The Relationship between Drug-Induced Neurogenesis and Pain Behavior in Mice.

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Abstract

This experiment aims to study the relationship between neurogenesis and pain by inducing varying amounts of neurogenesis in mice using a pharmacological manipulation and to evaluate the subsequent pain behavior. A muscarinic ACh receptor agonist, galantamine, was used to increase neurogenesis and a nicotinic ACh receptor agonist, nicotine, was intended to decrease neurogenesis. Injections were administered daily for two weeks to two different drug condition groups and a saline control group, with N = 10 male mice and N = 10 female mice in each group. It was hypothesized that rates of neurogenesis (measured by the number of BrdU positive cells/μm^3) would vary across condition, with animals receiving nicotine expressing the lowest rate of neurogenesis and animals receiving galantamine expressing the highest rate of neurogenesis. It was additionally hypothesized that pain behavior on the formalin test would vary across condition in the same direction as neurogenesis. Although the directionality was not as hypothesized, there was a significant effect of the drug conditions in the late phase of pain, with saline-treated animals expressing the lowest amount of pain behavior and galantamine-treated animals expressing the highest amount of pain behavior on average. There was also a significant main effect of condition on our measure of neurogenesis, and the directionality of the effect was the same as that of pain behavior. Our results provide evidence for a relationship between drug-induced neurogenesis and pain behavior in mice, which holds implications for further neurogenesis research.

Introduction

Pain

Pain is an unfortunate component of one's sensory experience of the world. Unique from the other sensory experiences, pain not only conveys information about our environment (as do the touch, taste, sound, and visual modalities), but it does so with negative hedonic properties. Intrinsic in pain is the idea that it is unpleasant. Pain has been defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"(Melzack and Wall, 1982). Despite being the only sensory modality with a hedonically negative experience (Sternberg, 2007), it has only been studied rigorously over the past few decades (Melzack and Wall, 1982). However, it has been noted for millennia the ways in which pain has caused suffering for people (Melzack and Wall, 1982). In studying pain, it is important to learn what factors contribute to pain and in what ways the experience of pain can be altered. This study aims to explore the potential of neural growth in the dentate gyrus of the brain for altering the pain experience in mice.

Pain as a sensory experience has many interesting characteristics. Aside from the hedonic value of pain, if it persists it also has the ability to lessen the experience of the other senses by rendering the sufferer unable to focus on anything else. Pain is also arguably more adaptive than the other senses because it serves as a warning and helps to direct behavior to avoid painful stimuli. Those individuals who are born with one of a handful of rare conditions in which they lack nociceptors (sensory neurons that are

specific to pain perception) tend to lead very short lives due to their inability to recognize the painful stimuli that signal impending or ongoing tissue damage. Their deaths often result from commonly occurring conditions that go unnoticed, such as appendicitis. One individual suffering from congenital insensitivity died from an infection of the joints because she did not experience the discomfort that causes normal persons to shift their weight to relieve the joints from pressure (Melzack and Wall, 1982). While pain is highly adaptive and necessary for a healthy life, it can also be highly debilitating. It is the incapacitating nature, and general unpleasantness, that makes pain relief so important.

Because the experience of pain is necessary for survival, it is reasonable to assume that the mechanisms of pain perception are highly conserved across species. This assumption has proven especially useful in laboratory settings and has led to a vast array of important findings concerning pain (Liebeskind, 1991) via experiments conducted in rodents. For example, a mouse's pain behavior can be observed according to several different measures. Acute or transient pain stimuli can be used to measure a mouse's pain threshold by placing a paw on a hotplate at a constant temperature. Once the stimulus becomes intense enough to the mouse, it will pick up its paw and make a flicking motion, which is operationally defined as pain behavior. Similarly, the hot water latency test involves dipping the tail into hot water and measuring the amount of time that it takes the mouse to flick its tail out (again providing a measure of pain threshold) (Malmberg and Bannon, 1999).

Experiments can also be performed in which mice are exposed to persistent, unavoidable noxious stimuli. One of the most common tests of this type is the formalin test, in which formalin is injected into the hindpaw, causing tissue damage, and pain

behavior (licking and flicking of the paw) that is recorded over time. This test is considered a good model for persistent pain, and represents both the early phase and late phase of pain. While the early phase contains the immediate, intense response, the late phase is characterized by the dull, throbbing pain, and is more representative of an on-going chronic pain experience (Abbott, Franklin, and Westbrook, 1995; Malmberg and Bannon, 1999). Use of animal models is possible only because the neural organization is highly conserved from humans to other species, and this conservation arises from the necessity of pain for survival.

The extent to which pain experiences vary, both between and within individuals, is relevant to our goal of altering the pain experiences of mice. In accordance with the highly variable nature of pain, there is no one-to-one ratio of a stimulus to its perception (Melzack and Wall, 1982). If a person has just made it off of the battlefield alive, they may not feel any pain from a gunshot wound, although they may complain about the pain associated with the insertion of a needle (Melzack and Wall, 1982). If an athlete sprains an ankle in the middle of a game, they may not experience any pain associated with the injury until after the game ends. At the other end of the spectrum, a person with depression may experience severe back pain with seemingly no source of injury (Melzack and Wall, 1982). Because pain can vary between individuals and between situations, better understanding the relationship between different modulating variables and subsequent pain enables us to implement what we know to alter pain experiences. By understanding the factors that can alter the pain experience, we can better learn how to control pain and relieve pain symptoms.

We hope to modulate the pain experience in mice, and consideration of ways to alter pain experience is thus relevant. Presently, many external and internal changes or conditions are known to modulate pain behavior, such as genetic, hormonal, and psychological variables. For example, females are known to have lower pain threshold levels and tolerance levels than men, and this finding holds true for lab rodents as well as humans (Berkley, 1997). Studies have shown that minorities in the US have lower pain threshold and tolerance levels that vary according to how strongly they identify with their ethnicity (Rahim-Williams et al., 2007), and also that individuals of a lower socio-economic status have lower pain thresholds (Portenoy, Ugarte, Fuller, and Haas, 2004). Pain sensitivity varies according to mental state, too. Depressed individuals experience increased pain sensitivity as well as having a higher prevalence of chronic pain conditions (Delgado, 2004). Stress is known to both decrease pain sensitivity in an immediate situation, and to heighten pain sensitivity if it persists chronically (Sternberg, 2007; Chipkin, Latranyi, and Iorio, 1982; McEwen, 2001). Interestingly, patients with a frontal lobotomy can still perceive pain, but they no longer mind it, and this was an early treatment for sufferers of chronic pain (Farrar, 2007). This observation points to the dissociation of pain into two separate components, intensity and unpleasantness. All of these factors modulate the pain experience, and knowledge of ways in which to modulate the pain experience leads to the potential to alter the pain experience to improve the conditions of its sufferers. In order to better understand the mechanisms by which such internal and external factors can alter the pain experience, one must better understand the mechanisms by which the pain experience arises.

Mechanisms Involved in Pain Perception and Modulation

Various theories of the pain experience have arisen over the years, but the most widely supported theory is the Gate Control Theory proposed by Melzack and Wall in 1965. The Gate Control Theory accounts for the lack of a one-to-one correspondence between the severity of the stimulus and the severity of the pain experience. According to this theory, the nociceptors in the periphery have inputs into the spinal cord. It proposes a much more complicated series of interactions at both the peripheral and central divisions of the pain pathway rather than having a single excitatory "pain neuron" that subsequently ascends to the brain, and whose activation in turn activates the brain structures involved in the sensation of pain intensity and unpleasantness,. In this way the Gate Control Theory accounts for the more elaborate interactions of cells that result in modulation at the level of the spinal cord (Melzack and Wall, 1965). The spinal cord receives input from both small fibers and large fibers. The small fibers consist of myelinated A delta fibers, which are sensitive to strong pressure and temperature, as well as unmyelinated C fibers, which are sensitive to pressure, temperature, and chemicals as shown by nociceptive fiber studies in monkeys. Additionally, A delta fibers were found to respond more robustly to noxious stimuli than the C fibers (Davis, Meyer, and Campbell, 1993). The small fibers serve as the nociceptors and are specific for providing information about noxious, tissue damaging stimuli. The spinal cord also receives input from large fibers, such as A beta fibers, which are myelinated and transmit sensory information much more quickly than small fibers. The A beta fibers are activated by low

intensity mechanical pressure, and are responsive to both noxious and innocuous stimuli (Light, Trevino, and Perl, 1979; Meyer, Ringkamp, Campbell, and Raja, 2005).

The cell bodies of the nociceptors are contained in the dorsal root ganglion just outside of the spinal cord. These nociceptive cells conduct action potentials when stimulated by noxious stimuli at the peripheral terminal and ultimately transmit this signal by the release of neurotransmitters at synapses in the dorsal horn of the spinal cord (Light, Trevino, and Perl, 1979; Meyer, Ringkamp, Campbell, and Raja, 2005). The cells within the dorsal horn of the spinal cord that receive input from the periphery have been termed "transmission cells" by Melzack and Wall. Because the transmission cells receive input from both small fibers that respond to noxious stimuli and also from large fibers that respond to innocuous fibers, Melzack and Wall proposed a relationship that accounts for the transmission cells' ability to decipher noxious from innocuous stimuli before transmitting a signal further along the ascending pathway up to the brain. It is the relative input from both the nociceptors and the large fibers as well as their relationship with interneurons in the spinal cord that ultimately results in the activation or inhibition of the transmission cells (Melzack and Wall, 1965).

Nociceptors enter the spinal cord in the superficial layers of the dorsal horn closest to the entry point, the lamina I and lamina II (Meyer, Ringkamp, Campbell, and Raja, 2005). It is within the lamina II, the region which is referred to as the substantia gelatinosa, where the nociceptors synapse with interneurons, and by inhibiting the interneurons the nociceptors ultimately have an excitatory effect on the transmission cells. Similarly, the large fibers synapse with interneurons. The activation of interneurons by large fibers is excitatory, but the excited interneurons inhibit transmission

cells, therefore the large fiber input ultimately results in an inhibitory effect upon the transmission cells. By considering both input from peripheral sensory neurons and the role of interneurons, the Gate Control Theory accounts for the potential of pain modulation at the level of the spinal cord (Melzack and Wall, 1965).

Upon the summation of the excitatory and inhibitory inputs from the large and small fibers as well as the interneurons that are excited or inhibited by the large and small fibers if there is substantial excitatory input to activate a given transmission cell, it conducts an action potential up to the brain by way of the spinothalamic tract and dorsal columns. The transmission cells ascend into the medulla, pons, cerebellum, and midbrain before reaching the thalamus (Wall, 2000), which is considered to be the sensory gateway to the brain (Melzack and Wall, 1982). Numerous lesion studies have implicated specific nuclei in the thalamus as necessary for the central processing of pain (Kim, Greenspan, Coghill, Ohara, and Lenz, 2007). From the thalamus, the signal resulting from a painful stimulus is relayed on to many structures, which as a whole are referred to as the "pain neuromatrix." In the presence of noxious stimuli, several structures in the brain become active, not all functions of all of the participating structures and their collective interactions are yet known. However, the functions of several of the main players can be inferred from the existing literature. For example, the pain message is relayed from the thalamus to the primary and secondary somatosensory cortex, which is responsible for the sensory aspect of pain perception (Carlson, 2007; Rainville, Duncan, Price, Carrier, and Bushnell, 1997; Hofbauer, Rainville, Duncan, and Bushnell, 2001). The anterior cingulate cortex is believed to play a role in the perception of pain as unpleasant, as opposed to the perception of the intensity of pain. The roles of the somatosensory cortex

and the anterior cingulate cortex in dissociating between the intensity component and the unpleasantness component of pain are experimentally supported by two brain imaging studies. The first study used PET technology to observe the activation of the somatosensory cortex and the anterior cingulate cortex while subjects were hypnotized to experience pain as less unpleasant while reporting the same intensity. A less unpleasant experience of pain correlated with decreased activity in the anterior cingulate cortex (Rainville, Duncan, Price, Carrier, and Bushnell, 1997). In contrast, a study involving hypnosis to reduce perceived intensity found that a less intense experience of pain correlated with lower activity in the somatosensory cortex rather than the anterior cingulate cortex (Hofbauer, Rainville, Duncan, and Bushnell, 2001).

The involvement of other brain structures in pain experience accounts for the many characteristics of pain sensation. For example, activation of the motor cortex is also likely to be involved in the role that pain has in modifying behavior to avoid noxious stimuli. This theory is supported by a study that found continuous avoidance of painful stimuli to alter the activation of the motor cortex during the avoidance reaction in dogs (Dolbaikian, 1976). Frontal lobe structures, such as the orbitofrontal cortex and the prefrontal cortex contribute to one's ability to think about their pain and put it in context of their other experiences. One study investigating the role of the prefrontal cortex in pain found that magnetic stimulation of the dorsolateral prefrontal cortex decreases pain perception (Graff, et al., 2005). This contribution also explains why early frontal lobotomy techniques did not prevent patients from experiencing pain, but rather resulted in the patients not caring about their pain (Farrar, 2007). Brain involvement is crucial for

the experience of pain, and is in itself able to further modulate pain perception through descending modulation.

Descending modulation occurs when the brain sends excitatory or inhibitory signals downward towards the spinal cord, where signals interact with the transmission cells to enhance or inhibit the ascending message. Descending modulation thus works to alter the continuing experience of pain. The process of descending modulation can be activated by multiple natural mechanisms. The autonomic fight or flight response modulates pain by lessening the pain experience when an individual is physically threatened (Sternberg, 2007). Stress induced analgesia is another natural phenomenon by which the descending pathway is activated and endogenous opioids decrease the pain experience, again when an individual is physically or psychologically threatened (Drolet, et al., 2001; Chipkin, Latranyi, and Iorio, 1982). Descending modulation can also occur in response to pharmacological methods or experimental stimulation methods. For example, electrical stimulation of particular brain areas such as the periaqueductal gray matter results in analgesia (Mayer, Wolfle, Akil, Carder, and Liebeskind, 1971). One can also pharmacologically induce analgesia by introducing drugs that stimulate the descending pathway to inhibit pain transmission. For example, administering opioid agonists in the central nervous system results in peripheral pain inhibition in rats (Czlonkowski, Millan, and Herz, 1987; Miaskowski and Levine, 1992; Sternberg, 2007).

The current study seeks to further understand the ways in which altering a brain structure known to be involved in the pain experience may alter the pain behavior of mice. Injections of the local anesthetic, lidocaine, into the dentate gyrus of the hippocampus produce analgesia. This observation verifies the role of the dentate gyrus in

the pain experience (McKenna and Melzack, 1992). Furthermore, the dentate gyrus is known to be highly subject to alterations in the rate of neurogenesis. It is thus reasonable to hypothesize that altering the structure of the dentate gyrus (through methods aimed to change levels of neurogenesis) may result in altered pain behavior in mice. Thus the primary goal of our experiment is to examine the relationship between neurogenesis and pain by observing pain behavior and relative amounts of neurogenesis in the dentate gyrus of the hippocampus in response to a pharmacological manipulation.

Neurogenesis

It was long believed that all mammalian species are born with all of the neurons that they will ever have, and that brain development cannot extend beyond adulthood (Gould, and Gross, 2002). This thought was such a pervasive idea that anecdotal quotes concerning this theory were commonplace outside of the realm of neurology and even the natural sciences. Believing that "you can't teach an old dog new tricks" is just one example of the belief held by many that brain development increases after birth until it reaches a particular limit and then the growth terminates altogether.

This idea persisted despite evidence that alluded otherwise. Doctors observed evidence of neural plasticity in the form of clinical patients who following severe brain trauma or surgery were able to regain some function over time. For example, hemispherectomy patients show evidence of compensation by the remaining half of the brain. The remaining hemisphere compensates by performing some of the duties that are general functions of the half of the brain that had been removed. Patients' ability to

recover some of the skills involved in language processing over an extended time period that they are not capable of performing immediately post-surgery is indicative of brain plasticity (Banich, 2004). As early as 1975, lesion studies in the motor cortex of monkeys resulted in diminished motor function that could be recovered to some extent both with and without training (Black, Markowitz, and Cianci, 1975). Despite the evidence of the brain's ability to restructure the way that it processes information, and thus to change the manner in which it functions in order to complete necessary daily tasks after trauma, the belief that the brain remains static after the initial development to maturity persisted.

Evidence of the brain's capacity to generate new cells through adulthood continued to mount as research of the brain and neural processes extended into the mid to late 90's. Experimenters found that the brains of birds undergo neurogenesis, or the birth of new neural cells, even in adulthood. The neurogenesis in the brains of birds occurs to compensate for the learning of others' birdsongs or landmarks for migration (Goldman and Nottebohm, 1983; Barnea and Nottebohm, 1994; Barnea and Nottebohm, 1996). And although older humans (in addition to other mammals) can "learn new tricks," the idea that the mammalian brain may undergo neurogenesis as well was not readily accepted. In the 1960's, a method by which one can label cells undergoing mitosis was used to show that cells in adult rat and cat brains do in fact divide. Altman exposed the test subjects' brains to a radioactive substance that is taken up by cells that are in the process of replicating their DNA during cell division, ^{3}H-thymidine. Using autoradiography techniques he was able to identify the dividing neurons and their daughter cells, thus implicating neurogenesis in mammalian species (Gould and Gross, 2002).

Even when faced with this evidence, the status quo belief remained a dominant force. It was not until new neural markers enabled the typing of new cells to identify them specifically as neurons rather than glial cells (which ^{3}H-thymidine autoradiography techniques alone did not allow). Novel techniques using 5-bromo-2-deoxyuridine (BrdU), a substance that binds to replicating DNA, provided a manner with which to label dividing neurons. Immunohistochemistry techniques enable the BrdU-labeled cells (i.e. newly replicated cells) to be counted individually. It was using BrdU paired with cell-type specific neural markers that Elizabeth Gould was able to show that neurogenesis occurs in regions of the brain of primates, thus providing the evidence that tipped the scale and changed the primary view of the field of neurology concerning neurogenesis (Gould, and Gross, 2002). Following Gould's work with primates, Eriksson provided evidence of human adult neurogenesis in humans using BrdU techniques. Because BrdU was given to cancer patients in order to monitor tumor cell growth, Eriksson received permission from patients diagnosed with cancer to examine their brains post-mortem. BrdU labeling within structures of the brains of his subjects indicates human adult neurogenesis (Eriksson, et al., 1998).

The evidence demonstrating neurogenesis in mammals has led to an explosion in the topic of adulthood cell growth within neuroscience, and subsequent studies have taken a multitude of directions. Today there is a great deal of knowledge about where in the brain neurogenesis happens, how it happens, various factors that can modulate neurogenesis, and functional changes of the brain upon neurogenesis and it's modulation. There is also a great deal more to be learned about the intricacies of the mechanisms controlling neurogenesis and the mediators that can alter the normal process of

neurogenesis. Furthermore, there are an infinite number of additional untested endogenous and exogenous factors that may affect neurogenesis and the resulting effects of neurogenesis are not all known. It is a field that will continue to grow as new ideas about the roles of neurogenesis are explored further.

Because of the ease of use in vivo and the relatively inexpensive price of BrdU, a great deal of the current literature on neurogenesis was developed using BrdU labeling for dividing cells in the brain. Although BrdU labels all dividing cells and their progeny, this by itself is not sufficient to identify a newly born cell in the brain as a neuron (as opposed to a glial cell) and it is not capable of measuring the rate of cell survival. However, it is very useful in identifying regions in which cells are actively undergoing mitosis and the extent to which they are doing so. If further knowledge about the type of newly born cells is necessary, the combination of BrdU with Neu-N labeling (which labels the neuronal nuclei) or other cell markers can accomplish this.

With the pervasive use of BrdU come issues of varying techniques that may lead to conflicting results between labs. For example, because it was believed that administration of high doses of BrdU may lead to non-specific labeling, the dose most commonly used is 50 mg/kg. However, Cameron and McKay found that upon administration of higher doses, a greater percentage of cells displayed the labeling and there was not any labeling of cells other than those that should be labeled. Thus the standard protocol has likely resulted in a gross underestimation of neurogenesis in most studies using BrdU, and it is possible that more subtle differences of BrdU between conditions in experimental studies were not observed (Gould and Gross, 2002). Due to the highly prevalent use of BrdU in varying labs, it is important to adopt a protocol that

uses doses of BrdU high enough to determine that a lack of effect between conditions is in fact due to a lack of difference in the number of dividing cells rather than using an ineffective dose that may only label a percentage of the newly born cells and may result in an inaccurate interpretation of the amount of neurogenesis.

For neurogenesis to occur, neural progenitor cells must be present. Neural progenitor cells are the neural stem cells and neuroblasts that are capable of proliferating and differentiating into neurons. Although neural stem cells have been identified throughout the brain, only a few structures of the mammalian brain display neurogenesis after brain trauma (Johansson, 2007). However, additional regions of the brain do display repair via plasticity (other cellular changes, such as dendritic lengthening and formation of new synapses) (Banich, 2004). Altman's work with rats and cats provided evidence of neurogenesis in the hippocampus, cerebral cortex, and olfactory bulb (Gould, and Gross, 2002). Current research in the field now focuses on two structures known to be highly generative of new neurons: the dentate gyrus (DG) of the hippocampus and the subventricular zone of the forebrain (SVZ).

Neurogenesis in the subventricular zone accounts for the cells in the olfactory bulb of rodents and monkeys via migration, and it may also be the location of the progenitor cells that give rise to the newly born neurons in the cortex (which occur at a much lower frequency than in the SVZ or DG, and are thus studied less) (Johansson, 2007). Within the dentate gyrus, progenitor cells proliferate in the subgranular zone and then migrate to the granule cell layer where they differentiate into neurons (Gould, Tanapta, Rydel, and Hastings, 2000). There has been a great deal of research concerning neurogenesis in the dentate gyrus of the hippocampus with memory and learning because

the hippocampus has been greatly implicated as having a significant role in memory function. According to a comprehensive literature review by Leuner, Gould, and Shors (2006), the results of multiple experimental studies indicate neurogenesis in the hippocampus as having a functional role with regard to learning and memory. It is highly relevant that there is little investigation into the impact that neurogenesis may have in altering aspects of the pain experience while the hippocampus, and the dentate gyrus in particular, has been implicated as having a role pain perception. Devor proposed a link between neurogenesis and chronic pain in the elderly in 1991, and an experimental study displayed chronic exposure to pain as decreasing neurogenesis (Ducric, and McCarson, 2006). However, there are no studies in the present literature that look at the effect of neurogenesis on pain, for which the present study aims to provide supporting evidence (although a causal relationship cannot be determined at present).

There are many factors that either regulate or modulate the amount of neurogenesis in the brain, which include both endogenous and exogenous methods. For example, sex hormones represent an endogenous factor capable of altering the rate of neurogenesis. It has been observed that female rats display higher rates of neurogenesis in the dentate gyrus than males, and that these effects were dependent on estrogen. The effects were diminished during periods of the estrous cycle with lower levels of estrogen and upon ovariectomy, and estrogen replacement restored the rate of cell proliferation (Tanapat, Hastings, Reeves, and Gould, 1999). Exposure to various environments or learning paradigms increases neurogenesis, and this exemplifies a manner in which exogenous forces can alter the rate of neurogenesis (Leuner, Gould, and Shors, 2006). The forces that modulate neurogenesis do not all work via the same mechanism, and

some of the better understood mechanisms will be discussed along with consideration of several of the modulating factors.

Because the neural stem cells that develop into neurons and glial cells exist throughout the brain (Jagasia, Song, Gage, and Lie, 2006), but neurogenesis is nearly entirely contained to the subventricular zone and the dentate gyrus, it is inferred that there are factors that continuously suppress neurogenesis in the other areas of the brain. It has been noted by Pearse that inhibiting cAMP hydrolysis, and thus increasing the amount of cAMP in present in the synapses, resulted in the increase of synaptogenesis and myelination of axons in the spinal cord. Because this happens naturally after damage to the axons in the spinal cord, it is reasonable to hypothesize that the body regulates the amount of synaptic cAMP to prevent continuous neural outgrowth and myelination when neural tissue damage is not present (Pearse, et al. 2004). Another research group observed that in the brain, a protein called Nogo-A, which is released for myelin repair in the central nervous system, also works to prevent axonal regeneration (Chen, et al., 2000; Donovan, et al., 2006).

Stress has been extensively studied with regard to the potential to modulate the rate of neurogenesis, and it has been observed that the two are negatively correlated. The fight or flight autonomic response occurs to suppress activities in the body that use energy so that as much energy as possible is readily available for the fighting or fleeing of imminent danger, and this includes cellular activities such as mitosis. Thus when presented with stressors, it is adaptive for the body to halt neurogenesis. Gould, Tanapat, McEwen, Flügge, and Fuchs (1998) found that upon the presence of an intruder monkey for one hour (a stressful experience for the resident monkey), the rate of neurogenesis in

the brain of the resident monkey decreased significantly due to the reduction in proliferation of precursor cells. The decrease in the rate of neurogenesis has been observed to occur in response to both transient and chronic stress, and suppression of neurogenesis in response to chronic stress likely has deleterious effects on the brain (McEwen, 1999). In fact, the consideration of the alteration of the structure of the hippocampus with chronic stress led McEwen to state that "because the hippocampus in implicated in the perception of pain, it is likely that alterations in hippocampal structure and function,… lead to changes in pain perception." (McEwen, 2001)

The effects of stress on neurogenesis are likely mediated by elevated stress hormones, and glucocorticoids in particular have been studied extensively. Glucocorticoids seem the most likely candidate for a number of reasons, which are considered in a comprehensive review of stress and neurogenesis by Mirescu and Gould (2006). One such reason is that there is a high prevalence of glucocorticoids receptors within the hippocampus, and it is necessary that the hippocampus be sensitive to the chemical substances responsible for mediating the effects of stress on neurogenesis. The idea that glucocorticoids play a major role is further evidenced by the observations that experimentally altering glucocorticoid levels in the brain results in structural and functional changes of the hippocampus (McEwen, 1999), and also that adrenalectomy increases neurogenesis while administration of corticosterone decreases the rate of neurogenesis in rats (Gould, Cameron, Daniels, Woolley, and McEwen, 1992; Cameron and Gould, 1994) . Another manner in which stress modulated neurogenesis is through the increase of endogenous glutamate levels. Increasing the amount of glutamate enhances neurotransmission across synapses and activation of NMDA receptors, which

can decrease neurogenesis (through a complex mechanism) (Mirescu and Gould, 2006). Observations of decreased neurogenesis in the adult tree shrew in stressful situations and the reversal of that effect with an NMDA receptor antagonist provides evidence for the role of NMDA receptor activation in decreasing neurogenesis (Gould, McEwen, Tanapat, Galea, and Fuchs, 1997).

Additional factors that correlate with neurogenesis have been identified, and old age negatively correlates with the rate of neurogenesis. While neurogenesis does occur throughout one's lifetime, the rate of neurogenesis begins to decrease upon reaching middle age, and decreases at a greater rate as the aging process continues (Emsley, Mitchell, Kempermann, and Macklis, 2005). Therefore, it is not surprising that neurodegenerative diseases such as Huntington's Disease and Alzheimer's Disease are associated with both a readily visible decrease in cell density, and also a decrease in neurogenesis (Johansson, 2007). Two major studies by one research group provided evidence that there is an increase of neurogenesis in an animal model of Alzheimer's Disease relative to control mice (Jin, Galvan, et al., 2004; Jin, Peel, et al., 2004). However, follow-up studies by Donovan clarified the results by finding that while the rate of cell proliferation does increase in the early stages of Alzheimer's in mice, the central nervous system protein Nogo-A prevents axonal growth and the newly born cells have a very low survival rate. The final result was that in the older model mice, there was a net decrease in neurogenesis by about 50% when a lack of survival or integration into the neural network was considered (Donovan, et al., 2006).

After considering factors that can decrease neurogenesis, especially given the evidence that a decrease in neurogenesis is associated with neurodegenerative diseases, it

is important to understand what factors have the potential to increase neurogenesis (as well as the functional impact of doing so). It has been observed that neurogenesis is controlled by growth factors. It is the presence of these growth factors, FGF-2, IGF-1, and VEGF, within the brain that enables the neural stem cells to differentiate into neurons. In the absence of these growth factors, the progenitor cells do not become mature neurons, and it is notable that the presence of these growth factors is reduced at middle age and is correlated with a decreased amount of neurogenesis (Emsley, Mitchell, Kempermann, and Macklis, 2005; Brinton and Wang, 2006b).

A great deal of research concerning the relationship between neurogenesis and memory/learning has also been conducted. Although there have been paradoxical findings, there is support for the idea that learning tasks involving activation of the hippocampus result in an increase in the number of new neurons. Furthermore, studies have shown that a decrease in new neurons after learning tasks has a deleterious effect on hippocampal-dependent learning, although there is not strong evidence that newly born cells are active in hippocampal-dependent learning. Glucocorticoids and stress are also negatively correlated with learning, and theses hormones may thus be mediating the effects via a decrease in neurogenesis (Leuner, Gould, and Shors, 2006).

The correlation between learning and neurogenesis led to the idea that mental stimulation may promote neurogenesis, which paved the way for enriched environment studies (Kempermann, Kuhn, and Gage, 1997). Enriched environments within the context of a lab often include small toys, various housing structures such as crawl tubes and igloos, and other novel stimuli that enable the animals to actively engage in their surroundings beyond what is possible in standard lab conditions. Experimental studies

have revealed that mammals display greater rates of neurogenesis when housed in conditions in which they receive an increased amount of mental stimulation in the form of environmental enrichment (Kempermann, Kuhn, and Gage, 1997). Studies that paired BrdU with other neural markers have pointed to an increase in the rate of cell survival (as opposed to an increase in proliferation of progenitor cells) as the driving force in the overall increase in neurogenesis (Banich, 2004). That is to say that while the rates of cell migration, development and differentiation remain stable, a positive net increase in the number of newly born neurons in the dentate gyrus is observed following enrichment because of enhanced survival rate. Enriched environments are now one of the most commonly used methods for inducing neurogenesis to examine the relationship between neurogenesis and a dependent variable, such as learning or memory function.

Kempermann, Kuhn, and Gage's environmental enrichment included not only one, but two factors that are now known to induce neurogenesis. The enriched environments in which their mice were housed also contained running wheels. Exercising on running wheels has been shown to increase neurogenesis in mice (van Praag, Kempermann, and Gage, 1999). A recent article considered two modulating factors with opposite effects on neurogenesis, one that promotes neurogenesis and one that decreases the rate of neurogenesis. This experiment enabled the reasearchers to observe the ultimate directionality of the neurogenesis (Stranahan, Khalil, and Gould, 2006). All rats were given access to running wheels, but some of the rats were housed with each other while other rats were housed in isolated conditions. Isolation is known to decrease neurogenesis by causing an increased hormonal response (higher levels of corticosterone) in response to stressors (Stranahan, Khalil, and Gould, 2006). The

finding that social isolation prevents the benefits of exercising on neurogenesis when rats are exposed to stressful stimuli is interesting because it provides evidence that alterations in the stress conditions of animals can prevent neurogenesis promoted by other modulating factors. Thus stress may ultimately confound any results of a dependent variable (such as pain behavior) in animals exposed to an enriched environment, preventing accurate conclusions about the relationship between neurogenesis and the dependent variable.

A preliminary study recently carried out in our laboratory aimed to study the relationship between exposure to an enriched environment, neurogenesis, and pain behavior. The data contains trends for variations in pain behavior according to housing condition, as well as an interesting decrease in relative pain sensitivity for females housed in an enriched environment. However, the results were likely mediated by altered stress responses and cannot be attributed to differences in neurogenesis. Interestingly, exposure to enriched environments has also been observed to alter opioid sensitivity in rats, although neurogenesis was not considered in these studies (Smith, Bryant, and McClean, 2003; Smith, et al, 2005). Furthermore, a recent study by Shum et al (2007) found that mice housed in an enriched environment displayed elevated pain behavior in the late phase of the formalin hindpaw test as well as inflammation pain incurred over a period of several days. These results correlated with measures of synaptic plasticity in the brain (they did not examine the dentate gyrus), but again cannot be attributed to the structural changes in regions involved in response to injury as their may be other mediating factors (Shum, et al., 2007). While these studies have linked neurogenesis (or mediating factors known to alter neurogenesis) with altered pain responses, they also highlight the need to

isolate variables in order to better understand the relationship between neurogenesis and pain. The current study aims to do precisely that.

Pharmacological Manipulations of Neurogenesis

It has been also been observed that drugs used to treat Alzheimer's Disease symptoms result in increased levels of neurogenesis (Brinton and Wang, 2006a; Brinton and Wang, 2006b,; Jin, Xie, Mao, and Greenberg, 2006). Because of the cell loss associated with Alzheimer's Disease and the resulting cognitive deficits such as memory impairment, it is reasonable to hypothesize that some of the improvements seen with Alzheimer's drugs may be due to a potentially compensatory role of increased cell growth. This consideration about the potential impact of certain drugs on neurogenesis has led multiple lab groups to investigate the effects of different Alzheimer's drugs on neurogenesis.

Two different lab groups have demonstrated the potential of Alzheimer's Disease drugs to increase the rate of neurogenesis. Because of the discovery of neurogenesis in the adult brain and the observation that cognitive impairments observed in neurodegenerative disease increase as cell loss becomes greater, it is a reasonable belief that promoting cell growth in the brains of patients may have a restorative effect by replacing lost cells. This idea led to the investigation of the potential of Alzheimer's drugs to not only halt the progression of the disease in terms of neural degeneration, but also to promote neurogenesis and subsequently enable recovery of cognitive function to some extent (Brinton and Wang, 2006a; Brinton and Wang, 2006b). Brinton and Wang's

lab examined the effects of allopregnanolone on the growth of new cells. Allopregnanolone is a steroid that is derived from progesterone. It has been observed that allopregnanolone is produced in the central nervous system during development of progenitor cells. As a result, it has been hypothesized that this molecule holds the potential to supplement the development of progenitor cells into neurons, thus boosting the rate of neurogenesis in the adult brain. It was found that allopregnanolone does have the capability to promote the proliferation of progenitor cells, thus increasing neurogenesis (Brinton and Wang, 2006a). Because this drug is a progesterone derivative, it's role as a sex hormone may have other confounding effects on the brain, as these hormones are known to have a modulating role in the central nervous system resulting in sex differences (such as in the perception of pain) (Aloisi and Bonifazi, 2006). It is relevant that other studies have been conducted that implicate other sex hormones, such as estrogen, in promoting neurogenesis as well (Tanapat, Hastings, Reeves, and Gould, 1999). Although the ability of allopregnanolone to promote neurogenesis is of interest in the context of considering pharmacological agents that can alter levels of cell growth in the brain, its potential for causing sex differences in pain perception, neurogenesis, or some other unconsidered and potentially confounding variable renders it unsuitable for the present study.

For this reason, a focus on the Alzheimer's drugs and neurogenesis research conducted by Jin, Xie, Mao, and Greenberg is particularly relevant. Jin et al. conducted an experiment in which adult male CD-1 mice were administered either one of three Alzheimer's drugs or PBS (a solution primarily consisting of saline) to serve as a control. All of the mice received a daily injection of the drug to which they were assigned for a

two week period. For the last three days of the two week period, mice were also administered BrdU. At the end of the entire two week injection period, the mice were sacrificed and their brains were examined using immunohistochemistry techniques to label BrdU. It was found that each of the different drug conditions increased the amount of BrdU labeling (and thus neurogenesis) by 26-45%. This experiment is important because it displayed the ability of commonly used Alzheimer's drugs to induce and promote neurogenesis, despite the fact that each of the three drugs works via a different mechanism (Jin, Xie, Mao, and Greenberg, 2006).

The three drugs that were considered in this experiment were tacrine, galantamine, and memantine. Tacrine and galantamine represent the major class of Alzheimer's drugs, which are acetylcholinesterase inhibitors. The cholinergic hypothesis of Alzheimer's Disease points to a decrease in regular ACh activity due to a loss of Ach containing cells of the basal forebrain as a major source of the deficits present with the disease (Terry and Buccafusco, 2003). As such ACh agonists have been used to treat Alzheimer's (Arneric, et al., 1995), and the observation of their ability to promote neurogenesis suggests that their role in treatment may be two-fold. Memantine on the other hand represents the other major class of drugs used for the treatment of Alzheimer's Disease, which consists of NMDA antagonists.

Of primary importance is how and to what extent these drugs were able to promote neurogenesis in vivo in CD-1 mice. Tacrine administered at a dose of 5mg/kg promoted neurogenesis in the subventricular zone of the forebrain by 41%, although it had no difference on neurogenesis in the dentate gyrus of the mice. Galantamine (5mg/kg) promoted neurogenesis in the SVZ by 45% and also in the DG by 36%, while

memantine (7.5mg/kg) increased neurogenesis in the SVZ by 27% and in the DG by 26% as compared to PBS injection controls. The in vivo experiment was also supplemented with an in vitro study, in which cortical cultures were exposed to the drugs and incorporation of BrdU into the cells was measured. In this study, the basal levels of BrdU incorporation increased by about 40% for each of the drug conditions, providing more evidence for the theory that these drugs are able to promote neurogenesis (Jin, Xie, Mao, and Greenberg, 2006).

In addition to the potential for treatment of Alzheimer's Disease, these drugs hold the potential for use in research on neurogenesis. Currently, most research concerning neurogenesis in relation with other behavioral variables, such as learning and memory, utilizes exposure to an enriched environment in order to promote neurogenesis so as to give rise to two different levels of neurogenesis for comparison across a different variable. Housing animals in an enriched environment may require a lengthy amount of time of environmental exposure to yield the desired effects on neurogenesis. Additionally, exposure to an enriched environment likely alters other aspects of psychological experiences for the animals (such as altering glucocorticoid levels in response to stress) that may in turn have a mediating or confounding effect on the variables being studied. Because of these characteristics of enriched environment exposure, a more direct way of inducing neurogenesis is desirable. The use of Alzheimer's drugs in a laboratory setting may enable us to draw a more direct relationship between neurogenesis and independent variables by eliminating the confounds of stress and the other changes that an animal's brain undergoes upon

exposure to an enriched environment. It is with this information that we aim to study the effects of drug induced neurogenesis on pain.

In order to use drugs in this manner within a laboratory setting, it is important to gain a better understanding of the effects of these drugs and whether the effects are likely to confuse the relationship between the independent and dependent variables being studied. Memantine, as an NMDA receptor antagonist, prevents the second phase of the pain experience (the dull, throbbing pain due to the inflammatory response of the body in the area of damaged tissue) by mediating activity in the first pain phase (the immediate painful response to noxious stimuli due to the excitation of nociceptors) and as such is not a drug that should be used to promote neurogenesis for the examination of neurogenesis and pain (Vaccarino, et al., 1993). For this reason, the mechanisms of NMDA receptor antagonist elevated neurogenesis will not be considered here.

In addition to the neural growth factors previously discussed as being necessary for the proliferation and differentiation of neural stem cells, it has been proposed that neurotransmitters are also responsible for the regulation of neurogenesis. In a study by Zhou, Wen, Shi, and Xie (2004), researchers were able to demonstrate that ACh enhanced proliferation and induced single cell differentiation in vitro. Additionally, the use of calcium imaging enabled the researchers to observe the depolarization of neural stem cells in the presence of ACh. They also analyzed the neural stem cells as well as their progeny for neurotransmitter receptors. It was observed that the receptors consisted primarily of ACh receptors (97.2%), and the remaining receptors were glutamate, GABA, and 5-HT. While the other receptors may very well contribute to the regulation of activity of the neural stem cells, the high incidence of ACh receptors (AChRs) and the

observation of depolarization of neural stem cells in the presence of ACh points to ACh as the neurotransmitter likely to be most influential. In addition to the observations that ACh increases the proliferation and differentiation as well as causing depolarization of the neural stem cells, Zhou et al. also found that presenting the cells with atropine, an ACh antagonist, prevented the effects of ACh on the cells.

In an additional study, the role of forebrain acetylcholine was considered as a potential mediator for the finding that hippocampal learning promotes neurogenesis. Selectively lesioning a source of ACh in the forebrain results in decreased neurogenesis in the dentate gyrus of the brains of rats, and also leads to impairment in spatial memory. Additionally, administration of physostigmine, an ACh agonist, yielded increased neurogenesis in the dentate gyrus. This study was also able to contribute to the current understanding of the role of ACh on neurogenesis by displaying that increasing ACh levels promotes proliferation and short-term survival of the newly born neurons, as opposed to increasing neurogenesis by promoting long-term survival or differentiation (Mohapel, Leanza, Kokaia, and Lindvall, 2005).

Because it is known that ACh agonists can promote neurogenesis by activation of neural stem cells and their progeny via the relatively numerous ACh receptors present on these cells, additional known ACh effects should be considered. It is important to know whether or not ACh has other effects on the brain that may mediate results in using these drugs to observe the relationship between neurogenesis and variables such as pain behavior. That is to say that if we are going to administer ACh agonists to promote neurogenesis in the brains of mice so that we can compare varying levels of neurogenesis with pain behavior, we need to know whether or not ACh agonists themselves alter pain

behavior. A review of the literature yields the conclusion that they are in fact capable of altering pain perception.

In 2004 a study found that ACh agonists had an antinociceptive (analgesic) effect, which was prevented with ACh antagonists. In this study by Dussor, Helesic, Hargreaves, and Flores, several ACh agonists and antagonists, which work via both muscarinic and nicotinic ACh receptor types, were tested using an orofacial formalin test (with formalin injected into a region of the face rather than the hindpaw technique previously discussed). Pain behavior was decreased in the presence of ACh agonists, and was restored with administration of ACh antagonists. Their results were also supported by in vitro stimulation of sensory neurons with ACh agonists and antagonists, with the finding that ACh agonists decreased the release of a neurotransmitter (CGRP) and that this effect was blocked by ACh antagonists. They also noted that the antinociceptive role of Ach was consistent upon the activation of either muscarinic or nicotinic Ach receptors, which will be of importance to our study and will be discussed later. Building on this study, in 2007 Yang, Xiao, and Xu extended the consideration of ACh on nociceptive neurons to the central nervous system. Because of the findings by another research group that injection of a 5-HT receptor agonist (which blocks one of the neurotransmitter receptors previously mentioned as occurring in low frequency on neural stem cells and their progeny) into the hippocampus of adult rats has a nociceptive effect (Soleimannejad, Semnanian, Fathollahi, and Naghdi, 2006), Yang Xiao, and Xu explored the role of ACh within the hippocampus (as opposed to the previous studies examining the role of ACh in the periphery). This experiment yielded results that implicate both

endogenous and exogenous ACh as having an antinociceptive effect when administered to the hippocampus of adult rats by activating muscarinic ACh receptors (mAChRs).

In the paper by Yang Xiao, and Xu (2007), the authors state that the effects of ACh within the hippocampus are immediate and last only for several minutes. It is further hypothesized that the timeline of this effect is due to the location of the hippocampus within close proximity of the lateral ventricle, thereby accelerating the metabolism of ACh. For this reason, it is likely that the effects of ACh on nociception are not relevant if the ACh agonists are not administered near the time of the pain testing. However, because the effects of chronic administration of ACh agonists (such as that of daily administration of galantamine used by Jin et al. in their study of AD drugs on neurogenesis) on the pain processing pathway are currently unknown, it is important to consider treatment with another drug to provide a further control for the role of ACh agonists. Fortunately nicotine provides just such a control. While it has been reported that nicotine has protective cellular effects in small doses, it has also been noted that in high doses, administration of nicotine, an ACh agonist that binds to nicotinic AChRs, results in a decrease in neurogenesis (Abrous, et al., 2002; Shingo and Kito, 2005). Rats allowed to self-administer varying doses of nicotine displayed decreased neurogenesis in the dentate gyrus, but not the subventricular zone, in the two groups with the highest doses (.4mg/kg and .8mg/kg) (Abrous, et al., 2002). A subsequent study by Shingo and Kito in 2005 found that intraperitoneal injection of nicotine over the course of two weeks also yielded significant decreases in the amount of neurogenesis in a dose-dependent manner. The greatest dose used yielded the greatest decrease in neurogenesis, which was 1mg/kg in rats (Shingo and Kito, 2005).

Within the context of pain and Alzheimer's, the role of ACh agonists as both having analgesic properties and being capable of promoting neurogenesis is important. The literature on pain and Alzheimer's is relatively robust and studies consistently report that clinical pain reports are lower in patients with Alzheimer's Disease, even when an inability to report pain in advanced cases is controlled for (Scherder, et al., 2001). Experimental pain studies have been conducted with the consistent finding that Alzheimer's Disease is associated with an increased pain tolerance, although normal stimulus detection and pain threshold are maintained as strongly evidenced by a study using electrical stimulation and ischemia (Bendetti, et al., 1999). Further research has implicated that the cognitive and affective components deteriorate with Alzheimer's Disease, but that the sensory-discriminative component remains intact. This conclusion arises from the observation that autonomic responses (blood pressure and heart rate) were blunted for stimuli just above threshold (perception of pain) but normal with painful stimuli twice that of threshold while stimulus perception was normal for stimuli just above threshold and blunted for stimuli twice that of threshold (Rainero, Vighetti, Bergamasco, Pinessi, and Benedetti, 2000). These findings vary according to the severity of the disease, that is to say that the worse the symptoms of Alzheimer's Disease are (often measured using MMSE scores), the higher the pain tolerance level becomes (Bendetti, et al., 1999; Rainero, Vighetti, Bergamasco, Pinessi, and Benedetti, 2000). These findings hold true over time, with consideration of impaired ability to communicate pain (Scherder, et al., 2001), and do not seem to have altered the management of pain (Pickering, Eschalier, and Dubray, 2000); the finding that there is relatively less pain treatment prescribed for patients with Alzheimer's Disease as

compared to elderly that do not have the disease is due to the lower incidence of pain reporting which is most likely reflective of the higher pain threshold and altered pain perception.

Given this information, it is interesting to consider that although patients with Alzheimer's Disease have a decreased pain experience overall, and they are often treated with ACh agonists, which have a further antinociceptive role, no consideration has been paid to the potential impacts of treatment with ACh agonists on pain over time. As previously mentioned, the effect of chronic administration on the pain pathway is currently unknown. However, if a decline in neurogenesis results in a desensitization to pain by increasing pain tolerance as suggested by observation of pain behavior in Alzheimer's patients, then it is reasonable to suggest that neurogenesis in the dentate gyrus may increase one's sensitivity to pain. If so, it is possible that by treating the cognitive symptoms of Alzheimer's patients, over time we may be increasing patients' susceptibility to pain despite the immediate role of the drugs in antinociception. This consideration of pain and Alzheimer's Disease only serves to highlight the importance of understanding the relationship between neurogenesis and pain.

The Current Experiment

The logical next step in neurogenesis research is to study neurogenesis with regard to the potential to alter pain behavior. The observation of the role of the dentate gyrus in pain perception paired with the observation of the ability of neurogenesis to result in structural changes within the dentate gyrus makes the idea that there may be a

relationship between neurogenesis and pain a reasonable one. Furthermore, the ability to promote and decrease neurogenesis in the dentate gyrus using galantamine and nicotine respectively, which are both ACh agonists, enables us to compare varying amounts of neurogenesis across conditions with pain behavior, without introducing animals to an enriched environment which likely mediates pain behavior by altering stress responses. Thus our experiment will involve the administration of nicotine, galantamine, or a saline control along with BrdU to animals in order to induce varying levels of observable neurogenesis followed by presentation of a noxious stimulus (a hindpaw formalin injection) and pain behavior observation.

We have two hypotheses concerning the potential outcomes of our experiment. It was first hypothesized that the amount of neurogenesis will vary across conditions. We expected that the galantamine condition would yield relatively more neurogenesis than the saline control condition, which would in turn yield relatively more neurogenesis than the nicotine condition. The amount of neurogenesis was measured by counting the number of cells labeled with BrdU in the subgranular zone of the dentate gyrus. Our other hypothesis concerns the pain behavior of the mice during the formalin test. If there is no role of neurogenesis in mediating pain behavior (i.e. the null hypothesis), then it would be expected that both the galantamine and the nicotine groups displayed the same amount of pain behavior (which was predicted for both groups to potentially be lower than that of the control group due to the analgesic effects of ACh agonists, if these drugs have a long-term effect when administered chronically). If however there is a relationship between neurogenesis and pain behavior, then it was hypothesized that the amount of pain behavior displayed for the two drug conditions would vary from one

another. This is due to the fact that while the role of both drugs as ACh agonists potentially lessen pain behavior for both groups from that of the control group, a decrease in neurogenesis may further lessen pain behavior (as is observed in Alzheimer's Disease) while an increase in neurogenesis may increase displayed pain behavior for the galantamine group relative to the nicotine group. If this is the case, then the galantamine group would have exhibited more or less pain behavior than the control group depending on the strength of the two opposing factors (analgesia from the ACh agonist vs. increased pain sensitivity due to neurogenesis if there is a bidirectional effect). In either case, the finding of a significant difference between the two drug conditions with significantly less pain behavior for the nicotine group than either of the other two groups would provide evidence in support of a relationship between neurogenesis and the pain experience.

Methods

The Haverford College Animal Care and Use Committee approved each of the procedures of this study.

Subjects

The subjects consisted of 30 male and 30 female adult CD-1® mice. Equal numbers of mice were randomly assigned to one of the three conditions (saline, galantamine, and nicotine) on the day of arrival. Each condition contained equal

numbers of males and females, with N = 10 for each drug and sex group. All animals were housed in identical cages in a constant temperature facility (20°C) on a 12:12h light-dark cycle (lights on at 20:00). Food pellets (Harlan Teklad 8604) and tap water were freely available in each of the cages.

Injection Protocol

On the first day of injections, each animal received an injection of both BrdU and the first injection for their drug condition. When assigning mice to the various day 1 injection days, careful attention was paid so that on each day at least one male and female from each condition received injections, preventing any unforeseen confounding variables by ensuring animals in each condition were treated equally.

Mice were weighed and each mouse received a 300 mg/kg intraperitoneal (abdominal) injection of BrdU (Sigma product, 5-bromo-2'-deoxyuridine, minimum 99% HPLC) dissolved in saline. The BrdU injection consists of 10mg of BrdU with 7μL 1M NaOH per mL of saline (recommended by Elizabeth Gould, personal communication).

After injection of BrdU, the mice then received their first injection of their drug condition. Drugs were administered in 10mg/mL injection volume and the doses selected have been shown to maximally affect the amount of neurogenesis while avoiding toxicity characteristic of high doses. We used a dose of 5mg/kg galantamine and a dose of 1mg/kg nicotine. An isotonic saline solution (.9%) to serve as a control was administered in a 10mg/mL injection volume as well. Each of these injections was also administered

into the abdominal cavity, and all intraperitoneal injections (BrdU and drugs) were injected using a 1mL syringe and a 25 gauge needle.

Following the day 1 injections for a given group of mice, each mouse was subsequently administered a 10mL/kg intraperitoneal injection according their assigned drug condition daily for two weeks. On the day following the final drug injection, the mice underwent pain testing. The pain testing did not occur on the final day of drug injection so that the pain behavior of the mouse would not be altered due to the immediate (analgesic) effects of the drugs. By waiting until the following day, we hypothesize that the pain behavior will reflect any changes in neurogenesis rather than the other effects of the drugs administered.

Formalin Test

On the day of pain testing, mice were placed in a plastic rectangular box on an elevated glass surface. In the event that multiple mice underwent pain testing simultaneously, an opaque divider was placed between enclosures to prevent mice from seeing one another. The mice were left to habituate to this environment for 20 minutes. After the habituation period, the mouse was placed into a cardboard and fabric pouch, which enables the experimenter to restrain the mouse and maintain access to the hindpaws. A 1mL syringe with a 25 gauge needle was used to inject .02mL of a 5% formalin solution into one of the hindpaws (randomly assigned). After this injection the mouse was immediately returned to the cylinder for observation of pain behavior. The entire observation period was videotaped from below the observation apparatus to be

coded at a later time for pain behavior. This method allowed for no humans to be present in the room with the mice, preventing an alteration in pain behavior due to the presence of an experimenter- as recent research has suggested (MacIntyre, et al., submitted).

Pain behavior is defined as any licking or flicking of the affected (injected) paw. Additionally, lifting and twitching of the affected paw were also recorded as pain behavior. These behaviors are recorded as either a yes (observation of one of the pain behaviors) or no (absence of pain behavior). The observation periods consisted of a 5 second time period every 20 seconds. This pattern of observation (5 seconds observe, 15 seconds rest) continues from time = zero until time = ten minutes, with time = zero at the time of return to the cylinder immediately after injection. During this ten minute time period, pain behavior is believed to reflect the first phase of pain. After the first 10 minute observation period, there is a 10 minute no-observation period, and the 20 second observation pattern resumes from time = 20 minutes to time = 40 minutes. The second phase of pain is observed via pain behavior in the 20-40 minute time period.

10 mice for each condition (5 males and 5 females) were randomly selected ahead of time to be examined for neurogenesis and were perfused after the observation period. All other mice were sacrificed immediately in a CO_2 chamber.

Perfusion

Mice were deeply anesthetized with an intraperitoneal injection of ~ .6-.8mL pentobarbital (100mg/kg) depending on the size of the mouse. The pentobarbital is known to be effective when the mouse is totally unresponsive to a strong, forceful pinch

of the tail. The tail pinch test was performed before any incisions were made. Once the mouse was sufficiently anesthetized, an incision was made using surgical scissors across the abdomen. From this incision, another incision was then made up the length of the body along the midline and terminating at the uppermost ribs. The scissors were used to separate the diaphragm tissue from the ribs and a cut through the middle of the ribs was made allowing access to the lungs and heart.

The mice were then transcardiacally perfused by injecting a large volume of PBS (about 30-40mL) into the heart which was cut for drainage, thus forcing the PBS throughout the body and flushing the circulatory system. The PBS was followed by approximately 60mL of paraformaldehyde to fix the neural tissue. After the perfusion had been performed and the tissues were rigid, the brains were extracted and refrigerated in fixative for later immunohistochemistry use.

Immunohistochemistry

The region of the brain containing the hippocampus was be sliced into 40μm slices using a Vibratome apparatus, and slices were alternated into 12 wells. The slices were stored in PBS until the slices in one well were plated onto slides. Once the slices were mounted they were treated with a mouse anti-BrdU primary antibody (Becton Dickinson, Pure with gelatin and .1% sodium azide) and a horse anti-mouse secondary antibody (Vector Labs, BA-2000) according to a BrdU peroxidase immunolabeling protocol (Gould, personal communication). This labeling protocol and subsequent staining with cresyl violet (a protocol also obtained from Elizabeth Gould's lab) labels

BrdU positive cells and stains the subgranular zone. This process enables visualization of the cells containing BrdU within the subgranular zone of the dentate gyrus, which can then be identified and quantitatively counted to be compared across conditions as a measure of neurogenesis.

Microscopy

Slides containing one set of slices (of the 12 possible sets per animal) were observed under a 100x power oil-immersion lens. Because the immunohistochemistry techniques yielded a high degree of background staining and the intensity of cresyl violet staining varies between slides, our identification of BrdU positive cells relied on relative staining. An absolute count of BrdU positive cells in the subgranular zone was obtained for each slice on a given slide. While coding the slides, experimenters were blind to the drug condition of the mice from which the slices were obtained. After cell counts were obtained, each slice was then photographed using a 4x power objective lens and SPOT Basic software. The area (in μm^2) of the subgranular zone for each slice was determined using ImageJ software. Multiplying the area value by 40μm, the thickness of each slice, provides the volume of the subgranular zone, subsequently enabling the cell count to be reported as a cells/volume(μm^3) value for comparison of density across conditions.

Data Analysis

Data analysis was performed using SPSS software. A 2 x 2 x 3 factorial ANOVA was performed for pain behavior with experimenter, sex, and drug condition as the independent variables and the summed periods observed pain behavior as the dependent variable. There were two experimenters coding the behavior videos, two sexes of mice, and three drug conditions (galantamine, nicotine, or saline control) represented in the data. This repeated measures ANOVA was performed to determine the presence of any main effects or interactions of sex and condition for early phase pain behavior and late phase pain behavior. Including experimenter in this test enables us to ensure that having two experimenters coding videos did not introduce a confounding variable. It should be noted that the late phase pain behavior for one of the saline male animals was omitted from statistical analysis. The number of observation periods (during the late phase only) in which the animal exhibited pain was greater than 2 standard deviations from the mean value of the saline males, warranting his identification as an outlier and removal from analysis. Analysis of neurogenesis was performed with a 2 x 2 x 3 factorial ANOVA with sex, condition, and experimenter as the independent variables and cells/ μm^3 as the dependent variable. Additionally, correlations were performed to determine the presence of a relationship between pain behavior and density of BrdU positive cells. Together these analyses will help determine if there is a relationship between neurogenesis and pain.

Results

Behavioral Data

A repeated measures ANOVA for sex, condition, experimenter, and early vs. late phase yielded significant results. The most relevant finding to our hypotheses was the observation of a significant interaction of phase and condition, $F(2, 36) = 5.615$, $p<.05$, with pain behavior exhibited increasing by phase more so for the two drug conditions than for the saline control. Scores for pain observation in the first phase are not significantly different across condition, with means for saline, nicotine, and galantamine-treated animals of 21.475 (*SE* = 1.254), 19.042 (*SE* =1.332), and 20.221 (*SE* = 1.223), respectively, $F(2,37) = 0.887$, *ns*. Similarly, the scores were not significantly different across condition in the late phase, $F(2,36) = 2.935$, *ns*(although there was a strong trend in the data, with $p=.066$). The significant interaction results from differences between the early phase pain values and the late phase pain values between drug conditions. The mean scores for saline (M = 29.150, *SE* = 2.872), nicotine (M = 38.583, *SE* = 2.832), and galantamine-treated animals (M = 38.092, *SE* = 2.600) in the late phase do however result in a significant interaction, because the scores for nicotine and galantamine increase more so from the early to late phase than for saline. Indeed, in the late phase, post hoc tests reveal that the mean difference between saline and galantamine-treated animals (M = -8.942, *SE* = 3.874) is significant, with $p < .05$ and the mean difference between saline and nicotine-treated animals (M = -7.433, *SE* = 4.033) is nearly significant, with $p = .074$.

The mean difference between nicotine and galantamine-treated animals (M = -1.508, *SE* = 3.844) is not near significant, with $p = .697$. These results are illustrated in *Figure 1*.

There was also a main effect of sex on pain behavior, with the mean number of time bins containing pain behavior greater for males than females. This main effect is illustrated in *Figure 2*. The repeated measures ANOVA yielded significantly different pain behavior for males and females, $F(1,36) = 4.754$, $p<.05$. The mean number of 5 second observation periods in which males exhibited pain behavior ($M = 29.708$, $SE = 1.418$) was significantly greater than the mean for female pain behavior periods ($M = 25.187$, $SE = 1.513$).

Additionally, there was a significant difference between the total number of pain observation periods for the early and late phases across gender and condition, $F(1, 36) = 103.610$, $p<.001$. This finding is spurious, with significantly more observation periods containing pain behavior in the 20 minute late phase period than the 10 minute early phase period ($M = 34.608$, $SE = 1.599$ and $M = 20.287$, $SE = 0.762$, respectively). Refer to *Figure 3* for a display of the main effect of phase.

There were two additional significant interactions present in the data and revealed by the repeated measures ANOVA test. There was a significant phase by sex interaction, $F(1, 36) = 4.368$, $p = < .01$, with females displaying a greater increase of mean pain behavior values in the late phase than the males. Females' values increased from the early phase ($M = 21.078$, $SE = 1.043$) to the late phase ($M = 38.339$, $SE = 2.188$), while males' values, which increase from the early phase ($M = 19.497$, $SE = 1.112$) to the late phase ($M = 30.878$, $SE = 2.334$), increased significantly less. This interaction is displayed in *Figure 4*.

There was also a significant three-way interaction of phase, sex, and condition, $F(2, 36) = 4.858$, $p = <.05$. This interaction arises from a significant increase of pain behavior scores from early to late phase for all three injection conditions of the females, but only for the nicotine and galantamine-treated males, while the mean behavior score for saline-treated males did not change significantly by phase. A Bonferroni post hoc test revealed a significant increase ($p<.05$) in pain behavior from the early to late phase in every sex by condition group except for the saline-treated males, whose pain behavior values did not differ significantly from the early to the late phase of pain. Refer to *Figure 5* for a display of the means with regard to the interaction of phase by sex by condition.

Because videos were coded by two experimenters, the factor of experimenter was considered in the statistical tests to ensure that the presence of multiple experimenters didn't confound our results. While there was a significant difference in scores of behavior between experimenters, with one experimenter noting significantly more observation periods containing pain behavior ($M = 32.075$, $SE = 1.477$) than the other experimenter ($M = 22.821$, $SE = 1.455$), $F(1,36) = 19.920$, $p<.001$, there were not any significant interactions between experimenter with sex and/or condition: experimenter with condition, $F(2,36) = 0.282$, *ns*, experimenter with sex, $F(1,36) = 1.711$, *ns*, and experimenter with condition and sex, $F(2,36) = 1.878$, *ns*.

Neurogenesis Data

A 2 x 2 x 3 one-way factorial ANOVA test was performed with the data obtained from cell counts of BrdU positive cells in the subgranular zone of the dentate gyrus for

each of the 30 animals perfused. Experimenter (the same two experimenters who examined behavioral data), sex, and condition were the between-group factors considered in this data analysis. The most relevant finding in our data is a significant main effect of condition on the number of observed BrdU cells per μm^3, $F(2, 18) = 4.252$, $p < .05$. The mean number of BrdU positive cells per μm^3 for saline, nicotine, and galantamine-treated animals are 8.399×10^{-7} (*SE* = 1.938), 1.1014×10^{-6} (*SE* = 1.938), and 1.6418×10^{-6}, respectively. While the mean difference of -8.020×10^{-7} between the values for saline and galantamine-treated mice is significant, $p < .05$, and the mean difference of -5.404×10^{-7} between nicotine and galantamine-treated animals is nearly significant, $p = .070$, the mean difference between saline and nicotine-treated animals, -2.615×10^{-7}, does not approach significance, $p = .363$. The main effect of condition on the number of BrdU positive cells per μm^3 can be seen in *Figure 6*.

There was no significant main effect of sex on our measurement for neurogenesis, $F(1, 18) = 0.002$, *ns*, nor was there a significant interaction between condition and sex, $F(2, 18) = 0.259$, *ns*. Similarly to the behavioral data, there is a significant main effect of the experimenter, $F(1, 18) = 5.507$, $p < .05$, with one experimenter noting significantly more positively labeled cells per μm^3 (M = 14.631, *SE* = 1.619) than the other experimenter (M = 9.256, *SE* = 1.619). However, there were no significant interactions involving the experimenter effects, including experimenter by condition, $F(2, 18) = 0.218$, *ns*, experimenter by sex, $F(1, 18) = 0.318$, *ns*, and experimenter by condition by sex, $F(2, 18) = 2.329$, *ns*.

A 2-tailed Bivariate correlational analysis was performed to compare the early phase and late phase pain behavior data with physiological data for the animals for which

both were available (i.e. the animals that were perfused after undergoing the formalin pain test). This analysis provides information about whether or not the range of pain behaviors observed correlates with the range of neurogenesis observed regardless of the condition of the animals. The number of observation periods containing pain during the early phase correlated significantly with the number of observation periods containing pain in the late phase, $r(27) = .579$, *ns*. However, there was no significant correlation between the numbers of BrdU positive cells per μm^3 for either the early phase values, $r(28) = -.113$, *ns*, or the late phase values, $r(27) = .100$, *ns*. These values are illustrated in *Table 1*.

Discussion

Neurogenesis Results

It was hypothesized that we would alter the rate of neurogenesis across the drug conditions relative to the saline condition. Accordingly, we observed an increase in the rate of neurogenesis for both drug conditions relative to the saline control animals. The galantamine and nicotine-treated animals had on average a higher ratio of cells containing BrdU per volume than the saline-treated animals, indicating a higher amount of neurogenesis in the dentate gyrus from the time of BrdU injection to perfusion. This finding supports the hypothesis that the drugs administered would alter the amount of neurogenesis. However, it was hypothesized that the nicotine condition animals would exhibit a decrease in the amount of neurogenesis relative to the saline control group.

Instead we observed an increase in the amount of neurogenesis in nicotine-treated animals, yielding a mean that is an intermediate between the mean neurogenesis values for the saline and galantamine conditions. This finding is not particularly surprising as nicotine is known to have neuro-protective effects at low doses (Abrous, et al., 2002), and has even been used to boost memory performance in mice (Platel and Porsolt, 1982). Because nicotine has been shown previously to decrease neurogenesis (Shingo and Kito, 2005), it is possible that we did not administer a high enough dose to effectively overcome the neuro-protective effects and instead provided animals with a dose capable of increasing neurogenesis. It is reasonable based on previous findings to assume that increasing the nicotine dose would yield a rate of neurogenesis lower than that of the control group. Although the directionality of the physiological results do not reflect our hypothesis, it is relevant that we were able to alter the rate of neurogenesis across conditions using two different ACh receptor agonists. The ability to induce varying rates of neurogenesis using these drugs or others like them has implications for the potential use as a means of comparing other dependent variables across conditions of various rates of neurogenesis.

We did not find a significant difference for the amount of neurogenesis in the dentate gyrus between males and females that was hypothesized in accordance with research concerning neurogenesis and sex hormones (i.e. there was no significant main effect of sex) (Tanapat, Hastings, Reeves, and Gould, 1999). While we had predicted a main effect of sex on the rate of neurogenesis, it is possible that any hormone effects weren't strong enough to overcome the effects of the drugs in a significant way. That is to say that while the hormonal differences may have altered the rate of neurogenesis to

some extent between the sexes, the drug effects may have been robust enough overcome the hormone effects. Nonetheless it should be noted that we were unable to replicate findings with regard to sex differences in the rates of neurogenesis that has been observed in previous mammal studies. Additionally, the main effect dependent on experimenter suggests that the coding process is to some extent a subjective technique and that what constitutes a BrdU positive cell was not operationally defined well enough to have greater agreement between experimenters. However, while the significant effect of experimenter did not yield any significant interactions with other factors evaluated, it was an additional source of variability and may have made it more difficult to observe effects. This suggests that the effects that were observed despite experimenter variability may be more robust than reported here.

Behavioral Results

The most pertinent behavioral result with regard to our hypotheses is the finding of a significant main effect of condition in the late phase of pain, as indicated by the significant phase by condition interaction. As hypothesized, we were able to alter the pain behavior of the two drug conditions relative to the control group. Similar to our neurogenesis findings, the effects were not in the hypothesized direction. Rather, the behavioral results across condition occur in the same direction as the physiological results. That is to say that while we initially hypothesized that nicotine-treated animals would have lower rates of neurogenesis than saline controls and decreased pain behavior relative to saline control animals (who would in turn exhibit less neurogenesis and pain

behavior than the galantamine-treated animals), the direction of the difference was reversed with regard to both neurogenesis and pain behavior. The effect on pain was observed in the same direction as the effect on neurogenesis. Thus while pain behavior did not vary across condition as hypothesized, it did vary across condition with the same directionality as the neurogenesis values. This finding is important because it affirms that we did alter the pain behavior of animals by administering two different ACh agonists, which also altered the rates of neurogenesis. This finding would have been greatly supported by a significant correlation for amount of neurogenesis and amount of pain behavior, which was not present in our data. So while there are significant between group differences for both neurogenesis and pain behavior with a positive relationship, the within group variance is such that this relationship is not applicable to individual animals. Furthermore it should be noted that the mean for the nicotine-treated group was closer to that of the galantamine-treated group on the measure of pain behavior, while the mean for the nicotine-treated group was closer to that of the saline-treated group on the measure of neurogenesis. This observation signifies that there does not appear to be a strict relationship between the trends for pain behavior and the trends for neurogenesis, although the directionality was the same for both. In addition, it should be considered that our significant experimenter effect may have increased the within-group variability, further inhibiting the correlation of individual animals and again representing a measure with an operational definition that was not effectively communicated between experimenters.

It is also not unexpected that there was a significant main effect of phase, as the second phase of pain represents a 20 minute period while the early phase of pain

represents only a 10 minute period for mice undergoing the formalin test. The different types of pain are also expressed differently, and it is not at all remarkable that the mice on average exhibited more pain in a 20 minute period than in a 10 minute period given our broad operational definition of pain behavior. The condition by phase interaction is however important with this consideration, because the condition effects were only observed with the more chronic late pain response and not the early pain response. This implies that the effects of the drugs (and potentially of neurogenesis) do not have significant differences for the immediate pain response, but may have different implications for chronic pain.

The pain behavior results indicate that we did replicate the expected sex differences across all other factors. This is not particularly interesting as it is a commonly observed effect, and it is also not surprising that females displayed a greater increase in mean pain behavior from the early phase to the late phase, as females tend to show more pain behavior than males in chronic pain conditions and in this experiment the two phases represent different lengths of time (Berkley, 1997). It is however of interest that there was a significant sex, phase, and condition interaction. The saline-treated males did not display the same increase in pain values from early to late phase that the other animals exhibited, thus the main effect of condition in the late phase is largely driven by the males. It is noteworthy that the female saline-treated animals exhibited pain increases similar to those of the nicotine and galantamine-treated animals, while the saline-treated males were distinguished from the other males by their drug condition.

Implications and Future Directions

This study provides support for a relationship between neurogenesis and pain behavior that is mediated by ACh agonists administered peripherally. Our evidence does not provide information about a particularly strong relationship without a robust correlation for the two measures. However, the corresponding directionality for both the pain behavior and neurogenesis results suggest a relationship between the two, and these findings merit further research into this relationship. There are ways in which the current methods could be improved upon, as well as novel directions that this experiment may lead other research efforts.

Because there were coding discrepancies between experimenters for both the pain behavior and the physiological data, efforts should be made to minimize the experimenter effects. The pain behavior needs to be better operationally defined so as to reduce variability between experimenters, and either a strict or lenient definition of pain behavior should be applied by all experimenters. Additionally, this effect would be eliminated if all data are analyzed by one individual. As previously mentioned, having a strong experimenter effect did not introduce experimental confounds, as both experimenters coded across all conditions and there were no significant experimenter interactions with sex or condition. Nonetheless, the potential for a significant difference in variable scores between experimenters highlights a need for a clearer definition of pain behavior and the level of relative staining necessary for a BrdU positive assignment.

An additional problem with our experimental design arises from our labeling of the mice to track data for individuals. During the pain testing, all animals' behavior was

videotaped and multiple animals were often coded side-by-side on the same tape. In order to ensure that animals were properly identified on the tape for coding purposes, each animal's designated area was labeled with the animal identification information. When animals were randomly assigned on the day of their arrival, they were each given an identification code containing the condition, gender, cage, and an individual number within the cage. For example, S4BM was the male labeled number 4 in the saline "B" cage, because each cage contained 5 animals of the same sex and there were two cages of each sex within a condition. When animals underwent pain testing, the platform on which they were contained was labeled and is visible throughout the video of the pain behavior. Thus in order to prevent confusing animals, we sacrificed the blindness of the pain behavior coding. For each animal, the experimenter coding pain behavior knew the condition of drug exposure. Consequently, the results for pain behavior may to some extent reflect experimenter bias, and should be considered with caution. It is however important to note that our behavioral results do not match our hypothesis, which provides evidence that any experimenter bias likely did not affect the data in a significant manner. Had there been a significant effect of experimenter bias, one would expect the data to reflect pain scores for nicotine animals less than those of saline animals. If in fact our results do reflect a tendency to underreport pain behavior for nicotine animals, then the actual difference between conditions should be even greater than the currently reported significant difference between nicotine and saline. While all efforts should be made to maintain experimenter blindness to condition in the future, it is unlikely that a lack of blindness compromises the data reported here by overestimating effects.

In designing this experiment, it was considered that drugs that alter the pain experience of mice should be either avoided completely or controlled for. Because it is known that ACh agonists have an antinociceptive effect, we controlled for the antinociceptive effects of muscarinic ACh receptor agonist galantamine with the nicotinic ACh agonist nicotine, which were predicted to have opposite effects on neurogenesis. Additionally, it is reasonable to assume that any acute antinociceptive effects did not alter the pain behavior of the mice as they underwent the formalin test 24 hours after the last injection. While nicotine does have an analgesic effect, it also appears to be a noxious stimulus when injected peripherally. Upon administration of nicotine, it was observed that occasionally some of the smaller females would exhibit a slight abdominal constriction, which is consistent with writhing pain behavior. Writhing pain behavior is observed upon the administration of a known noxious stimulus, such as a small volume of diluted acetic acid. Because this pain behavior is readily visible as an abdominal contraction with a hind leg kick that mimics the motion of stretching out a muscle cramp, acetic acid exposure with behavior observation is referred to as a writhing test and is commonly practiced using mice as an animal model for pain (Mogil, 2006). The observation of writhing behavior upon the administration of nicotine is of interest as it indicates that one of the drugs may have been peripherally noxious while the other drugs were not. To unintentionally provide one of the drug condition groups with a daily noxious stimulus may have confounded our results. It should be noted that writhing is an indicator of relatively mild pain, as writhing is readily overcome with very small doses of morphine (Mogil, 2006). Thus the occurrence of one brief writhe in only a few animals is not indicative of chronic or severe pain, but it does warrant the recommendation that

nicotine administration be considered in future experiments of this type. For example, nicotine could be replaced by a non-noxious nicotinic ACh receptor agonist, and in doses high enough to decrease neurogenesis. The issue of nociceptor stimulation in the periphery could also be overcome by administering all drugs centrally, although this may lead to a more complicated protocol. Using cannulated animals would enable direct administration into the ventricles of the brain without confounding peripheral effects, although the procedure of cannulating mice would likely necessitate the use of fewer animals per condition. Ultimately, if our experiment is to lead to the development of an experimental model of drug induced neurogenesis for testing other potentially related variables, the chronic administration of a noxious stimulus should be eliminated to prevent confounding the results.

Because of our use of ACh agonists, it cannot be determined whether the variations in pain behavior across conditions reflect the various rates of neurogenesis or other effects of the drugs. It is possible that chronic administration of ACh agonists led to receptor down-regulation in the brains of the animals receiving galantamine and nicotine. Because stimulating ACh receptors (AChRs) has an anti-nociceptive effect, having fewer of these receptors may lead to increased sensitivity to nociceptive stimuli. Thus injecting drugs regularly that decrease pain perception may increase pain sensitivity over time by initiating the body's own feedback system to regulate AChRs (Meyer and Quenzer, 2005). Although we controlled for the use of ACh agonists as best possible by using two different agonist that have different effects on neurogenesis and are assumed to have the same effects on pain processing, the potential for these drugs to mediate pain processing in different manners is impossible to discount.

Consequently, it cannot be determined conclusively that the differences in pain behavior were the result of neurogenesis rather than other effects of the drugs. The observation of pain behavior that varies across condition in the same direction as neurogenesis augmented by several other means (such as hormone treatment or enriched environment exposure) will provide further supporting evidence for the relationship between neurogenesis and pain behavior. However, there are not currently any known means of altering the levels of neurogenesis that do not also interact with the pain perception pathway. Furthermore, there are some implications of our research findings that are relevant regardless of the role of AChRs. For example, the use of galantamine in treating Alzheimer's Disease was discussed earlier. Our results suggest that galantamine, when administered chronically, may not only increase neurogenesis but also increases pain behavior in mice. Doctors giving this drug to Alzheimer's Disease patients may be inadvertently increasing their sensitivity to pain. This is finding is particularly important as Alzheimer's patients are less able to self-report their pain levels (Pickering, Eschalier, and Dubray, 2000). Knowing that galantamine, an important Alzheimer's drug, may alter the pain perception of patients should lead doctors to provide more analgesics for pain relief even if pain reporting does not increase.

One of the more critical limitations of our study in examining the relationship between neurogenesis and pain behavior is an inability to determine a causal relationship between the two. As previously mentioned, we cannot rule out the possibility that there were unobserved mediating effects of the drugs administered that altered the pain behavior of the mice, rather than the neurogenesis itself. Furthermore, there is evidence that suggests that the hypothalamic-pituitary-adrenal (HPA) axis is subject to functional

changes upon alterations of neurogenesis. The HPA axis, which is largely involved in stress response, is also implicated in modifying pain behavior. Thus increasing the number of cells in the hippocampus, which communicates in the HPA axis's negative feedback system in the presence of stress hormones, has the potential to increase efficiency of the negative feedback system. In the context of a painful manipulation, such as the formalin test, stress hormones would increase and the hippocampus would disable the HPA axis to decrease stress and pain behavior (Sternberg and Al-Chaer, 2006). Participation of this mechanism should yield results in the opposite direction of that observed, so it is reasonable to assume any mediating HPA axis effects were not strong enough to overcome the drug effects. Nonetheless, consideration of the HPA axis highlights an inability to conclude a *causal* relationship between neurogenesis and pain behavior, although our data does provide evidence for a relationship between the two.

Another future direction for the research of the relationship between pain and neurogenesis should involve investigation of new neurons' activity during pain perception. A co-labeling procedure to identify both new neurons and cells that are active during a period of pain behavior could implicate new cells as directly participating in the pain process. Although it seems unlikely that all novel cells in the dentate gyrus would be active during pain behavior, the identification of even a single active newly developed neuron during pain behavior would greatly strengthen evidence for a relationship between neurogenesis and pain. This co-labeling procedure could take advantage of labeling for general markers of cell activity, such as the transcription factor *c-fos* (Herrera and Robertson, 1996; Ceccarelli, Scaramuzzino, and Aloisi, 1999).

Furthermore, cell plasticity other than neurogenesis is prevalent throughout the central nervous system and is not as restricted to specific regions as neurogenesis is. For example, the formation of new synapses (synaptogenesis) and lengthening or shortening of cellular projections are commonly occurring cellular activities relative to neurogenesis (Johansson, 2007). Thus it is possible that other forms of cell plasticity may also alter the pain response to noxious stimuli. A new area of research could investigate the relationship between pain behavior and cell plasticity exhibited in various manners or in other structures of the brain.

Finally, one of the most relevant future applications of this line of research is the potential for a new manner of altering neurogenesis across conditions. As previously mentioned, this experiment may lead to the implementation of drug induced neurogenesis for the evaluation of various dependent variables. A great deal of research has been conducted concerning neurogenesis and cognitive variables such as learning and memory. Thus the use of pharmaceutical manipulations to alter levels of neurogenesis could contribute to the current understanding of the relationship between neurogenesis and learning, as current literature depends entirely on enriched environment to alter neurogenesis. For example, the dentate gyrus of the hippocampus is highly implicated in response to stressors, as well as to pain behavior (McEwen 1999; McEwen 2001). This knowledge, paired with our finding of altered pain behavior upon the administration of neurogenesis-altering drugs, suggests that stress behavior is also likely to be altered, and as such would be a reasonable variable to evaluate across drug conditions. In conclusion, we have developed a new method for altering neurogenesis for the study of additional variables, where previous studies have either measured neurogenesis as the only

dependent variable across conditions, or used environmental factors to study additional variables across conditions of altered neurogenesis. Additionally, we were able to observe behavioral changes in accordance with the administration of drugs that alter neurogenesis, indicating a possible relationship between neurogenesis and pain behavior.

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Tables & Figures

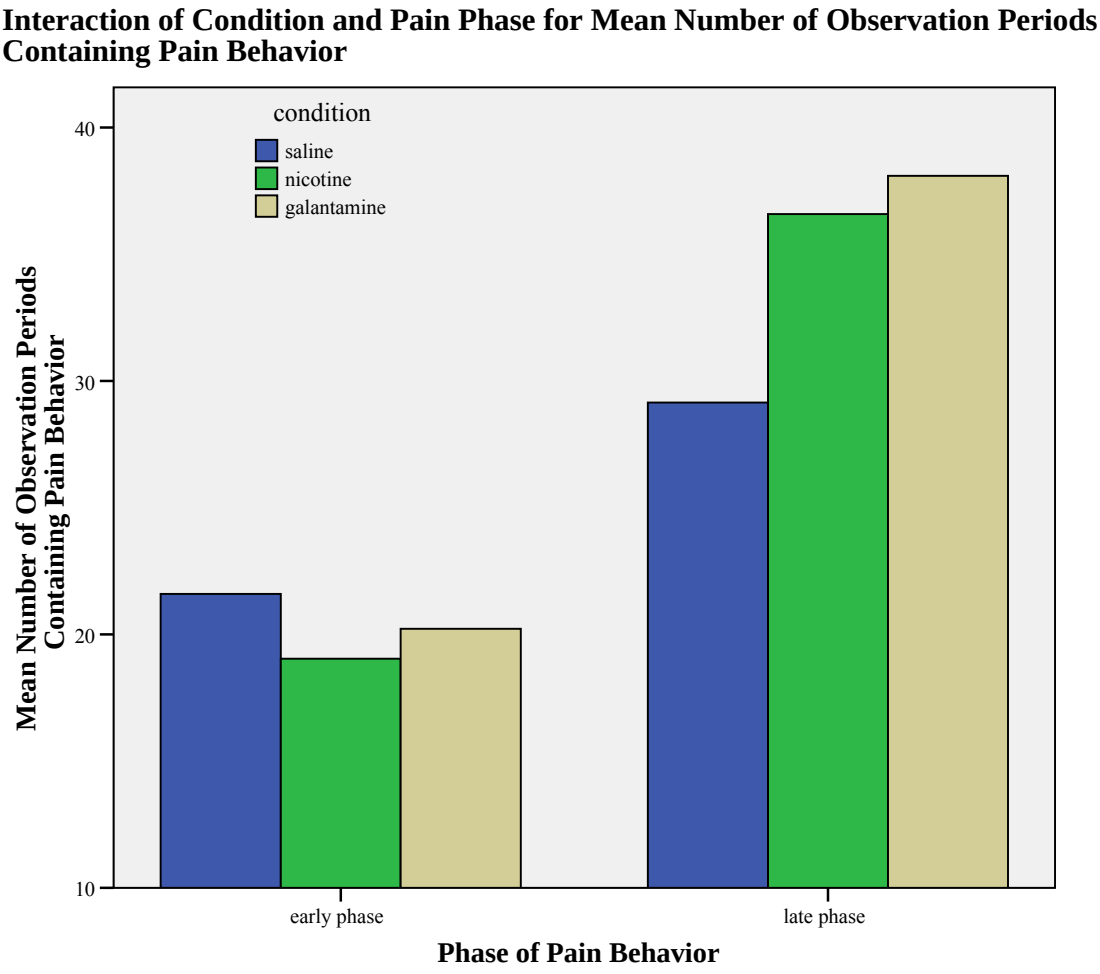

Figure 1. This figure illustrates the significant differences between the conditions that were observed in the late phase of pain but not the early phase of pain. Galantamine animals exhibited more pain behavior on average than the nicotine animals, and significantly more pain behavior than saline animals during the late phase.

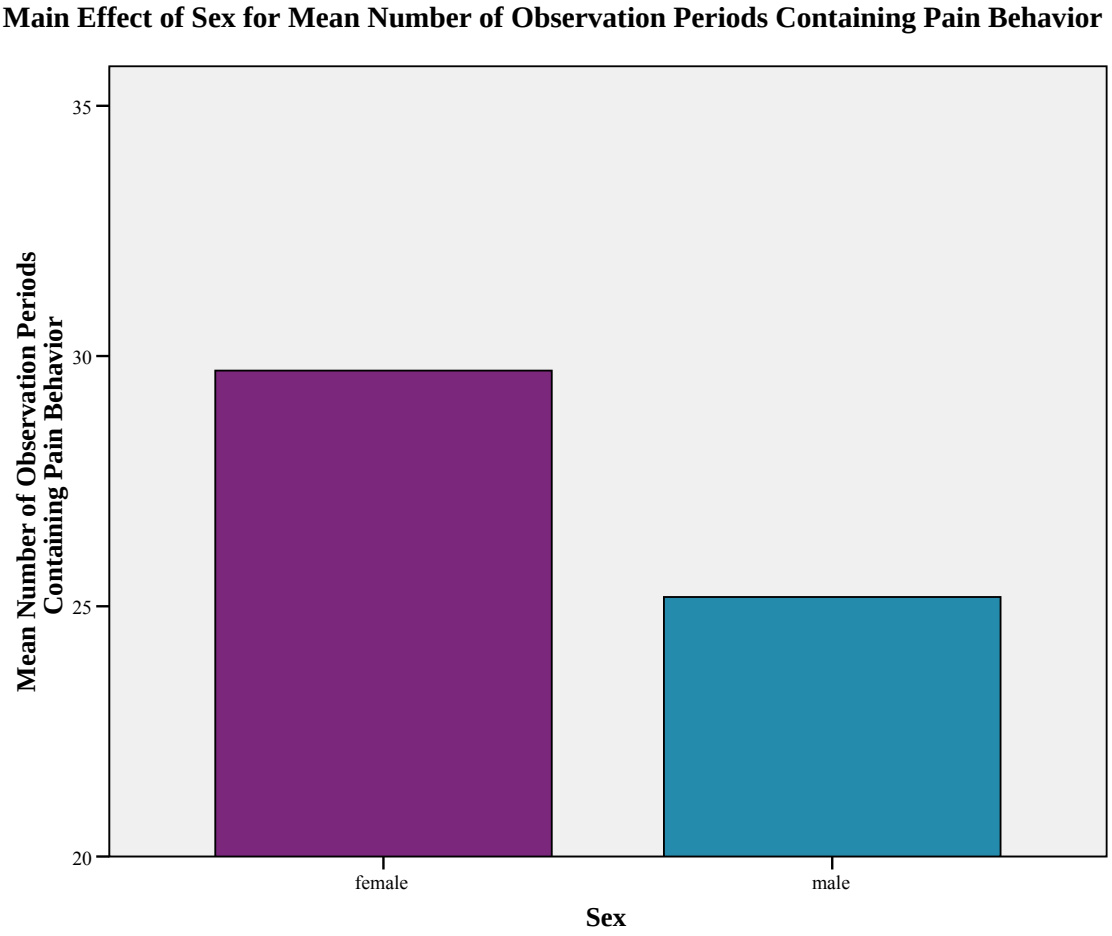

Figure 2. There was an observed main effect of sex on a pain behavior measure, with female mice on average exhibiting more pain behavior than males, across all other factors.

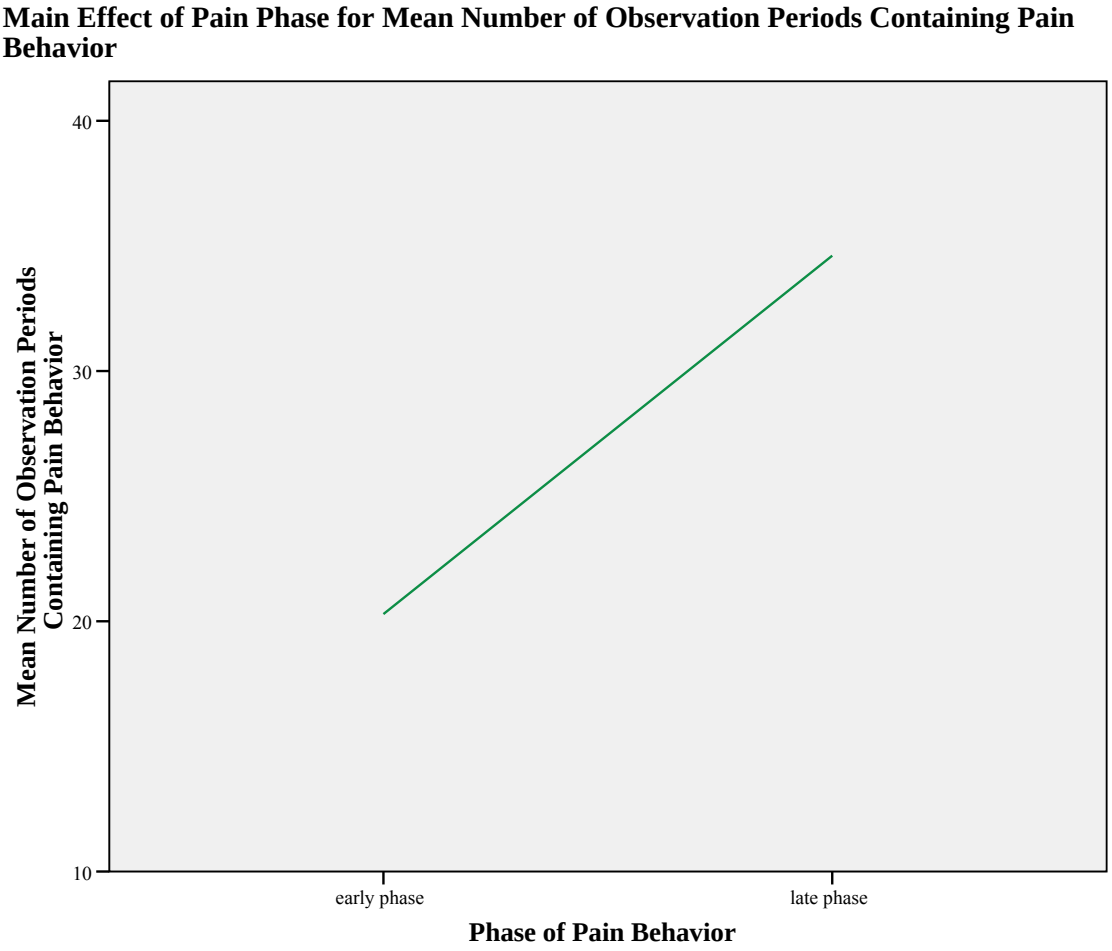

Figure 3. A main effect of phase was observed for pain behavior of all subjects. Mice displayed more pain behavior on average in the 20 minute late pain phase period than in the 10 minute early pain phase period.

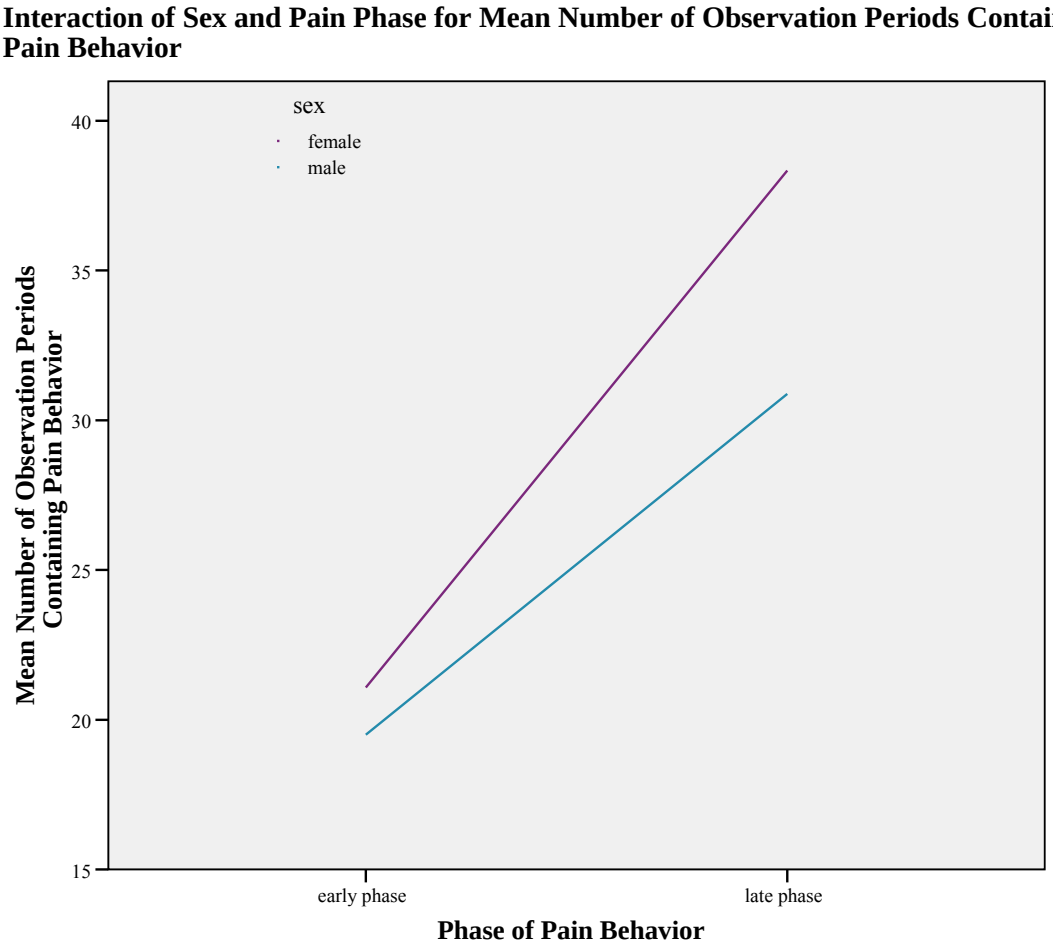

Figure 4. This figure illustrates the sex and phase interaction. Although females always displayed more pain on average than males, there was a significantly greater mean increase from the early phase to the late phase for the females than for the males.

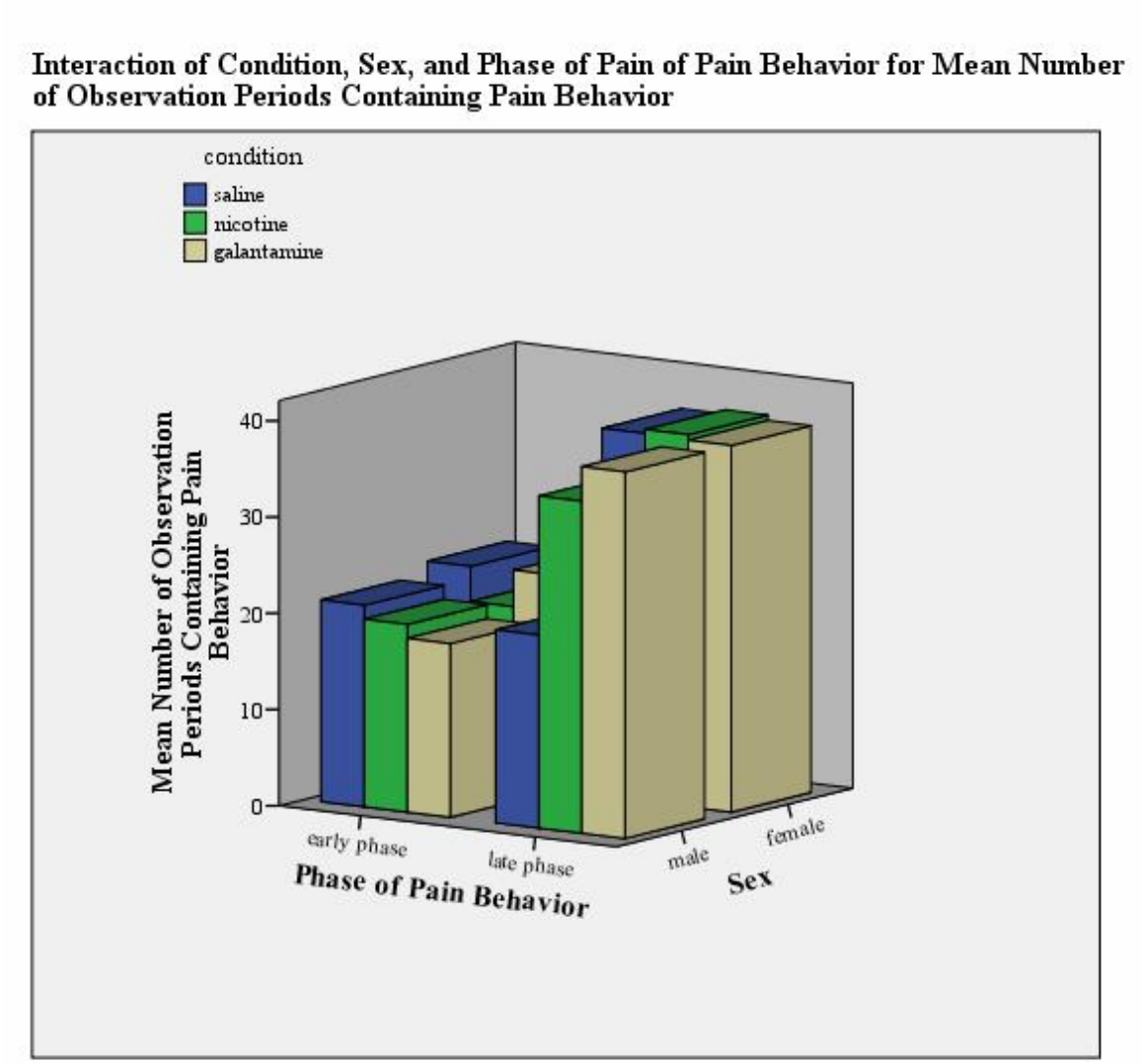

Figure 5. In this graph, it can be seen that saline male animals did not exhibit an increase from the early to the late phase of pain in the same manner as the other animals. Thus it is the males that drive the main effect of condition during the late phase observed and illustrated in *Figure 1*.

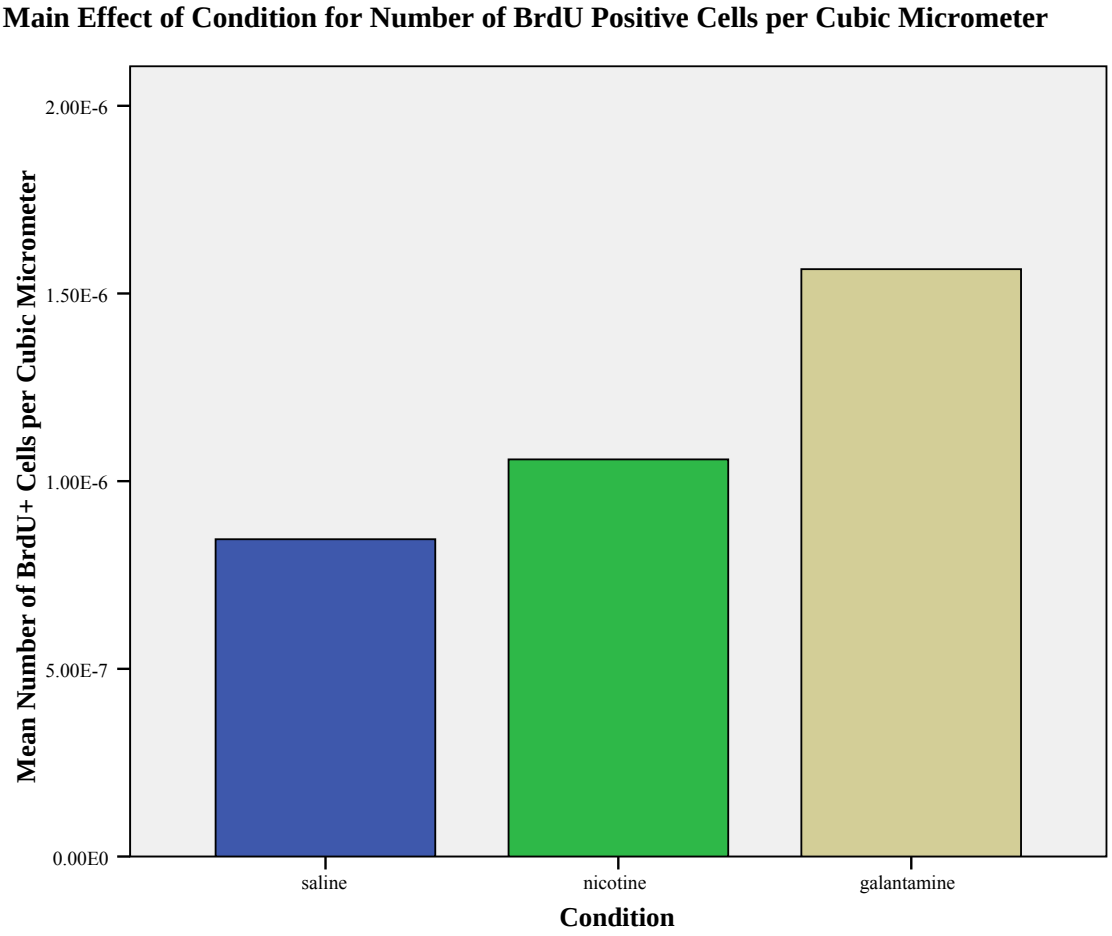

Figure 6. The main effect of condition on the measure for neurogenesis (the ratio of BrdU positive cells per μm^3) is depicted in the graph above. Animals in the galantamine drug condition had a significantly higher mean ratio of BrdU+ cells per volume than the saline animals, and a nearly significantly higher mean ratio than that of the nicotine animals. Refer to *Figure 1* for this relationship between conditions in the late phase with a measure of pain behavior as the dependent variable.

2-tailed Bivariate Correlational Analysis of the Number of Observation Periods Containing Pain Behavior and the Number of BrdU Positive Cells per μm³

Correlation	N	*r*	significance
Early Phase Pain Value and Late Phase Pain Value	29	0.579	$p < .01$
Early Phase Pain Value and BrdU+ Cells/μm³ Value	30	-0.113	ns
Late Phase Pain Value and BrdU+ Cells/μm³ Value	29	0.100	ns

Table 1. This table represents data obtained through a correlational analysis for measures of pain behavior and measures of neurogenesis for individual animals. There was not a significant relationship between the pain behavior and neurogenesis values of individuals, despite the correlating directionality of these measures for the groups of animals.