Robust stress response does not alter cell proliferation and survival in adult rat hippocampus after acute predator stress

By

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Abstract

Traumatic events contribute to a variety of neuropsychiatric disorders including post-traumatic stress disorder (PTSD). Identifying the mechanisms underlying the stress response may aid in understanding the development of, or improving treatment options for, these debilitating disorders. Neurogenesis, the production of new neurons, is known to occur in the subgranular zone (SGZ) of the adult mammalian hippocampus. While the reduction in adult neurogenesis following chronic stress is largely supported, acute stress models, particularly predator stress, have yielded inconsistent results. Thus, the goal of the current study was to help elucidate the effects of predator stress on adult hippocampal neurogenesis. This study implements a single, unprotected cat exposure which produces anxiety-like behaviors and hyperarousal in rats for up to three weeks. Despite a robust stress response detected by elevated corticosterone (CORT) in predator stressed rats, predator stress had no effect on the total number of 5-bromo-2'-deoxyuridine immunoreactive (BrdU-IR) cells in the SGZ at 2 hours or 4 weeks post-stressor. However, acoustic startle (measure of hyperarousal behaviour) along with predator stress significantly reduced BrdU-IR cells in comparison to rats that were only predator stressed.

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Table of Contents

List of Figures

List of Abbreviations

ABC	Avidin-Biotin Complex
ANOVA	Analysis of Variance
B#	Bin Number
BrdU	5-bromo-2'-deoxyuridine
CORT	Corticosterone
DAB	3,3'-diaminobenzidine
DG	Dentate Gyrus
EPM	Elevated Plus Maze
GCL	Granular Cell Layer
GR	Glucocorticoid Receptor
HC	Handled Control
HPA	Hypothalamic-Pituitary-Adrenal axis
IHC	Immunohistochemistry
i.p.	Intraperitoneal
LA, C, I, M, AC	Amygdala Regions (Lateral, Central, Intercalated, Medial, Anterior Cortical)
NaN_3	Sodium Azide
NDS	Normal Donkey Serum
PBS	Phosphate Buffered Saline
PS	Predator Stressed
PTSD	Post-traumatic Stress Disorder
RIA	Radioimmunoassay
RPM	Revolutions per minute
SGZ	Sub-granular zone
TMT	2,3,5-Trimethyl-3-thiazoline
U, L	Hippocampus (Upper, Lower blade)
VMH(r,d,l)	Ventral Medial Hypothalamus (region, dorsal, lateral

1.0 Introduction

1.1 Post-traumatic stress disorder (PTSD)

A single traumatic psychological or physical event can be sufficient to cause the onset of stress-related disorders such as post-traumatic stress disorder (PTSD). Now classified as a trauma- and stress-related disorder, the diagnosis of PTSD focuses on four behavioural criteria which include re-experiencing memories of the traumatic event, avoiding trauma-associative stimuli, negative cognition or mood and arousal (5th ed; DSM-V, American Psychiatric Association, 2013). More than two-thirds of the general population are estimated to be exposed to trauma in their lifetime (Breslau et al., 1998; Breslau, Davis, Andreski, & Peterson, 1991), although this may be more prevalent in geographical areas vulnerable to wars, terrorism and natural disasters. Prevalence of PTSD also increases depending on level of exposure; direct victims of disasters range from 30-40%, rescue workers range from 10-20%, while the general population shows the lowest range of prevalence from 5-10% (Neria, Nandi, & Galea, 2008). In the United States and Canada, the lifetime prevalence of PTSD is at 6.8% and 9.2% respectively (Kessler et al., 2005; Van Ameringen, Mancini, Patterson, & Boyle, 2008). Arguably one of the most violent mass attacks in North America was the terrorist attack on September 11, 2001 in New York, which increased the prevalence of PTSD immediately following the attack (Silver, Holman, McIntosh, Poulin, & Gil-Rivas, 2002). Over the past decade, the world has experienced some of the largest natural disasters from Hurricane Katrina which hit New Orleans in 2005 to the more recent earthquake and tsunami in Japan in

2011. Several reports showed an increase of PTSD symptoms in many victims, survivors and rescue team members in both these traumatic events (Kessler et al., 2008; Kukihara, Yamawaki, Uchiyama, Arai, & Horikawa, 2014). Current treatments such as cognitive behavioural therapy and selective serotonin reuptake inhibitors improve PTSD symptoms in patients with a wide range of traumas (Ehlers et al., 2013; Steckler & Risbrough, 2012). However, the efficacy of current PTSD treatments is variable among individuals and therefore, recovery is not guaranteed. Thus, understanding the mechanisms underlying the stress response is critical. An ongoing phenomenon called adult hippocampal neurogenesis could be a promising target for treatments in mental disorders including PTSD, due to the important role of the hippocampus in maintaining healthy and adaptive responses to stress.

1.2 Adult hippocampal neurogenesis

One of the most striking changes following exposure to chronic stress is a reduction of newly born cells within the subgranular zone (SGZ) in the adult hippocampus (Mirescu & Gould, 2006). The postnatal generation of new neurons in the dentate gyrus (DG) of the hippocampus (Altman & Das, 1965) is now a widely accepted phenomenon. Neurogenesis can be measured by three main stages: cell proliferation, differentiation and survival (Schoenfeld & Gould, 2012). Cell proliferation occurs at the border of the granule cell layer and hilus (also known as the SGZ), where stem cells (Type 1 cells) divide to produce daughter progenitor cells (Type 2 cells). Progenitor cells are then programmed to become neurons, astrocytes or oligodendrocytes (differentiation),

although a majority of surviving adult born cells become neurons in vivo (DeCarolis & Eisch, 2010), while a small population become glia (Cameron, Woolley, McEwen, & Gould, 1993). Progenitor cells then become neuroblasts and develop into immature neurons and eventually migrate into the molecular layer to make connections in the perforant pathway and also extend through the hilus to the CA3 region in the hippocampus. The point at which progenitor cells become neurons is still unclear, however it takes several weeks to months before adult born neurons become fully incorporated into the hippocampal circuit (DeCarolis & Eisch, 2010). A variety of mammals demonstrate this process well into adulthood, suggesting a functional role for neurogenesis, although this has yet to be elucidated.

Much more is known about neurogenesis in the SGZ, however recent studies suggest that neurogenesis and cell proliferation occur in the amygdala and ventral medial hypothalamus (VMH) (Fowler, Liu, & Wang, 2008). This is not surprising given the critical involvement of both the amgygdala and VMH in the stress response (Anthony et al., 2014).

1.3 The link between stress and adult hippocampal neurogenesis

Along with the hypothalamus, the hippocampus is involved in the negative feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis which regulates the level of stress hormones known as glucocorticoids; cortisol in humans and corticosterone (CORT) in rodents. Upon a stressful encounter, glucocorticoids along with epinephrine and norepinephrine are released from the adrenal glands as part of the body's fight or

flight response. Under chronic stress, the negative feedback loop is disrupted and glucocorticoid levels increase to a toxic level which can stunt the immune response and promote cell death (Behl et al., 1997). Such a dysregulation of glucocorticoids is associated with cognitive impairments, stress-disorders and depression (Holsboer & Ising, 2010; McEwen, 2007).

Both stress and exogenous glucocorticoid administration can produce adverse effects on the hippocampus. Chronic CORT administration (10mg daily for 21 days) reduces the number of apical dendritic branches in CA3 pyramidal cells (Woolley, Gould, & McEwen, 1990). In addition, exogenous CORT decreases cell proliferation and survival in the DG (Brummelte & Galea, 2010; Wong & Herbert, 2004). This is consistent with a study that showed that new neurons in the hippocampus are required for regulating endocrine and behavioural stress responses (Snyder, Soumier, Brewer, Pickel & Cameron, 2011). Neurogenesis-deficient mice demonstrate a slower recovery of glucocorticoid levels after an acute stressor and are unable to regulate glucocorticoids after dexamethasone (synthetic glucocorticoid) administration. Furthermore, these mice show increased food avoidance in a novel environment, increased despair in the forced swim test and a decreased sucrose preference indicative of a depression-like phenotype. Chronic CORT administration, as well as restraint stress, increases depression-like behaviours without altering anxiety (Gregus, Wintink, Davis, & Kalynchuk, 2005). In contrast, acute injection of CORT produces neither depression-like behaviors nor changes in the HPA axis response to a novel stressor (Johnson, Fournier, & Kalynchuk, 2006).

Glucocorticoids have been strongly suggested to be responsible for mediating the stress induced decrease in cell proliferation (Cameron & Gould, 1994; Gould, Cameron, Daniels, Woolley, & McEwen, 1992), as other peripheral hormones from the adrenal gland do not cross the blood brain barrier (Weil-Malherbe, Axelrod, & Tomchick, 1959). Adrenal steroid regulation of cell proliferation is not direct, as progenitor cells do not express glucocorticoid receptors. However, glucocorticoids may be involved in an indirect way via N-methyl-D-aspartate (NMDA) receptors which are a part of an excitatory pathway (Cameron, Tanapat, & Gould, 1998). Along with an increase of glucocorticoids, stress stimulates an excitatory electrophysiological response in the DG (Heale, Vanderwolp, & Kavaliers, 1994). Thus, exciting this pathway via NMDA receptors may be one of the mechanisms that reduces cell proliferation.

1.4 Implications of adult neurogenesis for mental illness

Measures of neurogenesis in humans have previously been done in post-mortem analysis (Eriksson et al., 1998). Thus, for obvious ethical reasons, measures of neurogenesis following stress in humans are not done. However, correlates of neurogenesis have been established to obtain indirect measures of neurogenesis, such as DG blood volume using neuroimaging techniques and neuropsychological test batteries (Déry et al., 2013). Individuals that score high on the Beck Depression Inventory or Perceived Stress Scale show poor performance on cognitive tasks that are suggested to be linked to lower neurogenesis levels (Becker & Wojtowicz, 2007).

Computational and cognitive neuroscientists have developed models in order to understand the functional purpose of neurogenesis. One computational process that has long been associated with neurogenesis in the DG is pattern separation (Deng, Aimone, & Gage, 2010). Pattern separation is a process where similar episodes are characterized as discrete non-overlapping representations, thus providing a means to discriminate highly similar experiences (Yassa & Stark, 2011). Computational models of the hippocampus suggest that DG granule cells are responsible for separating overlapping representations arriving from the entorhinal cortex before projecting the signal to the CA3 region (Becker & Wojtowicz, 2007; O'Reilly & Norman, 2002). Such a process may be relevant for understanding PTSD, as impairments in pattern separation in the DG could be responsible for the overgeneralization of fear responses to emotional stimuli apparent in PTSD and other anxiety-related disorders (Kheirbek et al., 2012). For example, a soldier with PTSD may show heightened arousal to a cue that is associated with a traumatic war experience (such as a campfire) despite other contextual cues in the environment that would indicate otherwise (park setting). This failure to discriminate between similar episodes could be due to low neurogenesis levels.

1.5 The importance of animal models in stress and neurogenesis

Hippocampal neurogenesis is particularly difficult to study in humans since there is no non-invasive method to accurately measure new neurons in the DG. Thus, empirical studies rely heavily on animal models to further understand the functional role of neurogenesis. The overlap in similar mechanisms between experimental animals and

humans can help neuroscientists identify potential mechanisms involved in a range of mental illnesses, with the goal of finding effective treatments for these debilitating diseases. Many scientific discoveries have been made first in animals, including non-human mammals. In fact, the discovery of neurogenesis was first shown in rats (Altman & Das, 1965) before being shown in humans (Eriksson et al., 1998). The use of animals in stress models has allowed scientists to measure and manipulate types of stress, which fall under the broad categories of chronic or acute.

1.6 Chronic stress models and adult hippocampal neurogenesis

Chronic stress typically involves repetitive exposures that can have damaging effects on physiology and behaviour. Specifically, stressors such as electric shock and restraint (inescapable) stress decrease cell proliferation, neuronal differentiation and survival (Dagyte et al., 2009; Pham, Nacher, Hof, & McEwen, 2003; Rosenbrock, Koros, Bloching, Podhorna, & Borsini, 2005). These same results can also be produced by unpredictable stressors which use a combination of conditions including restraint, food/water deprivation, group housing, shaker stress and cold swims (Heine, Maslam, Zareno, Joels, & Lucassen, 2004; Li et al., 2006; Xu et al., 2007). Furthermore, prolonged maternal separation during early life decreases cell proliferation and immature neurons in the DG of adult rats (Mirescu, Peters, & Gould, 2004). In contrast, others have found a decrease in survival but no change in proliferating cells (Lee et al., 2006) or no effect at all (Hanson, Owens, Boss-Williams, Weiss, & Nemeroff, 2011) following chronic stress. Housing conditions following chronic stress may help explain these

discrepancies. Male rats housed individually following chronic stress show a decrease in neurogenesis compared to those that are socially housed, while females housed individually following chronic stress show increased neurogenesis compared to those socially housed (Westenbroek, Den Boer, Veenhuis, & Ter Horst, 2004). In addition to the adverse consequences of chronic stress on adult neurogenesis, an increase in adrenal gland size (Ulrich-lai, Arnhold, Engeland, Yvonne, & William, 2006), anxiety levels (Mineur, Belzung, & Crusio, 2007) and memory deficits (Yun et al., 2010) have been reported after chronic exposures.

One model which is particularly relevant to PTSD is the social defeat paradigm. During this stressor, experimental animals (intruders) are subjected to an aggressive conspecific (resident). Social defeat is observed when the intruders respond with submissive body postures or freezing behaviour. Following the stressful encounter, subordinated animals demonstrate signs of stress, such as social avoidance, anxiety and hyperactivity (Venzala, García-García, Elizalde, Delagrange, & Tordera, 2012). Defeat-induced social avoidance can last for weeks and even months, but can be reversed by repetitive antidepressant treatment (Yan et al., 2010). Early studies using chronic social defeat showed alterations in hippocampal pyramidal neurons (Nissl staining intensity of nucleoplasm) (Fuchs, Uno, & Flügge, 1995) as well as a suppression of adult neurogenesis and a decrease in granule cell layer volume in tree shrews (Fuchs, Flugge, McEwen, Tanapat & Gould, 1997). Similarly, adult male rats and mice show a reduction in both proliferation and survival of newly generated hippocampal granule cells following social defeat stress (Czéh et al., 2002; Mitra, Sundlass, Parker, Schatzberg, & Lyons, 2006; Yap et al., 2006).

Not all animals will have the same stress response during social defeat. Animals that are more resilient show fewer social defeat characteristics in comparison to their susceptible counterparts. By separating animals into susceptible and resilient populations, Krishnan et al. (2007) were able to identify molecular mechanisms underlying resilience in the mesolimbic dopamine circuit, including specific genes which are significantly upregulated in the ventral tegmental area of unsusceptible mice. A subsequent study followed suit by isolating populations based on vulnerability and showed that mice showing persistent stress-induced avoidance behaviour during the social defeat had more surviving neurons in the DG. When neurogenesis was ablated using X-ray irradiation, this behaviour was inhibited, suggesting a functional role for adult hippocampal neurogenesis in producing stress-induced social avoidance (Lagace et al., 2010). Defining susceptible populations during the social defeat paradigm provides a way to consider individual differences that are observed in mental disorders such as PTSD. Overall, chronic stress models provide a stress intensity which can cause severe physiological and behavioural changes that can persist for long durations. However, stress does not necessarily need to be chronic for individuals to show symptoms of PTSD; sometimes acute exposure is sufficient.

1.7 Acute stress models and adult neurogenesis

Acute stress typically involves a single short exposure to a stressor. Early studies showed that acute social defeat paradigms were sufficient to suppress hippocampal neurogenesis in tree shrews (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997) and

decrease proliferating granule cell precursors in adult marmoset monkeys (Gould, Tanapat, McEwen, Flügge, & Fuchs, 1998). A single exposure to unpredictable stressors such as (cold) immobilization or forced swim tests can also suppress proliferation in the DG as well as cell survival (Heine et al., 2004; Koo, Russo, Ferguson, Nestler, & Duman, 2010; Vega-Rivera, Fernández-Guasti, Ramírez-Rodríguez, & Estrada-Camarena, 2013). As demonstrated by restraint and electric shock studies, acute stressors may have a greater impact on cell proliferation than cell survival (Bain, Dwyer, & Rusak, 2004; Malberg & Duman, 2003). This may be due to the relatively short exposure of stress, which limits the amount of time to impact cell survival. However, there are some inconsistent results in the acute stress and neurogenesis literature. For example, acute social defeat stress results in a decrease of surviving cells but no difference in cell proliferation in the DG (Thomas, Hotsenpiller, & Peterson, 2007), while a more recent study demonstrated an increase of neurogenesis in the dorsal hippocampus following acute (3 hour) immobilization stress (Kirby et al., 2013). Studies comparing both chronic and acute restraint stress have also reported no differences in cell proliferation after an acute stressor (Pham et al., 2003; Rosenbrock et al., 2005). One study using acute restraint and tail shock found no significant effect on neurogenesis, as well as no reduction in brain derived neurtrophic factor (BDNF) mRNA in stressed animals (Hanson et al., 2011). BDNF contributes to neuronal survival, morphology and plasticity in CNS (Lewin, 1996; Thoenen, 1995) therefore any suppression of neuron generation is associated with lower levels of BDNF. This has been shown in both chronic and acute restraint stress (Murakami, Imbe, Morikawa, Kubo, & Senba, 2005).

In comparison to chronic stress, fewer studies have examined stress-induced behavioral changes following acute stress. This is particularly important because: 1) a single stressful exposure can be enough to produce severe symptoms of PTSD and 2) models should reliably produce similar symptoms to those seen in the human condition. These types of behavioural changes are well established in predator stress models.

1.8 Predator stress and neurogenesis

Predator stress (PS) is both fear provoking and stressful (Adamec, Kent, Anisman, Shallow, & Merali, 1998; Blanchard & Blanchard, 1989; McGregor, Schrama, Ambermoon, & Dielenberg, 2002) and typically involves unprotected exposure of a rodent to a predator or predator odor (Adamec & Shallow, 1993; Adamec, Walling, & Burton, 2004; Cohen, Zohar, & Matar, 2003;Muñoz-Abellán, Andero, Nadal, & Armario, 2008; Muñoz-Abellán, Daviu, Rabasa, Nadal, & Armario, 2009). This traumatic experience is ecologically valid as it exposes the animal to an event which could be encountered in their natural environment. In addition, PS reliably induces hyperarousal (enhanced acoustic startle response) which closely resembles one of the core symptoms in PTSD patients (Adamec, Blundell, & Burton, 2003; Adamec, Head, Soreq & Blundell, 2008; Adamec & Shallow, 1993; Cohen et al., 2003). This result is relevant as increased generalized anxiety is co-morbid with PTSD (Pitman, Orr, & Shalev, 1993).

The most common predator odors used in rodent studies are 2,3,5-Trimethyl-3-thiazoline (TMT; a component of fox feces) and cat odor, although ferret odor can have similar stress and/or anxiety effects (Campeau, Nyhuis, Sasse, Day, & Masini, 2008). Cat

odor produces reliable measures of behaviours related to PTSD in rodents, such as increases of defensive responses including avoidance (e.g. flight and hiding), risk assessment (cautious investigation of potential threat) and decreases of non-defensive responses , including feeding and grooming (Staples, 2010). TMT is often used at high concentrations which may not necessarily be observed in the animal's natural habitat. However, a recent study conducted by Hacquemand, Choffat, Jacquot, and Brand (2013) compared low doses of TMT with cat odor and found that solutions containing 1% or 0.1% TMT produced similar anxiety and fear related behaviours as seen in responses to cat odor. The efficacy of each odor is variable across laboratories, with odors from the fur/skin generally producing more robust defensive responses compared to urine/feces (D. C. Blanchard et al., 2003; Masini, Sauer, & Campeau, 2005). Odors derived from urine/feces may not be as predictive of a predatory threat, since predators may selectively defecate or urinate in areas where they do not hunt (Staples, 2010). However, some studies have produced consistent anxiety-like behavior and hyperarousal following the use of cat urine/feces (Hagit Cohen et al., 2004, 2003; Goswami, Samuel, Sierra, Cascardi, & Paré, 2012).

To our knowledge, adult hippocampal neurogenesis in rats has only been examined following exposure to the predator odor, TMT. A single exposure of TMT can suppress proliferation of cells in the DG (Galea, Tanapat & Gould, 1996). This was further supported by Tanapat, Hastings, Rydel, Galea, and Gould (2001), however this effect was brief as the decrease in granule cells was not seen three weeks after predator odor exposure. The study also showed that the suppression of cell proliferation was driven by the stress-induced rise in CORT, as adrenalectomies prevented the transient

decrease of granule cells and immature neurons. Interestingly, the inhibition of cell proliferation in the hippocampus may be dependent on the sex of the animal as Falconer and Galea (2003) demonstrated a decrease in neurogenesis in adult male rats but not in females. Although there is a general consensus of the suppressing effects of TMT on proliferating cells in the DG, Thomas, Urban, and Peterson (2006) demonstrated that despite a rise in CORT, there was no difference in cell proliferation following exposure to the fox odor. However, a lower concentration of 5-bromo-2'-deoxyuridine (BrdU) was used for labeling proliferating cells and injections were given prior to the stressor, thus cells labeled may not have given an accurate representation of the effect of predator stress. Neither the effects of cat odor nor exposure to cats on neurogenesis have been investigated despite the robust behavioral responses following cat odor/exposure (Adamec & Shallow, 1993, Blanchard, Blanchard, Tom & Rodgers, 1990; Cohen et al., 2004). Thus, the goal of our study was

to examine the effects of cat exposure on proliferation and survival of newly born cells in the adult rat brain.

Overall, the use of predator stress as a valid PTSD animal model has been recently emphasized due to its representation of: 1) realistic life and death circumstances compared to a physical stressor (e.g. restraint) and 2) long –term outcomes or symptoms of psychological trauma (Goswami, Rodríguez-Sierra, Cascardi, & Paré, 2013). Therefore, understanding the mechanisms underlying stress-induced changes following predator stress may aid in the development of novel, more effective treatment options for stress-related disorders such as PTSD.

1.9 Examining adult hippocampal neurogenesis by using a cat exposure

There are very few labs in the world that use direct cat exposures to model PTSD. Those that do often keep animals protected in an enclosure during the exposure (Zoladz, Conrad, Fleshner, & Diamond, 2008). Our lab uses a single unprotected cat exposure to capture an experience which best corresponds to an exposure that could occur in the animal's natural environment. This PTSD model has been shown to generate consistent anxiety-like behaviours and hyperarousal for up to three weeks following the predator stress (Adamec & Shallow, 1993; Adamec et al., 2004). Despite this, to our knowledge, there are no studies that have examined the effects of cat exposure (in any form) on adult hippocampal neurogenesis (proliferation or survival). Given that stressful exposures can decrease neurons in the DG by downregulating cell proliferation (Gould & Gross, 2002), we hypothesized that predator stressed rats would show a reduction in both cell proliferation and survival in comparison to controls.

2.0 Methods

2.1 Animals

A total of 73 six week old, male Long Evans rats (Charles River, Canada) were used in the three studies. All rats were housed individually in transparent plastic cages with wire covers, which held food (standard rat chow) and water that was available ad libitum with lights on at 7am. Rats were randomly labelled as predator stressed or handle control animals and were handled daily for five days before treatment. After this, labelled predator stressed animals would receive the cat exposure and then moved into a separate room. This was done to ensure that the any olfactory cues from predator stressed animals

would not affect control animals. This method for housing animals has consistently been done in previous studies using the same paradigm (Adamec et al., 2004; Adamec & Shallow, 1993). Procedures for Studies 1-3 adhered to the guidelines of the Canadian Council on Animal care, and were approved by the Institutional Animal Care committee of Memorial University.

2.2 Experiment 1: The effects of predator stress on cell proliferation

A total of 18 male Long Evans (Charles River, Canada) rats were used in the study. There were two groups of rats (n=9): handled control (HC) and predator stressed (PS). During the first day of handling, rats were picked up and stroked gently. However, for the latter days, rats were habituated to a handling procedure that was used during blood collection from the tail. This involved wrapping them gently but firmly in a towel. Rats in the PS group were exposed to the cat for ten minutes. A full description of the predator stress encounter can be found in section 2.5 entitled "Predator Stress Paradigm and Behavioural Measures". Following predator stress, rats were firmly held in a towel (as described above) and blood was collected from the end of the tail. Blood collection was used for a CORT assay. A detailed procedure can be found in section 2.7 entitled "Collection of blood and determination of CORT levels". After this procedure, rats were given an intraperitoneal (i.p.) injection of 5-bromo-2'-deoxyuridine (BrdU) and placed back into their cage. After 2 hours, animals were given an overdose of sodium pentobarbital (150mg/kg) and perfused transcardially with 50 mL of phosphate buffered saline (PBS) (7mL/min) followed by 120 mL of 4% paraformaldehyde (7mL/min) using

a peristaltic pump. Two hours is sufficient time for BrdU to become incorporated into the DNA of new cells. The HC group followed a similar procedure except the rats were handled only (not exposed to a cat) on treatment day.

2.3 Experiment 2: The effects of predator stress on cell survival

A total of 15 male Long Evans rats were used in the study (5 HC and 10 PS). The PS group was exposed to a ten minute cat exposure, followed by an i.p. BrdU injection. Rats were placed back into their designated cage and left undisturbed for the next four weeks. The HC group underwent the same procedure; however they were handled only on treatment day. After four weeks, rats were given the same deep anesthetic and perfused following the same protocol as described above.

2.4 Experiment 3: The effects of predator stress on startle response and cell survival

A total of 40 male Long Evans rats were used in the study (20 HC and 20 PS). The PS group was exposed to a ten minute cat exposure, followed by an i.p. BrdU injection. Rats were returned to their cages and left undisturbed until the acoustic startle test which occurred seven days after the predator stress. Following the startle testing, rats were returned to their home cages and left undisturbed for the next three weeks, at which point they underwent the same endpoint as noted previously. The HC group was handled only on treatment day, then underwent the same procedures as the PS group. A summary of all studies can be found in Figure 1.

2.5 Predator Stress Paradigm and Behavioural Measures

All cat exposures occurred in an enclosed room with a floor area of 3.25 square meters (0.91m by 2.1 m) divided into 30.5cm squares with masking tape. To minimize human contact prior to the exposure, rats were placed in a small chamber (used for transferring the animal to the room) for approximately five minutes. This chamber fit into a small opening to the room and was opened to allow the rat to enter the room once the cat and all apparatuses were prepared (Adamec & Shallow, 1993, See Figure 2 for schematic). Exposures lasted 10 minutes and were videotaped to capture the behaviour of both the rat and the cat. Cat responses consisted of the number of approaches to the rat, vocalizations, pawing and the occasional mild attack. No rats were injured during the exposure and all were exposed to the same adult male cat. Rat behaviour measures included the number of approaches to the cat, flights from the cat, locomotor activity (number of masking tape lines crossed) and the frequency of proximity to the cat (within one square from cat).

2.6 Acoustic Startle Testing

Acoustic startle responses were calculated as previously described (Adamec et al., 2004). For startle testing, rats were placed into a cylindrical enclosure (measuring 12.7cm long and 3.7cm in diameter) inside the startle chamber (San Diego Instruments). The animal enclosure sat on a piezo electric transducer that produced an electrical signal sampled by a computer, providing a measure of rat movement. During the test, the chamber was closed and rats acclimated to the startle apparatus (5 minutes of 50dB white

noise) in the dark. Following this, rats were exposed to 30 pulses of 50 millisecond bursts of 105dB white noise from the 50dB background for 15 minutes. There was a 30s inter-trial interval. For each trial, startle amplitude was obtained by subtracting the transducer output at the beginning of the noise burst (Vstart) from the maximum transducer output (Vmax) during the 150 ms recording window (Vmax-Vstart).

2.7 Collection of blood and determination of CORT levels

One hour before the predator exposure or handling, lidocaine cream was put on the end of each rat's tail to ensure blood collection would be as painless as possible. While the animal was firmly held with a towel, a tail nick was performed with a scalpel. The blood was collected into a 1mL Eppendorf tube with (20 µl) Heparin for one minute (following the tail nick) by gently milking the tail and then set on ice. All samples were centrifuged at 1500 revolutions per minute (RPM) for 20 minutes before aliquoting at least 30 µl of serum into a new Eppendorf tube. Serum samples were then stored at -20°C. The CORT assay was completed at Carlton University, Ottawa by Dr. Shawn Hayley. CORT levels were measured by a commercial radioimmunoassay (RIA) kit (ICN Biomedicals, CA, USA). Inter-assay variability was avoided by assaying all samples (in duplicate) within a single run.

2.8 BrdU injection and processing

For all experiments, rats received an i.p. injection of BrdU (150 ug/g, Roche Diagnostics) immediately after the predator stress or handling. BrdU labels proliferating

cells in the S-phase of the cell cycle after the exposure. During the S-phase, DNA is replicated and since BrdU acts as a thymidine analog, it can replace the specific nucleoside that pairs with adenosine. In doing so, it becomes incorporated with the DNA of new born cells and can be detected using immunohistochemistry (Magavi & Macklis, 2008).

2.8.1 Immunohistochemistry procedures

After animals were perfused, either two hours (Experiment 1) or four weeks (Experiments 2 and 3) following BrdU injection, the brains were placed into 50 mL falcon tubes with 4% paraformaldehyde. They remained in the fixative for 24 hours before being transferred into 30% sucrose with 0.1% sodium azide (NaN_3) in 0.1M PBS and stored at 4°C. The brains were shipped to the University of Ottawa for further processing. The brains were sectioned coronally on a freezing microtome at a 30 µm thickness. Nine serial sets of sections were stored in 0.1% NaN_3 in 1XPBS at 4°C until processing. One series of sections (sixth series) from the most anterior part of the hippocampus to most posterior was mounted onto glass slides (Superfrost/Plus; Fisher) and set aside to dry overnight. Slides were specifically coded to ensure objectivity during cell counting. Sections underwent pretreatment which consisted of antigen unmasking (heated in 0.01M citric acid, pH 6.0, 15 minutes), membrane permeabilization (0.1% trypsin in 0.1M Tris and 0.1% $CaCl_2$, 10 minutes) and DNA denaturation in 2N hydrochloric acid in 1X Tris base saline (TBS) for 30 minutes. Following pretreatment, nonspecific staining was blocked with 3% normal donkey serum (NDS; vol/vol) in 0.3% TritonX-100 in 1X TBS for 60 minutes. Between each step, sections were rinsed with

1XTBS. Sections were then incubated with rat anti-BrdU primary antibody (1:300; Accurate Chemical) in 3% NDS (vol/vol) and 0.3% Tween-20 in 1X TBS) overnight. The following day, sections were incubated with biotinylated-donkey anti-rat secondary antibody (1:200; Sigma Laboratories) in 1.5% NDS (vol/vol) for 60 minutes, followed by 0.3% hydrogen peroxide (Sigma Laboratories) for 30 minutes and avidin-biotin complex (ABC; Vector Laboratories) for 90 minutes. BrdU staining was visualized by using 3,3'-diaminobenzidine (DAB; Pierce) for 20-30 minutes, counterstained with Nuclear Fast Red (Vector Laboratories) and coverslipped with DPX. See Appendix II: Protocols - A for full immunhistochemistry protocol.

2.82. Cell counting procedures

Brightfield sections were visualized and quantified with an Olympus DP-72 microscope. Staining was examined and quantified at 40x magnification in the SGZ of the dentate gyrus of the hippocampus (bregma:-1.92 to -6.6mm). The SGZ was defined as the region between the hilus and the granular cell layer (GCL): two GCL cell widths into the hilus and the inner half of the GCL. Cells and clusters were quantified using a procedure by the Lagace lab at the University of Ottawa (Appendix II: Protocols- B). Sections from rats sacrificed 2 hours after BrdU injection were quantified as cell proliferation, whereas section from rats sacrificed after four weeks were quantified as cell survival.

For the purpose of consistency, only the dorsal (bregma: -2.52 to -4.36 mm) CA1 region was measured as the boundaries became more challenging to set in posterior regions. Proliferating cells were counted in the region above the molecular layer of the

upper DG arm (entire length), past the pyramidal cell layer but underneath the corpus callosum (Appendix II: Protocols- C).

Regions in both the amygdala (bregma: -2.28 to -3.72mm) and the ventral medial hypothalamus (VMH) (bregma: -1.92 to -3.36mm) were also analyzed in the cell proliferation group. The amygdala was divided into the following regions: lateral, central, intercalated, medial and anterior cortical. These areas changed across the bregma boundary, thus two types of templates were made, one for the anterior and another for the posterior areas. All templates were constructed using the Image-Pro Plus 7.0 software using the AOI tool. Corresponding worksheets (Appendix II: Protocols- D) were made to aid in the counting process as there was no way to have access to a template while changing magnification. Due to tissue damage in the amygdala or lack of BrdU positive staining, only the cell counts of 3 HC and 4 PS rats were counted. The VMH was divided into three areas which included the VMH (before it diverges into specific regions), VMH dorsal and VMH lateral. Since the VMH was smaller, a simple template was used to outline the general area of the VMH regions on either side of the brain (Appendix II: Protocols-E). Only one subject was excluded in the VMH analysis due to damage.

2.9 Statistical Analysis

In Experiment one, 4 HC and 3 PS animals were excluded due to lack of positive staining in the DG. Thus, the total number of animals was reduced to 11 (5 HC and 6 PS). In experiment two, 2 HC and 4 PS animals were excluded due to lack of BrdU positive staining. Thus, the total number of animals was reduced to 3 in HC condition and 6 in the

PS condition. In study three, 8 animals in both HC and PS conditions were excluded due to lack of BrdU positive staining. One animal in each condition was also excluded due to a malfunctioning acoustic startle box, which resulted in incorrect output. Thus, the total number of animals was reduced to 22 with 11 in both HC and PS conditions.

Cell/cluster count differences and startle responses between PS and HC were conducted by using Independent-samples T tests and Repeated Measures two-way Analysis of Variance (ANOVA) for analyses across AP plane. AP boundaries, which included five bins, were used to organize cell and clusters. The bins corresponded to the following AP ranges: Bin 1 = -1.92 to -2.4mm, Bin 2 = -2.64 to -3.72mm, Bin 3 = -3.84 to -4.68mm, Bin 4 = -4.8 to -5.88mm , Bin 5 = -6 to -6.6mm. The amygdala and VMH were not divided into AP bins as the boundaries were smaller (approximately 1mm difference). It was judged not to be worthwhile to separate and analyze cells/clusters in this manner. One-way ANOVAs were done to compare cells and clusters in different areas of the same brain region (e.g., amygdala) as well as counts across proliferation and survival groups. Post-Hoc Bonferroni tests were conducted if ANOVAs were significant. The Pearson's r correlation was used to compare CORT levels, cell/cluster counts, PS behaviour during the cat exposure and acoustic startle responses.

For the acoustic startle analysis, peak startle amplitude was calculated by taking the average startle amplitude across 30 trials. However, for the habituation analysis, trials were placed into 10 bins to summarize the overall trend seen across 30 trials. Each bin was an average of startle amplitudes of three trials. For example, the averages of trials 1, 2, and 3 were placed in Bin 1. For the startle habituation analysis, percentages of binned trials were calculated as follows:

Percent habituation = 100*(Bin1-Bin2)/Bin1

A positive percentage indicates that habituation (decrease in startle amplitude from previous trial) occurred. Negative percentages indicate an increase in startle amplitude from the previous trial.

3.0 Results

3.1 Experiment 1: Acute predator stress increases corticosterone, but does not alter cell proliferation

3.1.1 Robust stress effect following predator stress exposure.

Serum was obtained for CORT measures to verify whether predator stressed animals displayed a physiological stress response during the cat exposure. As expected, PS increased serum CORT levels (24.9 ± 1.2 ug/dL) above that of the handled controls HC (6.6 ± 1.2 ug/dL, $t(9.2)= -6.02$, $p<0.001$, Figure 4).

3.1.2 Predator stress does not alter the number of BrdU positive cells or clusters in the sub-granular zone of the dentate gyrus.

Despite the strong CORT response, there was no overall difference in proliferating cells($t(9) = -0.92$, $p=0.38$, Figure 5a) or clusters($t(9) = -1.37$, $p=0.21$, Figure 5b) in the SGZ between PS and HC animals (see Figure 3 for tissue comparison). Consistent with previous work (Lagace, Yee, Bolaños, & Eisch, 2006), analysis of BrdU positive cells across the anterior-posterior (AP) plane of the hippocampus revealed an increase in the number of proliferating cells ($F(2.09, 18.79)= 40.30$, $p<0.001$, Figure 6a)

and clusters ($F(2.17, 19.51) = 49.62$, $p<0.001$, Figure 6b) in posterior sections of the hippocampus relative to anterior sections with the exception of the last bin (AP boundary), where the hippocampus reaches its end and the number of cells decrease. There were no significant interactions for the HC and PS cells ($F(2.09, 18.79)= 0.21$, $p=0.82$) or clusters ($F(2.17, 19.51)= 0.307$, $p=0.756$) across the AP plane demonstrating that proliferation numbers between HC and PS were not significantly different across bins.

Previous work has also shown more neural stem cells in the upper blade of the dorsal dentate gyrus (DG) in comparison to the lower blade in standard housing conditions (Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006), while others have shown that the opposite is true and that experiences such as social isolation can change the fate of neural stem cells in mice (Dranovsky et al., 2011). In the current study, we compared cells and clusters in upper and lower blades of the DG. There were no differences within group [HC: cells ($t(8)=-1.53$, $p=0.164$) or clusters ($t(8)= -1.88$, $p=0.097$), PS: cells ($t(10)= -1.62$, $p=0.136$) and clusters ($t(10)= -1.09$, $p=0.302$)] or between groups in upper blades [HC vs PS: cells ($t(9)= -0.537$, $p=0.608$) or clusters; ($t(9)= -1.193$, $p=0.264$)] or lower blades [HC vs PS: cells ($t(9)= 0.204$, $p=0.843$) or clusters ($t(9)= -0.886$, $p=0.399$)]. Previous studies were done on mice, therefore differences in upper and lower blades may not occur in rats. Alternatively, predator stress or handling could have affected the proliferation distribution; standard housing without handling was not done in any of our studies.

Lagace et al. (2010) previously showed that there is a significant negative correlation between CORT levels and proliferating cells, thus higher levels of CORT

were associated with fewer labeled cells in the SGZ. We did not see this relationship, as shown by no significant correlation between CORT and BrdU-positive cells or clusters respectively ($r(9)= 0.35$, $p=0.30$; $r(9)=0.5$, $p=0.118$). This correlation was also conducted separately for HC and PS. However, no significant relationship was found in cells or clusters in HC ($r(3)= -0.071$, $p=0.910$,; $r(3)= 0.248$, $p=0.688$) or PS animals ($r(4)= 0.295$, $p=0.571$; $r(4)= 0.348$, $p=0.499$).

3.1.3 Predator stress does not alter the number of BrdU positive cells or clusters in the CA1 Region (Dorsal)

The CA1 in the hippocampus is vulnerable to glucocorticoid-induced oxidative stress (Wang et al., 2005; You et al., 2009) and this can have deleterious effects on proliferating cells and neurons. Given a robust CORT response following PS (Figure 4), we examined cell proliferation in the CA1. Only the dorsal part of the CA1 was counted to avoid ambiguity in the ventral and posterior sections. No difference in cells ($t(6.48) = -0.363$, $p=0.728$, Figure 7a) or clusters ($t(7)= -0.163$, $p=0.875$, Figure 7b) between HC and PS animals were observed in the dorsal CA1. Additionally, no significant correlations between cells/clusters and CORT were found (Appendix I: Table 2).

3.1.4 Predator stress does not alter the number of BrdU positive cells or clusters in amygdala

Reports of newly born neurons in the amygdala of prairie voles (Fowler, Liu, Ouimet, & Wang, 2001) has influenced studies to consider examining this area in other mammals (Keilhoff, Becker, Grecksch, Bernstein, & Wolf, 2006) especially following

stressful experiences (Mitra et al., 2006). Given the extensive research showing a critical role of the amygdala in stress-induced changes following predator stress, we examined proliferating cells and clusters in the amygdala.

Overall, cell counts ($t(5)=-0.125$, $p=0.906$, Figure 8a) and cluster counts ($t(5)=-0.622$, $p=0.561$, Figure 8b) between PS and HC in the amygdala did not differ, nor were there differences in other amygdala regions (Appendix I: Table 3). Cell counts were not statistically different across regions within HC ($F(4, 10)= 3.03$, $p=0.071$) or PS ($F(4,15)= 1.57$, $p=0.234$), indicating a similar cell distribution in all regions (Figure 9a). However, in the cluster analysis for PS animals, there were significant differences in various regions ($F(4,15)= 7.79$, $p=0.001$, Figure 9b). More clusters were found in the lateral region compared to the intercalated ($p=0.023$) and cortical area ($p=0.029$). A greater number of clusters were also found in the medial region in comparison to the intercalated ($p=0.007$) and cortical ($p=0.008$) area. However, we found no significant differences in cluster counts in HC rats ($F(4,10)= 3.26$, $p=0.059$). Additionally, there were no significant correlations between CORT and cell or cluster counts (Appendix I: Table 3). Correlations between CORT and cells in specific regions of the amygdala were not conducted due to insufficient numbers of subjects.

3.1.5. Predator stress does not alter the number of BrdU positive cells or clusters in the Ventral Medial Hypothalamus (VMH)

In addition to the hippocampus and amygdala, neurogenesis has been previously reported in the VMH an area sensitive to stress (Kokoeva, Yin, & Flier, 2005). Overall, PS and HC animals did not show significantly different cell ($t(8)= -1.52$, $p=0.168$, Figure

10a) or cluster counts ($t(8)= -1.54$, $p=0.162$, Figure 10b). No significant changes in cells or clusters between condition were reported across all VMH regions (Appendix I: Table 4), demonstrating that each region had similar numbers of proliferating cells or clusters. Similarly, counts of the cell and cluster distribution in the HC ($F(2,12)= 0.440$, $p=0.654$, $F(2,12)= 0.472$, $p=0.635$) and PS ($F(2,12)= 0.209$, $p=0.814$, $F(2,12)=0.868$, $p=0.445$) groups did not show statistically significant differences, indicating consistent counts in all regions within condition (Figure 11a,b). In addition, CORT levels did not correlate with proliferating cells or clusters in the VMH (Appendix I: Table 4).

3.2. Predator stress behaviour in cell proliferation groups

Cat behaviour (e.g. vocal calls, cat approaches) was not included in the analysis due to insufficient data. CORT levels did not correlate with rat behaviour during the PS encounter (Appendix I: Table 5). In addition, the correlation between cell or cluster counts and PS behaviour were analyzed to look for PS behaviours that could be potential predictors of proliferation. However, there were no significant correlations between cells or clusters in the SGZ, CA1, or VMH (Appendix I: Table 5). The amygdala data could not be properly analyzed due to a low number of animals.

3.3. Experiment 2: Predator stress does not affect cell survival

Given that previous studies have found differences in survival of newly born cells, despite no difference in cell proliferation (Lee et al., 2006; Thomaset al., 2007), we examined survival of newly born cells in the SGZ four weeks after predator stress. The

results revealed no differences in either cells ($t(7)= -0.126$, $p=0.903$, Figure 12a) or clusters ($t(7)= -0.215$, $p=0.836$, Figure 12b) between PS and HC groups.

Similar to Experiment 1, we examined BrdU positive cells across the AP plane. A similar trend (comparable to proliferation group, Experiment 1) was demonstrated in the survival group, with most cells and clusters found in the middle bins (Bregma: -2.64 to -5.88mm) compared to the most anterior and posterior bins. Both HC and PS groups demonstrated this trend in cells ($F(1.67, 11.70) = 6.35$, $p=0.017$) and clusters ($F(1.61, 11.24)= 5.47$, $p=0.027$). There were no significant differences in survival counts between HC and PS across the AP plane in cells ($F(1.67, 11.70)= 0.709$, $p= 0.49$, Figure 13a) or clusters ($F(1.61, 11.24)= 0.64$, $p=0.51$, Figure 13b). There were no significant correlations between cell or cluster counts and rat behaviour during the cat exposure in the survival group (Appendix I: Table 6).

3.4. Experiment 3: Predator stress does not affect cell survival in startled animals

3.4.1 Predator stress does not alter response to acoustic startle

Given that we did not see a difference in cell survival in the SGZ (Experiment 2), Experiment 3 included a measure of response to acoustic startle one week after predator stress to verify that predator stress produced a change in a behavioral response. Many previous studies show predator stress potentiates startle response (Adamec & Shallow, 1993; Adamec et al., 2003; Adamec et al., 2004; Cohen et al., 2004). Surprisingly, there was no difference in peak startle amplitude (overall average of startle responses in 30 trials) ($t(20)= 0.70$, $p=0.49$, Figure 14) and no change in startle responses between HC

and PS for each binned trial ($F(2.06, 8.23)= 2.80$, $p=0.118$, Figure 15). However, PS animals showed a faster habituation response to the startle stimulus specifically from Bin 1 to Bin 2 ($t(20)= -2.36$, $p=0.029$, Figure 16).

3.4.2. Predator stress does not alter survival of newly born cells in the SGZ of acoustic startled animals

Overall, there were no differences in either cells ($t(20)= 0.594$, $p=0.559$, Figure 17a) or clusters ($t(20)= 0.687$, $p=0.50$, Figure 17b) between PS or HC. Across AP plane, more cells ($F(2.58, 51.53)= 24.42$, $p<0.001$) and clusters ($F(2.51, 50.26)= 28.0$, $p<0.001$) were found in the later bins with the exception of Bin 5 in both HC and PS groups. Additionally, no significant changes in cells ($F(2.57, 51.53)=0.48$, $p=0.669$, Figure 18a) or clusters ($F(2.51, 50.26)=0.56$, $p=0.617$, Figure 18b) were observed between HC and PS groups across the AP plane. There was no significant relationship between cell or cluster counts and rat behaviour during the cat exposure in the survival startle group (Appendix I: Table 7).

3.5 Differences in cell proliferation and survival (across experiments)

As expected, a noted difference in cell counts was observed across the proliferation, survival and survival/startle group for both HC ($F(2,17)= 18.0$ $p<0.001$) and PS groups($F(2, 21)= 31.27$, $p<0.001$, Figure 19a). More (detectable) cells are labelled closer to the BrdU administration (2 hours) compared to cells analyzed 4 weeks later, since not all cells survive and become functional neurons. BrdU is a reliable marker for

proliferating cells, while NeuN is generally used to mark mature cells. This study was unable to double label for both BrdU and NeuN due to difficulties with the protocol. It is possible that there may have been differences between the number of mature neurons in predator stressed and control animals. A future study will be conducted to address this. Additionally, lower cell counts could be accounted for by the single injection of BrdU; however this was done to accurately label cells following stress. Lower numbers of cells were observed in the survival group compared to proliferation for both HC and PS ($p<0.001$). However, the PS startle group showed significantly lower numbers compared to the PS non-startle group ($p=0.013$). This was not seen in the HC startle group and non-startle group ($p=0.218$), demonstrating a selective effect for PS animals only. In terms of clusters, there were no significant differences observed across studies for HC animals ($F(2,17)= 0.870$, $p=0.437$). This was in contrast to clusters found in PS animals ($F(2,21)= 5.175$, $p=0.015$, Figure 19b), where there was a significant reduction in clusters in the startle group compared to the non-startle survival group ($p=0.025$). These data suggest that the addition of acoustic startle with predator stress has an effect on cell survival numbers.

3.6 Acoustic startle reveals potential resiliency in predator stressed animals

A correlation analysis with Bin 2 (where the drop in startle response was seen) of the PS group revealed that lower total cell ($r(9)= -0.68$, $p=0.021$, Figure 20) and cluster ($r(8)= -0.66$, $p=0.03$, Figure 21) counts were associated with higher average startle amplitudes. This was not observed in the cells or clusters of the HC group ($r(9)=0.19$,

p=0.59; r(9)= -0.025, p=0.94, Appendix I: Table 7). In addition, flights r(9)= 0.7, p=0.017, Figure 22) and number of lines (r(9)= 0.67, p=0.024, Figure 23) crossed during cat exposure were positively correlated with startle. This demonstrates that increased locomotion during the exposure is associated with a larger startle response. Given the significant relationship between flights and startle as well as cell survival and startle, a correlation between flights and cells was conducted to determine whether flight behaviour could be a potential predictor of cell or cluster counts. Unfortunately, the results revealed no significant correlation in cells (r(9)= -0.35, p=0.29) and clusters (r(9)= -0.34, p=0.30). Thus, flight behaviour was not strongly associated with cell or cluster counts. PS animals with higher startle amplitudes at Bin 2 demonstrated higher peak startle amplitudes (average of startle responses across 30 trials) compared to those with lower startle amplitudes at Bin 2 (r(9)= 0.77, p=0.006, Figure 24). This same trend was observed in the HC group at Bin 2 (r(9)= 0.95, p<0.001, Figure 25), despite no significant correlation with cell or cluster counts and startle amplitude observed in the HC condition. This suggests that the startle response behaviour in the first few trials can potentially predict the animal's overall startle response.

A median split was done to separate rats that showed a high or low response to acoustic startle in Bin 2. This was done to confirm whether the relationships found previously continued after the categorization. Despite the trend seen in the correlations, there were no significant differences in cells (t(8)= 1.428, p=0.191, Figure 26) or clusters (t(8)=1.23, p=0.25 Figure 27) of rats that responded more to startle. This suggests that the average number of cells or clusters in the low startle response group is not necessarily higher than in the high startle response group. However, PS animals that showed an

increased startle response demonstrated more flights ($t(8)= 0.369$, $p= 0.005$, Figure 28) and number of lines crossed ($t(8)= 0.651$, $p=0.008$, Figure 29) compared to those that showed a low startle response). Thus, locomotion during the cat exposure is an important contributing factor for how the animal will respond to acoustic startle. Consistent with the previous correlation data, low startle animals had significantly lower peak startle amplitude compared to high startle animals ($t(4.352) = -6.356$, $p=0.002$, Figure 30), suggesting that animals showing an early low startle response will likely show the same response across trials; the reverse pattern was seen in early high startle response animals.

3.7 Predator stress behaviour between proliferation and survival groups

Predator stress behaviour across the current studies was compared to ensure that the behaviours during the exposure were consistent in each study. The results supported this, as there were no differences between proliferation and survival (non-startle/startle) groups in relation to approaches, flights, number of lines crossed and proximity frequency (Appendix I: Table 8).

4.0 Discussion

Over the past decade, numerous studies have shown that physiological and psychological (acute and chronic) stressors can suppress neural progenitor cell proliferation and survival in the adult rodent (See Schoenfeld & Gould, 2012 for review). Furthermore, treatment protecting or increasing adult hippocampal neurogenesis such as exercise (Déry et al., 2013) and antidepressants (Malberg, Eisch, Nestler, & Duman,

2000) have been implicated in several neuropsychiatric disorders, including depression, anxiety and post-traumatic stress disorder (PTSD). Thus, the purpose of this study was to assess whether predator stress, an animal model of PTSD inhibits adult neurogenesis. Despite a robust stress effect, we showed that acute predator stress does not alter the proliferation or survival of newly born cells in the adult rat brain.

4.1 Predator stress and hippocampal cell proliferation

The current study showed that serum CORT levels are increased in immediately following predator stress (Figure 4), suggesting a robust stress effect. This is consistent with previous studies that have found elevated CORT in rats following exposure to predator odor using cat litter, fur (Cohen et al., 2009; Munoz-Albellan et al., 2008; Wright, Muir, & Perrot, 2013) or TMT exposure (Tanapat et al., 2001; Thomas et al., 2006). Despite evidence of a strong stress effect (i.e., elevated CORT levels), our study showed that an acute cat exposure did not alter cell proliferation in the subgranular zone (SGZ) of the hippocampus in adult rats (Figures 5a). Our findings are consistent with Thomas et al. (2006) who showed that acute exposure to TMT, which caused a robust CORT response, did not alter proliferation in the SGZ of the hippocampus. These findings are in contrast to others who found that acute stress altered proliferation (Falconer & Galea, 2003; Galea et al., 1996; Tanapat et al., 2001).

At least two possibilities may explain this discrepancy. First, with acute stressors that demonstrate an effect on cell proliferation (Falconer & Galea, 2003; Tanapat et al., 2001), exposure times were much longer than in the current study (10 minutes) or that of Thomas et al.(2006) (20 minutes). For example, one hour exposure of TMT or three

hours of restraint stress suppressed proliferation of newly born cells in the DG (Falconer & Galea, 2003; Bain et al., 2004). It is possible that our study, along with Thomas et al. (2006), may not have used a sufficient stress duration to alter proliferation. However, despite shorter exposure times, robust stress responses were observed (elevated CORT) and have previously produced reliable long-lasting behavioural changes following cat exposure (Adamec & Shallow, 1993; Adamec, Strasser, Blundell, Burton, & McKay, 2006) or TMT (Dielenberg & McGregor, 2001; Hotsenpiller & Williams, 1997). Second, the variability of the cat exposure may have also contributed to the inconsistent results. In contrast to predator odor which exposes animals to a constant stressor, the cat and rat interaction (predator stress) is not the same across animals. For example, rat and cat behavior across trials may differ if the cat approaches one rat more than another. In addition, the large exposure room allows rats to avoid the cat. To improve the acute cat exposure model, longer exposure times to the cat as well as a smaller exposure area may produce effects on cell proliferation. Alternatively, the cat exposure could be used in conjunction with subsequent acute stressors to potentially enhance behavioural symptoms of PTSD (e.g., hyperarousal and anxiety-like behaviour). A recent study by Roth et al. (2012) combined both chronic and acute stressors in a model called Chronic plus Acute Prolonged Stress (CAPS) to represent the complexity of stress responses during war, specifically the chronic state of stress in the environment and acute but severe traumatic experiences. CAPS was able to increase anxiety, change active coping behavior to a passive approach and reduced acute HPA stress activity which falls in line with characteristics of human PTSD. Thus, it may be worthwhile to create a multiple stressor

model to enhance the representation of the stressful experiences (following trauma) that PTSD patients are likely to encounter.

Along with the SGZ in the hippocampus, other areas of the brain are vulnerable to stress, including the amygdala and ventral medial hypothalamus (VMH). Specifically, the amygdala plays an important role in contributing to the anxiety-like behaviour following predator stress (Adamec, Burton, Shallow, & Budgell, 1999) and its dendritic morphology is altered following chronic immobilization or predator stress (Adamec, Hebert, Blundell, & Mervis, 2012; Mitra, Adamec, & Sapolsky, 2009; Vyas, Mitra, Shankaranarayana, Rao & Chatarji). A decrease in proliferation in the hypothalamus of male (but not female) rats has been shown following prenatal stress (García-Cáceres et al., 2010). Additionally, social-isolation housing for 6 weeks in prairie voles can reduce both cell proliferation in amygdala and cell survival in the VMH (Lieberwirth, Liu, Jia, & Wang, 2012). Despite the above findings, our results showed no change in proliferation within the amygdala (Figure 8a) or VMH (Figure 10a) between PS and HC animals. This is consistent with previous studies that examined cell proliferation following stress within the amygdala (Mitra et al., 2006) in mice or both amygdala and VMH (Pan, Li, Lieberwirth, Wang, & Zhang, 2014) in long-tailed hamsters.

4.2 CORT levels and hippocampal cell proliferation

Adrenal steroids such as CORT mediate the rate of neurogenesis in the DG of the adult male rat (Cameron & Gould, 1994). Indeed, CORT administration can decrease both cell proliferation and survival (Brummelte & Galea, 2010; Wong & Herbert, 2004),

while suppression of the CORT surge following stress via an adrenalectomy (with low-CORT replacement) eliminates the stress effect on cell proliferation (Tanapat et al., 2001). Furthermore, high serum CORT measured after a chronic social defeat is associated with fewer proliferating cells in the SGZ (Lagace et al., 2010). In light of these data, and the fact that predator stress produces elevated CORT levels (Figure 4), we hypothesized that CORT would negatively correlate with cell proliferation. However, this was not shown. CORT levels following predator stress did not correlate with proliferation in the SGZ. Our results are consistent with previous studies using acute TMT exposure (Falconer and Galea, 2003; Holmes and Galea, 2002; Tanapat et al., 2001; Thomas et al., 2006). Inconsistencies across studies may be due to the nature of the stressor. Chronic stressors reveal changes in other systems such as serotonergic signaling and other transmitter systems, which may play a role in altering cell proliferation and survival in the hippocampus (Saaltink & Vreugdenhil, 2014).

It is possible that the surge of CORT following stress in the current study was not sufficient to suppress cell proliferation. CORT levels may have normalized by the time BrdU started to label proliferating cells and even if CORT did have an effect on cell generation, it may have been too transient to have been captured. However, PS animals can demonstrate persistent high levels of CORT for up to 180 minutes post-stressor (Adamec et al., 2006). Thus, normalized CORT levels may not occur as quickly, despite the acute nature of the stressor. Interestingly, studies showing significant stress responses (including the current study) report CORT levels within the same range for controls and stressed animals (Tanapat et al., 2001; Thomas et al., 2006; Yun et al., 2010) and yet have obtained different results on cell proliferation or neurogenesis. Therefore, it would

be worthwhile to take multiple measures of CORT to establish levels at different time points to observe if levels of CORT are maintained.

Alternatively, the absence or small proportion of glucocorticoid receptors (GRs) in newly generated cells could also have an impact on our findings (Cameron et al., 1998; Mirescu et al., 2004) as any direct effects of elevated CORT could be delayed until after cells are generated (Garcia, Steiner, Kronenberg, Bick-Sander, & Kempermann, 2004). This suggests that CORT may induce changes in cell survival, which has been previously shown (Lee et al., 2006; Thomas et al., 2007). Thus, an important area to investigate, that is particularly relevant to PTSD, is the sensitivity of GRs. It has been well established that circulating levels of cortisol in PTSD patients are low (Yehuda, 2001; Yehuda, 2009). Such low levels of cortisol indicate a dysfunction in GRs as suggested by dexamethsasone (synthetic glucocorticoid) administration, where PTSD patients show increased cortisol suppression in comparison to healthy individuals (Newport, Heim, Bonsall, Miller, & Nemeroff, 2004). Further understanding of GRs in PTSD has been revealed by epigenetics, where environmental stress responses can be detected through DNA methylation, which can in turn affect the expression of GRs in animals (See Meaney & Szyf, 2005 for review). Not all individuals exposed to traumatic events will show clear signs of PTSD (Yehuda, 2001), thus genetic changes due to traumatic stress will be important for effective diagnosis and treatment processes. Most recently, a human study demonstrated that traumatic events can induce DNA methylation changes in specific promoters on a human GR variant which can lead to transcriptional modifications related to the HPA activity in PTSD individuals (Labonté, Azoulay, Yerko,

Turecki, & Brunet, 2014). However, studies have yet to link this finding to its effects on adult hippocampal neurogenesis.

Although CORT is a widely accepted measure of stress and has been shown to alter neurogenesis (Cameron & Gould, 1994; Cameron et al., 1998), there have been instances where elevated CORT levels have not produced changes in behaviour. Previous studies using TMT exposure demonstrated an increase in CORT but no change in exploratory behaviour (Perrot-Sinal, Ossenkopp, & Kavaliers, 1999) or hippocampal serotonin metabolism, a well-documented system affected by stress (Soares, Fernandez, Aguerre, Foury, & Mormede, 2003). It has also been suggested that high levels of serum CORT do not directly correlate with the stress state of an animal (Figueiredo et al., 2003). Interestingly, Malberg and Duman (2003) showed that the decrease in cell proliferation in animals exposed to inescapable shock in a learned helplessness model of depression was not due to elevated levels of CORT. Thus, CORT is unlikely to be the single driving mechanism in the stress effect on different aspects of hippocampal neurogenesis. Future studies need to extend to other measures such as GR sensitivity, neurotransmitter signaling and synaptic plasticity along with CORT levels to obtain a better grasp of how stress response affects hippocampal cell proliferation.

4.3 Stress and cell survival in the hippocampus

As mentioned above, some reports show that stress can decrease cell survival without altering cell proliferation (Lee et al., 2006; Thomas et al., 2007). This may be because surviving cells are more susceptible to the effects of CORT due to (more)

glucocorticoid receptors formed at a later stage in the cell's life (Garcia et al., 2004). Indeed, Thomas et al. (2007) demonstrated high levels of CORT following the stressor and a decrease in cell survival 4 weeks later. Stress response from social defeat may take over 24 hours for changes to be apparent, thus leaving cell proliferation unchanged. Similarly, Lee et al. (2006) found a decrease in cell survival but not proliferation following chronic mild stress for 19 days. However, the only measure of CORT was obtained 6-8 hours after the last stressor and no difference was found between stressed and control animals. CORT levels during the stressor were not measured, thus it is possible an earlier increase of CORT affected cell survival. Despite these findings, we did not see changes in cell survival following predator stress (Figure 12a). We did not obtain CORT levels in Experiment 2 (survival study) but given the robust CORT effect in experiment 1 and the similar methodology in both studies, we assume that CORT was elevated following predator stress in the survival study (Experiment 2). Given that studies show that predator stress produces long lasting anxiety-like behavior and hyperarousal (Adamec & Shallow, 1993; Adamec et al., 2004; Cohen, Friedberg, Matar, Kotler, & Keev, 1997; Cohen et al., 2003) and the fact that predator stress increases CORT (Experiment 1, Figure 4), it is likely that predator stress produces a strong stress response which ultimately does not alter adult neurogenesis (proliferation or survival) in the adult rat brain.

One potential confound to the neurogenesis data across studies is stress susceptibility. Lagace et al. (2010) showed a significant increase in CORT following social defeat in both susceptible (choosing to avoid the dominant con-specific) and unsusceptible (choosing to spend more time around the dominant con-specific) mice.

However, CORT levels were normalized 24 hours after stress. Interestingly, the susceptible mice demonstrated an increase of surviving cells 4 weeks after the last social defeat compared to control or unsusceptible mice. This finding contrasts with the aforementioned studies (Lee et al., 2006; Thomas et al., 2007) and demonstrates how CORT may not be the sole determinant of cell survival. Additionally, this study is a prime example of how findings may differ when stress susceptibility is taken into account. This effect may not have been revealed had unsusceptible and susceptible mice been grouped together. Stress susceptibility has been examined in the cat predator stress model where predator stressed rodents were grouped based on responses from an anxiety test (elevated plus maze) (Mitra et al., 2009). Well-adapted animals (less anxious) demonstrated shorter dendrites in basolateral amygdala neurons in comparison to mal-adapted animals (more anxious) or control unstressed animals. This suggests that there are structural changes that differ within the stressed population. However, whether or not this occurs in adult hippocampal neurogenesis has yet to be examined. Clearly, this type of classification is important as not all animals will respond or cope with the stressor equally. This may (in part) explain why there are inconsistent results in the literature on the effects of stress on cell survival.

BrdU positive cells provide a strong indication of whether or not neurogenesis has been affected, since 70-90% of proliferating cells go on to develop into neurons (DeCarolis & Eisch, 2010). However, our study did not co-label with neuronal markers, thus we cannot verify if there were changes in neurogenesis. Future experiments should co-label BrdU with markers of immature neurons (Doublecortin) or mature neurons (NeuN) to confirm this. It would also be worthwhile to label for glial cells, as there is a

possibility that proliferating cells marked with BrdU are glia. In addition, recent studies have demonstrated an interesting role for glial cells in adult hippocampal neurogenesis following stress. One study showed that immobilization stress decreased neurogenesis but increased oligodendrogenesis in the DG (Chetty et al., 2014), while another demonstrated that acute stress enhanced adult rat hippocamapal neurogenesis (dorsal region only) and astrocytic fibroblast growth factor 2 expression (Kirby et al., 2013).

4.4 Acoustic startle

Given that there were no changes in cell proliferation and survival in Experiment 1 and 2, we wanted to verify that the expected stress-induced behavioral changes were produced following the predator stress. Acoustic startle response was assessed because it tests a core behavioral symptom of PTSD, hyperarousal. In light of past studies (Adamec & Shallow, 1993; Adamec et al., 2004), we expected predator stress to increase peak startle amplitude and delay habituation (Experiment 3). Surprisingly, predator stress had no effect on peak startle amplitude or habituation (Figures 15, 16). Although hyperarousal is often seen following predator stress, there are studies showing no effect or an opposing effect of predator stress on startle response (Adamec et al., 2006; Blundell, Adamec & Burton, 2005). A potential explanation for the lack of hyperarousal in predator stress rats is the order of testing. In all of the previous studies, elevated plus maze (EPM), light-dark box and open field were run prior to startle. However, some studies using only the EPM following stress (repeated restraint or predator stress) have had difficulty demonstrating an anxious profile (Berardi et al., 2014; Gregus et al., 2005).

It is possible that an anxiogenic test such as EPM could drive the increased hyperarousal in predator stressed animals, thus researchers must take additional precaution when implementing a specific test for a desired behavioural measure. To properly control for this, future experiments should compare PS animals tested in the EPM prior to startle with those that receive only acoustic startle following the stressor.

4.5 Acoustic startle and cell survival in the hippocampus

The challenge with measuring behaviour and neurogenesis following a stressor in the same experiment is that the behavioural test itself may impact neurogenesis. In such a situation, it may be best to test behaviour and neurogenesis separately. In the current study, behavioral changes in Experiment 1 (proliferation study) were not assessed because the behavioral tests themselves may have affected proliferation (given that the animals were sacrificed 2 hours post stress). In Experiment 3, cell survival was analyzed three weeks following acoustic startle and was compared to Experiment 2, where no behaviour test was implemented. Interestingly, following the predator stress exposure, acoustic startle significantly reduced surviving cells in the DG compared to animals that simply received predator stress (Figure 19a). These results suggest that subsequent stressors post-trauma can affect cell survival and potentially cell proliferation. Implementing mild stressors (i.e., behavioral tests) following trauma may provide a more realistic representation of PTSD in the human condition, as individuals are bound to face other stressors especially if they are confined to an already stressful environment (e.g., war zone). Successive stressors may increase the severity or prolong behavioural

symptoms for PTSD. Another way to avoid the potential problem with behavioural measures affecting cell counts is to use the behavioural test following a manipulation to ablate or suppress neurogenesis. This can be done through transgenic means or through X-ray irradiation (Jaholkowski et al., 2009; Lagace et al., 2010; Snyder et al., 2011). Using such a method would also show whether new neurons are necessary for specific behaviours. Despite its merits, suppressing neurogenesis has produced mixed results, with one study showing an increase in anxiety-like behaviour in the EPM (Revest et al., 2009) and another demonstrating no differences (Jaholkowski et al., 2009).

4.6 Acoustic startle reveals potential resiliency in predator stressed animals

Although there was no difference in peak startle amplitude between PS and HC animals, PS animals showed a faster habituation to acoustic startle (Figure 16) from Bin 1 (average of first three trials) to Bin 2 (average of subsequent three trials). This was not expected, since PS animals typically show both higher peak startle amplitude and slower habituation to the stimuli. Upon further analysis of the PS startle group, a high startle (approximately 600 mV or greater) and low startle (lower than 600 mV) response group was identified. The animals classified in the low startle response drove the habituation effect that was observed. Without this group, the high startle response group (797.4 ±64.7 mV) averaged about the same as the HC startle group (811.8 ±127.6 mV). This suggests that animals in the PS condition have different ways to cope with stress as seen by the divergent responses to acoustic startle. Such a finding provides an important reason for screening rats in terms of behaviour prior to specific tests. Similar to humans, not all rats

will respond the same way, thus a screening process may identify some individual differences. Several studies using PTSD models, including social defeat and predator stress have already taken this approach by using the EPM to separate those that demonstrate resilient or vulnerable behaviours right from the beginning (Cohen et al., 2004; Lagace et al., 2010; Mitra et al., 2009).

Our study also showed that higher PS startle responses (in Bin 2) were strongly associated with a lower number of surviving cells (Figure 20), increased flight from the cat during the exposure (Figure 22) and overall locomotion, as measured by number of lines crossed (Figure 23). Thus, the type of response observed during acoustic startle (following predator stress) could potentially predict the quantity of surviving cells. In addition, increased flight and movement during the cat exposure may be used to predict a higher response (amplitude) to startle. Once more, these types of behaviours during the cat exposure may be useful for future experiments to screen rats that could be more or less vulnerable to startle.

Conclusion

Our study has used an ecologically valid animal model of PTSD to examine adult neurogenesis. Despite a robust stress response, predator stress did not affect cell proliferation in the hippocampus, amygdala or VMH or cell survival in the hippocampus. Our data suggest that the CORT increase following acute predator stress is not, by itself, sufficient to alter adult neurogenesis. Our data also highlight the consequences of behavioural tests following stress, as the test itself can impact cell survival. Finally,

individual differences in the response to stress (measured during the cat/rat interaction or during behavioral tests such as startle) may be critical for developing effective treatment for stress-related disorders such as PTSD.

Increasing neurogenesis via exercise (Rhodes et al., 2003; van Praag, Kempermann, & Gage, 1999), environmental enrichment (Olson, Eadie, Ernst, & Christie, 2006) and antidepressants (Dranovsky & Hen, 2006; Malberg et al., 2000) in animal studies have demonstrated positive results such as memory improvements and a decrease in anxiety and depressive-like behaviour (Bruel-Jungerman, Laroche, & Rampon, 2005; David et al., 2009; Erickson et al., 2011). Although finding ways to upregulate hippocampal neurogenesis could be a promising treatment, it may not be useful for acute stress situations as revealed by inconsistencies in the literature, where neurogenesis may not necessarily decrease following stress. Given that individual differences can complicate efficient treatments for individuals with PTSD, perhaps the best approach to understanding PTSD is to examine the mechanisms involved in resilience and vulnerability. Furthermore, improving the animal model itself by implementing subsequent stressors following the traumatic event would provide a better representation of the stress that individuals with PTSD may face after trauma.

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Figures

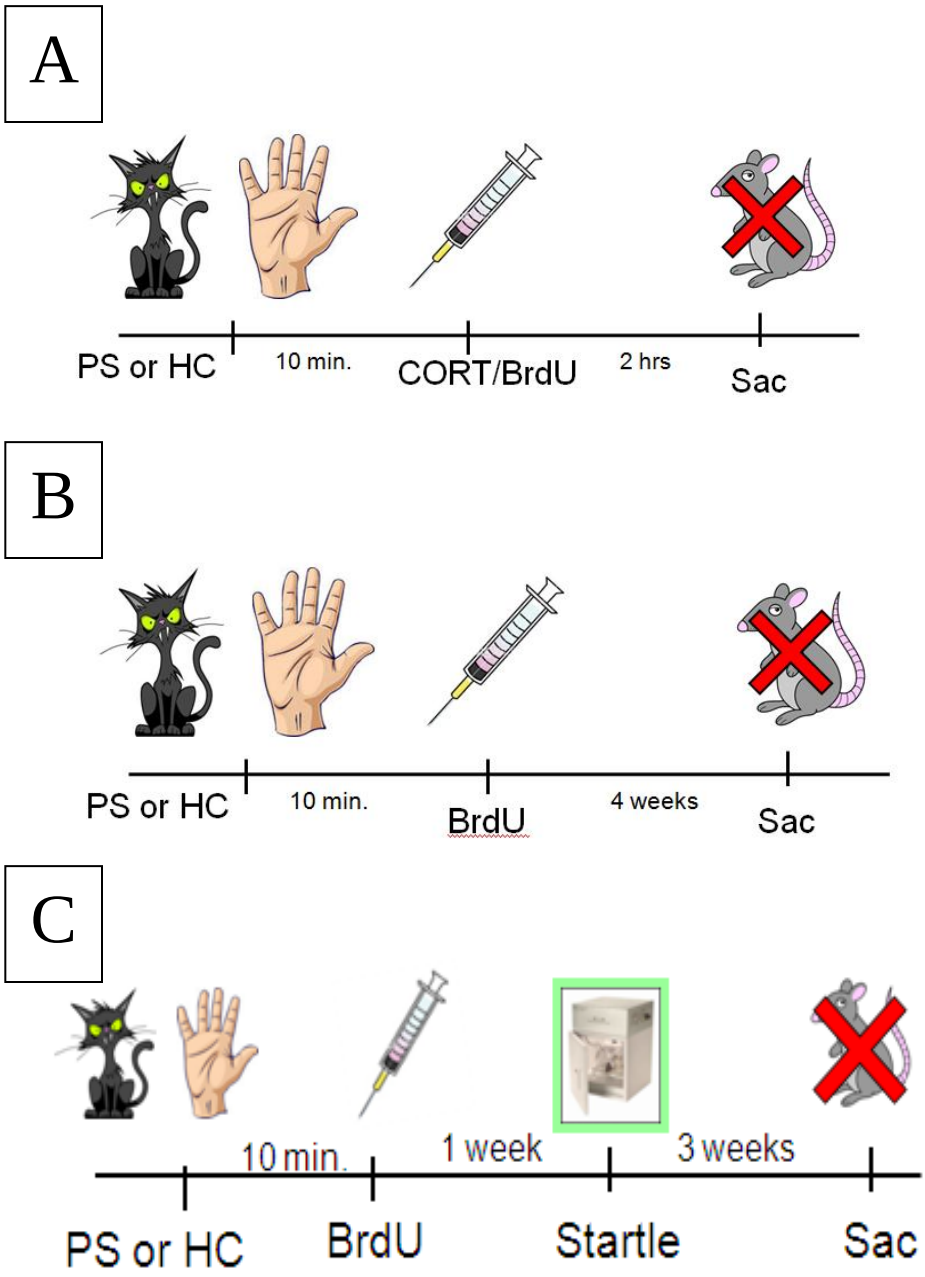


Figure 1: Timelines for all experiments. (Predator stressed (PS) and handled control (HC)): A) Experiment 1- Proliferation, B) Experiment 2 – Survival, C) Experiment 3 – Survival/Startle

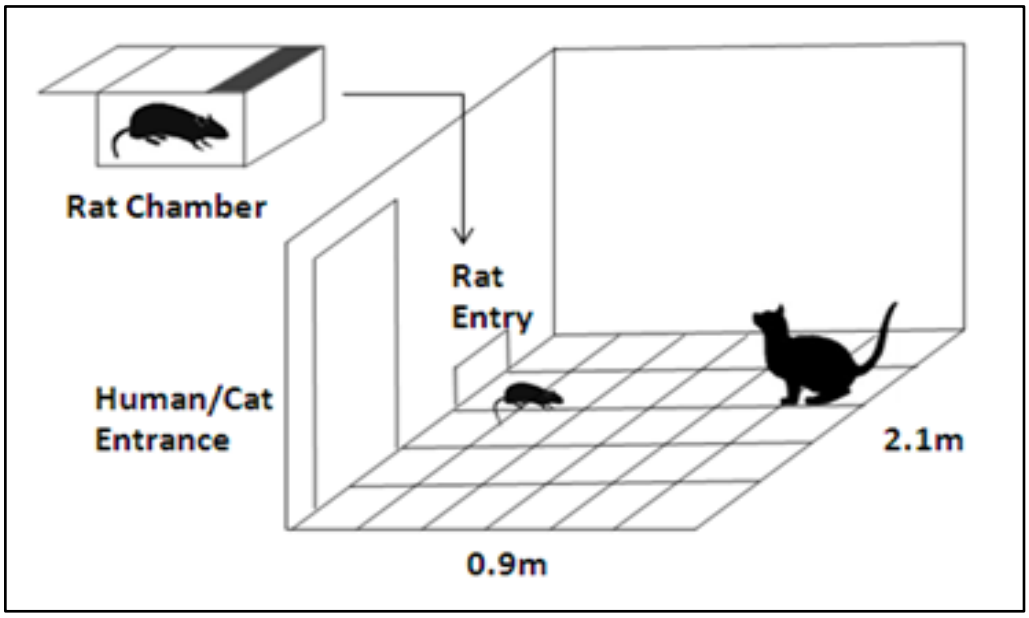


Figure 2: Predator stress schematic. The cat is first placed into the room. The rat is put into a chamber to habituate in for 5 minutes before the exposure begins. The entire exposure is videotaped and scored for behaviour. The stressor lasts for 10 minutes.

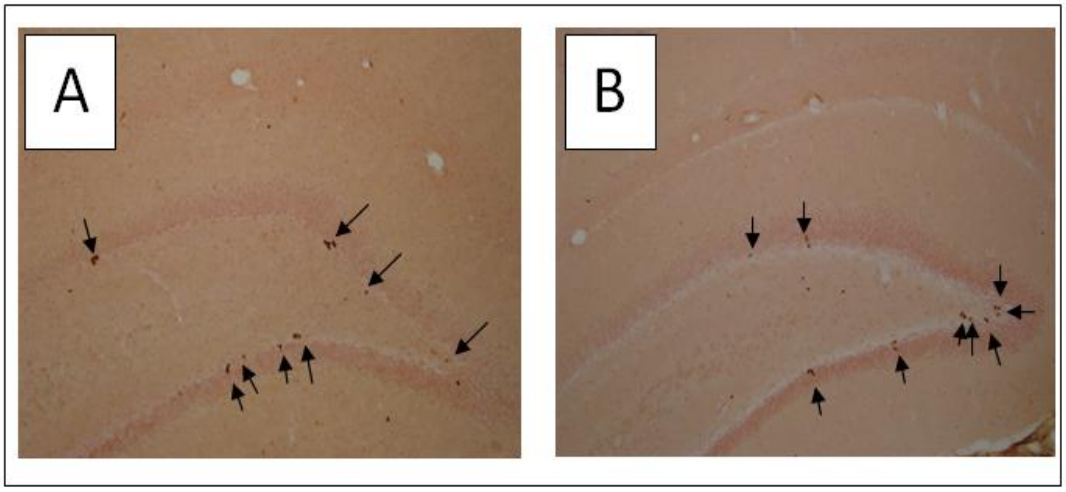


Figure 3: Photo of BrdU positive clusters in the SGZ. A) Predator stress (PS) and B) handled controls (HC) show no difference in numbers of cells or clusters. Clusters are marked by black arrows.

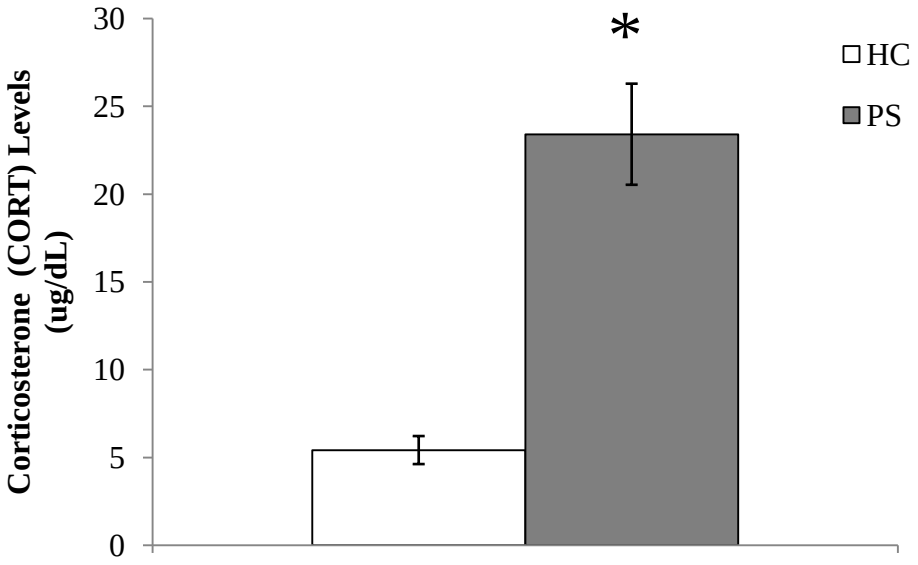


Figure 4: Experiment 1 (Proliferation): CORT response following predator stress (PS) or handled control (HC). PS animals show a significantly higher CORT level compared to HC ($t(9.2) = -6.02$, $p<0.001$).

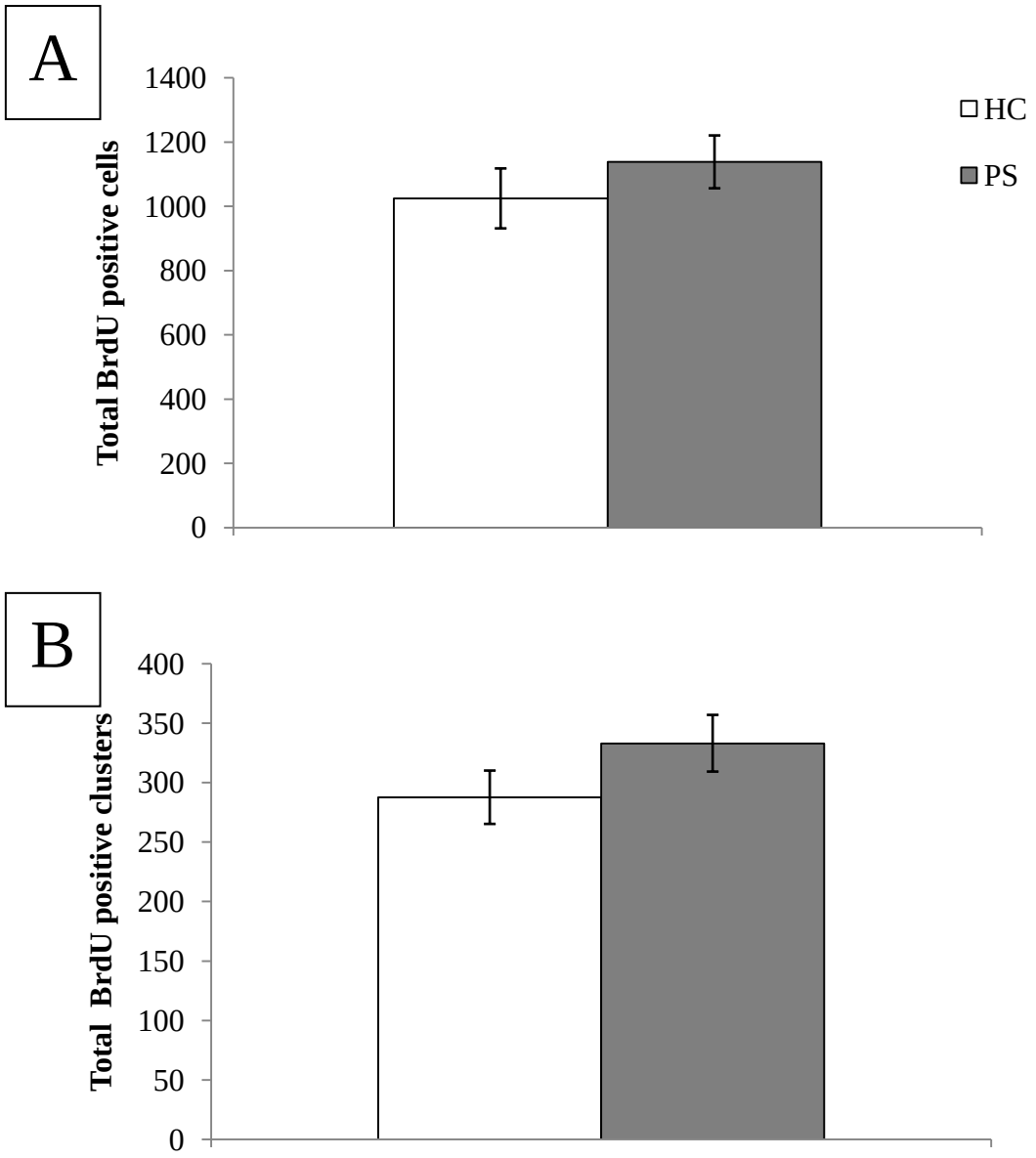


Figure 5: Experiment 1 (Proliferation): Total number of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the SGZ of the hippocampus. A) No difference in cell count between the PS and HC were observed ($t(9) = -0.92$, $p=0.38$). B) No difference between cluster count between the PS and HC was observed ($t(9) = -1.37$, $p=0.21$).

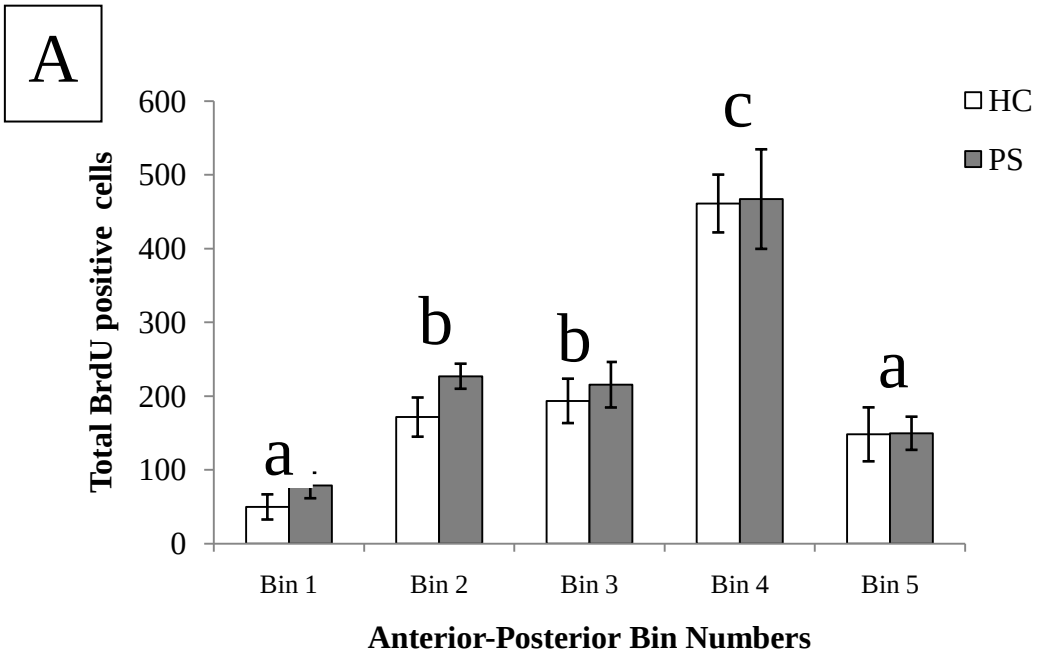


Figure 6A: Experiment 1 (Proliferation): Total number of BrdU positive cells across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

A) No difference was observed in cell counts between predator stressed (PS) and handled controls (HC) at any of the bins ($F(2.09, 18.79) = 0.21$, $p=0.82$). Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.001$, except for **a** and **b** where $p=0.04$).

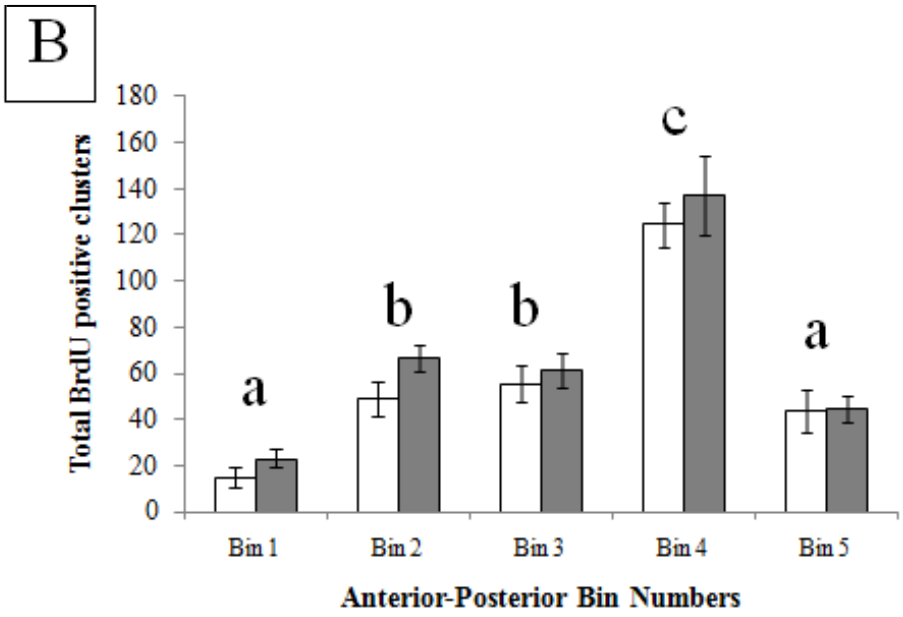


Figure 6B: Experiment 1 (Proliferation): Total number of BrdU positive clusters across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

B) No difference was observed in cluster count between predator stressed (PS) and handled controls (HC) at any of the bins ($F(2.17, 19.51)= 0.31$, $p=0.76$). Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.001$, except for **a** and **b** where $p=0.02$)

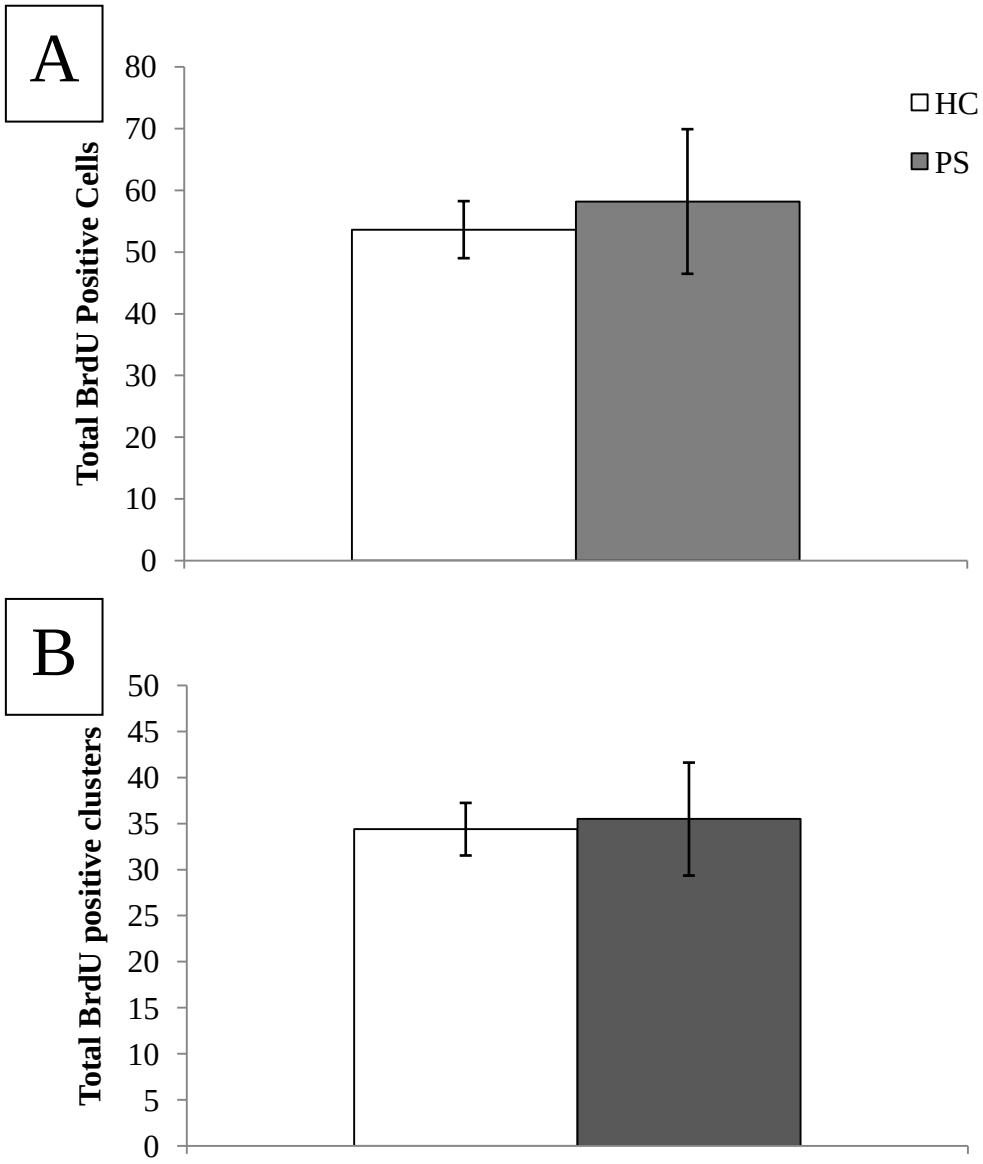


Figure 7: Experiment 1 (Proliferation): Total number of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the CA1 of the hippocampus.

A) No difference in cell count between the PS and HC were observed ($t(6.48) = -0.36$, $p=0.73$). B) No difference in cluster count between the PS and HC was observed ($t(7)= -0.16$, $p=0.88$).

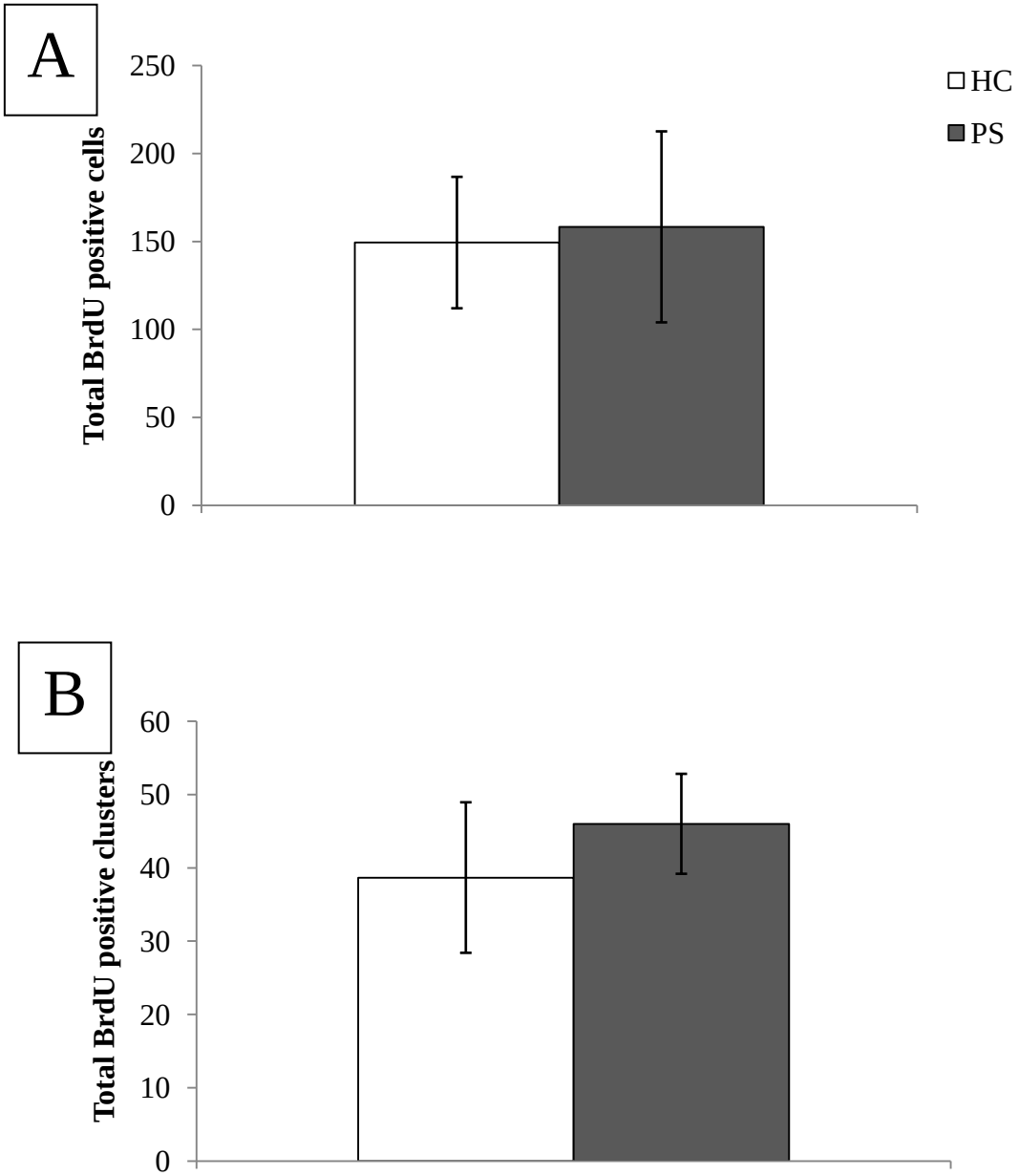


Figure 8: Experiment 1 (Proliferation): Total number of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the amygdala.

A) No difference in cell count between the PS and HC was observed ($t(5)=-0.13$, $p=0.91$). B) No difference in cluster count between the PS and HC was observed ($t(5)= -0.62$, $p=0.56$).

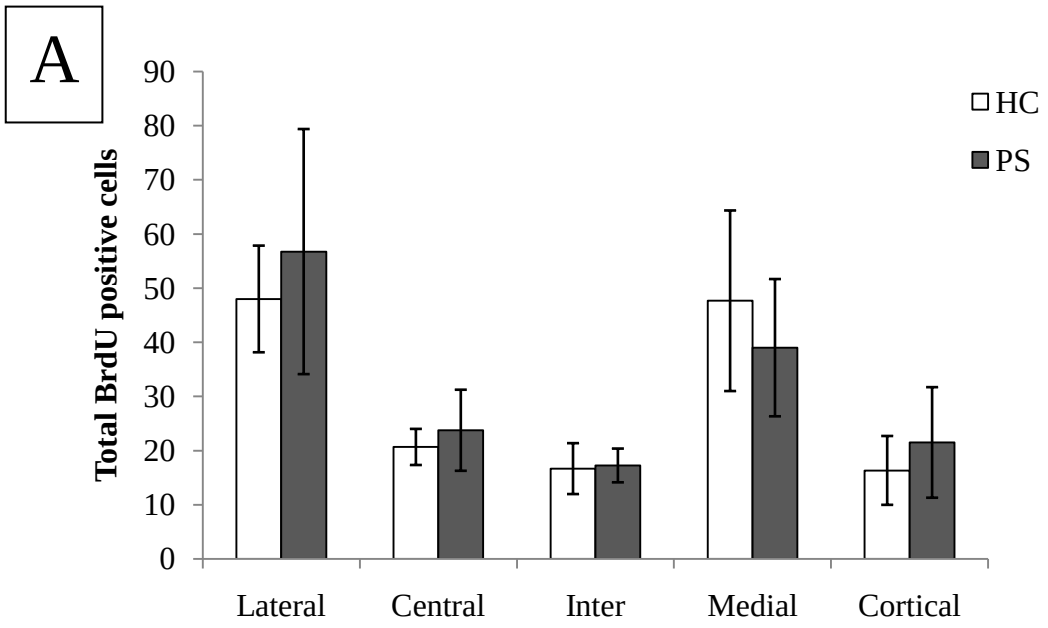


Figure 9A: Experiment 1 (Proliferation): Distribution of BrdU positive cells in different regions in the amygdala between predator stressed (PS) and handled control (HC) animals.

A)There were no significant differences in cell count between PS and HC in each of the regions ($F(2.3, 11.3)= 0.17$, $p=0.87$). A strong trend was observed in the HC condition with fewer cells found in the intercalated and anterior cortical regions ($F(4,10)= 3.3$, $p=0.06$). Significant differences were observed in the PS condition ($F(4,15)= 7.8$, $p=0.001$). Bins marked with the same letter(s) do not differ, bins marked with different letters show a significant difference ($p<0.05$).

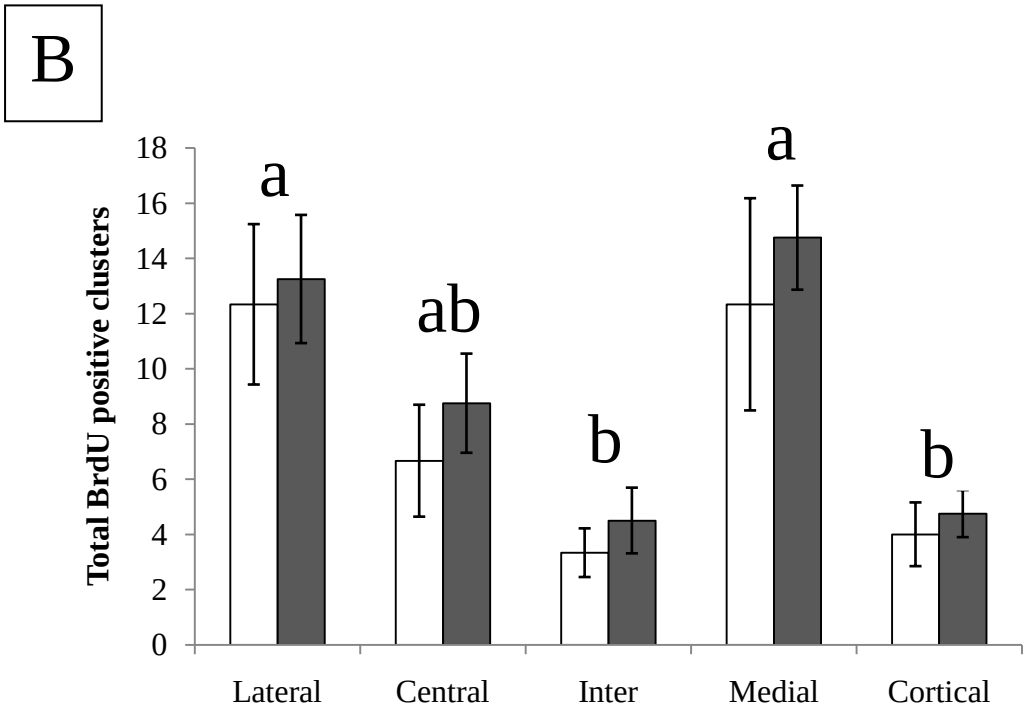


Figure 9B: Experiment 1 (Proliferation): Distribution of BrdU positive clusters in different regions in the amygdala between predator stressed (PS) and handled control (HC) animals.

B) There were no significant differences between PS and HC in each of the regions ($F(1.6, 8.0)= 0.36$, $p=0.66$). A strong trend was observed in the HC condition with fewer cells found in the central, intercalated and anterior cortical regions ($F(4,10)=3.0$, $p=0.07$). No significant differences were observed within the PS condition across regions ($F(4,15)= 1.6$, $p=0.23$).

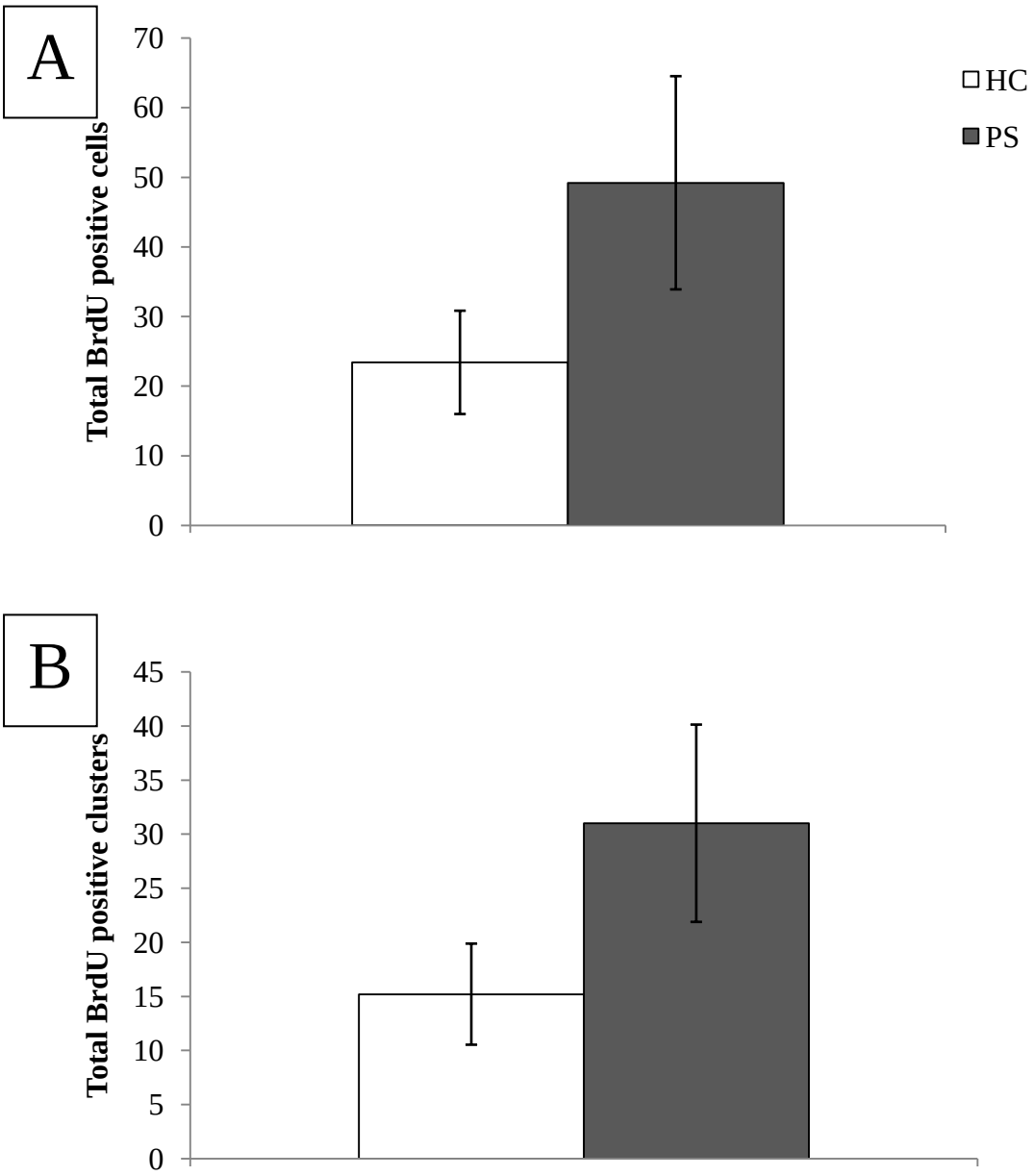


Figure 10: Experiment 1 (Proliferation): Total number of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the VMH.

A) No difference between the PS and HC was observed ($t(8)= -1.5$, $p=0.16$). B) No difference between the PS and HC was observed ($t(8)= -1.5$, $p= 0.17$).

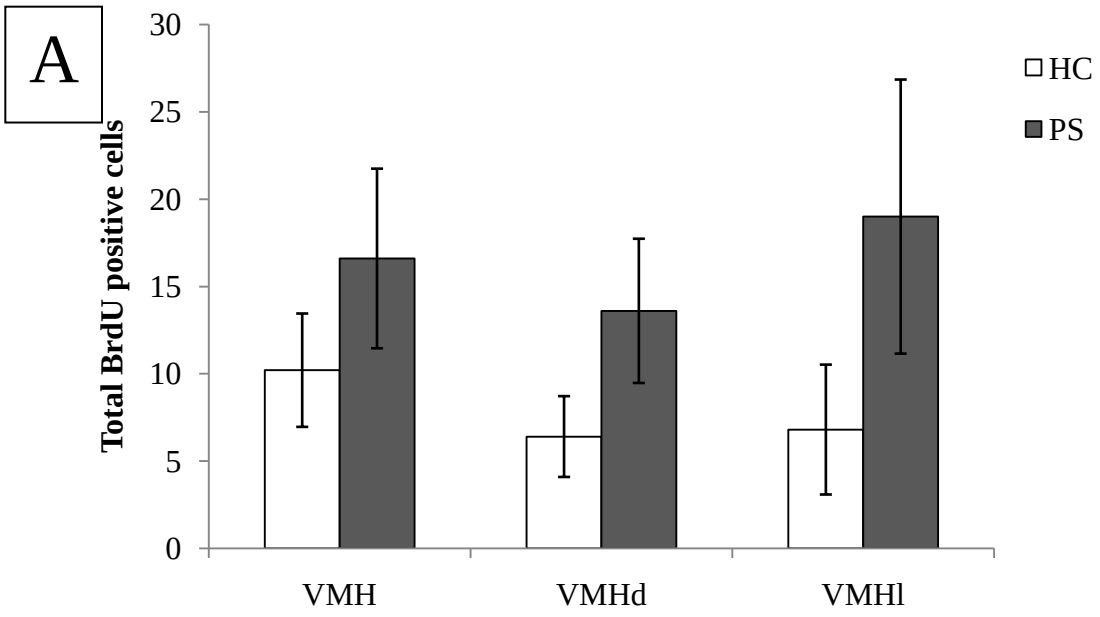


Figure 11A: Experiment 1 (Proliferation): Distribution of BrdU positive cells between the predator stressed (PS) and handled control (HC) animals in the VMH.

A) No difference between the PS and HC were observed in any of the regions (VMH, VMH dorsal, VMH lateral) ($p>0.1$). No differences were observed within PS or HC condition across regions ($p>0.1$). See Table 4 for summary of statistics.

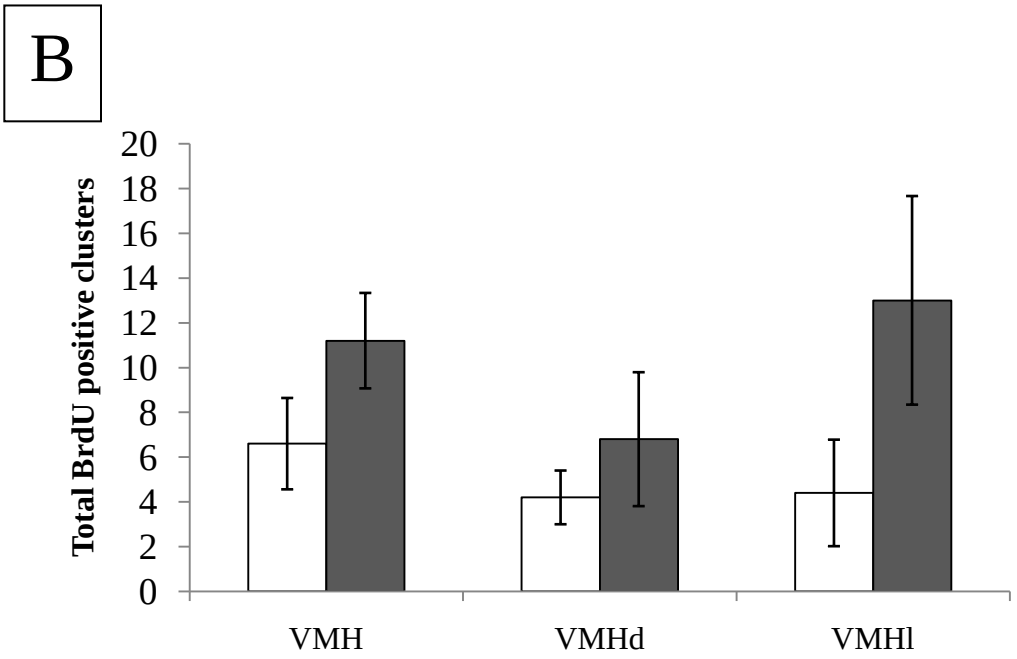


Figure 11B: Experiment 1 (Proliferation): Distribution of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the VMH.

B) No difference between the PS and HC were observed in any of the regions (VMH, VMH dorsal, VMH lateral) ($p>0.1$). No differences were observed within PS or HC condition across regions ($p>0.1$). See Table 4 for summary of statistics.

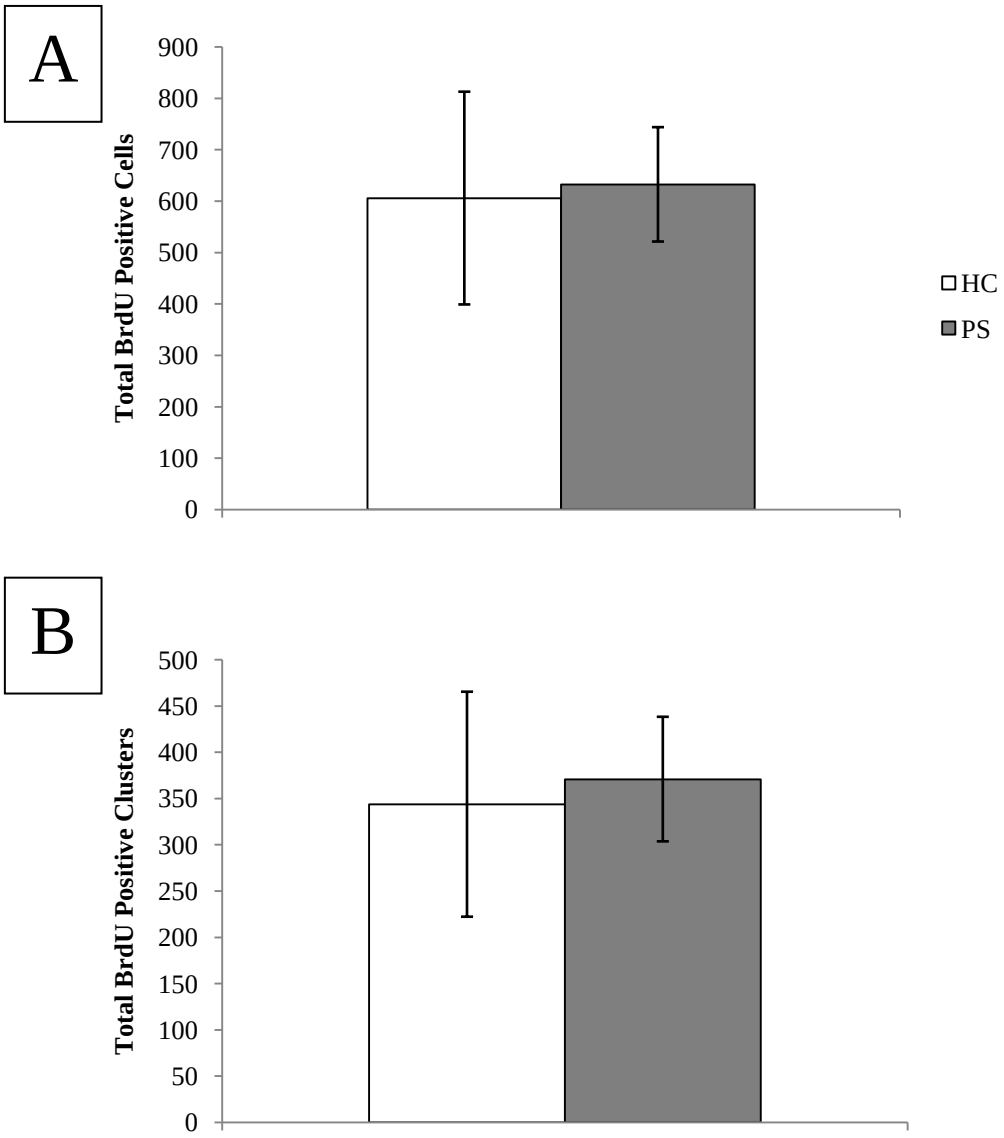


Figure 12: Experiment 2 (Survival): Total number of BrdU positive clusters between the predator stressed (PS) and handled control (HC) animals in the SGZ. A) No difference between the PS and HC was observed ($t(7)= -0.215$, $p=0.836$). B) No difference between the PS and HC was observed ($t(7)= -0.126$, $p=0.903$).

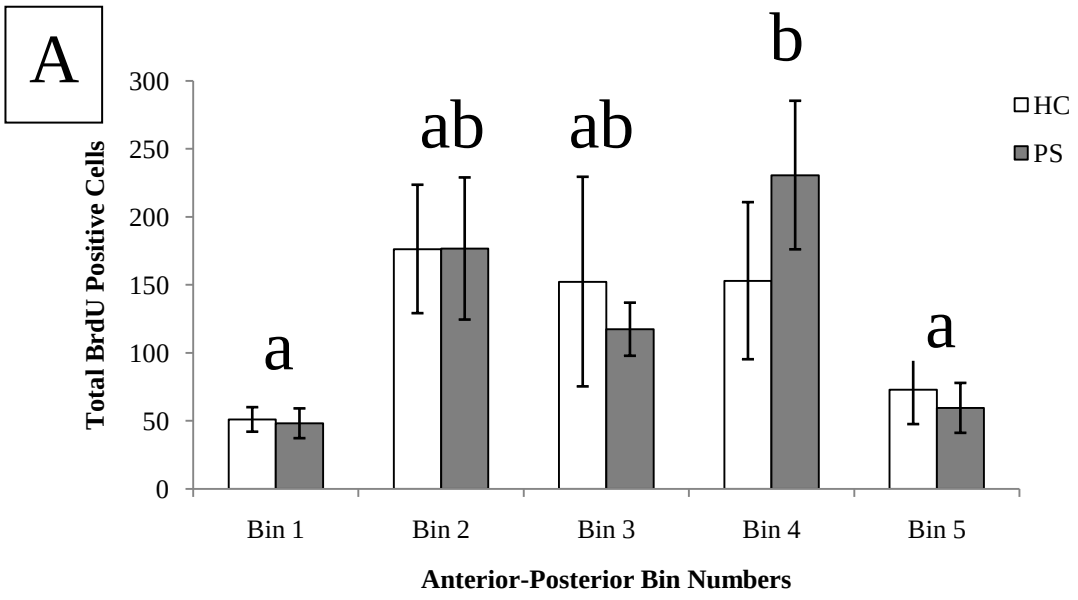


Figure 13A: Experiment 2 (Survival): Distribution of BrdU positive cells across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

A) No difference in cell count was observed between predator stressed (PS) and handled controls (HC) at any of the bins ($F(1.67, 11.70)=0.71$, $p=0.49$). Within the HC condition, there were no significant differences across bins ($F(4,10)= 1.26$, $p=0.35$). Within the PS group, there were significant differences ($F(4,25)= 4.6$, $p=0.006$). Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.05$).

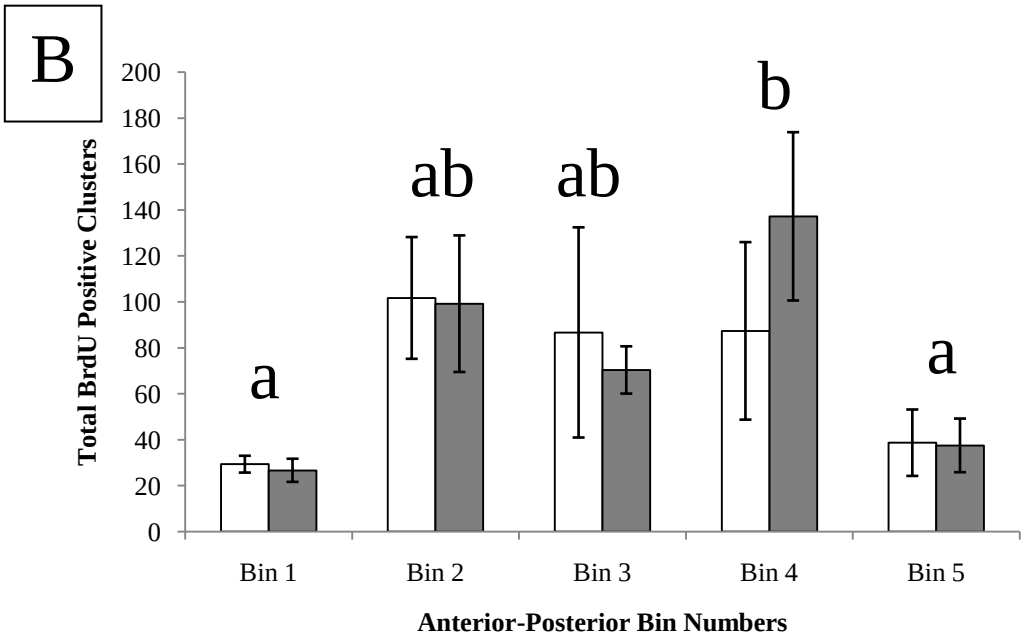


Figure 13B: Experiment 2 (Survival): Distribution of BrdU positive clusters across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

B) No difference in cluster count was observed between predator stressed (PS) and handled controls (HC) at any of the bins ($F(1.61, 11.24)= 0.64$, $p=0.51$). Within the HC condition, there were no significant differences across bins ($F(4,10)= 1.17$, $p=0.38$). Within the PS group, there were significant differences ($F(4,25)= 4.1$, $p=0.01$). Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.05$).

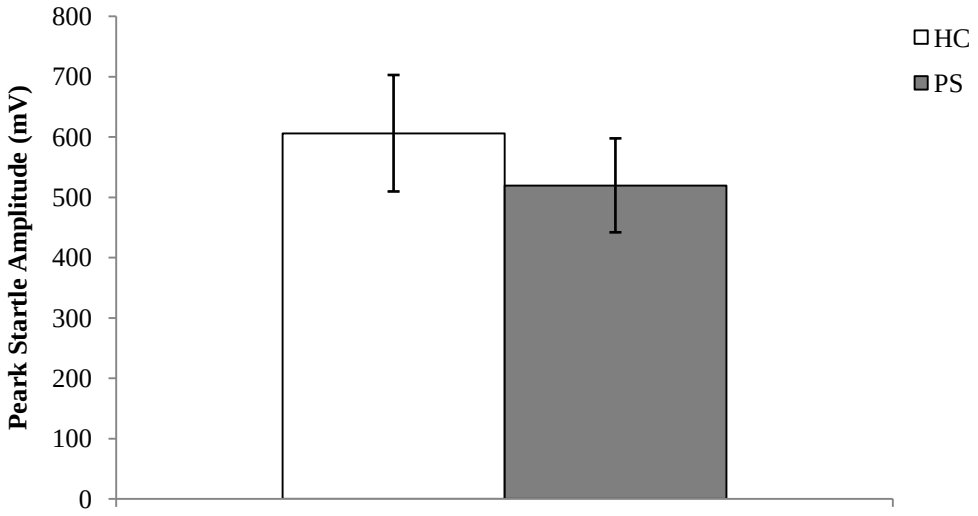


Figure 14: Experiment 3 (Survival/Startle): Peak startle amplitude between predator stressed (PS) and handled controls (HC). No difference was observed between PS and HC ($t(20)= 0.70$, $p=0.49$).

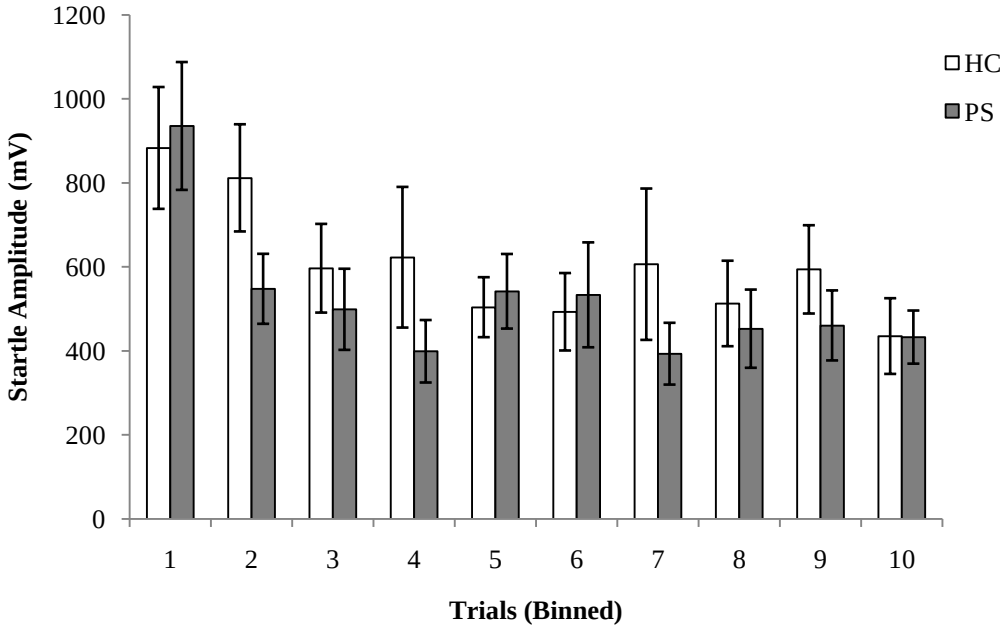


Figure 15: Experiment 3 (Survival/Startle): Average startle amplitude placed into 10 bins (Bin 1 = Average of Trial 1 + 2 + 3) for all conditions. No differences were observed between PS and HC across all bins ($F(2.06, 8.23)= 2.80$, $p=0.118$).

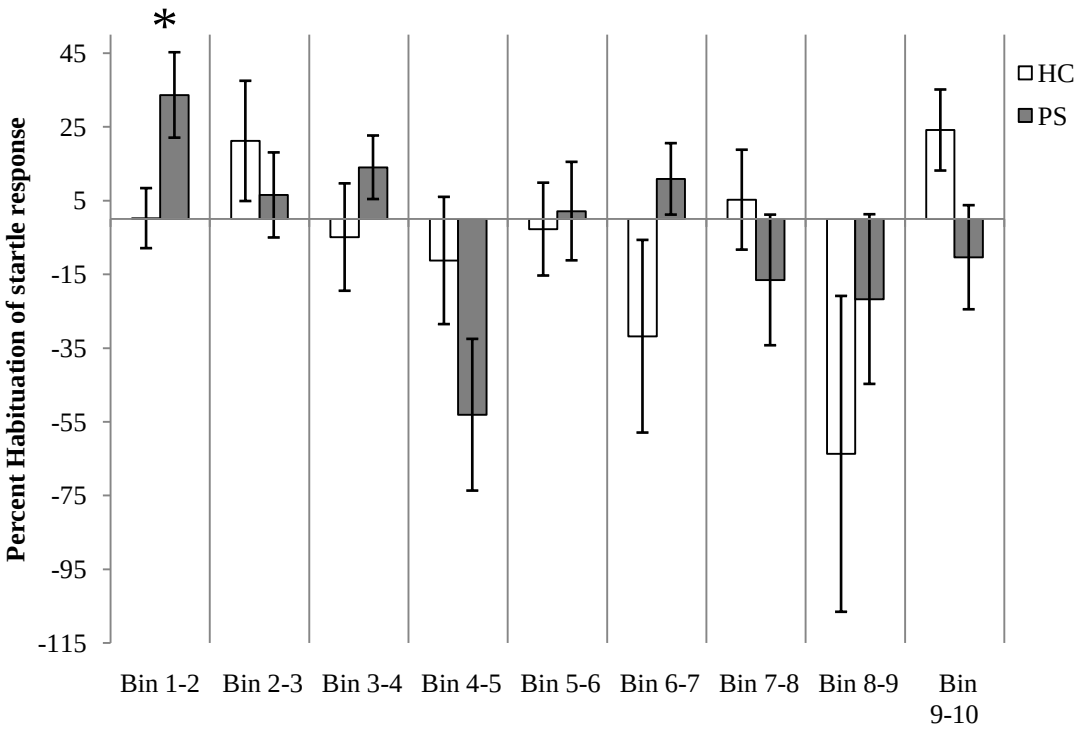


Figure 16: Experiment 3 (Survival/Startle): Percentage of habituation between binned startle data for both predator stressed (PS) and handled control (HC) animals. Percent habituation was calculated using the formula found in Section 2.9 – Statistical Analysis. Positive percentages indicate habituation, where negative percentages indicate an increase in startle from the previous trial. A significant difference in habituation was only observed between bin 1 and 2 ($t(20)= -2.36$, $p=0.029$), as indicated by the asterisk(*).

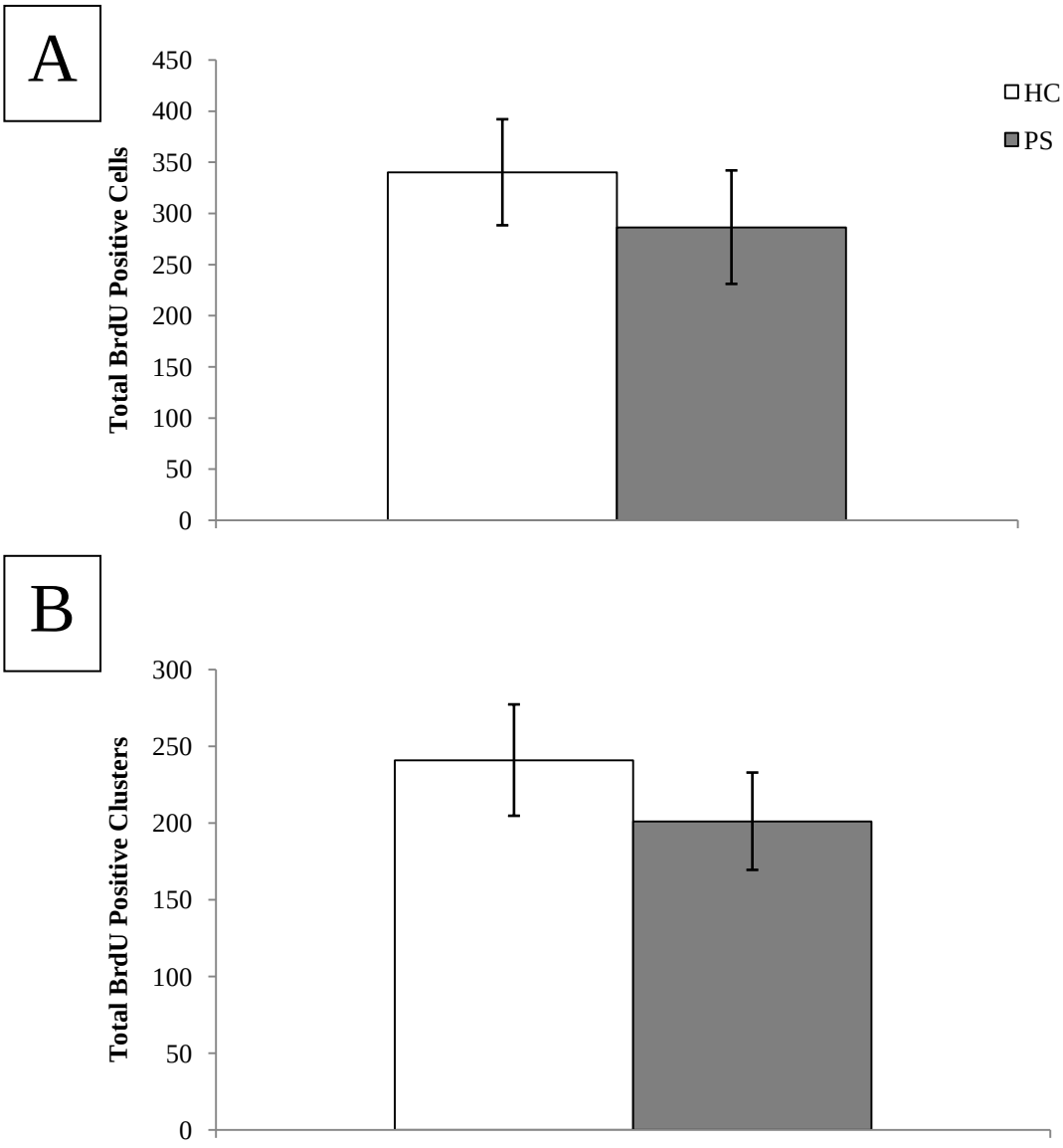


Figure 17: Experiment 3 (Survival/Startle): Total number of BrdU positive cells and clusters between the predator stressed (PS) and handled control (HC) animals in the SGZ. A) No difference between the PS and HC was observed ($t(7)= -0.126$, $p=0.903$). B) No difference between the PS and HC was observed ($t(7)= -0.215$, $p=0.836$).

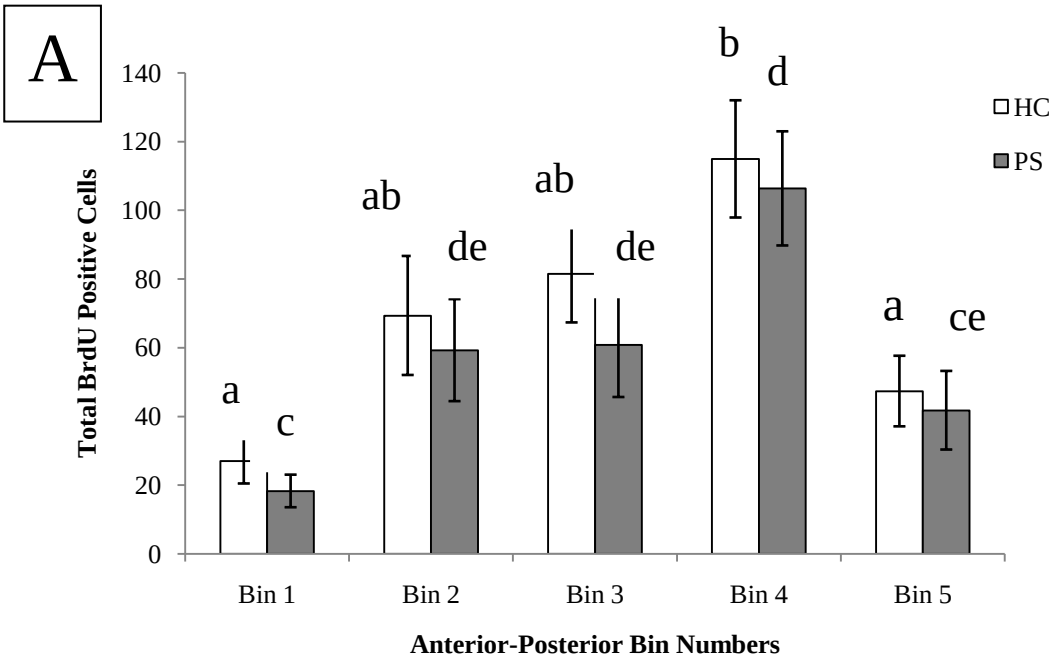


Figure 18A: Experiment 3 (Survival/Startle): Total number of BrdU positive cells across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

A) No difference was observed in cell count between predator stressed (PS) and handled controls (HC) at any of the bins ($F(2.57, 51.53) = 0.48$, $p=0.67$). Within the HC condition, there were significant differences across the bins ($F(4, 50)= 4.9$ $p=0.002$). Within the PS condition, there were significant differences across bins ($F(4,50)=8.6$, $p<0.001$). Letters **a-b** correspond with the HC group only and **c-d** correspond with PS only. Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.05$).

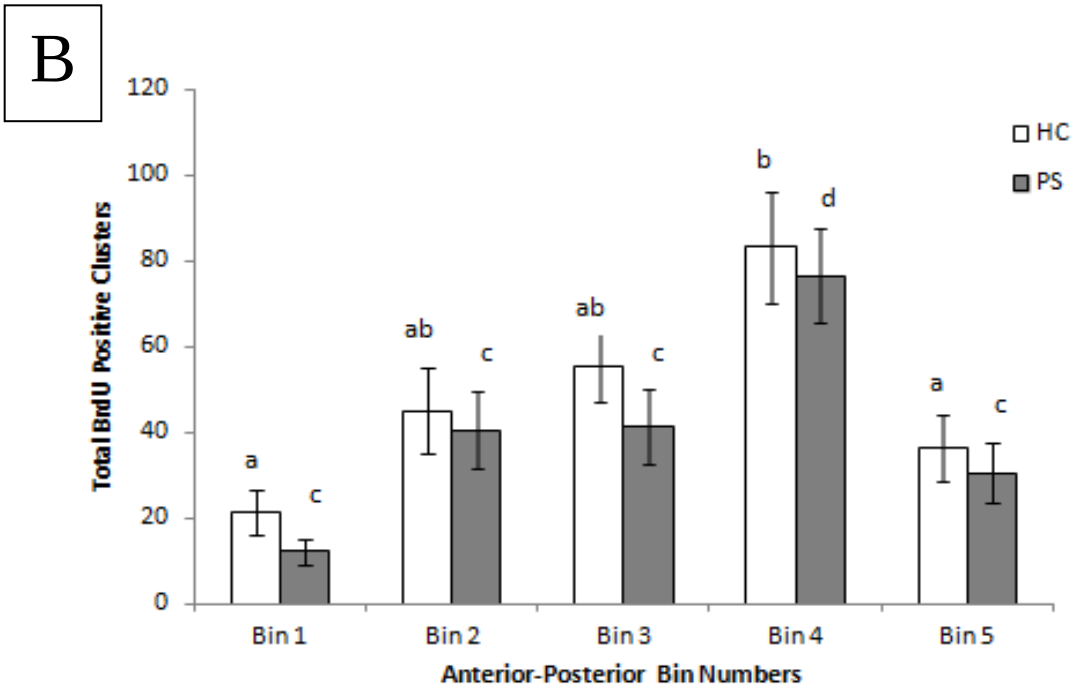


Figure 18B: Experiment 3 (Survival/Startle): Total number of BrdU positive clusters across AP plane (Anterior-Posterior: Bin 1-5) in the SGZ.

B) No difference was observed in cluster count between predator stressed (PS) and handled controls (HC) at any of the bins ($F(2.51, 50.26)= 0.56$, $p=0.62$). Within the HC group, there were significant differences across bins ($F(4, 50)= 5.12$, $p=0.002$). Within the PS group, there were significant differences across bins ($F(4,50)= 8.56$, $p<0.001$). Letters **a-b** correspond with the HC group only and **c-d** correspond with PS only. Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.05$).

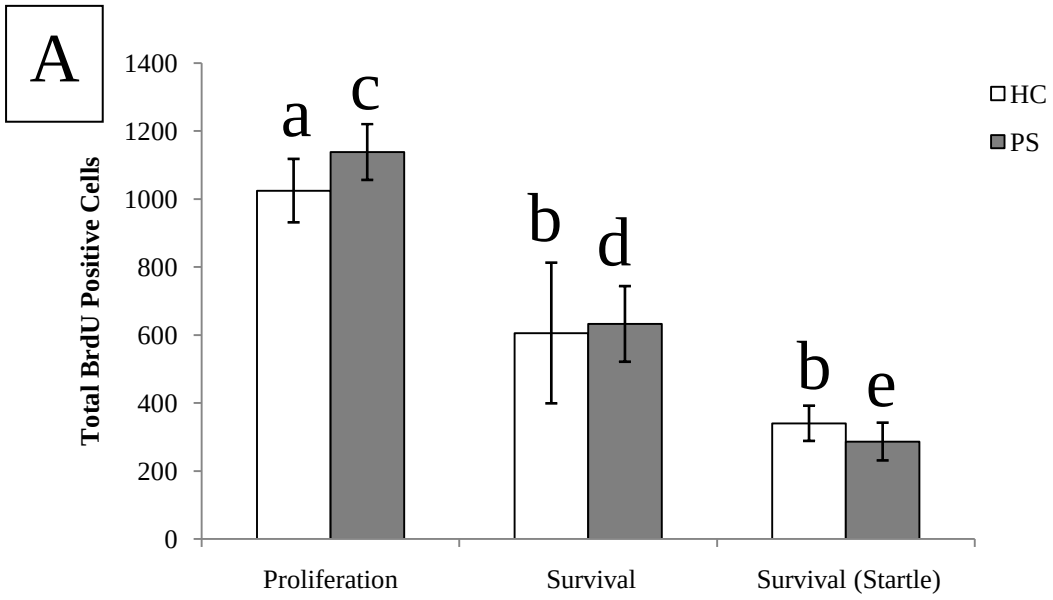


Figure 19A: Total number of BrdU positive cells across different experiments between the predator stressed (PS) and handled control (HC) animals in the SGZ.

A) Within the HC condition, there were significant differences observed ($F(2,17)= 18.0$, $p<0.001$). Within the PS condition, there were significant differences observed ($F(2,21)= 31.3$, $p<0.001$). Letters **a-b** correspond with the HC group only and **c-e** correspond with PS only. Bins marked with the same letter do not differ, bins marked with different letters are significantly different ($p<0.001$).

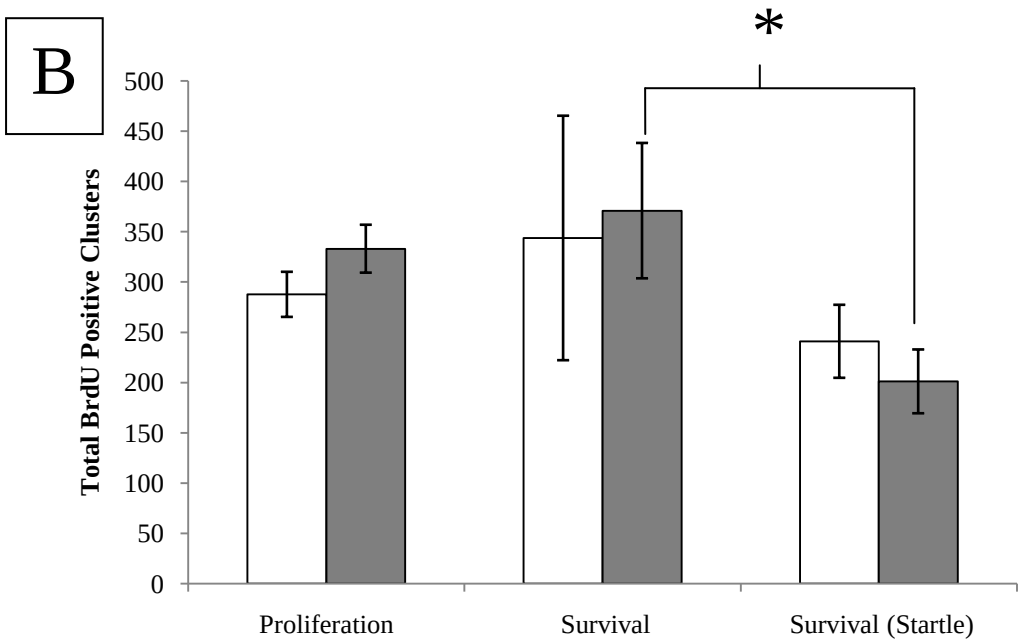


Figure 19B: Total number of BrdU positive clusters across different experiments between the predator stressed (PS) and handled control (HC) animals in the SGZ.

B) Within the HC condition, no significant differences were observed ($F(2,17)= 0.87$, $p=0.44$). Within the PS condition, a significant difference was observed between the survival and survival startle study ($p=0.025$).

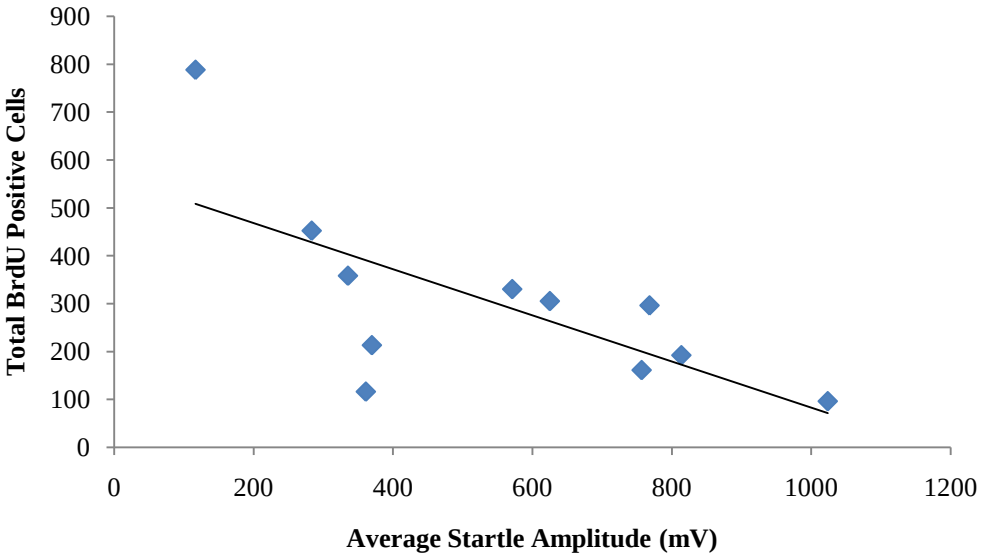


Figure 20: Experiment 3 (Survival/Startle): Correlation between BrdU positive cells and startle amplitude. Fewer total BrdU positive cells are strongly associated with higher average startle amplitudes from Bin 2 in the predator stressed condition ($r(9)= -0.68$, $p=0.021$).

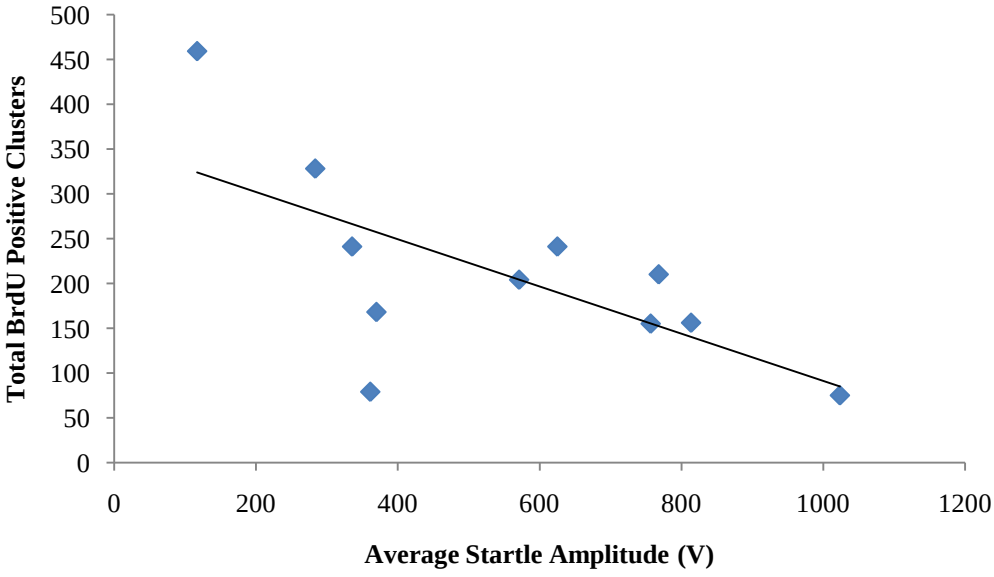


Figure 21: Experiment 3 (Survival/Startle): Correlation between BrdU positive clusters and startle amplitude. Fewer total BrdU positive clusters are strongly associated with higher average startle amplitudes from Bin 2 in the predator stressed condition ($r(8)= -0.69$, $p=0.03$).

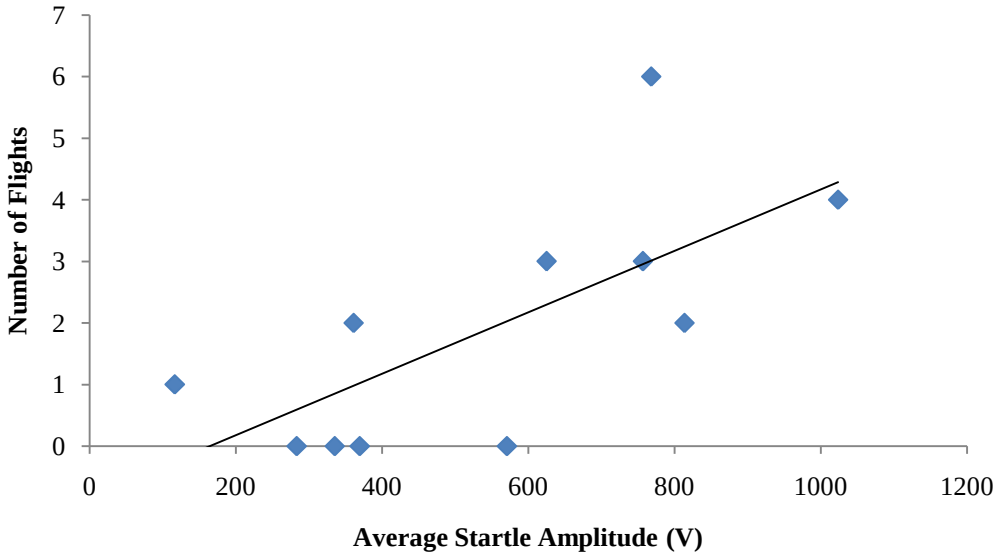


Figure 22: Experiment 3 (Survival/Startle): Correlation between flights and startle amplitude. A higher number of flights from the cat are strongly associated with higher average startle amplitudes from Bin 2 in the predator stressed condition ($r(9)= 0.7$, $p=0.017$).

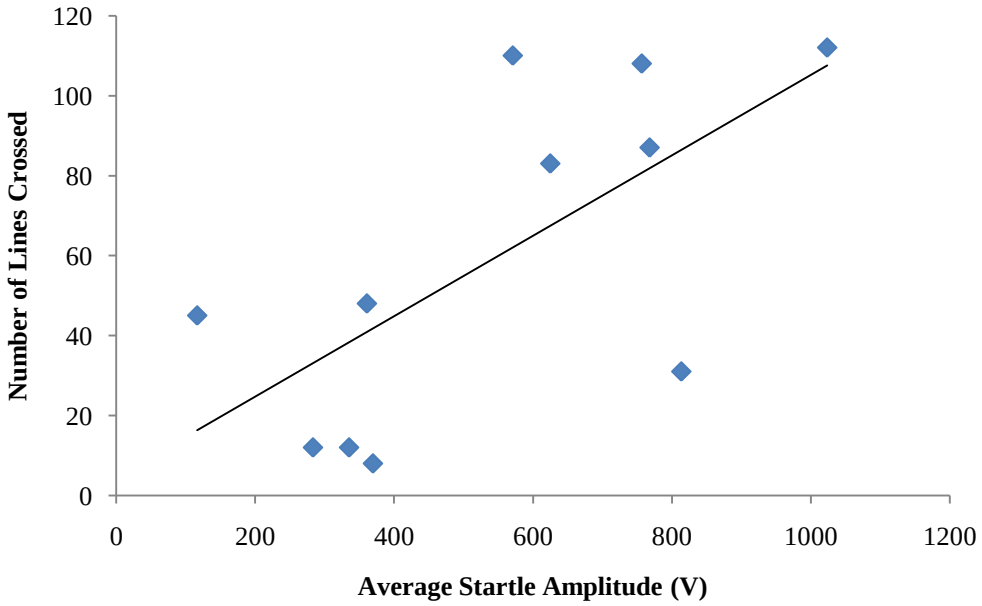


Figure 23: Experiment 3 (Survival/Startle): Correlation between lines and startle amplitude. High levels of locomotion as measured by number of lines crossed are strongly associated with higher average startle amplitudes from Bin 2 in the predator stressed condition ($r(9)= 0.67$, $p=0.024$).

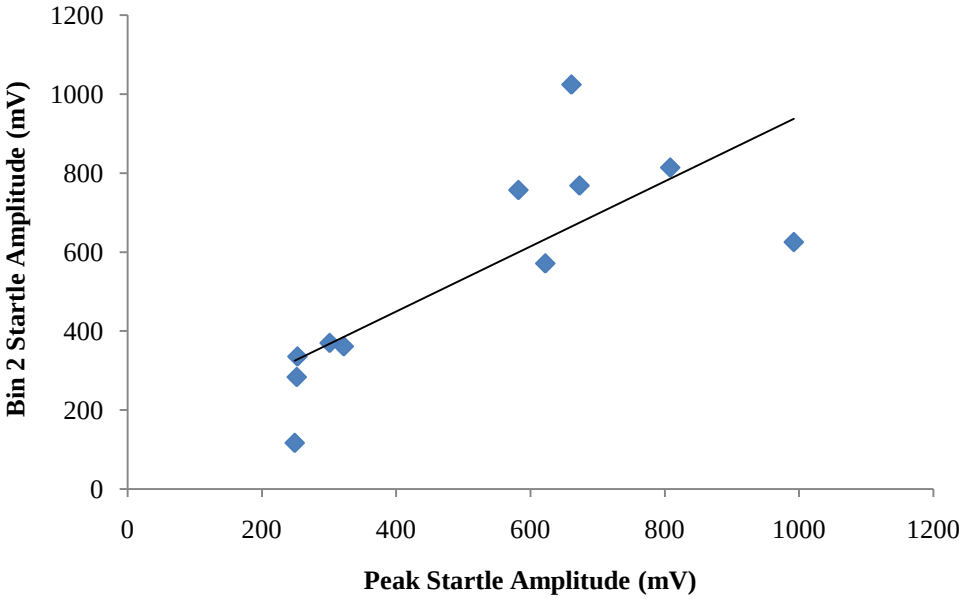


Figure 24: Experiment 3 (Survival/Startle): Correlation between predator stressed (PS) startle amplitudes. Startle amplitude from Bin 2 in the predator stressed group is positively correlated with peak startle amplitude ($r(9)= 0.77$, $p=0.006$).

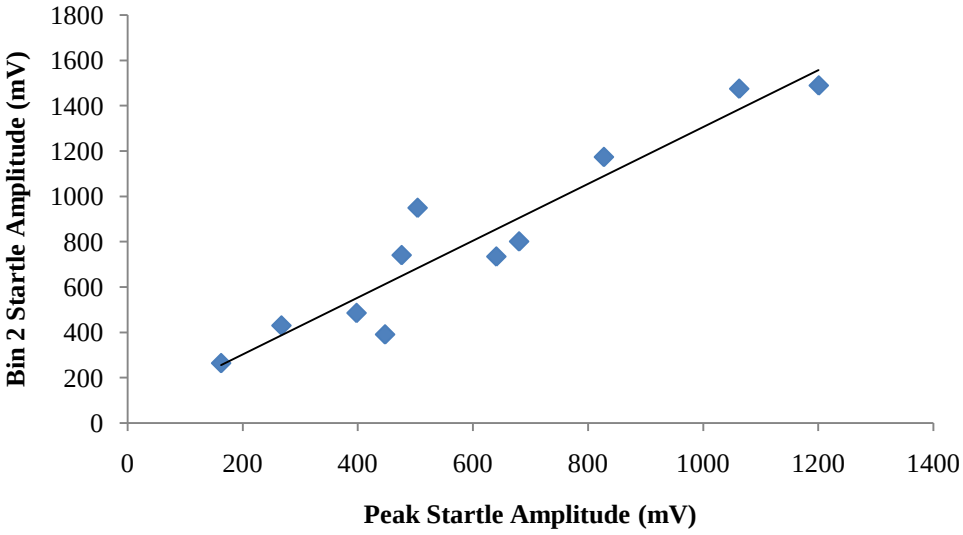


Figure 25: Experiment 3 (Survival/Startle): Correlation between handle controlled (HC) startle amplitudes. Startle amplitude from Bin 2 in the handled control group is positively correlated with peak startle amplitude ($r(9)= 0.95$, $p<0.001$).

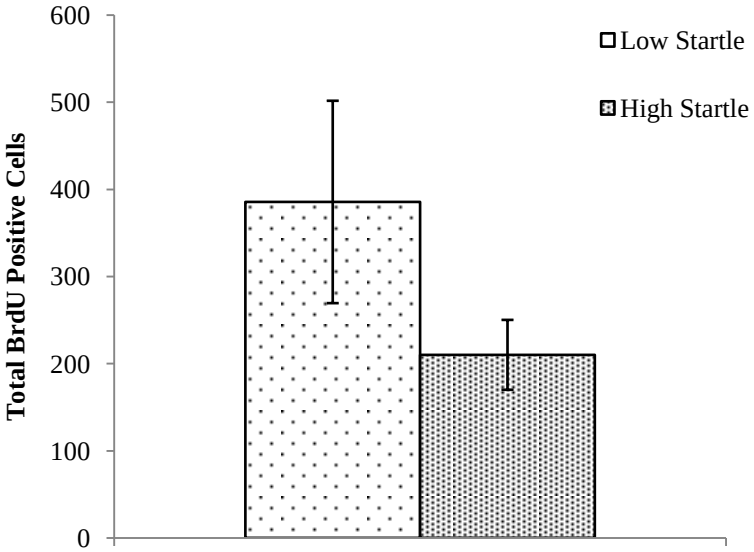


Figure 26: Experiment 3 (Survival/Startle): Median split of response to startle (low or high) in terms of total BrdU positive cells in the SGZ of the predator stressed condition. No significant difference is observed based on startle amplitude (t(8)= 1.43, p=0.19).

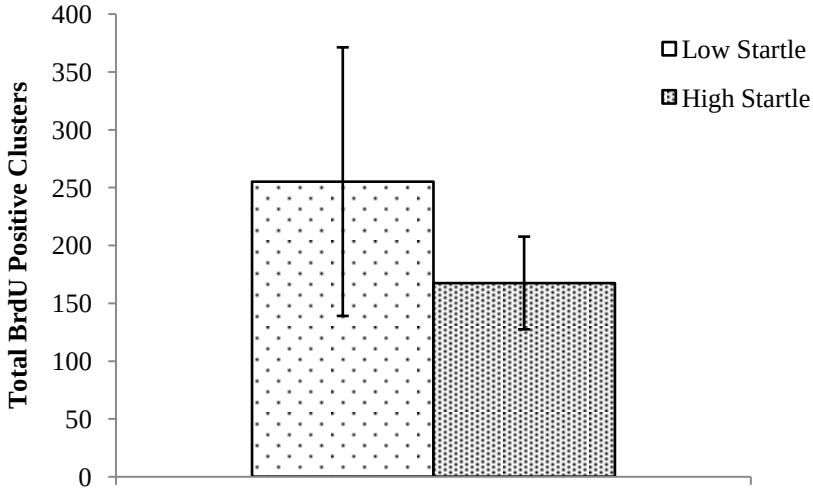


Figure 27: Experiment 3 (Survival/Startle): Median split of response to startle (low or high) in terms of total BrdU positive clusters in the SGZ of the predator stressed condition. No significant difference is observed between conditions (t(8)= 1.23, p=0.25).

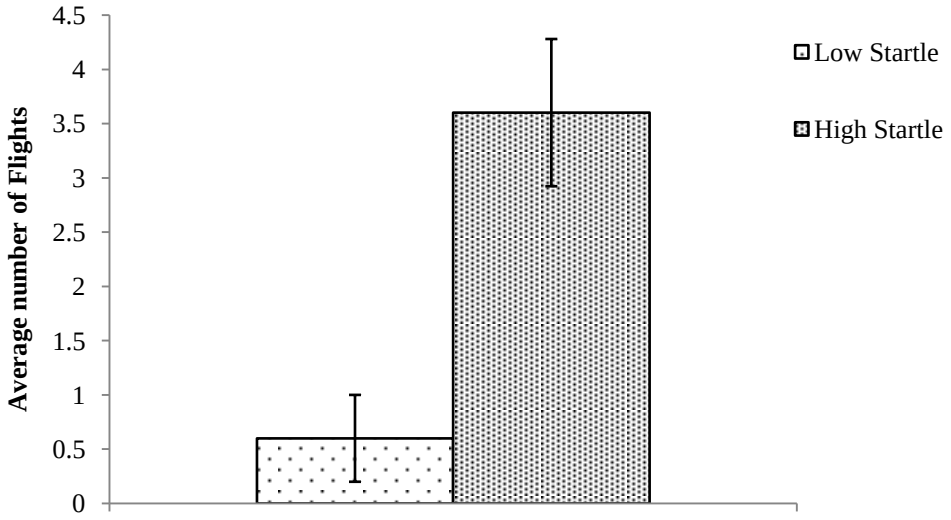


Figure 28: Experiment 3 (Survival/Startle): Median split of response to startle (low or high) in terms of average number of flights in the predator stressed condition. A significant difference between conditions was observed ($t(8)= 0.369$, $p= 0.005$).

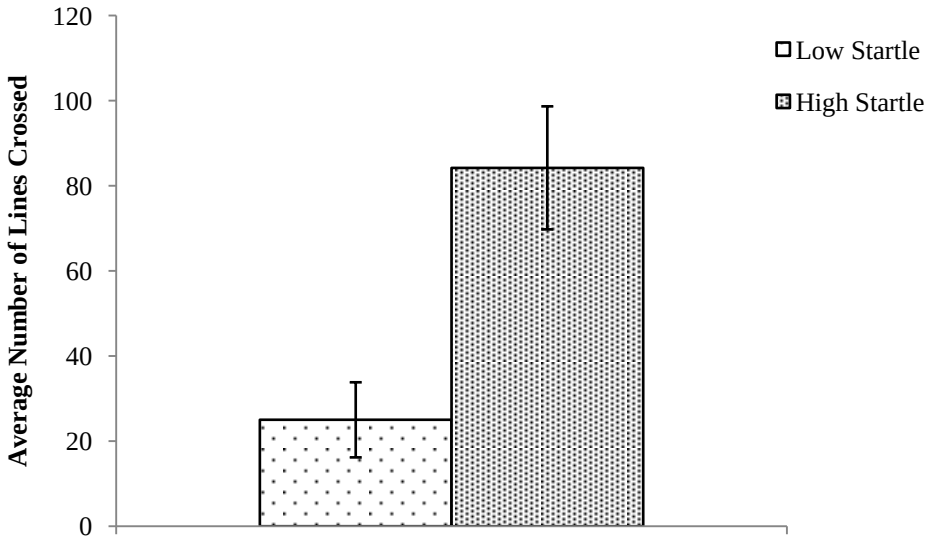


Figure 29: Experiment 3 (Survival/Startle): Median split of response to startle (low or high) in terms of average number of lines crossed in the predator stressed condition. A significant difference between conditions was observed ($t(8)= 0.651$, $p=0.008$).

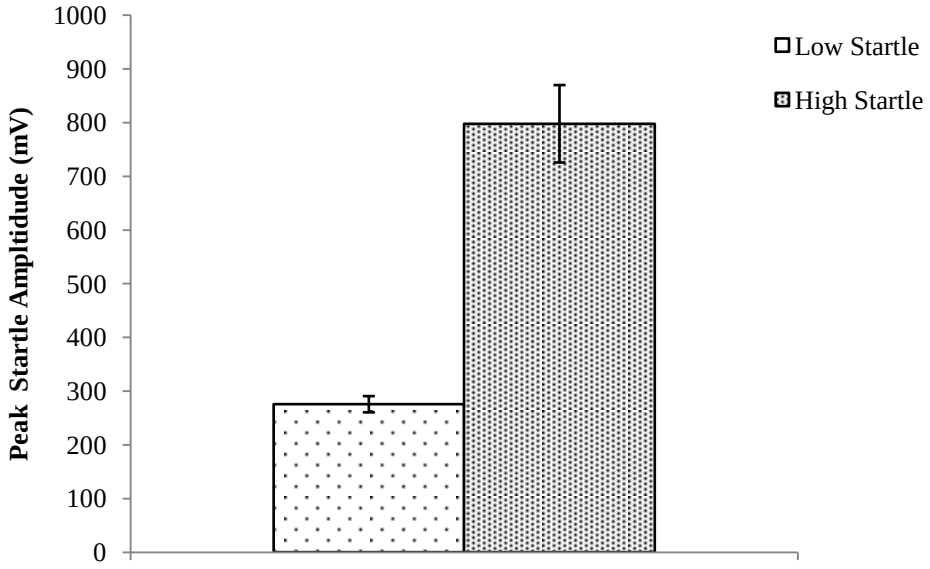


Figure 30: Experiment 3 (Survival/Startle): Median split of response to startle (low or high) in terms of peak startle amplitude for the predator stressed condition. A significant difference between conditions was observed (t(4.352) = -6.356, p=0.002).

Appendix I: Summary of Statistical Tests

Table 1: Experiment 1- The effects of predator stress on cell proliferation in the SGZ

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
CORT Levels	PS vs. HC	PS: 6 HC: 5	Mean= 24.9, SD= 8.2 Mean= 6.6, SD=2.6	Independent-samples T Test: **t(9.2)= -6.02, p<0.001**
Total cell Count	PS vs. HC	PS: 6 HC: 5	Mean= 1138, SD= 201.4 Mean= 1024, SD= 208.6	Independent-samples T Test: t(9)= -0.92, p=0.38
Total cluster Count	PS vs. HC	PS: 6 HC: 5	Mean= 333, SD= 58.4 Mean= 288, SD= 50.1	Independent-samples T Test: t(9)= -1.37, p=0.21
Cells across AP plane	PS vs. HC	PS: 6 HC: 5	B1: Mean=79, SD=42.3 B2: Mean=227, SD=41.6 B3: Mean=216, SD=75.6 B4: Mean=467, SD=165.3 B5: Mean=150, SD=55.1 B1: Mean=50, SD=38.1 B2: Mean=172, SD=59.2 B3: Mean=194, SD=67.3 B4: Mean=461, SD=87.6 B5: Mean=148.2, SD=81.8	Repeated Measure two-way ANOVA: Main Effect – **F(2.09, 18.79)= 40.30, p<0.001** Interaction – F(2.09, 18.79)= 0.21, p=0.82
Clusters across AP plane	PS vs. HC	PS: 6 HC: 5	B1: Mean=23, SD=10.1 B2: Mean=66, SD=14.3 B3: Mean=61, SD=19.0 B4: Mean=137, SD=41.9 B5: Mean=45, SD=13.7 B1: Mean=15, SD=9.5 B2: Mean=49, SD=16 B3: Mean=56, SD=18.3 B4: Mean=124, SD=21.1 B5: Mean=44, SD=19.9	Repeated Measure two-way ANOVA: Main Effect – **F (2.17, 19.51) = 49.62, p<0.001** Interaction – F(2.17, 19.51)= 0.307, p=0.756

Cells in DG blades	HC: Upper vs. Lower blade	5	U: Mean=461, SD=45.0 L: Mean=601, SD=199.5	Independent-samples T Test: t(8)=-1.53, p=0.164
	PS: Upper vs. Lower blade	6	U: Mean=485, SD=99.5 L: Mean=582, SD=106.5	Independent-samples T Test: t(10)= -1.62, p=0.136
Clusters in DG blades	HC: Upper vs. Lower blade	5	U: Mean=130, SD=18.1 L: Mean=171, SD=45.6	Independent-samples T Test: t(8)= -1.88, p=0.097
	PS: Upper vs. Lower blade	6	U: Mean=163, SD=59.1 L: Mean=204, SD=71.0	Independent-samples T Test: t(10)= -1.09, p=0.302
Cells in upper blade of DG	PS vs. HC	PS: 6 HC: 5	PS: Mean=485, SD=99.5 HC: Mean=461, SD=45.0	Independent-samples T Test: t(7.22)= -0.537, p=0.608
Clusters in upper blade of DG	PS vs. HC	PS: 6 HC: 5	PS: Mean=163, SD=59.1 HC: Mean=130, SD=18.1	Independent-samples T Test: t(9)= -1.193, p=0.264
Cells in lower blade of DG	PS vs. HC	PS: 6 HC: 5	PS: Mean=582, SD=106.5 HC: Mean=601, SD=199.5	Independent-samples T Test: t(9)= 0.204, p=0.843
Clusters in lower blade of DG	PS vs. HC	PS: 6 HC: 5	PS: Mean=204, SD=71.0 HC: Mean=171, SD=45.6	Independent-samples T Test: t(9)= -0.886, p=0.399
SGZ cells in relation to CORT	PS + HC vs. CORT	11	N/A	Pearson's r Correlation: r(9)= 0.35, p=0.30
	PS vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.295, p=0.571

	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.071, p=0.910
SGZ clusters in relation to CORT	PS + HC vs. CORT	11	N/A	Pearson's r Correlation: r(9)=0.5, p=0.118
	PS vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.348, p=0.499
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= 0.248, p=0.688

Table 2: Experiment 1- The effects of predator stress on cell proliferation in the CA1

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cells in CA1	PS vs. HC	PS: 6 HC: 5	Mean= 58, SD=28.7 Mean=54, SD=10.3	Independent-samples T Test: t(6.48) = -0.363, p=0.728
Clusters in CA1	PS vs. HC	PS: 6 HC: 5	Mean=36, SD=15.0 Mean=34, SD=6.4	Independent-samples T Test: t(7)= -0.163, p=0.875
Cells in CA1 in relation to CORT	PS + HC vs. CORT	11	N/A	Pearson's r Correlation: r(9)= 0.109, p=0.75
	PS vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.061, p=0.909
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.322, p=0.597
Clusters in CA1 in relation to CORT	PS + HC vs. CORT	11	N/A	Pearson's r Correlation: r(9)= 0.076, p=0.86
	PS vs. CORT	6	N/A	Pearson's r Correlation: r(4)= -0.344, p= 0.571
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= 0.108, p=0.84

Table 3: Experiment 1- The effects of predator stress on cell proliferation in the amygdala

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cells in Amygdala (Total)	PS vs. HC	PS: 4 HC: 3	Mean=158, SD=108.6 Mean=149, SD=64.7	Independent-samples T Test: t (5)=-0.125, p=0.906
Clusters in Amygdala (Total)	PS vs. HC	PS: 4 HC: 3	Mean=46, SD=13.6 Mean=39, SD=17.8	Independent-samples T Test: t(5)=-0.622, p=0.561
Cells in Amygdala (Lateral)	PS vs. HC	PS: 4 HC: 3	Mean=57, SD=45.3 Mean=48, SD=17.1	Independent-samples T Test: t(5)= -0.312, p=0.767
Clusters in Amygdala (Lateral)	PS vs. HC	PS: 4 HC: 3	Mean=13, SD=4.6 Mean=12, SD=5.0	Independent-samples T Test: t(5)= -0.250, p=0.819
Cells in Amygdala (Central)	PS vs. HC	PS: 4 HC: 3	Mean=24, SD=15.0 Mean=21, SD=5.8	Independent-samples T Test: t(5)= -0.332, p=0.753
Clusters in Amygdala (Central)	PS vs. HC	PS: 4 HC: 3	Mean=9, SD=3.6 Mean=7, SD=3.5	Independent-samples T Test: t(5)=-0.766, p=0.478
Cells in Amygdala (Intercalated)	PS vs. HC	PS: 4 HC: 3	Mean=17, SD=6.2 Mean=17, SD=8.1	Independent-samples T Test: t(5)= -0.108, p=0.918
Clusters in Amygdala (Intercalated)	PS vs. HC	PS: 4 HC: 3	Mean=5, SD=2.4 Mean=3, SD=1.5	Independent-samples T Test: t(5)= -0.734, p=0.496
Cells in Amygdala (Medial)	PS vs. HC	PS: 4 HC: 3	Mean=39, SD=25.4 Mean=48, SD=28.9	Independent-samples T Test: t(5)= 0.423, p=0.690

Clusters in Amygdala (Medial)	PS vs. HC	PS: 4 HC: 3	Mean=15, SD=3.8 Mean=12, SD=6.7	Independent-samples T Test: t(5)= -0.617, p=0.564
Cells in Amygdala (Cortical)	PS vs. HC	PS: 4 HC: 3	Mean=22, SD=20.4 Mean=16, SD=11.0	Independent-samples T Test: t(5)= -0.391, p=0.712
Clusters in Amygdala (Cortical)	PS vs. HC	PS: 4 HC: 3	Mean=4.8, SD=1.7 Mean=4, SD=2.0	Independent-samples T Test: t(5)= -0.537, p=0.615
Cells across Amygdala regions	HC	3	LA: Mean=48, SD=17.1 C: Mean=21, SD=5.8 I: Mean=17, SD=8.1 M: Mean=48, SD=28.9 AC: Mean=16, SD=11.0	One-way ANOVA: F(4, 10)= 3.03, p=0.071
	PS	4	LA: Mean=57, SD=45.3 C: Mean=24, SD=15.0 I: Mean=17, SD=6.2 M: Mean=39, SD=25.4 AC: Mean=22, SD=20.4	One-way ANOVA: F(4,15)= 1.57, p=0.234
Clusters across Amygdala regions	HC	3	LA: Mean=12, SD=5.0 C: Mean=7, SD=3.5 I: Mean=3, SD=1.5 M: Mean=12, SD=6.7 AC: Mean=4, SD=2	One-way ANOVA: F(4,10)= 3.26, p=0.059
	PS	4	LA: Mean=13, SD=4.6 C: Mean=9, SD=3.6 I: Mean=5, SD=2.4 M: Mean=15, SD=3.8 AC: Mean=5, SD=1.7	One-way ANOVA: **F(4,15)= 7.79,** **p=0.001** Post-Hoc (Bonferroni) Test: **L vs. I: p=0.023** **L vs. AC: p=0.029** **M vs. I: p=0.007** **M vs. AC: p=0.008**

Cells in amygdala vs. CORT	PS and HC	7	N/A	Pearson's r Correlation: r(5)= -0.081, p=0.86
Clusters in amygdala vs. CORT	PS and HC	7	N/A	Pearson's r Correlation: r(5)= -0.067, p=0.89

Table 4: Experiment 1- The effects of predator stress on cell proliferation in the VMH

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cells in VMH (Total)	PS vs. HC	5	PS: Mean=49, SD=34.2 HC: Mean=23, SD=16.6	Independent-samples T Test: t(8)= -1.52, p= 0.168
Clusters in VMH (Total)	PS vs. HC	5	PS: Mean=31, SD=20.4 HC: Mean=15, SD=10.4	Independent-samples T Test: t(8)= -1.54, p=0.162
Cells in VMH (region)	PS vs. HC	5	PS: Mean=17, SD=11.5 HC: Mean=10, SD=7.3	Independent-samples T Test: t(8)= -1.05, p=0.323
Clusters in VMH (region)	PS vs. HC	5	PS: Mean=11, SD=4.8 HC: Mean=7, SD=4.6	Independent-samples T Test: t(8)= -1.56, p=0.157
Cells in VMH (dorsal)	PS vs. HC	5	PS: Mean=14, SD=9.2 HC: Mean=6, SD=5.2	Independent-samples T Test: t(8)= -1.52, p=0.167
Clusters in VMH (dorsal)	PS vs. HC	5	PS: Mean=7, SD=6.7 HC: Mean=4, SD=2.7	Independent-samples T Test: t(8)= -0.807, p=0.443
Cells in VMH (lateral)	PS vs. HC	5	PS: Mean=19, SD=17.5 HC: Mean=7, SD=8.3	Independent-samples T Test: t(8)= -1.41, p=0.198
Clusters in VMH (lateral)	PS vs. HC	5	PS: Mean=13, SD=10.4 HC: Mean=4, SD=5.3	Independent-samples T Test: t(8)= -.1.64, p=0.139
Cells across VMH regions	HC	5	r: Mean=10, SD=7.3 d: Mean=6, SD=5.2 l: Mean=7, SD=8.3	One-way ANOVA: F(2,12)= 0.440, p=0.654
	PS	5	r: Mean=17, SD=11.5 d: Mean=14, SD=9.2 l: Mean=19, SD=17.5	One-way ANOVA: F(2,12)= 0.209, p=0.814
Clusters across VMH regions	HC	5	r: Mean=7, SD=4.6 d: Mean=4, SD=2.7 l: Mean=4, SD=5.3	One-way ANOVA: F(2,12)= 0.472, p=0.635
	PS	5	r: Mean=11, SD=4.8 d: Mean=7, SD=6.7 l: Mean=13, SD=10.4	One-way ANOVA: F(2,12)=0.868, p=0.445

Cells in VMH (total) in relation to CORT	PS + HC vs. CORT	10	N/A	Pearson's r Correlation: r(8)= 0.275, p=0.443
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.307, p=0.616
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.488, p=0.405
Clusters in VMH (total) in relation to CORT	PS + HC vs. CORT	10	N/A	Pearson's r Correlation: r(8)= 0.344, p=0.331
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.333, p=0.584
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.463, p=0.432
Cells in VMH (region) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.45 ,p=0.44
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.453, p=0.443
	HC vs. CORT	5	N/A	Pearson's r Correlation: (r(3)= -0.467, p=0.428
Clusters in VMH (region) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.59 ,p=0.30

	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.587, p=0.298
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.392, p=0.514
Cells in VMH (dorsal) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.24 ,p=0.69
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.244, p=0.693
	HC vs. CORT	5	N/A	Pearson's r Correlation: (r(3)= 0.142, p=0.820
Clusters in VMH (dorsal) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.19 ,p=0.77
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.186, p=0.765
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= 0.115, p=0.854
Cells in VMH (lateral) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.17 ,p=0.78

	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.173, p=0.781
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.653, p=0.232
Clusters in VMH (lateral) in relation to CORT	PS + HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)=-0.26, p=0.67
	PS vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.264, p=0.668
	HC vs. CORT	5	N/A	Pearson's r Correlation: r(3)= -0.632, p=0.252

Table 5: Experiment 1- Predator stress behaviour

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
PS behaviour	Approach vs. Flights	6	N/A	Pearson's r Correlation: r(4)= 0.712, p=0.113
	Approach vs. Lines crossed	6	N/A	Pearson's r Correlation: r(4)= 0.407, p=0.423
	Flights vs. Lines crossed	6	N/A	Pearson's r Correlation: r(4)=0.049, p=0.926
	Approach vs. Proximity frequency	6	N/A	Pearson's r Correlation: r(4)= 0.791, p=0.061
	Lines crossed vs. Proximity frequency	6	N/A	Pearson's r Correlation: r(4)= 0.083, p=0.876
	Flights vs. Proximity frequency	6	N/A	Pearson's r Correlation: **r(4)= 0.917, p=0.01**
PS behaviour in relation to CORT	Approaches vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.385, p=0.450
	Flights vs. CORT	6	N/A	Pearson's r Correlation: r(4)= -0.206, p=0.696
	Lines crossed vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.510, p=0.301
	Proximity frequency vs. CORT	6	N/A	Pearson's r Correlation: r(4)= 0.157, p=0.766
PS behaviour in relation to cells in DG	Approaches vs. Cells	6	N/A	Pearson's r Correlation: r(4)=0.295, p=0.571

	Flights vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.610, p=0.199
	Lines crossed vs. Cells	6	N/A	Pearson's r Correlation: (r(4)= 0.322, p=0.53
	Proximity Frequency vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.57, p=0.234
PS behaviour in relation to clusters in DG	Approaches vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= 0.130, p=0.806
	Flights vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.518, p=0.293
	Lines crossed vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= 0.273, p=0.601
	Proximity Frequency vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.491, p=0.322
PS behaviour in relation to cells in CA1	Approaches vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.244, p=0.641
	Flights vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.704, p=0.118
	Lines crossed vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.072, p=0.892
	Proximity Frequency vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.687, p=0.131

PS behaviour in relation to clusters in CA1	Approaches vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.244, p=0.641
	Flights vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.704, p=0.118
	Lines crossed vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.072, p=0.892
	Proximity Frequency vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.687, p=0.131
PS behaviour in relation to cells in VMH	Approaches vs. Cells	6	N/A	Pearson's r Correlation: r(3)=-0.127, p=0.839
	Flights vs. Cells	6	N/A	Pearson's r Correlation: r(3)= -0.118, p=0.850
	Lines crossed vs. Cells	6	N/A	Pearson's r Correlation: (r(3)= 0.468, p=0.427
	Proximity Frequency vs. Cells	6	N/A	Pearson's r Correlation: (r(3)= -0.130, p=0.835
PS behaviour in relation to clusters in VMH	Approaches vs. Clusters	6	N/A	Pearson's r Correlation: r(3)=-0.162, p=0.79
	Flights vs. Clusters	6	N/A	Pearson's r Correlation: r(3)= -0.127, p=0.838
	Lines crossed vs. Clusters	6	N/A	Pearson's r Correlation: r(3)= -0.066, p=0.916

	Proximity Frequency vs. Clusters	6	N/A	Pearson's r Correlation: r(3)= -0.149, p=0.811

Table 6: Experiment 2- The effects of predator stress on cell survival in the SGZ

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cell count	PS vs. HC	PS: 6 HC: 3	Mean=632, SD=272.4 Mean=606, SD=358.3	Independent-samples T test: (t(7)= -0.126, p=0.903
Cluster count	PS vs. HC	PS: 6 HC: 3	Mean=371, SD=164.8 Mean=344, SD=210.5	Independent-samples T test: t(7)= -0.215, p=0.836
Cells across AP plane	PS vs. HC	PS: 6 HC: 3	B1:Mean=48, SD=26.8 B2:Mean=177,SD=128 B3:Mean=117,SD=47.8 B4:Mean=231,SD=133.8 B5:Mean=60,SD=44.9 B1:Mean=51, SD=15.6 B2:Mean=176,SD=81.8 B3:Mean=152,SD=133.4 B4:Mean=153,SD=100.0 B5:Mean=73,SD=44.0	Repeated Measure two-way ANOVA: Main Effect – **F(1.67, 11.70) = 6.35, p=0.017** Interaction – F(1.67, 11.70)= 0.709, p= 0.49
Clusters across AP plane	PS vs. HC	PS: 6 HC: 3	B1:Mean=27,SD=12.3 B2:Mean=99,SD=72.8 B3:Mean=70,SD=25.2 B4:Mean=137,SD=89.7 B5:Mean=38,SD=28.6 B1:Mean=29,SD=6.4 B2:Mean=102,SD=45.9 B3:Mean=87,SD=79.2 B4:Mean=87,SD=66.9 B5:Mean=39,SD=25.0	Repeated Measure two-way ANOVA: Main Effect – **F(1.61, 11.24)= 5.47, p=0.027** Interaction – F(1.61, 11.24)= 0.64, p=0.51
PS behaviour	Approach vs. Flights	6	N/A	Pearson's r Correlation: r(4)=0.74 ,p=0.09
	Approach vs. Lines crossed	6	N/A	Pearson's r Correlation: r(4)=0.79,p=0.06

	Flights vs. Lines crossed	6	N/A	Pearson's r Correlation: r(4)=0.40 ,p=0.43
	Approach vs. Proximity frequency	6	N/A	Pearson's r Correlation: r(4)=0.73 ,p=0.1
	Lines crossed vs. Proximity frequency	6	N/A	Pearson's r Correlation: r(4)=0.20 ,p=0.71
	Flights vs. Proximity frequency	6	N/A	Pearson's r Correlation: **r(4)= 0.878, p=0.021**
PS behaviour in relation to cells in DG	Approaches vs. Cells	6	N/A	Pearson's r Correlation: r(4)= -0.523, p=0.287
	Flights vs. Cells	6	N/A	Pearson's r Correlation: r(4)= 0.042, p=0.938
	Lines crossed vs. Cells	6	N/A	Pearson's r Correlation: r(10)= -0.445, p=0.376
	Proximity Frequency vs. Cells	6	N/A	Pearson's r Correlation: r(4) = -0.173, p=0.743
PS behaviour in relation to clusters in DG	Approaches vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.606, p=0.202
	Flights vs. Clusters	6	N/A	Pearson's r Correlation: r(4)=-0.011, p=0.984
	Lines crossed vs. Clusters	6	N/A	Pearson's r Correlation: (r)= -0.499, p=0.313
	Proximity Frequency vs. Clusters	6	N/A	Pearson's r Correlation: r(4)= -0.246, p=0.638

Table 7: Experiment 3- The effects of predator stress on cell survival (startle) in the SGZ

Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cell count	PS vs. HC	11	PS:Mean=301,SD=194.8 HC:Mean=349,SD=185.6	Independent-samples T test: t(20)= 0.594, p=0.559
Cluster count	PS vs. HC	11	PS:Mean=211,SD=110 HC:Mean=246,SD=130.6	Independent-samples T test: t(20)= 0.687, p=0.50
Cells across AP plane	PS vs. HC	PS=11 HC=11	B1: Mean=18, SD=17.2 B2: Mean=63, SD=51.7 B3: Mean=62, SD=55.1 B4: Mean=113, SD=55.3 B5: Mean=43, SD=41.2 B1: Mean=28, SD=23.7 B2: Mean=71, SD=62.6 B3: Mean=87, SD=47.4 B4: Mean=112, SD=61.1 B5: Mean=51, SD=35.5	Repeated Measure two-way ANOVA: Main Effect – **F(2.58, 51.53)= 24.42, p<0.001** Interaction – F(2.57, 51.53)=0.48, p=0.669
Clusters across AP plane	PS vs. HC	PS=11 HC=11	B1: Mean=12, SD=10.7 B2: Mean=43, SD=31.4 B3: Mean=42, SD=31.5 B4: Mean=81, SD=36.6 B5: Mean=32, SD=25.0 B1: Mean=22, SD=18.9 B2: Mean=46, SD=35.7 B3: Mean=59, SD=26.3 B4: Mean=81, SD=46.4 B5: Mean=39, SD=27.4	Repeated Measure two-way ANOVA: Main Effect – **F(2.51, 50.26)= 28.0, p<0.001** Interaction – F(2.51, 50.26)=0.56, p=0.617
Acoustic Startle (peak startle amplitude)	PS vs. HC	11	PS: Mean=519.5, SD=258.3 HC: Mean=605.9, SD=320.2	Independent-samples T test: t(20)= 0.70, p=0.49

Acoustic Startle (Habituation)	PS vs. HC	PS=11	Mean differences and standard deviations between bins:	Independent-samples T test:
			B1-2:Mean=33.6, SD=38.4	**t(20)= -2.4, p=0.03**
			B2-3:Mean=6.5, SD=38.3	t(20)= 0.73 , p=0.47
			B3-4:Mean=14.0, SD=28.6	t(20)=-1.1 , p=0.28
			B4-5:Mean= -53.1, SD=68.2	t(20)=1.6 , p=0.14
			B5-6:Mean=2.1, SD=44.3	t(20)= -0.2 , p=0.79
			B6-7:Mean=10.8, SD=32.1	t(20)=-1.5 , p=0.14
			B7-8:Mean= -16.6, SD=58.8	t(20)=0.98 , p=0.34
			B8-9:Mean=-21.7, SD=76.3	t(20)=-0.86 , p=0.40
			B9-10:Mean=24.1, SD=36.5	t(20)=1.9, p=0.07
		HC=11	B1-2:Mean=0.20, SD=27.0	
			B2-3:Mean=21.2, SD=54.1	
			B3-4:Mean=-4.9, SD=48.3	
			B4-5:Mean=-11.3, SD=57.2	

			B5-6:Mean=-2.8, SD=41.8 B6-7:Mean=-31.8, SD=86.7 B7-8:Mean=5.2, SD=44.9 B8-9:Mean=-63.7, SD=142.1 B9-10:Mean=24.1, SD=36.5	
Cell counts vs. startle amplitude	PS	11	N/A	Pearson's r Correlation: **r(9)= -0.68, p=0.021**
	HC	11	N/A	Pearson's r Correlation: r(9)=0.19, p=0.59
Cluster counts vs. startle amplitude	PS	11	N/A	Pearson's r Correlation: r(8)= -0.66, p=0.03
	HC	11	N/A	Pearson's r Correlation: r(9)= -0.025, p=0.94
Startle (Bin 2) vs. peak startle amplitude	PS	11	N/A	Pearson's r Correlation: **r(9)=0.77, p=0.006**
	HC	11	N/A	Pearson's r Correlation: **r(9)= 0.95, p<0.001**
Acoustic Startle (Bin 2) in relation with PS behaviours	Flights vs. startle amplitude	11	N/A	Pearson's r Correlation: **r(9)= 0.7, p=0.017**

	Lines crossed vs. startle amplitude	11	N/A	Pearson's r Correlation: **r(9)= 0.67, p=0.024**
	Approaches vs. startle amplitude	11	N/A	Pearson's r Correlation: r(9)= 0.23, p=0.53
	Proximity Frequency vs. startle amplitude	11	N/A	Pearson's r Correlation: r(9)= 0.29, p=0.41
Median Split based on level of startle for PS group	Low vs. High startle cell count	10	N/A	Independent-samples T test: t(8)= 1.428, p=0.191
	Low vs. High startle flights	10	N/A	Independent-samples T test: t(8)= 0.369, p= 0.005
	Low vs. High startle lines crossed	10	N/A	Independent-samples T test: t(8)= 0.651, p=0.008
	Low vs. High startle peak startle amplitude	10	N/A	Independent-samples T test: (t(4.352) = -6.356, p=0.002
	Low vs. High startle cluster count	10	N/A	Independent-samples T test: t(8)=1.23, p=0.25
PS Behaviour	Approach vs. Flights	11	N/A	Pearson's r Correlation: r(9)= 0.11, p=0.75
	Approach vs. Lines crossed	11	N/A	Pearson's r Correlation: r(9)= 0.50, p=0.12
	Flights vs. Lines crossed	11	N/A	Pearson's r Correlation: r(9)= 0.60, p=0.05
	Approach vs. Proximity frequency	11	N/A	Pearson's r Correlation: **r(9)= 0.87, p<0.001**

	Lines crossed vs. Proximity frequency	11	N/A	Pearson's r Correlation: r(9)= 0.47, p=0.15
	Flights vs. Proximity frequency	11	N/A	Pearson's r Correlation: r(9)=0.45, p=0.17
PS behaviour in relation to cells in DG	Approaches vs. Cells	11	N/A	Pearson's r Correlation: r(9)= -0.08, p=0.81
	Flights vs. Cells	11	N/A	Pearson's r Correlation: r(9)=-0.35, p=0.29
	Lines crossed vs. Cells	11	N/A	Pearson's r Correlation: r(9)=-0.29, p=0.39
	Proximity Frequency vs. Cells	11	N/A	Pearson's r Correlation: r(9)= -0.12, p=0.75
PS behaviour in relation to clusters in DG	Approaches vs. Clusters	11	N/A	Pearson's r Correlation: r(9)= -0.04, p=0.91
	Flights vs. Clusters	11	N/A	Pearson's r Correlation: r(9)= -0.34, p=0.30
	Lines crossed vs. Clusters	11	N/A	Pearson's r Correlation: r(9)= -0.32, p=0.33
	Proximity Frequency vs. Clusters	11	N/A	Pearson's r Correlation: r(9)= -0.045, p=0.90

Table 8: Comparing cell proliferation and survival groups

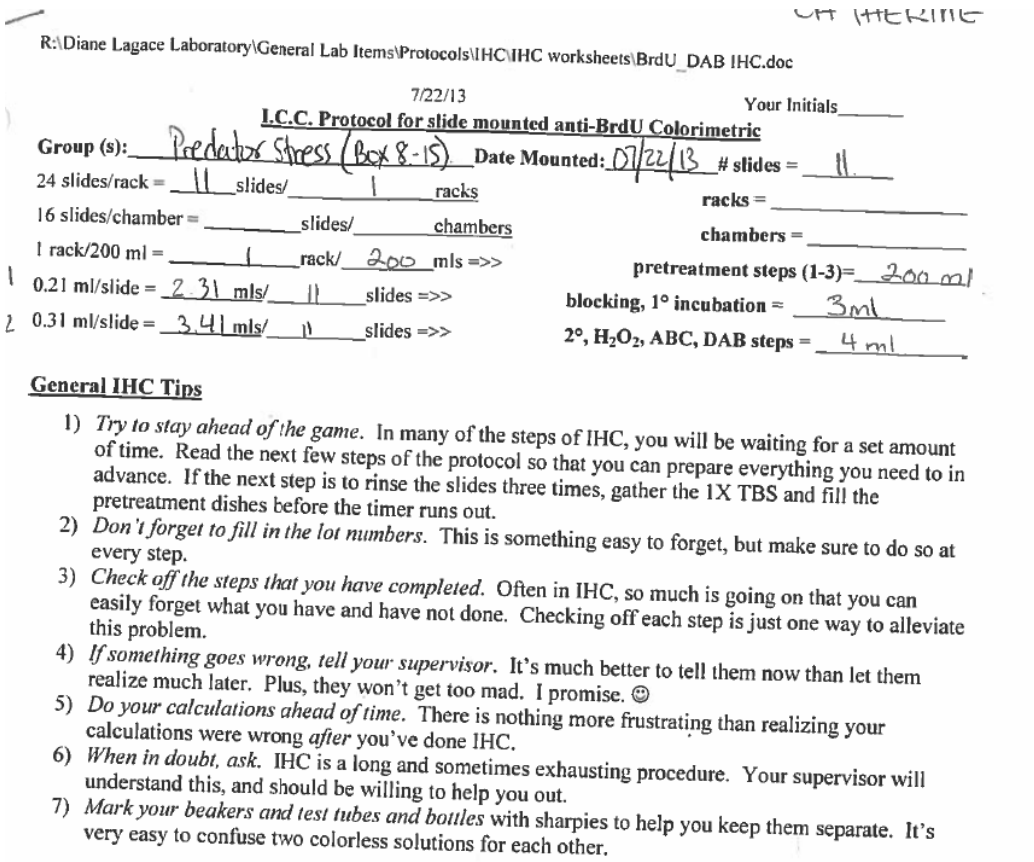
Analysis	Comparison	n of each group	Mean and Standard Deviation	Results
Cell counts in SGZ	HC2H vs. HC4W vs. HCST4W	HC2H=5 HC4W=3 HCST4W=11	HC2H Mean=1024, SD=208.6 HC4W Mean=606, SD=358.3 HCST4W Mean=340, SD=179.5	One-way ANOVA: **F(2,17)= 18.0 p<0.001** Post Hoc (Bonferroni) Test: **HC2H vs. HC4W: p<0.001** **HC2H vs. HCST4W: p<0.001** HC4W vs. HCST4W: P=0.218
	PS2H vs. PS4W vs. PSST4W	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=1138, SD=201.4 PS4W Mean=632, SD=272.4 PSST4W Mean=286, SD=192.2	One-way ANOVA: **F(2, 21)= 31.27, p<0.001** Post Hoc (Bonferroni) Test: **PS2H vs. PS4W: p<0.001** **PS2H vs. PSST4W: p<0.001** **PS4W vs. PSST4W: p=0.013**
Cluster counts in SGZ	HC2H vs. HC4W vs. HCST4W	HC2H=5 HC4W=3 HCST4W=11	HC2H Mean=288, SD=50.1 HC4W Mean=344, SD=210.5 HCST4W Mean=241, SD=125.7	One-way ANOVA: F(2,17)= 0.870, p=0.437

	PS2H vs. PS4W vs. PSST4W	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=333, SD=58.4 PS4W Mean=371, SD=164.8 PSST4W Mean=201, SD=109.9	One-way ANOVA: **F(2,21)= 5.175, p=0.015** Post Hoc (Bonferroni) Test: PS2H vs. PS4W: P=1.0 PS2H vs. PSST4W: P=0.10 **PS4W vs. PSST4W: p=0.025**
PS Behaviour	Approaches (2H, 4W, ST4W)	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=1.7, SD=1.6 PS4W Mean=1.0, SD=0.6 PSST4W Mean=1.5, SD=2.5	One-way ANOVA: F(2,21)= 0.187, p=0.831
	Flights 2H, 4W, ST4W	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=1.0, SD=1.5 PS4W Mean=1.8, SD=2.1 PSST4W Mean=1.8, SD=2.0	One-way ANOVA: F(2,21)=0.375, p=0.692

	Lines Crossed 2H, 4W, ST4W	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=86.2, SD=47.3 PS4W Mean=56.5, SD=31.9 PSST4W Mean=56.8, SD=40.9	One-way ANOVA: (F(2,21)= 1.19, p=0.325
	Proximity frequency 2H, 4W, ST4W	PS2H= 6 PS4W =6 PSST4W=11	PS2H Mean=1.0, SD=1.5 PS4W Mean=2.2, SD=1.7 PSST4W Mean=1.8, SD=2.2	One-way ANOVA: F(2,21)= 0.550, p=0.585

Appendix II: Protocols

A) IHC Protocol for BrdU



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7/22/13 Your Initials_____

I.C.C. Protocol for slide mounted anti-BrdU Colorimetric

Group (s): Predator Stress (Box 8-15) **Date Mounted:** 07/22/13 **# slides =** 11

24 slides/rack = 11 slides/ 1 racks **racks =** _____

16 slides/chamber = _____ slides/_____ chambers **chambers =** _____

1 rack/200 ml = 1 rack/ 200 mls =>> **pretreatment steps (1-3)=** 200 ml

1 0.21 ml/slide = 2.31 mls/ 11 slides =>> **blocking, 1° incubation =** 3ml

2 0.31 ml/slide = 3.41 mls/ 11 slides =>> **2°, H_2O_2, ABC, DAB steps =** 4 ml

General IHC Tips

1) *Try to stay ahead of the game.* In many of the steps of IHC, you will be waiting for a set amount of time. Read the next few steps of the protocol so that you can prepare everything you need to in advance. If the next step is to rinse the slides three times, gather the 1X TBS and fill the pretreatment dishes before the timer runs out.
2) *Don't forget to fill in the lot numbers.* This is something easy to forget, but make sure to do so at every step.
3) *Check off the steps that you have completed.* Often in IHC, so much is going on that you can easily forget what you have and have not done. Checking off each step is just one way to alleviate this problem.
4) *If something goes wrong, tell your supervisor.* It's much better to tell them now than let them realize much later. Plus, they won't get too mad. I promise. ☺
5) *Do your calculations ahead of time.* There is nothing more frustrating than realizing your calculations were wrong *after* you've done IHC.
6) *When in doubt, ask.* IHC is a long and sometimes exhausting procedure. Your supervisor will understand this, and should be willing to help you out.
7) *Mark your beakers and test tubes and bottles* with sharpies to help you keep them separate. It's very easy to confuse two colorless solutions for each other.

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7/22/13 Your Initials_____

1a. Antigen unmasking: 0.01M citric acid in MilliQ (MQ) H_2O, pH 6.0 (make fresh); 15 min 100°C.

Racks needed: 1 **Volume needed:** 200 ml.

0.01M citric acid: 1M = 192.1 g/1000 ml

=>> 0.01M = 1.92 g/1000 ml = (x) 0.384 g/ 200 mls MQ H_2O

Add 0.384 g citric acid to 200. mls MQ H_2O

pH to 6.00 Use 10N NaOH to adjust the pH until you reach close to pH 6. 0, then continue adjusting with 1N NaOH LOT # Citric Acid 0019815

Time in: 2:17 pm Time Out: 2:31.

Comments:_____

After preparation of CA:

1) Find a pretreatment dish (small green rectangular boxes) marked on the sides with a number and straight line "| "on the side. The dishes marked with a + are *not* pretreatment dishes. They are dehydration dishes.
2) Find a pretreatment slide rack. These racks are marked with a straight line on their handles.
3) Load the slides back-to-back in every other row of the slide rack. If you have any unused rows, fill them with dummy slides. This allows for even heating of the slides in the citric acid.
4) Pour the CA in the pretreatment dish.
5) *Without the slides loaded in,* heat the citric acid in the microwave until it is hot, *but not boiling*. Power levels and times will vary depending on the microwave, but about one minute and 30 sec on power level nine is usually a step in the right direction.
6) Continue to heat the CA *slowly* in the microwave on a lower power setting (usually power level five) in fifteen second increments until small bubbles appear on the sides of the pretreatment dishes.
7) Once the CA is sufficiently heated (but not boiling), place the slides in the CA. Continue to heat the CA *slowly* in the microwave as needed to keep the CA at a relatively even, pre-boiling temperature (another 20 sec in power level one would be sufficient).
8) Set a timer for fifteen minutes and press start.
9) Repeat step six as needed to keep the CA at a relatively even, pre-boiling temperature.
10) Remove the slides when the timer goes off.

Comments: 1:30 (9), :15 (5), :15(5), :15(5), :20(15)

15 minutes start.

12 min check OK.

Diane

4 min check — :15 s(5).

date_____

R:\Diane Lagace Laboratory\General Lab Items\Protocols\IHC\IHC worksheets\BrdU_DAB IHC.doc

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1b. 1XTBS: quick rinse to cool; two long rinses (each 2min)

2a. Permeabilization: 0.1% Trypsin in 0.1M Tris and 0.1% $CaCl_2$, 10 min RT

Volume needed: 200ml **check if Tris, $CaCl_2$ need to be made**

DATE of 0.1M Tris 07/16/13 MS If none available, make 0.1M Tris:

(0.1M Tris, **pH 7.4**: 1M = 121.14 g/1000ml 0.1M = >> 12.1 g /1000ml dH_2O = _____g/_____mls dH_2O LOT # Tris used_____)

DATE OF 1M $CaCl_2$ 4/22/09 KK. If none available, make 1M $CaCl_2$

(To make 1M: 1M = 147 g/1000 ml = _____g/_____mls dH_2O LOT # $CaCl_2$ used_____)

1 M $CaCl_2$ = 14.7%; To make 0.1% $CaCl_2$ = >> (x)(14.7) = (200 mls Tris)(0.1)

0.1% $CaCl_2$: add 1.36 **mls of 1M $CaCl_2$ to** 200 **mls of 0.1M Tris, pH7.4**

Trypsin: 0.1% Trypsin: 0.1g/100ml = 0.2 g/ 200 mls of Tris/$CaCl_2$ graduated cylinder

LOT # TRYPSIN 1st container. 040M7353V. / 2nd container. SLBB2490V.

Add 0.2 **g Trypsin to** 200 **mls $CaCl_2$/Tris.**

Time in: 2:48 pm Time Out: 2:58 pm

2b. 1XTBS: Three rinses (1 min each)

3a. Denaturation: 2N HCl in 1XTBS, 30 min RT fume hood

Note: Make more 2N.

Volume needed: 200

(x)(12N) = (200 mls)(2N) LOT # MS+AD June 18/13 ~~200mls/L~~ 160

Bring 33.3 **mls of 12N HCl to** 166.7 **mls with 1XTBS**

Time in: 5:03pm Time Out:_____

200./ 800mls

160/800mls.

Procedure

Use 2N HCl

3b. 1XTBS : Three rinses (1 min each)

Circle with PAP pen.

Leave in 1XTBS on slide until blocking

Time to PAP 3:40 pm slides: 11

date_____

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4. Blocking: 3% NDS and 0.3%TritonX100 in 1XTBS, 20-60 min RT ? Looked at only value.

Volume needed: 4ml X slides/covered?

3% NDS: 3/100 = x/ 4 mls =>> 0.12 mls NDS =>> 120 µl NDS

* Lot# NDS 108574

0.3% TX: 0.3/100 = x/ 4 mls; (x) 10 = 0.12 mls of TX 10% (0.0012)

=>> 120 µl Triton-X 10%

Bring 120 µls NDS and 120 µls TX 10% to 4 mls with 1XTBS

Time in: 4 10, Time Out: 5 10 pm

Total blocking: 60 mins Remainder:______

Reminder: Do not rinse with 1X TBS between blocking and adding the primary.

While in blocking, make map of chambers.

Include chamber numbers.

R:\Diane Lagace Laboratory\General Lab Items\Protocols\IHC\IHC worksheets\BrdU_DAB IHC.doc

7/22/13 Your Initials______

5. Primary Carrier: in 3%NDS and 0.3% Tween 20 in 1XTBS, 12-24 h RT

Volume of carrier needed: 4

3% NDS: 3/100 = x/ 4 mls =>> 120 µl NDS

0.3% Tween-20: 0.3/100 = x/ 4 mls; (x)10 = 0.120 mls of Tween-20 10%

=>> 120 µl Tween-20 10%

Antibody amount $C_1V_1 = C_2V_2$ Lot # 1°______

$1x = (1/300)(4)$ Lot # 1°______

$= 13.3\ \mu l$

Bring 0.120 µls NDS, 120 µls Tween-20 10%, 13.3µls anti-BrdU to 4 mls with 1XTBS

Additional Serum used? ______ µls NDS

Time in: 5 30 pm Time Out: 9 30 am.

Total primary: 15:56 hrs. Dates applied/removed: July 22 / July 23

Reminder: When you leave the slides overnight, remember to place another box on top of your slides. Then place a weight on top of these boxes, usually a couple of full slide boxes. Also make sure there is water in the four orange caps in the chamber.

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7/22/13 Your Initials_____

Second Day ICC:

6. 1XTBS : two-three quick rinses

7a. Secondary: 1:200 biotinylated- D -α- R **IgG in 1.5% NDS, 60 min RT**

Reminder: Make up ABC now also – since needs to be made ahead of time

Volume needed: 4ml **Antibody Source /Company:**_____

1.5% NDS : 1.5/100 = x/ 4 mls =>> 0.06 mls NDS =>> 60 □ l NDS

b- D -α- R IgG: 1/200= x/ 4 mls =>> 0.02 □ l b- D -α- R IgG

LOT # 2° 99449 20 µl

Bring 60 **µls N S and** 20 **µls b-** D **-α-** R **IgG to** 4 **mls with 1XTBS**

Time in: 10:09am Time Out: 11:09am

Total secondary: 60mins Remainder:_____

7b. 1XTBS : two-three quick rinses

8a. Quench endogenous peroxidases: 0.3% H_2O_2 in 1XTBS, 30 min RT

Volume needed: 4ml Dilute 30% H_2O_2 1:100 40µl

0.3% H_2O_2: 1/100 = x/ 4 mls =>> 0.04 ls 30% H_2O_2

Bring 40 **µls H_2O_2 to** 4 **mls with 1XTBS**

LOT # H_2O_2_____

Time in 11:37am Time Out: 12:07 pm

Total H_2O_2: 30m Remainder:_____

8b. 1XTBS : two-three quick rinses

9a. ABC: Vector Elite avidin-biotin complex, 60-90 min RT

Volume needed: 4 Made at least 30 min ahead of time? Yes

20 ul A or B/ ml 1XTBS = 20 ul A or B / 4 ml 1XTBS 80µl

Add 80 **µls A to** 4 **mls 1XTBS, VORTEX.**

Add 80 **µls B, VORTEX.**

LOT # ABC_____

Time in:_____ Time Out: 1:59

date_____

R:\Diane Lagace Laboratory\General Lab Items\Protocols\IHC\IHC worksheets\BrdU_DAB IHC.doc

7/22/13

Total ABC:_____ Remainder:_____ Your Initials_____

9b. 1XTBS : two-three quick rinses

10a. Visualization: Pierce DAB, about 20-30 min RT

Volume needed: 4ml Purple DAB (-20°C) 1:10 with Pierce DAB Buffer (4°C)

1/10 = x purple/ 4 mls =>> 0.4 mls purple 400µl

Lot # Buffer_____ Lot#DAB_____

Bring 0.4 **mls purple to** 3.6 **mls with Thermo scientific buffer.**

Total DAB time: 0.4 Remainder:_____

Look at sections under microscope to make sure the staining worked.

10b. 1XTBS : two-three rinses (Use DAB waste container to suction off DAB and the first rinse, Use regular waste container to suction the second rinse, Put the slides in the green dish filled with 1XTBS for the third rinse)

Counter stain: Vector Fast Red

MQH_2O: dip

Fast Red: 2-10 min (See sticker on bottle for suggested time) Exact duration: 2min. Lot #/Date of Fast Red_____

MQH_2O: rinse gently until dH_2O runs clear

Dehydrate: use clean racks and dishes and fresh Citrisolv.

70% EtOH	5 min
95% EtOH	5 min
100% EtOH	5 min
100% EtOH	10 min
Citrosolv	1 min
Citrosolv	5 min
Citrosolv	10 min – several days

Coverslip with DPX and #1.5 coverslips Date coverslipped_____

Cleaned grey racks, chambers, work space?_____ Map complete?_____

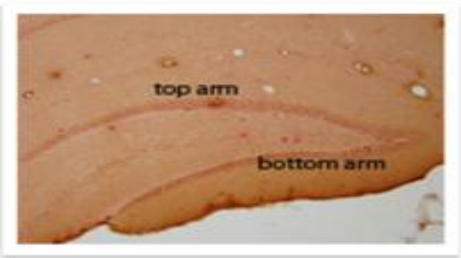
DAB waste put in hood?_____ Refilled ethanols if needed?_____

Cleaned coverslip station?_____ Refilled TBS, Tris if needed?__

date_____

B) Cell counting procedure (provided by Lagace lab)

How to Count Brdu Cells



Slide Layout

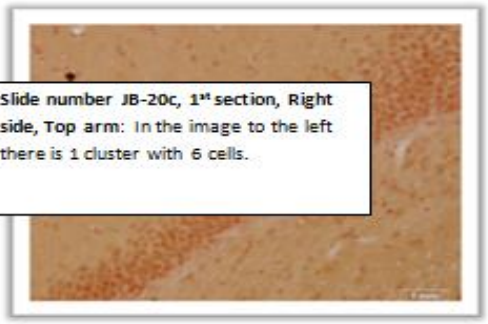
Animal ID Section Date	SECTIONS 1 2 3 Left Right

Examples

Slide number JB-20c, 2nd section, Left side, Top arm: In the image to the left there are 2 clusters, one with 3 cells and one with 2 cells

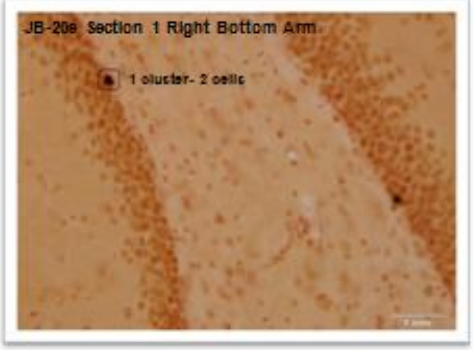
JB-20c Section 1 Right Top Arm

1 cluster- 6 cells



Slide number JB-20c, 1st section, Right side, Top arm: In the image to the left there is 1 cluster with 6 cells.

Slide number JB-18b, 1st section, Right side, Bottom arm: In the image to the left there are 2 clusters circled, one with 5 cells and one with 2 cells.



Slide number JB-20e, 1st section, Right side, Bottom arm: In the image to the left 1 cluster is circled with 2 cells.

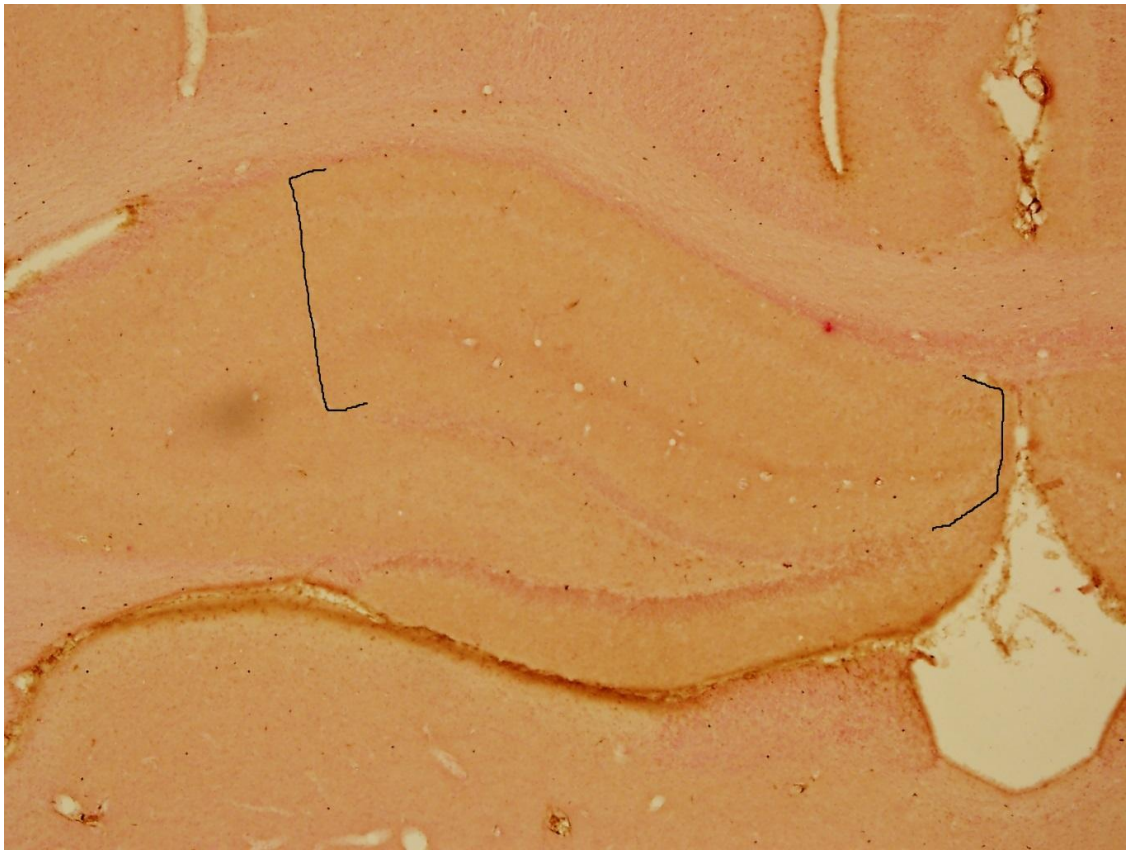
The slides are labelled with animal numbers and letters.

Example: animal **JB-18** has 6 slides labelled JB-18a through JB-18f.

Reminders

- Always count at **40x** magnification
- Constantly adjust the fine focus so as not to miss any cells
- In the examples above not all the clusters are circled, the information given is only for the clusters which are circled.

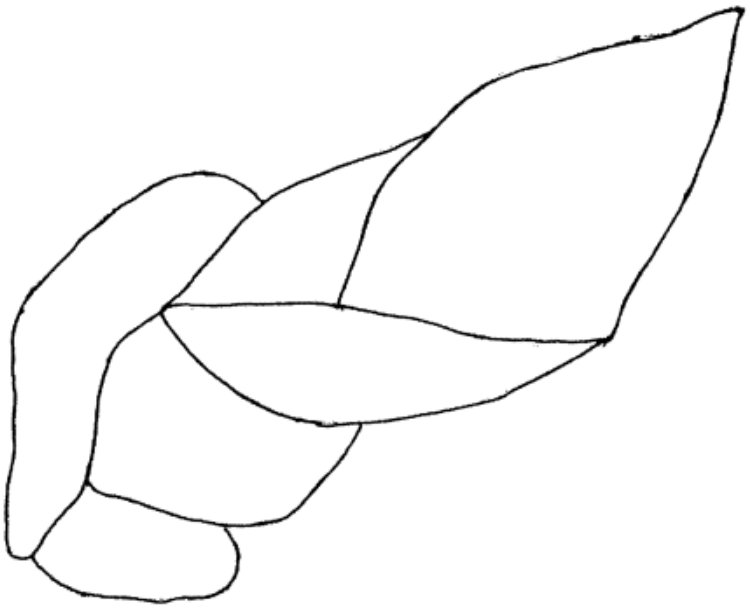
C) Counting boundary for CA1 as marked by black lines



D) Counting templates – Amygdala

Bregma:

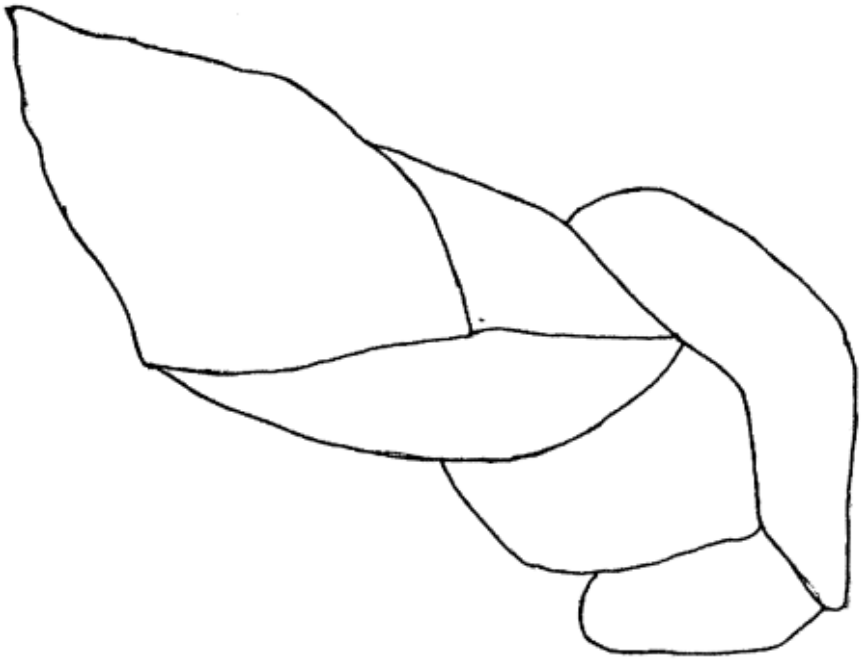
Date: Subject: Section:



Cluster	Cells	Cluster	Cells	Cluster	Cells	Cluster	Cells

Posterior Right Amygdala

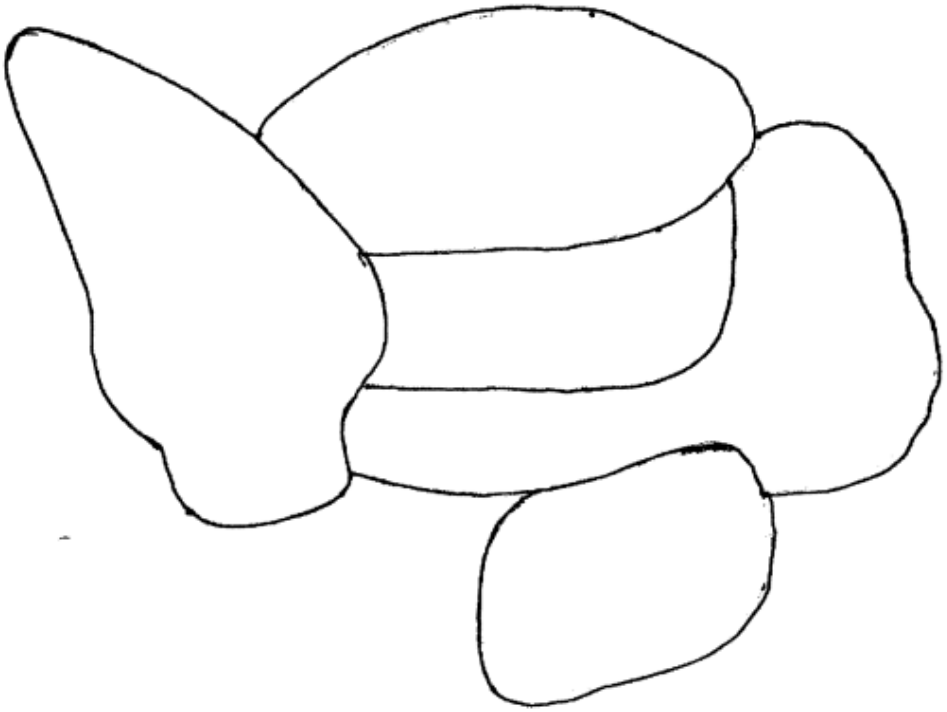
Date: Subject: Section: Bregma:



Cluster	Cells	Cluster	Cells	Cluster	Cells	Cluster	Cells

Posterior Left Amygdala

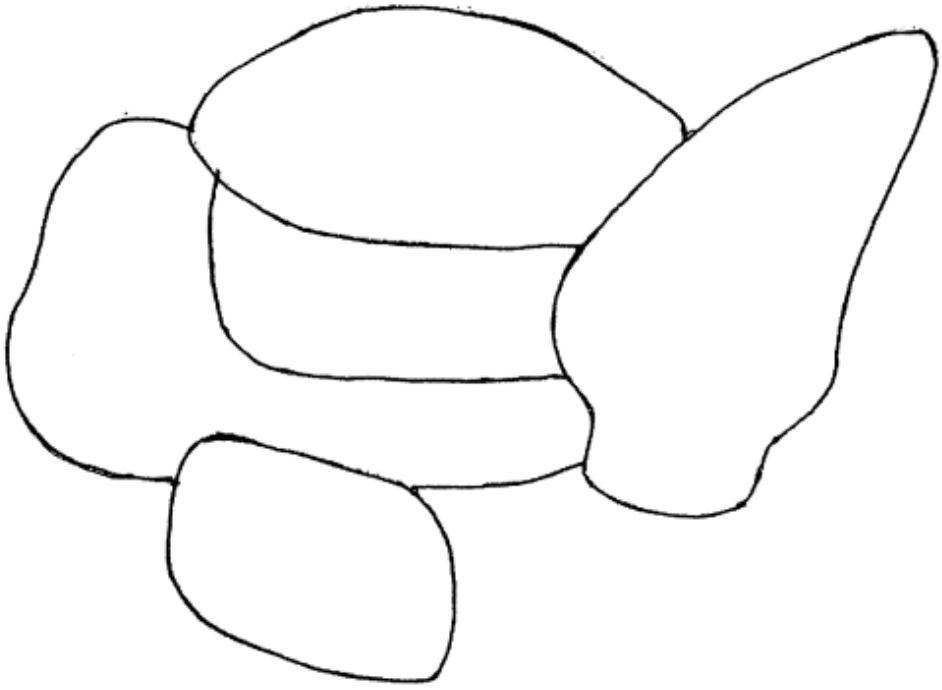
Date: Subject: Section: Bregma:



Cluster	Cells	Cluster	Cells	Cluster	Cells	Cluster	Cells

Anterior Left Amygdala

Date: Subject: Section: Bregma:



Cluster	Cells	Cluster	Cells	Cluster	Cells	Cluster	Cells

Anterior Right Amygdala

E) VMH Template on Image-Pro Plus 7.0