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Involvement of the 5-HT2A receptor in the regulation of hippocampal-dependent learning and neurogenesis

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Involvement of 5-HT2A Receptor in the Regulation of Hippocampal-Dependent Learning and Neurogenesis

by

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To anyone who overcomes obstacles to live out their dreams…

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ABSTRACT

Aberrations in brain serotonin (5-HT) neurotransmission have been implicated in psychiatric disorders including anxiety, depression and deficits in learning and memory. Many of these disorders are treated with drugs which promote the availability of 5-HT in the synapse. Selective serotonin uptake inhibitors (SSRIs) are known to stimulate the production of new neurons in the hippocampus (HPC) by increasing synaptic concentration of serotonin (5-HT). However, it is not clear which of the 5-HT receptors are involved in behavioral improvements and enhanced neurogenesis. The current study aimed to investigate the effects of $5HT_{2A}$ agonists psilocybin and 251-NBMeO and the $5HT_{2A/C}$ antagonist ketanserin on neurogenesis and hippocampal-dependent learning. Agonists and an antagonist to the 5-HT2A receptor produced alterations in hippocampal neurogenesis and trace fear conditioning. Future studies should examine the temporal effects of acute and chronic psilocybin administration on hippocampal-dependent learning and neurogenesis.

Chapter One

Introduction

The idea of new neurons forming in the adult central nervous system (CNS) is a relatively new one. In the 1960's Joseph Altman published the first evidence of neurogenesis, or the birth of new neurons in the adult mammalian brain (Altman, 1962; Altman, 1963; Altman & Das, 1965). Utilizing the tritiated thymidine method (Sidman et al., 1959; Messier et al., 1958; Messier & Leblond, 1960) Joseph Altman was able to demonstrate that the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG) of the hippocampus (HPC) produce new neurons throughout the lifespan (Altman, 1962; Altman & Das, 1965; Altman, 1969). For years following Altman's discovery scientists acknowledged the possibility of the generation of new glial cells in the adult brain but rejected the concept of new born neurons. With the advent of new technologies such as the bromodeoxyuridine (BrdU) method of birth dating cells and double labeling using immunofluorescence, adult neurogenesis has been identified in many mammalian species including mice (Kempermann et al., 1998), rats (Kaplan & Hinds, 1977), hamsters (Huang et al., 1998), tree shrews (Gould et al., 1997), nonhuman primates (Gould et al., 1999b; Bernier et al., 2002) and humans (Eriksson et al., 1998). Peter Eriksson's discovery of new neurons in the human HPC changed the perception of neurogenesis in the scientific community

so now the fact that new neurons are produced in the adult brain is firmly established.

The Anatomy of Hippocampal Neurogenesis

The HPC is divided into four areas: DG (also called area dentate, or fascia dentata), cornu ammonis (CA, further divided into CA1, CA2, CA3 and CA4), the presubiculum and the subiculum. This anatomical description of the HPC has been confirmed by both gene expression and fiber connections. The DG and areas CA form a trisynaptic circuitry within the HPC (see Figure 1.1). Neurons in the entorhinal cortex (EC) project to dendrites of the granule cells in the DG forming the perforant pathway. The granule cells extend their axons (Ramon, 1952) to pyramidal neurons in area CA3, forming the mossy fiber tract (Ribak et al., 1985). CA3 pyramidal neurons project to the contralateral (via associational commissural pathway) and the ipsilateral CA1 region forming the Shaffer collateral pathway. Pyramidal neurons in CA1 extend axons to the subiculum and from the subiculum back to EC (for detailed descriptions of hippocampal circuitry see (Witter, 1993)).

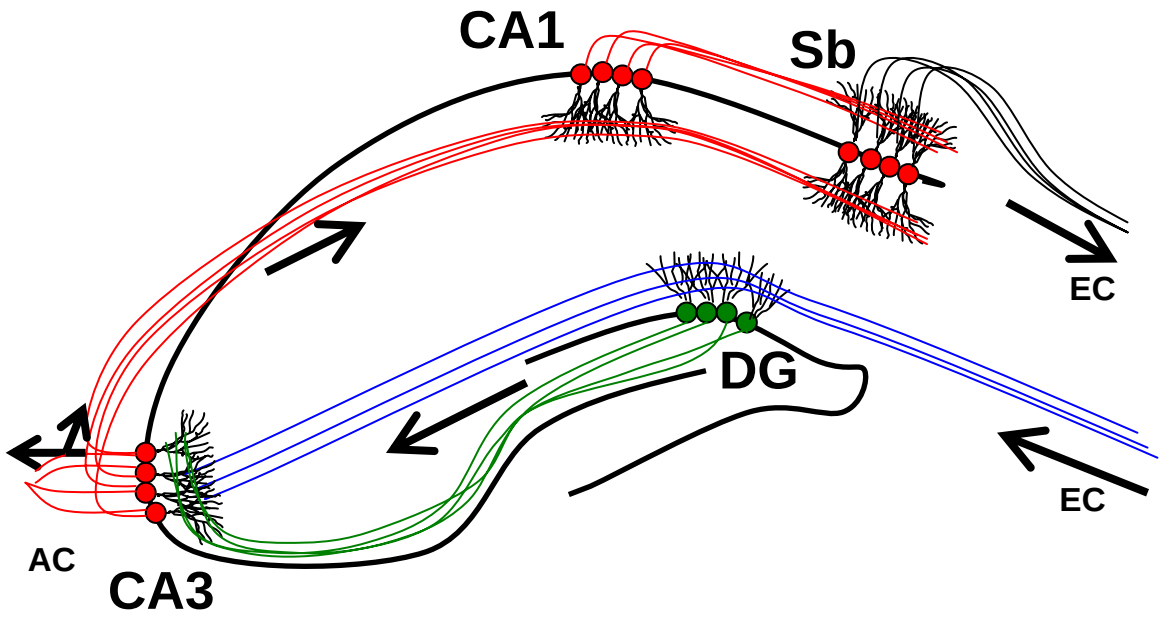


Figure 1.1. Anatomy of the Hippocampus. The HPC forms a trisynaptic pathway with inputs from the Entorhinal Cortex (EC) that projects to the Dentate Gyrus (DG) and CA3 pyramidal neurons via the perforant pathway. Granule cells in the DG project to CA3 via the mossy fiber pathway. Pyramidal neurons in CA3 project to both the contralateral (associational commissural pathway) and the ipsilateral CA1 region via the Schaffer Collateral Pathway. CA1 pyramidal neurons send their axons to the Subiculum (Sb) which in turn projects out of the HPC back to the EC.

In normal physiological conditions, neurogenesis that occurs in the HPC is found only in the DG and results in the generation of new granule cells. Within the DG, progenitor cells reside in a narrow band between the DG and the hilus (also called CA4 or plexiform layer) called the subgranular zone (SGZ) which is

approximately 2-3 cells thick (20-25 μM) (see Figure 1.2). Neural progenitor cells (1) divide and form clusters of proliferating cells (2). Proliferating cells exit from the cell cycle and begin to differentiate into immature neurons (3). The immature granule cell forms sodium currents, extends dendrites and an axon to make connections with other cells and form synapses to become a mature neuron (4). The SGZ contains many cell types including astrocytes (Seri et al., 2001; Filippov et al., 2003; Fukuda et al., 2003), several types of glial and neuronal progenitor cells (Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003; Seri et al., 2004) and neurons in all stages of differentiation and maturation (Brandt et al., 2003; Ambrogini et al., 2004).

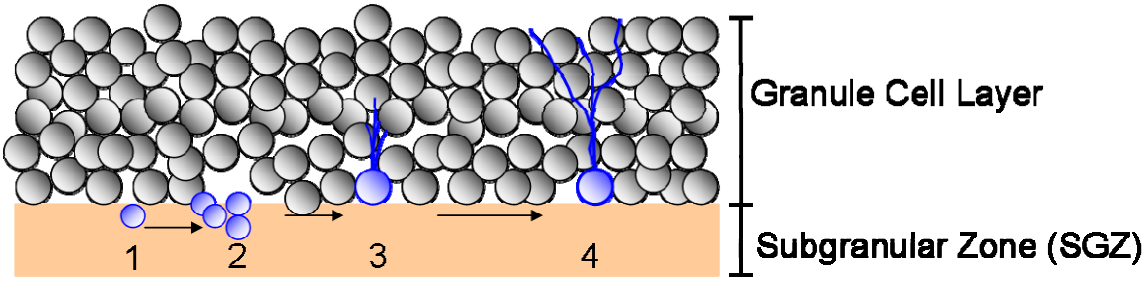


Figure 1.2. Neurogenesis in the Dentate Gyrus of the Hippocampus. Neural stem cells exist the SGZ of the DG, these cells then divide, differentiate and mature into their phenotypic fate. Neural progenitor cells (1) divide and form clusters of proliferating cells (2). Proliferating cells exit from the cell cycle and begin to differentiate into immature neurons (3). The immature granule cell forms sodium currents, dendrites and extends an axon out to make connections with other cells and form synapses to become a mature granule cell (4).

Regulation of Neurogenesis in the Dentate Gyrus

The proliferation and survival of neural progenitors in the adult HPC can be influenced in a positive and negative manner by a variety of stimuli. Factors as diverse as stress, odors, neurotrophins, psychoactive drugs such as antidepressants, opioids and alcohol, electroconvulsive therapy, seizures, ischemia, cranial irradiation, physical activity, learning, hormones and age amongst many others have been linked to the regulation of neurogenesis (Kempermann et al., 1998; Van et al., 1999b; Malberg et al., 2000; Malberg & Duman, 2003; Tanapat et al., 2001). Some of these factors have been studied extensively and their role in the regulation of neurogenesis is well defined. For example, environmental enrichment and physical activity are strong positive regulators of neurogenesis (Van et al., 1999b; Kempermann et al., 1997), while stress and age (Cameron et al., 1993; Kempermann et al., 1998) appear to be negative regulators of neurogenesis.

The first report of any factor increasing neurogenesis in the mammalian brain was an enriched environment. In an experimental setting a rodent enriched environment typically consists of a large cage, a large number of animals, toys and a tunnel system. In order to maintain the enrichment aspect novel toys are introduced and tunnel system is rearranged on a regular basis. Mice (Kempermann et al., 1997) and rats (Nilsson et al., 1999) living in an enriched environment exhibited a strong up-regulation of cell proliferation and

neurogenesis in the DG of the HPC. The proneurogenic effects of environmental enrichment can be increased further depending on the age of the animal when exposure occurs. When late adolescent/young adult animals live in an enriched environment it enhances the ability of environmental enrichment to up-regulate cellular proliferation and neurogenesis in the DG. In fact, when the morphology of the HPC was examined later in life, a greater number of absolute granule cells was observed (Kempermann et al., 1997). In aged animals (typically 18 months or older in rodents) lower levels of neurogenesis have been observed, however living in an enriched environment counteracts the effects of aging (Kempermann et al., 1998). Kempermann and colleagues demonstrated environment enrichment during aging increases cell proliferation and neurogenesis in the DG (Kempermann et al., 1998). Furthermore, if animals live in an enriched environment during mid age, basal levels of neurogenesis increase as much as five fold in old age.

When experimentation with environmental enrichment began, novel foods were included as apart of the environmental enrichment experience. When similar food was given to mice living either an enriched or control environment, the effect of environmental enrichment was still present. One type of diet however, has been found to have positive effects on neurogenesis specifically, caloric restriction (Lee et al., 2000). As an experimental manipulation caloric restriction usually consists of limiting the amount of food an animal can eat by a third. Caloric restriction is the only factor that has been shown experimentally to

increase the life span of animals and it is thought that caloric restriction actually acts as a mild stressor. It is of interest to note while environmental enrichment is a strong positive regulator of adult hippocampal neurogenesis, it does not affect adult neurogenesis in the olfactory system (Brown et al., 2003).

Exposure to an enriched environment increases neurogenesis in the DG of adult rodents, however, environmental enrichment typically includes a running wheel and increased physical activity. Physical activity is known to up-regulate cell proliferation and neurogenesis in the DG. Rodents will take full advantage of the opportunity to exercise on a running wheel during their active phase of their day. Mice have been reported to run between 3 and 8 km per night on a running wheel (Van et al., 1999a; Van et al., 1999b). Voluntary physical activity has been shown to increase the number of progenitor cells and new neurons in the DG of the HPC (Van et al., 1999a; Van et al., 1999b). The effect of running on neurogenesis is acute so that running must continue to effect neurogenesis and once the animal no longer uses the running wheel the effect on neurogenesis will decline. The up-regulation of adult neurogenesis by physical activity also increases long term potentiation (LTP) in the DG and enhances performance on the Morris water maze (MWM) (Van et al., 1999a). The MWM is a behavioral task that assesses memory and learning, but as part of the task the animals are placed into a pool of water and forced to swim. Some have argued that swimming, being physical activity, could also influence the outcome of the task. This point was addressed by Ehninger et al who found involuntary physical

activity (swimming in radial arm water maze (RAWM)) had no effect on hippocampal neurogenesis (Ehninger & Kempermann, 2003).

The mechanisms underlying the increase in neurogenesis by physical activity are unknown however, growth factors such as insulin growth factor -1 (IGF-1), vascular endothelial growth factor (VEGF), and brain derived neurotrophic factor (BDNF) have been strongly implicated. IGF-1 levels are increased in the HPC of running animals and running induced increases in cellular proliferation and neurogenesis (Carro et al., 2001; Carro et al., 2000; Trejo et al., 2001). This increase in neurogenesis is blocked by scavenging circulating IGF-1 absent in IGF-1 mutants (Carro et al., 2000). VEGF is necessary for the effects of running on adult hippocampal neurogenesis. Blocking peripheral VEGF abolished the running-induced induction of neurogenesis, however there were no detectable effects on baseline neurogenesis in non-running animal (Fabel et al., 2003). Quantitative polymerase chain reaction analysis revealed BDNF mRNA levels are significantly increased in the DG of running rats (Farmer et al., 2004). BDNF is a key factor involved in modulating neuroplasticity including LTP and neurogenesis. Infusions of BDNF into the lateral ventricles induced neurogenesis originating in the SVZ (Pencea et al., 2001) and BDNF knockout (KO) mice have diminished levels of neurogenesis in the DG (Lee et al., 2002). Like environmental enrichment, physical activity up-regulates adult hippocampal neurogenesis, however, it does not affect adult neurogenesis in the olfactory system (Brown et al., 2003).

Stress severely impairs hippocampal neurogenesis. One of the first studies to link stress to hippocampal neurogenesis was conducted by Gould and colleagues (1992). They found stress increased the number of dying cells in the HPC but that the total number of granule cells in the dentate was not different from non-stressed controls and concluded neurogenesis must be occurring to maintain cellular balance (Gould et al., 1992). They postulated the stress hormone, cortisol in humans and corticosterone in rodents mediates the stress effect on neurogenesis and went on to discover adrenalectomy (removing the adrenal gland hence the source of endogenous corticosterone) led to an up-regulation of neurogenesis and exogenous corticosterone down-regulated cellular proliferation and neurogenesis in the DG (Cameron et al., 1993). Since these early experiments, severe stress has been shown to downregulate cell proliferation and consecutive stages of neuronal development using many different paradigms. Prenatal stress caused learning deficits and had detrimental effects on neurogenesis that lasted well into adulthood (Lemaire et al., 2000). The effects of psychosocial stress on neurogenesis were demonstrated using the resident-intruder model of territorial tree shrews (Gould et al., 1997). Tree shrews are extremely territorial and guard their environment so the introduction of an intruder to the resident's cage is extremely stressful. The territorial tree shrews compete for dominance and soon after the introduction of an intruder a dominant-subordinate relationship is established resulting in elevated cortisol and decreased neurogenesis in the subordinate tree shrew (Gould et al., 1997).

Predator odor triggered a stress response in prey and had detrimental effects on cell proliferation. In rodent models, fox odor has been shown to decrease cell proliferation and neurogenesis in the DG (Tanapat et al., 2001). Both acute and chronic restraint stress have been shown to affect the rate of adult hippocampal neurogenesis. Pham and colleagues demonstrated that 6 weeks of daily restraint stress suppressed cell proliferation and attenuated survival of the newly born cells, resulting in a 47% reduction of granule cell neurogenesis (Pham et al., 2003). Neurogenesis is not only affected by environmental stimuli, the absence of stimuli, such as social isolation, negatively regulated neurogenesis. Young rats reared in social isolation for 4-8 weeks showed decreased performance on the MWM and decreased hippocampal neurogenesis (Lu et al., 2003). In the learned helplessness model of depression animals are exposed to an inescapable foot shock using avoidance testing. Exposure to inescapable shock decreased cell proliferation in the HPC, extending previous studies demonstrating downregulation of neurogenesis by exposure to acute stressors (Malberg & Duman, 2003).

The key mechanism underlying the negative impact that stress has on neuroplasticity appears to be stress hormone (glucocorticoid) secretion (Cameron et al., 1993). Acute, severe and sometimes traumatic stress leads to chronically high levels of glucocorticoids and alters the functioning of the hypothalamic-adrenal-pituitary (HPA) axis resulting in disregulation of glucocorticoid secretion and receptor expression. Depression is an example of a

clinical condition associated with disturbed regulation of the HPA axis which upsets the circadian rhythm of hormone secretion resulting in chronically elevated glucocorticoid levels and decreased neurogenesis (Jacobs et al., 2000).

Aging is another factor known to have a strong negative influence on neurogenesis. This has been known since the discovery of adult neurogenesis by Altman and Das in 1965. In the original study a progressive decrease in the levels of neurogenesis was observed after puberty and continued into old age (Altman & Das, 1965) and this finding has been since replicated in both rats (Seki & Arai, 1995; Kuhn et al., 1996; Cameron & McKay, 1999; Bizon & Gallagher, 2003), mice (Kempermann et al., 1998) and humans (Eriksson et al., 1998). The highest levels of adult neurogenesis occurred in young adulthood and steadily decreased over the lifespan. In old age (typically 18 months or older in rodents) baseline levels of neurogenesis are extremely low, however, there are ways to enhance neurogenesis in the aging hippocampus. Environment enrichment during aging increases cell proliferation and neurogenesis in the DG, however, the effect of an enriched environment is more robust in young animals (Kempermann et al., 1998). Animals that lived in an enriched environment starting at mid age had five fold increases in basal levels of neurogenesis in old age (Kempermann et al., 1998). Cortisol (or corticosterone in rodents) levels are elevated in aging which likely reduces baseline proliferation and neurogenesis. Adrenalectomy in aged animals restored adult neurogenesis in the DG to a level comparable to that of a much younger age, demonstrating corticosterone is at

least in part responsible for the decline in neurogenesis observed in aging (Cameron & McKay, 1999). IGF-1 levels are increased in the HPC running animals and running induced increases in cellular proliferation and neurogenesis (Carro et al., 2001; Carro et al., 2000; Trejo et al., 2001). Similarly, aged animals administered exogenous IGF-1 to restore endogenous IGF-1 levels to that of a younger age and induced neurogenesis above controls thus counteracted the negative effect of aging on neurogenesis (Lichtenwalner et al., 2001).

BDNF is considered a critical secreted factor modulating brain plasticity. Physical activity, which is known to positively regulate neurogenesis and induce LTP, induces hippocampal BDNF mRNA expression. It is thought that BDNF may modulate the effect that physical activity has on LTP and neurogenesis (Farmer et al., 2004). Infusions of BDNF into the lateral ventricles induced neurogenesis originating in the SVZ (Pencea et al., 2001) and BDNF KO mice have diminished levels of hippocampal neurogenesis (Lee et al., 2002). In pathological conditions such as depression, BDNF blocks neurogenesis (which is opposite to healthy animals) and it is now understood one of the critical functions of BDNF is to keep neurogenesis within a physiological range. BDNF function has been implicated in the neurogenesis hypothesis of depression, the idea being that the antidepressants enhance neurogenesis, and BDNF is a key regulator of this mechanism (Jacobs et al., 2000; D'Sa & Duman, 2002). Antidepressants (including selective serotonin reuptake inhibitors (SSRIs)) induce the phosphorylation of CREB, after which CREB binds to the BDNF

promoter and induces BDNF transcription. 5-HT_{2A} receptor agonists, such as 2,5-dimethoxy-4-iodoamphetamine (DOI), increase BDNF mRNA expression in the HPC (Vaidya et al., 1997). BDNF is involved in inducing neuronal differentiation possibly through the induction of neuronal nitric oxide synthase (nNOS) which has been shown to stop proliferation and promote differentiation. In vitro BDNF is a differentiation factor that can down-regulate precursor cell proliferation (Cheng et al., 2003).

Hippocampal Neurogenesis and Learning

Memory involves the encoding, storing and recalling of information. The HPC plays a critical role in learning and memory by converting short-term memories into long-term memories and is pivotal in the encoding, consolidation and retrieval of episodic memory (Squire et al., 1992; Squire, 1992). Several studies have investigated the connection between learning and hippocampal neurogenesis. Hippocampal mediated learning and memory has been shown to be related to the generation of new neurons in the adult DG (Van et al., 2002; Nilsson et al., 1999).

It has been postulated only learning tasks which are hippocampal dependant affect progenitor cell proliferation and neurogenesis in the DG (Gould et al., 1999a). This idea has since been demonstrated using a learning task that is easily manipulated to be either hippocampal dependent or independent. Hippocampal-dependent learning can be assessed using trace eye blink conditioning. In trace conditioning the conditioned stimulus (CS), a tone, sounds

for 5 seconds, then after a 100-1000 ms interval, the unconditioned stimulus (US) an airpuff or eyelid shock is activated. In this way the CS and US do not overlap. Hippocampal-independent learning can be assessed using delay eyeblink conditioning. In delay eyeblink conditioning the tone (CS) sounds for 5 seconds and in the last 20 ms of the tone sounding the airpuff (US) is activated. In this way the CS and US overlap. Shors and colleagues used both trace and delay eyeblink conditioning to demonstrate that trace eyeblink conditioning, a hippocampal dependent task, is affected by neurogenesis whereas delay eyeblink conditioning is not. Mice were treated with methylazoxymethanol acetate (MAM), an anti-mitotic agent which wipes out the progenitor cell population in the DG and administered BrdU to birth date the cells then performed either trace or delay eyeblink conditioning. In both trace and delay eyeblink conditioning, saline treated mice performed well on the task and had similar numbers of BrdU positive cells in the DG. This is in contrast to mice treated with MAM which produced different results for trace and delay eyeblink conditioning. In trace eyeblink conditioning MAM severely impaired learning and obliterated BrdU incorporation in the DG, whereas, no impairment in learning was observed after delay eyeblink conditioning despite mice being treated with MAM, thus obliterating the progenitor pool and resulted in a dramatic reduction of BrdU positive cells in the DG (Shors et al., 2001). These results clearly indicate that newly generated neurons in the adult DG are affected by the formation of hippocampal-dependent memory.

Only certain types of hippocampal dependent tasks have been shown to be involved in hippocampal neurogenesis (Shors et al., 2002). This was demonstrated using two different learning paradigms known to require the HPC, the spatial navigation task and trace fear conditioning. Similar to the study mentioned earlier, mice were treated with MAM and BrdU then performance on either behavioral task was assessed. The spatial navigation task is performed in the MWM and required the mouse to use spatial cues in the environment (like a black square on a wall) to navigate to and find the platform. Over trials mice learned where the platform was located and spent less time trying to find it. MAM failed to result in impairment in escape latency but did significantly decreased BrdU+ cells in the SGZ, demonstrating that hippocampal progenitor cell proliferation is not essential for this hippocampal-dependent task (Shors et al., 2002). In a separate group of mice trace fear conditioning, which like trace eyeblink conditioning involves a time gap between CS and US presentation was performed. In trace fear conditioning MAM severely impaired learning and significantly diminished BrdU incorporation in the DG, thus providing more evidence for the involvement of trace conditioning in hippocampal neurogenesis (Shors et al., 2002). The above experiments clearly demonstrate that some forms of learning are dependent on the HPC but not all hippocampal-dependent learning tasks require neurogenesis.

The HPC is involved in the formation and expression of memory in the passive avoidance task in rats (Cahill & McGaugh, 1998). The logic underlying

the passive avoidance (PA) task is that animals associate a particular environment with an unpleasant foot shock and learn by avoiding the environment they can avoid the aversive foot shock. Consequently, an increase in response latency is thought to reflect the strength of the memory for the aversive event (Sahgal & Mason, 1985). Specifically, the multi-herbal formula BR003 increased response latency, and hence the memory of the foot shock while also increasing the number of BrdU positive cells in the DG (Oh et al., 2006). The PA task is relatively quick and simple but is limited in the information it provides regarding memory, that latencies increase following shock. A modified version of PA, the active avoidance paradigm measures acquisition (learning), retention (memory) and the extinction of the conditioned response.

Active avoidance is a fear-motivated associative avoidance task. In this task the mouse has to learn to predict the occurrence of an aversive event (shock) based on the presentation of a specific stimulus (tone), in order to avoid the aversive event by moving to a different compartment. The measures recorded include number of avoidances (the mouse crossing to the other compartment during the warning signal), number of non-responses (the mouse failing to cross to the other compartment during the trial), response latency (latency to avoid or escape), number of intertrial responses (i.e., crossing the barrier within the intertrial interval), and serve as an index of learning which allows memory to be assessed.

Many studies supported the role of the HPC in active avoidance learning.

LTP via electrical stimulation to the perforant pathway is negatively correlated to learning in the shuttle box avoidance task, suggesting active avoidance training lowered the threshold frequency to induce LTP in the DG (Ramirez & Carrer, 1989). Active avoidance learning increased the length of the postsynaptic density in the molecular cell layer of the DG (Van et al., 1992) and increased immunoreactivity for muscarinic receptors in the granular cell layer (Van der Zee & Luiten, 1999). Two-way Active avoidance also increased synthesis of BDNF (Ulloor & Datta, 2005) and cAMP response element binding (CREB) in the dorsal HPC (Saha & Datta, 2005). Rats that learned the active shock avoidance task (responders) had similar levels of Brdu positive and Ki67 positive cells in the DG as non-responders, suggesting ASA has no effect on hippocampal progenitor cell proliferation (Van der et al., 2005).

Active avoidance testing is commonly used following exposure to severe inescapable foot shock in the learned helplessness model of depression. Exposure to inescapable foot shock decreased progenitor cell proliferation in the DG and this effect is reversed by chronic treatment with fluoxetine (Malberg & Duman, 2003). One target of antidepressant treatment is BDNF since antidepressants not only increase the expression of CREB in the rat HPC (Nibuya et al., 1996) but also increase the expression of BDNF (Nibuya et al., 1995). BDNF produced antidepressant like effects in the learned helplessness model of depression (Shirayama et al., 2002).

Assessing Neurogenesis

The systemic injection of thymidine, radioactively labeled with tritium was the first method developed to label dividing cells (Messier et al., 1958). Once in the bloodstream, tritiated thymidine competes with endogenous thymidine in all cells in the S phase of cell division and is permanently incorporated into the DNA. Labeled thymidine has a short half-life *in vivo* and labels all cells in the process of cell division when the label is injected. At a later time point, tissue sections are prepared and coated with a photo emulsion. The radiation from the labeled thymidine molecules blackens the photo emulsion, thus making visible the typical grains of thymidine autoradiography. Utilizing the tritiated thymidine method (Sidman et al., 1959; Messier et al., 1958; Messier & Leblond, 1960) Joseph Altman was able to demonstrate that the SVZ and the DG of the HPC produce new neurons throughout the lifespan (Altman, 1962; Altman & Das, 1965; Altman, 1969).

BrdU is a false base that competes with endogenous thymidine and becomes permanently incorporated in the DNA during the S phase of the cell cycle. BrdU is typically administered via injections of usually 50 - 250 mg/kg in a single bout or over several days depending on the experimental paradigm (Corotto et al., 1993). BrdU is advantageous because it is a permanent marker so any cells that express BrdU can be directly related to the time BrdU was administered thus providing the birth date of the cell. It is important to note BrdU can be incorporated into cells that are on the verge of dying when cell death

related mechanisms trigger DNA repair, therefore proper controls need to be included such as immunohistochemical stains for apoptosis (caspase-3 or TUNEL) and proliferative markers (Ki67) to determine if a cell is truly proliferative.

The rate of proliferation can be differentiated from the rate of survival by manipulating time between BrdU injection and sacrifice so proliferating cells can be determined by sacrificing animals 24 hours after a BrdU injection. In this way BrdU has time to incorporate into the cell but the cell does not have time to differentiate into a neuron, a process which takes a minimum of 72 hours. Survival can be determined by taking the brains of animals days, weeks or even months after BrdU injection. The phenotypic fate of the cell is determined in the survival condition by double labeling BrdU with another marker. Table 1 presents a summary of the common markers used to determine the phenotype of neural progenitors.

Marker	Significance	Reference
Ki67	Proliferation; late G1, S, G2 and M phases nuclear	(Scholzen & Gerdes, 2000)
Doublecortin (DCX)	Immature neuron; microtubule-associated protein enriched in migratory neuronal cells. Early neuronal marker with lineage determined and limited self-renewal dendritic	(Meyer et al., 2002)
III β-tubulin (Tuj1)	Immature neuron; Tubulin protein soma and processes	(Uittenbogaard & Chiaramello, 2002)
Calretinin (CRT)	Immature neurons; calcium binding protein transiently	(Brandt et al., 2003)
Neuronal nuclei (NeuN)	Mature neurons; mostly in nuclei but can be detected in cytoplasm nucleus	(Mullen et al., 1992)
Glial Fibrillary Acidic Protein (GFAP)	Intermediate filament protein expressed in astrocytes.	(Fuchs & Weber, 1994)

Table 1. Antibodies used to assess phenotypic fate of progenitors

If the cell expresses Ki67 it is an early progenitor since Ki67 is a protein expressed the G1, S, G2 and M phases of the cell cycle (Scholzen & Gerdes, 2000). Cells that express doublecortin (DCX), β -tubulin III (IIIβ-tubulin, Tuj1), or calretinin (CRT) are immature neurons. DCX is a microtubule associated protein transiently expressed in immature neurons (Meyer et al., 2002), Tuj1 marks tubulin in microtubules (Uittenbogaard & Chiaramello, 2002) and CRT is a

calcium binding protein transiently expressed in immature neurons and is expressed in the developing neuron at a stage where DCX expression dissipates (Brandt et al., 2003). The best and most widely used marker to identify mature neurons is neuronal nuclei (NeuN) (Mullen et al., 1992). The expression of NeuN is restricted to post-mitotic neurons and is predominately located in the nucleus of neurons although it can occasionally be observed in the neurites. In order to convincingly demonstrate neurogenesis, cells are double labeled with BrdU plus NeuN, which clearly demonstrates that the cell was born around the time of BrdU injection and survived to differentiate into a neuron. Cell survival depends on many factors including the ability of the cell to form dendrites, an axon, synthesize neurotransmitter, receptors and establish functional connections with other cells. Cells that don't establish functional connections will most likely die.

It is possible to assess neurogenesis using methods other than the BrdU and tritiated thymidine methods. Using immunohistochemical and immunofluorescent techniques, cells can be stained for markers of immature neurons that are transient and only present in newly formed neurons. Brandt and colleagues (2003) elegantly demonstrated this method by defining time periods of development in which cells express particular markers double-labeled with BrdU (Brandt et al., 2003). BrdU is still the only way to birth date cells so for establishing the method it was essential to know the exact age of cells. The expression of CRT plus BrdU positive cells was greatest 1 to 2.5 weeks after BrdU injection and the number of double-labeled cells was negligible at 4 weeks,

demonstrating CRT is transient. If a cell expressed DCX or CRT, that cell can be positively identified as an immature neuron, thus estimates of DCX or CRT positive cells in the DG represent estimates of neurogenesis.

Serotonergic Innervation in the Dentate Gyrus

Serotonin (5-HT) is a modulatory neurotransmitter in the central nervous system which is important in the regulation of vital brain functions such as feeding (Lucki, 1992), thermoregulation (Feldberg & Myers, 1964), sleep (Jouvet, 1967) and aggression (Sheard, 1969). In psychopathological states such as depression (Pinder & Wieringa, 1993), eating disorders (Leibowitz & Shor-Posner, 1986) and anxiety serotonergic signaling is disturbed.

In the mammalian brain 5-HT is produced by neurons in the raphe nucleus (RN) which project to many areas of the brain via the medial forebrain bundle (MFB) (Azmitia & Segal, 1978; Parent et al., 1981). Neurons from RN innervate virtually all brain areas with dense innervation occurring in the HPC, cerebral cortex, striatum, hypothalamus, thalamus, septum and olfactory bulb (Jacobs & Azmitia, 1992; Leger et al., 2001). The innervation of serotonergic fibers to areas within the HPC is variable (Moore & Halaris, 1975; Vertes et al., 1999; Bjarkam et al., 2003). The DG is innervated with serotonergic fibers in both the molecular layer and the hilus with particularly dense innervation projecting to the SGZ where they synapse with interneurons (Halasy & Somogyi, 1993).

5-HT activates fifteen known receptors, many of which are expressed in the DG (el et al., 1989; Tecott et al., 1993; Vilaro et al., 1996; Djavadian et al.,

1999; Clemett et al., 2000; Kinsey et al., 2001). Most of the 5-HT receptors interact with G proteins except for the 5-HT_{3A} receptors, which are ligand-gated ion channel receptors. The 5-HT_3 receptors (subtypes 5-HT_{3A} and 5-HT_{3B}) are ligand-gated Na^+ ion channels and their activation leads to the depolarization of neurons (Barnes & Sharp, 1999). The 5-HT_1 family of receptors (including subtypes 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}) are coupled to the G_i protein which, when activated decreases the activity of adenylyl cyclase thus decreasing the rate of formation of cyclic adenosine monophosphate (cAMP). Activation of 5-HT_1 receptors can lead indirectly to the opening of K^+ channels therefore increasing the conductance of the cell membrane for K^+ ions. Activation of 5-HT_4, 5-HT_6, 5-HT_7, receptors are coupled to G_s proteins which have the opposite effect. They increase the activity of adenylyl cyclase, increase the rate of cAMP formation and decrease K^+ conductance (Thomas et al., 2000; Raymond et al., 2001). The 5-HT_2 receptors (including subtypes 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}) are coupled to G_q proteins and activate phospholipase C (PLC), increasing the rate of formation of inositol triphosphate (IP_3) and diacylglyerol leading to the increased formation of protein kinase C (PKC) (Kurrasch-Orbaugh et al., 2003; Ananth et al., 1987).

Serotonin and Neurogenesis in the Dentate Gyrus

While several factors regulate the rate of generation of new cells in the adult DG, one of the most important known factors is 5-HT. Malberg and colleagues found increased levels of 5-HT resulted in the increased rate of

proliferation of neural progenitors in the DG (Malberg et al., 2000). Administering 5,7-dihydrosytryptamine (5,7-DHT), a serotonergic neurotoxin into the RN and caused the destruction of axons and serotonergic cells and resulted in a decreased in the number of BrdU-labeled cells in the DG (Brezun & Daszuta, 1999). The 5,7-DHT lesion resulted in around a 60% depletion of the serotonergic innervation to the DG which lasted for one month. After two months, reinnervation to the DG was observed with the sprouting of serotonergic axons so that by the third month there was no observable difference between the 5,7-DHT and vehicle in newly generated cells or serotonergic innervation (Brezun & Daszuta, 2000).

Many serotonergic receptors have been implicated in the regulation of neurogenesis in the DG. In vitro, when the 5-HT_{1A} receptor agonist, 8-OH-DPAT was added to a medium in which cultured fibroblasts transfected with the 5-HT_{1A} receptor were present, the rate of cell divisions increased (Varrault et al., 1992). In vivo, 5-HT_{1A} receptor antagonists (NAN-190, *p*-MPPI and WAY-100635) decreased the number of progenitors in the DG by approximately 30% (Radley & Jacobs, 2002) and injections of 5-HT_{1A} receptor agonists increased the number of BrdU positive cells in the DG (Santarelli et al., 2003). Similarly, Banasr and colleagues showed various 5-HT_1 receptor agonists increase the number of BrdU labeled cells in the subgranular layer. Acute administration of the 5-$HT_{2A/C}$ receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI), 5-HT_{2C} receptor agonist RO 600175, and 5-HT_{2C} receptor antagonist SB 206553 had no effect of

cell proliferation in the HPC, whereas the 5-$HT_{2A/C}$ receptor antagonist ketanserin produced a 63% decrease in BrdU incorporation (Banasr et al., 2004). A recent study found acute ketanserin decreased proliferation whereas chronic ketanserin increased proliferation in the DG (Jha et al., 2008). No effect on proliferation in the DG was observed after DOI or lysergic acid diethylamide (LSD) were administered either acutely or once daily for seven consecutive days (chronic) (Jha et al., 2008).

The 5-HT_{2A} receptor is involved in the regulation of BDNF in the HPC (Vaidya et al., 1997). DOI alone and in combination with selective 5-HT_{2A} and 5-HT_{2C} receptor antagonists decreased the expression of BDNF mRNA in the HPC. Interestingly, the decrease in BDNF mRNA expression was blocked by the 5-HT_{2A} receptor antagonist but not the 5-HT_{2C} receptor antagonist, implicating the 5-HT_{2A} receptor in the regulation of BDNF expression. In addition, the stress-induced reduction in BDNF expression in the HPC was blocked by a 5-$HT_{2A/C}$ receptor antagonist (Vaidya et al., 1997).

Psilocybin (PSOP)

PSOP (4-phosphoryloxy-N,N-dimethyltryptamine) is the main active agent in “magic mushrooms” and is categorized as a indole hallucinogen. First isolated from *psilocybe mexicana*, a mushroom from Central America by Albert Hofmann in 1957, PSOP was then produced synthetically in 1958 (Hofmann et al., 1958a; Hofmann et al., 1958b). PSOP is converted into the active metabolite psilocin (4-hydroxy-N,N-dimethyltryptamine) which may produce some of the psychoactive

effects of PSOP. The chemical structure of PSOP ($C_{12}H_{17}N_2O_4P$) and the metabolite, psilocin ($C_{12}H_{16}N_2O$) are similar to 5-HT ($C_{10}H_{12}N_2O$), the main neurotransmitter which they affect (see Figure 1.3).

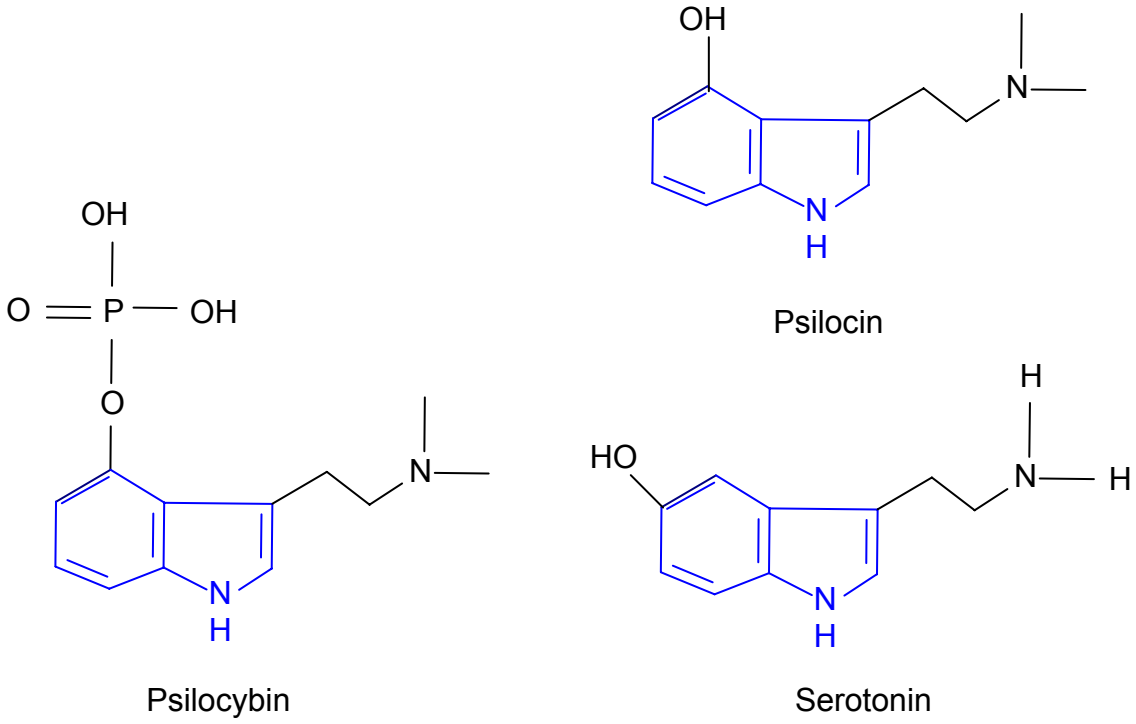


Figure 1.3. Chemical structure of Psilocybin, Psilocin and Serotonin.

In vivo studies in mice have shown the LD_{50} of PSOP via intravenous administration to be 280 mg/kg (Cerletti & Konzett, 1956; Cerletti, 1959). Autonomic effects of 10 mg/kg/sc in mice, rats, rabbits, cats and dogs include mydriasis, piloerection, irregularities in heart and breathing rate and hyperglycemic and hypertonic effects (Cerletti & Konzett, 1956; Cerletti, 1959).

These effects were interpreted as an excitatory syndrome caused by stimulation of the sympathetic nervous system with one large exception being the absence of hyperlocomotion (Monnier, 1959).

PSOP exerts psychoactive effects by altering serotonergic neurotransmission by binding to 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A} and 5-HT_{2C} receptor subtypes (Passie et al., 2002). PSOP binds to the 5-HT_{2A} receptor (Ki = 6 nM) with high affinity and to a much lesser extent to the 5-HT_{1A} receptor subtype (Ki = 190 nM) (McKenna et al., 1990). However, PSOP has a lower affinity for 5-HT_{2A} and 5-HT_{2C} receptors compared to lysergic acid diethylamide (LSD), a similar indole hallucinogen (Nichols, 2004). In contrast to LSD, PSOP has a very low affinity to DA receptors and only extremely high doses affect NE receptors. PSOP has been shown to induce schizophrenia-like psychosis in humans, a phenomenon attributed to the action of PSOP through 5-HT_{2A} receptor action. Specifically, human volunteers were pretreated with ketanserin, an antagonist to the 5-$HT_{2A/C}$ receptor, then administered 0.25 mg/kg p.o. PSOP and the psychotomimetic effects of PSOP were completely blocked (Vollenweider et al., 1998). Since blocking the 5-HT_{2A} receptor prevented the psychotropic effect of PSOP it appears as though the actions of PSOP are mediated via the activation of 5-HT_{2A} receptors. A recent study tested PSOP-induced stimulus control and found 5-HT_{2A} receptor antagonists prevented rats from recognizing PSOP in a drug discrimination task, an effect which was not observed with 5-HT_{1A} receptor antagonists (Winter et al., 2007). Repeated daily administration of PSOP

selectively downregulated 5-HT_{2A} receptors in the rat brain (Buckholtz et al., 1990; Buckholtz et al., 1988; Buckholtz et al., 1985). PSOP binds to the 5-HT_{2A} receptor and stimulates arachidonic acid and consequently, the PI pathway resulting in the activation of PKC (Kurrasch-Orbaugh et al., 2003). This dissertation sought to evaluate the involvement of the 5-HT_{2A} receptor in the regulation of hippocampal neurogenesis and hippocampal-dependent learning.

Specific Aims

The present study investigated the role of 5-HT_{2A} receptor on hippocampal neurogenesis and hippocampal-dependent learning. The effects of acute and chronic 5-HT_{2A} receptor agonists and an antagonist on the survival and phenotypic fate of progenitor cells in the DG were assessed using immunofluroescent techniques. In addition the effects of acute PSOP on trace fear conditioning were used to assess learning and memory.

Specific Aim 1. To evaluate the effect of acute 5-HT_{2A} receptor agonists and an antagonist on the survival and phenotypic fate of hippocampal progenitor cells. It was hypothesized PSOP and 251-NBMeO, both 5-HT_{2A} receptor agonists positively regulate neurogenesis in the DG of the HPC, and ketanserin, a 5-$HT_{2A/C}$ receptor antagonist downregulates hippocampal neurogenesis.

Specific Aim 2. To elucidate whether PSOP affects learning and memory using the trace fear conditioning paradigm. It was hypothesized acute exposure to PSOP would enhance hippocampal-dependent learning and ketanserin would impair learning on the trace fear conditioning task.

Chapter Two

Involvement of the $5HT_{2A}$ receptor in the Regulation of Adult Neurogenesis in the Mouse Hippocampus

Abstract

Selective serotonin uptake inhibitors (SSRIs) are known to stimulate the production of new neurons in the hippocampus (HPC) by increasing synaptic concentration of serotonin (5-HT). The delay in the appearance of anti-depressant effects corresponds to the time required to generate new neurons. However, it is not clear which of the many serotonergic receptors in the HPC are responsible for the enhanced neurogenesis. The current study evaluated the effects of the acute and chronic administration of $5HT_{2A}$ agonists psilocybin and 251-NBMeO and the $5HT_{2A/C}$ antagonist ketanserin on hippocampal neurogenesis. To investigate the effects of acute drug administration mice received a single injection of varying doses of psilocybin, 251-NBMeO, ketanserin or saline followed by i.p. injections of 75 mg/kg bromodeoxyuridine (BrdU) for 4 consecutive days followed by euthanasia two weeks later. For chronic administration 4 injections of psilocybin, ketanserin or saline were administered weekly over the course of one month. On days following drug injections mice received an injection of 75 mg/kg BrdU and were euthanized two weeks after the last drug injection. Unbiased estimates of BrdU+ and

BrdU/NeuN+ cells in the dentate gyrus (DG) revealed a significant dose dependent reduction in the level of neurogenesis after acute $5HT_{2A}$ receptor agonist or antagonist administration. Interestingly, chronic administration of psilocybin increased the number of newborn neurons in the DG while the antagonist suppressed hippocampal neurogenesis, suggesting the $5HT_{2A}$ receptor appears to be involved in the regulation of hippocampal neurogenesis.

Introduction

Evidence suggests neurogenesis occurs throughout the lifespan in two specific regions of the adult brain, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the DG (Altman J, 1962; Altman & Das, 1965; Altman J, 1969). The proliferation and survival of neural progenitors in the adult HPC can be influenced by a variety of stimuli including stress, age, physical activity and depression (Gould et al., 1992; Kempermann et al., 1998; Van et al., 1999b; Malberg et al., 2000). Antidepressant medications such as selective 5-HT uptake inhibitors (SSRIs) enhance the production of new born neurons in the DG of the HPC (Malberg et al., 2000). However, this effect is time specific with chronic administration (14 days or more) enhancing neurogenesis but not acute treatment (Malberg et al., 2000). Interestingly, there is a delay in the appearance of antidepressant effects which corresponds to the time required to generate new neurons (Santarelli et al., 2003) suggesting an enhancement of neurogenesis may mediate the behavioral effects of antidepressants.

The requirement of chronic administration of antidepressant medications

to enhance neurogenesis is likely due to number of factors. Antidepressant treatments upregulated the expression of brain-derived neurotrophic factor (BDNF) in the HPC (Nibuya et al., 1995). BDNF knockout mice have diminished levels of neurogenesis in the DG (Lee et al., 2002) and infusions of BDNF into the lateral ventricles induced neurogenesis originating in the SVZ (Pencea et al., 2001).

The involvement of 5-HT in the regulation of neurogenesis may be mediated through different 5-HT receptor subtypes expressed on cells in the neurogenic microniche (Barnes & Sharp, 1999). The 5-HT_{2A} receptor is involved in the regulation of BDNF in the HPC (Vaidya et al., 1997). 2,5-dimethoxy-4-iodoamphetamine (DOI), a 5-$HT_{2A/C}$ receptor agonist decreased the expression of BDNF mRNA in the HPC (Vaidya et al., 1997). Interestingly, the decrease in BDNF mRNA expression was blocked by the 5-HT_{2A} receptor antagonist but not the 5-HT_{2C} receptor antagonist, implicating the 5-HT_{2A} receptor in the regulation of BDNF expression in the HPC (Vaidya et al., 1997). Acute administration of DOI, 5-HT_{2C} receptor agonist RO 600175, or the 5-HT_{2C} receptor antagonist SB-206553 had no effect on cell proliferation, whereas the 5-$HT_{2A/C}$ receptor antagonist ketanserin produced a 63% decrease in BrdU incorporation (Banasr et al., 2004). A recent study found acute ketanserin decreased proliferation whereas chronic ketanserin increased proliferation in the DG (Jha et al., 2008). No effect on proliferation in the DG was observed after DOI or lysergic acid diethylamide (LSD) were administered either acutely or once daily for seven

consecutive days (chronic) (Jha et al., 2008). However, daily doses of LSD or psilocybin (PSOP) produce rapid tolerance to the drug and resulted in a selective downregulation of the $5HT_{2A}$ receptor (Buckholtz et al., 1990; Buckholtz et al., 1985). Therefore, in order to investigate the role of the $5HT_{2A}$ receptor in the regulation of hippocampal neurogenesis the current study evaluated the effects of acute and repeated intermittent administration of $5HT_{2A}$ agonists and the $5HT_{2A/C}$ antagonist ketanserin on hippocampal neurogenesis.

Materials and Methods

Subjects. C57BL/6J male mice (30-40g) were housed in standard laboratory cages and left undisturbed for 1 week after arrival at the animal facility. All mice had unlimited access to water and laboratory chow and were maintained in a temperature and humidity controlled room on a 12:12 light/dark cycle with light onset at 7:00 $_{AM}$. All National Institutes for Health (NIH) guidelines for the Care and Use of Laboratory Animals were followed (National Institutes of Health, 2002).

Drugs. 251-NBMeO was synthesized in the laboratory of Dr David Nichols (Braden et al., 2006). PSOP was provided by Dr Francisco Moreno from University of Arizona. Ketanserin (+)-tartrate salt (#S006, St. Louis, MO) and 5-Bromo-2′-deoxyuridine (#B5002, St. Louis, MO) were supplied by Sigma-Aldrich Inc.

General Procedure. Acute Administration: A total of 48 C57BL/6 mice received a single injection of 0.1 mg/kg PSOP (n=6), 0.5 mg/kg PSOP (n=6), 1.0

mg/kg PSOP (n=6), 0.1 mg/kg 251-NBMeO (n=6), 0.3 mg/kg 251-NBMeO (n=6), 1.0 mg/kg 251-NBMeO (n=6), 1.0 mg/kg ketanserin (n=6) or saline (n=6). Mice received an intraperitoneal (i.p.) injection of 75 mg/kg BrdU once daily for 4 days following drug administration and were euthanized two weeks after the last drug injection. Mice were euthanized with nembutal then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were stored in 4% paraformaldehyde, transferred to 20% sucrose solution and sectioned coronally using a cryostat (Leica, Germany) at 30μM in a 1:6 series and stored in 24-well plates in cryoprotectant at -20ºC. Repeated Intermittent Administration: A total of 31 C57BL/6 mice received 4 i.p. injections of either 0.5 mg/kg PSOP (n=6), 1.0 mg/kg PSOP (n=7), 1.5 mg/kg PSOP (n=6), 1.0 mg/kg ketanserin (n=6) or 0.9% saline solution (n=6) over the course of one month on days 1, 8, 15, and 22. Each day following drug administration 75 mg/kg BrdU was injected i.p. All mice were euthanized two weeks after the last drug injection according to the above procedures.

Immunofluorescence. For the double labeling of progenitor cells in the DG free-floating sections were denatured using 2N HCl and neutralized in 0.15M borate buffer then washed in PBS. Tissue was blocked in PBS+ (PBS, 10% normal goat serum, 1% 100x Triton X, 10% BSA) for 1 hour at 4ºC and incubated for 48 hours at 4ºC in an antibody cocktail of rat monoclonal anti-BrdU (AbD Serotec, Raleigh NC, #OBT0030G, 1:100) plus mouse anti-NeuN (Chemicon, 1:100) in PBS. Sections were washed in PBS and incubated in a secondary

antibody cocktail of goat anti-rat IgG Alexa Fluor 594 (1:1000, Invitrogen, Eugene OR) plus goat anti-mouse (1:400, Invitrogen) and coated with vectorshield mounting medium (Invitrogen).

Quantitation. For the quantification of doubled labeled cells using immunofluroescence, the number of BrdU+ and BrdU/NeuN+ labeled cells were estimated using every 6^{th} section taken throughout the DG (every 180 microns). To avoid counting partial cells a modification to the optical dissector method was used so that cells on the upper and lower planes were not counted. The number of BrdU+ cells counted in every 6^{th} section was multiplied by 6 to get the total number of BrdU+ or BrdU/NeuN+ cells in the DG (Shors et al 2002). Positive labeling was verified by confocal microscopy (Zeiss).

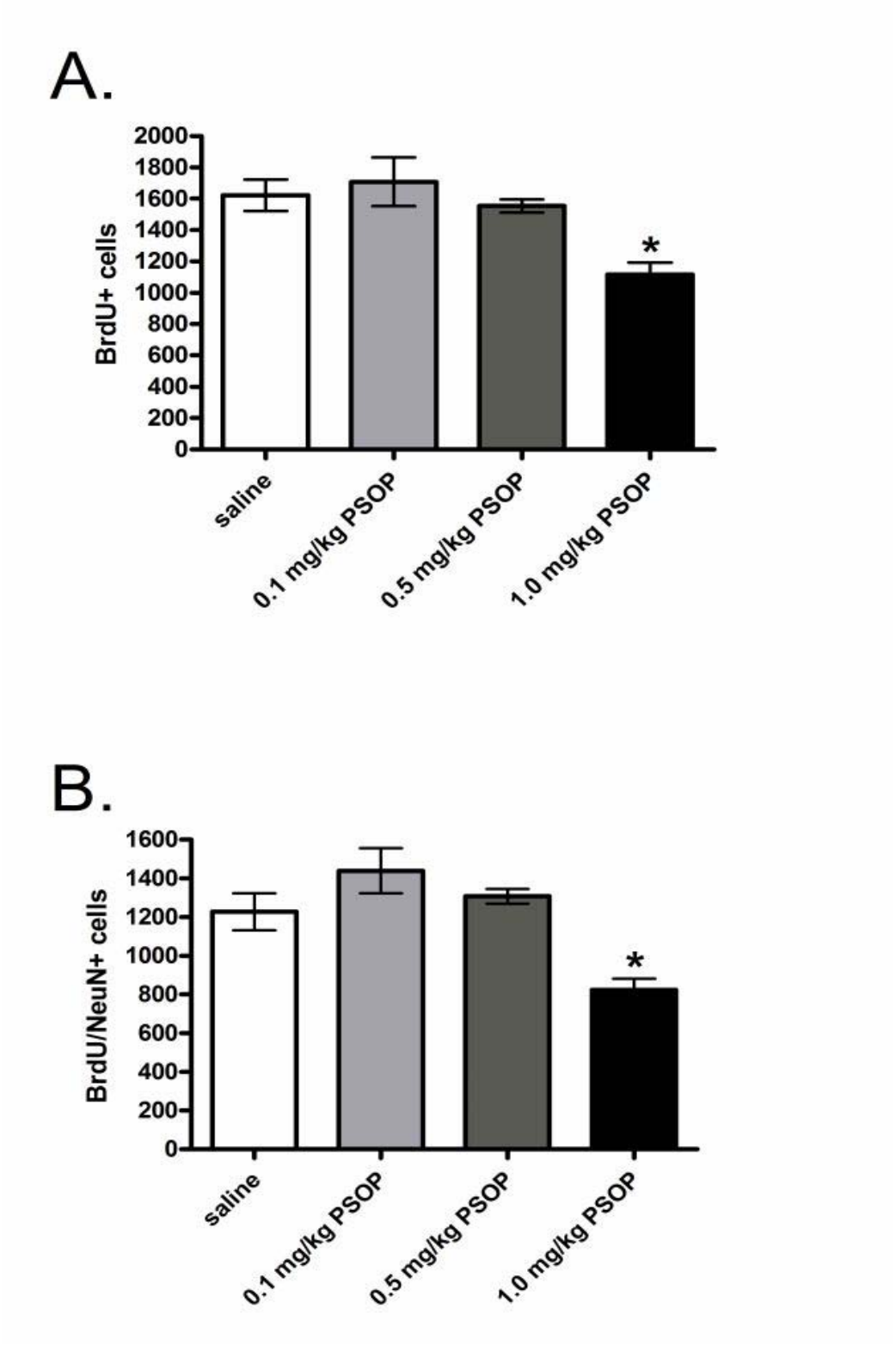
Design and analyses. Separate one-way analyses of variance (ANOVA) were used to evaluate the acute and chronic effects of 5-HT_{2A} receptor agonists and an antagonist on hippocampal neurogenesis. For acute drug administration separate one-way ANOVA was used to determine the effects of Drug [PSOP (saline, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg), 251-NBMeO (saline, 0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg)] and a two-tailed t-test (saline, 1.0 mg/kg ketanserin) was used to establish differences in cell survival and neurogenesis. For chronic drug administration a separate one-way ANOVA was used to determine the effects of Dose (saline, 0.5 mg/kg PSOP, 1.0 mg/kg PSOP, 1.5 mg/kg PSOP, 1.0 mg/kg ketanserin) on cell survival and neurogenesis. When appropriate, post hoc

analyses such as Bonferroni were used to isolate Drug effects. All statistical analyses were determined significant at the 0.05 alpha level.

Results

Effects of Acute Administration of 5-HT_{2A} receptor agonists and an antagonist in vivo on Cell Survival and Neurogenesis in the Hippocampus. In order to investigate the effects of acute PSOP administration on cell survival and neurogenesis mice (n = 6-7 per condition) were injected with PSOP (0.1, 0.5, or 1.0 mg/kg), 251-NBMeO (0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg), ketanserin (1.0 mg/kg) or 0.9% saline solution then received 75 mg/kg BrdU once daily for 4 days following drug administration followed by euthanasia two weeks after the last drug injection. A one-way ANOVA detected significant differences in the total number of surviving BrdU+ cells in the DG as a result of acute PSOP treatment [$F(3,20)=6.64$, $p=0.003$]. As can be seen in Figure 2.1A a significant decrease in the number of surviving BrdU+ cells was observed after 1.0 mg/kg PSOP compared to saline (indicated by *).

The phenotypic fate of surviving cells was determined by immunofluorescent labeling of BrdU and NeuN. A one way ANOVA revealed a significant effect of dose on the number of double labeled neurons in the DG [$F(3,20)=10.26$, $p=0.0003$]. As can be seen in Figure 2.1B, acute administration of 1.0 mg/kg PSOP significantly diminished the number of BrdU/NeuN+ cells compared to saline ($p<0.05$). These data suggest acute administration of 1.0 mg/kg PSOP, a $5HT_{2A}$ agonist downregulated neurogenesis in the DG.


A.
BrdU+ cells
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saline
0.1 mg/kg PSOP
0.5 mg/kg PSOP
1.0 mg/kg PSOP
B.
BrdU/NeuN+ cells
1600
1400
1200
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800
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400
200
0
*
saline
0.1 mg/kg PSOP
0.5 mg/kg PSOP
1.0 mg/kg PSOP

Figure 2.1. Effect of Acute PSOP Administration on Hippocampal Neurogenesis. Mice (n = 6 per condition) were injected with PSOP (0.1, 0.5, or 1.0 mg/kg) or saline then received 75 mg/kg BrdU once daily for 4 days following drug administration followed by euthanasia two weeks after drug injection. A) The total number of BrdU+ cells in the DG were significant diminished after a single injection of 1.0 mg/kg PSOP ($p<0.05$). B) Acute administration of 1.0 mg/kg PSOP significantly diminished the number of BrdU/NeuN+ cells compared to saline ($p<0.05$). These data suggest acute administration of 1.0 mg/kg PSOP, a $5HT_{2A}$ agonist downregulated neurogenesis in the DG of the HPC. * indicates a significant difference from saline.

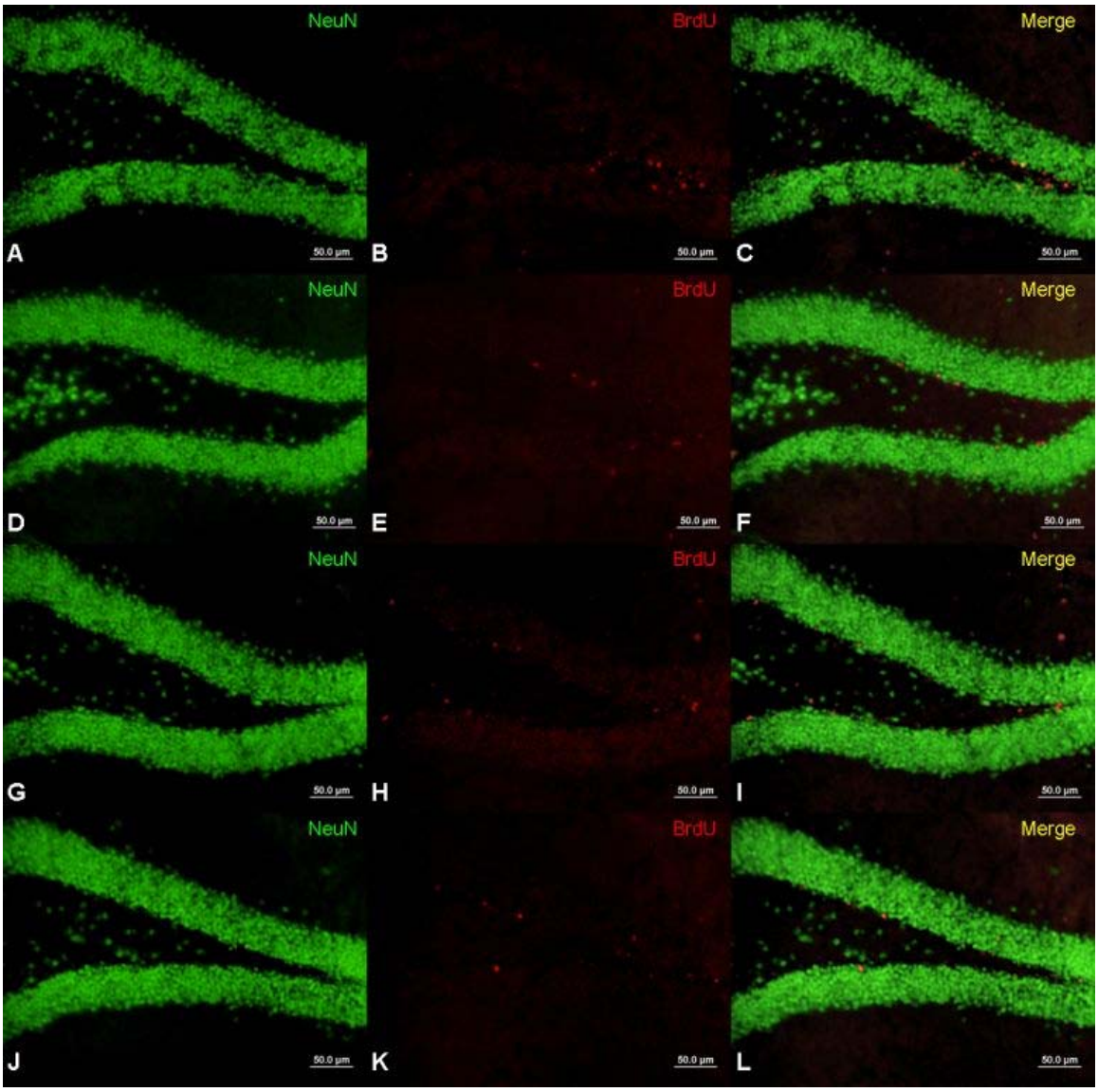
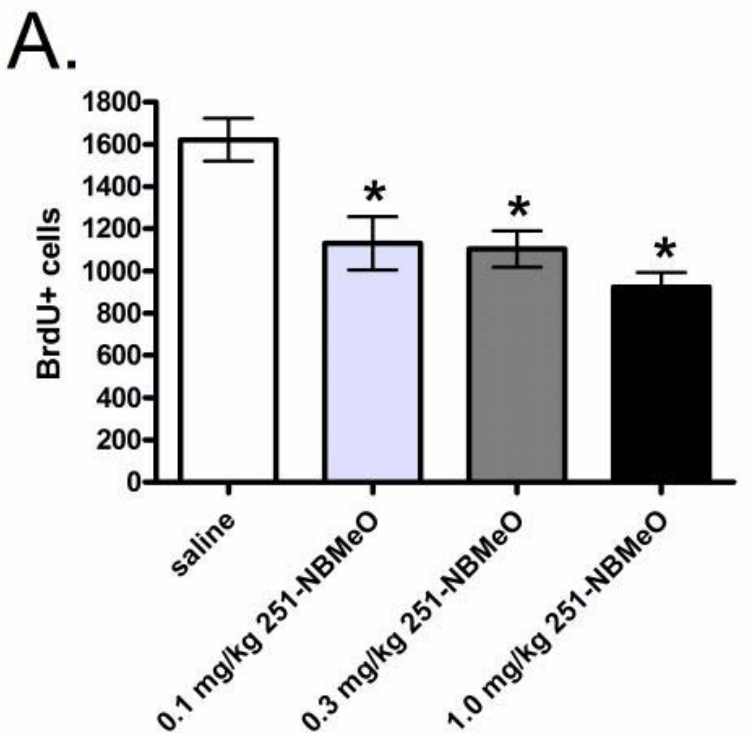


Figure 2.2. Representative photomicrographs showing the effects of acute PSOP on hippocampal neurogenesis. NeuN+ cells (left), BrdU+ cells (center) and NeuN/BrdU+ cells (right). Saline (A-C), 0.1 mg/kg PSOP (D-F), 0.5 mg/kg PSOP (G-I) and 1.0 mg/kg PSOP (J-L). Scale = 50 µM

A one-way ANOVA detected significant differences in the total number of surviving BrdU+ cells in the DG as a result of acute 251-NBMeO treatment [$F(3,20)=9.00$, $p=0.0004$]. There was a significant decrease in the number of surviving BrdU+ cells after acute administration of 0.1, 0.3 and 1.0 mg/kg 251-NBMeO compared to saline (see figure 2.3A, indicated by *).

In addition, there was a significant effect of drug on the number of double labeled neurons in the DG [$F(3,20)=3.00$, $p=0.03$]. As can be seen in Figure 2.3B, 1.0 mg/kg 251-NBMeO significantly diminished the number of new born neurons in the DG compared to saline ($p<0.05$, indicated by *). These data suggest acute administration of the selective 5-HT_{2A} receptor agonist, 251-NBMeO, attenuated hippocampal neurogenesis.


A.
BrdU+ cells
1800
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1400
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800
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saline
0.1 mg/kg 251-NBMeO
0.3 mg/kg 251-NBMeO
1.0 mg/kg 251-NBMeO

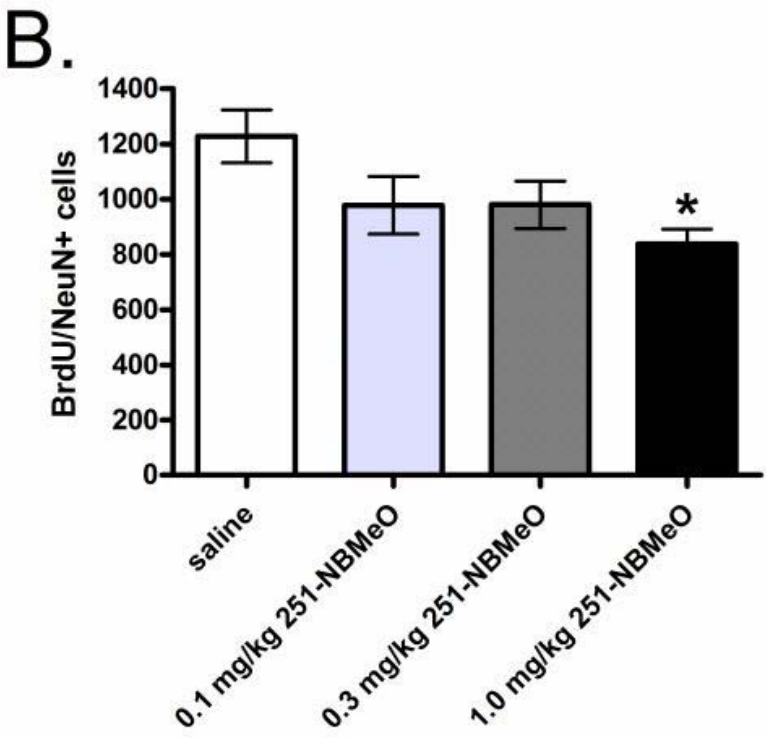

B.
BrdU/NeuN+ cells
1400
1200
1000
800
600
400
200
0
*
saline
0.1 mg/kg 251-NBMeO
0.3 mg/kg 251-NBMeO
1.0 mg/kg 251-NBMeO

Figure 2.3. Effect of the selective 5-HT_{2A} receptor agonist, 251-NBMeO on hippocampal neurogenesis. Mice (n = 6 per condition) were injected with 251-NBMeO (0.1, 0.3, or 1.0 mg/kg) or saline then received 75 mg/kg BrdU once daily for 4 days following drug administration followed by euthanasia two weeks after drug injection. A) There was a significant decrease in the total number of BrdU+ cells in the DG after the administration of 251-NBMeO ($p<0.05$). B) The number of new born neurons was significantly decreased after 1.0 mg/kg of 251-NBMeO compared to saline ($p<0.05$), suggesting acute administration of the $5HT_{2A}$ receptor agonist downregulated neurogenesis in the DG of the HPC.

* indicates a significant difference from saline.

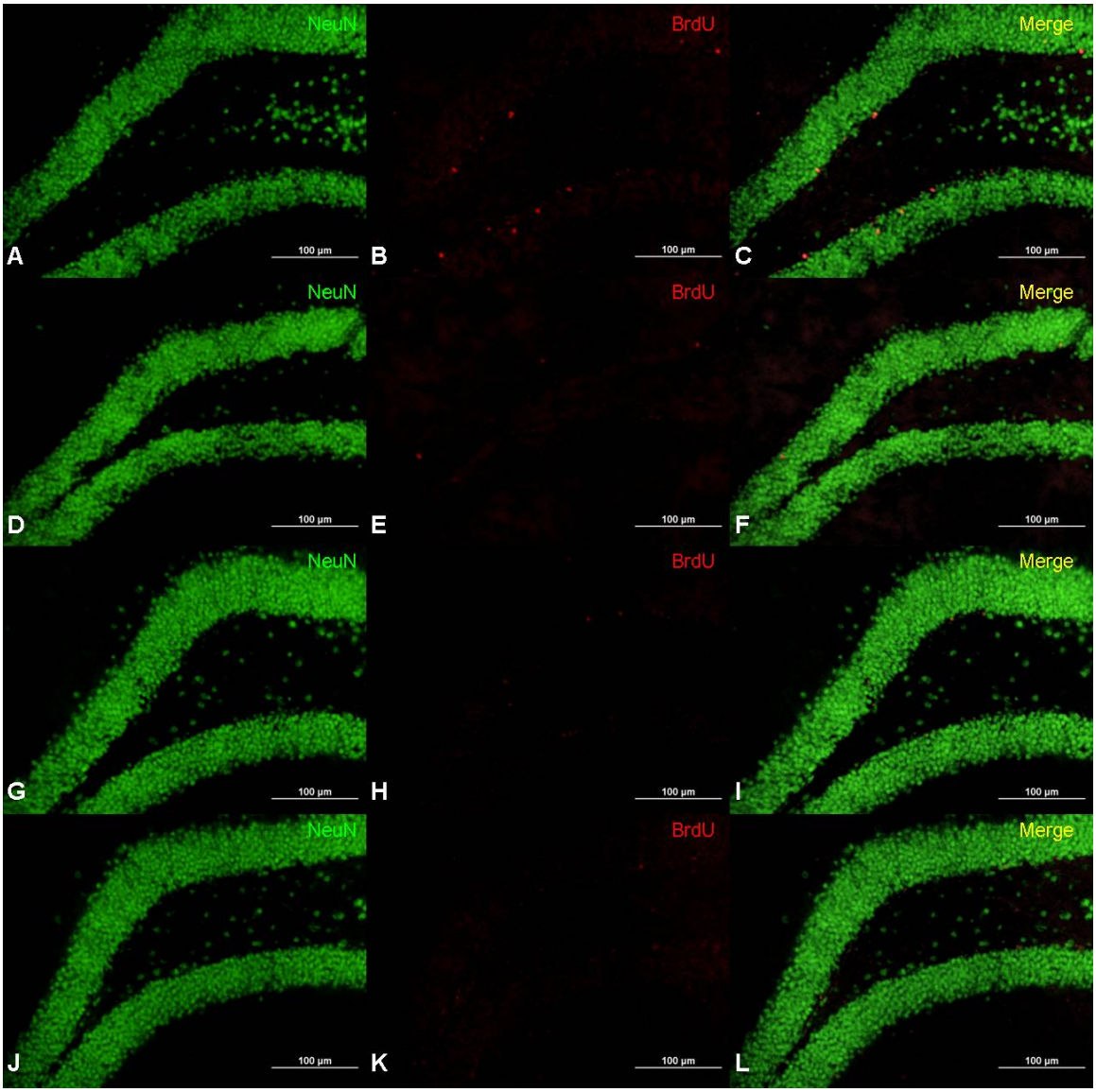


Figure 2.4. Representative photomicrographs showing the effects of the selective 5-HT_{2A} receptor agonist 251-NBMeO on hippocampal neurogenesis. NeuN+ cells (left), BrdU+ cells (center) and NeuN/BrdU+ cells (right). Saline (A-C), 0.1 mg/kg 251-NBMeO (D-F), 0.3 mg/kg 251-NBMeO (G-I) and 1.0 mg/kg 251-NBMeO (J-L). Scale = 100 µM

Interestingly, acute administration of the 5-$HT_{2A/C}$ receptor antagonist ketanserin produced similar effects on neurogenesis as high doses of the 5-HT_{2A} receptor agonists. As can be seen in figure 2.5, the total number of BrdU+ cells in the DG was significantly decreased after 1.0 mg/kg ketanserin [$t(10)=3.0$, $p=0.008$], suggesting that the 5-$HT_{2A/C}$ receptor is involved in the regulation of cell survival in the HPC. Furthermore, acute ketanserin decreased the total number of BrdU/NeuN positive cells compared to saline [$t(10)=3.0$, $p=0.02$] demonstrating that antagonism of the 5-$HT_{2A/C}$ receptor negatively regulated the number of new born neurons generated in the DG of the HPC.

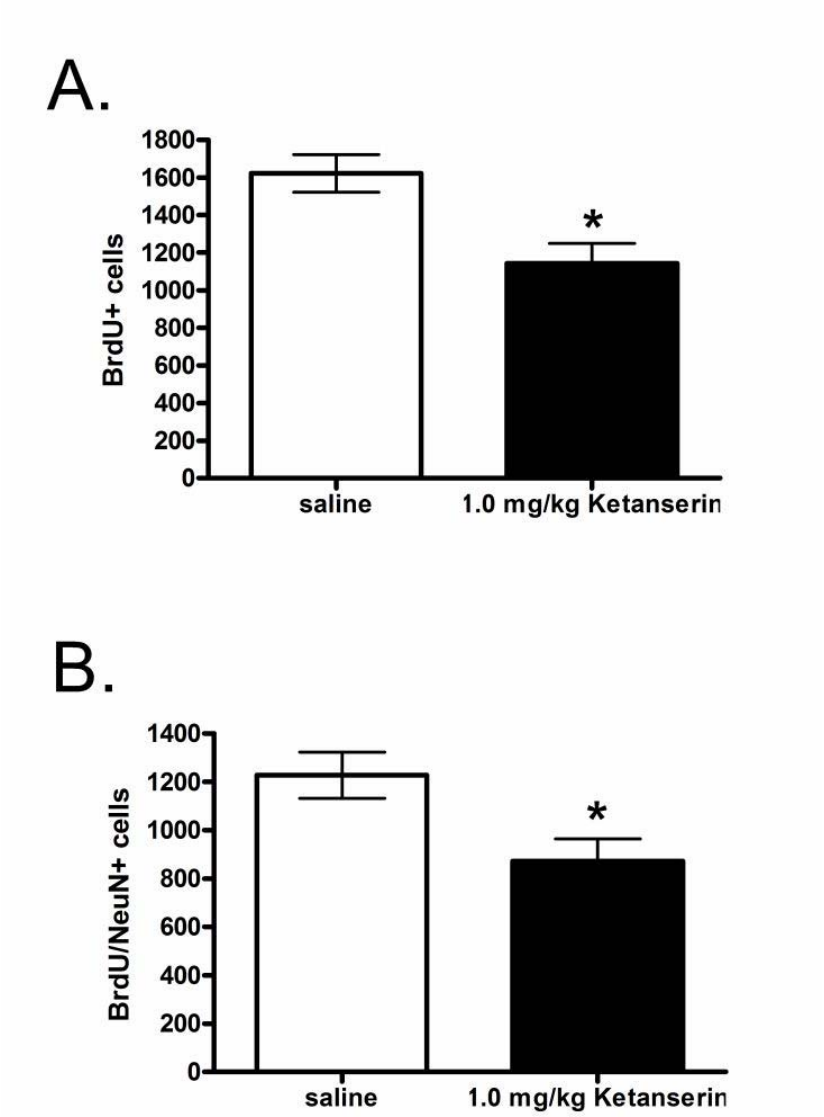


Figure 2.5. Acute Administration of the 5-$HT_{2A/C}$ receptor antagonist ketanserin negatively regulated cell survival and neurogenesis in the HPC. Mice (n = 6 per condition) were injected with 1.0 mg/kg ketanserin or saline then received 75 mg/kg BrdU once daily for 4 days following drug followed by euthanasia two weeks after drug injection. Ketanserin decreased the total number of BrdU+ (A) and BrdU/NeuN positive cells (B) suggesting that antagonism of the 5-$HT_{2A/C}$ receptor negatively regulated cell survival and neurogenesis in the HPC.

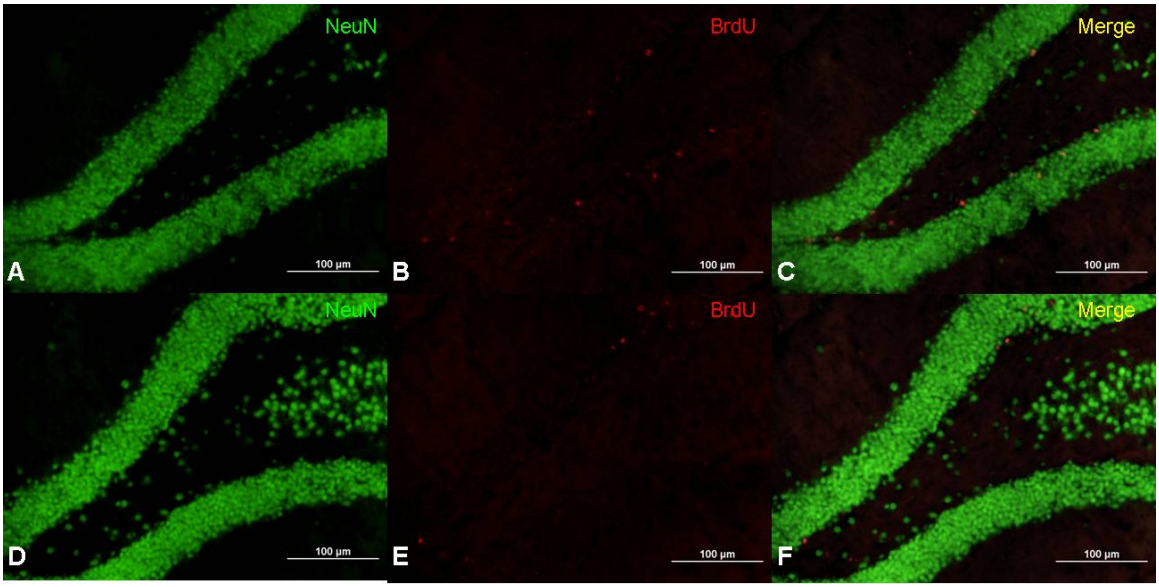


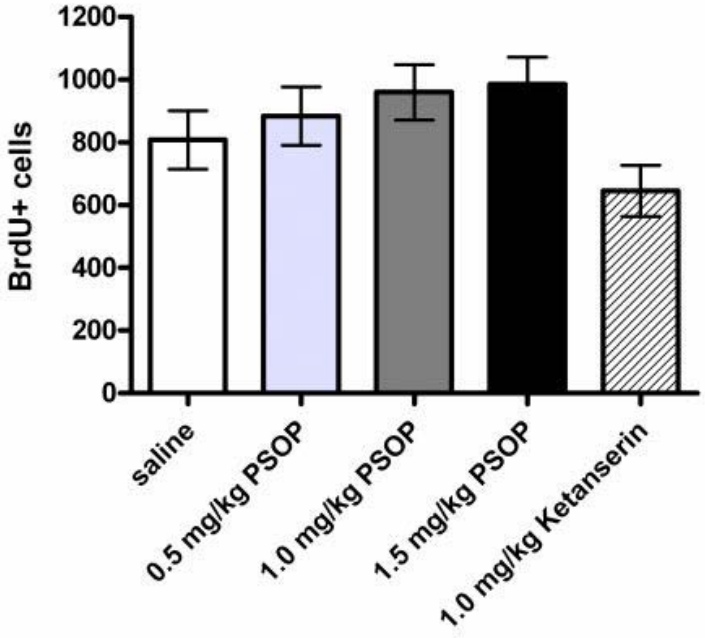
Figure 2.6. Representative photomicrographs showing the effects of the 5-$HT_{2A/C}$ receptor antagonist ketanserin on neurogenesis in the dentate gyrus. NeuN+ cells (left), BrdU+ cells (center) and NeuN/BrdU+ cells (right). Saline (A-C), 1.0 mg/kg ketanserin (D-F). Scale = 100 µM

Effects of Repeated Intermittent PSOP Administration on Progenitor Cell Survival and Neurogenesis in the Hippocampus. In order to investigate the effects of repeated intermittent PSOP administration on cell survival mice (n = 6-7 per condition) were injected with PSOP (0.5, 1.0, or 1.5 mg/kg), 1.0 mg/kg ketanserin or 0.9% saline solution once weekly for 4 weeks. Each day following drug administration, mice were administered 75 mg/kg BrdU and sacrificed two weeks after last drug injection. ANOVA failed to reveal differences in the total number of BrdU+ cells in the DG as a result of repeated intermittent drug

treatment [$F(4,26)=2.20$, $p=0.09$]. As can be seen in Figure 2.7A a trend toward an increase in the number of surviving cells was observed after high doses of PSOP.

The phenotypic fate of surviving cells was determined by immunofluorescent labeling of BrdU and NeuN. ANOVA revealed a significant effect of Dose on the number of double labeled neurons in the DG [$F(4,26)=3.15$, $p=0.02$]. As can be seen in Figure 2.7B, chronic administration of 1.5 mg/kg PSOP significantly increased the number of BrdU/NeuN+ cells compared to saline and ketanserin ($p<0.05$) (indicated by *). These data suggest repeated intermittent administration of PSOP, a $5HT_{2A}$ agonist upregulated neurogenesis in the DG of the HPC.

A.


BrdU+ cells
1200
1000
800
600
400
200
0
saline
0.5 mg/kg PSOP
1.0 mg/kg PSOP
1.5 mg/kg PSOP
1.0 mg/kg Ketanserin

B.

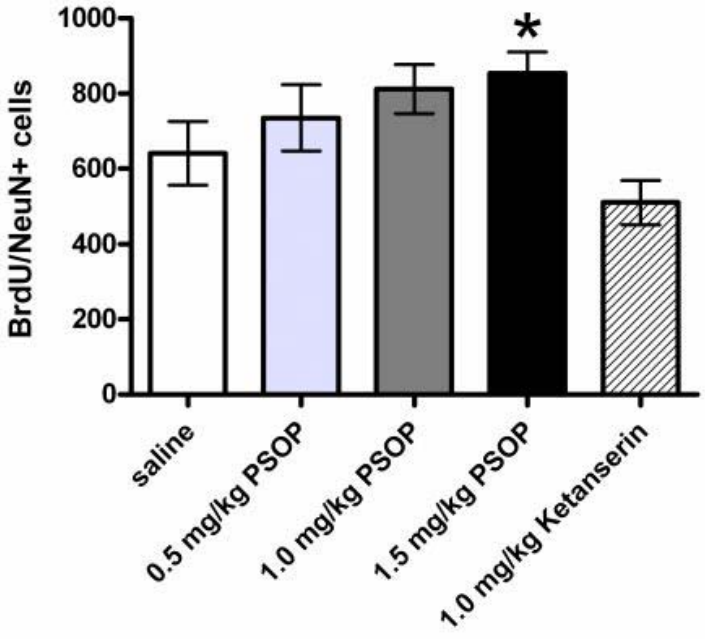

BrdU/NeuN+ cells
1000
800
600
400
200
0
*
saline
0.5 mg/kg PSOP
1.0 mg/kg PSOP
1.5 mg/kg PSOP
1.0 mg/kg Ketanserin

Figure 2.7. Effect of Repeated Intermittent PSOP Administration on Hippocampal Neurogenesis. Mice (n = 6-7 per condition) were injected with PSOP (0.5, 1.0, or 1.5 mg/kg), 1.0 mg/kg ketanserin or saline once weekly for 4 weeks. Each day following drug administration, mice were administered 75 mg/kg BrdU and sacrificed two weeks after last drug injection. A) The total number of BrdU+ cells in the DG did not differ as a result of repeated intermittent drug treatment, however, a trend toward an increase in the number of surviving cells was observed after high doses of PSOP. B) Repeated intermittent administration of 1.5 mg/kg PSOP significantly increased the number of BrdU/NeuN+ cells compared to saline and ketanserin ($p<0.05$). These data suggest repeated intermittent administration of high doses of PSOP, a $5HT_{2A}$ agonist upregulated neurogenesis in the DG.

* indicates a significant difference from saline and ketanserin.

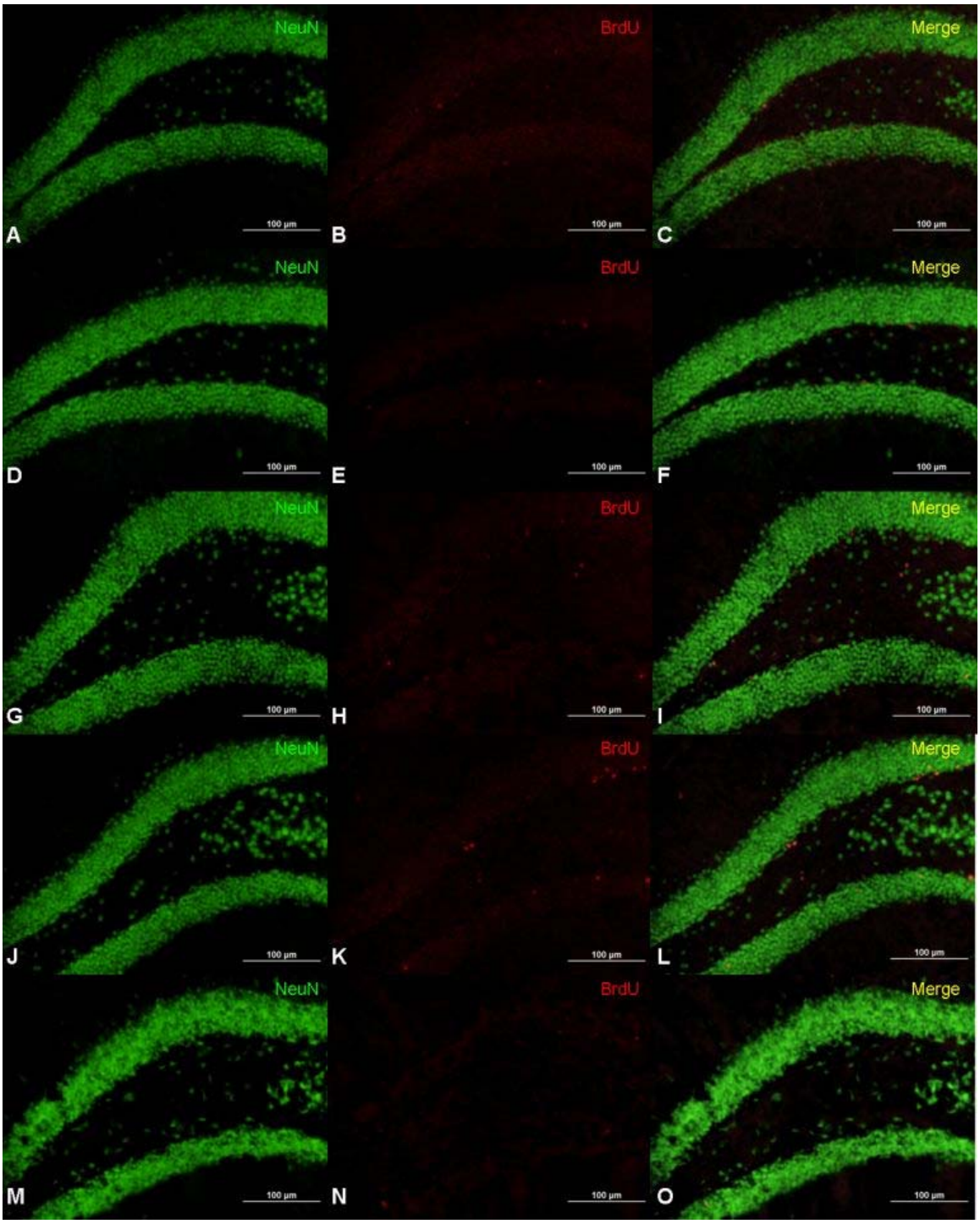


Figure 2.8. Representative photomicrographs showing the effects of repeated intermittent PSOP or ketanserin administration on neurogenesis in the DG.

NeuN+ cells (left), BrdU+ cells (center) and NeuN/BrdU+ cells (right). Saline (A-C), 0.5 mg/kg PSOP (D-F), 1.0 mg/kg PSOP (G-I), 1.5 mg/kg PSOP (J-L), and 1.0 mg/kg Ketanserin (M-O). Scale = 100 μM

Discussion

The present investigation illustrates the involvement of the 5-HT_{2A} receptor in the regulation of neurogenesis in the DG of the HPC. Acute administration of low doses of PSOP (0.1 and 0.5 mg/kg) did not alter neurogenesis, however, higher doses of PSOP (1.0 mg/kg) decreased neurogenesis two weeks after drug exposure (Figure 2.1). In addition, acute administration of the potent 5-HT_{2A} receptor agonist 251-NBMeO (Figure 2.3) and the 5-$HT_{2A/C}$ receptor antagonist ketanserin (Figure 2.5) decreased hippocampal neurogenesis. Acute ketanserin (1-5 mg/kg) administered within 4 hours of sacrifice decreased the number of BrdU+ cells in the DG, indicating a reduction in cell proliferation (Banasr et al., 2004; Jha et al., 2008). The present study extends these findings by demonstrating that acute ketanserin decreases the number of BrdU+ and BrdU/NeuN+ cells 2 weeks after drug administration, indicating a reduction in cell survival and neurogenesis after exposure to acute ketanserin.

The current study reports that repeated intermittent administration of high doses of PSOP (1.5 mg/kg) increased neurogenesis in the DG (see Figure 2.7). A recent study investigated the effects of chronic DOI, LSD and ketanserin administration on the number of Brdu+ cells in the DG (Jha et al., 2008). They

report no effect of chronic DOI or LSD on progenitor cell proliferation but observed an increase in the number of BrdU+ cells in the DG after chronic ketanserin. There are several methodological differences between the studies which may account for the different results, namely drug administration protocol, doses of compounds administered and administration protocol of BrdU. Jha and colleagues administered DOI (8 mg/kg), LSD (0.5 mg/kg) or ketanserin (5 mg/kg) once daily for seven consecutive days for the chronic drug administration protocol (Jha et al., 2008). The current investigation administered PSOP (0.5, 1.0 or 1.5 mg/kg) or ketanserin (1.0 mg/kg) 4 times over the course of one month so that injections were given one week apart. This was a critical consideration in our experimental design given that daily doses of LSD, PSOP or other 5-HT_{2A} receptor agonists produce rapid tolerance to the drug and results in a selective downregulation of the $5HT_{2A}$ receptor (Buckholtz et al., 1990; Buckholtz et al., 1985; Buckholtz et al., 1988). Jha and colleagues administered BrdU (200 mg/kg) 2 hours after the last injection of DOI, LSD or ketanserin and sacrificed the animals 24 hours later (Jha et al., 2008). In the current study BrdU (75 mg/kg) was administered 24 hours after each drug injection and mice were sacrificed two weeks after the last drug injection so that time between the first BrdU injection and sacrifice was 6 weeks. This allowed for the assessment of neurogenesis giving time for the birth-dated cells (BrdU labeled) to mature into neurons.

Brain derived neurotropic factor (BDNF) has been implicated in synaptic

plasticity (Kang et al., 1997; Pang et al., 2004; Tyler et al., 2002) through the modulation of synapse formation and dendritic spine growth in the HPC (Bamji et al., 2006; Tyler & Pozzo-Miller, 2001; Tyler & Pozzo-Miller, 2003). Chronic administration of 5-HT agonists (including SSRIs) upregulate BDNF mRNA expression in the HPC (Nibuya et al., 1995; Nibuya et al., 1996). Evidence suggests that the 5-HT_{2A} receptor is involved in the regulation of BDNF in the HPC (Vaidya et al., 1997). Specifically DOI, a 5-$HT_{2A/C}$ receptor agonist decreased BDNF mRNA expression in the granule cell layer of the DG but not in the CA subfields of the HPC. Interestingly, the decrease in BDNF mRNA expression was blocked by the 5-HT_{2A} receptor antagonist but not the 5-HT_{2C} receptor antagonist, implicating the 5-HT_{2A} receptor in the regulation of BDNF expression (Vaidya et al., 1997).

PSOP and 251-NBMeO exert their effects through binding to 5-HT receptors. PSOP binds to the 5-HT_{2A} receptor (Ki = 6 nM) with high affinity and to a much lesser extent to the 5-HT_{1A} receptor subtype (Ki = 190 nM) (McKenna et al., 1990). The synthetic phenethylamine 251-NBMeO binds to 5-HT_{2A} receptors (Ki = 0.044 nM) with an extremely high affinity (Braden et al., 2006).

5-HT_{2A} receptors are highly expressed throughout the HPC in the DG, hilus, CA1, and CA3 and are colocalized on GABAergic neurons, pyramidal and granular cells (Cornea-Hebert et al., 1999; Morilak et al., 1993; Pompeiano et al., 1994; Shen & Andrade, 1998; Luttgen et al., 2004; Morilak et al., 1994). 5-HT_{2A} receptor agonists stimulate arachidonic acid and consequently, the

phosphoinositide (PI) pathway resulting in the activation of protein kinase C (PKC) (Kurrasch-Orbaugh et al., 2003; Ananth et al., 1987). Electrophysiological evidence suggests that 5-HT_{2A} receptors stimulate GABAergic interneurons in the HPC (Shen & Andrade, 1998) and GABAergic interneurons in the hilus form connections with progenitor cells in the SGZ (Wang et al., 2005). When progenitor cells are less than 2 weeks old the GABAergic input exerts an excitatory influence on the progenitor cells and as the cells establish glutamatergic synapses the GABAergic interneurons become inhibitory (Wang et al., 2005; Zhao et al., 2006; Aimone et al., 2006). Given that 5-HT_{2A} receptor agonists administered chronically downregulates receptor expression, and evidence suggests that the 5-HT_{2A} receptor excites the GABAergic interneurons which stimulate progenitor cells in the SGZ, one might anticipate a reduction in neurogenesis after chronic PSOP. On the contrary, the present study reports high doses of PSOP upregulates neurogenesis. Based on this finding it is plausible to suggest the increase in neurogenesis observed may be attributed to the administration paradigm in which PSOP was given 4 times over the course of one month so that injections were given once a week. In this administration paradigm alterations in receptor levels may not have occurred to the extent that occurs with daily exposure and give this highly plastic microniche time to adapt between drug exposures.

The results shown provide evidence that the 5-HT_{2A} receptor is involved in the regulation of hippocampal neurogenesis. The data suggest that acute

administration of 5-HT_{2A} receptor agonists and an antagonist downregulated neurogenesis in the DG. Whereas, chronic administration of high doses of 5-HT_{2A} receptor agonists enhance hippocampal neurogenesis in the DG. Future studies should investigate the effects of chronic administration of PSOP and ketanserin on 5-HT_{2A} receptor levels and neuroplasticity in the HPC.

Acknowledgments: This work was supported by the Helen Ellis Research Endowment (JSR). Thanks to David Nichols Ph.D. for donating the selective 5-HT_{2A} agonist 251-NBMeO and Dr Francisco Moreno from University of Arizona and Rick Doblin Ph.D. of the Multidisciplinary Association for Psychedelic Studies (MAPS) for donating the PSOP.

Chapter Three

The Effects of Psilocybin on Trace Fear Conditioning

Abstract

Aberrations in brain serotonin (5-HT) neurotransmission have been implicated in psychiatric disorders including anxiety, depression and deficits in learning and memory. Many of these disorders are treated with drugs which promote the availability of 5-HT in the synapse. However, it is not clear which of the 5-HT receptors are involved in behavioral improvements. The current study aimed to investigate the effects of psilocybin, a $5HT_{2A}$ receptor agonist on hippocampal-dependent learning. Mice received a single injection of psilocybin (0.1, 0.5, 1.0 or 1.5 mg/kg), ketanserin (a $5HT_{2A/C}$ antagonist) or saline 24 hours before habituation to the environment and subsequent training and testing on the fear conditioning task. Trace fear conditioning is a hippocampal-dependent task in which the presentation of the conditioned stimulus (CS, tone) is separated in time by a trace interval to the unconditioned stimulus (US, shock). All mice developed contextual and cued fear conditioning; however, mice treated with psilocybin extinguished the cued fear conditioning more rapidly than saline treated mice. Interestingly, mice given the $5HT_{2A/C}$ receptor antagonist ketanserin showed less of cued fear response than saline and psilocybin treated

mice. Future studies should examine the temporal effects of acute and chronic psilocybin administration on hippocampal-dependent learning tasks.

Introduction

The hippocampus (HPC) plays a critical role in learning tasks that involve temporal encoding of stimuli (Squire et al., 1992; Squire, 1992). The trace classical conditioning paradigm requires temporal processing because the conditioned stimulus (CS) and the unconditioned stimulus (US) are separated in time by a trace interval. Lesions to the HPC prevent trace conditioning, indicating that it is a hippocampal-dependent task (McEchron et al., 1998; Weiss et al., 1999).

The serotonergic system has been implicated in hippocampal-dependent learning. Administration of selective serotonin (5-HT) uptake inhibitors (SSRIs) produce alterations in performance on learning tasks that require the HPC (Flood & Cherkin, 1987; Huang et al., 2004). In a knockout (KO) mouse model, central 5-HT deficient mice developed heightened contextual fear conditioning which was reversed by intracerebroventricular microinjection of 5-HT (Dai et al., 2008). An impairment in learning on the morris water maze was observed in 5-HT_{1A} KO mice along with functional abnormalities in the HPC (Sarnyai et al., 2000). Activation of 5-HT_{1A} receptors in the medial septum alters encoding and consolidation in a hippocampal-dependent memory task (Koenig et al., 2008). In addition, Lysergic acid diethylamide (LSD), a 5-HT_{2A} receptor agonist facilitated

learning of a brightness discrimination reversal problem (King et al., 1972; King et al., 1974).

Evidence suggests that performance on hippocampal-dependent learning tasks is influenced by neurogenesis in the dentate gyrus (DG) of the HPC (Van et al., 2002; Nilsson et al., 1999; Shors et al., 2001; Shors et al., 2002; Gould et al., 1999a; Gould et al., 1999c). This was elegantly demonstrated by Shors and colleagues by treating animals with methylazoxymethanol acetate (MAM), an anti-mitotic agent which eradicates the progenitor cell population in the DG before testing mice on hippocampal-dependent and hippocampal-independent learning tasks (Shors et al., 2001; Shors et al., 2002). MAM treated animals had significantly fewer BrdU+ cells in the subgranular zone (SGZ) of the DG but showed no impairment in the spatial navigation task (HPC-dependent) or delay eyeblink conditioning task (HPC-independent) demonstrating that the hippocampal progenitor cell population is not essential for these particular tasks (Shors et al., 2002; Shors et al., 2001). In contrast, MAM severely impaired performance on trace fear conditioning and trace eyeblink conditioning, providing evidence for the involvement of progenitor cells in the DG in trace classical conditioning (Shors et al., 2002; Shors et al., 2001). In addition, hippocampal neurogenesis is influenced by serotonergic agonists. Specifically, SSRIs enhance the production of new born neurons in the DG of the HPC (Malberg et al., 2000; Santarelli et al., 2003).

Psilocybin (PSOP), a tryptamine alkaloid, exerts psychoactive effects by

altering serotonergic neurotransmission (Passie et al., 2002). PSOP binds to the 5-HT_{2A} receptor (Ki = 6 nM) with high affinity and to a much lesser extent to the 5-HT_{1A} receptor subtype (Ki = 190 nM) (McKenna et al., 1990). 5-HT_{2A} receptors are highly expressed throughout the HPC in the DG, hilus, CA1, and CA3 (Cornea-Hebert et al., 1999; Morilak et al., 1993; Pompeiano et al., 1994; Shen & Andrade, 1998; Luttgen et al., 2004; Morilak et al., 1994). 5-HT_{2A} receptor agonists, including PSOP, stimulate arachidonic acid (AA) and consequently, the phosphoinositide (PI) pathway resulting in the activation of protein kinase C (PKC) (Kurrasch-Orbaugh et al., 2003; Ananth et al., 1987). Electrophysiological evidence suggests that 5-HT_{2A} receptors stimulate GABAergic interneurons in the HPC (Shen & Andrade, 1998) and GABAergic interneurons in the hilus form connections with progenitor cells in the SGZ (Wang et al., 2005).

The present study aimed to investigate the effects of the $5HT_{2A}$ receptor agonist PSOP on hippocampal-dependent learning. Mice received a single injection of psilocybin (0.1, 0.5, 1.0 or 1.5 mg/kg), 1.0 mg/kg ketanserin (a $5HT_{2A/C}$ antagonist) or saline 24 hours before habituation to the environment and subsequent training and testing on the trace fear conditioning task.

Materials and Methods

Subjects. C57BL/6J male mice (30-40g) were housed in standard laboratory cages and left undisturbed for 1 week after arrival at the animal facility. All mice had unlimited access to water and laboratory chow and were maintained in a temperature and humidity controlled room on a 12:12 light/dark cycle with

light onset at 7:00 $_{AM}$. All National Institutes for Health (NIH) guidelines for the Care and Use of Laboratory Animals were followed (National Institutes of Health, 2002).

General Procedure. Mice (n=9-10/condition) received an intraperitoneal (i.p.) injection of PSOP (0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg), ketanserin (1.0 mg/kg) or 0.9% saline and 24 h later were habituated to the testing chamber for 30 min. The fear conditioning environment consisted of two chambers each placed inside a larger soundproof chamber. The 35.6 (W) x 38.1 (D) x 31.8 (H) cm freeze monitor box (San Diego Instruments, San Diego, CA) is a clear Plexiglas chamber with a removable lid which contains a metal grid floor (0.3 cm grids spaced 0.8 cm apart) through which a foot shock can be delivered. Photo-beam activity within the chamber recorded the vertical and horizontal movements of mice. Two minutes into the habituation period a baseline (BL) measure of movement was recorded for 3 minutes and served as the habituation BL measure. Freeze monitor boxes were cleaned with quatricide between each mouse to prevent olfactory cues. Mice were returned to their home cage after habituation.

The next day mice were returned to the same freeze monitor chamber and underwent training to form CS – US associations. After a 2 minute acclimation period, mice were exposed to 10 trials of trace fear conditioning which is illustrated in Figure 3.1. Each trial consisted of the CS (tone, 82 dB, 15 s) followed by a trace interval (30 s) and ended with the presentation of the US

(shock, 0.5 s, 1 mA) delivered through the grid flooring. After each trial ended there was a 210 s intertrial interval (ITI). Freeze monitor boxes were cleaned with quatricide between each mouse to prevent olfactory cues and mice were returned to their home cage after training.

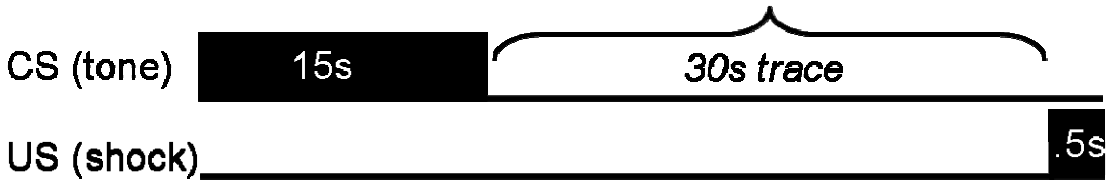


Figure 3.1. Schematic representation of the Trace Fear Conditioning Paradigm. Trace fear conditioning is a hippocampal-dependent task in which the presentation of the conditioned stimulus (CS, tone) is separated in time by a trace interval to the unconditioned stimulus (US, shock)

On day 3 of the task, testing of the fear conditioning response was assessed in 2 phases. First, mice were placed in the freeze monitor box for 5 minutes and movement was recorded for the last 3 minutes and used to assess the measure of fear associated to the training context. Mice were then returned to the home cage for 1 hour. Second, the context was altered by replacing the grid floors with black Plexiglas flooring and adding a cotton ball with 1ml vanilla essence inside the sound attenuated chamber. Mice were placed inside the novel chamber and after 2 minutes, movements were recorded for the next 3 minutes. Next 10 trials with the presentation of the CS only were delivered with an ITI of 240 s. Cue fear conditioning was measure by the percent freezing

during the CS (tone; 15s), during the trace interval (30s) and after the trace when the US would have occurred. Conditioned fear was defined as an increase in percent immobility during the cue test. Percent immobility was calculated by dividing time spent immobile during stimuli (CS, trace or after trace) by the length of time the stimuli lasted multiplied by 100.

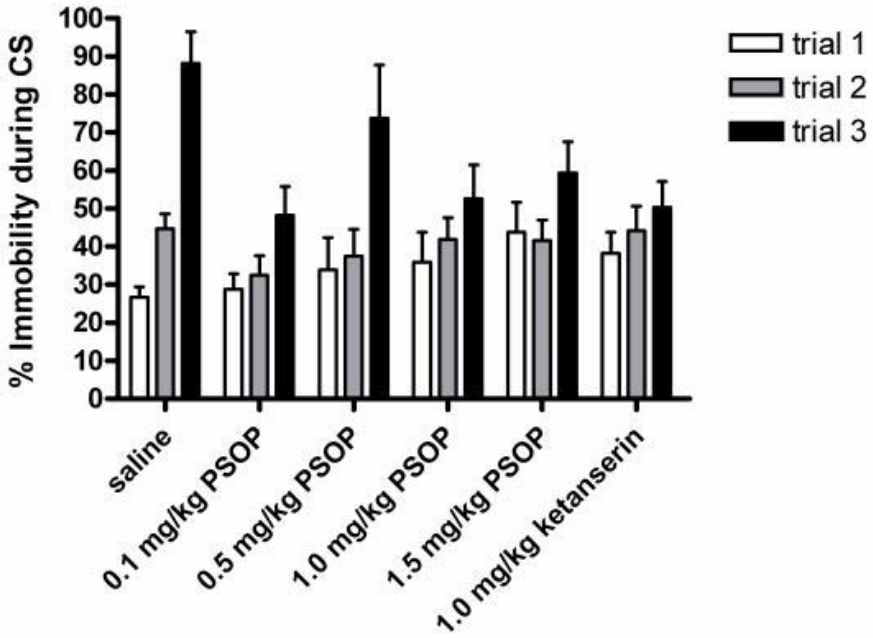
*Design and analyses. Se*parate two-way repeated measure analyses of variance (ANOVA) were used to evaluate the effect of Dose and Trial on each dependent variable in the trace fear conditioning task. Dependent measures recorded included percent freezing during CS, during trace, after trace, during habituation BL, during the context text and in response to the novel environment. When appropriate, post hoc analyses such as Bonforreoni were used to isolate effects. All statistical analyses will be determined significant at the 0.05 alpha level.

Results

Acquisition. The acquisition of the freezing response is displayed in Figure 3.2 showing both the response to the CS (Figure 3.2A) and during the trace (Figure 3.2B). Using percent immobility in response to the CS for the first 3 trials of training, ANOVA showed that regardless of drug treatment there was a significant improvement across trial [$F(2, 108) = 40.0$, $p<0.0001$]. Specifically, immobility in response to the CS increased from trial 1 to trial 3 indicating the learned association between the stimuli during training ($p<0.05$). Additionally, ANOVA of the percent immobility during the trace interval revealed a significant

effect of trial [$F(2, 108) = 20.0$, $p<0.0001$]. There was a striking increase in the amount of time spent immobile during the trace period from trial 1 to trial 3 demonstrating that the association between the CS and US promoted immobility in anticipation of the shock.

A.


% Immobility during CS
100
90
80
70
60
50
40
30
20
10
0
trial 1
trial 2
trial 3
saline
0.1 mg/kg PSOP
0.5 mg/kg PSOP
1.0 mg/kg PSOP
1.5 mg/kg PSOP
1.0 mg/kg ketanserin

B.

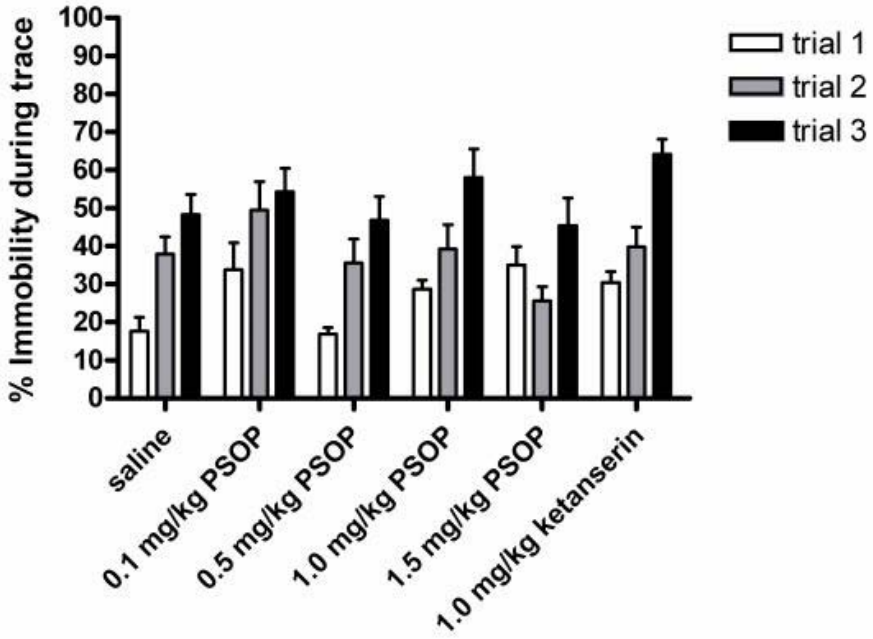

% Immobility during trace
100
90
80
70
60
50
40
30
20
10
0
trial 1
trial 2
trial 3
saline
0.1 mg/kg PSOP
0.5 mg/kg PSOP
1.0 mg/kg PSOP
1.5 mg/kg PSOP
1.0 mg/kg ketanserin

Figure 3.2. Effects of Psilocybin on the Acquisition of Trace Fear Conditioning. Mice underwent training to form CS – US associations by exposure to 10 trials of trace fear conditioning. Each trial consisted of the presentation of the CS (tone, 15-s) followed by a trace interval (30-s) and ended with the US (shock, 0.5-s). A) Percent immobility during presentation of the 15-s CS during the first three trials of CS – US pairing. B) Percent immobility during the 30-s trace interval during the first three trials of CS – US pairing.

Contextual Fear Conditioning. Contextual fear conditioning was assessed by comparing percent immobility in the freeze monitor box on habituation day to percent immobility during the context test. There was no interaction between Dose and Trial [$F(5,90) = 0.95$, $p=0.45$] and no effect of Dose during the habituation BL or context test [$F(5,90) = 1.15$, $p=0.34$]. However, there was a significant effect of Trial [$F(1,90) = 105.85$, $p<0.0001$] indicating that mice spent significantly more time freezing after the CS – US pairings during the context test compared to habituation BL. Figure 3.3 illustrates percent immobility during exposure to the freeze monitor box during the habituation test (A) and during the contextual fear conditioning test (B).

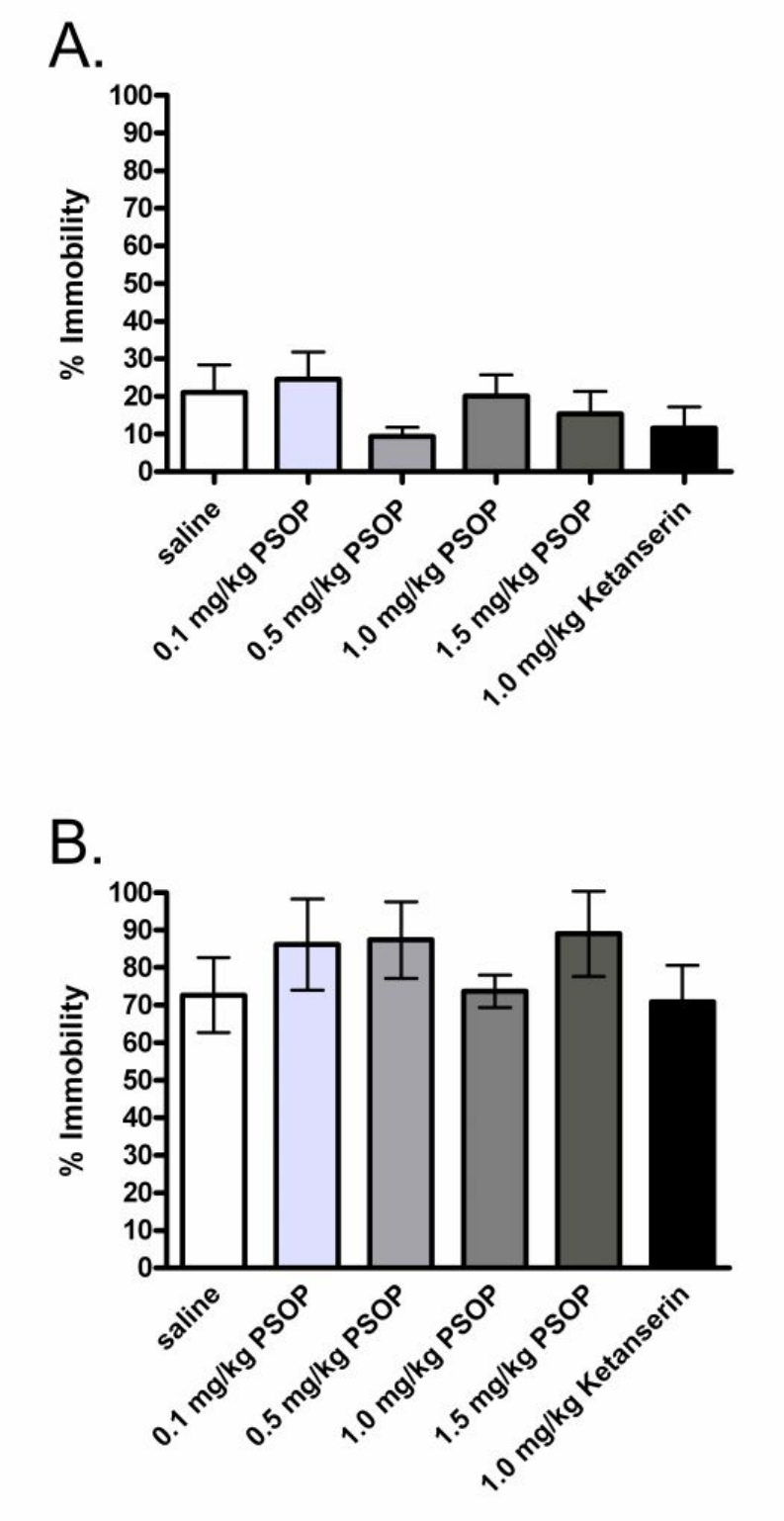


Figure 3.3. Contextual Fear Conditioning. Percent immobility expressed during

exposure to the freeze monitor box during habituation (A) and during contextual fear conditioning (B). All mice expressed contextual fear conditioning as indicated by a significant increase in percent immobility during the context test ($p<0.05$).

Cue Fear Conditioning. Freezing responses (% immobility) during the CS only (tone) test are illustrated in Figure 3.4. There was a significant effect of Trial [$F(2,102) = 7.83$, $p<0.0007$] with trials 2 and 3 eliciting significantly more freezing in response to the CS compared to trial 1 (Figure 3.4A). There was also a significant effect of Dose [$F(5,51) = 4.96$, $p<0.0009$] revealing that control mice showed a greater fear response to the cue compared to 0.1 mg/kg PSOP, 1.0 mg/kg PSOP, 1.5 mg/kg PSOP and 1.0 mg/kg ketanserin. ANOVA revealed a significant effect of Dose during the trace interval [$F(5,51) = 2.41$, $p<0.05$]. The fear associated with the trace interval during the first three trials on test day was reduced in mice treated with 1.0 mg/kg PSOP compared to saline and 1.5 mg/kg PSOP (Figure 3.4B). Figure 3.4C illustrates the fear response after the trace interval which coincides with the timing the US (shock) was delivered during the acquisition of the CS - US pairing phase. A two-way repeated measures ANOVA revealed a significant interaction between Dose and Trial [$F(10,100) = 3.53$, $p<0.0005$]. Interestingly, mice administered low doses of PSOP (0.1 and 0.5 mg/kg) froze more on trial 1 compared to trial 2 and 3 suggesting they are more apt to adapt to the absence of the US so that the fear response is diminished as

the US is extinguished. This pattern was reversed in mice treated with ketanserin who increased fear responses from trials 1 to 3, indicating the robust memory for the US even in its absence. Taken together, these data suggest differential effects of PSOP on trace fear conditioning.

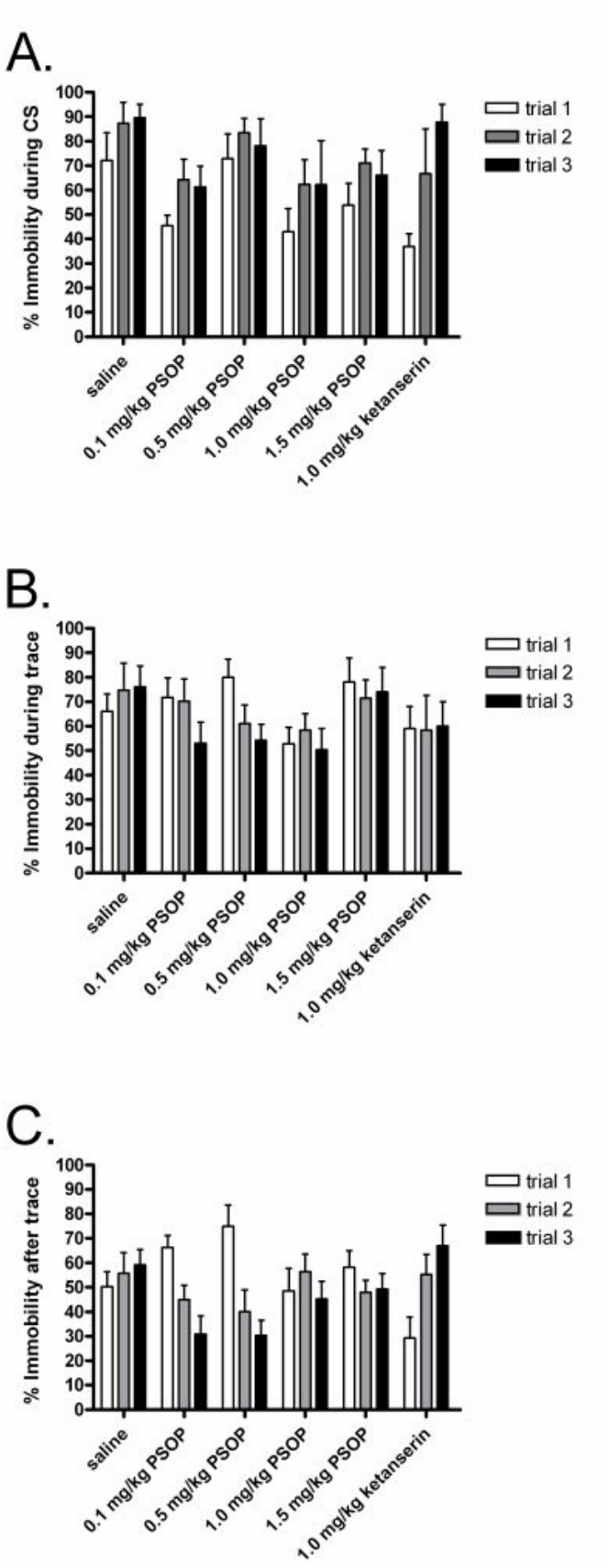

A.
% Immobility during CS
100
90
80
70
60
50
40
30
20
10
0
trial 1
trial 2
trial 3
saline
0.1 mg/kg PSOP
0.5 mg/kg PSOP
1.0 mg/kg PSOP
1.5 mg/kg PSOP
1.0 mg/kg ketanserin
B.
% Immobility during trace
C.
% Immobility after trace

Figure 3.4. Effect of Acute PSOP on Cue Fear Conditioning. Cue fear conditioning was examined 24 hours after the training period in which mice were exposed to 10 trials of CS – US pairings. A) Percent immobility during presentation of the 15-s CS during the first three CS-only trials. B) Percent immobility during the 30-s trace interval during the first three trials. C) Percent immobility after the trace interval which coincides with the timing the US (shock) was delivered during the acquisition of the CS - US pairing phase.

Discussion

The present investigation illustrates the involvement of the 5-HT_{2A} receptor in trace fear conditioning, a hippocampal-dependent learning task. During the acquisition of the freezing response there was a striking increase in the amount of time spent freezing during the presentation of the CS and during the trace period from trial 1 to trial 3 (see Figure 3.2). These data demonstrate that the association between the CS and US promoted freezing in anticipation of the shock, however, the acquisition of learning was not altered by acute administration of PSOP or ketanserin.

All conditions displayed similar locomotor activity levels in the freeze monitor box during the habituation baseline exposure and during re-exposure to the same environment during the contextual fear conditioning test. As expected mice froze substantially more during re-exposure to the same context after CS – US associations were formed compared to the habituation baseline, indicating

that all groups formed contextual fear conditioning (Figure 3.3). It is well known that contextual fear conditioning is a hippocampal-dependent learning task (Kim & Fanselow, 1992; McNish et al., 1997; Hirsh, 1974; Esclassan et al., 2008; Frohardt et al., 1999). The serotonergic system has been implicated in performance on the contextual fear conditioning task (Dai et al., 2008). The present investigation found that the 5-HT_{2A} receptor does not alter contextual fear conditioning since no differences were observed between controls and mice treated with PSOP or ketanserin.

The current study reports alterations in cue associated fear conditioning mediated by the 5-HT_{2A} receptor. Independent of drug administered all mice developed cue-induced freezing during the presentation of the tone on the CS-only trial (see Figure 3.4). At the time coinciding with the expected US (shock) presentation low doses of PSOP (0.1 and 0.5 mg/kg) elicited a heightened freezing response on trial 1 compared to other trials suggesting they are more apt to adapt to the absence of the US so that the fear response is diminished as the US is extinguished. This pattern was reversed in mice treated with ketanserin who increased fear responses from trials 1 to 3, indicating the robust memory for the US even in its absence.

Synaptic plasticity in the HPC is critical for the acquisition of learning and memory. Brain derived neurotropic factor (BDNF) has been implicated in synaptic plasticity and memory processing (Kang et al., 1997; Pang et al., 2004; Tyler et al., 2002) through the modulation of synapse formation and dendritic

spine growth in the HPC (Bamji et al., 2006; Tyler & Pozzo-Miller, 2001; Tyler & Pozzo-Miller, 2003). Chronic administration of 5-HT agonists (including SSRIs) upregulate BDNF mRNA expression in the HPC (Nibuya et al., 1995; Nibuya et al., 1996).

Evidence suggests that the 5-HT_{2A} receptor is involved in the regulation of BDNF in the HPC (Vaidya et al., 1997). Specifically DOI, a 5-$HT_{2A/C}$ receptor agonist decreased BDNF mRNA expression in the granule cell layer of the DG but not in the CA subfields of the HPC. Interestingly, the decrease in BDNF mRNA expression was blocked by the 5-HT_{2A} receptor antagonist but not the 5-HT_{2C} receptor antagonist, implicating the 5-HT_{2A} receptor in the regulation of BDNF expression (Vaidya et al., 1997).

5-HT_{2A} receptors are highly expressed throughout the HPC in the DG, hilus, CA1, and CA3 and are colocalized on GABAergic neurons, pyramidal and granular cells (Cornea-Hebert et al., 1999; Morilak et al., 1993; Pompeiano et al., 1994; Shen & Andrade, 1998; Luttgen et al., 2004; Morilak et al., 1994). Agonists to the 5-HT_{2A} receptor stimulate AA and consequently, the PI pathway resulting in the activation of PKC (Kurrasch-Orbaugh et al., 2003; Ananth et al., 1987). Electrophysiological evidence suggests that 5-HT_{2A} receptors stimulate GABAergic interneurons in the HPC (Shen & Andrade, 1998). Aberrations in GABAergic function in the HPC has been implicated in learning and memory due to the role of hippocampal GABA in temporospatial integration (Wallenstein et al., 1998)

Taken together, the data reported in the present investigation implicate the 5-HT_{2A} receptor in hippocampal-dependent learning. The present study reports that prior exposure to PSOP altered responsivity in a novel environment indicating an absence of a fear response, an effect not elicited by control mice. Furthermore, low doses of PSOP heightened cue elicited fear conditioning and antagonists to the 5-$HT_{2A/C}$ receptor diminished fear conditioning to the cue. Results of this study raise the possibility that 5-HT_{2A} receptor activity could lead alterations hippocampal-dependent learning and memory.

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