The Role of the Endocannabinoid System in Learning-Induced Neurogenesis

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Senior Honors Thesis

May 2011

Author Note:

This honors project was done in collaboration with another undergraduate student, Ana Florencia Lopezulloa, with the assistance of graduate student, Orianna Sill. The background research and experimental idea were contributed by Jegath Athilingam. Surgical procedures and perfusions were performed by Orianna Sill. Behavioral data was collected by all three contributors (10 rats by Ana, 4 rats by Jegath, and 1 by Orianna). Immunohistochemistry, cell counting, and statistical analysis of both behavioral and immunohistochemical data was performed by Jegath Athilingam.

Abstract

Though it was previously thought that the birth of new neurons stopped after development, we now know that neurogenesis continues throughout life in some areas of the brain (i.e. the olfactory bulb and the dentate gyrus of the hippocampus). Learning, in particular, has been shown to facilitate the survival of newborn neurons in the dentate gyrus. The endocannabinoid system, famous for its activation by the illicit drug cannabis (marijuana), is known to play a role in learning as well as both developmental and normal adult hippocampal neurogenesis. This study aimed to test whether the role of the endocannabinoid system extends to the specific neuronal survival that is induced by learning. Fifteen male Long-Evans rats were injected with bromodeoxyuridine (a marker for newborn cells; BrdU), implanted with hippocampal injection cannulae, and trained on a hippocampal-dependent odor discrimination learning task while being infused with either rimonabant, which effectively blocks the endocannabinoid system, vehicle (DMSO), or saline control. After the rats were sacrificed, brains were removed and newborn cells were visualized using immunohistochemical labeling of BrdU. Our behavioral results show that rimonabant rats tend to learn slower than saline control rats but no slower than the DMSO vehicle controls. This may imply that our vehicle impairs learning such that any effect of the CB1 antagonist cannot be distinguished. Though there were not enough subjects for formal statistical tests, preliminary histology data shows that vehicle, rimonabant, and saline rats have the most to least new cells numerically. It turns out that DMSO can cause neural damage and therefore may have led to gliosis and resulted in inflated cell counts. Future studies should continue to explore the questions of this study using a different vehicle, a larger sample size, and fluorescent double-labelling for neurons.

Key words: neurogenesis, endocannabinoids, extinction learning

The role of the endocannabinoid system on learning-induced neurogenesis

For a long time, it was widely believed in the scientific community that the birth of neurons ended with development. However, we now know that neurogenesis (the birth of new neurons) continues throughout life in some specific areas of the brain, particularly the olfactory bulb (Luskin, 1993) and the dentate gyrus of the hippocampus (Eriksson et al., 1998). Adult neurogenesis and learning have been shown to have a very complex and reciprocal relationship and both learning and neurogenesis seem to be modulated at least in some part by the endocannabinoid system. The endocannabinoid system, learning, and neurogenesis have a complex interconnected relationship; however, no research to date has tried to put together these puzzle pieces.

Adult Hippocampal Neurogenesis

In the adult hippocampus, new neurons are born in the subgranular zone (SGZ) of the dentate gyrus. After birth, these neurons migrate to the granule cell layer of the dentate gyrus where they mature and, after 4 – 10 days, begin sending axonal projections along the mossy fiber pathway to reach targets in the CA3 region, to effectively integrate themselves into existing neuronal circuits (Hastings & Gould, 1999, Fig 1). These newborn granule cells also tend to have special membrane properties that allow them to slowly join existing circuits. For example, new neurons seem to have a subthreshold Ca^{2+} conductance which allows for action potential firing with only very small excitatory currents (Schmidt-Hieber, Jonas, & Bischofberger, 2004). This may contribute to the observed lower threshold for long term potentiation (LTP) of new neurons which may facilitate synaptic plasticity, an ease of integration into circuits, and

contribute to the role of these new neurons in learning as will be discussed (see Ming & Song, 1993 for review).

Studying neurogenesis. The phenomenon of neurogenesis has been most widely studied in rats who produce between 8,000 to 10,000 new neurons each day (Cameron & McKay, 2001), 40% of which survive and mature into fully functional neurons over the course of about 4 weeks (Becker & Wojtowicz, 2006). The study of neurogenesis has been possible with the help of a compound called bromodeoxyuridine (BrdU), a synthetic nucleoside that acts as an analog of thymidine during mitosis and incorporates itself into replicating DNA. After injecting BrdU into a live rat, newborn cells can be identified postmortem through immunocytochemical measures where BrdU is stained and visualized using specific antibodies which bind to BrdU (Magavi & Macklis, 1984; von Bohlen und Halbach, 2007; Magavi & Macklis, 2008).

Why study neurogenesis? A major clinical implication of neurogenesis is its hypothesized role in depression. Interest in the relationship between neurogenesis and depression came about for several reasons (see Warner-Schmidt & Duman, 2006 for review). To briefly review, many treatments both pharmacological (e.g. SSRIs) and behavioral (e.g. exercise) that were known to help treat depression also increased neurogenesis, blocking neurogenesis in animal models via irradiation of the dentate gyrus also blocked the antidepressant effects of SSRIs, and postmortem studies showed that hippocampal volume was decreased in depressed subjects. This relationship between neurogenesis and depression is thought to be modulated by stress which is known to be a factor in depression and is also known to decrease hippocampal neurogenesis (Gould & Tanapat, 1999). By studying the function and mechanisms of neurogenesis in the normal adult brain, we can better understand when neurogenesis goes wrong, such as in depression.

While dysfunctional neurogenesis may be in a position to explain some aspects of psychiatric disorders, normal adult hippocampus is important in the normal adult brain for everyday learning and memory as discussed below.

Neurogenesis & Learning

Since the adult hippocampus is constantly producing neurons, neurons at all stages of development are continually available, which may contribute to hippocampal plasticity and thereby take a part in learning and memory. Several hypotheses have been put forward regarding the relationship between neurogenesis and learning; so far the literature paints a somewhat circular relationship with neurogenesis being necessary for successful learning but learning also being said to influence levels of neurogenesis (see Leuner, Gould, & Shors, 2006 for review).

Is neurogenesis crucial for learning? When neurogenesis is blocked with treatment of an anti-mitotic agent or via x-ray irradiaton of the hippocampus, rats show a detriment in the learning of hippocampal-dependent tasks. For example, rats with methylazoxymethanol (MAM) antimitotic treatment showed difficulties in learning hippocampal-dependent trace conditioning, a task where unconditioned and conditioned stimuli are separated in time, but not in hippocampus-independent delay conditioning, a task where unconditioned and conditioned stimuli overlap temporally (Shors, Miesegaes, Beylin, Zhao, Rydel, & Gould, 2001). Similarly, rats that have been subjected to hippocampal irradiation have been shown to do worse on hippocampus-dependent tasks such as place recognition (Madsen, Kristjansen, Bolwig, & Wortwein, 2003), non-match-to-sample with a long delay (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006), contextual fear conditioning (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006), and a Morris water maze (Rola et al., 2004). However, this detriment has not been

seen in tasks that do not require the hippocampus such as object recognition (Madsen, Kristjansen, Bolwig, & Wortwein, 2003) and a non-match-to-sample task with a short delay (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Overall, the majority of studies indicate an important role of newborn neurons in hippocampal learning; however, learning also seems to affect the rate of proliferation or survival of these new neurons.

Does learning affect the rate of neurogenesis? Just as researchers blocked neurogenesis to see its effect on learning, scientists have also trained animals on learning tasks to test their effects on neurogenesis. Several studies have shown that rats trained on hippocampus-dependent tasks (e.g. trace eyeblink conditioning, Morris water maze, and social transmission of food preference) showed increases in BrdU labeled cells (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Dobrossy, Drapeau, Aurousseau, Le Moal, Piazza, & Abrous, 2003; Hairston et al., 2005; Olariu, Cleaver, Shore, Brewer, & Cameron, 2005). However, one study showed no difference in neurogenesis between rats trained on a hippocampus-dependent task versus rats trained on a task that did not require the hippocampus (Dalla, Bangasser, Edgecomb & Shors, 2007). But many other studies found that rats trained on tasks that may involve the hippocampus but not require it (e.g. delay eyeblink conditioning, cue maze training, active shock avoidance) did not show any difference in the number of BrdU labeled cells (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Van der Borght, Meerlo, Luiten, Eggen, & Van der Zee, 2005), implying that hippocampal-dependence in learning tasks is necessary for the neurogenic effect. It also seems that the actual learning of the task and not just training is crucial to the neurogenic effect since performance (rather than the amount of training) correlated with the number of BrdU labeled cells in a trace eyeblink conditioning task (Dalla, Bangasser, Edgecomb, & Shors, 2007; Dalla, Papachristos, Whetstone & Shors, 2009). Though the results from these studies are not crystal clear, it does

seem that tasks requiring the hippocampus do affect neurogenesis and that this effect is dependent on the actual learning and not just training of the task.

Does learning facilitate the survival of new neurons? There has been a bit of debate over whether learning induces an increase in the proliferation of new neurons or enhances the survival of previously born neurons. Differences in procedure (i.e. date of BrdU injection, number of BrdU injections, length of training, and date of brain removal) makes this question a bit difficult to answer and many researchers seem to blur the distinction between proliferation and survival (Prickaerts, Koopmans, Blokland, & Scheepens,2004). However, learning on the Morris water maze and trace eyeblink conditioning have been shown to increase the survival of neurons that were born prior to learning the task (Dobrossy, Drapeau, Aurousseau, Le Moal, Piazza, & Abrous, 2003; Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Specifically, learning seems to facilitate the survival of neurons that were born approximately one week prior to learning (Ambrogini, et al., 2000). Since seven days after mitosis is approximately the time when new neurons tend to die off (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003), this seems to be the 'sensitive period' during which learning could rescue newborn neurons. This 'sensitive period' also corresponds with the time during which surviving granule cells begin to make connections with the CA3 region of the hippocampus (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003), so perhaps learning during this period is able to not only save new neurons but also facilitate their integration into circuits.

The Endocannabinoid System & Learning

Cannabinoid (CB) receptors were initially of interest to scientists because the psychoactive component of cannabis (i.e. delta-9-tetrahydrocannabinol) was known to bind to

them (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988). But by the logic of evolution, these receptors could not just simply exist for exogenous compounds; there had to be native molecules which bound to these receptors implying some evolutionary purpose for the endocannabinoid system. Eventually scientists discovered a native group of ligands, such as anandemide and 2-arachidonoylglycerol (2-AG), which came to be known collectively as endocannabinoids (eCBs) (Mechoulam & Fride, 1993; Stella, Schweitzer, & Piomelli, 1997). But due to the illegality of cannabis, the endogenous cannabinoid system has been poorly researched and its role is still somewhat shrouded in mystery.

In recent years, scientists have begun to study the endocannabinoid system and its relationship to learning and memory. Rimonabant (SR141716), a potent antagonist of the cannabinoid receptor (CB1), has been shown to eliminate the inherent encoding bias in the hippocampus based on similarity in a delay-match-to-nonsample (DMNS) task (Deadwyler & Hampson, 2009), inhibit spatial learning when administered intraperitoneally (Robinson et al., 2008), and enhance learning when administered intrahippocampally (Robinson et al., 2008). These studies are a good start to uncovering the role of the eCB system in learning but are contradictory.

A more promising area of research regarding the eCB system and learning is in the area of extinction, the process of learning when previous memories or associations are no longer valid. Research into this topic began when Marsicano and colleagues (2002) found that genetically altered mice that were missing the CB1 receptor (CB1 “knockout” mice) as well as wild-type mice administered the CB1 antagonist, rimonabant (SR141716A), showed an impairment in both short and long term extinction in an auditory fear conditioning task. To further support this idea of the eCB system’s role in extinction, Pamploma and colleagues (2008)

established that the extinction of contextual fear conditioning memories was enhanced in rats that were administered the CB1 agonist WIN55212-2 or the eCB reuptake inhibitor AM404, which effectively increases the levels of eCBs by blocking their degradation (Pamploma, Bitencourt, & Takahashi, 2008). Furthermore, this group also replicated the previous findings that the CB1 antagonist (SR141716, rimonabant) inhibited extinction capabilities and that the effects of all pharmacological manipulations were still persistent when extinction was measured seven days later (Pamploma, Bitencourt, & Takahashi, 2008). Other studies have followed up with findings of impaired extinction in CB1 "knockout" (KO) mice in a Morris water maze task (Varvel, Anum, & Lichtman, 2005) and enhanced extinction with a reuptake inhibitor in a fear conditioning task (Chhatwal, Davis, Maguschak, & Ressler, 2005).

Since an effect of endocannabinoids was demonstrated in aversive extinction learning, researchers turned to examine its role in appetitive learning paradigms. Several of these studies proposed that the eCB system did not play a crucial role in the extinction of appetitive conditioning with a food reward (Niyuhire, Varvel, Thorpe, Stokes, Wiley, & Lichtman, 2009; Harloe, Thorpe, & Lichtman, 2008; Holter et al., 2005). However, these studies failed to take into account that eCBs have been implicated in the mediation of pleasure from food evidenced by anandamide (an endogenous CB1 agonist) infusions into the medial nucleus accumbens, the brains "reward center," increasing eating behavior, food intake, as well as the rewarding nature of food (Mahler, Smith, & Berridge, 2007). Since the previously mentioned studies of appetitive conditioning utilized systemic rimonabant injections or CB1 genetic deletions, CB1 receptors in the nucleus accumbens would have been blocked or missing leading to a possible lack of motivation in the task. One paper even explicitly mentioned a lack of motivation in a food reward task in CB1 KO mice (Holter et al., 2005). This disinterest in the reward may have

masked any effects on extinction that drug administration may have had. This idea seems highly plausible in light of a study that used cocaine and amphetamine as the appetitive reward in a conditioned place preference task and discovered that a CB1 agonist (i.e. delta-9-tetrahydrocannabinol) potentiated the extinction of this drug-induced conditioned place preference (Parker, Burton, Sorge, Yakiwchuk, & Mechoulam, 2004). There have been no studies to date that examine extinction in an appetitively motivated learning using intracranial infusions so it is not possible to know whether the lack of effect seen is due to the aforementioned methodological problem. It is clear, however, that the eCB system does play a definitive role in learning and memory.

The Endocannabinoid System & Neurogenesis

In understanding the process of normal adult neurogenesis, some signs have pointed towards a role of the endocannabinoid system. It is known that neurogenesis is increased following injury or insult and both *in vitro* and *in vivo* studies have implicated that the eCB system is necessary for this effect. Administration of kainic acid induces excitotoxity which in turn provokes neural stem cell generation. Cells that have been administered the CB1 antagonist rimonabant do not show this excitotoxicity-induced neurogenesis; likewise, administration of the endocannabinoids anandamide and 2-AG leads to increased neural generation (Aguado et al., 2007). The same group used kainic acid to induce excitotoxic epileptiform seizures in living mice and found that both CB1 KO mice and wild type mice that have been administered rimonabant showed severely impaired neural progenitor proliferation in response to excitotoxicity. This effect may be modulated by basic fibroblast growth factor (bFGF) which was shown to be upregulated following excitotoxicity in controls but not in CB1 KO mice (Aguado et

al., 2007). In addition to excitotoxic effects on neurogenesis, the reduction in neurogenesis caused by stress was prevented by the eCB uptake inhibitor AM404 in mice (Hill, Kambo, Sun, Gorzalka, & Galea, 2006). AM404 showed no effect when administered alone, implying a role of eCBs in promoting survival of newborn cells rather than the direct proliferation of neural progenitors (NPs) (Hill, Kambo, Sun, Gorzalka, & Galea, 2006). The CB1 receptor is coupled to two cell survival pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the extracellular signal-regulated (ERK) pathway, which could contribute to the neuroprotective role of eCBs (Galve-Roperh, Aguado, Palazuelos, & Guzman, 2008). There is also evidence of crosstalk between the eCB system and brain-derived neurotrophic factor (BDNF), which is a key player in neuronal survival; BDNF has been shown to increase neuronal sensitivity to eCBs (Maison, Walker, Walsh, Williams, & Doherty, 2009).

Though the eCB system has been shown to play a role in excitotoxicity-driven neurogenesis, it has also been implicated in normal adult hippocampal neurogenesis. *In vitro* studies have shown that CB1 receptors are clearly expressed in adult hippocampal neural progenitors (NPs) and that NPs incubated with the CB1 agonist HU210 show an increase in NP proliferation (Jiang, Zhang, Xiao, & Cleemput, 2005). CB1 KO mice also have shown reductions in BrdU labeled cells but contrarily, wild type mice given systemic rimonabant showed an increase in BrdU labeled cells (Jin et al., 2004). Mice that were genetically lacking the gene for fatty acid amide hydrolase (FAAH), which participates in the breakdown of eCBs, show increased neurogenesis (Aguado et al., 2005) and mice lacking the proteins that synthesize 2-AG, diacylglycerol lipases α and β, show significant reductions in BrdU labeled cells in the dentate gyrus (Gao et al., 2010). Mice that have been chronically administered the agonist HU210 showed increased proliferation in NPs, which are then shown to survive one month later to be

integrated into neural circuits (Jiang, Zhang, Xiao, & Cleemput, 2005). In this same study, measures of depression such as novelty-suppressed feeding (NSF) and the forced swimming test (FST) decreased with agonist HU210 administration and increased BrdU counts. HU210 rats that were irradiated to block neurogenesis showed both a decrease in BrdU cells and HU210-induced antidepressant effects (Jiang, Zhang, Xiao, & Cleemput, 2005). The results of these studies point to some role of the eCB system in varying types of neurogenesis.

Missing Links

While it's been shown that the endocannabinoid system is involved in normal adult neurogenesis, neurogenesis following toxicity, and can reverse the deficit in neurogenesis caused by stress, no study to date has explored the eCB system in relation to learning-induced neuronal survival. Since endocannabinoids seem to play a facilitative role in the survival of newborn neurons via crosstalk with BDNF and the PI3K/Akt and ERK pathways and learning itself is also known to assist in the survival of new neurons, this study aims to examine whether the eCB system plays a crucial role in this learning-induced neuronal survival. We hypothesize that the eCB system is implicated in this and that therefore rats treated with an eCB system blocker (antagonist) would show lower levels of neuronal survival (as indicated by fewer numbers of BrdU labeled cells). We also hypothesize that the eCB system may be involved in the learning of this task and that therefore these drug-treated rats would show poorer performance the learning task (as indicated by a lower accuracy). These hypotheses were tested with the pharmacological manipulation of rats trained on a contextual learning paradigm which is known to require the hippocampus.

Method

Subjects

Fifteen male Long-Evans rats (Charles River Laboratories, Wilmington, MA) were individually housed and kept on a 12 hour light/dark cycle with food restriction of 80-85% of their free feeding weight to ensure motivation for the food reward during the learning task. All procedures complied with guidelines established by the Cornell University Animal Care and Use Committee.

Materials

Cannulation surgery. Rats were anesthetized with sodium pentobarbital (40mg/kg) before undergoing stereotaxic surgery where bilateral cannulae (28 gauge/2.5cm) were implanted for intrahippocampal infusions. Cannulae were positioned at the dentate gyrus (DG) of the hippocampus (-3.6mm posterior to Bregma, ±2.3mm lateral from midline, and -3.6 from top of the skull implying a depth of 2.6mm since the skull is 1mm thick, at angle of 8º inward) and affixed to the skull using dental cement. Rats were given an antibiotic (5mg/kg Baytril), an analgesic (5mg/kg ketoprofen), and one week to recover. Rats were only included in the study if cannulae placement was verified post-mortem with Nissl staining.

BrdU injections. Rats were intraperitoneally injected with one dose of BrdU (200mg/kg) one week prior to training on List 2 (see odor discrimination task) in order to be consistent with research indicating that one week after neuronal birth is the critical period where learning can facilitate survival (Gould, Beylin, Tanapat, Reeves & Shors, 1999). BrdU incorporates into the DNA of dividing cells during the S phase of the cell cycle by substituting for thymidine and is therefore an ideal marker of neurogenesis.

Intrahippocampal infusions. The cannabinoid antagonist Rimonabant (SR141716A, Cayman Chemicals, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 10mg/mL. During infusion, subjects were restrained by hand and a 28 gauge needle attached via a length of tubing to a 10μL Hamilton syringe was inserted into the cannulae. The tubing allowed for the subjects to move around while 0.8μL was infused into each hemisphere over the course of 2 minutes. Rats were infused with either rimonabant, vehicle (100% DMSO, used since rimonabant is hydrophobic and cannot be dissolved directly in saline), or saline 15 minutes prior to their experimental session on each day of List 2 as well as the half-lists in the interim period before rat euthanasia.

Procedure

Odor discrimination task. The learning task utilized in this experiment is a task of odor discrimination where rats are presented with lists of odor pairs. Within each pair, one of the odors is consistently rewarded and the rats must learn the association between odor and reward. When rats successfully learn the first list of odor pairs, the rats enter a new context and must learn a new list of items, which involves a high level of interference due to the two lists having common items. While learning the initial list of odors does not require the hippocampus, the coupling of contextual learning and high interference in the second part of the task has been shown to be hippocampus-dependent (Butterly, Petroccione, & Smith, 2011) and is ideal for this study for its ability to likely induce neurogenesis.

The experimental apparatus was a 45cm X 60cm X 40cm (width X length X depth) box made of Plexiglass which was divided across the center with a removable piece of Plexiglass to separate the odor presentation area from the inter-trial waiting area. In the odor discrimination

task, rats were presented with two cups (8.5cm diameter, 4.5cm deep), each filled a different odor mixed with corncob bedding material. A sucrose pellet reward (45mg) was consistently buried in one odor of the pair and the rat had to learn to dig in the correct cup for the reward. Initially, the rat digs in the first cup he approaches but as learning occurs he only digs in the rewarded cup. Thirty-two total odors were used in the experiment and included: propyl butyrate, citronellal, ethyl isovalerate, furfuryl proprionate, n-butyl glycidyl ether, methyl salicylate, n-amyl acetate, ethyl butyrate, propionic acid, benzaldehyde, 1-octanol, pentanol, trans-2-hexenyl acetate, propenoic acid, heptanol, ethyl valerate, 1,8-cineole, anisole, 5-methylfurfural, ethyl acetate, +/- limonene, methyl butyrate, 2-phenylethanol, 1-butanol, methyl 2-furoate, butyl butyrate, cis-3-hexenyl acetate, pentyl butyrate, benzyl benzoate, 2-furyl methyl ketone, 1-nonanol, butyl pentanoate.

Rats were first acclimated to the testing environment and procedure for approximately one week following surgery recovery. During acclimation, two ceramic cups were filled with neutral odor and a sucrose pellet was initially placed on top for the rat to retrieve. Over the course of the week, the pellet was slowly buried deeper and deeper in the bedding until the rat successfully learned to dig to the bottom to retrieve the pellet. When the rat had successfully learned the digging procedure, training on List 1 began.

List 1 consisted of 8 odor pairs (16 total odors) and was associated with a specific context (context A) consisting of a white box cleaned with unscented baby wipes, black walls in the room, 65dB white noise in the background, and the rat's cage being wheeled to the experiment room on a cart. Rats were trained at the same time each day for 64 trials. In each trial, the experimenter buried the reward in one cup, lowered both cups into one side of the testing box, raised the divider, and recorded the rat's digging responses and latency to dig. The position of

the cups on the left or right of the apparatus was counterbalanced and order of odors presented was randomized. Rats were trained on List 1 until they reached a behavioral criterion of 90% correct choices for two consecutive days.

Rats then began training on List 2 which was presented in a different context (context B) consisting of a black box cleaned with scented baby wipes, white walls in the room, 65dB pink noise in the background, and the rat's cage being carried into the experiment room under the experimenter's arm. In each pair of List 2 odors, one odor from List 1 was paired with a novel odor and the previously encountered odor was given its opposite reward value (i.e. unrewarded odors in List 1 were rewarded in List 2 and rewarded odors in List 1 were unrewarded in List 2). This part of the experiment contains a large amount of interference and has been shown to be hippocampus-dependent (Butterly, Petroccione, & Smith, 2011) and therefore likely to promote neuronal survival. All rats were tested on List 2 for five consecutive days.

Due to the desire to test whether surviving neurons would become integrated into the existing neural circuitry, it was necessary to sacrifice the animals 21 days following BrdU administration since previous literature has shown that the cells which survive for three weeks are those that become integrated into circuits (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003). Research has shown that lack of activity can be detrimental to newborn cells (Kempermann, Kuhn, & Gage, 1997) so the rats were trained on a 32 trials (half of List 2) every three days in the interim period between the end of List 2 and euthanasia in order to continue learning but minimize the risk of infection from many infusions. A pellet detection task was given prior to perfusion to ensure that the rats could not smell the pellet directly through the bedding. In this task, rats were presented with two ramekins containing the same odorized

bedding and did not choose the correct cup above chance (50%) implying that they were unable to smell the pellet through the bedding. (See Fig. 2 for schedule of experimental procedures)

Immunohistochemistry. Rats were perfused 21 days following the initial BrdU injection. For perfusion, rats were deeply anesthetized with isofluorane gas and their brains were intracardially perfused with saline followed by 10% paraformaldehyde. Brains were extracted, postfixed in paraformaldehyde followed by 30% sucrose in PBS, sliced into 40µm coronal sections, and mounted to slides for immunostaining. The tissue of nine rats was used to learn and optimize the complicated immunohistochemistry techniques. When the procedure was perfected, the tissue of the six remaining rats (two from each condition) was processed for immunostaining of BrdU.

As formalin-fixed tissue often forms protein cross-links that can mask antigen sites, the immunohistochemical procedure began with two antigen unmasking steps: First, the slides were microwaved in a bath of 0.1M citric acid in water for 10 minutes and then incubated in 0.001% trypsin for 10 minutes. After 3 consecutive PBS washes, the tissue was subjected to 2N HCl (in PBS) for 60 minutes (to denature the DNA), washed with PBS three times, and incubated in blocking serum for 20 minutes (to prevent nonspecific binding), and mouse anti-Brdu primary antibody in blocking serum (1:100) overnight. The next day, after washing, slices were incubated with secondary biotinylated goat anti-mouse secondary antibody (1:200) for one hour, followed by 0.3% H_2O_2 in PBS (to block endogenous peroxidase activity) for 30 minutes, Elite ABC peroxidase (the enzyme which binds to the secondary antibody) for one hour, and lastly DAB (peroxidase's chemical substrate that allows for visualization by turning tagged cells brown). After washing with phosphate buffer, slices were dehydrated with 70%, 95%, and 100% ethanol, and counterstained with cresyl violet before coverslipping. Undamaged slices between -1.6mm

and -5.7mm posterior to Bregma were identified at equivalent locations for counting from each cohort of three rats (29 slices from one cohort of rats and 25 slices from the other cohort of rats). BrdU labeled cells (stained brown) were counted in the entire hippocampus blindly at 20x magnification. Cells were included in the count if they were round, occurred in the correct plane of focus, and matched the size of neighboring counterstained cells. Figure 3 shows examples of immunostained tissue.

Results

Behavioral Analysis

To test the hypothesis that drug-treated rats would show a slower rate of learning than control rats, a repeated measures ANOVA was performed on behavioral performance (percent correct) with training session (five days of List 2) as the within-subjects factor and injection condition (rimonabant, DMSO, saline) as the between subjects factor. The ANOVA showed a significant main effect of training session ($F[4,48]=101.135$, $p<.000$), indicating that the performance of the rats improved with training, but no significant effect of injection condition ($F[2,12]=1.132$, $p=.354$). Figure 4 diagrams the percent correct data for the three groups across sessions.

However, for some unknown reason it seemed that our saline control group performed more poorly than saline controls in previous studies using the same learning paradigm, which might have obscured any effects of the rimonabant or DMSO treatment. Though it is not acceptable to simply substitute a control group from another experiment, to examine the possibility that our control rats did perform poorly, we ran another repeated measures ANOVA including a saline control group from a previous study in our lab (Butterly, Petroccione, &

Smith, 2011) along with the three groups from the present study. This ANOVA showed a significant main effect of training session (F[4,12]= 129.385, p<.000), injection condition (F[3,19]=7.924, p<.001), and an interaction of training session and injection condition factors (F[12,76]=3.085, p<.005). Figure 5 shows percent correct data across time for all groups including comparison saline group. Pairwise comparisons adjusted by Tukey HSD criteria showed a difference between the saline control group of the other study and rimonabant-treated rats (p<.001) as well as vehicle-treated rats (p<.05). No other between-group differences were significant.

Immunohistochemical Analysis

Since we only obtained immunohistochemical data for six animals (n = 2 per group), any statistical test that yielded significance would not be meaningful. However, for the sake of analysis, a simple one-way ANOVA was performed to compare BrdU cell counts between the three groups and found no significant difference between the groups (F[2,3]=3.628, p=.158). Table 1 gives BrdU cell counts for each animal and figure 6 shows a bar graph of average BrdU cell counts for the three groups.

Discussion

Endocannabinoids and Learning

We did not find an effect of rimonabant on learning, although this may have been due, in part, to poorly performing control subjects. The ECB system has been implicated in several types of learning and memory (Marsicano et al, 2002; Varvel, Anum, & Lichtman, 2005; Chhatwal, Davis, Maguschak, & Ressler, 2005; Pamploma Bitencourt, & Takahashi, 2008). In addition, all

previous studies of appetitively-motivated learning which found no effect of the eCB system utilized systemic antagonist injections or CB1 genetic deletions (Holter et al., 2005; Harloe, Thorpe, & Lichtman, 2008; Niyuhire et al., 2009), and are thereby confounded by the role of ECBs in the mediation of pleasure from food (Mahler, Smith, & Berridge, 2007). In fact, our study initially intended to utilize systemic injections as well, but during piloting, we noticed a severe detriment in motivation in our pilot rats. They did not seem interested in the food reward at all and we had serious difficulties getting them to complete 64 trials of the task. A previous study also commented on a lack of motivation in CB1 KO mice (Holter et al., 2005). Though our study was not exactly an extinction task, it was important to address this methodological issue using intracranial infusions since it was a task with appetitive motivation (i.e. food reward). We predicted that the eCB system was involved in our learning task and that this would be manifested in poorer performance on List 2 in the CB1 antagonist-treated rats. We saw an effect of training indicating that all our subjects did indeed learn the task with performance increasing with training session. However, we saw no effect of the drug on performance on the high interference learning task (List 2 of the odor discrimination task). There are several possible explanations for this lack of effect.

As previously discussed, the saline control rats of this experiment seemed to perform more poorly than those in other experiments using the same learning paradigm. We are unsure of the exact reason for this, but it may be that these rats were just not as good at learning or that novice experimenters who assisted in this study did not run the task correctly. When comparing our data with control rats of a previous study from our laboratory (Butterly, Petroccione, & Smith, 2011) we did see that rimonabant and vehicle-treated rats performed significantly worse than these controls. However, we still saw no difference between the rimonabant and vehicle rats

indicating that rimonabant did not cause impairment above and beyond the effect of the vehicle. The issues regarding our vehicle (DMSO) are discussed below but it is possible that the vehicle affected performance so drastically that any effect of the rimonabant was masked.

Endocannabinoids in Learning-Induced Neuronal Survival

In addition to the role in learning, our study also aimed to test eCB involvement in learning-induced neuronal survival since eCBs have previously been implicated in excitotoxicity-induced (Aguado et al., 2007) and normal adult neurogenesis (Jiang et al., 2005; Aguado et al., 2005; Gao et al., 2010). We hypothesized that rimonabant-treated rats would show marked deficits in BrdU labeled cells in the hippocampus indicating lower levels of neuronal survival induced by learning. We found no significant differences in BrdU cell counts between the experimental groups. However, we actually found the opposite of our prediction since numerically, vehicle, rimonabant, and saline rats had the most to least number of BrdU-labeled cells respectively though this was not statistically significant. Consistent with our behavioral results, it seemed that our vehicle was having some kind of adverse effect that could have masked any result of rimonabant. The effects of our vehicle (DMSO) are likely due to its very high concentration (100%) in our infusion solution. Since very few studies administer rimonabant intracranially, we based our solvent concentration on one of the groups that had successfully used DMSO as a solvent for intracranial infusions in several studies without adverse effects and had dissolved rimonabant in a 100% concentration (Hough, Svokos, Nalwalk, 2009). However, this study infused into completely different brain regions (pariaqueductal gray and raphe magnus), was testing a completely different question (cannabinoid involvement in antinociception), and did not test for the toxicity of the drug.

As it turns out, DMSO has been shown to cause peripheral nerve damage at a concentration of 7.2% (Cavaletti et al., 2000), as well as central neural damage in developing mice at concentrations as low as 0.5% (Hanslick et al., 2009). In light of these findings, it seems very plausible that DMSO-induced neural damage in our rats could have occurred and possibly caused gliosis, the proliferation of astrocytes seen in response to damage of the central nervous system. Since BrdU incorporates into all dividing cells by substituting for thymidine, glial cells are also labeled by BrdU immunostaining, but astrocytes (which can be double-labeled for their marker GFAP) usually only comprise about 11% of the total BrdU-labeled cell population in the dentate gyrus (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). But since neural damage could be seen at concentrations as low as 0.5% in developing mice, our concentration of 100% could have led to large scale gliosis, thereby inflating our cell counts for both rimonabant and vehicle-treated rats.

Methodological Limitations & Future Directions

The data from this study is very preliminary in addressing the question we aimed to answer, namely the role of endocannabinoids in the learning-induced neuronal survival. Due to methodological limitations, these questions deserve to be further explored in future experiments which address the weaknesses of our study design. Though our learning task was well formulated with intracranial infusion rather than systemic injection, our saline controls showed a very high variance among them in their behavioral performance and overall performed more poorly than normal. This issue could be overcome in a future experiment by including a larger sample size of subjects.

One of the biggest issues in this study was the choice of DMSO as the vehicle for our drug of interest, rimonabant. In this case, our 'control' did not truly function as a control since it caused its own effects and may have obscured the effects of the drug of interest, rimonabant. Due to its high concentration at 100%, DMSO may have elicited such a drastic response that the effects of rimonabant were overshadowed. Future studies should investigate a less harmful vehicle or use DMSO in extremely low concentrations to avoid this problem.

And lastly, since BrdU stains all new cells it was impossible for us to distinguish between neurons and glial cells. If we had doublestained for a neuronal marker (e.g. DCX, NeuN, calbindin) using fluorescence immunostaining, we could have readily differentiated astrocytes from neurons and ascertained whether DMSO was indeed inducing gliosis as we hypothesize. With neurons clearly labeled, we would have been more readily able to see the effect of rimonabant on neuronal survival.

Conclusion

The data from this experiment is inconclusive and a preliminary foray into the question of the ECB system's involvement in both learning and neurogenesis. This is still an area worth exploring and we hope that future scientists will expand upon this data to leap one step forward in understanding the mysterious role of endocannabinoids in the relationship between learning and neurogenesis.

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FIGURE 1

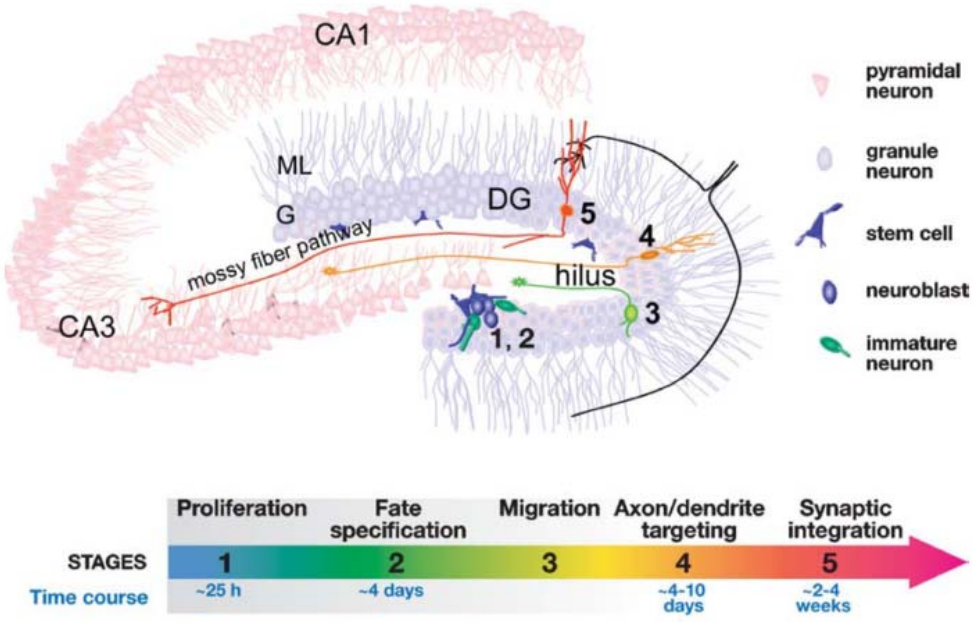

Figure 1: Diagram showing the stages of neurogenesis in the hippocampus from proliferation in the subgranular zone to synaptic integration into circuits with CA3. (from Ming & Song, 2005).

FIGURE 2

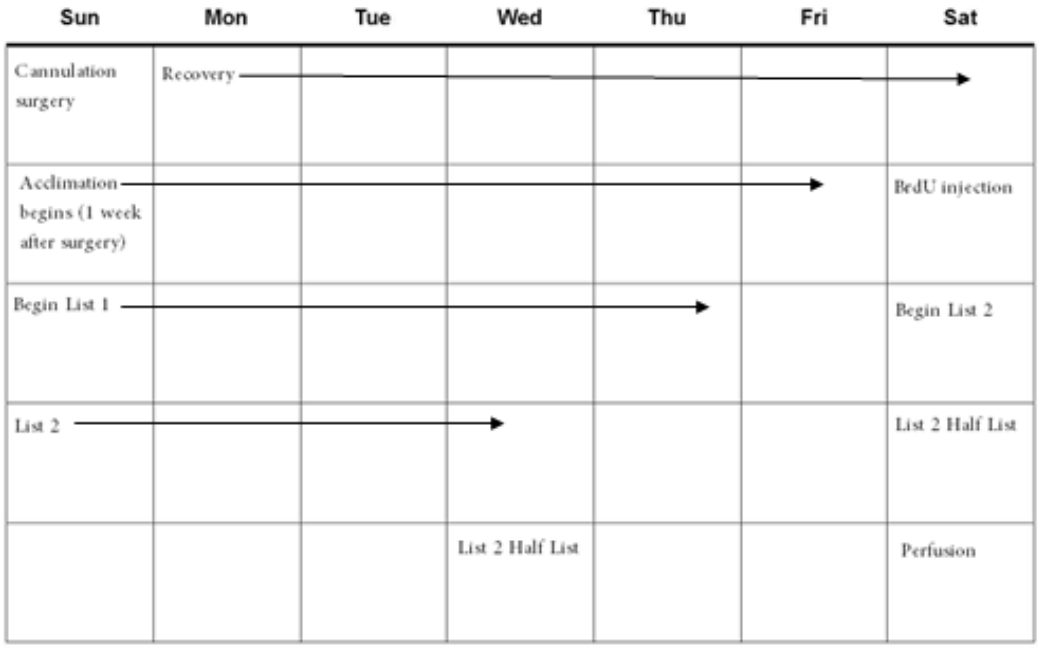

Figure 2. Schedule of experimental procedures. Sequentially, rats were implanted with hippocampal cannulae and allowed one week to recover before the acclimation period where they were trained on the experimental procedure. Once successfully acclimated, rats were injected with BrdU the day before beginning List 1 and then trained on List 1 until critical performance was reached. List 2 began one week after BrdU injection and continued for 5 days. Rats were trained intermittently (approximately every 3-4 days) on half List 2 sessions until perfusion which occurred 21 days after BrdU injection.

FIGURE 3

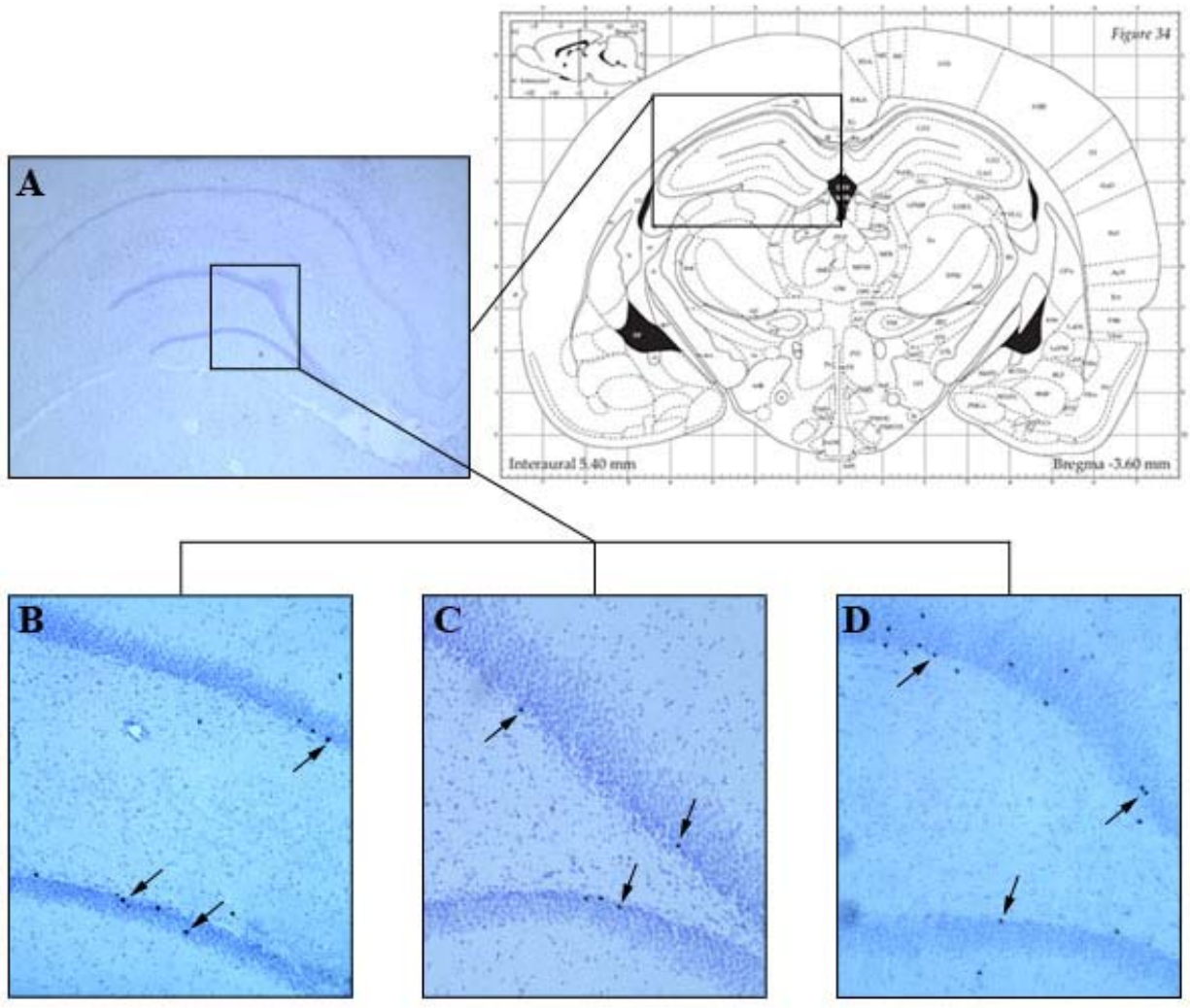

Figure 3. Images of immunostained sections located at -4.0 posterior to Bregma for subjects from each condition. A) The entire hippocampus visualized at 4x. B-D) Vehicle, rimonabant, and saline respectively visualized at 20x. Arrowheads indicate examples of cells counted.

FIGURE 4

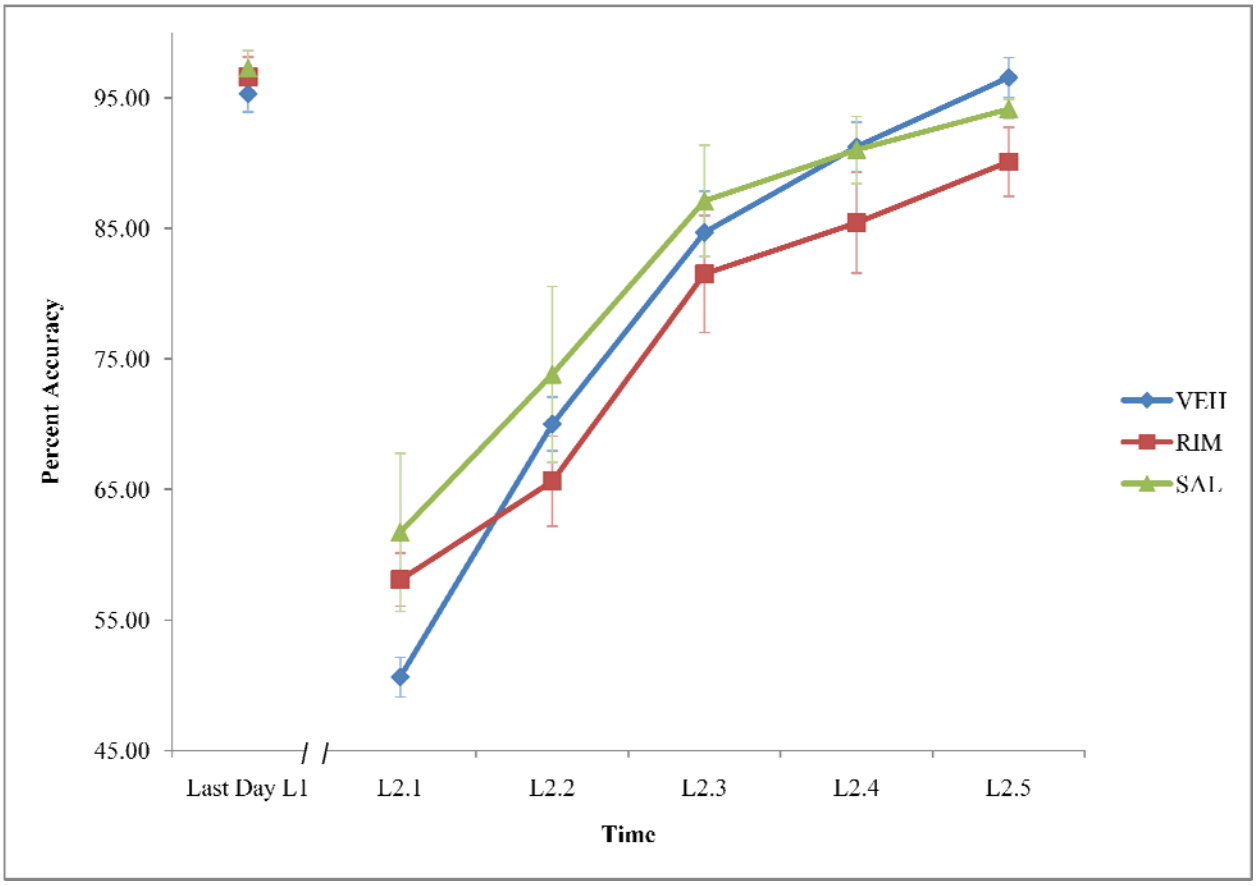

Figure 4. Percent correct from last day of List 1 through last day of List 2 for all three groups. The repeated measures ANOVA of this data showed a significant main effect of time (F[4,48]=101.135, p <.000), indicating that the performance of the rats improved with training, but no significant effect of injection condition (F[2,12]=1.132, p =.354).

FIGURE 5

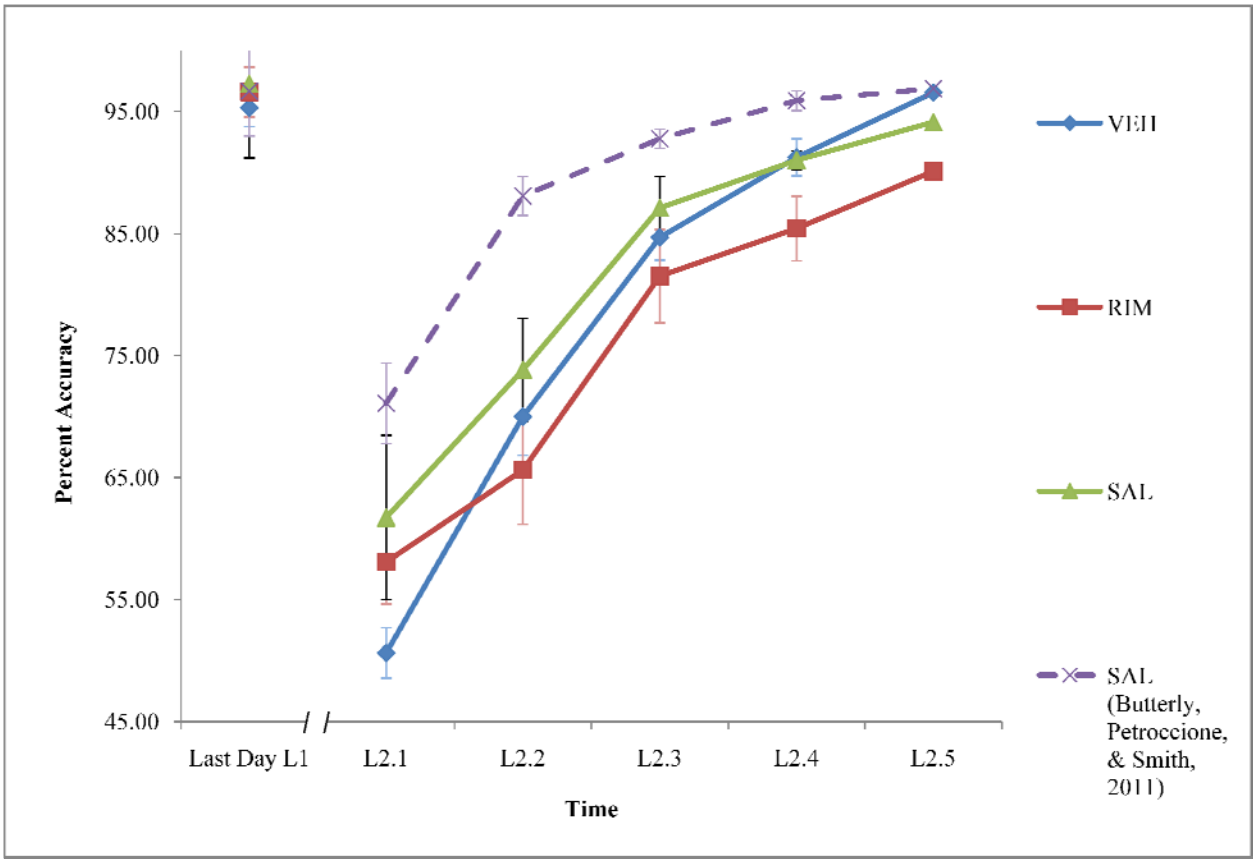

Figure 5. Average percent correct in List 2 for the three groups in our study as well as an additional saline control group from a previous study in our lab (Butterly, Petroccione, & Smith, 2011). We included this comparison since our saline group seemed to perform more poorly than saline controls from other experiments. The repeated measures ANOVA of this data showed a significant main effect of time (F[4,12]= 129.385, p<.000), injection condition (F[3,19]=7.924, p<.001), and an interaction of training session and injection condition factors (F[12,76]=3.085, p<.005). Pairwise comparisons adjusted by Tukey HSD criteria showed a difference between the saline control group of the other study and rimonabant-treated rats (p<.001) as well as vehicle-treated rats (p<.05).

TABLE 1

Numbers of BrdU-labelled Cells in All Rats

Cohort.rat	Conditon	Total BrdU Cell Count	Average Number Cells Per Slice
1.1	VEH	1066	36.76
1.2	RIM	989	34.10
1.3	SAL	627	21.62
2.1	VEH	1107	44.28
2.2	RIM	843	33.72
2.3	SAL	886	35.44

Table 1. Number of BrdU-labelled cells for all rats.

FIGURE 6

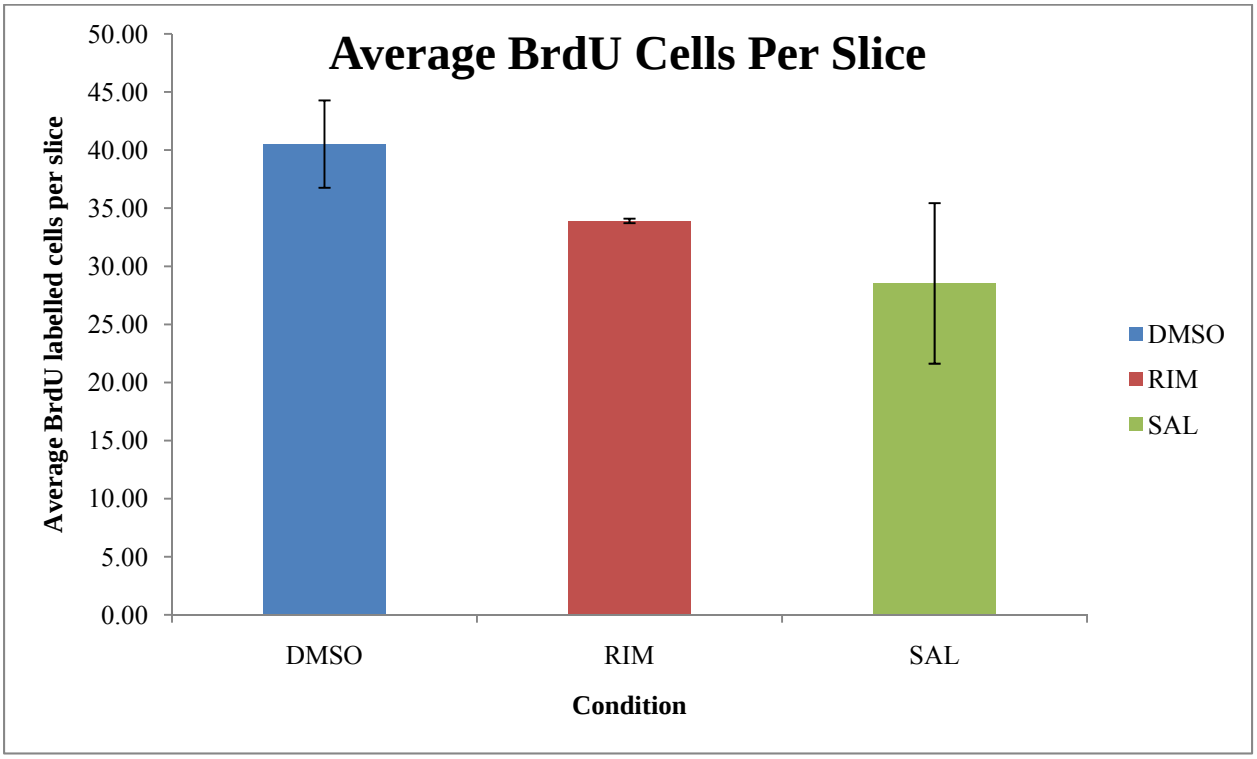

Figure 6. Bar graph of average BrdU cells per slice for each condition. Vehicle rats show the highest number of cells, followed by rimonabant rats, and saline rats show the fewest cells. There is no significant difference between the three conditions (p=.154).