HUMAN CHORIONIC GONADOTROPIN REDUCES PROLIFERATION BUT NOT SURVIVAL OF BRDU-LABELED CELLS IN THE DENTATE GYRUS IN A RAT MODEL OF PREGNANCY

by

Jesse Robert Taylor
B.Sc. (Molec. Biology & Biochem.), Simon Fraser University, 2003
B.A. (Hons., Psychology), Simon Fraser University, 2007

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Name: **Jesse Robert Taylor**

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Examining Committee:

Chair: **Dr. S. Zaitsoff**
Assistant Professor of Psychology

Dr. N. V. Watson
Senior Supervisor
Professor of Psychology

Dr. R. E. Mistlberger
Supervisor
Professor of Psychology

Dr. L. A. M. Galea
External Examiner
Professor of Psychology
University of British Columbia

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ABSTRACT

Human chorionic gonadotropin (hCG) and luteinizing hormone (LH) are proluteotropic hormones that signal via a common receptor (LH/CG receptor), which is expressed throughout the brain. hCG has also been linked to changes in cognition during human pregnancy, and to parallel behavioural effects in rat models. Additionally, LH treatment increases cell proliferation in the dentate gyrus in some non-pregnancy rat models. This suggests a possible role for hCG in regulating neurogenesis during pregnancy. To test this hypothesis, ovariectomized rats were implanted with silastic capsules to mimic the levels of estrogen and progesterone present in early pregnancy, and injected with BrdU to label dividing cells. Treatment with hCG resulted in significantly lowered cell proliferation in the dentate gyrus, but had no impact on 21 day cell survival. These results also suggest a mechanism underlying the relationship between hCG levels and changes in cognition during pregnancy.

Keywords: Neurogenesis; Gonadotropins; Pregnancy

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1: INTRODUCTION

Pregnancy in mammals is a spectacular display of maternal endocrine plasticity. The production of steroid and protein hormones by the human placenta is greater in amount and in diversity than any other known mammalian endocrine tissue, and the maternal system must cope with this influence on virtually every physiological process. Although the female body is adapted to normally produce significant variations in steroid hormone and gonadotropin release, as well as major alterations in the endometrial tissue and ovarian function during the menstrual cycle, these pale in comparison to the changes that take place during pregnancy. As the luteal phase of the menstrual cycle begins post-ovulation, continued LH signalling is necessary to maintain the corpus luteum and thus maintain estrogen and progesterone synthesis and release. If blastocyst implantation occurs during the early luteal phase window, as LH maintenance of the corpus luteum fails, human chorionic gonadotropin (hCG) secreted by the trophoblast (the precursor to the placenta) takes over proluteotropic signalling, estrogen and progesterone levels are maintained or increased, and menstruation does not occur. As pregnancy progresses, the placenta releases significant levels of a vast number of biologically active substances, including hCG, gonadotropin-releasing hormone (GnRH), and very high levels of progesterone and estrogen (Cunningham et al., 2010). Given the intimate intermingling of the circulatory systems of mother and fetus at the

placental interface, and the diversity of secretions by the placenta during pregnancy, it should be little wonder that there are many physiological, cognitive and behavioural implications of pregnancy for the mother.

1.1 Pregnancy and Fetal Sex: Impacts on Cognition and Behaviour

Although there are dramatic differences between taxa with regards to the process of pregnancy, much of what is known in this field has necessarily been elucidated using animal models. A commonly studied process in behavioural research is the regulation of anxiety, but in this area the results have been inconsistent with respect to pregnancy. Some studies report decreases in anxiety related behaviours over the course of pregnancy, as assessed using the elevated plus maze (EPM) (de Brito Faturi, Teixeira-Silva & Leite, 2006), while others show the opposite pattern, with the highest levels of anxious behaviour on the EPM on pregnancy day 18 (Macbeth, Gautreaux & Luine, 2008). Postpartum, a number of studies have demonstrated that pup presence decreases maternal anxiety (reviewed in Macbeth & Luine, 2010), but this does not appear to be sufficient to reduce anxiety in pup-sensitized virgin females, as compared to normal virgin females, in the EPM (Pawluski, Brummelte, Barha, Crozier, & Galea, 2009).

In contrast to the anxiety research discussed above, one relatively consistent finding in the rodent literature has been a positive effect of pregnancy on spatial memory. Galea et al. (2000) demonstrated enhancements in spatial memory as assessed on the Morris water maze (MWM) early in pregnancy (7-10

days), but noted that this effect reversed itself by the third week of pregnancy (day 21), at which point the pregnant rats were displaying more memory difficulties than the non-pregnant controls. A study by Bodensteiner, Cain, Ray, and Hamula (2006) failed to find differences between pregnant and non-pregnant female rats in the ability to acquire, consolidate, or recall platform location information in the MWM, although in pregnant animals some benefit was detected with regards to finding the platform if it was moved to a novel location. However, there appears to be less variability in the persistence of the memory enhancement postpartum. It has been demonstrated that there is a slight decrement immediately after parturition (1-4 days), but by 10 days postpartum the spatial memory benefits seem to have returned (Darnaudery et al., 2007). Similarly, Pawluski, Walker and Galea (2006) demonstrated an advantage of primiparous females over nulliparous females on performance on the radial arm maze after weaning, and Lemaire et al. (2006) have demonstrated that the memory enhancement from pregnancy is “lifelong”, and still remains 16 months after weaning, when the mothers were 22 months old. In sum, it appears that the effects of pregnancy and the postpartum period on spatial memory function varies across this time period, and this may be linked to the rapid hormonal changes that are occurring during these processes.

Similar to the data from the rodent literature, there is debate as to the pattern of pregnancy-related anxiety in humans, with some groups presenting evidence to demonstrate that anxiety is at its peak in the first trimester and declines through the rest of pregnancy (Esimai, Fatoye, Quiah, Vidal & Momoh,

2008) or vice versa, stating that pregnancies generally begin with little anxiety, and it builds through to parturition (Da Costa, Larouche, Drista & Brender, 1999; Rofe, Blittner & Lewin, 1993). Most studies examining postpartum anxiety have noted decreases from antepartum levels, but some groups have reported observing anxiety related symptoms for as long as a year after delivery (reviewed in Macbeth & Luine, 2010). Irrespective of the rate and direction of change, it does appear that pregnancy is having an effect on anxiety, although more research is needed to clarify many fundamental questions that still remain about this relationship.

In the context of cognition and memory, it actually appears that the effects in human mothers are somewhat different from those displayed by rodents. Subjective reports abound of the so-called “pregnancy brain”, where mothers rate themselves as having poorer memory as compared to self-reports from non-pregnant controls (Brett & Baxendale, 2001; Buckwalter et al., 1999; Henry & Rendell, 2007; Macbeth & Luine, 2010). Furthermore, it appears that most women report these changes as beginning by the second or third month of gestation, rather than in the second or third trimester (Brett & Baxendale, 2001). A number of studies attempting to address the issue objectively have also demonstrated that the memory effects are independent of mood disturbances (Buckwalter et al., 1999; Macbeth & Luine, 2010), and that they do continue postpartum, although the effects are subtle and small improvements are generally seen shortly after parturition (Buckwalter et al., 1999; Henry & Rendell, 2007). A recent meta-analysis by Henry and Rendell (2007) demonstrated an

unequivocal effect of pregnancy on memory performance, but only for constructs that require high levels of cognitive effort; in this case, specifically free recall and the executive component of working memory were negatively affected. Also of import is that the effects identified by Henry and Rendell (2007) were of similar magnitude to those reported subjectively by pregnant women, thereby providing convergent evidence of what many human mothers are already sure of during pregnancy; higher order memory functions are being impaired.

In the process of studying these cognitive and behavioural phenomena in a longitudinal study, Vanston and Watson (2005) unexpectedly identified a fetal sex effect on maternal memory, which was present at 12 weeks of gestation (approximately equivalent to 10 weeks of fetal age), and persisted through to the final testing session (after menstruation resumed post-parturition for each subject, ranging from 4-19 months after giving birth). Specifically, on tasks where a difference between the groups was noted, mothers carrying a male fetus performed better than mothers bearing a female fetus. Of note is the fact that although the cognitive test battery used spanned tests of perceptual speed and accuracy, visual-motor coordination and motor skills, and various types of memory tasks, the fetal sex effect was only discernable for the most difficult tests given. The tests in question (listening span, computation span, and mental rotation, measuring verbal working memory, arithmetic working memory, and spatial visualization/spatial working memory, respectively) are all demanding tasks that specifically challenge working memory. This effect has subsequently

been replicated in a cross-sectional sample of pregnant women as well (Palmer & Watson, 2011, unpublished data).

Given the nature of the tests on which a sex difference was present, it seems that the most obvious culprit for the effect would be fetal steroid secretion. Certainly, in adults, on average males have a clear advantage on spatial orientation tasks compared to females, and this effect has also been linked to optimal levels of circulating testosterone (Kimura, 2002). However, the fetal sex effect was also seen in a computational task and in a verbal memory task; these are both tasks which, on average, women outperform men, and this difference has been correlated with increased serum estrogen (Kimura, 2002). However, as was previously mentioned, a significant female fetus advantage was not reported; mothers carrying males performed better on all of the cognitive tasks where there was a significant difference (Vanston & Watson, 2005). In addition, there is another major caveat to the fetal steroid explanation; namely, that radioimmunoassays have not revealed significant differences in circulating maternal testosterone based on the sex of the fetus at 12 weeks of gestation (Glass & Klein, 1981; Meulenberg & Hofman, 1991). This is in no small part due to the incomplete development of the hypothalamic-pituitary-gonadal (HPG) axis in the fetus during this gestational stage. At 12 weeks of gestation, gonadal differentiation has occurred, but expression of testosterone in male fetuses has not peaked, and will not do so until well into the second trimester (approximately 15-18 weeks of gestation). Therefore, it seems unlikely that the causative

mechanism underlying the fetal sex effect on maternal memory task performance is related to sex steroids..

If the difference in memory task performance due to fetal sex cannot be attributed to gonadal steroids, less prominent candidates must be considered. One such possibility is hCG; there are numerous placental, uterine and fetal functions of hCG during pregnancy, and perhaps most importantly in the current context, it is expressed by the trophoblast virtually from the time of implantation onwards (Cole, 2010). In support of this possibility, fetal sex differences in maternal hCG titres have been identified in analyses of women at all stages of pregnancy (Obiekwe & Chard, 1982; Santolaya-Forgas, Meyer, Burton & Scommegna, 1997; Yaron et al., 2002) and it has been consistently found that mothers carrying a female fetus display higher serum levels of hCG on average than those with a male fetus. Given the temporal expression profile and the sex difference in expression, there is a strong case to be made for hCG as the factor underlying the previously discussed fetal sex effect on maternal memory.

1.2 hCG: Biochemistry, Signalling, and Behaviour

The prevailing dogma underlying early gonadotropin research suggested that the singular target of these hormones was the regulation of gonadal production of sex steroids, and that any peripheral effects in other systems were a secondary effect related to modulation of steroid hormone release (Rao & Lei, 2007). However, it is now known that hCG has a much wider breadth of functions, of which the regulation of gonadal secretions is but one. The glycoprotein hormone hCG has four known isoforms, all with different biological

functions, although one variant is only expressed under pathological conditions. The “standard” form of the hormone that is found in the highest concentration during pregnancy is produced by the syncytiotrophoblast. Structurally, hCG is typically a heterodimer composed of an α-subunit and a β-subunit which are held together via non-covalent hydrophobic and ionic interactions. The α-subunit is a 14.5 kDa protein that is common to hCG, it’s hyperglycosylated and pituitary variants, LH, FSH, and to thyroid stimulating hormone (TSH) (Cole, 2010). The 22.2 kDa β-subunit is unique to the isoforms of hCG, although it is structurally similar to the β-subunit of LH. All variants of hCG are glycosylated to a significant degree, and in the case of hCG proper, glycosylation amounts to approximately 25%-30% of the molecular weight of the hormone. Functionally, the oldest recognized purpose for hCG is in driving progesterone production during early pregnancy by taking over maintenance of the corpus luteum from LH. However, hCG only promotes progesterone production in this manner for the first three to four weeks of pregnancy, and given that maternal hCG serum levels continue to increase until they peak in the tenth to eleventh week of gestation, and are subsequently maintained at a moderate level through until parturition (Cole, 2010), maintenance of the corpus luteum is certainly not the sole function of hCG during this time frame. Other major functions in the development of the placenta and the fetus have also been linked to hCG, such as promotion of angiogenesis in the uterus and induction of immunological tolerance by the maternal immune system (Tsampalas et al., 2010). The myometrium of the uterus is also sensitive to hCG, and as such the hormone has

also been implicated in maintaining the quiescence of uterine muscle contraction during pregnancy, as well as relaxation of the muscle wall to allow uterine expansion as the fetus grows (Cole, 2010).

Hyperglycosylated hCG is a variant of hCG produced by cytotrophoblast cells and shares its primary protein structure with hCG, but has a divergent tertiary structure based on the increased levels of glycosylation present, which account for 35%-41% of the molecular weight of the hormone (Cole, 2010). This change in glycosylation prevents complete folding of the heterodimer, which exposes a cystine-knot motif that is otherwise occluded in the protein interior in other isoforms and this allows hyperglycosylated hCG to have functions divergent from those of normal hCG. For example, hyperglycosylated hCG has been shown to be critical for successful implantation and avoidance of miscarriage (Sasaki, Ladner, & Cole, 2008), presumably via its antagonistic effects on the receptor for a tumor suppressor (Cole, 2007). The other two isoforms of hCG are not typically associated with the pregnant state. The free β-subunit of hyperglycosylated hCG is a monomeric protein produced in gestational trophoblastic disease and virtually all other non-trophoblastic cancers, and there is a strong association between free β-subunit detection in serum and urine samples and advanced stage cancer with poor prognoses (Cole, 2010). Indications are that the free β-subunit is able to act as an anti-apoptotic factor (Cole, 2007). Similarly, pituitary hCG is also not thought to play a significant role in pregnancy, although it varies from the other isoforms only in terms of the type of glycosylation present, and this results in a significantly shorter half life for the

molecule. In the context of the menstrual cycle, pituitary hCG circulates at approximately 1/120 the concentration of LH, and yet, due to it's greater potency, has in the range of one third the biological activity of LH (Cole, 2010), leaving open the possibility that pituitary hCG has a significant impact on the biological processes involved in female gonadal regulation. Having said this, no independent function of pituitary hCG has yet been elucidated.

Although, as described above, hCG had been demonstrated to have a multitude of effects, in mammals, the proluteotropic hormones (hCG and LH) both bind and signal via a single common receptor (Cole et al., 1973): the luteinizing hormone/chorionic gonadotropin receptor (LH/CGR). It is now known that the LH/CGR is a member of a large family of guanine nucleotide-binding protein (G protein) coupled receptors (McFarland et al., 1989), and that the subfamily of G protein receptors that includes the LH/CGR also encompasses the follicle-stimulating hormone receptor, the thyroid-stimulating hormone receptor, and several orphan receptors with unknown ligands (Apaja, Harju, Aatsinki, Petaja-Repo, & Rajaniemi, 2004). As G protein linked receptors, unsurprisingly stimulation of the LH/CGR by an appropriate ligand causes activation of both the cAMP and PLC second messenger signalling cascades (Segaloff & Ascoli, 1993).

The first definitive demonstration of the presence of LH/CGRs in the brain came from Lei, Rao, Kornyei, Licht and Hiatt (1993), who provided confirmation of the presence of receptor mRNA transcripts throughout the rat brain, including in the cortex, the hippocampal formation, the hypothalamus, the cerebellum, the

brainstem, and the anterior pituitary. Concurrently, Lei et al., (1993) also demonstrated that in both males and females, all regions of the hippocampal formation contained cells that expressed LH/CG mRNA, displayed proteins that were immunoreactive with an anti-LH/CGR antibody and contained proteins that were able to actively bind hCG, implying that functional LH/CGRs were present. Furthermore, expression of LH/CGR was demonstrated in cultured rat fetal neurons using the same set of techniques (Al-Hader, Lei, & Rao, 1997a). Apaja et al. (2004) extended these experiments using transgenic mice expressing lacZ under the control of the LH/CGR promoter. In this manner, whole-mount staining of fetuses showed LH/CGR expression in the sensory, cranial and spinal ganglia, the olfactory bulbs, the thalamus, and in the brain stem prior to 16.5 days post coitus, although after this time point, there was deceased lacZ expression in all areas except for the olfactory bulbs. Comparatively, in intact adult mouse brains, the majority of LH/CGR expression was detected in the olfactory bulbs, in the auditory, visual and somatosensory cortices, and in the dentate gyrus and hilus of the hippocampal formation. In sum, these temporal and spatial patterns of LH/CGR expression suggest the potential for hCG to have both organizational and activational effects on the CNS, and indicate that expression of the LH/CGR begins early in development and is maintained through adulthood.

Evidence to support the functionality of the LH/CGR at a cellular level has substantially come from research using cell culture. For instance, it has been shown that LH/CGR activation can produce dose-dependent functional effects in immortalized rat hypothalamic GT1-7 neurons (Lei & Rao, 1994), in cultured rat

glial cells (Al-Hader, Lei, and Rao, 1997b, although see Apaja et al., 2004), and in immortalized HN33p hippocampal cell culture (Zhang, Lei, & Rao, 1999). Al-Hader et al. (1997a) also reported dose-dependent increases in outgrowth of neurite processes, increases in total cellular protein levels, and a decrease in DNA fragmentation in cultured fetal rat neurons in response to hCG treatment. As a whole, these studies indicate that the luteotropic hormones are able to directly impact the cellular functions of neurons, even in the absence of gonadal steroids. However, for hCG signalling in the brain to be possible *in vivo*, especially in the case of pregnancy, where the source of the vast majority of hCG is external to the CNS, hCG must be able to cross the blood-brain barrier. Lukacs, Hiatt, Lei, and Rao (1995) demonstrated that hCG has this capability; approximately 1/100 of radioactively labelled, peripherally injected hCG was found in the CSF of live rats after thirty minutes, and was detected in the choroid plexus, in blood vessels of the brain, and in the hippocampus post-mortem. Perhaps most importantly, it was determined that the hCG in the CSF was intact hormone, rather than protein fragments.

Ultimately however, the crux of whether or not hCG is a plausible effector of the fetal sex effect on memory reported by Vanston and Watson (2005) lies in whether or not there are discernable behavioural and cognitive changes in response to the physiological effects described above. The data that has been reported on this topic mainly focuses on behaviours in rodent models that parallel the cognitive changes seen in pregnant women. For example, Toth et al., (1994) assessed the response to hCG using electroencephalograpy (EEG) and

electromyography (EMG) in gonadally intact and normally cycling female rats, and found that hCG treatment produced decreases in active awake phases, increases in high and low amplitude sleep, and decreases in walking, sniffing, and chewing behaviours. Although the latter behaviours may be reduced due to their interdependency with the decreases in the general activity of the rats, the changes in sleep patterns were found to be attributable to an hCG mediated increase in soporific prostaglandin synthesis, and a reduction in sleep-inhibiting prostaglandin synthesis. This finding may have particular relevance to the changes in sleeping patterns experienced by women during pregnancy. Often, women sleep much more during the first trimester, before sleep returns to normal during the second trimester, and eventually sleep becomes difficult in the third trimester, presumably due to physical discomfort (Toth et al., 1994). Historically this effect has been attributed to the soporific nature of progesterone, but levels of this hormone during pregnancy do not correlate well with the changes of sleep patterns experienced. However, as previously described, hCG levels are highest in the first trimester before dropping to a constant-but-elevated level in the second trimester; this pattern correlates well with sleep changes during pregnancy (Cole, 2010). However, the animals used in this study were gonadally intact, and therefore it cannot be determined with absolute certainty that the effects of hCG were directly due to actions of the hormone in the CNS, rather than via inducing steroid hormone release from the ovaries, although the sum of this evidence makes for a convincing argument attributing sleep disturbances to hCG via regulation of prostaglandin synthesis.

In addition to the effects on sleep discussed above, it has also been demonstrated that hCG administration, both by peripheral injection and intracerebroventricular (ICV) injection, results in the inhibition of locomotion and rearing in an open-field test, and decreases in aversion to novel food in the form of high fat crackers (Lukacs et al., 1995), as well as increases in the time spent in the odour marked arms of a Y-maze (Lukacs, 2001). All of these results can be interpreted in terms of hCG impacting anxiety levels; higher anxiety in an open-field task should present as more thigmotaxis and lower levels of movement, decreases in anxiety should lessen the rats' natural avoidance of novel foods, and decreased anxiety could account for the preference of "stranger" smells in the odour preference task. However, alternative explanations are also equally possible, and perhaps more consistent with the results of other published studies. For example, it may be that the results of the open-field test are due to inherently lower levels of activity in the hCG treated animals; this interpretation would be consistent with the effects of hCG on sleep and activity levels described by Toth et al. (1994). The preference for novel foods could instead be attributable to the effects of hCG on the olfactory bulbs, where LH/CGRs are present (Apaja et al., 2004). Finally, the preference for the odour of other animals may be related to something other than anxiety, as one of the smells presented was that of a lactating rat's nest, and maternal behaviour is known to be induced by hCG treatment (Lukacs, 2001). An important side note to these results was that the behavioural changes witnessed by Lukacs et al. (1995) were for the most part identical between the peripheral and ICV injections, despite the

fact that the ICV injection concentrations were 1/50 that of the peripheral injections. Even if all of the hCG delivered via ICV injection escaped into the periphery, once diluted in the much larger volume of peripheral fluids, the hCG concentrations would be so low as to make it very unlikely they could have a significant impact on the gonads, offering further evidence implicating a direct effect of hCG on the brain.

In a set of experiments using OVXed female rats with hormone replacement to mimic pregnancy levels of estrogen and progesterone, daily peripheral injections of hCG resulted in dose-dependent increases in anxiety related behaviours in the EPM (Turner, Jones, & Watson, 2011). Furthermore, Telegdy, Tanaka, and Schally (2009) demonstrated a clear anxiolytic effect in EPM testing with mice treated with a GnRH antagonist (thereby blocking natural secretion of LH). This result appears consistent with the premise that hCG (and LH) have anxiogenic effects, although GnRH antagonists also block gonadal steroid release, so the implications of this particular study are not clear in showing a direct influence of gonadotropins on behaviour. Interestingly, replication of this study using rats as the model organism (Telegdy, Adamik, Tanaka & Schally, 2010) uncovered a biphasic relationship between dose of GnRH antagonist and anxiety; only the middle dose had a anxiolytic effect, while the high and low doses were anxiogenic in nature. However, the consensus of these studies generally indicate a discernable anxiogenic effect of hCG administration, and in the case of the OVXed animals, it is of course not

mediated by steroids released by the gonads and therefore almost certainly must be due to direct signalling via the LH/CGR.

Having said this, the general pattern of anxiety described in animal studies is not so clearly mirrored in human pregnancy. An anxiogenic effect of hCG during pregnancy would be consistent with the result reported by Esimai et al. (2008) indicating that anxiety peaks in the first trimester and declines through the rest of pregnancy, roughly following changes in the circulating levels of hCG in the mother. However, studies by Rofe et al. (1993) and Da Costa et al. (1999) would correlate with an anxiolytic effect of hCG in humans, although the methodologies used by these latter groups to measure anxiety are more subjective and less thorough (and therefore less convincing) than the effect reported by Esimai et al. (2008). Furthermore, there may be a parallel to be drawn between the hCG driven suppression of the rats' normal avoidance of novel foods and altered food preferences during human pregnancy. Although the rationale for viewing this process in humans as a proxy for anxiety is likely suspect, the argument can be made that the rats' preference for high-fat crackers (Lukacs et al., 1995) might be paralleled by pregnant women desiring foods of higher caloric content than usually consumed; this would make evolutionary sense, given the higher metabolic demands on mothers during pregnancy.

Also of note is that a minor inhibitory effect on learning was found by Turner et al. (2011), using the MWM and the previously described endocrine model of rat pregnancy. This is somewhat in contrast with Lukacs et al., (1995), who found no impairment of memory function in intact rats treated with hCG that

were assessed using a T-maze, but is consistent with Berry, Tomidokoro, Ghiso, and Thornton (2008) who found hCG induced impairments of spatial memory in estrogen treated OVXed rats on an object location memory task and in the Barnes maze task. Casadesus et al. (2007) also demonstrated that in a transgenic mouse line (Tg-LHβ) that overexpresses LH, Y-maze performance was impaired compared to age-matched controls. This same effect was not identified in transgenic mice (LHRKO) that also overexpressed LH, but did not have functional LH/CGRs, indicating fairly convincingly that gonadotropins play a central role in memory impairment. However, since the animals were gonadally intact, again this does not allow discrimination between direct and indirect effects of hCG and LH. In a recent study, Bryan et al., (2010) addressed this issue, showing that leuprolide acetate (which blocks steroid and gonadotropin secretion) improved Y-maze performance in OVXed mice, as compared to OVXed controls, thereby eliminating the possibility that the cognitive improvements were due to the actions of gonadotropins on the gonads. As previously discussed, most of the literature indicates that specific memory impairments are the norm during human pregnancy. As well, gonadotropin activity has been implicated in the pathogenesis of Alzheimer's disease (Casadesus et al., 2006; Gregory & Bowen, 2005), and although the inhibitory results of hCG treatment of memory function in rodents is in opposition to the normal pattern of memory improvement during pregnancy, it does appear to be in line with the human pattern of memory dysfunction during this time period.

In one of the only studies in the literature to directly provide a gonad-independent demonstration of behavioural modification in response to gonadotropins, Yang, Nasipak, and Kelley (2007) examined the implications of hCG treatment on the reproductive behaviours of the South African clawed frog, *Xenopus laevis*. In *X. laevis*, male song plays a central role in courtship, and although this behaviour has been demonstrated to be androgen dependent, androgen replacement in castrated animals is not sufficient to return song production to the levels of intact males. Similarly, injections of castrates with hCG alone did not rescue calling behaviour, but hCG treatment combined with androgen replacement in castrates produced dose dependent male song behaviour indistinguishable from intact males indicating a separate, non-gonadal function for gondaotropins in the regulation of this behaviour.

It should be noted at this juncture that since LH and hCG are bound by the same receptor (Cole et al., 1973), and therefore activate the same signalling pathways (McFarland et al., 1989; Lee & Silva, 2009), the biological functions induced in this fashion should be very similar. However, the two hormones do not necessarily have redundant biological activity. LH has a circulating half-life of 25-30 minutes, whereas hCG has a circulating half-life of approximately 37 hours, as well as a four-fold greater binding affinity for the LH/CGR (Rao & Lei, 2007). The implication of this disparity is that hCG has approximately eighty times the biological activity, per molecule, as LH (Cole, 2010); it appears that it is not inappropriate to think of hCG as a hyper-potent version of LH. Also of note is that hCG is only produced in primates, and is not naturally present in other

mammals (Maston & Ruvolo, 2002). However, the processes regulated by hCG in humans are induced in other non-primate mammals by LH, and since both hormones signal via the common LH/CGR, it is unlikely that there are fundamental differences between the physiological effects of hCG in humans and the physiological effects seen as a result of experimental administration of hCG in other mammalian model organisms. Certainly, the validity of this experimental practice is supported by the biochemical evidence *in vitro*, and by the experimentally induced behavioural changes by hCG treatments in organisms that do not produce it naturally (Berry et al., 2008; Lukacs et al., 1995; Lukacs, 2001; Toth et al., 1994; Yang et al., 2007).

1.3 Influence of hCG, LH, and Pregnancy on Neurogenesis

Although the previous two sections made the case for hCG as a plausible effector of cognitive and behavioural change during pregnancy, the physiological basis which might form the substrate for these changes has, as of yet, not been discussed. In short, a likely mechanism by which hCG might be able to exert its demonstrated influence on behaviour and cognition during pregnancy could include alterations in any of the processes of proliferation, differentiation, migration and survival that comprise the developmental pathway for the birth of new neurons from progenitor cells. However, to date adult neurogenesis has been demonstrated to occur predominantly in the dentate gyrus (DG) of the hippocampal formation and in the subventricular zone (SVZ) of the lateral ventricular walls (in the latter case, leading to new neurons in the olfactory bulbs) (Koehl & Abrous, 2011). The DG in particular is in a unique position to modulate

some types of memory as it receives multiple sensory inputs from both cortical and sub cortical afferent pathways. The main incoming pathway, the perforant pathway, originates in layer 2 of the entorhinal cortex and carries spatial information from the cortex along its medial branch and non-spatial information on its lateral branch (Koehl & Abrous, 2011). Furthermore, the DG also receives subcortical inputs from the septum, the posterior hypothalamus, and from the monoaminergic nuclei of the brainstem. On the efferent side, the DG only has one output; it projects solely to the CA3 region of the hippocampus proper, and this puts it in a prime position to have a significant impact on hippocampal function. This proposition is borne out by data from lesion studies performed with rats that demonstrate impairment of spatial reference memory and working memory as assessed on the MWM and on the radial arm maze (RAM) in response to abalation of the DG (Xavier & Costa, 2009). These results have been supported by convergent evidence from the analysis of immediate-early gene expression and by autoradiography with radioactive 2-deoxyglucose, which have demonstrated activation of the DG during learning tasks in the RAM, as well as during a recall session 5 days after training (Ros et al., 2006). As a final piece of evidence supporting the importance of the DG in memory function, reversible inactivation of the mossy fibres comprising the DG-CA3 connection results in similar memory impairments as discussed above in MWM testing (Lassalle, Bataille & Halley, 2000).

Although the importance of the hippocampal formation for normal memory function has been supported by a long history of research, adult neurogenesis

has become a widely accepted phenomenon much more recently. New and refined experimental techniques have allowed the identification of the division of neural progenitor cells in the adult brain, and indeed, many studies have since linked modification of neurogenesis in the DG to the alterations in behavioural and cognitive performance described above. For example, Kempermann, Brandon and Gage (1998) elucidated a relationship between enriched environments and both increases in neurogenesis in the DG and improved reference memory abilities. In a similar vein, cytotoxic chemotherapy drugs, other drugs with antimitotic effects, and irradiation have all been shown to reduce neurogenesis and thereby alter behaviour on some hippocampal dependent tasks (reviewed in Koehl & Abrous, 2011). Although these types of study have provided substantial evidence elucidating the role of neurogenesis in memory function, they also suffer from methodological limitations related to collateral damage to other processes and structures outside of the intended target. As such, many researchers have developed transgenic knockout models with which to study the link between neurogenesis and memory and the results have been more consistent; inducible disruption of neurogenesis in these transgenic mice lines invariably leads to deficits in spatial memory (Deng, Saxe, Gallina & Gage, 2009; Imayoshi et al., 2008). In addition, it has been demonstrated that superior memory performance on a learning task is correlated positively with increased cell proliferation and cell survival, not only in “middle-age” rats (Driscoll et al., 2006), but also in aged rats (Drapeau et al., 2003), and in humans (Coras et al., 2010). Finally, Kee, Teixeira, Wang and Frankland (2007) have demonstrated

that new neurons are preferentially recruited during the acquisition and retrieval phases of a spatial reference memory task, providing further evidence for the importance of adult neurogenesis in the normal functioning of memory processes.

Despite the links between hippocampal neurogenesis and cognitive and behavioural functions, there are still gaps in the literature regarding the mechanisms responsible for regulating normal adult neurogenesis. One area that has received quite a bit of attention is the influence of steroid hormones, specifically estrogen, on rates of neurogenesis in the DG. For example, OVXed rats show decreases in cell proliferation within 6-7 days compared to sham operated controls, although longer term periods without hormone replacement (3-4 weeks) do not appear to maintain these effects (Barha & Galea, 2010), indicating some type of mechanism for recovery of normal rates of neurogenesis in the long term absence of ovarian steroids. Therefore, as would be expected, it has also been demonstrated that estrogen replacement shortly after ovariectomy can rescue normal levels of cell proliferation (Tanapat, Hastings, Reeves, & Gould, 1999). However, in the case of OVX without estrogen replacement for 4 weeks, cell proliferation patterns become unalterable by these same estrogen treatments (Tanapat, Hastings, & Gould, 2005), raising the possibility that long-term ovarian hormone deprivation can result in desensitization of the neural progenitor cells in the SGZ to estrogen (Barha & Galea, 2010). Furthermore, it appears that administration of exogenous estrogen to OVXed female rats in doses within the normal physiological range result in increases in cell

proliferation as compared to controls, but doses higher or lower than this range result in equivalent levels between groups (Tanapat et al., 2005). In sum, these results clearly show that estrogen has a mitogenic effect in the DG. However, given the use of OVXed animals, with estrogen replacement, in the experiments being presented in this current report, it seems unlikely that there would be differential effects of estrogen on neurogenesis across the treatment groups. Hence, the significant literature covering estrogenic influences on neurogenesis will not be discussed in more depth here (for a review, see Barha & Galea, 2010).

In any case, as it seems that hormonal modulation can have a significant impact on neurogenesis in both the DG and the SVZ, it appears reasonable to propose that the dramatic variations in hormone levels during pregnancy might induce similar effects on cell division and survival in the neurogeneic areas of the brain. However, surprisingly little published research exists addressing neurogenesis in this particular context. In one of the few relevant studies available, Shingo et al. (2003) demonstrated that pregnancy in mice results in increased cell proliferation in the SVZ at gestational day 7 (GD7), and that this difference was dependent on prolactin (PRL), although by GD14 cell counts were indistinguishable from the non-pregnant controls. However, no changes in cell proliferation were found in the dentate gyrus at GD7 (Shingo et al., 2003), and a similar lack of effect of pregnancy on hippocampal neurogenesis at GD21 has also been reported (Furuta & Bridges, 2005). Similarly, Pawluski, Barakauskas, and Galea (2010) found no differences in the number of BrdU (an exogenous

marker of cell division) labeled cells on GD1 or GD21 of primigravid or multigravid rats, although decreases in the number of pyknotic cells (these cells have altered morphology of the nucleus that is representative of cellular necrosis or apoptosis) were observed during early pregnancy. In contrast, Rolls, Schori, London, & Schwartz (2008) have reported that cell survival is depressed during the second week of gestation in mice, but by postpartum day 21 (PD21), the effect has disappeared. Similarly, Galea and McEwen (1999) have demonstrated decreased levels of hippocampal cell proliferation in pregnant wild meadow voles captured during the breeding season, as compared to non-pregnant voles caught during the non-breeding season. In a recent comprehensive study, Kim et al. (2010) demonstrated that at GD16.5 there were significantly fewer Ki67-labeled cells (endogenous marker for cell division) and doublecortin-labeled cells (DCX; marker of neuroblast differentiation) in the SGZ of the DG in pregnant C57BL/6 mice as compared to virgin controls. In the same study, no effect of pregnancy was found on the number of NeuN-labeled cells (marker of mature neurons), on terminal deoxynucleotidyl dUTP nick-end labeling or on Fluoro-Jade B labeling (TUNEL and F-JB, respectively; markers of neuronal death), indicating a specific effect of pregnancy on cell proliferation and neuroblast differentiation. Although somewhat inconsistent in their results, in sum these few available published studies appear to offer some support for the premise that neurogenesis in the dentate gyrus is altered during rodent pregnancy, although these effects seem to differ based on the species studied, and the time point during pregnancy at which samples are taken.

Postpartum, suppression of neurogenesis has been generally been the pattern demonstrated. Darnaudery et al. (2007) reported a decrease in cell proliferation in the GCL on PD1, although the number of BrdU-labeled cells was not significantly different from controls two weeks later. Data presented by Leuner, Mirescu, Noiman and Gould (2007) showed a similar depressive effect on cell proliferation on PD2 and PD8 in postpartum female rats that was mediated by the increased levels of corticosterone present during this time frame. Furthermore, Pawluski and Galea (2007) demonstrated decreases in cell proliferation in the DG in the early postpartum period for both primiparous and multiparous female rats, but corresponding decreases in cell survival 21 days later were only seen in the primiparous group. As a whole, the reported effects of the postpartum experience on neurogenesis seem to closely mirror those changes linked to pregnancy and furthermore indicate that the period extending from early pregnancy well into postpartum is a time of altered neuroplasticity.

Given the dramatic fluctuations of gonadotropin levels during pregnancy and postpartum, it is somewhat surprising that there is such an absence of research examining the possible relationship of these hormones and changes in the rate of neurogenesis in the SVZ and the DG. Although not a classical proluteotropic hormone, PRL does have weak luteotropic activity, and PRL levels peak early in pregnancy, and again in the early postpartum period, which mirrors the pattern of increases in neurogenesis in the SVZ described by Shingo et al. (2003). It should be noted at this juncture that this lack of effect on the DG is most certainly due to the fact that the PRL receptor is not expressed in the

female hippocampus (Mak et al., 2007). However, PRL receptor expression by neural progenitor cells has been demonstrated in the DG of male mice, and knockout of these receptors resulted in elimination of the increases in neurogenesis induced by paternal interactions with their pups (Mak & Weiss, 2010).

Additionally, research examining the impact of male pheromones on female mice has provided convergent evidence indicating a role for gonadotropins in regulating neurogenesis; exposure to dominant male pheromones has been shown to lead to increased levels of cell proliferation in the SVZ and cell survival in the olfactory bulb, compared to controls (Larsen, Kokay, & Grattan, 2008; Mak et al., 2007). Furthermore, blocking PRL by administering bromocriptine (Larsen et al., 2007) or knocking out the PRL receptor (Mak et al., 2007) both result in suppression of the pheromone effect on neurogenesis in the SVZ. However, in the study by Mak et al. (2007), male pheromone exposure also increased cell proliferation in the DG, and this effect was mediated by LH rather than by PRL. Specifically, a high dose of LH resulted in increases in BrdU-labeled cell proliferation and in the number of DCX-labeled cells in the DG and in the SVZ. However, administration of a lower dose of LH abolished the neurogenic effect in the SVZ, but not in the DG. Knockout of the LH/CGR blocked the previously described effect of male pheromone exposure and the impact of LH administration on neurogenesis in the DG of female mice, but had no impact on these effects in the SVZ, where increases in cell proliferation persisted. This indicates that LH is the likely mediator of the

pheromone-neurogenesis effect in the DG, whereas in the SVZ, PRL is the apparent culprit. LH and PRL have both subsequently been demonstrated to increase cell proliferation in the DG in male mice using a similar paradigm (Mak & Weiss, 2010). However, these studies comprise the limited extent of research examining the role of gonadotropins on neurogenesis, and to date no published studies have examined the impact of hCG (or LH) on neurogenesis during pregnancy. Given the demonstrated impact of LH on neurogenesis in the DG described above, in addition to the known variation in hCG during pregnancy and the confirmed expression of LH/CGRs in the hippocampus, a reasonable expectation exists that hCG will have a discernable influence on neurogenesis in the context of pregnancy. Hence, to address this gap in the literature, in the study presented here a rat endocrine model of pregnancy was used to examine the role of hCG in the regulation of neurogenesis.

2: MATERIALS & METHODS

2.1 Animals

Forty nulliparous adult female (80-90 days old) Sprague-Dawley rats (Charles River, Saint Constant, QC) were kept on a 12:12-hour light-dark cycle (lights off at 11am), and were group housed in polysulphone cages (PSU) after recovery from surgery until the initiation of the study. Upon commencement of the experiment, and for its duration, all animals were single housed in an individually ventilated cage (IVU) rack, in PSU cages with autoclaved Enrich-o

'Cobs bedding (The Andersons Inc., Maumee, OH). Food (Maintenance Diet 5001; Lab Diet, Richmond, IN) and sterile sipper sacs (Edstrom, Waterford, WI) containing reverse osmosis filtered water were provided *ad libitum*, and the room temperature was held at a constant 21ºC. All animals were treated in accordance with the Canadian Council on Animal Care guidelines, and all protocols were approved by the University Animal Care Committee at Simon Fraser University.

2.2 Hormone Manipulations

All subjects were deeply anesthetized via isoflurane inhalation, bilaterally ovariectomized (OVXed) following standard operating procedures (Appendix A) and received Silastic implants (1.57mm inner diameter, 3.18mm outer diameter; Dow Corning, Midland, MI), which were inserted between the scapulae subcutaneously. Because the permeability, the diameter, the wall thickness, and the concentration gradient of the implants do not vary, capsule length is the determining factor for the amount of hormone diffusing into the animal (Smith, Damassa, & Davidson, 1977). Each animal received three 30mm implants packed with crystalline progesterone (Sigma-Aldrich, Oakville, ON), and one 2mm implant containing crystalline 17β-estradiol (E2) (Sigma-Aldrich, Oakville, ON). This set of implants was chosen as it has been demonstrated to produce mean serum concentrations of progesterone ranging from 48-57 ng/mL and E2 ranging from 47-59 pg/mL in OVXed rats (Bridges, 1984). Not only are these titres similar to circulating hormone levels in intact pregnant rats, but these implants also are also able to induce maternal behaviour in nulliparous OVXed

animals, and thus represent a validated experimental rat model for mimicking the normal hormonal profile of estrogen and progesterone during early pregnancy (Bridges, 1984). The use of such a model, in comparison to using intact pregnant animals, is necessitated by the gonadotropic effects of hCG. As hCG is able to stimulate estrogen release from the ovaries, which in turn is known to have a significant impact on neurogenesis in the dentate gyrus (Barha & Galea, 2010), use of intact animals would have presented a major confound in interpreting the data from these manipulations. All animals received post-operative care, and were given 21 days of recovery time before initiation of the experiments.

2.2.1 Experiment 1: Cell Proliferation

Animals in the cell proliferation study (n=10 per group) received intraperitoneal (IP) injections of either 200 IU of hCG (Calbiochem, San Diego, CA), dissolved in 0.1 mL of physiological saline (0.9% NaCl w/v) or of 0.9% saline (vehicle, 0.1mL), each day for 2 consecutive days, between 9:30 and 10:30 each morning. This dose of hCG was the most effective in inducing behavioural changes in previous work from our lab (Turner et al., 2011), and it falls within the high end of the physiological range of hCG levels seen during human pregnancy (Cole, 2010; Lukacs et al., 1995). A single injection (per animal) of 5-bromo-2'-deoxyuridine (BrdU; 200mg/kg, dissolved in 0.9% saline, IP; Sigma-Aldrich, Oakville, ON) was then administered 2 hours after the final hCG/saline injection on day 2; this delay was to account for the pharmacokinetics of hCG uptake from peripheral injections (Saal, Glowaina, Hengst & Happ, 1991).

BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells during the S-phase of the cell cycle, and administration of this substance allows for the identification (via immunohistochemistry) of any cells in the CNS that have divided during an approximately 2-hour post-injection window (Taupin, 2007), as well as the progeny resulting from any subsequent mitoses of these newly labeled daughter cells. Administering the BrdU concurrently with the hCG or saline allowed for detection of any changes in the rate of cell division due to treatment. Twenty-four hours after the BrdU injection (day 3), animals were euthanized using CO_2, and perfusions were performed as described in Section 2.3.

2.2.2 Experiment 2: Cell Survival

Animals assigned to the cell survival study (n=10 per group) received an injection of BrdU (200 mg/kg, dissolved in 0.9% saline, IP) on the first day of the experiment (day 1). Beginning 24 hours post-BrdU injection (day 2), daily IP injections of either 200 IU of hCG (dissolved in 0.1mL of 0.9% saline) or 0.9% saline (vehicle, 0.1mL) were administered for 21 consecutive days. As the purpose of this experiment was to assess the effect of hCG on the survival of new cells, BrdU was administered first, so as to label a homogeneous set of cells across groups. Initiating the experimental manipulation (hCG/saline treatment) after the administration and clearance of BrdU from the subjects allowed for evaluation of the effect of treatment on cell survival without the possible confounding influence of treatment induced changes in rates of cell proliferation (see Pawluski et al., 2009). All hCG (and vehicle) injections were performed

between 9:30 and 10:30 each morning. Twenty-four hours following the final hCG/saline injection (day 23), animals in the survival study were euthanized and perfused in the same manner described for Experiment 1.

2.3 Tissue Preparation

All animals were euthanized using CO_2, and upon cessation of breathing were transcardially perfused, first with cold 0.1M phosphate buffered saline (PBS), and subsequently with cold paraformaldehyde (adjusted to pH 7.4; 8% for proliferation, 4% for survival) in PBS. Immediately after removal from the skull, the brains were post-fixed for 24 hours in 4% paraformaldehyde to assist in rigid tissue fixation. The tissue was then stored at 4ºC in 30% sucrose in PBS until sectioning. The brains were subsequently blocked with a razor blade by cutting at the base of the cerebellum and in front of the optic chiasm, and sectioned into 50µm coronal slices using a freezing sledge microtome (American Optical Company, Model 860). Samples were collected beginning with the first section in which the anterior hippocampus was visible, and ended once the posterior aspect of the hippocampus had been passed. Sections were divided into tenths as slicing of the tissue progressed, resulting in 10 samples per animal, each containing 10-12 sections spanning from the anterior to the posterior of the hippocampus. One of the 10 samples per animal was chosen at random for analysis. The tissue was stored at -20ºC in DeOlmos solution for cryoprotection and to preserve antigenicity (Watson, Wiegand, Clough, & Hoffman, 1986), and the sections were stored under these conditions until they were subjected to the immunohistochemical protocols.

2.4 Immunohistochemistry

All reactions were performed at room temperature, and all rinses were for ten minutes each, unless otherwise indicated (see Appendix A for the complete protocol). To identify cells which had incorporated BrdU, brain sections were first washed three times in Tris-buffered saline (TBS), followed by treatment with 0.6% hydrogen peroxide for 30 minutes (to quench endogenous peroxidase activity in the tissue) and another three washes in TBS. The sections were then incubated in 2N hydrochloric acid in a 37ºC water bath (to denature the DNA) for 30 minutes, followed by a 10 minute rinse in 0.1M borate buffer (0.1M boric acid, adjusted to pH 8.5), and three more rinses in TBS. The tissue was exposed to 3% normal horse serum (NHS) in 0.1% Triton-X in TBS for 30 minutes (to block non-specific antibody-antigen interactions), and then incubated at 4ºC for 24 hours with mouse anti-BrdU IgG primary antibody (Roche, Laval, QC) in 3% NHS/0.1% Triton-X/TBS at a dilution of 1:200. Following three more TBS rinses, sections were incubated in horse anti-mouse biotinylated IgG secondary antibody (Vector Laboratories, Burlington, ON), in 3% NHS/0.1% Triton-X/TBS, also at a dilution of 1:200, for 4 hours. Subsequent to three more TBS washes, the tissue was incubated with avidin-biotin peroxidase complex (Vectastain Elite; Vector Laboratories, Burlington, ON) for 90 minutes. After three further TBS washes, BrdU labeling was accomplished using 3,3' diaminobenzidine (DAB) in the presence of hydrogen peroxide, resulting in a honey-brown coloured stain. Sections were given three final TBS rinses to remove any remaining DAB solution and then mounted on gelatin-coated slides, and allowed to dry overnight.

Finally, the tissue was counter-stained using 8% thionine, dehydrated with graded alcohols (ranging from 70% to 100% ethyl alcohol), cleared in xylene, and cover-slipped with Permount (Fisher Scientific, Ottawa, ON).

2.5 Light Microscopy

The slides for all groups were coded to ensure that researchers would be blind to the experimental condition for each tissue sample. The brain sections were examined using a Nikon Eclipse E600 light microscope. Cells were counted as being in the granule cell layer (GCL) if they fell within the cell layer proper, or within the subgranular zone (SGZ; a 50μm zone extending from the inner edge of the GCL). Cells that were found inside the dentate gyrus, but outside of the GCL/SGZ and not within the CA3 region of the hippocampus were counted as being in the hilus (Figure 1). Cell counts were made at 1000x magnification, and the counts were recorded manually using a pen and paper method. Quantification criteria for counting a labelled object as a cell included i) appropriate size (the labelled object was of similar size to the counter-stained granule cell nuclei), ii) appropriate location (in the GCL/SGZ or the hilus), iii) appropriate shape (round or oval), iv) appropriate texture (punctate), and v) appropriate colour (honey-brown) (Figure 2). BrdU immunoreactive nuclei in focus in the uppermost focal plane in each section were not counted, in line with the optical disector principle (Coggeshall & Lekan, 1996; Gundersen et al., 1988b, West, Slomianka, & Gundersen, 1991), which was followed to avoid overestimation of the number of cells present. Digital photographs of the dentate

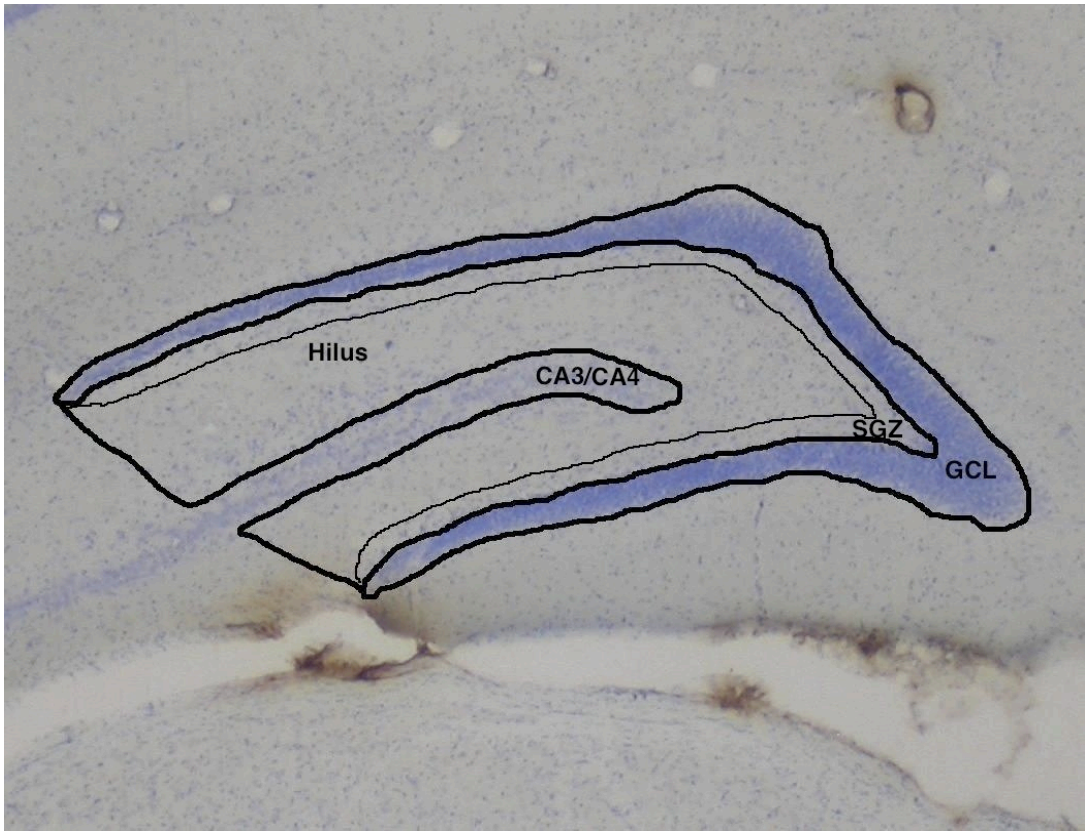

Figure 1. Representative bright-field photomicrograph of the subdivisions of the DG of the hippocampal formation. Cells were counted in the GCL, which included the SGZ (not to scale), and the hilus, but not CA3/CA4.

gyrus for each brain section counted were taken at 200x magnification, using a Nikon E4500 digital camera. These photographs were analyzed using ImageJ (National Institutes of Health, Bethesda, MD) to measure the areas of both the GCL and the hilus. All assessments made by the software were based on freehand tracings of the structures in question by the experimenters using a digitizing tablet.

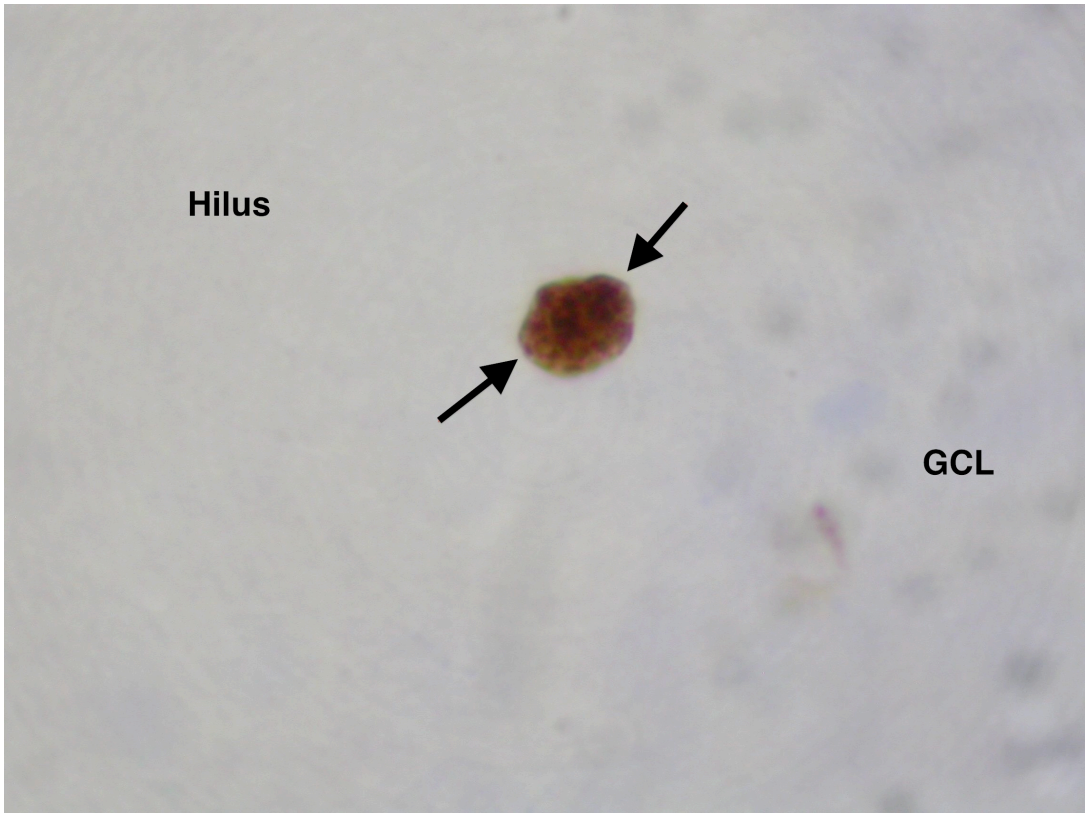

Figure 2. Representative BrdU labelling in the dentate gyrus at 1000x magnification. This sample was taken from the cell survival study (experiment 2). Arrows indicate BrdU-labeled cells; two overlapping cells are present.

2.6 Data Analyses

Applying the fractionator principle, the raw cell counts were multiplied by 10 (as every tenth section was counted) to produce a stereological estimate of cell number per subject (Coggeshall & Lekan, 1996; Gundersen et al., 1988b; West et al., 1991). To account for the possibility of unequal dentate gyrus volumes between groups, the volume of the GCL and hilus in each subject was estimated using Cavalieri's principle (Gundersen et al., 1988a), by multiplying the sum of the section areas for a given subject by the distance between sections

(500μm, calculated as the section thickness as determined by the microtome settings, divided by the fraction of sections counted). Possible differences in DG volumes were assessed using a mixed measures ANOVA for each experiment, with region (GCL, hilus) as the within subject variable, and treatment (hCG, saline) as the between subjects variable. Post hoc tests for the volume analyses consisted of t-tests (independent groups or paired samples, as appropriate) with a Bonferroni correction to control the family-wise error rate. Similarly, differences between groups in the total number of BrdU-labelled cells were also analyzed using a mixed measures ANOVA, with region (GCL, hilus) as the within subjects variable, and treatment (hCG, saline) as the between subjects variable. As the question of interest in these experiments focused on the effects of hCG on neurogenesis, a priori planned comparisons (independent samples t-tests) were performed to assess differences between the cell counts across treatment groups, rather than using a blanket post-hoc analysis.

3: RESULTS

3.1 Experiment 1: Cell Proliferation

To account for the possibility of systematic differences in volume within each brain region across treatment groups, estimates of GCL and hilus volumes (Table 1) were calculated as previously described and subjected to a mixed measures ANOVA. The ANOVA revealed an unsurprising effect of region on

volume ($F_{1, 18}$ = 1312.193, p < 0.001), along with a statistically non-significant effect of treatment condition on volume ($F_{1, 18}$ = 1.517, p = 0.234) (Figure 3).

Table 1. Effects of hCG treatment on dentate gyrus volume

	Proliferation		**Survival**	
Treatment	GCL Volume (mm^3 ± SEM)	Hilus Volume (mm^3 ± SEM)	GCL Volume (mm^3 ± SEM)	Hilus Volume (mm^3 ± SEM)
hCG	1.460 ± 0.066	3.110 ± 0.096	1.230 ± 0.040	2.836 ± 0.113
Saline	1.421 ± 0.079	2.855 ± 0.111	1.213 ± 0.101	2.837 ± 0.214

However, unexpectedly there was an interaction effect for region x treatment ($F_{1, 18}$ = 6.471, p = 0.020). Post-hoc t-tests indicated the expected significant differences between volume of the GCL and hilus within both the hCG (t_9 = -31.896, p < 0.001) and the control (t_9 = -21.205, p < 0.001) groups. The test of the effect of treatment group on GCL volume also provided equivalent results (t_{18} = -0.376, p = 0.711). However, the analysis of the effect of treatment group on hilus volume displayed a trend (t_{18} = -1.740, p = 0.099) in the direction of increased volume with hCG treatment, although statistical significance was not reached. Given the disparity in the trend of hCG effects on volume between the GCL and the hilus, it seems likely that this is the source of the interaction effect

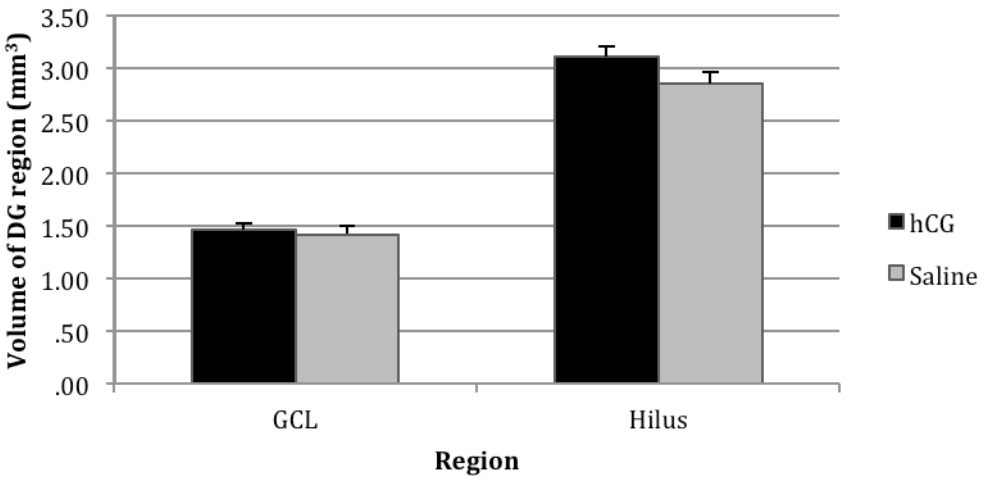

Figure 3. Volume of the granule cell layer (including the subgranular zone) and the hilus after 2 days of treatment in the cell proliferation study. No statistically significant differences were found between treatment groups within each region. Error bars represent the mean + SEM.

identified by the omnibus ANOVA. Further analysis of the overall DG volume, as calculated by adding together the GCL/SGZ volume and the hilus volume for each animal, was undertaken (Figure 4). The mean DG volume was 4.570 ± 0.156 mm^3 for the hCG group and 4.276 ± 0.181 mm^3 for the control group. Statistical comparison between the groups was performed using an independent samples t-test, and no significant differences were found between the groups (t_{18} = -1.232, p = 0.234).

Based on these statistical inferences, to account for the differences in DG volumes related to treatment condition, both unadjusted cell counts and cell densities, as calculated based on the stereological estimates of BrdU+ cell

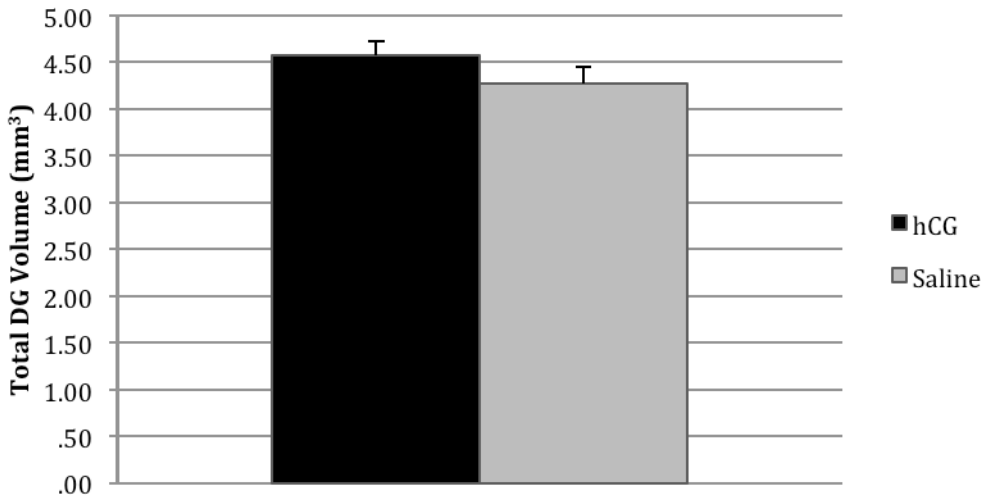

Figure 4. Total DG volume after 2 days of treatment in the cell proliferation study. No statistically significant differences were found between treatment groups within each region. Error bars represent the mean + SEM.

number and the calculated volumes for each brain region analyzed, were used for further analyses. Mean counts of BrdU-labeled cells for the hCG group were 1267 ± 179.33 cells in the GCL and 121 ± 15.09 cells in the hilus. In the control group, the GCL contained 1814 ± 146.11 cells whereas the hilus had a mean of 199 ± 19.86 cells. The ANOVA performed on the unadjusted cell counts showed the expected main effect of region ($F_{1, 18} = 143.122$, $p < 0.001$), as well as a main effect of treatment ($F_{1, 18} = 7.103$, $p = 0.016$). Additionally, there was a trend towards a region x treatment interaction ($F_{1, 18} = 4.130$, $p = 0.057$), although this was not statistically significant at the chosen alpha level. The planned a priori comparisons demonstrated statistically significant differences in BrdU-labeled cells between treatment groups for both the GCL ($t_{18} = 2.365$, $p = 0.029$) (Figure 5) and the hilus ($t_{18} = 3.128$, $p = 0.006$) (Figure 6).

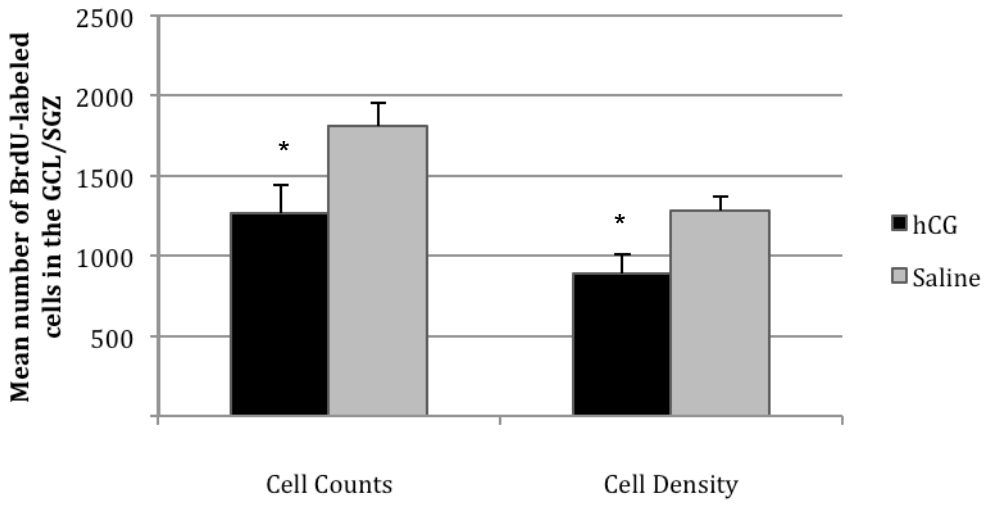

Figure 5. The unadjusted number of BrdU-labeled cells and the density of BrdU-labeled cells (per mm^3) in the GCL and SGZ after 2 days of treatment in the cell proliferation study. There were significantly higher cell counts and densities in the control group as compared to the hCG treatment group. Error bars represent the mean + SEM. * indicates $p < 0.05$.

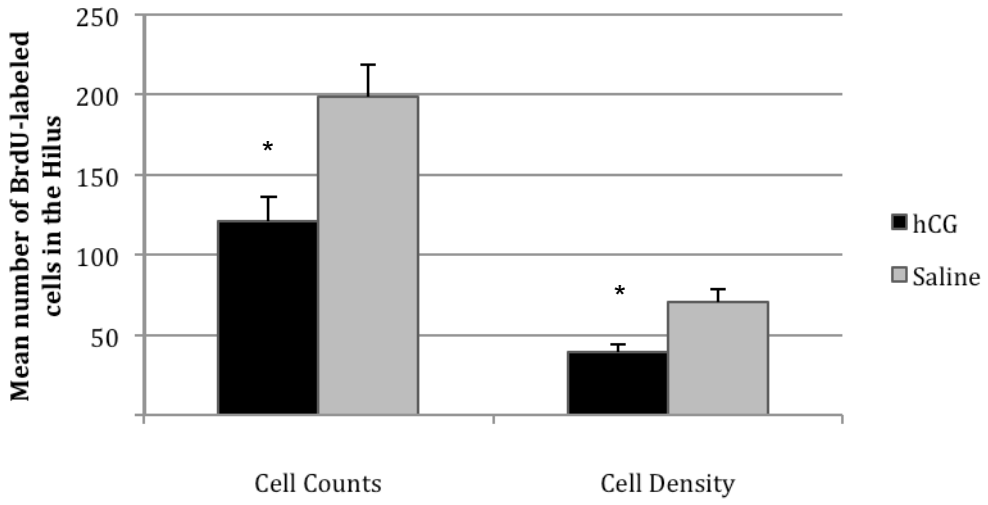

Figure 6. The unadjusted number of BrdU-labeled cells and the density of BrdU-labeled cells (per mm^3) in the hilus after 2 days of treatment in the cell proliferation study. There were significantly higher cell counts and densities in the control group as compared to the hCG treatment group. Error bars represent the mean + SEM. * indicates $p < 0.05$.

When normalized using the region volumes, the mean cell densities for the hCG group were 888.94 ± 126.61 cells/mm^3 for the GCL, 39.22 ± 4.89 cells/mm^3 for the hilus, and 1281.98 ± 88.26 cells/mm^3 and 120.51 ± 7.71 cells/mm^3 for the GCL and the hilus in the control group, respectively. Similar analysis of the calculated cell densities using a mixed measures ANOVA showed the expected main effect of region ($F_{1, 18}$ = 184.941, p < 0.001), along with a main effect of treatment ($F_{1, 18}$ = 7.264, p = 0.015), but in this case the region x treatment interaction ($F_{1, 18}$ = 5.683, p =0.028) was also statistically significant. The planned a priori comparisons performed on the cell densities also revealed significant differences in densities between treatment groups for both the GCL (t_{18} = 2.547, p = 0.020) (Figure 5) and the hilus (t_{18} = 3.482, p = 0.003) (Figure 6).

3.2 Experiment 2: Cell Survival

Overall, as expected, the volume of the hilus was significantly greater than that of the GCL (Figure 7); there was a main effect of region on volume ($F_{1, 18}$ = 431.20, p < 0.001), but no effect of treatment on volume ($F_{1, 18}$ = 0.002, p = 0.961), nor a treatment x region interaction ($F_{1, 18}$ = 0.014, p = 0.906). Based on this information, further analyses were performed using the stereological estimates of BrdU immunoreactive cells only, and did not include the calculated cell densities.

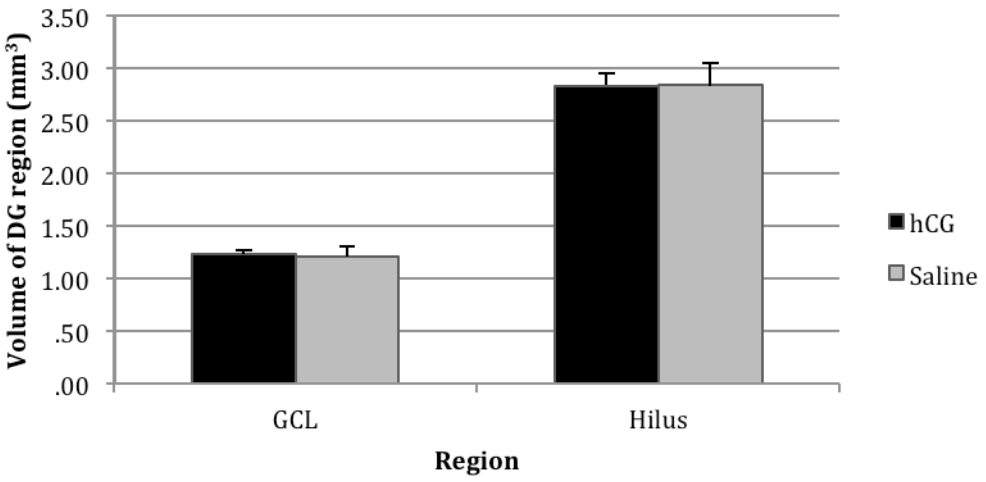

Figure 7. Volume of the granule cell layer (including the subgranular zone) and the hilus after 21 days of treatment in the cell survival study. There were no significant differences in volume within each region across treatment groups. Error bars represent the mean + SEM.

Analysis of the cell counts did reveal a predictable difference between the number of BrdU-labelled cells in the GCL and hilus ($F_{1, 18} = 110.348$, $p < 0.001$), but did not reveal a significant difference based on treatment ($F_{1, 18} = 0.320$, $p = 0.578$), or a treatment x region interaction ($F_{1, 18} = 0.615$, $p = 0.443$). This is not surprising given the equivalent nature of the group means; in the GCL, the hCG group average was 389 ± 50.07 cells, whereas the control group mean was 435 ± 46.07 cells, and in the hilus, the hCG group mean was 141 ± 22.77 cells, as compared to 147 ± 19.33 cells in the control condition (Figure 8). The results of the a priori comparisons were consistent with the outcome of the omnibus test; there were no significant effects of treatment on BrdU+ cell counts in the GCL ($t_{18} = 0.676$, $p = 0.899$) or in the hilus ($t_{18} = 0.201$, $p = 0.696$).

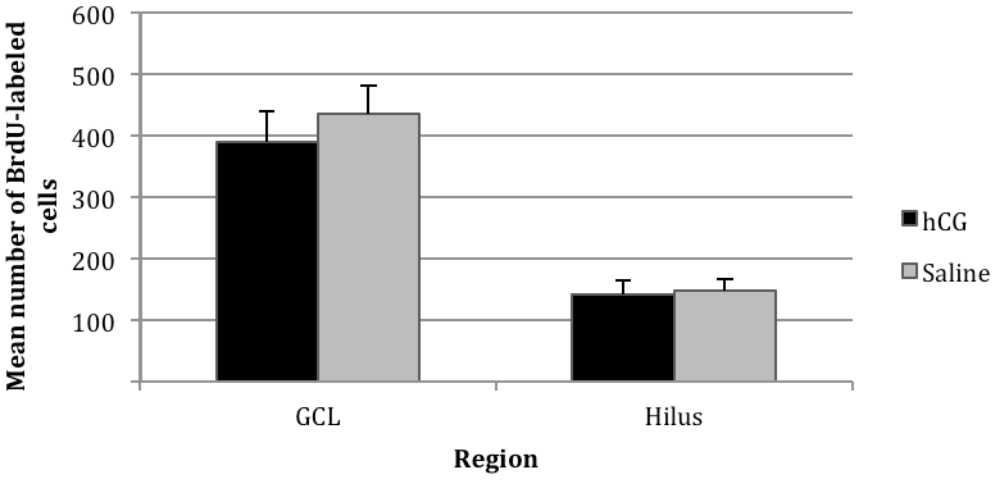

Figure 8. The total number of BrdU-labeled cells surviving in the granule cell layer (including the subgranular zone) and the hilus after 21 days of treatment. There were no significant differences in the cell counts within each region. Error bars represent the mean + SEM.

4: DISCUSSION

The results of these experiments demonstrate that, in an endocrine model of pregnancy, hCG decreases cell proliferation but has no impact on 21 day cell survival. Specifically, the number of BrdU-labeled cells counted in the GCL and the hilus of hCG treated animals was lower than the number of such cells counted in these regions in saline injected animals when sampling occurred 24 hours post-labeling, but these differences were not present after 21 days of treatment. Furthermore, analysis of the cell densities in each group indicated a significant interaction for region x treatment, and based on the group means, this

is appears to be a result of a larger depressive effect of hCG on cell proliferation in the hilus than in the GCL.

Having said this, the purpose of this particular set of experimental designs was to disentangle the effects of hCG on cell proliferation and cell survival. By using this methodology, this study showed that hCG does not appear to impact cell survival independent of cell proliferation during pregnancy. However, *in vivo*, these processes are inexorably linked, with changes in cell proliferation able to have a direct impact on the number of precursor cells that are available to differentiate and mature into functional neurons. Thus, in an intact and functioning CNS, decreases in the rates of cell proliferation may influence a commensurate change in the number of surviving cells as deviations from baseline rates of cell proliferation in either direction will result in alterations in cell survival, if no changes in the proportion of new cells that survive occur (proliferation dependent survival). In this context, this newly discovered effect of hCG on neurogenesis would appear to be one possible mechanism related to the fetal sex effect on memory described by Vanston and Watson (2005). Given the lower levels of circulating hCG in mothers carrying a male, as compared to a female, fetus (Obiekwe & Chard, 1982; Santolaya-Forgas et al., 1997, Yaron et al., 2002), the anti-mitogenic effect of hCG may result in differential cognitive effects in hippocampal dependent processes in women carrying a female fetus; the higher levels of circulating hCG in these women should result in a more severe repression of cell proliferation in their respective hippocampal formations. As previously described, decreases in cell proliferation in the DG have been

linked with some types of memory impairment (see Koehl & Abrous, 2011), specifically spatial memory function and associative memory (contextual fear conditioning). However, in the case of the working memory deficits reported by Vanston and Watson (2005), other brain regions, specifically the prefrontal cortex, have been widely implicated, indicating the possibility of the involvement of other brain systems in producing the effect. However, in any case, to test the theory that hCG may be related to fetal sex linked differences in cognition during pregnancy, further research is necessary, in the form of a multi-group study in which different doses of hCG are administered, representing the circulating levels of the hormone during pregnancy in mothers carrying either a male fetus or a female fetus. This design would eliminate the possibility that in both cases, hCG is present in sufficient quantities to saturate the signalling system, and would allow confirmation that the difference in circulating levels of hCG between the two fetal sexes results in a significantly different alteration in the rate of cell proliferation across groups. Of course, in addition a direct measurement of maternal hCG serum levels at the time points concurrent with the cognitive assessments needs to be undertaken to demonstrate a direct correlation between these two factors in individual human mothers. As such studies have not yet been reported, any links between hCG and the working memory impairments demonstrated by Vanston and Watson (2005) are still entirely speculative in nature.

In addition, this newly elucidated relationship between hCG and neurogenesis may very well be related to both the timing of the onset of the fetal

sex memory effect, which occurs by 12 weeks of gestational age, (which is also just after the peak in hCG levels), and the persistence of the effect throughout pregnancy and beyond. As hCG is present in the maternal circulation at moderate to high levels until parturition (Cole, 2010), the concurrent and lengthy suppression of cell proliferation in pregnant women may result in altered numbers of surviving cells, so as to significantly deplete the number of neurons in the DG by the end of pregnancy, although this remains to be tested directly. Furthermore, as previously mentioned, given the higher circulating levels of hCG in mothers carrying a female fetus, this possible indirect effect of hCG on cell survival (via changes in cell proliferation) would be expected to be more severe in these particular women. It is not until several days post-parturition that hCG levels decline to pre-pregnancy titres, due to the fact that the primary source of hCG is the placenta, and with this source removed at birth, hCG is metabolically cleared from circulation by the kidneys and the liver (Cunningham et al., 2010). This change would presumably allow for relative increases of cell proliferation (as compared to rates in the presence of hCG), eventually leading to recovery of normal memory function, although other factors, such as cortisol/corticosterone (Leuner et al., 2007) can continue to repress neurogenesis postpartum. However, it must be noted that this difference in maternal memory as reported by Vanston and Watson (2005) did not examine cognition before about 10 weeks weeks of gestational age (12 weeks since last menstrual period), and as such it is possible that the memory effect predates even the differences in hCG serum titres. If this is the case, other existing factors related to the sex of the fetus

(such as the Y chromosome genes) or lasting maternal changes due to previous pregnancies may be underlying the fetal sex memory effect instead. In any case, the initial peak of hCG in the first trimester of pregnancy also correlates well with the onset of memory difficulties self-reported by pregnant women (Brett & Baxendale, 2001), although again, it should be noted that tests of working memory are generally not considered hippocampal dependent tasks per se, and as such this hCG induced inhibition of cell proliferation in the dentate gyrus may be more closely related to variations in spatial memory and in the levels of anxiety experienced during pregnancy, rather than to changes in working memory in general.

Another important implication of the results of this study is that as we used ovariectomized animals, it appears that the depressive effect of hCG on cell division is not regulated via gonadotropin induced modulation of steroid hormone release from the ovaries (although this specific effect of hCG on neurogenesis may require their presence in the proportions seen during pregnancy, as discussed subsequently). Controlling the circulating levels of estrogen and progesterone identically in both groups via hormone implants allowed alterations in rates of cell proliferation to be attributed to the actions of hCG via its cognate receptor in the CNS. However, it has been demonstrated that LH signalling in the brain modulates the expression of the cholesterol transport protein StAR, and that this change results in increased production in neuro-sex steroids (NSS) (Liu, Wimalasena, Bowen & Atwood, 2007). As steroids present in the CNS are a mixture of peripherally derived sex steroids, locally converted peripheral steroids,

and steroids produced *de novo* in the CNS, the possibility that hCG is having its effect on neurogenesis through regulation of NSS production cannot be eliminated. However, at a minimum, the results of the experiments reported here indicate that hCG is not inducing these changes in cell proliferation via effects on the gonads, but instead is having an impact on the CNS directly.

Interestingly, analysis of the volumes of the GCL and hilus revealed an interaction for region x treatment after only 2 days of hCG administration, and although post hoc comparisons did not indicate any statistically significant differences, a trend in the volume data for the hilus pointed to increased hilar volumes in hCG treated animals. However, this trend was not seen in the GCL, leading to the significant interaction for region x treatment. In addition, this trend disappeared within 21 days, as no significant effects or trends in the DG volume data were seen in the cell survival study. Also of interest is that there were no significant differences between groups in terms of overall DG volume, despite equivalent GCL volumes in conjunction with this trend towards larger hilar volumes in response to hCG treatment. It is known that hippocampal volumes decrease during pregnancy (Galea et al., 2000), and as such, it may be that hCG is having a subtle, but apparently specific, influence on the maintenance of hilar volume under these endocrine conditions. However, this result is somewhat paradoxical in light of the demonstrated depressive impact of hCG treatment on cell proliferation; it seems that hCG is reducing levels of cell division (and thus the relative number of cells present), but is concurrently maintaining (or increasing) the volume of the hilus. If not due to cell proliferation or proliferation

independent cell survival, this trend towards larger hilar volumes may be attributable to other processes, such as altered migration of unlabeled cells into the region, or changes in the number or the size of processes from unlabeled existing cells passing through the area. Also, as the volume of the CA3/CA4 region (Figure 1) was not analyzed, we cannot discount the possibility that the reductions in the volume of this region may account for the aforementioned changes in hilar volume. In any case, the reasons for this trend in the data are not clear, and further studies to systematically examine possible alterations in DG volumes in response to hCG administration are necessary to replicate and confirm this pattern, and to isolate the underlying physiological changes responsible for it.

Although mitosis in the adult DG is thought to typically occur in the SGZ, and newly produced daughter cells in this region subsequently migrate into the GCL proper, during development loci of proliferative cells are located in the hilar region, before formation of the SGZ occurs (Seress, Abraham, Tornoczky & Kosztolanyi, 2001). In rodents, proliferation of cells in the hilus drops off considerably in the postnatal period, although dividing progenitor cells remain in this region into adulthood (Seress et al., 2001), and neurogenesis in SGZ and the hilus has been demonstrated in human adults (Eriksson et al., 1998). Although most of the research on hippocampal neurogenesis focuses on effects in the GCL, and has linked the cognitive and behavioural impacts of altered neurogenesis to this region, in our study hCG was shown to affect rates of cell division in both the GCL and the hilus. Having said this, BrdU immunoreactivity

demonstrates a recent mitotic event for the cell in question, but does not distinguish between cells of a neuronal phenotype and other associated cell types, such as glial cells. Endogenous markers can be used to distinguish between these groups of cells, but it is difficult to assess cell phenotype only 24 hours post-mitosis as differentiation into a mature phenotype has not yet occurred. Endogenous markers of neuronal phenotypes, such as nestin, DCX, and NeuN are only expressed in the daughter cell 2 days, 2-10 days, and 7-10 days and onwards after division, respectively (Taupin, 2007). In studies of cell survival, NeuN, a protein marker associated with mature neurons, can be used in conjunction with BrdU to co-label surviving cells that have taken on a neuronal phenotype. In general, the majority of the surviving BrdU-labeled cells in the GCL co-label with NeuN (eg. Barker & Galea, 2008) or other neuronal markers (eg. Darnaudery et al., 2007), and as such, it appears likely, although not a certainty, that most of the new daughter cells produced in the GCL/SGZ will become neurons. However, as few studies analyze neurogenesis in the hilus, it is unclear whether the changes in cell proliferation in this region as demonstrated in this report will be reflected in terms of changes in the number of neurons present, or more accurately represent changes in other cell types, such as glial cells. In any case, without a predictive marker of eventual neuronal fate to be used in conjunction with BrdU labeling in proliferation studies, this uncertainty will remain.

If these newly produced daughter cells in fact go on to follow a neuronal developmental pathway, it is important to note that there is some evidence to

indicate that they play a functional role in learning and memory, although this premise is still controversial. As previously mentioned, Kee et al. (2007) have demonstrated that adult-born neurons are preferentially recruited over neurons generated during development by tasks that require spatial learning. Furthermore, immature neurons display unique characteristics, such as lower activation thresholds and increased potential for long term potentiation (LTP) (Koehl & Abrous, 2011). As well, once mature, adult born neurons become indistinguishable from those neurons produced developmentally (Deng, Aimone & Gage, 2010). However, Wojtowicz, Askew and Winocur (2008) found no changes in spatial learning in rats with irradiation-induced reductions in neurogenesis, indicating the possible presence of compensatory mechanisms to protect this particular set of cognitive functions, although another hippocampal dependent task, contextual fear conditioning, was severely impaired. As a whole, these studies do seem to indicate that adult born neurons, particularly in their immature state, exhibit a higher plasticity than existing mature neurons, and this malleability may at least partially underlie the modifications of the DG that are necessary to form some types of new memories. In this context, a reduction in the number of proliferating cells, which presumably includes a majority of cells that will become new neurons, in response to hCG treatment may reduce the ability of the DG to respond to environmental inputs via the normal involvement of immature neurons, and thereby impair related memory function.

Proceeding under the assumption that at least a significant proportion of the BrdU-labeled cells will become functional neurons, the impact of hCG on cell

proliferation may be mediated via the known signalling pathways activated in neurons as a result of ligand binding with the LH/CGR. As previously discussed, activation of the LH/CGR results in initiation of the cAMP second messenger signalling cascade through G protein activation. Up-regulation of cAMP causes the downstream phosphorylation of various transcription factors, which are in a position to directly affect gene expression. The actual physiological effects of this pathway vary depending on a vast number other extracellular and intracellular cues, as well as cell and tissue type; effects range from the production of enzymes that cleave cholesterol side-chains in the biosynthesis of progesterone in the corpus lutem (Cole, 2010) to activation of the c-fos and brain-derived neurotrophic factor (BDNF) genes in the brain, as well as direct involvement in neuroplasticity and long term facilitation (Lee & Silva, 2009). Activation of the LH/CGR is also associated with activation of the G protein linked phospholipase C (PLC) signalling pathway; activation of this cascade results in calcium release from the endoplasmic reticulum. This cytosolic increase in Ca^{2+} concentration results in the downstream activation of calmodulin-dependent kinases (CaMKs), which have been shown to be necessary for long-term potentiation to occur, and are therefore an important molecular correlate of memory formation (Lee & Silva, 2009). Activation of this pathway also impacts on neuronal excitation as well as acetylcholine mediated memory functions. However, it has been argued that the PLC signalling pathways are only activated by pharmacological doses of hCG (Menon, Menon, Wang, Gulappa, & Harada, 2010), although the evidence is not yet definitive. Regardless, these effects of

activation of the LH/CGR for the most part involve processes and factors that lead to increases in neurogenesis and improvements in memory formation, which is not consistent with the demonstrated effect of hCG in supressing cell proliferation. Given the extraordinary number of convergent and divergent inputs on a cell that can modulate these two basic cascades, the apparent inconsistencies with respect to the impact of hCG on neurogenesis seem to be indicative of a differential effect of hCG during pregnancy as compared to other contexts. There is evidence to support this hypothesis, as it has been demonstrated that administration of progesterone with estrogen can result in supressed cell proliferation, in comparison to the increases in proliferation normally seen with estrogen treatment alone (Pawluski et al., 2009; Tanapat et al., 2005). If alterations in the levels of these two sex hormones can completely reverse the pattern of cell division in the DG, it is not unreasonable to propose that the particular hormonal milieu during pregnancy allows for a differential effect of gonadotropin signalling on neurogenesis as compared to the non-pregnant case, either through a direct mechanism or via the aforementioned modulation of NSS production in the brain.

Another possible mechanism by which hCG may be inducing its regulatory effects on neurogenesis is the reduction of sensitivity to the hormone via down-regulation of its cognate receptor. Indeed, continued exposure to LH can induce receptor desensitization *in vitro* and *in vivo*, via decoupling of the receptor from its linked G proteins (Segaloff & Ascoli, 1993). This process may explain the lack of an effect of hCG on cell survival, as cells may be initially sensitive to hCG and

LH signalling immediately after cell division, but over time may lose the ability to react to the hormone. In the shorter term, reductions in LH/CGR mRNA have been demonstrated to last for approximately 24 hours after a single exposure to LH in the rat corpus luteum, although recovery to pre-supression levels occurs within 72 hours (Menon et al., 2010), and these effects do not seem to occur during pregnancy, at least in humans (Duncan, McNeilly, Fraser & Illingworth, 1996). However, these regulatory effects of gonadotropin administration on mRNA stability may not directly translate into differences in active receptor present on the cell surface, at least over short time periods. Two different isoforms of the LH/CGR have been identified; both a 90 kDa “mature” form, and a 73 kDa precursor variant have been isolated. Furthermore, this difference has been attributed to variable glycosylation and it has been suggested that the precursor form is not functional (Apaja et al., 2004). There are significant levels of the immature isoform present in the CNS, and it is thought that this is due to inefficient conversion to the mature variant; it has been proposed that this may be a mechanism of regulation that controls the amount of active receptor at the cell surface at any given time (Apaja et al., 2004). In this regard, cells that express the LH/CGR may have a reserve of immature but non-functional receptors that can be brought into service, and although mRNA levels are shown to be down-regulated briefly in response to surges of LH or hCG, this does not necessarily indicate that there is any change in the number of active receptors present on the cell surface. This mechanism would be consistent with the lack of down-regulation of the LH/CGR demonstrated by Duncan et al. (1996) during

pregnancy, when gonadotropin levels are consistently elevated for long periods of time. Given our use of an endocrine model of pregnancy, it seems unlikely that differential regulation of the LH/CGR is the mechanism responsible for the hCG induced suppression of cell proliferation reported here, although desensitization of the receptor in response to long term gonadotropin exposure may explain the lack of group differences in the cell survival study.

Adding further evidence in support of the suggestion that hCG may have divergent effects on neurogenesis dependent on the reproductive status of the person or animal in question, the results of the current study are in contrast to the impact of LH on neurogenesis demonstrated by Mak et al. (2007), where LH was shown to have a mitogenic effect in intact and OVXed female mice. However, Mak et al. (2007) were examining alterations in neurogenesis in female mice in response to exposure to male pheromones, and as such were not using a hormonal model representative of pregnancy. In the case of the experiments presented here, which indicate that hCG has an anti-mitotic effect, such a model was employed, and thus the elevated levels of progesterone and estrogen may be the source of these divergent results, as previously discussed. Conversely, these disparities may be due to a simple difference in response characteristics between mice and rats with respect to gonadotropin manipulation, the possibility of which was discussed previously with regards to the effects of a GnRH antagonist on anxiety (Telegdy et al., 2009; Telegdy et al., 2010), or perhaps a possible, albeit unlikely, qualitative difference in the effects of LH and hCG due to the differing strength of their biological activities. These differences would be

easily resolved by replication of the study reported here with the substitution of LH for hCG, thereby isolating the effect to either the hormonal state of pregnancy, or to one of the other causes listed.

Finally, the finding in this study that hCG depresses cell proliferation may have relevance to the pathogenesis of Alzheimer's disease (AD), specifically, the "gonadotropin hypothesis" of AD. The classical view of AD pathogenesis as it relates to hormonal changes is that estrogen replacement therapy (ERT) during menopause provides a protective effect against development of the disease that is no longer being maintained by ovarian function (Gregory & Bowen, 2005). This ovarian failure results in the body's remaining steroid production being limited to the small amount of androgens produced by the adrenal cortex, and the even smaller concentration of estrogens produced by peripheral conversion from testosterone. Epidemiologically, these menopausal changes have long been known to correlate with a steep jump in the incidence of AD. Therefore, there has been significant momentum behind the idea that estrogen provides a protective barrier against AD prior to menopause. However, in the absence of estrogen, there is a concurrent and dramatic elevation in LH levels due to the homeostatic regulation of gonadal steroid release by the gonadotropins. As estrogen levels are low due to the quiescence of the ovaries during menopause, the hypothalamus responds by releasing more GnRH, which in turn induces the pituitary to release more LH to try to induce the now non-responsive ovaries to release more estrogen. Given these regulatory mechanisms, all the factors discussed above lend credence to the idea that the estrogen provided in ERT

does not confer a protective effect via direct action on neurons, but rather that sufficient levels of circulating estrogen from ERT prevent the excess of LH seen during menopause from being released into circulation. If AD is attributable to excessively high levels of gonadotropins, the LH surge post-menopause may account for the 2:1 ratio in incidence of AD between women and men.

Support for this hypothesis, in contrast to support for the neuroprotective effects of estrogen proposed by clinical reports, came from a rigorous, large scale study run by the Women's Health Initiative (reviewed in Casadesus et al., 2006), in which it was demonstrated that ERT was only helpful if initiated during menopause transition or in early menopause and had a significant negative effect on dementia incidence rates in older post-menopausal patients (so much so that the study had to be stopped early). Other studies have indicated that ERT is not helpful in patients who had already developed AD (Gregory & Bowen, 2005). These observations are indicative of a critical period for ERT to be successful; it appears that the negative impacts of the menopausal LH surge can only be moderated early on, before years of constitutively low estrogen levels have rendered the HPG axis feedback loop inoperative. It is also recognized that all abnormalities of AD known thus far are consistent with aberrant re-entry of neurons into the cell cycle, although the mitogenic factors underlying the condition have not been positively identified (Gregory & Bowen, 2005). Mitotic alterations are one of the earliest known signs of AD pathology, so the fact that LH and hCG are known to activate mitogen signalling pathways and mitogen activated protein kinase (MAPK) via cAMP and PKA signalling (Gregory and

Bowen, 2005; Casadesus et al., 2006) provides further support for the gonadotropin hypothesis of AD..

When considering the sum of this evidence, the gonadotropins seem well positioned to be important signalling molecules factoring into the development of AD, independent of any impact they have on gonadal steroid release, especially considering that post-menopause the gonads are in a state of quiescence. However, the possible mitotic effect of the gonadotropins in this context is in contrast to the anti-mitotic effect demonstrated in this current study. As previously discussed, when OVXed rats given estrogen replacement were treated with hCG, the result was impairments in spatial memory as measured by performance on an object location memory task and in the Barnes maze task with concurrent increases in amyloid-β levels in both whole brain homogenates and in the hippocampus specifically (Berry et al., 2008). This behavioural impact is in line with the impairments associated with the pathogenesis of Alzheimer's disease, the cognitive deficits demonstrated during human pregnancy, and the behavioural changes in response to hCG administration in endocrine models of pregnancy in rodents. This adds further support to the premise that there may be a differential physiological effect of hCG (and the gonadotropins in general) during the pregnant state as compared to other contexts, as pregnancy is characterized by elevated levels of ovarian hormones, whereas Alzheimer's disease is associated with the opposite pattern, despite elevated gonadotropin levels in both cases. It has been suggested that rates of neurogenesis that are either too high or too low may similarly impair memory function (Koehl & Abrous,

2011) and if so, this may help explain the similar cognitive and behavioural outcomes for hCG mediated repression of neurogenesis during pregnancy and the hypothesized mitogenic effect of the gonadotropins in AD pathogenesis.

In sum, this study is unique in its use of an endocrine model of pregnancy to assess the influence of gonadotropins, specifically hCG, on neurogenesis. The results of these experiments demonstrate a clear anti-mitotic effect of hCG, resulting in decreased cell proliferation in both the GCL and hilus. However, no effect of hCG treatment was discernable on the survival of the newly produced daughter cells. This depression of cell division in the dentate gyrus due to hCG provides a possible explanation for the impairment of memory seen in pregnant women from the end of the first trimester onwards that is not otherwise definitively attributable to steroid hormones. As well, these results provide a possible mechanism by which fetal sex differentially impacts memory function in pregnant women, and also adds new information relevant to the pathogenesis of Alzheimer's disease.

REFERENCES

Al-Hader, A. A., Lei, Z. M., & Rao, C. V. (1997). Neurons from fetal rat brains contain functional luteinizing hormone/chorionic gonadotropin receptors. *Biology of Reproduction, 56*(5), 1071-1076.

Al-Hader, A. A., Lei, Z. M., & Rao, C. V. (1997). Novel expression of functional luteinizing hormone/chorionic gonadotropin receptors in cultured glial cells from neonatal rat brains. *Biology of Reproduction, 56*(2), 501-507.

Apaja, P. M., Harju, K. T., Aatsinki, J. T., Petaja-Repo, U. E., & Rajaniemi, H. J. (2004). Identification and structural characterization of the neuronal luteinizing hormone receptor associated with sensory systems. *The Journal of Biological Chemistry, 279*(3), 1899-1906.

Barha, C. K., & Galea, L. A. (2010). Influence of different estrogens on neuroplasticity and cognition in the hippocampus. *Biochimica Et Biophysica Acta, 1800*(10), 1056-1067.

Barker, J. M., & Galea, L. A. (2008). Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats. *Neuroscience, 152*(4), 888-902.

Berry, A., Tomidokoro, Y., Ghiso, J., & Thornton, J. (2008). Human chorionic gonadotropin (a luteinizing hormone homologue) decreases spatial memory and increases brain amyloid-beta levels in female rats. *Hormones and Behavior, 54*(1), 143-152.

Bodensteiner, K. J., Cain, P., Ray, A. S., & Hamula, L. A. (2006). Effects of pregnancy on spatial cognition in female hooded long-evans rats. *Hormones and Behavior, 49*(3), 303-314.

Brett, M., & Baxendale, S. (2001). Motherhood and memory: A review. *Psychoneuroendocrinology, 26*(4), 339-362.

Bridges, R. S. (1984). A quantitative analysis of the roles of dosage, sequence, and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. *Endocrinology, 114*(3), 930-940.

Bryan, K. J., Mudd, J. C., Richardson, S. L., Chang, J., Lee, H. G., Zhu, X., . . . Casadesus, G. (2010). Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. *Journal of Neurochemistry, 112*(4), 870-881.

Buckwalter, J. G., Stanczyk, F. Z., McCleary, C. A., Bluestein, B. W., Buckwalter, D. K., Rankin, K. P., . . . Goodwin, T. M. (1999). Pregnancy, the postpartum, and steroid hormones: Effects on cognition and mood. *Psychoneuroendocrinology, 24*(1), 69-84.

Casadesus, G., Garrett, M. R., Webber, K. M., Hartzler, A. W., Atwood, C. S., Perry, G., . . . Smith, M. A. (2006). The estrogen myth: Potential use of gonadotropin-releasing hormone agonists for the treatment of alzheimer's disease. *Drugs in R&D, 7*(3), 187-193.

Casadesus, G., Milliken, E. L., Webber, K. M., Bowen, R. L., Lei, Z., Rao, C. V., . . . Smith, M. A. (2007). Increases in luteinizing hormone are associated with declines in cognitive performance. *Molecular and Cellular Endocrinology, 269*(1-2), 107-111.

Coggeshall, R. E., & Lekan, H. A. (1996). Methods for determining numbers of cells and synapses: A case for more uniform standards of review. *The Journal of Comparative Neurology, 364*(1), 6-15.

Cole, F. E., Weed, J. C., Schneider, G. T., Holland, J. B., Geary, W. L., & Rice, B. F. (1973). The gonadotropin receptor of the human corpus luteum. *American Journal of Obstetrics and Gynecology, 117*(1), 87-95.

Cole, L. A. (2007). Hyperglycosylated hCG. *Placenta, 28*(10), 977-986.

Cole, L. A. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology : RB&E, 8*, 102.

Coras, R., Siebzehnrubl, F. A., Pauli, E., Huttner, H. B., Njunting, M., Kobow, K., . . . Blumcke, I. (2010). Low proliferation and differentiation capacities of adult hippocampal stem cells correlate with memory dysfunction in humans. *Brain : A Journal of Neurology, 133*(11), 3359-3372.

Cunningham, F. G., Leveno, K. J., Bloom, S. L., Hauth, J. C., Rouse, D. J., & Spong, C. Y. (2010). *Williams obstetrics.* New York: McGraw Hill Medical.

Da Costa, D., Larouche, J., Dritsa, M., & Brender, W. (1999). Variations in stress levels over the course of pregnancy: Factors associated with elevated hassles, state anxiety and pregnancy-specific stress. *Journal of Psychosomatic Research, 47*(6), 609-621.

Darnaudery, M., Perez-Martin, M., Del Favero, F., Gomez-Roldan, C., Garcia-Segura, L. M., & Maccari, S. (2007). Early motherhood in rats is associated with a modification of hippocampal function. *Psychoneuroendocrinology, 32*(7), 803-812.

de Brito Faturi, C., Teixeira-Silva, F., & Leite, J. R. (2006). The anxiolytic effect of pregnancy in rats is reversed by finasteride. *Pharmacology, Biochemistry, and Behavior, 85*(3), 569-574.

Deng, W., Aimone, J. B., & Gage, F. H. (2010). New neurons and new memories: How does adult hippocampal neurogenesis affect learning and memory? *Nature Reviews.Neuroscience, 11*(5), 339-350.

Deng, W., Saxe, M. D., Gallina, I. S., & Gage, F. H. (2009). Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 29*(43), 13532-13542.

Drapeau, E., Mayo, W., Aurousseau, C., Le Moal, M., Piazza, P. V., & Abrous, D. N. (2003). Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 100*(24), 14385-14390.

Driscoll, I., Howard, S. R., Stone, J. C., Monfils, M. H., Tomanek, B., Brooks, W. M., & Sutherland, R. J. (2006). The aging hippocampus: A multi-level analysis in the rat. *Neuroscience, 139*(4), 1173-1185.

Duncan, W. C., McNeilly, A. S., Fraser, H. M., & Illingworth, P. J. (1996). Luteinizing hormone receptor in the human corpus luteum: Lack of down-regulation during maternal recognition of pregnancy. *Human Reproduction, 11*(10), 2291-2297.

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nature Medicine, 4*(11), 1313-1317.

Esimai, O. A., Fatoye, F. O., Quiah, A. G., Vidal, O. E., & Momoh, R. M. (2008). Antepartum anxiety and depressive symptoms: A study of nigerian women during the three trimesters of pregnancy. *Journal of Obstetrics and Gynaecology : The Journal of the Institute of Obstetrics and Gynaecology, 28*(2), 202-203.

Furuta, M., & Bridges, R. S. (2005). Gestation-induced cell proliferation in the rat brain. *Brain Research.Developmental Brain Research, 156*(1), 61-66.

Galea, L. A., & McEwen, B. S. (1999). Sex and seasonal differences in the rate of cell proliferation in the dentate gyrus of adult wild meadow voles. *Neuroscience, 89*(3), 955-964.

Galea, L. A., Ormerod, B. K., Sampath, S., Kostaras, X., Wilkie, D. M., & Phelps, M. T. (2000). Spatial working memory and hippocampal size across pregnancy in rats. *Hormones and Behavior, 37*(1), 86-95.

Glass, A. R., & Klein, T. (1981). Changes in maternal serum total and free androgen levels in early pregnancy: Lack of correlation with fetal sex. *American Journal of Obstetrics and Gynecology, 140*(6), 656-660.

Gregory, C. W., & Bowen, R. L. (2005). Novel therapeutic strategies for alzheimer's disease based on the forgotten reproductive hormones. *Cellular and Molecular Life Sciences : CMLS, 62*(3), 313-319.

Gundersen, H. J., Bagger, P., Bendtsen, T. F., Evans, S. M., Korbo, L., Marcussen, N., . . . Pakkenberg, B. (1988). The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 96*(10), 857-881.

Gundersen, H. J., Bendtsen, T. F., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., . . . Vesterby, A. (1988). Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 96*(5), 379-394.

Henry, J. D., & Rendell, P. G. (2007). A review of the impact of pregnancy on memory function. *Journal of Clinical and Experimental Neuropsychology, 29*(8), 793-803.

Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., . . . Kageyama, R. (2008). Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nature Neuroscience, 11*(10), 1153-1161.

Kee, N., Teixeira, C. M., Wang, A. H., & Frankland, P. W. (2007). Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nature Neuroscience, 10*(3), 355-362.

Kempermann, G., Brandon, E. P., & Gage, F. H. (1998). Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. *Current Biology : CB, 8*(16), 939-942.

Kim, S. K., Hwang, I. K., Yoo, K. Y., Yoo, D. Y., Bae, E., Lee, C. H., . . . Won, M. H. (2010). Pregnancy inhibits cell proliferation and neuroblast differentiation without neuronal damage in the hippocampal dentate gyrus in C57BL/6N mice. *Brain Research, 1315*, 25-32.

Kimura, D. (2002). Sex hormones influence human cognitive pattern. *Neuro Endocrinology Letters, 23 Suppl 4*, 67-77.

Koehl, M., & Abrous, D. N. (2011). A new chapter in the field of memory: Adult hippocampal neurogenesis. *The European Journal of Neuroscience, 33*(6), 1101-1114.

Larsen, C. M., Kokay, I. C., & Grattan, D. R. (2008). Male pheromones initiate prolactin-induced neurogenesis and advance maternal behavior in female mice. *Hormones and Behavior, 53*(4), 509-517.

Lassalle, J. M., Bataille, T., & Halley, H. (2000). Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the morris navigation task. *Neurobiology of Learning and Memory, 73*(3), 243-257.

Lee, Y. S., & Silva, A. J. (2009). The molecular and cellular biology of enhanced cognition. *Nature Reviews.Neuroscience, 10*(2), 126-140.

Lei, Z. M., & Rao, C. V. (1994). Novel presence of luteinizing hormone/human chorionic gonadotropin (hCG) receptors and the down-regulating action of hCG on gonadotropin-releasing hormone gene expression in immortalized hypothalamic GT1-7 neurons. *Molecular Endocrinology, 8*(8), 1111-1121.

Lei, Z. M., Rao, C. V., Kornyei, J. L., Licht, P., & Hiatt, E. S. (1993). Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain. *Endocrinology, 132*(5), 2262-2270.

Lemaire, V., Billard, J. M., Dutar, P., George, O., Piazza, P. V., Epelbaum, J., . . . Mayo, W. (2006). Motherhood-induced memory improvement persists across lifespan in rats but is abolished by a gestational stress. *The European Journal of Neuroscience, 23*(12), 3368-3374.

Leuner, B., Mirescu, C., Noiman, L., & Gould, E. (2007). Maternal experience inhibits the production of immature neurons in the hippocampus during the postpartum period through elevations in adrenal steroids. *Hippocampus, 17*(6), 434-442.

Liu, T., Wimalasena, J., Bowen, R. L., & Atwood, C. S. (2007). Luteinizing hormone receptor mediates neuronal pregnenolone production via up-regulation of steroidogenic acute regulatory protein expression. *Journal of Neurochemistry, 100*(5), 1329-1339.

Lukacs, H. (2001). Rat as model for studying behavior effects of hCG. *Seminars in Reproductive Medicine, 19*(1), 111-119.

Lukacs, H., Hiatt, E. S., Lei, Z. M., & Rao, C. V. (1995). Peripheral and intracerebroventricular administration of human chorionic gonadotropin alters several hippocampus-associated behaviors in cycling female rats. *Hormones and Behavior, 29*(1), 42-58.

Macbeth, A. H., & Luine, V. N. (2010). Changes in anxiety and cognition due to reproductive experience: A review of data from rodent and human mothers. *Neuroscience and Biobehavioral Reviews, 34*(3), 452-467.

Macbeth, A. H., Gautreaux, C., & Luine, V. N. (2008). Pregnant rats show enhanced spatial memory, decreased anxiety, and altered levels of monoaminergic neurotransmitters. *Brain Research, 1241*, 136-147.

Mak, G. K., & Weiss, S. (2010). Paternal recognition of adult offspring mediated by newly generated CNS neurons. *Nature Neuroscience, 13*(6), 753-758.

Mak, G. K., Enwere, E. K., Gregg, C., Pakarainen, T., Poutanen, M., Huhtaniemi, I., & Weiss, S. (2007). Male pheromone-stimulated neurogenesis in the adult female brain: Possible role in mating behavior. *Nature Neuroscience, 10*(8), 1003-1011.

Maston, G. A., & Ruvolo, M. (2002). Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. *Molecular Biology and Evolution, 19*(3), 320-335.

McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., . . . Seeburg, P. H. (1989). Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. *Science (New York, N.Y.), 245*(4917), 494-499.

Menon, K. M., Menon, B., Wang, L., Gulappa, T., & Harada, M. (2010). Molecular regulation of gonadotropin receptor expression: Relationship to sterol metabolism. *Molecular and Cellular Endocrinology, 329*(1-2), 26-32.

Meulenberg, P. M., & Hofman, J. A. (1991). Maternal testosterone and fetal sex. *The Journal of Steroid Biochemistry and Molecular Biology, 39*(1), 51-54.

Obiekwe, B. C., & Chard, T. (1982). Human chorionic gonadotropin levels in maternal blood in late pregnancy: Relation to birthweight, sex and condition of the infant at birth. *British Journal of Obstetrics and Gynaecology, 89*(7), 543-546.

Palmer, J., & Watson, N. V. (2011). [The relationship between stage of pregnancy and performance on cognitive tasks in a cross-sectional sample]. Unpublished raw data.

Pawluski, J. L., & Galea, L. A. (2007). Reproductive experience alters hippocampal neurogenesis during the postpartum period in the dam. *Neuroscience, 149*(1), 53-67.

Pawluski, J. L., Barakauskas, V. E., & Galea, L. A. (2010). Pregnancy decreases oestrogen receptor alpha expression and pyknosis, but not cell proliferation or survival, in the hippocampus. *Journal of Neuroendocrinology, 22*(4), 248-257.

Pawluski, J. L., Brummelte, S., Barha, C. K., Crozier, T. M., & Galea, L. A. (2009). Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. *Frontiers in Neuroendocrinology, 30*(3), 343-357.

Pawluski, J. L., Walker, S. K., & Galea, L. A. (2006). Reproductive experience differentially affects spatial reference and working memory performance in the mother. *Hormones and Behavior, 49*(2), 143-149.

Rao, C. V., & Lei, Z. M. (2007). The past, present and future of nongonadal LH/hCG actions in reproductive biology and medicine. *Molecular and Cellular Endocrinology, 269*(1-2), 2-8.

Rofe, Y., Blittner, M., & Lewin, I. (1993). Emotional experiences during the three trimesters of pregnancy. *Journal of Clinical Psychology, 49*(1), 3-12.

Rolls, A., Schori, H., London, A., & Schwartz, M. (2008). Decrease in hippocampal neurogenesis during pregnancy: A link to immunity. *Molecular Psychiatry, 13*(5), 468-469.

Ros, J., Pellerin, L., Magara, F., Dauguet, J., Schenk, F., & Magistretti, P. J. (2006). Metabolic activation pattern of distinct hippocampal subregions during spatial learning and memory retrieval. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism, 26*(4), 468-477.

Saal, W., Glowania, H. J., Hengst, W., & Happ, J. (1991). Pharmacodynamics and pharmacokinetics after subcutaneous and intramuscular injection of human chorionic gonadotropin. *Fertility and Sterility, 56*(2), 225-229.

Santolaya-Forgas, J., Meyer, W. J., Burton, B. K., & Scommegna, A. (1997). Altered newborn gender distribution in patients with low mid-trimester maternal serum human chorionic gonadotropin (MShCG). *The Journal of Maternal-Fetal Medicine, 6*(2), 111-114.

Sasaki, Y., Ladner, D. G., & Cole, L. A. (2008). Hyperglycosylated human chorionic gonadotropin and the source of pregnancy failures. *Fertility and Sterility, 89*(6), 1781-1786.

Segaloff, D. L., & Ascoli, M. (1993). The lutropin/choriogonadotropin receptor ... 4 years later. *Endocrine Reviews, 14*(3), 324-347.

Seress, L., Abraham, H., Tornoczky, T., & Kosztolanyi, G. (2001). Cell formation in the human hippocampal formation from mid-gestation to the late postnatal period. *Neuroscience, 105*(4), 831-843.

Shingo, T., Gregg, C., Enwere, E., Fujikawa, H., Hassam, R., Geary, C., . . . Weiss, S. (2003). Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. *Science (New York, N.Y.), 299*(5603), 117-120.

Smith, E. R., Damassa, D. A., & Davidson, J. M. (1977). Hormone administration: Peripheral and intracranial implants. In R. D. Myers (Ed.), *Methods in psychobiology (Volume 3): Advanced laboratory techniques in neuropsychology and neurobiology* (pp. 259-279). London: Academic Press.

Tanapat, P., Hastings, N. B., & Gould, E. (2005). Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. *The Journal of Comparative Neurology, 481*(3), 252-265.

Tanapat, P., Hastings, N. B., Reeves, A. J., & Gould, E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 19*(14), 5792-5801.

Taupin, P. (2007). BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation. *Brain Research Reviews, 53*(1), 198-214.

Telegdy, G., Adamik, A., Tanaka, M., & Schally, A. V. (2010). Effects of the LHRH antagonist cetrorelix on affective and cognitive functions in rats. *Regulatory Peptides, 159*(1-3), 142-147.

Telegdy, G., Tanaka, M., & Schally, A. V. (2009). Effects of the LHRH antagonist cetrorelix on the brain function in mice. *Neuropeptides, 43*(3), 229-234.

Toth, P., Lukacs, H., Hiatt, E. S., Reid, K. H., Iyer, V., & Rao, C. V. (1994). Administration of human chorionic gonadotropin affects sleep-wake phases and other associated behaviors in cycling female rats. *Brain Research, 654*(2), 181-190.

Tsampalas, M., Gridelet, V., Berndt, S., Foidart, J. M., Geenen, V., & Perrier d'Hauterive, S. (2010). Human chorionic gonadotropin: A hormone with immunological and angiogenic properties. *Journal of Reproductive Immunology, 85*(1), 93-98.

Turner, B. J., Jones, B. A., & Watson, N. V. (2011). *Effects of human chorionic gonadotropin (hCG) on spatial and emotional processing in a rat model of pregnancy.* Manuscript submitted for publication.

Vanston, C. M., & Watson, N. V. (2005). Selective and persistent effect of foetal sex on cognition in pregnant women. *Neuroreport, 16*(7), 779-782.

Watson, R. E.,Jr, Wiegand, S. J., Clough, R. W., & Hoffman, G. E. (1986). Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides, 7*(1), 155-159.

West, M. J., Slomianka, L., & Gundersen, H. J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *The Anatomical Record, 231*(4), 482-497.

Wojtowicz, J. M., Askew, M. L., & Winocur, G. (2008). The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. *The European Journal of Neuroscience, 27*(6), 1494-1502.

Xavier, G. F., & Costa, V. C. (2009). Dentate gyrus and spatial behaviour. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 33*(5), 762-773.

Yang, E. J., Nasipak, B. T., & Kelley, D. B. (2007). Direct action of gonadotropin in brain integrates behavioral and reproductive functions. *Proceedings of the National Academy of Sciences of the United States of America, 104*(7), 2477-2482.

Yaron, Y., Lehavi, O., Orr-Urtreger, A., Gull, I., Lessing, J. B., Amit, A., & Ben-Yosef, D. (2002). Maternal serum HCG is higher in the presence of a female fetus as early as week 3 post-fertilization. *Human Reproduction (Oxford, England), 17*(2), 485-489.

Zhang, W., Lei, Z. M., & Rao, C. V. (1999). Immortalized hippocampal cells contain functional luteinizing hormone/human chorionic gonadotropin receptors. *Life Sciences, 65*(20), 2083-2098.

APPENDICES

Appendix A: Protocols and Procedures

Bromodeoxyuridine (BrdU) Preparation

Galea lab 2007

Calculation:

1. Determine amount of BrdU needed

****Adult Rats 200 mg/kg; Voles & Mice 50 mg/kg****

To make 200 mg/kg dose:
For example, 10 rats * 500 g (average mass of each rat) = 5000 g (5 kg) of rat.

You need 200 mg/kg * 5 kg rat = 1000 mg of BrdU

For every 100 g (0.1 kg) of rat: 200 mg/kg = X? mg / 0.1 kg

X= 20 mg

Injection volume is 1 ml per 100g of rat. Calculate the volume you need:
Therefore, for every 100 g of rat you need 20 mg BrdU in 1 ml solution.

Your concentration will be 20 mg BrdU / ml of solution; therefore:

1000 mg BrdU * 1 ml / 20 mg BrdU = 50 ml of saline solution.

Note: always make more than the exact amount needed. There is always loss in needle head.

Procedure:

2. a. Mix 50 ml of 0.9% saline:

50 ml dH_2O
0.45 g NaCl

Mix over low heat on stir plate (not above 40°C)
Always prepare saline just before use—BrdU dissolves better in warm saline.

b. Mix 50 ml 200 mg/kg BrdU solution:

Place 1000 mg of BrdU (from freezer-record lot #) in a 100 ml clean beaker with a stir bar (or in a 50 ml centrifuge tube when making less than 50 ml). Wrap beaker in foil to protect BrdU from light.
Add 350 µl of 1*N* NaOH (0.7%) and mix or vortex

Add 50 ml of 0.9% NaCl and mix or vortex until completely dissolved- may take a while.

Store solution at room temperature protected from light until use.
Use solution within a few hrs of preparation (to a max of 4 hrs).
Inject i.p. 1 ml of BrdU solution per 100 g of rat:
i.e. for a 455 g rat, inject 4.6 ml.

BrdU/DAB Immunohistochemistry Protocol

(updated by Galea lab April 2008)

0.1*M* TBS (pH 7.4); 2 L
2 L dH_2O
26.44 g Trizma HCl
3.88 g Trizma Base
18 g NaCl
pH to 7.4

Day 1

Procedure:

All steps are done at RT and on a rotator, and tissue is in nets unless otherwise stated.

0. Preheat water bath to 37°C
1. Rinse tissue in 0.1*M* TBS (pH 7.4) 3x 10 min to equilibrate tissue
2. Incubate tissue in 0.6% H_2O_2 for 30 min

   0.6% H_2O_2; 50 mL
   1 mL 30% H_2O_2
   49 mL dH_2O
   ** H_2O_2 should be stored < 1 month at 4°C and longer at -20°C. If you do not see robust bubbling on tissue then H_2O_2 needs to be replaced

3. Rinse tissue 3x 0.1*M* TBS 10 min each
4. Incubate in 2*N* HCl and maintain at 37°C in a hot water bath for 30 minutes (to denature DNA)
5. Incubate in **0.1*M*** Borate Buffer for 10 min

   This means dilute the 0.5*M* Borate Buffer stock to 0.1*M*:
   20 mL stock Borate Buffer
   80 mL dH_2O

   0.5 *M* Borate Buffer Stock Solution; 1 L
   1 L dH_2O
   30.9 g Boric Acid
   pH to 8.5 with NaOH

6. Rinse tissue 3x 0.1*M* TBS 10 min each (to remove background staining)
7. Block in TBS+ (made up of TBS, 0.1% Triton-X, and 3% NHS) and let incubate for 30 min

   TBS + (3% NHS and 1% of 10% Triton-X in TBS); 100 mL
   96 mL TBS
   1 mL 10% Triton-X [**1 mL Triton-X and 9 mL dH_2O; vortex**] (0.1%)
   3 mL Normal Horse Serum (3%)

8. Incubate in primary antibody in TBS+ (remove from nets) at 4°C for 20-48 hours

   Primary Antibody (BrdU) Solution
   1:200 (1.25 mL per well)

50 mL TBS+
250 µL Ms anti-BrdU (Roche)

Day 2

Procedure:

9. Transfer tissue back into net wells and wash in TBS 3x for 10 min each (to remove any anti-BrdU that did not attach to the BrdU)
10. Incubate (out of net wells) in secondary antibody for 4 hours

    Secondary antibody (anti Ms) solution
    1:200 (1.25 per well)
    50 mL TBS+
    250 µL anti-mouse IgG (Vector) (50µL per drop)

11. Rinse 3x in TBS 10 min each (back into net wells)
12. Incubate in ABC reagent for 1.5 hours (prepare as specified in the Vector Kit insert *at least* ½ hour in advance)

    ABC solution (prepare at least ½ hour in advance)
    50 mL TBS
    1.0 mL Reagent A (20 drops)
    1.0 mL Reagent B (20 drops)

13. Rinse 3x in TBS (can leave in fridge overnight, or go straight to 14.)

14. Under fume hood, add DAB to each well. Remove tissue from net wells into DAB wells for 5 min (watch the rxn and look under the microscope to be sure you see good staining).

    DAB 5 mg/mL; 10 mL
    ***Work under a fume hood with personal protection equipme*nt**
    8.975 mL TBS
    0.975 mL DAB (Sigma) (carcinogenic and mutagenic! light sensitive!)
    50 µL H_2O_2
    Mix for 1 min
    ****Neutralize used DAB dishes with bleach****
    Note: Wear protective goggles, lab coat, two layers of gloves and a protective mask. Work under the FUME HOOD.

15. Rinse 3x in TBS quick, 3 x 10 min (back into net wells for rinses)
16. Mount tissue on microscope slides. Ideally, you should do the DAB reaction the day you mount the tissue because the DAB reaction can proceed even after several washes.
17. Allow mounted sections to dry overnight (up to 2 days) at RT (cover to protect from dust).

Following drying, you can counter stain with cresyl violet

18. Cresyl violet stain, dehydrate, clear, and coverslip the sections with permount (Cresyl violet is optional). Otherwise, dehydrate: 50%-100% EtOH; 2x 10 min dips in Xylene. Coverslip with permount. Allow to dry several days prior to microscopy.

SIMON FRASER UNIVERSITY
ANIMAL CARE SERVICES

S5: OVARIECTOMY IN RODENTS

DRAFT: Revised July 2006

PURPOSE: To describe the surgical procedure for performing an ovariectomy in rats under general anaesthesia.

POLICY: To provide detailed instructions on performing specific aseptic surgical procedures successfully and which will minimize complications such as pain, infection and closure dehiscence.

RESPONSIBILITY: Technical personnel or Researcher under Veterinary supervision

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ANATOMY BASICS:

Surgical removal of the ovaries is known as ovariectomy. The layman's term "spaying" usually refers to removal of both the ovaries and the uterus, or ovariohysterectomy.

The female reproductive organs consist of paired ovaries and fallopian tubes (oviducts), followed by the uterus, the cervix, the vagina, and the vulva opening to the exterior. The two ovaries are small, round, irregular-shaped organs found on either side of the abdomen, slightly below (ventral to) the kidneys, and produce eggs (ova) and hormones, such as estrogen. The uterus is bicornuate, meaning it has two horns or branches. Each ovary is connected to one uterine horn by the highly convoluted Fallopian tube. This tube carries the eggs released by the ovary into the uterus. If fertilization occurred in the fallopian tube by the union of the egg with one sperm, the egg implants in the uterus for fetal development.

The ovaries are surrounded by fat making it sometimes difficult to initially identify them, but this movable fat can be used as a landmark.

EQUIPMENT:

1 #3 scalpel handle
1 #15 blade
2 mosquito hemostats (can replace with small Carmalts, if available)
1 blunt atraumatic forceps
1 rat tooth forceps
1 iris retractors-optional
1 blunt/sharp scissors
1 Metzenbaum (blunt ended) scissors
1 Olsen-Hagar needle driver
1 slit drape
2-4 towel clamps
5 2 inch square gauze
1 small cutting needle

4/0 absorbable suture material-catgut, vicryl or PDS suggested (do not use catgut if using on skin as well)

Skin clips and applicator if not using skin sutures

PROCEDURE:

A. ANIMAL PREPARATION

1. Disinfect the surgery table and preparation table with disinfectant located in the room. See **SOP S1: MINIMUM STANDARDS FOR PERFORMING ASEPTIC SURGERY.**
2. Place a hot water circulating blanket on both the preparation and the surgery table and turn on ½ hour before surgery. Cover blanket with a clean towel.
3. Bring rat in cage to the animal preparation room ½ hour before anaesthesia is to begin to allow it to become accustomed to its new environment.
4. Prepare surgery room as per **SOP S1: MINIMUM STANDARDS FOR PERFORMING ASEPTIC SURGERY**.
5. Weigh rat and record weight on anaesthesia record.
6. Administer rat its anesthetic drugs as per **SOP A5: RAT ANAESTHESIA** and return rat to its cage. Monitor closely.
7. Once fully anesthetized, transfer rat to preparation table.
8. Prepare rat for surgery as described in **SOP S2: ANIMAL PREPARATION FOR SURGERY**.
9. Surgeon can prepare himself/herself for surgery as described in SOP S3: Surgeon preparation for surgery while technician is preparing the rat for surgery.

   If working alone, surgeon must delay this step until after the rat has had its final prep (step 10) in the surgery room.
10. Rat is transferred to surgery table and placed on the hot water blanket in the appropriate position for the surgical procedure to be performed. This is described below.
11. A final surgical prep is performed as per **SOP S2: ANIMAL PREPARATION FOR SURGERY,** Animal is now ready for surgery.

B. SURGICAL PROCEDURE

1. Aseptic technique must be used. Instruments and surgical gloves used must be sterile. Surgeon should be wearing a surgical mask, hair bonnet and a clean gown or scrub top along with sterile surgical gloves. A sterile gown/scrub top is preferred but not required until the move to the new facility.
2. With the rat lying on its belly and its tail facing you, place a sterile drape across the rat's back with the opening centered over the middle of the spine and running parallel. Attach the drape to the rat's skin with towel clamps to prevent the drape moving (alternatively, attach the drape to the towel on which the animal lies).

   NOTE: Do not lay the towel clamps (or other instruments) across the animal's chest as the weight of the instruments can prevent the animal from breathing.

3. Make a small (1 inch) dorsal (back) midline skin incision (cut) with a #15 scalpel blade, approximately half way between the middle of the back (the hump) and the base of the tail. This is just cranial to the pelvis and where the flank "sucks in."

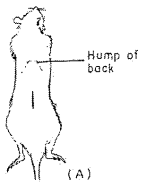

Fig. A . Site of incision for ovariectomy.

4. Blunt dissect through the subcutaneous layer with Metzenbaum (blunt ended) scissors down either side of the abdomen until the muscle layer below is visible. Do not dissect more than is necessary as it can slow down healing (increased dead space and potential for seroma formation).
5. Rotate rat slightly away from the side on which the incision is to be made to facilitate reaching the muscle. The skin can be retracted with forceps or iris retractors can be used to keep the skin incision open and improve visibility, if necessary.

   NOTE: It is important that the muscle incision be made correctly to minimize any anatomical confusion.

6. Grasp the muscle wall with atraumatic forceps and lift up so the muscle wall is pulled away from the organs within the peritoneal cavity.
7. Make a small muscle incision with a scalpel or pointed scissors half to two thirds of the way down the side of the body to enter the abdominal (peritoneal) cavity. This incision is made just dorsal to a fatty area present in the muscle wall and should be made between the muscle fibres. Be careful to not damage organs on the other side of the abdominal wall. Widen the muscle opening by expanding the scissors just large enough to allow forceps to go through the incision.

NOTE: If the incision is correctly placed, the ovaries surrounded by fat will be just underneath or within easy reach.

8. Grasp the periovarian fat (the freely movable fat surrounding the ovary) to exteriorize the ovary through the muscle incision. Do not touch the ovary itself as it may result in pieces of the ovary becoming detached and remaining in the abdomen to reimplant and carry on normal function.

If the fat is not freely movable, fat found under and attached to the midline spinal muscles may have been incorrectly grasped. This occurs if the muscle incision is made too dorsally. If this occurs, make another attempt to find the periovarian fat. The muscle incision may have to be extended ventrally.

9. Clamp the tissue at the junction of the fallopian tube and the uterine horn with hemostats (incorporating all accompanying blood vessels and fat). If it is difficult to identify tissue due to a large amount of fat, it is safer to remove some of the fat before clamping to improve visibility.

10. Place a second hemostat distal to the first hemostat (closer to the ovary). Loosely place a single absorbable suture/stitch (simple interrupted pattern, SIP) by the first hemostat. Suggested suture material can be 4-0 catgut, Vicryl, or PDS.
11. Remove the first hemostat, keeping one hand on the suture ties so the tissues cannot fall back into the abdomen, and then tighten the suture over where the clamp was located.
12. Excise (cut and remove) the tissues above (distal to) the second hemostat and suture to remove the ovary, remove the hemostat and return the uterine horn to the abdominal cavity.

(Although textbooks indicate that severing this junction with scissors is sufficient for hemostasis, clamping and suturing to prevent any bleeding is preferred).

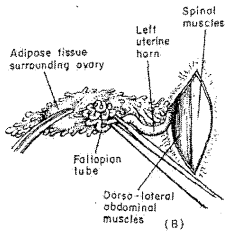

Fig. B . Removal of ovary and Fallopian tube.

13. The muscle incision requires no suturing unless it is large enough that tissues, such as intestines or fat, can pass through. In that case, or if unsure, a single SIP suture with the same absorbable suture used above will usually suffice. Place enough sutures (1-2) that the muscle edges are apposed. When placing sutures through muscle, whenever possible, grasp the fascia (shiny white tissue) along with the muscle to add strength to the suture pattern.
14. Repeat steps 4 to 13 on the other side of the abdominal wall to remove the second ovary.

Close the skin layer with skin clips/staples, skin sutures (stitches), or a subcuticular (buried skin) pattern.

Clips are fast, easy to use and are not easily removed by the rat. However, they do not appose the skin edges well, seem to irritate the skin more than sutures and need to be removed in 1-2 weeks time. Skin sutures are more time consuming and easily chewed out by the animals, but provide a nicer skin closure, seem less irritating and may or may not need to be removed once the skin has healed. Subcuticular sutures are preferred as they have the advantages of skin sutures but are not visible, so they cannot be chewed out and do not need to be removed. However, this pattern takes practice to learn..

15. If using skin sutures, use a SIP suture and the number of stitches used should be sufficient (about 2-3) to appose the incision edges without any openings evident. Either nonabsorbable sutures (nylon) or absorbable sutures (vicryl or PDS) can be used. For subcuticular closure, only absorbable suture material can be used. Nonabsorbable sutures should be removed after 4 weeks if not sloughed off with skin.

16. Once surgery is complete, monitor the animal closely post-operatively and do not leave the animal alone until full motor activity is regained - i.e., the animal is able to move into sternal recumbancy (onto its belly).
17. Ensure any post-operative duties have been followed, such as analgesics (pain relievers), fluids, heat source, new cage/bedding/food/water, etc. Read **SOP S4: POST OPERATIVE CARE.**
18. We encourage researchers to involve the animal care staff in assisting them with pre- and post-operative care. However, if staff has not been involved, the surgeon must inform them of animals recovering and their status so that the animal care staff can also monitor the animals.

References

1. Waynforth, H.B. and Flecknell, P.A. (1992) *Experimental and Surgical Technique in the Rat, Second Edition*, London, UK: Academic Press Limited.

2. LabDiet, Introduction to Laboratory Animals: Laboratory Animal Care Course, Richmond, IN: Renaissance Research Associates, Inc.

3. Lane, D.R. and Cooper, B.C. (1999). Veterinary Nursing, Second Edition. Oxford, UK: Butterworth-Heinemann

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Director's Signature: _____
Date Approved by UACC: _____
UACC Chair's Signature: _____

Note: In step 3 of Part B: Surgical Procedures, instead of a single skin incision on the midline, bilateral skin incisions on the flanks were used instead.

Appendix B: Data

Experiment 1: Cell Proliferation

Note: All volumes are reported in mm^3, and densities are reported as cells/mm^3

Animal ID	Group	GCL Cell Count	GCL Corrected Cell Count	GCL Volume	Hilus Cell Count	Hilus Corrected Cell Count	Hilus Volume
1	hCG	80	800	1.4533	10	100	3.0736
2	Control	223	2230	1.845	13	130	3.1901
3	hCG	130	1300	1.5972	22	220	3.3637
4	Control	143	1430	1.4062	16	160	2.5919
5	hCG	140	1400	1.2673	18	180	2.9033
6	Control	120	1200	1.1706	18	180	2.5782
7	hCG	238	2380	1.8008	9	90	3.5908
8	hCG	177	1770	1.1439	13	130	2.66
9	Control	156	1560	1.4841	15	150	3.0946
10	hCG	23	230	1.6535	9	90	3.517
11	Control	211	2110	1.4492	19	190	2.9083
12	Control	206	2060	1.1533	33	330	2.7385
13	Control	163	1630	1.6092	29	290	3.0015
14	hCG	134	1340	1.2145	11	110	3.0786
15	Control	128	1280	1.0901	19	190	2.5619
16	Control	262	2620	1.7021	17	170	3.5183
17	hCG	98	980	1.3697	8	80	2.7828
18	hCG	125	1250	1.5757	7	70	3.0391
19	hCG	122	1220	1.525	14	140	3.095
20	Control	202	2020	1.304	20	200	2.3676

Animal ID	Group	GCL Cell Density	Hilus Cell Density	Total DG Volume
1	hCG	550.4903	32.5357	4.5268
2	Control	1208.7048	40.7517	5.035
3	hCG	813.9498	65.4051	4.9608
4	Control	1016.9612	61.732	3.998
5	hCG	1104.7108	61.9984	4.1706
6	Control	1025.1153	69.8162	3.7488
7	hCG	1321.6348	25.0641	5.3916
8	hCG	1547.3381	48.8731	3.8039
9	Control	1051.1421	48.4715	4.5787
10	hCG	139.0989	25.5904	5.1705
11	Control	1456.0259	65.3303	4.3575
12	Control	1786.2562	120.5061	3.8917
13	Control	1012.9572	96.6184	4.6107
14	hCG	1103.3347	35.7305	4.2931
15	Control	1174.2042	74.1637	3.652
16	Control	1539.3202	48.3188	5.2204
17	hCG	715.5113	28.7485	4.1524
18	hCG	793.2982	23.0331	4.6148
19	hCG	800	45.2342	4.62
20	Control	1549.0798	84.4755	3.6716

Experiment 2: Cell Survival

Note: All volumes are reported in mm^3, and densities are reported as cells/mm^3

Animal ID	Group	GCL Cell Count	GCL Corrected Cell Count	GCL Volume	Hilus Cell Count	Hilus Corrected Cell Count	Hilus Volume
21	hCG	44	440	10	100	1.2069	2.6569
22	Control	59	590	12	120	1.6562	3.2843
23	hCG	34	340	9	90	0.9075	2.1538
24	hCG	67	670	18	180	1.3355	3.0372
25	Control	53	530	23	230	1.0999	3.1403
26	Control	37	370	17	170	1.1408	2.7104
27	hCG	21	210	8	80	1.3775	3.1375
28	Control	64	640	17	170	1.4591	3.1782
29	hCG	46	460	20	200	1.278	3.0321
30	Control	42	420	13	130	1.5303	3.4859
31	hCG	51	510	27	270	1.248	3.0328
32	Control	56	560	26	260	1.4901	3.5433
33	hCG	32	320	14	140	1.2336	2.8798
35	Control	30	300	12	120	1.0358	2.4541
36	hCG	36	360	14	140	1.2699	3.0178
37	hCG	11	110	2	20	1.2433	3.1333
38	Control	42	420	12	120	0.8492	1.6746
39	hCG	47	470	19	190	1.2033	2.2784
40	Control	17	170	7	70	0.66	1.746
41	Control	35	350	8	80	1.2055	3.151