Is Prolactin Action on Forebrain Neurons During Pregnancy Important for Maternal Neurogenesis and Behaviours?

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Abstract:

Pregnancy is associated with a dramatic increase in serum prolactin levels that is essential for the normal increase in neurogenesis in the maternal brain, and for the associated expression of maternal behaviours after birth. As yet, it is not known which neurons prolactin acts on in the maternal brain to induce these changes, but it has been shown that prolactin receptors are present on GABAergic neurons in forebrain nuclei implicated in the regulation of maternal behaviour. This study aimed to examine the effect that prolactin action on forebrain neurons has on expression of maternal behaviour and on levels of neurogenesis, employing conditional deletion of the prolactin receptor using cre-lox technology. This technology allowed the creation of mice with prolactin receptors removed from CaM kinase II containing neurons (most forebrain neurons; CKC mice), and more specifically from neurons containing the vesicular GABA transporter (vGAT mice). When cre positive mice and controls (wildtype C57BL/6J and cre negative mice), were reproductively mature (6-8 weeks old), some mice were placed in individual cages and mated with a stud male. Neurogenesis was assessed on day seven of pregnancy, using injections of BrdU, which labels newly dividing cells. Other groups of mice were allowed to continue the pregnancy until term, and the day of parturition was designated as postpartum day one (PPD1). Maternal behaviour was tested on PPD2 by placing the mouse and three pups in a clean novel cage and observing all behaviours. On PPD3 maternal behaviour was tested in a similar manner in the home cage. Mice were also tested for anxiety on PPD4 in the light-dark box. Both CKC and vGAT cre positive mice showed significantly impaired maternal behaviour in the home cage compared with control mice, but the impairment was greater in the CKC positive mice than the vGAT cre positive mice. While all mice showed impairment in the novel cage, maternal behaviour was once again significantly more impaired in the CKC positive mice. CKC positive mice mostly ignored the pups, but at times were aggressive towards them, either eating pups before, or after death. Consequently, pup survival was dramatically reduced in CKC cre positive mice, compared with controls. There was no difference in levels of anxiety between the groups postpartum, suggesting that prolactin acts elsewhere to

exert the postpartum anxiolytic effect. Surprisingly, given the maternal behaviour results, the levels of neurogenesis on day seven of pregnancy in the transgenic mice were not reduced as expected. There was no significant decrease in neurogenesis in either cre positive group compared with the controls. These findings suggest that prolactin acts through receptors on GABAergic neurons to stimulate some aspects of maternal behaviour, but other neurons must also contribute, and that prolactin acts somewhere other than forebrain neurons to induce neurogenesis.

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ACTH: adrenocorticotropic hormone

ANOVA: one-way analysis of variance

AVPV: anteroventral periventricular nucleus

BNST: bed nucleus of the stria terminalis

BrdU: bromodeoxyuridine

CaM: Calmodulin/ calcium modulated protein

CKC: CaM kinase IIa cre

CSF: cerebrospinal fluid

FSH: follicle-stimulating hormone

GABA: gamma-Aminobutyric acid

GFP: green fluorescent protein

GnRH: gonadotropin-releasing hormone

HPA axis: hypothalamo-pituitary-adrenal axis

JAK: Janus kinase

LH: luteinising hormone

LV: lateral ventricle

MPN: median preoptic nucleus

mPOA: medial preoptic area

mRNA: messenger ribonucleic acid

opt: optical tract

PBS: phosphate buffered saline

PPD: postpartum day, PPD1 being the day of parturition

PRL-R: prolactin receptor

$PRLR^{flox/flox}$: prolactin receptor flox mouse

pSTAT: phosphorylated signal transducer and activator of transcription

SEM: standard error of mean

SVZ: sub-ventricular zone

TIDA: tubero-infundibular dopaminergic (neurons)

vGAT: vesicular GABA transporter

3V: third ventricle

1.0 Literature Review

1.1 Introduction

Anxiety and depression, both during pregnancy and the post partum period, have enormous repercussions for the mother, the child, and for the bond between them. Common symptoms are difficulty concentrating, disturbances in appetite and sleep, low mood, fatigue, extreme anxiety, panic attacks, insomnia and restlessness [1]. These are commonly associated with guilt and feelings of inadequacy [2]. The majority of research has focused on the post partum period, with anxiety and/or depression during pregnancy used as a predictor of postnatal mood disturbances [3, 4]. However, another study found that depression and anxiety are more prevalent in the prenatal period than the postnatal period [5], suggesting that while risk factors are similar, prenatal mood disorders need to be considered as an affliction in their own right rather than a precursor to postpartum mood disorders. Around 20% of New Zealand mothers suffer from depression in the postpartum period [6-8], with higher rates in Maori [8]. Of these, very few are aware that the symptoms they are experiencing are classed as depression [7] and the majority of cases go untreated [6]. Less prevalent is post partum psychosis, affecting approximately one in every thousand new mothers. It presents as sudden onset of mania, depression, delusions and/or hallucinations shortly after parturition [9].

Crucial to understanding these disorders is a comprehensive knowledge of the changes that occur in the maternal brain during this time. There is substantial evidence from animal models that prolactin adapts the maternal brain to promote rapid responses to offspring, in part by decreasing maternal anxiety postpartum [10, 11]. The normal increase in prolactin early in pregnancy stimulates an increase in neurogenesis in the maternal sub-ventricular zone (SVZ) [10, 12]. These new neurons migrate to the olfactory bulb where they become incorporated as olfactory interneurons [13]. The prolactin-mediated increase in neurogenesis is essential for the normal decrease in postpartum maternal anxiety to occur. As maternal behaviour is dependent on the decrease in anxiety, the prolactin-induced increase in neurogenesis early in pregnancy is essential for normal expression of postpartum maternal behaviours [10, 12]. To generate methods to prevent and treat these disorders, it is first necessary to understand

the mechanisms by which prolactin produces these effects. In this review I will discuss prolactin, its role in maternal behaviours, and methods to explore how prolactin acts to induce maternal responses to offspring.

1.2 Prolactin

Discovered in the 1930's as a hormone stimulating milk production and named for that function, prolactin is produced in the anterior pituitary gland by the lactotroph cells. These cells store large amounts of the hormone, and there is a relatively high basal rate of spontaneous prolactin secretion by exocytosis [14]. The structure of prolactin is closely related to that of growth hormone and placental lactogen [15]; they are all single chain polypeptides that are part of the haematopoietic superfamily, which is characterised by a tertiary structure consisting of four alpha-helices (figure 1.1). Dependent on the species, these hormones also bind to the prolactin receptor and stimulate the same downstream pathways (for review see [14] or [16]).

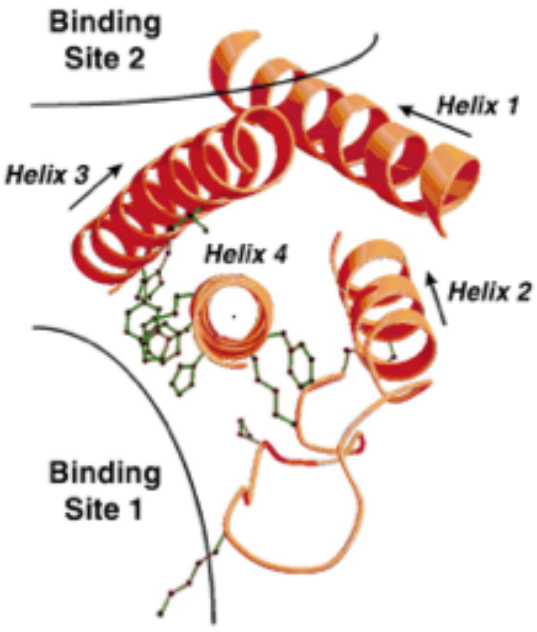

Figure 1.1 | *The 3D structure of human prolactin, containing four alpha-helices and two binding sites [17].*

Since it's discovery, many more functions have been discovered, and prolactin is now known to have over 300 biological functions [14]. The most widely studied of these, and the one this review concentrates on, is prolactin's role in the regulation of reproduction [14]. Other roles of prolactin include regulation of water and salt balance,

metabolism, growth and development, brain and behaviour, and the immune system [17, 18].

1.2.1 Functions specific to reproduction

Prolactin has a wide range of effects during pregnancy itself, as outlined in figure 1.2.

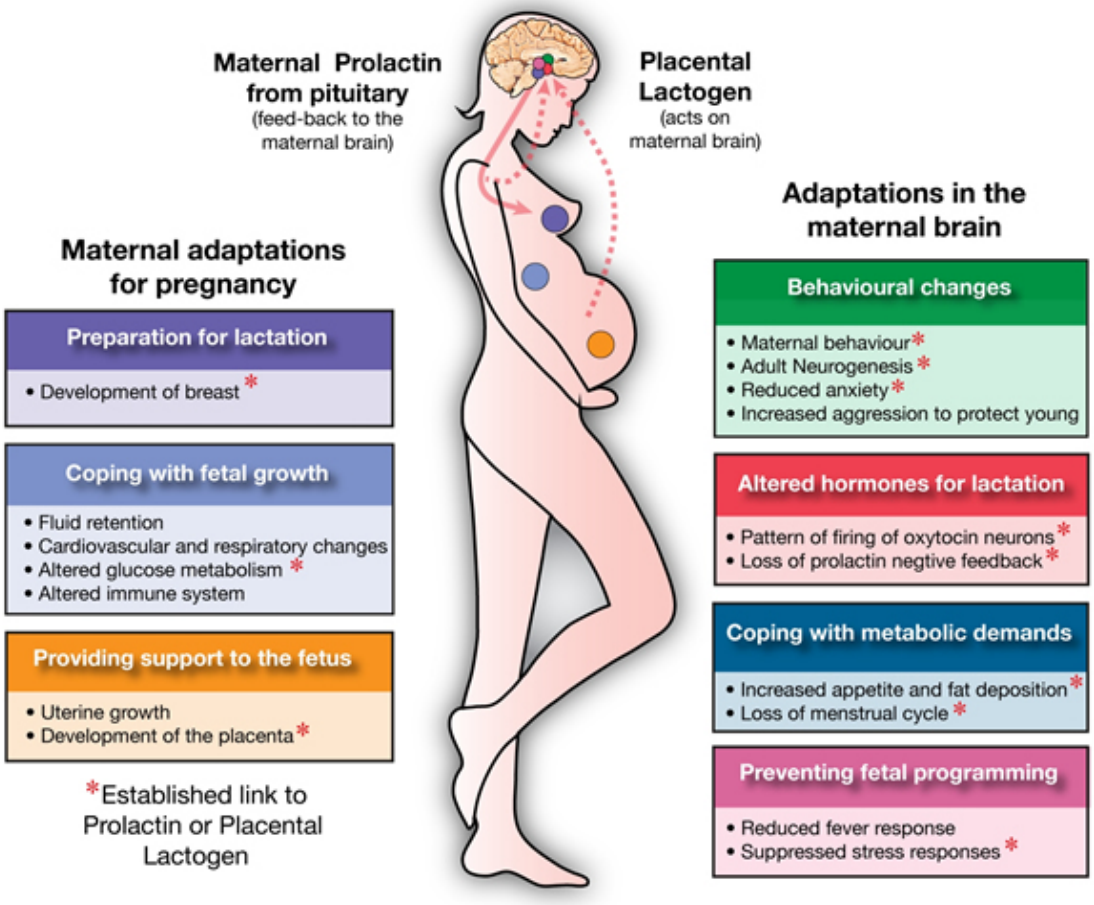

Figure 1.2 | *Adaptations that occur during pregnancy, and the roles prolactin plays. Diagram from the British Society for Neuroendocrinology website [19]*

Female prolactin receptor knock out ($PRLR^{-/-}$) mice are infertile [20, 21], despite mating more frequently than wild-type littermates. This increased frequency of mating can be explained by their failure to enter a pseudopregnant state following mating [21]. Investigation into the cause of infertility showed that prolactin receptor knock out mice ovulate fewer oocytes than the controls, and that once fertilised, the ova develop poorly. Further investigation revealed that the ova themselves are viable when implanted into a control mother; therefore the problem lies in the maintenance of pregnancy [21]. In rodents, prolactin plays a crucial role in the maintenance of the corpus luteum, and thus the continuation of the pregnancy [14]. This suggests that

prolactin action through its receptor on the ovary is directly responsible for maintenance of the corpus luteum. Prolactin is not required for the maintenance of the corpus luteum in humans, and may not be critical for maintenance of gestation [14]. Despite this, prolactin appears to play a role in the maintenance of human pregnancy, with both hyper- [22, 23] and hypo-prolactinaemia [24] being linked to miscarriage. The PRL-R has been localised to the granulosa cells in the human ovary [25] and PRL has been shown to be an anti-apoptotic factor for granulosa cells [26], indicating a potential role in the development and survival of the corpus luteum. Hyperprolactinaemia can lead to reproductive problems such as infertility and impotence [14]. During lactation, gonadotrophin-releasing hormone (GnRH) secretion is reduced, leading to a decrease in LH and FSH levels. This is believed to contribute to lactation-related infertility (for review see [27]). Although there is variation, in some species this has been linked to the inhibitory effects of prolactin [27]. Many patients with amenorrhoea have been shown to have a decrease in the frequency of GnRH surges due to elevated prolactin levels, which could be corrected by bromocriptine treatment to suppress prolactin [28, 29]. In addition, the normal increase in prolactin early in pregnancy mediates a large increase in neurogenesis in the subventricular zone (SVZ) of the maternal brain [12], which is essential for normal maternal behaviours postpartum [10]. Furthermore, prolactin is also involved during pregnancy in the suppression of fertility, the suppression of the hypothalamo-pituitary-adrenal axis (HPA) stress response, and appetite changes [30].

1.2.2 Prolactin as an anxiolytic

In late pregnancy and the postpartum period, both behavioural (dark/light box, elevated plus maze [10]) and physiological (circulating levels of ACTH, corticosterone, oxytocin [31]) tests demonstrate that anxiety and the normal stress response are significantly decreased. Prolactin has a strong inhibitory effect on the hypothalamo-pituitary-adrenal (HPA) axis [31]. As pregnancy and the postpartum period are characterised by a state of sustained hyperprolactinaemia, the increase in circulating prolactin may continuously decrease the HPA response, and thus alter perception of stressful stimuli. Virgin female mice and male mice given an intracerebral infusion of prolactin display reduced anxiety, and chronic infusion reduces the levels of

corticotropin released in response to stress [32]. Stress-induced prolactin production and release in neurons of the hypothalamus has been detected, suggesting that prolactin acts as an endogenous anxiolytic even in the non-pregnant state [33]. Down-regulation of the long form prolactin receptor had the opposite effect both behaviourally and physiologically [32]. These results strongly suggest that prolactin in specific circumstances acts as an endogenous anxiolytic, however as prolactin showed anxiolytic effects in such a short time period, it is highly unlikely that this effect is due to the effect on neurogenesis (described above). It is possible that the induction of neurogenesis and this more rapid mechanism are complementary.

1.3 Prolactin Receptors

Prolactin receptors (PRL-R) belong to the class 1 cytokine receptor superfamily and are present in most vertebrates. They are specific, membrane bound, high affinity receptors that vary greatly both between, and within, a species [17]. In mice, one long and three short isoforms have been identified [34] compared with long, short and intermediate isoforms in rats [17]. Humans have even more variation, a number of isoforms of all three types have been discovered as well as a soluble prolactin-binding protein [14]. (For review of the prolactin receptor see [17]).

Within a species, all the isoforms have an identical extracellular domain made up of about 200 amino acids, to which prolactin binds, and a single-pass chain transmembrane region [14]. The variation within a species comes from differences in length and amino acids in the intracellular domain (figure 1.3) [17]. This consists of two main parts, box one and box two. Box one is proximal to the membrane, hydrophobic and consists of eight amino acids and is the only part of the intracellular domain present in the short form PRL-R. It is rich in proline, leading to folding believed to assist recognition by transducing molecules [17, 35]. Box two is made up of hydrophobic residues with a negative charge followed by residues with a positive charge [35].

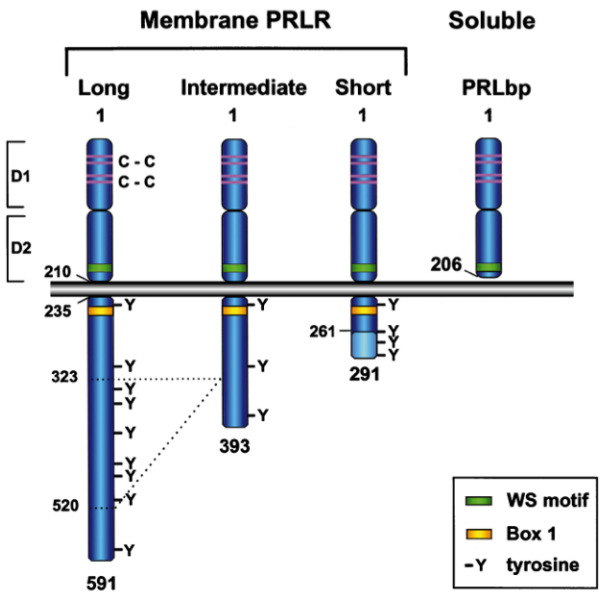

Figure 1.3 | *Various isoforms of the prolactin receptor. Box one, which is highly conserved between species, and the more variable box two are intracellular. Box two is not found in short form receptors. The extracellular domain is identical for all isoforms within a species. A soluble isoform, otherwise known as prolactin binding protein, has also been identified in humans but is less well studied. [17]*

1.3.1 Distribution of receptors in the forebrain

In many mammalian species, prolactin receptor mRNA is widely expressed throughout the forebrain. The periventricular regions, the arcuate nucleus and the ventromedial nuclei of the hypothalamus in particular have high expression of PRL-R mRNA [36, 37] and display immunoreactivity for the PRL-R [38]. The lateral septum, stria terminalis and medial amygdala have the strongest expression of PRL-R mRNA external to the hypothalamus [36]. Most of the areas where PRL-R mRNA has been detected also express pSTAT5, a marker that is induced by prolactin acting on the long form of the PRL-R [36]. The dorsolateral corner of the SVZ, where neuronal progenitors leave the SVZ to begin their migration to the olfactory bulb, has been shown to have immunoreactivity for the PRL-R in mice [12]. However, PRL-R mRNA has not been detected in this region, suggesting that the immunohistochemistry may have been non-specific [36].

Many of these regions show an increase in both PRL-R mRNA expression and PRL-R protein expression during pregnancy and lactation [37]. For example, the supraoptic nucleus, paraventricular nucleus and ventromedial nucleus of the hypothalamus display

a significant increase in both long and short form PRL-R mRNA [37], and immunoreactivity for the PRL-R protein itself [38], in lactating rats compared with diestrous controls. The increase in mRNA expression is not significant in the arcuate nucleus [37], but the increase in immunostaining of the receptor suggests a decrease in the rate of turnover of the protein [38]. There is also an increase in PRL-R immunoreactivity in the medial preoptic area lactating rats [38]. However there is no change in some brain areas, such as the parietal cortex in lactation [37, 38].

Following pregnancy and lactation, "reproductive experience", there is an increased responsiveness to offspring, even after a sustained period of no contact [39]. A small injection of prolactin evokes a much larger response in the medial preoptic area and the arcuate nucleus of reproductively experienced rats, compared with virgins [40]. This suggests that pregnancy and lactation increases the responsiveness of the brain to prolactin [40]. As prolactin action in the medial preoptic area regulates maternal behaviours [41], this evidence implies that the reproductively experienced maternal brain has been primed to respond more rapidly to future offspring by being more sensitive to increases in prolactin levels.

The choroid plexus is particularly rich in prolactin receptors and becomes even more so during pregnancy and lactation, although the function of PRL-R in this region is not yet known. Both long and short form of the PRL-R mRNA have been detected at relatively high levels in the choroid plexus of diestrous rats, with levels increasing significantly during lactation [37]. Correspondingly, immunostaining for the PRL-R in the choroid plexus in diestrous rats is strong, but even more so in lactation, to the extent where individual epithelial cells can not be identified [38]. The up-regulation of the long form PRL-R occurs by day seven of pregnancy and mRNA expression continues to increase, reaching levels about five times higher than those seen in diestrous by day seven of lactation [42]. Short form PRL-R mRNA increases significantly also, by day fourteen and reaches levels about 2.5 times those of diestrous on day seven of lactation [42]. After weaning both types of PRL-R mRNA levels decrease to levels not significantly different from diestrous [42].

1.4 Patterns and regulation of secretion

Prolactin is produced and released spontaneously, at a relatively high rate by lactotroph cells in the anterior pituitary gland [43]. Control of secretion is by a negative feedback loop, allowing prolactin to control its own release (figure 1.4). When prolactin binds to its receptor on the tubulo-infundibular dopamine neurons, located in the arcuate nucleus of the hypothalamus, it stimulates dopamine synthesis, and release from the terminals into the median eminence [44, 45]. From there, dopamine travels in the portal vessels to the anterior pituitary gland where it binds to D_2 receptors on the lactotroph cells and suppresses prolactin secretion by increasing potassium conductance [16, 45]. This leads to inactivation of voltage dependent calcium channels, in turn causing decreased intracellular calcium, which is crucial for exocytosis of prolactin [14]. In rodents, the tuberohypophysial (THDA) and periventricular (PHDA) dopamine neurons also play a role in the regulation of prolactin secretion, but this is not the case in humans [16, 45].

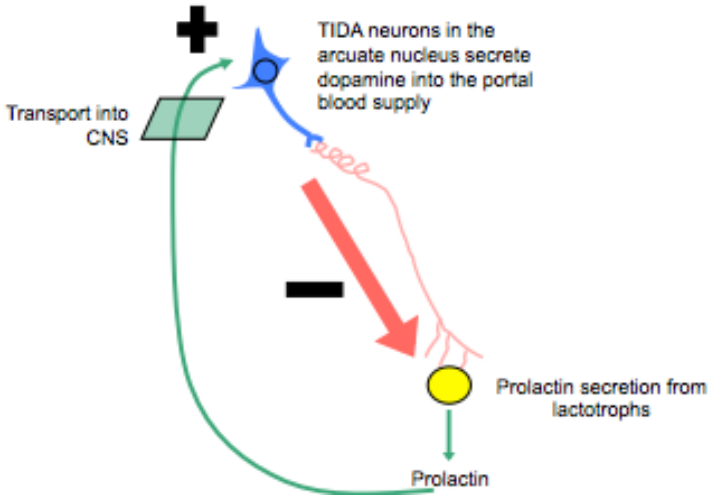

Figure 1.4 | *Prolactin secretion is regulated by a negative feedback loop. Prolactin binds to PRL-R on the TIDA neurons of the arcuate nucleus, stimulating the release of dopamine into the portal blood supply. The dopamine travels down to the anterior pituitary gland where it binds to the lactotroph cells and supresses further prolactin secretion.*

A number of prolactin- releasing factors such as epidermal growth factor, prolactin-releasing peptide and oxytocin contribute to modifying prolactin secretion. Pre-menopausal women have higher circulating levels of prolactin than men and post-menopausal women do, as estrogen promotes secretion and transcription of the prolactin gene [46]. The levels of prolactin vary during the menstrual cycle in humans,

with a slight increase around ovulation, but a much greater rise in prolactin levels occurs in rodents at the start of proestrus due to the preceding rise in estrogen [14] (figure 1.5).

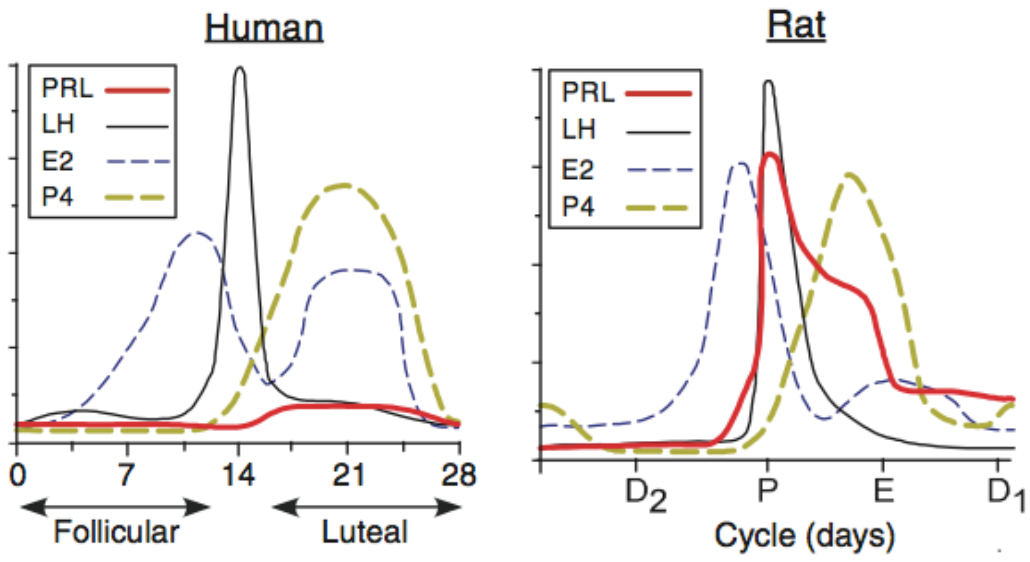

Figure 1.5 | *Graphs showing the change in prolactin levels during the menstrual cycle in humans and the estrous cycle in rats. A slight increase occurs during the luteal cycle in humans, but not nearly as dramatic as the preovulatory rise seen during proestrous in rats. The mouse cycle is divided in to diestrous (D), proestrous (P) and estrous (E) in this graph. LH = luteinizing hormone, E2=estrogen, P4 =progesterone [14].*

However, the most dramatic alteration in prolactin secretion is caused by pregnancy. In humans, at approximately six to eight weeks of pregnancy prolactin levels rise and remain elevated for the duration of pregnancy, reaching levels about ten times those seen in non-pregnant reproductively active females (200 ng/ml compared with less than 25 ng/ml)(figure 1.6)[46]. These levels will remain high for at least three months if the mother breastfeeds, averaging about 100 ng/ml [47], but if she does not breastfeed they will return to normal about seven days postpartum [48]. If menstruation resumes before six months postpartum, but the woman is still breastfeeding, levels reduce to about 70 ng/ml, which is lower than levels during pregnancy but still higher than levels in a non-pregnant woman [49].

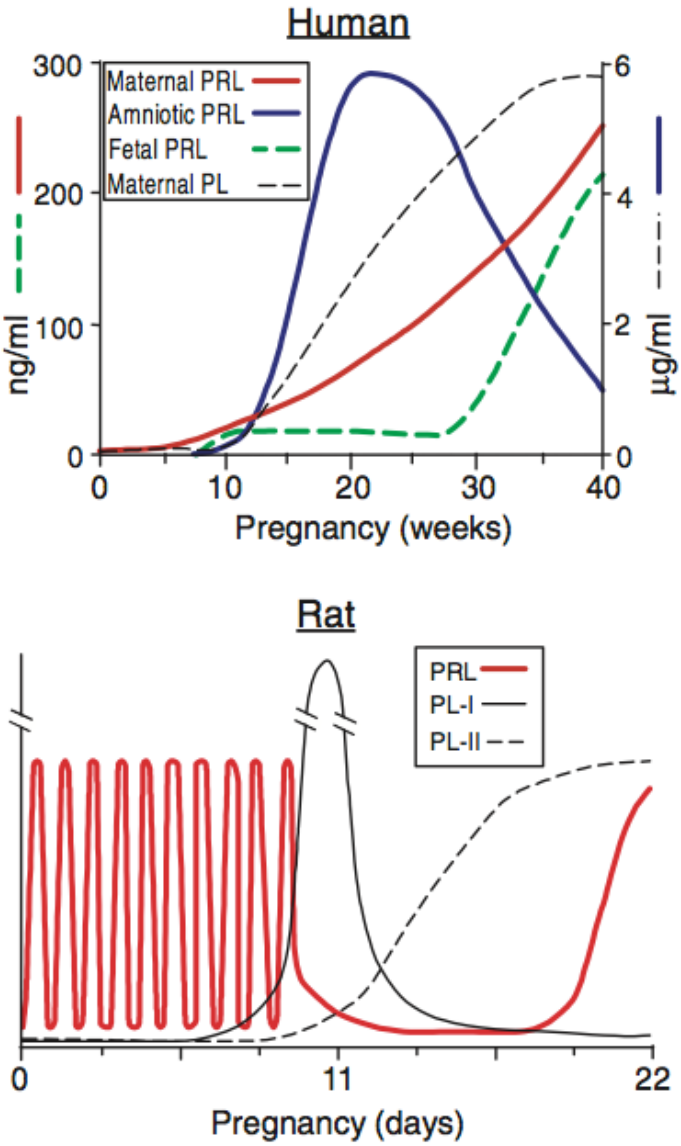

Figure 1.6 | *Graphs showing the change in levels of prolactin produced by the pituitary gland (maternal prolactin) and levels of other lactogenic hormones during pregnancy in both humans and rats. In humans maternal prolactin rises gradually from about six to eight weeks gestation, with a steep increase after thirty weeks. Rodent pregnancy begins with twice-daily surges until rising placental lactogen (PL) levels cause a decrease on about day ten. Pituitary prolactin increases greatly the day preceding parturition [14].*

Rodents also have a significant increase in prolactin levels during pregnancy. The cervical stimulation that occurs during mating triggers a dramatic twice-daily surge in prolactin levels that is essential for maintenance of the corpus luteum, as described earlier (figure 1.6 and1.7) [10, 50, 51].

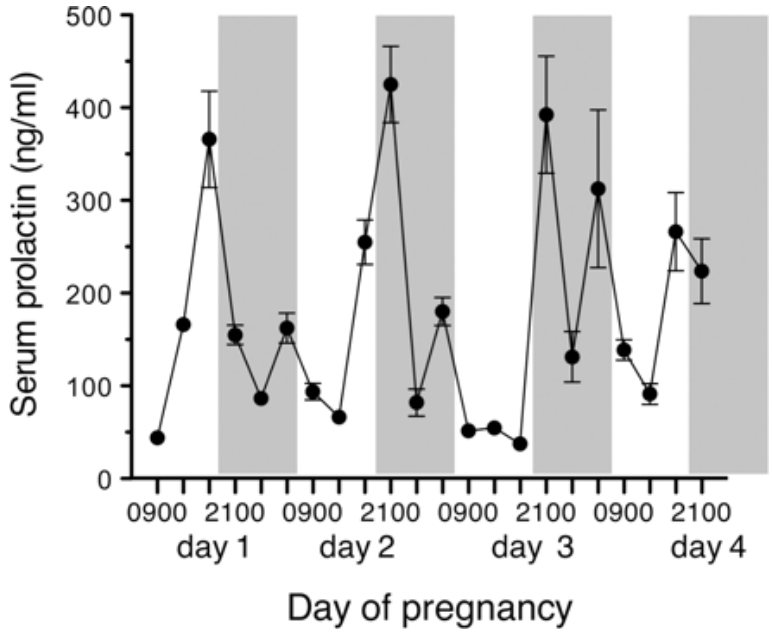

Figure 1.7 | *Prolactin secretion measured by radioimmunoassay in mice during the first four days of pregnancy. Grey bars indicate lights off, white bars indicate lights on. A large peak occurs just prior to lights off, and a smaller peak just before lights on [10].*

Lactogenic hormones produced by the placenta from day seven of pregnancy cause a decrease in prolactin levels between days seven to ten of a mouse's pregnancy, as they bind with equal affinity to the PRL-R on TIDA neurons, and thus suppress prolactin secretion [52]. Prolactin secretion increases dramatically late in pregnancy, despite the continuing secretion of placental lactogens, pointing to a desensitization of the TIDA neurons and/or loss of negative feedback [53]. Changes in the ratio of short and long forms of the receptor may be responsible for this loss of sensitivity [42]. Alternatively, as there is an increase in cytokine inducible SH2 domain-containing protein at this time, this may lead to suppression of prolactin-induced signalling [54]. The diurnal prolactin surges seen in rodents in early in pregnancy reflect its role in corpus luteum maintenance, a role it does not play in human pregnancy thus the delayed increase in PRL levels [14]. The decrease in PRL levels due to placental lactogens is another stark contrast between rodents and humans; why PRL and placental lactogens can rise concomitantly in human pregnancy is poorly understood but suggests a decrease in sensitivity of the feedback mechanisms to both PRL and placental lactogens (for review see [14]).

1.5 Transport into the brain

The route by which prolactin is transported into the brain remains unclear. It is known to be a saturable receptor-mediated process, which may occur in the choroid plexus [42, 55]. It is possible that transport is via the short form of the prolactin receptor, which has high expression in the choroid plexus. The high levels of prolactin receptor expression in the choroid plexus of diestrous rodents, and even higher levels in that of pregnant and lactating rodents [37, 42] support this hypothesis. The increase in prolactin receptors in the choroid plexus during pregnancy might underlie the increase in prolactin levels in the maternal brain during pregnancy, although recently this has been shown to be unlikely as prolactin receptor knock out mice have the same rate of transport into the brain as mice with normal prolactin receptor expression (courtesy of Dr Rosie Brown, unpublished data). If a different, as yet unknown, transporter molecule is responsible for prolactin transport, regulation of that molecule by serum prolactin levels, or prolactin binding to the PRL-R mediating changes in the pertinent transporter molecule could explain the correlation between increasing serum prolactin levels and the corresponding increase in prolactin levels in the brain. Conversely, the increase in prolactin levels in the brain during pregnancy and lactation may simply be due to the increased serum levels of prolactin, binding to receptors that are not saturated under non-pregnant conditions.

1.6 The Sub-ventricular Zone

Located in the lateral wall of the lateral ventricles, the sub-ventricular zone (SVZ) is a paired brain structure. In humans it is arranged in four layers of varying thickness and cellular arrangement; with layer one being most proximal to the ventricle [56]. Layer one consists of a single layer of ependymal cells, which regulate transport of molecules from the CSF to the brain parenchyma, and line the ventricle, although clusters of them are found within the SVZ [56].

Layer two is a gap that has very few cells, a feature that appears to be unique to the human SVZ (figure 1.8) [56, 57].

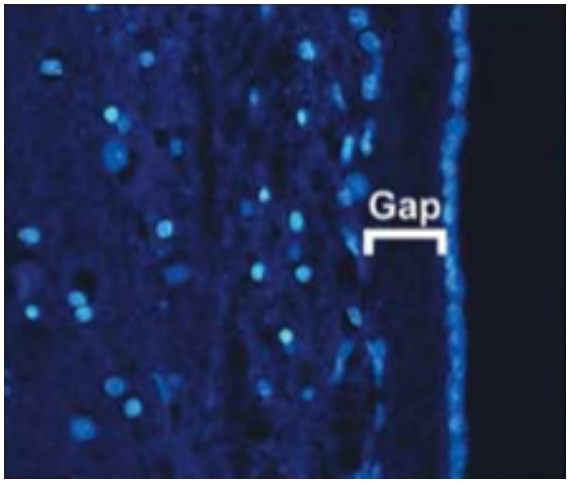

Figure 1.8 | *6 µm coronal section stained with nuclear marker DAPI to show the gap separating layer one (ependymal cells) and three (ribbon of SVZ astrocytes) in the human SVZ [57]*

The gap contains processes from astrocytes connected by gap junctions and desmosomes, some of which come from the SVZ astrocytes, however it has not yet been determined if some come from astrocytes elsewhere in the brain [56]. An occasional astrocyte cell body is also present, and they give off processes, which appear to penetrate through the first layer towards the lumen of the ventricle [57]. Few dendrites, axons and synapses are present in this hypo-cellular layer. The function of this gap is not yet known, but it may be involved in the regulation of neural stem cells [56].

Layer three is made up of a ribbon of SVZ astrocytes, the function of which is also unknown (figure 1.9). It is most prominent in the wall of the lateral ventricle's anterior

horn and in the regions of the ventricle wall facing the caudate nucleus, and in this site mitosis has been observed [56].

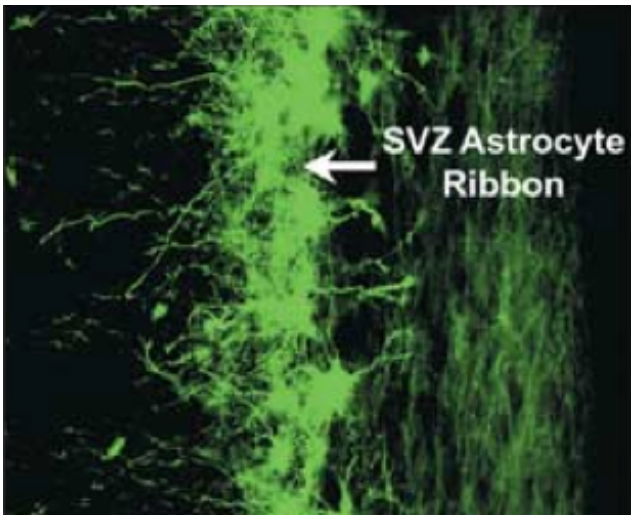

Figure 1.9 | *The SVZ astrocyte ribbon, showing stellate morphology and positively staining for GFAP (glial fibrillary acidic protein, expressed by astrocytes). Fibres penetrating the second (gap) layer are also staining positive for GFAP. [57]*

Layer four is the most lateral and is the transitional zone between the SVZ and other brain tissue, defined by the presence of myelinated axons [56].

The arrangement of the SVZ in rodents is unlike that seen in humans; neuronal precursor cells are located directly adjacent to the ependymal layer and surround chains of migrating immature neurons, there is no gap layer (figure 1.10) [58]. Astrocytes separate the chains of migrating neuroblasts from the ependymal cells and the striatum, and some penetrate the ependymal layer [59].

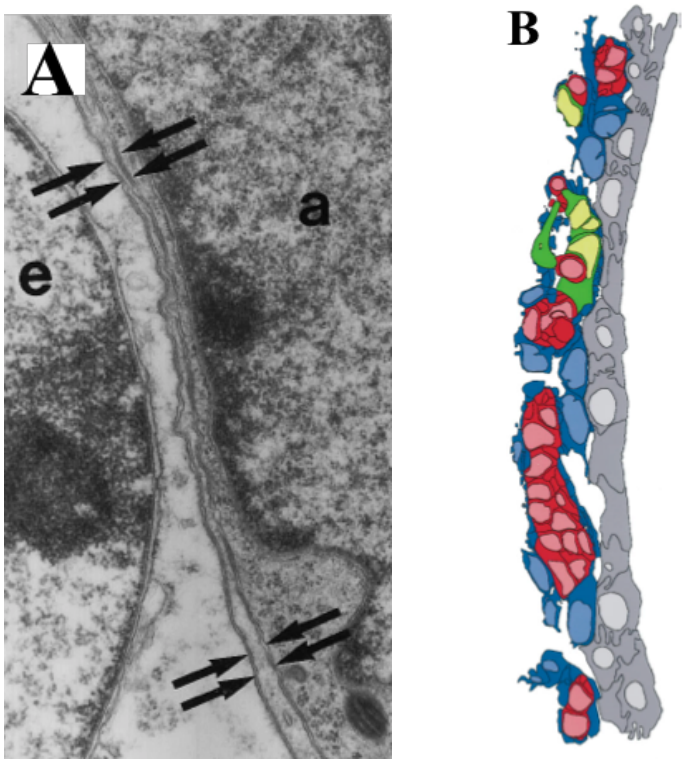

Figure 1.10 | *A) Ependymal cell (e) and migrating neuronal precursor cell (a) separated by a process from an astrocyte (arrows) in the adult mouse SVZ. B) Diagram of the cellular arrangement in the mouse SVZ. The grey cells represent the ependymal layer lining the ventricle, the red are the neuroblasts, astrocytes are the blue cells surrounding these neuroblasts and the green are thought to be pluripotent precursors that give rise to both glia and neurons [59].*

Some studies have detected no evidence of migrating chains of new neurons in the human brain, unlike in most other mammals [56, 57]. More recent evidence suggests that in humans, like rodents, adult neurogenesis occurs in the SVZ producing immature neurons destined for the olfactory bulb [60, 61].

1.7 Prolactin and Neurogenesis

During pregnancy there is an increase the formation of new neurons in the SVZ, which in rodents peaks on day seven of pregnancy (figure 1.11)[12].

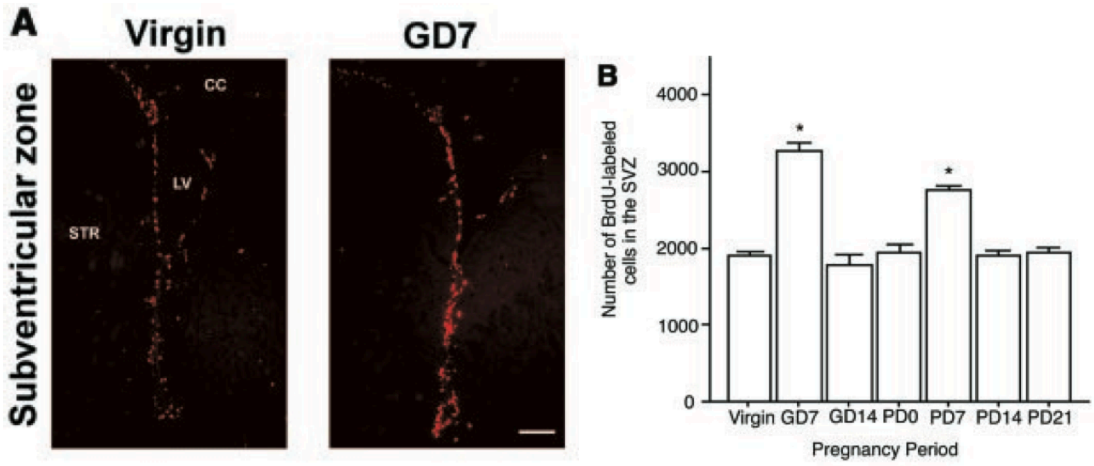

Figure 1.11| ***A)*** *Immunofluorescent staining of BrdU in the SVZ of a virgin rat and a rat on day seven of gestation, indicating that neurogenesis increases significantly during pregnancy. STR = striatum, CC = corpus callosum and LV = lateral ventricle.* ***B)*** *Quantification of the BrdU labelled cells in a virgin rat, on day seven and fourteen of gestation, the day of parturition and on days seven, fourteen and twenty-one postpartum showing that the peak in neurogenesis occurs on day seven of gestation [12].*

The new neurons are destined to migrate to the olfactory bulb and become olfactory interneurons [13], and are crucial for maternal behaviours in rodents [10], potentially because of the role they play in developing new olfactory memories [12]. The ability to form new olfactory memories is important for offspring recognition, as well as formation of the mother-offspring bond [62, 63]. There is strong evidence that the increase in neurogenesis during pregnancy is dependent on increased prolactin very early in pregnancy [10, 12]. This is possibly due to the lag between the formation of new neurons in the SVZ, and their arrival at the olfactory bulb immediately prior to parturition. The question that remains to be answered is whether prolactin is acting directly on neural progenitor cells in the SVZ, or acting indirectly; binding elsewhere and inducing a chain of events that stimulate neurogenesis.

1.8 Rodents as models

Replicating human anxiety disorders in a rodent model of anxiety and depression is challenging, and as yet there is no definitive completely objective way of mimicking the complexity of the human condition. As previously discussed, there are many significant differences between mice and humans. Patterns of prolactin secretion both in pregnant and non-pregnant females are very different. The destiny of new neurons in the SVZ is most likely different, as is the SVZ itself. The receptors and prolactin itself are different, mouse and human prolactin having approximately 61% homology [14]. However, behavioural tests, such as the elevated plus maze or the dark light box have been well validated as being able to represent some aspects of human disorders [64]. As it is not yet possible to see inside a living human brain at a cellular level, rodents remain the best available model to explore the mechanisms that underlie the regulation of the normal and pathological maternal brain.

1.8.1 Transgenic mouse models

Initially a mouse with a global knockout of the prolactin receptor was generated, offering an opportunity to study the role of prolactin in maternal behaviours, and a clear link was established [21]. However these mice are not ideal for studies of post partum maternal behaviour, or neurogenesis during pregnancy, as they are unable to carry pregnancy to term due to prolactin's crucial role in maintaining the corpus luteum. They also have disturbed estrous cycles, and a reduced rate of fertilisation [65]. Virgin homozygotes and postpartum heterozygotes were both shown to have impaired maternal behaviours [20], however, due to the prolactin receptor being knocked out from every tissue in the body, it is not known whether this was caused by a lack of prolactin action in the brain, or by some downstream consequence of the lack of prolactin action throughout the body. In order to overcome this, a gene-targeting mouse enabling PRL-R to be knocked out only in specific populations of cells was developed by our group. This was achieved by cre-lox recombination [66, 67]. The prolactin receptor flox mouse (PRL-$R^{flox/flox}$) has the GFP (green fluorescent protein) gene inserted in the reverse direction beside exon five of the PRL-R gene. LoxP sites were inserted either side of a segment of the gene containing both exon five and the newly incorporated GFP sequence. When loxP comes into contact with cre recombinase, a

inversion of the segment it flanks occurs and a stop codon is introduced, leading to a non-functional exon five and a functional GFP gene (figure 1.12). Exon five is present in all forms of the receptor and codes for part of the intramembranous domain, so when it is reversed a truncated form of the receptor with no signalling capacity is produced.

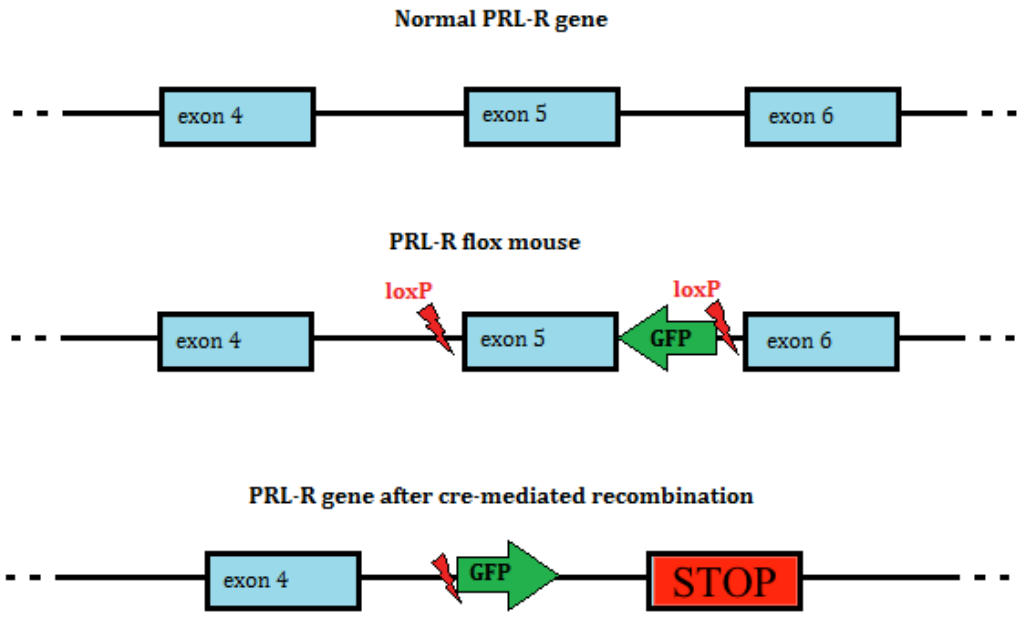

Figure 1.12 | *Diagram showing the basic concept of cre-mediated recombination. The gene for green fluorescent protein (GFP) is inserted in reverse next to exon five, and the two are flanked by loxP. When loxP comes into contact with cre, exon five becomes non functional, a stop codon is introduced and GFP is expressed.*

Immunohistochemistry for expression of GFP can then be used to show where recombination has occurred as can be seen in figure 1.13.

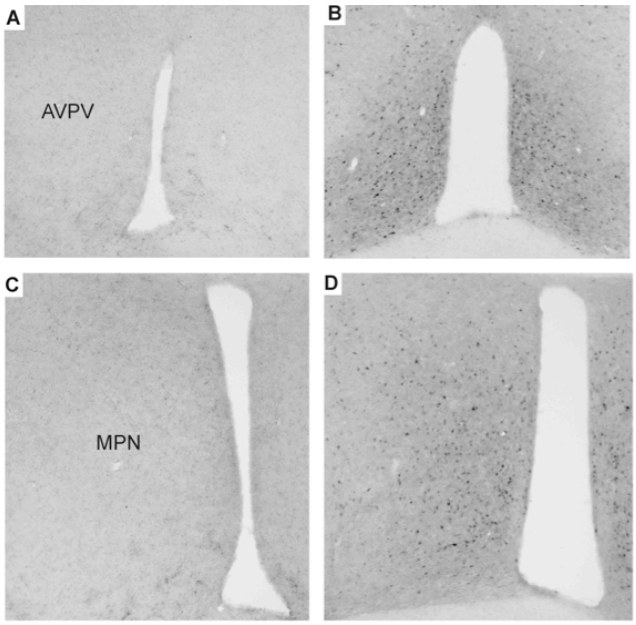

Figure 1.13 | ***A*** *and* ***C*** *show negative immunoreactivity staining for GFP in the anteroventral periventricular nucleus (AVPV) and medial preoptic nucleus (MPN) of wildtype mice.* ***B*** *and* ***D*** *show the AVPV and MPN of CKC positive mice. Many small black spots are present indicating the presence of GFP. Courtesy of Dr Rosie Brown, unpublished data.*

The PRL-R$^{flox/flox}$ mice can be crossed with mice expressing cre only in specific neuronal populations. For example, the CKC positive mouse has cre only in cells containing CaM kinase II, which is present in most forebrain neurons. Thus, when the PRL-R flox mouse is mated with the CKC mouse the cre-mediated recombination occurs only in forebrain neurons that contain CaM kinase II. When the PRL-R flox mouse is mated with the vGAT cre positive mouse, the offspring will only have the truncated receptor in neurons containing the vesicular GABA transporter (vGAT).

1.9 Summary

The current literature establishes a clear link between prolactin and SVZ neurogenesis in the maternal brain and the development of maternal behaviours. However, it is not yet known how prolactin induces these adaptations. We will use the recently developed gene-targeting mouse models with conditional knockouts of the prolactin receptor in the forebrain, to explore which neuronal populations prolactin acts on to induce maternal behaviours and stimulate neurogenesis.

2.0 Introduction

2.1 Postpartum anxiety and depression

Approximately 30% of women experience pathological anxiety and/or depression during pregnancy or after giving birth [68-70]. Symptoms may include trouble concentrating, disturbances in appetite and sleep, low mood, fatigue and extreme anxiety, panic attacks, insomnia and restlessness [1]. Women commonly feel guilty or inadequate about not being able to care for their child adequately [2]. Much less common is post partum psychosis, occurring in approximately one in every thousand new mothers. Postpartum psychosis has a sudden onset days or weeks after birth, and is characterized by mania, depression, delusions and/or hallucinations [9]. A decrease in prolactin-induced neurogenesis during pregnancy may be a contributing factor to alterations in mood postpartum; mice with suppressed prolactin in early pregnancy showed significantly increased anxiety post partum and impaired maternal behaviors compared with control mice [10, 12].

2.2 Prolactin, Neurogenesis and Anxiety

Prolactin is an endogenous anxiolytic that interacts with the hypothalamo-pituitary-adrenal axis (HPA). Rats given an intracerebral prolactin infusion have markedly decreased anxiety-like behaviour [32]. Low prolactin during early pregnancy prevents the normal increase in maternal gestational neurogenesis on day seven of pregnancy [10, 12]. When the increase in neurogenesis is supressed, independent of prolactin levels, anxiety postpartum is increased. Thus, the increase in maternal neurogenesis during pregnancy, which is dependent on prolactin, is essential for normal mood postpartum [10]. Therefore, the lag between supression of prolactin and the change in mood two to three weeks later may be due to the two to three week timecourse necessary for new neurons to migrate and become functionally incorporated into neuronal networks [13]. However, the impaired postpartum maternal behaviour does not appear to be solely caused by an increase in anxiety. When neurogenesis was supressed only for days three to seven of pregnancy, but prolactin levels remained normal, the maternal behaviours were impaired but anxiety levels were not increased [10]. This suggests that the normal increase in prolactin levels during pregnancy is

essential for normal mood postpartum independent of neurogenesis, whereas postpartum maternal behavior is critically dependent on the prolactin-induced increase in neurogenesis.

Some studies suggest full maternal behavior, is dependent on the development of new olfactory memories [12]. Neurogenesis must occur for the new memories to be made; if it is disrupted there is a decrease in the ability to discriminate between odours [71]. Olfactory interneurons originate from the neural stem cells in the sub ventricular zone (SVZ) and migrate to the olfactory bulb [13]. During pregnancy this neurogenesis is enhanced, a phenomenon that it is important for normal maternal behavior [10, 12]. The increase in neurogenesis during pregnancy is dependent on prolactin [10, 12] as both estrogen and progesterone were shown to be incapable of stimulating a similar phenomenon [12]. This strongly suggests that prolactin-induced neurogenesis during pregnancy is a prerequisite for the formation of new olfactory hormones postpartum which is essential for normal postpartum maternal behaviours.

2.3 Hypotheses and Aims

While an increase in prolactin-induced neurogenesis during pregnancy is essential for normal postpartum maternal behaviours, there is no strong evidence of high levels of the prolactin receptor's presence in the SVZ [36]. One study did detect PRL-R protein in the SVZ, in the dorsolateral corner [12], but it has not been detected during pregnancy or lactation and no mRNA has been detected [36]. Therefore, we hypothesised that prolactin regulates SVZ neurogenesis indirectly and that prolactin acts on neurons or glia located elsewhere in the brain, or cells in the choroid plexus, to indirectly stimulate SVZ neurogenesis. It is known that the neurons of the mPOA, which is a critical area for maternal behaviour development, that are active during interaction with pups are largely GABAergic [72] and that at least some of these express PRL-R [73]. Furthermore, non-responsiveness to GABA has been implicated in anxiety and depression in the postpartum period [74]. Hence, we hypothesised that prolactin action through GABAergic neurons at least partially contributes to the prolactin-stimulated induction of neurogenesis and maternal behaviour development. Alternatively, prolactin may act on cells in the choroid plexus to stimulate the release of trophic factors, which cross into the brain to regulate neurogenesis.

The aims of this project were as follows:

Aim 1: Validate the CKC and vGAT cre mice as an animal model for studying postpartum behaviour.

This was done by assessing the efficacy of the genetic recombination using GFP as an indicator of successful cre-mediated recombination and pSTAT5 as an indicator of PRL-R activation. We also looked at the estrous cycles of these mice to see if the absence of PRL-R in specific forebrain neurons would cause irregularity.

Aim 2: Test the hypothesis that a selective knockout of PRL-R in forebrain neurons would result in impaired maternal behaviours.

This was tested by carrying out behavioural tests on days two and three postpartum. We hypothesised that both the CKC positive and the vGAT cre positive mice would have impaired maternal behaviour expression in the postpartum period, reflected by poor pup survival.

Aim 3: If maternal behaviour is impaired, to begin to investigate the mechanism of this impairment, focussing on prolactin induced neurogenesis.

The lack of PRL-R in the SVZ suggests that the neurogenesis that occurs here in the maternal brain during pregnancy is induced indirectly, potentially by prolactin's action on forebrain neurons.

3.0 Methods

3.1 Animals

To test the role of prolactin in the onset and continuation of postpartum maternal behaviour, this study used a mouse model where the prolactin receptor had been made non-functional using cre-lox genetic modification of specific populations of forebrain neurons. Mice with total ablation of the prolactin receptor exist, but are not suitable for this study as homozygote mice cannot carry a pregnancy to term, and heterozygotes only have a partial knockout of the PRL-R gene. For similar reasons pharmacological methods of supressing prolactin, or pituitary gland ablation, were not suitable, as supression may be incomplete, and ablation would deprive all organs of prolactin rather than just forebrain neurons. In mice, prolactin acts on the corpus luteum in the ovary to maintain the pregnancy [21]. These experiments used CaM kinase II cre (CKC) and vesicular GABA transporter (vGAT) cre mice, which have a brain specific knockout of the PRL-R, and thus prolactin's actions on the ovary to maintain the corpus luteum can continue and successful pregnancy is possible. Therefore, the CKC positive mice should have non-functional prolactin receptors in nearly all forebrain neurons. Whereas, more specific vGAT cre positive mice only have recombination in the majority of GABAergic forebrain neurons, and thus, the prolactin receptor is non-functional in a more limited population. The controls used were cre negative mice and C57BL/6J wildtype mice.

Mice were ordered from the Taieri Resource Facility and brought into the Hercus Resource Facility for the duration of the experiment. Prior to the start of an experiment virgin females were housed in communal cages with other virgin female mice. Stud C57BL/6J males were housed in individual cages when not required for mating. Virgin females were placed in individual 32 x16 x18 cm cages at the start of the experiment. Stud males were placed in some individually-housed females' cages until mating had occurred. The stud males were then placed back into their home cages for a rest period of at least twenty-four hours. The female mice remained in their own cage for the duration of the experiment. Cages were cleaned on a fortnightly basis solely by the experimenter unless the female became pregnant, in which case they were left

undisturbed until day seven of lactation. The mice were exposed to a twelve hour light/ twelve hour dark cycle with lights on at 0600 hours. Mice had ad-libitum access to food and water. All animal work was approved by the University of Otago Ethics committee (AEC 52/10 and AEC 106/10).

3.1.1 Transgenic Mice

CKC and vGAT cre mice were mated with a PRL-R^{flox} mouse to develop the animals used in the experiments. PRL-R^{flox} mice have loxP sites inserted either side of exon five (figure 1.12). GFP is inserted into the PRL-R gene alongside exon five, but in reverse orientation. When the loxP sites come into contact with cre, exon five and GFP are inverted, leading to the introduction of a stop codon and GFP becoming functional. The result is a truncated form of the PRL-R that is non-functional, but that expresses GFP, which serves as a useful marker of successful cre-mediated recombination. For more detail see (Feil, 2009) and (Zhang, 2002). Genotyping of the mice was carried out using PCR on collected tail tips by Pene Knowles, a technician in our laboratory.

3.2 Mating and Pregnancy

All of the mated mice used in this experiment were bred with C57BL/6J stud males, regardless of genotype. After the male had been placed into a female's cage, the female mice were checked each morning for a vaginal mucous plug using a small wire loop. The presence of a mucous plug indicated that successful mating had occurred. The male was removed when a mucous plug was detected.

The day the plug was detected was considered Day 1 of pregnancy. Parturition typically occurred on day twenty of pregnancy. During pregnancy the mice were left undisturbed until sacrifice or parturition. The cages were checked for the presence of pups from day eighteen onwards, with as little disturbance as possible. Some mice were left to carry their pregnancy to term, and tested for maternal behaviours and anxiety (see sections 3.3 and 3.4 below), while others were sacrificed on day seven of pregnancy and their brains harvested to carry out immunohistochemistry (see section 3.7 below).

	C57BL/6J	Cre negative	vGAT cre positive	CKC positive
Estrous cyclicity	n = 17 virgin	n = 7 virgin	n = 12 virgin	n = 7 virgin
Brain collected for BrdU, GFP and pSTAT5 staining	n = 6 D7 pregnant	n = 4 D7 pregnant	n = 4 D7 pregnant	n = 6 D7 pregnant
Home cage maternal behaviour	n = 8 PPD 3	n = 6 PPD 3	n = 7 PPD 3	n = 6 PPD 3
Novel cage maternal behaviour	n = 6 PPD 2	n = 5 PPD 2	n = 6 PPD 3	n = 7 PPD 3
Pup survival	n = 12	n = 7	n = 6	n = 9
Anxiety Testing	n = 5 PPD4	n = 7 PPD4	n = 6 PPD4	n = 6 PPD4

Table 3.1 | *Table showing the strain of mice used in the experiments, how many of each were used and the stage of pregnancy they were at. PPD = post partum day.*

3.3 Vaginal smears

Virgin CKC x PRLR$^{flox/flox}$ positive (CKC positive) and vGAT x PRLR$^{flox/flox}$ cre positive (vGAT cre positive), and their respective cre negative mice were smeared every morning for twenty-one days to study the effect of the PRL-R's absence from specific forebrain neuronal populations on the estrous cycle. A small wire loop was dipped in water then inserted into the vagina, rotated two to three times, and then the collected cells were smeared onto a microscope slide. The slides were then air dried and subsequently stained with toluidine blue and observed under a microscope to determine the cytology, and thus, what stage of the cycle the mouse was in. The mouse

estrous cycle consists of four stages; proestrous, estrous, metestrous and diestrous (figure 3.1).

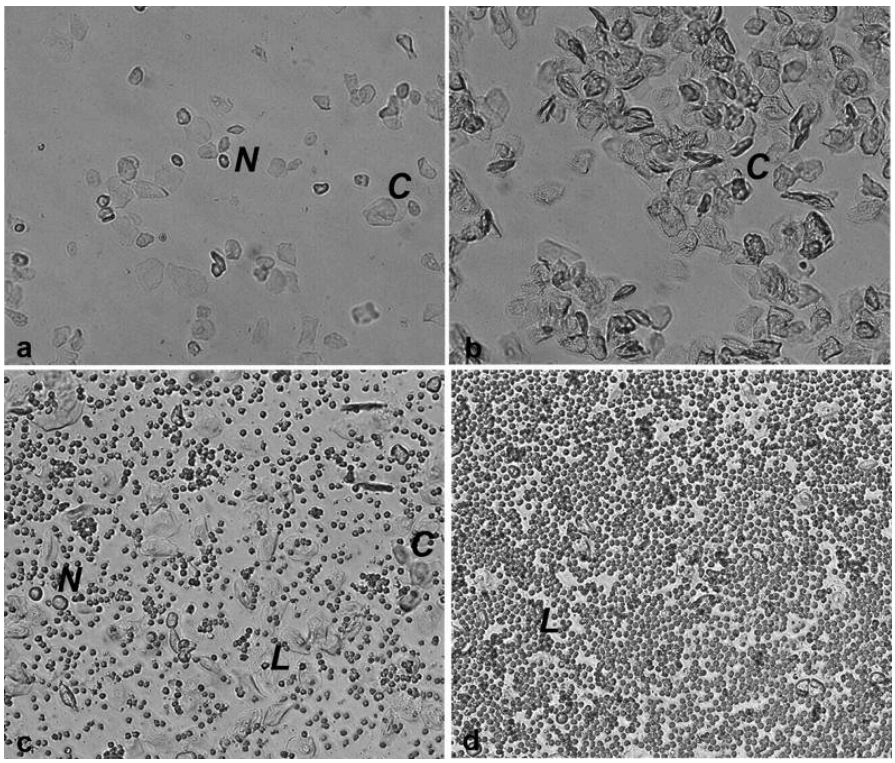

Figure 3.1 | The 4 stages of the mouse estrous cycle. Proestrous (a) where mainly nucleated epithelial cells are present, estrous (b) where mainly cornified cells are present, metestrous (c) characterised by numerous leucocytes and some nucleated and cornified cells, and diestrous (d) recognisable by almost solely having leucocytes present. [75].

3.4 Maternal behaviour assessment

Maternal behaviours were tested by observing all behaviour to pups in a setting that was familiar to the mouse i.e. the home cage, and in a setting unfamiliar to the mouse i.e. a clean novel cage. Testing was carried out both in the novel cage and in the home cage, as we have previously shown the stress of being in a clean novel cage unmasks the effect of an anxiogenic stimuli on maternal behaviour, whereas, testing in the home cage examines aspects of routine maternal behaviour in a known and familiar environment [10]. We hypothesised that an anxiogenic stress would impair maternal behaviour in the PRL-R deficient mice as prolactin has been shown to have a role in onset of maternal behaviour [76], and in supressing the stress response [32]. On day two postpartum (PPD2, the day following parturition) the mouse was placed in a clean (novel) cage, and immediately subsequent to this three pups were placed at the opposite end of the cage (figure 3.2). The mouse was then observed for forty minutes or until the

lactating position, kyphosis, was reached. Forty minutes was chosen as the cut off point, as a longer time period might be detrimental to the pups' health, and we have previously seen that non-anxious mice retrieve their pups within this time period [10]. The time taken to retrieve each pup to the nest, and to reach kyphosis, was recorded. General observations about the mouse's other behaviour, such as pup grooming and nest building, were noted. During testing, the home cage was placed away from the testing cage to minimise the risk of olfactory or auditory contact with any pups in the home cage nest. Testing was carried out between 0900 hours and 1300 hours. The pup retrieval test was repeated on day three postpartum (PPD3) in the home cage. All pups were removed from the cage immediately prior to the test, and then three pups were placed at the opposite end of the cage to the nest, and maternal behaviours observed and recorded.

Figure 3.2 | *Images of maternal behaviour testing*

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3.5 Anxiety Testing

The light-dark box is a box divided into two sections; one third is the dark portion (18 x 27 x 27 cm section constructed out of black Perspex) and two thirds is the light portion (27 x 27 x 27 cm section constructed out of clear Perspex with ventilation holes in the roof) with a small opening between the two (figure 3.3).

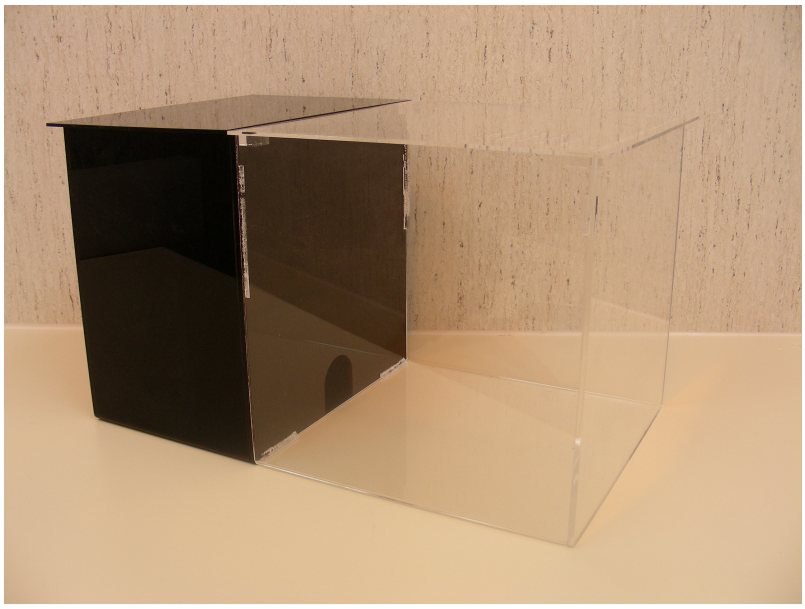


Figure 3.3 | *The light/dark box.*

The light/dark box is one of many 'classical' (developed at the same time as, or prior to, the elevated-plus maze) tests for anxiety in rodents, and these classical tests are strongly favoured by researchers, being used in around two thirds of studies [77]. Anxiolytic drugs such as diazepam increase the amount of time mice spent in the light portion [64], providing further evidence that this is an accurate way to quantify anxiety. Testing was carried out between 0900 hours and 1300 hours. Five minutes prior to each test the light-dark box was cleaned thoroughly with 95 % ethanol. For each test, an individual mouse was placed in the centre of the dark section, the lid replaced and the time spent in each section over the course of the five-minute experiment recorded. Two front paws in a section are counted as an entry, four paws out are considered an exit. The total time spent in the dark section, the number of rears and the number of entries can all be used as an indicator of anxiety [64]. Each mouse was only tested once. After the test was complete, the mouse was returned to the home cage, and any faeces or urine removed, prior to the box being cleaned with 95 % ethanol.

3.6 Tissue Collection and Preparation

At the time of sacrifice mice were injected intraperitoneally with an anaesthetic overdose of 0.15 ml of pentobarbitone 15. Once pedal and corneal reflexes were absent the mouse was transported to the fume hood. Mice were then perfused transcardially with 15 ml of 4 % paraformaldehyde (appendix A). Brains were subsequently removed and stored in 4 % paraformaldehyde at 4^{o} C for at least 24 hours. The brains were then immersed in 30 % sucrose solution (appendix B) at 4^{o} C for at least 48 hours until the brains had sunk, once they had sunk brains were frozen on dry ice and stored at - 80^{o} C until required. Day seven pregnant mice were given three injections of BrdU at two hours apart, starting at approximately 0900 hours. These mice were killed one hour after the last injection at approximately 1400 hours. Prior to perfusion, mice were checked for a successful pregnancy by an examination of the uterine horns for the presence of at least three embryos, mice with less than three embryos were not included in the experiment.

Brains were cut into 30 μm sections using a cryostat [Leica Microsystems (Germany), CM3050S] and mounted on to gelatin coated slides. The chamber temperature was set to - 20^{o} C and the outer chamber to - 19^{o} C. The brains were removed from the - 80^{o} C freezer and placed in the cryostat (still wrapped in aluminium foil) for twenty minutes to come up to – 20^{o} C. The brain stem was cut off to make a flat surface at the caudal end of the brain, then the brain was mounted on a metal plate flat surface down using Surgipath FSC 22 frozen section compound, ensuring the brain was positioned as perpendicular to the plate as possible and that the folds were symetrical. Serial 30 μm sections were collected from between bregma 1.94 mm and -2.92 mm, and cut in a series of one-in-five (ie each brain was serially divided between 5 slides). Once cut, the slides were air dried for at least thirty minutes and then stored in cryoprotectant (appendix C) at -20^{o} C.

3.7 Immunohistochemistry

3.7.1 BrdU staining

Mice were given three BrdU injections on the day of sacrifice, as described in section 3.6. BrdU is a synthetic nucleotide that is an analogue of thymidine, and is taken up by dividing cells, phosphorylated to BrdUTP and incorporated into new DNA [78]. Thus, it makes a useful marker of proliferating cells and a good way to indicate levels of neurogenesis.

As the BrdU is located in the nucleus, prior to successful labelling of the BrdU with an antibody, two antigen retrieval steps are carried out; one to break down the cell membrane and one to break down the nuclear membrane. Slides from each of the BrdU treated mice were removed from the cryoprotectant and washed three times in phosphate buffered saline solution (PBS) (appendix D), for ten minutes the first time and then for five minutes twice. We then carried out the first stage of antigen retrieval by applying approximately 200 μL of 5 μg/ml proteinase kinase K solution (appendix E) to each slide to break down the cell membranes. After twenty-five minutes at room temperature, the sections were rinsed in PBS with 0.133g/L of $CaCl_2$ for five minutes to halt the proteinase kinase K reaction. They were then put through three five-minute PBS rinses. The second step of antigen retrieval, breaking down the nuclear membrane, was carried out by applying approximately 200 μL of 4 mol HCl (appendix F) to each slide for ten minutes at room temperature. Sodium tetraborate was used to neutralise the acid and the slides were washed in it for ten minutes, followed by another three five-minute PBS washes. Approximately 400 μL of primary antibody (rat anti-BrdU, clone: BU1/75, Oxford Biotechnology Ltd, England), diluted 1:200 in PBS containing 1% bovine serum albumin (BSA), was then added to each slide and they were left to incubate at 4^{o} C for forty-eight hours. The BSA reduces background staining by increasing the specificity of the reaction [79]. Following this incubation period the slides were washed in PBS for ten minutes three more times. The secondary antibody (alexa fluor 546 goat anti-rat IgG, Life Technologies Ltd, CA, USA) was diluted 1:200, was applied in a darkened room and left to incubate in a light proof container for twenty-four hours at 4^{o} C. A few drops of Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc, CA, USA) were applied to

each slide, a cover slip applied and the edges sealed by nail polish. Slides were stored in a light proof box at 4^{o} C.

The numbers of positively staining cells in the sub ventricular zone (SVZ) were counted on one section from each brain, at x 20 magnification. The number of positively staining cells from one SVZ in five consecutive sections was counted.

3.7.2 GFP staining

GFP, or green fluorescent protein, is an indicator of successful cre-mediated recombination in the transgenic groups of mice, as explained earlier. A slide from each brain was removed from the cryoprotectant solution and washed in PBS solution for ten minutes and then twice more for five minutes each time. Approximately 400 µL of primary antibody (GFP rabbit serum polyclonal antibody, Life Technologies Ltd, CA, USA) diluted 1:20,000 in PBS with 1% BSA was then applied to each slide, and the slides were then incubated at 4^{o} C. After forty-eight hours, the slides were washed three times in PBS for ten minutes. They were then incubated in 1:200 diluted biotinylated goat anti-rabbit IgG antibody (Vector Laboratories Inc., CA, USA). Approximately 400 µL was applied to each slide, and then left for ninety minutes. This was followed by another three ten minute washes in PBS. During this time A/B vectastain elite (ABC) solution (appendix G) was prepared and mixed on the shaker for 30 minutes. After the slides had been washed, approximately 200 µL of the ABC solution was applied to each slide and left at room temperature for thirty minutes for biotinylation of the secondary antibody with the reporter enzyme horseradish peroxidase to occur. Next, three ten-minute PBS washes were carried out, following which approximately 200 µL of nickel-diaminobenzidine tetrahydrochloride (DAB) reporter dye (DAB substrate kit for peroxidase, Vector Laboratories Inc., CA, USA) was applied to each slide. This turns blue-black in the presence of horseradish peroxidase. The DAB was left on the slides for around twenty minutes, until the black dots indicating the presence of GFP were clearly visible under the light microscope in the brains of mice with genetic recombination. Slides were then washed three times in PBS for ten minutes each time, before having dibutyl phthalate xylene (DPX) mounting medium and cover slips applied.

The number of positively staining nuclei in the sections were not counted, but rather noted as being present or not present.

3.7.3 pSTAT5 Staining

This staining was carried out to investigate the levels of long form PRL-R activation, as the long form of the PRL-R predominantly signals through the JAK2/STAT5 pathway [36]. When prolactin binds to the long form of the PRL-R, a signalling pathway leading to the phosphorylation of STAT5 is initiated [17] (figure 3.4). While immunohistochemistry measures levels of pSTAT5 staining, this staining does not indicate levels of activation of the short form PRL-R, as this activates alternative pathways (figure 3.4).

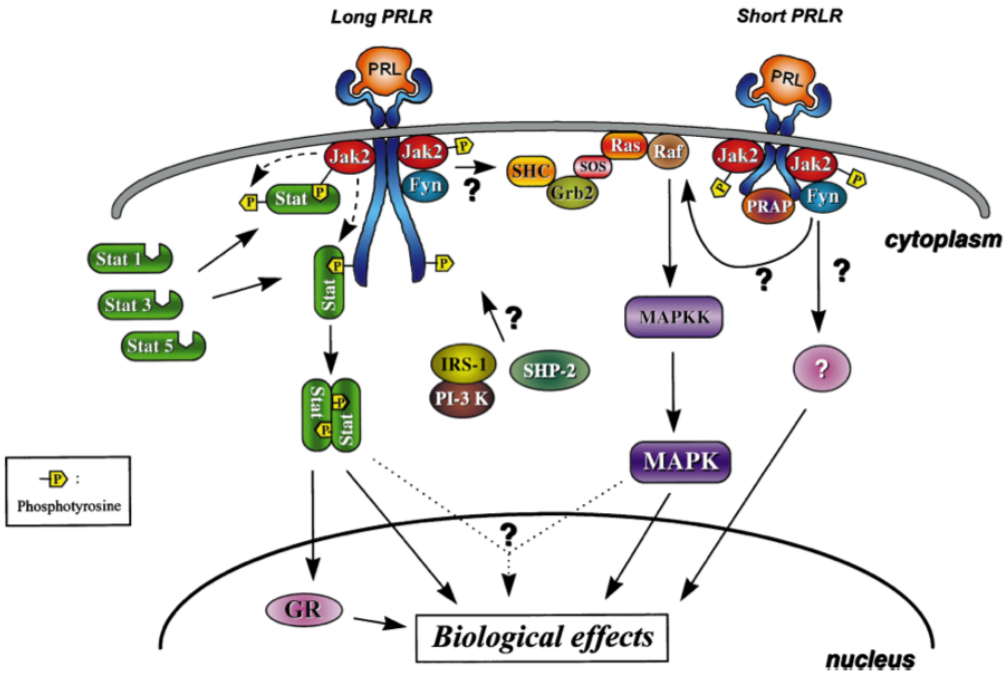


Figure 3.4 | *Diagram showing the downstream signalling pathways of the long and short forms of prolactin receptor. STAT proteins, mainly STAT5, are predominantly involved in the signalling pathway of the long form PRL-R, and are phosphorylated by JAK2 [17].*

Once again one slide of sections from each brain was removed from storage in cryoprotectant and washed in a PBS bath three times for ten minutes. Following this the slides were immersed in 0.01M Tris-HCl at 95^{o} C (appendix H) for fifteen minutes for antigen retrieval. They were then washed in incubation solution (PB-TX with BSA, appendix I) for ten minutes. This was rinsed off by three ten minute washes in PBS. Approximately 400 µL of the primary antibody, rabbit polyclonal anti-pSTAT5 [Cell

Signalling Technology (MA, USA), batch 9351L] diluted 1:400 with the incubation solution and 2 % goat serum, was applied to each slide and left to incubate at 4^{o} C for seventy-two hours. The slides were washed three more times in PBS, following which approximately 400 μL of secondary antibody was applied to each slide and left to incubate for 120 minutes. This antibody was biotinylated goat anti-rabbit IgG [Vector Laboratories Inc. (CA, USA), batch BA-1000], diluted 1:200 in the incubation solution used previously. The slides were then once again washed three times, for ten minutes each time, in PBS. Approximately 200 μL of ABC solution was applied to each slide and left to incubate for thirty minutes before being washed off by three ten minute washes in PBS. Approximately 200 μL of nickel-diaminobenzidine tetrahydrochloride (DAB) reporter dye (DAB substrate kit for peroxidase, Vector Laboratories, CA, USA) was then applied to each slide and left until blue/black staining was visible under a microscope. The slides were then coverslipped with DPX mounting medium.

After being left for a few days on the downdraft bench to allow the DPX to dry, the number of stained cells in a range of areas known to express the prolactin receptor were counted at x 10 magnification. The anteroventral periventricular nucleus (AVPV) was counted at ~bregma 0.38 mm, the bed nucleus of the stria terminalis (BNST) was counted at ~bregma 0.14 mm, the medial preoptic area (mPOA) was also counted at ~bregma 0.14 mm, the arcuate nucleus at ~bregma -1.58 mm and the medial amygdala at the same level as the arcuate.

3.8 Statistics

The data are expressed as mean +/- SEM (standard error of the mean). Data were compared by one-way ANOVA and where a significant F statistic was obtained, *post hoc* comparisons were made using Newman-Keuls multiple-comparison test (Graphpad Prism Software, La Jolle, CA). $P < 0.05$ was used as the level of significance.

4.0 Results

4.1 Validation of the animal models

GFP staining

The presence of positive staining for GFP in all of the CKC and vGAT cre positive mice, visible as black dots, confirms that cre-mediated recombination did occur, so the PRL-R in these cells is successfully rendered non-functional (figure 4.1).

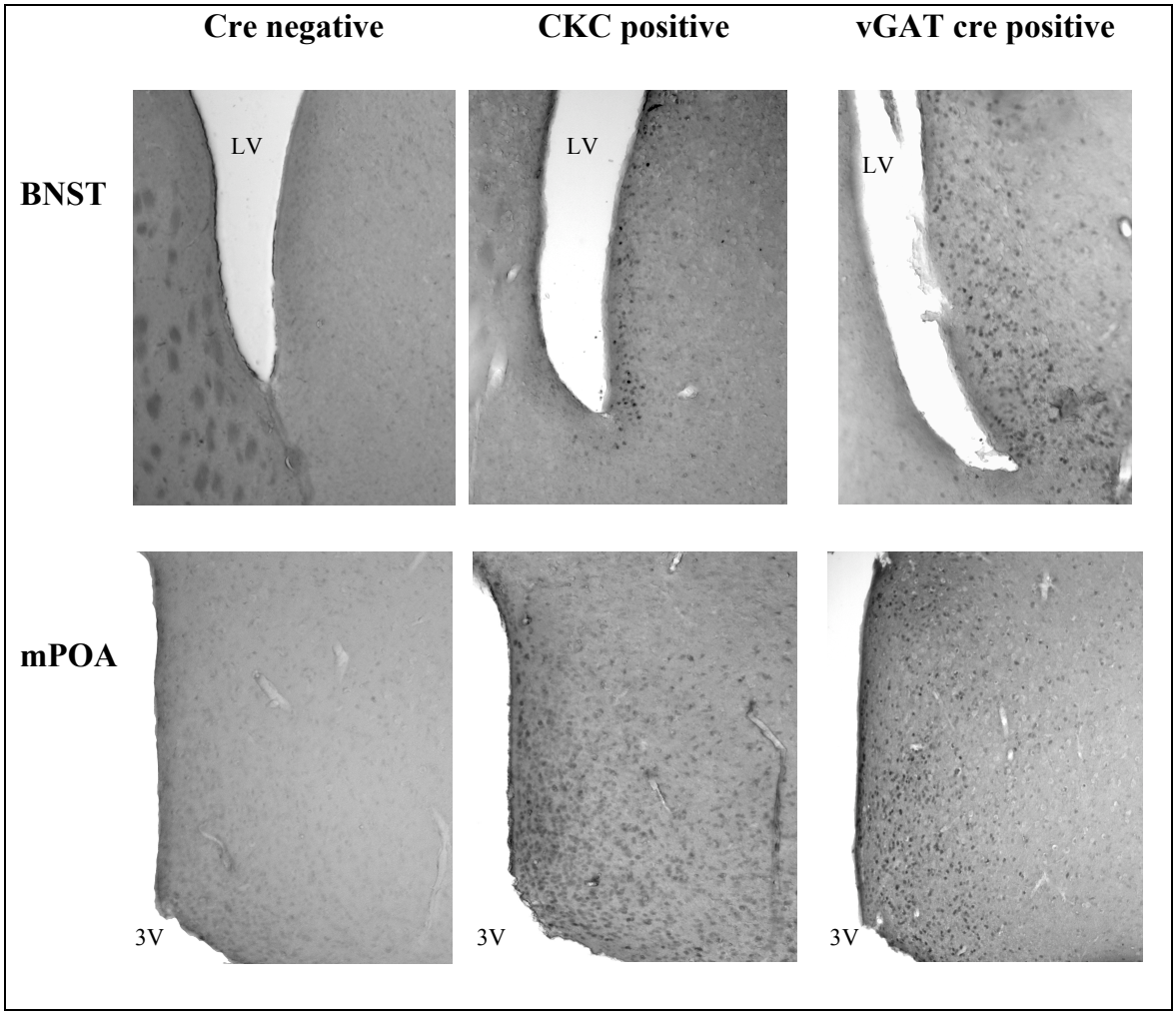


Figure 4.1 | *Representative sections of immuno-staining for GFP in the BNST and the mPOA on day seven of pregnancy. The brains collected from the CKC negative mice did not stain positively for GFP, whereas the CKC and vGAT cre positive mice had positive immuno-staining for GFP. 3V = third ventricle, LV = lateral ventricle.*

pSTAT5 staining

All mice showed evidence of staining for pSTAT5, however, the CKC and vGAT cre positive mice had significantly lower levels of staining than both cre negative and C57BL/6J controls in the AVPV and mPOA (figures 4.2 and 4.4, and for representative images see figures 4.3 and 4.5). In the arcuate, the cre negative mice also showed a trend to lower levels of pSTAT5. The lower level of positive staining for pSTAT5 in the cre positive mice offers further evidence that the PRL-R is non-functional, as the receptor is not being activated to the same extent despite elevated serum prolactin levels.

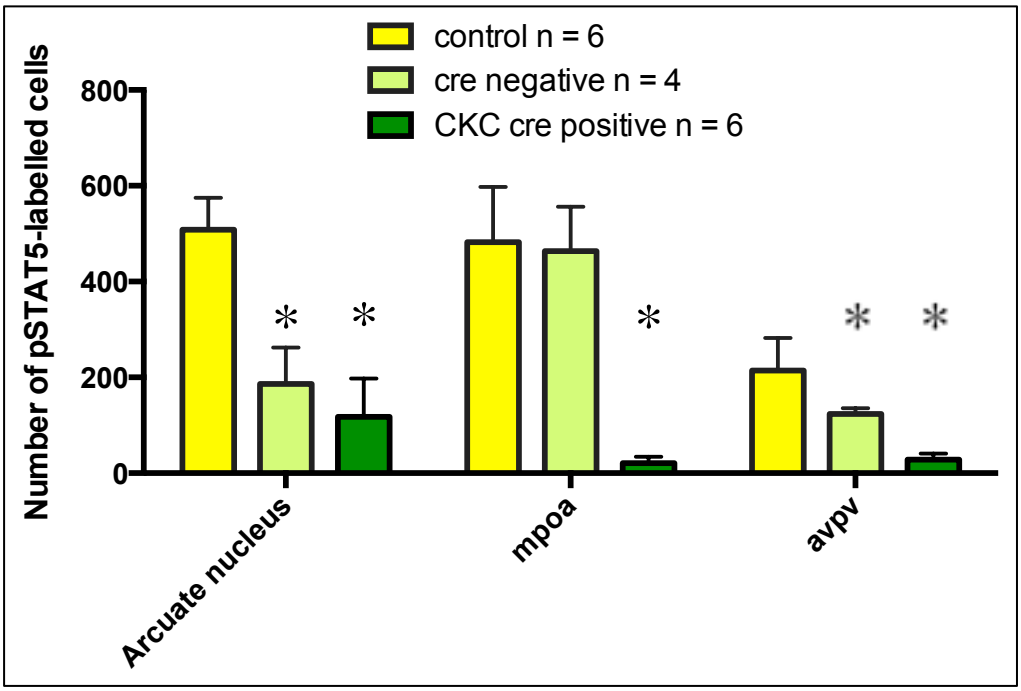
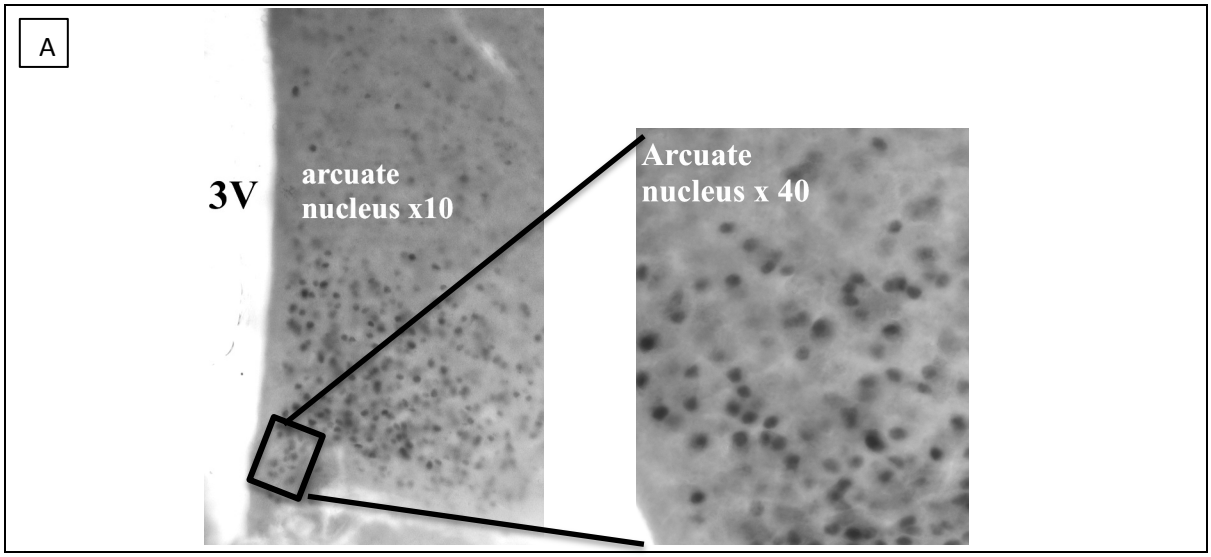


Figure 4.2 | *Numbers of cells that positively stained for pSTAT5 in the arcuate nucleus, AVPV and mPOA, counted at x 10 magnification. There was significantly more positive staining for pSTAT5 in the arcuate nucleus, the MPOA and AVPV of the CB57BL/6J mice than the CKC positive mice on day seven of pregnancy. * indicates statistically significant results ($p < 0.05$).*



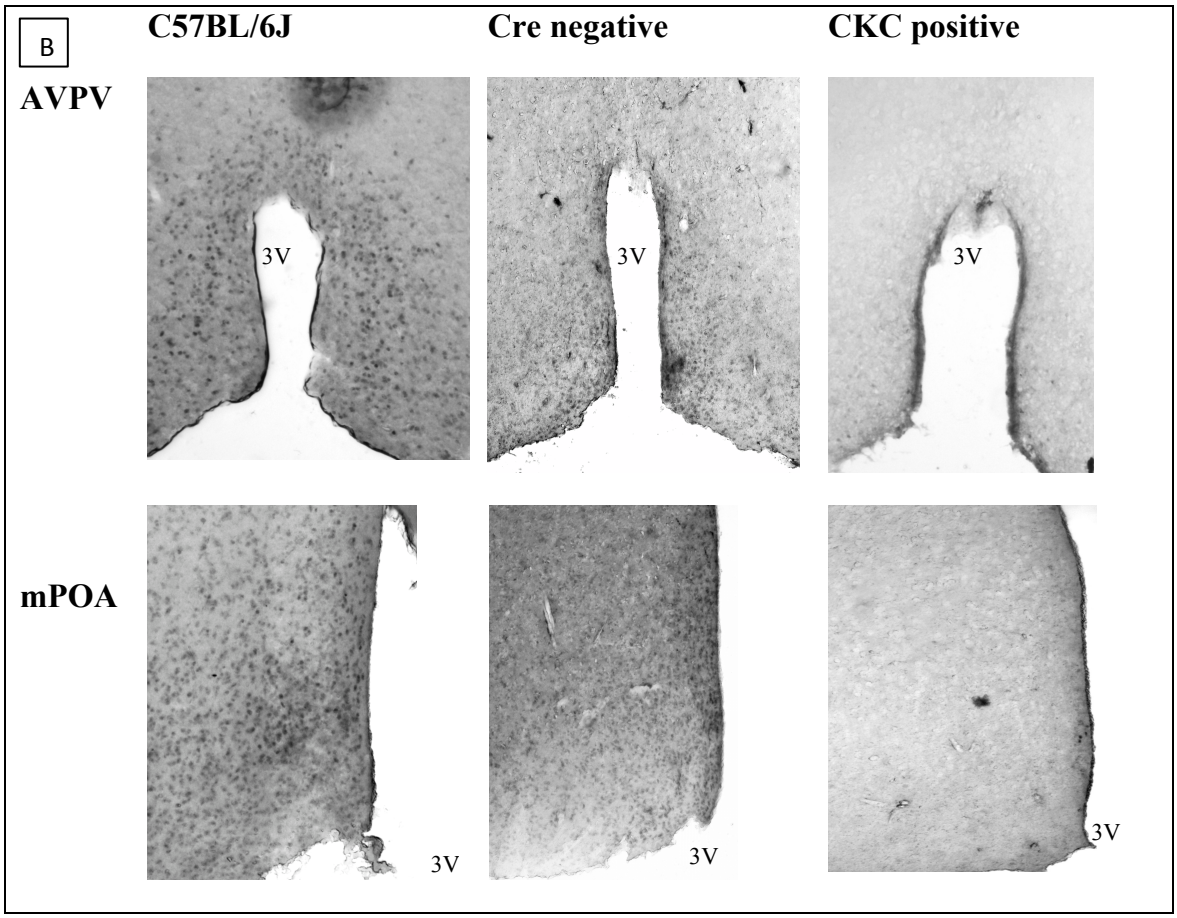


Figure 4.3 | ***A)*** *The arcuate nucleus of a C57BL/6J mouse magnified to x 40 to demonstrate that the staining for pSTAT5 is nuclear.* ***B)*** *Images taken at x10 magnification of the arcuate nucleus, AVPV and the mPOA in CKC positive, cre negative and C57BL/6J mice. Black dots are cells that have been labelled for pSTAT5. All three groups were sacrificed on day seven of pregnancy. The CKC positive mice show very little positive staining for pSTAT5 compared with the other two groups, indicating decreased PRL-R activation. 3V = third ventricle.*

The amount of positive staining present in the vGAT cre positive mice was extremely low (figure 4.4). Unfortunately this was most likely due to sub-optimal staining and although we are confident that the decrease in pSTAT5 was somewhat due to the loss of PRL-R from the GABAergic neurons, a repeat of this staining would be necessary for us to be confident in our quantification.

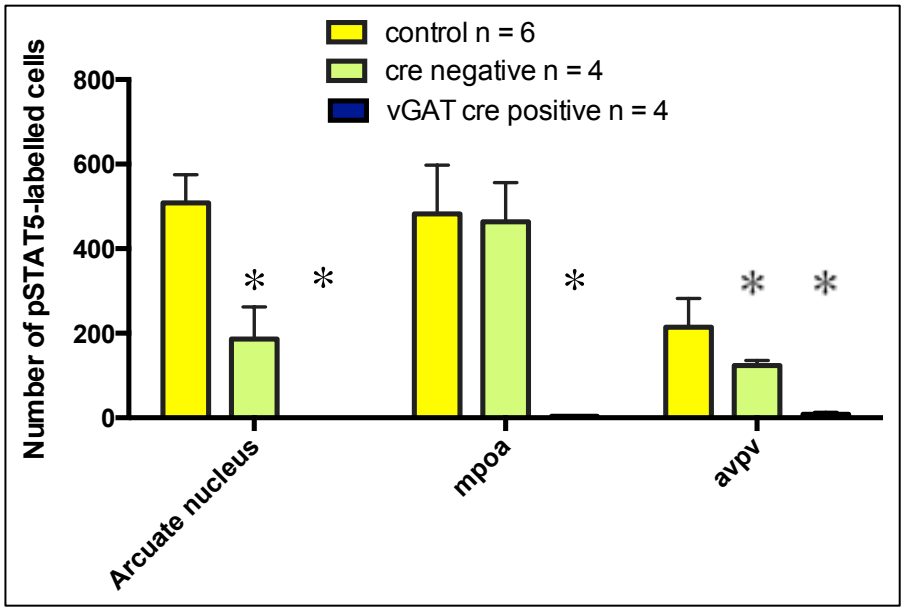


Figure 4.4| *Numbers of cells that positively stained for pSTAT5 in the arcuate nucleus, AVPV, and mPOA counted at x 10 magnification. There was significantly more positive staining for pSTAT5 in the forebrain of the day seven pregnant CB57BL/6J mice than the day seven pregnant vGAT cre positive mice. * indicates statistically significant results ($p < 0.05$).*

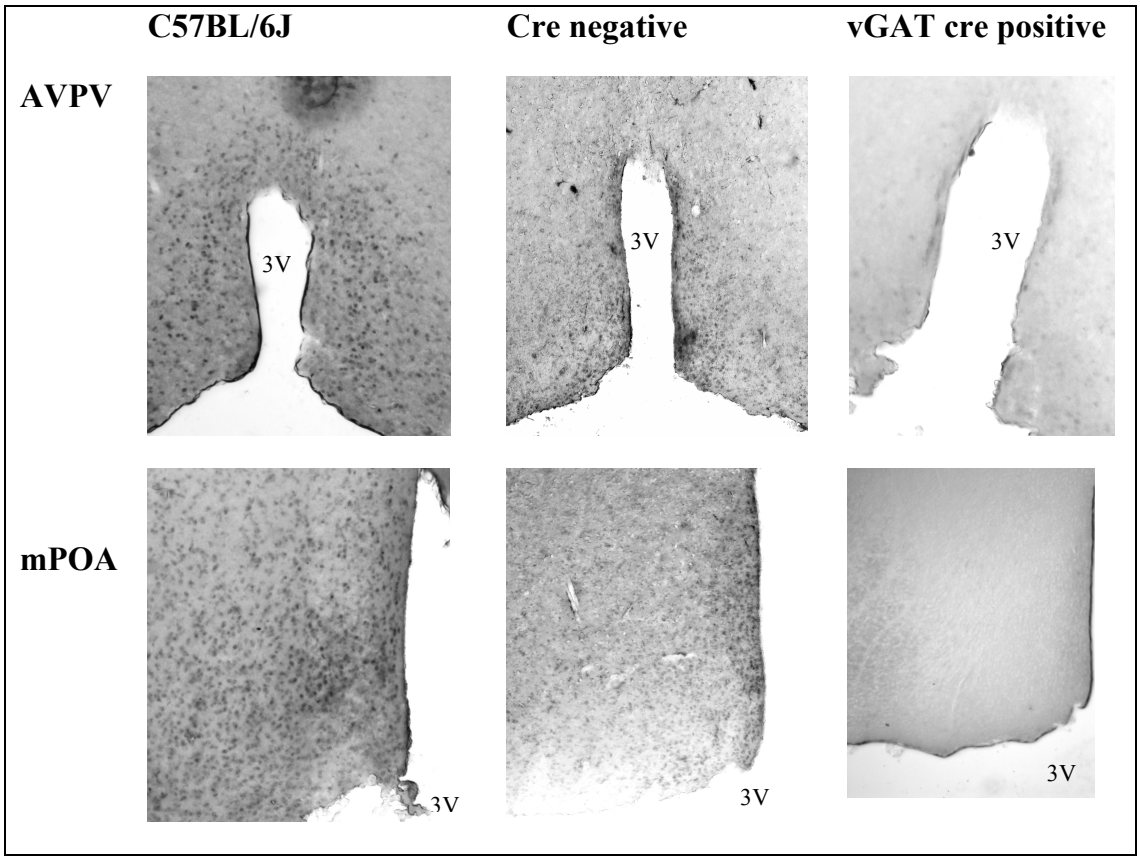


Figure 4.5 | *Images taken of pSTAT5 staining at x10 magnification of the anteroventral periventricular nucleus (AVPV) and the medial preoptic area (mPOA) in vGAT cre positive, cre negative and C57BL/6J mice. All three groups were sacrificed on day seven of pregnancy. The vGAT cre positive mice show very little positive staining for pSTAT5 compared with the other two groups, indicating decreased PRL-R activation.*

Estrous cyclicity

Estrous cycles were investigated in virgin mice by analysing vaginal smears for twenty-one days to determine any effect of the absence of functional prolactin receptors in forebrain neurons.

There were no significant differences between the CKC negative mice and the C57BL/6J control mice, with a full cycle length from proestrous through to diestrous of approximately seven days in both groups, and with less than half of each cycle spent in diestrous (figure 4.6).

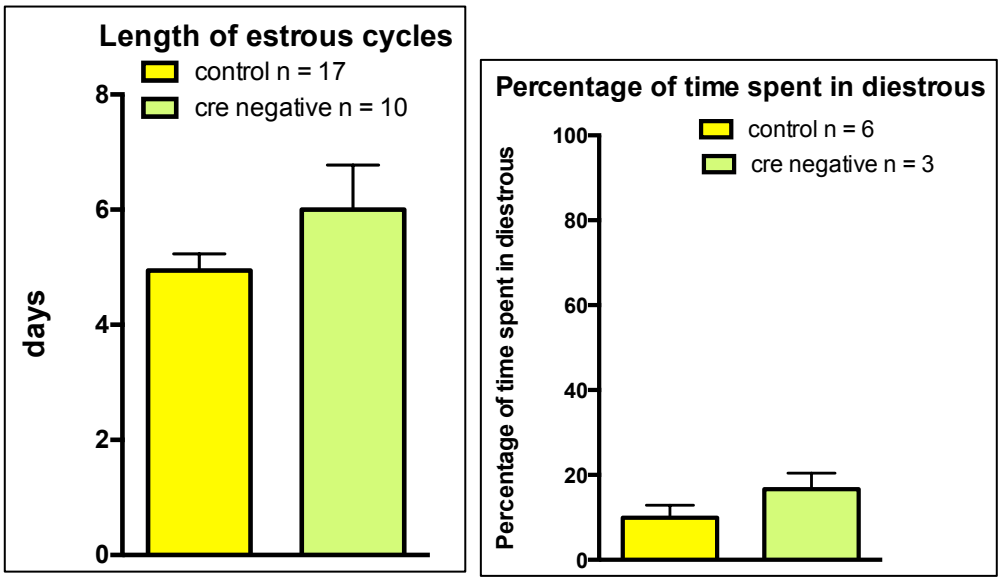


Figure 4.6 | *The length of virgin C57BL/6J and cre negative mice's estrous cycles and the percentage of time spent in diestrus are similar.*

By comparison, the CKC positive mice had significantly longer estrous cycles than those seen in the CKC negative animals, taking more than twice as long to complete a full cycle (figure 4.7). The CKC positive mice also spent a larger percentage of each cycle in diestrous than the CKC negative mice (figure 4.7). However, there were no significant differences between the C57BL/6J, CKC negative and CKC positive mice in the time to get pregnant (within 4 days of being mated), the duration of pregnancy (twenty days), or the number of pups born (between six to nine).

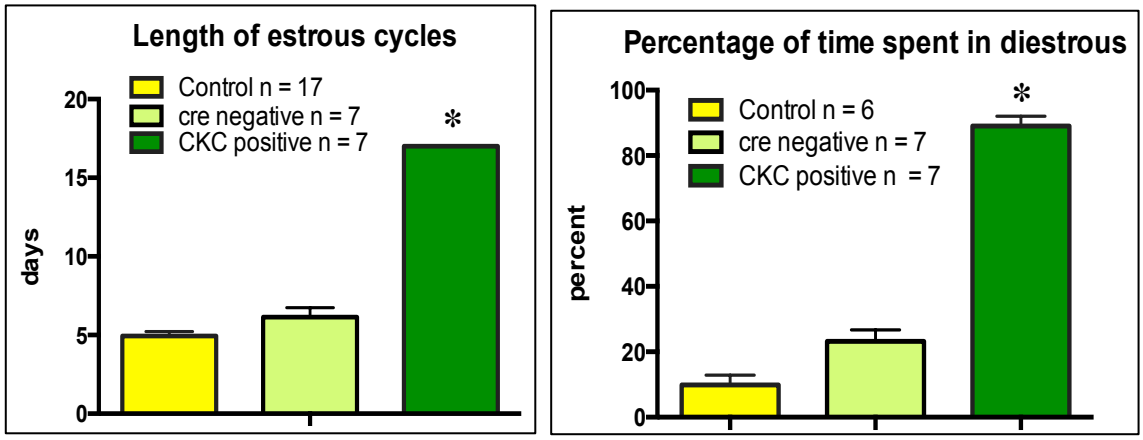


Figure 4.7| *Estrous cycles of CKC positive mice compared with cre negative mice. CKC positive mice have significantly longer cycles than CKC negative controls. CKC positive mice spend a significantly larger percentage of the cycle in diestrus than the CKC negative mice. * indicates statistically significant results ($p < 0.05$).*

There was no significant difference in the length of the estrous cycle, or in the percentage of time spent in diestrous, between C5BL/6J controls, vGAT cre negative mice, and vGAT cre positive mice (figure 4.8). There was also no difference in the time taken to get pregnant (approximately four days), the length of pregnancy (20 days), or the litter size (six to nine pups) between the three groups.

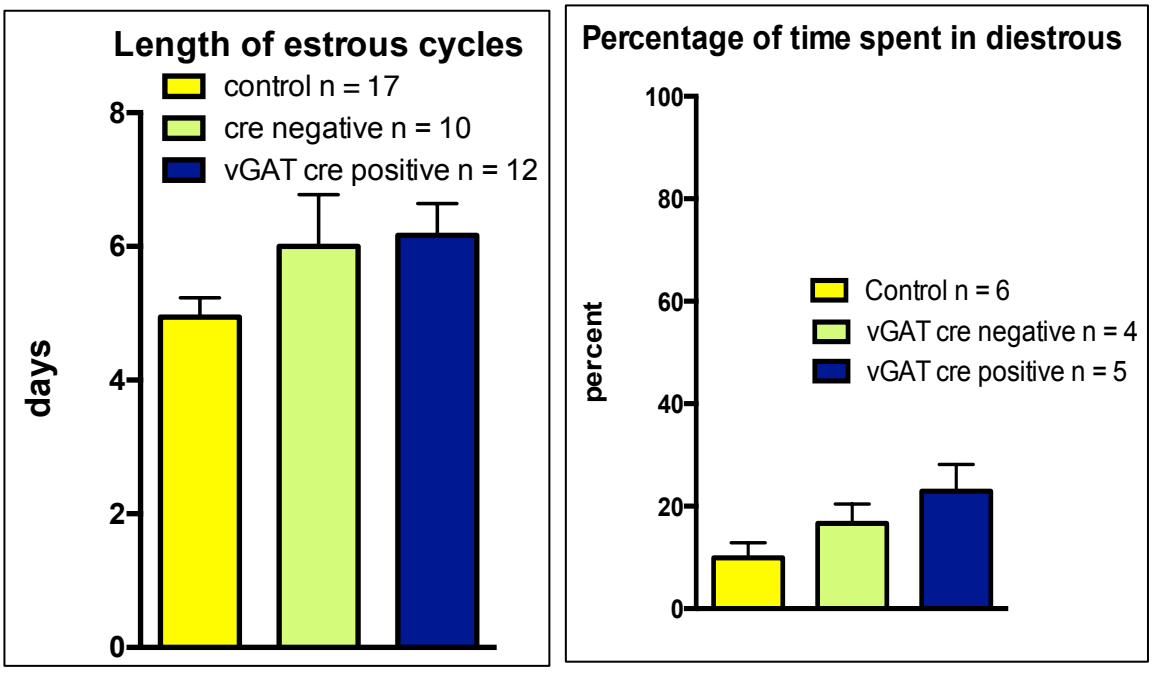


Figure 4.8 | *Estrous cycles of virgin C57BL/6J, vGAT cre negative and vGAT cre positive mice. There is no significant difference, in either length of cycle, or percentage of the cycle spent in diestrous, between any of the groups of mice.*

4.2 Maternal behaviour

Maternal behaviour was assessed on post partum day two in a clean (novel) cage, and in the home cage on postpartum day three. Mice were observed for forty minutes and the time taken to retrieve each pup, and subsequently reach kyphosis, was observed.

In the home cage, there were no significant differences in maternal behaviour between C57BL/6J control mice and CKC negative mice (figure 4.9). Both the C57BL/6J control mice, and the CKC negative control mice, retrieved their pups to the nest and reached kyphosis within a short period of time. These mice also displayed other maternal behaviours such as nest building and grooming during the observation period. By contrast, most of the CKC positive mice retrieved none or only one of the pups, and displayed very little nest building behaviour, or interest in grooming pups. All of the CKC positive mice took significantly longer to retrieve pups than both control groups (figure 4.9).

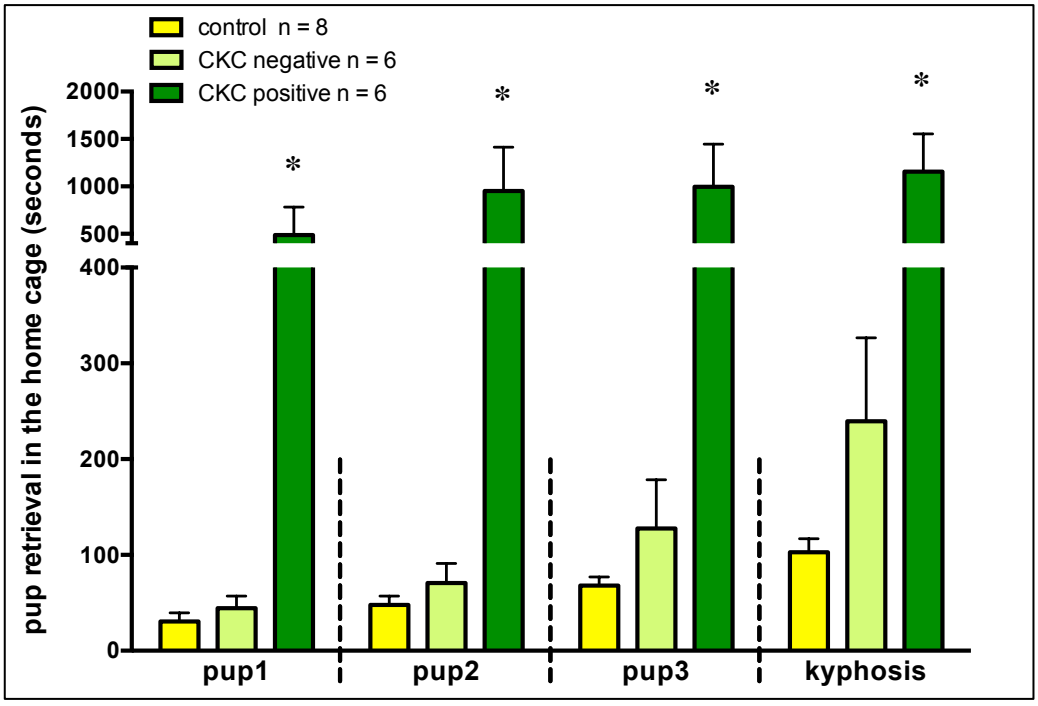


Figure 4.9 | *Graph showing the difference in time taken (in seconds) to retrieve each pup and reach kyphosis in the home cage on postpartum day three. The CKC positive mice took significantly longer to retrieve pups than both C57BL/6J controls and CKC negative mice. * indicates statistically significant results ($p < 0.05$).*

All mice took significantly longer to retrieve pups in the novel cage, compared with the time taken to retrieve pups in the home cage, and many did not retrieve pups in the

forty minute period. Hence, it was not possible to analyse and clearly present the retrieval times (as in figure 4.9). However, analysis of the total number of pups retrieved during the forty minute observation period shows that the CKC positive mice still displayed a significant impairment in maternal behaviour, compared with both cre negative and C57BL/6J mice (figure 4.10).

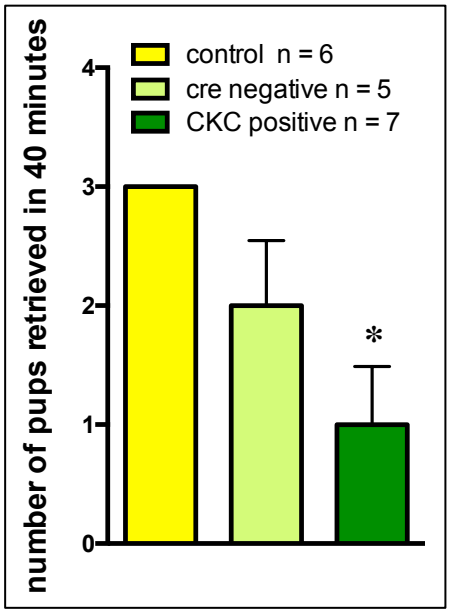


Figure 4.10 | *Postpartum day 2 CKC positive mice retrieved significantly fewer pups than both the B6 controls and the cre negative mice in the forty minute time period in the novel cage than the C57BL/6J controls and the CKC negative mice. * indicates statistically significant results ($p < 0.05$).*

In the home cage, there were no significant differences in maternal behaviour between C57BL/6J control mice and vGAT cre negative mice (figure 4.11). There was also no significant difference in the time taken to retrieve pups between any of the groups. However, the vGAT cre positive mice took significantly longer to reach kyphosis, compared with both the C57BL/6J control mice and the vGAT cre negative mice (figure 4.11). The vGAT cre positive mothers appeared to be very interested in the pups, grooming them often, and also displaying nest building behaviour early in the observation period, similar to the control mothers. However, despite bringing the pups to the nest, the vGAT cre positive mothers rarely settled down in the nest with the pups.

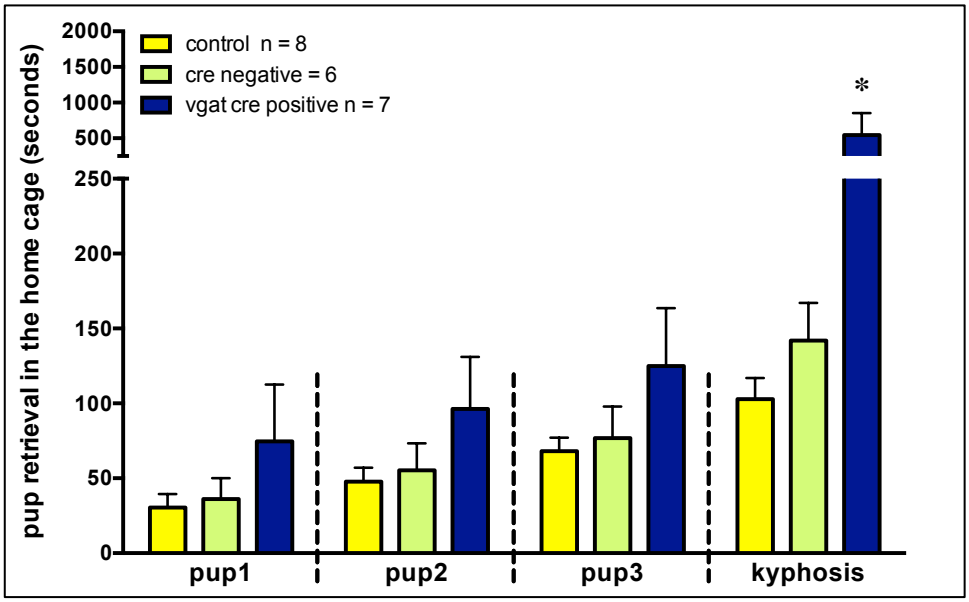


Figure 4.11 | *Time taken for C57BL/6J controls, vGAT cre negative, and vGAT cre positive mice to retrieve three pups and get into kyphosis in the home cage on postpartum day three. vGAT cre positive mice took significantly longer to reach kyphosis than control mice. * indicates statistically significant results ($p < 0.05$).*

Once again, all mice took significantly longer to retrieve pups in the novel cage meaning analysis of the total number of pups retreived during the observation period was the best way to display these results. There was no difference between the controls and the vGAT cre positive mice in the amount of pups retrieved in forty minutes (figure 4.12).

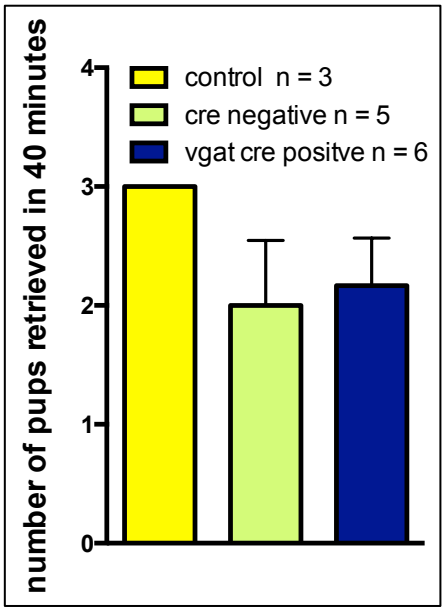


Figure 4.12 | *There was no significant difference in the number of pups retrieved in the novel cage on postpartum day two in forty minutes in the novel cage.*

4.3 Pup survival

The number of live pups was recorded on the first four days after parturition, postpartum day one being the day of parturition, and at weaning on postpartum day 21. The majority of C57BL/6J control and CKC negative pups survived to weaning (figure 4.13). In contrast, very few pups of CKC positive mothers survived to weaning, with few pups alive by day three postpartum (figure 4.13). CKC positive dams often ate the pups, although it is not possible to say whether this was before or after the pups died.

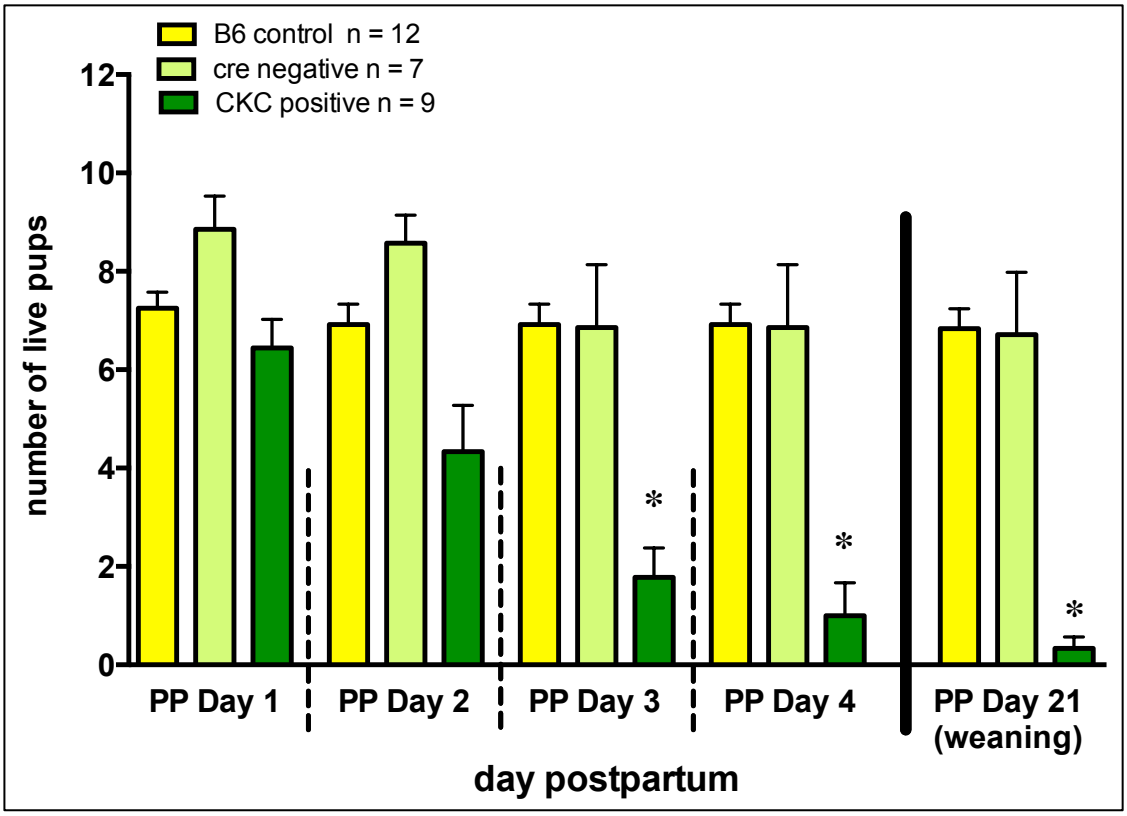


Figure 4.13 | *Survival rates of pups of C57BL/6J controls, CKC negative, and CKC positive dams. There was no difference in the pup survival rate between the C57BL/6J control dams and the CKC negative dams. The CKC positive dams had a significantly lower pup survival rate compared with both the C57BL/6J and the CKC cre negative dams. * indicates statistically significant results ($p < 0.05$).*

There was no significant difference in pup survival rates between C57BL/6J control mice, vGAT cre negative mice, and vGAT cre positive mice. However, there was a trend for a lower survival rate in the vGAT cre positive mice (figure 4.14). Reduced survival of the pups did not appear to be due to aggression from the mother, as the dead pups were still generally present in the cage and did not usually have any signs of injury.

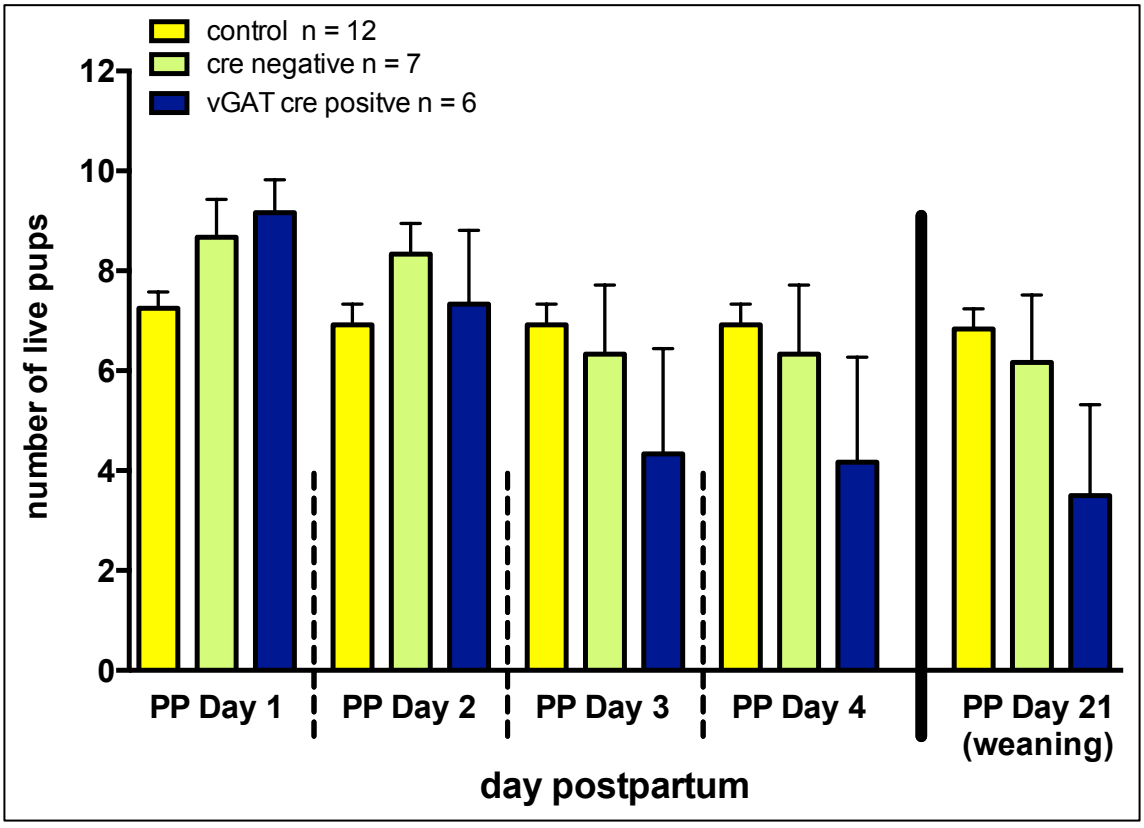


Figure 4.14 | *The number of pups still alive on postpartum days one to four and at weaning on day twenty-one for C57BL/6J controls, vGAT cre negative, and vGAT cre positive mothers. Although there is not a significant difference, the offspring of vGAT cre positive mice appear to have a poorer chance of survival. * indicates statistically significant results ($p < 0.05$).*

4.4 Anxiety levels

There were no significant differences in anxiety levels between the mice when assessed using the light/dark box (figure 4.15).

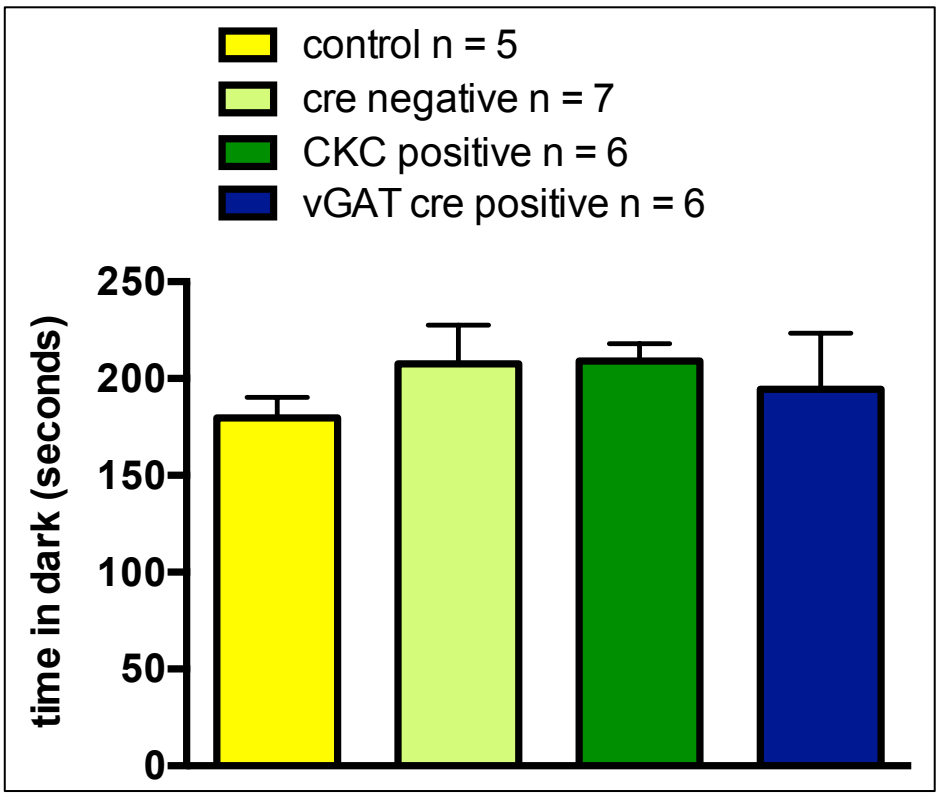


Figure 4.15| *Testing using the light/dark box indicates that there is no significant difference in anxiety between C57BL/6J control, cre negative, CKC positive and vGAT cre positive mice on day four postpartum. * indicates statistically significant results ($p < 0.05$).*

4.5 Neurogenesis

BrdU was injected on day seven of pregnancy. BrdU is incorporated into the DNA of newly dividing cells, and as such, is an indicator of levels of neurogenesis. There was no difference in levels of BrdU-labelled cells between the day seven pregnant C57BL/6J control mice, the day seven pregnant cre negative mice and the day seven pregnant CKC and vGAT cre positive mice (figure 4.16 and 4.17).

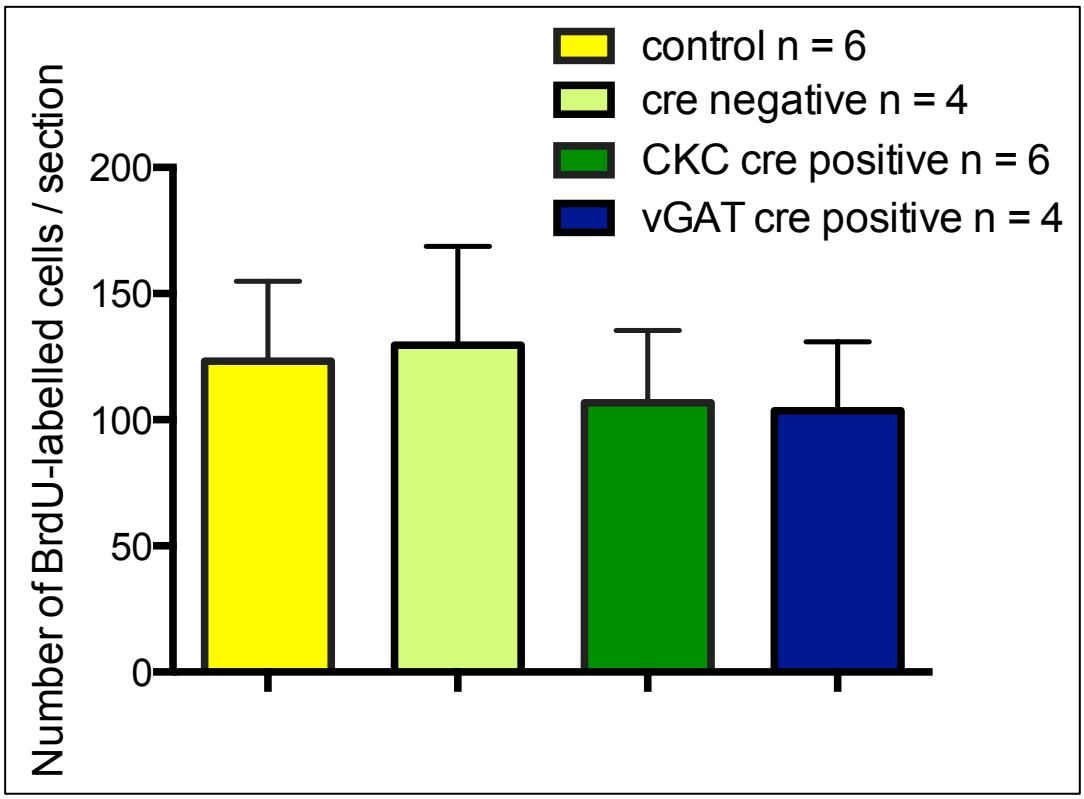


Figure 4.16 | *There is no difference in the number of BrdU-labelled cells in the SVZ of C57BL/6J control mice, cre negative mice, CKC positive mice and vGAT cre positive mice have similar numbers of cells staining positively for BrdU on day seven of pregnancy. Cells were counted at x 20 magnification.*

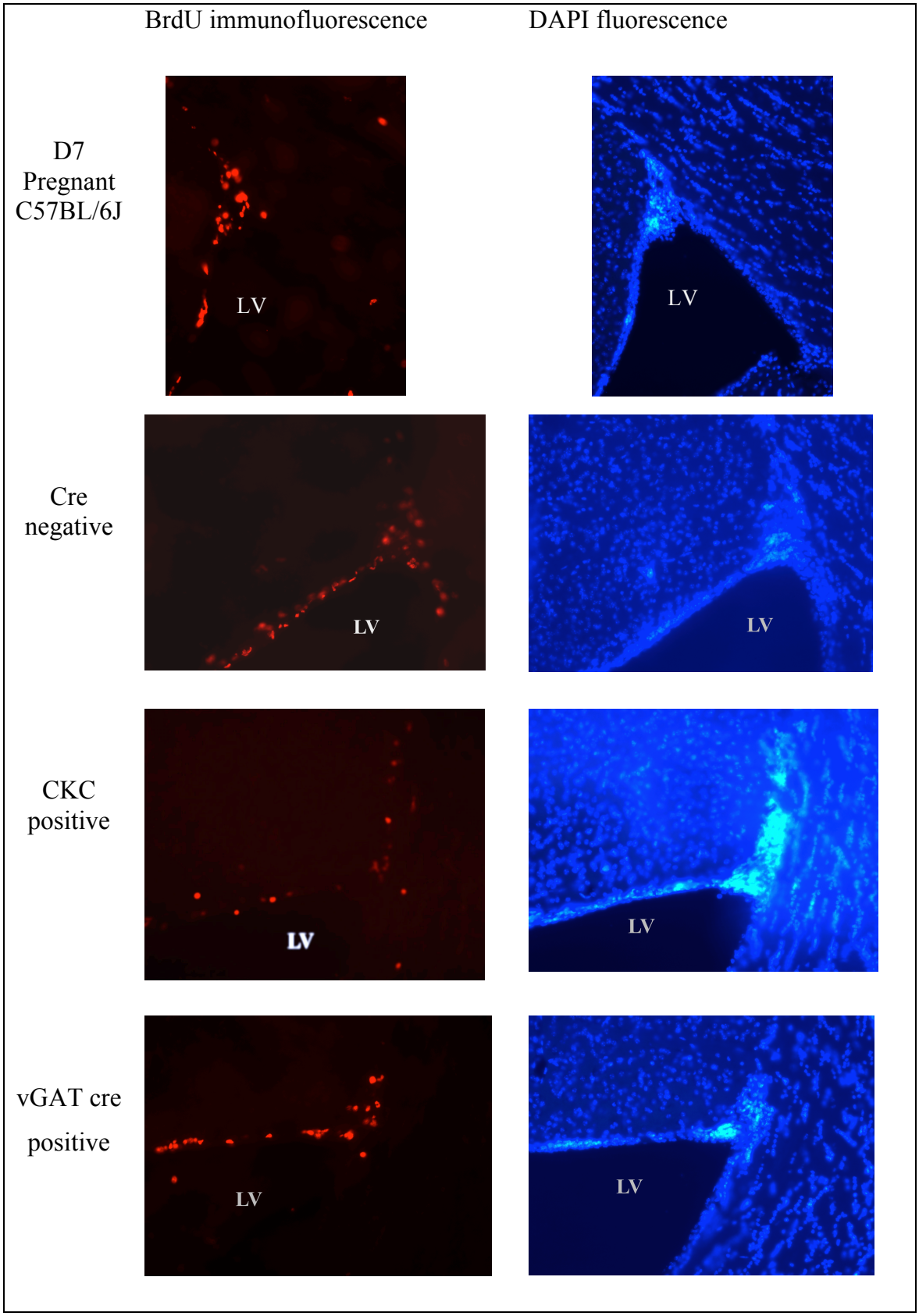


Figure 4.17 | *Representative images of BrdU immunofluorescent staining and DAPI fluorescent staining in the SVZ of day seven pregnant C57BL/6J, cre negative, CKC positive and vGAT cre positive mouse brains. The DAPI stain is taken up by all cell nuclei.*

5.0 Discussion

Maternal behaviour and levels of neurogenesis were examined in CaM-kinase cre x $PRLR^{flox/flox}$ mice and vGAT cre x $PRLR^{flox/flox}$ mice. CKC positive mice had significantly impaired maternal behaviour, compared with the controls, and this was reflected by poor pup survival. vGAT cre positive mice had a lesser, but still significant, impairment in maternal behaviour. This indicates that while prolactin's action on GABAergic neurons does contribute to the expression of maternal behaviours, other populations of neurons must also contribute. The levels of neurogenesis in the two groups of transgenic mice were not significantly different to the controls, suggesting that the majority of prolactin's effect on neurogenesis is not mediated through adult neurons.

It is important to consider the success of the recombination that occurred in the genetically modified mice prior to discussion of the results. One population of mice had the PRL-R rendered non-functional in all neurons containing CaM-kinase II (CKC positive mice). CaM-kinase II is purported to be present in the majority of forebrain neurons, and thus, in this group the PRL-R should be non-functional in most forebrain neurons. The other transgenic group of mice had the receptor more specifically truncated in any neuron containing the vesicular GABA transporter (vGAT cre positive mice), and therefore, in this group of mice the PRL-R should have been rendered non-functional in most GABAergic neurons. In these populations of mice GFP became activated if the PRL-R gene was inverted to become non-functional. Therefore, to investigate how successful recombination of the transgene was, we used immunostaining techniques to look at GFP. As staining for pSTAT5 is only present in nuclei that contain functional PRL-R mRNA [36], immunostaining techniques to look at the presence of pSTAT5 were also used to further examine the extent of recombination of the transgene. GFP was present in both CKC positive and vGAT cre positive mice in all areas of the forebrain known to express PRL-R, indicating that cre-mediated genetic recombination had occurred successfully. However, in the CKC positive mice, low levels of positive staining for pSTAT5 was still seen in the arcuate nucleus, mPOA, and AVPV of CKC positive mice. These are areas where GFP was also seen, and the prolactin receptor is densely expressed. The staining was

significantly less than in the control groups, and present in variable levels, indicating that the CamK-cre x PRLR $^{flox/flox}$ positive mice would have had variable, but overall reduced, levels of responsiveness to prolactin in these areas. Previous investigations by Dr Brown in our laboratory using these techniques obtained similar results (unpublished data). It is possible that neurons in these areas express the PRL-R but not CaM-kinase II, that non-neural cells such as astrocytes express PRL-R or that the cre-mediated recombination was inconsistent. As pSTAT5 is an indirect measure of PRL-R expression, it is also possible that another cytokine is activating STAT5 and the presence of pSTAT5 is not an indication of PRL-R. Levels in the vGAT cre positive mice have the PRL-R knocked out of a more specific group of neurons than the CKC positive mice; therefore we would expect to see similar or greater evidence of pSTAT5 in the forebrains of these mice. The extremely low levels of positive staining for pSTAT5 we detected in the vGAT cre positive mice were unexpected and are most likely due to suboptimal staining. A significantly lower level of pSTAT5 staining was also unexpectedly seen in the arcuate nucleus of the cre negative controls, compared with the CB57BL/6J controls, suggesting that either these mice were incorrectly genotyped (which is unlikely as other areas showed normal levels of pSTAT5 and GFP was not detected) or the staining was once again suboptimal. The immunostaining for pSTAT5 will need to be repeated before a reliable quantification of pSTAT5 in the forebrain of cre negative and vGAT cre positive mice can be acquired. However, we are confident that the CKC positive mice had significantly decreased pSTAT5 expression and, based on this and the presence of GFP in both groups, that both the CKC and the vGAT cre positive mice are still useful animal models for observing the role of prolactin's action on forebrain neurons, albeit with the proviso that some prolactin activation is still occurring in these areas.

As previously discussed in chapter one, rodents experience changing hormone levels throughout their estrous cycle (figure 1.5). The loss of regulation of prolactin levels disturbs the carefully controlled cycle, and chronic hyperprolactinaemia has been shown to suppress LH secretion [80]. As a result of the hormonal imbalance, the mice enter a pseudopregnant state due to stimulation of the corpus luteum, and ovulation cannot occur normally and the diestrous portion of the estrous cycle is greatly prolonged. The CKC positive mice are severely hyperprolactinaemic (figure 5.1),

which probably underlies the changes that were seen in estrous cyclicity, where they spent ninety percent of the cycle in diestrous.

Prolactin secretion is controlled by a negative feedback loop, where prolactin binds to the prolactin receptor on TIDA neurons to stimulate the secretion of dopamine, which in turn binds to its cognate receptor on pituitary lactotroph cells to suppress prolactin secretion (figure 1.4). In the CKC positive mice we saw a great attenuation of pSTAT5 in the arcuate nucleus, the location of the TIDA neurons. The hyperprolactinaemia seen in the cre positive mice suggests that the prolactin receptor was expressed to a greatly reduced extent on the TIDA neurons, and thus, that they were not as responsive to prolactin. Therefore, the hyperprolactinaemia seen in the CKC cre positive mice was evidence of successful recombination of the transgene in the TIDA neurons. In contrast, vGAT cre positive mice were not hyperprolactinaemic, which was as we expected, as it is unlikely that the dopaminergic neurons involved in the control of prolactin secretion are also GABAergic.

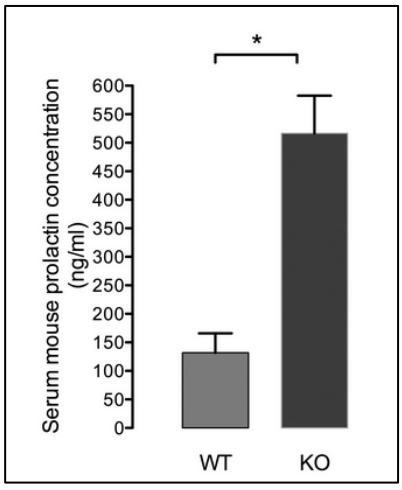


Figure 5.1 | *Serum prolactin levels of non-pregnant CKC positive mice (KO), compared with those of non pregnant C57BL/6J wildtype (WT) mice as measured by radioimmunoassay. The CKC positive mice have significantly elevated prolactin levels, indicating that the TIDA neurons are no longer responsive to prolactin. Courtesy of Dr Rosemary Brown, unpublished data.*

The CKC positive mice appeared to have normal fertility, despite the extended time virgin females spent in diestrous. The normal fertility observed in these mice despite their abnormal estrous cycles may be due to the Bruce Effect. This is a phenomenon observed in mice, where the smell of an unfamiliar male results in a change in hormone

levels, and in miscarriage if they are pregnant [81]. In this experiment, being paired with an unfamiliar C57BL/6J stud male may have caused a change in hormone levels, precipitating proestrus, allowing the mice to ovulate, mate and become pregnant.

Both transgenic groups of mice displayed impaired maternal behaviours, but the deficit was much more pronounced in the CKC positive mice. Furthermore, the behaviour observed towards pups appeared to be distinctly different to that of the vGAT cre positive mice. While the CKC positive mice typically explored the cage and paid little attention to their pups, the vGAT cre positive mice groomed the pups and built a nest, and this was often followed by retrieval. However, both groups of mice seldom settled into the lactating kyphotic position. The lack of maternal behaviours in the CKC positive mice was reflected in the low number of pups that survived to weaning on day twenty-one, and the carcasses were often absent or showed evidence of aggression. The offspring of the vGAT cre positive mice had better survival rates, and once dead the pups were left undisturbed. This suggests that the CKC positive mice kill their pups, or at least severely neglect them, and eat them once dead. Whereas, the vGAT cre positive mothers did not appear to have aggressive tendencies, and their pups most likely died of starvation or hypothermia. Therefore, we conclude that the maternal instinct seems to be somewhat preserved in the vGAT cre positive mice, whereas it is almost completely abolished in the CKC positive mice. These findings in the CKC positive mice are similar to those seen in $PRLR^{-/-}$ mice in a previous study, where the prolactin receptor was knocked out of all cells in the body and the mice also displayed significantly impaired maternal behaviours [20]. This evidence suggests that the severe deficit in maternal behaviour was caused by a lack of prolactin action on CaM-kinase II containing forebrain neurons. Therefore, we conclude that prolactin regulates maternal behaviour by acting on these neurons. What the experiments do not show is whether this effect happens during pregnancy, where pregnancy induced changes alter the future responsiveness of nuclei, or by acting postpartum to alter immediate responses when prolactin is induced by suckling, or both.

The vGAT cre positive mice had a specific knockdown of the PRL-R only in vGAT containing neurons, and had a lesser deficit in maternal behaviours than the CKC cre positive mice, which had a more complete knockdown of the PRL-R in many brain

regions. These results suggest that vGAT containing neurons do play a part in the onset and continuance of maternal behaviours, but other neuronal populations contribute significantly. Which part of maternal behaviour development the GABAergic neurons are involved in remains to be investigated further, but based on our observations it appears to be the development of the suckling instinct. The mPOA has been implicated in the expression of maternal behaviours, as bilateral lesions lead to impaired maternal behaviour expression and infusions of the mPOA with prolactin stimulated maternal behaviour, and the majority of neurons that are active in this area during interaction with pups are GABAergic [72, 82]. This, along with the presence of high levels of positive staining for GFP in the mPOA, suggests that the absence of functional PRL-R from these GABAergic neurons in both the CKC and vGAT cre positive mice was quite possibly a major cause of the decrease in maternal behaviour expression we saw in these mice. Moreover, as the vGAT cre positive mice showed a specific impairment in remaining settled over the pups in the nest, the results suggest that prolactin action on GABAergic neurons, potentially in this area, predominantly underlies the development of this behaviour. The neurons expressing CaM-kinase II seem to be involved in regulating a much wider range of maternal behaviour expression such as the retrieving, grooming and suckling instincts, and suppression of aggressive behaviour.

We have previously seen that postpartum maternal behaviour is dependent on the normal increase in maternal neurogenesis that occurs on day seven of pregnancy [10]. Hence, one possibility was that PRL-R deletion from forebrain neurons, which we have shown leads to impaired maternal behaviours, would lead to a decrease in neurogenesis. However, this does not appear to be the case; the day seven pregnant CKC and vGAT cre positive mice had similar numbers of BrdU containing cells to the day seven pregnant cre negative and C57BL/6J control mice, and significantly more than the previously reported levels seen in diestrous virgin mice and day seven pregnant bromocriptine treated mice [10] . One possibility is that in the previous study where prolactin responsiveness of forebrain neurons was normal, the increase in anxiety that resulted from inhibition of neurogenesis contributed to the mother's inability to adequately care for their pups [10]. As neither the CKC nor the vGAT cre positive mice had abnormal anxiety levels or decreased neurogenesis, the mice in the

two studies appear to have a separate failure of full maternal behaviour development. This strongly suggests that while the prolactin-mediated increase in maternal neurogenesis during pregnancy is important for some aspects of maternal behaviour, other prolactin-mediated changes in the maternal brain are also crucial for normal expression of maternal behaviours. The normal anxiety levels observed in the CKC and vGAT cre positive mice, despite having the PRL-R knocked out of forebrain neurons, supports the previous findings that SVZ neurogenesis in the maternal brain is crucial for the prolactin-induced decrease in anxiety observed in mice postpartum.

It could be speculated that the maternal behaviours of both the CKC and vGAT cre positive mice that were retained to some extent, such as pup retrieval, are regulated by levels of neurogenesis that are increased by either prolactin's actions elsewhere, such as glial cells in the forebrain, or cells in the choroid plexus, or potentially by an increase in neurogenesis that is mediated by a different pathway. More work would need to be done to establish this.

Ideally the transgenic animals used in these experiments would have a 100 % knockout of the PRL-R in the neurons targeted, and the CKC x $PRLR^{flox/flox}$ mice would have no positive staining for pSTAT5 confirming the complete absence of PRL-R from forebrain neurons. Some of the effects we observed, such as some animals displaying more maternal behaviours than others of the same group, may be due to the inconsistency of the recombination. The small decrease in neurogenesis observed in the two populations of mice may be due to there being some target neurons which still have functional prolactin receptors, although this is unlikely due to the low level of PRL-R-induced activity. Working with rodents presents more problems than just their genetics; even if the genetic recombination works perfectly they need to be kept in a constant, stress-free environment and all of the animals need to be exposed to the exact same variables. While the mice were kept in an environment as strictly monitored as possible, perfection is unfortunately impossible and small fluctuations in temperature among other things are bound to have happened. Other researchers sharing the facility and the animal facility workers may potentially introduce stressful stimuli such as noise or disease that could alter the maternal behaviours and/or anxiety levels of the mice. Data collection is a huge issue in itself; it is not possible to survey the mice to discover

how anxious they are or how well they are coping with motherhood as you would a human, and maternal behaviour testing must be observed, and anxiety tested using behavioural tests. This creates potential for human error when recording the observations and variations in testing conditions from one day to the next. Similarly, human error among various other factors can lead to poor quality of immunostaining which can also impair results. The best way to ensure that results are affected as little as possible by all the challenges that working with animals presents is to have a large sample size, unfortunately time constraints meant that the sample size in some of these experiments was not as large as would be ideal. For this reason, and to reinforce the validity of the results, it would be worth repeating the experiments conducted in this study in the future. In our maternal behaviour assessment we used the mother's own pups, unless she did not have three live pups in which case foster pups were used. This potentially affects how rapidly a mother responds to the pups, so in the future it would be best to carry out all experiments using foster pups, as has been done in previous studies [10, 11, 20].

It would be interesting to clarify and further develop our findings by creating a mouse model with the prolactin receptor knocked out of other cells, such as dopaminergic neurons or glial cells, or from the choroid plexus, to confirm or deny whether this is the mechanism by which prolactin is inducing neurogenesis and regulating maternal behaviour. In addition, experiments using a GABA agonist would be interesting to confirm the effects of the GABAergic neuron specific knockout.

We hypothesised that both CKC positive and vGAT cre positive mice would exhibit impaired maternal behaviour. The results of this study have proven that hypothesis correct, although to varying degrees. The CKC positive mice have a more severe impairment, indicating that while prolactin action on GABAergic neurons is important, prolactin's action on more than one type of forebrain neurons contributes to full maternal behaviour expression in mice. We then wanted to begin to explore the mechanism by which this impairment arises, firstly by looking at the effect the absence of prolactin action on forebrain neurons has on neurogenesis. We found that prolactin does not act on these neurons to induce this effect, as the CKC and vGAT positive mice

both had similar levels of neurogenesis to the controls, reflected by the normal levels of anxiety observed in these mice.

Postpartum depression and anxiety affect a large number of women, and can be severely detrimental to both mother and child. We wished to investigate whether or not it is plausible that a lack of responsiveness of forebrain neurons to prolactin may contribute to the development of these disorders, to guide future research into prevention and treatment. This results of this study suggest that this may be the case and that future research in this area would be worthwhile.

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Appendices

A. Paraformaldehyde solution

Phosphate buffer (0.2M):

$NaH_2PO_4H_2O$	13.8g	[BDH Prolabo (PA, USA), batch 07F140008]
$Na_2HPO_4H_2O$	17.799g	[AppliChem (Germany), batch 2O003967]

Made up to 500ml with Milli-Q H_2O.

Paraformaldehyde:

Paraformaldehyde	20 g	[ProSciTech (Australia), 12103]
Milli-Q H_2O	500ml	
NaOH	1-3 drops	

Phosphate buffer (0.2M) 500ml

Paraformaldehyde was added to the Milli-Q H_2O and heated to 50^oC while being stirred continuously. A few drops of NaOH were added to aid the dissolving of the paraformaldehyde. Once it was fully dissolved it was poured through filter paper into a collection bottle. Once cooled phosphate buffer was added.

B. Sucrose Solution

NaH_2PO_4	13.8g	[BDH Prolabo (PA, USA), batch 07F140008]
Na_2HPO_4	17.779g	[Applichem (Germany), batch 2O003967]

Make up to 1L with Milli-Q H_2O

Sucrose solution:

Sucrose	150g	[Applichem (Germany), batch 2J003104]

Make up to 500ml using phosphate buffer (0.1M).

C. Cryoprotectant

Phosphate buffer (0.1M)	500ml	
Sodium chloride	9g	[Thermo Fisher Scientific (Australia), batch 1110327]
Sucrose	300g	[Applichem (Germany), batch 2J003104]
Polyvinylpyrrolidone	10g	[BDH Chemicals Ltd (England), batch K34069883]
Ethylene glycol	300ml	[Sigma-Aldrich (MO, USA), batch SHBC5686V]

NaCl, sucrose and polyvinylpyrrolidone were dissolved in the phosphate buffer. Once they were completely dissolved, the ethylene glycol was added. Total volume was 1L.

D. 1x Phosphate buffered saline solution (PBS)

NaCl	8g	[Scharlau (Spain), batch 13806002]
KCl	0.2g	[BDH chemicals ltd (England), batch TA714938]
Na_2HPO_4	1.44g	[AppliChem (Germany), batch 2D007934]
KH_2PO_4	0.24g	[BDH Chemicals ltd (England), 1201350]

Dissolve in 800 mL of milli-Q H_2O. Adjust the pH to 7.4, then make up to 1 L with milli-Q H_2O.

E. 5µg/ml Proteinase kinase K

proteinase K	1mg	[Gibco-BRL (Ca, USA), batch 1096756]

Add proteinase K to 20ml of 0.01M tris-HCl and mix

F. 4M HCl

HCl [Ajax Finechem Pty ltd (Australia), batch 1012312]

G. A/B Vectastain elite solution

VECTASTAIN elite ABC kit [Vector Laboratories (CA, USA),]

1 drop reagent A and B added to 2.5ml PBS, then left on the slow shaker for 30 minutes.

H. Tris-HCl (0.01M)

Tris-HCl 0.788g [AppliChem (Germany), batch 1S008001]

Make up to 500mL with Milli-Q H_2O, adjust pH to 10.0.

I. PB-TX + BSA (incubation solution)

Incubation solution:

1 x PBS	500ml	
BSA	1.25g	[Sigma, batch 040MI756]
Triton-X-100	1.5ml	[Acros Organics, batch AO290154]

Dissolve the BSA and triton-X-100 in the PBS.