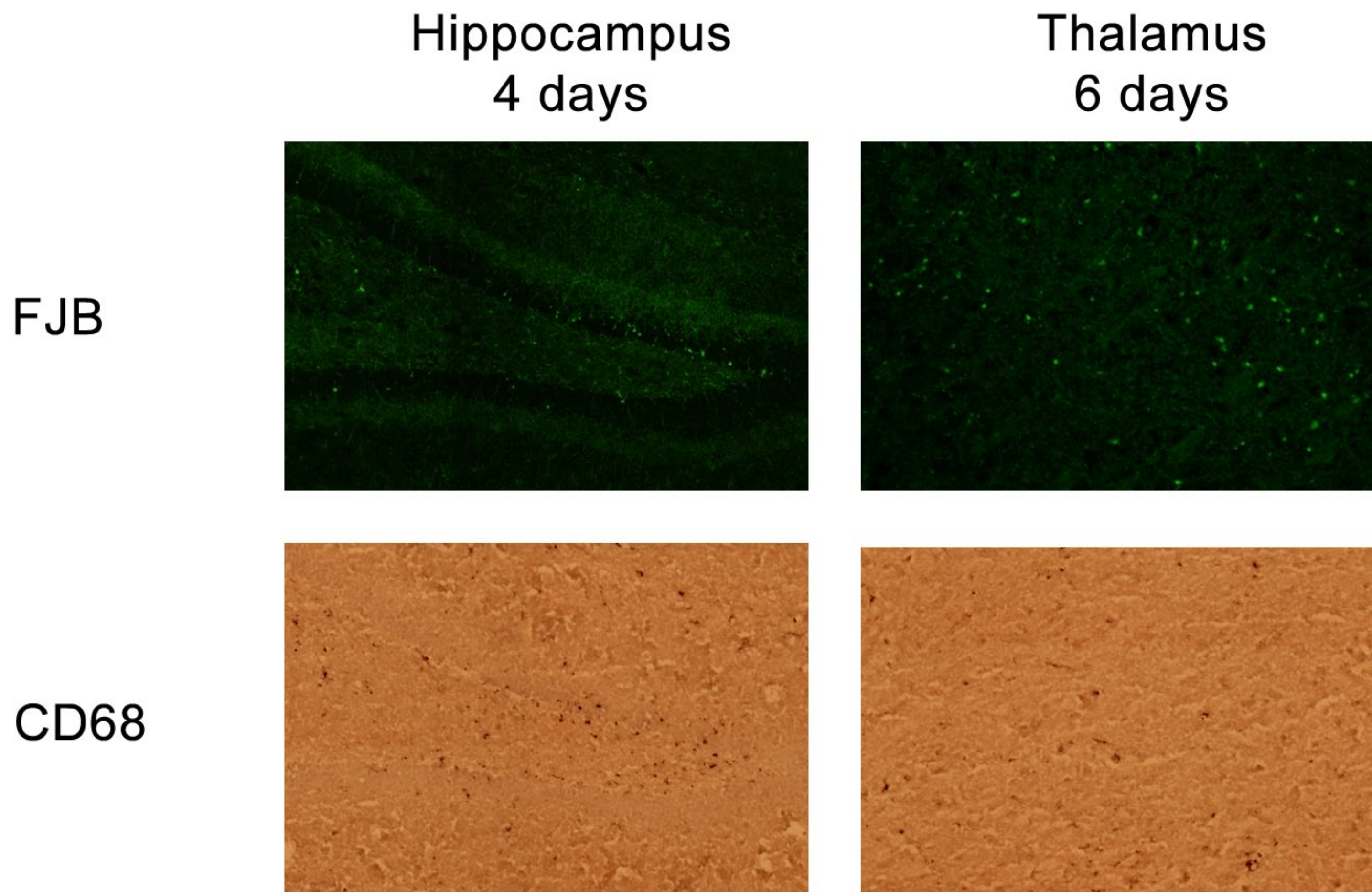

Figure 2-14 – FJ-B and CD68 staining in the hippocampus and thalamus following CCI

In the hippocampus at 4 days FJ-B staining was primarily seen in the dentate gyrus and hilus, which corresponded with increased numbers of CD68 positive cells found In that region at the same time-point. In the thalamus at 6 days, very significant numbers of FJ-B positive cells were found, but interestingly very few CD68 positive cells were present demonstrating difference in the pattern of TSPO expression and CD68 expression in this brain region.

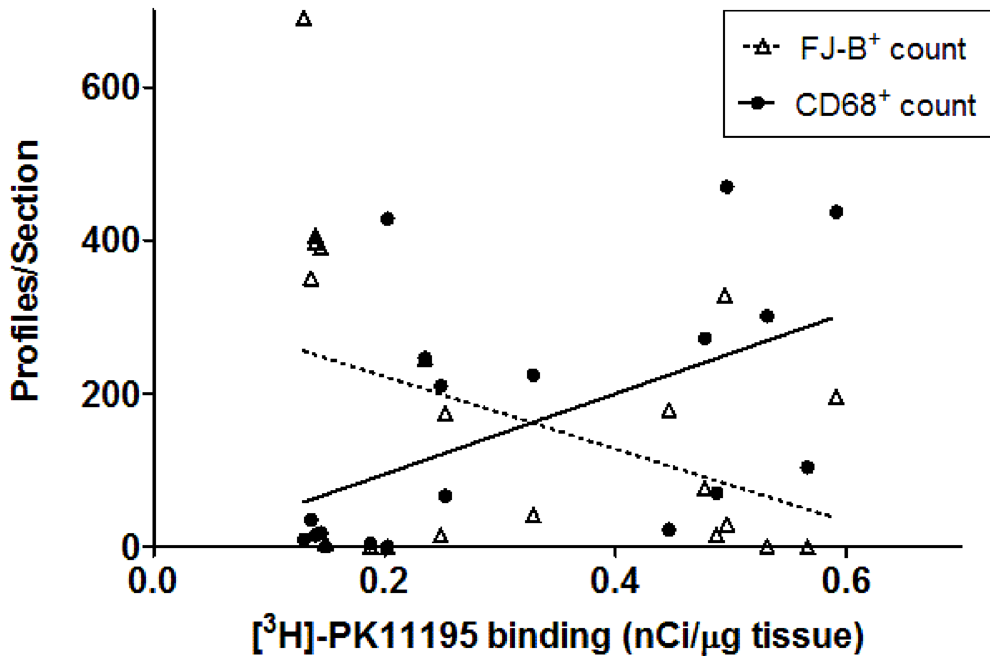

Figure 2-15 - Correlation of FJ-B and CD68 positive cell counts to [^{3}H]-PK11195 binding data

There is a moderate positive correlation of CD68 cell counts with PK11195 binding data. Data for FJ-B and CD68 staining were plotted against PK11195 binding data and a Spearman's correlation coefficient was calculated (r = 0.687).

2.4 Discussion

A thorough evaluation of the time-course of molecular and cellular events following TBI is essential to support the ongoing efforts to develop efficacious treatments for this condition. The vast differences between laboratory animal and human brains in terms of size, organization, and complexity require that detailed assessments be made in both animal and human brain injury. PK11195 is a molecule of particular interest for this task because it has been utilized successfully to make assessments of inflammation in human patients as well as in post-mortem studies in laboratory animals (Chen and Guilarte, 2008). PK11195 can be used as a sensitive marker to assess and track inflammation in neurodegenerative conditions including TBI. Correlations to cell specific markers (Raghavendra Rao et al., 2000; Venneti et al., 2008a; Vowinckel et al., 1997), and co-localization studies (Myers et al., 1991a; Venneti et al., 2007a), support a binding site in activated microglia and/or macrophages. Furthermore, radiolabelled PK11195 has been successfully used to visualize inflammation in positron emission tomography (PET) studies (Bartels and Leenders, 2007; Gerhard et al., 2006; Venneti et al., 2008a; Vowinckel et al., 1997). In rodent brain injury models, sham-operated animals show the expected pattern of [^{3}H]-PK11195 binding, i.e. binding is largely restricted to the choroid plexus, ependymal cells lining the ventricles, and olfactory bulb (Raghavendra Rao et al., 2000). Previous studies that used PK11195 to visualize neuroinflammation following TBI found significant increases in PK11195 binding sites in the hippocampus and thalamus at 3 and 14 days post-injury (Raghavendra Rao et al., 2000), and a co-localization of PK11195 binding sites with CD68-positive cells (Venneti et al., 2007a). Furthermore, previously published data from other groups have demonstrated that increases in [^{3}H]-PK11195 binding following brain injury are not due to a change in receptor affinity (Pedersen et al., 2006; Raghavendra Rao et al., 2000). At all time points assessed, TBI had no effect on K_d values in either the ipsilateral or contralateral brain regions. This study builds on previous research from other laboratories by providing a detailed assessment of [^{3}H]-PK11195 binding in multiple brain regions following CCI.

There is a clear difference between individual brain regions with respect to the time-course of [^{3}H]-PK11195 binding following injury. As expected, in sham-operated animals [^{3}H]-PK11195 binding was very low with the expression only detected in the choroid plexus lining the ventricles. In the cortex relatively modest increases were found at 4 and 6 days post-injury. While it was not possible to quantify binding in discrete regions of the cortex due to the nature of the injury, visual assessment of the autoradiographs (Figure 2-5) reveals that the highest densities are found surrounding the lesion. An additional observation was that at 2, 4, and 6 days post-injury [^{3}H]-PK11195 binding was elevated throughout the ipsilateral cortex. This could represent the disruption of neuronal networks associated with the lesioned portion of the cortex.

The most obvious increase in [^{3}H]-PK11195 binding density began in the dentate gyrus at just 12 hours after injury, and reached significance at 2 days. Binding increases in this region occurred before any significant increases were measured in other brain regions, including the contused cortex. In a previous study in our laboratory we were able to detect a significant increase in [^{3}H]-PK11195 binding in the dentate gyrus just 6 hours after injury (Figure 2-16). The sensitivity of the dentate gyrus to CCI is further highlighted by the detection of significant increases in [^{3}H]-PK11195 binding in the contralateral dentate gyrus 6 days after injury (Figure 2-3). The sensitivity of cells in the region to injury has been reported previously (Baudry and Altar, 1991). In that study, chemical lesioning of the entorhinal cortex resulted in a significant increase in [^{3}H]-Ro5-4864 (a TSPO ligand) binding to ipsilateral dentate gyrus homogenates (Deller et al., 1996). One other interesting observation was a small but significant increase in binding to contralateral dentate gyrus homogenates 4 days after the lesion was induced. The vulnerability of dentate gyrus cells could be due to excitotoxicity brought about by the release of glutamate as a result of injury to neurons in the entorhinal cortex that project into the hippocampus.

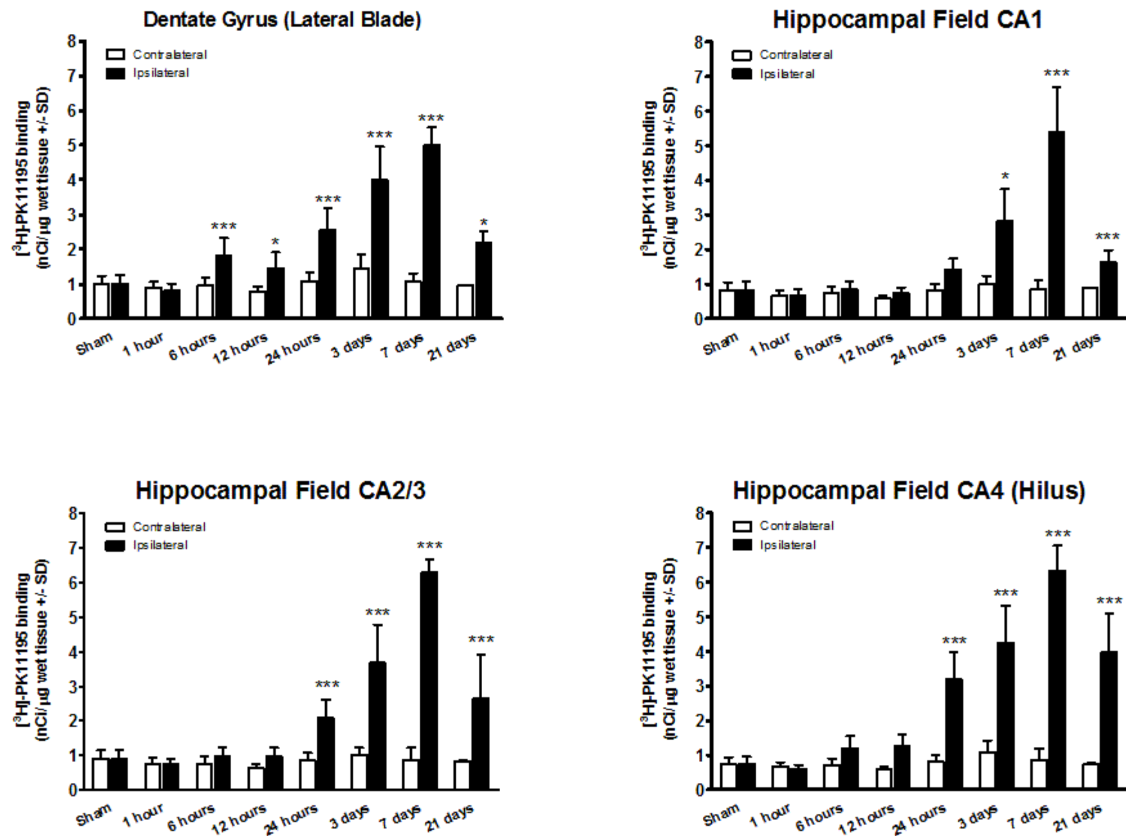

Figure 2-16 - [^{3}H]-PK11195 binding following a 2 mm CCI

In the dentate gyrus of the hippocampus significant increases in [^{3}H]-PK11195 binding were found just 6 hours after a 2.0 mm CCI. Maximal [^{3}H]-PK11195 binding in all hippocampal regions occurred on day 7, and by day 21 was returning to sham levels. Significant differences from contralateral regions were found by Tukey's HSD ($p < 0.05$, **$p < 0.01$, ***$p < 0.005$). Data plotted are means +/- standard deviations ($n = 8$).*

In the thalamus we found that binding of [^{3}H]-PK11195 does not robustly increase until approximately 3 days after CCI, and increases through all subsequent time points examined. Delayed microglial activation in the thalamus may represent retrograde cell death of thalamocortical neurons as a result of deafferentation in the projection region of the somatosensory cortex. This finding fits well with previous studies from our laboratory that have shown a delayed loss of nicotinic receptor binding in the thalamus, starting at 21 days post injury (Verbois et al., 2002). Further support for this mechanism as the basis of delayed microglial activation in the thalamus comes from studies in which lesions of the thalamus lead to a delayed loss of nicotinic binding in the cortex (Gioanni et al., 1999; Lavine et al., 1997). In addition, we have collected data in a previous study which shows that 3 weeks after injury [^{3}H]-PK11195 binding is returning to sham levels in all regions except for the thalamus (Figure 2-16, and 2-17).

The anionic fluorescein derivative FJ-B was utilized in brain sections adjacent to sections used for [^{3}H]-PK11195 autoradiography to assess the numbers of degenerating neurons. It has been shown that FJ-B staining correlates to a high degree with silver stains; a traditional marker of neuronal degeneration (Schmued and Hopkins, 2000a). In this study the time-course of FJ-B staining in the post-CCI rat brain was found to be different to the time-course of [^{3}H]-PK11195 binding. In the cortex a significant amount of FJ-B staining was seen at 12 hours after injury, but less at 24 hours and 2 days, and very little staining thereafter. In contrast, [^{3}H]-PK11195 binding begins increasing in the cortex at 2 days and peaks at 6 days.

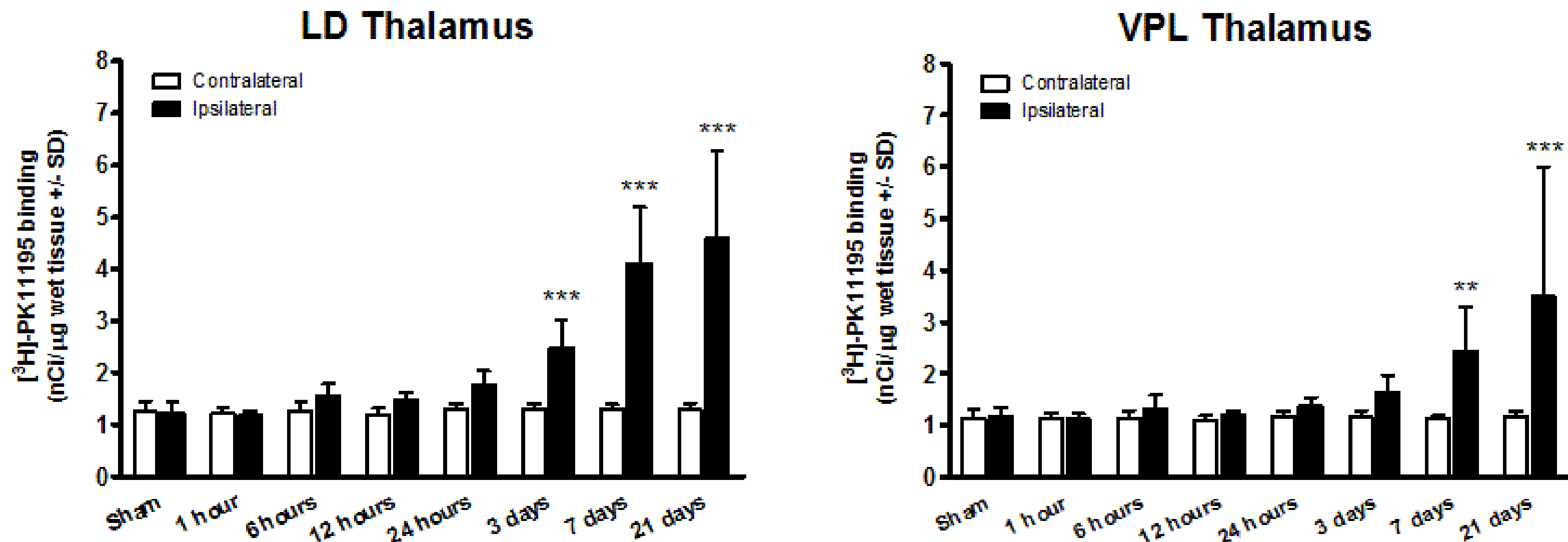

Figure 2-17 - [^{3}H]-PK11195 binding in the thalamus following a 2 mm CCI

In the thalamic regions, CCI results in significant increases in [^{3}H]-PK11195 binding that persist for at least 21 days. Unlike other regions measured, there was an increase in [^{3}H]-PK11195 binding between 7 and 21 days post injury. All data are displayed as means +/- standard deviation, with significant differences from contralateral measurements as found by Tukey.s HSD shown by asterisks ($p < 0.05$, **$p < 0.01$, ***$p < 0.005$).*

In the hippocampus numerous FJ-B stained neurons were visible in the dentate gyrus, CA4 (hilus), and in the CA2/3 field at 12 hours after injury. Significant numbers of degenerating neurons persisted in the CA2/3 and CA4 regions at the 1 day time-point and at later time points fewer were seen. In comparison, a large number of FJ-B stained neurons remained visible in the dentate gyrus at 2, 4 and to a lesser extent 6 days after CCI. A previous assessment of FJ-B staining in the hippocampus after CCI using stereological techniques made similar findings (Anderson et al., 2005). In the dentate gyrus FJ-B staining was confined to the granule cell layer at all time-points. The significant amount of neurons degenerating in this region for a prolonged length of time could significantly affect cognitive performance of these head injured animals. In addition to the dentate gyrus, the CA1, CA2/3, and CA4 (hilus) fields of the hippocampus also contain areas of neuronal degeneration. In the CA1 a small population of neurons in the medial portion of the field was stained at 12 hours and to a lesser extent at later time points. Then in the CA2/3 field of the hippocampus a large number of cells are stained starting just 12 hours with the highest numbers counted 24 hours after CCI. It has been suggested that proportionately, the amount of dying neurons in this region of the hippocampus is greater than any other (Anderson et al., 2005).

Finally, in the thalamus no significant numbers of FJ-B stained cells were seen until 4 and 6 days after CCI. The presence of degenerating neurons in this region at these time points lends credence to the hypothesis that the increased [^{3}H]-PK11195 binding is due to retrograde degeneration of thalamocortical projections. More FJ-B cells were counted in the thalamus at 4 days than at 6 days, although the difference was not significant. In comparison, [^{3}H]-PK11195 binding increases begin on day 2 and increase at each subsequent time-point. There was a significant increase in [^{3}H]-PK11195 binding between day 4 and 6 when assessed by Tukey's HSD ($p < 0.01$).

In an effort to correlate CD68 staining with [^{3}H]-PK11195 binding we quantified the numbers of CD68 positive cell profiles in different brain regions. In

the cortex, as with [^{3}H]-PK11195 binding data, significant increases in the numbers of CD68 positive cells were found at 4 and 6 days post injury. Interestingly, a larger number of CD68 positive cells were found on day 4 versus day 6 after CCI, this may represent a difference in the expression of each of these markers. In the hippocampus the time points with the most CD68 staining appeared to be 1 and 2 days post injury. However, staining persisted in all regions at 4 and 6 days, and overall there were no significant differences between time-points. Interestingly the pattern of CD68 staining in the hippocampus seems to follow the pattern of FJ-B staining, in that an increase in FJ-B staining at one time-point is followed by an increase in CD68 staining at the subsequent time-point. This observation was also made in the cortex, where large numbers of FJ-B staining at 12, 24 and 48 hours is followed by increased CD68 staining at 24 and 48 hours, and increasing till 4 days post-CCI. Since the post-CCI cortex contained large numbers of CD68 positive cells and FJ-B positive neurons, [^{3}H]-PK11195 binding was assessed in this area and correlated with these markers. While there was no positive correlation of [^{3}H]-PK11195 binding with FJ-B staining, there was a moderate positive correlation with CD68 staining ($r = 0.69$). By squaring the r-value one can make a crude assessment of the amount of variation in [^{3}H]-PK11195 binding that CD68 positive cells are responsible for, in this case ~48%. It is likely that other changes in [^{3}H]-PK11195 binding can be explained by sub-phagocytic activation of microglia, as has already been suggested (Banati et al., 1997). This fits with our assessment of CD68 staining in the thalamus, where very few CD68 positive cell profiles were counted despite the increases in [^{3}H]-PK11195 binding in this region.

Indeed, this previous assessment of [^{3}H]-PK11195 determined that [^{3}H]-PK11195 binding was associated with an 'activated' microglial phonotype in addition to the 'phagocytic' phenotype without any difference in the binding density of [^{3}H]-PK11195 (Banati et al., 1997). One might hypothesize that the neurons in the thalamus are distressed/dysfunctional but not dying at the same rate as neurons in the cortex or hippocampus. Therefore, microglia in the thalamus are becoming activated to provide support to these neurons thereby

expressing [^{3}H]-PK11195 binding sites. Since the CD68 antigen is a marker of phagocytic cells it may suggest that [^{3}H]-PK11195 is binding to microglial cells that are activated, but have not made the transition to a phagocytic phenotype.

A final consideration in these studies pertains to the possibility of increases in [^{3}H]-PK11195 binding being related to the process of astrocytosis. Astrocytosis is defined as an activation and accumulation of astrocytes as a response to injury. It is a key element of neuroinflammation and as such increased astrocyte activation and accumulation would be expected to occur at the same times as increases in microglial activation. Indeed, one previous study has found a moderate positive correlation of [^{3}H]-PK11195 binding with GFAP immunoreactivity following CNS injury (Raghavendra Rao et al., 2000). It may therefore, have been beneficial to perform some co-labelling experiment to determine the contribution of astrocyte activation to increases in [^{3}H]-PK11195 binding in the CCI model.

In conclusion, this study provides a detailed assessment of [^{3}H]-PK11195 binding following a mild/moderate CCI in rat brain. The correlation of [^{3}H]-PK11195 binding with ED-1 immunohistochemistry adds to a growing body of evidence that this ligand binds to activated microglia and macrophages (Banati et al., 1997; Myers et al., 1991b; Raghavendra Rao et al., 2000; Venneti et al., 2007a; Venneti et al., 2008b; Vowinckel et al., 1997). Microglia are the brains' injury surveillance cells and are very sensitive to tissue damage. When resting they are observed as having many long thin processes to 'listen' for signs of neuronal distress or death. Upon activation the processes become thicker and often polarized towards the site of injury. At this point they are believed to begin expressing the TSPO, most likely on the mitochondrial membrane. Then, if they receive the appropriate signals they can take on a phagocytic role to clear cellular debris. It is at this point that they start expressing the CD68 antigen, but no changes in the level of TSPO expression has been associated with this shift in phenotype. Therefore, although PK11195 cannot discriminate between the

various activation stages of microglia, it can detect changes in microglial cells that conventional analysis by CD68 immunohistochemistry cannot.

In addition, our study has highlighted the differences in the timing and magnitude of the microglial response, and indeed the pattern of neuronal degeneration following TBI. This should be an important consideration for targeting of pharmacological therapies. Moreover, PK11195 represents a useful marker for the assessment inflammation in animal models and human patients, and has significant potential for the evaluation of neuroprotective effects of pharmacological interventions.

2.5 Acknowledgements

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3. CHAPTER 3

Neuroprotective Actions of the Nicotinic Receptor Antagonist Methyllycaconitine Following Experimental Brain Injury

3.1 Introduction

It is estimated that over 1.4 million Americans experience a TBI every year (Sosin et al., 1996), however only a small fraction of these individuals sustain a injury that results in death or significant long term morbidity (Thurman et al., 1999). The remaining milder TBI patients also suffer from a wide variety of neuropsychological, behavioral and cognitive problems. These deficits are frequently left untreated, can persist for years following TBI and significantly detract from the quality of daily life for those affected (Oddy et al., 1985; Rao and Lyketsos, 2002).

It is well accepted that excessive synaptic levels of the excitatory amino acid glutamate can lead to a significant increase in neuronal dysfunction and cell death. The neurotoxic properties of glutamate are thought to be mediated primarily through calcium permeable ionotrophic NMDA receptors that are widely expressed throughout the nervous system (Olney, 1969). After traumatic injury sustained activation of NMDA receptors increases intra-neuronal calcium, leading directly or indirectly to mitochondrial impairment, an increase in ROS production, protein degradation, DNA damage, lipid peroxidation and ultimately neuronal death by necrosis and/or apoptosis. Thus it is not surprising that NMDA receptor antagonists have drawn substantial attention as potential neuroprotective drugs that could be useful for the CNS disorders involving excitotoxicity including TBI, spinal cord injury and stroke. However, human clinical trials of NMDA receptor antagonists for the acute and/or chronic treatment of these disorders have consistently ended in failure (Albers et al., 1999; Bullock et al., 1999; Haley et al., 2005; Ikonomidou and Turski, 2002). One significant problem with the use of NMDA receptor antagonists for the treatment of neurotrauma may be an inadequate understanding of the

therapeutic window of opportunity for successful treatment. While the acute actions of NMDA receptor antagonists might block some trauma-induced excitotoxicity, extended treatment might inhibit other critical functions of endogenous glutamate, including promotion of synaptic activity, neuronal survival and cognitive processing (Biegon et al., 2004). One study found that administering NMDA itself to mice 24 or 48 hours after head injury improved cellular and functional outcomes (Biegon et al., 2004), challenging the dogma regarding NMDA blockers and neuroprotection. Considering the short window of opportunity of NMDA receptor antagonist treatment and the importance of NMDA receptor stimulation in synaptic plasticity, an alternative approach to the acute treatment of TBI might be useful.

Targeting the brain cholinergic system could be one such approach; persistent deficits in neuropsychological and cognitive function are common following mild or moderate head injury, and research has supported involvement of the brain cholinergic system (Dixon et al., 1994b; Dixon et al., 1999; Griffin et al., 2003; Pike and Hamm, 1997a, b; Pike et al., 1997). Indeed, TBI has been shown to cause long-term changes in cholinergic function in the brains of both animals and humans (Dewar and Graham, 1996; Dixon et al., 1995b; Murdoch et al., 1998). Previous studies from our laboratory have demonstrated a time-dependant and consistent loss of α7 nAChR expression in rat cortical and hippocampal regions following experimental TBI (Verbois et al., 2003a; Verbois et al., 2002, 2003b; Verbois et al., 2000). The mechanism underlying selective loss of α7 nAChR expression following CCI remains unknown, but we hypothesize an elevated level of cholinergic activity immediately following CCI may play a crucial role. Similar to NMDA receptors, the α7 nAChR has high permeability to calcium ions (Zhao et al., 2003) and plays a role in hippocampal dependant cognitive processing. Furthermore, studies support a predominant presynaptic localization of α7 nAChRs, where they regulate the release of other neurotransmitters including ACh and glutamate (Albuquerque et al., 1997; Lagostena et al., 2008; McGehee et al., 1995).

In the current study we evaluated the neuroprotective properties of the α7 nAChR antagonist MLA. MLA was originally isolated from the seeds of *Delphinium brownii*, and has been found to be a potent inhibitor of α-BTX binding to nAChRs (Macallan et al., 1988). MLA is one of the most potent diterpenoid alkaloids found in *Delphinium brownii,* and is known to cause poisoning of livestock in the American Midwest due to its' actions on the neuromuscular nAChR (Pfister et al., 1999). As a potent and competitive antagonist of the CNS α7 nAChr, MLA has been found to be neuroprotective in models of β-amyloid and methamphetamine-induced toxicity (Escubedo et al., 2005; Martin et al., 2004b). The current study tested the hypothesis that antagonism of α7 nAChRs acutely following TBI will be associated with improved anatomical, histological and behavioral outcomes.

3.2 <u>Materials and methods</u>

3.2.1 Animals and surgery

All experiments were conducted in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee (IACUC) that follows the guidelines set forth by the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize the number of animals used in this study and to minimize animal suffering. Male Sprague-Dawley rats weighing 275-300 g were obtained from Harlan Breeding Laboratories (Indianapolis, IN) housed 3 per cage and randomly assigned into five treatment groups (n = 8 per group). Animals were anaesthetized with 3% isoflorane and immobilized in a stereotaxic frame. A 6 mm craniotomy was performed using a Michele trephine just above the somatosensory cortex (Bregma –2.8, 2.5 mm lateral). The stereotaxic frame was then positioned under a TBI 0310 impaction device (Precision Systems & Instrumentation, Fairfax Station, VA) that was used to administer a 1.5 mm cortical deformation at a velocity of 3.5 m/s (Baldwin et al., 1996; 5 mm impactor diameter). Following the injury the skull disc was replaced and sealed with dental acrylic. Sham animals underwent the same procedure with the exception of the administration of the

cortical contusion injury CCI. Five minutes after surgery the animals were given an intraperitoneal injection of MLA (1, 5, or 10 mg/kg) or saline; animals received three additional injections of saline or MLA 8, 24 and 32 hours post-CCI.

3.2.2 Behavioral testing

Cognitive function was assessed on days 11-15 post-CCI, using a standard MWM procedure (Morris, 1984). The behavioral testing room contained a circular 150 cm diameter pool with a 13.5 cm diameter submerged escape platform in the center of one quadrant. Visual cues distributed throughout the room helped to aid spatial orientation. All cognitive evaluations were videotaped and analyzed using Videomax software (Columbus Instruments, Columbus, OH). Quadrant entry was randomized for different starting positions, and animals were allowed to swim until they found the platform, up to a maximum of 60 seconds. Rats that were unable to locate the platform in 60 seconds were guided to and manually placed on the platform, and then allowed observe their position within the pool 15 seconds. The acquisition phase of MWM testing consisted of four trials conducted each day for five consecutive days, with a 5 minute inter-trial interval. Four hours following the last acquisition trial, the platform was removed and a 30 second memory retention trial was performed. Videomax software was used to analyze several aspects of each animals search strategy on the retention trial. MWM acquisition phase data were analyzed using the two-way repeated measure ANOVA (treatment $\times$ day), where the day of training was used as a repeated measure. A Tukey's HSD test was used to assess differences between the individual groups. MWM retention data were analyzed by one-way ANOVA, and a Tukey's HSD post-hoc test to determine individual group differences.

3.2.3 Cortical tissue sparing analysis

Following the completion of behavioral testing the animals were euthanized and their brains were immediately removed and frozen in isopentane. Brains sections (16 μm) were prepared using a Lecia CM1850 cryostat and were mounted onto slides coated with gelatin, chromium potassium sulfate and poly-L-lysine to ensure tissue adhesion. A cortical tissue sparing

analysis was performed to assess the potential neuroprotective actions of MLA administration. Briefly, fourteen equally spaced cryostat sections through the damaged area were obtained from each animal and stained with cresyl violet. Images of each stained section were captured and digitized, then, using NIH image calibrated to measure area, the ipsilateral and contralateral cortices were outlined separately and measured. The percentage of cortical tissue spared was calculated by dividing the mean cortical area for the ipsilateral cortex by the mean cortical area for the contralateral cortex (×100) (Scheff and Sullivan, 1999).

3.2.4 Receptor autoradiography

α7 nAChR density was measured using [^{125}I]-α-BTX autoradiography, as previously described (Sparks and Pauly, 1999) using a ligand concentration of 2.5 nmol [^{125}I]-Tyr_{54}-α-BTX (PerkinElmer Life Sciences, Inc., Boston, MA; specific activity = 102.9 Ci/mmol). Sections adjacent to those used for [^{125}I]-α-BTX binding were labeled with [^{3}H]-PK11195 to identify the TPSO, formerly known as the PBR (Papadopoulos et al., 2006a). This protein is thought to be primarily associated with mitochondrial cholesterol transport in activated microglia. Previous studies have used autoradiographic localization of [^{3}H]-PK11195 binding to detect the presence of activated microglia as a marker of neuroinflammation. Briefly, slides were incubated in buffer containing 1 nM [^{3}H]-PK11195 for 2 hours, washed in buffer, and dried overnight. RayMax Beta High Performance Autoradiography Film (ICN Biomedicals Inc., Aurora, Ohio) was used to visualize the areas of ligand binding. Radioactive rat brain tissue standards were included with each film X-ray cassette in order to determine the response of the film to the increasing amounts of radioactivity. Binding data were analyzed using NIH image v1.59 on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. Molar quantities of bound ligand were determined by constructing a standard curve from radioactivity tissue standards fitted to a third degree polynomial curve. Changes in [^{125}I]-α-BTX binding following CCI and MLA treatment were quantified in several hippocampal sub-regions. [^{125}I]-α-BTX binding data were normalized relative to levels measured in sham animals since two separate binding experiments had to be

performed on these sections. Based on the results from Chapter 2, [^{3}H]-PK11195 binding was measured in the cortex, the dentate gyrus, the hilus, the CA1 and CA2/3 fields, and in the lateral dorsal and ventral posterolateral thalamus. Binding data were analyzed by a 2-way ANOVA where treatment and hemisphere were the independent variables and binding density the dependant variable. Tukey's HSD post-hoc tests were used when the ANOVA found a significant overall effect.

3.3 <u>Results</u>

3.3.1 Post-CCI MLA administration produces modest improvement in MWM performance

Figure 3-1 depicts the acquisition of spatial learning in groups of animals tested in the MWM following CCI and MLA administration. ANOVA results indicated a significant main effects of training day ($F_{(4,112)} = 19.65$, $p < 0.001$) and treatment ($F_{(4,112)} = 20.99$, $p < 0.001$), as well as a significant treatment X day interaction ($F_{(16,112)} = 1.76$, $p < 0.05$). Each dose of MLA administered resulted in a slight decrease in escape latency on each test day of acquisition testing. However, Tukey's HSD post-hoc analysis revealed significant group differences on day 4 of testing only. Compared to CCI animals treated with saline, treatment with 1 or 5 mg/kg MLA significantly decreased distance traveled to platform on day 4 of acquisition testing ($p < 0.05$). However on day 5 of acquisition testing MLA-treated animals performed much more poorly than would be expected by the trajectory of learning observed on days 1 to 4. This was unexpected, and the reasons for this apparent relapse of behavior on day 5 of testing are unclear.

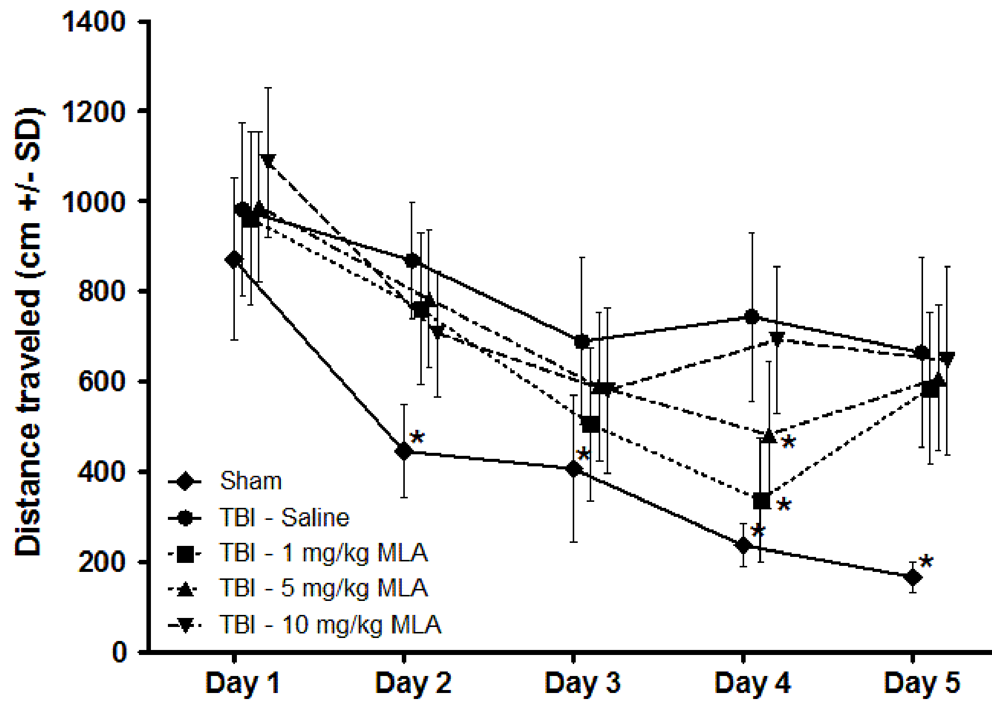

Figure 3-1 - The effect of MLA treatment on MWM acquisition phase performance

*MLA treatment partially attenuates cognitive deficits following CCI in the acquisition phase of Morris water maze testing. Day 1 represents the day of the first trial and also the 11th day after injury. The mean distance traveled by animals in each group was calculated and plotted against day of trial (mean ± SD). Animals treated with 1 mg/kg or 5 mg/kg MLA performed better than saline treated animals on day 4 of testing (*p<0.01).*

Four hours following the completion of the acquisition phase of the MWM test, the platform was removed from the pool and animals swim strategy was assessed for 30 seconds (Figure 3-2). One-way ANOVA found a significant effect of treatment on entries made into target quadrant ($F_{(4,36)} = 3.05$, $p < 0.05$), time spent in the target quadrant ($F_{(4,36)} = 3.35$, $p < 0.05$), and distance traveled in target quadrant ($F_{(4,35)} = 4.26$, $p < 0.01$). However post-hoc analysis showed that sham operated rats performed better than injured animals in each measure, and there were no improvements associated with MLA treatment. The lack of clear cognitive enhancement in the retention phase of MWM testing was not surprising since the performance of these groups in acquisition trials on day 5 was also unexpectedly poor.

3.3.2 Post-CCI MLA administration results in dose-dependent cortical tissue sparing

One-way ANOVA analysis of cortical tissue sparing data revealed a significant effect of treatment, $F_{(4,32)} = 5.44$, $p < 0.005$; Figures 3-3, 3-4). Fifteen days following CCI, saline treated animals had 76% of their cortical tissue spared ipsilateral to the site of impact. In contrast, animals treated 4 times over 2 days with 1 mg/kg (85%) or 5 mg/kg (87%) MLA has significantly greater tissue sparing as compared to saline treated animals ($p < 0.05$). In rats that treated with the highest dose of MLA, 93% of cortical tissue was spared, which was not significantly different from estimated cortical volume in sham-operated animals.

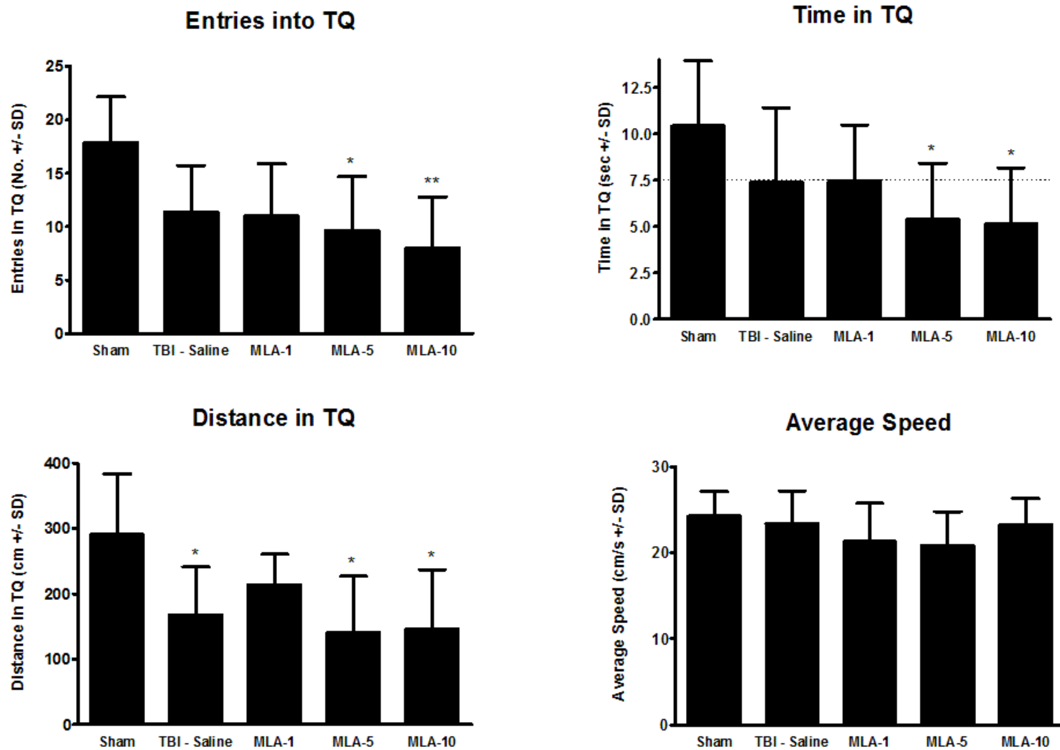

Figure 3-2 - The effect of MLA treatment on memory retention in the MWM

*High dose MLA generally impairs memory performance in a 30 second retention test, conducted one day following the completion of MWM acquisition training. CCI animals treated with saline surprisingly had only deficits in distance traversed in the target quadrant (TQ) compared to sham operated controls (mean ± SD). In contrast animals treated with 5 mg/kg and 10 mg/kg MLA were significantly impaired compared to sham operated animals in terms of entries into, time spent in, and distanced traveled in the target quadrant (*p < 0.05, **p < 0.01). Animals treated with the lowest MLA dose (1 mg/kg) performed at the same level as sham-operated controls on each measure of memory retention, and there were no group differences in swim speed.*

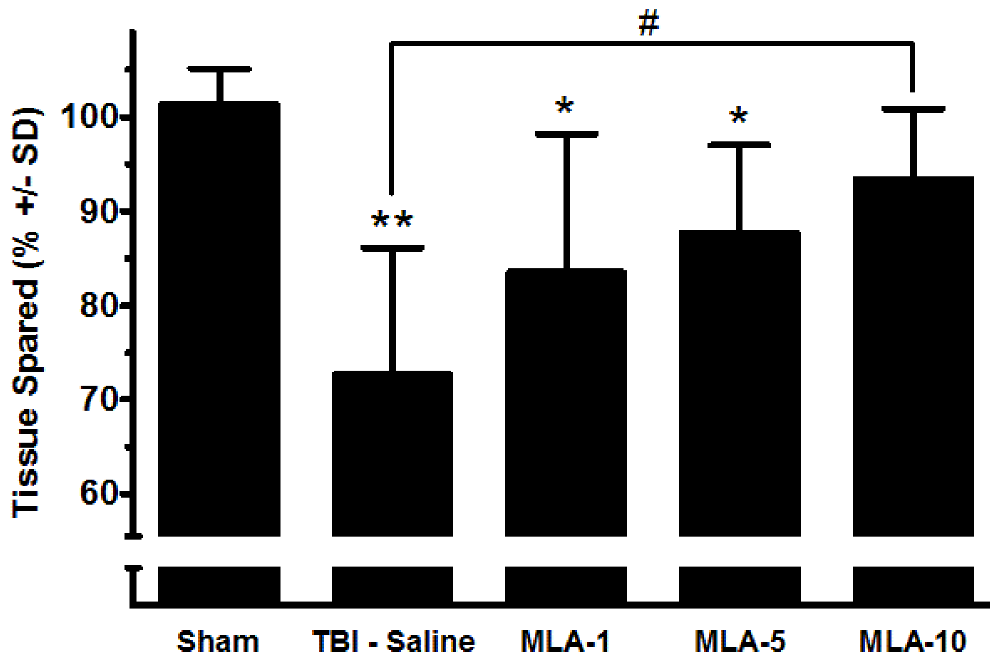

Figure 3-3 - Cortical tissue sparing in rats treated with MLA following CCI

*There is a dose-related cortical tissue sparing following post-CCI MLA administration. Saline treated animals had 76% (mean ± SD) cortical tissue spared (**p < 0.005 versus sham operated animals) compared to 83% sparing in animals that received 1 mg/kg MLA (*p < 0.05 versus sham operated animals), 88% sparing after 5 mg/kg MLA (*p<0.05 versus sham operated animals) and 93% spared following 10 mg/kg (not significantly different from sham operated controls). Furthermore, the animals treated with 10 mg/kg MLA had a significantly higher tissue sparing than saline treated animals following CCI (#p < 0.01).*

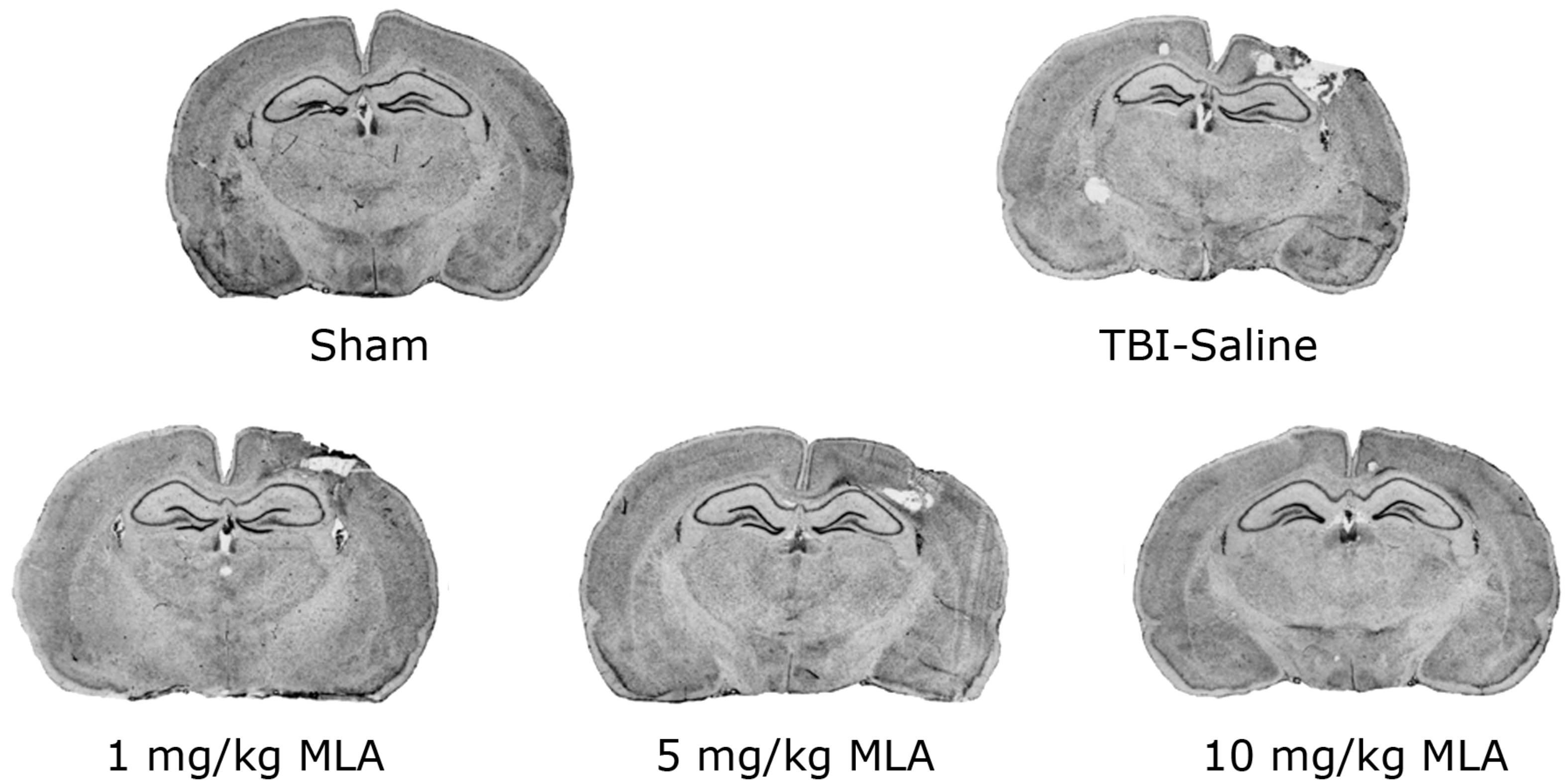

Figure 3-4 - Representative images of cresyl violet-stained rat brain sections

Representative nissl-stained tissue sections illustrating dose-related cortical tissue sparing following post-CCI saline or MLA administration. All images were obtained from the epicenter of the cortical tissue loss in each group (approximately Bregma -2.8 mm).

3.3.3 *MLA administration exacerbates CCI-induced loss of hippocampal α7 nAChR expression*

In saline injected animals, CCI was associated with a significant loss of α7 nAChR expression in the ipsilateral stratum oriens and CA2/3 regions of the hippocampus (Table 3-1), consistent with previous studies from our lab. Surprisingly, all animals treated sub-acutely with MLA had significant reductions in hippocampal α7 nAChR binding. In MLA treated rats, bilateral reductions in [^{125}I]-α-BTX binding were seen throughout the hippocampus.

3.3.4 *Post-CCI administration of MLA reduces regional brain inflammation*

Previous studies have shown that CCI produces a widespread and significant increase in [^{3}H]-PK11195 binding beginning within hours in areas proximal to the injury, and persisting for days to weeks in brain regions more distal to the site of injury (Banati et al., 1997; Miyazawa et al., 1995; Raghavendra Rao et al., 2000). Our analyses focused on hippocampal, cortical and thalamic neuroinflammation. The cortex adjacent to the impaction site and the hippocampus have early expression of inflammatory markers following TBI, and thalamic inflammation is more delayed and may occur only secondary to cortical tissue encavitation (Chapter 2). Table 3-2 shows the effects of TBI and MLA administration on changes in the density of [^{3}H]-PK11195 binding (Representative tissue sections are depicted in Figure 3-5). In saline treated animals subjected to a 1.5 mm TBI, significant increases in [^{3}H]-PK11195 were observed on the ipsilateral side of the brain, in each area evaluated ($p < 0.001$) consistent with previous studies. Animals treated with the lowest dose of MLA had no significant changes in regional brain inflammation compared to saline treated TBI animals, except that a significant increase in [^{3}H]-PK11195 binding was observed in the laterodorsal nucleus of the thalamus. In comparison, 5 mg/kg and 10 mg/kg MLA both caused a significant attenuation of neuroinflammation in the somatosensory cortex adjacent to the injury, and the CA1 and CA2/3 sub-regions of the hippocampus. Post TBI administration of MLA did not affect [^{3}H]-PK11195 binding in the dentate gyrus of the hippocampus or thalamus.

	Sham	0 mg/kg MLA		1 mg/kg MLA		5 mg/kg MLA		10 mg/kg MLA	
	Ipsi	*Contra*	*Ipsi*	*Contra*	*Ipsi*	*Contra*	*Ipsi*	*Contra*	*Ipsi*
Stratum oriens	1.00±0.21	1.02±0.09	0.84±0.08**,†	0.84±0.28**	0.82±0.20**	0.78±0.20**	0.71±0.14**	0.86±0.25**	0.87±0.23**
Hippocampal CA1 field	0.99±0.15	0.97±0.08	0.98±0.06	0.76±0.28**	0.75±0.28**	0.83±0.23**	0.86±0.17**	0.85±0.31**	0.85±0.25**
Hippocampal CA2/3 field	0.99±0.12	0.97±0.11	0.88±0.08**,†	0.77±0.28**	0.79±0.22**	0.84±0.17**	0.82±0.17**	0.86±0.23**	0.88±0.17**
Hippocampal CA4 field (Hilus)	0.99±0.15	0.94±0.14	0.94±0.11	0.90±0.22*	0.98±0.22	0.79±0.25**	0.80±0.20**	0.95±0.23	0.98±0.20
Dentate gyrus (dorsal blade)	0.95±0.15	0.88±0.11	0.90±0.06*	0.73±0.25**	0.73±0.22**	0.79±0.20**	0.79±0.20**	0.85±0.23**	0.86±0.11**

Table 3-1 - The effect of post-CCI MLA administration on binding density of [^{125}I]-α-BTX in selected brain regions

[^{125}I]-α-BTX binding to α7 nAChRs following CCI and MLA administration. In sham-operated animals there were no hemispheric differences in the density of [^{125}I]-α-BTX binding, so regional data were averaged (mean ± SD), and the remainder of the data were expressed as a percent of sham-operated control values. CCI followed by saline administration resulted in a unilateral deficit in [^{125}I]-α-BTX binding in the stratum oriens, CA2/3 and dorsal blade of the dentate gyrus. Surprisingly acute post-CCI administration of MLA caused significant bilateral reductions in α7 nAChR expression in all areas measured, persisting for up to 15 days post-injury.

	Sham		0 mg/kg MLA		1 mg/kg MLA		5 mg/kg MLA		10 mg/kg MLA	
	Contra	*Ipsi*	*Contra*	*Ipsi*	*Contra*	*Ipsi*	*Contra*	*Ipsi*	*Contra*	*Ipsi*
Cortex	0.78±0.10	1.02±0.16	0.78±0.12	3.44±0.65*	0.99±0.31	3.37±0.69*	1.00±0.23	2.12±0.83*†	0.91±0.16	2.29±1.25*†
Hippocampal CA1 field	0.79±0.07	0.82±0.08	0.94±0.16	4.19±1.14*	0.90±0.16	3.38±1.37*	0.84±0.12	2.72±1.12*†	0.90±0.12	3.10±1.28*†
Hippocampal CA2/3 field	0.81±0.09	0.84±0.08	0.85±0.15	4.85±1.52*	0.86±0.17	4.55±1.18*	0.80±0.15	3.27±0.61*†	0.86±0.14	3.69±1.03*†
Hippocampal CA4 field (Hilus)	0.83±0.08	0.85±0.08	1.01±0.30	5.21±1.55*	1.05±0.26	5.09±0.97*	0.88±0.12	4.64±0.69*	1.03±0.26	5.23±1.45*
Dentate gyrus (dorsal blade)	1.09±0.11	1.11±0.11	1.28±0.36	4.02±0.99*	1.45±0.35	4.06±0.86*	1.17±0.15	3.34±0.99*	1.38±0.30	3.39±1.17*
Dentate gyrus (ventral blade)	1.11±0.10	1.12±0.13	1.46±0.36	4.11±0.73*	1.64±0.38	3.72±1.21*	1.30±0.17	3.56±0.79*	1.63±0.40	4.31±1.00*
Lateral dorsal thalamus	0.72±0.06	0.73±0.06	0.67±0.12	2.75±0.63*	0.78±0.14	3.49±0.79*†	0.73±0.15	2.80±0.57*	0.81±0.19	3.14±0.42*
Ventroposterolateral thalamus	0.73±0.07	0.74±0.07	0.64±0.06	2.60±0.52*	0.67±0.12	2.65±0.71*	0.66±0.13	2.42±0.51*	0.79±0.15	2.48±0.61*

Table 3-2 - The effect of post-CCI MLA administration on binding density of [^{3}H]-PK11195 in selected brain regions

Neuroinflammation following CCI and MLA administration as assessed by [^{3}H]-PK11195 autoradiography. All CCI animals had significant increases in [^{3}H]-PK11195 binding compared to sham-operated controls ($p < 0.01$). However, animals treated with 5 or 10 mg/kg MLA had significantly less neuroinflammation in several regions evaluated (†$p < 0.01$ compared to the ipsilateral side in saline-treated controls)*

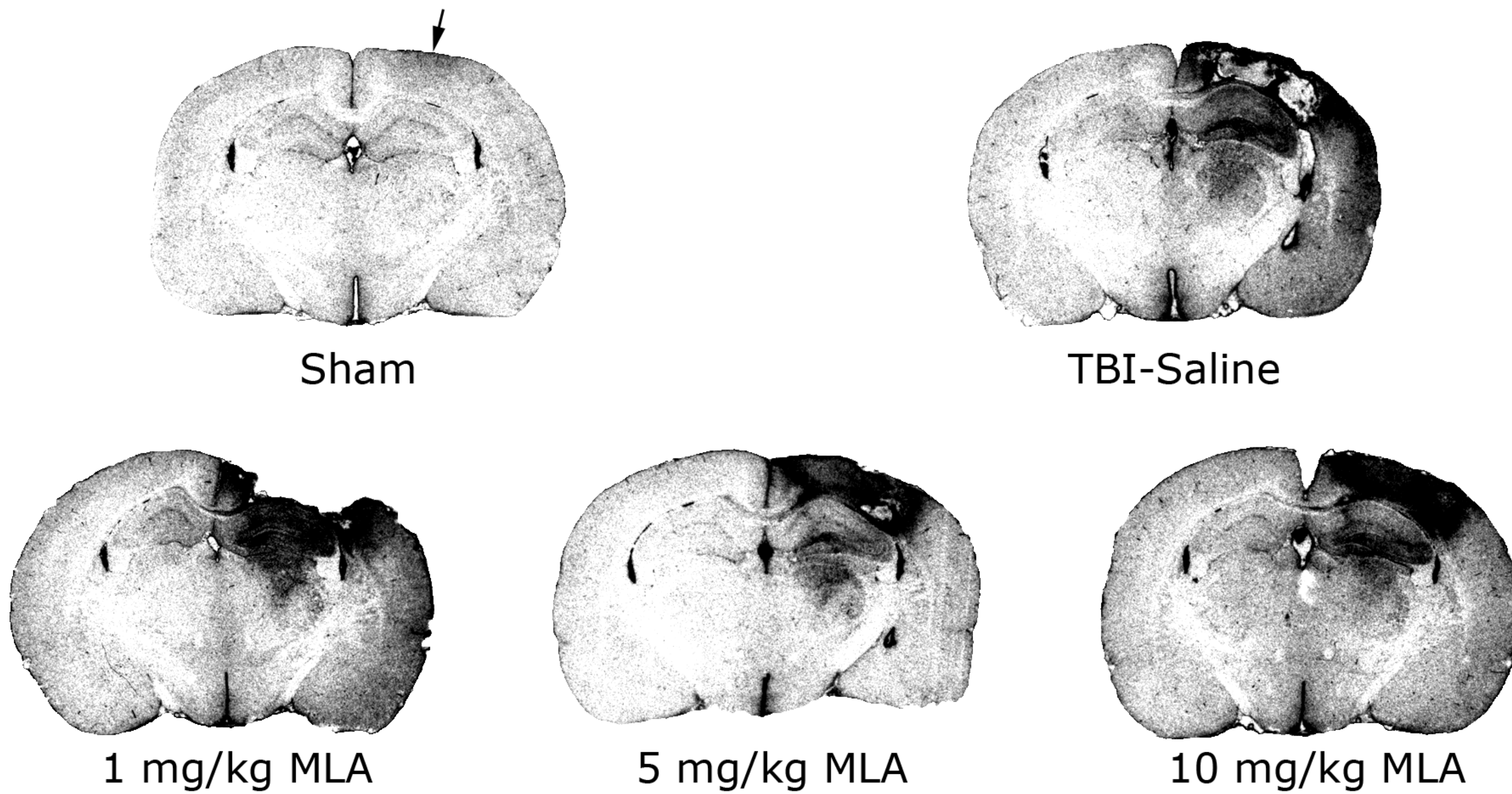

Figure 3-5 - Autoradiographs of rat brain sections bound with [^{3}H]-PK11195

Representative sections demonstrating [^{3}H]-PK11195 binding in animals treated with saline or MLA following CCI. The sensitivity of [^{3}H]-PK11195 binding as a marker of neuroinflammation following brain injury is illustrated by the increased binding seen ventral to the craniotomy site in sham-operated animals (arrow). In CCI-saline animals a highly significant increase in binding was pervasive in the cerebral cortex, hippocampus, and thalamus. The 5 mg/kg and 10 mg/kg doses of MLA significantly attenuated TBI-induced increases in neuroinflammation in the cerebral cortex, and the CA fields of the hippocampus.

3.4 Discussion

Previous studies from our lab have postulated that α7 nAChR activation may contribute to calcium-mediated neurotoxicity following TBI. The high calcium permeability of α7 nAChR (Gilbert et al., 2009) and their prominent location in brain regions affected indirectly and directly by TBI make these receptors plausible targets for neuroprotection. Pharmacological antagonism of these receptors during the first 32 hours following injury produced some beneficial results, but other outcome measures were unaffected or exacerbated. The most consistent outcome measure in this study was cortical tissue sparing, where post-injury MLA administration was associated with dose dependant preservation of tissue. In saline treated animals, a 1.5 mm CCI was associated with loss of 26% of ipsilateral cortical volume, 15 days following the initial surgery. Loss of cortical mass was attenuated by post-surgery MLA administration, with the highest dose (10 mg/kg) producing a level of tissue sparing that was not significantly different from sham-operated animals. However neuroprotection evidenced by preservation of cortical mass was generally not correlated with improved outcomes in the hippocampal-dependant MWM spatial memory task. In fact animals treated with 10 mg/kg MLA, had the greatest cortical tissue sparing, but no cognitive improvement compared to CCI animals treated with saline in the acquisition phase of testing. Furthermore animals treated with the higher doses of MLA performed worse than saline treated CCI rats in the memory retention phase of cognitive testing. The dose inversion of the MWM results suggests that higher doses of MLA may produce pharmacological actions that are inconsistent with improved functional outcomes, in spite of significant cortical tissue preservation.

Studies from the Levin laboratory have conclusively shown that acute, intracerebral injection of nicotinic receptor antagonists impairs performance in cognitive tests including the radial arm maze. When the α4β2 nAChR antagonist dihydro-β-erythroidine (DHβE) or MLA were injected into the basolateral amygdala or ventral hippocampus, cognitive deficits were observed (Addy et al., 2003; Nott and Levin, 2006). There were no additive effects of DHβE and MLA

when administered simultaneously, however DHβE co-administration prevented the amnestic actions of MLA delivered into the ventral hippocampus, suggesting a more complicated interaction between nicotinic receptor subtypes and regulation of cognitive function. Also in the Levin studies, behavioral performance was assessed 10 minutes following injection of nAChR antagonists. In our experiment, rats received 4 injections of MLA in the first 32 hours following experimental injury, and no further drug treatments. Behavioral testing ensued 9 days later, and continued across days 11 to 15 post-injury. Since the half-life of MLA is only approximately 20 minutes in rodents (Stegelmeier et al., 2003; Turek et al., 1995) it seems highly unlikely that residual MLA caused cognitive impairment by direct blockade of α7 nAChRs. However long-term functional changes in nicotinic receptor sensitivity following acute administration of nAChR antagonists are not without precedence. Several studies have previously shown that a single injection of the nicotinic receptor antagonist chlorisondamine blocks the actions of a nicotine challenge dose for up to 5 weeks, or more (Clarke, 1984; el-Bizri and Clarke, 1994). Thus it is possible that the nAChR blocking actions of MLA greatly outlast receptor occupancy, and confounded the MWM results in our study, particularly at the higher doses that were employed. Another surprising action of MLA that was observed in these studies was a persistent down regulation of hippocampal α7 nAChR expression. In saline treated animals, 1.5 mm CCI caused ipsilateral down-regulation of α7 nAChR expression in the stratum oriens, CA2/3 and the dentate gyrus, consistent with previous findings from our lab (Verbois et al., 2003a; Verbois et al., 2002, 2003b; Verbois et al., 2000). Each dose of MLA administered caused a bilateral decrease in hippocampal α7 nAChR expression that persisted for up to 13 days following the last MLA injection. However [^{125}I]-α-BTX deficits caused by MLA administration were generally not dose-dependent.

Interestingly in spite of MLA-associated deficits in α7 nAChR expression, each dose of MLA administered to rats after trauma was associated with some degree of improvement in the acquisition phase of MWM testing. This effect was most pronounced in rats given 1 mg/kg MLA on the fourth day of testing. On the

same day rats administered 5 mg/kg MLA showed some improvement over saline animals, and the 10 mg/kg MLA group did not perform significantly better than saline-injected animals. This inverse-dose response suggests that while 1 mg/kg MLA is therapeutic, higher doses of MLA are detrimental to the function of neurons involved in acquisition of the MWM task. It is possible that the higher doses of MLA impeded the recovery of neurons by inhibiting basal levels of signaling required for survival or for the induction of LTP. On the fifth day of testing there were no significant differences in performance of MLA-treated animals versus saline-treated animals. MLA treated animals took longer to find the platform [data not shown], and traveled further around the pool before finding the escape platform. There is no obvious explanation for this sudden relapse in performance. Coincidentally, the animals also performed poorly on the memory-retention phase of the MWM that was carried out later on the same day. In this phase of the MWM the search strategy of each animal was assessed without the submerged platform for 30 seconds. The distance traveled in, entries into, and time spent in the quadrant of the pool that had contained the platform were measured. Sham animals performed well by all measures, and saline treated CCI animals performed poorly as expected. Unexpectedly, animals which had received MLA injections did not perform any better than saline-treated controls. The apparent lack of effect of MLA in this memory retention task could be linked to the poor performance of these animals on the fifth acquisition trial carried out on the same day.

In contrast to the inverse-dose response found in the MWM, cortical tissue sparing analysis revealed a direct relationship between dose and tissue sparing. Indeed, the 10 mg/kg dose of MLA spared 93% of cortical tissue; a figure that was not significantly different from sham-operated animals. A dose of 5 mg/kg spared 88% of cortical tissue, 1 mg/kg spared 84%, and saline spared 76%. Clearly MLA is sparing cortical neurons in a dose dependant manner. We believe this effect is due to the inhibition of the α7 nAChRs by MLA, since the reduced calcium flow into cells expressing these receptors will attenuate the calcium load and protect them from cellular death.

The lack of concordance between the dose response curves for tissue sparing and cognitive recovery in the MWM was intriguing. Animals given 1 or 5 mg/kg of MLA performed better than animals that received a dose of 10 mg/kg, while the highest level of tissue sparing was achieved in animals that received the highest dose of MLA. While the highest dose may protect neurons from excitotoxicity, it may also cause long-term changes in neuronal function that affect the learning and memory process. Importantly, it should be noted that the MWM is a hippocampal dependant task (Liang et al., 1994) and that we only measured tissue loss in the cerebral cortex. The use of a cortical-dependant test such as pre-pulse inhibition could have yielded a positive correlation between functional and tissue sparing results.

Autoradiographic study of TSPO expression (a marker of inflammation and cellular damage) revealed a marked increase in [^{3}H]-PK11195 binding in the ipsilateral cortex, hippocampus and thalamus of all injured animals. However, treatment with 5 and 10 mg/kg MLA significantly reduced binding in the CA1, CA2/3 and cortex. This reduction corresponds well with results from the tissue sparing analysis, supporting the notion that inhibition of the α7 nAChR by MLA is protecting cells from apoptosis and necrosis. In contrast to the attenuation of [^{3}H]-PK11195 binding in the cortex and hippocampus, no measurable effect of MLA was found in the thalamus. This could merely be a timing issue, since increases in thalamic [^{3}H]-PK11195 binding are delayed compared to those in the cortex and hippocampus (Chapter 2). Elevated [^{3}H]-PK11195 binding first appears in the thalamus about 3 days after injury and may still be increasing 2 weeks after injury. It is possible that any changes in thalamic binding of [^{3}H]-PK11195 would not be seen until later time points.

Our results show that MLA attenuates cognitive deficits following CCI, spares tissue, and reduces tissue damage as assessed by [^{3}H]-PK11195 binding. While these actions are most likely to be mediated via the α7 nAChR, it should be noted that MLA has affinity for other nicotinic subunits including α3 and α6 (Anderson et al., 2008; Mogg et al., 2002). Furthermore, MLA has a relatively

large molecule with an atomic mass of 683, so there are some questions about its ability to cross the BBB. To date there has only been one detailed pharmacokinetic analysis of plasma and brain levels of MLA following intravenous or intraperitoneal administration (Turek et al., 1995). In this study peak plasma levels of MLA were attained approximately 15 minutes following IP MLA administration, and brain levels peaked about 15 minutes later. Brain levels of MLA were approximately 95% lower than that observed in plasma, indicating minimal BBB penetration. However, we were undeterred by these results since CCI is known to cause transient and delayed opening of the BBB (Baldwin et al., 1996). The primary insult leads to a compromise of the BBB from the time of impact until about 3 hours post-injury. The BBB then opens again between 12 and 48 hours after injury, possibly caused by a secondary wave of brain damage (Baldwin et al., 1996).

Another consideration in this study is the possibility of MLA acting indirectly via the cerebrovascular response to generate positive functional outcomes. For example, stimulation of basal forebrain cholinbergic receptors can increase cerebral cortical blood flow (Linville et al., 1993). In addition, there is some evidence to suggest that acetylcholine receptors located on the vessel walls could modulate vascular tone, though current data support a role for muscarinic receptors only (Elhusseiny and Hamel, 2000). However, the expression of α7 nAChR on cortical vessel walls has been demonstrated (Clifford et al., 2008), so a role in regulation of cerebral blood flow cannot be ruled out. Control of cerebral blood flow via these mechanisms by a nicotinic antagonist and resultant changes in the supply of oxygenated blood could potentially effect outcome from CCI.

An additional concern in the use of MLA is inhibition of a cholinergic anti-inflammatory pathway (Wang et al., 2003). Electrical stimulation of the vagus nerve has been shown to cause a significant decrease in tumor necrosis factor (TNF) release from macrophages, which express α7 nAChRs; the effects of vagal activation were blocked by administration of MLA and absent in α7 nAChR

knockout mice. More recently it has been found that in the CNS microglia express α7 nAChRs and that exposure to ACh or nicotine reduces inflammatory markers following the administration of lipopolysaccharide (Shytle et al., 2004). α7 nAChR antagonists attenuated the effects of nicotine and ACh, directly implicating the α7 nAChR as a mediator of these responses. Thus α7 nAChRs are key players in the cholinergically mediated anti-inflammatory pathway in the periphery and CNS, and MLA inhibition of this system might limit the usefulness of this compound, especially in long-term studies.

Taken together, these results show that blockade of α7 nAChR stimulation immediately following CCI can be neuroprotective. This is the first study aiming to assess the effects of MLA in an in vivo animal model of TBI; some important issues have been raised. The validation of our hypothesis relies on MLA being administered early in the acute phase of injury in which cholinergic activity is elevated. In rat brain the acute period of cholinergic excitotoxicity appears to be followed by a more chronic period of cholinergic hypofunction (DeAngelis et al., 1994; Dixon et al., 1994a; Dixon et al., 1995a; Dixon et al., 1996; Dixon et al., 1997; Saija et al., 1988). The precise timing of the post CCI-changes in cholinergic activity have yet to be fully elucidated. The first MLA injection was administered immediately following surgery, and then subsequent injections were administered at 8, 24 and 32 hours thereafter. It is possible that the window of opportunity for efficacious MLA treatment is relatively short and that administering drug at later time-points is detrimental to neuronal survival. An improved understanding of the time frame of cholinergic hyperactivity is required to guide future cholinergic treatment regimens.

3.5 Acknowledgements

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4. CHAPTER 4

Alpha-7 Nicotinic Acetylcholine Receptor Partial Agonists Improve Functional Outcome Following Controlled Cortical Impact in the Rat

4.1 Introduction

Increased public awareness of TBI has been brought about in recent years by the wars in Afghanistan and Iraq (Galarneau et al., 2008). While reports are widespread on the effects of TBI on the many soldiers and civilians who are victims of improvied explosive devices (IEDs), many more people fall victim to TBIs each year in the USA as a result of motor vehicle accidents or falls (Langlois et al., 2006). The vast majority of TBI sufferers recover from the physical effects of their injuries, but many are left with significant behavioral, emotional, and cognitive deficits that remain untreated (Corrigan et al., 2004; Resch et al., 2009). These deficits often result in significant reductions in quality of life measures for patients, place a great burden on families and care-givers, and make the transition back into the workforce very difficult (Oddy et al., 1985; Rao and Lyketsos, 2002). Despite an estimated 1.5 million people suffering a TBI in the USA alone each year (Sosin et al., 1996; Thurman et al., 1999), and the huge financial burden imposed by this condition, there is no pharmacological treatment for TBI. There is a great need for new and novel approaches aimed at reducing secondary damage and improving functional and cognitive outcomes.

While there has been widespread interest in researching the role of excitatory amino acids (EAAs) in brain injury, the roles played by nAChRs have been largely ignored until recently. These receptors are known to be widely distributed and highly expressed in the brain (Sargent, 1993), and are intimately involved in cognition and other neurobiological processes (Kadir et al., 2006). Among nAChRs, the α7 nAChR is of particular interest due to its high levels of expression in the hippocampus; a region known to be vitally important for learning and memory, and also very sensitive to brain injury related pathology (Verbois et al., 2000). Furthermore, the α7 nAChR possesses a uniquely high

permeability to calcium ions (Delbono et al., 1997), which play a significant role in the well characterized secondary injury cascades. Studies into the expression of the α7 nAChR in AD and Parkinson's disease have demonstrated a decreased α7 nAChR expression with increased severity of disease (Perry et al., 2000; Schmaljohann et al., 2004; Wevers et al., 1999).

Our laboratory has been investigating the potential role played by the α7 nAChR in brain injury induced post-traumatic cognitive impairment. Initial studies found that the α7 nAChR is particularly sensitive to experimental brain injury and exhibits significant reductions in expression in several brain regions that can persist for weeks after injury (Verbois et al., 2002; Verbois et al., 2000). These significant reductions in α7 nAChR expression were of particular interest because the α7 nAChR deficit was of greater magnitude than the NMDA receptor deficit after injury (Verbois et al., 2000). Twice-daily nicotine administration following experimental brain injury attenuated some cognitive deficits in animals subjected to brain injury, but did not normalize deficits in α7 nAChR expression (Verbois et al., 2003a). In a separate experiment chronic nicotine infusions were associated with an increase in post-surgical convulsions, and failed to spare cortical tissue (Verbois et al., 2003b).

It is thought that immediately after a brain injury there is a period of increased extracellular ACh and excessive cholinergic receptor stimulation (Saija et al., 1988). The excessive entry of cations into neurons overloads natural buffering systems and leads directly or indirectly to necrotic or apoptotic cell death. We have shown that targeting the initial period of elevated cholinergic activity with an α7 nAChR antagonist results in significant tissue-sparing and a modest improvement in cognitive performance (Chapter 3). At some-point after injury there is a transition to a more chronic hypocholinergic state (Arciniegas, 2003). Studies in our lab have shown that animals maintained on a diet containing choline, a selective α7 nAChR agonist, showed modest improvements in MWM performance, increased tissue-sparing, and reduced brain inflammation compared to rats on normal chow (Guseva et al., 2008).

The studies in Chapter 3 of this dissertation showed some beneficial effects of short-term treatment with nicotinic antagonists following CCI. Other studies from our laboratory have shown that long-term dietary supplementation with the low affinity, but selective α7 nAChR full agonist choline, results in neuroprotection (Guseva et al., 2008). However, the most efficacious time-points for administration of these compounds remains to be elucidated, and beyond the technical expertise of our research group. While a detailed analysis of post-CCI cholinergic activity in rats could be useful for these rodent studies the time-course may not accurately reflect the time-course in human head injury. Indeed, the failures of several NMDA antagonists in clinical trials for TBI have been blamed in part on poorly predicted therapeutic windows in human versus rodent TBI (Albensi et al., 2004; Muir, 2006). A potential solution to the animal versus human therapeutic "window of cholinergic hyper/hypofunction" conundrum may be to administer a partial agonist. A partial agonist is simply an agonist that binds to a receptor but only has partial efficacy (compared to the full agonist of that receptor). Theoretically, in the presence high concentrations of the endogenous full agonist ACh, a partial agonist would serve as a "functional" antagonist of α7 nAChR, possibly contributing to an attenuation of calcium mediated excitotoxicity. Conversely, when endogenous levels of ACh are low, a partial agonist would provide some level of receptor agonism.

The administration of a partial nAChR agonist during the initial period of hypercholinergic activity could be neuroprotective. A high affinity partial agonist would compete with ACh for binding sites, but because it lacks full efficacy would lead to a relative reduction in the activation of receptors. At later time-points when there is a deficit in cholinergic signaling, a partial agonist may help to maintain α7 nAChR cholinergic signaling. Although a partial agonist may not be as effective as targeting a full antagonist to the period of hypercholinergic activity, and treating the deficit with a full agonist, it negates the need for these treatments to be carefully timed to avoid exacerbating the condition.

In this study we assessed the neuroprotective and cognitive-enhancing potential of three α7 nAChR partial agonists tropisetron (Navoban®), ondansetron (Zofran®), and DMXB-A (also known as GTS-21) in TBI. Tropisetron is best known for its use as an anti-emetic following chemotherapy, and acts by antagonizing 5-HT_3 receptors in the chemoreceptor trigger zone (CTZ). In addition to its action on 5-HT_3 receptors, tropisetron has been found to have a high affinity for α7 nAChRs at which it acts as a partial agonist (Macor et al., 2001). Ondansetron is another 5-HT_3 antagonist which acts as a partial agonist at α7 nAChRs, but with much lower affinity (Macor et al., 2001). Ondansetron is also used to reduce nausea and vomiting in chemotherapy patients (Framarino dei Malatesta et al., 1995). A lot of interest currently surrounds the potential of DMXB-A to treat the symptoms of schizophrenia via its actions on the α7 nAChR (Freedman et al., 2008; Martin et al., 2004a; Olincy and Stevens, 2007). It is also being investigated for treatment of AD (Kem, 2000; Wei et al., 2005).

Given the beneficial effects of α7 nAChR agonists and antagonists in TBI we hypothesize that a partial agonist of the α7 nAChR will be neuroprotective and improve functional outcomes following brain injury. The need for a well defined therapeutic window is negated by the partial agonist's ability to reduce α7 nAChR activation immediately after injury, while maintaining some cholinergic activity at later time points. To test this hypothesis we began by assessing the effect of post-CCI tropisetron treatment on MWM performance, tissue sparing, and neuroinflammation. We then tested two other α7 nAChR partial agonists in the same paradigm in order to demonstrate the therapeutic potential of α7 nAChR partial agonists following brain injury.

4.2 <u>Materials and methods</u>

4.2.1 Animals and surgery

All experiments were conducted in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee (IACUC), which follow the guidelines set forth by the National Institutes of Health guide for

the care and use of laboratory animals. All efforts were made to minimize the number of animals used in this study and to minimize animal suffering.

4.2.1.1 *Experiment 1 – Post-CCI tropisetron administration*

A total of 35 male rats were obtained from Harlan Breeding Laboratories (Indianapolis IN) for the experiments. Sprague-Dawley rats weighing 275-300 g were divided into 4 groups (8-10 animals per group). All animals underwent surgery to administer a CCI of moderate severity as described previously (Verbois et al., 2000). Briefly, animals were anesthetized in 5% isofluorane and placed in a Kropf stereotaxic frame (David Kopf Instruments, CA), then maintained at 2.5% isofluorane for the rest of the surgical procedures. A longitudinal incision was made to expose the skull, and a 6 mm diameter craniotomy was made using a Michelle trephine laterally, midway between bregma and lambda. The animal was then placed beneath the CCI device and oriented so that the impactor tip was aligned with the craniotomy. A calibration was the performed to accurately set the position of the dural membrane and the device was programmed to perform a 1.5 mm deformation of the cortex at 3.5 m/s with a dwell time of 400 ms. Following impact, the animal was removed from the device and a piece of Surgiseal was placed on the wound. The skull disk was then replaced and sealed with dental acrylic before stapling the wound closed. Immediately after surgery the animals received 1, 2, or 5 mg/kg tropisetron (Sigma-Aldrich, MO), or saline via i.p. injections. Animals received additional injections of tropisetron or saline 3, 24, 27, 48, 51, 72, and 75 hours later. This dosing regimen was selected to target the initial period of cholinergic hyperactivity, and to coincide with the increases in microglial activation in all brain regions assessed in Chapter 2. Behavioral testing began one day after the last injection had been administered. The doses of tropisetron administered in this experiment were selected based on previously published data in rodents (Candelario-Jalil et al., 2008). In this study sham-operated animals were not included in the interest of reducing animal usage and because our aim was to demonstrate an enhanced cellular and functional recovery versus saline-treated animals.

4.2.1.2 *Experiment 2 – Post-CCI Ondansetron and DMXB-A treatment*

In addition to the groups described above, a further 14 male rats were obtained from Harlan Breeding Laboratories (Indianapolis IN) for this experiment. Rats weighing 275-300 g were divided into 2 groups (7 animals per group) and underwent surgery to administer a CCI of moderate severity as described above. Ondansetron (2 mg/kg) (Sigma-Aldrich, MO) or DMXB-A (10 mg/kg) (kindly provided by Dr. William Kem, University of Florida) was administered at 0, 3, 24, 27, 48, 51, 72, and 75 hours after CCI. Doses of these drugs were selected based on previous studies demonstrating beneficial effects in rodents (Fontana et al., 1995; Kem, 2000).

4.2.2 Behavioral testing

The MWM was used to assess cognitive performance in all animals following CCI (Brandeis et al., 1989; Vorhees and Williams, 2006). Acquisition testing began in the MWM 4 days after surgeries and continued for a total of 4 days, with a 'retention' test carried out on the fifth day. The test room contained a 1.5 m diameter black plastic pool approximately 60 cm deep, filled with water to a depth of 30 cm. The pool was arbitrarily divided into 4 quadrants by assigning the points N, E, S, and W at equidistant points around the circumference of the pool. In the NE quadrant a 13.5 cm diameter platform was hidden by submerging it approximately 1 cm below the water's surface. The addition of black water-based powder paint to the water helped to conceal the platform from view and improved the contrast of the animal in the pool. Visual cues were placed around the pool to enable the rats to orient themselves within the pool and facilitate spatial learning. All tests were recorded on a digital camera placed above the pool and analyzed with Videomex software (Columbus Instruments, Columbus, OH).

Each trial consisted of placing the animals one at a time in the pool at a randomly assigned point of the circumference (N, E, S, or W). The animal was allowed to swim until it found and climbed onto the hidden platform, or until 60 seconds had passed. At this point any animal that had not found the platform

was placed onto the hidden platform and was allowed to remain on the platform for 15 seconds to gain awareness of the platform's location. The rat was then returned to its home cage for 5 minutes and then tested again. A total of 4 such trials were carried out each day for 4 days to assess learning and memory. Although there was no significant difference in the swim speed of animals in each group the distance each animal traveled to the platform from the edge of the pool was used as a measure of performance to nullify possible intra-animal differences.

On day 5 of cognitive testing the platform was removed from the pool and each animal was placed at the SW edge of the pool and allowed to swim for 60 seconds. This test is used to assess the animal's search strategy and therefore memory retention. Measures of performance in the retention test were 'distance traveled in target quadrant', 'time spent in target quadrant', and 'entries made into target quadrant'.

4.2.3 Cortical tissue sparing analysis

Following completion of the MWM testing animals were euthanatized by rapid decapitation. The brains were quickly removed, frozen in isopentane, and stored at -80˚C until further processing could be performed. The brains were sliced on a Leica 1850 cryostat (Nussloch, Germany) at 16 µm thick and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Eight serial sets of sections were cut for each brain to enable comparisons to be made between subsequent autoradiographical and staining procedures. For tissue sparing analysis a set of slides were stained with Cresyl Violet to visualize cell bodies and enable assessment of spared tissue (Scheff and Sullivan, 1999). Analysis was performed with ImageJ v1.34 (National Institutes of Health, MA) calibrated to measure area. Twelve equally spaced sections throughout the lesion were chosen for analysis. In each section the area of the ipsilateral and the contralateral cortex were measured separately and averaged over the 12 sections. To calculate tissue sparing, the area of the ipsilateral cortex remaining

was divided by the area of the contralateral cortex and multiplied by one hundred.

4.2.4 Receptor autoradiography

Autoradiography was used to assess nicotinic receptor expression and neuroinflammation/microglial activation following experimental brain injury and drug treatment. Slides were removed from the freezer and thawed overnight in a desiccator at 4˚C. For assessment of α7 nAChR expression [^{125}I]-α-BTX binding was carried out as previously described (Sparks and Pauly, 1999) using 2.5 nM of [^{125}I]-Tyr_{54}-α-BTX (PerkinElmer Life Sciences Inc., Boston, MA). A ligand of the TSPO, [^{3}H]-PK11195 was used to assess neuroinflammation following injury. PK11195 binding has been shown to correlate well with the activation of microglia and macrophages (Stephenson et al., 1995; Vowinckel et al., 1997), which in turn are useful indicators of tissue damage and repair. Briefly, slides were pre-incubated in 50 mM Tris-HCl buffer for 15 minutes at 4˚C, and then transferred to fresh buffer containing 2 nM [^{3}H]-PK11195 (PerkinElmer Life Sciences Inc., Boston, MA) for 2 hours at 4˚C. Slides were then washed, and dried overnight before being exposed to RayMax Beta High Performance Autoradiography Film (ICN Biomedicals Inc., Aurora, Ohio) for 8 weeks.

4.2.5 Analysis of autoradiographs

Films were imaged using a Northern Lights trans-illuminator with a monochrome digital camera (a CFW-1310M, Scion Corp) mounted directly above. Settings on the lightbox and camera were adjusted to produce a high resolution image with the best possible contrast and then left unchanged during subsequent image captures. Images were captured on an iMac G5 using Visi-capture software (Scion Corp.) and saved as lossless TIFF files. Images from the same animal were combined into image ‘stacks’ without modification of individual images using ImageJ for ease of storage and analysis. Image stacks were analyzed with ImageJ v1.34 with the ‘Measure Stack’ plug-in (OptiNav Inc., Bellevue, WA). Briefly, the area of interest was outlined on each slice in the stack and then the mean pixel value was measured for each slice. An average

pixel value for each region was then calculated for every animal in the study. Binding in each ipsilateral region was expressed as a percentage of the same region in the contralateral hemisphere. Based on the results from Chapter 2, [^{3}H]-PK11195 binding was measured in the cortex, the dentate gyrus, the hilus, the CA1 and CA2/3 fields, and in the lateral dorsal and ventral posterolateral thalamus.

4.3 <u>Results</u>

4.3.1 Tropisetron improves MWM performance

Four days following CCI the rats began testing in the MWM to assess their spatial memory performance (Figure 4-1). Two-way ANOVA analysis of the data revealed a significant effect of treatment ($F_{[3,119]} = 8.34$, $p < 0.001$) and day ($F_{[3,119]} = 18.75$, $p < 0.001$), but no significant interaction ($F_{[9,119]} = 1.00$, $p = 0.45$). On average, saline-treated animals performed worse on all days, traveling further in the pool before reaching the platform than any other group. All treated animals had shorter path-length traces for every day of the test, but only animals administered 2 mg/kg tropisetron performed significantly better than saline-treated controls. Post-hoc analysis using Tukey's HSD test showed that on days 3 and 4 of testing, animals treated with 2 mg/kg tropisetron performed significantly better than saline controls ($p < 0.01$).

Analysis of memory retention-test data did not reveal conclusive evidence of improved memory retention in any group (Figure 4-2). Overall, one-way ANOVA found a significant difference between groups in terms of distance traveled in target quadrant ($F_{[3,32]} = 4.03$, $p = 0.016$), entries made into the target quadrant ($F_{[3,32]} = 4.71$, $p = 0.008$), and time spent in target quadrant ($F_{[3,32]} = 1.62$, $p = 0.203$). Despite their performance in the acquisition phase of the test, animals treated with 2 mg/kg of tropisetron only performed marginally better than saline-treated animals in terms of distance traveled in the target quad, and the difference was non-significant.

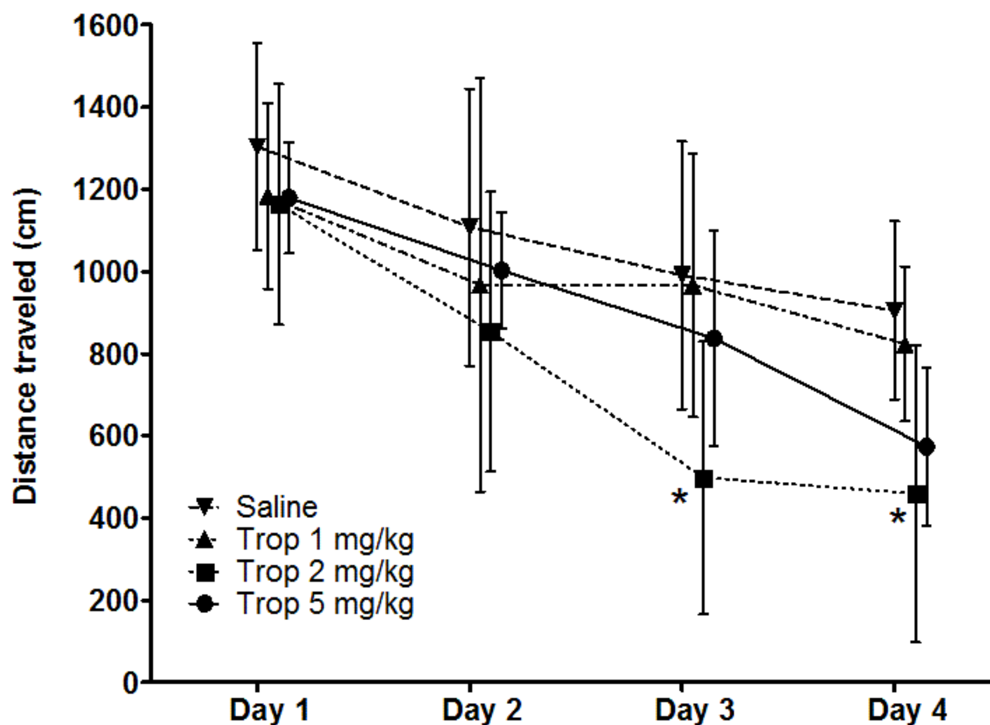

Figure 4-1 - The effect of α7 nAChR partial agonist treatment on MWM acquisition performance

*Cognitive performance of each animal was assessed in the MWM beginning 4 days after injury. Partial agonist treatment reduced distance traveled to platform versus saline treatment on all days. A significant effect of 2 mg/kg tropisetron treatment was observed on days 3 and 4. Data have been plotted as means plus or minus standard deviations (n = 8). Data-points have been slightly displaced for clarity and do not represent different testing times. An asterisk represents a significant difference from saline-treated controls as determined by Tukey's HSD (*p < 0.01).*

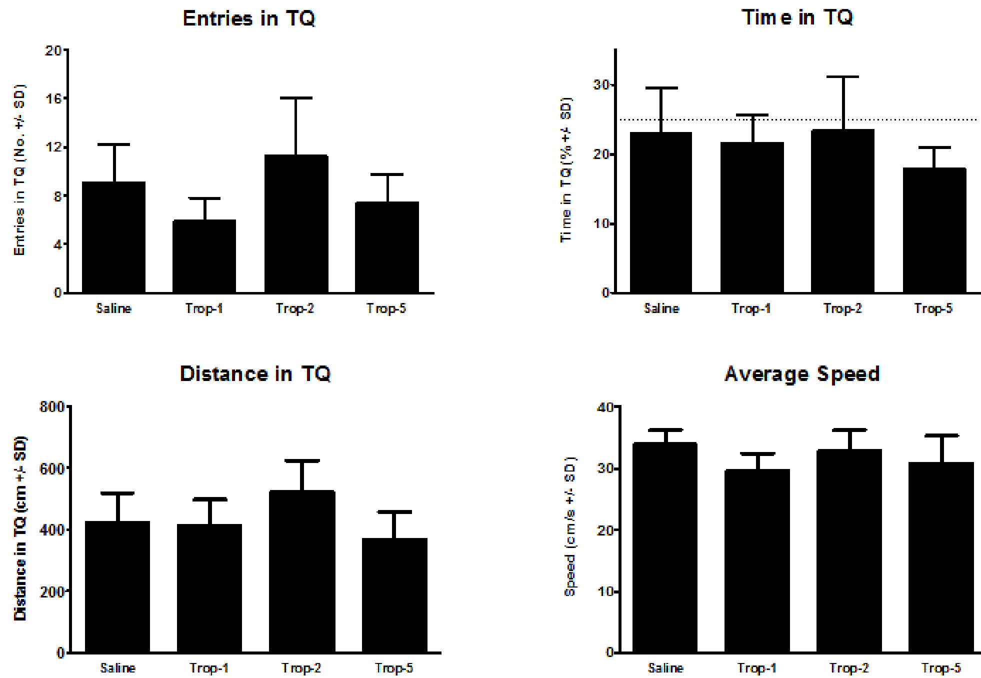

Figure 4-2 - MWM retention task in brain injured animals treated with Tropisetron

Spatial memory retention was assessed in injured animals by analyzing various parameters of their search strategy after the escape platform had been removed. Mean values are plotted for each group with bars representing the standard deviation of the sample (n = 8). Despite an overall effect of treatment found by one-way ANOVA, Tukey's HSD tests did not reveal any differences between saline treated animals and tropisetron treated animals. There was no significant difference in the swim speeds of animals between treatment groups.

4.3.2 There is no clear effect of tropisetron treatment on tissue sparing

Following completion of behavioral testing the rats were euthanatized and their brains removed and processed for histological analysis (Figure 4-3). Cresyl violet stained sections were used for assessment of cortical tissue sparing. There was a slight trend for animals treated with 2 mg/kg tropisetron to exhibit higher tissue sparing than saline-treated controls. However, one-way ANOVA reported no significant difference between groups ($F_{[3,36]} = 2.32$, $p = 0.094$).

4.3.3 Post-CCI tropisetron does not have an effect on α7 nAChR expression

For analysis of [^{125}I]-α-BTX autoradiographs the binding density in the contralateral hemisphere was used as a baseline to which the effect of injury in the ipsilateral (injured) hemisphere was compared. Binding in each brain region was therefore expressed as a proportion of the binding density in the contralateral hemisphere so that each brain acted as its own control (Table 4-1). One-way ANOVA did not report a significant effect of post-CCI tropisetron treatment on α7 nAChR expression in any of the regions analyzed. Further analysis of the data using one-sample t-tests to determine differences between ipsilateral means and the theoretical contralateral mean binding density (100%) revealed significant reductions in the hilus and superior colliculus ($p < 0.05$) of saline treated animals.

4.3.4 No effect of α7-nAChR partial agonist tropisetron on [^{3}H]-PK11195 binding

As with the [^{125}I]-α-BTX binding data, [^{3}H]-PK11195 binding was expressed as a proportion relative to the contralateral side of each brain (Table 4-2). Analysis of these data by one-way ANOVA failed to find a significant effect of tropisetron treatment on [^{3}H]-PK11195 binding in any brain region. However, as expected there were very significant increases in [^{3}H]-PK11195 binding in all ipsilateral regions compared to contralateral (control) regions (assessed by t-test, $p < 0.01$ in every region).

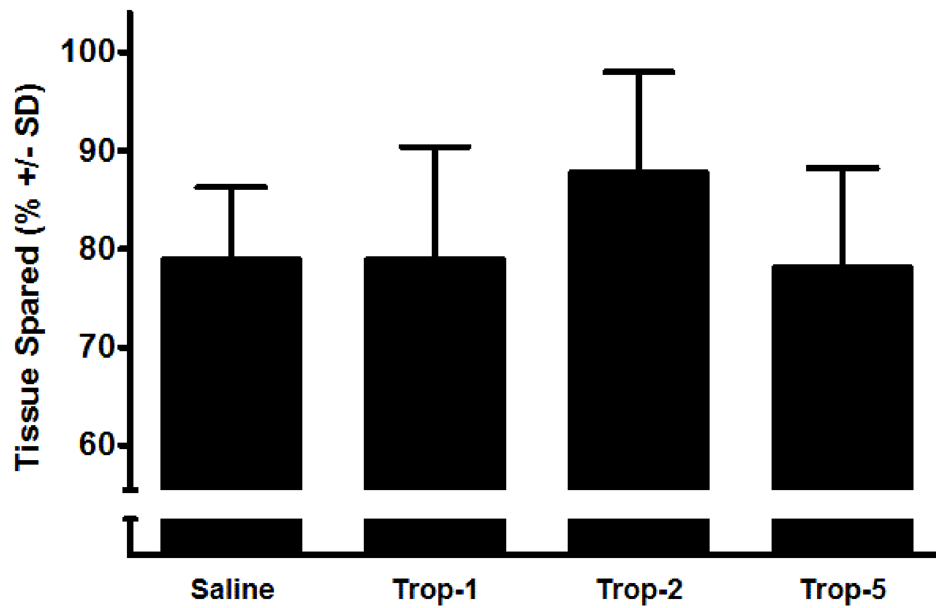

Figure 4-3 - Cortical tissue sparing following CCI and tropisetron treatment

There was no significant effect of treatment on cortical tissue sparing measured 9 days after CCI. Saline-treated controls had a 79% tissue sparing, and although 2 mg/kg tropisetron resulted in 88% tissue sparing, analysis of the data by one-way ANOVA reported no effect of treatment. Data are plotted as mean ± standard deviation (n = 8).

	Saline	1 mg/kg Tropisetron	2 mg/kg Tropisetron	5 mg/kg Tropisetron
Cortex layers 1-4	0.97±0.08	1.05±0.10	0.97±0.07	0.96±0.03*
Stratum oriens	0.98±0.07	0.92±0.08*	0.97±0.07	0.97±0.09
Hippocampal field CA1	0.97±0.07	0.99±0.04	0.98±0.07	0.93±0.12
Hippocampal field CA2/3	0.97±0.05	0.95±0.07	0.96±0.05*	0.94±0.10
Hippocampal field CA4 (Hilus)	0.94±0.03*	0.95±0.08	0.95±0.07*	0.91±0.05*
Dentate gyrus (lateral blade)	0.95±0.05	0.99±0.04	0.92±0.05*	0.95±0.05*
Superior colliculus	0.97±0.02*	0.96±0.04	0.98±0.03*	0.95±0.05

Table 4-1 - The effect of CCI and tropisetron treatment on α7 nAChR expression in the cortex and hippocampus

[^{125}I]-α-BTX binding density was measured in both ipsilateral and contralateral regions of the cortex and hippocampus in order to assess post injury α7 nAChR expression and any possible effects of tropisetron treatment. Since more than one binding was performed data for the ipsilateral (hit) side were expressed as a proportion of the contralateral binding density. In this way each brain served as its own control. One-way ANOVA did not find any effect of treatment on α7 nAChR expression. Significant reductions in α7 nAChR expression in the ipsilateral hemisphere versus a theoretical mean of 1 are signified with an asterisk ($p < 0.05$).*

	Saline	1 mg/kg Tropisetron	2 mg/kg Tropisetron	5 mg/kg Tropisetron
Cortex	1.93±0.65	2.21±0.31	1.75±0.52	2.03±0.58
Hippocampal field CA1	2.34±0.82	2.95±0.76	2.18±0.95	3.17±0.71
Hippocampal field CA2/3	3.10±1.51	3.99±0.93	2.58±1.29	3.80±0.68
Hippocampal field CA4 (Hilus)	3.42±0.80	4.29±0.81	3.40±1.29	4.06±0.82
Dentate gyrus (lateral blade)	2.16±0.48	2.72±0.34	2.14±0.57	2.58±0.43
Thalamus (Lateral dorsal)	2.40±0.81	2.84±0.44	2.07±0.48	2.76±0.62
Thalamus (Ventral posterolateral)	2.38±0.74	2.37±0.46	1.89±0.38	2.71±0.31

Table 4-2 - Effect of injury and post-CCI tropisetron treatment on [^{3}H]-PK11195 binding in the cortex, hippocampus and thalamus

Data are presented are binding in ipsilateral regions expressed as a proportion of the binding density in the corresponding contralateral region. Binding density in the ipsilateral hemisphere was increased in all regions analyzed, however, tropisetron treatment did not have any significant effect on [^{3}H]-PK11195 binding when compared to saline.

4.3.5 Animals treated with other α7 nAChR partial agonists after CCI show modest improvement in the MWM

Two other α7 nAChR partial agonists; DMXB-A and ondansetron were tested for efficacy in this model. In the MWM acquisition phase animals treated with either partial agonist traveled a shorter distance to the platform on every day of the test (Figure 4-4). Two-way ANOVA revealed a significant effect of treatment ($F_{[2,78]} = 6.28$, $p < 0.005$) and test-day ($F_{[3,78]} = 13.45$, $p < 0.001$) on the distance travelled, but no significant interaction term ($F_{[6,78]} = 0.47$, $p = 0.83$). Post-hoc analysis by Tukey's HSD revealed that on the fourth day of testing animals treated post-CCI with DMXB-A traveled a significantly shorter distance tp the platform than saline-treated animals ($p < 0.05$).

In the memory retention test (Figure 4-5) one-way ANOVA returned a significant effect of treatment with regard to both distance traveled in the target quadrant ($F_{[2,19]} = 4.57$, $p < 0.05$) and entries made into the target quadrant ($F_{[2,19]} = 9.19$, $p < 0.005$). Animals treated with DMXB-A traveled significantly further in the target quadrant than saline-treated animals (Tukey's HSD, $p < 0.05$). However, when entries into the target quadrant and time spent in the target quadrant were analyzed DMXB-A treated animals did not perform significantly better than saline-treated controls. Also of note is the fact that ondansetron-treated animals performed significantly better than saline-treated animals with regard to the number of entries they made into the target quadrant during the memory retention trial (Tukey's HSD, $p < 0.05$). There were no significant differences in the mean swim speeds of animals in each treatment group ($F_{[2,19]} = 1.23$, $p = 0.31$).

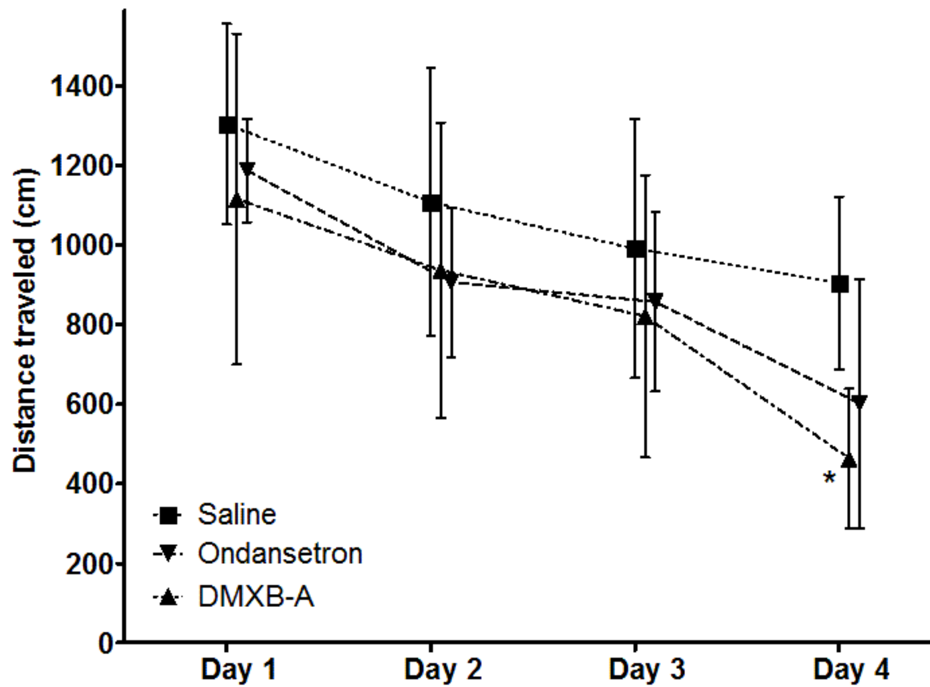

Figure 4-4 - The effect of α7 nAChR partial agonist treatment on CCI-induced cognitive deficits measured in the MWM acuisition phase

Partial agonist treated animals exhibited shorter path lengths to the platform versus saline treated animals on all days of testing. However, a significantly reduced distance traveled to platform was only found in DMXB-A treated animals on the fourth day of acquisition testing. Data plotted are means plus or minus standard deviation ($n = 8$). An asterisk denotes a significant difference from saline-treated animals ($p < 0.05$).

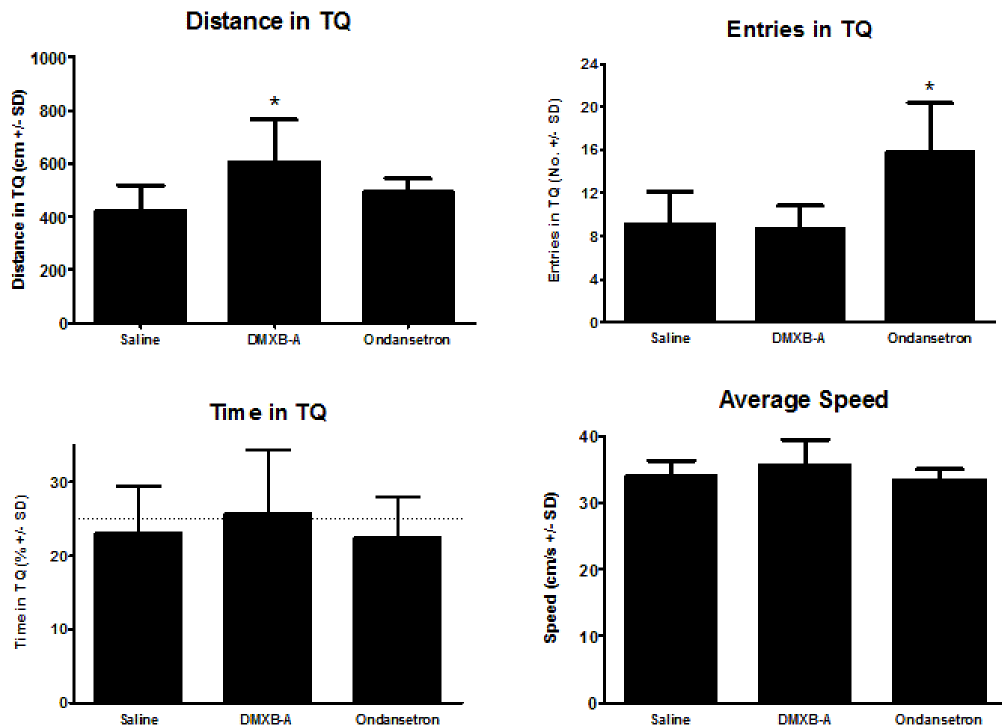

Figure 4-5 - Post-CCI differences in memory retention in animals treated with α7 nAChR partial agonists

Post-CCI DMXB-A and ondansetron treatment resulted in a modest improvements in memory recall in some measures. For example, animals treated with DMXB-A travelled significantly further than saline-treated animals in the target quadrant (which had contained the escape platform in preceding acquisition trials. In addition, animals treated with ondansetron made significantly more entries into the target quadrant compared to saline-treated animals. A significant difference from saline-treated controls is denoted with an asterisk ($p < 0.05$).

4.3.6 Neither DMXB-A nor ondansetron have any effect on cortical tissue sparing following CCI

Cortical tissue sparing analysis did not reveal any significant effect of partial agonist treatment (Figure 4-6). Saline animals has an 83% tissue sparing, and although DMXB-A treatment resulted in an 88% tissue sparing it was not statistically significant ($p = 0.06$). Data were analysed by one-way ANOVA which reported no significant differences ($F_{[2,19]} = 1.85$, $p = 0.19$).

4.3.7 Ondansetron exacerbates post-CCI α7 nAChR deficits

In saline treated animals there were significant reductions in α7 nAChR expression in the hilus and superior colliculus following injury (t-test versus theoretical mean of 1; $p < 0.05$). Similar deficits were observed in animals treated with DMXB-A and ondansetron. One-way ANOVA reported a significant effect of treatment in the CA2/3 field of the hippocampus ($F_{[2,16]} = 4.60$, $p < 0.05$), and in the superior colliculus ($F_{[2,16]} = 6.90$, $p < 0.05$). Post-hoc analysis revealed significant differences between ondansetron and saline treated animals in the superior colliculus ($p < 0.05$), and between DMXB-A and ondansetron treated animals in the CA2/3 and superior colliculus ($p < 0.05$).

4.3.8 Ondansetron treatment exacerbates neuroinflammation in the hippocampus following CCI

As expected, [^{3}H]-PK11195 binding was significantly increased following injury in all regions analyzed (Table 4-4). Analysis of the data by one-way ANOVA revealed a significant effect of treatment in the hilus only ($F_{[2,20]} = 4.52$, $p < 0.05$). Further post-hoc analysis revealed a significantly higher binding density in ondansetron-treated animals versus saline treated animals ($p < 0.05$). Interestingly, [^{3}H]-PK11195 binding was higher in ondansetron treated animals than in saline or DMXB-A treated animals all regions of the hippocampus. An additional observation was the trend towards a reduction in PK11195 binding in DMXB-A treated animals versus saline treatment. However, there was no significant difference in any of the regions assessed.

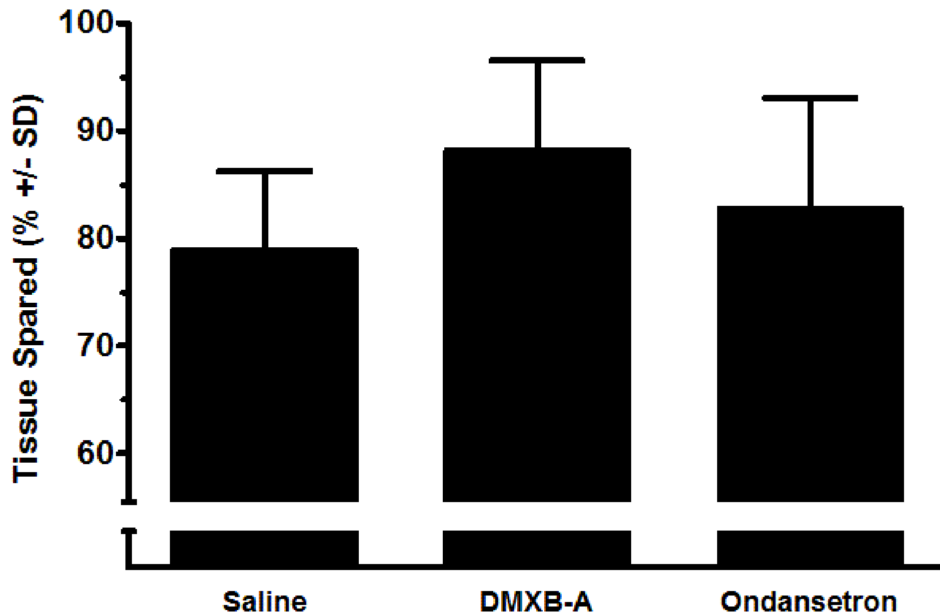

Figure 4-6 - Tissue sparing analysis in animals treated with DMXB-A or ondansetron

Neither DMXB-A, nor ondansetron has any effect on cortical tissue sparing following experimental brain injury. Saline treated animals had an 83% tissue sparing, while DMXB-A and ondansetron resulted in 88% and 82% sparing, respectively. Data are presented as means plus or minus standard deviation (n = 6).

	Saline	10 mg/kg DMXB-A	2 mg/kg Ondansetron
Cortex layers 1-4	0.97±0.08	1.00±0.10	0.94±0.06
Stratum oriens	0.98±0.07	1.00±0.03	0.99±0.05
Hippocampal field CA1	0.97±0.07	0.97±0.05	0.92±0.09
Hippocampal field CA2/3	0.97±0.05	0.99±0.02	0.92±0.04*#
Hippocampal field CA4 (Hilus)	0.94±0.03*	0.93±0.04*	0.90±0.03*
Dentate gyrus (lateral blade)	0.95±0.05	0.97±0.05	0.91±0.08
Superior colliculus	0.97±0.02*	0.98±0.02*	0.93±0.03*#

Table 4-3 - The effect of DMXB-A or ondansetron on α7 nAChR expression following CCI

α7 nAChR expression was assessed by [^{125}I]-α-BTX binding density and results are presented for the ipsilateral regions only expressed as a proportion of binding in the contralateral regions. An asterisk indicates a significant difference from the theoretical mean of 1 as assessed using a t-test ($p < 0.05$), and the pound sign (#) denotes a significant difference from saline as found using Tukey's HSD ($p < 0.05$).*

	Saline	10 mg/kg DMXB-A	2 mg/kg Ondansetron
Cortex	1.93±0.65**	1.83±0.56*	1.53±0.44*
Hippocampal field CA1	2.34±0.82***	1.84±0.35**	2.97±1.14**
Hippocampal field CA2/3	3.10±1.51***	2.40±0.66**	3.57±0.99***
Hippocampal field CA4 (Hilus)	3.42±0.80***	3.03±0.66**	4.49±1.09***#
Dentate gyrus (lateral blade)	2.16±0.48***	1.91±0.29***	2.55±0.60***
Thalamus (Lateral dorsal)	2.40±0.81***	2.05±0.44**	2.51±0.52***
Thalamus (Ventral posterolateral)	2.38±0.74***	1.75±0.35**	2.43±0.46***

Table 4-4 - Post-CCI [^{3}H]-PK11195 binding density in animals treated with DMXB-A or Ondansetron

[^{3}H]-PK11195 binding density in the ipsilateral hemisphere expressed as a proportion of the binding density in equivalent contralateral region. Significant differences from the theoretical mean of 1 are shown with an asterisk ($p < 0.05$, **$p < 0.01$, and ***$p < 0.005$). A significant effect of treatment was found in the hilus only, and Tukey's HSD found a significant difference between the saline and ondansetron groups (#$p < 0.05$).*

4.4 Discussion

In chapter 3 we demonstrated the therapeutic potential of the α7 nAChR antagonist MLA following experimental brain injury. However, the highest dose of MLA was not the most efficacious for reducing cognitive deficits in the MWM despite sparing tissue and reducing neuroinflammation. Bilateral reductions in α7 nAChR binding in all brain regions following MLA treatment could have effected MWM performance. This result suggests the need for some α7 nAChR stimulation, and therefore the possible utility of a α7 nAChR partial agonist. The use of partial agonists as therapeutic agents is well established; buprenorphine, for example, is a μ-opioid receptor partial agonist that has been used since the 1980s as an analgesic, and more recently was approved for the treatment of opioid dependence (McClellan, 2002). Other approved partial agonists include aripiprazole, a D_2 partial agonist used to treat schizophrenia and bipolar disorder, and buspirone, a 5-HT_{1A} partial agonist approved as an anxiolytic and antidepressant. More recently the $\alpha_4\beta_2$ partial agonist varenicline (Chantix) has been approved for smoking cessation treatment (Cahill et al., 2008; FDA, 2006).

This study has demonstrated that partial agonists of the α7 nAChR have therapeutic potential in treating TBI. Rats that were treated with tropisetron or DMXB-A performed significantly better in the MWM acquisition task than did animals given saline-only injections. Attenuation in cognitive deficits resulting from CCI was most pronounced in animals treated with 2 mg/kg of tropisetron, while animals treated with DMXB-A preformed significantly better than saline-treated controls on day 4 only. It is however, interesting to note that the mean distance traveled to the escape platform by all treatment groups was lower than for saline-treated animals. Despite this result in the acquisition phase of the MWM task there was no clear trend of improved performance in the retention test for any treatment group. The finding that tropisetron attenuates cognitive deficits in a model of TBI adds to other findings in which tropisetron has reduced cognitive deficits in a mouse model of schizophrenia (Hashimoto et al., 2006; Pitsikas and Borsini, 1997). In addition, the more modest effect of DMXB-A in attenuating cognitive deficits is in agreement with other studies that have

demonstrated DMXB-As cognitive enhancing effects (Arendash et al., 1995b; Nanri et al., 1998a). The lack of any cognitive-enhancing effect for ondansetron is surprising, since several previous studies in rodents (Boast et al., 1999; Diez-Ariza et al., 2003; Fontana et al., 1995; Harder and Kelly, 1997; Hodges et al., 1996; Kumar and Kela, 2004; Reeta et al., 1999; Roychoudhury and Kulkarni, 1997), and primates (Barnes et al., 1989; Barnes et al., 1990) have reported improvements in cognition. While cognitive enhancement after insult has been reported for doses up to 10 mg/kg, one study assessing the effect of ondansetron in ameliorating cognitive deficits after atropine injection found a loss of efficacy at doses above 3 mg/kg (Fontana et al., 1995). It is possible that had a lower dose of ondansetron been tested beneficial effects may have been found. Interestingly, ondansetron treatment exacerbated post-CCI α7 nAChR binding deficits, a finding which could help to explain the lack of a favorable functional outcome. In chapter 3 of this dissertation a similar exacerbation of α7 nAChR binding deficits was found following α7 nAChR antagonist treatment in the same model. A more thorough investigation of ondansetron in TBI will be required to assess its effects on α7 nAChR expression and cognition following injury.

In this study there was no significant effect of any treatment or dose on tissue sparing. In contrast, a recent study from our laboratory with an α7 nAChR full agonist (choline) produced a marked increase in tissue sparing following injury (Guseva et al., 2008). Another study in which rats were treated with a full α7 nAChR antagonist immediately after CCI also showed significant increases in tissue sparing versus saline-treated animals (Woodcock et al., 2008). The absence of an effect here could be due to these molecules lacking full efficacy at α7 nAChRs, or could be due to some other mechanism possibly involving their antagonism of 5-HT_3 receptors. It should also be noted here that a recent study found that tropisetron administered to rats prior to permanent middle cerebral artery occlusion (pMCAO) failed to reduce infarct size, and actually led to a higher mortality rate and exacerbated neurological deficits (Candelario-Jalil et al., 2008). However, this does not deter us from our results since the doses used in

that study were relatively high (5-10 mg/kg), and the mechanism of injury in pMCAO is very different to that in the CCI injury model we used.

In this study [^{3}H]-PK11195 autoradiography was utilized as a more sensitive marker of tissue damage. PK11195 binds to the TSPO, which is thought to located on the outer mitochondrial membrane within activated microglia and macrophages. We have previously shown that increases in [^{3}H]-PK11195 binding can be detected just 6 hours after CCI in the hippocampus, while significant increases are not detected in the cortex until 12-24 hours after injury (refer to chapter 2). Interestingly, there is a delayed increase in binding in the thalamus, possibly due to retrograde degeneration of axonal projections to the injured cortex. The pattern of [^{3}H]-PK11195 binding was in agreement with our previous studies using this tool. In our past study we found that [^{3}H]-PK11195 binding peaks in the cortex and hippocampus at 3-7 days, so the 8 day time-point in this study comes just after the peak. In this study there was no significant effect of treatment in the cortex, thalamus or any hippocampal region measured. Furthermore, cortical tissue sparing was used to assess neuroprotective effects of α7 nAChR partial agonist treatment but failed to detect any significant effect of treatment. There was a trend for animals treated with 2 mg/kg tropisetron to have a greater tissue sparing, but this did not reach significance. The lack of a neuroprotective effect for any of the partial agonists tested in this study was unexpected. MLA treatment following CCI resulted in a significant neuroprotective effect and a modest improvement in functional outcomes. It is possible that a full agonist is needed immediately following injury in order to facilitate neuroprotection. The lack of efficacy of partial agonists in this model may dictate the need for combination therapies with which to treat TBI.

As mentioned earlier, tropisetron and ondansetron are both approved for clinical use as antiemetics that function via their competitive antagonism of the 5-HT$_3$ receptor, but it is recognized that they also act on α7 nAChRs. Both α7 nAChRs and 5-HT$_3$ receptors are members of the cys-loop ligand-gated ion

channel superfamily, which also includes other nAChRs, $GABA_A$ receptors and glycine receptors (Sine and Engel, 2006). All receptors in the superfamily form pentameric ion channels and each subunit has a characteristic loop formed by a disulphide bond. The receptors can be homomeric (made up of 5 identical subunits) like the α7 nAChR and $5\text{-}HT_{3A}$, or heteromeric as can be the case for some $5\text{-}HT_3$ receptors. For example, the $5\text{-}HT_3$ subunit subtypes $5\text{-}HT_{3B}$, $5\text{-}HT_{3C}$, $5\text{-}HT_{3D}$ and $5\text{-}HT_{3E}$ can form functional receptors in combination with the $5\text{-}HT_{3A}$ subtype (Barnes et al., 2009). While the $5\text{-}HT_3$ receptor does not bind ACh it does share more structural homology with nAChRs than it does to other serotonin receptors. It is this homology that makes it unsurprising that some ligands with affinity for the $5\text{-}HT_3$ receptor also have affinity for the α7 nAChR and vice-versa. Tropisetron and ondansetron are both competitive partial agonists of α7 nAChRs, although ondansetron seems to have a lower affinity for these receptors (Macor et al., 2001). DMXB-A is slightly different, having been developed as a partial agonist of α7 nAChRs (Briggs et al., 1995; de Fiebre et al., 1995), it is also a weak competitive antagonist of $\alpha_4\beta_2$ nAChRs. *In vivo* experiments have since demonstrated that DMXB-A is also a weak antagonist of $5\text{-}HT_3$ receptors (Machu et al., 2001), though possibly through a non-competitive mechanism (Gurley and Lanthorn, 1998; Kem, 2000). Given our results it is possible that both antagonism of the $5\text{-}HT_3$ receptor and partial agonism of the α7 nAChR are beneficial in TBI.

Interestingly, there is evidence that $5\text{-}HT_3$ antagonists might be able to increase cholinergic tone via an indirect mechanism. It has been shown that $5\text{-}HT_3$ receptors in the entorhinal cortex are located on GABAergic neurons, which inhibit cholinergic function (Barnes et al., 1989). Therefore, antagonism of the $5\text{-}HT_3$ receptor could potentially improve cholinergic function independent of the α7 nAChR. Equally, $5\text{-}HT_3$ antagonism immediately after injury could exacerbate excitotoxicity via this mechanism, and could explain the detrimental effects of pretreatment with tropisetron in pMCAO (Candelario-Jalil et al., 2008). This emerging evidence suggests that the $5\text{-}HT_3$ receptor itself could be a novel target for neuroprotective strategies. Also worthy of further study is the

selectivity for each 5-HT_3 antagonist for different isoforms of the receptor, and the roles that these different 5-HT_3 receptors might play in the CNS. Different affinities and efficacies of ondansetron and tropisetron for the various 5-HT_3 receptors may help to explain the different effects we observed in this study.

An interesting aspect of this study was with regard to the results obtained for DMXB-A treatment following CCI. DMXB-A has been shown to be neuroprotective in several studies and continues to be extensively evaluated, especially with regard to its potential therapeutic effects in AD (Kem, 2000; Meyer et al., 1998a; Nanri et al., 1998a; Nanri et al., 1998b; Ren et al., 2007). In this study DMXB-A significantly improved MWM performance versus saline, and although not statistically significant it also resulted in a greater degree of tissue sparing and reduced [^{3}H]-PK11195 binding in all regions assessed when compared to saline treatment. Further assessment of this molecule in neurotrauma studies, including a refinement of the dosing and administration regimen might uncover neuroprotective effects for this drug following brain injury.

In conclusion, our results have demonstrated that α7 nAChR partial agonist treatment following CCI can produce modest improvements in functional outcomes. This study adds to previously reported findings that tropisetron can reverse cognitive deficits (Hashimoto et al., 2006; Koike et al., 2005; Pitsikas and Borsini, 1997). The precise mechanism by which these drugs act to improve cognition remains to be determined. The role of 5-HT_3 receptors cannot be ruled out, and their involvement in excitotoxicity and neuroprotection will require further research.

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5. CHAPTER 5

Discussion and Conclusions

5.1 Review

The wars in Afghanistan and Iraq have brought traumatic brain injuries into the limelight. Blast injuries from IEDs are increasingly common among combat soldiers (Galarneau et al., 2008), however, significant numbers of Americans also suffer TBIs, primarily as a result of motor vehicle accidents (Langlois et al., 2006). In addition to the physical effects of trauma many TBI patients are left with behavioral and cognitive deficits that frequently go untreated (Corrigan et al., 2004; Resch et al., 2009). As a result, many patients suffer significant quality of life reductions and make the transition back into society and the workforce difficult (Rao and Lyketsos, 2002). It is estimated that more than 1.5 million people sustain a TBI every year in the USA, which creates an immense financial burden (Thurman et al., 1999). Despite the scale of the problem there are no successful pharmacological treatments for TBI, and therefore there is a great need for new and novel treatment strategies aimed at improving outcomes.

The present lack of efficacious treatments targeted at secondary injury mechanisms in TBI means that injury prevention is of utmost importance. This can be achieved by raising awareness of the condition and encouraging people to take preventive or safety measures (such as wearing seatbelts and helmets). Raised awareness, improvements in automobile safety, and advances in other safety technologies must remain a focus if the impact of TBI on society and the emotional wellbeing of individuals is to be reduced (Kraus and Peek, 1995). A future pharmacological intervention could potentially improve the lives of thousands of people by attenuating the behavioral and cognitive deficits brought about by TBI. The studies outlined in this dissertation add to the current understanding of the time-course of secondary injury, and demonstrate the

potential therapeutic effects of targeting α7 nAChRs of the cholinergic system following a TBI.

The experiments detailed within this dissertation have demonstrated that (1) [^{3}H]-PK11195 is a sensitive and selective marker of microglial and macrophage activation within the injured brain, which can be used to track neuronal damage and potentially repair in many brain regions following injury (Chapter 2); (2) Short-term administration of an α7 nAChR antagonist after CCI is neuroprotective and leads to some improvement in cognitive performance (Chapter 3); and (3), partial agonists of the α7 nAChR can attenuate cognitive deficits associated with brain injury to a degree, but do not produce any measurable neuroprotective effect (Chapter 4).

5.1.1 The time-course of damage and repair in TBI

It is difficult to generalize the time-course of tissue, cellular and molecular changes that occur following a brain injury because of the heterogeneity of the condition (Saatman et al., 2008). Affected brain regions can vary considerably by injury method, severity and location. The injury paradigm we used in these studies models a focal closed-head injury (immediately following injury the skull cap is replaced and fixed in place with dental acrylic). We have found that a small change in injury severity (1 mm cortical deformation versus 2 mm cortical deformation) produces a markedly different pattern and intensity of [^{3}H]-PK11195 binding throughout the brain (Figure 5-1). However, in order to assess the efficacy of any treatment in TBI and models of TBI there need to be clear assessments made of the effect of injury on various measures of tissue and cellular damage as well as the functional and cognitive consequences of the injury. These assessments need to be validated in human TBI so that meaningful evaluations of TBI treatments can be made in our various models.

The easiest and most commonly employed methods for assessing injury severity and neuroprotective effects are lesion volume analysis and tissue sparing analysis, respectively. While it is excellent as an initial validation of injury severity and uniformity and can be used to detect neuroprotective effects, its

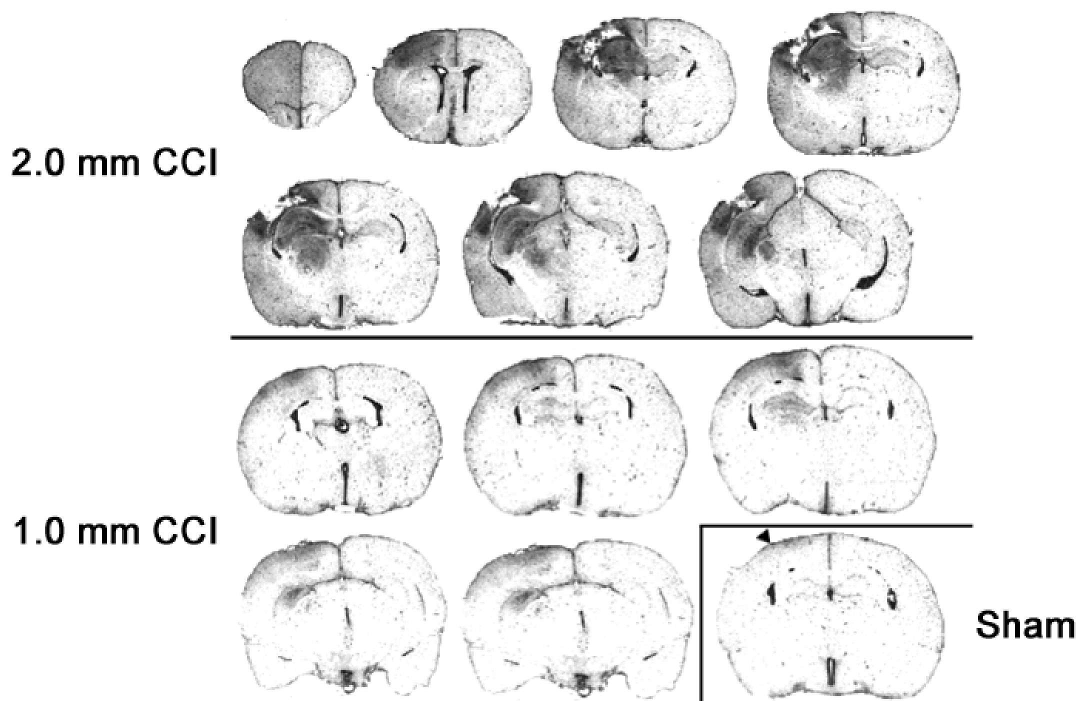

Figure 5-1 - Comparison of [^{3}H]-PK11195 binding in rat brain following different injury severities

[^{3}H]-PK11195 binding to the TSPO 14 days after CCI surgery shows great sensitivity to the degree of injury. Following a 2 mm CCI increased [^{3}H]-PK11195 binding is found throughout the injured cortex, in the hippocampus, and in parts of the thalamus. In contrast, a 1 mm CCI only leads to modest increases in binding to the ipsilateral cortex, small increases in the dentate gyrus and hilus of the hippocampus, and no significant increases in binding in the thalamus. Furthermore, [^{3}H]-PK11195 autoradiography can detect microglial activation in the cortex directly beneath the site of craniotomy following sham surgery (see arrowhead, ▼).

utility in relating to clinical results is very limited. Barring the fact that nissl staining cannot be carried out on live patients, this is chiefly due to significant differences in size, shape, and structure of the human cortex compared to rat brain. Furthermore, an analysis of this type would be restricted to assessment of injury in focal brain injured patients (possibly by computerized tomography [CT]), who represent a relatively small proportion of the total brain injuries (Langlois et al., 2006). In addition, analysis of this type would not pick up smaller, but potentially more significant disruption of other brain regions.

A more pertinent analysis of post-injury damage and possible neuroprotective effects is to assess neuronal dysfunction and death (e.g. FJ-B staining or de Olmos silver staining), or neuronal survival (e.g. NeuN immunohistochemistry). Although more laborious than tissue sparing analysis, a thorough appraisal of neuronal survival following injury and experimental therapies can provide more detailed information on the brain areas affected and the relative severity of injury that together could help to explain physical and neurological alterations. Again though, there is no way of accurately measuring neuronal death in live patients.

Glial cells, which account for more than half of the cells in the primate brain (Azevedo et al., 2009) may be the key to relating injury in animals to human patients. Microglia in particular have been attracting a lot of attention in recent years as the critical role played by inflammation in many neurodegenerative conditions becomes apparent. Microglia are the brain's resident immune cells, and play an important role in detecting damage to the brain parenchyma (Carbonell et al., 2005). Following injury microglia quickly become activated, taking on a different morphology and expressing a range of neurotrophic, proinflammatory, and potentially neurotoxic factors (Ransohoff and Perry, 2009). Whether these activated cells are detrimental to neuronal survival or promote recovery from injury is still a topic of much contention, however, what is clear is that their presence corresponds well to areas of neuronal dysfunction and death. We therefore set about evaluating the potential of the microglial marker [^{3}H]-

PK11195 to image tissue damage and repair in a model of TBI (Chapter 2). Our experiments have demonstrated the potential of [^{3}H]-PK11195 as a marker of microglial and macrophage activation, which in turn are markers of tissue damage and neuronal dysfunction.

[^{3}H]-PK11195 is believed to bind to the TSPO within activated microglia and macrophages; a conclusion supported by the discovery of correlations between [^{3}H]-PK11195 binding density and immunohistochemical detection of macrophages/microglia (Fujimura et al., 2008; Myers et al., 1991a; Venneti et al., 2008a; Venneti et al., 2007a; Venneti et al., 2007b). In our study we were able to confirm this correlation of [^{3}H]-PK11195 binding to CD68, a marker of phagocytosis. However, this correlation only held up in the injured cortex and not in the thalamus where increases in [^{3}H]-PK11195 binding were not accompanied by any substantial increase in the numbers of CD68 positive cell profiles. This finding could be due to the presence of activated, but not phagocytic, microglia. As mentioned earlier, CD68 is a marker of phagocytic cells, yet microglia are known to go through distinct ‘phases; of activation. Our study therefore, has shown that the PK11195 binding site is expressed before significant numbers of phagocytic microglia can be detected. Thus [^{3}H]-PK11195 is a very sensitive marker of tissue damage because it increases in injured regions before neuronal death occurs.

The potential for use of the TSPO as a marker of neuroinflammation is heightened by the development of PK11195 and other TSPO ligands for use in PET. PET is a 3D imaging technique that uses a positron-emitting radionucleotide tracer introduced into the body of a patient or animal. PET is often performed alongside a CT or MRI scan to give anatomical information in addition to the biochemical information afforded by PET imaging. The use of [^{11}C]-(R)-PK11195 in PET has been demonstrated in AD (Edison et al., 2008), Parkinson’s disease (Gerhard et al., 2006), Huntington’s disease (Pavese et al., 2006; Tai et al., 2007a, b), schizophrenia (van Berckel et al., 2008), individuals with human immunodeficiency virus (Hammoud et al., 2005), multiple sclerosis

(Debruyne et al., 2003; Vas et al., 2008; Versijpt et al., 2005), ALS (Turner et al., 2004a), stroke (Gerhard et al., 2000; Gerhard et al., 2005), and others (Banati et al., 1999; Leong and Butterworth, 1996; Leong et al., 1994; Leong et al., 1996). Most recently a study has found that PK11195 binding in AD brain correlates with mini mental state exam (MMSE) scores (while amyloid load did not) (Edison et al., 2008). Other interesting findings have been a correlation with HD severity (Pavese et al., 2006), a correlation with brain atrophy in MS (Versijpt et al., 2005), and a correlation with burden of upper motor neuron signs in ALS (Turner et al., 2004a). Other studies have proposed that the utility of PK11195 in PET is not limited to the nervous system, but may also be a valuable tool to detect inflammation in atherosclerotic plaques (Fujimura et al., 2008), or in the lungs (Hardwick et al., 2005). Despite the promising clinical results obtained using PK11195 in PET studies, the molecule does have some disadvantages which have prompted the development of new TSPO ligands. These disadvantages include a relatively high level of non-specific binding in humans and primates (Petit-Taboue et al., 1991; Shah et al., 1994), poor signal to noise ratio, pharmacological effects independent of TSPO binding, and the necessity of labeling PK11195 with carbon-11 which has a very short half-life (~20 min) meaning that tracers must be synthesized very close to the point of use (Chauveau et al., 2008).

PET is not limited to human patients. Recent advances in technology have made PET with PK11195 possible in animal models, and a mini-PET device is now available that can be attached to a rat's head for 'live' measurements (RatCAP, Brookhaven National Laboratory, NY). PK11195 binding, detected by PET has been studied in rat transient focal ischemia (Rojas et al., 2007), a rat model of ethanol injury (Toyama et al., 2008), a transgenic mouse model of AD (Venneti et al., 2008a), and a macaque model of HIV encephalitis (Venneti et al., 2007b).

While this technique promises great things for the future of animal studies, technology is still expensive and out of reach for most labs. In the meantime

[^{3}H]-PK11195 autoradiography allows us to make detailed assessments of microglial activation in post-mortem human and animal brain. Our studies have revealed widespread increases in [^{3}H]-PK11195 binding in the rat brain during the days following CCI. Furthermore, we reported that these increases vary significantly in timing and magnitude between distinct brain regions. Increased binding became apparent in the dentate gyrus of the hippocampus just 6 hours after injury. This observation highlights two important aspects of this technique; firstly, it demonstrates the sensitivity of [^{3}H]-PK11195 autoradiography in detecting cellular disruption at a time when no significant changes were found in the impacted cortex. Secondly, it demonstrates how PK11195 binding can be used to detect small and discrete areas of cellular damage and dysfunction distal from the injury. The latter inference is further supported by our other finding that [^{3}H]-PK11195 binding sites are expressed in distinct thalamic nuclei after injury, but increases are not observed until later time points and a peak is not reached until after at least 6 days. We have hypothesized that this is due to lesioning of the cortex as a result of the injury, and subsequent retrograde degeneration of thalamocortical afferents culminating in neuronal death in the thalamus. Support for this hypothesis comes from studies involving lesioning of the thalamus, in which a delayed loss of nicotinic binding was observed in the cortex (Gioanni et al., 1999; Lavine et al., 1997). In addition, more recent studies have demonstrated secondary thalamic damage following cortical injuries (Langen et al., 2007), and delayed increases in thalamic PK11195 binding in stroke patients in a PET study (Pappata et al., 2000). One similar study concluded that PET with PK11195 would be a useful way in which to investigate remote changes in neuroinflammation (i.e. via retrograde or anterograde degeneration) following ischemic stroke (Gerhard et al., 2005). The most recent, and most compelling evidence in support of this hypothesis found that the thalamic nuclei that exhibited the greatest increases in inflammation varied depending on the area of the cortex that was lesioned (Langen et al., 2007).

A further interesting observation in the thalamus from our study was the presence of significant increases in PK11195 binding without any significant

increase in CD68 staining in that region at the same time-point. This was surprising but not without possible explanations. First and foremost CD68 is an antigen found on activated microglia and peripheral macrophages, while PK11195 binds to a completely different site on these cells not necessarily related to CD68. Secondly, PK11195 binding is not limited to microglia and macrophages. For example, in brain displaying a normal pathology PK11195 binding can still be observed in the choroid plexus and endothelium. It is also expressed to some extent on erythrocytes. However, the most likely candidates for TSPO expression in the brain besides microglia and macrophages are astrocytes. It is now established that astrocytes express TSPO (Butterworth, 1990; Cosenza-Nashat et al., 2009; Panickar et al., 2007; Veiga et al., 2007; Vlodavsky and Soustiel, 2007), and since they also lack CD68 this implicates them in PK11195 binding increases seen in the thalamus. Evidence to support this hypothesis comes from a recent study of a novel TSPO ligand (DPA-714) in a rat model of focal cerebral ischemia (Martin et al., 2009). In this study immunohistochemistry was used to stain microglia (OX-42/CD11b), astrocytes (GFAP), and the TSPO (NP155). Following the ischemic insult DPA-714 binding increased, peaked at 11 days, and then decreased but not to control levels by 30 days. This pattern matched well the temporal changes in TSPO-positive microglia. Furthermore, numbers of TSPO-positive astrocytes increased at each consecutive time-point to 30 days, but there was no resultant increase in DPA-714 binding. The lack of concordance between DPA-714 and TSPO/GFAP staining in this study could be due to a lower level of TSPO expression by astrocytes or possibly the presence of another subtype of TSPO with differing affinity for the ligand. Regardless, astrocytic expression of TSPO following brain injury, especially at later time-points may help to explain the increased PK11195 binding seen in the thalamus. In order to delineate the cellular expression profile of TSPO in brain injury it might be interesting to double-stain brain sections with GFAP and TSPO antibodies and look for double labeled cells within the thalamus.

Having assessed PK11195 binding in untreated rats after subjecting them to a CCI we applied this technique to our pharmacological studies. In the first study, 5 or 10 mg/kg MLA was found to significantly reduce [^{3}H]-PK11195 binding versus saline-treated controls (Table 3-2). These differences were found in the cortex and hippocampus, suggesting that either MLA is having a direct effect on neuroinflammation, or that these changes in [^{3}H]-PK11195 binding are secondary to the neuroprotective effect of MLA that we observed. The first scenario seems unlikely, because current evidence suggests an inhibitory role for nicotinic agonists on inflammation (Gallowitsch-Puerta and Tracey, 2005), in fact, the anti-inflammatory effects of nicotine in the brain are thought to be mediated by the α7 nAChR receptor (Nizri et al., 2009; Shytle et al., 2004). Therefore, we would certainly not expect an α7 nAChR antagonist to reduce inflammation, but rather exacerbate microglial activation. Further evidence suggesting the reductions in [^{3}H]-PK11195 binding we observed are not due to a direct effect of MLA comes from a recent *in vivo* study in which rats were injected Intracerebroventricularly with LPS to induce an inflammatory response and cytokine expression was measured following drug treatment (Tyagi et al., 2009). Nicotine treatment reduced cytokine expression, and pretreatment with MLA antagonized nicotine's effect, while DHβE (an α4β2 antagonist) had no effect.

In chapter 4 of this dissertation we tested partial agonists of the α7 nAChR for neuroprotective effects and for their ability to improve functional outcomes. We also measured neuroinflammation in these animals using [^{3}H]-PK11195 autoradiography. In this study none of the partial agonists reduced [^{3}H]-PK11195 binding, but ondansetron treatment did significantly increase [^{3}H]-PK11195 binding in the hilus. This increase could be due to ondansetron's agonistic effect, since MLA reduced inflammation. A second important observation from this study was the lack of any tissue sparing with partial agonist treatment. This could be the reason why there was no effect on neuroinflammation, suggesting that the reduction in [^{3}H]-PK11195 we observed in Chapter 3 with MLA treatment was secondary to the tissue sparing effect.

We conclude that [^{3}H]-PK11195 autoradiography is an excellent tool with which to assess the time-course of tissue disruption, which combines low cost with sensitivity and resolution. It has already been utilized in an array of animals models (Giannaccini et al., 2000; Katchanov et al., 2003; Mankowski et al., 2003; Sitte et al., 2001), including brain injury (Grossman et al., 2003; Raghavendra Rao et al., 2000), although our study was the first to fully characterize it in a CCI model. In addition, we have demonstrated in each of our studies that autoradiography with this ligand is a very sensitive technique for detecting injury and neuroprotective effects (Chapter 2, Chapter 3.3.4, and Chapter 4.3.4). We demonstrated large and robust increases in [^{3}H]-PK11195 binding in several brain regions that were very consistent between animals (Chapter 2). Furthermore, significant reductions in [^{3}H]-PK11195 binding were detected in some brain regions following CCI and MLA treatment (Chapter 3.3.4), demonstrating its potential in detecting the effects of drug treatment. Additionally, it has not escaped our attention that while PET studies with PK11195 are becoming more common, an assessment of PK11195 binding sites in human brain injury patients has yet to be conducted. Such a study would help to completely validate the technique as a method to assess the effectiveness of experimental therapies. Coupled with CT scanning, it could also be a valuable method for the determination of injury type and severity in a clinical setting to help direct treatments and therapies to individual TBI victims. Indeed, a recent workshop was convened with the purpose of recommending ways in which to develop a classification system for TBI that could be used to better target therapies (Saatman et al., 2008).

In summary, imaging of the TSPO has great potential in providing a detailed and quantitative assessment of neuroinflammation both in the laboratory and in the clinic. TSPO binding could be a valuable biomarker for use in determination of inflammation in drug development and early clinical trials.

5.1.2 Excitotoxicity mediated by the α7 nAChR

Studies of excitotoxicity are generally focused on the NMDA receptor because of its high degree of calcium permeability. However, despite promising drug candidates all clinical trials to date have failed (Albers et al., 1999; Bullock et al., 1999; Haley et al., 2005; Ikonomidou and Turski, 2002). While the difficulties in translating doses and therapeutic windows from well controlled rodent models to the heterologous human condition could be partly to blame, it would be worthwhile to investigate alternative strategies to the treatment of excitotoxicity in TBI.

We chose to investigate the effects of α7 nAChR blockade on CCI induced neuropathologies and functional recovery due to its interesting parallels with the NMDA receptor. Firstly, the α7 nAChR is selectively and highly permeable to calcium like the NMDA receptor. Studies in Xenopus oocytes have shown that α7 subunits form functional homopentamers, and that stimulation of the receptors causes a rapid influx of calcium (Hovda et al., 1995; Wolf et al., 2001; Young, 1992). Calcium flux through the α7 nAChR could lead directly to excitotoxicity and may also lead to an increased probability of glutamate release from pre-synaptic terminals (Dickinson et al., 2008; Jiang and Role, 2008; Marchi et al., 2002). Secondly, the α7 nAChR is widely expressed in the brain, and is thought to be involved in cognitive processes. α7 knockout mice have been produced (Orr-Urtreger et al., 1997), and although they do not display cognitive deficits, antisense knockdown of the α7 gene in rats does result in loss of α7 nAChR expression and cognitive deficits as measured in the MWM (Curzon et al., 2006). In our laboratory we tested the hypothesis that these α7 nAChR knockout mice would be less sensitive to post-CCI tissue loss and neuroinflammation (Kelso et al., 2006). However, there were no differences in tissue sparing or neuroinflammation between wildtype, heterozygous, and knockout mice. This result was surprising since we believe the α7 nAChR is involved in calcium-mediated excitotoxicity following brain injury. However, when interpreting results from genetic knockout animals care must be taken since compensatory

mechanisms may confound the results. All together, these results suggest that α7 knockout mice may exhibit some compensatory developmental features.

Since we believe that the α7 nAChR is integral to induction of excitotoxicity following a brain injury, modulation of this receptor might be therapeutic. Fortunately, the well recognized therapeutic potential of nAChRs as targets for treating neurodegenerative conditions has led to a number of companies that are developing selective nicotinic agonists, antagonists and PAMs (positive allosteric modulators, which increase receptor activity indirectly via an allosteric site) for the α7 nAChR (Cincotta et al., 2008; Faghih et al., 2007). Some recent compounds of interest are A-867744, an α7 nAChR PAM which has been shown to potentiate ACh-evoked currents in recombinant α7 nAChR expressed in oocytes (Malysz et al., 2009), and SEN12333, another α7 nAChR PAM with demonstrated procognitive and neuroprotective properties (Roncarati et al., 2009). Continued progress in the area promises to provide researchers with a multitude of novel pharmacologically active compounds with which to target nAChRs.

In chapters 3 and 4 of this dissertation we tested the neuroprotective potential of an α7 nAChR antagonist and three different α7 nAChR partial agonists for neuroprotective potential when administered after CCI. We hypothesized that the increased cholinergic activity in the brain after an injury could be damaging by adding to calcium-mediated excitotoxicity via α7 nAChRs. A full antagonist would be expected to block α7 nAChR activation, while a partial agonist would behave as an antagonist in the presence of high levels of endogenous agonist. We found that MLA treatment had a significant effect on tissue sparing, an effect that was dose dependent and clearly demonstrated a neuroprotective effect. In contrast, the α7 nAChR partial agonists tropisetron, ondansetron and DMXB-A all failed to produce any tissue sparing effect. This was unexpected, but it is possible that in order to produce neuroprotection a full antagonist is required.

We chose MLA as a full antagonist for investigation in these studies because it is readily available and represents a potent and selective antagonist of α7 nAChRs (Alkondon et al., 1992; Davies et al., 1999; Ward et al., 1990). MLA is a plant alkaloid found in the larkspur, and its actions on the NMJ acetylcholine receptor implicate it in animal poisonings (Pfister et al., 1999). In these experiments MLA was administered at 1, 5 or 10 mg/kg beginning immediately after CCI surgery, again 8 hours later and then again at 24 and 32 hours following CCI. The doses were selected based on previous studies in which MLA was administered to rats in which no adverse effects were observed (Grottick and Higgins, 2000). Since we were targeting excitotoxicity with this treatment we began the regimen as soon as the rat's wound had been stapled closed. Because we were administering MLA via an intraperitoneal injection it is important to know that it is able to penetrate the BBB. Based on previously published pharmacokinetic studies in rodents, MLA exhibits relatively poor brain penetration (approximately 5% of an IP dose) after peripheral injection (Turek et al., 1995). However, since we are testing this molecule as a treatment for TBI its access to the brain will be improved by a disruption of the BBB. Studies in the CCI model of TBI have shown significant BBB disruption following injury (Baldwin et al., 1996), which should allow or drug to reach its target receptors. This disruption appears to be biphasic, with maximal breach occurring in the minutes immediately following injury. The BBB appears to recover by 3 hours, but increased disruption was observed between 1 and 2 days. Based on these findings only the injection of MLA administered 8 hours after injury would have been impeded by the BBB. Even so, the amount of MLA that is able to cross the BBB may still be therapeutic. Despite these considerations, the ability of MLA to access the brain is supported by the detection of significant central effects in our study. There was a clear and obvious dose-dependent effect of MLA treatment on cortical tissue sparing, such that animals receiving the highest dose of MLA (10 mg/kg) did not differ significantly from sham operated controls (Chapter 3.3.2). This neuroprotective effect is what would be expected of a treatment that attenuates excitotoxicity after injury. In addition to this neuroprotection there was

some attenuation of cognitive deficits as measured in the MWM swim test. The results of this test were mixed; all animals treated with MLA performed consistently better than untreated animals during the acquisition phase of the test. However, significant differences were only found on day 4 (of 5) and occurred in animals treated with 5 or 1 mg/kg MLA (Chapter 3.3.1). This finding was interesting in the regard that 10 mg/kg of MLA had yielded the greatest tissue sparing, but had failed to produce a significant attenuation of cognitive deficits. There are several possible explanations for this finding; firstly, the MWM is a hippocampal dependant task, whereas our tissue sparing analysis exclusively measures changes in cortical tissue volume. Secondly, it is conceivable that while α7 nAChR blockade is beneficial in the cortex it is detrimental to neuronal survival at higher doses in the hippocampus. Indeed, when we extended MLA treatment to 4 days there was no significant improvement in MWM performance [unpublished data]. Thirdly, it is possible that while MLA was able to save cells from an excitotoxic fate it was not able to prevent the depression of cholinergic function that occurs in TBI. Indeed, our assessment of α7 nAChR expression by [^{125}I]-α-BTX binding actually revealed a reduction in binding in MLA treated animals. This was unexpected, but could help to explain the lack of well defined cognitive improvements in these animals. Of course, there are other explanations for the apparent deficit in [^{125}I]-α-BTX binding in these animals, including a change in affinity of the α7 nAChRs for [^{125}I]-α-BTX. However, we consider this scenario unlikely for two reasons, (1) the animals were euthanatized 15 days after injury, 13 days after the last MLA injection had been administered. It is improbable that MLA-induced changes in affinity of the α7 nAChR would persist this long after cessation of treatment. (2) We have previously carried out a saturation analysis of [^{125}I]-α-BTX binding to rat brain tissue from animals euthanatized 2 days after CCI. The analyses confirmed that changes in binding were due to changes in the number of binding sites (B_{max}) rather than affinity (K_D) of [^{125}I]-α-BTX for the receptor (Verbois et al., 2002). A separate scenario could be the accumulation of a pharmacologically active MLA metabolite in the brain, which is unlikely but previously published

work has demonstrated such a scenario with nicotine (Ghosheh et al., 2001). It would have been useful to include a sham-operated group treated with MLA, but this effect was not anticipated. An appropriate control group would have helped us to explain the reduction in [^{125}I]-α-BTX binding that we observed. A final consideration is with regard to the selectivity of MLA for the α7 nAChR, since recent studies have determined that at certain concentrations it can interact with α4β2 and α6β2 nAChRs (Mogg et al., 2002). With this in mind, the involvement of other nAChRs in the effects we observed cannot be ruled out. Finally, the peripheral effects of MLA on the NMJ would be a concern for its administration clinically. This work could be expanded upon by testing more selective α7 antagonists as they become available, preferably with more favorable pharmacokinetic profiles.

From these experiments we surmise that the α7 nAChR plays a significant role in post-traumatic cell death and possibly cognitive deficits. Antagonism of the α7 nAChR at early time points after injury promotes cell survival in the cortex, and leads to small improvements in the MWM versus saline treatment. We believe this effect is mediated by the blockade of α7 nAChRs immediately following injury when high levels of ACh are present. This α7 nAChR blockade will directly reduce calcium-mediated excitotoxicity, and may also indirectly attenuate excitotoxicity by reducing α7 nAChR-mediated glutamate release (Jiang and Role, 2008; Konradsson-Geuken et al., 2009). In any case, treatment strategies utilizing α7 nAChR antagonists would be better directed by a well defined time-course of cholinergic function after injury. Furthermore, antagonists with better BBB permeability, pharmacokinetic profile, and selectivity for the α7 nAChR would greatly benefit these studies.

5.1.3 Cholinergic deficits following TBI

Studies in our laboratory have consistently reported a reduced expression of α7 nAChRs following experimental brain injury in rodents (Guseva et al., 2008; Kelso et al., 2006; Verbois et al., 2003a; Verbois et al., 2002, 2003b; Verbois et al., 2000, Chapter 3.3.3, Chapter 4.3.3). This deficit is most prominent 24 hours

after injury, and persists in many brain regions until at least 3 weeks after injury. Because there is a certain level of recovery from this deficit at later time points it is unlikely that the deficit is a result of cell death in those regions. Furthermore, these deficits can be reversed by administration of nicotine via osmotic mini-pumps (Verbois et al., 2003b), making cell loss an unlikely cause of reduced α7 nAChR binding. In addition, the involvement of the cholinergic system in cognition implicates the reduced expression of these receptors in the cognitive deficits observed after TBI. As mentioned above, antisense knockdown of the α7 nAChR in rat produces impairments in MWM acquisition and retention tests (Curzon et al., 2006). Examination of cholinergic tone following brain injury has determined that there is an initial period of hyperactivity following brain injury (Saija et al., 1988), similar to the hyperactivity observed in the glutaminergic system that is responsible for excitotoxic injury. We therefore hypothesized that an overactivation of the calcium-permeable α7 nAChR could lead to excitotoxicity and neuronal dysfunction. Our hypothesis is supported by the finding that blockade of the α7 nAChR following CCI increases tissue sparing and reduces [^{3}H]-PK11195 binding in some brain regions (Chapter 3). Then, at some time point the excess cholinergic activity becomes a deficit that could affect cognitive function (Arciniegas, 2003; Murdoch et al., 1998). This finding is validated by animal models in which significant reductions in ChAT (Leonard et al., 1994), reduced basal and evoked ACh release (Dixon et al., 1996), and reduced choline uptake (Dixon et al., 1994a) have been reported. Past studies in our laboratory have shown that treatment with a non-specific nAChR agonist, nicotine, can restore α7 nAChR binding following injury (Verbois et al., 2003b) and when administered as a series of chronic intermittent injection can attenuate cognitive deficits (Verbois et al., 2003a). Interestingly, chronic intermittent nicotine treatment did not restore α7 binding, suggesting that stimulation of the remaining receptors could be sufficient to improve cognitive function. However, despite the benefits of nicotine there are some limitations to these studies. Firstly, nicotine is not a selective agonist and therefore has numerous effects. By binding to receptors in the adrenal medulla it can stimulate the release of epinephrine and

norepinephrine, which among other effects increase heart rate and blood pressure. It can also promote the release of the neurotransmitter norepinephrine via its action on nAChRs in the hippocampus and amygdala (Fu et al., 1998). Furthermore, nicotine is a psychoactive drug and is well known to lead to dependence; unwanted qualities in a therapeutic compound. In addition to the problems with the drug, there is an issue of when is the best time to administer the drug. In the previous two studies conducted in our laboratory nicotine was administered beginning immediately after injury. However, heightened levels of cholinergic activity occurring at this time combined with the nicotine being administered could have exacerbated excitotoxicity. For these reasons we chose to explore the use of selective nAChR agonists and antagonists in TBI. Having demonstrated the neuroprotective capability of an α7 antagonist, we explored the possibility of improving functional recovery using a selective α7 agonist.

We have previously shown that α7 nAChR agonist choline, when administered in chow to male rats does not result in any significant phenotypic differences in terms of MWM performance but does significantly increase [^{125}I]-α-BTX binding in the hippocampus and cortex (Guseva et al., 2006). We therefore hypothesized that dietary choline supplementation would improve functional outcomes by improving cholinergic tone following a TBI. Choline supplementation in rats subjected to a CCI resulted in significant cortical tissue sparing, reduced neuroinflammation, and attenuated some TBI-induced deficits in nAChR expression (Guseva et al., 2008). The results from these experiments, combined with the positive results garnered from the MLA study prompted us to test the therapeutic potential of α7 nAChR partial agonists.

5.1.4 Partial agonists of the α7 nAChR

In recent years a number of relatively selective α7 nAChR ligands have been discovered and developed. While the emphasis on developing these ligands is driven by their therapeutic roles in AD and schizophrenia, parallels between these conditions and TBI led us to test these novel ligands in a CCI model of TBI. Among these new ligands is DMXB-A, otherwise known as GTS-

21, which along with its active metabolite 4-OH-GTS-21 have been found to be a selective partial agonists of the α7 nAChR (Meyer et al., 1998b). DMXB-A has been found to enhance cognition (nootrophic effect) in healthy male volunteers (Kitagawa et al., 2003). In laboratory studies DMXB-A has demonstrated neuroprotective effects in a rat model of neocortical atrophy (Meyer et al., 1998a), ischemia (Shimohama et al., 1998), and ethanol-induced neurotoxicity (de Fiebre and de Fiebre, 2003; Li et al., 2002). Most importantly, DMXB-A has shown great potential for the treatment of symptoms of schizophrenia (Freedman et al., 2008; Martin et al., 2004a; Olincy and Stevens, 2007; Simosky et al., 2002). In addition, there is evidence to suggest that DMXB-A may also be useful in treating AD (Kem, 2000) and nicotine dependence (Foulds et al., 2004). We also tested two other α7 nAChR partial agonists, tropisetron and ondansetron, which unlike DMXB-A are high affinity antagonists of the 5-HT_3 receptor. Both are approved for clinical use, primarily used to treat chemotherapy induced vomiting, although they may also be administered to TBI patients as part of their treatment or following surgery. The established safety of these drugs combined with their selective partial agonism at α7 nAChRs made them exciting molecules for investigation in a model of TBI. Furthermore, as partial agonists they have the potential to be therapeutic regardless of the level of cholinergic activity when administered. For instance, a full antagonist might be more efficacious in attenuating nAChR-mediated excitotoxicity, but could worsen cognitive deficits if administered outside its therapeutic window. Alternatively, a full agonist could exacerbate excitotoxicity if administered before, or immediately after injury. A partial agonist therefore would eliminate the need to carefully time treatment with a full agonist or antagonist. In our study we administered partial agonists for 4 days following injury and began cognitive testing the day after the last injection. Tissue sparing results did not demonstrate a significant neuroprotective effect of any partial agonist, and there was no reduction in neuroinflammation, this is possibly due to the agonist activity of the drugs. It is also possible we could have produced a neuroprotective effect had we continued administering the drugs for a longer time-period after trauma. However, we did find that some of our partial

agonist treatments attenuated cognitive deficits in the MWM (Chapter 4.3.1). This finding was most pronounced in animals that received 2 mg/kg of tropisetron or 10 mg/kg of DMXB-A. Furthermore, these improvements were observed subsequent to the cessation of treatment, suggesting they result from neuroprotective effects of treatment rather than a direct enhancement of cognition. It would be interesting to test whether accentuated effects can be achieved with extended treatment with these partial agonists.

As in the antagonist study, it was discovered that the most efficacious dose of tropisetron was not the highest dose. Neither 1 mg/kg, nor 5 mg/kg of tropisetron significantly improved MWM performance, or reduced [^{3}H]-PK11195 binding. In addition, there was discrepancy between the effects of the different drugs. It is possible that the doses we chose in the study are not optimal, and that a more thorough assessment of their use in TBI could achieve therapeutic effects. It is also possible that the differences are due to differences in their pharmacological profiles. For instance, potent antagonism of 5-HT_3 receptors is well documented for ondansetron and tropisetron, while DMXB-A may be less potent at these sites (Gurley and Lanthorn, 1998). In addition, their affinities for the α7 nAChR vary significantly, with DMXB-A and tropisetron displaying high affinity for the α7 nAChR, and ondansetron having much lower affinity. Clearly there is a lot of difficulty in finding a molecule with good selectivity for the α7 nAChR. The action of 5-HT_3 ligands at α7 nAChRs should be expected since they share a great deal of structural homology (Karlin and Akabas, 1995). There is still hope for selective ligands though, since the potent α7 agonist α-anatoxin, the α7 antagonist MLA, and [^{125}I]-α-BTX have been shown to be ineffective at 5-HT_3 receptors (Gurley and Lanthorn, 1998; Yang et al., 1992).

5.1.5 The role of the 5-HT_3 receptor

An important consideration in our third study is the possible role that antagonism of the 5-HT_3 receptor plays in neuroprotection and the recovery of function that we observed. All three of the compounds tested in this study are known to be antagonists of the 5-HT_3 receptor, and two were specifically

developed to target these receptors in the CTZ of the brainstem. 5-HT_3 receptors are also found in the amygdala, hippocampus, and entorhinal cortex (Fletcher and Barnes, 1999; Miquel et al., 2002). They are known to be located predominantly presynaptically (similar to α7 nAChRs), where they can regulate the release of neurotransmitters (Doucet et al., 2000; Turner et al., 2004b). Indeed, 5-HT_3 receptors have been shown to regulate GABA release, which in turn inhibits ACh release (Bolanos et al., 2002; Ramirez et al., 1996). This observation has been used as a rationale for treating AD with 5-HT_3 antagonists to improve cholinergic activity (Gil-Bea et al., 2004). Based on these findings it is conceivable that α7 nAChR partial agonists with 5-HT_3 antagonist activity could be of higher value than more selective α7 nAChR partial agonists.

5.2 Concluding Remarks

The pharmacological studies described within this dissertation, combined with other pharmacological studies from our laboratory described in Chapter 1 are summarized in Table 5-1. Initial studies from our laboratory focused on the administration of the agonist nicotine, with the goal of reversing [^{125}I]-α-BTX binding deficits and thereby facilitating improved MWM performance. Chronic nicotine infusions succeeded in reversing [^{125}I]-α-BTX binding deficits, but resulted in increased post-CCI seizures and did not spare cortical tissue. When MWM performance was assessed in animals administered a series of chronic intermittent nicotine injections there was a clear improvement in MWM performance. However, no reversal of [^{125}I]-α-BTX binding deficits was detected. A more selective α7 nAChR agonist was next administered in the chow of rats that underwent CCI. Choline administration improved cognitive function, spared tissue, and reduced inflammation, but did not reverse [^{125}I]-α-BTX deficits. Questions remain as to the window of therapeutic opportunity for choline in order to achieve these results. As described in detail in this dissertation, MLA produced significant improvements in MWM performance, spared cortical tissue, and reduced inflammation, but exacerbated [^{125}I]-α-BTX binding deficits. Partial agonists of the α7 nAChR improved cognitive function, but failed to significantly spare tissue, reduce neuroinflammation, or reverse [^{125}I]-α-BTX binding deficits.

Treatment	Cognitive improvement?	Tissue Sparing?	Reduced Inflammation	Reversal of BTX deficits	Notes
Nicotine infusions	**ND**	**X**	**ND**	**+**	Caused seizures
Nicotine injections	**+**	**X**	**ND**	**X**	
Choline pre/post-treatment	**+**	**+**	**+**	**X**	Window of opportunity?
MLA	**+**	**+**	**+**	**XX**	Reduced BTX binding
Partial agonists	**+**	**X**	**X**	**X**	Dose? Window? Specificity?

Table 5-1 - Summary of pharmacological studies from our laboratory

Experimental brain injury causes cognitive impairment, cortical tissue loss, microglial activation, and BTX binding deficits. Chronic nicotine infusions following CCI reversed deficits in BTX binding, but did not have any effect on cortical tissue sparing. Additionally, the nicotine infusions resulted in a higher rate of post-CCI seizures. Chronic intermittent nicotine injections were found to attenuate cognitive deficits as measured in the MWM, but again did not spare tissue, and no reversal of BTX deficits was found. Choline, an α7 nAChR agonist, when administered in chow before and after CCI attenuated cognitive deficits, spared cortical tissue, and reduced inflammation, but no reversal of BTX deficits was found. There are still questions as to whether pre-treatment is necessary to yield these positive effects. As described in this dissertation, MLA improves MWM performance, spares tissue, and reduces inflammation, but exacerbates BTX binding deficits. Finally, partial agonists of the α7 nAChR attenuated MWM deficits, but tissue sparing, inflammation, and BTX binding data did not show a definitive positive effect. (ND) Not done, (X) No effect, (XX) Exacerbated effect of CCI, (+) Positive effect.

Despite a lack of knowledge concerning the precise time-course of cholinergic changes, and pharmacokinetics/pharmakodynamics of the pharmacological agents chosen for these studies, our results are nevertheless encouraging and merit further investigation. While a lot of work remains to be done to characterize TBI related changes following injury, our results have helped to describe the binding profile of a promising marker of neuroinflammation, PK11195. We have used this tool to assess neuroinflammation in two pharmacological studies that aimed to demonstrate that α7 nAChR modulation improves functional and cellular outcomes from. Overall, the studies outlined herein have demonstrated that (1) the TSPO ligand PK11195 can measure differences in the timing and magnitude of microglial activation following injury, making it an excellent translational instrument; (2) antagonism of the α7 nAChR after TBI is neuroprotective and moderately improves functional outcomes; (3) partial agonists of the α7 nAChR administered after an experimental brain injury only marginally improve spatial memory and do not significantly spare cortical tissue.

Based on our findings, the most promising route for future research in this area is likely to be in the development of α7 nAChR antagonists with more favorable pharmacological characteristics. The potential of potent and selective nicotinic agonists and antagonists is well recognized, and therefore drug discovery research in the field is progressing quickly (Haydar et al., 2009; Lopez-Hernandez et al., 2009; Smith et al., 2009; Tasso et al., 2009). Further studies with MLA or a novel α7 nAChR antagonist should firstly seek to dissect the α7 nAChR binding reductions we found by including an appropriate sham + antagonist control group. Studies could aim to refine the therapeutic window for α7 nAChR antagonist treatment and further elucidate treatment effects on other measures of cellular damage such as FJ-B or silver staining (Hall et al., 2008). Furthermore, an effect of these treatments of neurogenesis cannot be ruled out since has been shown to occur following CNS injury such as ischemia (Jin et al., 2006; Jung et al., 2009), alcohol withdrawal (Nixon and Crews, 2004; Nixon et al., 2008), and TBI (Gao et al., 2009; Lu et al., 2005). It would be very interesting

to determine whether neurogenesis is affected by our pharmacological interventions. Finally, the disappointing results of our partial agonist study suggest that there may not be a single ‘silver bullet’ pharmacological agent with which to treat TBI. The studies outlined in this dissertation, and the results of other studies from our lab suggest that a combination of α7 nAChR antagonists and agonists administered at the right times might provide most benefit.

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APPENDICES

APPENDIX I: list of acronyms and abbreviations

5-HT	-	5-hydroxytryptamine
ABC	-	Avidin: Biotinylated enzyme complex
ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AD	-	Alzheimer's disease
ALS	-	Amyotrophic lateral sclerosis
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	-	Analysis of variance
AP5	-	(2R)-amino-5-phosphonovaleric acid
ATP	-	Adenosine-5'-triphosphate
BBB	-	Blood-brain barrier
BTX	-	Bungarotoxin
Ca^{2+}	-	Calcium (ion)
CCI	-	Controlled cortical injury
ChAT	-	Choline acetyltransferase
CNS	-	Central nervous system
CPP	-	Cerebral perfusion pressure
CT	-	Computerized tomography
CTZ	-	Chemoreceptor trigger zone
DAB	-	3,3' Diaminobenzidine
DAI	-	Diffuse axonal injury
DHβE	-	Dihydro-β-erythroidine
DMXB-A	-	3-2,4 dimethoxybenzylidene anabaseine (a.k.a. GTS-21)
DNA	-	Deoxyribonucleic acid
EAA	-	Excitatory amino acid
FITC	-	Fluorescein isothiocyanate
FJ-B	-	Fluoro-Jade B
FPI	-	Fluid percussion injury
GCS	-	Glasgow coma score
GFAP	-	Glial fibrillary acidic protein
HSD	-	Honestly significant difference
IACUC	-	Institutional Animal Care and Use Committee
ICP	-	Intracranial pressure
IED	-	Improvised explosive device
LD	-	Lateral dorsal
LTP	-	Long-term potentiation
mAChR	-	Muscarinic acetylcholine receptor
MLA	-	Methyllycaconitine
MMSE	-	Mini mental state exam
MWM	-	Morris water maze
Na^{+}	-	Sodium (ion)
nAChR	-	Nicotinic acetylcholine receptor

NeuN	-	Neuronal nuclei
NMDA	-	N-methyl-D-aspartic acid
NMJ	-	Neuromuscular junction
PAM	-	Positive allosteric modulator
PBR	-	Peripheral benzodiazepine receptor
PBS	-	Phosphate-buffered saline
PET	-	Positron emission tomography
pMCAO	-	Permanent middle cerebral artery occlusion
PNS	-	Peripheral nervous system
ROS	-	Reactive oxygen species
TBI	-	Traumatic brain injury
TUNEL	-	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VPL	-	Ventral posterolateral

APPENDIX II: supplemental figures

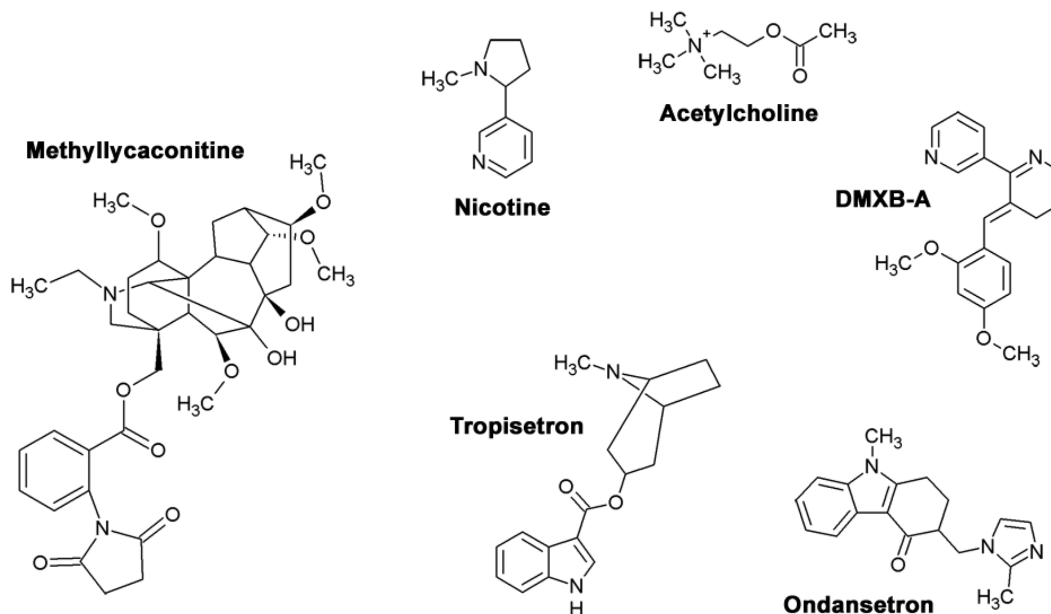

Figure A1 - Molecular structure of six ligands that bind the α7 nAChR

Skeletal diagrams showing the molecular structures of α7 nAChR ligands Methyllycaconitine (MLA), Nicotine, Acetylcholine (ACh), 3-(2,4-dimethoxybenzylidene)anabaseine (DMXB-A), ondansetron, and tropisetron.

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