The Effects of Nicotine Treatment on Striatal Dopamine Neurotransmission after Traumatic Brain Injury

by

Samuel Sang-Hyun Shin

Bachelor of Science, University of California, San Diego, 2006

Submitted to the Graduate Faculty of

University of Pittsburgh in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

Center for Neuroscience at University of Pittsburgh

This thesis was presented

by

Samuel Sang-Hyun Shin

It was defended on

March 1, 2011

and approved by

Steven H. Graham, Ph.D. M.D., Professor, Neurology

Teresa G. Hastings, Ph.D., Associate Professor, Neurology, Neuroscience

Anthony E. Kline, Ph.D., Associate Professor, Psychology, Physical Medicine & Rehabilitation

Ruth G. Perez, Ph.D., Assistant Professor, Neurology, Pharmacology & Chemical Biology

Thesis Director: C. Edward Dixon, Professor, Neurological Surgery, Anesthesiology, Neurobiology, Physical Medicine & Rehabilitation

The Effects of Nicotine Treatment on Striatal Dopamine Neurotransmission after Traumatic Brain Injury

Samuel S. Shin, Ph.D.

University of Pittsburgh, 2011

ABSTRACT

Traumatic brain injury (TBI) is a widespread problem in the United States affecting thousands of individuals annually. Due to our lack of understanding of the mechanisms of TBI, medical management of the functional deficits in these patients is difficult. In this study, injury induced deficits in striatal dopamine neurotransmission was studied using rats injured by controlled cortical impact. We identified specific decrease in the levels of phosphorylated tyrosine hydroxylase (TH) measured by Western blots in the striatum and substantia nigra at 1 week following TBI, suggesting a decrease in TH activity. A direct measurement of TH activity by an in-vivo TH activity assay showed a correlating deficit in the injured animals. Striatal dopamine release evoked by potassium stimulus using microdialysis probes was decreased in injured animals compared to shams at 1 week. These results suggest deficits in presynaptic dopamine synthesis and release. To reverse these deficits, nicotine which was previously demonstrated to enhance striatal dopamine signaling, was administered for 1 week following injury. Rats that were treated with nicotine showed recovery of dopamine release and TH activity deficits.

We have previously identified that TBI induces deficits in phosphorylation of striatal posynaptic protein: dopamine and cAMP regulated phosphoprotein 32 (DARPP-32), an important regulator of striatal dopamine signaling. To assess if nicotine treatment can also reverse this deficit in DARPP-32 phosphorylation at threonine 34 (pDARPP-32-T34), Western blot was used. There was no enhancement of pDARPP-32-T34 levels by nicotine treatment compared to saline controls. Also, phosphorylation levels of molecules downstream of pDARPP-32-T34: extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB) were not affected by nicotine treatment. Behavioral experiments testing motor function by Beam Balance Test and Beam Walking Test and cognitive function by Morris Water Maze test showed no benefits of nicotine treatment. The molecular results in this study suggest that nicotine may lead to activativation of multiple receptor signaling pathways that have opposite modulation of pDARPP-32-T34. This study gives us a better understanding of the complex signaling pathways of striatal dopamine neurotransmission in the setting of TBI.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

My work with Dr. Dixon began in the summer of 2006 when I arrived in Pittsburgh. At the time I had just attained my undergraduate degree in Bioengineering with 2 years of part time research in immunology, and neuroscience was a novel subject for me. Although I've invested much time in learning bioengineering and immunology, the curiosity of neuroscience was what drove me to switch fields. I was a bit apprehensive to make the switch, but deep inside my mind I felt that understanding the mechanism of consciousness was my greatest desire that I've had for years.

Five years have quickly passed since then, and I truly feel that the decision was correct. Over the years Dr. Dixon has guided me and transformed me in various ways. Grantsmanship, oral presentation skills, and scientific writing skills are key skills I have gained under Dr. Dixon's guidance. Also, with the help of the thesis committee, I have grown to think as a scientist. Scientific facts are something one can gain from reading a book, but the skills to critically analyze data and communicate thoughts to others are not something attainable without mentorship.

Other members of my lab James Bales, Youming Li, Michelle Ma, Hong Yan, Sherman Culver, and Kristin McFarlane have taught me various techniques over the years. I appreciate their dedication as well as their patience to tolerate my somewhat obsessive personality. In particular, I will miss the presence of Dr. James William Bales, who introduced an ounce of sanity in my research endeavors during the last three years.

Lastly, I'd like to close this preface with words of wisdom I will borrow from my father and Zhu Chi, a great Chinese philosopher. My father had quoted this philosopher in his final lecture as a retiring professor of Seoul National University, Korea. The few memorable lines were:

> "Do not say you will learn tomorrow what you must learn today.
> Do not say you will learn next year what you must learn this year.
> It is easy for a young boy to gain age, but it is difficult for him to gain knowledge.
> Therefore, do not take even one second lightly."

As time passes by and I gain more knowledge, I realize that it is inevitable that I gain age, but impossible to learn to the degree I desire. Yet, I will pursue learning until the last day of my life. This is the scholarly spirit that my father has passed on to me and the ideals that I will live by.

1. Introduction and Background

1.1 Traumatic Brain Injury in the United States

Traumatic brain injury (TBI) is a widespread problem in the United States, causing 1.5 million cases of death or emergency care annually (Nolan S, 2005). Approximately 5.3 million individuals live with varying degrees of disability resulting from TBI in the U.S., and 70,000 to 90,000 (Consensus Conference, 1999) have substantial loss of functioning as a long term outcome. TBI occurs at an incidence of 200 per 100,000 people per year, most commonly by motor vehicle accidents, falls, and violence (Shames J et al., 2007). The majority of patients may recover rapidly, but a large proportion of moderate to severely injured patients sustain permanent cognitive, emotional, and physical deficits. In addition, TBI can result in various devastating complications such as seizures, hydrocephalus, infections, and behavioral and functional deficits. As a result, there is a tremendous cost of medical care for these individuals not only for the immediate management but also for the long term rehabilitation. Moreover, the loss of potential human resources among these individuals is incalculable.

Because the causes of TBI are varied, such as motor vehicle accident, falls, violence, and sports related injury, the mechanisms of injury are also diverse. The current primary classification system for TBI is the Glasgow Coma Scale (GCS), which categorizes cases into mild, moderate, and severe injury. However, these categories do not explain the mechanism of injury, and patients with the same GCS score may have very different pathophysiology ranging from epidural hematoma, subdural hematoma, subarachnoid hemorrhage, and diffuse axonal

injury (Saatman et al., 2008). The heterogeneity of injury and difficulty of categorization and management is thus a major issue for researchers and clinicians who specialize in TBI.

Although TBI is a heterogeneous disease of various pathophysiological mechanisms, almost all cases of TBI cause deficits in cognitive function. The long lasting consequences of cognitive impairment are often underreported and the mechanisms are often unclear. Neuropsychological tests looking at various aspects of behavior such as social function, cognitive abilities, and psychiatric symptoms at 10-20 years after TBI show significant behavioral impairment at such chronic time points (Hoofien D et al., 2001). Individuals who have suffered TBI in the past have high incidence of personality changes and psychiatric issues such as depression, hostility, anxiety disorders, and post traumatic stress disorder. These individuals also have decreased information processing speed and lower intellectual functioning amounting to difficulty in learning and social interaction.

The economic burden resulting from TBI is estimated to be around $10 billion (Consensus Conference, 1999) annually for emergent care and rehabilitation. On average, the cost for medical management of a person with severe TBI is estimated to range from $600,000 to $1,875,000. This figure is likely an underestimate not including calculated lost earnings or cost to social service systems. Moreover, there is an even larger loss in the economic value of human potential from the decreased contribution of these individuals to society due to the impairments in intellectual and social functioning capacity—a point that is often overlooked.

1.2 Current treatments for TBI

Current treatment strategies for severe TBI focus on the control of cerebral edema and raised intracranial pressure, performed by administration of osmotic agents such as mannitol or hypertonic saline and decompressive craniectomy (Meyer et al., 2010). The principle strategy behind using osmotic agents is to withdraw water from cerebral tissue, thereby reducing brain volume and pressure. Surgical interventions allow decompression, which provides room for the swollen injured brain to reduce intracranial pressure when medical management fails. Also, surgical interventions are useful for evacation of intracranial mass lesions such as hematomas. Hyperventilation was commonly used in the past in order to rapidly reduce intracranial pressure. However, aggressive or prolonged hyperventilation may in fact worsen outcomes because it reduces vascular perfusion and depletes bicarbonate levels. Thus, only moderate hyperventilation is currently used in the clinical setting while cerebral oxygenation is being closely monitored (Muizelaar et al., 1991; Ralph and Lowes, 2009).

Pharmacological treatments are also used in various settings of TBI. Sedatives and antiepileptic drugs such as phenytoin and benzodiazepenes are used to prevent post traumatic seizures (Losiniecki and Shutter, 2010). Barbiturates also prevent seizures and are used for management of uncontrollable intracranial pressures by reducing cerebral metabolism and cerebral blood volume. However, adverse effects such as hypotension, adrenal insufficiency, and bone marrow suppression have been reported, and there is conflicting evidence as to whether the above treatments are more effective than conventional strategies of reducing intracranial pressure such as the use of mannitol (Meyer et al., 2010)

Corticosteroids were commonly used during the last decade in order to reduce post-traumatic inflammation, which was believed to be one of the major mechanisms of secondary injury in TBI. Although spinal cord injury studies have reported positive outcomes in motor and sensory function after corticosteroid treatment (Bracken et al., 1990, 1997), a large randomized trial of corticosteroids showed no beneficial effect and even increased the risk of death during the 2 weeks following TBI (Roberts et al., 2004).

The use of hypothermia for therapeutic purposes in traumatic brain injury has gained much attention in the last decade. It is believed to reduce inflammation and metabolic consumptions after injury. In addition, it can attenuate excitoxic effects by attenuating glutamate release (Mueller-Burke et al., 2007) and alter the activity of matrix metalloproteinases thereby reducing blood-brain barrier permeability (Nagel et al., 2007). A meta-analysis of eight studies (Peterson et al., 2008), however, showed no statistically significant effect on mortality or neurological outcome. Prolonged hypothermia also has been associated with adverse effects such as sepsis, coagulopathy, and pneumonia. Much research on hypothermia is now focused on the details of the protocol, as optimizing the duration and rate of cooling may result in positive outcome of the patients (De Deyne, 2010).

In addition to these therapies, various pharmacological agents for TBI management have been attempted previously, ranging from cannabinoids, antioxidants, N-Methyl-D-aspartic acid (NMDA) receptor antagonists, and calcium channel blockers (Jain, 2008). These agents were shown to be beneficial in animal models, but most clinical trials in humans found no efficacy. In

a recent study comparing eleven pharmacological agents, only amantadine (a dopamine agonist and NMDA receptor antagonist) and CP-0127 (a bradykinin receptor antagonist) markedly improved behavioral and cognitive outcomes after TBI (Wheaton et al., 2009). Pharmacological management of patients suffering from TBI is challenging due to the complexity of the mechanism of injury and heterogeneity of patients. More research targeting specific mechanisms of injury or post injury deficits are needed for future development of pharmacological agents to treat TBI.

1.3 Dopamine Deficits after TBI

Changes in the dopamine system after TBI have been demonstrated in various studies in the past (Bales et al., 2009). The pharmacological agents targeting the dopamine system can improve cognitive function in animals and humans after TBI. Catecholamine agonist therapy has shown motor and cognitive improvement in both humans and animals (Phillips et al., 2003). Also, L-DOPA treatment which increases dopamine synthesis, enhances cognitive and motor function following TBI (Kraus and Maki, 1997; Koeda and Takeshita, 1998; .

Methylphenidate (Kline et al., 1994, 2000) and D-amphetamine (Feeney et al., 1981; Sutton et al., 1989; Hovda et al., 1989; Hornstein et al., 1996), which increase synaptic dopamine levels by inhibiting dopamine transporter (DAT) function, enhance functional outcomes after experimental TBI. Benefits in memory and attention (Evans et al., 1987), as well as increased information processing speeds were also previously reported (Willmott and Ponsford, 2009).

Moreover, amphetamine can facilitate neuronal plasticity and regeneration (Ramic et al., 2006). Outside the striatum, these agents not only inhibit the function of DAT, but also norephinephrine transporters. This leads to an increase in synaptic norepinephrine levels in the neocortex receiving input from the neurons in the locus ceruleus and reportedly contributing to cognitive benefit.

Amantadine is an agent that was originally used as an antiviral agent for Influenza A. Its function as a psychostimulant gained wide interest in its use in neuropsychiatric diseases over time. In the central nervous system, amantadine primarily functions as a noncompetitive antagonist of the NMDA receptor and is believed to increase extracellular dopamine concentrations by inhibiting reuptake and facilitating dopamine synthesis (Von Voightlander and Moore, 1971; Bak et al., 1972). It also induces postsynaptic effects, possibly by increasing postsynaptic dopamine receptor activation or by altering their conformation (Gianutsos et al., 1985; Beers et al., 2005). Amantadine administration following TBI has been shown to induce dopamine release when infused into the striatum by microdialysis (Takahashi et al., 1996). In both clinical data (Sawyer et al., 2008) and animal models of TBI (Dixon et al., 1999), amantadine was demonstrated to facilitate neurorecovery. As mentioned previously in Section 1.2, meta-analysis of recent clinical trials showed marked cognitive benefit for using amantadine in TBI.

In addition, alterations in the levels of striatal dopamine and proteins that synthesize and transport dopamine after injury have been reported in neurochemical studies. Dopamine levels increase at acute time points in several regions such as the striatum, hypothalamus, and frontal

cortex (Massucci et al., 2004; McIntosh et al., 1994). The metabolism of dopamine, measured by the ratio of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) to dopamine, was increased at 1 hour after injury in the striatum (Massucci et al., 2004).

There have been only a few reports on the levels of striatal dopamine receptors after TBI. One study demonstrated no changes in D_2 receptors, but decreased numbers of D_1 receptors immediately after TBI followed by an increase at 24 hours (Henry et al., 1997). However, at 14 days following TBI, no changes in D_2 receptors were reported (Wagner et al., 2005, 2009). These studies also showed that the levels of striatal DAT is decreased at 14 days after TBI and that electrically evoked dopamine release, asmeasured by fast scanning cyclic voltammetry, is also decreased at that time.

1.4 Dopamine Signaling in the Striatum

The major dopamine projections in the CNS arise from the midbrain regions of the ventral tegmental area and substantia nigra pars compacta. The dopaminergic axons arising from the ventral tegmental area project to nucleus accumbens and form mesolimbic pathway, whereas the dopaminergic axons arising from substantia nigra project to the dorsolateral striatum to form the nigrostriatal pathway. Midbrain dopamine projections from ventral tegmental area also reach into cortical regions such as the prefrontal cortex.

The striatum functions as the major input structure of the basal ganglia, a subcortical structure that has an important role in cognitive, emotional, and movement control (Hauber, 2010). In the striatum, nigrostriatal axons synapse onto medium spiny neurons that synthesize γ-Aminobutyric acid (GABA). Because medium spiny neurons represent 95% of neurons in the striatum, striatal dopamine signaling is regulated mostly by activation of these GABA-ergic postsynaptic neurons. Once medium spiny neurons receive inputs, two output pathways can be activated: 1. Direct pathway projecting to substantia nigra and globus pallidus pars interna, and 2. Indirect pathway projecting to subthalamic nucleus and globus pallidus pars externa (Hauber, 1998). Activation of the direct pathway (also known as striatonigral pathway) facilitates movement, whereas activation of the indirect pathway (also known as striatopallidal pathway) inhibits movement. The activation of direct and indirect pathways occurs by dopamine release from striatal dopamine terminals activating different subtypes of dopamine receptors: D_1 and D_2 receptors, respectively.

Dopamine receptors belong to the G protein-coupled receptor superfamily, and these receptors are located on dendrites and cell bodies of neurons as well as axons and nerve terminals. Dopamine receptors are categorized into either D_1-like or D_2-like receptors, with D_1-like receptors consisting of D_1 and D_5 receptors and D_2-like receptors consisting of D_2, D_3, and D_4 receptors. In the striatum, D_1 and D_2 receptors were identified in the cell body and dendrites of medium spiny neurons (Levey et al., 1993). Activation of D_1-like receptors leads to activation of adenylate cyclase, which increases synthesis of 3'-5'-cyclic adenosine monophosphate (cAMP). In turn, cAMP activates a second messenger important in activation various functions

of medium spiny neurons: protein kinase A (PKA). However, D_2-like receptors reduce adenylate cyclase activation by coupling to G_i/G_o proteins (Vizi and Lajtha, 2008).

Dopamine neurotransmission in the striatum has a significant role in brain function such as voluntary movement (Korchounov, 2008). Dopamine receptors D_1 and D_2 influence long-term potentiation and long-term depression, important mechanisms of synaptic plasticity in motor skill learning (Vizi and Lajtha, 2008). Striatal dopaminergic signaling also plays a major role in spatial learning and memory (Mura A and Feldon J, 2003): rats that had bilateral lesions of nigrostrial dopaminergic system had profound deficits in acquisition task of Morris water maze. These findings indicate the importance of the striatal dopamine system in both memory and motor function.

1.5 Downstream signaling: DARPP-32, ERK, CREB

When dopamine release activates receptors on medium spiny neurons, adenylate cyclase is activated and increase in PKA activity subsequently leads to phosphorylation of a phosphoprotein dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein, 32kDa (DARPP-32). The protein DARPP-32 is present in 97% of medium spiny neurons (Matamales et al., 2009) and is involved in virtually all of dopamine mediated events ranging in biochemical, electrophysiological, and transcriptional events (Greengard et al., 1999; Svenningsson et al., 2004). It has a central role in regulating neuronal excitability and induction

of long-term depression (LTD) and long-term potentiation (LTP), which form synaptic plasticity and serve as the basis of learning and memory (Calabresi et al., 2000).

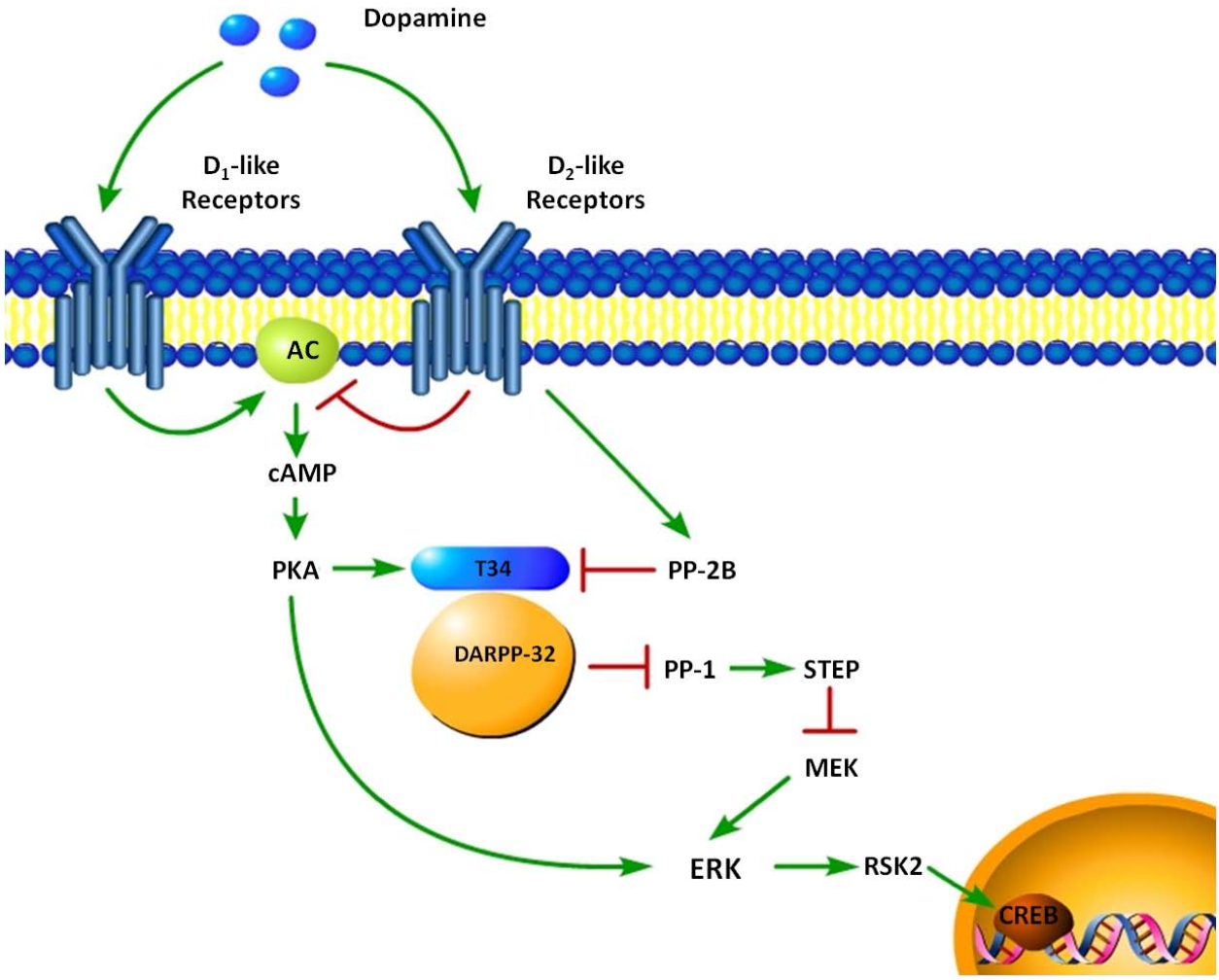


Figure 1. Striatal dopamine signaling in medium spiny neurons. Dopamine activation of D_1 receptors activates PKA signaling pathway and activation of D_2 receptors inhibits PKA signaling pathway. Activation of PKA leads to phosphorylation of T34 site of DARPP-32, which then inhibits PP-1. D_1 and D_2 receptors are depicted in the same neuron for conceptual

simplicity; most striatal medium spiny neurons express only D_1-like receptors or D_2-like receptors, and not both.

DARPP-32 is present in both striatonigral neurons of the direct pathway activated by D_1 receptors and striatopallidal neurons of the indirect pathway activated by D_2 receptors. As previously mentioned, D_1 receptor activation by dopamine is followed by an increase in activity of adenylate cyclase (**Fig. 1**). This further leads to activation of PKA, which phosphorylates DARPP-32 in one of its two major phosphorylation sites: threonine-34 (T34). This would in turn cause strong inhibition of protein phosphatase 1 (PP-1), which has numerous substrates, such as neurotransmitter receptors, ion channels, and other phosphatases. Inhibition of PP-1 will activate transcription factors such as cAMP response element binding protein (CREB) via extracellular regulated kinase (ERK) pathway. This in turn would induce activation of genes important for synaptic plasticity.

Activation of D_2 receptor by dopamine will inhibit adenylate cyclase as well as cause phospholipase C and protein phosphatase 2B (PP-2B) activation (Nishi et al., 1997). Since PP-2B is a strong phosphatase that can dephosphorylate DARPP-32 phosphorylated at T34 (pDARPP-32-T34), this will lift the inhibition on PP-1. Thus, depending on the activation of D_1 or D_2 receptors, there is a differential regulation in the activity of DARPP-32. This is in agreement with previous electrophysiological and gene transcriptional data that demonstrate dopamine's stimulatory effect via D_1 receptors and inhibitory effect via D_2 receptors (Gerfen et al., 1990; Robertson et al., 1990; West et al., 2002).

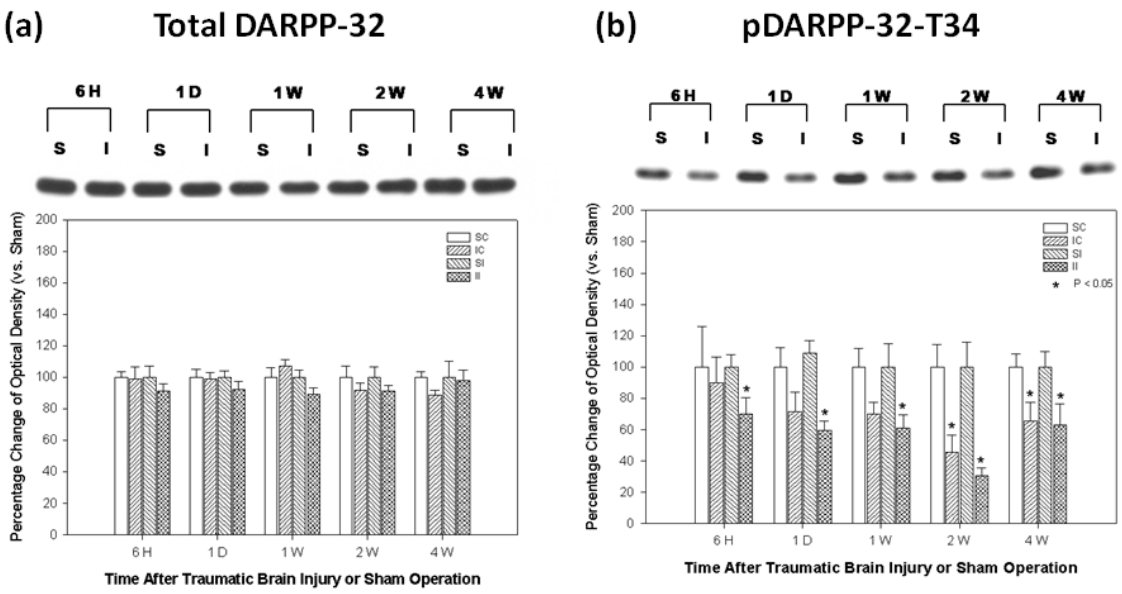


Figure 2. Western blot time course of striatal DARPP-32 (a) and pDARPP-32-T34 (b). From 6 hours to 4 weeks, there is no change in protein levels of DARPP-32 (a), but there is a decrease in p-DARPP-32-T34 (b) in the injured animals' striata that are both ipsilateral and contralateral to the side of injury. (SC = sham contralateral, IC = injured contralateral, SI = sham ipsilateral, II = injured ipsilateral, *p≤0.05) Figure from (Bales, 2010).

Data previously generated by other members of the Dixon laboratory have shown that TBI decreases the levels of pDARPP-32-T34 from 6 hours to 4 weeks after injury in rats (**Fig. 2**). Although phosphorylation of DARPP-32 is altered, there is no alteration in total DARPP-32 protein levels. Since pDARPP-32-T34 has an inhibitory effect on PP-1 activity, the decrease in pDARPP-32-T34 may cause an increase in PP-1 activity. In accord with these data, the activity of striatal PP-1 increases at 1 day and 2 weeks post injury (Bales, 2010). In addition, other previous data showed that intraperitoneal injection of PP-2B inhibitor FK506 after TBI can

reverse this deficit in pDARPP-32-T34 (**Fig. 3**). Thus, there is a major role of PP-2B in reducing pDARPP-32-T34 following TBI.

Another major regulation site for activity of DARPP-32 is threonine-75 (T75). Phosphorylation of T75 by cyclin dependent kinase 5 (cdk5) causes DARPP-32 to function as an inhibitor to PKA. This in turn will prevent T34 phosphorylation. Thus, DARPP-32 acts either as an inhibitor of PKA or PP-1 depending on the phosphorylation of T34 or T75. This differential function of DARPP-32 depending on the phosphorylation of two sites makes it a bifunctional signaling molecule.

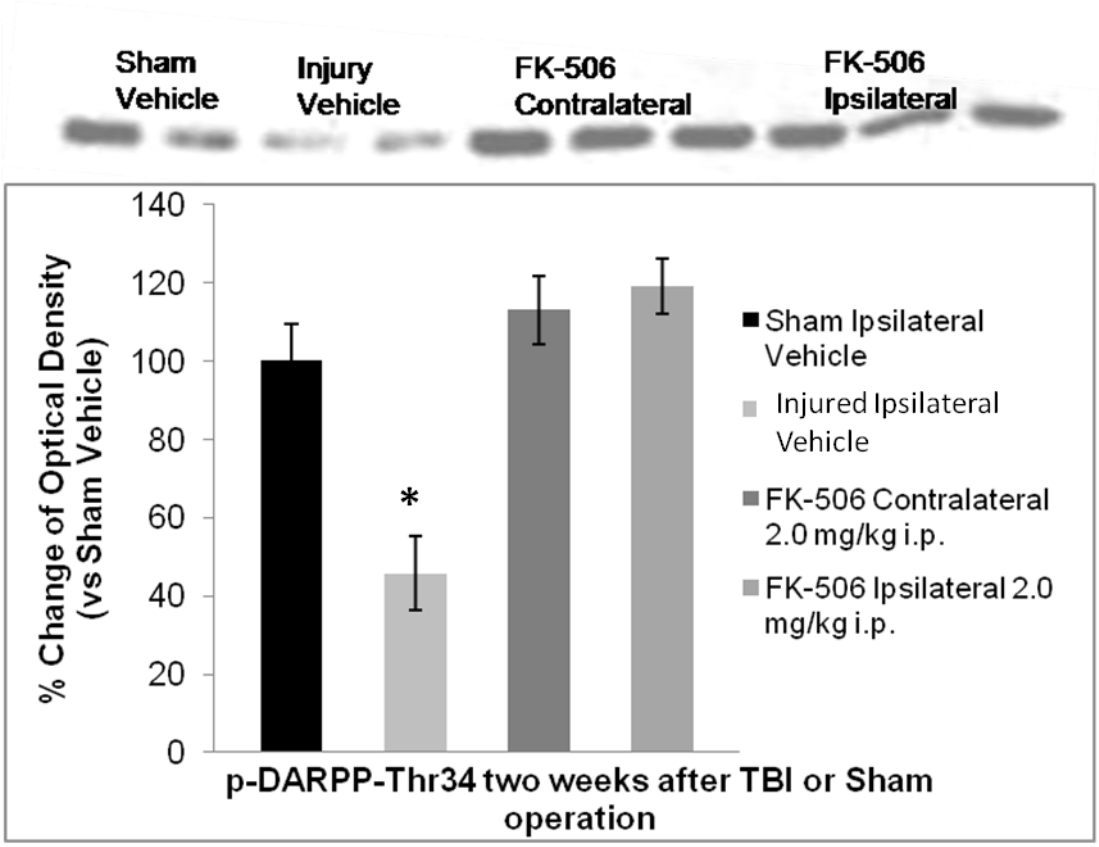


Figure 3. FK506 treatment following TBI. Animals were injected with single dose of FK506 (2 mg/kg, suspended in 0.9% saline) 5 minutes after injury. Western blots of pDARPP-32-T34 demonstrated full reversal of TBI induced deficit with FK506 treatment ($p \leq 0.05$* compared to sham ipsilateral vehicle). Figure from (Bales, 2010).

Downstream of DARPP-32 is the extracellular signal-regulated kinase (ERK), which has a major role in learning and memory. ERK is activated by phosphorylation, leading to its subsequent phosphorylation of CREB. Although ERK activation can occur downstream of DARPP-32, PKA can also directly activate ERK, as shown in (**Fig. 1**). The importance of ERK in learning and memory is evidenced by its necessity in LTP induction and behavioral tests for learning and memory.

The role of ERK in learning and memory was most extensively investigated in the hippocampus in the past. In hippocampal slices, LTP-inducing high frequency stimulation activates ERK (English and Sweatt, 1996). Inhibition of the ERK cascade (English and Sweatt, 1997; Impey et al, 1998) attenuates LTP formed by this high frequency stimulus. Behaviorally, rats subjected to fear conditioning tasks, a form of associative learning, had increased levels of phosphorylated ERK (Atkins et al., 1998). Pharmacological inhibition of MEK, the kinase upstream of ERK, also inhibited the ERK cascade and abolished fear conditioning. This study also showed inhibition of LTP induction when MEK inhibitor was applied. Rats subjected to spatial learning in Morris Water Maze also show increased phosphorylated ERK immediately after training (Blum et al., 1999). Moreover, infusion of a MEK inhibitor decreased long term spatial learning in these animals, again verifying the importance of ERK in memory formation.

Similarly, treatment of mice with SL327, a MEK inhibitor inhibited cocaine induced locomotion (Valjent et al., 2005).

The role of ERK in striatal learning has also been demonstrated in the past by electrophysiological and behavioral studies. The formation of LTP by in vivo electrical stimulus of cortico-striatal circuit (Carpier and Deniau, 1997) induces ERK activation (Sgambato et al., 1998) and immediate early genes such as c-fos, zif268, MAP Kinase phosphatase-1. Activation of ERK leads to activation of downstream transcription factors such as CREB and Elk-1, which induce immediate early gene transcription (Deng and Karin, 1994). In rodent behavioral studies, molecular changes in the striatum followed several forms of learning such as associations between action and outcome and motor tasks. Increases in phosphorylation of striatal ERK and *c-fos* occurred when mice were subject to motor skill learning using accelerating rotarod task (Bureau et al., 2010). Injection of inhibitors of ERK signaling such as PD98059 or SCH237 inhibited motor skill learning, demonstrating the importance of ERK signaling in memory involving the striatum. Similarly, ERK phosphorylation (Shiftlett et al., 2008) as well as the activation of transcription factor CREB by phosphorylation (Shiftlett et al., 2009) in the nucleus accumbens increases after training to produce associations between an auditory tone and food.

1.6 TBI and dysregulation of α-synuclein

Alpha-synuclein (α-syn) is a soluble protein found in presynaptic terminals of various parts of the brain. It is believed to play a particularly important role in synucleinopathies such as Parkinson's Disease, dementia with Lewy bodies, and multiple system atrophy, though the exact mechanism has not been demonstrated thus far. Past studies have shown that α-syn may inhibit activity of tyrosine hydroxylase (TH) (Perez et al., 2002) and aromatic amino acid decarboxylase (AADC) (Tehranian et al., 2006), enzymes responsible for dopamine synthesis, by direct physical interaction as demonstrated by immunoprecipitation. Thus, disruptions in α-syn levels by its increased expression or aggregation can cause dysregulation of dopamine biosynthesis. Also, α-syn has been shown to inhibit DAT function by trafficking DAT away from plasma membrane into the cytoplasm (Wersinger et al., 2003, 2004). Overall, pathologic processes that lead to loss of α-syn equilibrium then can lead to dysregulation of both biosynthesis and transport of dopamine. Studies on α-syn knock out mice show nigrostriatal dopaminergic deficits in terms of its release, tissue content, and amphetamine- induced locomotion (Abeliovich et al., 2000), supporting the idea that α-syn is an important protein in the modulation of dopamine activity.

In a previous study that I have performed, α-syn levels in the striatum of TBI rats were shown to be drastically elevated compared to the levels in sham rats at 1 week post injury (**Fig. 4**). By 4 weeks, the levels of α-syn in injured animals were reduced to levels comparable to those seen in sham animals. Because α-syn can modulate the activity of dopamine regulating proteins, this temporary upregulation of α-syn protein may be related to dopamine dysregulation.

Increased levels of α-syn may inhibit the activity of TH and AADC, decreasing dopamine synthesis and possibly contributing to deficits in striatal dopamine neurotransmission previously explained in sections 1.4 and 1.5.

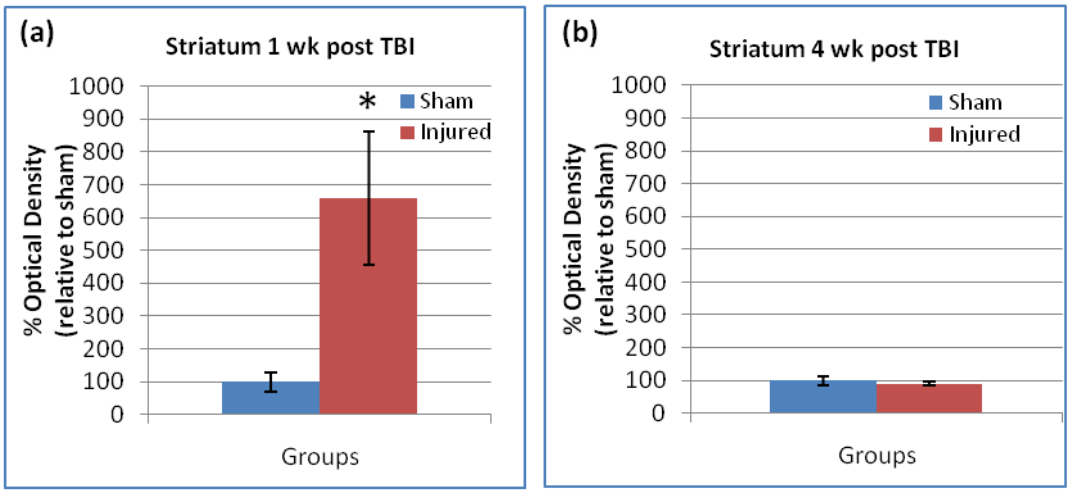


Figure 4. α-syn levels in striatum measured by Western blot. There is a drastic upregulation of striatal α-syn levels at 1 week post TBI. By 4 weeks, the increases are reversed. (* = $p \leq 0.05$)

Although the level of α-syn is increased in the injured animals at 1 week, the tissue level of dopamine in injured animals at 1 week is not different from those of sham animals (Massucci et al., 2004). However, α-syn increase may still inhibit dopamine synthesis, decreasing potassium stimulus evoked dopamine release which will be shown in Chapter 2. In addition, the recovery of potassium stimulus evoked dopamine release in the injured striatum at 4 weeks will be shown, correlating with the return of α-syn to sham levels. However, whether this α-syn increase occurs specifically in dopamine terminals or other regions of the striatum has not been

demonstrated. This is a question left for future studies using immunohistochemistry experiments.

1.7 The Effects of Nicotine and Nicotinic Agonists: Behavioral Aspect

Nicotine is a major neurostimulant that enhances cognitive function in both humans and animal models (Poorthuis et al., 2009). Activation of nicotinic acetylcholine receptors (nAChRs) affects cholinergic signaling and other neurotransmitter systems that interact with the cholinergic system such as dopamine, leading to improvements in learning, memory, and attention. Acute injection of nicotine improved rats' performance on 16-arm radial maze, a measure of working memory (Levin et al., 1997). Similarly, synthetic nAChR agonists such as dimethylaminoethanol (Levin et al., 1995), AR-R17779 (Levin et al., 1999), ABT-418 (Decker et al., 1994), and TC-1734 (Gatto et al., 2004) were all shown to improve working memory. Even with chronic nicotine infusion, this benefit in memory function was not diminished, and the benefits persisted even up to 4 weeks after withdrawal (Levin et al., 1990, 1992). As chronic treatment would be necessary in managing neurodegenerative diseases or acute brain injuries, this property was clinically valuable.

Nicotine and nAChR agonists have been shown to improve not only working memory, but also attention in more recent studies. These experiments tested the rats' abilities to pay attention to visual signals then to make correct choices by pressing on the levers that correlated with the signals, and demonstrated a benefit of nicotine and nAChR agonist (Rezvani et al.,

2002; Rezvani and Levin, 2003; McGaughy et al., 1999). In a similar attention test known as the five-choice serial reaction time task, a nicotine agonist SIB-1553A improved performance of rats as well as monkeys (Terry et al., 2002).

Nicotine treatment also improves symptoms in several human diseases characterized by cognitive deficits. In Alzheimer's Disease, nicotine skin patch or intravenous delivery improves memory function (Newhouse et al., 1999; Parks et al., 1996) and learning (Wilson et al., 1995). In addition, nicotine application by skin patch (White and Levin, 1999) or subcutaneous injection (Jones et al., 1992) improved attention in Alzheimer's Disease patients. Similarly, in patients with Schizophrenia, cigarette smoking was shown to improve auditory gating (Adler et al., 1993). Schizophrenia patients have decreased nAChR in the hippocampus (Freedman et al., 1995) and self-medication is a prevalent among them. These findings support the notion of cognitive benefit of nicotine administration for Schizophrenia.

Application of nicotine or nAChR agonists in the setting of acute brain injury improves cognitive function. In rats with cortical lesion induced by electrical current, nicotine injection either pre or post injury resulted in improvement of memory and cognitive function (Brown et al., 2000). These rats had better performance in the Morris Water Maze than the vehicle treated group for both the acquisition (learning test) and probe (memory test) trials, demonstrating improvement in both learning and memory. In the setting of TBI, dietary supplementation of choline, which specifically activates α7 nAChRs, improved Morris Water Maze performance, and increases the levels of α7 nAChRs and tissue survival (Guseva et al., 2008). Similarly,

intermittent injection of nicotine treatment for 11 days after TBI also improved acquisition and retention of spatial memory (Verbois et al., 2003a).

Post TBI injection of cytidine-5'-diphosphate-choline (CDP-choline), a compound closely related to choline, also result in improvement of Morris Water Maze performance (Dixon et al., 1997). CDP-choline treatment has also been shown to improve outcomes following stroke in clinical trials (Clark, 2009) and reduce memory problems and cognitive dysfunction in elderly patients with Alzheimer's Disease (Conant et al., 2004). However, it is not clear if these beneficial effects of CDP-choline treatment were due to increased choline to activate nAChRs or increased acetylcholine to activate all acetylcholine receptors. Treatments using other specific agonists for nAChRs also induce cognitive benefit. An agonist for nAChRs, AR-R1779 increases social recognition memory in rats (Van Kampen et al., 2004) and nAChR agonist GTS-21 improves learning and memory in aged rats (Young et al., 2007). Also AZD0328, a nAChR agonist, was shown to improve learning and attentional process in rats (Sydserff et al., 2009).

1.8 The Effects of Nicotine and Nicotinic Agonists: Nicotinic Receptors

Nicotinic acetylcholine receptors are ligand-gated ion channel receptors that increase cellular permeability to Na^+ and Ca^{2+} when activated. As pentameric structures, there are 5 types of α subunits and 3 types of β subunits that nAChRs can be composed of. The majority of striatal nAChRs are α4 and β2 containing nAChRs (α4β2), and this subtype is also most commonly found in dopaminergic terminals (Wonnacott et al., 2000). Depending on the

composition of these receptors, they can have different sensitivities to nicotine (Salminen et al., 2007) and firing patterns (Meyer et al., 2008). Another prevalent nAChR is the α7 receptor expressed at glutamateric terminals (Salminen et al., 2004; Grady et al., 1992). Since medium spiny neurons receive their major inputs from both glutamate and dopamine terminals, nicotine activates striatal signaling by both glutamate and dopamine **(Fig. 5)**.

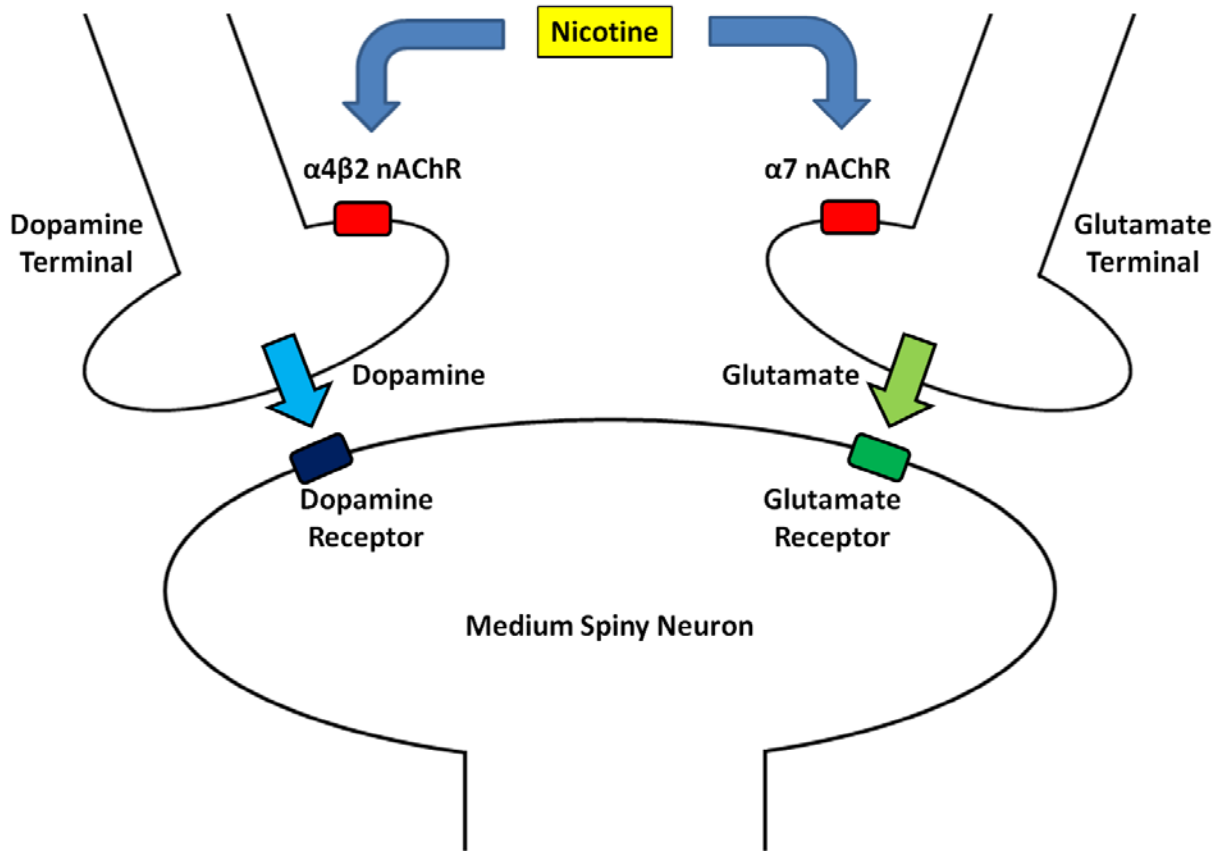


Figure 5. Simplified diagram of nicotine's effect on striatal neurotransmitter release. Nicotine in the striatum activates α7 receptors on glutamate terminals and α4β2 receptors on dopamine terminals. Thus, both neurotransmitters are released to activate the medium spiny neurons when nicotine is administered.

One of the molecular findings after chronic nicotine or nicotinic agonist treatment is the upregulation of nAChR. In vitro, chronic nicotine treatment of SH-SY5Y cells upregulates the expression of α7 and α3 nAChR (Peng et al., 1997). This upregulation by nAChR can also occur in vivo in rats treated with nicotine for 7 days post TBI using osmotic minipumps (Verbois et al., 2003b). Moreover, the TBI induced deficits in nAChR expression are attenuated with choline treatment (Guseva et al., 2008). Delivering nicotine by intraperitoneal injection also increased the number of nAChRs (Verbois et al., 2003a).

1.9 The Effects of Nicotine and Nicotinic Agonists: Striatal Dopamine

Nicotine treatment has been previously shown to induce upregulation of the levels of tyrosine hydroxylase (TH). The contribution of intracellular Ca^{2+} in TH synthesis was shown by a study where blockade of L-type Ca^{2+} channel prevented c-fos and TH mRNA upregulation after nicotinic receptor activation (Craviso et al., 1995). These studies demonstrated that nicotine increases intracellular Ca^{2+} and that TH gene expression was Ca^{2+} dependent. Previous studies also show that dopamine release in the striatum can be enhanced by nicotine (Marshall DL et al., 1997; Rahman SJ et al., 2003) or nAChR agonist treatment (Sydserff et al., 2009).

Activation of nAChRs by nicotine administration in striatal slices leads to either decrease or increase of pDARPP-32-T34 in a dose dependent manner (Hamada et al., 2004). At a lower dose of nicotine (1μM) in mouse neostriatal slices, there was a decrease in pDARPP-32-T34,

which is blocked by pretreatment with D_2 receptor antagonist raclopride. However, at a higher dose of nicotine (100μM), there was an increase in pDARPP-32-T34, which is blocked by pretreatment with D_1 receptor antagonist SCH23390. Thus, this study supports the idea that low concentrations of nicotine induce D_2 receptor dependent decreases in pDARPP-32-T34, and high concentration nicotine induces D_1 receptor dependent increase in pDARPP-32-T34. This dose dependence of nicotine is possible since α7 receptors and α4β2 receptors have different affinities for nicotine. Since α4β2 receptors have much higher binding affinity (K_i = 0.6-10 nM) than α7 receptors (K_i = 400-15,000 nM), low dose nicotine will activate mostly α4β2 receptors, and thus affect dopaminergic terminals. This will stimulate low level dopamine release onto the medium spiny neurons and activate D_2 receptors, which have a higher affinity for dopamine. However, high dose nicotine will activate both α7 and α4β2 receptors. The activation of α7 receptors may lead to release of glutamate from the glutamatergic terminals synapsing on to dopaminergic terminals, causing a more robust release of dopamine. At higher levels of dopamine release, primarily D_1 receptors are activated. Thus, nicotine concentration will determine which of the dopamine receptors become activated and whether pDARPP-32-T34 levels increase or decrease.

Aside from enhancing striatal dopamine signaling, nicotine has been demonstrated to have neuroprotective function in the setting of nigrostriatal injury and Parkinson's disease. Epidemiological studies showed that nicotine administration has a protective effect in the dopaminergic system. The incidence of Parkinson's disease in cigarette smokers is approximately one-half of that of age-matched non smokers (Quik and Kulak, 2002). In parkinsonian animals, nicotine is neuroprotective against degeneration of the nigrostriatal system, and in patients with Parkinson's disease, nicotine attenuates dyskinesia (Quik M et al.,

2009; Huang LZ et al., 2009, Parain K et al., 2003). In animals treated with nicotine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced nigrostriatal dopaminergic neuronal loss was greatly reduced (Janson et al., 1988b, Maggio et al., 1998, Parain et al., 2003). Nicotine administration also reduced the loss of striatal dopamine after hemitransection of nigrostriatal fibers (Janson et al., 1988a; Janson et al., 1991). In a rat model of Parkinson's disease by rotenone, nicotine attenuates motor deficits and dopamine neuronal cell loss in substantia nigra (Takeuchi et al., 2009). Nicotine treatment also prevented striatal dopamine loss, increased dopamine turnover, and dopamine terminal loss induced by 6-hydroxydopamine (Costa et al., 2001; Ryan et al., 2001). Thus, the beneficial effect of nicotine in the setting of striatonigral injury has drawn much interest in its possible use as a therapy.

1.10 Aims

Various studies in the past using pharmacological agents that affect the dopamine system have been found to hasten functional recovery following TBI in both humans and animals. Moreover, proteins that regulate dopamine synthesis, release, and transport are altered following TBI at different time points. Because dopamine signaling has a major role in both motor and cognitive function, this study investigated the specific mechanisms of striatal dopamine deficit and the possibility of reversing this deficit by a pharmacological agent, nicotine. This study was conducted in four phases to answer the major hypothesis: ***TBI induces deficits in striatal dopamine synthesis and release, which impairs postsynaptic signaling, factors that can be reversed by treatment with nicotine.***

To answer the major hypothesis, 4 specific aims were followed throughout this study:

- Aim 1 (Chapter 2): Determine if there are deficits in striatal TH activity and dopamine release after TBI.
- Aim 2 (Chapter 3): Determine if TH activity and dopamine release deficits after TBI are restored by nicotine treatment.
- Aim 3 (Chapter 4): Determine if enhancing TH activity and dopamine release can reverse the deficits in DARPP-32 activation and its downstream molecules.
- Aim 4 (Chapter 4): Determine if enhancing striatal dopamine signaling by nicotine treatment results in cognitive and motor improvement in rats.

2. Alterations in Tyrosine Hydroxylase and Dopamine Release After TBI

2.1 Introduction

Tyrosine hydroxylase is a rate-limiting synthesis enzyme for the catecholamines such as dopamine and norepinephrine. Though TH is responsible for synthesis of both catecholamines, striatal tissue contains very small levels of norepinephrine. Thus, striatal TH represents the presence of dopaminergic fibers (Hokfelt et al., 1977; Anden et al., 1964). Tyrosine hydroxylase is responsible for the conversion of amino acid tyrosine, which is transported across the blood-brain barrier, into L-DOPA inside catecholaminergic neurons. Subsequently, L-DOPA is converted into dopamine by the enzyme AADC. Since the activity level of AADC is much greater than that of TH, L-DOPA is instantaneously converted into dopamine upon synthesis by TH (Vizi and Lajtha, 2008). Due to its rate-limiting activity in dopamine synthesis, TH is one of the most commonly monitored enzymes for studying dopaminergic neuron's function.

Tyrosine hydroxylase regulates catecholamine biosynthesis either by phosphorylation or transcription. Phosphorylation changes the activity levels of TH immediately, but transcriptional regulation to change the protein levels of TH increases the capacity for catecholamine synthesis in the long term. There are four commonly reported sites of phosphorylation in TH: serine residues 8, 19, 31, and 40. Each site has a specific major kinase that regulates its phosphorylation: serine 19 is regulated by $Ca^{2+/}$calmodulin-dependent protein kinase II

(Campbell et al., 1986), serine 31 is regulated by ERK1/2 (Haycock et al., 1992), and serine 40 is regulated by protein kinase A (PKA) (Haycock and Haycock, 1991).

Increased activity of TH has previously been associated with phosphorylation of TH at the serine 40 (pser40TH), and direct activation does not occur by phosphorylation of serine 19 (pser19TH) (Lindgren et al., 2000), (Harada et al., 1996), although the binding of 14-3-3 proteins to pser19TH enhances pser40TH phosphorylation and TH activity (Kleppe et al, 2001; Bobrovskaya et al. 2004). Phosphorylation of serine 40 leads to an increase in TH affinity to tetrahydrobiopterin, a cofactor required for L-DOPA synthesis, causing increased TH activity (Dunkley et al., 2004). Also, phosphorylation of TH leads to changes in its interaction with dopamine: dopamine binds to unphosphorylated TH and inhibits its activity (Daubner et al., 1992), (Gordon et al., 2008). However, when phosphorylation at serine 40 occurs, catecholamine is released from TH. This relieves dopamine's inhibitory feedback on TH, increasing the activity of TH. The activity of TH is also complicated by the possible interactions between phosphorylation sites: serine 19 phosphorylation may alter the conformation of TH such that serine 40 phosphorylation occurs at an increased rate (Dunkley et al., 2004). Thus, a better understanding of TH activity can be gained by investigation of both pser19TH and pser40TH.

The experiments in this chapter focused on **Aim 1:** ***Determine if there are deficits in striatal TH activity and dopamine release after TBI.*** The alteration in dopamine synthesis was first studied by assessing TH levels and TH activity. To investigate both short term and long term changes in dopamine synthesis, total TH levels and phosphorylated TH levels were studied. Striatal TH, pser40TH, and pser19TH levels were monitored by Western blot at 1 day, 1 week,

and 4 weeks after injury. Then, it was followed up using an in-vivo TH activity. A PKA activity assay was also used to associate its role in modulating pser40TH levels. In addition, potassium stimulus-evoked dopamine release was assessed using a combination of microdialysis and high performance liquid chromatography (HPLC).

2.2 Materials and Methods

2.2.1 Animals

One hundred and three male Sprague-Dawley rats (Harlan Laboratories) weighing 280-350 grams were used for this study. All experiments were performed according to the guidelines for Care and Use of Laboratory Animals set by the University of Pittsburgh. The Institutional Animal Care and Use Committee approved all the experiments in this study. Animals were housed in 12 h light/dark cycle with food and water given *ad libitum*.

2.2.2 Surgery

Animals were injured by the controlled cortical impact (CCI) device as previously described (Dixon et al., 1991). The rats were first anesthetized using 5% isoflurane in 2:1 ratio of nitrous oxide: oxygen and endotracheally intubated. After intubation, animals were mounted on a stereotaxic frame and secured by incisor bar and ear bars. Mechanical ventilation maintained anesthesia with 2% isoflurane in 2:1 N_2O/O_2 while the animals were prepared for

surgical exposure of the skull. The head was shaved then cleaned using povidone-iodine. After a midline incision exposed the skull from bregma to lambda, a retractor was placed to expose the skull. Then, parasagittal craniectomy was made with the center of craniectomy at (AP: +4.0 mm, L: +2.8 mm), making a window large enough for 6mm tip of the CCI device. For injured groups, 2.6-3.2 mm deformation depth (severe injury) at 4 m/s was given while sham groups received only craniectomy. After the impact, the incision site was closed with silk sutures and animals were taken off the anesthesia. Animal recovery was monitored until tail pinch and righting reflex returned. After recovery, the animals were returned to the home cages in the animal facility.

2.2.3 Western blot

Rats were anesthetized using sodium pentobarbital (100 mg/kg) and sacrificed by decapitation at 1 day, 1 week, or 4 weeks after surgery (n=6 for each group). Brains were dissected on a chilled ice plate and striata and substantia nigra ipsilateral and contralateral to the injury were frozen in liquid nitrogen and stored at -70˚C until preparation. Tissues were prepared by sonicating in a lysis buffer (0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 μg/ml phenylmethylsulfonyl fluoride, Phosphatase Inhibitor Cocktails 1 and 2 (1:100, Sigma, St. Louis, MO), Protease Inhibitor Cocktail (Complete Mini, Roche Applied Science, Mannheim, Germany)). The sonicated tissues were centrifuged at 13,000 x g for 30 min and supernatants were used for Western blot. Using a BCA protein assay kit (Pierce, Rockford, IL), samples containing 40 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and blocked by 5% bovine serum albumin

(BSA) (Sigma, St. Louis, MO) in 0.05 M TBS with 0.05% Tween-20 (TBST) for 1 h. The membranes were incubated with anti-TH (1:10,000, Millipore) anti-pser19TH (1:1,000, Millipore), or anti-pser40TH (1:1,000, Millipore) overnight, then washed with TBST and incubated for 1 hour at room temperature with anti-mouse or anti-rabbit immunoglobulin G conjugated to peroxidase (1:20,000, Pierce). Membranes were treated for chemiluminescence (Western Lighting, Perkins Elmer, Boston, MA) and TH, pser19TH, and pser40TH signals were visualized by exposing the membranes to autoradiographic X-ray film from 10s to 1 min. Afterwards, membranes were stripped using 100 mM glycine pH 2.3 at 55°C for 1 hour, incubated with anti-β-actin monoclonal antibody (1:10,000, Sigma-Aldrich) for 1 hour, then incubated with anti-mouse immunoglobulin G conjugated to peroxidase. The same steps were taken as described above to visualize β-actin. To measure the optical density of Western blots, Scion Image PC software (Frederick, MD) was used. Optical densities of TH, pser19TH, and pser40TH were normalized by β-actin of each blot, and the values displayed are given as a percentage compared to sham tissue levels for each respective side.

2.2.4 PKA Activity Assay

The activity level of PKA was measured using a commercially available kit (Promega, Madison, WI). Briefly, the ipsilateral tissue homogenates used for Western blots were suspended in PepTag PKA 5x reaction buffer, Peptag A1 peptide, and PKA activator 5x solutions. These mixtures were incubated at room temperature for 30 minutes. The reaction was stopped by placing the mixture in boiling water for 10 minutes. Glycerol (30%, 1 μL) was added to the mixture, and samples were loaded onto 0.8% agarose/Tris-HCl gel. The gels were run at

100 V until separation of phosphorylated and unphosphorylated samples became apparent. The gels were then scanned using Kodak Image Station 440CF. The optical densities of the phosphorylated product from the PKA reaction were then normalized to total protein level in each sample using NIH Image J software. PKA activity levels were reported with respect to sham striatal PKA activity levels.

2.2.5 In-vivo TH Activity Assay

As described in (Urbanavicius et al., 2007), in vivo TH activity was assessed by quantifying L-DOPA accumulation in striatal tissue after inhibiting AADC with 3-hydroxybenzylhydrazine (NSD-1015) (Sigma, St. Louis, MO). Thirty minutes before sacrifice, rats were intraperitoneally injected with NSD-1015 (100 mg/kg, suspended in 0.9% saline). Ipsilateral striata were dissected out, immediately frozen in liquid nitrogen, and stored at -70˚C until neurochemical analysis. On the day of analysis, the tissues were weighed and sonicated in 0.2 M $HClO_2$ at 0.2 mg/μL concentration. The samples were then centrifuged at 13,000 x g for 30 min and supernatants were used to quantify L-DOPA levels by HPLC.

2.2.6 Microdialysis

Artificial cerebrospinal fluid (ACSF) containing 126.5 mM NaCl, 2.4 mM KCl, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 27.5 mM $NaHCO_3$, and 0.5 mM KH_2PO_4 was used for this experiment. The microdialysis probe (SciPro, Sanborn, NY) with the following specification (membrane length: 3mm, diameter: 0.6mm, permeability cut-off: 35kDA) was implanted 1 hour before the

experiment for the long duration stimulus experiment. In the short duration stimulus experiment, the microdialysis probe was implanted one day before the microdialysis experiment. The probe was implanted into the striatum (AP: +0.0 mm, L: +2.8 mm, DV: -4.0 mm, reference point: bregma) and secured with dental cement. The animals were then disconnected from the anesthesia apparatus and placed in a Plexiglas chamber as previously described (Dixon et al., 1997). Microdialysates were collected in awake, freely moving animals. Overnight, ACSF was continuously perfused at 0.2 µl/min. On the day of the experiment, flow rate was adjusted to 2.0 µl/min for 1 hour then samples were collected every 20 minutes into a tube containing 5 µL of 0.3 M $HClO_2$. Samples were immediately analyzed by HPLC. At 60 minutes (4^{th} collection time), ACSF was switched to a high potassium ACSF solution. For short duration stimulus, potassium challenge (80 mM K^+) was stopped and ACSF was infused forty minutes after beginning of stimulus (6^{th} collection time). For long duration stimulus, potassium challenge (100 mM K^+) continued for 180 minutes. After microdialysis, the rats were sacrificed and the locations of the probes were verified. For data analysis, micromolar concentrations of dopamine and dopamine metabolites are reported.

2.2.7 Neurochemical analysis

Neurochemical measurements were made by HPLC with CoulArray Detector using two four-channel analytical cells (ESA, Chelmsford, MA, USA). Eight coulometric electrodes with potentials from -120 to +300 mV in 60 mV increments were used, and a C_{18} column was used to separate the analytes. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were monitored in the microdialysates (Sigma, St. Louis, MO). For TH activity

assay, L-DOPA was monitored by analyzing its levels from striatal homogenates using HPLC. Baseline values of dopamine below the linear range of detection were recorded as 0 μM.

2.2.8 Statistical analysis

The normalized TH activity levels and optical densities of TH, pser19TH, and pser40TH were analyzed by using unpaired Student's T test to compare each group's sham ipsilateral side to injured ipsilateral side and sham contralateral side to injured contralateral side. Microdialysis data for DOPAC and HVA were analyzed using repeated measures ANOVA. For potassium evoked dopamine release, the peak values were used to compare the sham injured and CCI injured animals. Statistical analysis for microdialysis data was performed using a Mann-Whitney U test. All statistical calculations were performed by using PASW Statistics 19 (SPSS Inc., Chicago, IL) software.

2.3 Results

2.3.1 Total TH Levels in Striatum and Substantia Nigra After TBI:

Striatal levels of TH did not show significant changes at any of the time points for both ipsilateral and contralateral sides (**Fig. 6**). However, there was a decrease in TH levels in the ipsilateral injured substantia nigra compared to sham substantia nigra at 1 week after injury (ipsilateral sham: 100.0±10.6%, ipsilateral injured:72.6±8.7%, $p \leq 0.05$).

For pser19TH levels, no statistically significant differences were found at any of the time points in striatal tissues (**Fig. 7**). For substantia nigra, no difference between sham and injured groups were found for the 1 day time point. However, substantia nigra showed a significant decrease at 1 week (ipsilateral sham: 100.0±11.6%, ipsilateral Injured: 62.7±3.6%, $p \leq 0.05$), similar to the pattern of TH deficit specific to substantia nigra at 1 week (**Fig. 6**). This deficit is reversed by 4 weeks, with no significant differences seen in both ipsilateral and contralateral sides.

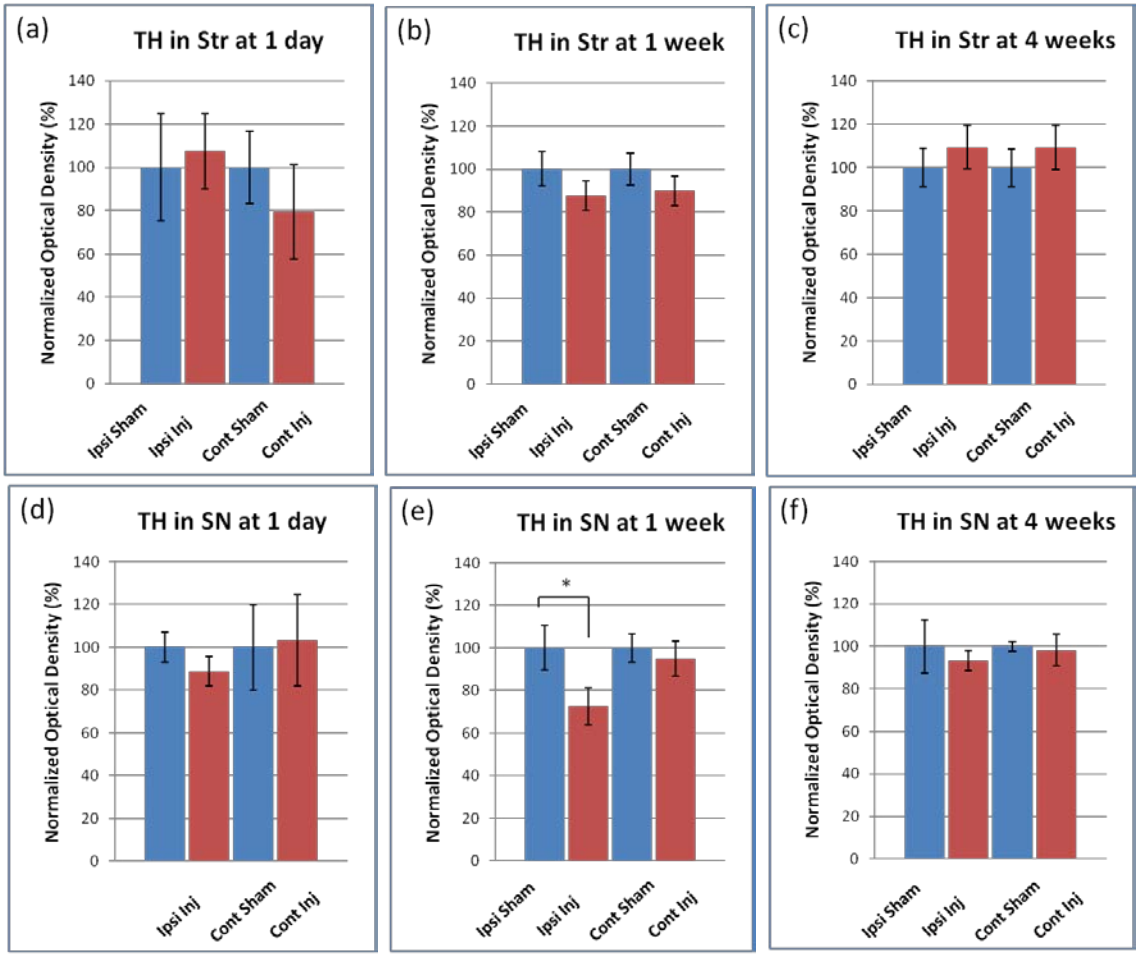


Figure 6. Western blot measurement of TH protein levels were analyzed for the striatum (a-c) and substantia nigra (d-f). The striatal protein levels of TH at 1 day, 1 week, and 4 weeks showed no significant changes in injured groups compared to sham groups for ipsilateral and contralateral sides. There was a significant decrease in TH in ipsilateral injured substantia nigra at 1 week after TBI, but not at 1 day and 4 weeks. Ipsi=ipsilateral, Contra=contralateral, Inj=injured, *$p \leq 0.05$.

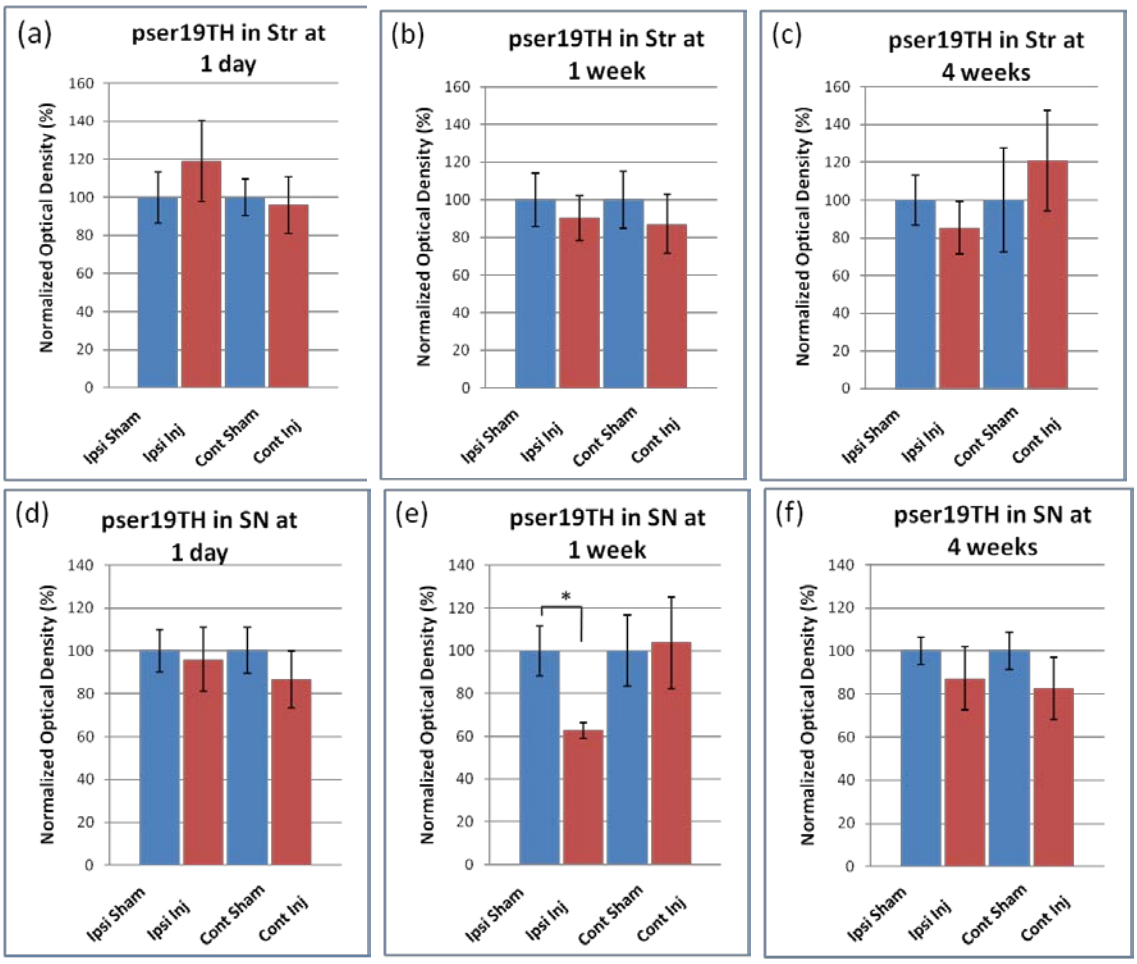


Figure 7. Western blot measurement of levels of pser19TH in the striatum (a-c) and substantia nigra (d-f). The only significant decrease was found in ipsilateral injured substantia nigra compared to sham substantia nigra at 1 week post injury. This TBI induced deficit is transient as there is recovery of pser19TH at 4 week time point. Ipsi=ipsilateral, Contra=contralateral, Inj=injured, *$p \leq 0.05$.

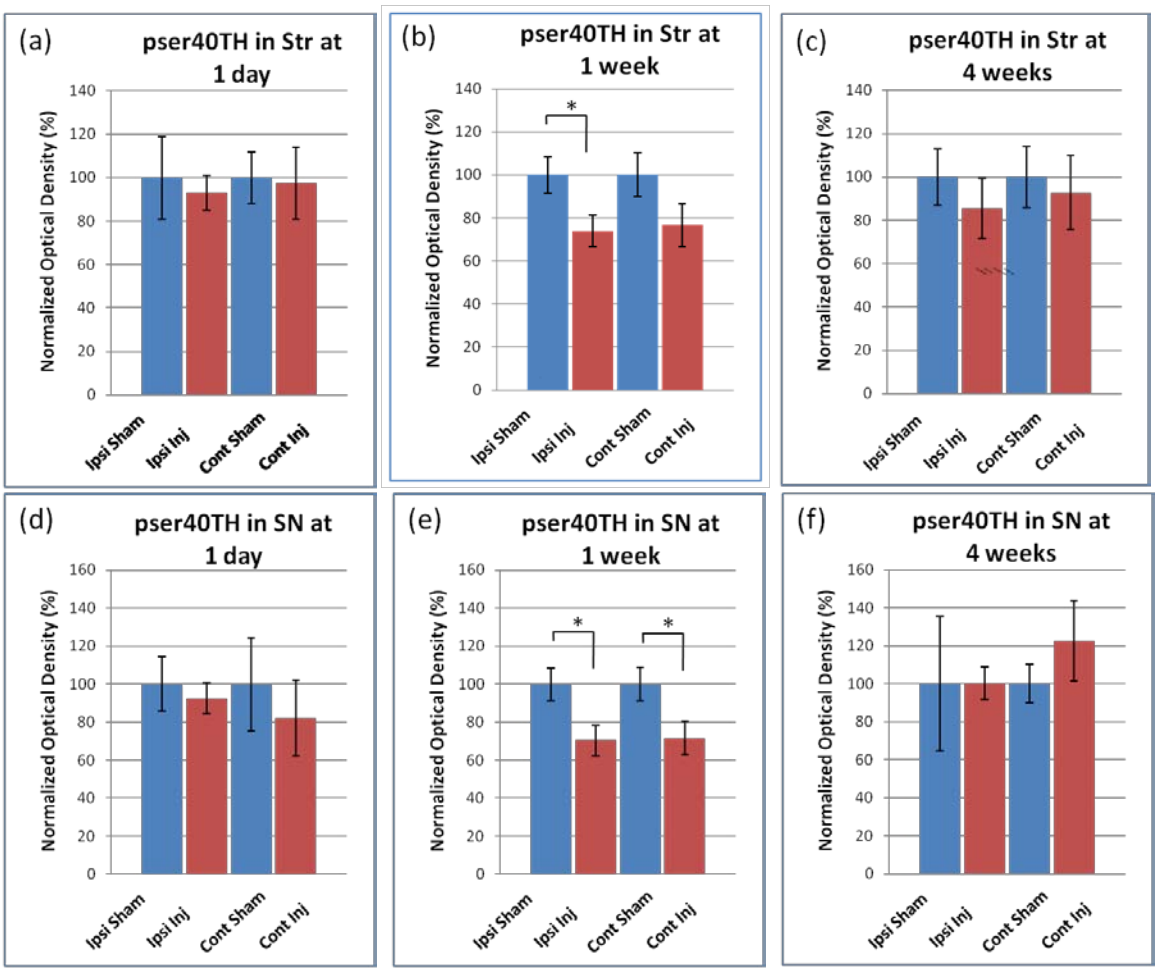


Figure 8. Western blot data showing pser40TH levels in the striatum (a-c) and substantia nigra (d-f). At 1 week, significant decreases in pser40TH levels were seen in the injured striatum (b) and substantia nigra (e). Ipsi=ipsilateral, Contra=contralateral, Inj=injured, *$p \leq 0.05$.

For pser40TH levels (**Fig. 8**), there were no alterations at 1 day. At 1 week, pser40TH of ipsilateral injured striatum showed a significant decrease (ipsilateral sham: 100 ± 8.5 %, ipsilateral injured: 73.9 ± 7.3%, $p \leq 0.05$). By 4 weeks, t his difference was no longer significant. Similarly, ipsilateral substantia nigra showed decreased pser40TH levels at 1 week (ipsilateral

sham: 100.0±10.3%, ipsilateral injured: 71.6±8.8%, p≤0.05). Contralateral side also showed a similar decrease (contralateral sham: 100.0±10.3%, contralateral injured: 71.6±8.8%, p≤0.05).

2.3.2 Timecourse of TH Activity Assay

After Inhibition of AADC activity by NSD-1015 injection, L-DOPA accumulation in striatal tissue was detected by HPLC. The accumulation of L-DOPA was used to assess TH activity level. At 1 day after CCI (**Fig. 9a**), there is a no statistically significant change in TH activity (sham: 100.0 ± 9.7 %, injured: 138.0 ± 17.9 %). At 1 week after CCI (**Fig. 9b**), TH activity was decreased significantly in the CCI injured animals compared to sham animals. (sham: 100.0 ± 10.6%, injured: 62.1 ± 8.2%, p≤0.05). The activity levels of TH remains decreased up to 4 weeks (sham: 100.0 ± 5.2%, injured: 68.8 ± 6.2%, p≤0.05), (**Fig. 9c**).

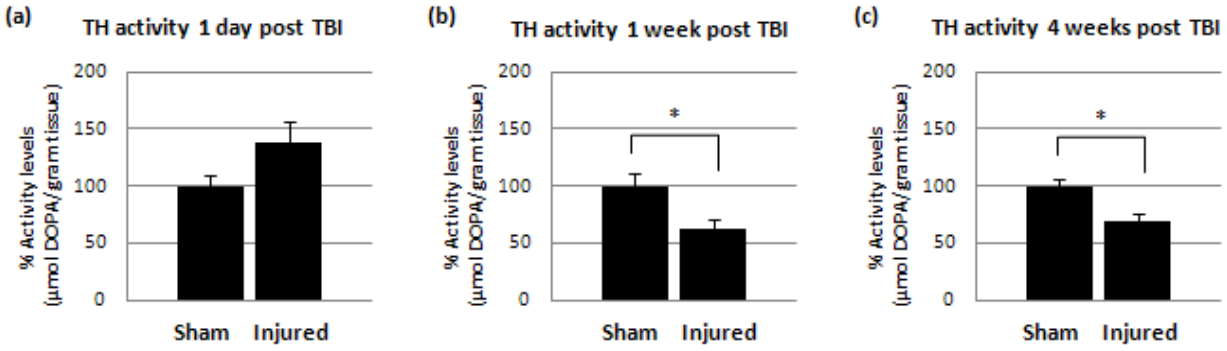


Figure 9. TH activity assay. The results of in-vivo TH activity assay at 1 day (a), 1 week (b), and 4 weeks (c) after injury are shown. Each group's striatal DOPA levels are displayed ± SEM (n=6). At 1 day after injury, there is a trend but no statistically significant

increase in TH activity of CCI injured rats compared to sham rats. At 1 week and 4 weeks after injury, there is a decrease in TH activity in injured rats compared to sham rats ($p \leq 0.05$). *$p \leq 0.05$.

2.3.3 Timecourse of PKA activity assay

The ipsilateral activity levels of PKA in the striatum after TBI was determined at 1 day, 1 week, and 4 weeks (**Fig. 10**). There was no significant difference between sham and injured striatum at 1 day (sham: 100.0 ± 5.9 %, injured: 110.0 ± 5.2 %). At 1 week, there is decreased PKA activity in injured rats (sham: 100.0 ± 4.2 %, injured: 87.8 ± 2.8%). This decrease was reversed by 4 weeks, and no significant changes were shown in injured rats compared to sham rats (sham: 100.0 ± 3.2%, injured: 102.3 ± 6.0%).

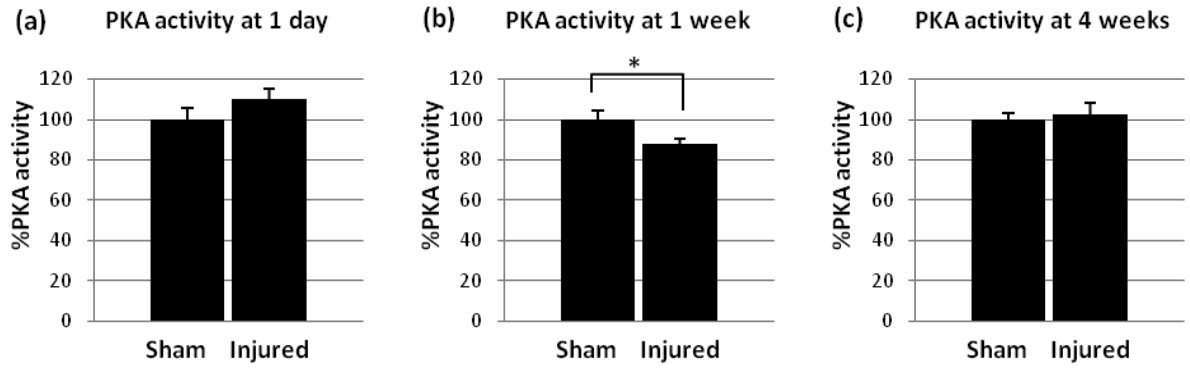


Figure 10. PKA activity assay for striatal tissue. PKA activity levels at 1 day (a), 1 week (b), and 4 weeks (c) after injury are normalized by sham striatal PKA activity. There were no significant differences between injured and sham rats at 1 day and 4 weeks. However, 1 week time point showed significant decrease in injured PKA activity levels compared to shams. *$p \leq 0.05$.

2.3.4 Microdialysis: Long Duration Potassium Stimulus

Striatal dopamine release and extracellular dopamine metabolite levels at 1 week time point was first studied to characterize injury induced dopamine dysregulation. Microdialysis was used in conjunction with high performance liquid chromatography (HPLC). One hour after implantation of the microdialysis probe, the experiment began. A K^+ stimulus of 100 mM was given for 180 minutes. Maximal dopamine release was achieved at 40 minutes after stimulus application (**Fig. 11**). However, no statistically significant difference was found between sham and injured animals (sham: 0.268 ± 0.039 μM, injured: 0.276 ± 0.058 μM). Also, analysis of DOPAC and HVA levels showed no significant differences between sham and injured groups.

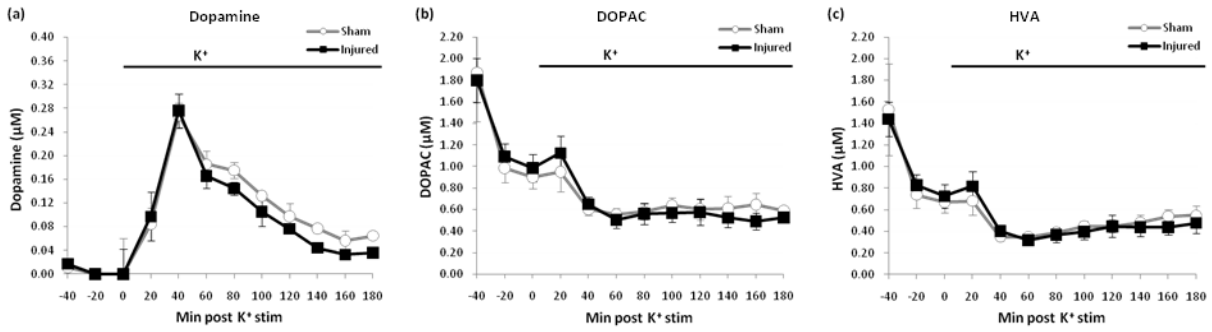


Figure 11. Long duration stimulus at 1 week. Animals were compared for their response to 180 minute long 100mM K^+ stimulus after sham or CCI injury. There were no significant differences between the two groups for dopamine (a), DOPAC (b), and HVA (c). Black bar represents duration of K^+ stimulus.

2.3.5 Microdialysis: Short Duration Potassium Stimulus

In the second attempt to characterize the dopamine release, the protocol was refined to implant the probe the day before the experiment in order to reduce the effects of acute injury by probe placement. On the day of the experiment, 80mM K^+ stimulus was given for 40 minutes to induce dopamine release.

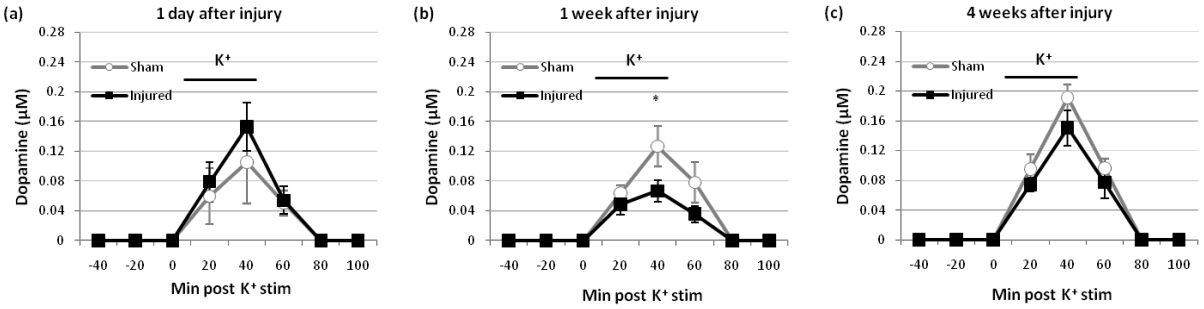


Figure 12. Potassium stimulated dopamine release. The levels of dopamine in microdialysates were quantified by HPLC at 1 day (a), 1 week (b), and 4 weeks (c). Potassium stimulus duration is labeled as a line between 4th and 5th collection times. There is no significant alteration at 1 day between CCI injured rats (n=8) and sham injured rats (n=8). At 1 week, there is a statistically significant decrease in peak dopamine levels of CCI injured rats (n=10) compared to sham injured rats (n=10). By 4 weeks, there is no difference between CCI injured (n=7) and sham rats (n=7). *$p \leq 0.05$.

At 1 day after CCI (**Fig. 12a**), the peak dopamine levels of injured striata after 80 mM potassium stimulus were not statistically different from the peak dopamine levels of sham striata (sham: 0.105 ± 0.032 μM, injured: 0.153 ± 0.056 μM). At 1 week after CCI (**Fig. 12b**), the peak

dopamine levels of injured striata showed a statistically significant decrease compared to that of sham striata (sham: 0.127 ± 0.027 μM, injured: 0.067 ± 0.015 μM, $p \leq 0.05$). By 4 weeks after CCI, there are no significant differences between the sham and injured groups for peak dopamine levels (sham: 0.191 ± 0.017 μM, injured: 0.151 ± 0.024 μM) (**Fig. 12c**).

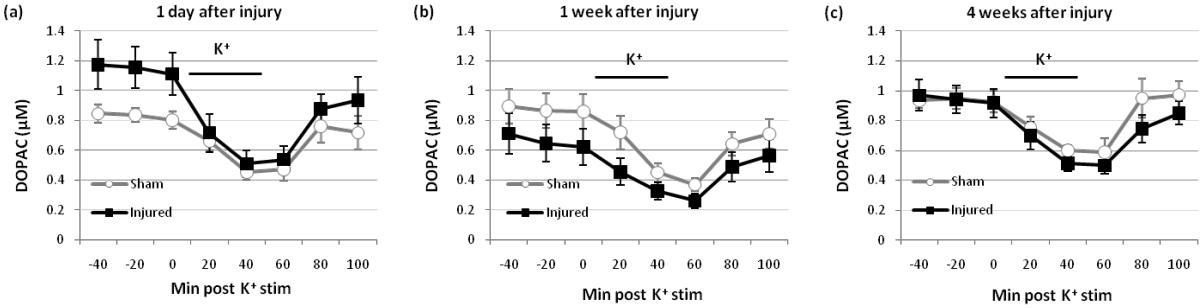


Figure 13. DOPAC measured by microdialysis. The levels of dopamine metabolite DOPAC are quantified by HPLC at 1 day (a), 1 week (b), and 4 weeks (c) after injury. There were no significant differences between CCI injured rats and sham rats at any time point.

There were no significant differences between sham and injured striatal levels of DOPAC (**Fig. 13a**) and HVA (**Fig. 14a**) as compared by repeated measures ANOVA at 1 day. These dopamine metabolites also showed no significant differences between sham and injured animals at 1 week (**Fig. 13b, 13c**) or 4 weeks after injury (**Fig. 13c, 14c**).

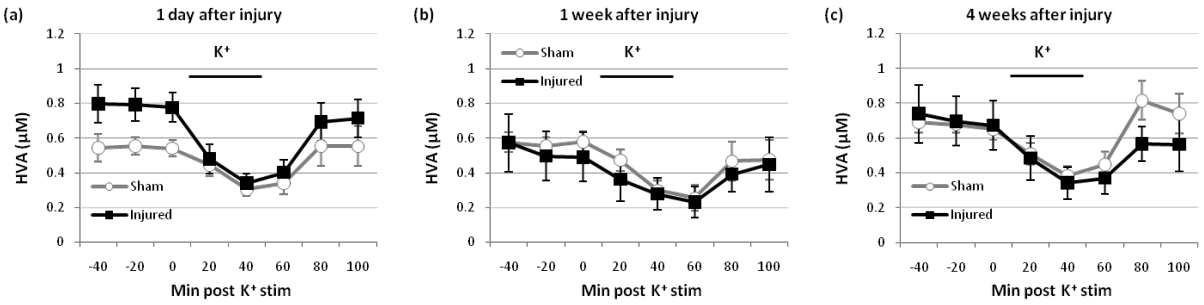


Figure 14. HVA measured by microdialysis. The levels of dopamine metabolite HVA are quantified by HPLC at 1 day (a), 1 week (b), and 4 weeks (c) after injury. Repeated measures ANOVA found no significant differences between sham and injured groups at any of the 3 time points.

2.4 Discussion

In this chapter, we demonstrated for the first time a deficit in striatal TH activity 1 week (subacute) and 4 weeks (chronic) after TBI in rats as indicated by decreased tissue levels of L-DOPA. There was decreased pser40TH in the injured striatum at 1 week but not at 4 weeks. Neither pser19TH nor TH levels in striatum were altered by injury at any of the time points. However, substantia nigra content of pser40TH, pser19TH, and TH levels were all decreased in injured animals at 1 week. Since TH is a rate limiting enzyme in dopamine synthesis, the decrease in its activity suggests a dopamine synthesis deficit. However, a previous report of striatal dopamine content after TBI demonstrates no significant change at 1 week or 4 weeks after injury (Massucci et al., 2004). Dopamine levels depend on both synthesis and degradation. Therefore, activities of monoamine oxidase and catechol-O-methyl transferase may be decreased

at these time points, reducing the degradation of dopamine. Future studies are needed to elucidate TBI induced alterations of various synthesis and metabolizing enzymes for dopamine. The Western blot results of striatal pser40TH suggest decreased activity of TH, since the level of pser40TH correlates with enzymatic TH activity (Lindgren et al., 2000; Harada et al., 1996; Waymire et al., 1988). However, the level of striatal pser19TH showed no significant decrease. Because a decrease in pser40TH level is consistent with a decreased activity of TH, these data suggest that there is TH activity deficit in injured animals at 1 week time point.

The role of pser40TH in TH activity levels are also supported by dopamine autoreceptor studies. Activation of autoreceptors at dopamine terminals reduces TH phosphorylation and dopamine synthesis (Wolf and Roth, 1990). Specifically pser40TH is reduced, but not pser19TH or pser31TH (Lindgren et al., 2001), indicating that pser40TH is responsible for TH activity. Also, some studies show that TH activity does not correlate with the level of pser19TH (Jedynak et al., 2002), but a stronger correlation with pser40TH levels (Sutherland et al., 1993). However, pser19TH levels may induce conformational change in TH such that phosphorylation at serine 40 site increases (Bevilaqua et al., 2001; Dunkley et al., 2004). Thus, pser19TH levels may contribute indirectly to TH activity levels. The current Western blot data shows decrease in TH, pser19TH, and pser40TH levels in substantia nigra after injury. Collectively, these results suggest the possibility of dopamine synthesis deficit in nigrostriatal neurons.

In this study, decreased activity of PKA is demonstrated at 1 week after TBI. However at 1 day and 4 weeks, there are no significant changes. Because PKA is a major regulator of the serine 40 phosphorylation of TH, the activity level of PKA is in agreement with pser40TH levels

at 1 day, 1 week, and 4 weeks after injury. Deficits in PKA activity at 1 week after injury may cause decreased TH phosphorylation, which could then reduce TH activity. It should also be noted that other kinases such as protein kinase C and protein kinase G may also regulate pser40TH levels (Dunkley et al., 2004), although less extensively characterized than PKA's effect is on pser40TH. At 4 weeks, there is a recovery of PKA activity and pser40TH levels in injured striatum, but TH activity deficits are still present (**Fig. 9c**). This suggests that the TH activity assay may be more sensitive to detecting injury induced changes compared to phosphorylation of TH by Western blotting.

A decrease in PKA activity has been previously reported in the parietal cortex and hippocampus at acute time points (15 minutes to 48 hours) using a fluid percussion injury model (Atkins et al., 2007). In contrast, pser40TH level and PKA activity in our study does not decrease at 1 day after injury. However, this study and our current study are not directly comparable due to the differences in brain regions studied (hippocampus and cortex vs. striatum) and injury models (fluid percussion vs. CCI). The directionality of PKA activity change may depend strongly on each of these factors.

Dopamine release induced by potassium stimulation has been used to compare differences in young and aged rats (Shui et al., 1998; Stanford et al., 2000) and different dietary treatments (Agut et al., 2000; Wang et al., 2005). Consistent with these previous studies, potassium stimulation induces dopamine release and decreases extracellular DOPAC and HVA concentrations in our current data. Dopamine release is not significantly altered at 1 day after injury but shows a decrease at 1 week after CCI injury compared to sham injured animals. These

data are in agreement with a previous study showing differences in dopamine release evoked by electrical stimulation of the medial forebrain bundle 2 weeks after CCI injury compared to naïve animals. In that study, dopamine levels were detected by fast scanning cyclic voltammetry, and injured animals have reduced dopamine release compared to sham animals (Wagner et al., 2005). By 4 weeks, there is a recovery of dopamine release in the injured animals in the current study.

Potassium stimulated dopamine release depends on newly synthesized dopamine, since depletion of vesicular stores of dopamine using reserpine does not alter the potassium evoked dopamine release (Fairbrother et al., 1990). Thus, our microdialysis data showing decrease in dopamine release associated with a dopamine synthesis deficit at 1 week is consistent with a decrease in TH activity shown by in vivo activity assay and pser40TH Western blot results.

A high intensity stimulus was applied for the microdialysis experiment in section 2.3.4: 100mM K^+ stimulus for 3 hours. Since high potassium concentration and long duration of stimulus would drive the striatal dopamine synthesis and release to the maximum capacity, this experiment was attempted to maximize the differences between sham and injured groups. The dopamine release levels gradually decreases throughout the duration of the stimulus, similar to the previous microdialysis experiment using multiple K^+ stimulus showing gradual decrease in dopamine release with each stimulus (Dluzen et al., 1991; Kematani et al., 1995). This decrease occurs due to depletion of the dopamine stores. However, the high intensity potassium stimulus did not show any difference between sham and injured groups.

The protocol for potassium evoked dopamine release was changed because there was a concern that acute injury to the brain tissue upon implantation may be a confounding variable that reduced the difference in dopamine release between the two groups. Because microdialysis probe implantation is damaging to the brain tissue, sufficient time is necessary for the dopamine system to recover before starting the experiment (Shui et al., 1998). It was previously demonstrated that dopamine release on the day of the probe implantation or the day after implantation had different responses to tetrodotoxin sensitivity, an indicator of damage for brain tissue (Westerink and De Vries, 1988).

In the current study, there was a significant difference between sham and injured groups in the short duration experiment, demonstrating that this recovery time is important for distinguishing TBI induced effects in dopamine release. In addition, the stimulus was adjusted to a shortened duration like the previous studies (Shui et al., 1998; Stanford et al., 2000; Agut et al., 2000; Wang et al., 2005) in order to compare the results to these studies. The dialysate levels of dopamine and dopamine metabolites followed the same pattern of release as reported in these studies, with the dopamine levels increasing and metabolite levels decreasing upon potassium stimulus.

The results of this chapter indicate that there are decreased striatal TH activity and dopamine release at a subacute time point (1 week) after TBI. These may be important contributors to downstream deficits in postsynaptic mediators of dopamine signaling such as pDARPP-32-T34, leading to functional deficits after TBI. In the following chapter, experiments using nicotine to improve presynaptic dopamine neurotransmission will be described.

3. The Effects of Nicotine on Presynaptic Dopamine Signaling After TBI

3.1 Introduction

In the previous chapter, the presynaptic deficits in nigrostriatal dopamine signaling have been identified to be 1) decreased nigrostriatal TH phosphorylation and activity, and 2) decreased striatal dopamine release. Other members of the Dixon laboratory have previously demonstrated postsynaptic deficits in pDARPP32-T34 and increased PP-1 activity in striatum at 2 weeks after TBI as discussed in Chapter 1. The goal this study at this point was to further examine the role of dopamine synthesis and release deficit contributing to postsynaptic dopamine signaling deficits after TBI by using a pharmacological intervention that normally enhances dopamine signaling.

Nicotine's effect on the dopamine system has been demonstrated in the past studies. Experiments using both striatal slices (Wonnacott et al., 2000) and synaptosomes (Grady et al., 1992) showed nicotine to induce dopamine release. Nicotinic receptors' presence in presynaptic locations with diversity in subunit composition has been demonstrated (Wonnacott, 1997). Different subtypes of nicotinic receptors are present in dopaminergic terminals compared to nondopaminergic terminals, possibly causing different pharmacological characteristics (Zoli et al., 2002). As ligand-gated ion channels, nicotinic receptor activation leads to Na^+ influx and neuronal depolarization. This depolarization will then lead to activation of voltage gated Ca^{2+} channels and eventually induce dopamine release into the synapse (Grady et al., 2007).

Nicotine treatment also increases TH activity and TH levels in vivo as well as in vitro (Carr et al., 1989; Sabban et al., 2002; Hiremagalur et al., 1993). Even in the setting of chemical insult to the nigrostriatal neurons by 6-hydroxydopamine, dopamine levels (Costa et al., 2001) and TH activity (Urbanavicius et al., 2007) were protected by nicotine treatment. In addition, chronic nicotine injections have been demonstrated to enhance nicotine induced dopamine release in the striatum (Marshall et al., 1997) and VTA (Rahman et al., 2003) as measured by microdialysis.

In this chapter, the effect of nicotine treatment on striatal presynaptic dopamine signaling was assessed. The experiments focused on achieving **Aim 2:** ***Determine if TH activity and dopamine release deficits after TBI are restored by nicotine treatment.*** Rats underwent nicotine treatment following injury in order to reverse TBI induced deficits in TH phosphorylation and activity, as well as dopamine release.

3.2 Materials and Methods

3.2.1 Animals

One hundred and thirteen Sprague-Dawley rats (Harlan Laboratories) weighing 280-350g were used in this study under the guidelines set by the Institutional Animal Care and Use Committee of University of Pittsburgh. Animals were housed in 12-h light/dark cycle with food

and water given *ad libitum.* Animals were grouped into 4 conditions: Sham Saline, Injured Saline, Sham Nicotine, and Injured Nicotine. Each group had n = 6-7 (TH activity assay), n = 5-6 (microdialysis), and n=6-7 (Western blot).

3.2.2 Surgery and Drug Administration

Animals assigned to injury groups received TBI using the controlled cortical impact (CCI) device as previously described (Dixon et al., 1991). Briefly, animals were first anesthetized using 5% isoflurane. Intubation was performed, and animals were mounted on a stereotaxic frame. Anesthesia was maintained using 2% isoflurane in 2:1 N_2O/O_2 and parasagittal craniectomy was performed to expose the brain to allow access for the impactor tip of CCI device. TBI was induced at 4 m/s and 2.6 mm deformation (severe injury). After injury, the surgical area was closed by silk sutures and animal recovery was monitored by watching for tail pinch and righting reflexes. Sham injured animals underwent craniectomy only and no CCI. After the surgery and postoperative recovery, animals were returned to the housing facility.

Rats were intraperitoneally injected with either 0.9% saline or nicotine (2.0 mg/kg, suspended in 0.9% saline) starting 24 hours after surgery. The injection schedule continued twice daily for 7 days. At the end of the 7 days, animals were sacrificed and striata were dissected out and immediately frozen in liquid nitrogen.

3.2.3 Microdialysis, TH Activity Assay, Western Blot

All procedures were the same as those described in Chapter 2. In addition, microdialysis data for dopamine was analyzed by calculating the area under the curve for the dopamine peaks after potassium stimulus.

3.3 Results

3.3.1. Dose Determination

Since the major regulator of dopamine signaling in medium spiny neurons is DARPP-32, the phosphorylation of striatal DARPP-32 was evaluated as the major outcome of nicotine induced enhancement of dopamine signaling. The preliminary experiment to decide the optimal dose began by using previously reported doses of nicotine that increased striatal TH activity (Carr et al., 1989), dopamine release using microdialysis (Bednar et al., 2004), (Marshall et al., 1997), and improvement in locomotor activity (Ksir et al., 1987; Ksir et al., 1985) and spatial memory (Verbois et al., 2003) ranging from 0.1-0.5 mg/kg.

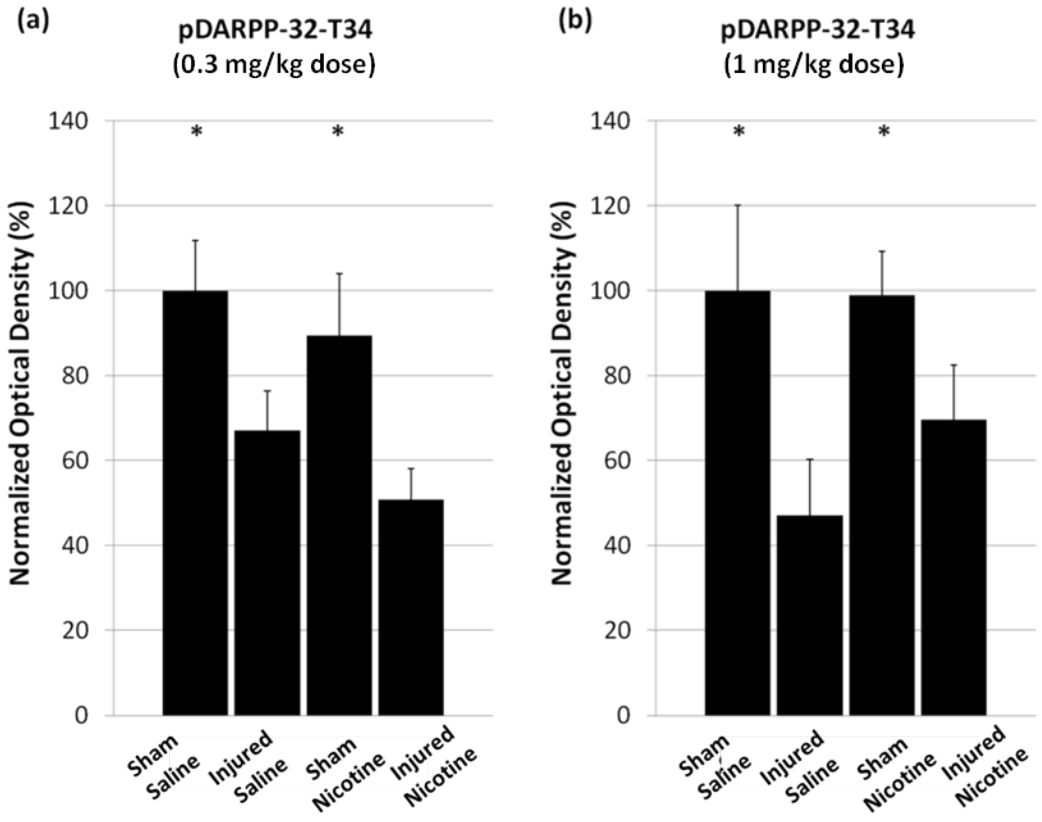


Figure 15. Nicotine at 0.3 mg/kg (a) and 1 mg/kg (b) were intraperitoneally injected into rats for 7 days, twice daily. There were significant decreases in the level of pDARPP-32-T34 for injured animals that were treated with saline or nicotine. (*$\leq$0.05 compared to Injured Saline group)

This preliminary experiment using either 0.3 mg/kg dose or 1.0 mg/kg dose showed a minor improvement in the level of striatal pDARPP-32-T34 at a higher dose of 1.0 mg/kg. Although the Injured Nicotine group was not significantly different from the Injured Saline group at either concentration, there was a modest trend of increased pDARPP-32-T34. Based on these data, and previously reported studies showing increased pDARPP-32-T34 levels in striatal slice culture only when high nicotine concentration was applied (100μM) (Hamada et al., 2004),

nicotine at 3.0 mg/kg was used for one animal. However, this dose induced temporary changes in the respiratory pattern of the animal lasting minutes. Due to the fact that high dose of nicotine can affect neuromuscular junctions, possibly affecting respiratory muscles of rats, the test dose had to be lowered below 3.0 mg/kg. A dose of 2.0 mg/kg was tested, and no respiratory pattern changes were noted in the animals with 2.0 mg/kg nicotine injection. In the following experiments using nicotine injection, 2.0 mg/kg dose was used.

3.3.2 Microdialysis Analysis of Dopamine and Dopamine Metabolites

Dopamine levels in the microdialysates were analyzed by two different methods: by peak dopamine levels and dopamine area under the curve. Peak dopamine levels showed significant between-group differences ($F_{3,17}$=4.372; $p \leq 0.05$). The Injured Saline group had a significant decrease in dopamine release compared to the Sham Saline group, confirming the findings shown in Chapter 2. Nicotine treatment in the Sham Nicotine group induced no significant change from Sham Saline. Also, the Injured Nicotine group showed no significant difference in peak dopamine levels compared to the Sham Saline group, demonstrating nicotine's effect in increasing striatal dopamine release.

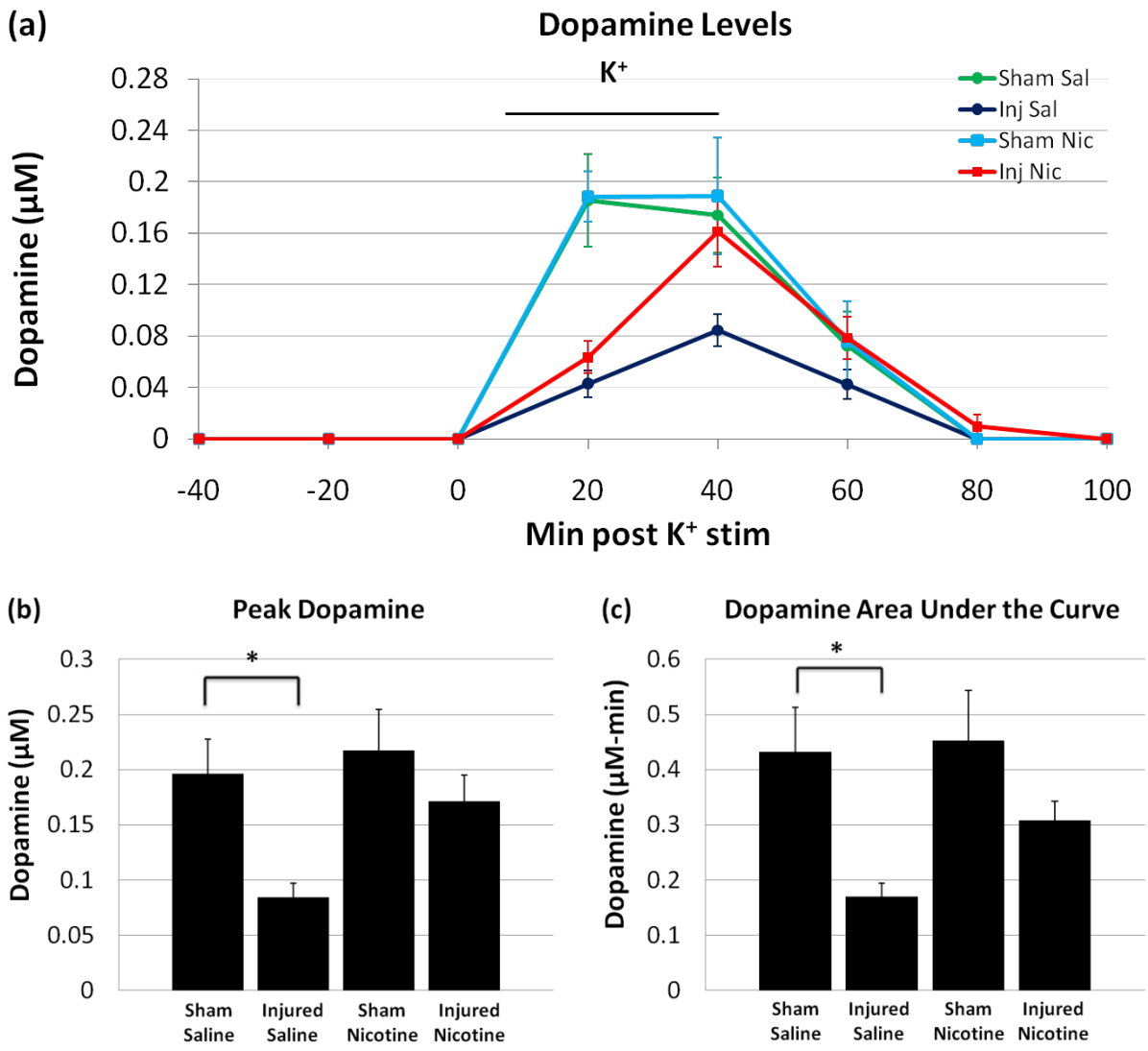


Figure 16. Microdialysis for dopamine. High potassium ACSF was infused for 40 minutes (represented by a black line). Injured Saline group had decreased peak dopamine release compared to Sham Saline group (a), (b). The dopamine area under the curve showed the same result, with Injured Saline group having significantly decreased value compared to Sham Saline group (c). (Inj = Injured, Sal = Saline, Nic = Nicotine, * $p \leq 0.05$ compared to Injured Saline group)

Dopamine area under the curve was analyzed for each of the four groups to assess for differences in dopamine levels over the entire duration of the potassium stimulus. There were overall significant differences among the groups ($F_{3,17}$=4.241; $p \leq 0.05$). The Injured Saline group had significantly lower dopamine area under the curve compared to the Sham Saline group ($p \leq 0.05$). However, the Injured Nicotine group was not significantly different from the Sham Saline group, similar to the dopamine peak level comparisons.

Microdialysis of dopamine metabolites showed no significant changeamong the four groups (DOPAC: $F_{3,17}$=0.824, $p>0.05$; HVA: $F_{3,16}$=0.316, $p>0.05$). Thus, nicotine reverses the dopamine deficit induced by TBI but induces no effect on extracellular dopamine metabolite levels.

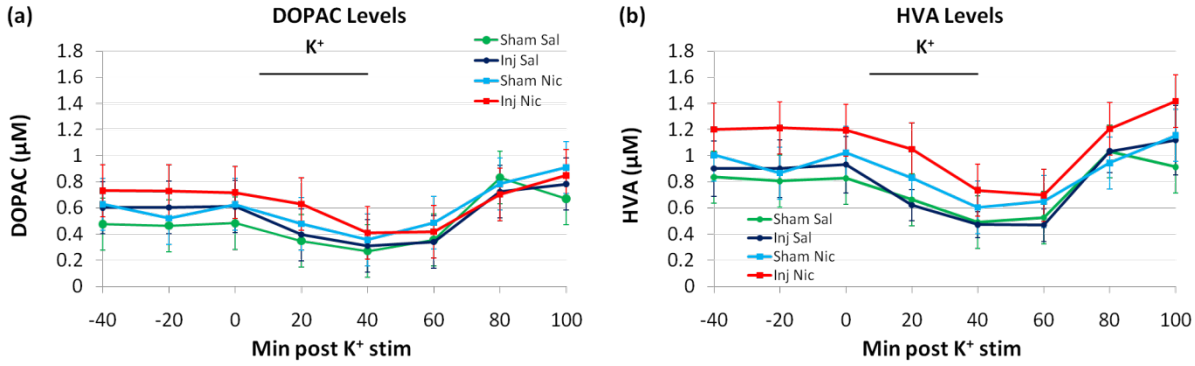


Figure 17. Microdialysis for dopamine metabolites. High potassium ACSF infusion induced transient decreases in DOPAC (a) and HVA (b) levels in microdialysates. When the stimulus was stopped, the levels of metabolites returned to baseline. Repeated measures ANOVA found no significant between group differences for both DOPAC and HVA. (Inj = Injured, Sal = Saline, Nic = Nicotine)

3.3.3 Striatal TH Activity After Nicotine Treatment

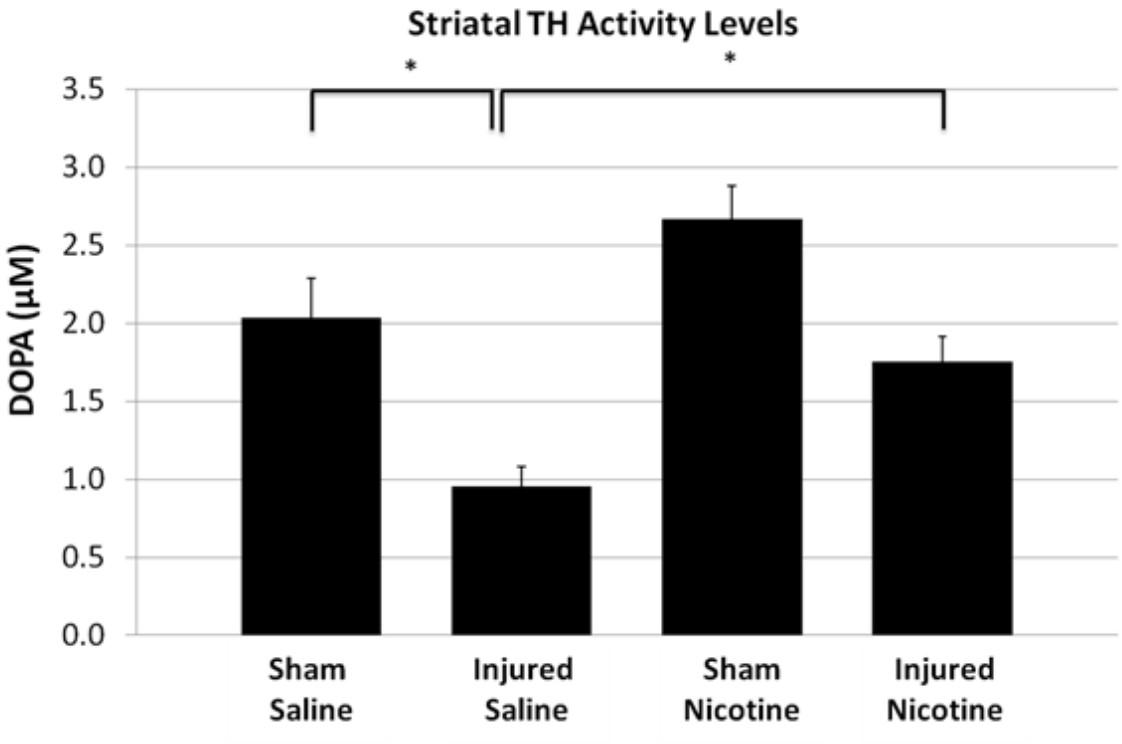


Figure 18. TH activity assay. DOPA accumulation in striatal tissue after injection of NSD-1015 was monitored using HPLC. Injured Saline group had significant decrease in TH activity level compared to Sham Saline group, and Injured Nicotine had statistically significant increase compared to Injured Saline group. (* $p \leq 0.05$)

The between group statistics showed significant differences in TH activity among the four groups ($F_{3,23}=14.360$, $p \leq 0.05$). The TH activity level of Injured Saline group was decreased compared to Sham Saline group ($p \leq 0.05$). With nicotine treatment, injury induced deficit in TH

activity was reversed: Injured Nicotine group had significantly higher TH activity levels compared to Injured Saline group ($p \leq 0.05$).

3.3.4 Tyrosine Hydroxylase Protein and Phosphorylation Levels

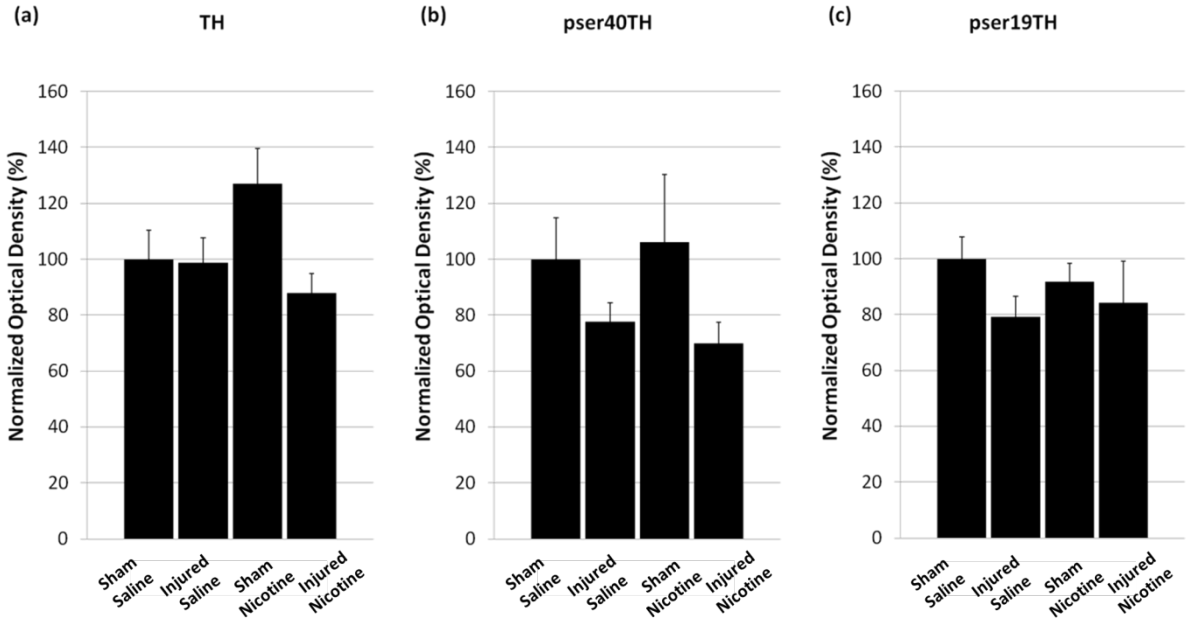


Figure 19. Western blots of TH, pser40TH, and pser19TH. There was no significant effect of nicotine in the levels of TH or its phosphorylation at 1 week after injury.

The protein levels of TH in Sham Nicotine group had an increased trend compared to the rest of the groups, but there was no statistically significant differences among the four groups ($F_{3,20}=3.068$; $p>0.05$). Similarly, there were no significant between group differences for pser19TH ($F_{3,21}=0.740$; $p>0.05$) and pser40TH ($F_{3,16}=1.329$; $p>0.05$).

3.4 Discussion

In this chapter, it was demonstrated that nicotine treatment following TBI induces recovery of TH activity and dopamine release as measured by microdialysis. However, phosphorylation of TH at both serine 19 and serine 40 was not altered, this apparent discrepancy may be due to the fact that the in-vivo activity assay is a more sensitive measure of TBI effects on dopamine compared to TH phosphoryaltion as measured by Western blots. This difference will be explained in more detail below. Overall, the current nicotine regimen following TBI appeared to improve presynaptic dopamine signaling: synthesis and release.

The peak dopamine levels showed that nicotine treatment induces recovery of dopamine release in severly CCI injured animals, and that nicotine treatment of sham animals does not significantly change potassium evoked dopamine release as compared to saline treated sham animals. Also, nicotine treatment induced partial recovery of dopamine release measured by dopamine peak and dopamine area under the curve. This recovery was not large enough to show a significant difference in the Injured Nicotine group when compared to the Injured Saline group, but there was no statistically significant difference between Injured Nicotine and Sham Saline groups. The graph showing the time course of dopamine release (**Fig. 16a**) demonstrates this partial recovery: although maximum dopamine level for the Injured Nicotine group was close to that of Sham Saline group at 40 minutes after potassium stimulus began, the delay in dopamine release can be seen at 20 minutes in the Injured Nicotine group (0.063±0.012μM), which shows much lower dopamine release compared to the Sham Saline group (0.186±0.036μM)

($F_{3,17}$=13.163 $p \leq 0.05$). Thus, despite the improvement in peak dopamine release by nicotine, there is still a partial temporal delay in dopamine release as a result of injury.

Past studies showed increased nicotine evoked striatal dopamine release in nicotine treated animals (Marshall et al, 1997). Unlike our current experiment utilizing potassium stimulus, the authors in this study infused nicotine by the microdialysis probes to stimulate dopamine release. *Our current study showed for the first time that potassium evoked dopamine release in nicotine treated animals and the effect of nicotine treatment in the setting of TBI.* Potassium evoked release was used in the current study since it has been well characterized by previous microdialysis studies (Stanford et al., 2000; Agut et al., 2000; Shui et al., 1998; Gerhardt and Maloney, 1998). In addition, potassium stimulus has been used to stimulate dopamine release in striatal slices (Delanoy et al., 1982) and synaptosomal preparations (Bowyer et al., 1987). Unlike nicotine evoked dopamine release, which will specifically activate synapses via nAChRs, potassium evoked dopamine release occurs by activating neurons in the vicinity of the probe by depolarization. Various terminals in the striatum are activated by depolarization of acting by constitutively open potassium channels (Stone, 1995), which may include not just dopaminergic terminals but also glutamatergic, cholinergic, and GABAeric interneurons. Thus, it provides a general functional assessment of striatal dopaminergic terminals.

In addition to increased striatal dopamine release, in-vivo TH activity assay showed decreased activity in the Injured Saline group compared to Sham Saline group, and a reversal of this deficit was noted in the Injured Nicotine group (**Fig. 18**). Since TH activity and phosphorylation decreases after injury as shown in Chapter 2, nicotine treatment was aimed to

reverse this loss. Past studies report that nicotine increases TH phosphorylation in situ (Haycock, 1991; Haycock et al., 1993) and upregulates TH transcription (Osterhout et al., 2005). In vitro cell culture studies also showed nicotinic receptor activation increasing TH phosphorylation, activity, and protein levels (Bobrovskaya et al., 2007; Craviso et al., 1992; Sabban and Gueorguiev, 2002).

There are several suggested mechanisms of TH activation by nicotine treatment. Regulation of TH activity levels by intracellular Ca^{2+} has been previously reported (Bustos et al., 1980). Since tyrosine hydroxylase is mainly phosphorylated by CaMKII at serine 19 (Dunkley et al., 2004), increased intracellular Ca^{2+} can lead to CaMKII activation, which will subsequently lead to pser19TH increase. Increased pser19TH may then induce a priming effect on TH conformational, allowing phosphorylation at serine 40 to occur at an increased rate. In addition, Ca^{2+} activation of CaMKII can lead to activation of specific subtypes of adenylate cyclase (Wong et al., 1999), which can further contribute to pser40TH increase.

Nicotine has also been demonstrated to induce PKC activation (Tang et al., 1997), which can phosphorylate TH and induce activation. Increase in pser40TH was dependent on protein kinase C (PKC) 24 hours after nicotine stimulus, whereas other kinases may contribute to pser40TH levels at earlier times (Bobrovskaya et al., 2007). Thus, PKC and CAMKII activation may be the major contributors of increased TH activity by nicotine treatment. This reversal of TH activity deficit can then contribute to increased dopamine release when striatum is stimulated by potassium, since potassium evoked dopamine release depends on newly synthesized dopamine (Fairbrother et al., 1990)

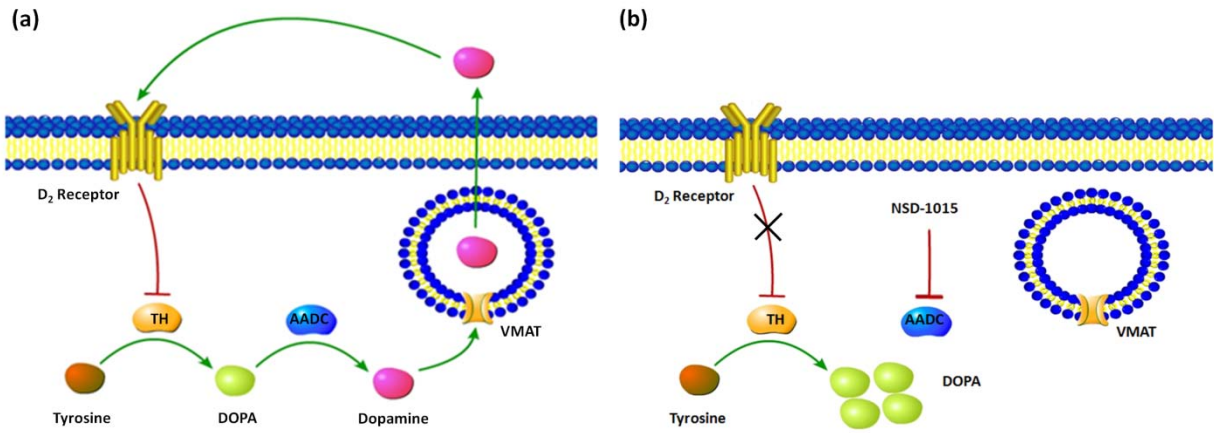


Figure 20. Dopamine regulation and TH activity assay. Under physiological settings, AADC converts DOPA to dopamine, and dopamine release in the synapse reduces TH activity by the inhibitiory action of D_2 autoreceptors (a). NSD-1015 inhibits AADC, inducing accumulation of DOPA which can be quantified to correlate with TH activity (b). However, dopamine synthesis is subsequently inhibited and inhibitory effect of D_2 receptors on TH activity will also be decreased. Thus, TH activity may be higher than baseline TH activity when NSD-1015 is applied **(Fig. 18)**.

Contrary to the in-vivo TH activity data, Western blots showed no change in pser19TH and pser40TH levels in injured animals treated with nicotine (**Fig. 19**). Also, despite an increased trend in TH levels in Sham Nicotine group, no change in protein levels were found. One possible reason for the lack of nicotine's effect on acute changes in TH phosphorylation may be due to the activation of NMDA receptors. Activation of nAChR at glutamate terminals can lead to glutamate release, which can then activate NMDA receptors on dopamine terminals. Striatal slice studies have shown that activation of NMDA receptors inhibit forskolin activated

TH activity and pser40TH increase (Lindgren et al., 2000). Similarly, NMDA receptor activation in rat striatal synaptosomes also inhibited dopamine synthesis (Chowdhury and Fillenz, 1991; Desce et al., 1994) in a Ca^{2+} dependent manner.

The discrepancy between our TH activity and Western blot data suggests that the TH assay may be a more sensitive measure of nicotine induced changes. In the setting of TH activity assay, NSD-1015 is intraperitoneally injected into rats 30 minutes before tissue collection. Since NSD-1015 inhibits AADC activity, DOPA accumulates and dopamine synthesis is inhibited. Dopamine depletion may occur and this may lead to reduced activation of presynaptic dopamine autoreceptors and consequently reduce inhibition of TH activity (**Fig. 20**). Therefore in the setting of NSD-1015 injections, all four groups analyzed in Section 3.2.3 may have increased TH activity compared to baseline activity levels. However, the striatal tissues from all four groups used for Western blotting may equally be subject to reduced TH activity as explained. It is possible that only under increased TH activity levels as in the setting of TH activity assay, differences between sham and injured groups become apparent.

A dose determination study initially used 0.3 mg/kg and 1.0 mg/kg doses, which did not achieve significant improvement in the levels of striatal pDARPP-32T34 after injury. A higher dose at 2.0 mg/kg nicotine was used since previous reported studies showed increased pDARPP-32T34 levels in striatal slices and improvement of motor function in behavioral studies with higher nicotine doses.

When low concentration of nicotine of 1.0 μM was applied to striatal slices, pDARPP-32T34 levels decreased (Hamada et al., 2004). This effect was abolished by treatment with dopamine D_2 receptor antagonist raclopride. When a higher concentration of nicotine at 100 μM was applied, pDARPP-32T34 level increased. This effect was prevented by pretreatment using D_1 receptor antagonist SCH23390. Thus the authors of this study concluded that nicotine at 1.0 μM activates D_2 receptors, but nicotine at 100 μM activates D_1 receptors. This may occur by low concentration of nicotine inducing a low level of dopamine release, which is sufficient to activate D_2 receptors since they have higher affinity for dopamine. However, nicotine at high concentrations can induce higher levels of dopamine release, which will predominantly activate D_1 receptor signaling.

Also, when a range of nicotine was used in experiments testing locomotor activity (0.2 - 5.0 mg/kg/hr) (Marks et al., 1983) and motor function (0.01 – 0.3 mg/kg/day) (Ksir et al., 1987), improved function was seen when animals were treated with higher doses of nicotine compared to lower doses. These previous findings indicate that molecular changes and behavioral responses were dependent on the dose of nicotine given to animals.

In this chapter, rats were treated with nicotine for 1 week after injury. Nicotine treatment was shown to reverse TBI induced deficits in striatal dopamine release and TH activity. Thus, presynaptic dopamine synthesis and release in striatum can be recovered by nicotine treatment after injury. The preliminary data in Chapter 1 showed deficits in pDARPP-32-T34 levels after TBI, as well as PP-1 activity, which may be in part due to upstream dopamine release deficit. Thus, nicotine's improvement of presynaptic dopamine signaling may also reverse postsynaptic

deficits in DARPP-32 signaling. The next chapter will further investigate the effects of nicotine on downstream dopamine signaling after TBI.

4. The effects of nicotine on postsynaptic dopamine signaling and behavior after TBI

4.1 Introduction

Striatal postsynaptic dopamine terminals are regulated by various receptors, kinases, and second messenger proteins. The key regulators that were monitored in this chapter are: PKA, DARPP-32, ERK, and CREB, all implicated in the functions of learning and memory. As explained in Chapter 1: Introduction, dopamine release from the terminals of nigrostriatal neurons will activate dopamine receptors. Activation of D_1 receptors will lead to increased PKA activity, which will increase pDARPP-32-T34 levels. The activity of PP-1 is regulated by pDARPP-32-T34, which functions as its inhibitor. Inhibition of PP-1 activity will induce increased phosphorylation of ERK, which will eventually lead to an increase in the phosphorylation of CREB.

Previous chapters showed decreases in TH activity and dopamine release after TBI, which may subsequently reduce the activation of D_1 receptors and contribute to decreased pDARPP-32-T34 levels. Also, the effect of TBI on presynaptic dopamine synthesis and release was reversed by nicotine treatment for 1 week after TBI. In this chapter, the effect of nicotine on reversing the postsynaptic deficits in striatal dopamine signaling as measured by changes in DARPP-32 phosphorylation was investigated. Previous studies in striatal slices have shown that nicotine can modulate pDARPP-32-T34 levels in vitro in a D_1 or D_2 receptor dependent manner (Hamada et al., 2004, 2005). Whether D_1 or D_2 receptors were activated depended on the dose of nicotine applied, which controlled the amount of dopamine release. Thus in this chapter, the

animals were treated with nicotine, and striatal tissues were analyzed for the activation of DARPP-32, ERK, and CREB. Furthermore, the activity of PKA was monitored to understand the possible causes of pDARPP-32-T34 changes. In addition, phosphorylation of DARPP-32 at another site threonine 75 (pDARPP-32-T75) was monitored, since pDARPP-32-T75 can inhibit PKA activity.

Nicotine's effect on striatal dopamine signaling also affects various behavioral measures. Animal studies have shown that rats with striatal lesions have spatial memory deficits (Block et al., 1993; Devan et al., 1999) and motor deficits (Erinoff et al., 1979; Ungerstedt 1971). Such deficits were also characterized in animal models of traumatic brain injury (Smith et al., 1991; Dixon et al., 1999). Previous experiments using the Morris Water Maze showed improved memory acquisition and retention after nicotine treatment in both physiological states (Riekkinen and Riekkinen, 1997; Socci et al., 1995) as well as in the setting of brain injury (Yamada et al., 2010; Sharifzadeh et al., 2005; Brown et al., 2000). Moreover, repeated nicotine treatments increase locomotor activity in rats (Smith et al., 2010) and transgenic mice expressing hypersensitive nAChRs show increased ambulatory behavior (Drenan et al., 2010). Thus, behavioral experiments in the literature substantiate the possibility of functional improvement by nicotine treatment in the setting of TBI.

These previous findings collectively suggest that nicotine treatment of rodents enhances dopamine release, DARPP-32 signaling, and result in functional improvement in behavior. The activity of DARPP-32 has a key role in striatal function, and ERK and CREB are implicated in

functions of synaptic plasticity and memory. Thus, nicotine induced enhancement of dopamine signaling after TBI may lead to recovery of both learning and motor deficits.

The experiments in this chapter focused on **Aim 3:** ***Determine if enhancing TH activity and dopamine release by nicotine treatment can reverse the deficits in DARPP-32 activation and its downstream molecules***, and **Aim 4:** ***Determine if enhancing striatal dopamine signaling by nicotine treatment results in cognitive and motor improvement in rats.*** After molecular changes of striatum were monitored, the behavioral effect of nicotine was monitored by testing animals using a combination of motor and cognitive assessments.

4.2 Materials and Methods

4.2.1 Animals

Eighty Sprague-Dawley rats (Harlan Laboratories) weighing 280-350g were used following the guidelines of the Institutional Animal Care and Use Committee of University of Pittsburgh. The same feeding and housing conditions were provided for the animals as described in previous chapters. Animals were grouped into 4 conditions: Sham Saline, Injured Saline, Sham Nicotine, and Injured Nicotine. Each group had n = 8-10 (motor and spatial memory test) or n = 5-7 (Western blot and PKA activity assay).

4.2.2 Western Blot

All procedures were identical to those described in Chapter 2. The antibodies for pDARPP-32-T34, pDARPP-32-T75, pCREB, and pERK were from (Cell Signaling, Boston, MA).

4.2.3 Surgery and Drug Administration

Animals were injured using the CCI device or sham injured as described in previous chapters. For Western blot and PKA activity assay, nicotine injection schedule was also identical to the ones described in Chapter 3: 0.9% saline or 2mg/kg nicotine injection twice daily for 7 days, since this regimen was able to reverse deficits in TH activity and dopamine release. However, animals used for motor and spatial memory tests were treated with saline or nicotine until 21 days post injury, at which point they were sacrificed. During the interval days (days 6-13) between motor testing (days 1-5) and cognitive testing (days 14-20), animals were injected with saline or nicotine once daily.

For the initial motor testing reported in Chapter 4.3.4, nicotine injection schedule consisted of evening injection around 7:00 pm and morning injection at 8:00 am. When the animals were tested by Beam Balance and Beam Walking Tests, the time of injection was at least 5 hours after the last injection. Regarding the fact that nicotine's half life in the rat brain is 50 minutes (Sastry et al., 1995; Ghosheh et al., 1999), 5 hours was considered sufficient time to avoid acute effects of nicotine treatment.

For the additional behavioral testing reported in Chapter 4.3.5 consisting of motor testing (days 1-5) and cognitive testing (days 14-20), injections were performed at 7:00 pm and 1 hour prior to testing. The goal of injecting the animals 1 hour before testing was to have active effects of nicotine in the brain while the animals were being tested.

4.2.4 Motor Test

On post injury days 1-5, the motor function of animals were tested using beam-balance and beam-walking test, as previously described (Dixon et al., 1987; Singleton et al., 2010). Briefly, the animals were placed on a 1.5 cm wide beam and their duration of balance was recorded for up to 60 seconds 3 times daily. The beam balance latency was assessed before the injury (preassessment), and for 5 days following the injury. For beam walking test, the animals were trained the day before surgery to escape brightness and noise by traversing a wooden beam (2.5 x100cm) into a dark goal box placed at the opposite end. On the wooden beam, there were four pegs between the starting location and goal box to challenge motor function as the rats moved from one end to the other.

Like the beam balance test, animals were preassessed before the injury, and their performances were also recorded on post operative days 1-5. The time taken for the animals to traverse the beam was recorded 4 times daily. If the animals took longer than 60 minutes or fell, they were guided back to the goal box manually. Once after reaching a goal box, animals remained there for 30 seconds before the next session. The beam walking score was given by the

following criteria: reaching the goal box = 5 points, reaching the zone between goal box and 4th peg = 4 points, reaching the zone between 4th and 3rd peg = 3 points, reaching the zone between 3rd and 2nd peg = 2 points, reaching to zone between 2nd and 1st peg = 1 point, unable to pass the 1st peg = 0 point. Beam-balance latency, beam-walking latency, and beam-walking score for each day were the mean values of all the sessions each day.

4.2.5 Cognitive Test

Rats were subjected to cognitive function tests beginning on postoperative day 14 for 5 consecutive days (days 14-18). The Morris Water Maze pool is 180 cm in diameter and 60 cm in depth. Water level was 28cm deep, and a clear plastic platform of 26 cm in height was placed in one of the quadrants below the surface of the water. Visual cues were displayed on the walls outside the pool for spatial guidance. The position of the platform was constant throughout the experiment at a Southwest quadrant. The animals were placed in the pool in one of the four directions randomly selected (east, west, north, and south). They were allowed to swim in the pool until they found the platform, at which point the latency was recorded. For the animals that did not find the platform in 120 seconds, they were manually guided to the platform and 120 seconds were recorded as the latency. After the animals located the platform or were guided to it, they were left to stand on the platform for 30 seconds before they were moved to a 30 incubator to rest between trials for 4 minutes. Each day, a total of four trials were performed for each animal, and the mean latency was recorded. On day 19, the probe trial was performed. The platform was removed from the pool and the duration of swimming in the quadrant where the platform was previously present was recorded. Visible platform tests were also performed for

days 19 and 20 to monitor the contributions of factors other than spatial memory, such as motor performance, motivation, and visual acuity. For this test, the platform was raised 2.0 cm above the water surface, and the latency until platform was found recorded. Only the latency to find the visible platform on day 20 is reported, since latency data from day 19 is considered variable from the past experiences of the laboratory. A video analysis system (Any-maze, Stoelting Co., Wood Dale, IL) was used to record each animal's swim speed and distance traveled.

4.2.6 Statistical Analysis

One way ANOVA was used to test between group differences for PKA activity assay and all the Western blot data using PASW software. Motor testing (Beam Balance Duration, Beam Walking Latency, Beam Walking Score) as well as cognitive test (Morris Water Maze) were analyzed using repeated measures ANOVA. For all statistical tests, post hoc analysis was performed using Tukey's Test once between group significance was found. For all statistical tests, a p-value of$\leq$ 0.05 was considered significant. The PKA activity levels and optical densities of Western blots were reported as percentage of Sham Saline group mean ± standard error of the mean. Behavioral test data were reported as latency (sec), score, or % time in quadrant ± standard error of the mean.

4.3 Results

4.3.1 Phosphorylation of DARPP-32

The level of pDARPP-32-T34 showed significant differences between the groups ($F_{3,16}$=8.816; $p \leq 0.001$), with Injured Saline (44.2±9.4%) and Injured Nicotine (31.9±7.3%) groups having significantly lower levels compared to Sham Saline group ($p \leq 0.05$). This follows the previous results from the Dixon laboratory showing reducted pDARPP-32-t34 levels after injury, but also shows that nicotine did not reverse this loss. When pDARPP-32-T75 levels were monitored, no significant between groups difference was found ($F_{3,21}$=2.171; $p > 0.05$). However, a modest decrease without statistical significance was noted in the Injured Nicotine group .

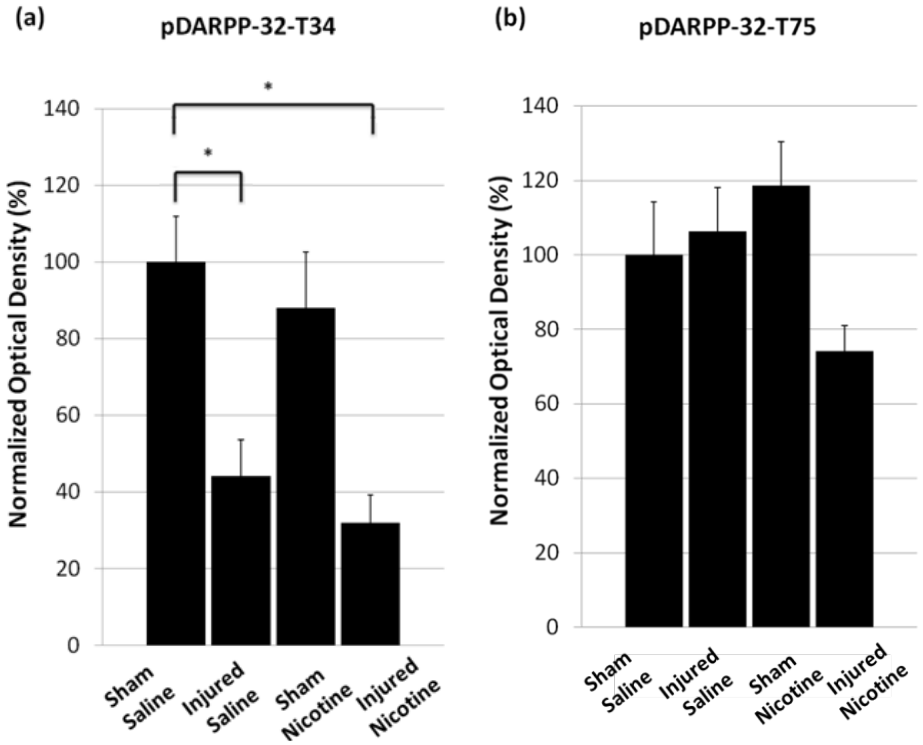


Figure 21. Western blot data for the effects of nicotine treatment on phosphorylation of DARPP-32. Two phosphorylation sites were monitored using Western blot: pDARPP-32-T34 (a) and pDARPP-32-T75 (b). There were significant decreases in Injured Saline and Injured Nicotine groups compared to Sham Saline group for pDARPP-32-T34, but no changes were seen for pDARPP-32-T75.

4.3.2 Striatal PKA Activity After Nicotine or Saline Treatment

When PKA activity assay was performed for the four groups, a significant group effect was found ($F_{3,21}$=4.869; $p \leq 0.01$). Nicotine treatment did not increase PKA activity in any of the groups compared to the saline treated groups. Instead, there was a decrease in PKA activity of Injured Nicotine group compared to Sham Saline group (100.0±2.4% and 88.3±1.5% respectively; $p \leq 0.01$). No statistically significant differences were found between any other groups.

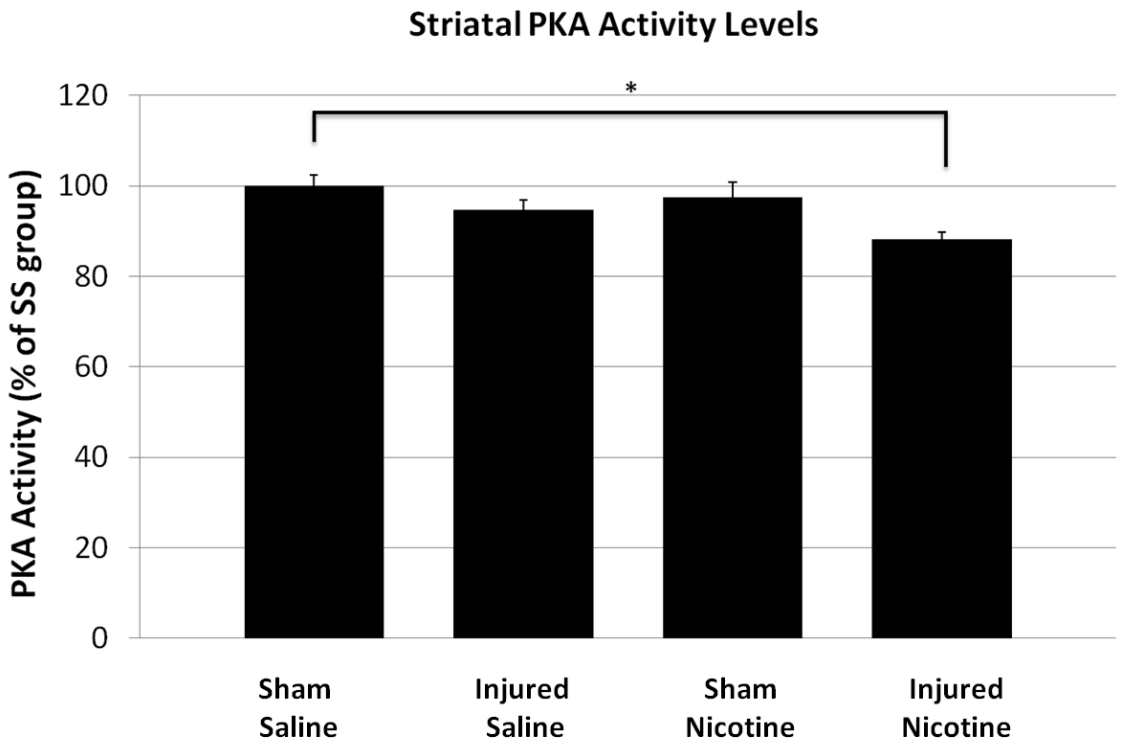


Figure 22. PKA activity assay. Injured groups consistently showed decreased trend, although only the Injured Nicotine group was significantly different from Sham Saline group. (* $p \leq 0.05$)

4.3.3 The effects of nicotine on phosphorylation of ERK and CREB

Western blots showed no statistically significant differences among the four groups for the level of pERK ($F_{3,20}=1.557$; $p>0.05$). Similarly, there were no between groups differences for pCREB ($F_{3,20}=2.823$; $p>0.05$). There were no effects of injury or nicotine on phosphorylation of these proteins. Although Sham Nicotine group showed a trend of increased pCREB, this was not statistically significant.

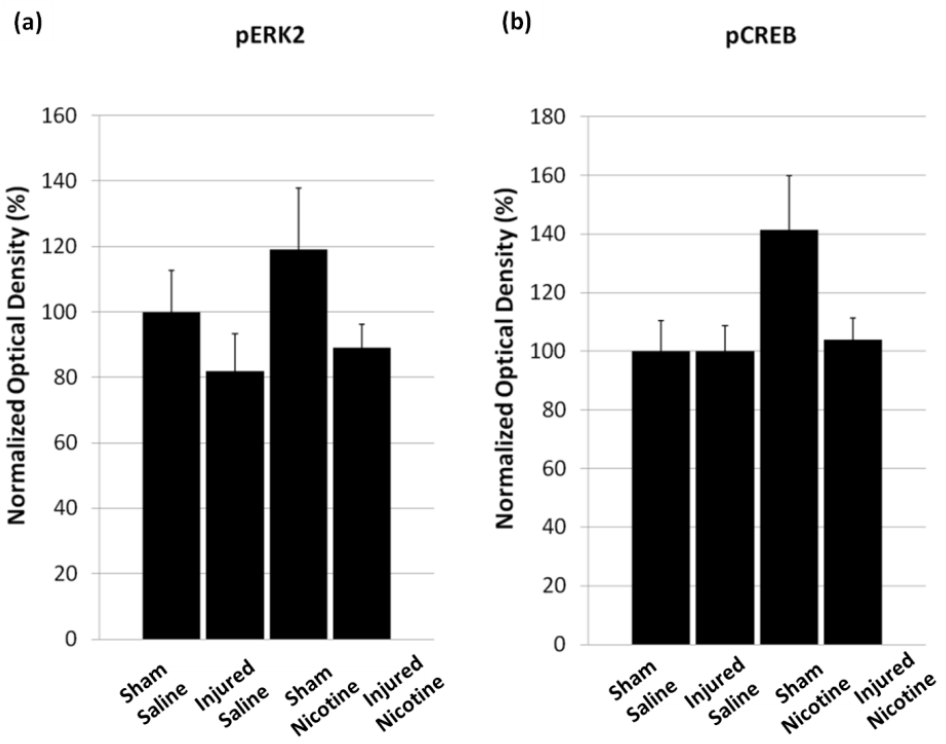
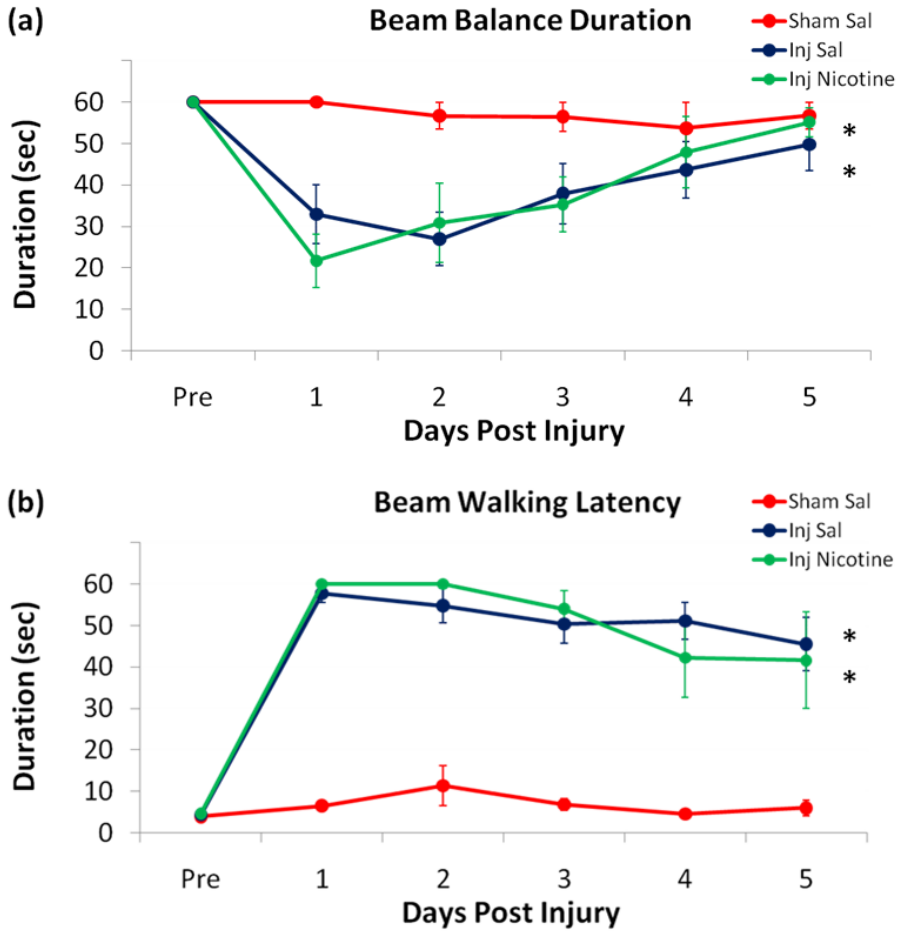


Figure 23. Western blots of pERK2 (a) and pCREB (b). No significant changes were induced by either injury or nicotine treatment for pERK2 and pCREB.

4.3.4 Motor Testing Without Acute Nicotine Dose

Nicotine (2.0 mg/kg) or saline injections took place at least 5 hours prior to the test and second injections took place approximately 5 hours after the test to avoid acute effects of the drugs. Two tests were used to assess motor function in the animals at 1 week post injury: Beam Balance Test (**Fig. 24a**) and Beam Walking Test (**Fig. 24b,c**). The Beam Walking Test measures 2 parameters: latency to cross the beam (**Fig. 24b**) and farthest distance traveled on the beam (**Fig. 24c**) thus a total of 3 parameters are displayed for the motor testing. In all three measures of motor function, significant differences between groups were found by repeated

measures ANOVA: Beam Balance Duration ($F_{2,18}$=3.998, $p \leq 0.05$), Beam Walking Latency ($F_{2,18}$=40.648, $p \leq 0.001$), and Beam Walking Score ($F_{2,18}$=21.441, $p \leq 0.001$). Post hoc analysis showed significant motor deficits for all three measures in Injured Saline and Injured Nicotine groups from Sham Saline group, but there were no significant differences between Injured Saline and Injured Nicotine groups. Thus, there was no benefit of nicotine on motor function.



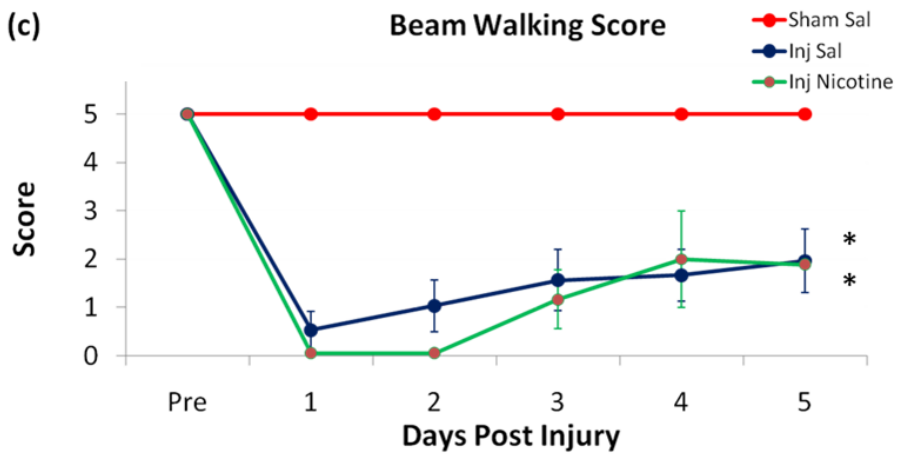


Figure 24. Motor function test with nicotine or saline injection 5 hours prior. Beam Balance Test (a), Beam Walking Duration (b), and Beam Walking Score (c) showed significant between groups difference. (Inj = Injured, Sal = Saline, Nic = Nicotine , $*p \leq 0.05$ compared to Sham Saline group)

4.3.5 Motor testing with acute nicotine dose

After finding no improvement in Injured Nicotine group, the injection regimen was changed to test the animals while nicotine was still active in the brain. Nicotine's half life is reported as approximately 50 minutes (Sastry et al., 1995; Ghosheh et al., 1999). The animals were previously observed to be hyperactive and have increased locomotor function up to 30 minutes after injection, so motor testing at a time point that had no acute effects yet without complete metabolism of nicotine was sought. Thus the injection schedule took place 1 hour prior to testing each animal. When motor testing was completed, second daily injections were performed approximately 5 hours after the test.

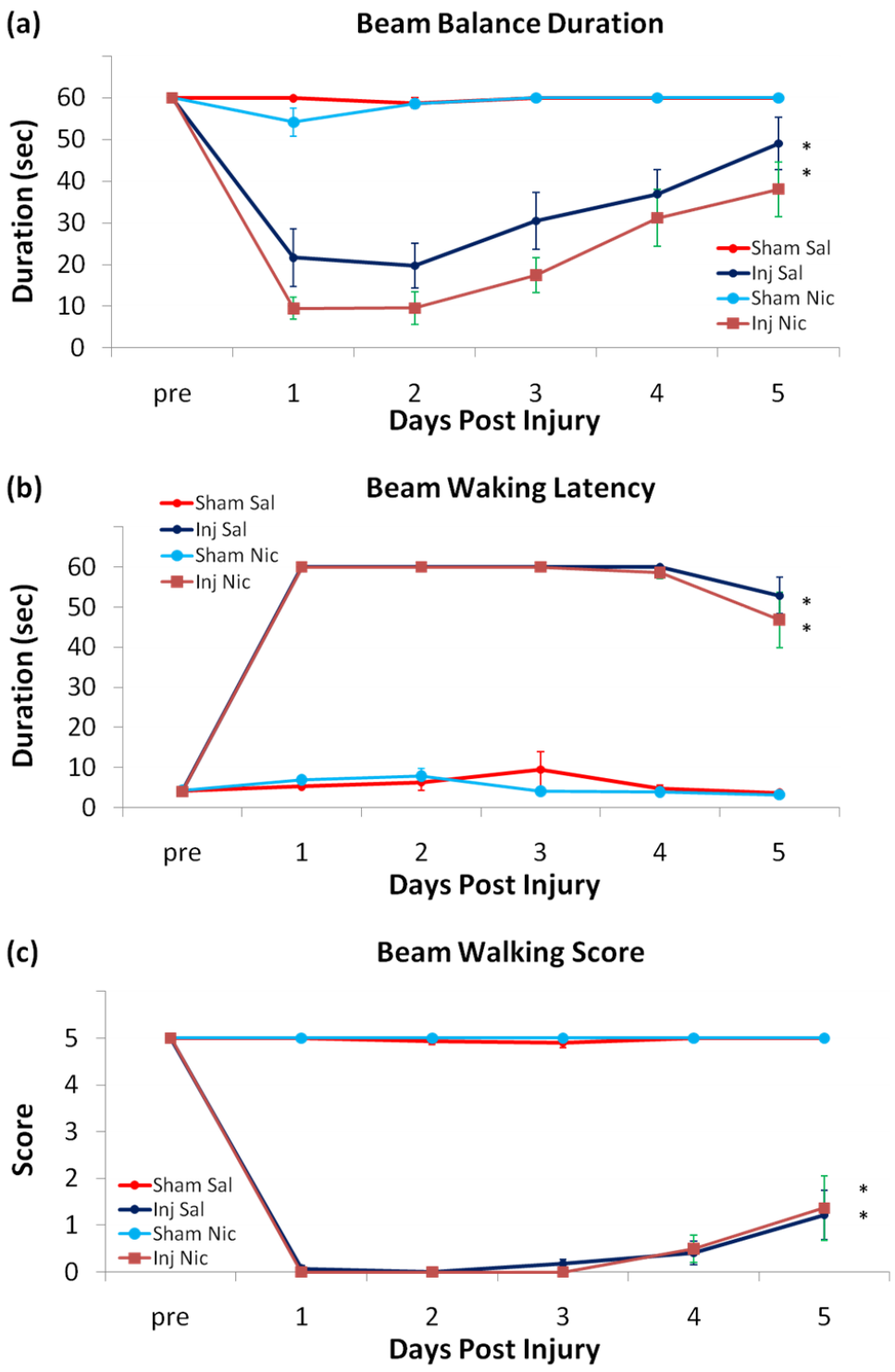


Figure 25. Motor testing with nicotine or saline injection 1 hour prior to experiment. Injured Saline and Injured Nicotine groups were both significantly different from Sham Saline,

and there was no difference between Injured Saline and Injured Nicotine groups. (Inj = Injured, Sal = Saline, Nic = Nicotine ,* $p \leq 0.05$ compared to Sham Saline group)

Statistically significant between-groups differences were noted for Beam Balance Test ($F_{3,35}=47.527$, $p \leq 0.001$), Beam Walking Latency ($F_{2,18}=643.03$, $p \leq 0.001$), and Beam Walking Score ($F_{2,18}=453.87$, $p \leq 0.001$). Similar to the prior motor test, Injured Saline and Injured Nicotine groups showed significantly lower duration on the Beam Balance Test and higher latency on Beam Walking Test, as well as shorter distance traveled on Beam Walking Score ($p \leq 0.001$ for all motor tests). The Injured Nicotine group showed no improvement of motor function compared to Injured Saline group in all three measures.

4.3.6 The Effects of Nicotine on Cognitive Function After TBI

The data for cognitive function of animals were similar to that of motor function tests. There were significant between groups differences ($F_{3,32}=63.110$, $p \leq 0.001$), with Injured Saline and Injured Nicotine groups having significantly higher platform finding latency than Sham Saline group ($p \leq 0.001$). No significant differences were found between Injured Saline and Injured Nicotine groups, showing that nicotine does not improve cognitive function after TBI.

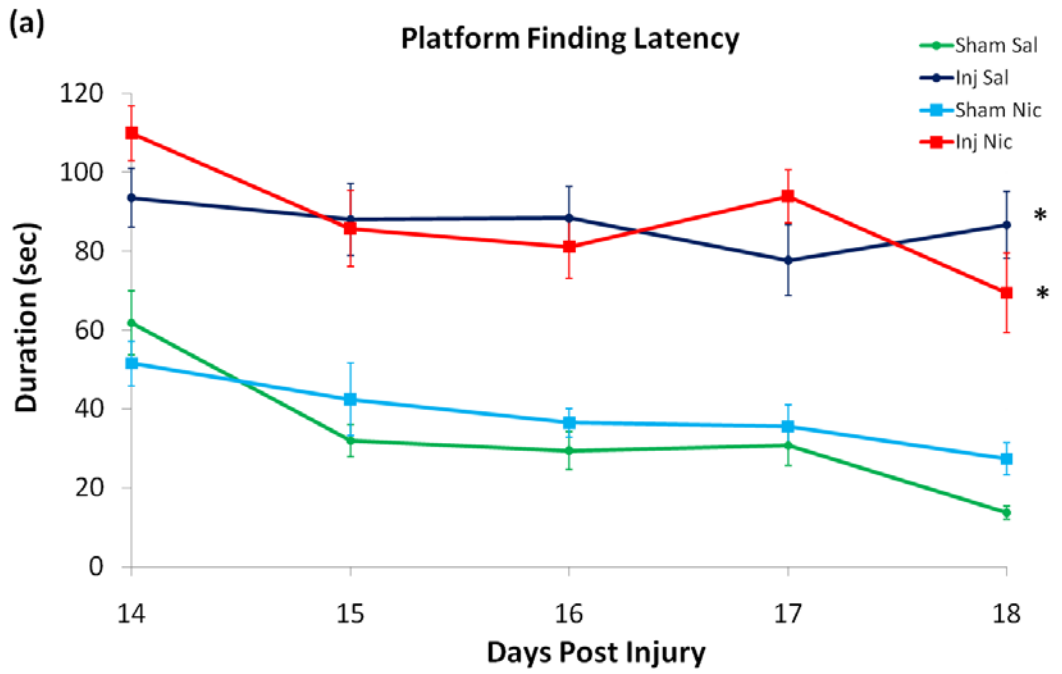


Figure 26. Morris Water Maze data. The animals in Injured Saline and Injured Nicotine groups showed significantly higher latency for finding the platform compared to Sham Saline group. There was no difference between Injured Saline and Injured Nicotine groups (Inj = Injured, Sal = Saline, Nic = Nicotine ,* $p \leq 0.05$ compared to Sham Saline group)

There were significant between group differences for the time spent in target quadrant in the probe phase of the experiment ($F_{3,32}=6.81$, $p \leq 0.01$) (**Fig. 27a**). Specifically, Injured Saline and Injured Nicotine groups spent less time compared to Sham Saline group in the quadrant that previously had the platform. There was no significant difference between Injured Saline and Injured Nicotine groups. Swim speed did not differ among the groups ($F_{3,32}=0.375$, $p>0.05$) during the probe phase of the experiment (**Fig. 27b**). The groups showed significant differences in latency for finding visible platform ($F_{3,32}=10.342$, $p \leq 0.001$) (**Fig. 27c**).

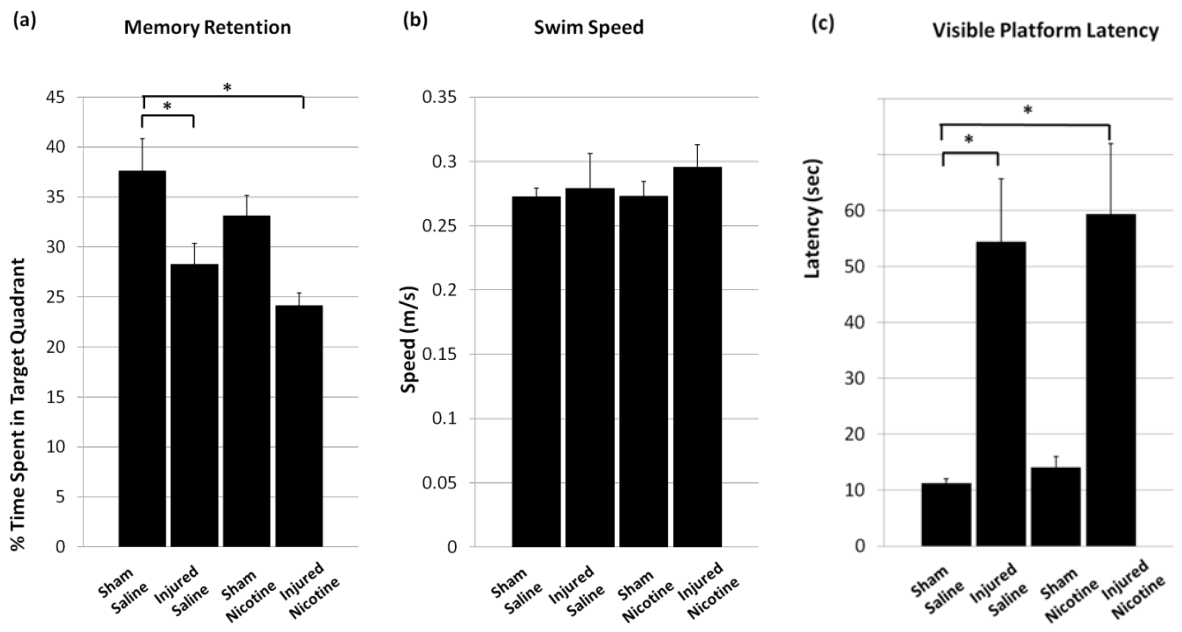


Figure 27. Assessments from probe and visible platform phases. On days 19 and 20, the animals were subject to probe phase of the experiment (a) and visible platform phase (c), respectively. The swim speed data were from probe phase of the experiment (b).

4.4. Discussion

The aim of nicotine treatment was to reverse dopamine signaling deficits after TBI. It was originally hypothesized that pDARPP-32-T34 deficits following injury were in part due to dopamine synthesis and release deficits upstream. Nicotine increases dopamine release in the striatum, and activation of D_1 receptors on medium spiny neurons can lead to activation of PKA which phosphorylates DARPP-32 to form pDARPP-32T34 (Svenningsson et al., 1998). By nicotine treatment increasing dopamine synthesis and release, I had hypothesized that it would reverse the pDARPP-32-T34 deficit. However, the results in this chapter show no benefit of

nicotine treatment (**Fig. 21a**). The goal of the rest of chapter was to clarify the reason for this lack of nicotine's effect on DARPP-32, by monitoring the activity of molecules upstream and downstream to DARPP-32.

Because phosphorylation of DARPP-32 at threonine 34 site is regulated by PKA, the activity of PKA was monitored for the four treatment groups to further understand the lack of nicotine's effect. If nicotine induced increased dopamine signaling by activation of D_1 receptors, PKA activity would be increased, leading to increased pDARPP-32-T34. However, the result for the PKA activity assay showed a significant decrease in the activity of PKA for Injured Nicotine group compared to Sham Saline group, showing that nicotine treatment in fact worsened the injury induced deficit on the activity level of PKA (**Fig. 22**).

In Chapter 2, the activity levels of PKA was interpreted in the context of presynaptic dopamine synthesis: it's ability to phosphorylate serine 40 site of TH and thereby activating it. However, the majority of striatal PKA content is either glial or from medium spiny neurons postsynaptic to dopamine terminals since they compose about 90% of striatal neurons. In this context, PKA activity will be dependent on glutamate and dopamine receptor activation on medium spiny neurons. Nicotine in striatum induces both glutamate and dopamine release, which leads to a complex sequence of events involving multiple signaling cascades which can both activate or inhibit PKA (Nishi et al., 2005; Shiftlet and Balleine, 2011).

Depending on the phosphorylation state, DARPP-32 can function as a bidirectional signal transduction molecule (**Fig. 29**). When DARPP-32 is phosphorylated at threonine 75 site

(pDARPP-32-T75), it functions as an inhibitor of PKA, indirectly reducing pDARPP-32-T34 and modulating PP-1 inhibition (Bibb et al., 1999). The levels of pDARPP-32-T75 is dependent on the activity of cyclin depedent kinase (cdk5), which is activated by metabotropic glutamate receptors (mGluR) (Liu et al., 2001). Thus, one possible reason for the lack pDARPP-32T34 increase after nicotine treatment was the opposing effects of glutamate receptors and D_1 receptors in medium spiny neurons. Since nicotine can also activate glutamate release by activating nAChRs on glutamateric terminals synapsing on to medium spiny neurons, mGluR can be activated, subsequently increasing pDARPP-32-T75 levels. This may explain the inhibition of PKA activity and reduction of pDARPP-32-T34 levels noted. However, the result in (**Fig. 21b**) showed no increase in pDARPP-32-T75 levels in Injured Nicotine group. In fact, there was a modest decrease in pDARPP-32T-75 although no statistically significant difference was found. Thus, the decreased PKA activity in Injured Nicotine group could not be explained by nicotine's possible effect on pDARPP-32-T75 levels by mGluR activation.

A likely reason for the decrease in PKA activity was the activity of D_2 receptors (**Fig. 28**). The activation of D_2 receptors leads to inhibition of adenylate cyclase via G-protein coupled process (Huff, 1996; Stoof and Kebabian, 1981) as well as possible activation of PP-2B, which can dephosphorylate pDARPP-32-T34 (Nishi et al., 1997). Subsequently, decreased PKA activity and pDARPP-32-T34 levels can occur. It is possible that the level of dopamine release induced by the current nicotine treatment regimen was not high enough to activate D_1 receptors mainly. Low level of dopamine release by nicotine at 1.0 μM in the striatum may activate mainly D_2 receptors, which are fewer but have higher affinity (Hamada et al., 2004). Only the

high nicotine concentration at 100 μM induced higher dopamine release which activated mainly D_1 receptors.

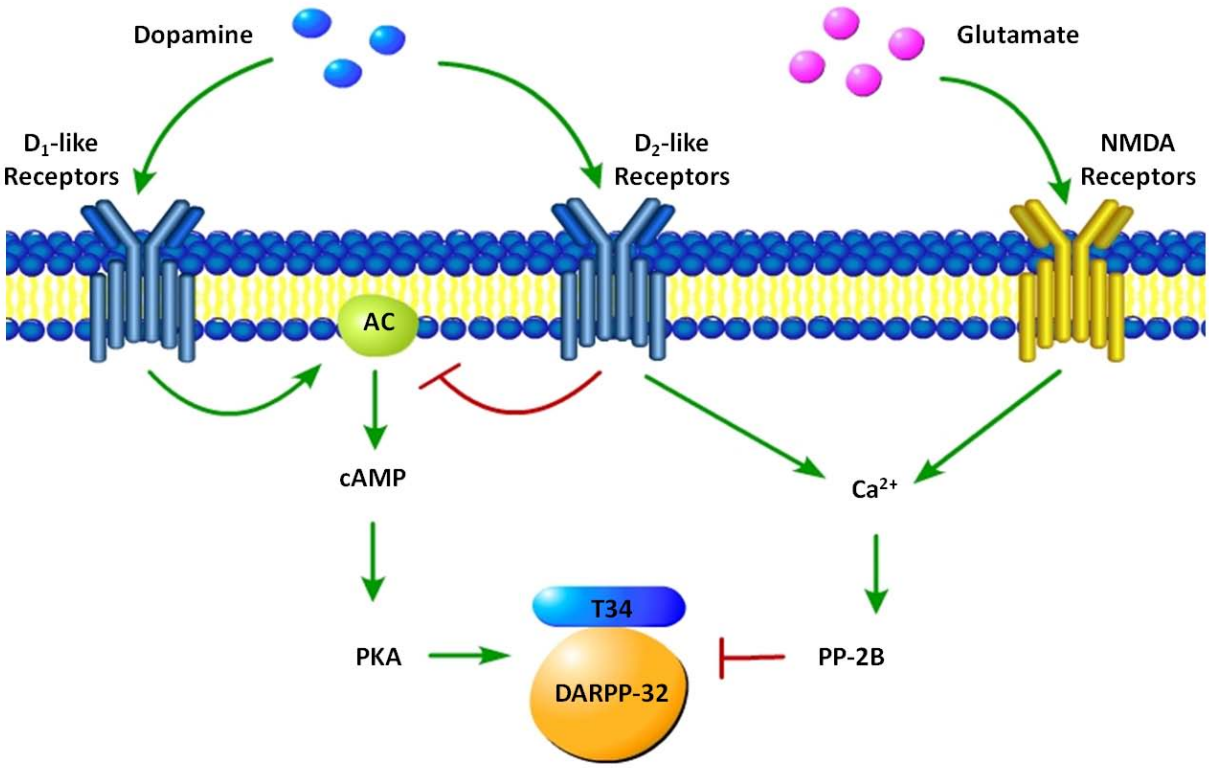


Figure 28. Simplified conceptual diagram of pathways modulating pDARPP-32-T34 levels. Activation of D_1 receptors will induce pDARPP-32-T34 increase, whereas activation of D_2 receptors and NMDA receptors will induce pDARPP-32-T34 decrease. The activation of these two receptors may take place in separate neurons, but they are depicted here in the same cell for easy conceptual understanding.

Previous reports of nicotine metabolism show relatively low levels of nicotine in the brain after injection. When 0.8 mg/kg dose was injected subcutaneously, brain nicotine

concentration reached the maximum value of approximately 2000 pmol/g tissue (Ghosheh et al., 1999). When 3.0 mg/kg dose was applied by series of 10 injections, a maximum value of approximately 2500 pmol/g tissue was reached (Ghosheh et al., 2001). Since these doses are close to the dose used in the current experiment (2.0 mg/kg), it can be assumed that the maximum value of approximately 2500pmol/g tissue occurred with this dose. It is not clear how these nicotine tissue concentrations compare to the concentration of nicotine in striatal slice culture. However, even after grossly underestimating brain tissue density to be 1.0 g/ml (density of water), the nicotine concentration will calculate out to be 2.0 μM. The actual concentration of nicotine in striatal synapses will likely be lower, since the brain tissue density is higher than 1.0 g/ml. But it can be rationalized that the 2.0 mg/kg injection of nicotine will induce local striatal nicotine concentration closer to 1.0 μM rather than 100 μM levels, resulting in activating D_2 receptors primarily. A weakness in this current regimen is that only one dose was used. In order to provide a more comprehensive understanding of nicotine's effects on striatal dopamine signaling, the experiments presented in this chapter and Chapter 3 could be repeated using multiple doses. Moreover, different delivery methods such as using an osmotic pump (Verbois et al., 2003b) could be tested for optimal nicotine administration in future studies.

The levels of pDARPP-32-T75 is not only regulated by mGluR activation, but also indirectly regulated by PKA (Nishi et al., 2000). When PKA is activated, it can activate PP-2A, which will reduce pDARPP-32-T75 levels by dephosphorylation. Thus, pDARPP-32-T5 and PKA have mutually inhibitory roles. The current results show that neither pDARPP-32-T75 nor PKA activity were dominant, since both were decreased in the Injured Nicotine groups. The roles of other kinases and phosphatases need to be considered to better understand these data.

The decreased trend of pDARPP-32-T75 and pDARPP-32-T34 in the Injured Nicotine group may be due to increased PP-2A and PP-2B activity, respectively. Glutamate release induced by nicotine can activate NMDA receptors on medium spiny neurons, inducing Ca^{2+} influx and activation of PP-2A and PP-2B. The activation of NMDA receptors have been shown to reduce pDARPP-32-T34 levels by possibly activating protein phosphatase PP-2B (King et al., 1984; Halpain et al., 1990). In the setting of TBI, PP-2B has an important role in regulating pDARPP-32-T34 levels. Previous experiments in the Dixon laboratory have shown that TBI induced decreases in pDARPP-32-T34 can be reversed by FK506, a PP-2B inhibitor (Chapter 1).

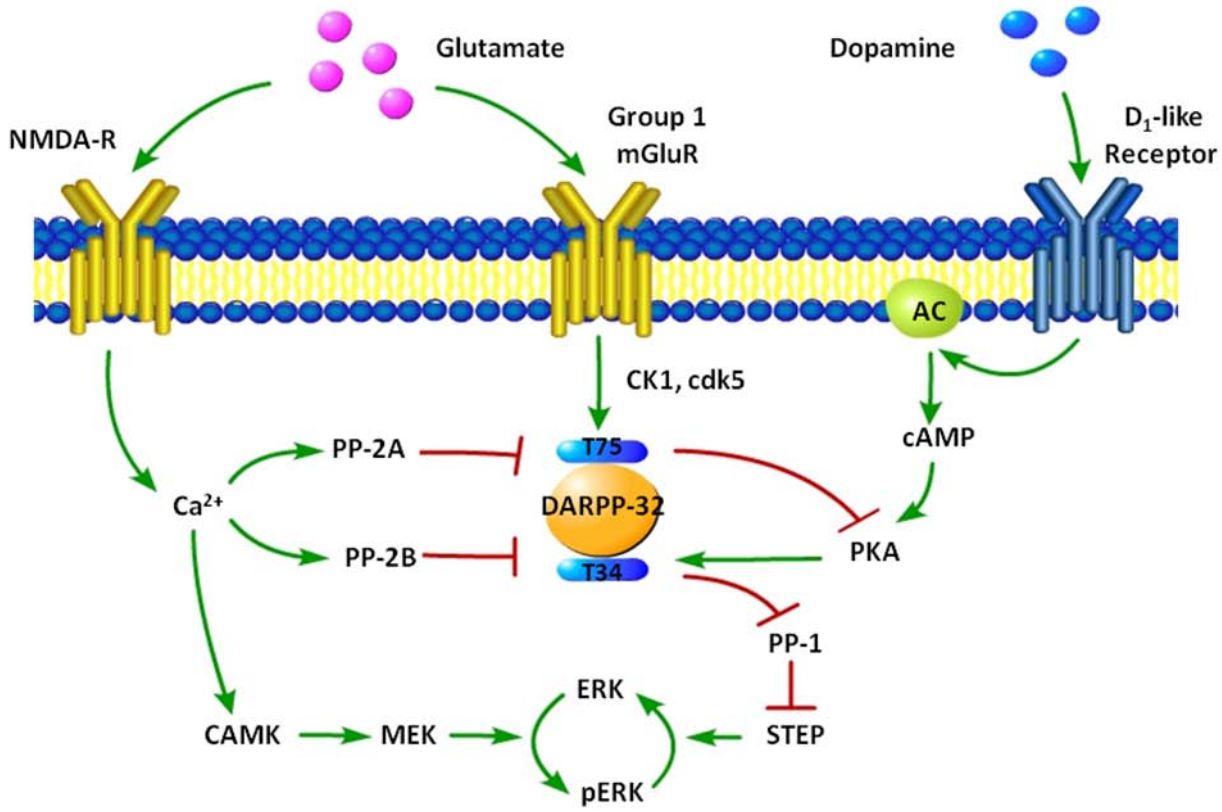


Figure 29. Glutamate and dopamine interaction in medium spiny neurons. The activity of DARPP-32 is regulated by phosphorylation at threonine 75 and threonine 34, by complex interactions of glutamate and dopamine signaling. Also, ERK is dually modulated by glutamate and dopamine signaling.

The high intracellular Ca^{2+} can also reduce PKA levels, since striatum is enriched with a subtype of adenylate cyclase that is inhibited by Ca^{2+}: type V calcium-inhibitable adenylate cyclase (Cooper et al., 1994, 1995). Also, calcium-dependent phosphodiesterase is prevalent in striatal tissue (Polli and Kincaid, 1994). These phosphodiesterases can be activated upon increased intracellular Ca^{2+}, decreasing the levels of cAMP thereby reducing PKA activity. Thus influx of Ca^{2+} via NMDA receptor activation can lead to secondary decreases in PKA activity levels.

To further monitor postsynaptic striatal dopamine signaling, Western blots were performed for 2 molecules downstream of pDARPP-32T34: pERK, and pCREB, due to their important roles in learning and memory. Similar to the lack of nicotine's effect on pDARPP-32-T34 levels, no changes were noted for pERK and pCREB. The activation of ERK occurs by several upstream regulators, making it as complicated as DARPP-32 activation. It has been explained in Chapter 1 that activation of D_1 receptors and the following pDARPP-32-T34 increase can lead to increased pERK levels. In fact, ERK is modulated also by glutamate and its function is known as an integrator of both glutamate and dopamine signals, which can serve to increase or decrease ERK phosphorylation.

Glutamate release can activate NMDA receptors of medium spiny neurons, which will activate Ras/Raf pathway, leading to activation of MAPK kinase (MEK), which phosphorylates ERK (Shiftlet and Balleine, 2011). However, this activation of ERK is transient since Ca^{2+} influx by NMDA receptor leads to PP-2B activation, which dephosphorylates and activates striatal enriched phosphatase (STEP) (Paul et al., 2003). This activation of STEP then leads to dephosphorylation of ERK (Shiftlet and Balleine, 2011). Also, D_2 receptor activation can induce decreased pDARPP-32-T34, which will reduce the activation of ERK. Thus, the lack of nicotine's effect on pERK levels and downstream pCREB may be due to these opposing effects of upstream signaling pathways.

The performance of rats on Beam Balance Test and Beam Walking Test can be attributed to different functional measures of motor skill, whereas the Morris Water Maze tests both the learning and retention of spatial memory. Beam Balance Test showed both Injured Saline and Injured Nicotine groups to have a decreased motor performance compared to Sham Saline and Sham Nicotine groups. Post hoc analysis revealed no improvement in Injured Nicotine group compared to Injured Saline group for Beam Walking Score and Beam Walking Latency. In fact, the Beam Balance Duration for Injured Nicotine group at each of the days were lower compared to Injured Saline group (**Fig. 25a**), but there was no significant difference ($p=0.067$). Thus, nicotine treatment did not benefit, and may even have possibly worsened performance Beam Balance Test in the setting of TBI. This result is in agreement with the molecular data: PKA, pDARPP32-T34, pERK, and pCREB which showed no improvement by nicotine treatment.

Cognitive testing by the Morris Water Maze also showed lack of benefit by nicotine treatment in injured animals. There was no effect of nicotine for learning of platform location during the acquisition phase (days 14-19). Also, retention of memory, tested during probe phase (day 20) showed no benefit of nicotine treatment. The swim speed test showed no difference among the four groups, showing that the motor deficit of the injured animals was not the cause of higher latency to find the platform. The data for visible platform phase (day 21) showed significant deficit in latency to find platform among Injured Saline group and Injured Nicotine group even when it was visible. This showed that not only spatial memory, but also other cognitive functions such as motivation, attention, and visual recognition may have been damaged in injured animals and nicotine did not improve this deficit. Similar to the motor testing, the cognitive behavioral data are in agreement with the lack of benefits by nicotine treatment in molecular experiments.

Postsynaptic dopamine signaling in the striatum is regulated by complex interaction of multiple pathways by both glutamate and dopamine release activating various receptors and downstream mediators. Although the current nicotine treatment regimen was able to induce recovery of TH activity and increased dopamine release as demonstrated in Chapter 3, no postsynaptic signaling deficits were reversed at the dose of nicotine used in these studies. As described in this section, this may be due to a combination of factors: 1. nicotine inducing primarily D_2 receptor dominant signaling, 2. Influx of Ca^{2+} via NMDA receptor activating PP-2A and PP-2B, and 3. The opposing effects of glutamate and dopamine signaling on ERK phosphorylation. In addition, both cognitive and motor deficits following TBI were not reversed by nicotine treatment, correlating with the deficits in postsynaptic dopamine signaling.

5. General Discussions

5.1 Further Insights into Nicotine Treatment and TBI

The previous chapters demonstrated that nicotine treatment enhanced TH activity and dopamine release after TBI but did not improve pDARPP-32-T34 signaling and behavioral deficits. In each chapter's conclusion section, the possible causes of the current data were discussed and related to the previous studies in the literature. However, there are certain additional issues that were not discussed in these chapters due to the lack of direct relevance to the data. Nevertheless, these issues are important to review in order to better understand the complexity of events that we are investigating. In this chapter, these additional issues will be explored for a potentially deeper understanding of TBI and the effects of nicotine treatment.

5.1.1 Nicotine Receptor Desensitization

Nicotine receptors are present on the soma, presynaptic area, and postsynaptic area. Although agonist application can lead to nAChRs activation, they are then easily desensitized (Wang and Sun, 2005). Different subtypes of receptors also exhibit differences in their vulnerability to desensitization: α7 receptors have more rapid desensitization compared to non-α7 receptors, and different mechanisms of desensitization occur with different concentrations of nAChR agonists applied (McGehee and Role, 1995; Quick and Lester, 2002). With high level of

agonists ranging from micromolar to millimolar concentrations, a phenomenon known as classical desensitization can occur, where receptors are activated but desensitized within milliseconds following activation (Giniatullin et al., 2005). Then, sensitivity will recover once the agonist is cleared. In the current experiments, the local concentration of nicotine at striatal dopamine synapse was estimated to be in the order of μM in Chapter 3. If desensitization of nAChRs occurred in the synapse, this could be a postulated to be the mechanism by which nicotine administration in the current project failed to enhance postsynaptic dopamine signaling via DARPP-32. However, the current nicotine regimen was still able to reverse dopamine release and TH activity deficits.

Resting tonic firing of midbrain dopamine neurons in awake, freely moving rats occurs at 2-5 Hz, whereas action potentials will drive up the firing rate to 15-100 Hz (Hyland et al., 2002). Experiments with mouse striatal slices show that nicotine desensitization induces decreases in dopamine release under tonic firing of the neuron that is lower than in control rats. However, higher frequency stimuli with bursts of action potentials induces increased levels of dopamine release (Exley et al., 2008). Thus, by desensitization of nAChR, nicotine paradoxically enhances dopamine release in neurons undergoing phasic bursts of action potentials (Rice and Cragg, 2004). Even in animals chronically treated with nicotine, this enhancement of dopamine release occurs (Perez et al., 2008).

A possible mechanism behind this increased dopamine release despite desensitization is by differential action of nicotine on α7 and α4β2 receptors in midbrain neurons. In the VTA and substantia nigra, GABAeric neurons express α4β2 receptors and glutamatergic neurons express

α7 receptors. Since α4β2 receptors are more prone to nicotine desensitization (α4β2 receptors IC_{50}: ~1-60 nM, α7 receptor IC_{50}: ~0.5-7 μM), nicotine treatment may reduce inhibitory effects of GABAergic interneurons on dopaminergic neurons projecting to the striatum (Dani and Zhou, 2001; Wooltorton et al., 2003). However, glutamate release onto dopaminergic neurons and subsequent activation may not be affected by desensitization due to α7 receptors being less prone to desensitization. Thus, desensitization would not attenuate dopamine release but in fact may enhance dopamine release.

5.1.2 Nicotine's Effects on Other Neurotransmitters

Although this study focused on the effects of nicotine treatment on striatal dopamine neurotransmission, nicotine can in fact affect various other neurotransmitter systems in different regions of the brain as well as the periphery. Systemically, nicotine activates the sympathetic system by inducing the release of epinephrine and norepinephrine from the adrenal medulla, increasing heart rate and arterial blood pressure (Haas and Kuber, 1997). However, earlier studies looking at rats treated with chronic nicotine administration for 4 weeks showed no effect on growth rate and water intake of (Bhagat, 1970). There were no changes in the norepinephrine content of the brain, although the turnover rate was increased. Chronic treatment has been shown to have no effect on body temperature but reduces heart rates (Marks et al., 1983). These findings suggest that acute nicotine administration affects physiological parameters, but chronic treatment leads to tolerance development.

Since administration of pharmacological agents by intraperitoneal injection will affect all areas of the brain, nicotine will affect not just striatal dopamine and glutamate release as previously described but also other neurotransmitter systems. Nicotine modulates the release of neurotransmitters in the hippocampus such as norepinephrine and acetylcholine, contributing to functions such as spatial memory and attention. Norepinephrine release occurs by activation of nAChR on terminals projecting from the locus ceruleus to the hippocampus and acetylcholine release occurs by activation of nAChR autoreceptors on terminals projecting from the medial septum to the hippocampus (Wonnacott, 1997). A synaptosomal study showed that norepinephrine release from hippocampal synaptosomes was 40-fold less sensitive to nicotine than dopamine release from striatal synaptosomes, possibly due to different composition of nAChR subtypes (Clarke and Reuben, 1996). In this study, the EC_{50} of nicotine induced dopamine release in striatal synaptosomes was 0.16 μM, whereas the EC_{50} of nicotine induced norepinephrine in hippocampal synaptosomes was 6.5 μM. Another study showed that the EC_{50} of nicotine induced acetylcholine release in hippocampal synpatosomes was 0.9 μM (Wilkie et al., 1996). Thus, the previous studies showing behavioral benefits of nicotine after injury is attributed to not only nicotine's effects on striatal dopamine signaling, but also to a combination of effects on various regions of the brain and multiple neurotransmitter signaling.

Nicotinic receptors are also present in the prefrontal cortex, as demonstrated by an autoradiography using radiolabeled nicotine binding (Clarke et al., 1985). Since prefrontal cortex has important roles in cognitive functions such as working memory, planning, and attention (Hahn et al., 2003), nicotine's effect in this region may explain how it improves cognitive function. Also, nicotine has been demonstrated to induce dopamine release in

prefrontal cortex when administered locally (Marshall et al., 1997) or systemically (Nisell et al., 1996). Thus, cognitive enhancement of nicotine in previous TBI study (Verbois et al., 2003) as well as other modes of brain injury (Yamada et al., 2010; Sharifzadeh et al., 2005; Brown et al., 2000) may be due to a combination of effects on prefrontal cortex, hippocampus, striatum, as well as other regions. If the current study showed cognitive benefit of nicotine treatment, the next goal would have been to study nicotine's effect on dopamine, norepinephrine, glutamate, and acetylcholine neurotransmission in hippocampus and frontal cortex. The current findings further demonstrate that neurorecovery following TBI by a pharmacological management is challenging due to the involvement of multiple neurotransmitter systems and complexity of injury.

5.1.3 The effects of nicotine on dopamine transporters

Synaptic levels of dopamine are not only regulated by release but also by uptake. Dopamine transporter (DAT) is a membrane protein that provides the primary mechanism of dopamine clearance from the synapse. It functions as a symporter that co-transports two Na^+ and one Cl^- ion down the concentration gradient along with dopamine. Previously, other drugs that induce dopamine release such as cocaine and amphetamines have been reported to alter the surface expression of DAT (Daws et al., 2002; Kahlig et al., 2006). In the nucleus accumbens, in vivo voltammetry was used to show that nicotine increases dopamine clearance (Hart and Ksir, 1996). Another study using the same method showed that nicotine induced faster dopamine clearance in the dorsal striatum as well as prefrontal cortex (Middleton et al., 2004). Striatal synaptosome experiments showed that nicotine treatment increases dopamine uptake, but surface

expression of striatal DAT using subcellular fractionation and Western blots showed no changes (Middleton et al., 2007). Since the level of DAT was not altered but the activity has increased with nicotine treatment, the authors proposed that phosphorylation levels of DAT may be changed by a kinase or a phosphatase, altering its activity. Another molecule that modulates DAT is α-syn, a small chaperone like protein that binds to DAT to inhibit its activity.

The current study reported TH activity deficits after TBI and showed that nicotine treatment can reverse this. Also, dopamine release deficits occur after TBI, and nicotine improves dopamine release. If nicotine reduces DAT activity, striatal extracellular dopamine could increase. However, previous studies have shown that nicotine does not reduce DAT activity but in fact may increase it. Since dopamine levels are increased with nicotine administration in previous studies using microdialysis in striatum (Marshall et al., 1997) and VTA (Rahman et al., 2003), the effect of nicotine on dopamine release overcomes the opposing effect increasing DAT activity.

5.1.4 The Role of ERK in TBI

Aside from its role in memory and learning, ERK is also an important mediator of differentiation, proliferation, and apoptosis at acute time points following TBI (Neary, 2005). The levels of pERK were previously reported to be increased in pericontusional cortex as early as 5 minutes after TBI, returning to baseline values by 6 hours (Otani et al., 2002). Another report showed increased pERK levels in the cortex, subcortical white matter, thalamus, and hippocampus ranging from 2 hours to 72 hours post injury (Raghupathi et al., 2003). Although TBI induced activation of ERK has a major role in apoptosis, it also has a major role in neuronal

plasticity. Inhibition of pERK upregulation acutely after TBI is detrimental to behavioral outcome after TBI (Dash et al., 2002). Application of PD98059 by intracerebroventribular injection 20 minutes prior to injury inhibited this upregulation of pERK but also induced worsening motor performance and spatial memory. The fact that this increase in pERK was specific to neurons rather than astroglia (Mori et al., 2002; Dash et al., 2002) also supports the possibility that pERK contributes to neurogenesis and neuronal plasticity after injury, especially in TBI.

The effects of ERK activity at chronic time points following TBI have not been commonly reported. At 2-8 weeks after TBI, hippocampal levels of pERK and pCREB upon glutamate or potassium activation were reduced (Atkins et al., 2009). The chronic hypofunction of ERK after TBI demonstrated by this study may be a contributing factor for functional deficits after injury. These studies indicate that ERK activity is upregulated at acute time points but downregulated at chronic time points following TBI.

In the current study, no changes in pERK were seen in the striatum after injury. The time point used in the current study is 1 week, which is neither an acute nor a chronic time point. The acute increase in pERK is possibly decreased to the baseline at this point, since both experiments by Raghupathi et al. and Otani et al., showed reversal of injury induced pERK upregulation by 6 hours and 72 hours, respectively.

The experimental settings in the current study were different from the setting of Atkins et al., in which a hippocampal slice was stimulated by glutamate or potassium. Without glutamate

or potassium stimulus, basal pERK and pCREB levels were not different between sham and injured animals in this study. However, when the stimulus was applied, injured animals displayed deficits in upregulation of pERK and pCREB. The striatal tissue that is assessed in the current study was dissected from animals without glutamate or potassium stimulation. This may explain why no differences were seen among the four groups for pERK and pCREB in Western blot data.

5.1.5 Limitations of the Study and Future Suggestions

One of the weaknesses of this study is the usage of only one nicotine regimen. A full dose-response study for behavior has not been performed. Since nicotine at different dose can have different effects on DARPP-32 phosphorylation (Hamada et al., 2004) as well as behavior (Ksir et al., 1987), a dose-response study may add insight into optimal treatment dose of nicotine for recovery from TBI induced deficits. Also, the effects of once daily or twice daily injection were not compared. The previous study using nicotine in the setting of TBI (Verbois et al., 2003) utilized twice daily injection regimen, whereas other studies assessing the effects on nicotine on cognitive enhancement (Riekkinen and Riekkinen, 1997) and dopamine release (Rahman et al., 2003; Marshall et al., 1997) utilized once daily injection regimen.

Moreover, only intraperitoneal administration of nicotine was performed, and no experiment was performed using subcutaneous injection or delivery by osmotic pump. The current dose and delivery method was based on the same regimen that enhanced dopamine release and TH activity in Chapter 3. Thus, the regimen was not optimized to yield behavioral

benefit. Currently, it is not clear which regimen, dose, and delivery of nicotine is optimal for enhancing dopamine signaling and functional outcome after TBI. This limitation can be overcome by additional experiments thoroughly characterizing the effects of each method of nicotine administration.

Another possible limitation is the lack of characterization of different injury severities. In our current study, only severe injury was performed at a velocity of 4 m/s and injury depth: 2.6 mm. However, previously reported studies using the CCI technique used lower values: velocity: 2.32 m/s, depth: 2 mm (Moro et al., 2011), velocity: 3.5 m/s, depth: 1.5 mm (Verbois et al., 2003), velocity: 1.5 m/s, depth: 2 mm (Nishibe et al., 2010). Also, compared to the Verbois' article from 2003 as well as other behavioral experimental data from our lab, the Injured Saline group's motor and cognitive deficits are more pronounced.

In Chapter 4, the performance of Injured Saline group on Beam Walking Latency and Beam Walking Score showed no signs of recovery until the last two days of the testing. The final Latency and Score values were very small improvements compared to first day following injury. It is possible that the severity of injury was too high, and that nicotine is not effective in the setting of severe injury. Lowering the injury severity may show benefits of nicotine treatment in motor and cognitive function.

Although we have seen enhancement of TH activity and dopamine release after nicotine administration, this did not enhance DARPP-32 phosphorylation. It is not clear if TBI induced deficits in DARPP-32 phosphorylation is reversible or not by increasing dopamine release. In

order to investigate this, chronic dopamine receptor agonist treatment should be performed following injury in rats. The study should use delivery method and dose optimized by a pilot study. If dopamine agonist treatment can reverse DARPP-32 phosphorylation deficit, then multiple agents that enhance dopamine signaling that are currently used in clinical practice such as methylphenidate and amphetamine can then be tested to reverse DARPP-32 phosphorylation deficit.

As shown in Chapter 1, our lab has previously shown that FK-506 administration reverses TBI induced deficits in DARPP-32 phosphorylation. Thus, combined treatment of nicotine and FK-506 may be able to increase DARPP-32 phosphorylation even further. Another strategy is to use nicotine in conjunction with D_2 receptor antagonist. This will reduce the possible inhibition of cAMP and PKA signaling pathway induced by D_2 receptor activation. As striatal dopamine signaling is a complex phenomena involving multiple kinase cascades, combined drug administration may have added benefit.

An additional study that can help us gain a deeper understanding of striatal dopamine signaling is an experiment using DARPP-32 knockout mice. Other than nicotine, dopamine signal enhancing agents such as amphetamine (Evans et al., 1987), methylphenidate (Wagnet et al., 2009), and L-DOPA (Koeda and Takeshita, 1998; Kraus and Maki, 1997) induce functional benefit in the setting of TBI. A direct experiment to test if DARPP-32 signaling has an important role in this functional benefit is to administer nicotine or other dopamine enhancing agent to DARPP-32 knockout mice following TBI. If the deficit in pDARPP-32-T34 is an

important contributor to functional deficits following TBI, the functional benefits induced by these agents will not be seen in DARPP-32 knockout mice.

5.2 Summary and Conclusion

After identification of specific parameters of deficit in striatal dopamine signaling following TBI, this project pursued a possible therapeutic regimen using nicotine with the intention of enhancing dopamine synthesis and release. The presynaptic deficits in dopamine signaling such as TH activity and dopamine release are recovered by nicotine treatment. However, activity of downstream signaling molecules such as PKA, DARPP-32, ERK, and CREB show no significant changes in activation after nicotine treatment, at least at the time points that we evaluated. In agreement with this, the behavioral data showed no benefit of nicotine treatment. Thus, the data in this study suggest that: ***Traumatic brain injury induces striatal dopamine synthesis and release deficit, and a post injury nicotine treatment that was sufficient to induce recovery of striatal presynaptic dopamine signaling was not sufficient to induce striatal postsynaptic dopamine signaling or behavioral recovery.***

Although the original hope of this project was to identify the therapeutic potential for nicotine after TBI, the current data show the complexity of dopamine neurotransmission in the striatum. The knowledge gained from this study can be applied to devising more sophisticated pharmacological treatments following TBI in future studies, using a combination of agents such as calcineurin inhibitors and/or glutamate and dopamine receptor antagonists/agonists.

BIBLIOGRAPHY

Abeliovich A, Schmitz Y, Fariñas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron. 2000 Jan;25(1):239-52.

Adler LE, Hoffer LD, Wiser A, Freedman R. Normalization of auditory physiology by cigarette smoking in schizophrenic patients. Am J Psychiatry. 1993 Dec;150(12):1856-61.

Agut J, Ortiz JA, Wurtman RJ. Ann N Y Acad Sci. 2000;920:332-5. Cytidine (5')diphosphocholine modulates dopamine K(+)-evoked release in striatum measured by microdialysis.

Anden AE, Carlsson A, Dahlstroem A, Fuxe K, Hillarp NA, Larsson K. Demonstration and mapping out of nigrostriatal dopamine neurons, *Life Sci.* **3** (1964), pp. 523–530.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD. The MAPK cascade is required for mammalian associative learning. Nat Neurosci. 1998 Nov;1(7):602-9.

Atkins CM, Falo MC, Alonso OF, Bramlett HM, Dietrich WD. Deficits in ERK and CREB activation in the hippocampus after traumatic brain injury. Neurosci Lett. 2009 Aug 7;459(2):52-6. Epub 2009 May 3.

Bales JW, Wagner AK, Kline AE, Dixon CE. Persistent cognitive dysfunction after traumatic brain injury: A dopamine hypothesis. Neurosci Biobehav Rev. 2009 Jul;33(7):981-1003. Epub 2009 Apr 1. Review.

Bales JW. The DARPP-32/PP-1 Signaling Pathway: A Novel Therapeutic Target Following Traumatic Brain Injury. 2010; Doctoral dissertation, University of Pittsburgh, Pittsburgh, PA.

Bhagat B. Effects of chronic administration of nicotine on storage and synthesis of noradrenaline in rat brain. Br J Pharmacol. 1970 Jan;38(1):86-92.

Bak IJ, Hassler R, Kim JS, Kataoka K. Amantadine actions on acetylcholine and GABA in striatum and substantia nigra of rat in relation to behavioral changes. J Neural Transm. 1972; 33(1):45–61.

Bednar I, Friberg L, Nordberg A. Modulation of dopamine release by the nicotinic agonist epibatidine in the frontal cortex and the nucleus accumbens of naive and chronic nicotine treated rats. Neurochem Int. 2004 Dec;45(7):1049-55.

Beers SR, Skold A, Dixon CE, Adelson PD. Neurobehavioral effects of amantadine after pediatric traumatic brain injury: a preliminary report. J Head Trauma Rehabil. 2005 Sep-Oct;20(5):450-63.

Bevilaqua LR, Graham ME, Dunkley PR, von Nagy-Felsobuki EI, Dickson PW. Phosphorylation of Ser(19) alters the conformation of tyrosine hydroxylase to increase the rate of phosphorylation of Ser(40). J Biol Chem. 2001 Nov 2;276(44):40411-6. Epub 2001 Aug 13.

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC Jr, Nairn AC, Greengard P. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature. 1999 Dec 9;402(6762):669-71.

Block F, Kunkel M, Schwarz M. Quinolinic acid lesion of the striatum induces impairment in spatial learning and motor performance in rats. Neurosci Lett. 1993 Jan 12;149(2):126-8.

Blum S, Moore AN, Adams F, Dash PK. A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. J Neurosci. 1999 May 1;19(9):3535-44.

Bobrovskaya L, Gilligan C, Bolster EK, Flaherty JJ, Dickson PW, Dunkley PR. Sustained phosphorylation of tyrosine hydroxylase at serine 40: a novel mechanism for maintenance of catecholamine synthesis. J Neurochem. 2007 Jan;100(2):479-89. Epub 2006 Oct 25.

Bowyer JF, Masserano JM, Weiner N. Inhibitory effects of amphetamine on potassium-stimulated release of [3H]dopamine from striatal slices and synaptosomes. J Pharmacol Exp Ther. 1987 Jan;240(1):177-86.

Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon J, et al. A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. N Engl J Med. 1990 May 17;322(20):1405-11.

Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, Fehlings M, Herr DL, Hitchon PW, Marshall LF, Nockels RP, Pascale V, Perot PL Jr, Piepmeier J, Sonntag VK, Wagner F, Wilberger JE, Winn HR, Young W. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. JAMA. 1997 May 28;277(20):1597-604.

Brown RW, Gonzalez CL, Kolb B. Nicotine improves Morris water task performance in rats given medial frontal cortex lesions. Pharmacol Biochem Behav. 2000 Nov;67(3):473-8.

Bureau G, Carrier M, Lebel M, Cyr M. Intrastriatal inhibition of extracellular signal-regulated

kinases impaired the consolidation phase of motor skill learning. Neurobiol Learn Mem. 2010 Jul;94(1):107-15. Epub 2010 May 4.

Calabresi P, Gubellini P, Centonze D, Picconi B, Bernardi G, Chergui K, Svenningsson P, Fienberg AA, Greengard P. Dopamine and cAMP-regulated phosphoprotein 32 kDa controls both striatal long-term depression and long-term potentiation, opposing forms of synaptic plasticity. J Neurosci. 2000 Nov 15;20(22):8443-51.

Carr LA, Rowell PP, Pierce WM Jr. Effects of subchronic nicotine administration on central dopaminergic mechanisms in the rat. Neurochem Res. 1989 Jun;14(6):511-5.

Campbell DG, Hardie DG, Vulliet PR. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. J Biol Chem. 1986 Aug 15;261(23):10489-92.

Charpier S, Deniau JM. In vivo activity-dependent plasticity at cortico-striatal connections: evidence for physiological long-term potentiation. Proc Natl Acad Sci U S A. 1997 Jun 24;94(13):7036-40.

Chéramy A, Desce JM, Godeheu G, Glowinski J. Presynaptic control of dopamine synthesis and release by excitatory amino acids in rat striatal synaptosomes. Neurochem Int. 1994 Aug;25(2):145-54.

Chowdhury M, Fillenz M. Presynaptic adenosine A2 and N-methyl-D-aspartate receptors regulate dopamine synthesis in rat striatal synaptosomes. J Neurochem. 1991 May;56(5):1783-8.

Clark WM. Efficacy of citicoline as an acute stroke treatment. Expert Opin Pharmacother. 2009 Apr;10(5):839-46.

Clarke PB, Reuben M. Release of [3H]-noradrenaline from rat hippocampal synaptosomes by nicotine: mediation by different nicotinic receptor subtypes from striatal [3H]-dopamine release. Br J Pharmacol. 1996 Feb;117(4):595-606.

Clarke PB, Schwartz RD, Paul SM, Pert CB, Pert A. Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. J Neurosci. 1985 May;5(5):1307-15.

Conant R, Schauss AG. Therapeutic applications of citicoline for stroke and cognitive dysfunction in the elderly: a review of the literature. Altern Med Rev. 2004 Mar;9(1):17-31.

Consensus conference. Rehabilitation of persons with traumatic brain injury. NIH consensus development panel on rehabilitation of persons with traumatic brain injury. JAMA. 1999, Vols. 282(10):974-83.

Cooper DM, Mons N, Fagan K. Ca(2+)-sensitive adenylyl cyclases. Cell Signal. 1994 Nov;6(8):823-40. Review

Cooper DM, Mons N, Karpen JW. Adenylyl cyclases and the interaction between calcium and cAMP signalling. Nature. 1995 Mar 30;374(6521):421-4. Review

Costa G, Abin-Carriquiry JA, Dajas F. Nicotine prevents striatal dopamine loss produced by 6-hydroxydopamine lesion in the substantia nigra. Brain Res. 2001 Jan 12;888(2):336-342.

Craviso GL, Hemelt VB, Waymire JC. Nicotinic cholinergic regulation of tyrosine hydroxylase gene expression and catecholamine synthesis in isolated bovine adrenal chromaffin cells. J Neurochem. 1992 Dec;59(6):2285-96.

Craviso GL, Hemelt VB, Waymire JC. The transient nicotinic stimulation of tyrosine hydroxylase gene transcription in bovine adrenal chromaffin cells is independent of c-fos gene activation. Brain Res Mol Brain Res. 1995 Apr;29(2):233-44.

Dani JA, Ji D, Zhou FM. Synaptic plasticity and nicotine addiction. Neuron. 2001 Aug 16;31(3):349-52.

Dash PK, Mach SA, Moore AN. The role of extracellular signal-regulated kinase in cognitive and motor deficits following experimental traumatic brain injury. Neuroscience. 2002;114(3):755-67.

Daubner SC, Lauriano C, Haycock JW, Fitzpatrick PF. Site-directed mutagenesis of serine 40 of rat tyrosine hydroxylase. Effects of dopamine and cAMP-dependent phosphorylation on enzyme activity. J Biol Chem. 1992 Jun 25;267(18):12639-46.

Daws LC, Callaghan PD, Morón JA, Kahlig KM, Shippenberg TS, Javitch JA, Galli A. Cocaine increases dopamine uptake and cell surface expression of dopamine transporters. Biochem Biophys Res Commun. 2002 Feb 8;290(5):1545-50.

Decker MW, Curzon P, Brioni JD, Arnerić SP. Effects of ABT-418, a novel cholinergic channel ligand, on place learning in septal-lesioned rats. Eur J Pharmacol. 1994 Aug 11;261(1-2):217-22.

De Deyne CS. Therapeutic hypothermia and traumatic brain injury. Curr Opin Anaesthesiol. 2010 Apr;23(2):258-62.

Delanoy RL, Hunter GD, Dunn AJ. Catecholamine metabolism in brain slices. Determination of relevant precursor pool and the effects of elevated K+. Biochem Pharmacol. 1982 Oct 15;31(20):3289-96.

Deng T, Karin M. c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK.. Nature. 1994 Sep 8;371(6493):171-5.

Desce JM, Godeheu G, Galli T, Glowinski J, Chéramy A. Opposite presynaptic regulations by glutamate through NMDA receptors of dopamine synthesis and release in rat striatal synaptosomes. Brain Res. 1994 Mar 21;640(1-2):205-14.

Devan BD, McDonald RJ, White NM. Effects of medial and lateral caudate-putamen lesions on place- and cue-guided behaviors in the water maze: relation to thigmotaxis. Behav Brain Res. 1999 Apr;100(1-2):5-14.

Dixon CE, Lyeth BG, Povlishock JT, Findling RL, Hamm RJ, Marmarou A, Young HF, Hayes RL. A fluid percussion model of experimental brain injury in the rat. J Neurosurg. 1987 Jul;67(1):110-9.

Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL. A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Methods. 1991 Oct;39(3):253-62.

Dixon CE, Ma X, Marion DW. Effects of CDP-choline treatment on neurobehavioral deficits after TBI and on hippocampal and neocortical acetylcholine release. J Neurotrauma. 1997 Mar;14(3):161-9.

Dixon CE, Kraus MF, Kline AE, Ma X, Yan HQ, Griffith RG, Wolfson BM, Marion DW. Amantadine improves water maze performance without affecting motor behavior following traumatic brain injury in rats. Restor Neurol Neurosci. 1999;14(4):285-294.

Dluzen DE, McDermott JL, Ramirez VD. Changes in dopamine release in vitro from the corpus striatum of young versus aged rats as a function of infusion modes of L-dopa, potassium, and amphetamine. Exp Neurol. 1991; 112:153–160.

Drenan RM, Grady SR, Steele AD, McKinney S, Patzlaff NE, McIntosh JM, Marks MJ, Miwa JM, Lester HA. Cholinergic modulation of locomotion and striatal dopamine release is mediated by alpha6alpha4* nicotinic acetylcholine receptors. J Neurosci. 2010 Jul 21;30(29):9877-89.

Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW. Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem. 2004; 91:1025-43. Review.

English JD, Sweatt JD. Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J Biol Chem. 1996 Oct 4;271(40):24329-32.

English JD, Sweatt JD. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem. 1997 Aug 1;272(31):19103-6.

Erinoff L, MacPhail RC, Heller A, Seiden LS. Age-dependent effects of 6-hydroxydopamine on locomotor activity in the rat. Brain Res. 1979 Mar 23;164:195-205.

Evans RW, Gualtieri CT, Patterson D. Treatment of chronic closed head injury with psychostimulant drugs: a controlled case study and an appropriate evaluation procedure. J Nerv Ment Dis. 1987 Feb;175(2):106-10.

Exley R, Clements MA, Hartung H, McIntosh JM, Cragg SJ. Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. Neuropsychopharmacology. 2008 Aug;33(9):2158-66. Epub 2007 Nov 21.

Feeney DM, Gonzales A, Law WA. Amphetamine restores locomotor function after motor cortex injury in the rat. Proc West Pharmacol Soc. 1981;24:15-7.

Fitch MT, Silver J. Activated macrophages and the blood-brain barrier: inflammation after CNS injury leads to increases in putative inhibitory molecules. Exp Neurol. 1997 Dec;148(2):587-603.

Freedman R, Hall M, Adler LE, Leonard S. Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. Biol Psychiatry. 1995 Jul 1;38(1):22-33.

Frizzo JK, Tramontina AC, Tramontina F, Gottfried C, Leal RB, Donato R, Gonçalves CA. Involvement of the S100B in cAMP-induced cytoskeleton remodeling in astrocytes: a study using TRTK-12 in digitonin-permeabilized cells. Cell Mol Neurobiol. 2004 Dec;24(6):833-40.

Gatto GJ, Bohme GA, Caldwell WS, Letchworth SR, Traina VM, Obinu MC, Laville M, Reibaud M, Pradier L, Dunbar G, Bencherif M. TC-1734: an orally active neuronal nicotinic acetylcholine receptor modulator with antidepressant, neuroprotective and long-lasting cognitive effects. CNS Drug Rev. 2004 Summer;10(2):147-66.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr, Sibley DR. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science. 1990 Dec 7;250(4986):1429-32.

Gerhardt GA, Maloney RE Jr. Brain Res. 1999 Jan 16;816(1):68-77. Microdialysis studies of basal levels and stimulus-evoked overflow of dopamine and metabolites in the striatum of young and aged Fischer 344 rats.

Ghosheh O, Dwoskin LP, Li WK, Crooks PA. Residence times and half-lives of nicotine metabolites in rat brain after acute peripheral administration of [2-(14)C]nicotine. Drug Metab Dispos. 1999 Dec;27(12):1448-55.

Ghosheh OA, Dwoskin LP, Miller DK, Crooks PA. Accumulation of nicotine and its metabolites in rat brain after intermittent or continuous peripheral administration of [2'-(14)C]nicotine. Drug Metab Dispos. 2001 May;29(5):645-51.

Giniatullin R, Nistri A, Yakel JL. Desensitization of nicotinic ACh receptors: shaping cholinergic signaling, Trends Neurosci. 2005 28:371–378.

Gordon SL, Quinsy NS, Dunkley PR, Dickson PW. Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites. J Neurochem. 2008 Aug;106(4):1614-23. Epub 2008 May 31.

Grady SR, Salminen O, Laverty DC, Whiteaker P, McIntosh JM, Collins AC, Marks MJ. The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. Biochem Pharmacol. 2007 Oct 15;74(8):1235-46. Epub 2007 Jul 27.

Grady S, Marks MJ, Wonnacott S, Collins AC. Characterization of nicotinic receptor-mediated [3H]dopamine release from synaptosomes prepared from mouse striatum. J Neurochem. 1992 Sep;59(3):848-56.

Greengard P, Allen PB, Nairn AC. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron. 1999 Jul;23(3):435-47. Review.

Guseva MV, Hopkins DM, Scheff SW, Pauly JR. Dietary choline supplementation improves behavioral, histological, and neurochemical outcomes in a rat model of traumatic brain injury. J Neurotrauma. 2008 Aug;25(8):975-83.

Haass M, Kübler W. Nicotine and sympathetic neurotransmission. Cardiovasc Drugs Ther. 1997 Jan;10(6):657-65. Review.

Halpain S, Girault JA, Greengard P. Activation of NMDA receptors induces dephosphorylation of DARPP-32 in rat striatal slices. Nature. 1990 Jan 25;343(6256):369-72.

Hamada M, Higashi H, Nairn AC, Greengard P, Nishi A. Differential regulation of dopamine D1 and D2 signaling by nicotine in neostriatal neurons. J Neurochem. 2004 Sep;90(5):1094-103.

Hamada M, Hendrick JP, Ryan GR, Kuroiwa M, Higashi H, Tanaka M, Nairn AC, Greengard P, Nishi A. Nicotine regulates DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) phosphorylation at multiple sites in neostriatal neurons. J Pharmacol Exp Ther. 2005 Nov;315(2):872-8. Epub 2005 Jul 22.

Harada L, Wu J, Haycock JW, Goldstein M. Regulation of l-DOPA biosynthesis by site-specific phosphorylation of tyrosine hydroxylase in AtT-20 cells expressing wild-type and serine 40-substituted enzyme. J Neurochem. **67** 1996. 67:629–635.

Hart C, Ksir C. Nicotine effects on dopamine clearance in rat nucleus accumbens. J Neurochem. 1996 Jan;66(1):216-21.

Hauber W. Dopamine release in the prefrontal cortex and striatum: temporal and behavioural aspects. Pharmacopsychiatry. 2010 May;43 Suppl 1:S32-41. Epub 2010 May 17. Review.

Hauber W. Involvement of basal ganglia transmitter systems in movement initiation. Prog Neurobiol. 1998 Dec;56(5):507-40. Review.

Haycock JW. Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, 40. J Biol Chem. 1990 Jul 15;265(20):11682-91.

Haycock JW, Haycock DA. Tyrosine hydroxylase in rat brain dopaminergic nerve terminals. Multiple-site phosphorylation in vivo and in synaptosomes. J Biol Chem. 1991 Mar 25;266(9):5650-7.

Haycock JW, Ahn NG, Cobb MH, Krebs EG. ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. Proc Natl Acad Sci U S A. 1992 Mar 15;89(6):2365-9.

Henry JM, Talukder NK, Lee AB Jr, Walker ML. Cerebral trauma-induced changes in corpus striatal dopamine receptor subtypes. J Invest Surg. 1997 Sep-Oct;10(5):281-6.

Hiremagalur B, Nankova B, Nitahara J, Zeman R, Sabban EL. Nicotine increases expression of tyrosine hydroxylase gene. Involvement of protein kinase A-mediated pathway. J Biol Chem. 1993 Nov 5;268(31):23704-11.

Hokfelt T, Johansson O, Fuxe K, Goldstein M, Park D. 1977. Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain II. Tyrosine hydroxylase in the telencephalon. Med Biol 55, 21-40.

Hoofien D, Gilboa A, Vakil E, Donovick PJ. Traumatic brain injury (TBI) 10-20 years later: a comprehensive outcome study of psychiatric symptomatology, cognitive abilities and psychosocial functioning. 2001, Brain Inj, Vols. 15(3):189-209.

Hovda DA, Sutton RL, Feeney DM. Amphetamine-induced recovery of visual cliff performance after bilateral visual cortex ablation in cats: measurements of depth perception thresholds. Behav Neurosci. 1989 Jun;103(3):574-84.

Hornstein A, Lennihan L, Seliger G, Lichtman S, Schroeder K. Amphetamine in recovery from brain injury. Brain Inj. 1996 Feb;10(2):145-8.

Huang LZ, Parameswaran N, Bordia T, Michael McIntosh J, Quik M. Nicotine is neuroprotective when administered before but not after nigrostriatal damage in rats and monkeys. J Neurochem. 2009 May;109(3):826-37. Epub 2009 Feb 24.

Huff RM. Signal transduction pathways modulated by the D2 subfamily of dopamine receptors. Cell Signal. 1996 Sep;8(6):453-9. Review.

Hyland BI, Reynolds JN, Hay J, Perk CG, Miller R. Firing modes of midbrain dopamine cells in the freely moving rat. Neuroscience. 2002;114(2):475-92.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR. Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron. 1998 Oct;21(4):869-83.

Jain KK. Neuroprotection in traumatic brain injury. Drug Discov Today. 2008 Dec;13(23-24):1082-9. Epub 2008 Oct 22. Review.

Janson AM, Fuxe K, Agnati LF, Kitayama I, Härfstrand A, Andersson K, Goldstein M. Chronic nicotine treatment counteracts the disappearance of tyrosine-hydroxylase-immunoreactive nerve cell bodies, dendrites and terminals in the mesostriatal dopamine system of the male rat after partial hemitransection. Brain Res. 1988 Jul 12;455(2):332-45.

Janson AM, Fuxe K, Sundström E, Agnati LF, Goldstein M. Chronic nicotine treatment partly protects against the 1-methyl-4-phenyl-2,3,6-tetrahydropyridine-induced degeneration of nigrostriatal dopamine neurons in the black mouse. Acta Physiol Scand. 1988 Apr;132(4):589-91.

Janson AM, Meana JJ, Goiny M, Herrera-Marschitz M. Chronic nicotine treatment counteracts the decrease in extracellular neostriatal dopamine induced by a unilateral transection at the mesodiencephalic junction in rats: a microdialysis study. Neurosci Lett. 1991 Dec 16;134(1):88-2.

Jones GM, Sahakian BJ, Levy R, Warburton DM, Gray JA.Effects of acute subcutaneous nicotine on attention, information processing and short-term memory in Alzheimer's disease. Psychopharmacology (Berl). 1992;108(4):485-94.

Kahlig KM, Lute BJ, Wei Y, Loland CJ, Gether U, Javitch JA, Galli A. Regulation of dopamine transporter trafficking by intracellular amphetamine. Mol Pharmacol. 2006 Aug;70(2):542-8. Epub 2006 May 9.

Kametani H, Iijima S, Spangler EL, Ingram DK, Joseph JA. In vivo assessment of striatal dopamine release in the aged male Fischer 344 rat. Neurobiol Aging. 1995; **16**:639–646.

Kihara T, Shimohama S, Sawada H, Honda K, Nakamizo T, Shibasaki H, Kume T, Akaike A. alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. J Biol Chem. 2001 Apr 27;276(17):13541-6. Epub 2001 Jan 19.

King MM, Huang CY, Chock PB, Nairn AC, Hemmings HC Jr, Chan KF, Greengard P. Mammalian brain phosphoproteins as substrates for calcineurin. J Biol Chem. 1984 Jul 10;259(13):8080-3.

Kleppe R, Toska K, Haavik J. Interaction of phosphorylated tyrosine hydroxylase with 14-3-3 proteins: evidence for a phosphoserine 40-dependent association. J Neurochem. 2001 May;77(4):1097-107.

Kline AE, Chen MJ, Tso-Olivas DY, Feeney DM. Methylphenidate treatment following ablation-induced hemiplegia in rat: experience during drug action alters effects on recovery of function. Pharmacol Biochem Behav. 1994 Jul;48(3):773-9

Kline AE, Yan HQ, Bao J, Marion DW, Dixon CE. Chronic methylphenidate treatment enhances water maze performance following traumatic brain injury in rats. Neurosci Lett. 2000 Feb 25;280(3):163-6.

Koeda T, Takeshita K. A case report of remarkable improvement of motor disturbances with L-dopa in a patient with post-diffuse axonal injury. Brain Dev. 1998 Mar;20(2):124-6.

Korchounov AM. Role of D1 and D2 receptors in the regulation of voluntary movements. Bull Exp Biol Med. 2008 Jul;146:14-7.

Kraus MF, Maki P. The combined use of amantadine and l-dopa/carbidopa in the treatment of chronic brain injury. Brain Inj. 1997 Jun;11(6):455-60.

Ksir C, Hakan R, Hall DP Jr, Kellar KJ. Exposure to nicotine enhances the behavioral stimulant effect of nicotine and increases binding of [3H]acetylcholine to nicotinic receptors. Neuropharmacology. 1985 Jun;24(6):527-31.

Ksir C, Hakan RL, Kellar KJ. Chronic nicotine and locomotor activity: influences of exposure dose and test dose. Psychopharmacology (Berl). 1987;92(1):25-9.

Lal S, Merbtiz CP, Grip JC. Modification of function in head-injured patients with Sinemet. Brain Inj. 1988 Jul-Sep;2(3):225-33.

Levey AI, Hersch SM, Rye DB, Sunahara RK, Niznik HB, Kitt CA, Price DL, Maggio R, Brann MR, Ciliax BJ. Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. Proc Natl Acad Sci U S A. 1993 Oct 1;90(19):8861-5.

Levin ED, Lee C, Rose JE, Reyes A, Ellison G, Jarvik M, Gritz E. Chronic nicotine and withdrawal effects on radial-arm maze performance in rats. Behav Neural Biol. 1990 Mar;53(2):269-76.

Levin ED, Briggs SJ, Christopher NC, Rose JE. Persistence of chronic nicotine-induced cognitive facilitation. Behav Neural Biol. 1992 Sep;58(2):152-8.

Levin ED, Rose JE, Abood L. Effects of nicotinic dimethylaminoethyl esters on working memory performance of rats in the radial-arm maze. Pharmacol Biochem Behav. 1995 Jun-Jul;51(2-3):369-73.

Levin ED, Kaplan S, Boardman A. Acute nicotine interactions with nicotinic and muscarinic antagonists: working and reference memory effects in the 16-arm radial maze. Behav Pharmacol. 1997 Jun;8(2-3):236-42.

Levin ED, Bettegowda C, Blosser J, Gordon J. AR-R17779, and alpha7 nicotinic agonist, improves learning and memory in rats. Behav Pharmacol. 1999 Nov;10(6-7):675-80.

Lindgren N, Xu ZQ, Lindskog M, Herrera-Marschitz M, Goiny M, Haycock J, Goldstein M, Hökfelt T, Fisone G. Regulation of tyrosine hydroxylase activity and phosphorylation at Ser(19) and Ser(40) via activation of glutamate NMDA receptors in rat striatum. J Neurochem. 2000 Jun;74(6):2470-7.

Lindgren N, Xu Z-Q D, Herrera-Marschitz m, Haycock J, Hokfelt T, Fisone G. Dopamine D2 receptors regulate tyrosine hydroxylase activity and phosphorylation at Ser40 in rat striatum. Eur J Neurosci. 2001. Feb;13(4):773-80.

Liu F, Ma XH, Ule J, Bibb JA, Nishi A, DeMaggio AJ, Yan Z, Nairn AC, Greengard P. Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11062-8.

Losiniecki A, Shutter L. Management of traumatic brain injury. Curr Treat Options Neurol. 2010 Mar;12(2):142-54.

Maggio R, Riva M, Vaglini F, Fornai F, Molteni R, Armogida M, Racagni G, Corsini GU. Nicotine prevents experimental parkinsonism in rodents and induces striatal increase of neurotrophic factors. J Neurochem. 1998 Dec;71(6):2439-46.

Marks MJ, Burch JB, Collins AC. Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. J Pharmacol Exp Ther. 1983 Sep;226(3):817-25.

Marshall DL, Redfern PH, Wonnacott S. Presynaptic nicotinic modulation of dopamine release in the three ascending pathways studied by in vivo microdialysis: comparison of naive and chronic nicotine-treated rats. J Neurochem. 1997 Apr;68(4):1511-9.

Massucci JL, Kline AE, Ma X, Zafonte RD, Dixon CE. Time dependent alterations in dopamine tissue levels and metabolism after experimental traumatic brain injury in rats. Neurosci Lett. 2004 Nov 30;372(1-2):127-31.

Matamales M, Bertran-Gonzalez J, Salomon L, Degos B, Deniau JM, Valjent E, Herve D, Girault JA. Striatal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in BAC transgenic mice. PLoS One. 2009;4(3):e4770. Epub 2009 Mar 10.

McGaughy J, Decker MW, Sarter M. Enhancement of sustained attention performance by the nicotinic acetylcholine receptor agonist ABT-418 in intact but not basal forebrain-lesioned rats. Psychopharmacology (Berl). 1999 May;144(2):175-82.

McGehee DS, Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol. 1995;57:521-46.

McIntosh et al., 1994 T.K. McIntosh, T. Yu and T.A. Gennarelli, Alterations in regional brain catecholamine concentrations after experimental brain injury in the rat, *J. Neurochem.* **63** (1994), pp. 1426–1433.

Meyer MJ, Megyesi J, Meythaler J, Murie-Fernandez M, Aubut JA, Foley N, Salter K, Bayley M, Marshall S, Teasell R. Acute management of acquired brain injury part II: an evidence-based review of pharmacological interventions. Brain Inj. 2010;24(5):706-21. Review.

Middleton LS, Apparsundaram S, King-Pospisil KP, Dwoskin LP, Nicotine increases dopamine transporter function in rat striatum through a trafficking-independent mechanism 2007 Eur J Pharmacol 554 :128–136.

Middleton LS, Cass WA, Dwoskin LP. Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex. J Pharmacol Exp Ther. 2004 Jan;308(1):367-77. Epub 2003 Oct 16.

Mori T, Wang X, Jung JC, Sumii T, Singhal AB, Fini ME, Dixon CE, Alessandrini A, Lo EH. Mitogen-activated protein kinase inhibition in traumatic brain injury: in vitro and in vivo effects. J Cereb Blood Flow Metab. 2002 Apr;22(4):444-52.

Moro N, Ghavim SS, Hovda DA, Sutton RL. Delayed sodium pyruvate treatment improves working memory following experimental traumatic brain injury. Neurosci Lett. 2011 Mar 17;491(2):158-62. Epub 2011 Jan 15.

Mueller-Burke D, Koehler RC, Martin LJ. Rapid NMDA receptor phosphorylation and oxidative stress precede striatal neurodegeneration after hypoxic ischemia in newborn piglets and are attenuated with hypothermia. Int J Dev Neurosci. 2008 Feb;26(1):67-76. Epub 2007 Sep 8.

Muizelaar JP, Marmarou A, Ward JD, Kontos HA, Choi SC, Becker DP, Gruemer H, Young HF. Adverse effects of prolonged hyperventilation in patients with severe head injury: a randomized clinical trial. J Neurosurg. 1991 Nov;75(5):731-9.

Mura A, Feldon J. Spatial learning in rats is impaired after degeneration of the nigrostriatal dopaminergic system. Mov Disord. 2003 Aug;18(8):860-71.

Nagel S, Su Y, Horstmann S, Heiland S, Gardner H, Koziol J, Martinez-Torres FJ, Wagner S. Minocycline and hypothermia for reperfusion injury after focal cerebral ischemia in the rat: effects on BBB breakdown and MMP expression in the acute and subacute phase. Brain Res. 2008 Jan 10;1188:198-206. Epub 2007 Nov 26.

Neary JT. Protein kinase signaling cascades in CNS trauma. IUBMB Life. 2005 Nov;57(11):711-8.

Newhouse PA, Sunderland T, Tariot PN, Blumhardt CL, Weingartner H, Mellow A, Murphy DL. Intravenous nicotine in Alzheimer's disease: a pilot study. Psychopharmacology (Berl). 1988;95(2):171-5.

Nisell M, Nomikos GG, Hertel P, Panagis G, Svensson TH. Condition-independent sensitization of locomotor stimulation and mesocortical dopamine release following chronic nicotine treatment in the rat. Synapse. 1996 Apr;22(4):369-81.

Nishi A, Snyder GL, Greengard P. Bidirectional regulation of DARPP-32 phosphorylation by dopamine. J Neurosci. 1997 Nov 1;17(21):8147-55.

Nishi A, Bibb JA, Matsuyama S, Hamada M, Higashi H, Nairn AC, Greengard P. Regulation of DARPP-32 dephosphorylation at PKA- and Cdk5-sites by NMDA and AMPA receptors: distinct roles of calcineurin and protein phosphatase-2A. J Neurochem. 2002 May;81(4):832-41.

Nishi A, Watanabe Y, Higashi H, Tanaka M, Nairn AC, Greengard P. Glutamate regulation of DARPP-32 phosphorylation in neostriatal neurons involves activation of multiple signaling cascades. Proc Natl Acad Sci U S A. 2005 Jan 25;102(4):1199-204. Epub 2005 Jan 18.

Nishibe M, Barbay S, Guggenmos D, Nudo RJ. Reorganization of motor cortex after controlled cortical impact in rats and implications for functional recovery. J Neurotrauma. 2010 Dec;27(12):2221-32. Epub 2010 Nov 22.

Nolan S. Traumatic brain injury: a review. Crit Care Nurs Q. 2005 Apr-Jun;28(2):188-94. Review.

Osterhout CA, Sterling CR, Chikaraishi DM, Tank AW. Induction of tyrosine hydroxylase in the locus coeruleus of transgenic mice in response to stress or nicotine treatment: lack of activation of tyrosine hydroxylase promoter activity. J Neurochem. 2005 Aug;94(3):731-41.

Otani N, Nawashiro H, Fukui S, Nomura N, Shima K. Temporal and spatial profile of phosphorylated mitogen-activated protein kinase pathways after lateral fluid percussion injury in the cortex of the rat brain. J Neurotrauma. 2002 Dec;19(12):1587-96.

Parain K, Hapdey C, Rousselet E, Marchand V, Dumery B, Hirsch EC. Cigarette smoke and nicotine protect dopaminergic neurons against the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine Parkinsonian toxin. Brain Res. 2003 Sep 12;984(1-2):224-32.

Parks RW, Becker RE, Rippey RF, Gilbert DG, Matthews JR, Kabatay E, Young CS, Vohs C, Danz V, Keim P, Collins GT, Zigler SS, Urycki PG. Increased regional cerebral glucose metabolism and semantic memory performance in Alzheimer's disease: a pilot double blind transdermal nicotine positron emission tomography study. Neuropsychol Rev. 1996 Jun;6(2):61-79.

Paul S, Nairn AC, Wang P, Lombroso PJ. NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. Nat Neurosci. 2003 Jan;6(1):34-42.

Peng X, Gerzanich V, Anand R, Wang F, Lindstrom J. Chronic nicotine treatment up-regulates alpha3 and alpha7 acetylcholine receptor subtypes expressed by the human neuroblastoma cell line SH-SY5Y. Mol Pharmacol. 1997 May;51(5):776-84.

Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. A role for alpha-synuclein in the regulation of dopamine biosynthesis. J Neurosci. 2002 Apr 15;22(8):3090-9.

Perez XA, Bordia T, McIntosh JM, Grady SR, Quik M. Long-term nicotine treatment differentially regulates striatal alpha6alpha4beta2* and alpha6(nonalpha4)beta2* nAChR expression and function. Mol Pharmacol. 2008 Sep;74(3):844-53. Epub 2008 Jun 26.

Peterson K, Carson S, Carney N. Hypothermia treatment for traumatic brain injury: a systematic review and meta-analysis. J Neurotrauma. 2008 Jan;25(1):62-71.

Phillips et al., 2003 J.P. Phillips, D.J. Devier and D.M. Feeney, Rehabilitation pharmacology: bridging laboratory work to clinical application, J. Head Trauma Rehabil. 2003. **18:**342–356.

Polli JW, Kincaid RL. Expression of a calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation. J Neurosci. 1994 Mar;14(3 Pt 1):1251-61.

Quik M, Kulak JM. Nicotine and nicotinic receptors; relevance to Parkinson's disease. Neurotoxicology. 2002. 23:581-594.

Quick MW, Lester RA. Desensitization of neuronal nicotinic receptors. J Neurobiol. 2002 Dec;53(4):457-78.

Raghupathi R, Muir JK, Fulp CT, Pittman RN, McIntosh TK. Acute activation of mitogen-activated protein kinases following traumatic brain injury in the rat: implications for posttraumatic cell death. Exp Neurol. 2003 Oct;183(2):438-48.

Rahman, S., J. Zhang, and W.A. Corrigall, Effects of acute and chronic nicotine on somatodendritic dopamine release of the rat ventral tegmental area: in vivo microdialysis study. Neurosci Lett, 2003. 348(2): p. 61-4.

Ralph JK, Lowes T. J R Army Med Corps. 2009 Jun;155(2):147-51. Review. Neurointensive care.

Ramic M, Emerick AJ, Bollnow MR, O'Brien TE, Tsai SY, Kartje GL. Axonal plasticity is associated with motor recovery following amphetamine treatment combined with rehabilitation after brain injury in the adult rat. Brain Res. 2006 Sep 21;1111(1):176-86. Epub 2006 Aug 21.

Rezvani AH, Bushnell PJ, Levin ED. Effects of nicotine and mecamylamine on choice accuracy in an operant visual signal detection task in female rats. Psychopharmacology (Berl). 2002 Dec;164(4):369-75. Epub 2002 Sep 20.

Rezvani AH, Levin ED. Nicotine-alcohol interactions and attentional performance on an operant visual signal detection task in female rats. Pharmacol Biochem Behav. 2003 Aug;76(1):75-83.

Rice ME, Cragg SJ. Nicotine amplifies reward-related dopamine signals in striatum. Nat Neurosci. 2004 Jun;7(6):583-4. Epub 2004 May 16.

Riekkinen M, Riekkinen P Jr. Nicotine and D-cycloserine enhance acquisition of water maze spatial navigation in aged rats. Neuroreport. 1997 Feb 10;8(3):699-703.

Roberts I, Yates D, Sandercock P, Farrell B, Wasserberg J, Lomas G, Cottingham R, Svoboda P, Brayley N, Mazairac G, Laloë V, Muñoz-Sánchez A, Arango M, Hartzenberg B, Khamis H, Yutthakasemsunt S, Komolafe E, Olldashi F, Yadav Y, Murillo-Cabezas F, Shakur H, Edwards P; CRASH trial collaborators. Effect of intravenous corticosteroids on death within 14 days in 10008 adults with clinically significant head injury (MRC CRASH trial): randomised placebo-controlled trial. Lancet. 2004 Oct 9-15;364(9442):1321-8.

Robertson GS, Vincent SR, Fibiger HC. Striatonigral projection neurons contain D1 dopamine receptor-activated c-fos. Brain Res. 1990 Jul 23;523(2):288-90.

Ryan RE, Ross SA, Drago J, Loiacono RE. Dose-related neuroprotective effects of chronic nicotine in 6-hydroxydopamine treated rats, and loss of neuroprotection in alpha4 nicotinic receptor subnit knockout mice. Br J Pharmacol. 2001. 132:1650-1656.

Saatman KE, Duhaime AC, Bullock R, Maas AI, Valadka A, Manley GT; Workshop Scientific Team and Advisory Panel Members. Classification of traumatic brain injury for targeted therapies. J Neurotrauma. 2008 Jul;25(7):719-38.

Sabban EL, Gueorguiev VD. Effects of short- and long-term nicotine treatment on intracellular calcium and tyrosine hydroxylase gene expression. Ann N Y Acad Sci. 2002 Oct;971:39-44.

Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, Grady SR. Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. Mol Pharmacol. 2004 Jun;65(6):1526-35.

Sastry BV, Chance MB, Singh G, Horn JL, Janson VE. Distribution and retention of nicotine and its metabolite, cotinine, in the rat as a function of time. Pharmacology. 1995 Feb;50(2):128-36.

Sawyer E, Mauro LS, Ohlinger MJ. Amantadine enhancement of arousal and cognition after traumatic brain injury. Ann Pharmacother. 2008 Feb;42(2):247-52. Epub 2008 Jan 22.

Sgambato V, Pagès C, Rogard M, Besson MJ, Caboche J. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J Neurosci. 1998 Nov 1;18(21):8814-25.

Shames J, Treger I, Ring H, Giaquinto S. Return to work following traumatic brain injury: trends and challenges. Disabil Rehabil. 2007 Sep 15;29(17):1387-95. Review.

Sharifzadeh M et Sharifzadeh M, Tavasoli M, Naghdi N, Ghanbari A, Amini M, Roghani A. Post-training intrahippocampal infusion of nicotine prevents spatial memory retention deficits induced by the cyclo-oxygenase-2-specific inhibitor celecoxib in rats. J Neurochem. 2005 Nov;95(4):1078-90. Epub 2005 Sep 7.

Shiflett MW, Martini RP, Mauna JC, Foster RL, Peet E, Thiels E. Cue-elicited reward-seeking requires extracellular signal-regulated kinase activation in the nucleus accumbens. J Neurosci. 2008 Feb 6;28(6):1434-43.

Shiflett MW, Mauna JC, Chipman AM, Peet E, Thiels E. Appetitive Pavlovian conditioned stimuli increase CREB phosphorylation in the nucleus accumbens. Neurobiol Learn Mem. 2009 Oct;92(3):451-4. Epub 2009 Feb 25.

Shiflett MW, Balleine BW. Contributions of ERK signaling in the striatum to instrumental learning and performance. Behav Brain Res. 2011 Mar 17;218(1):240-7. Epub 2010 Dec 13.

Shimohama, S. and T. Kihara, Nicotinic receptor-mediated protection against beta-amyloid neurotoxicity. Biol Psychiatry, 2001. 49(3): p. 233-9.

Shin SS, Bray ER, Zhang CQ, Dixon CE. Traumatic brain injury reduces striatal tyrosine hydroxylase activity and potassium-evoked dopamine release in rats. Brain Res. 2010 Nov 1. [Epub ahead of print]

Shui HA, Peng YI, Tsai YF. Neurosci Lett. 1998 Nov 20;257(1):1-4. Recovery of high potassium-evoked dopamine release after depolarization challenge in the striatum of young and old male rats.

Smith AM, Pivavarchyk M, Wooters TE, Zhang Z, Zheng G, McIntosh JM, Crooks PA, Bardo MT, Dwoskin LP. Repeated nicotine administration robustly increases bPiDDB inhibitory potency at alpha6beta2-containing nicotinic receptors mediating nicotine-evoked dopamine release. Biochem Pharmacol. 2010 Aug 1;80(3):402-9. Epub 2010 Mar 25.

Smith DH, Okiyama K, Thomas MJ, Claussen B, McIntosh TK. Evaluation of memory dysfunction following experimental brain injury using the Morris water maze. J Neurotrauma. 1991 Winter;8(4):259-69.

Socci DJ, Sanberg PR, Arendash GW. Nicotine enhances Morris water maze performance of young and aged rats. Neurobiol Aging. 1995 Sep-Oct;16(5):857-60.

Stanford JA, Giardina K, Gerhardt GA. Int J Dev Neurosci. 2000 Jul-Aug;18(4-5):411-6. In vivo microdialysis studies of age-related alterations in potassium-evoked overflow of dopamine in the dorsal striatum of Fischer 344 rats.

Stone TW. CNS neurotransmitters and neuromodulators: glutamate. 1995 CRC Press, Inc. Florida.

Strait KA, Kuczenski R. Dopamine autoreceptor regulation of the kinetic state of striatal tyrosine hydroxylase. Mol Pharmacol. 1986 Jun;29(6):561-9.

Sutherland C, Alterio J, Campbell DG, Le Bourdelles B, Mallet J, Haavik J, Cohen P. Phosphorylation and activation of human tyrosine hydroxylase in vitro by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2. Eur J Biochem. 1993 Oct 15;217(2):715-22.

Sutton RL, Hovda DA, Feeney DM. Amphetamine accelerates recovery of locomotor function following bilateral frontal cortex ablation in cats. Behav Neurosci. 1989 Aug;103(4):837-41.

Svenningsson P, Lindskog M, Rognoni F, Fredholm BB, Greengard P, Fisone G. Activation of adenosine A2A and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. Neuroscience. 1998 May;84(1):223-8.

Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC, Greengard P. DARPP-32: an integrator of neurotransmission. Annu Rev Pharmacol Toxicol. 2004;44:269-96. Review.

Sydserff S, Sutton EJ, Song D, Quirk MC, Maciag C, Li C, Jonak G, Gurley D, Gordon JC, Christian EP, Doherty JJ, Hudzik T, Johnson E, Mrzljak L, Piser T, Smagin GN, Wang Y, Widzowski D, Smith JS. Selective alpha7 nicotinic receptor activation by AZD0328 enhances cortical dopamine release and improves learning and attentional processes. Biochem Pharmacol. 2009 Oct 1;78(7):880-8. Epub 2009 Jul 16.

Takada-Takatori Y, Kume T, Ohgi Y, Izumi Y, Niidome T, Fujii T, Sugimoto H, Akaike A. Mechanism of neuroprotection by donepezil pretreatment in rat cortical neurons chronically treated with donepezil. J Neurosci Res. 2008 Dec;86(16):3575-83.

Takahashi T, Yamashita H, Zhang YX, Nakamura S. Inhibitory effect of MK-801 on amantadine-induced dopamine release in the rat striatum. Brain Res Bull. 1996;41(6):363-7.

Takeuchi H, Yanagida T, Inden M, Takata K, Kitamura Y, Yamakawa K, Sawada H, Izumi Y, Yamamoto N, Kihara T, Uemura K, Inoue H, Taniguchi T, Akaike A, Takahashi R, Shimohama S. Nicotinic receptor stimulation protects nigral dopaminergic neurons in rotenone-induced Parkinson's disease models. J Neurosci Res. 2009 Feb;87(2):576-85.

Tang K, Wu H, Mahata SK, Mahata M, Gill BM, Parmer RJ, O'Connor DT. Stimulus coupling to transcription versus secretion in pheochromocytoma cells. Convergent and divergent signal transduction pathways and the crucial roles for route of cytosolic calcium entry and protein kinase C. J Clin Invest. 1997 Sep 1;100(5):1180-92.

Tehranian R, Montoya SE, Van Laar AD, Hastings TG, Perez RG. Alpha-synuclein inhibits aromatic amino acid decarboxylase activity in dopaminergic cells. J Neurochem. 2006 Nov;99(4):1188-96. Epub 2006 Sep 18.

Terry AV Jr, Risbrough VB, Buccafusco JJ, Menzaghi F. Effects of (+/-)-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride (SIB-1553A), a selective ligand for nicotinic acetylcholine receptors, in tests of visual attention and distractibility in rats and monkeys. J Pharmacol Exp Ther. 2002 Apr;301(1):284-92.

Ungerstedt U. Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour. Acta Physiol Scand Suppl. 1971;367:49-68.

Urbanavicius J, Ferreira M, Costa G, Abin-Carriquiry JA, Wonnacott S, Dajas F. Nicotine induces tyrosine hydroxylase plasticity in the neurodegenerating striatum. J Neurochem. 2007; 102:723-30.

Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nairn AC, Greengard P, Hervé D, Girault JA. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc Natl Acad Sci U S A. 2005 Jan 11;102(2):491-6. Epub 2004 Dec 17.

Van Kampen M, Selbach K, Schneider R, Schiegel E, Boess F, Schreiber R. AR-R 17779 improves social recognition in rats by activation of nicotinic alpha7 receptors. Psychopharmacology (Berl). 2004 Apr;172(4):375-83. Epub 2004 Jan 15.

Verbois SL, Hopkins DM, Scheff SW, Pauly JR. Chronic intermittent nicotine administration attenuates traumatic brain injury-induced cognitive dysfunction. Neuroscience. 2003;119(4):1199-208.

Verbois SL., Scheff SW, Pauly JR. Chronic nicotine treatment attenuates alpha 7 nicotinic receptor deficits following traumatic brain injury. Neuropharmacology. 2003 Feb;44(2):224-33.

Vizi ES, Lajtha A (2008). Handbook of neurochemistry and molecular neurobiology, 3rd edition. New York: Springer.

Von Voigtlander PF, Moore KE. Dopamine: release from the brain in vivo by amantadine. Science. 1971 Oct 22;174(7):408-10.

Wagner AK, Sokoloski JE, Ren D, Chen X, Khan AS, Zafonte RD, Michael AC, Dixon CE. Controlled cortical impact injury affects dopaminergic transmission in the rat striatum. J Neurochem. 2005 Oct;95(2):457-65.

Wagner AK, Drewencki LL, Chen X, Santos FR, Khan AS, Harun R, Torres GE, Michael AC, Dixon CE. Chronic methylphenidate treatment enhances striatal dopamine neurotransmission after experimental traumatic brain injury. J Neurochem. 2009 Feb;108(4):986-97. Epub 2008 Dec 10.

Wang H, Sun X. Desensitized nicotinic receptors in brain. Brain Res Brain Res Rev. 2005 Jun;48(3):420-37.

Wersinger C, Prou D, Vernier P, Niznik HB, Sidhu A. Mutations in the lipid-binding domain of alpha-synuclein confer overlapping, yet distinct, functional properties in the regulation of dopamine transporter activity. Mol Cell Neurosci. 2003 Sep;24(1):91-105.

Wersinger C, Vernier P, Sidhu A. Trypsin disrupts the trafficking of the human dopamine transporter by alpha-synuclein and its A30P mutant. Biochemistry. 2004 Feb 10;43(5):1242-53.

West AR, Grace AA. Opposite influences of endogenous dopamine D1 and D2 receptor activation on activity states and electrophysiological properties of striatal neurons: studies combining in vivo intracellular recordings and reverse microdialysis. J Neurosci. 2002 Jan 1;22(1):294-304.

Westerink BH, De Vries JB. J Neurochem. 1988 Sep;51(3):683-7. Characterization of in vivo dopamine release as determined by brain microdialysis after acute and subchronic implantations: methodological aspects.

Wheaton P, Mathias JL, Vink R. Impact of early pharmacological treatment on cognitive and behavioral outcome after traumatic brain injury in adults: a meta-analysis. J Clin Psychopharmacol. 2009 Oct;29(5):468-77.

White HK, Levin ED. Four-week nicotine skin patch treatment effects on cognitive performance in Alzheimer's disease. Psychopharmacology (Berl). 1999 Apr;143(2):158-65.

Wilkie GI, Hutson P, Sullivan JP, Wonnacott S. Pharmacological characterization of a nicotinic autoreceptor in rat hippocampal synaptosomes. Neurochem Res. 1996 Sep;21(9):1141-8.

Willmott C, Ponsford J. Efficacy of methylphenidate in the rehabilitation of attention following traumatic brain injury: a randomised, crossover, double blind, placebo controlled inpatient trial. J Neurol Neurosurg Psychiatry. 2009 May;80(5):552-7. Epub 2008 Dec 5.

Wilson AL, Langley LK, Monley J, Bauer T, Rottunda S, McFalls E, Kovera C, McCarten JR. Nicotine patches in Alzheimer's disease: pilot study on learning, memory, and safety. Pharmacol Biochem Behav. 1995 Jun-Jul;51(2-3):509-14.

Wolf ME, Roth RH. Autoreceptor regulation of dopamine synthesis. *Ann NY Acad Sci* 1990;604:323–343.

Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron. 1999 Aug;23(4):787-98.

Wonnacott S. Presynaptic nicotinic ACh receptors. Trends Neurosci. 1997 Feb;20(2):92-8. Review.

Wonnacott S, Kaiser S, Mogg A, Soliakov L, Jones IW. Presynaptic nicotinic receptors modulating dopamine release in the rat striatum. Eur J Pharmacol. 2000 Mar 30;393(1-3):51-8.

Wooltorton JR, Pidoplichko VI, Broide RS, Dani JA. Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in midbrain dopamine areas. J Neurosci. 2003 Apr 15;23(8):3176-85.

Yamada K, Furukawa S, Iwasaki T, Ichitani Y. Nicotine improves AF64A-induced spatial memory deficits in Morris water maze in rats. 2010 Neurosci Lett; 469(1):88-92.

Yan HQ, Kline AE, Ma X, Hooghe-Peters EL, Marion DW, Dixon CE. Tyrosine hydroxylase, but not dopamine beta-hydroxylase, is increased in rat frontal cortex after traumatic brain injury. Neuroreport. 2001 Aug 8;12(11):2323-7.

Yan HQ, Ma X, Chen X, Li Y, Shao L, Dixon CE. Delayed increase of tyrosine hydroxylase expression in rat nigrostriatal system after traumatic brain injury. Brain Res. 2007 Feb 23;1134(1):171-9. Epub 2006 Dec 28.

Young JW, Crawford N, Kelly JS, Kerr LE, Marston HM, Spratt C, Finlayson K, Sharkey J. Eur Neuropsychopharmacol. Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice. 2007 Jan 15;17(2):145-55. Epub 2006 May 2.

Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J Neurosci. 2002 Oct 15;22(20):8785-9.